

WDR74 facilitates TGF- β /Smad pathway activation to promote M2 macrophage polarization and diabetic foot ulcer wound healing

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Abstract

Diabetes is a serious global health challenge that affects millions of people worldwide. One major complication is diabetic foot ulcer (DFU), leading to decreased quality of life and increased mortality in diabetic patients. The underlying mechanism of DFU is not completely understood. The mechanism involved in wound healing in diabetes was studied in vivo and in vitro. Gain-and loss-of function studies were performed to study the roles that WDR74 and TGF- β play in DFU. M1 and M2 macrophage phenotypic marker, and extracellular matrix (ECM) components, angiogenic makers were determined by RT-qPCR and/or Western blot analysis. Localization of these proteins were determined by immunofluorescence staining and/or immunohistochemistry. Interaction between WDR74 with Smad2/3 in macrophages was detected by co-immunoprecipitation. We found that WDR74 and M2 macrophages were decreased in wound tissues from DFU mice. TGF- β upregulation increased the expression of M2 macrophage markers (arginase-1, and YM1), IL-4, while decreased expression of M1 macrophage marker (iNOS). TGF- β upregulation also increased the production of ECM and promoted the wound closure in diabetic mice. We also noticed that WDR74 overexpression increased Smad2/3 phosphorylation elevated the population of M2 macrophage ECM production, and alleviated DFU. LY2109761 treatment normalized effects of TGF- β or WDR74 overexpression. In conclusion, WDR74 promoted M2 macrophage polarization, leading to improved DFU in mice, through the activation of TGF- β /Smad pathway.

Introduction

Diabetes is a severe pathological condition that affects about 387 million people worldwide, reaching over 8% of the world's population [1]. Among them, 1.5-5.1 million people died every year. Preventive measures and treatments are far from satisfactory. There will be more people dying of diabetes in the future, predicted to increase to 592 million by year 2035 [2]. This huge number of patients will be a tremendous economic burden on our society. Complications of diabetes severely affect the quality of life and life expectancy of patients. Recent studies demonstrated that diabetes affect many organs, including eye, kidney, heart, and brain [3-5]. It is also well-established that diabetic foot ulcer (DFU) is one serious complication. In China, over 8% of diabetic patients eventually developed foot ulcer [6]. Moreover, about 19-22% of patients with DFU had their foot amputated in China [7, 8]. Diabetic patients who have foot ulcers have a two-fold higher mortality rate than those who do not [9]. Therefore, it is important to further our understanding of DFU, which could lead to identifying improved treatments.

Several studies have shown that macrophages are involved in wound healing, including DFU [10, 11]. There are two phenotypes of macrophages, M1 and M2. M1 macrophage is considered to possess pro-inflammatory effects while M2 macrophages are considered to possess anti-inflammatory effects [12]. M2 macrophages have been shown to be involved in tissue repair and wound healing [13]. Moreover, macrophages are regulated by transforming growth factor-beta (TGF- β) [14]. Importantly, TGF- β has been shown to induce M2 macrophage polarization [15]. Furthermore, TGF- β is well-known to activate Small Mothers Against Decapentaplegic (SMAD) signaling pathway in various physiological and pathological processes [16-18], including the induction of M2 macrophage polarization [19]. More importantly, growth

factors, including TGF- β , have been implicated as potential treatments for DFU [20]. WD repeat 74 (WDR74) protein has initially been found to be involved in cancer metastases in 2010 [21, 22]. Importantly, WDR74 was recently discovered as a coactivator in TGF- β signaling [23]. To the best of our knowledge, WDR74 has never been linked to macrophage regulation and function, as well as DFU. Therefore, in the current study, we explored the potential effects of WDR74 in DFU with the involvement of TGF- β /Smad pathway using a mouse model.

Materials And Methods

Mice and diabetes induction

All mice were maintained in the pathogen-free animal facility and all animal studies have been approved by the Institutional Animal Care and Use Committee at The Affiliated Hospital of Southwest Medical University. Healthy male Kunming mice (3-5 weeks, 13-18 g, n=140) were provided by Laboratory Animal Center of The Affiliated Hospital of Southwest Medical University. Totally 15 mice received a saline injection and fed with normal diet as control, while 125 mice (n=125) received streptozotocin (45 mg/kg, i.p., 0.1 mmol/L sterile sodium citrate buffer, STZ, Sigma-Aldrich, St. Louis, MO, USA) after 6 weeks of high-sugar and high-fat diet to establish diabetic model. Seventy-two hours post STZ injection, blood glucose level was measured by a blood glucose monitoring system (Glucotrend 2, Roche Diagnostics, Mannheim, Germany). Mice with fasting blood glucose concentration over 16.7 mmol/L for at least 10 days were considered as diabetic mice. Five mice died, and therefore, success rate was 96.43%.

Excisional wound preparation

Two weeks after the establishment of diabetes model, mice were anesthetized with pentobarbital sodium (Sigma-Aldrich, St. Louis, MO, USA) at 50 mg/kg body weight. A 10 × 10 mm punch wound (excision of the skin and the underlying panniculus carnosus) were made on the instep of the mice. This wound was disinfected daily with 0.5% iodine. Control mice were defined as normal ulcer (NU, n=15). Diabetic mice further received different treatments (n=15/treatment): DFU, DFU + vehicle (veh), DFU + TGF- β , DFU + TGF- β + LY2109761, DFU + overexpression (oe)-negative control (NC), DFU + oe-WDR74, DFU + oe-WDR74 + veh, DFU + and oe-WDR74 + LY2109761 groups. Both oe-NC and oe-WDR74 were achieved by lentivirus injection (1×10^8 Tu/mL). Wound size was measured by tracking the edge of the wound on a transparent plastic film. Wound healing was observed under a light microscope during the experiment. On the 7th day after the wounding, 5 mice from each group were randomly selected and processed for hematoxylin and eosin (HE) staining, and 5 mice for immunohistochemistry and immunofluorescence assays while the rest were used for further studies.

Immunofluorescence

Paraffin-embedded tissue was cut into 5- μ m sections. Samples were then deparaffinized and quenched with 3% H₂O₂ for 10 min. For M2 macrophage immunofluorescence, sections were incubated with mouse primary antibody against F4/80 (1:100, Cat# 14-4801-82; Thermo Fisher Scientific, Waltham, MA, USA)

and CD206 (1:100, Cat# MA5-16871; Thermo Fisher Scientific, Waltham, MA, USA) overnight at 4°C. Samples were then probed with goat anti-mouse secondary antibody (1:200, ab150165 or ab150167; Abcam, Cambridge, UK). For colocalization between CD206 and WDR74, sections were incubated with primary mouse antibody against WDR74 (1:100, Cat# PA5-20924; Thermo Fisher Scientific, Waltham, MA, USA) and CD206 (1:100, Cat# MA5-16871; Thermo Fisher Scientific, Waltham, MA, USA) at 4°C overnight. Samples were then stained with goat anti-mouse immunoglobulin G (IgG) secondary antibody (ab150167, 1:200, Abcam, Cambridge, UK) or goat anti-rabbit IgG secondary antibody (1:200; ab150077; Abcam, Cambridge, UK). For angiogenesis, sections were incubated with primary antibody against CD31 (1:100, ab9498; Abcam, Cambridge, UK) overnight at 4°C. Samples were then incubated with goat anti-mouse secondary antibody (1:200, ab150167; Abcam, Cambridge, UK), followed by nuclear DAPI (Sigma-Aldrich, St. Louis, MO, USA) staining. Tissues were observed under an Olympus BX51 fluorescence microscope.

For immunofluorescence in cells, cells were permeabilized with 2% Triton X-100 for 15 min and with 2 M HCL for 20 min. Cells were blocked by 2% bovine serum albumin (BSA) for 45 min and then incubated with primary antibody against collagen 1A1 (COL1A1, 1:100, ab21286; Abcam, Cambridge, UK; rabbit), vascular endothelial growth factor (VEGF, 1:50, Cat#MA5-13182; Thermo Fisher Scientific, Waltham, MA, USA) or fibronectin (FN, 1:250, ab45688, Abcam, Cambridge, UK) overnight at 4°C. Cells were then incubated with goat anti-rabbit IgG H&L fluorescent secondary antibody (1:200, ab150165, 1:200; Abcam, Cambridge, UK), goat anti-mouse IgG H&L fluorescent secondary antibody (1:200, ab150117; Abcam, Cambridge, UK) or goat anti-mouse IgG H&L fluorescent secondary antibody (1:200, ab150115; Abcam, Cambridge, UK) for 2 h at room temperature, followed by DAPI (2 µg/mL) staining. Cells were mounted on a slide and observed under an Olympus BX51 fluorescence microscope.

Immunohistochemistry

Sections were deparaffinized with xylene and rehydrated by serial of gradient alcohol (100%, 100%, 95%, 85%, and 75%, 3 min each). Sections were then treated at high temperature and pressure for 2 min and allowed to cool down at room temperature. Sections were incubated with avidin for 10 min at room temperature, followed by 3 phosphate buffer saline (PBS)-washes with PBS. Sections were further incubated with d-biotin solution for 10 min at room temperature and washed 3 times with PBS. Endogenous peroxidase was eliminated by incubating with hydrogen peroxide for 15 min at room temperature. After washing with PBS the sections were blocked with 10% goat serum for 10 min at room temperature. Sections were incubated with primary antibodies (Abcam, Cambridge, UK) against TGF-β (1:100, ab215715, 1:100), Smad2/3 (1:100, ab217553) and phosphorylated Smad2/3 (1:100, ab63399) overnight at 4°C. After washing 3 times with PBS, sections were incubated with anti-biotin-labeled goat anti-rabbit IgG (1:1,000, ab150077; Abcam, Cambridge, UK) for 10 min at room temperature. Sections were then treated with streptomyacin avidin-peroxidase solution for 10 min at 37°C. Freshly prepared DAB were added and color change was observed under a microscope. After 20 s, sections were rinsed with water, counterstained with hematoxylin, dehydrated, and observed under a microscope.

Hematoxylin and eosin (HE) staining

Tissue sections were deparaffinized and rehydrated as mentioned above. Sections were then immersed in hematoxylin solution for 5 min, and washed with running water for 1 min. The sections were then immersed in 1% hydrochloric acid alcohol for 30 s and washed with running water for 1 min. After that, the sections were stained in 0.5% eosin solution for 3 min and washed with running water for 1 min. Then, the sections were dehydrated, mounted, and observed under a microscope.

Mouse primary macrophage culture

In order to isolate primary bone marrow-derived mouse macrophages, the tibias of mice were isolated and disinfected with 75% alcohol, then washed with precooling medium. The two ends of tibia were cut off, the bone marrow cells in tibia were washed out after absorbing culture medium, and red bone marrow could be seen. Bone marrow was slowly titrated into a single-cell suspension. The cell suspension was centrifuged at 4°C and 1600 rpm for 5 min with the supernatant discarded. The red blood cell lysis was used to lyse red blood cells for no more than one minute. Then, the red blood cells were washed twice with the pre-cooled RPMI 1640 medium with 5 times of the volume of red blood cell lysis at 4°C and 1600 rpm. After that, the cells were resuspended in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FBS) and 20ng/ml Recombinant Murine M-CSF and seeded into 6-well plate in the incubator at the concentration of 5×10^5 cells/well. After 3 days incubation, the medium and the unattached cells were removed, and the attached macrophages were cultured for 3 days for different treatments. Control macrophages were treated with interleukin (IL)-13 (10 ng/mL), IL-13 + TGF- β (5 ng/mL), TGF- β , over-Expression (oe)-WDR74, oe-NC, short hairpin (sh)-WDR74, sh-NC, IL-13 + oe-WDR74, or IL-13 + sh-WDR74. oe-NC, sh-NC, sh-WDR74 and oe-WDR74 were infected into macrophages by lentivirus at 2×10^6 TU/mL.

Effect of macrophage-derived conditioned medium on fibroblast

Mouse NIH3T3 fibroblasts were seeded onto culture plates with a density of 15,000 cells/cm² in DMEM containing 10 % FBS and high glucose. The next day, culture medium was replaced with conditioned medium (CM) derived from macrophages. CM was collected from primary macrophages (Mo-CM), IL-13-stimulated macrophages (M2-CM), TGF- β -stimulated macrophages (TGF- β -CM), IL-13 and TGF- β co-stimulated macrophages (TGF- β -M2-CM), IL-13-stimulated macrophages infected with oe-NC (oe-NC-M2-CM), IL-13-stimulated macrophages infected with oe-WDR74 (oe-WDR74-M2-CM), IL-13-stimulated macrophages infected with sh-NC (sh-NC-M2-CM), or IL-13-stimulated macrophages infected with sh-WDR74 (sh-WDR74-M2-CM). After cultured for 72 h, COL1A1, FN and VEGF expression in fibroblasts were detected by Western blot analysis.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted by Trizol RNA (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using Primescript RT reagent kit (RR037A, TaKaRa, Dalian, China). Target genes (primers are shown in Table 1) were amplified by a real-time PCR system (ABI 7500, Applied Biosystems, Foster City,

CA, USA). PCR reaction system consisted of 2.5 μ L 10 $\times$ PCR Buffer, 2.5 μ L 25 mmol/L $MgCl_2$, 1.5 μ L 10 mmol/L dNTP, 0.5 μ L 10 mmol/L primer, 0.25 μ L Taq, 2.5 μ L cDNA and 15.25 μ L sterile distilled water. Reaction conditions were as follows: 40 cycles of denaturation at 94 °C for 5 min, at 94 °C for 30 s, at 58 °C for 45 s and at 72 °C for 30 s, and extension at 72 °C for 10 min. Each sample was tested in triplicates. β -actin was used as an internal reference. mRNA expression level of target genes was calculated by the $2^{-\Delta\Delta Ct}$ method [24].

Western blot analysis

Wound tissues or cells were treated with lysis buffer containing phenylmethylsulfonyl fluoride and phosphatase inhibitor followed by centrifugation at 12,000 r/min for 30 min at 4°C. Total protein concentration in the supernatant was measured by a BCA kit. Protein (50 μ g) was dissolved in 2 $\times$ SDS loading buffer and boiled at 100°C for 5 min. Proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membrane. Membrane was blocked by skim milk (5%) at room temperature for 1 h and rinsed 3 times with tris-buffered saline with Tween 20 (TBST). Membrane was incubated with primary antibodies against COL1A1 (1:1,000, ab34710), FN (1:1,000, ab45688), VEGF (1:1,000, ab69479), CD31 (1:1,000, ab9498), Smad2/3 (1:500, ab217553), phosphorylated Smad2/3 (1:5,000, ab63399), arginase-1 (1:1,000; #93668; Cell Signaling, Danvers, MA, USA), YM1 (1:10,000; ab192029), inducible nitric oxide synthase (iNOS; 1:500, ab178945) and β -actin (1:1,000, ab8226). After washing 3 times with TBST, membranes were incubated with horseradish peroxidase-labeled secondary antibodies (1:10000, ab6721 and ab205719) for 1 h. The above antibodies except for arginase-1 were purchased from Abcam (Cambridge, UK). After rinsing 3 times with TBST, the membrane was developed by electrogenerated chemiluminescence (ECL) kit (BB-3501, Amersham, Buckinghamshire, UK) in Bio-Rad Image Analysis System (Bio-Rad, Hercules, CA USA), and analyzed by Quantity One v4.6.2 software. Relative protein expression was expressed by gray intensity of protein bands in target protein normalized by that of β -actin protein band. Each sample was tested in triplicates.

Co-immunoprecipitation

Cells were harvested and lysed in cell lysis containing protease (Roche, Basel, Switzerland) and phosphatase inhibitor (Thermo Fisher Scientific, Waltham, MA, USA) for 30 min at 4°C and cleared cell lysates were collected by centrifugation at 4°C (16,000 g, 30 min). Supernatant was immunoprecipitated overnight at 4°C with WDR74 antibody (1:50, Cat# PA5-83390; Thermo Fisher Scientific, Waltham, MA, USA). Lysate was then incubated with 30 μ L of protein G plus-sepharose (Amersham, Buckinghamshire, UK) for 2 h at 4°C. Beads were precipitated and washed three times with immunoprecipitation buffer. Western blot analysis was performed to determine Smad2, Smad3, and WDR74 protein expression.

Statistical analysis

All data were analyzed using SPSS 21.0 (IBM Corp. Armonk, NY, USA). Measurement data were expressed as mean $\pm$ standard deviation. Unpaired *t*-test was used to compare two groups of unpaired data obeying

normal distribution and homogeneity of variance. One-way analysis of variance (ANOVA) and Tukey's post hoc test was used for comparing data among multiple groups. Differences were considered significant when $p < 0.05$.

Results

M2 macrophages are decreased in mouse DFU model

Accumulating evidence has shown that macrophage polarization plays a critical role in the wound healing of DFU. Therefore, we established the murine model of DFU and evaluated the polarization status of macrophages. We have found that the number of M2 macrophages (CD206⁺ F4/80⁺ positive cells) in wounded tissues from DFU mice was significantly lower than that from NU mice (Figure 1A). Meanwhile, macrophage M1/M2 polarization status was determined by RT-qPCR and Western blot analysis. The results demonstrated that the macrophages from wounded tissue in DFU mice expressed significant higher level of M1 macrophage marker (iNOS) and lower level of M2 macrophage markers (arginase, YM1, and IL-4) compared with that from NU mice (Figure 1B, C). These results suggested that macrophages polarized to M1 instead of M2 in DFU mice.

Activation of TGF- β /Smad pathway promotes polarization of M2 macrophages to reduce DFU in mice

TGF- β has previously been shown to promote M2 macrophage polarization via regulating Smad2/3 phosphorylation status [25]. Moreover, abnormal macrophage phenotypical transition plays a critical role in the pathogenesis of diabetic foot ulcer (DFU) [26]. So, it is reasonable to speculate that in disrupted macrophage M2 polarization is caused by interrupted TGF- β /Smad2/3 signaling. In order to investigate if macrophage polarization is regulated by TGF- β in DFU, we established the murine DFU model and evaluated the expression levels of TGF- β and Smad2/3 phosphorylation status. We have noticed that the expression of TGF- β and Smad2/3 phosphorylation was significantly decreased in DFU mice (Figure 2AB). On the other hand, TGF- β treatment resulted in significantly increased Smad2/3 phosphorylation and elevated expression of M2 phenotypic markers as well as decreased M1 phenotypic markers. Moreover, these changes could be abolished by LY2109761, a TGF- β receptor inhibitor (Figure 2C). Consistently, immunofluorescence staining revealed that TGF- β resulted in an increase of M2 macrophage (CD206⁺ F4/80⁺) while LY2109761 neutralized the promotive effect of TGF- β (Figure 2D). These results suggested that TGF- β treatment activated Smad2/3 pathway and promoted M2 macrophage polarization. We further asked whether TGF- β and Smad2/3 is involved in the process of wound healing. We investigated the healing of the wounded tissue by evaluating the extracellular matrix (ECM) and angiogenesis-related proteins in DFU mice. We have found that Col1a1, FN, VEGF, and CD31 expression was increased by TGF- β treatment, while LY2109761 inhibited the effects of TGF- β (Figure 2E). Similarly, immunofluorescence staining showed that DFU mice displayed decreased CD31 at wounded tissue compared with that of NU mice. However, overexpressing TGF- β led to an increased CD31 in DFU mice, which was reversed by LY2109761 (Figure 2F). Furthermore, results from histological analysis of wounded tissue and wound closure observation showed that exogenous TGF- β treatment

promoted wound healing and this promotive effect could be neutralized by addition of LY2109761 (Figure 2GH). The results suggested that TGF- β /Smad pathway promoted polarization of M2 macrophages and promoted wound healing in DFU mice.

Activation of TGF- β /Smad pathway promotes M2 macrophage polarization and ECM production in fibroblasts in vitro

Our results have demonstrated that TGF- β treatment resulted in an increase of M2 macrophage population at wounded tissue in vivo, this finding suggested that TGF- β promotes M2 polarization of macrophage. In order to confirm this finding in vitro, primary macrophages were isolated from peritoneal cavity of mice were used. Macrophages were treated with IL-13 (a known stimulator of M2 macrophage polarization), TGF- β , or combination of IL-13 and TGF- β . Western blot analysis described that expression of arginase-1, YM1, and IL-4 was significantly increased while iNOS expression was significantly decreased by the treatment of TGF- β or IL-13. The effect of TGF- β or IL-13 was further promoted by treatment of IL-13 + TGF- β combination (Figure 3A).

Furthermore, we cultured fibroblasts with CM derived from macrophages. We found that expression of ECM-related proteins (Col1a1, FN and VEGF) was increased in fibroblasts cultured with TGF- β -Mo-CM or M2-CM, and further increased by co-culture of TGF- β -M2-CM (Figure 3B). Moreover, immunofluorescence images of fibroblasts showed similar results (Figure 3C). In conclusion, in vitro activation of TGF- β /Smad pathway promoted M2 macrophage polarization and ECM production in fibroblasts.

Upregulation of WDR74 in vitro activates TGF- β /Smad pathway to promote M2 macrophage polarization and fibroblast ECM production

A previous study showed that WDR74 bound to Smad to activate TGF- β /Smad pathway [23]. Therefore, we first determined the relationship between WDR74 and Smad2/3 *in vitro*. Immunoprecipitation study revealed that WDR74 interacted with Smad2/3 in mouse macrophages (Figure 4A). Interestingly, immunofluorescence depicted that WDR74 protein expression was significantly reduced in wound tissue from DFU mice versus NU mice ($p < 0.05$; Figure 4B). Similarly, RT-qPCR presented that WDR74 expression was also lower in isolated macrophages from DFU mice versus that from NU mice (Figure 4C). Moreover, we used overexpression or knockdown of WDR74 to determine the effect of WDR74 on macrophages. Treatment of oe-WDR74 elevated WDR74 expression, while treatment of sh-WDR74, sh-WDR74-1 or sh-WDR74-2 reduced WDR74 expression, among which the lowest expression occurred after treatment sh-WDR74. Therefore, sh-WDR74 was selected for following experiments (Figure 4C). Overexpression of WDR74 increased arginase-1, YM1 and IL-4 protein expressions, while decreased iNOS expression. In contrast, WDR74 silencing reduced protein expression of arginase-1, YM1 and IL-4, while enhanced iNOS expression. Moreover, the effects of treatment of IL-13 alone were promoted by treatment of IL-13 + oe-WDR74 but reversed by the treatment of IL-13 + sh-WDR74. In addition, overexpression of WDR74 significantly increased the expression of phosphorylated Smad2/3, suggesting an activation of Smad2/3 (Figure 4D). In fibroblasts co-cultured with CM, culture with oe-WDR74-M2-CM increased protein expression of COL1A1, FN and VEGF, while culture with sh-WDR74-M2-CM reduced expression of these

proteins (Figure 4E). In short, upregulation of WDR74 in macrophages activated TGF- β /Smad pathway to promote M2 macrophage polarization and fibroblast ECM production.

Upregulation of WDR74 facilitates TGF- β -mediated Smad pathway activation to promote polarization of M2 macrophages and alleviate DFU in vivo

To investigate the effect of WDR74 on DFU *in vivo*, we overexpressed WDR74 in DFU mice and the up-regulation was evaluated by RT-qPCR (Figure 5A) and immunofluorescence staining (Figure 5B). Moreover, WDR74 overexpression increased expression of phosphorylated Smad2/3 in DFU mice, suggesting activation of TGF- β /Smad pathway (Figure 5C). Immunofluorescence staining was applied to detect the number of M2 macrophages. The results showed that WDR74 overexpression also elevated the number of M2 macrophages in wound tissues from DFU mice, while addition of LY2109761 inhibited this effect (Figure 5D). Western blot analysis demonstrated that WDR74 overexpression increased expression of arginase-1, YM1, IL-4, COL1A1, FN, VEGF and CD31 in wound tissues from DFU mice, but decreased iNOS protein expression (Figure 5E). Conversely, the effects of WDR74 overexpression was neutralized by addition of LY2109761 (Figure 5E). Immunofluorescence staining also exhibited that upregulation of WDR74 promoted CD31 expression in wound tissue from DFU mice, whereas LY2109761 abolished this effect (Figure 5F). Lastly, upregulation of WDR74 facilitated wound closure in DFU mice, while LY2109761 neutralized this effect (Figure 5G,H). These results suggest that overexpression of WDR74 *in vivo* in macrophages activated TGF- β /Smad pathway to promote polarization of M2 macrophages and reduce DFU.

Discussion

Diabetes is one of the severe diseases in the world, and its incidence has increased dramatically in recent decades [27]. Despite tremendous efforts exerted on diabetes prevention and treatment, the number of diabetes patients is predicted to reach 592 million by 2035 [1, 2]. One serious complication of diabetes is DFU, which doubles the mortality rate of diabetes patients [9]. The current study investigated the mechanism of progression of this serious but not well understood complication. In wound tissue collected from diabetic mice, WDR74 expression and the amount of M2 macrophage were decreased. These results led to hypothesize that WDR74 would increase M2 polarization in macrophages, leading to improved wound healing in DFU. We found that overexpression of WDR74 *in vitro* activated TGF- β /Smad pathway, which further promoted M2 macrophage polarization and fibroblast ECM production. We further our investigation *in vivo* and found that WDR74 overexpression activated TGF- β /Smad pathway, improved M2 macrophage polarization, and enhanced wound closure in diabetic mice. These results provide a backbone for further research on the effects of WDR74 and TGF- β /Smad pathway on wound healing in DFU.

TGF- β , a multifunction cytokine, acts on protein kinase receptors at the plasma membrane to induce various biological functions including immune response, inflammation, angiogenesis, cell growth, and death [28]. These functions led to extensive research into the effects of TGF- β on biology, pathology, and

treatment of cancers [28-30]. Due to its function as a regulator of cell growth, TGF- β has been studied for tissue repair and wound healing [31]. In particular, a previous study has bridged the TGF- β /Smad signaling and diabetic wound healing [32]. A previous research have shown a number of related mediators that involves in TGF- β -mediated improvement of wound healing, including increased levels of ECM components (collagen and α -smooth muscle actin), decreased levels of proinflammatory cytokines (IL-1 β , IL-6 and tumor necrosis factor- α), and increased angiogenesis (vascular endothelial growth factor and platelet-derived growth factor) [33-36]. In addition, a recent study showed that Smad2/3 was also involved in in TGF- β -mediated wound healing in DFU [35]. Our result confirmed this finding and added a novel mechanistic insight in WDR74.

WDR74 is a relatively newly discovered protein among WD repeat proteins. In 2010, WDR72 was shown to be related to complications of diabetes [37]. WDR74 act as a co-activator of TGF- β /Smad pathway, which has been recently demonstrated [23]. Because there is no report suggesting that WDR72 is related to macrophages, ECM, and anti-inflammatory functions, we speculate the effects on WDR72 on M2 macrophage polarization, increased formation of ECM, and reduction of wound size are indirect and relied on the activation of TGF- β /Smad pathway.

M2 macrophages are alternatively activated and considered anti-inflammatory, which are generally preferred in alleviating disease states [12]. Our initial finding that polarization of macrophages to M1 rather than M2 in DFU led us to hypothesize that M2 macrophage polarization may be beneficial for DFU wound healing. We found that increased M2 macrophage polarization led to reduced wound size in diabetic mice, which is in alignment with a previous study [13]. In addition, we found that polarization to M2 macrophages were increased by TGF- β treatment both *in vitro* and *in vivo*. Similar results were also obtained showing that TGF- β regulated and induced M2 macrophage polarization [14, 15]. Therefore, our results solidified the hypothesis that TGF- β -mediated M2 macrophage polarization is critical in wound healing in DFU. In addition, it was reported that the cGAS/Sting pathway assumed a pivotal role in the inflammasome activation [38]. A prior study further indicated that the cGAS/Sting pathway could induce NLRP3 activation and cause the development of intervertebral disc degeneration [39]. More importantly, a prior research evidenced that the agonist of human Sting α -Mangostin could polarize human monocyte-derived M2 macrophages to the M1 phenotype [40]. Also, Martin *et al.* discovered that Sting activation was able to repolarized M2 macrophages towards an M1-like subtype in mice with experimental colitis [41]. These results indicated that the Sting pathway might augment M2-to-M1 polarization to promote inflammation response, thus assuming a role in wound healing of DFU. It was demonstrated that activated NLRP3 could promote macrophage M1 polarization [42], or that M1 polarization of macrophages could activate NLRP3 inflammatory bodies [43, 44]. Meanwhile, inflammasome-independent NLRP3 was capable of activating TGF- β pathway and increasing Smad3 phosphorylation and activity in kidney epithelium [45]. These results triggered our conjecture whether cGAS/Sting/NLRP3 pathway was involved in the effects of WDR74 on TGF- β and M2 polarization, which will be further explored in our future studies.

Conclusion

In conclusion, we demonstrated that WDR74 facilitates TGF- β mediated Smad2/3 activation in macrophage leading to increased M2 polarization, which, in turn, increased ECM formation, and improved wound healing in a mouse model of DFU (Figure 6). Our results further suggest that WDR74/TGF- β activation warrants further investigation as a therapeutic target for DFU.

Declarations

AUTHOR CONTRIBUTIONS

Youhua Xu provided the scope of the research, conceived the manuscript structure. Kang Geng and Xiumei Ma oversaw the writing of the manuscript and drafted the final version of the manuscript. Zongzhe Jiang, Huang Wei, Junling Gu, and Weiming Wang were involved in data collection. Xiumei Ma and Yong Xu performed the statistical analysis and preparation of the Figures. Kang Geng and Youhua Xu drafted the paper. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest

The authors have no declarations of interest.

Ethics approval

Ethical approval was obtained from the Institutional Animal Care and Use Committee at The Affiliated Hospital of Southwest Medical University.

Consent to participate

Informed consent was obtained where applicable.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/Supplementary Material.

Consent for publication

All authors have read and agreed to the published version of the manuscript.

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Tables

Table 1 is not available with this version.

Figures

Figure 1

Mouse DFU model exhibits decreased macrophage M2 polarization. A) Expression of CD206, F4/80 detected by immunofluorescence (200 ×) in mouse wound tissue. mRNA (B) and protein (C) expression of

M1 and M2 macrophage polarization markers. * $p < 0.05$ vs. NU mice. Measurement data were expressed as mean $\pm$ standard deviation. Unpaired t -test was used to compare two groups of data. $n = 5$.

Figure 2

TGF- β /Smad pathway activation induces wound healing by increasing polarization of M2

by immunohistochemistry (200 $\times$). B) Phosphorylated Smad2/3 protein expression in mice after model establishment determined by immunohistochemistry (200 $\times$). DFU mice were treated with veh, TGF- β or TGF- β + LY2109761. C) Expressions of M1 and M2 macrophage polarization markers in mice detected by Western blot analysis. D) Protein expression of CD206 and F4/80 (markers for M2 macrophages) in mice determined by immunofluorescence (200 $\times$). E) Protein expressions of Col1A1, FN, VEGF and CD31 in mice detected by Western blot analysis. F) CD31 expression in mice determined by immunofluorescence (200 $\times$). G) Morphological changes in wound tissues on 7th day of DFU determined by HE staining (200 $\times$). H) Wound size of mice. * $p < 0.05$ vs. NU mice; # $p < 0.05$ vs. DFU mice treated with veh; & $p < 0.05$ vs. DFU mice treated with TGF- β . Measurement data were expressed as mean $\pm$ standard deviation. Unpaired t -test was used to compare two groups of data. One-way analysis of variance (ANOVA) and Tukey's post hoc test was used for comparing data among multiple groups. $n = 5$.

Figure 3

In vitro activation of TGF- β induces M2 macrophage polarization and fibroblast ECM production. A) Protein expressions of arginase-1, YM1, IL-4 and iNOS in control macrophages treated with TGF- β and IL-13. * $p < 0.05$ vs. control macrophages; # $p < 0.05$ vs. control macrophages treated with IL-13. B) Protein expression of COL1A1, FN and VEGF in fibroblasts cultured with CM derived from macrophages determined by Western blot analysis. * $p < 0.05$ vs. fibroblasts cultured with Mo-CM; # $p < 0.05$ vs. fibroblasts cultured with M2-CM. C) Expression of COL1A1, FN and VEGF protein in fibroblasts cultured with CM derived from macrophages determined by immunofluorescence (200 $\times$). Measurement data were expressed as mean $\pm$ standard deviation. One-way analysis of variance (ANOVA) and Tukey's post hoc test was used for comparing data among multiple groups. The cell experiment was repeated 3 times.

Figure 4

WDR74 overexpression *in vitro* induces M2 macrophage polarization and fibroblast ECM production by activating TGF- β /Smad pathway. A) Interaction between WDR74 and Smad2/3 determined by immunoprecipitation. B) Expression of WDR74 in wound tissue after model establishment determined by immunofluorescence (200 $\times$, n = 5). * $p < 0.05$ vs. NU mice. C) mRNA expression of WDR74 in control macrophages with altered WDR74. * $p < 0.05$ vs. control macrophages; # $p < 0.05$ vs. control macrophages treated with oe-NC; & $p < 0.05$ vs. control macrophages treated with sh-NC. D) Protein expression of WDR74, arginase-1, YM1, IL-4, Smad2/3, phosphorylated Smad2/3 and iNOS in control macrophages with altered WDR74 and IL-13 determined by Western blot analysis. * $p < 0.05$ vs. control macrophages treated with oe-NC; # $p < 0.05$ vs. control macrophages treated with sh-NC; & $p < 0.05$ vs. control macrophages treated with IL-13. E) Protein expressions of COL1A1, FN and VEGF in fibroblasts cultured with CM derived from macrophages determined by Western blot analysis. * $p < 0.05$ vs. fibroblasts cultured with oe-NC-M2-CM; # $p < 0.05$ vs. fibroblasts cultured with sh-NC-M2-CM. Measurement data were expressed as mean $\pm$ standard deviation. Unpaired *t*-test was used to compare two groups of data. One-way analysis of variance (ANOVA) and Tukey's post hoc test was used for comparing data among multiple groups. The cell experiment was repeated 3 times.

Figure 5

WDR74 upregulation promotes M2 macrophage polarization and suppresses DFU *in vivo* by activating TGF- β /Smad pathway. A) mRNA expression of WDR74 in wound tissues of DFU mice after WDR74 overexpression. B) Colocalization of CD206 and WDR74 proteins in wound tissues of DFU mice after WDR74 overexpression determined by immunofluorescence (200 $\times$). C) Expression of Smad2/3 and phosphorylated Smad2/3 in wound tissues of DFU mice after WDR74 overexpression by immunohistochemistry (200 $\times$). D) CD206 positive cells in DFU mice after WDR74 overexpression and TGF- β inhibition determined by immunofluorescence (200 $\times$). E) Protein expression of M1, M2 macrophages, angiogenesis, and ECM related molecules in DFU mice after WDR74 overexpression and TGF- β inhibition determined by Western blot analysis. F) CD31 expression in DFU mice after WDR74 overexpression and TGF- β inhibition determined by immunofluorescence (200 $\times$). G: Morphological changes in wound tissues in DFU mice after WDR74 overexpression and TGF- β inhibition determined by HE staining (200 $\times$). H) Wound size in DFU mice after WDR74 overexpression and TGF- β inhibition. * $p < 0.05$ vs. DFU mice treated with oe-NC; # $p < 0.05$ vs. DFU mice treated with oe-WDR74 + veh. Measurement data were expressed as mean $\pm$ standard deviation. Unpaired *t*-test was used to compare two groups of data. One-way analysis of variance (ANOVA) and Tukey's post hoc test was used for comparing data among multiple groups. n = 5.

Figure 6

Schematic diagram showing the proposed mechanism that involves WDR74 and TGF- β /Smad pathway in wound healing in DFU. WDR74 expression and M2 macrophages were low in wound tissues from a mouse model of DFU. WDR74 overexpression activates TGF- β /Smad pathway to promote M2 macrophage polarization, leading to wound healing in DFU.