Mitochondrial Dysfunction and Morphological Abnormality in Keloid Fibroblasts

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Objective: As an aberrant response to cutaneous wound healing, keloid scarring is a challenge to cure, and its clinical management remains unsatisfactory. The purpose of this study was to investigate whether mitochondrial function and morphology were impaired in keloid pathology.

Approach: Keloid fibroblasts (KFs) and normal skin fibroblasts (NFs) were isolated and cultured from excised samples. A real-time extracellular flux analyzer was used to monitor the oxygen consumption rate for evaluating the mitochondrial respiratory function. Real-time PCR or Western blot was performed to detect the levels of mRNAs or proteins, respectively, including those associated with the tricarboxylic acid cycle, mitochondrial fission and fusion, and mitochondrial biogenesis. The reactive oxygen species (ROS) level and the mitochondrial mass were measured by flow cytometry. Mitochondrial ultrastructure was observed by transmission electron microscopy.

Results: Compared with NFs, KFs showed reduced basal oxidative respiration ($p < 0.05$), maximal oxidative respiratory capacity, ATP production, and coupling efficiency ($p < 0.01$) but increased proton leakage ($p < 0.01$). ROS were decreased ($p < 0.05$) in KFs, whereas the mitochondrial mass was increased ($p < 0.01$). The mRNA and protein expressions related to mitochondrial dynamics (fission and fusion) and biogenesis were significantly upregulated in KFs ($p < 0.05$). Mitochondria in both KFs and keloid tissue were distended and swollen, along with displaying vacuolization and effacement of the cristae.

Innovation: The possible involvement of mitochondrial abnormalities in keloid pathology may promote promising therapeutic strategies for wound healing disorders.

Conclusion: Collectively, our data suggested that both mitochondrial dysfunction and morphological impairment might play vital roles during keloid pathogenesis.

Keywords: keloid, scar, fibroblast, wound healing, mitochondrial dysfunction, fibrosis

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INTRODUCTION

KELOIDS, BENIGN FIBROPROLIFERATIVE tumors that only occur in humans, are characterized by abnormal proliferation of fibroblasts and excess extracellular matrix deposition. Keloids grow beyond the original wound boundary and do not regress sponta-

neously with time.¹ They are caused by almost every type of dermal injury, including trauma, surgery, infection, skin piercing, vaccination, and insect bite.² Given the presence in families and varied incidence among different ethnic populations, genetic susceptibility is also a key factor.³ Clinically,

treatments such as surgical resection, steroid injection, cryotherapy, and radiation therapy are not satisfactory to maintain low recurrence.⁴ Pain, itching, local deformity, and dysfunction are usually accompanied by the disease, which severely affect the quality of life of patients. Given a lack of a validated animal model for keloid disease,⁵ better knowledge of the etiology and molecular mechanisms of its pathogenesis presents a challenge for clinicians and researchers.

The mitochondrion is one of the most important organelles for cellular activity. It plays a key role in bioenergy metabolism, apoptosis, and autophagy. As the main location of oxidative phosphorylation and the tricarboxylic acid (TCA) cycle in the cell, the mitochondrion is called the “power factory” and provides energy for cellular activities. Changes in the function, number, or structure of mitochondria can impact cellular physiology and further promote disorders in the body.⁶ Some recent studies suggested involvement of dysfunctional mitochondria-mediated apoptosis in keloid pathology. Lu *et al.*⁷ reported that treatment with green tea polysaccharide in keloid fibroblasts (KFs) caused the activation of mitochondrial apoptotic pathway, including elevated caspase-3 activity, increased proapoptotic protein Bax expression, and decreased expression levels of antiapoptotic protein Bcl-2. Recent studies showed enhancement of microRNA-21 level in keloid tissue and inhibition of microRNA-21 induced mitochondria-mediated apoptosis in KFs.^{8,9} These evidences suggested that targeting mitochondria-mediated apoptotic pathway was effective and therapeutic to intervene and prevent keloid formation, indicating involvement of mitochondrial dysfunction in keloid pathology.

Our previous work reported that KFs underwent metabolic reprogramming into aerobic glycolysis.¹⁰ The shift to aerobic glycolysis, also called the Warburg effect, is a canonical metabolic characteristic of cancerous cells. However, accumulating studies have found that cells derived from other fibrotic diseases, such as laryngotracheal stenosis,¹¹ chronic kidney diseases,¹² and idiopathic pulmonary fibrosis,¹³ also exhibited this phenomenon, which indicates that aerobic glycolysis is not the exclusive metabolic phenotype of cancer cells.

Mitochondrial dysfunction is considered to be a vital cause of metabolic reprogramming. Here, we hypothesized that metabolically reprogrammed KFs, the main cell type in keloids, may fall within the category of mitochondrial abnormalities. In this study, we compared mitochondrial oxidative respiration, gene expression related to the TCA cycle, mitochondrial dynamics (fission and fusion),

mitochondrial mass, and mitochondrial morphology between KFs and normal skin fibroblasts (NFs).

CLINICAL PROBLEM ADDRESSED

Caused by an abnormal response of the wound healing process, keloid scarring markedly disfigures the patient, resulting in reduced quality of life. Despite multiple kinds of treatments available, none of these treatments is satisfying or guarantees a cure.¹⁴ Furthermore, as an excessively hyperfibrotic dermal disorder that occurs on the surface of the human body, keloids also provide a superior model to investigate fibrotic disease that occurs in other tissues and organs. Disclosing the mechanisms that underlie keloid scarring is of great significance to promote the development of novel therapies for keloids and other fibrous diseases.

MATERIALS AND METHODS

Ethics statement and tissue samples

This study was performed under approval of the Medical Ethics Committee of Peking University Third Hospital. All subjects were provided information about the purpose of the study and gave full, written informed consent. Human keloid tissue specimens ($n=9$) and normal skin tissue specimens ($n=8$) from different participants were obtained from keloid patients with no previous treatment and healthy volunteers from the Plastic Surgery Department of Peking University Third Hospital. The collected skin samples were immersed in 0.9% normal saline or 2.5% glutaraldehyde and then immediately transported in an ice-cold box for primary cell culture or ultrastructure observation. Information about each specimen is listed in Supplementary Table S1.

Cell isolation and culture

Primary fibroblasts derived from keloid and normal skin were isolated and cultured as reported previously.¹⁵ Epidermis and subcutaneous fat were cut and washed away from specimens using phosphate buffered saline. Samples were cut into 1–3 mm³ pieces and incubated in 75 cm² culture flasks containing Dulbecco's modified Eagle's medium (DMEM; HyClone, South Logan, UT) with 10% fetal bovine serum and 1% penicillin and streptomycin. Cells were maintained at 37°C in a humidified incubator containing 5% CO₂. The culture medium was changed every 3 days, and cells at passages 4–6 were used for the following experiments.

Measurement of oxygen consumption rate

Oxidative phosphorylation was evaluated using the oxygen consumption rate (OCR), which was

determined using the XF Cell Mito Stress Test with a Seahorse XFp Real-time Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA), according to the manufacturer's protocol instructions as previously described.¹⁶ Briefly, 2×10^4 cells per well were seeded in triplicate in normal growth medium and allowed to attach overnight before measurement. Seahorse XF Base Medium (Seahorse Bioscience) supplemented with 10 mM glucose, 1 mM sodium pyruvate, and 2 mM glutamine (Sigma-Aldrich, St. Louis, MO) was used in the Mito Stress Test. Cells were washed three times and preincubated for 1 h in this medium in a non-CO₂ humidified incubator at 37°C. OCR measurements were detected under basal conditions over time and after the successive addition of 1 μ M oligomycin, an inhibitor of ATP synthase, 0.75 μ M fluoro-carbonyl cyanide phenylhydrazone (FCCP), and 0.5 μ M rotenone plus 0.5 μ M antimycin A, inhibitors of electron transport chain complexes I and III, respectively. Data were normalized by the cell number in each well.

RNA extraction and quantitative real-time PCR

Total RNA was prepared using TRIzol reagent (Invitrogen, Carlsbad, CA), and cDNA was synthesized using the FastKing RT Kit (with gDNase) (Tiangen Biotech, Beijing, China). Quantitative real-time PCR (qRT-PCR) was performed according to the Talent qPCR PreMix (SYBR Green) kit protocol (Tiangen Biotech) with an ABI QuantStudio 5 Real-Time PCR System (Applied Biosystems, Foster City, CA). The relative change in mRNA expression was calculated by the $2^{-\Delta\Delta CT}$ method, and housekeeping gene β -actin mRNA expression was used for normalization. Primer sequences are detailed in Supplementary Table S2.

Western blot analysis

Cells were extracted in ice-cold RIPA lysis buffer supplemented with phosphatase and protease inhibitor cocktail (Applygen Technologies, Beijing, China). Protein concentration of lysates was determined by a BCA Protein Assay Kit (CWbiotech, Beijing, China), and Western blotting was performed as previously described.¹⁰ Briefly, proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a 0.45 μ m polyvinylidene fluoride membrane (Millipore, Billerica, MA). Membranes were blocked in Tris-buffered saline containing 0.002 g/L Tween 20 (TBST) with 5% bovine serum albumin for 1 h and incubated overnight at 4°C in TBST with primary antibodies against citrate synthase (CS), isocitrate dehydrogenase 2 (IDH2), succinate

dehydrogenase complex flavoprotein subunit A (SDHA) (1:1,000; Abcam, Cambridge, UK), mitochondrial fission 1 (FIS1), dynamin-related protein 1 (DRP1), mitofusin 1 (MFN1), MFN2, PGCl α , TFAM (1:500; Abcam), oxoglutarate dehydrogenase (OGDH) (1:1,000; Proteintech, Chicago, IL), and β -actin (1:2,000; Cell Signaling Technology, Danvers, MA). Then, IRDye 800CW secondary antibody (1:10,000; LI-COR Biosciences, Lincoln, NE) and an Odyssey Infrared Imaging System (LI-COR Bioscience) were used to detect protein bands. Signal was quantified with the ImageJ software (National Institutes of Health).

Mitochondrial mass assessment

Mitochondrial mass was assessed by MitoTracker Green (Beyotime, Nanjing, Jiangsu, China). In brief, cells were harvested with 0.25% trypsin and centrifuged at 300 *g* at 4°C for 5 min and then incubated with prewarmed 200 nM MitoTracker Green staining solution in DMEM for 20 min at 37°C in the dark. After centrifugation at 300 *g* at 4°C for 5 min, cells were resuspended in 500 μ L DMEM. Fluorescence was detected by a BD FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) flow cytometer. Data acquisition and analysis were processed using the FlowJo software (Tree Star, Ashland, OR).

Measurement of intracellular reactive oxygen species and mitochondrial reactive oxygen species

The change in intracellular reactive oxygen species (ROS) was measured with a Reactive Oxygen Species Assay Kit (Beyotime) according to the manufacturer's instructions. Briefly, 2'-7'-dichlorofluorescein diacetate (DCFH-DA) probe stock solution was diluted with DMEM to obtain a 10 μ M DCFH-DA working solution. Cells were washed and incubated in 10 μ M DCFH-DA working solution at 37°C for 30 min in the dark. Intracellular ROS oxidize nonfluorescent DCFH-DA into fluorescent 2'-7'-dichlorofluorescein (DCF), reflecting the ROS production.¹⁷ Then, the fluorescence intensity was measured on a BD FACSCalibur (Becton Dickinson) flow cytometer. MitoSOX Red reagent (Invitrogen) was used for detecting mitochondrial ROS (mtROS). Briefly, cells were harvested with 0.25% trypsin and incubated in 5 μ M MitoSOX Red in Hanks' balanced salt solution with calcium and magnesium at 37°C for 15 min in the dark. The fluorescence intensity was measured by a flow cytometer. Data acquisition and analysis were processed using the FlowJo software (Tree Star).

Superoxide dismutase activity detection

Superoxide dismutase (SOD) activity was measured by using a SOD Assay Kit (Dojindo Molecular Technologies, Kumamoto, Japan). Briefly, 20 μ L cell lysis or ddH₂O were added into 200 μ L water-soluble tetrazolium working solution. Then, 20 μ L dilution buffer or enzyme working buffer was added with mixtures. After incubation for 30 min at 37°C, the absorbance at 450 nm was detected using a microplate reader. In the end, the SOD activity (U/mL) was calculated using the equation.

Transmission electron microscope imaging

Cells and tissue samples were fixed with 2.5% glutaraldehyde and postfixed in 1% osmium tetroxide for 2 h at 4°C. After dehydration in a gradient series of ethanol, the cells were embedded in epoxy resin and polymerized at 60°C. Ultrathin sections were cut and further stained with uranyl acetate and lead citrate. The samples were then examined under a transmission electron microscope (TEM) (JEM1400 plus; JEOL, Tokyo, Japan), and photographs were randomly taken in each sample.

Statistical analyses

Data are expressed as the mean $\pm$ standard deviation from three independent experiments. Statistical analyses were performed using the SPSS 22.0 software, and Student's *t*-tests were used to evaluate the differences between groups. *p*-values <0.05 were considered to be statistically significant.

RESULTS

KFs undergo an impaired mitochondrial oxidative respiration compared with NFs

The OCR can be employed as an indicator of oxidative phosphorylation of mitochondrial respiratory function and energy production to assess mitochondrial bioenergetics.¹⁸ We measured OCR in NF cells and KF cells under baseline conditions, following the consecutive addition of oligomycin, FCCP, and rotenone/antimycin A (Fig. 1A). Oligomycin is an inhibitor that suppresses ATP synthase, causing a decrease in OCR in relation to mitochondrial ATP production. FCCP is a protonophore that uncouples ATP synthesis and induces maximal turnover of mitochondrial OCR. Rotenone and antimycin A are inhibitors of electron transport chain complexes, which lead to a complete abolition of mitochondrial respiration. Figure 1A shows a representative graph of the mitochondrial stress test to measure the OCR readouts over time in NF and KF cell cultures. It was found that KFs exhibited significantly greater non-mitochondrial

oxygen consumption compared with NFs (Fig. 1B). Meanwhile, the basal respiration (Fig. 1C), maximal respiration (Fig. 1D), and spare respiratory capacity (Fig. 1E) were significantly lower in KFs than in NFs, meaning that the mitochondria of KFs could play a less compensatory role in the case of increased energy demand. Compared with NFs, mitochondrial ATP production (Fig. 1F) and coupling efficiency (Fig. 1G) in KFs were significantly reduced, whereas proton leak (Fig. 1H) was significantly increased in KFs, which indicated that KF cells utilized less energy from oxidative phosphorylation. These data demonstrated that KF cells exhibited diminished mitochondrial oxidative respiration.

Alterations in gene and protein expression related to the TCA cycle in KFs and NFs

The TCA cycle is a key process composed of a series of enzymatic reactions that provide reducing equivalents for mitochondrial oxidative respiration. To uncover whether a diminished mitochondrial metabolic function is related to changes in the influx of the TCA cycle, we detected the expression levels of TCA cycle-related genes and proteins, including pyruvate dehydrogenase kinase (PDK) 1, PDK2, CS, aconitase (ACO) 1, ACO2, IDH1, IDH2, OGDH, succinate-CoA ligase GDP-forming beta subunit 2 (SUCLG2), SDHA, fumarate hydratase (FH), and malate dehydrogenase (MDH) 1 and MDH2. The mRNA levels of PDK1, CS, ACO2, IDH1, IDH2, OGDH, and SDHA were markedly augmented in KFs versus those in NFs, whereas the SUCLG2 mRNA level in KFs was significantly decreased compared with that in NFs, and there was no difference in the expression levels of PDK2, ACO1, FH, MDH1, and MDH2 (Fig. 2A). We further measured the protein levels of the key enzymes in the TCA cycle. Our data showed that KFs exhibited lower protein levels of CS, OGDH, and SDHA, but a greater level of IDH2 protein compared with that in NFs (Fig. 2B).

KFs exhibit decreased ROS production and increased SOD activity

Mitochondrial oxidative phosphorylation is the primary cellular source of ROS generation.¹⁹ We used DCFH-DA probe and MitoSOX Red reagent to assess intracellular ROS and mtROS, respectively. Figure 3A shows a significantly higher fluorescence intensity of DCF in NFs, indicating that the cellular ROS level was lower in KFs than in NFs. Meanwhile, KFs indicated a significant decrease level of mtROS (Fig. 3B), whereas a significant increase level of the SOD activity (Fig. 3C).

Figure 1. Mitochondrial oxidative respiration is impaired in KFs. **(A)** Representative mitochondrial stress test performed on a Seahorse Bioscience XFp Extracellular Flux Analyzer in KFs and NFs. **(B)** KFs demonstrate increased non-mitochondrial oxygen consumption. **(C)** Basal respiration, **(D)** maximal respiration, and **(E)** spare respiratory capacity are reduced in KFs. KFs exhibit lower ATP production **(F)** and coupling efficiency **(G)**, whereas a higher proton leak **(H)** is observed compared with NFs. **(B–H)** $n=6$ in KFs and NFs. * $p<0.05$, ** $p<0.01$. KFs, keloid fibroblasts; NFs, normal skin fibroblasts; OCR, oxygen consumption rate; FCCP, fluoro-carbonyl cyanide phenylhydrazone.

KFs suggest altered mitochondrial dynamics

Mitochondria are dynamic organelles continuously undergoing fission and fusion,²⁰ which play a vital role in the maintenance of a healthy mitochondrial network.²¹ Next, we detected the expression levels of fission-associated genes, such as FIS1 and DRP1, and fusion-related genes, MFN1/2. qRT-PCR results demonstrated that FIS1 (Fig. 4A), DRP1 (Fig. 4B), MFN1 (Fig. 4C), and MFN2 (Fig. 4D) mRNA expressions were higher in KFs than in NFs. As shown in Fig. 4E, the results

of Western blot analysis were in accordance with qRT-PCR tests. Altogether, the results presented here indicate possibilities of higher mitochondrial dynamics in KFs.

Comparison of mitochondrial mass and biogenesis between KFs and NFs

Mitochondrial dynamics could affect their mass and degree of biogenesis.²² We next detected the mitochondrial content and biogenesis to assess whether these were associated with dysfunction of

Figure 2. Comparison of gene and protein expressions related to the TCA cycle in KFs and NFs. **(A)** qRT-PCR analysis for genes in the TCA cycle. **(B)** Western blot analysis for the key enzymes. **(A, B)** $n=6$ in KFs and NFs. * $p < 0.05$, ** $p < 0.01$. qRT-PCR, quantitative real-time PCR; TCA, tricarboxylic acid.

Figure 3. KFs demonstrate lower ROS production and higher SOD activity. **(A)** Flow cytometry for the detection of DCF intensity in KFs and NFs and quantitative analysis of ROS generation. $n=6$ in KFs and NFs. **(B)** MitoSOX Red intensity is measured by flow cytometry to reveal mitochondrial ROS level, which is decreased in KFs compared with that in NFs. **(C)** KFs show a higher SOD activity. **(B, C)** $n=5$ in KFs and NFs. * $p<0.05$. ROS, reactive oxygen species; DCF, 2',7'-dichlorofluorescein; SOD, superoxide dismutase.

mitochondria in KFs. As shown in Fig. 5A, the MitoTracker fluorescence intensity was significantly higher in KFs than in NFs, which suggested an increased mitochondrial mass. Peroxisome proliferator-activated receptor- γ coactivator 1 α (PGC1 α) and mitochondrial transcription factor A (TFAM), a downstream target of PGC1 α , are major regulators and indicators of mitochondrial biogenesis.²³ Our data indicated that both PGC1 α and TFAM mRNA levels (Fig. 5B, C) and protein expressions (Fig. 5D) were significantly upregulated in KFs compared with those in NFs, meaning that KFs might embrace an increased mitochondrial biogenesis through PGC1 α /TFAM signaling pathway.

Ultrastructure is abnormal in KFs and keloid tissue

Mitochondrial ultrastructure and the cellular bioenergetic status are intimately linked.²⁴ In the end, we observed mitochondrial morphology in NFs, KFs, and tissue samples by TEM. In healthy cells, mitochondria have a bilayer membrane structure composed of outer and inner membranes, with the inner membrane folded into cristae,²⁵ which is important to maintain proper

mitochondrial function.²⁶ In NFs and normal skin tissue, elongated mitochondria were found, and their bilayer membrane ultrastructure clearly presented wrinkled cristae (Fig. 6A, C and Supplementary Fig. S1A). However, when we examined the morphology of mitochondria in KFs and keloid tissue, we found that distended and swollen mitochondria superseded normal elongated mitochondria, with cristae disarrangements, effacement, and vacuolization noted, as well as an influx of cytoplasmic material into the mitochondria (Fig. 6B, D and Supplementary Fig. S1B, C). Our results demonstrated the existence of morphologically abnormal mitochondria in both KFs and keloid intact tissue. These abnormalities were also described in other mitochondria-related diseases,^{27,28} implicating mitochondrial alterations in the pathogenesis of keloids (Fig. 7).

DISCUSSION

Keloid disease is a benign fibroproliferative tumor occurring in human skin. As reported in the literature, keloids exhibit complicated formation progress. Existing treatments, including surgical resection, steroid injection, radiation therapy, and

Figure 4. KFs exhibit higher mitochondrial fission and fusion gene expression. Analysis of mRNA expression of (A) FIS1, (B) DRP1, (C) MFN1, and (D) MFN2 in KFs and NFs using qRT-PCR assay. (A–D) $n=5$ in KFs and NFs. (E) Representative Western blot images and quantitative analysis for FIS1, DRP1, MFN1, and MFN2 protein levels. $n=6$ in KFs and NFs. * $p<0.05$, ** $p<0.01$. FIS1, mitochondrial fission 1; DRP1, dynamin-related protein 1; MFN, mitofusin.

cryotherapy, are not fully effective for all patients. At the same time, no specific factors have been identified in the incidence of keloid disease to date.⁴ Furthermore, although great efforts and advances in the keloid tissue implantation model and tissue-engineered keloid animal models have been made to research mechanisms and therapies,^{14,29} the etiology of keloids has not been fully understood. Our identification of fibroblasts derived from keloids indicates that they are undergoing a metabolic variation and resorting to aerobic glycolysis,¹⁰ which suggested that metabolic reprogramming and mitochondrial function closely participate in the pathogenesis of keloid disease.

With KFs actively expressing genes involved in the glycolytic pathway, a higher glucose influx and lactate production, we speculated that KFs used the alternative pathway of glycolysis¹⁰ partly be-

cause of an impaired oxidative phosphorylation in mitochondria. In this study, KFs showed significantly decreased basal respiration, maximal respiration, and spare respiratory capacity, suggesting impaired mitochondrial oxidative phosphorylation in KFs. Meanwhile, the observation of diminished mitochondrial ATP biosynthesis and coupling efficiency in KFs in this work demonstrated that KFs could utilize less energy from their mitochondria and possess a less effective mitochondrial network. There is still potential for KFs to activate more oxidative phosphorylation through a comparatively lower spare respiratory capacity in KFs. Furthermore, KFs are capable of generating more ATP when glycolysis is inhibited and respiratory substrates are added,³⁰ which also means that the mitochondria in KFs are not fully motivated.

Figure 5. Mitochondrial mass and biogenesis are elevated in KFs. **(A)** MitoTracker Green intensity is detected with flow cytometry, and KFs demonstrate higher mitochondrial mass. **(B)** PGC1 α and **(C)** TFAM mRNA levels are assessed with qRT-PCR assays. **(A–C)** $n=5$ in KFs and NFs. **(D)** Representative Western blot images and quantitative analysis for PGC1 α and TFAM protein expression. $n=6$ in KFs and NFs. * $p<0.05$, ** $p<0.01$. PGC1 α , peroxisome proliferator-activated receptor- γ coactivator 1 α ; TFAM, mitochondrial transcription factor A.

The TCA cycle is an important part of mitochondrial oxidative respiration and provides a large number of intermediate substances for cellular biosynthesis. The process also provides reducing equivalents (*i.e.*, NADH and FADH₂) that pass through the mitochondrial electron transport chain and produce ATP through oxidative phosphorylation. Abnormal mitochondrial oxidative respiration and the preferential use of aerobic glycolysis suggest a potential for abnormality in the TCA cycle.^{31,32} Here, we found significant changes in enzymes involved in the TCA cycle. Interestingly, not all protein expression trends are consistent with their mRNA levels, which indicate that the final expression of the TCA cycle-related enzymes is further regulated at the post-transcriptional level. The mRNA and protein levels related to the TCA cycle are indirect indi-

cators for TCA disruption, thus future work should adopt advanced metabolic tracing experiments to explore changes of intermediate products in the TCA cycle. In addition to participating in metabolism, many studies have found that enzymes involved in the TCA cycle can regulate cellular biological functions, such as cell proliferation, apoptosis, migration, and invasion.^{33,34} It has been well recognized that frequent mutations of NADP⁺-dependent isocitrate dehydrogenases encoded by IDH1 and IDH2 occur in multiple types of human cancer, such as glioma and acute myeloid leukemia.^{35,36} Catalyzing the oxidative decarboxylation of isocitrate and yielding α -ketoglutarate (α -KG) and NADPH,³⁷ IDH1 and IDH2 play a role in the cytosol and in the mitochondria, respectively.³⁸ As a coenzyme of glutathione (GSH) reductase, NADPH is required to

Figure 6. Mitochondrial morphology is abnormal in KFs and keloid tissue. Mitochondrial ultrastructure is analyzed by TEM. Representative images are displayed. **(A)** Mitochondrial morphology in NFs ($n=5$) is within normal limits. **(B)** In KFs ($n=5$), elongated mitochondria cannot be observed, and mitochondria are distended and swollen, along with displaying vacuolization and effacement of the cristae. **(C)** TEM images show tubular cristae of mitochondria from normal skin tissue (sample 7, $n=2$). **(D)** Keloid tissue (sample 7, $n=3$) shows the existence of mitochondrial swelling, vacuolization, and cristae degeneration. Scale bars are indicated on each image. Mitochondria are indicated by arrows. TEM, transmission electron microscope.

maintain the reduced state of GSH, which protects cells from redox stress and promotes resistance to apoptosis. Thus, increased expression of isocitrate dehydrogenase (IDH) in KFs could contribute to reducing the attack by ROS and apoptosis.

As one of the most important strategies for inducing apoptosis, intracellular ROS accumulation is mainly generated from mitochondrial respiration. A switch of metabolic phenotype from oxidative phosphorylation to aerobic glycolysis may minimize the production of ROS. In line with our previous study and Vincent *et al.*,^{30,39} we detected

decreased ROS generation in KFs, which indicated reduced induction of apoptosis in KFs reported in many studies. However, several reports found elevation of ROS levels in keloids, which indicated the increased oxidative stress.^{40,41} The discrepancy of different ROS results suggested that KFs were under regulation of oxidative stress, which was a complex balancing process. Mitochondrial respiration is a potential origin for ROS; lower ROS found in this study might be caused by decreased respiration and increased scavenger of ROS. Here, decline of mtROS reflected a diminishment of

Figure 7. Overview of mitochondrial dysfunction and ultrastructural abnormalities in the pathogenesis of keloids.

mitochondria-derived ROS. Consistent with a recent report that copper-zinc SOD activity was higher in keloids,⁴² the upregulation of the SOD activity in this study suggested the response to oxidative stress in KFs. Aerobic glycolysis by proliferating cells is a means to minimize its oxidative stress and maximally enhances the biosynthesis.⁴³ Different results of ROS level might be caused by different stage of microenvironments, sites of sampling, and ethnic differences, and further researches are needed to perform in the future to clarify.

Mitochondrial dynamics (fission and fusion) not only influence the entire mitochondrial number but also influence nearly every aspect of mitochondrial function, such as respiration, ROS generation, and ATP production.⁴⁴ Mitochondrial fission and fusion are highly dynamic and precisely regulated through FIS1, DRP1, and MFN1/2, respectively, to sustain sufficient numbers of functional mitochondria and to cope with the energy demand.⁴⁵ From this perspective, the elevated expression of mitochondrial fission and fusion genes in KFs ob-

served in this work may represent a compensatory mechanism that protects against diminished mitochondrial oxidative respiration. Both mRNA and protein levels are not direct indexes for mitochondrial fission and fusion, which highlights a limitation of the current study. Future work should also explore the effects of intervention for mitochondrial fission/fusion by utilizing dynamic monitoring technique and investigate possible therapeutics and mechanisms.

Mitochondrial mass and biogenesis are key factors that could affect the function of mitochondria. Although KFs showed decreased mitochondrial function, it was not clear whether this decreased function was caused by a diminishment of mitochondrial mass and biogenesis. Thus, we presumed that KFs exhibited decreased mitochondrial mass and biogenesis. PGC1 α and TFAM are significant regulators of mitochondrial biogenesis. Interestingly, both mitochondrial number and biogenesis are significantly upregulated in KFs compared with those in NFs. In a recent study, researchers found that mitochondria-dysfunctional fibroblasts,

caused by deficiency of cytochrome c oxidase enzyme (complex IV) of the respiratory chain, presented an upregulation of mitochondrial mass.⁴⁶ Additional studies also reported that mitochondrial dysfunction was accompanied by increased mitochondrial biogenesis, which is considered a compensatory mechanism triggered by the oxidative phosphorylation defect.^{47,48}

Mitochondrial functions including oxidative phosphorylation have been linked to their morphology, which is shaped by fission and fusion of outer and inner membranes.⁴⁹ To further investigate whether mitochondrial morphology was affected, ultrastructure was observed by TEM. Unfortunately, cristae disarrangements, effacement, and vacuolization were obvious in the mitochondria of KFs. Noly *et al.*²⁸ reported that accompanied by a decrease in myocardial fiber mitochondrial oxidative respiration, mitochondrial structure of cardiomyocytes appeared abnormalities (vacuole, swelling, and cristae disruption) in ventricular hypertrophy. To the best of our knowledge, our results indicated the first evidence of mitochondrial ultrastructural damage in both KFs and keloid tissue. Furthermore, our findings linked the evidence that mitochondrial structural abnormalities, impaired mitochondrial oxidative respiration, and aerobic glycolysis simultaneously involved in the pathogenesis of the disease.

In summary, our current study has comprehensively demonstrated mitochondrial dysfunction and morphological abnormality in KFs, suggesting the significance of mitochondria in the pathogenesis of keloid disease. More importantly, our findings provide novel insight to improve our understanding of fibroproliferative disorders and potential therapeutic strategies. However, further studies are required to elucidate the pathways and mechanisms by which mitochondria were affected in keloid scarring.

This study is not without limitations. One major limitation is that the KFs are isolated from diseased tissue, and therefore, it is not possible to be sure if mitochondrial dysfunction is a consequence of keloids or causative in the pathological process itself. Future studies will explore the influences of cutaneous wound healing process or scarring formation in mitochondrial dysfunctional animal models. Cells derived from 11 females of 12 patients or controls limit the substantiality of the results. Another limitation is the absence of strict matching for body site, which may be a factor that affects cell phenotype. Future studies

KEY FINDINGS

- Compared with NFs, KFs demonstrate decreased mitochondrial oxidative phosphorylation.
- There is disorder of the TCA cycle in KFs.
- KFs exhibit higher mitochondrial dynamics, mitochondrial mass, and biogenesis.
- The morphology of mitochondria is impaired in KFs and keloid tissue.

should avoid these controllable issues and incorporate other pathological scars, such as hypertrophic scar.

INNOVATION

To the best of our knowledge, this is the first time that both mitochondrial dysfunction and morphological impairments were proven in keloid scarring. Here, our data provide evidence that mitochondria play a vital role in keloid pathology. The novel insight of mitochondrial abnormality in keloids may promote promising therapeutic strategies for wound healing disorders and other fibrotic diseases.

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AUTHOR DISCLOSURE AND GHOSTWRITING

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SUPPLEMENTARY MATERIAL

Supplementary Figure S1
Supplementary Table S1
Supplementary Table S2

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Abbreviations and Acronyms

CS	=	citrate synthase
DCF	=	2'-7'-dichlorofluorescein
DCFH-DA	=	2'-7'-dichlorofluorescein diacetate
DMEM	=	Dulbecco's modified Eagle's medium
DRP1	=	dynamins-related protein 1
FCCP	=	fluoro-carbonyl cyanide phenylhydrazone
FH	=	fumarate hydratase
FIS1	=	mitochondrial fission 1
KFs	=	keloid fibroblasts
MFN	=	mitofusin
mtROS	=	mitochondrial reactive oxygen species
NFs	=	normal skin fibroblasts
OCR	=	oxygen consumption rate
OGDH	=	oxoglutarate dehydrogenase
PGC1 α	=	peroxisome proliferator-activated receptor- γ coactivator 1 α
qRT-PCR	=	quantitative real-time PCR
ROS	=	reactive oxygen species
SDHA	=	succinate dehydrogenase complex flavoprotein subunit A
SOD	=	superoxide dismutase
SUCLG2	=	succinate-CoA ligase GDP-forming beta subunit 2
TCA	=	tricarboxylic acid
TEM	=	transmission electron microscope
TFAM	=	mitochondrial transcription factor A