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Original Paper

Prodigiosin Alleviates Pulmonary Fibrosis Through Inhibiting miRNA-410 and TGF-β1/ADAMTS-1 Signaling Pathway

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Abstract

Background/Aims: Pulmonary fibrosis is a common outcome of various interstitial lung diseases. Prodigiosin (PG) is a series of red pigment with methoxypyrrole ring. This study investigates the role of prodigiosin in pulmonary fibrosis and its underlying mechanisms. ***Methods:*** A pulmonary fibrosis rat model was established by intra-tracheal injection of bleomycin A5. Rats were divided into 4 groups: Normal group, pulmonary fibrosis Model group, Prodigiosin treatment group and hydrocortisone treatment group. HE and Masson staining were carried out to evaluate histopathological changes. The content of hydroxyproline in lung tissue was determined by alkaline hydrolysis. The expression of PICP and PIIINP was examined by ELISA. The mRNA expression of miR-410, TGF-β1 and ADAMTS1 in lung homogenate were detected by RT-PCR. The bronchoalveolar lavage fluid (BALF) and lung tissues of rats were collected and analyzed. Human embryonic pulmonary fibroblast (HEPF) was used for study *in vitro*. A dual-luciferase reporter assay was conducted to examine the effect of miR-410 on ADAMTS1 expression. Cell transfection was conducted to inhibit miR-410. MTT assay was performed to investigate cell proliferation. The expressions of miR-410, TGF-β1, ADAMTS1 and other fibrosis related biomarkers (Col I, Col III, and α-SMA) were examined by RT-PCR and Western Blot. ***Results:*** HE and Masson staining showed thickened alveolar septum, hyperplastic capillaries, and large areas of collagen fiber deposition in pulmonary fibrosis model rats. Rats in prodigiosin and hydrocortisone treatment groups had alleviated symptoms. There was high hydroxyproline expression in model rats, whereas the expression of hydroxyproline reduced after prodigiosin or hydrocortisone treatments. RT-PCR results showed high miR-410, high TGF-β1 and low ADAMTS1 in lung tissue of model rats. The expression of PICP and PIIINP were higher in BALF of model group than in treatment groups. Prodigiosin and hydrocortisone treatment significantly reduced PICP and PIIINP content. RT-PCR and Western Blot analysis showed that prodigiosin inhibited expression of miR-410 and TGF-β1, but up-regulated

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ADAMTS1 expression. MTT assay indicated that prodigiosin inhibited HEPF proliferation induced by miR-410 overexpression. **Conclusion:** Prodigiosin down-regulates the expression of miR-410 and TGF-β1, up-regulates ADAMTS1, leading to decrease accumulation of fibrotic proteins. It could be used in alleviating pulmonary fibrosis.

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Introduction

Pulmonary fibrosis is a common outcome of various interstitial lung diseases, including idiopathic pulmonary fibrosis, pneumoconiosis and drug-induced fibrosis. Collagen is the major component of extracellular matrix, and its excess accumulation is a characteristic pathological change of pulmonary fibrosis.Type I collagen (Col I) and type III collagen (Col III)are the main components of lung collagen and are often usedas indicators of pulmonary fibrosis [1]. Transforming growth factor-β1 (TGF-β1) is a common fibrotic factor, which plays an important role in development of pulmonary fibrosis [2]. A disintegrin and metalloproteinase with a thrombospondin type 1 motif (ADAMTS1) is a metalloproteinase with secretory function. It is secreted into extracellular matrix and specificallydegrades Col I and Col III [3, 4]. Studies have shown that [5, 6], ADAMTS-1 plays important roles in liver cirrhosis and renal fibrosis. It is reported that expression of ADAMTS-1 decreased in pulmonary fibrosis [7]. Recent study indicatedthat captopril degrades Col I and Col III through TGF-β1/ADAMTS-1 pathway, thereby inhibiting myocardial fibrosis [8].

Prodigiosin (PG) is a series of red pigment with methoxypyrrole ring, such as metacycloprodigiosin, undecylprodigiosin, nonylprodigiosin and cycloprodigiosin. They are secondary metabolites from actinomycetes, serratia marcescens, and other bacteria. PG was first separated and purified from serratia marcescens in 1929 [9, 10]. It has various biological functions. However, the effect of prodigiosin on pulmonary fibrosis has not been investigated.

MiRNAs are short (18~25 nucleotides), endogenous non-coding molecules that cause translational repression or mRNA cleavage by binding to the 3'-untranslated regions (3'-UTRs) of target mRNAs [11, 12]. MiRNAs have been demonstrated to regulate many cellular processes, including differentiation, proliferation and apoptosis [13, 14].

In this study, weestablish a rat model of pulmonary fibrosis by intra-tracheal injection of bleomycinin order to explore the effect of prodigiosinin pulmonary fibrosis. The effect of miR-410 on TGF-β1/ADAMTS1 signaling pathway and the effect of prodigiosin on accumulation of fibrotic proteinsare investigatedusingHuman embryonic pulmonary fibroblast(HEPF) cells. This study provides a new insight and experimental basis for treatment of pulmonary fibrosis.

Materials and Methods

Establishing a pulmonary fibrosis rat model

A total of 48 healthy male Sprague-Dawley rats aged 3 - 4 months and weight 220±7.5g were purchased from Shanghai SLAC Experimental Animal Co., Ltd, Rats were randomly sub-caged in SPF-grade environment. All animals received humane care in compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. The rats were randomly divided into 4 groups: Normal group (n = 12), Model group (n = 12), Prodigiosin (PG) group (n = 12) and Hydrocortisone (HC) group (n = 12). After intraperitoneal injection of 10% chloral hydrate (3ml/kg), an incision was made in the middle of the neck and the trachea was exposed. 0.2ml saline containing bleomycin A5 (Tianjin Taihe Pharmaceutical Limited, 5mg / kg) was injected into trachea of Model rats and drug treatment rats (PG and HC groups). The rats in Normal group were given the same amount of saline, and then rats were erected and rotated, so that the drug distributed evenly in the lungs. The Normal group and the Model group were given 10ml/kg of saline from the second day of modeling, the rats of hydrocortisone group was injected intraperitoneally with 10mg/kg hydrocortisone (Tianjin Jinyao Amino Acid Co., Ltd.). The rats in prodigiosin group was orally administered prodigiosin at

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a daily dose of 200 μg/kg. Rats in each group were sacrificed on the 28th day after modeling. The thoracic cavity was opened, ligation of the right hilum was applied. The left lung was lavaged. 10mL bronchoalveolar lavage fluid was collected from each rat, and preserved under -70°C. The upper lobe of the right lung was fixed in neutral buffered formaldehyde, paraffin-embedded, andsectioned before HE and Masson staining. Right lower lobe of the lung was kept inliquid nitrogen and transferred to -70°C for preservation.

Table 1. Pulmonary alveolar inflammation scores

Score	Description
0	No lung tissue inflammation
1	Mild pulmonary inflammation, inflammatory cell infiltration is limited to local or near the pleura, the area is less than 20% of the whole lung
2	Moderate pulmonary tissue inflammation, the affected area of the lung accounted for 20% to 50%
3	Severe pulmonary tissue inflammation, involved area was greater than 50%

Table 2. Pulmonary fibrosis scores

Score	Description
0	No pulmonary fibrosis
1	Mild pulmonary fibrosis, the affected area less than 20% of the whole lung
2	Moderate pulmonary fibrosis, the affected area of the lung accounted for 20% to 50%
3	Severe pulmonary fibrosis, involved area was greater than 50%

Histopathological analysis

The upper lobe of the right lung was fixed in 10% buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin, and Masson's staining. For quantitative histologic analysis, a numeric inflammatory and fibrotic scale was used (Szapiel et al., Table 1 and 2) [15]. The mean score was considered as the inflammatory and fibrotic score.

Detecting hydroxyproline (HYP) in lung tissue by alkaline hydrolysis

40-80mg lung tissue was taken and put into the test tube. Tissues were mixed with 1ml hydrolysase and put in boiledwater bath for 20min. The supernatant was centrifuged at 3500rpm/min for 10min according to the instructions of HYP detection kit. The absorbance at 550nm was measured by a spectrophotometer, and the HYP content of each lung tissue was calculated.

ELISA for PICP and PIIINP detection in BALF

In order to confirm whether the anti-fibrosis effect of prodigiosin is related to its promotion of ColI and ColIII degradation, the concentration of PICP and PIIINP in rats BALF were measured by double antibody sandwich ELISA.

Cell culture

Human embryoic pulmonary fibroblast（HEPF）was purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in an incubator (37°C, 5% CO_2 and saturated humidity) with RPMI 1640 medium (Sigma, United States) supplemented with 10% fetal bovine serum (Invitrogen, United States). The culture medium was replaced every two days. When the cells reached 90% confluence, they were passaged at a ratio of 1:2.

Dual-luciferase reporter assay

According to the biological information online software microRNA.org, miR-410 was predicted to bind to the 3'UTR of ADAMTS-1 mRNA. The target sequence and mutation sequence were designed based on the binding sequence of the ADAMTS-1 3'UTR and miR-410. Then, the target sequence was chemically synthesized, and sites cleaved by Xho *I* and Not *I* enzymes were added to both ends of the sequence. The synthesized fragment was cloned into a PUC57 vector. After the positive clones were identified, the recombinant plasmids were identified by DNA sequencing, sub-cloned into a psiCHECK-2 vector, and then transformed into Escherichia coli DH5α cells for amplification. Plasmid extraction was performed in accordance with the instructions of the Omega Plasmid Extraction Kit (Omega Bio-tek Inc, Norcross, GA, USA). The cells were inoculated into a 6-well plate (2×10^5 cells/well), and after the cells adhered to the wells, transfection was performed according to the aforementioned method. Subsequently, the cells were cultured

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for 48 h and then collected. The experimental cells were divided into the miR-410-NC + psi-CHECK2- ADAMTS1-3'UTR group, the miR-410-NC + psi-CHECK2- ADAMTS1-3' UTR mut group, the miR-410 mimics + psi-CHECK2- ADAMTS1-3'UTR group and the miR-410 mimics + psi-CHECK2- ADAMTS1-3'UTR mut group. The effect of miR-410 on the luciferase activity of the ADAMTS1 3'-UTR was detected using a dual-luciferase reporter assay kit (Genecopoeia, Rockville, MD, USA). A fluorescence detector (type: Glomax20/20; Promega Corporation, Madison, WI, USA) was used to determine the fluorescence intensity. This experiment was repeated 3 times for each group.

Table 3. Primer sequences for RT-PCR

Name	seq:5'-3'
α-SMA	F:TCAAATACCCCATTGAACACGG
	R:GGTGCTCTTCAGGTGCTACA
Vimentin	F:TGCGTGAAATGGAAGAGAACT
	R:TGCGTGAAATGGAAGAGAACT
Col I	F:TCTGACTGGAAGAGTGGAGAGTAC
	R:ATCCATCGGTCATGCTCTCG
Col III	F:GAATGGTGGCTTTCAGTTCAGC
	R:GCTGTTTTTGCAGTGGTATGTAATG
TGF-β1	F:CCTTGCCCTCTACAACCAACAC
	R:CTTGCAGGAGCGCACGATC
ADAMTS1	F:GTTTGTGGAGGAAATGGCTCCA
	R:CACTTCAATGTTGGTGGCTCC
GAPDH	F:GGATTTGGTCGTATTGGG
	R:GGAAGATGGTGATGGGATT

Cell transfection and grouping

For the cell transfection experiments, the cells were divided into the following groups: (1) Blank group (no sequence transfected) (2) miR-410 mimics group (transfected with artificially synthesized miR-410 mimics); (3) miR-410-NC group (transfected with a scrambled sequence); (4) anti-miR-410 (transfected with miR-410 inhibitors); (5) anti-miR-410+ADAMTS1 siRNA (transfected with miR-410 inhibitors and ADAMTS1 siRNA); (6) Prodigiosin+ADAMTS1 siRNA (transfected with ADAMTS1 siRNA and cultured with 40mg/L Prodigiosin); and (7) Prodigiosin+miR-410 mimics (transfected with artificially synthesized miR-410 mimics and cultured with 20mg/L Prodigiosin). The chemically synthesized miR-410 mimics, miR-410 inhibitors, negative control and siRNA sequences were all purchased from Shanghai Gene Pharma Co., Ltd., (Shanghai, China). The cells were inoculated into a 6-well plate 24 h before transfection. When the cells reached approximately 50% confluence, they were transfected using Lipofectamine2000 (Invitrogen Inc., Carlsbad, CA, USA). After 6 hours, the culture medium was replaced. The cells were collected for further experiments after 48 h in culture.

MTT assay for cell proliferation

Cells were seeded for 1×10^4/well in 96-well plate. When cell fusion reached 60% -70%, cells were cultured in medium without serum for 24h for synchronization. 20µl MTT (5mg/ml) was then added in each well for 4h. Supernatant was discarded, and 150 µl dimethyl sulfoxide (DMSO) was added in each well. The plate was shaken at room temperature for 15min.After dissolution of yellow crystals, the absorbance value (OD) at 570 nm was measured by a microplate reader. Detections were repeated for at least three times. Cell proliferation rate (%) = (experimental group absorbance / blank control group absorbance) × 100%, was calculated and a cell growth curve was drawn based on the results.

Real-time PCR for detection of miR-410 and mRNA of TGF-β1, ADAMTS1, Col I, Col III, α- SMA and Vimentin

Total RNA was extracted from tissue samples using TRIZOL (Invitrogen Inc., Carlsbad, CA, USA) A Nano Drop 2000 (Thermo Fisher Scientific Inc., Waltham, MA) was used to detect the concentration and purity of total RNA. In accordance with the published gene sequences in GenBank, PCR primers were designed with Primer5.0 software (Table 3) and were synthesized by Shanghai GenePharma Company (Shanghai, China). An ABI PRISM 7500 real-time PCR System (ABI Company, Oyster Bay, NY) and SYBR Green I fluorescence kit (Takara Biotechnology Ltd., Dalian, China) were used for the PCR reaction. The reliability of the results was evaluated with a standard curvewith *GAPDH* as an internal reference. The CT value (amplification power curve inflection point) was obtained, ΔCt = CT (target gene) – CT (internal reference), ΔΔCt = ΔCt (treatment group) – ΔCt (control group); the relative expression of target genes was calculated using $2^{-\Delta\Delta Ct}$.

Western blotting for detecting proteins of TGF-β1, ADAMTS1, Col I, Col III, α- SMA and Vimentin

Proteins were extracted from tissue samples, and a bicinchoninic acid (BCA) kit (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China) was used to detect the protein concentration. After sample buffer was added (30 µg per sample each well), proteins were boiled at 95°C for 10 min. Then, the proteins were separated using 10% polyacrylamide gel (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China) electrophoresis, with 80 V electrophoretic voltage converted to 120 V. After electrophoresis, proteins were transferred to polyvinylidene fluoride (PVDF) membranes with 100 V transfer-molded voltage lasting for 45

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to 70 min. Afterwards, samples were incubated at room temperature for 1 h with 5% bovine serum albumin (BSA), and were incubated with primary antibodies (1: 1000 dilution) (all from Abcam Inc., Cambridge, MA, USA), overnight at 4°C. Then, samples were washed with tris-buffered saline Tween 20 (TBST) 3 times (5 min/time). The corresponding secondary antibody was added for incubation at room temperature for 1 h, after which membranes were washed 3 times (5 min/time). Development was completed with chemiluminescence reagents. *GAPDH* was used as an internal reference. Bands were visualized with a Bio-Rad Gel Doc EZ imager (GEL DOC EZ IMAGER, Bio-Rad, California, USA). ImageJ software was applied to analyze the intensity of the target bands.

Statistical analysis

All data were analyzed by GraphPad Prism version 6 statistical software. Measurement data were expressed by mean ± standard deviation. The t test was used for comparisons between two groups. One-way analysis of variance was applied for comparisons ofmultiple groups. *P* < 0.05 was considered as of statistical significance.

Results

Histopathological evaluation of lung tissues

HE staining showed normal lung tissue structure and intact alveolar cavitywith few inflammatory cellsin the Normal group. In the Model group, there was significant infiltration of inflammatory cells in alveolar space and pulmonary interstitium. The alveolar septum was thickened, the capillary was hyperplastic and the lesion was extensive. The difference between normal and model groups was statistically significant (*P*< 0.05). The alveolar septum was less thickened in the Prodigiosin and the hydrocortisone treated group. The mononuclear macrophage infiltration and hemorrhage in the alveolar lumen were observed in some of the lung tissues. The difference was statistically significant (*P* < 0.05). Masson staining showed the lung tissue of the normal group had no obvious fibrous tissue. In the model group, the structure of lung tissue was damaged, the inflammatory cells infiltrated, and there were large area of collagen fiber bundles. Moreover, the alveolar wall thickened. There were a lot of collagen deposition in bronchus and blood vessels, and the fibrosis score was significantly higher than that of the normal group, *P*<0.05. In the prodigiosin and hydrocortisone treatment groups, the alveolar wall was less thickened and inflammatory cell infiltration decreased. Furthermore, there was decreased pulmonary tissue damage and only a small amount of collagen fiber deposition. The fibrosis score decreased compared with the model group, *P*<0.05 (Fig. 1, Table 4).
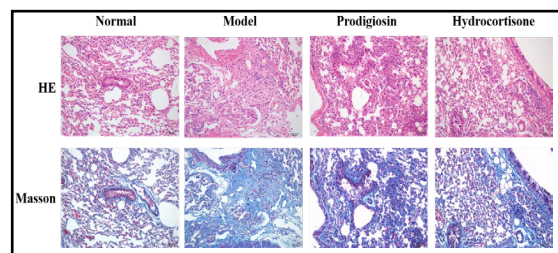

Fig. 1. HE staining and Masson's staining of lung tissues(×200)

Hydroxyproline concentration in lung tissue

Hydroxyproline concentration in lung tissue homogenate of model group was significantly higher than that in normal group. In drug treatment groups, hydroxyproline concentration were significantly lower than that of model group (P < 0.05), but there was no significant difference in hydroxyproline concentration between the Prodigiosin and Hydrocortisonetreatment groups, (P> 0.05). This result was consistent with the Masson staining results (Fig. 2).

Table 4. Lung inflammatory and fibrosis scores. * indicated P< 0.05 compared with Normal group, # indicated P< 0.05 compared with Model group

Group	n	Inflammatory scores				Fibrosis scores			
		0	1	2	3	0	1	2	3
Normal#	12	12	0	0	0	12	0	0	0
Model*	12	0	0	1	11	0	0	2	10
Prodigiosin#*	12	0	5	5	2	0	2	5	5
Hydrocortisone#*	12	0	6	5	1	0	3	4	5

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Effect of Prodigiosin on PICP and PIIINP concentration in BALF

In order to confirm whether the anti-fibrosis effect of prodigiosin is related to its promotion of Col I and Col III degradation, the precursor polypeptides of collagens PICP and PIIINP were detected by double antibody sandwich ELISA. The results showed that the concentration of PICP and PIIINP in BALF of the model group was significantly higher than that of the other three groups. Both prodigiosin and hydrocortisone significantly reduce PICP and PIIINP concentrations in BALF of pulmonary fibrosis rats (Fig. 3, P <0.05). There was no significant difference in PICP and PIIINP concentration between prodigiosin and the hydrocortisone groups (P> 0.05).

Fig. 2. Hydroxyproline concentration in lung tissue (* indicated P<0.05 compared with Normal group, # indicated P< 0.05 compared with Model group.)

miR-410 and TGF-β1/ADAMTS1 expression in lung tissue

Tissue homogenates were prepared from lung tissues of each group. The expression of miR-410 and TGF-β1/ ADAMTS1 mRNA were detected by RT-PCR in these lung tissues. Additionally, fibrosis biomakers including CoI, CoIII and α-SMA were also detected. Indeed, the expression of CoI, CoIII and α-SMA increased in model group. Prodigiosin and hydrocortisone reduced the expression of these makers as shown in Fig. 4A, B & C. Moreover, the expression of miR-410 and TGF-β1 mRNA in normal rats was significantly lower, while the expression of ADAMTS1 mRNA was significantly higher than that in other groups (P <0.05). The expression of miR-410 and TGF-β1 mRNA in lung tissue of pulmonary fibrosis rats were significantly decreased after treatment with prodigiosin and hydrocortisone (P <0.05). There was no significant difference between the two treatment groups (P> 0.05).

ADAMTS1 is the potential target gene of miR-410

ADAMTS1 was identified to be a target gene of miR-410 according to the biological information online analysis software (TargetScan, miRDB, microrna, PicTar and starBase). The luciferase reporter vector, which contained ADAMTS1 3'UTR fragments with wild-type (psi-CHECK2- ADAMTS1-3'UTR) or mutant (psi-
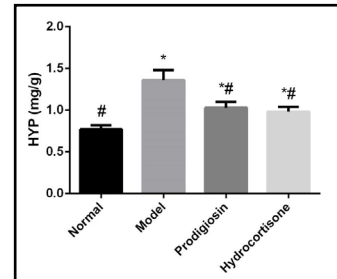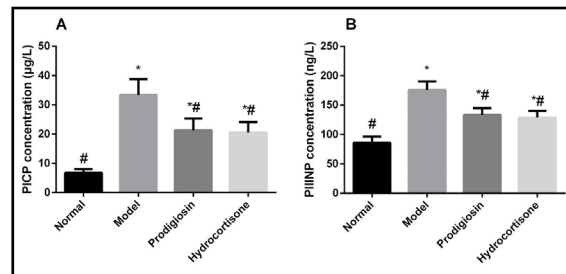

Fig. 3. PICP (A) and PIIINP (B) concentration in BALF (* indicated P< 0.05 compared with Normal group, # indicated P< 0.05 compared with Model group.)
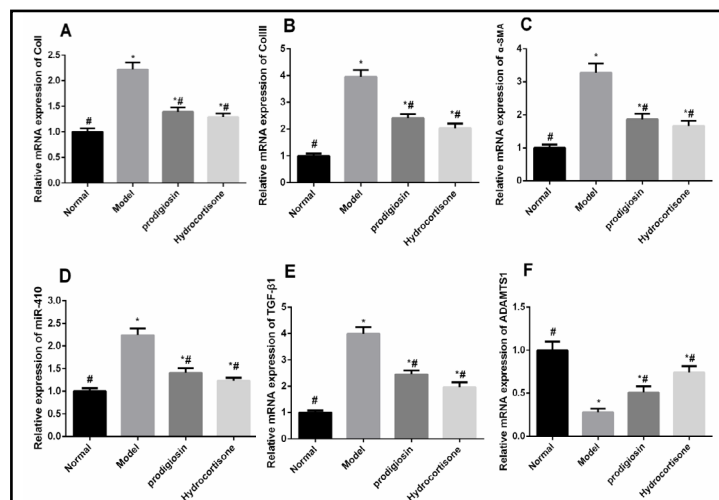

Fig. 4. Col I (A), Col III (B), α-SMA (C), miR-410 (D), TGF-β1 (E) and ADAMTS1 (F) mRNA expressionin lung tissues(* indicated P< 0.05 compared with Normal group, # indicated P< 0.05 compared with Model group.)

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Fig. 5. A: screen capture from microrna.org showing relationship of miR-410 and ADAMTS1; B: dual luciferase reporter assay (* indicated P< 0.05 compared with Normal group, # indicated P< 0.05 compared with Model group.)
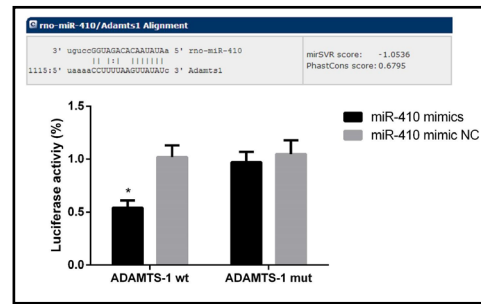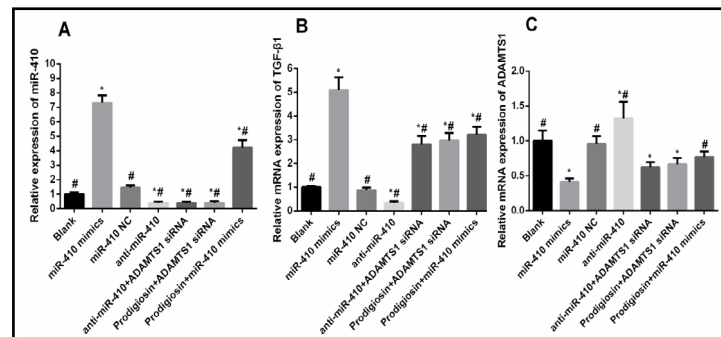

Fig. 6. RT-PCR on relative expression of miR-410 (A), TGF-β1 mRNA (B) and ADAMTS1 mRNA (C) (* indicated P< 0.05 compared with Normal group, # indicated P< 0.05 compared with Model group.)

CHECK2-ADAMTS1-3'UTR mut) miR-410 complementary sites, is shown in Fig. 5A. The dual-luciferase reporter assay (Fig. 5B) demonstrated a significant difference in the luciferase activity among the miR-410-NC + psi-CHECK2-ADAMTS1-3'UTR, miR-410-NC + psi-CHECK2-ADAMTS1-3' UTR mut, miR-410 mimics + psi-CHECK2-ADAMTS1-3' UTR and miR-410 mimics + psi-CHECK2-ADAMTS1-3' UTR mut groups (F = 91.72, P< 0.001). Compared with the miR-410-NC + psi-CHECK2-ADAMTS1-3'UTR, miR-410-NC + psi-CHECK2-ADAMTS1-3' UTR mut and miR-410 mimics + psi-CHECK2-ADAMTS1-3'UTR mut groups, the luciferase activity was significantly decreased in the miR-410 mimics + psi-CHECK2-ADAMTS1-3'UTR group (P< 0.05).

Prodigiosin inhibits miR-410 and TGF-β1 expression and up-regulates ADAMTS1 expression

RT-PCR results were shown in Fig. 6.

Fig. 7. Western Blot on relative protein expression of TGF-β1 (A) and ADAMTS1 (B) (* indicated P< 0.05 compared with Normal group, # indicated P< 0.05 compared with Model group.)

The miR-410 expression of miR-410 mimics group was significantly higher than those in Blank and miR-410 NC group. The expression of miR-410 in HEPF cells transfected with miR-410 inhibitors was significantly lower than that in Blank and miR-410 NC group. There was no significant difference among groups of anti-miR-410+ADAMTS1 siRNA group, Prodigiosin+ADAMTS1 siRNA group and anti-miR-410 group, P>0.05. In Prodigiosin+miR-410 mimics group, miR-410 expression was significantly higher than in Blank group but lower than in miR-410 mimics group, P < 0.05. The expression of TGF-β1 was presented in Fig. 7A. Indeed, miR-410 mimics group had the highest but anti-miR-410 group had the lowest TGF-β1 expression. There was no significant difference between blank group and miR-410 NC
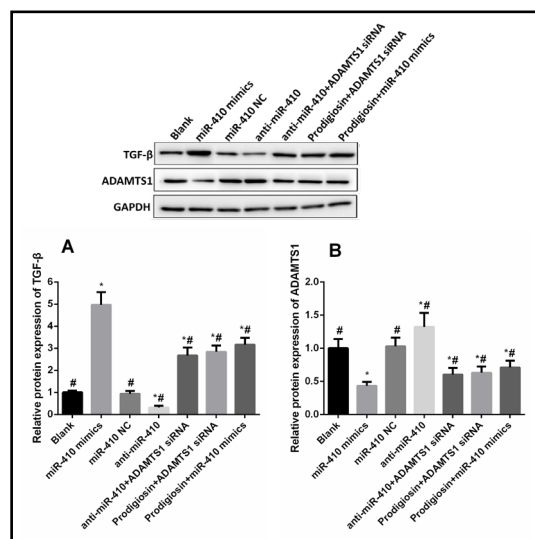
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group (P > 0.05). Moreover, TGF-β1 expression in the other 3 groups were significantly lower than that in miR-410 mimics group and significantly higher than thatin Blank group, P < 0.05. However, the ADAMTS1 expression hasthe opposite trend. miR-410 mimics group had the lowest, while anti-miR-410 group had the highestexpression, P < 0.05. There was no significant difference among anti-miR-410+ADAMTS1 siRNA group, Prodigiosin+ADAMTS1 siRNA group, and miR-410 mimics group, P > 0.05.

Fig. 8. RT-PCR onrelative mRNA expression of Col I (A), Col III (B), α-SMA (C)and Vimentin (D), (* indicated P<0.05 compared with Normal group, # indicated P< 0.05 compared with Model group.)

Prodigiosin inhibits fibroblast differentiation

The change of expression of Col I, Col III, α-SMA and Vimentinhad the same trends with TGF-β1: highest in miR-410 mimics group and lowest in anti-miR-410 group, P < 0.05. There was no significant difference between Blank and miR-410 NC group, P > 0.05. There was no significant difference among anti-miR-410+ADAMTS1 siRNA group, Prodigiosin+ADAMTS1 siRNA, and Prodigiosin+miR-410

Fig. 9. Western blotonrelative Protein expression of Col I (A), Col III (B), α-SMA (C)and Vimentin (D) (* indicated P< 0.05 compared with Normal group, # indicated P< 0.05 compared with Model group.)

mimics group, P > 0.05. However, their expression were higher than thatin Blank group and lower than that in miR-410 mimics group, P < 0.05. The expression of these mRNA and proteins were significantly lower than that in Blank group and higher than thatin miR-410 mimics group, P < 0.05 (Fig. 8 and 9).

Prodigiosin inhibits HEPF proliferation induced by miR-410 up-regulation

A significant phenomenon in the process of pulmonary fibrosis is proliferation of fibroblasts. In this study, HEPF transfected with miR-410 mimics resulted in a dramatic increase in cell proliferation compared with the Blank group, P < 0.05. The transfection of anti-miR-410 significantly inhibited the proliferation of HEPF, P < 0.05. The effect of miR-

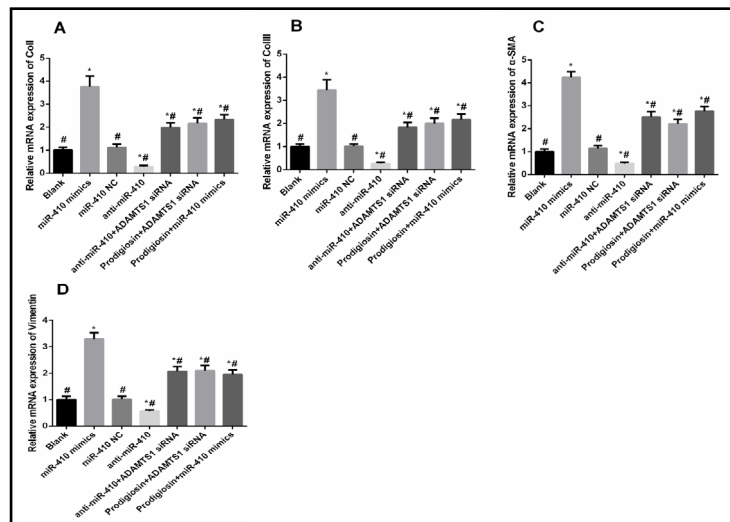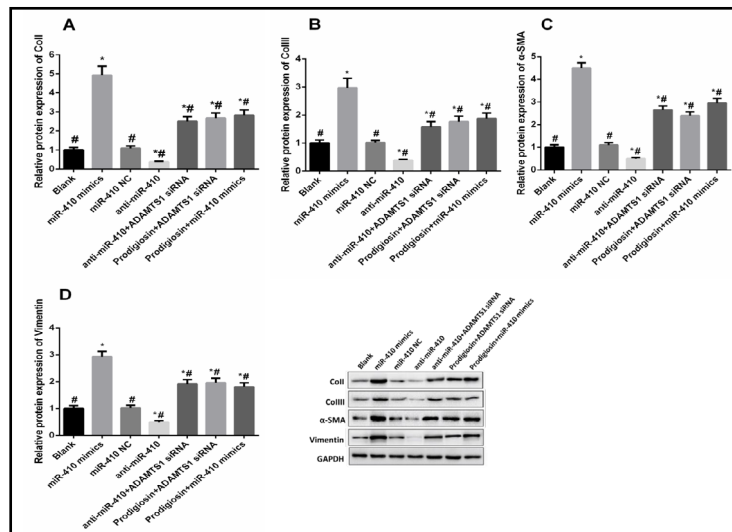**Cellular Physiology and Biochemistry**

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410 mimics on the proliferation of HEPF was significantly inhibited by Prodigiosin, P < 0.05 (Fig. 10).

Discussion

Pulmonary fibrosis is manifested as progressive dyspnea, and patients eventually died from respiratory failure. Mechanism of pathogenesis of the disease is not entirely clear and effective treatment has not been well developed. Therefore, understanding its pathogenesis, on the purpose of seeking curative therapy, has become the current focus of medical research. This study found that the pulmonary fibrosis model rats manifested as lung alveolar inflammation at early stage and pulmonary interstitial fibrosis at late stage, which was in consistent with reports in literatures [16], indicating that pulmonary fibrosis model has been successfully established. In this study, we found that prodigiosin significantly improved alveolar inflammation and reduced pulmonary fibrosis. The effect of prodigiosin was similar to that of hydrocortisone which is a conventional treatment of pulmonary fibrosis, indicating that Prodigiosinhas a good therapeutic effect on pulmonary fibrosis.

Fig. 10. MTT assay. * indicated P< 0.05 compared with Normal group, # indicated P< 0.05 compared with Model group.

Previous studies have shown that miR-410 plays a key role in the differentiation of bone marrow mesenchymal stem cells into osteoblasts induced by TGF-β3 [17]. MiR-410 regulates cartilage differentiation by targeting Wnt signaling pathway. The relationship between miR-410 and pulmonary fibrosis was not clear. Abnormal accumulation of extracellular matrix plays an important role in the process of pulmonary fibrosis. TGF-β1 is one of the most common cytokines that cause fibrosis, and plays a key role in accumulation of extracellular matrix. The mechanisms include: (1) induce fibroblasts, alveolar macrophages and other cells to synthesize and secrete extracellular matrix; (2) reduce degradation of extracellular matrix by stimulating the production of matrix metalloproteinase inhibitors; and (3) increases expression of integrin receptors on extracellular matrix and promotes adhesion and deposition of extracellular matrix [18]. ADAMTS-1 is the first member of the ADAMTS family and is produced by macrophages, vascular endothelial cells, fibroblasts and other cells. After secretion, ADAMTS-1 anchors in extracellular matrix mostly through the C-terminal 3 platelet binding protein motif and spacer region, which involved in regulation of extracellular matrix protein. Ng et al. [19] found that TGF-β1 can directly down-regulate ADAMTS-1 expression from *in vitro* experiments. Guo et al. [8]. reported that captopril increased expression of ADAMTS-1 by reducing expression of TGF-β1 thereby played a role of anti-myocardial fibrosis. In order to determine whether the anti-fibrosis effect of prodigiosin is related to TGF-β1/ADAMTS-1 signaling pathway, we detected the expression of TGF-β1 and ADAMTS-1 mRNA in lung tissue of rats.The results showed that the expression of TGF-β1mRNA in drug treatment groups were significantly lower than that in the model group, and the expression of ADAMTS-1 mRNA increased, suggesting that prodigiosin could decrease the expression of TGF-β1 and increase the expression of ADAMTS-1 thereby playing a role in anti-pulmonary fibrosis. MiR-410 was confirmed to be able to regulate expression of ADAMTS1 through dual luciferase reporter assay. The upregulation of miR-410 was directly involved in activation of TGF-β1/ADAMTS-1 signaling pathway, leading to pulmonary fibrosis.

Col Iand Col III are the main components of extracellular matrix in lung tissue and are important protein markers of fibroblasts. Col I and Col III increased significantly during pulmonary fibrosis, particularly ColI increased prominently. Shoda et al. [20] found that stimulation on rat lung fibroblasts can increase Col I and Col III synthesis, which increased extracellular matrix accumulation, causing pulmonary fibrosis in rats. Studies have shown that ADAMTS-1 plays a role in anti-fibrosis by degrading Col I and Col III [8]. In our study,
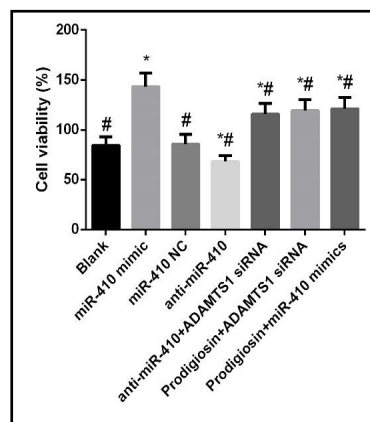
the expression of Col I and Col III mRNA in lung tissue wasdetected by qRT- PCR. The results showed that the expressions of Col I and Col III mRNA in model group were significantly higher than that in control group, which was in consistent with literature report [21]. After prodigiosin treatment, the expression of Col I and Col III mRNA was significantly decreased than that of the model group, which indicated that prodigiosin could promote degradation of Col I and Col III through TGF-β1/ADAMTS-1 signaling pathway. PICP and PIIINP are precursor polypeptides of Col I and Col III, which are free fragments released from precollagen into blood during precollagen-collagen transformation under action of specific peptide endopeptidase. They increase during collagen synthesis, as such it can reflect the levels of Col I and Col III, and can be used as an indirect indicator of fibrosis [22].It was found that the level of PICP and PIIINP was similar to that of Col I and Col III, demonstrating that prodigiosin could degrade Col I and Col III through TGF-β1/ADAMTS-1 signaling pathway thus playingarole in anti-pulmonary fibrosis [23].

Pulmonary fibrosis is the result of abnormal repair of alveolar epithelial cell [24]. In this process, epithelial cells were damaged, leading tothe release of growth factors, cytokines, causing fibroblasts to proliferate and transform into myofibroblasts. This results in extracellular matrix deposition, leading to destruction of the lung parenchyma [25, 26]. In this study, we used miR-410 mimics, miR-410 inhibitors and ADAMTS1 siRNA to transfect into HEPF cells, and observed cell proliferation after transfection and prodigiosin treatment. We found that HEPF cells transfected with miR-410 mimics had the strongest proliferative ability, and prodigiosin could significantly inhibit this abnormal proliferation.

Conclusion

In conclusion, our results suggest that prodigiosin could down-regulate miR-410 expression, inhibit the expression of TGF-β1 and up-regulate the expression of ADAMTS1, leading to decrease proliferation of fibroblasts. Prodigiosin alleviates pulmonary fibrosis through inhibiting miRNA-410 and TGF-β1/ADAMTS-1 signaling pathway.

Disclosure Statement

The authors declare to have no competing interests.

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