Pathology - Research and Practice 215 (2019) 235–242

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

# Actin related protein 3 (ARP3) promotes apoptosis of intestinal epithelial cells in ulcerative colitis

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ARTICLE INFO

*Keywords:*
IBD
Apoptosis
ARP3

ABSTRACT

Apoptosis in intestinal epithelial cells (IECs) promotes the development of ulcerative colitis (UC), a type of inflammatory bowel disease (IBD). Efficient clearance of apoptotic cells is essential for tissue homeostasis in metazoans. Actin related protein 3 (ARP3) promotes endothelial dysfunction. The expression and function of ARP3 in UC remains unclear. In this study, the expression of apoptotic markers as p53, Bax, Cleaved-Caspase9 and Cleaved-Caspase3 were proved to be increased in the intestinal epithelial cells (IECs) of UC patients and in a mouse disuccinimidyl suberate(DSS)-induced colitis model; meanwhile, ARP3 expression was elevated. ARP3 expression levels and the severity of symptoms in patients with UC were positively correlated. By knocking down ARP3 in a TNF-α-treated NCM-460 cell colitis model, the apoptotic markers described above were all decreased. In conclusion, our data indicates that ARP3 might promote the apoptosis of IECs in UC, revealing a potential molecular target for treating UC.

## 1. Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder with unknown etiology, manifesting two main clinical forms: Crohn,s disease (CD) and ulcerative colitis (UC) [1]. Recent years, there is a growing trend of rising morbidity of IBD worldwide, it has been proved that IBD have increased significantly during the last 50 years in Western countries and more frequently in Asia, Africa, and the Middle East. However, the majority of newly diagnosed cases turn out to be UC (approximately 54%) [2]. UC starts from young adulthood and sustains throughout life, the process of the UC is commonly characterized by an alternating flare and remission period except for some cases showing a continuous disease activity. The clinical feature and the response to treatment of UC vary a lot, ranging from mild symptoms of systemic manifestations to life-threatening fulminant colitis that does not respond to treatment and requires colon resection [3]. And UC patients are prone to suffer from recurrent attacks, leading to aggravated condition and seriously decreased working ability as well as life quality [4].

Intestinal epithelial cells (IECs) regulate gut immune homeostasis, and impaired epithelial responses are implicated in the pathogenesis of inflammatory bowel diseases (IBD) [5]. Increased IEC apoptosis has also been detected in acute inflammatory colonic tissue from the biopsies of UC patients [6], Abnormal IEC apoptosis impairs mucosal barrier, leads to high intestinal permeability, invasion of symbiotic microenvironment, and initiation of proinflammatory cytokines such as TNF-α and IFN-γ which further induce IEC apoptosis [7]. Apparently, IEC apoptosis plays an important role in the pathogenesis of UC.

The ARP2/3 complex consists of seven evolutionarily conserved subunits (ARP2, ARP3 and ARPC1-5) and influences many different cellular processes [8]. ARP2/3 complex leads to endothelial dysfunction [9] and its overexpression also makes keratinocytes hypersensitive to inflammatory stimuli both *in vitro* and *in vivo* [10]. In colon diseases, ARP2/3 complex can modulates Nrf2-dependent gene transcription and knockout of ARP2/3 complex in mouse model leads to hyperactivation of transcription factor Nrf2 [11] while activated Nrf2 signaling has been demonstrated to increase proteasome activity and apoptosis resistance which protects the colic tissue from the inflammatory damage [12,13]. Thus, according to the previous studies, the ARP2/3 complex

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https://doi.org/10.1016/j.prp.2018.10.011
Received 2 August 2018; Received in revised form 15 September 2018; Accepted 17 October 2018

is an important factor during the inflammatory and apoptotic procession in UC, however, the precise role and function of the ARP3 subuit remains unclear. Based on the fact that deleting ARP3 significantly inactivates the ARP2/3 complex [14,15], we hypersize that ARP3 may be associated with the process of IEC apoptosis and UC progression. In the present study, we used human UC samples and contructed mice UC models to reveal the relationship between ARP3 and apoptosis during the development and progression of UC.

## 2. Materials and methods

### 2.1. Clinical specimens

Colon tissue samples were collected from UC patients (n = 10) and healthy subjects (n = 10) by endoscopy at the Affiliated Hospital of Nantong University (Nantong, Jiangsu, China) from 2011 to 2016. Our experimental protocols were subject to approval by the ethics committee of the hospital, as well as fully understood and signed by all participating patients. A portion of each specimen was used for western blot, RT-PCR, and the other part was immediately fixed in formalin and embedded in paraffin section for immunohistochemistry. The informed consent was obtained before specimen collection.

### 2.2. Colitis animal model

The care and handling of animals were in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Research Council in 1996 and the Chinese National Committee for the Use of Experimental Animals for Medical Purposes, Jiangsu Branch. C57BL/6 J mice (females, aged 6–8 weeks) were supplied by the Department of Laboratory Animals Center, Nantong University. All mice were maintained under specific pathogen-free (SPF) conditions in which mice were housed in a pathogen-free animal facility at 22℃ under controlled 12-h light and12-h dark cycles. To create a ulcerative colitis model, enteritis was induced by adding 2.5% [w/v] dextran sodium sulphate [DSS] [MP Biomedicals; Santa Ana, CA, USA] as previously described [16].

### 2.3. Evaluation of experimental colitis

Mouse body weights were recorded at the beginning of the study and before they were sacrificed, and the change rate was calculated as a percentage compared with the initial weight. Assessment of the colitis severity was based on a previously published standard [17].

### 2.4. Cell culture and treatment

NCM460 is a human colon epithelial cell line (Oulu Biotechnology, Shanghai, China). Cells were cultured in low-glucose Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS) with 100 μg/ml penicillin and 100 μg/ml streptomycin at 37 ˚C with 95% air/5% CO2. The culture medium was replaced every other day. For subsequent experiments, the cells were stimulated with TNF-α (Sigma Chemical Co, USA) at different concentrations and times.

### 2.5. Western blot

Colon samples were lysed in lysis buffer and then centrifuged at 12,000 *g* at 4 ˚C for 20 min to collect the supernatant. The protein extracts were harvested with lysis buffer. Protein concentrations were determined by Bradford protein assays (Pierce, USA). Equal amounts of protein were separated by 10% SDS-PAGE and transferred onto PVDF membranes (Roche, USA) using the semidry transfer method; the membranes were blocked with skim milk in Tris－HCl-buffered saline for 2 h. Then, the membranes were incubated overnight at 4 ˚C with the primary antibodies, including anti-ARP3 (rabbit, 1:5000; Cell Signaling Technology, USA), anti-cleaved caspase-9, anti-Bax, anti-cleaved caspase-3,anti-Heme Oxygenase 1, anti-GAPDH, (rabbit, 1:1000; Abcam, USA), anti-p53 (mouse, 1:200; BOSTER, China). Then, the membranes were incubated with HRP-conjugated secondary antibodies (Jackson ImmunoResearch, USA) for 1.5 h at room temperature. The membranes were visualized using an enhanced chemiluminescence system (ECL, Pierce Company, USA).

### 2.6. TUNEL assay

Apoptotic DNA fragmentation was examined using One Step TUNEL Apoptosis Assay Kit (Beyotime, Shanghai, China) according to the manufacturer's protocol. The localized green fluorescence of the apoptotic cells were detected by fluorescence microscopy (Olympus BX41, Olympus Corporation, Japan)

### 2.7. Frozen sections and immunohistochemistry (IHC)

The mouse colon samples from above the anus were cut and fixed in 4% paraformaldehyde for 12 h and subsequently subjected to 20% and 30% sucrose gradient dehydration for one day until the colon sample sunk completely to the bottom. Then, the specimens were embedded in OCT and cut into 5 μm slices. Finally, the sections were stored at −20 ˚C. The paraffin sections (human colon tissue) were baked in an oven at 60 ˚C for 6 −8 h, and the frozen sections (mice colon tissue) were baked in an oven at 37 ˚C for 1–2 h. Then, the antigens in the specimens were detected with an IHC kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instructions.

### 2.8. Apoptosis detection

The Annexin V-FITC Apoptosis Detection Kit (Beyotime, Shanghai, China) was used to detect NCM460 cell apoptosis according to the manufacturer's protocol.

### 2.9. Plasmids and transfection

The cDNA templates for human ARP3 (Origene, Beijing, China) and HA tag were cloned into the pcDNA3.1 (+) plasmid. NCM460 cells were digested with trypsin for 2 min and then plated at the density of 90% before transfection. Serum-free medium was used to culture cells for 24 h. The same amount of medium was used to dilute the plasmids and Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany). The mixture of plasmids and Lipofectamine 2000 were sustained for 20 min before added to plates. Replace serum-free medium with serum-containing medium 4–6 hours after the transfection.

### 2.10. Real time PCR

The RNA was extracted using Trizol (Invitrogen) and the RNeasy Mini kit (Qiagen) [18]. cDNA was then prepared using the iscript DNA synthesis kit (Bio-Rad, USA). SYBR Green dye-based PCR amplification was used to measure gene expression; qPCR was performed using the ABI StepOnePlus apparatus. mRNA expression was determined using the standard curve method recommended by the manufacturer's protocol (Sangon Biotech,-Shanghai,China). The primerslist: ARP3 Forward, 5'GGA GAA CGG ACG TTG ACC G 3′; ARP3 Reverse, 5'TCC TGC GAT TGG AAT GTG TTT3′; P53 Forward, 5'CAG CAC ATG ACG GAG GTT GT 3′; P53 Reverse, 5' TCA TCC AAA TAC TCC ACA CGC 3′; Caspeased 9 Forward, 5' CTT CGT TTC TGC GAA CTA ACA GG 3′; Caspeased 9 Reverse, 5' GCA CCA CTG GGG TAA GGT TT 3′; Caspase3 Forward, 5'CAT GGA AGC GAA TCA ATG GAC T 3′; Caspeased 3 Reverse, 5'CTG TAC CAG ACC GAG ATG TCA 3′; Bax Forward, 5'CCC GAG AGG TCT TTT TCCG AG 3′; Bax Reverse, 5' CCA GCC CAT GAT GGT TCT GAT 3′; HO-1 Forward, 5'AAG ACT GCG TTC CTG CTC AAC 3′; HO-1 Reverse, 5' AAA GCC CTA CAG CAA CTG TCG 3′;

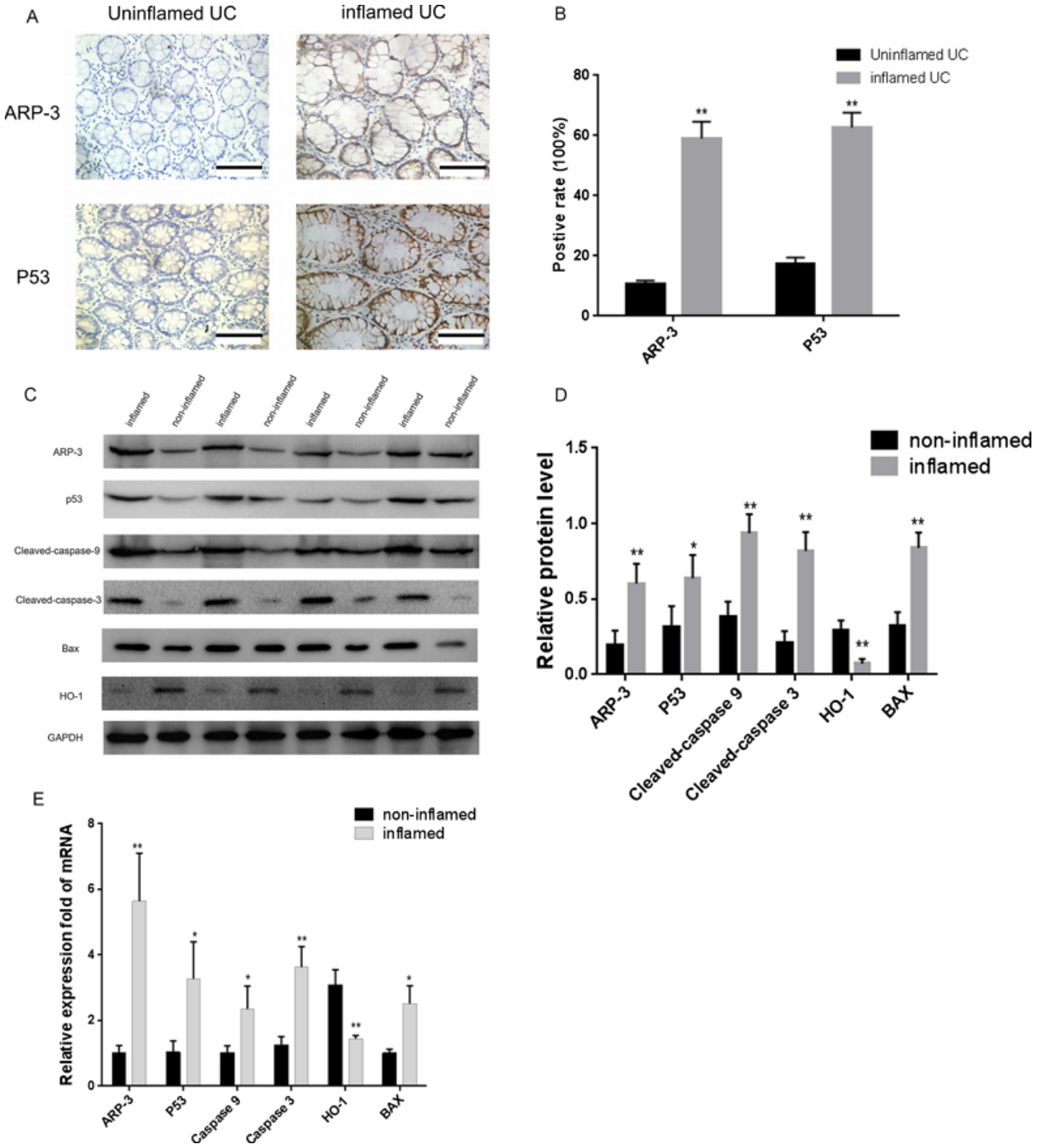


**Fig. 1.** ARP3 expression level and location in non-inflamed and inflamed colon samples from UC patients.
(A) IHC analysis of ARP3 and p53 in mucosal biopsies from inflamed tissues (n = 10) and non-inflamed control tissues (n = 10). Scale bar = 200 μm. (B) The bar graph indicates the positive staining rate of ARP3 and p53 in tissues. (C) ARP3, p53, Cleaved-caspase 9, Cleaved-caspase 3, Bax and HO-1 protein levels in non-inflamed and inflamed colon tissues from UC patients were detected by western blot. Each pair of non-inflamed and inflamed colon tissue samples came from the same patient. (D) Quantification of the ARP3, p53, Cleaved-caspase 9, Cleaved-caspase 3, Bax and HO-1 protein levels relative to GAPDH. (E) ARP3、p53, Caspase 9, Caspase 3, Bax and HO-1 mRNA levels were detected by RT-PCR. $^{*}P < 0.05$ and $^{**}P < 0.01$.

### *2.11. Statistical analysis*

All the data were shown as the mean ± standard deviation. Comparisons of the data were conducted using Student's t-tests. Significance was indicated by P values of less than 0.05. Each experiment was conducted in duplicate at least 3 times.

## 3. Results

### *3.1. ARP3 and apoptotic protein markers expression in inflamed UC samples*

IHC assay was used to determine the expression and location of ARP3 and P53 in inflamed and non-inflamed UC samples. As shown in

Fig. 1A and B, ARP3 staining was stronger in the inflamed samples than the non-inflamed samples accompany with the higher p53 expression which was a most frequently used apoptotic maker in the inflammatory reaction. Western blot and RT-PCR also revealed that both the protein and mRNA level of ARP3 was up-regulated in the inflamed samples. Our colon tissue samples were collected from UC patients which have abdominal pain, blood in the stool, weight loss, urgency, vomiting, etc clinical symptoms. ARP3 expression levels and the severity of symptoms in patients with UC were positively correlated. Similar to the earlier research [19], the simultaneous detection of protein and mRNA levels proved that besides P53, other inflammation-related apoptotic genes including Caspase 9 、Caspase3 and Bax were overexpressed meanwhile HO-1 the Nrf2 target genes was reduced in the inflamed samples. (Fig. 1C–E).These data suggested ARP3 was co-expressed with apoptosis related genes and might be involved in the pathogenesis of UC.

### 3.2. Establishment of the colitis model

To better explore the role of ARP3 in UC, a DSS-induced mice UC model was established. During the model construction, alterations to the faecal properties, activity levels and body weights of the mice were recorded to assess the disease activity. The DSS-induced mice demonstrated severe weight loss, bloody stools, and low activity compared to the control groups and showed a time-related increasing disease activity index. Since the 7 day had been indicated to be a proper time point for evaluating acute colic damage when tissues always acquired most serious inflammation in this time, our consistent results verified a remarkable body weight loss and disease activity index boosting after 7 days DSS treatment(Fig. 2A and B). To identify the pathological changes in this model, HE staining was applied in both control and 7d DSS-treatment samples and drug induced group showed increased inflammatory cell accumulation and destruction of the architecture of the intestinal epithelium, including intrinsic glands (Fig. 2C and D); All these results suggested a successfully constructed mice UC model, in addition, the 7 day of this model was chosen as an appropriate time point for subsequent experiments.

### 3.3. ARP3 and apoptotic protein markers expression in colitis model

Consistent with the results from the inflamed tissues from UC patients, ARP3 expression was also found to be upregulated in DSS-induced UC models compared with the untreated groups. IHC assay manifested the expression as well as the distribution of ARP3 and p53 were notably increased in the colitic mice (Fig. 2E and F). Western blots in three respective pairs confirmed the phenomenon and showed Cleaved-caspase 3、Bax upregulation and HO-1 reduction in the DSS group in accordance with the trend in human UC samples (Fig. 2G and H). These results implied that ARP3 might play a essential role in the process of inflammation-related apoptosis in intestinal epithelial cells and further affect the development of UC.

### 3.4. ARP3 promote TNF-α mediated apoptosis in colonic epithelial cells

TNF-α is a vital pro-apoptotic cytokine secreted by inflammatory cells. It has been reported that TNF-α secretion in DSS-induced colitis reaches its peak at 7d [20] which corresponds with the previous results that the UC models acquired the most severe features after 7 days DSS induction. So it is reasonable to speculate that TNF-α mediated intestinal epithelial cells apoptosis may participate in the progresson of UC and ARP3 may influence the process. To verify this, the colonic cell line NCM 460 was treated with TNF-α for both different concentration (0 ng/ml, 0.5 ng/ml, 10 ng/ml, 50 ng/ml, 100 ng/ml) and time (0 h, 1 h, 2 h, 6 h, 12 h, 24 h) and the results showed that with the induction of TNF-α, the protein levels of ARP3 as well as apoptotic related genes p53、Cleaved-caspase 9, Cleaved-caspase 3 and Bax increased significantly in a time or concentration dependent manner. (Fig. 3A–F) Further knockdown of ARP3 significantly attenuated TNF-α mediated activation of P53、Cleaved-caspase 9、 Cleaved-caspase 3 and Bax in protein level and attenuated TNF-α mediated of P53、Caspase 9、Caspase 3 and Bax in mRNA level (Fig. 4A–C) along with the decreased apoptotic cells analyzed by flow cytometry and TUNEL (Fig. 4D–F), indicating ARP3 plays an auxo-apoptotic role in intestinal epithelial cells.

## 4. Discussion

Ulcerative colitis (UC) is one of the two well-defined subtypes of IBD and is becoming increasingly common in Asian countries. Unlike Crohn's disease (CD), the pathological characteristics of UC is often described by limited mucosal and submucosal ulcers with severe mucosal inflammation. Although many therapies including the TNF-α antagonist infliximab, immunosuppressor and corticosteroids have been proved to be partly effective to induce clinical remission, the long-term efficacy remains poor as well as the exact etiology and mechanism has not been well illustrated [21]. The ARP 2/3 complex was first discovered as a polyprotein complex containing actin-related proteins [22] and playing an important role in nucleation [28]. In addition, ARP 2/3 complex as well as its subunits has also been reported to take part in carcinogenesis and mediate tumour metastasis [23–25]. In the keratinocytes, ARP2/3 complex is a key regulator to maintain the epidermal morphogenesis and homeostasis, knockout of the ARP2/3 complex leads to hyperactivation of Nrf2 signaling pathway causing a severe psoriasis-like disease [11]. Considering the Nrf2 signaling pathway is capable to attenuate colonic inflammation and apoptosis [27], it is sensible to hypothesize that as a part of ARP2/3 complex, ARP3 may be a crucial factor positively influencing the apoptosis of IECs and development of UC. Subsequent experiments confirmed the inference that ARP3 was higher expressed and co-expressed with apoptic related genes such as P53, Bax、Cleaved-caspase 3 and Cleaved-caspase9 in inflamed UC samples. Then, a DSS induced mice UC model [28] was successfully constructed to investigate the alteration of ARP3 among the generation of UC. Similar phenomenon was detected as the DSS induction leading to severe weight loss and disease activity index also brought about the higher expression of ARP3, P53 and Bax as in human UC tissues.

Based on the above *in vivo* findings, we preliminarily conclude that ARP3 may positively correlate with the apoptosis of UC. As TNF-αis a classical inflammatory cytokine and has been reported to be oversecreted in DSS-induced UC model [20], we choose TNF-αas the stimulator to build an *in vitro* UC model in IECs. Consistent with the *in vivo* findings, ARP3, p53, Cleaved-caspase 9, Cleaved-caspase 3 and Bax expression significantly ascended in response to TNF-α exposure and knockdown of ARP3 remarkablely decreased the upregulation of p53, Cleaved-caspase 9, Cleaved-caspase 3, and Bax mediated by TNF-αstimulation in IECs, suggesting that ARP3 promote IECs apoptosis in UC.

Although our results are convincing to suggest ARP3 has a pro-apoptotic effect in IECs and may be an important regulator in the progression of UC, adverse proof has shown depletion or inhibition of ARP2 / 3 leads to activation of NF-κB [29] which is frequently hyperactivated in IBD [30]. And in human pulmonary the ARP2/3 complex protects endothelial barrier function [31]. This could be partially explained by the different functions of subunits compared to the complex or dual regulation through different cell signaling.

In summary, our study firstly investigated the role of ARP3 during the development of UC, revealing its pro-apoptosis effect *via* upregulation of apoptotic genes. However, how ARP3 affects apoptosis in UC development remains poorly understood and requires further study. Based on the previous research and our results, ARP3 could possibly serve as a novel molecular target for UC treatment.

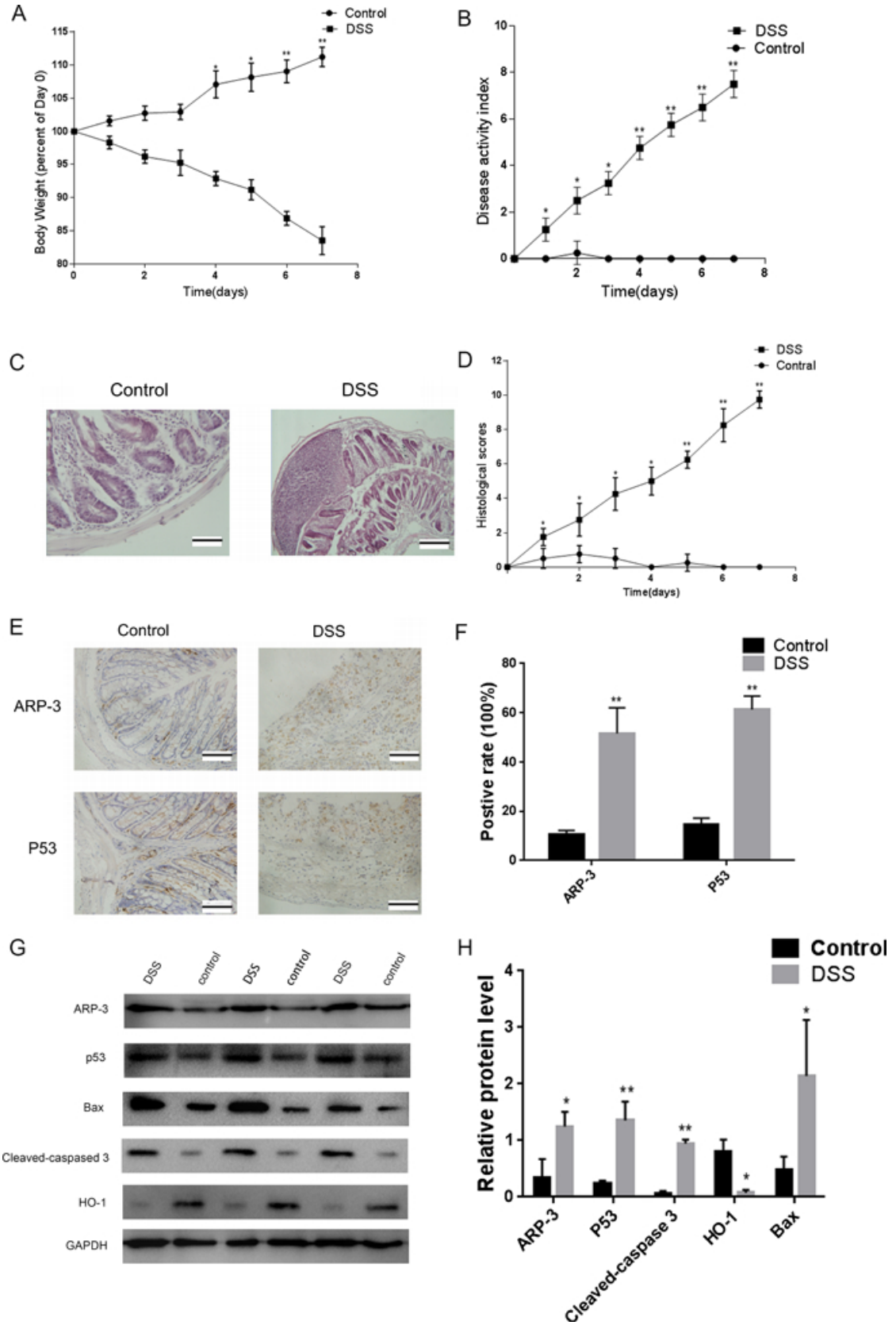


**Fig. 2.** Construction of the mouse experimental colitis model and the relationship between ARP3 and IEC apoptosis in the mouse experimental colitis model. (A) Body weights, (B) Disease activity index, (C) Histological scores and (D) HE staining of the colon structure in mice treated with DSS as well as control group. Scale bar = 200 μm. (E) IHC analysis of ARP3 and p53 in colon tissues from the DSS treated and Control groups. Scale bar = 200 μm. (F) The bar graph indicates the positive staining rate for ARP3 and p53 in the tissues. (G) ARP3、p53、Cleaved-caspase 3 and Bax protein levels were measured by western blot in different groups. (H) Relative protein levels of different groups. n = 3, * $P < 0.05$ and ** $P < 0.01$.

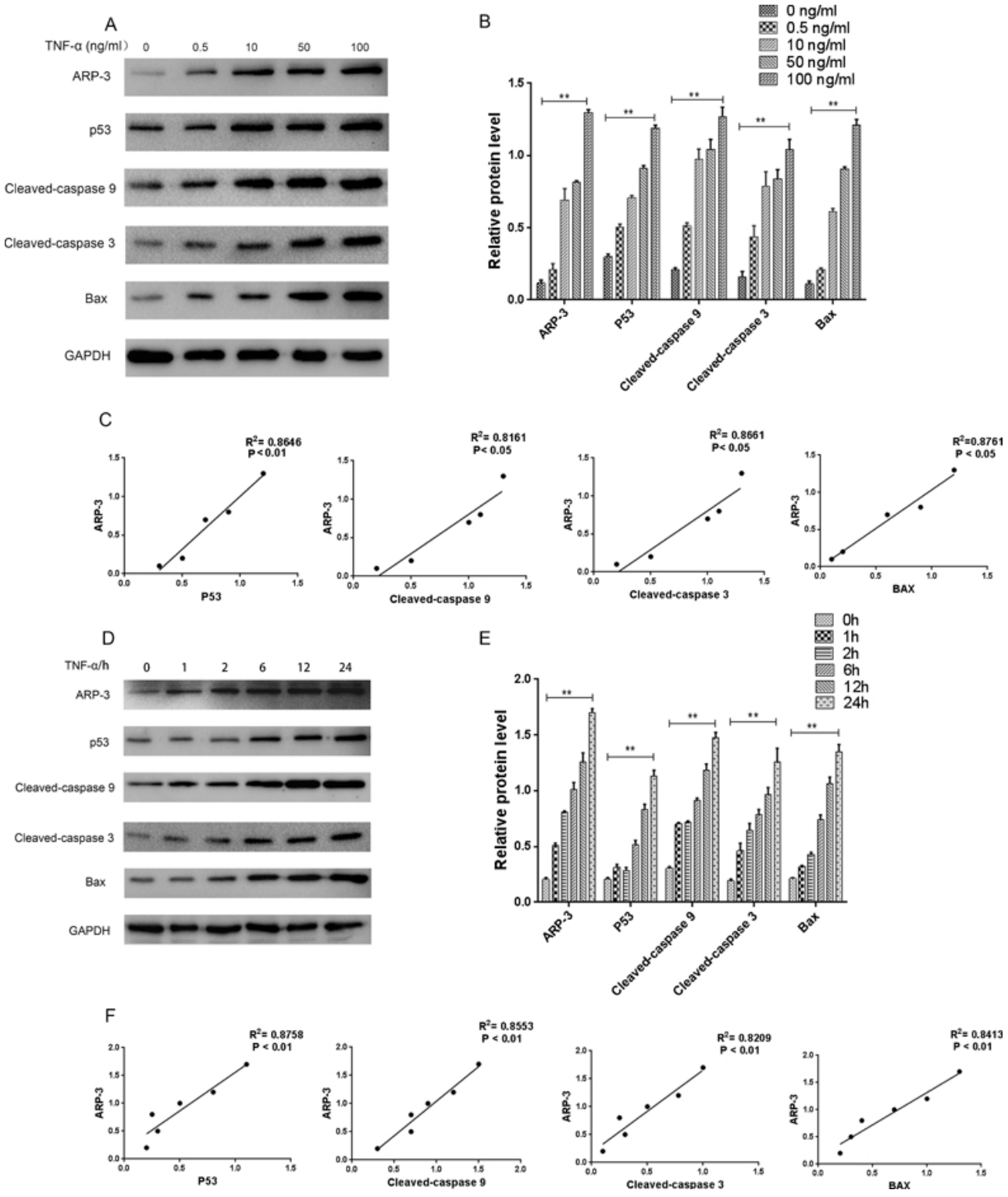


**Fig. 3.** ARP3 was positively correlated with IECs apoptosis.

ARP3, P53, Cleaved-caspase 9、Cleaved-caspase 3 and Bax protein levels were measured by western blot for (A) different concentrations and (D) time points of TNF-αtreatment. (B) Relative ARP3, Cleaved-caspase 9 、Cleaved-caspase 3 and Bax levels at diferent concentrations. n = 3,*$P <$ 0.05 and **$P < 0.01$. (E) Relative ARP3, Cleaved-caspase 9, Cleaved-caspase 3 and Bax levels at different times. n = 3, *$P < 0.05$ and **$P < 0.01$. (C) The correlation analysis of ARP3 expression with p53、 Cleaved-caspase 9, Cleaved-caspase 3 and Bax at different concentrations of TNF-αtreatment. **$P <$ 0.01, $R^2 = 0.8646$;*$P <$ 0.05, $R^2 = 0.8161$;*$P <$ 0.05, $R^2 = 0.8661$; *$P <$ 0.05, $R^2 = 0.8761$,respectively. (F) The correlation analysis of ARP3 expression with p53, Cleaved-caspase 9, Cleaved-caspase 3 and Bax at different times of TNF-αtreatment. **$P < 0.01$, $R^2 = 0.8758$; **$P < 0.01$, $R^2 = 0.9551$;**$P < 0.01$, $R^2 = 0.8209$; **$P <$ 0.01, $R^2 = 0.8431$,respectively.

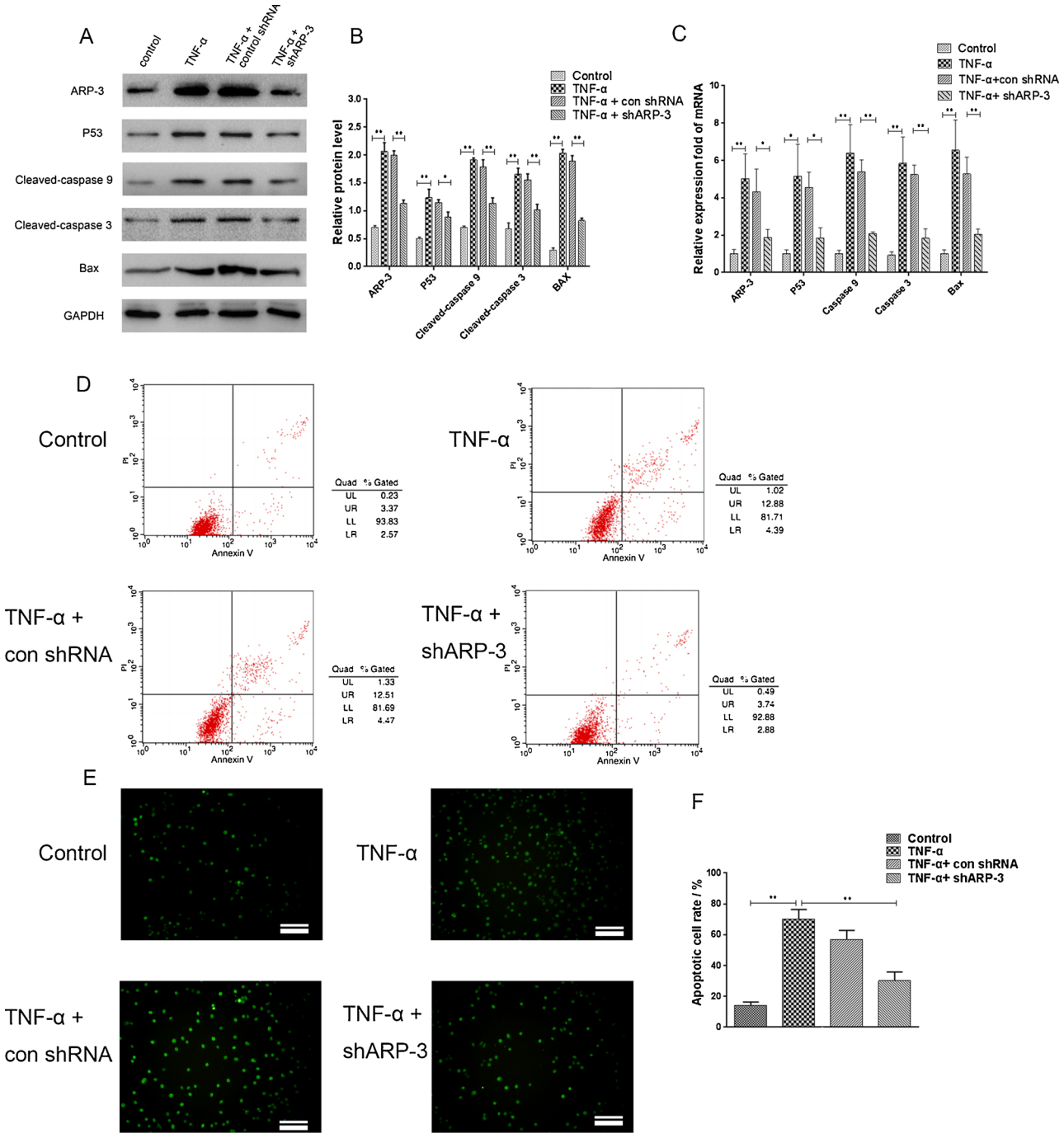


**Fig. 4.** ARP3 promote apoptosis in IECs.
(A) ARP3, p53, Cleaved-caspase 9, and Bax protein levels were measured by western blot after induction of shARP3 and control shRNA to NCM460 cells pretreated with TNF-αfor 24 h. (B) Relative levels of p53、Cleaved-caspase 9, Cleaved-caspase 3 and Bax compared to GAPDH. (C) mRNA levels of p53, Caspase 9 、Caspase 3 and Bax relative to GAPDH. (D) Flow cytometry assays were performed to measure the cells apoptosis in different groups. (E) TUNEL assay were performed to measure the cells apoptosis in different groups. (F) Quantification analysis of apoptotic cells in different groups. n = 3, *P < 0.05 and **P < 0.01.

## Conflict of interest

These authors declare that they have no conflict of interest.

## Acknowledgments

This work was supported in part by the National Nature Science Foundation of China (Nos. 81602114); Nantong Science and Technology Innovation Project (Nos. MS22015044).

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