Am J Transl Res 2017;9(11):5150-5159
www.ajtr.org /ISSN:1943-8141/AJTR0067915

Original Article

Triptolide protects podocytes from TGF-β-induced injury by preventing miR-30 downregulation

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Received October 25, 2017; Accepted November 11, 2017; Epub November 15, 2017; Published November 30, 2017

Abstract: Triptolide is known to have a strong anti-proteinuric effect through direct protection of podocytes from injury and is used to treat glomerular diseases. However, the mechanism underlying its protective effect on podocytes remains elusive. MiR-30 family has recently been shown to be essential for structural and functional homeostasis of podocytes but is downregulated by injurious factors, leading to podocyte injury. In the present study, we explore whether Triptolide protects podocytes through preventing miR-30 downregulation. Since TGF-β signaling is a critical mediator in various podocyte injuries and we previously found that TGF-β induces podocyte injury through downregulating miR-30s, we thus used TGF-β-induced podocyte injury model to address the issue. We found that Triptolide is capable of protecting cultured podocytes from TGF-β-induced cytoskeletal injury and apoptosis, as expected. Consistently, Triptolide also prevented TGF-β-induced signaling activation of MAPK p38, NFkB (p65) and calcineurin/NFATC3, which are known to be downstream mediators of podocyte injury. Meanwhile, Triptolide was found to completely prevent TGF-β-induced miR-30 downregulation, indicating that Triptolide protects podocytes by sustaining miR-30 expression. Mechanistically, we found that Triptolide can prevent TGF-β-induced Smad2/3 phosphorylation/activation, which likely underlies miR-30 restoration by Triptolide. We also performed *ex vivo* study and found that Triptolide prevented TGF-β-induced miR-30 downregulation and Smad2/3 phosphorylation in the isolated glomeruli of mice or rats. Thus, our study has provided novel insights into the mechanism underlying the therapeutic effectiveness of Triptolide on podocytopathies.

Keywords: Podocyte, Triptolide, miR-30, TGF-β, cytoskeleton, apoptosis

Introduction

Tripterygium wilfordii hook, a Chinese herb, and its bioactive component, Triptolide, have immunosuppressive, anti-inflammatory and anti-cancer effects and are used clinically [1]. They are also used to treat kidney diseases for its anti-proteinuric and anti-fibrotic effects [2-4]. Triptolide is the effective substance in Tripterygium wilfordii and capable of inhibiting immune activity in the body [5]. Triptolide has also been shown to directly act on podocytes to alleviate oxidative stress, apoptosis and cytoskeletal injury through suppressing signaling activation of p53, mitogen-activated protein kinase p38 and NFκB, etc. [6, 7]. Regardless of these studies, the mechanism underlying the protective effect of Triptolide on podocytes remains largely unknown.

MicroRNAs are involved in many cellular and molecular processes in the physiology and pathogenesis of organisms by regulating the expression of target genes [8]. We and others have shown that microRNAs are also essential for podocytes as the mice with podocyte-specific knockout of the gene encoding Dicer, which is required for miRNA synthesis, exhibited podocyte injury, glomerulosclerosis and proteinuria [9-11]. These studies also suggest that certain miRNAs are critical for podocyte homeostasis and it is their lack (due to Dicer deficiency) that causes disruption of structural and functional homeostasis of podocytes, leading to injury as observed in Dicer podocyte-specific knockout mice. We have further shown that miR-30 family represents such miRNAs in podocytes because miR-30 deficiency is sufficient to cause podocyte injury [12-14]. Unfortunately, miR-30

expression can be downregulated by various injurious factors, including TGF-β [12], puromycin aminonucleosides [13-14], and Angiotensin II [15], etc.

TGF-β signaling is known to be primary and central to the development of various kidney diseases [16-17]. It is also one of the major signaling pathways that mediate podocyte injury, including apoptosis and cytoskeletal injury [18-19]. TGF-β is known to induce the activation of several downstream mediators of podocyte injury, including MAPK p38 [18], NFκB [20, 21] and calcineurin/NFATC pathways [22, 23].

In the present study, we used TGF-β-induced podocyte injury model to explore the mechanism underlying the protective effect of Triptolide on podocytes. We found that Triptolide can prevent TGF-β-induced activation of injurious signaling of p38, NFκB and calcineurin/NFATC, and alleviate podocyte cytoskeletal injury and apoptosis. Importantly, Triptolide can also prevent TGF-β-induced miR-30 downregulation completely. These results indicate that sustaining miR-30 expression is the mechanism by which Triptolide protects podocytes under stresses. Finally, we found that Triptolide can prevent TGF-β-induced Smad2/3 phosphorylation and activation, indicating that Triptolide sustains miR-30s expression through blocking TGF-β signaling.

Materials and methods

The study that involved animals was performed to conform to the institutional regulations and requirements concerning the care and use of laboratory animals at Jinling hospital.

Culture and treatment of human immortalized podocyte cell line

The cell line was the gift from Dr. Moin Saleem at Bristol University, UK [24]. Podocytes were grown at 33°C and switched to and incubated at 37°C for 7-10 days. Cells were serum starved for overnight before treatment. Cells were treated with 5 ng/ml recombinant TGF-β1 (R&D Systems) for 24 h or as indicated, followed by analyses with immunoblotting, qRT-PCR, immunocytostaining, or flow cytometry analysis.

Immunoblotting

After treatment in a 6-well plate, podocytes were washed with cold PBS and then lysed with RIPA buffer which contained proteinase inhibitors cocktail (Roche) and phosphatase inhibitors. The lysates were incubated on ice and then centrifuged 12,000 g for 15 min at 4°C. The supernatant samples were transferred to fresh tubes and then subjected to protein concentration measurement with BCA protein kit (Bio-Rad). After loading buffer was added, the samples were boiled for 5 min. 10% SDS-PAGE was used for fractionation of the samples, followed by transfer of the protein from the gel to PVDF membrane. The blot was incubated with blocking solution that contained 5% milk in TBST solution (20 mM Tris-HCl, PH 7.14, 150 mM NaCl, 0.1% Tween-20) for 60 min at room temperature. The blot was then incubated with antibody overnight at 4°C. After washed in TBST for 10 min for 3 times, the blot was incubated with HRP-labeled secondary antibody for 1 h at room temperature. After washed, we used the ECL system (Millipore) to detect the protein of interest. The antibodies used in the study were purchased from Santa Cruz (NFATC3, Smad2/3, and Synaptopodin) or Cell Signaling Technology (pSmad2/3, total p65, phospho-p65, total p38 and phospho-p38).

qRT-PCR analysis of miR-30 expression

Total RNA of podocytes was prepared by using mirVana miRNA extraction kit (Ambion). The RNA samples were used for miR-30s quantification by a qRT-PCR kit (Takara Bio, Otsu, Japan). The primers specific for mature miR-30a, miR-30b, miR-30c, miR-30d, and miR-30e were synthesized by Qiagen. U6 RNA was used for normalization. SYBR Green dye was used in the qPCR. The thermal condition was 95°C/30 s for denaturation, followed by 40 cycles of 95°C/5 s-60°C/30 s on the ABI 7900HT Fast Real time System. Threshold cycle (CT) values were measured and the relative abundance of the miRNA was calculated with the formula $2^{-\Delta\Delta Ct}$.

Phylloidin staining of F-actin in podocyte and stress fiber quantification

We used phalloidin labeled with Rhodamine (Thermo Fisher Scientific) to stain F-actin stress fibers in the podocytes that were cultured and treated on a glass disc. The stained cells were visualized and images were taken with a confocal microscope. The images were digitized such that the areas of rhodamine-stained F-actin fibers could be converted to black pixels, followed by quantification using ImageJ software (NIH, Bethesda, MD) following the method described in the software. The mean F-actin

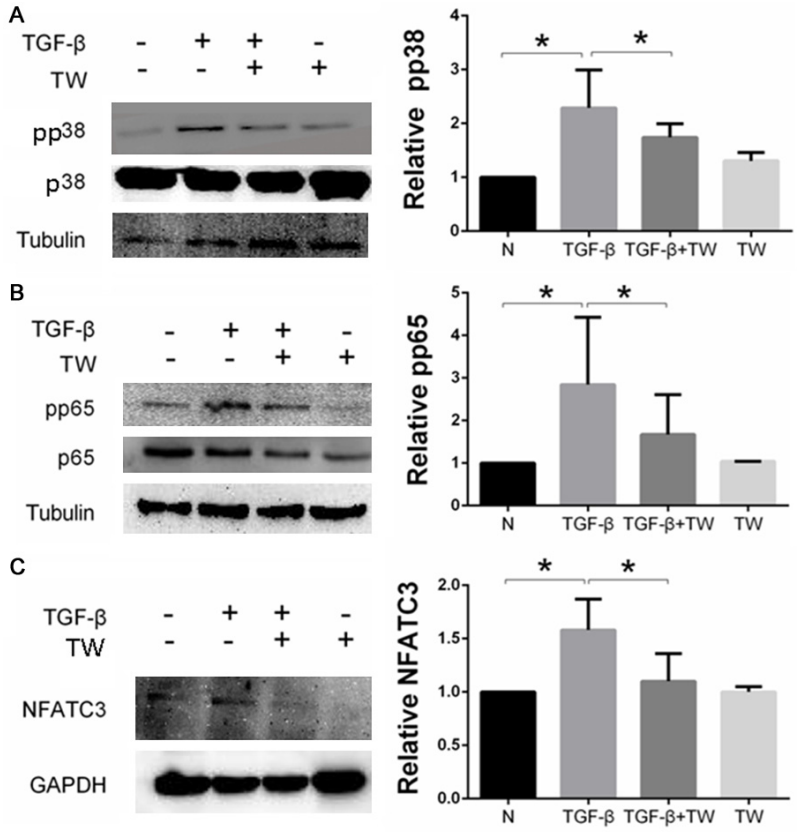

Figure 1. TGF-β-induced activation of proapoptotic p38, NFκB p65 and calcineurin/NFATC3 signaling pathways were blocked by Triptolide. Immunoblotting of pp38 (A), pp65 (B) and NFATC3 (C) in the podocytes treated with TGF-β in the absence or presence of Triptolide. N: normal untreated control; TGF-β: treated with TGF-β; TGF-β+TW: treated with TGF-β in the presence of Triptolide; and TW: Triptolide alone *p values were all <0.05: for pp38, p=0.03 (NC vs TGF-β) and 0.01 (TGF-β vs TGF-β+TW); for pp65, p=0.01 (NC vs TGF-β) and 0.04 (TGF-β vs TGF-β+TW); for NFATC3, p=0.003 (NC vs TGF-β) and 0.02 (TGF-β vs TGF-β+TW); n=3.

content per pixel and the total F-actin content per cell were calculated and expressed as arbitrary units.

Apoptosis analysis by flow cytometry

After treatment, cells were stained with Annexin V and PI or 7-AAD following the manufactures' instruction (Biouniquer) and then subjected to flow cytometry analysis (FACS ARIA, BD).

Fluorescent immunocytochemistry staining

After treatment, the cells were fixed in 4% paraformaldehyde for 10 min and blocked by incubation of 2% albumin solution for 30 min. Then the cells were incubated with primary antibody for 2 h at room temperature followed by secondary antibody conjugated with Rhodamine. After incubated with DAPI, images of cells were caught by a Zeiss fluorescence microscope.

Ex vivo study

We isolated glomeruli from mice following the method described [25]. Briefly, mice were anesthetized and then perfused cardiovascularly with PBS, followed by Dynabead perfusion. The kidneys were excised and cut into small pieces (~1 mm^3), followed by digestion of collagenase IV at 37 C for 10 min. The digests were gently squashed through a 100 μm sieve and the glomeruli were collected by magnetic concentrator. For rat glomeruli isolation, the cortex tissues were separated from the kidneys and cut into ~1 mm^3 pieces. The tissues were squashed through 200 μm sieve, and then passed a 70 μm sieve to retain the glomeruli on the sieve. The glomeruli were collected and aliquoted as desired, and they were treated as indicated in the Results, followed by cell lysate preparation or total RNA preparation.

Statistical analysis

The experiments described in the present study were performed for at least three times, and the results were given as mean±SD. SPSS18.0 software was used for the statistical analyses. Two-tailed Student's t test was used for comparison between two groups, and $p<0.05$ was considered to be significantly different.

Results

Triptolide prevented TGF-β-induced activation of MAPK p38, NFκB p65 and calcineurin/NFATC

TGF-β signaling is known to be injurious to podocytes. We examined the relationship of TGF-β signaling with several pathways, including MAPK p38, NFκB p65 and calcineurin/NFATC3, which are known to be commonly involved in a variety of podocyte injuries. We

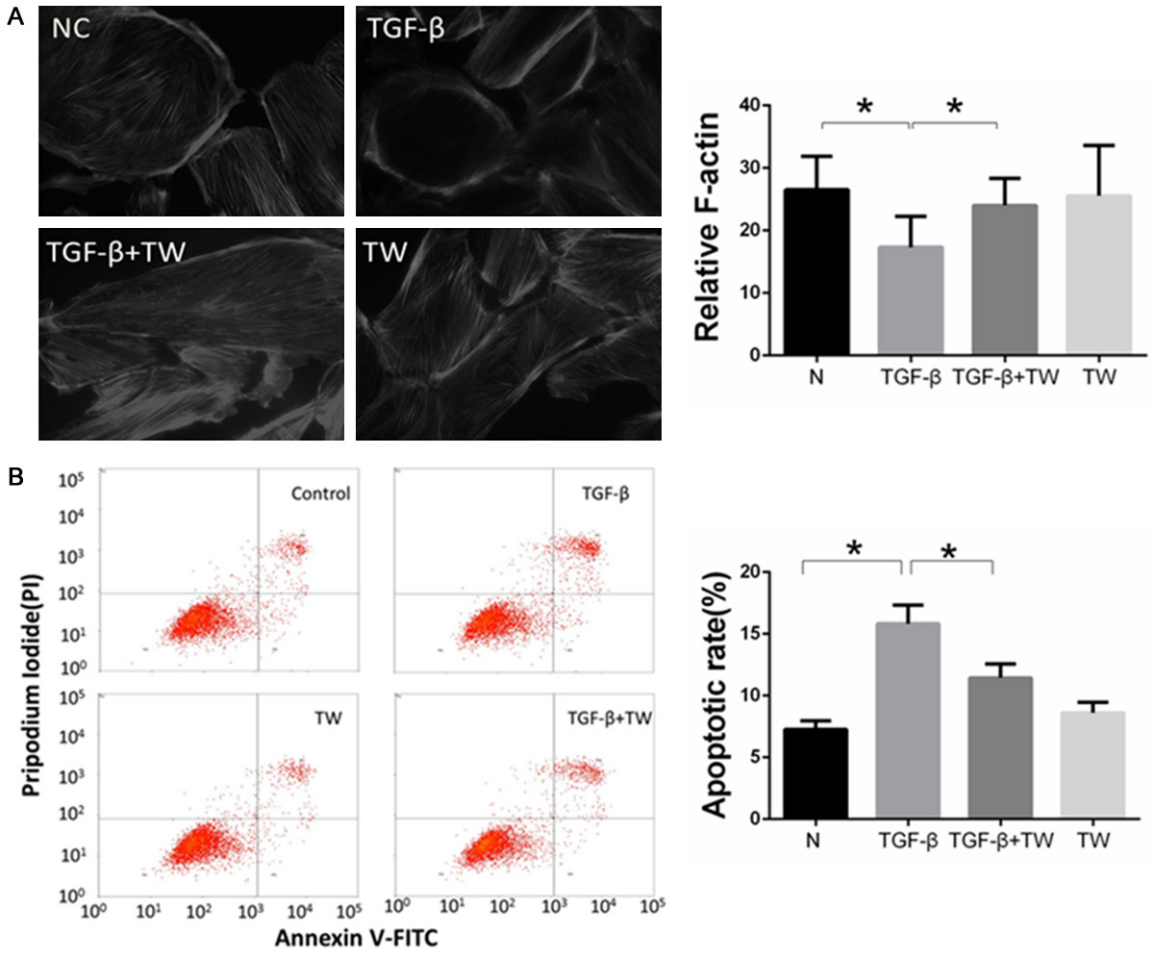

Figure 2. Triptolide prevented TGF-β-induced podocyte cytoskeletal injury and apoptosis. A. Phylloidin staining of actin stress fibers in the podocytes treated for 48 h as indicated. NC: normal untreated control; TGF-β: treated with TGF-β; TGF-β+TW: TGF-β-treated in the presence of Triptolide; and TW: Triptolide alone. Quantifications of F-actin stress fibers in above cells that were treated differently are shown on the right panel. *p<0.05 (p<0.001 (NC vs TGF-β) and <0.001 (TGF-β vs TGF-β+TW); n=3. B. TUNEL assays of the podocytes that were treated as indicated for 48 h. Quantification of the results is shown on the right panel. *p<0.05 (p=0.001 (NC vs TGF-β) and 0.002 (TGF-β vs TGF-β+TW); n=3.

found that TGF-β treatment activated p38, p65 and NFATC3 as shown in **Figure 1A-C**, consistent with previous studies [14, 18, 26] and indicating that TGF-β signaling induces podocyte injury through these pathways. However, in the presence of Triptolide the activation of these pathways was blocked (**Figure 1A-C**). These results suggest that Triptolide protects podocyte by inhibiting TGF-β signaling downstream pathways.

Triptolide prevented TGF-β-induced cytoskeletal injury and apoptosis

To confirm that TGF-β downstream pathway inhibition by Triptolide is associated with podocyte protection by Triptolide, we used TGF-β injury model of cultured podocytes treated with or without Triptolide. We observed that TGF-β induced podocyte cytoskeletal injury as shown by the reduced amount and disorganization of F-actin stress fibers compared with untreated control cells in the Phylloidin staining (**Figure 2A**). However, 10 ng/ml of Triptolide restored the amount of F-actin stress fibers and their structure prominently. It should be pointed out that Triptolide treatment alone (10 ng/ml) did not cause any change of the cytoskeletons (**Figure 2A**).

Meanwhile, in the TUNEL assay that tested anti-apoptotic effect of Triptolide on podocytes under the treatment of TGF-β1, we found that 5 ng/ml TGF-β1 induced apoptosis in podocytes,

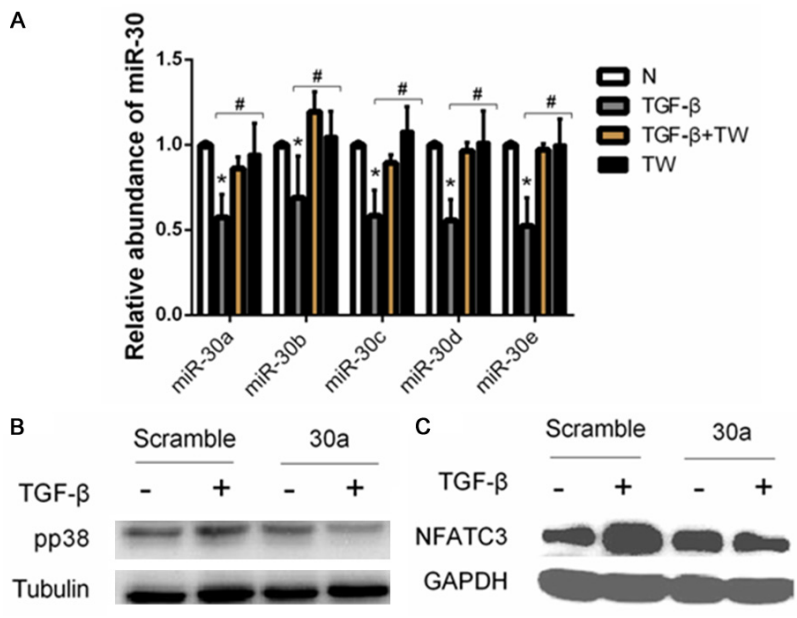

Figure 3. miR-30s mediated the protective effect of Triptolide on TGF-β-induced podocyte injury. (A) Triptolide prevented miR-30s downregulation induced by TGF-β. *p<0.05 (For miR-30a, p=0.001 (NC vs TGF-β) and 0.004 (TGF-β vs TGF-β+TW); for miR-30b, p=0.036 (NC vs TGF-β) and 0.004 (TGF-β vs TGF-β+TW); for miR-30c, p=0.003 (NC vs TGF-β) and 0.003 (TGF-β vs TGF-β+TW); for miR-30d, p=0.001 (NC vs TGF-β) and 0.001 (TGF-β vs TGF-β+TW); and for miR-30e, p=0.002 (NC vs TGF-β) and 0.001 (TGF-β vs TGF-β+TW); n=3. (B and C) Restoration of miR-30 level by miR-30a overexpression prevented TGF-β-induced activation of injurious p38 (B) and NFATC3 signaling (C).

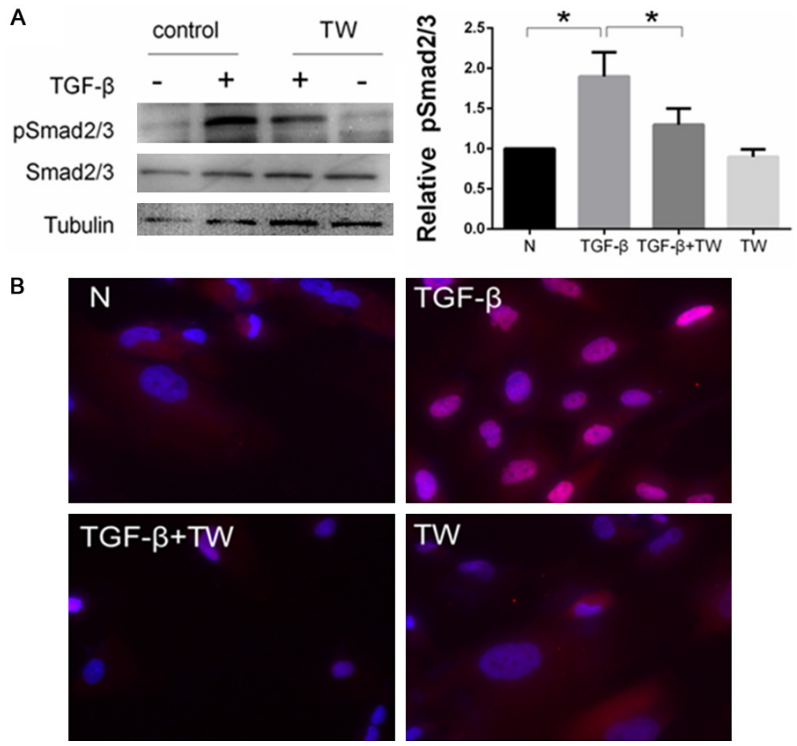

Figure 4. Triptolide prevented TGF-β-induced Smad2/3 phosphorylation/activation and nuclear translocation. A. Immunoblotting of Smad2/3 in the podocytes treated as indicated for 3 h. N: normal untreated control; TGF-β: treated with TGF-β; TGF-β+TW: TGF-β-treated in the presence of Triptolide and TW: Triptolide alone. Quantification of the results is shown on the right. *p<0.05 (p=0.002 (NC vs TGF-β) and 0.007 (TGF-β vs TGF-β+TW); n=3. B. Fluorescent immunocytochemistry staining of Smad2/3 in podocytes treated with TGF-β for 3 h in the absence or presence of Triptolide. This result supports that TW prevents Smad2/3 phosphorylation and nuclear translocation thereby lowering the strength of the signaling.

while the presence of Triptolide ameliorated the apoptosis of the cells (**Figure 2B**). Triptolide treatment alone did not cause significant apoptosis in the podocytes (**Figure 2B**).

These results were consistent with those shown in **Figure 1**, and together indicated that Triptolide protects podocytes by inhibiting TGF-β signaling and downstream pathways.

Triptolide prevented TGF-β-induced miR-30s downregulation

Since we had previously shown that the mechanism underlying TGF-β-induced podocyte injury is that TGF-β can downregulate the expression miR-30 family which is essential for podocyte structural and functional homeostasis, we speculated that Triptolide might protect podocytes through sustaining miR-30 expression in TGF-β treatment. We found that in the presence of Triptolide the downregulation of all miR-30 family members was

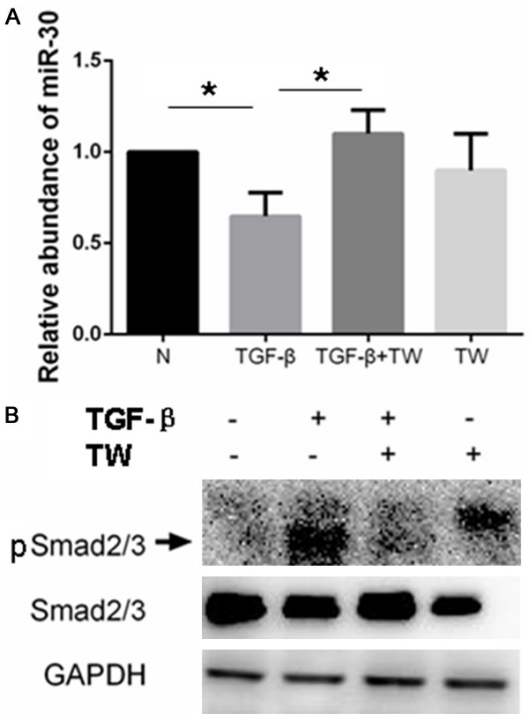

Figure 5. *Ex vivo* experiments showing the protective effects of Triptolide on podocytes treated with TGF-β. A. qPCR quantification of miR-30a in the isolated mouse glomeruli treated with TGF-β for 24 h in the absence or presence of Triptolide. *p<0.05 (p=0.001 (NC vs TGF-β) and <0.0001 (TGF-β vs TGF-β+TW); n=3. B. Immunoblotting of pSmad2/3 in the rat glomeruli treated with TGF-β in the absence or presence of Triptolide.

completely prevented in the treatment of TGF-β (**Figure 3A**), clearly demonstrating that the mechanism of the protective effect of Triptolide on podocytes is the prevention of miR-30 downregulation in the treatment of TGF-β. We further confirmed that prevention of miR-30 downregulation in the cells treated with TGF-β by overexpressing miR-30a prevented the activation of injurious sigaling, e.g., p38 and NFATC3 (**Figure 3B, 3C**), consistent with our earlier studies [12, 14] and supporting that Triptolide protects podocytes through sustaining miR-30 expression.

Triptolide prevented TGF-β-induced Smad2/3 phosphorylation and activation

We next explored how Triptolide prevents TGF-β-induced miR-30 downregulation. We found, to our surprise, that the presence of Triptolide could prevent Smad2/3 phosphorylation/activation induced by TGF-β (**Figure 4A**), indicating that Triptolide blocks TGF-β signaling at the very first upstream steps thereby preventing miR-30 downregulation. To further demonstrate that Triptolide inhibits TGF-β-induced phosphorylation of Smad2/3, we performed fluorescent immunocytochemistry staining of Smad2/3, and, consistently, we found that the presence of Triptolide prevented Smad2/3 nuclear translocation in the podocytes treated with TGF-β (**Figure 4B**), supporting above result and further demonstrating that Triptolide prevents TGF-β-induced Smad2/3 phosphorylation/activation and nuclear translocation.

Triptolide prevented miR-30 downregulation and blocked phosphorylation of Smad2/3 in isolated mouse glomeruli treated with TGF-β ex vivo

To test whether the *in vitro* studies above reflect *in vivo* situation in animals, we performed *ex vivo* study using isolated glomeruli from mice and treating them in dishes with TGF-β1 without or with Triptolide. We found that TGF-β significantly downregulated miR-30 expression as shown by miR-30a qPCR analysis (**Figure 5A**), consistent with previous study showing that TGF-β downregulates miR-30 expression in mice [12]. This result indicates that the *in vitro* model we used is valid and can represent *in vivo* situation. We next tested with this ex vivo system whether Triptolide can prevent Smad2/3 phosphorylation and activation as it does in cultured podocytes. We isolated glomeruli from rats for a better yield in glomeruli preparation, and then performed immunoblotting of pSmad2/3 in the glomeruli treated with TGF-β1 in the absence or presence of Triptolide. We found that Triptolide reduced TGF-β-induced phosphorylation of Smad2/3, as expected (**Figure 5B**).

Discussion

Direct protective effect of Triptolide has been observed both *in vitro* and *in vivo*. However, only several studies have provided limited insight into the underlying mechanism [6, 7]. These studies have shown that Triptolide is capable of restoring the expression of cytoskeleton- and slit-diaphram-associated proteins, e.g., synaptopodin, nephrin and podocin; that Triptolide can inactivate NADPH oxidase, there-

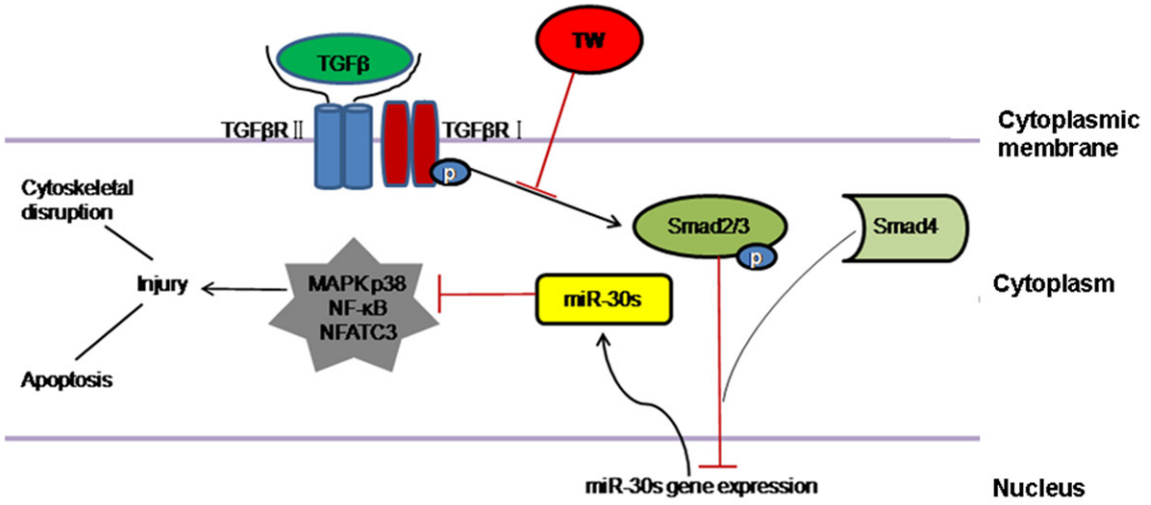

Figure 6. Working model of the relationship between Triptolide, TGF-β, Smad2/3 signaling, miR-30s, injurious signaling of NFATC3, MAPK p38 and NF-κB, and injury (cytoskeletal disruption and apoptosis) in podocytes.

by eliminating the generation of reactive oxygen species, which is known to be harmful to podocytes [26]; and that Triptolide is also able to prevent the activation of MAPK p38 which is known to mediate podocyte apoptosis [18] and to restore RhoA signaling which is essential for the homeostasis of podocyte cytoskeletons [6, 7]. Regardless of these studies, the fundamental mechanism of how Triptolide protects podocytes from injury remains elusive. In the present study, we show that Triptolide is capable of completely preventing TGF-β-induced miR-30 downregulation thereby protecting podocytes from TGF-β-induced injury. We further show that Triptolide can attenuate TGF-β signaling by preventing phosphorylation of Smad2/3 in podocytes, explaining how Triptolide prevents miR-30 downregulation by TGF-β. Thus, our study has revealed a novel mechanism underlying the protective effect of Triptolide on podocytes, which is illustrated in **Figure 6**.

We attempted to link the protective effect of Triptolide on podocytes with miR-30s. This was because we previously showed that miR-30 family members play a critical role in structural and functional homeostasis of podocytes [12, 13]; and multiple injurious factors, including TGF-β, AngII and PAN, can downregulate miR-30s, thereby activating several pathways that are known to be involved in podocyte injury [12-15]. Importantly, we found that glucocorticoids, which are anti-proteinuric and have a direct protective effect on podocytes, are capable of preventing miR-30 downregulation, thereby preventing harmful signaling activation induced by injurious factors [13]. Due to the similarity in podocyte protection between Triptolide and glucocorticoids, we postulated that Triptolide might also be capable of preventing miR-30 downregulation induced by injurious factors given the fact that Triptolide and glucocorticoids do have distinct molecular actions on cells [27, 28] with glucocorticoids acting on its receptor of transcriptional factor to regulate target gene expression, while Triptolide acting on several other putative target proteins, e.g., polycystin-2 [29], DCTPP1 [30], TAB1 [31], and XPB [32].

TGF-β signaling plays an essential role in kidney diseases [33]. It is also a key signaling in various podocyte injuries and podocytopathies [18] because TGF-β signaling activation has been observed in multiple podocyte injury models and diseases [19, 34-37], and its inhibition, as in Smad3-deficient mice, alleviates podocyte injury in these models [38, 39]. Importantly, miR-30s are downregulated in these podocyte injury models and podocytopathies and TGF-β signaling has been shown to directly downregulate miR-30s to induce podocyte injury [12-15, 40]. We therefore used the TGF-β-induced podocyte injury model to test our hypothesis that podocyte-protective effect of Triptolide lies in its capability of sustaining miR-30 expression under stress.

We found that Triptolide is able to completely prevent TGF-β-induced miR-30 downregulation

in podocytes and, accordingly, prevents podocyte injury, including cytoskeletal injury and apoptosis. Cytoskeletal damage and apoptosis are the major forms of podocyte injury [41, 42]. Consistent with these protective effects, we found that Triptolide suppressed the activation of injurious pathways, including MAPK p38, NFκB and calcineurin, which are activated by TGF-β. These results are expected and demonstrate that Triptolide protects podocytes by preventing the activation of these harmful signaling pathways. However, how Triptolide suppresses these pathways still needs to be clarified and miR-30 could be the possible link between them.

We found that Triptolide completely prevented miR-30 downregulation induced by TGF-β, similarly to glucocorticoids [13]. We have previously shown restoration of miR-30 expression by exogenous miR-30 expression is sufficient to prevent podocyte injury and activation of p38, p53, NFκB, calcineurin, etc. [12-15]. We therefore conclude that prevention of miR-30 downregulation induced by injurious factors is the underlying mechanism of the protective effect of Triptolide on podocytes.

We further found that Triptolide blocked Smad2/3 phosphorylation/activation induced by TGF-β in podocytes. Consistently, fluorescent immunocytochemistry staining showed that nuclear translocation of Smad2/3 induced by TGF-β was blocked by Triptolide (**Figure 4B**). These results indicate that Triptolide prevents miR-30 downregulation at least partly through attenuating TGF-β signaling. This is the first time that Triptolide is shown to be capable of suppressing upstream TGF-β signaling by disrupting Smad2/3 phosphorylation in podocytes. In contrast, glucocorticoids are not able to affect TGF-β-induced Smad2/3 phosphorylation (data not shown), suggesting that Triptolide and glucocorticoids use distinct mechanisms by which they both prevent miR-30 downregulation induced by injurious factors. At present, it is not known how glucocorticoids prevent miR-30 downregulation, an issue deserving to be explored. Meanwhile, the issue concerning how Triptolide disrupts TGF-β-induced phosphorylation of Smad2/3 is also important. It is known that TGF-β acts on receptor II and activates its kinase activity, and then the activated receptor II kinase phosphorylates receptor I to activate its kinase activity. The receptor I next phosphorylates Smad2/3. Triptolide must have interfered one of these steps to prevent Smad2/3 phosphorylation, resulting in their failure in binding to Smad4 and being translocated to nuclei where they act as transcription factors to promote target genes expression.

On the other hand, Triptolide blocks Smad2/3 phosphorylation mostly but not completely (**Figure 4A**). It is not known whether the extent of Smad2/3 phosphorylation inhibition is sufficient to completely restore miR-30 expression by Triptolide (**Figure 3**). Otherwise, attenuation of Smad2/3 phosphorylation may only partly contribute to the prevention of miR-30 downregulation by Triptolide, and therefore, there may be an additional pathway through which Triptolide sustains miR-30 expression. This issue should be clarified in future studies.

Finally, we determined whether the results observed *in vitro* with cultured podocytes can be reproduced *in vivo*. In this regard, we decided to isolate mouse or rat glomeruli and treat them with TGF-β in dish, hoping that this simplified and clean *ex vivo* experimental system would give rise to definitive results and conclusions. We observed that TGF-β treatment indeed induced Smad2/3 phosphorylation, accompanied by downregulation of miR-30s; whereas Triptolide reversed these consequences of TGF-β treatment (**Figure 5**). Since podocytes are the only cell type in the glomeruli that expresses miR-30s, therefore the changes of miR-30 levels in the glomeruli represent those in the podocytes. Although we did not perform all the experiments *in vitro* with isolated glomeruli due to technical difficulties, the available results from the *ex vivo* experiments have sufficiently proven that our hypothesis concerning the mechanism underlying Triptolide protection on podocytes is valid.

In conclusion, the mechanism by which Triptolide protects podocytes against TGF-β-induced injury at least partly involves the capability of Triptolide to sustain miR-30 expression. Additionally, we found that Triptolide is capable of attenuating TGF-β signaling by disrupting phosphorylation of Smad2/3, a novel mechanism by which Triptolide prevents TGF-β-induced miR-30 downregulation and thus podocyte injury. Our study has provided new insights into the mechanism underlying protective effect of Triptolide on podocytes.

Acknowledgements

This work was supported by the grants funded by National Natural Science Foundation of China to (81470944 to JW, and 81370827, 81670653 to SS).

Disclosure of conflict of interest

None.

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References

[1] Ziaei S and Halaby R. Immunosuppressive, anti-inflammatory and anti-cancer properties of triptolide: a mini review. Avicenna J Phytomed 2016; 6: 149-164.

[2] Ji SM, Wang QW, Chen JS, Sha GZ, Liu ZH and Li LS. Clinical trial of Tripterygium Wilfordii Hook F. in human kidney transplantation in China. Transplant Proc 2006; 38: 1274-1279.

[3] Wang J, Tang Z, Luo C, Hu Y, Zeng C, Chen H and Liu Z. Clinical and pathological features of dense deposit disease in Chinese patients. Clin Nephrol 2012; 78: 207-215.

[4] Tang X, Xu F, Chen DM, Zeng CH and Liu ZH. The clinical course and long-term outcome of primary focal segmental glomerulosclerosis in Chinese adults. Clin Nephrol 2013; 80: 130-139.

[5] Chen BJ. Triptolide, a novel immunosuppressive and anti-inflammatory agent purified from a Chinese herb Tripterygium wilfordii Hook F. Leuk Lymphoma 2001; 42: 253-265.

[6] Zheng CX, Chen ZH, Zeng CH, Qin WS, Li LS and Liu ZH. Triptolide protects podocytes from puromycin aminonucleoside induced injury in vivo and in vitro. Kidney Int 2008; 74: 596-612.

[7] Chen ZH, Qin WS, Zeng CH, Zheng CX, Hong YM, Lu YZ, Li LS and Liu ZH. Triptolide reduces proteinuria in experimental membranous nephropathy and protects against C5b-9-induced podocyte injury in vitro. Kidney Int 2010; 77: 974-988.

[8] Ambros V. The functions of animal microRNAs. Nature 2004; 431: 350-355.

[9] Shi S, Yu L, Chiu C, Sun Y, Chen J, Khitrov G, Merkenschlager M, Holzman LB, Zhang W, Mundel P and Bottinger EP. Podocyte-selective deletion of dicer induces proteinuria and glomerulosclerosis. J Am Soc Nephro 2008; 19: 2159-2169.

[10] Harvey SJ, Jarad G, Cunningham J, Goldberg S, Schermer B, Harfe BD, McManus MT, Benzing T and Miner JH. Podocyte-specific deletion of dicer alters cytoskeletal dynamics and causes glomerular disease. J Am Soc Nephrol 2008; 19: 2150-2158.

[11] Ho J, Ng KH, Rosen S, Dostal A, Gregory RI and Kreidberg JA. Podocyte-specific loss of functional microRNAs leads to rapid glomerular and tubular injury. J Am Soc Nephrol 2008; 19: 2069-2075.

[12] Shi S, Yu L, Zhang T, Qi H, Xavier S, Ju W and Bottinger E. Smad2-dependent downregulation of miR-30 is required for TGF-β-induced apoptosis in podocytes. PLoS One 2013; 8: e75572.

[13] Wu J, Zheng C, Fan Y, Zeng C, Chen Z, Qin W, Zhang C, Zhang W, Wang X, Zhu X, Zhang M, Zen K and Liu Z. Downregulation of microRNA-30 facilitates podocyte injury and is prevented by glucocorticoids. J Am Soc Nephrol 2014; 25: 92-104.

[14] Wu J, Zheng C, Wang X, Yun S, Zhao Y, Liu L, Lu Y, Ye Y, Zhu X, Zhang C, Shi S and Liu Z. MicroRNA-30 family members regulate calcium/calcineurin signaling in podocytes. J Clin Invest 2015; 125: 4091-4106.

[15] Zhao Y, Wu J, Zhang M, Zhou M, Xu F, Zhu X, Zhou X, Lang Y, Yang F, Yun S, Shi S and Liu Z. Angiotensin II induces calcium/calcineurin signaling and podocyte injury by downregulating microRNA-30 family members. J Mol Med (Berl) 2017; 95: 887-898.

[16] Border WA, Noble NA, Yamamoto T, Tomooka S and Kagami S. Antagonists of transforming growth factor-beta: a novel approach to treatment of glomerulonephritis and prevention of glomerulosclerosis. Kidney Int 1992; 41: 566-570.

[17] Sharma K and Ziyadeh FN. The transforming growth factor-beta system and the kidney. Semin Nephrol 1993; 13: 116-128.

[18] Schiffer M, Bitzer M, Roberts IS, Kopp JB, ten Dijke P, Mundel P and Böttinger EP. Apoptosis in podocytes induced by TGF-beta and Smad7. J Clin Invest 2001; 108: 807-816.

[19] Herman-Edelstein M, Weinstein T and Gafter U. TGFβ1-dependent podocyte dysfunction. Curr Opin Nephrol Hypertens 2013; 22: 93-99.

[20] Siebenlist U, Franzoso G and Brown K. Structure, regulation and function of NF-kappa B. Annu Rev Cell Bio 1994; 10: 405-55.

[21] Brähler S, Ising C, Hagmann H, Rasmus M, Hoehne M, Kurschat C, Kisner T, Goebel H,

Shankland S, Addicks K, Thaiss F, Schermer B, Pasparakis M, Benzing T and Brinkkoetter PT. Intrinsic proinflammatory signaling in podocytes contributes to podocyte damage and prolonged proteinuria. Am J Physiol Renal Physiol 2012; 303: F1473-1485.

[22] Wang Y, Jarad G, Tripathi P, Pan M, Cunningham J, Martin DR, Liapis H, Miner JH and Chen F. Activation of NFAT signaling in podocytes causes glomerulosclerosis. J Am Soc Nephrol 2010; 21: 1657-1666.

[23] Wang L, Chang JH, Paik SY, Tang Y, Eisner W and Spurney RF. Calcineurin (CN) activation promotes apoptosis of glomerular podocytes both in vitro and in vivo. Mol Endocrinol 2011; 25: 1376-1386.

[24] Saleem MA, O'Hare MJ, Reiser J, Coward RJ, Inward CD, Farren T, Xing CY, Ni L, Mathieson PW and Mundel P. A conditionally immortalized human podocyte cell line demonstrating nephrin and podocin expression. J Am Soc Nephrol 2002; 13: 630-638.

[25] Lu Y, Ye Y, Yang Q and Shi S. Single-cell RNA-sequence analysis of mouse glomerular mesangial cells uncovers mesangial cell essential genes. Kidney Int 2017; 92: 504-513.

[26] Susztak K, Raff AC, Schiffer M and Böttinger EP. Glucose-induced reactive oxygen species cause apoptosis of podocytes and podocyte depletion at the onset of diabetic nephropathy. Diabetes 2006; 55: 225-233.

[27] Krett N and Ma S. Overcoming glucocorticoid resistance: a new pathway from an ancient Chinese medicine? Leuk Lymphoma 2009; 50: 689-690.

[28] Wang J, Jiang Z, Ji J, Wang X, Wang T, Zhang Y, Tai T, Chen M, Sun L, Li X and Zhang L. Gene expression profiling and pathway analysis of hepatotoxicity induced by triptolide in Wistar rats. Food Chem Toxicol 2013; 58: 495-505.

[29] Leuenroth SJ, Okuhara D, Shotwell JD, Markowitz GS, Yu Z, Somlo S and Crews CM. Triptolide is a traditional Chinese medicine-derived inhibitor of polycystic kidney disease. Proc Natl Acad Sci U S A 2007; 104: 4389-4394.

[30] Corson TW, Cavga H, Aberle N and Crews CM. Triptolide directly inhibits dCTP pyrophosphatase. Chembiochem 2011; 12: 1767-1773.

[31] Lu Y, Zhang Y, Li L, Feng X, Ding S, Zheng W, Li J and Shen P. TAB1: A Target of Triptolide in Macrophages. Chem Biol 2014; 21: 246-256.

[32] Titov DV, Gilman B, He QL, Bhat S, Low WK, Dang Y, Smeaton M, Demain AL, Miller PS, Kugel JF, Goodrich JA and Liu JO. XPB, a subunit of TFIIH, is a target of the natural product triptolide. Nat Chem Biol 2011; 7: 182-188.

[33] Meng XM, Nikolic-Paterson DJ and Lan HY. TGF-β: the master regulator of fibrosis. Nat Rev Nephrol 2016; 12: 325-338.

[34] Lee HS. Mechanisms and consequences of TGF-ß overexpression by podocytes in progressive podocyte disease. Cell Tissue Res 2012; 347: 129-140.

[35] Ihm CG, Jeong KW, Lee SH, Lee TW and Park JK. Effects of therapeutic agents on the inflammatory and fibrogenic factors in IgA nephropathy. Nephrology (Carlton) 2007; 12 Suppl 3: S25-26.

[36] Chang AS, Hathaway CK, Smithies O and Kakoki M. Transforming growth factor-β1 and diabetic nephropathy. Am J Physiol Renal Physiol 2016; 310: F689-F696.

[37] Tossidou I and Schiffer M. TGF-β/BMP pathways and the podocyte. Semin Nephrol 2012; 32: 368-376.

[38] Sun YB, Qu X, Howard V, Dai L, Jiang X, Ren Y, Fu P, Puelles VG, Nikolic-Paterson DJ, Caruana G, Bertram JF, Sleeman MW and Li J. Smad3 deficiency protects mice from obesity-induced podocyte injury that precedes insulin resistance. Kidney Int 2015; 88: 286-298.

[39] Xavier S, Niranjan T, Krick S, Zhang T, Ju W, Shaw AS, Schiffer M and Böttinger EP. TbetaRI independently activates Smad- and CD2AP-dependent pathways in podocytes. J Am Soc Nephrol 2009; 20: 2127-2137.

[40] Liu L, Lin W, Zhang Q, Cao W and Liu Z. TGF-β induces miR-30d down-regulation and podocyte injury through Smad2/3 and HDAC3-associated transcriptional repression. J Mol Med (Berl) 2016; 94: 291-300.

[41] Loeffler I and Wolf G. Transforming growth factor-β and the progression of renal disease. Nephrol Dial Transplant 2014; 29 Suppl 1: i37-i45.

[42] Nagata M. Podocyte injury and its consequences. Kidney Int 2016; 89: 1221-1230.