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Original Article

GRK2 deletion improves the function of skin flap following ischemia-reperfusion injury by regulating Drp1

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Abstract: Skin flap ischemia-reperfusion (IR) injury is the key factor to the success rate of skin transplantation, the molecular mechanism of flap IR injury needs to be continuously explored to provide new ideas for its clinical treatment. G protein-coupled receptor kinase 2 (GRK2) was reported to be involved in regulating mitochondrial function, and mitochondria were essential in the process of flap IR. Thus, we aimed to investigate the function of GRK2 in flap ischemia-reperfusion injury and further explore the underlying mechanism. Sixty male C57BL/6 mice were randomly divided into four groups: sham, IR+sh-NC, IR+sh-GRK2 and IR+sh-GRK2+ dynamin-related GTPase 1 (Drp1). Flap function and mitochondrial function were determined after ischemia for 3 hours and reperfusion for 72 hours. Comparing with sham group, GRK2 was increased in flap after IR injury. Loss of GRK2 inhibited cell apoptosis and promoted cell proliferation of flap after IR injury. And deficiency of GRK2 promoted mitochondrial function in flap after IR injury. IR injury up-regulated Drp1 expression in flap, while sh-GRK2 down-regulated Drp1 expression. Furthermore, overexpression of Drp1 removed the protective effect of sh-GRK2. In conclusion, our study revealed that GRK2 deletion improved flap function and mitochondrial function by inhibiting Drp1 expression, which may provide a new insight for the clinical treatment of flap ischemia-reperfusion injury.

Keywords: Skin flap ischemia-reperfusion injury, ROS, GRK2, Drp1, mitochondrial function

Introduction

Skin transplantation is the main method to repair the defects and malformations of body surface tissues and organs caused by tumor resection and various trauma [1]. Therefore, the survival of transplanted tissue often determines the success or failure of the operation. After a period of flap ischemia, the restoration of blood flow in the flap not only fails to restore its function, but aggravates the structural damage and dysfunction, and results in ischemia-reperfusion (IR) injury [2]. After years of research, the survival rate of the flap has been improved after transplantation, but there are still some cases of partial or total tissue necrosis in the clinic [3]. Therefore, the molecular mechanism of skin flap IR injury needs to be continuously explored to provide new ideas for its clinical treatment.

A large number of reactive oxygen species (ROS) will be produced in the process of IR of the skin flap, and hypoxanthine will accumulate in the body during ischemia-ischemia [4]. During the process of restoring blood flow, xanthine oxidase will convert hypoxanthine into guanosine and superoxide ion [5]. Studies have shown that [6] IR injury of skin flap positively regulates xanthine oxidase, which can improve ROS level in tissues, and the positive regulation of xanthine oxidase also mediates the inflammatory response in tissues [7]. During IR, neutrophils are activated and release a variety of cytokines, which in turn activate NADPH, causing an oxygen burst and releasing a large number of oxygen free radicals [8]. Thus, mitochondria, the main place for producing ROS, were important in the process of skin flap IR [9].

G protein-coupled receptor kinase (GRKs) is a critical serine/threonine kinases in the body, which can phosphorylate G protein-coupled receptors (GPCR), and thus participate in the regulation of GPCR signal and the regulation of multiple ligands and drug effects [10]. In recent years, some studies have found that GRK2 is involved in regulating mitochondrial function

[11, 12]. During myocardial ischemia-reperfusion injury, GRK2 is transferred from cytoplasm to mitochondria, thereby promoting oxidative stress response and initiating pro-apoptosis procedures [12]. Meanwhile, GRK2 can promote the accumulation of superoxide and damage mitochondrial function [13]. Currently, little is known about the function of GRK2 in skin flap ischemia-reperfusion injury. Thus, we aimed to investigate the function of GRK2 in skin flap ischemia-reperfusion injury and further explore the underlying mechanism.

Materials and methods

Animal experiment and grouping

Sixty male C57BL/6 mice were randomly divided into four groups: sham, IR+sh-NC, IR+sh-GRK2 and IR+sh-GRK2+Drp1, n = 15 for each group. The mice were anesthetized by intraperitoneal injection of 1% pentobarbital sodium (80 mg/kg), and then fixed to the operating board after they lost consciousness. The abdominal hair was shaved with a razor blade, and the midline of the mice abdomen was used as the axis of symmetry. The protruding part of the sternum from the upper to the groin was designed and labeled as a 4 cm×1 cm island flap. The operation and the surrounding area were sterilized with iodine, and the flap was gradually stripped down along the marked line to separate it from the muscle layer. When the left and right inferior superficial arteries were peeled, the connection between the vessels on both sides and the flap should be ensured. To ensure that only one side of the superficial artery supplied blood to the flap, the inferior superficial artery of the right abdominal wall was cut off by ligation, and the inferior superficial artery of the left abdominal wall was closed by vascular clamp for 3 hours. After the preparation of the flap was completed, to prevent the implantation of new capillaries in the muscle layer, a 0.1 mm thick silicone membrane was inserted between the flap and the abdominal muscle layer, and then the flap was sutured. The sham group was not subjected to ischemia, but ligate the left superficial epigastric artery. Adeno-associated virus 9 (AAV9) vector containing GRK2-shRNA (AAV9-sh-GRK2) or AAV9-Drp1 (6×10^{11} GC) was injected into mice through tail vein for 4 weeks, then mice were suffered from IR surgery. The experiments were finished depended on the protocols and accordance to the National Institutes of Health guidelines. This study was reviewed and approved by the Institutional animal care and use committee of Wuxi No. 9 People's Hospital Affiliated to Soochow University.

TUNLE staining

We used the in situ Cell Death Detection Kit (TUNEL fluorescence FITC kit, Roche, Germany) detect apoptotic level of skin flap. DAPI was used to stain nuclei. IX73 fluorescence microscope (Olympus, Valley, PA) was used to analyze fluorescence staining. The total cells and TUNEL positive cells numbers was counted by Image-J.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from serum and culture medium according to a standard protocol. And then, the purity and concentration of RNA was detected and all the samples were converted into cDNA using reverse transcription kit. We used SYBR Green (Thermo Fisher Scientific) system to perform the qRT-PCR. Primers list: GRK2: Forward primer: 5'-CTTCCAGCCATACATTGAGGAG-3', Reverse primer: 5'-TTCGATGCACACTGAAGTCATT-3', Drp1: Forward primer: 5'-TCCCTAAACTCCATGATGCCATA-3', Reverse primer: 5'-CCACAGGCATCAGCAAAGTC-3', GAPDH: Forward primer: 5'-AGGTCGGAGTCAACGGATTTG-3', Reverse primer: 5'-CCTGGAAGATGGTGATGGGAT-3'.

Western blot analysis

Protein samples were blotted depended on standard protocol. And we used Odyssey Infrared Scanning System (Gene Co. Ltd., Hongkong, China) to scan the membranes. At last, we used Image J software to analyze the western bolt results. The primary antibodies are as list: GRK2 (SAB4500592, Sigma-Aldrich), Drp1 (10656-1-AP, Proteintech) and GADPH (12935-1-AP, Proteintech). The secondary antibodies IRDye700/800 Mouse or Rabbit were produced by LICOR (Lincoln, Nebraska, USA).

Immunohistochemical (IHC) staining

Frozen sections of tumors were fixated in 4% paraformaldehyde and washed using PBS. We penetrated sections using 0.5% Triton X-100.

After 3 times wash, we blocked sections with 50% goat serum. Then, sections were incubated with primary antibody overnight. Then, we incubated the sections using secondary antibody. Immunofluorescence was analyzed under an IX73 fluorescence microscope (Olympus, Valley, PA).

Hematoxylin and eosin (H&E) staining

The skin flap tissues were gathered and fixed in 4% paraformaldehyde for 24 h. Then the fixed tissues were embedded in paraffin. Next, Paraffin slicer machine was used to cut slices (5-mm cross-sectional). Skin flap sections were dewaxed with xylene and treated with ethanol at different concentrations for 5 minutes. Hematoxylin staining for 5 minutes, 5% acetic acid treatment for 1 minute, water rinse. Dye with eosin for 1 minute, rinse with running water. Dehydrate in 70%, 80%, 90%, 100% ethanol for 10 seconds, xylene for 1 minute. Drizzle with neutral gum and seal.

Mitochondrial assays

The mitochondria was isolated from skin (40-50 mg) as described [14]. ATP production was measured with ATP Bioluminescent Assay (Sigma). ATP content was determined by ATP Assay Kit (Beyotime, China). The final ATP content of each sample was normalized to its protein concentration with the BCA protein detection kit (Beyotime, China). Mitochondrial electron transport chain complex activities were calculated using MitoCheck Complex Activity Assay Kit (Cayman chemical, USA). Seahorse XF96 Extracellular Flux Analyzer (Seahorse Bioscience) was used to measure basal and maximum oxygen consumption rate (OCR) and proton leak.

Statistical analysis

All data is presented as a mean ± S.E.M. Statistical analysis was performed using Student's t-test or Wilcoxon test or a one-way ANOVA.

Results

GRK2 was decreased in the skin after IR injury

To evaluate the expression of GRK2 in skin after IR injury, we first established IR model (ischemia for 3 hours and reperfusion for 72 hours) in the mice skin. As shown in **Figure 1A**, the skins in sham group were pink and elastic, while they were brown and inelastic in IR group, indicating a significant decrease of flap survival rate in IR group. As well, TUNEL analysis showed the number of apoptotic positive cells increased significantly in the IR group comparing with sham group (**Figure 1B**). Then, we performed a series of experiments to determine GRK2 expression in skin flap during IR injury. And we found that the mRNA and protein levels of GRK2 was upregulated in IR group (**Figure 1C** and **1D**). Furthermore, IHC data showed IR injury induced GRK2 expression in skin flap (**Figure 1E**). Together, our results suggested that GRK2 was increased in skin flap after IR injury.

Knockdown of GRK2 enhanced skin function and cell proliferation after IR injury

To identify the function of GRK2 in the skin after IR injury, we injected adeno-associated virus 9 (AAV9) vector containing GRK2-shRNA (AAV9-sh-GRK2) into mice undergoing IR operation through tail vein. And the qRT-PCR analysis showed a significant decrease in the skin injected with AAV9-sh-GRK2 (**Figure 2A**). Then functional experiments showed that loss of GRK2 improved the color and elasticity, which showed an enhancive flap survival rate (**Figure 2B**). TUNEL staining showed sh-GRK2 reduced the number of apoptotic positive cells after IR operation (**Figure 2C**). H&E staining indicated that injection of sh-GRK2 suppressed the number of inflammatory cells after IR injury (**Figure 2D**). Then, inflammation cytokines were determined, and the data indicated that sh-GRK2 inhibited the expression of TNFα, IL 6 and IL 1β (**Figure 2E**). Proliferating cell nuclear antigen (PCNA) is a key marker for proliferation, and we evaluated cell proliferation in flap by detecting PCNA expression. IHC staining showed that sh-GRK2 promoted PCNA level (**Figure 2F**). And deletion of GRK2 accelerated Ki67 expression comparing with NC group upon IR injury (**Figure 2G**). These data indicated that loss of GRK2 improved skin flap function and cell proliferation after IR injury.

Loss of GRK2 increased the number of hair follicles after IR injury

Hair follicles are one of the most important skin appendages and play an important role in

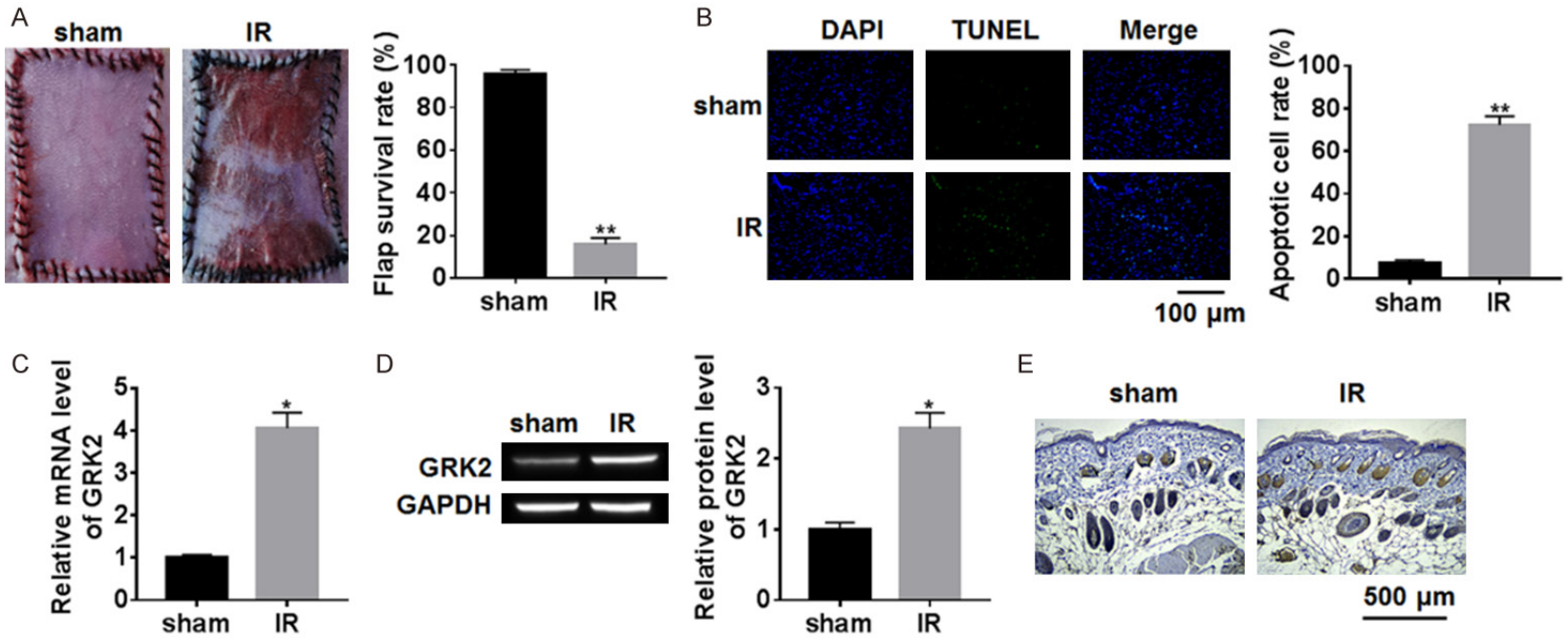

Figure 1. The expression of GRK2 in skin flap after IR injury. IR model (ischemia for 3 hours and reperfusion for 72 hours) was established in the abdominal skin of mice. A. Representative photographs of abdominal skin flap, and the survival rate of the total flap area was calculated. B. TUNEL staining was used to determine apoptosis level. Bar = 100 μm. C. qRT-PCR assay analyzed the expression of GRK2 in skin flaps. D. Western blot detected the protein expression of GRK2 in skin flaps. E. IHC staining for GRK2 in skin flaps. Bar = 500 μm. n = 10, *P<0.05, **P<0.01 vs sham. Data among multiple groups were analyzed by one-way ANOVA, followed by a Tukey post hoc test. The experiment was repeated in triplicate.

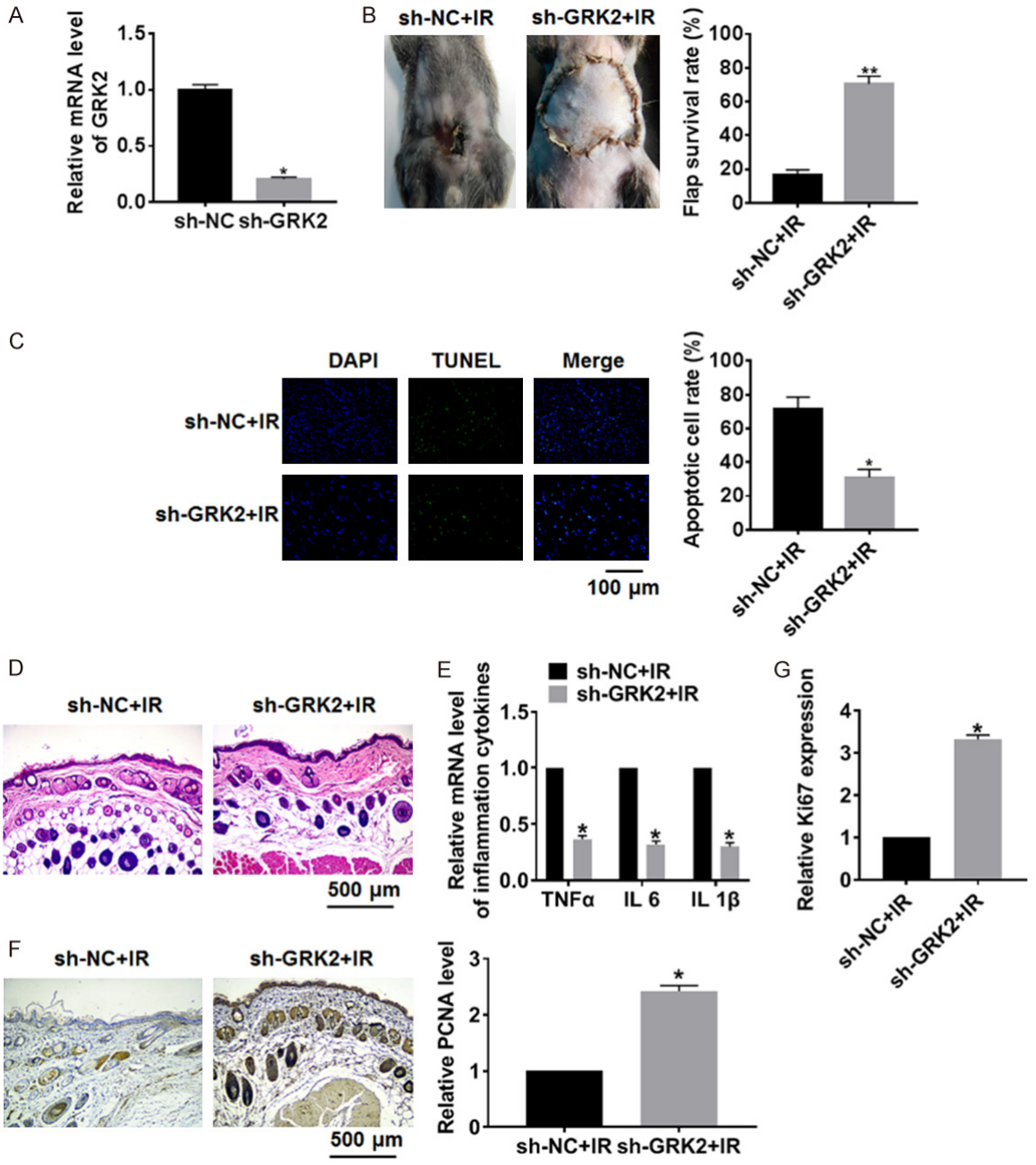

Figure 2. Knockdown of GRK2 promoted skin function and cell proliferation after IR injury. AAV9-sh-GRK2 or sh-NC was injected into mice through tail vein, then mice were suffered from IR operation after 4 weeks. A. qRT-PCR analysis for GRK2 expression. B. Representative photographs of skin flaps and the survival rate of the total flap area. C. TUNEL for skin flaps to detect apoptotic level. Bar = 100 μm. D. H&E staining was used to determine inflammation level. Bar = 500 μm. E. qRT-PCR analysis for inflammation cytokines TNFα, IL 6 and IL 1β. F. IHC staining for PCNA to determine cell proliferation. Bar = 500 μm. G. The mRNA level of Ki67 was detected using qRT-PCR. n = 10, *P<0.05, **P<0.01 vs sh-GRK2+IR. Data among multiple groups were analyzed by one-way ANOVA, followed by a Tukey post hoc test. The experiment was repeated in triplicate.

wound healing, and we evaluated the effect of sh-GRK2 on hair follicles after IR operation. The H&E images showed that the number of hair follicles was increased in sh-GRK2 group than that in sh-NC group after IR injury (**Figure 3A-C**).

Deficiency of GRK2 increased mitochondrial function

Considering the role of GRK2 in modulating mitochondrial function, we measured mitochondrial function in skin after IR injury. Comparing

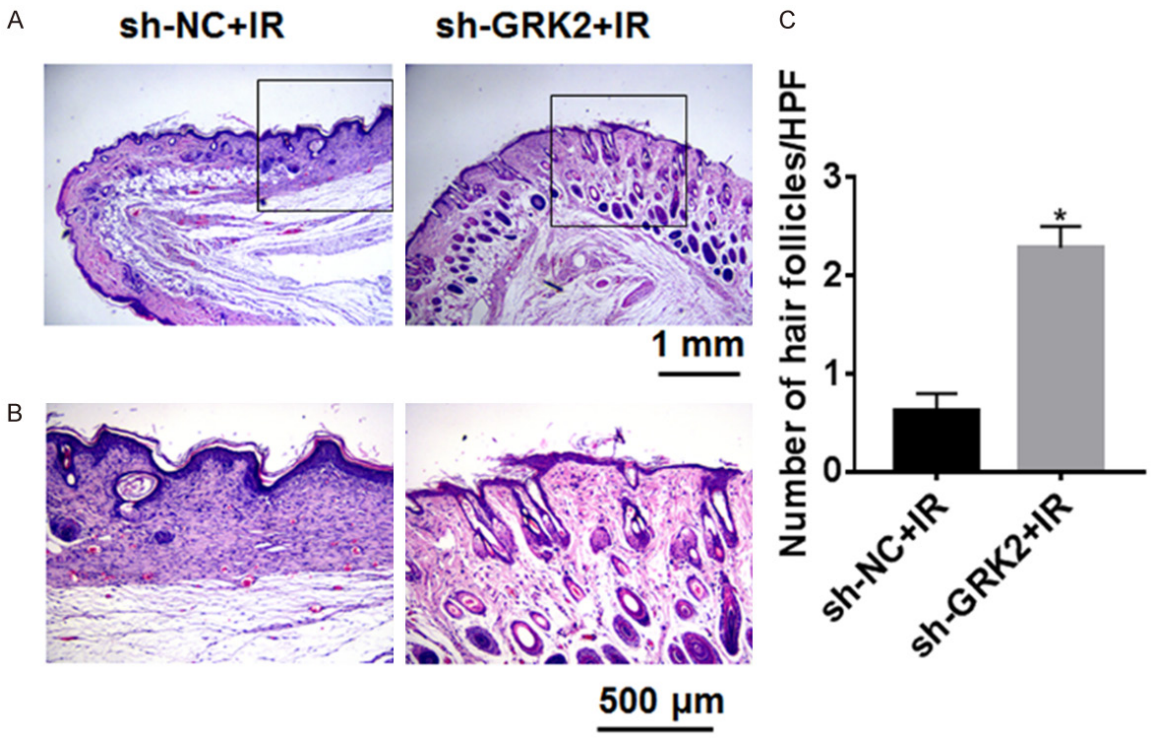

Figure 3. Knockdown of GRK2 increased the number of hair follicles after I/R operation. (A) Sections were stained with H&E staining. Bar = 1 mm. (B) Higher magnification of the area inside the rectangle in (A) is shown. Bar = 500 μm. (C) Statistical analysis of hair follicles under HPF. n = 6 for each group. n = 10, *P<0.05 vs sh-GRK2+IR. Data among multiple groups were analyzed by one-way ANOVA, followed by a Tukey post hoc test. The experiment was repeated in triplicate.

with sh-NC group, mitochondrial ATP production and ATP content were both increased in sh-GRK2 group upon IR operation (**Figure 4A** and **4B**). Knock-down of GRK2 promoted the activities of mitochondrial complexes I, II+III, and IV in comparison with sham (**Figure 4C-E**). Furthermore, the basal oxygen consumption rate (OCR) and the maximum OCR were increased in sh-GRK2 group upon IR operation than that in sham (**Figure 4F** and **4G**). As well, loss of GRK2 promoted the level of proton leak (**Figure 4H**). Taken together, these data revealed that deficiency of GRK2 promoted mitochondrial function in skin after IR injury.

Silencing of GRK2 suppressed Drp1 expression in skin after IR injury

Drp1 is a GTPase that regulates mitochondrial fission, and plays key role in mitochondrial function. And we found that the mRNA and protein level of Drp1 were induced in in skin upon IR stress, which was reversed after silencing of GRK2 (**Figure 5A** and **5B**). In addition, IHC staining showed IR injury up-regulated Drp1 expression in flap, while sh-GRK2 down-regulated Drp1 expression (**Figure 5C**).

Inhibition of GRK2 improved skin function and mitochondrial function by inhibiting Drp1

To investigate whether Drp1 was involved in GRK2 regulating skin function and mitochondrial function, we constructed AAV9-Drp1 and injected into mice. As shown in **Figure 6A**, injection of AAV9-Drp1 significantly promoted Drp1 expression in flap. Followed functional experiments showed that overexpression of Drp1 removed the protective effect of sh-GRK2. Comparing with sh-GRK2 group, Drp1 inhibited flap survival rate, promoted apoptosis level, suppressed cell proliferation and induced inflammation in flap upon IR stress (**Figure 6B-E**). As well, forced expression of Drp1 inhibited the number of hair follicles (**Figure 6F**). Moreover, sh-GRK2 improved mitochondrial function, which was reversed by Drp1, showing lower level of ATP production and basal OCR (**Figure 6G** and **6H**). Moreover, overexpressing Drp1 alone also aggravated IR-induced flap

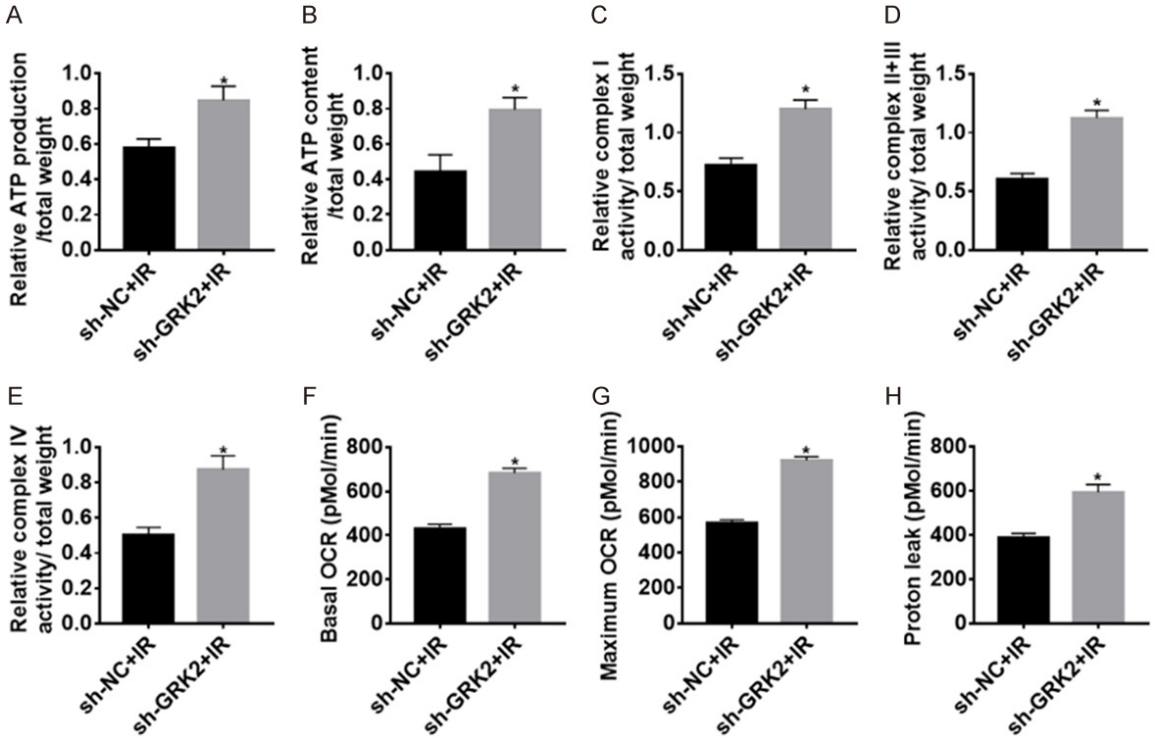

Figure 4. Mitochondrial function in skin flaps after I/R operation. A. Relative mitochondrial ATP production in skin flaps. B. Relative mitochondrial ATP content in skin flaps. C. Relative respiratory chain complex I activity in mitochondria. D. Relative respiratory chain complex II+III activity in mitochondria. E. Relative respiratory chain complex IV activity in mitochondria. The mitochondrial fraction prepared was subjected to Seahorse analyses. F. Basal respiration. G, H. Maximal respiration. OCR indicates oxygen consumption rate. n = 10, *P<0.05 vs sh-GRK2+IR. Data among multiple groups were analyzed by one-way ANOVA, followed by a Tukey post hoc test. The experiment was repeated in triplicate.

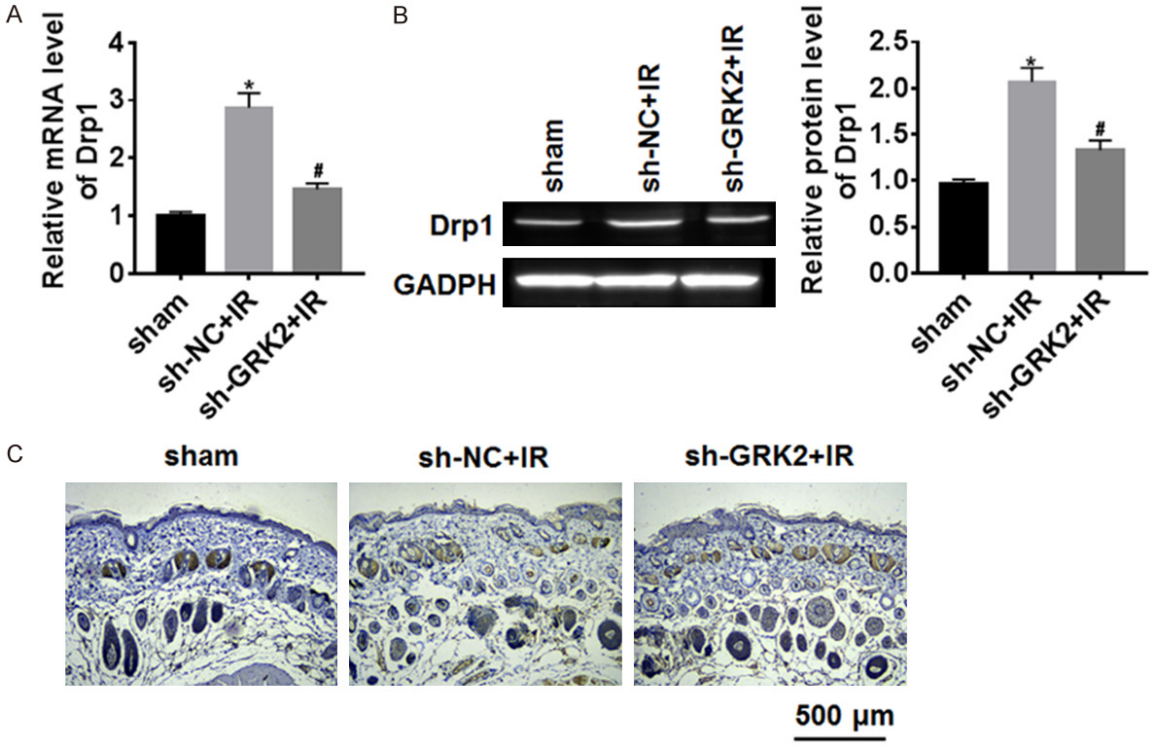

Figure 5. Knockdown of GRK2 inhibited Drp1 expression in skin flap after I/R operation. A. qRT-PCR assay analyzed the expression of Drp1 in skin flaps. B. Western blot detected the protein expression of Drp1 in skin flaps. C. IHC staining for Drp1 in skin flaps. Bar = 500 μm. n = 10, *P<0.05 vs sham, #P<0.05 vs sh-GRK2+IR. Data among multiple groups were analyzed by one-way ANOVA, followed by a Tukey post hoc test. The experiment was repeated in triplicate.

injury (Figure S1). These results showed that sh-GRK2 improved skin function and mitochondrial function by inhibiting Drp1 expression.

Discussion

Ischemia-reperfusion is one of the main factors leading to the necrosis of skin flap after transplantation [15]. The ischemia time of the flap is closely related to the reperfusion injury [16]. Therefore, surgeons are required to improve their own operation techniques and shorten the operation time. In addition, it is more important to actively seek drugs and other treatment methods to maintain the activity and function of skin cells and reduce the occurrence of ischemia-reperfusion injury of skin flap.

GRK2 is widely distributed in various tissues and organs, and it regulates the body's pathophysiological processes by phosphorylating related signaling pathways involved in GPCRs and non-GPCRs pathways [17]. GRK2 is closely related to the functions of multiple organs such as heart, liver, kidney and lung [18]. It has been reported that GRK2 plays a key role in IR injury of heart, and deletion of GRK2 enhanced cardiac function [19]. And in present study, we constructed an IR model of mice abdominal flap. In IR group, the skin flaps were brown and inelastic, and had a high apoptotic level. And the Comparing with sham group, GRK2 was increased in flap after IR injury. To explore the role of GRK2 in skin flap after IR injury, we injected AAV9-sh-GRK2 to inhibit GRK2 expression in mice. A normal proliferative ability of skin cell is necessary for repair after IR injury [20]. Further, hair follicles are appendages of skin, which reflect the secretory function of the skin [21]. And present study found that loss of GRK2 inhibited cell apoptosis, promoted cell proliferation of flap and increased the number of hair follicles after IR injury.

At present, it is believed that the resumption of blood supply of tissues and organs after a long period of ischemia can lead to the outbreak of ROS, the change of mitochondrial function, and eventually leads to tissue necrosis [22]. Excessive ROS in tissues will enhance lipid peroxidation and change the lipid microenvironment of ion channels, cell membrane receptors and proteases, thus damaging cell functions [23]. Oxygen free radicals can also cause vasoconstriction, increased capillary permeability, swelling in the endothelial cell, and damaged skin flap microcirculation process, causing necrosis of the organization [24]. As the main place of energy metabolism in eukaryotes, mitochondria play an important role in ROS production, further mitochondria are also key target organelles for oxidative stress [25]. Interestingly, our study showed that deletion of GRK2 improved mitochondria function in skin flaps after IR injury.

Drp1 is the main protein that mediates mitochondrial division [26]. The fusion and fission of mitochondria are the basic events in the cell, and maintaining the dynamic balance between them is very important for the normal morphology [27]. In recent years, it has been found that IR leads to the change of mitochondrial dynamics from fusion to division in heart, and the inhibition of excessive mitochondrial division is beneficial to the resistance to IR injury in heart [28]. Numerous studies indicate the mitochondrial division mediated by Drp1 is involved in IR injury [29]. IR disordered muscle fiber structure and increased mitochondrial fragmentation, while silencing of Drp1 significantly reduced the myocardial infarction area and improved cardiac function [30]. And our study showed that IR injury up-regulated Drp1 expression in skin flaps, while sh-GRK2 down-regulated Drp1 expression. Furthermore, overexpression of Drp1 removed the protective effect of sh-GRK2, which indicated that deletion of GRK2 improved skin flap function through inhibiting Drp1 in ischemia-reperfusion injury.

Conclusion

In conclusion, our study revealed that GRK2 deletion improved flap function and mitochondrial function by inhibiting Drp1 expression, which may provide a new insight for the clinical treatment of flap ischemia-reperfusion injury.

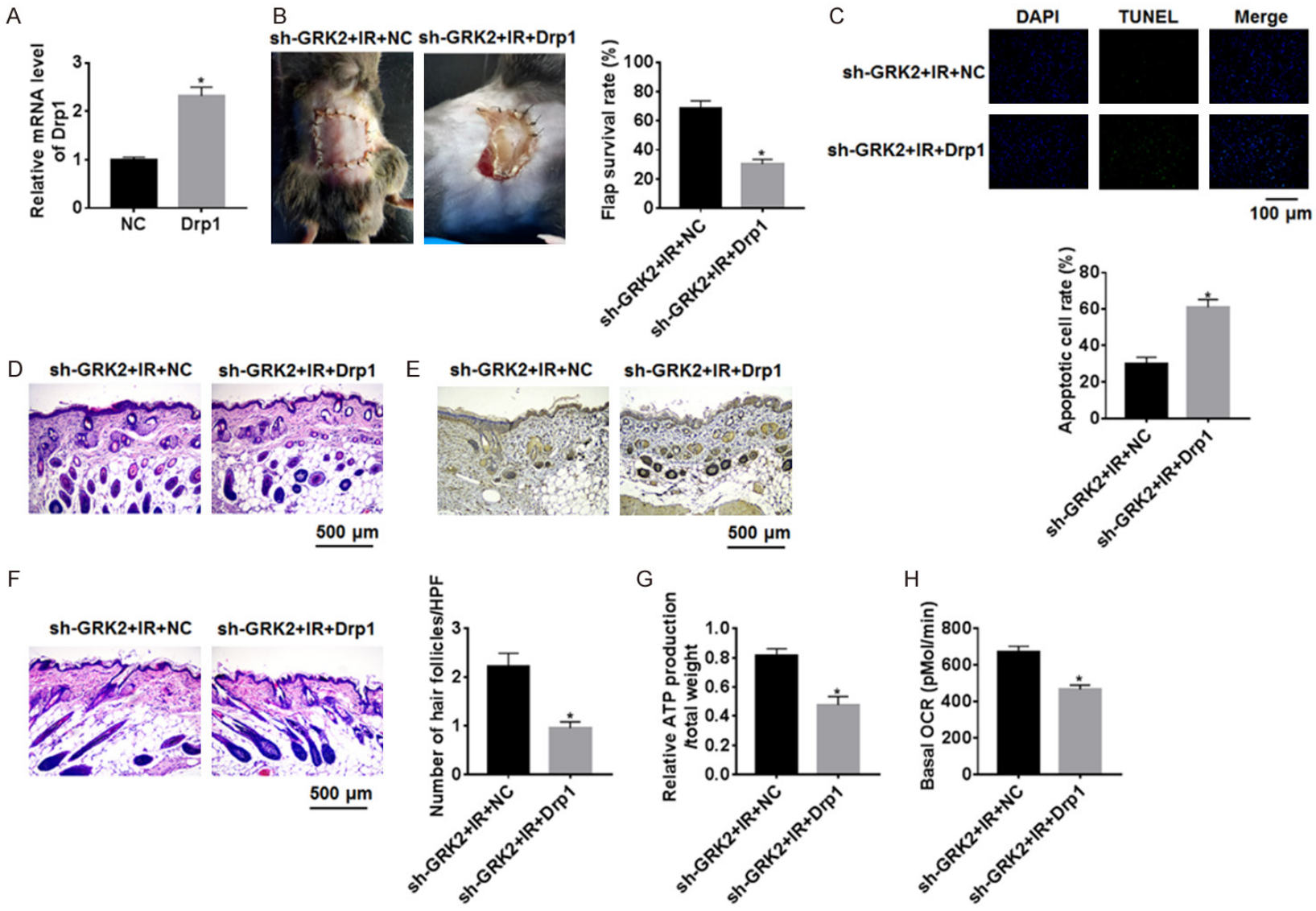

Figure 6. Overexpression of Drp1 reversed the protective effect of sh-GRK2 in skin flaps after I/R operation. AAV9-sh-GRK2 and AAV9-Drp1or AAV-NC was injected into mice through tail vein, then mice were suffered from IR operation after 4 weeks. A. qRT-PCR for Drp1 expression. B. Representative photographs of skin flaps and the survival rate of the total flap area. C. TUNEL for skin flaps. Bar = 100 μm. D. H&E staining for inflammatory cells. Bar = 500 μm. E. IHC staining for PCNA. Bar = 500 μm. F. H&E staining for hair follicles under HPF. Bar = 500 μm. G. Relative mitochondrial ATP production in skin flaps. H. Basal OCR of skin flaps. n = 10, *P<0.05 vs sh-GRK2+IR+NC. Data among multiple groups were analyzed by one-way ANOVA, followed by a Tukey post hoc test. The experiment was repeated in triplicate.

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Disclosure of conflict of interest

None.

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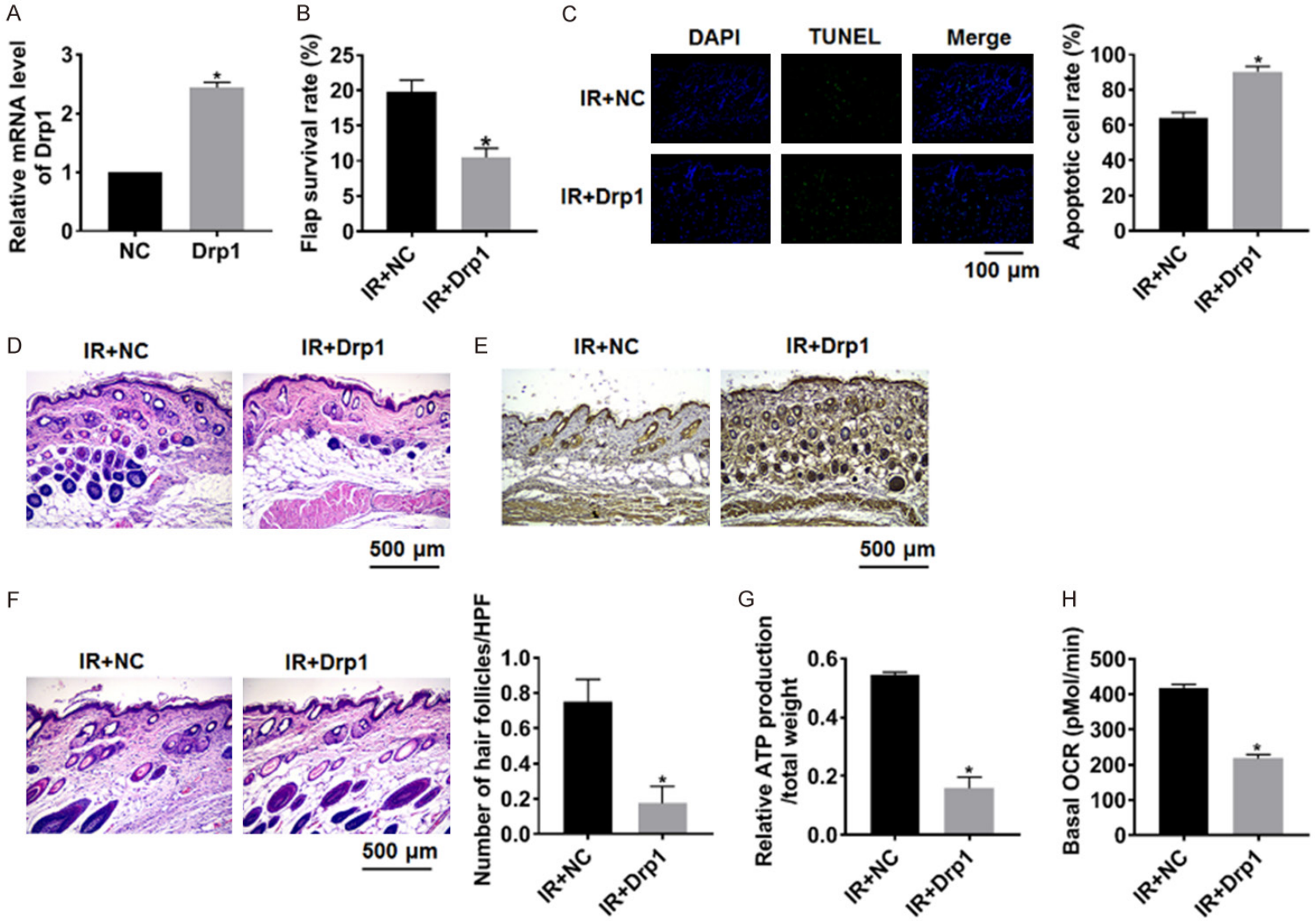

Figure S1. Overexpression of Drp1 aggravated IR-induced injury of skin flaps. AAV9-Drp1 or AAV-NC was injected into mice through tail vein, then mice were suffered from IR operation after 4 weeks. A. qRT-PCR for Drp1 expression. B. The survival rate of the total flap area. C. TUNEL for skin flaps. Bar = 100 μm. D. H&E staining for inflammatory cells. Bar = 100 μm. E. IHC staining for PCNA. Bar = 100 μm. F. H&E staining for hair follicles under HPF. Bar = 500 μm. G. Relative mitochondrial ATP production in skin flaps. H. Basal OCR of skin flaps. n = 10, *P<0.05 vs sh-GRK2+IR+NC. Data among multiple groups were analyzed by one-way ANOVA, followed by a Tukey post hoc test. The experiment was repeated in triplicate.