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Original Article

Overexpression of sortilin promotes pro-nerve growth factor-induced apoptosis in keloid fibroblasts

Gaosong Yang[1,2*], Xia Li[1,3*], Yongqian Cao[1], Liankui Zhao[2], Yibing Wang[1]

[1]Department of Burn and Plastic Surgery, Provincial Hospital Affiliated to Shandong University, Jinan, China; [2]Department of Burn and Plastic Surgery, The First Hospital of Shijiazhuang City, Shijiazhuang, China; [3]Department of Burn and Plastic Surgery, Weihai Municipal Hospital, Weihai, China. [*]Equal contributors.

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Abstract: Keloids are scars characterized by pathologically excessive dermal fibrosis and aberrant wound healing. Keloid fibroblasts (KFs) persistently proliferate and fail to undergo apoptosis, no treatment is completely effective against these lesions. Sortilin was found involved in pro-nerve growth factor (proNGF)-induced apoptosis of rat vascular smooth muscle cells. However, the role of sortilin in dermal fibrosis keloids is still unclear. Therefore, in this study, we investigated the effects of sortilin on KFs and proNGF-induced KFs apoptosis and explored the underlying mechanism. We found that sortilin was decreased in KFs, overexpression of sortilin significantly promoted proNGF-induced mitochondria-mediated internal apoptosis of KFs. In addition, sortilin obviously inhibited the activation of PI3K/Akt/NF-κB signaling pathway but increased bax expression. Taken together, our study suggested that sortilin potentiated proNGF-induced apoptosis in KFs through increasing the expression of bax and suppressing the activation of the PI3K/Akt/NF-κB signaling pathway, and sortilin is a potential target for treatment of the keloid disease.

Keywords: Sortilin, keloid fibroblasts (KFs), proNGF, apoptosis, bax, PI3K/Akt/NF-κB signaling pathway

Introduction

Wound healing is a complicated process includes three overlapping phases, inflammation, granulation and remodeling [1]. Any derailment in the process would lead to a variety of ailments, including chronic wound healing, hypertrophic scar and keloid [2-4].

keloids are pathologic proliferations of the dermal layer of the skin, resulting from an abnormal wound healing response and the excessive synthesis and deposition of extracellular matrix components. However, the treatment of keloid scars is extremely difficult because of the high recurrence of keloids after surgery, which spread beyond the original keloid margin. Therefore, although many strategies are available for the treatment of keloid scars, none is fully effective [5-7]. An imbalance between cell proliferation and apoptosis has been proposed as a possible mechanism for keloid formation [8-10].

Nerve growth factor (NGF) and its precursor (proNGF) are primarily considered as regulators of neuronal function [11]. NGF regulates cell survival and cell death via binding to two different receptors, TrkA and $p75^{NTR}$ [12]. In contrast, proNGF selectively induces apoptosis through $p75^{NTR}$ and sortilin but not TrkA [13, 14]. Sortilin, a member of the Vps10p-domain receptor family, acts as a co-receptor and molecular switch governing the $p75^{NTR}$-mediated pro-apoptotic signal induced by proNGF and was widely studied in the neuronal cells [14]. In addition, some experimental data indicated that sortilin, acted as an endocytic and intracellular sorting receptor, contributed to the targeting of ligands to lysosomes and the sorting between the Golgi apparatus and endosomes [15-17]. Recent study also showed that sortilin was involved in pro-nerve growth factor (proNGF)-induced apoptosis of rat vascular smooth muscle cells [18]. However, researches on the roles of sortilin and proNGF-induced apoptosis in the keloid fibroblasts (KFs) were rarely reported.

In this study, we found that sortilin was expressed at a low level in keloid tissues. In addition, we investigated the effects of sortilin on proNGF-induced apoptosis in KFs, and explored the underlying mechanism.

Material and methods

Keloid-derived and normal dermal fibroblast origin and cell culture

Six keloid tissue samples used in this study were obtained from six Chinese patients (three males and three females with age range from 20 to 42 years with a mean age of 31.8 years) after undergoing surgical excision. Normal skin tissue samples were obtained from six different Chinese volunteers (three males and three males with age range from 23 to 44 years with a mean age of 32.3 years). All experiments were performed after obtaining approval of the ethics committee of the Shandong Provincial Hospital, and in accordance with the Declaration of Helsinki Principles. Written informed consent was obtained from each individual. After specimen collection, the KFs were isolated and cultured as described [19]. The fibroblasts were grown to confluence and then to a maximum passage of three prior to further experiments.

RNA analysis

KFs were collected in cell lysis buffer. Total RNA was extracted with TRIzol reagent according to the manufacturer's instructions (Invitrogen). A LightCycler (ABI PRISM 7000; Applied Biosciences) and a SYBR RT-PCR kit (Takara Biotechnology.) were used for real time PCR analysis. RT-PCR and real time PCR were performed using the following primer sequences: Sortilin, forward were 5'-GGGGACCAAACAACATCATC-3' and reverse 5'-AAGGGCTCATGACCA CAGTC-3'; $p75^{NTR}$, forward 5'-TCGCCAGTGGACACTAACAG-3' and reverse 5'-AGGAGAGAACCTGCTGGTGA-3'; Actin, forward 5'-GGAAATCGTGCGTGACATT-AA-3' and reverse 5'-AGGAAGGAAGGCTGGAA-GAG-3'.

Western blot analysis

The protein in cell lysis was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by transference to a PVDF membrane (Millipore). The membrane was then blocked with 2.5% nonfat dry milk for 1 h. Primary antibodies (anti-Sortilin, anti-Bax, anti-Bcl2, anti-cleaved caspase3, anti-PARP, anti-cyt c, anti-p65, anti-$p75^{NTR}$, anti-p-PI3K, anti-PI3K, anti-Akt, anti-p-Akt, anti-Actin) (Santa Cruz Biotechnology) were added and incubated overnight at 4°C. After incubation with the corresponding horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology), the target protein was visualized by enhanced chemiluminescence (Thermo Fisher Scientific).

Caspase assay

The caspase-3 activity of cell extracts (~10^6 cells) was determined using the synthetic substrate, acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl coumarin (DEVD-AMC; Peptron), according to the manufacturer's instructions. The AMC fluorescence released by active caspase-3 was measured at an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

Analysis of apoptosis with flow cytometry

Keloid fibroblasts (KFs) were collected and suspended in 100 μL binding buffer. Five microliters of Annexin V-PE and 5 μL of 7-amino-actinomycin D (7-AAD) were added to each sample and the mixture was incubated in the dark at room temperature for 15 min. Apoptotic cells stained positive for Annexin V-PE.

Mitochondrial and cytosolic fractionation

The Cell Mitochondria Isolation kit (Beyotime Institute of Biotechnology, Haimen, China) was used to perform the isolation of the mitochondria and cytosol, according to the manufacturer's instructions. Samples of cytosol and mitochondria were dissolved in lysis buffer, and proteins were subjected to western blotting, respectively.

Lentivirus preparation and infection

Lentivirus containing empty plasmids or expression plasmids of sortilin were produced using pWPXL, psPAX2 and pMD2.G plasmids (addgene) with a proportion of 20:15:7 in HEK293T cells, 3 days later the culture was harvested and enriched by PEG8000. The enriched lentivirus particle (MOI, 50) was used to infect KFs for 4 days in the presence of polybrene (4 μg/mL) then subjected to puromycin (1 ug/mL) selection to establish stable overexpression cell lines.

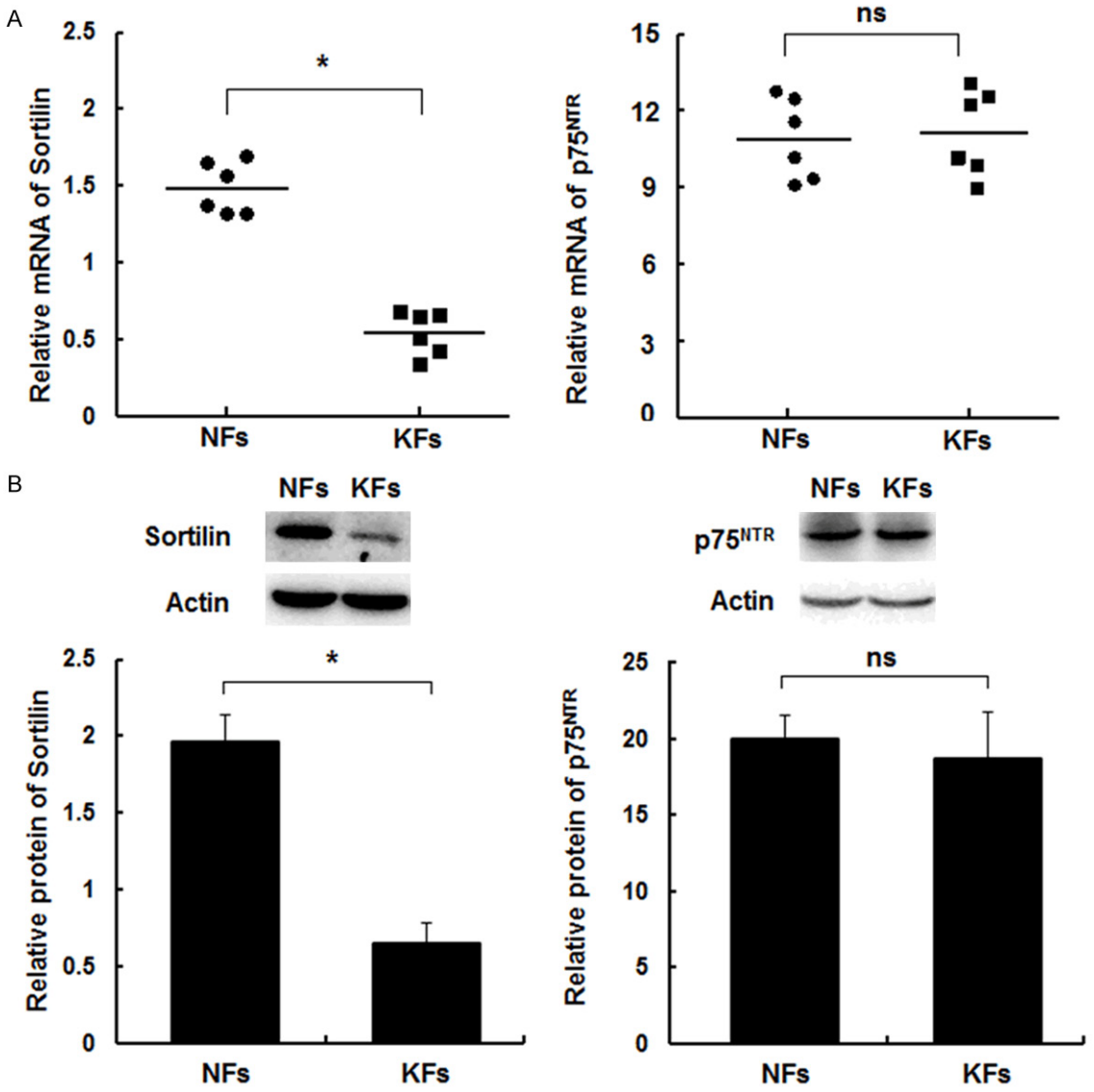

Figure 1. Expression of sortilin and p75^{NTR} in keloid fibroblasts. A. RNA was harvested from six keloid tissue samples and six normal skin tissue samples, sortilin expression and p75^{NTR} expression were assessed by qRT-PCR. B. Western blots of sortilin and p75^{NTR} in KFs and NFs. The levels of proteins were normalized based on the Actin levels. Data are shown as mean ± S.D. of three independent experiments. *, P<0.05. ns, not significant.

Statistical analysis

All data are presented as mean ± SD of three or four experiments. Statistical significance was determined with the two-tailed Student t test, with a *P* value <0.05 considered statistically significant.

Results

Sortilin expression in keloid fibroblasts (KFs)

To verify the expression of sortilin in KFs, we investigated the expression of sortilin in normal skin fibroblasts (NFs) and KFs. As shown in **Figure 1A**, the mRNA expression levels of sortilin in KFs was decreased significantly compared to that of controls (P<0.05). Consistent with the results of qRT-PCR, western blot analysis showed that sortilin protein expression was also downregulated in KFs, as compared with the control group (**Figure 1B**). Both **Figure 1A** and **1B** showed that there was no significant difference in the expression of p75^{NTR} between KFs and NFs. These data implied that sortilin expression was inhibited in KFs progression while p75^{NTR} expression was not affected.

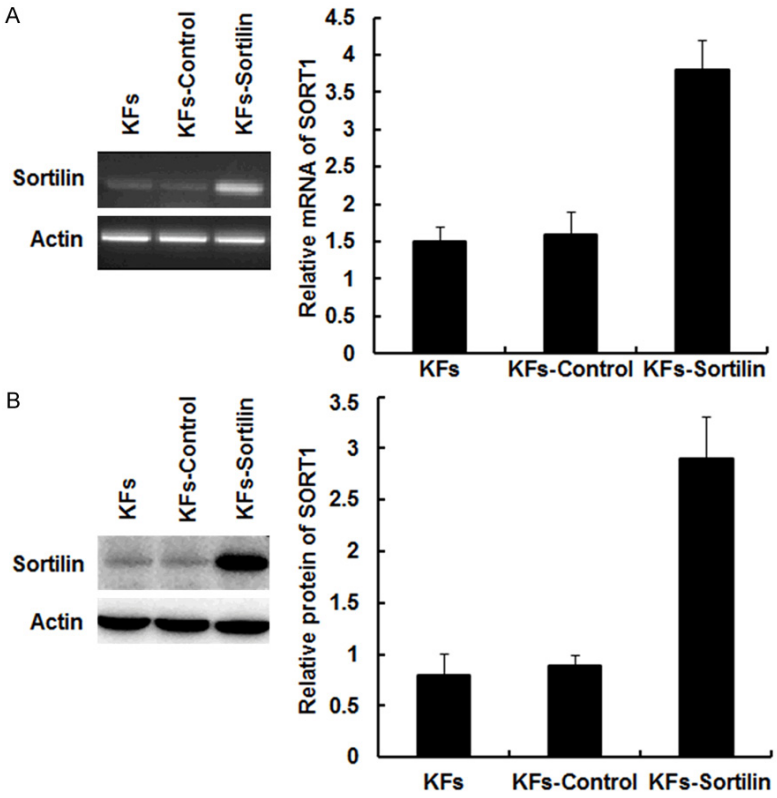

Figure 2. Stable overexpression of sortilin in KFs. (A and B) Lentivirus carried sortilin overexpression plasmid or control plasmid were constructed and transfected into KFs to build stable overexpression cell lines. The mRNA level (A) and protein level (B) of sortilin were measured. Data are shown as mean ± S.D. of three independent experiments.

Stable overexpression of sortilin in KFs by using lentivirus

We constructed recombinant lentivirus containing overexpression plasmid of sortilin for the further study. The stable expression of sortilin in KFs was analyzed. As shown in **Figure 2A**, sortilin mRNA expression was significantly increased after transfection of recombinant lentivirus expressing sortilin. Consistent with the results of RT-PCR, western blot analysis showed that sortilin protein expression was also increased by lentivirus compared to the control groups (**Figure 2B**). We named the cell line with sortilin overexpression for 'KFs-sortilin', and 'KFs-control' for the cell line which was transfected with lentivirus contained empty vector, 'KFs' as a negative control that not infected with lentivirus. These cell lines were using for the further studies.

Sortilin promotes proNGF-induced apoptosis in KFs

We then investigated the pathophysiological significance and its underlying mechanisms of the down-regulated sortilin in KFs. ProNGF was reported as a potent inducer of intimal smooth muscle cell apoptosis and sortilin expression was essential for this process [18], we suspected that sortilin contributed to the similar function in KFs. Indeed, as shown in flow cytometry results, overexpression sortilin promoted proNGF-induced apoptosis in KFs compared with the control groups (**Figure 3A** and **3B**).

Sortilin potentiates apoptosis mainly through the mitochondria-mediated pathway

Downstream of the apoptosis signaling pathways, there is significant cleavage activation of caspase-3 and poly (ADP-ribose) polymerase (PARP) (**Figure 4A** and **4B**). A significant release of cytochrome c from the mitochondria to the cytosol was observed in KFs-sortilin after the cells were treated with proNGF (**Figure 4C**). These results indicated that sortilin potentiated proNGF-induced apoptosis mainly through the mitochondria-mediated internal pathway in KFs.

Different regulation between PI3K/Akt/NF-κB signaling pathway and bax expression by sortilin

Bax and bcl-2 were essential for mitochondria-mediated internal apoptosis pathway, thus we investigated the expression of bax and bcl-2 in KFs after the cells were treated with proNGF, and the expression of bax was significantly increased while bcl-2 expression was not affected (**Figure 5**). ProNGF also reduced NF-κB

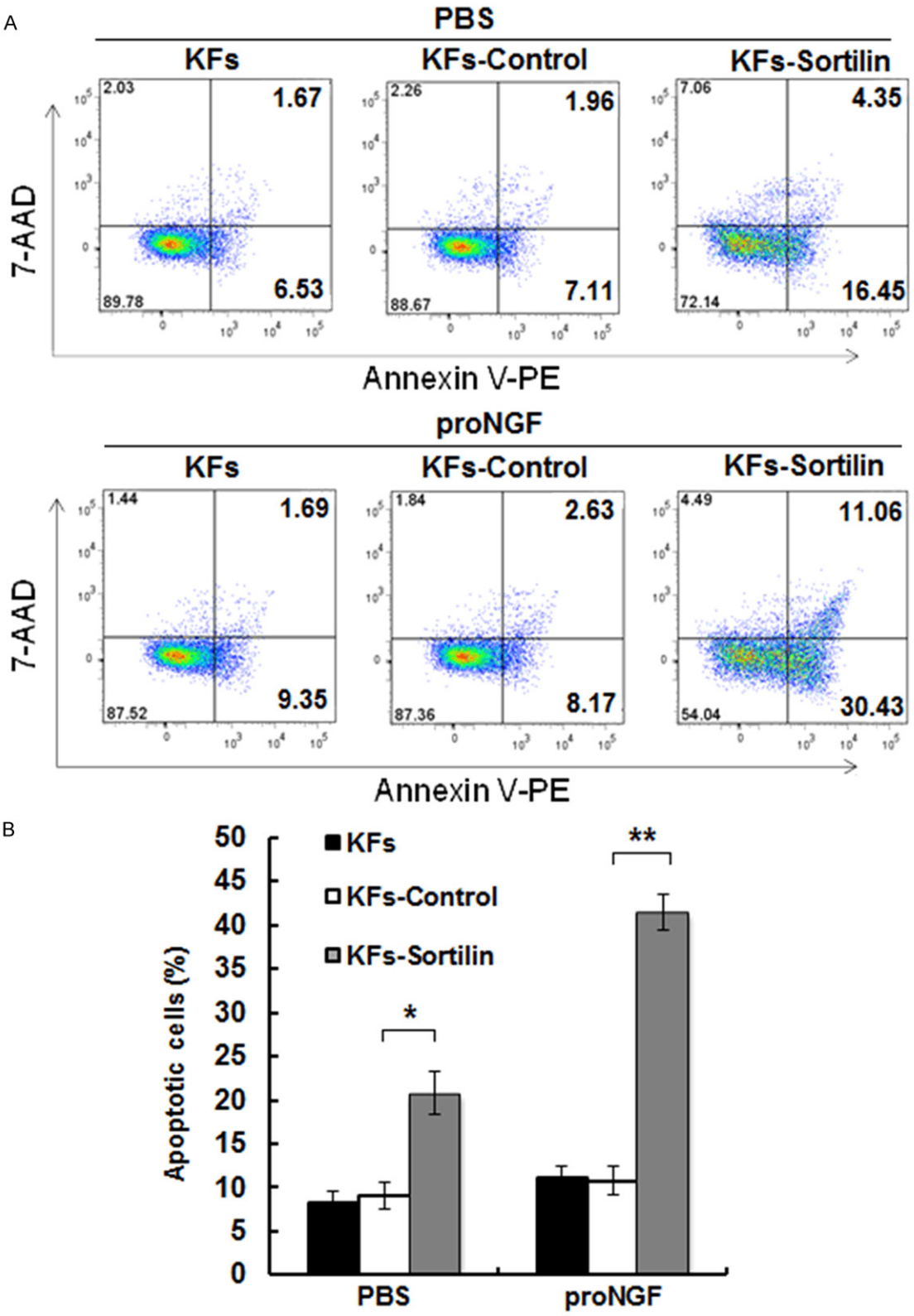

Figure 3. Sortilin promotes proNGF-induced apoptosis in KFs. A and B. KFs overexpression of sortilin or control KFs were treated with PBS or proNGF (5 ng/ml) for 18 h and stained with Annexin V and 7-AAD. The percentage of apoptotic cells was measured by flow cytometry analysis. The results are shown as the mean ± S.D. of three independent experiments. *, P<0.05; **, P<0.01.

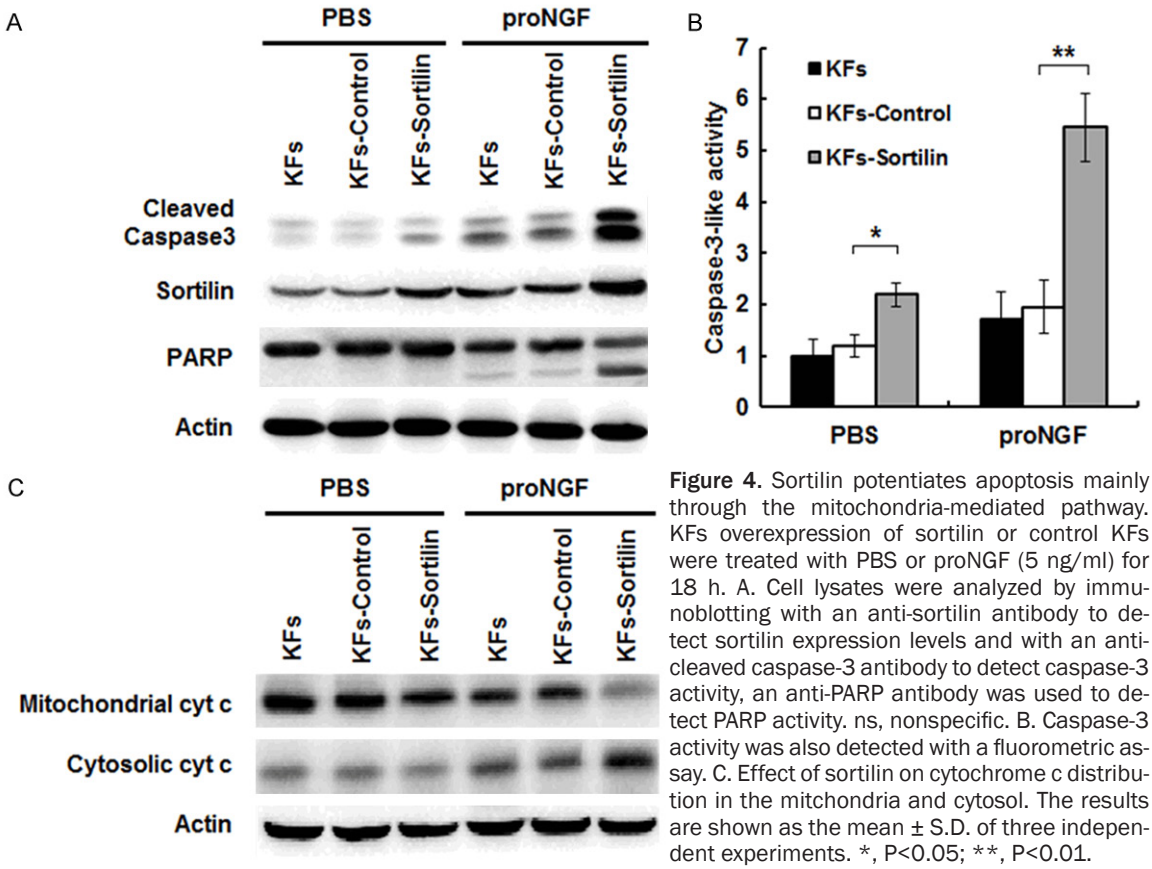

Figure 4. Sortilin potentiates apoptosis mainly through the mitochondria-mediated pathway. KFs overexpression of sortilin or control KFs were treated with PBS or proNGF (5 ng/ml) for 18 h. A. Cell lysates were analyzed by immunoblotting with an anti-sortilin antibody to detect sortilin expression levels and with an anti-cleaved caspase-3 antibody to detect caspase-3 activity, an anti-PARP antibody was used to detect PARP activity. ns, nonspecific. B. Caspase-3 activity was also detected with a fluorometric assay. C. Effect of sortilin on cytochrome c distribution in the mitchondria and cytosol. The results are shown as the mean ± S.D. of three independent experiments. *, P<0.05; **, P<0.01.

activity in IT cells [18], so we measured the protein level of p65 subunit of NF-κB, indeed, the expression of p65 was decreased (**Figure 5**). We further measured PI3K, Akt expression and their phosphorylation level, interestingly, the expression of PI3K and Akt were not affected but their phosphorylation level were significantly decreased in KFs overexpression of sortilin after treated with proNGF, compared with the control groups (**Figure 5**). Taken together, these results indicated that overexpression of sortilin increased bax expression which may contribute to KFs apoptosis, meanwhile, it can decrease p65 expression and suppress the activation of PI3K/Akt signaling pathway to inhibit the survival of KFs.

Discussion

To the best of our knowledge, this study is the first to demonstrate the critical role of sortilin in keloid fibroblasts. We found that the mRNA and protein of sortilin were significantly down-regulated in keloid tissue samples compared to the normal tissue samples. By using stable overexpression KFs of sortilin, we observed that overexpression of sortilin significantly promoted proNGF induced KFs apoptosis which may mainly through mitochondria-mediated internal pathway. We also found that overexpression of sortilin increased bax expression, but the expression of p65 was decreased and the activation of PI3K/Akt signaling pathway was inhibited.

Sortilin is widely expressed in neuronal and non-neuronal cells, and its expression is controlled by a variety of conditions [20, 21]. Treatment of Huh7 cells with Q3G reduced the intracellular level of sortilin [22]. Campagnolo et al. showed that sortilin expression is increased in rat aortic intimal thickening early after injury [18]. Sortilin plays diverse functions in multiple signaling pathways. It was well illustrated as an essential co-receptor for $p75^{NTR}$ that is required for proNGF to engage $p75^{NTR}$ with high affinity and transmit the apoptotic signal in neuronal cells [14, 23, 24]. Moreover, recent evidence suggests an important role for sortilin in facilitating anterograde Trk transport

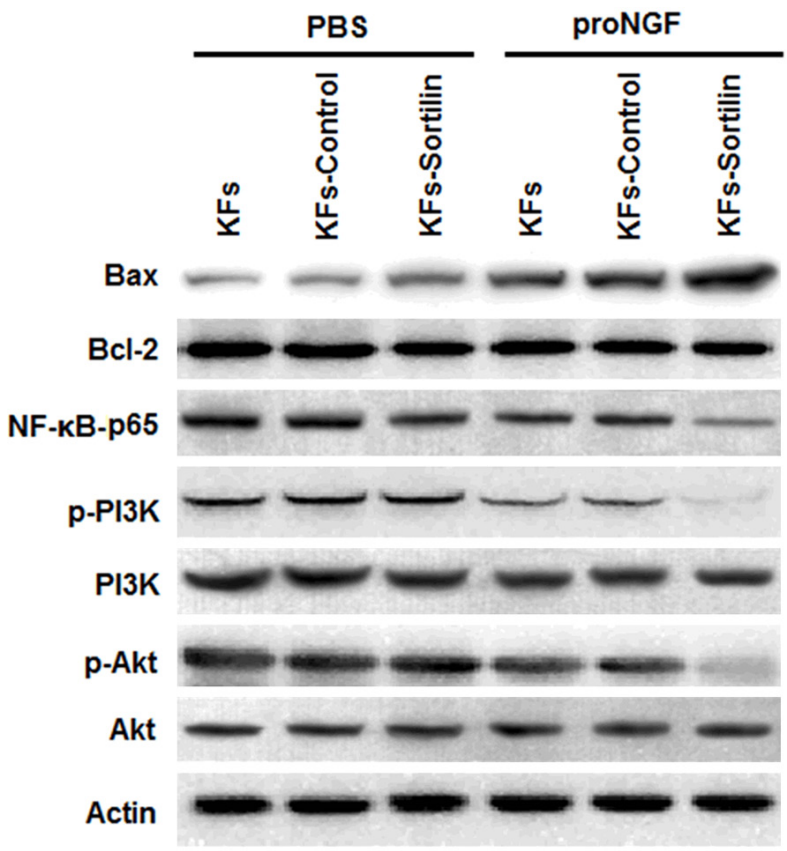

Figure 5. Sortilin increased bax expression and inhibited the activation of PI3K/Akt/NF-κB signaling pathway in KFs. KFs overexpression of sortilin or control KFs were treated with PBS or proNGF (5 ng/ml) for 18 h. Effect of sortilin on the expression of apoptosis regulating proteins, as assessed by western blotting. Protein fractions of total cells were assessed for expression of Bax, Bcl-2, NF-κB, p-PI3K, PI3K, p-Akt, Akt, and Actin. Similar results were obtained in at least three independent experiments.

along neurites [25]. It is noteworthy that soluble sortilin has been shown to prevent proBDNF cleavage *in vitro*, suggesting that it may function as a rheostat to balance synaptic input and regulate long-term depression (proBDNF) and long-term potentiation (BDNF) [26]. It has been observed that sortilin is essential for proNGF-induced apoptosis of rat vascular smooth muscle cells [18]. Similiar results were also found by us, we found that the mRNA and protein of sortilin were significantly down-regulated in KFs compared to the NFs, overexpression of sortilin significantly promoted proNGF induced KFs apoptosis. These data implied that sortilin may play an important role in the development of keloid fibroblasts.

Keloid, characterized by an abundant accumulation of extracellular matrix, are benign collagenous tumors that occur during abnormal wound healing. It has been reported that the growth potential of keloid fibroblasts could be higher than that of normal skin fibroblasts, and keloid fibroblasts are resistant to apoptosis induced by several stimuli such as antitumor necrosis factor receptor superfamily, member 6 (Fas) antibodies, staurosporin, and C2 ceramide [10, 27]. Apoptosis could mediate the transition from the granulation phase to the normal scar in the wound healing process [28]. These findings suggest that keloid formation could be a result of an abnormal wound healing process with prolonged cell growth and resistance to apoptosis, with subsequent collagen accumulation. Today, many studies found that TGFβ/smad signaling pathway played essential role in collagen accumulation of keloid [29-31], while the mechanism of regulating apoptosis induced by neurokines in keloid still remain unknown. In this article, we identified that overexpression of sortilin significantly promoted proNGF induced apoptosis in KFs, a significant release of cytochrome c from the mitochondria to the cytosol was observed and bax expression was obviously increased in KFs-sortilin groups upon proNGF treated. These results implied that soritlin may contribute to proNGF-induced mitochondria-mediated internal apoptosis pathways.

Cell survival factor inhibits cell apoptosis through activating specific signaling pathways, including the PI3K/Akt pathway. Akt can promote cell survival by indirectly activating the pro-survival transcription factor NF-κB through the phosphorylation of I-κB kinase (IKK) [32]. NF-κB, was reported as central dimeric transcription factor, regulated the expression of

genes responsible for innate and adaptive immunity, cell proliferation and apoptosis [32, 33]. Recently, many studies showed that inhibition of PI3K/Akt/NF-κB signaling pathway resulted in promoting cell apoptosis [34-36]. Similar with their researches, our data showed that overexpression of sortilin in KFs suppressed the activation of PI3K/Akt signaling, meanwhile, we found that the expression of p65 was decreased. These results suggested that sortilin can not only regulated mitochondria-mediated internal pathway in KFs, but also promoted KFs apoptosis via inhibition the PI3K/Akt/NF-κB signaling pathway.

In conclusion, these results suggest that sortilin potentiated proNGF-induced mitochondria-mediated internal apoptosis pathway through increasing expression of bax and suppressing activation of PI3K/Akt/NF-κB signaling pathway in KFs, and sortilin is a potential target for treatment of the keloid disease.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yi-Bing Wang, Department of Burn and Plastic Surgery, Provincial Hospital Affiliated to Shandong University, Jinan 250021, Shandong, People's Republic of China. E-mail: wang_yibing16@163.com

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