A study about the role of Wip1 in renal fibrosis by modulating macrophage phenotype

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Research Article

Keywords: Wip1, macrophage, phenotype transformation, renal fibrosis

Posted Date: February 7th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1222762/v1

Abstract

objective: To investigate the effect of wild type p53-induced phosphatase 1 (Wip1) on regulation the phenotype of macrophages and to participate in renal fibrosis.

Method: RAW264.7 macrophages were stimulated by lipopolysaccharide (LPS) and IFN-γ for 24h into M1 macrophages with high expression of iNOS and TNF-α. RAW264.7 macrophages were stimulated by interleukin 4(IL-4) for 24 h to induce M2 macrophages with high expression of Arg-1 and CD206. In the meantime, RAW264.7 macrophages were transduced with Wip1 lentivirus by overexpressing it, and transduced with Wip1 RNAi by reducing the Wip1 expression. Furthermore, after coculture the transformed macrophages and mice primary renal tubular epithelial cells, and the expression levels of E-Cadhrin, Vimentin and α-SMA were measured by RT-PCR.

Result: it was found that macrophages with Wip1 overexpression had no statistical changes on the expression of iNOS and showed the decreased expression of TNF-α; macrophages with Wip1 siRNA showed the increased expression of iNOS and TNF-α compared to control group. In the meantime, macrophages with Wip1 overexpression had increased expression of Arg-1 and CD206; macrophages with Wip1 siRNA showed decreased expression of Arg-1 and CD206 compared to control group. Macrophage RAW264.7could be transformed into the M2 macrophage after transducing with Wip1 lentivirus by overexpressing it, and the RAW264.7 macrophages could transform into the M1 macrophages by reducing the Wip1 expression via the Wip1 RNAi. Furthermore, after coculture the M2 macrophage and mice primary renal tubular epithelial cells, there was a decreased E-Cadherin expression, increased Vimentin, and α-SMA on the mRNA levels.

Conclusion: Wip1 in macrophages may participate in the pathophysiological process in the renal tubulointerstitial fibrosis by transforming them into the M2 macrophage phenotype.

Introduction

Renal fibrosis is mainly characterized by glomerulosclerosis and tubular interstitial fibrosis, and eventually develops into chronic renal failure, which is a serious threat to human health and life. The patients with chronic kidney failure have poor quality of life and high medical costs, which impose a severe burden on society and families. Therefore, to explore the pathogenesis of chronic renal fibrosis and to develop effective treatment methods has become an important challenge faced by health departments all over the world[1].

Clinical studies have found that most kidney diseases are characterized by macrophage accumulation, indicating that macrophages are involved in the occurrence and development of renal fibrosis[2]. Li et al. [3] modified macrophages to enable them to express the co-stimulating molecule VSIG4, thereby reducing the damage of renal tubulointerstitium by inhibiting T cell infiltration and secretion of inflammatory mediators. Zhao et al. [4-7] found that the mouse bone marrow and peripheral blood macrophages with Wip1 gene knockout secreted inflammatory factors, and the number of neutrophils increased

significantly, and their functions of phagocytosis, infiltration, inflammatory response and production of reactive oxygen species were also significantly enhanced, mainly through the p38MAPK-STAT1 and NF-κB pathways. In vitro, macrophage cell line Raw264.7 was transfected or RNAi interfered Wip1 gene and co-cultured with fibroblasts to establish microenvironment of renal fibrosis. The effect of Wip1 on the phenotype and function of macrophages and the mechanism of regulating renal fibrosis were studied, by inhibiting or slowing down the progression of renal fibrosis, the prevention and treatment of renal fibrosis may bring the new perspective.

Materials And Methods

Experimental reagent

RAW264.7 mouse mononuclear macrophage leukemia cells cells were purchased from the Kunming cell bank of the committee of typical culture preservation, Chinese academy of sciences. TGF-β1、 IFN-γ、TNF-α, purchased from Peprotech company; LPS purchased from Sigma; iNOS、 IL-4 、Arg-1、 CD206, purchased from Cell signaling technology. The genetic primer for Wip1 was synthesized by Takara. Plasmid extraction using the Omega plasmid large extraction kit. The protocol of study was approved by the ethics committee of Xi'an Jiaotong University.

Culture, Induction and identification of macrophages

The RAW264.7 macrophages in good condition were cultured and resuspended in 1640 culture medium of 1%FBS. The cell counting plate was used to count the cells, and the cell concentration was adjusted to 5×10^5/ml. The cells were seeded into a 6-well plate and 2ml of the above cell suspension was added into each well. Lipopolysaccharide (LPS 100 ng/ml) and IFN-γ (2.5 ng/ml) were added to each well for 12 hours to induce M1 macrophages. IL-4 (10ng/ml) was cultured in each well for 12 h to induce M2 macrophages. The cells in the 6-well plate were divided into 3 groups. After the induction period, the cells were gently washed with PBS for 3 times, replacing the liquid, and the changes in cell morphology were observed. The macrophage phenotype was detected by ELISA method: the level of iNOS, TNF-α, Arg-1 and CD206 in culture media were measured to determine the changes of macrophage phenotype. Take out the supernatant of the culture solution, adjust the sample gun to 100μl, add the standard sample and the sample to be tested, 100μl per hole. Add enzyme labeled coupling solution, 50μl per hole. After slightly shaking the pores, they were put into the water bath box and incubated for 60min. Wash plate 10 minutes before, prepare liquid A and liquid B and store them away from light. After incubation, take out 96-well plates and place them on the washing machine. Wash the plates 5 times. Add A, B liquid, after adding the sample, place the cover of the water bath box and incubate for 15 minutes in dark. Record time; At the end of incubation time, the enzyme plate was taken out and the water was sucked dry on the filter paper. Add termination fluid; OD value was measured on the enzyme marker for three times.

Construction of WIP1 overexpression and RNAi interference by lentivirus transfection

Construction and identification of lentiviral vector lenti-Wip1

The gene sequence of Wip1（Ppm1d-001）was searched in Genbank, the PCR primers(The sequence is Wip1 F 5’-ggttaattaaATGGCGGGGCTGTACTCGCTG-3’，Wip1 R 5’-ccgttaccTCAGCACACACACACTGTTTTCC-3’)were designed with Primer-3, and PacI and PmeI cleavage sites were added at the two ends of the primers, the fragment size was 1832 bp.

Transfection of 293T cells with lentiviral vector

Prepare 293T cells (cell density ~70%-80%) for transfection, and evenly spread 0.5~1×10^6 cells in a 5ml cell culture dish one day before transfection. The prepared transfection reaction system was added to the prepared culture dish of 293T cells, and the virus supernatant was collected after 48 hours of culture, then filtered and stored at-80 °C.

Construction of lentiviral vector LV-shWip1

According to the RNA interference (RNAI) sequence of Wip1 gene selected from websites, the first 3 pairs of the 10 pairs of sequences are usually selected in order to synthesize the target sequence (the approximate fragment size is 50 ~ 60 bp)

（1）shWip1-1#F：CCGGGCCCATCTTCTGAGTTGTAAACTCGAGTTTACAACTCAGAAGATGGGCTTTTTG

1#R:AATTCAAAAAGCCCATCTTCTGAGTTGTAAACTCGAGTTTACAACTCAGAAGATGGG

（2）shWip1-2#F：CCGGTCGAGTGAGGACGACGATTTACTCGAGTAAATCGTCGTCCTCACTCGATTTTTG

2#R:AATTCAAAAATCGAGTGAGGACGACGATTTACTCGAGTAAATCGTCGTCCTCACTCGA

（3）shWip1-3# F：CCGGGTATATTCTCACGCGGAATTTCTCGAGAAATTCCGCGTGAGAATATACTTTTTG

3#R:AATTCAAAAAGTATATTCTCACGCGGAATTTCTCGAGAAATTCCGCGTGAGAATATAC.

After enzyme digestion, the vector was linked to the synthetic target sequence, and the positive vector was confirmed by sequencing. The virus was packaged in 293T, the virus was collected, mononuclear macrophages were infected, the positive transfected cells were screened for drugs, RNA of positive cells was extracted, and the silencing efficiency of Wip1 gene was detected. Protein extraction, Western blot was used to detect the expression of lentiviral vector.

The phenotypic changes of macrophages were determined by ELISA

When mouse derived macrophage RAW264.7 was interfered with Wip1 overexpression and RNAi interference vector, it was found that macrophages with Wip1 overexpression had high expression of iNOS and TNF-α, while macrophages with RNAi interference had high expression of Arg-1 and CD206.

The Primary Cultured Renal Tubular epithelial cells were co-cultured with the above-mentioned macrophages, and the changes of fibrosis indexes were detected by RT-PCR

The kidney was taken from 5-6 weeks old healthy male BalB/C mice and put into a sterile petri dish; The kidney tissue was cut into pieces repeatedly with ophthalmic scissors until it was cut into 0.5 ~ 1mm^3 tissue pieces; 0.25% trypsin; Grind the kidney tissue on a sterile petri dish filter; 1ml was taken from the filtered cell suspension and the cell density after dilution was 5×105 ~ 1×106; The diluted cell suspensions were divided into culture vials, cultured in a CO2 incubator at 37 °C, and co-cultured with lentiviral over-expression vector Wip1 and RNAi vector to interfere with murine derived macrophages Raw264.7. After 72 hours, the cells were collected and the mRNA was extracted. The PCR product band was semi-quantitatively analyzed by gel image processing software and converted into numerical variables. Taking GADPH as internal reference, E-Cadhrin / GADPH, Vimentin / GADPH, α-SMA / GADPH, and computing the relative expression of E-Cadhrin, Vimentin, α-SMA.

Statistical

The data were processed by statistical analysis software SPSS13.0, and the measurement data were presented as mean ± standard deviation (). For inter-group comparison, the data were first tested for normality and homogeneity of variance. For multi-group comparison, one-way ANOVA was used, if the variance was equal to LSD test and if the variance was unequal to Dunnett t 3 tests, the difference was statistically significant ($p < 0.05$).

Results

Induction and identification of macrophages

RAW264.7 macrophages were cultured with lipopolysaccharide (LPS 100 ng/ml) and IFN-γ (2.5 ng/ml) for 12 h to induce M1 macrophages. The results showed that Raw264.7 macrophages were stimulated with LPS and IFN-γ for 24 hours to induce M1 macrophages with high expression of iNOS and TNF-α. RAW264.7 macrophages were stimulated with interleukin 4(IL-4) for 24 h to induce the M2 macrophages with high expression of Arg-1 and CD206 (figure 1).

Construction of Wip1 overexpression and RNAi interference by lentivirus transfection

Lentivirus was used to infect 293 cells and fibroblast cells respectively. After 24h of infection, the cells were obviously enlarged and spherical, the nuclei became larger, the adherence ability, decreased and the cells were easy to fall off. The infected positive cells were observed to be green under fluorescence microscope for 48h and 72h (figure 2A), and the infection efficiency was 83.28% as detected by flow cytometry. Western blot analysis of TIPE2 expression showed that the recombinant lentivirus infected 293T cells with Wip1 protein at 48h, and the infectious effect was the strongest at 72h, which was statistically different from the control group ($p<0.05$, see figure 2B,2D). The expression of Wip1 protein in constructing lentiviral vector LV-shWip1 infected 293T cells was decreased, which was statistically different from the control group ($p<0.05$, see figure 2C,2E).

Wip1 overexpression and RNAi vector were used to intervene macrophages, and the phenotypic changes of macrophages were detected by ELISA

When mouse derived macrophage RAW264.7 was interfered with Wip1 overexpression and RNAi interference vector, it was found that macrophages with Wip1 overexpression had no statistical changes on the expression of iNOS compared to the control group, and showed the decreased expression of TNF-α; macrophages with Wip1 siRNA showed the increased expression of iNOS and TNF-α compared to control group. In the meantime, macrophages with Wip1 overexpression had increased expression of Arg-1 and CD206 compared to the control group; macrophages with Wip1 siRNA showed decreased expression of Arg-1 and CD206 compared to control group. It was shown that after the intervention of Wip1 overexpression vector on mouse derived macrophages RAW264.7, macrophages transformed into m2-type macrophages, while after the intervention of RNAi interference vector on mouse derived macrophages RAW264.7, macrophages transformed into M1-type macrophages (figure 3).

Primary culture of mouse renal tubular epithelial cells, co-culture with above mentioned macrophages, and determination of changes in fibrosis indicators by RT-PCR

It was found that after co-culture with M2 and non-M1-type macrophages, the mRNA levels of E-Cadhrin in renal tubular epithelial cells decreased, and the mRNA levels of Vimentin and α-SMA increased, with statistically significant differences ($P < 0.01$, see figure 4).

Discussion

The early stage of renal fibrosis is mainly the infiltration and activation of T lymphocytes and macrophages to initiate renal fibrosis [8,9] . Macrophages can be divided into classically activated macrophages (M1) and activated macrophages (M2) types according to different characteristics such as inducible activation factors, secreted effector cytokines and phenotype. M1 macrophages release NO and promote the release of inflammatory factors through oxygen explosion, causing tissue damage and fibrosis. However, M2 macrophages were connected by FCγR, which combined with IgG immune complex, activates the ITAM motif and the downstream SyK, leads to the activation of PI3K, and converts the phenotype of the immune response to Th2 type, which has anti-inflammatory and tissue repair effects. Thus, the phenotypic and functional heterogeneity of macrophages, with strong plasticity, ultimately determines chronic inflammation and irreversible tissue damage [10,11].

Wip1 is a nucleoprotein belonging to the serine/threonine phosphatase family, encoded by the PPM1D (protein phosphatase magnesium-dependent 1 delta) gene, which is expressed in various organs of adult and embryonic mice [12]. Wip1 is mainly involved in DNA damage repair, apoptosis, inflammation, cell homeostasis and anti-aging by negatively regulating p38MAPK, p53, ATM, mdm2, Chk1/Chk2 and other kinases [13-17]. Chew et al [18] found that wip1-/-mice negatively modulate NF-κB signals, enhancing the body's inflammatory response; Wip1 can also inhibit NF-κB through negative regulation of P38MAPK, and reduce the expression of NF-κB-dependent inflammatory factors such as IL-1,6,8. Choi et al [19,20] found that wip1-/-mice had reduced T and B lymphocyte function and were more susceptible to pathogens. We transfected the macrophage RAW264.7 with lentivirus Wip1 to overexpress Wip1, and the macrophage transformed into M2-type macrophage, while the macrophage transformed into M1-type macrophage by interfering with Wip1 RNAi vector. Our results were consistent with the literature reports. It has been demonstrated that increasing the expression of Wip1 may play an important role in slowing the progression of renal fibrosis.

It was found that m1-type macrophage infiltration was dominant in the early 48 hours of renal ischemia injury, which aggravated the kidney injury. If macrophages were eliminated before injury, renal injury could be alleviated by promoting the proliferation and repair of renal tubular epithelial cells; During the recovery period, M2 type macrophages were predominant, which promoted the regeneration and repair of Renal Tubular epithelial cells [21]. There are also studies that suggest that M2-type macrophages produce a large amount of TGF-β, which promotes renal fibrosis. It is not completely clear whether this transformation of macrophage phenotype is beneficial or harmful to the body, but it is still difficult to confirm it in vivo [22]. Our study found that after co-culture with M2-type macrophages and non-M1-type macrophages, the mRNA level of E-Cadhrin in renal tubular epithelial cells decreased, and the mRNA levels of Vimentin and α-SMA increased, but the mechanism of M2-type macrophages and Wip1 in the role of renal fibrosis are still unclear. Specifically, which subtype of macrophages directly promotes renal fibrosis and the role of Wip1 still needs to be further explored. Next, we plan to study the anti-renal fibrosis mechanism of Wip1 through macrophage and verify our hypothesis.

Abbreviations

Wip1: wild type p53-induced phosphatase 1; LPS: lipopolysaccharide; IFN-γ: interferon-γ; IL-4: Interleukin 4; TNF-α: tumor necrosis factor-α; iNOS, Inducible Nitric Oxide Synthase; Arg-1: Arginase-1; NF-κB: Nuclear facto-κB; p38MAPK: *p38* mitogen activated protein kinases; α-SMA: *α*-smooth muscle actin; TGF-β: Transforming growth factor-β.

Declarations

Acknowledgements

We appreciate Drs. Fujian Lei and Jiamei Lu for their helpful discussions and critical review of the manuscript.This work was supported by theShanxi ProvinceFoundationfor Key R&D Projects (2020SF-158, J. LN).

Author contribution statement

Lining Jia: designed research and wrote thepaper; Hao Wang, Xiaotao Ma and Yinhong Wang: performed research and analyzed data; Rongguo Fu: analyzed data.

Funding Sources

This study was supported by the Shanxi Province Foundation for Key R&D Projects (2020SF-158, J. LN).

Availability of data and materials

The data of this study are available from the corresponding authorbut restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available.

Statement of Ethics

This study is an in vitro cell experiment without human participation.The study was performed in compliance with the Declaration of Helsinkiand approved by the ethics committee of Xi'an Jiaotong University. All experimental methods were performed in accordance with guidelines and regulations of the Ethics Committee of the second Affiliated Hospital of Xi’an Jiaotong University.

Consent for publication

Not applicable.

Conflict of Interest Statements

The authors declare that they have no competing interests.

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Figures

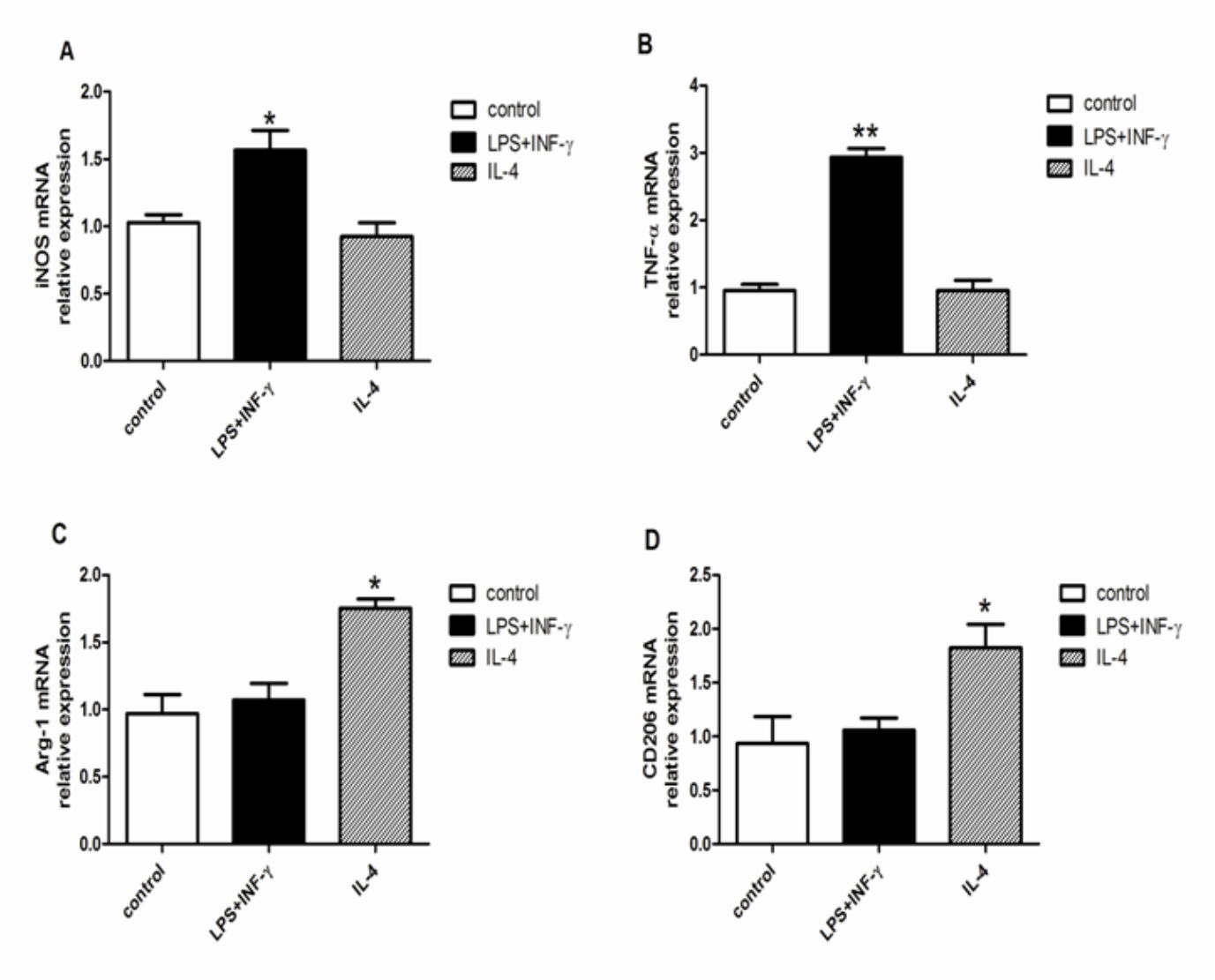

Figure 1

The changes of macrophage phenotype

RAW264.7 macrophages were stimulated by lipopolysaccharide (LPS) and IFN-γ for 12 h into M1 macrophages with high expression of iNOS and TNF-α). RAW264.7 macrophages were stimulated by interleukin 4(IL-4) for 12h to induce M2 macrophages with high expression of Arg-1 and CD206. (figure 1).

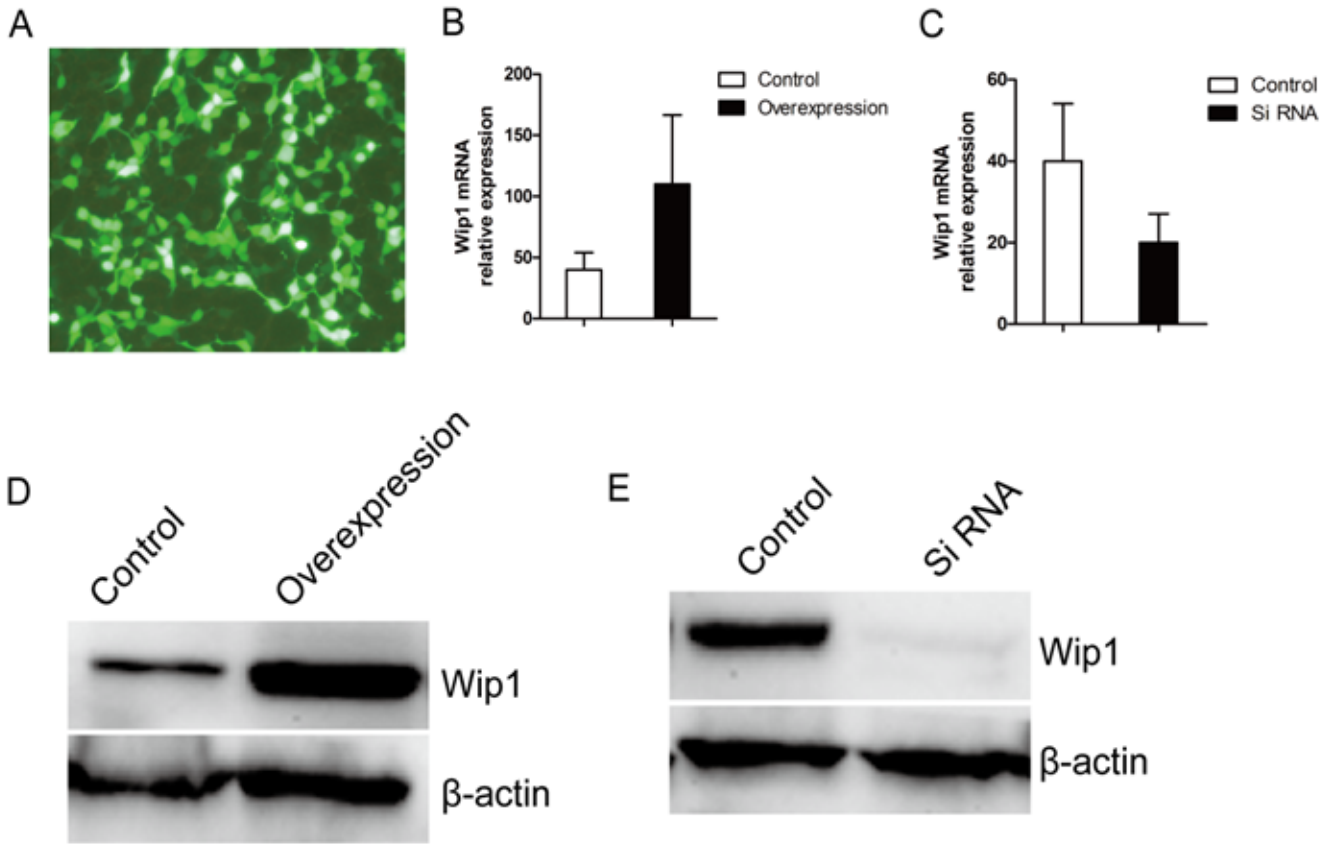

Figure 2

Construction and identification of lentiviral vector LV-shWip1

The 293 cells and fibroblast cells were infected by Lentivirus and the infected positive cells were observed to be green under fluorescence microscope for 48h and 72h (figure 2A). Wip1 expression was the strongest with the recombinant lentivirus infected 293T cells at 72h, which was statistically different from the control group ($p<0.05$, see figure 2B,2D). The expression of Wip1 protein in constructing lentiviral vector LV-shWip1 infected 293T cells was decreased, which was statistically different from the control group ($p<0.05$, figure 2C,2E).

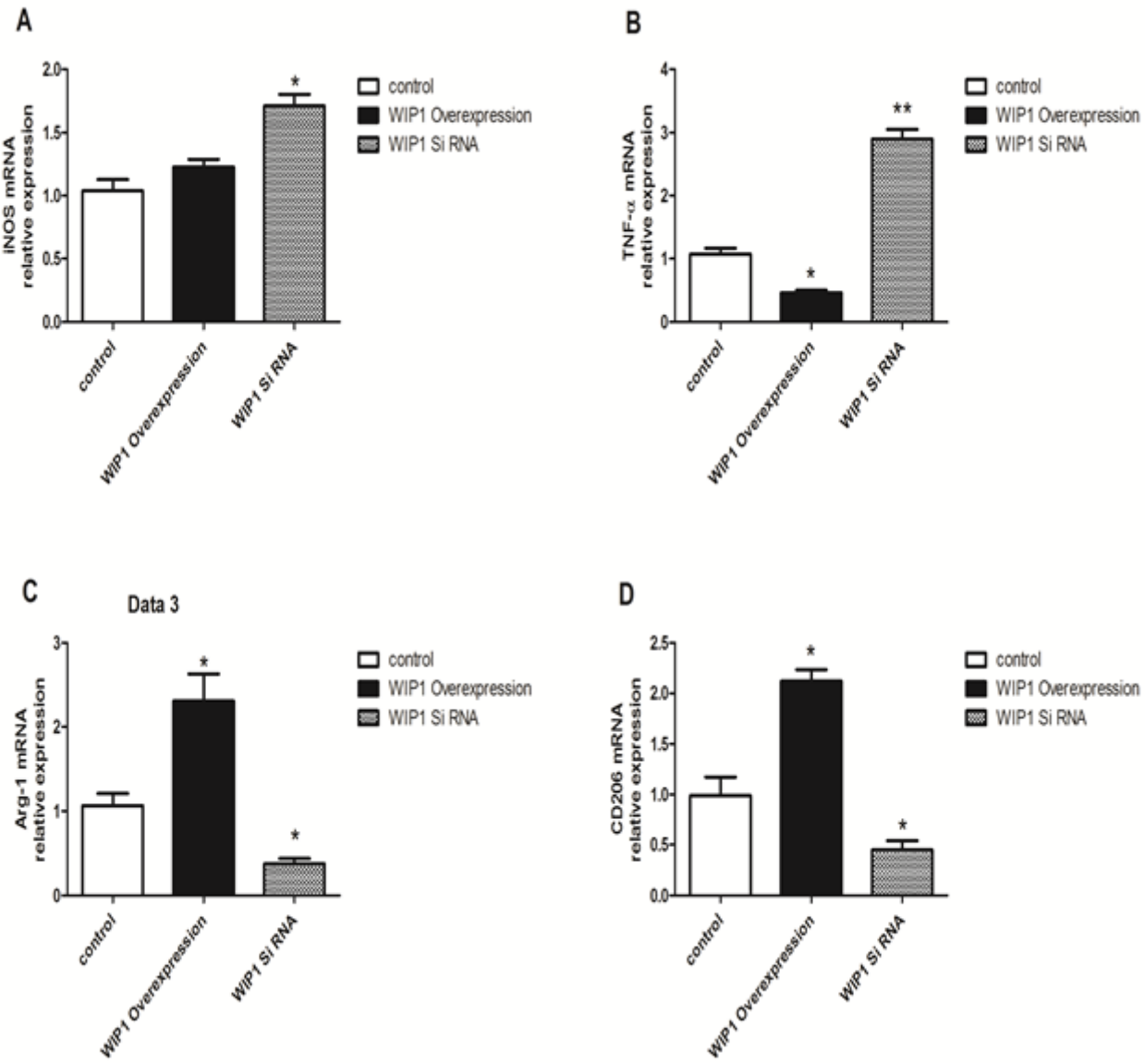

Figure 3

The phenotypic changes of macrophages were detected by ELISA

The macrophages with Wip1 overexpression had high expression of iNOS and TNF-α, while macrophages with RNAi interference had high expression of Arg-1 and CD206 after co-culture with the renal tubular epithelial cells and the macrophages transformed (figure 3).

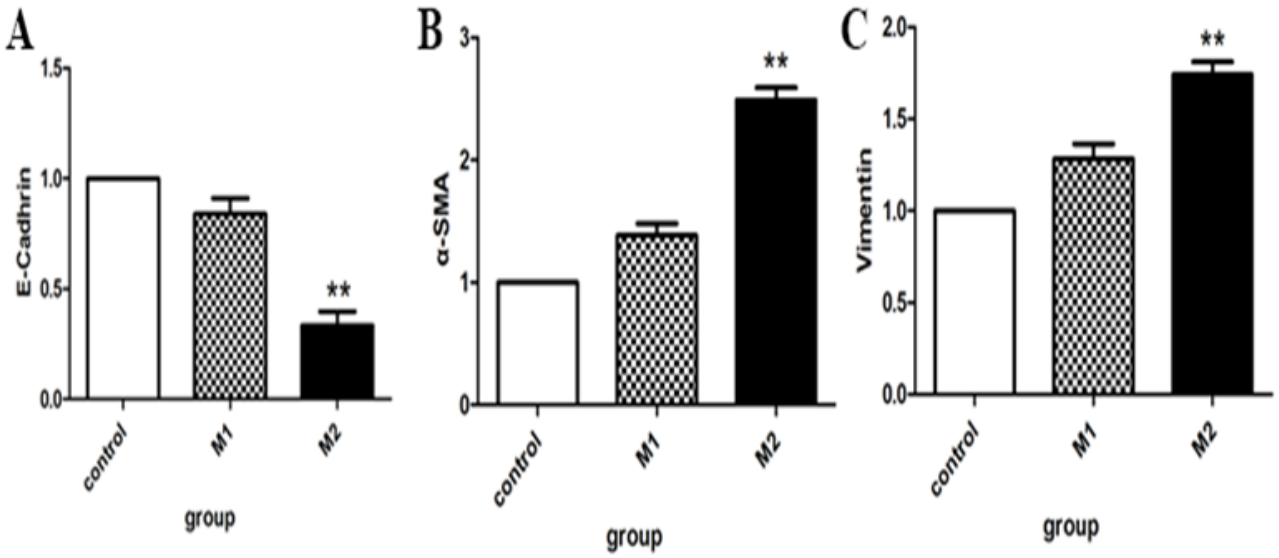

Figure 4

The fibrosis indicators were determined by RT-PCR

The mRNA levels of E-Cadhrin in renal tubular epithelial cells decreased, and the mRNA levels of Vimentin and α-SMA increased after, with statistically significant differences after co-culture with M2 and non-M1-type macrophages ($P < 0.01$, figure 4).