The signaling protein Wnt5a promotes TGFβ1-mediated macrophage polarization and kidney fibrosis by inducing the transcriptional regulators Yap/Taz

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M2 macrophage polarization is known to underlie kidney fibrosis. We previously reported that most of the members of the Wnt family of signaling proteins are induced in fibrotic kidneys. Dysregulation of the signaling protein Wnt5a is associated with fibrosis, but little is known about the role of Wnt5a in regulating M2 macrophage activation that results in kidney fibrosis. Here, using murine Raw 264.7 cells and bone marrow-derived macrophages, we found that Wnt5a enhanced transforming growth factor β1 (TGFβ1)-induced macrophage M2 polarization as well as expression of the transcriptional regulators Yes-associated protein (Yap)/transcriptional coactivator with PDZ-binding motif (Taz). Verteporfin blockade of Yap/Taz inhibited both Wnt5a- and TGFβ1-induced macrophage M2 polarization. In mouse models of kidney fibrosis, shRNA-mediated knockdown of Wnt5a expression diminished kidney fibrosis, macrophage Yap/Taz expression, and M2 polarization. Moreover, genetic ablation of Taz in macrophages attenuated kidney fibrosis and macrophage M2 polarization in mice. Collectively, these results indicate that Wnt5a promotes kidney fibrosis by stimulating Yap/Taz-mediated macrophage M2 polarization.

Monocyte-macrophage lineage may be activated and categorized as either classically activated (M1) or alternatively activated (M2) phenotype in response to various stimuli (1). Proliferative M1 macrophages causes acute tissue injury, whereas persistent accumulation of profibrotic M2 macrophages drives the fibrotic response (2, 3). During the past decade, great achievement has been made in defining the molecular networks involved in modulating the polarized activation of macrophages (4). However, the mechanisms for regulating macrophage polarization during kidney fibrosis remain to be further understood.

The Wnt family, containing at least 19 Wnt ligands in humans, is classified into canonical and noncanonical pathways based on whether or not β-catenin is activated (5, 6). Our published studies found that most of the Wnt family members are induced in fibrotic kidney tissue. We also demonstrated that the activation of canonical Wnt signaling can promote podocyte injury and macrophage M2 polarization through distinct molecular mechanisms (7, 8). Wnt5a, a ligand that binds Fizzled5 and triggers noncanonical Wnt signaling, is up-regulated in various fibrotic diseases (9–13). Furthermore, activation of Wnt5a is associated with epithelial-to-mesenchymal transition of tubular epithelial cells during renal fibrosis (13). In addition, many studies have found that Wnt5a can regulate macrophage function through distinct mechanisms (14–16). However, the role and mechanisms for Wnt5a in regulating macrophage M2 polarization as well as its contribution to kidney fibrosis are obscure.

Like Wnt signaling, Hippo signaling is evolutionarily conserved and pivotal in regulating embryonic development and disease progression (17). Yes-associated protein (Yap)2/tran-
scriptional coactivator with PDZ-binding motif (Taz) acts as the target and downstream effector of this pathway. When de-
phosphorylated, Yap/Taz accumulate and are relocated to the nucleus where it interacts with a number of transcription factors that may promote cell growth, differentiation, and survival (18). Yap and Taz have divergent but critical roles in nephro-
genesis (19). Ablation of Taz displays kidney cyst formation accompanied by enhanced Wnt/β-catenin signaling, suggesting that a cross-talk exists between Hippo and canonical Wnt signaling. Additionally, TGFβ1, a well-known profibrotic cyto-
kine, induces robust Taz but not Yap protein expression in both mesenchymal and epithelial cells. Sustained Taz activation pro-
 motes epithelial maladaptive repair, suggesting a potential role of Hippo signaling in promoting kidney fibrosis (20–23). In addition, it has been reported that Taz regulates reciprocal differen-
tiation of TH17 cells and Treg cells (24). Wnt5a may also

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2 The abbreviations used are: Yap, Yes-associated protein; TGFβ1, transforming growth factor β1; Taz, transcriptional coactivator with PDZ-binding motif; Tead, TEA domain family member; BMM, bone marrow-derived macrophage; Arg-1, arginase-1; Fizz1, found in inflammatory zone 1; Mr, mannose receptor; α-SMA, α-smooth muscle actin; UUO, unilateral ureter obstruction; IRI, ischemic/reperfusion injury; shRNA, short hairpin RNA; qRT-PCR, quantitative RT-PCR; FN, fibronectin; Stat, signal transducer and activator of transcription; p-Stat, phosphorylated Stat; DAPI, 4’,6-diamidino-2-phenylindole.
induce Yap/Taz activation through stimulating Yap dephosphorylation in bone marrow stromal cells (25). Moreover, blocking Yap/Taz prevents tumor-associated macrophage M2 polarization (26). Therefore, it is highly possible that Wnt5a may promote macrophage M2 polarization and contribute to kidney fibrosis by modulating Yap/Taz activity.

In this study, we found that Wnt5a could exacerbate TGFβ1-stimulated macrophage M2 polarization. In mouse models with kidney fibrosis, down-regulation of Wnt5a was able to reduce kidney fibrosis, macrophage M2 polarization, and Yap/Taz expression. Specific ablation of Taz gene in macrophages could markedly inhibit macrophage M2 polarization and kidney fibrosis and in mice.

Results

Wnt5a exacerbates TGFβ1-stimulated macrophage alternative activation

Wnt5a is one of the major components of the Wnt family of proteins. It is up-regulated in various fibrotic diseases. Activation of Wnt5a is associated with epithelial-to-mesenchymal transition of tubular epithelial cells during renal fibrosis (13). In addition to tubular epithelial cells, macrophage also plays a very important role in renal fibrosis. To explore the role of Wnt5a in regulating macrophage M2 polarization, we treated Raw 264.7 cells and bone marrow–derived macrophages (BMMs) with TGFβ1 with or without Wnt5a for 24 h. In Raw 264.7 cells, TGFβ1 could largely up-regulate arginase-1 (Arg-1), found in inflammatory zone 1 (Fizz1), and chitinase-like lectin (Ym1) mRNAs but not mannose receptor (MR) expression. Wnt5a plus TGFβ1 could significantly enhance Arg-1, MR, Fizz1, and Ym1 mRNA expression compared with treatment with TGFβ1 alone in both Raw 264.7 cells and BMMs (Fig. 1, A and B). In parallel, Wnt5a could further up-regulate TGFβ1-induced Arg-1 protein expression in both Raw 264.7 cells and BMMs in a time- and dose-dependent manner (Fig. 1, C–H). In addition, we found that Wnt5a could further up-regulate TGFβ1-induced α-SMA expression in both Raw 264.7 cells and BMMs (Fig. 1, E and H). Therefore, Wnt5a treatment could exacerbate TGFβ1-stimulated macrophage M2 polarization.

Yap/Taz mediate Wnt5a-exacerbated macrophage M2 polarization

We then wanted to elucidate the mechanisms for Wnt5a in promoting TGFβ1-stimulated macrophage M2 polarization. We first examined β-catenin signaling in Raw 264.7 cells. The results showed that Wnt5a could decrease β-catenin abundance and inhibit TOPFlash luciferase activity, suggesting the inactivation of β-catenin signaling in macrophages (Fig. 1, A and B). We then investigated the phosphorylation status for Stat3 or Stat6 in Wnt5a-treated Raw 264.7 cells. Western blot results showed that TGFβ1 could induce Stat3 phosphorylation at Tyr-705 in a time-dependent manner, whereas Wnt5a could not enhance it (Fig. 1C, D, F, and G).

Figure 1. Wnt5a exacerbates TGFβ1-induced macrophage alternative activation. A and B, real-time qRT-PCR analysis showing the mRNA abundance for Arg-1, MR, Fizz1, and Ym1 in Raw 264.7 cells (A) and BMMs (B) that were stimulated with TGFβ1 (2 ng/ml) with or without Wnt5a (100 ng/ml) for 24 h. The qRT-PCR data were normalized to a reference gene, Gapdh. *, p < 0.05 versus cells treated with vehicle (Veh.) alone, n = 3; #, p < 0.05 versus cells treated with TGFβ1 alone, n = 3. Error bars represent S.E.

C–H, Western blot assay showing the abundance for Arg-1 and α-SMA in Raw 264.7 cells (C–E) and BMMs (F–H) that were treated with TGFβ1 with or without Wnt5a for 12 or 24 h.

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19291
**Wnt5a promotes kidney fibrosis**

Figure 2. Yap/Taz mediates Wnt5a-exacerbated macrophage alternative activation. A and B, Western blot assay showing the induction of Yap/Taz in Raw 264.7 cells (A) and BMMs (B) that were treated with TGFβ1 (2 ng/ml) with or without Wnt5a (100 ng/ml) for various durations as indicated. In addition to Yap/Taz, the samples from Raw 264.7 cells were probed with p-Stat3, p-Stat6, Stat3 and Stat6 as shown in Fig. S1C; actin bands were shared between A and Fig. S1C. C, representative immunofluorescence staining images showing the nuclear localization of Yap/Taz in BMMs that were stimulated with TGFβ1 plus Wnt5a. Scale bar, 20 μm. D, Western blot assay showing that verteporfin inhibited TGFβ1 plus Wnt5a–induced Yap/Taz induction in Raw 264.7 cells. F, real-time qRT-PCR analysis showing that verteporfin inhibited TGFβ1 with or without Wnt5a-stimulated macrophage alternative activation. Cells were treated with TGFβ1 or TGFβ1 plus Wnt5a for 24 h. *, p < 0.05 versus cells treated with vehicle alone, n = 3; #, p < 0.05 versus cells treated with TGFβ1 alone, n = 3; $, p < 0.05 versus cells treated with TGFβ1 plus Wnt5a, respectively, n = 3. Error bars represent S.E. F, Western blot assay showing that deletion of Taz could reduce TGFβ1 plus Wnt5a–stimulated Arg-1 expression in BMMs. G, real-time qRT-PCR analysis showing the mRNA abundance for Arg-1 in Taz+/+ and Taz−/− BMMs. *, p < 0.05 versus Taz+/+ BMMs treated with vehicle alone, n = 3; #, p < 0.05 versus Taz−/− BMMs treated with TGFβ1 alone, n = 3; $, p < 0.05 versus Taz−/− BMMs treated with TGFβ1 plus Wnt5a, n = 3. Error bars represent S.E. H, Western blot assay showing the expression of exogenous TAZ(S89A) in BMMs after TAZ(S89A) plasmid transfection. I, real-time qRT-PCR analysis showing that expression of TAZ(S89A) exacerbates TGFβ1-induced Arg-1 up-regulation in Raw 264.7 cells. *, p < 0.05 versus cells transfected with pcDNA3 alone, n = 3; #, p < 0.05 versus cells treated with TGFβ1 alone for 24 h, n = 3. Error bars represent S.E. Veh., vehicle. 

Recently, it was reported that ovatodiolide prevents polarization of M2 tumor-associated macrophages through the Yap oncogenic pathway (26). Park et al. (25) also reported that alternative Wnt signaling could activate Yap/Taz. To investigate whether Wnt5a exacerbates TGFβ1-induced macrophage M2 polarization through Yap/Taz activation, we treated Raw 264.7 cells and BMMs with TGFβ1 with or without Wnt5a. Western blot results showed that TGFβ1 or Wnt5a treatment alone could not obviously increase Yap/Taz abundance, but TGFβ1 plus Wnt5a could largely up-regulate Yap/Taz expression (Fig. 2, A and B). In addition, TGFβ1 plus Wnt5a could promote Yap/Taz to undergo nuclear translocation in BMMs (Fig. 2C). To investigate the role of Yap/Taz induction in Wnt5a-exacerbated macrophage M2 polarization, we treated Raw 264.7 cells with verteporfin, a Yap inhibitor (28), followed by TGFβ1 or TGFβ1 plus Wnt5a treatment to induce macrophage M2 polarization. The results showed that verteporfin could largely inhibit Yap/Taz expression as well as macrophage M2 polarization, suggesting a pivotal role for Yap/Taz activation in modulating TGFβ1 plus Wnt5a–promoted macrophage M2 polarization (Fig. 2, D and E). Moreover, we generated BMMs with Taz ablation and examined macrophage M2 polarization. BMMs isolated from Csf1r-Cre−, Taz−/− mice were treated with 4-hydroxytamoxifen for 5 days to induce Taz gene deletion and stimulated with TGFβ1 with or without Wnt5a to induce macrophage M2 polarization (Fig. 2F). Ablation of Taz could markedly inhibit both protein and mRNA expression of Arg-1 stimulated by TGFβ1 plus Wnt5a in BMMs (Fig. 2, F and G). To further explore whether Taz induction is sufficient to enhance TGFβ1-stimulated macrophage M2 polarization, we transfected the BMMs with Taz(S89A) expression plasmid followed by TGFβ1 treatment for 24 h (Fig. 2H). The results showed that expression of exogenous Taz could enhance TGFβ1-stimulated Arg-1 expression, which mimicked the effect of Wnt5a in exacerbating TGFβ1-induced macrophage M2 polarization (Fig. 2I). Together, these results demonstrated that Wnt5a may exacerbate TGFβ1-induced macrophage M2 polarization through up-regulating Yap/Taz expression.

**Induction of Wnt5a/b and Yap/Taz in the fibrotic kidneys**

To explore the role of Wnt5a in kidney fibrosis, we generated mouse models of kidney fibrosis by unilateral ureter obstruct-
tion (UOU) and ischemic/reperfusion injury (IRI), respectively. Western blot results showed that Wnt3a protein abundance in UUUO kidneys was slightly decreased, whereas it was increased in IRI kidneys. Wnt5a/b protein abundance was largely increased in the fibrotic kidneys after UUO (Fig. 3A) or IRI (Fig. 3B). Immunohistochemical staining showed that Wnt5a/b abundance was increased in the fibrotic area of UUO or IRI kidneys (Fig. 3C). Of note, the abundance of Yap/Taz was also largely elevated in kidneys at different time points as indicated after UUO (Fig. 3A) or IRI (Fig. 3B). In addition, coimmunostaining results showed that, in the UUO or IRI kidneys, a large amount of Yap/Taz was detected to undergo nuclear translocation in F4/80-positive macrophages (Fig. 3C). Thus, these data suggest that Wnt5a/b and Yap/Taz are concurrently up-regulated in the fibrotic kidneys after UUO or IRI in mice.

**Down-regulation of Wnt5a ameliorates kidney fibrosis in mice**

The above data showed that Wnt5a was up-regulated in the fibrotic kidneys. To further explore whether Wnt5a induction promotes kidney fibrosis, we generated a mouse model with short hairpin RNA (shRNA)-mediated knockdown of Wnt5a gene. Western blot and real-time qRT-PCR analyses demonstrated the down-regulation of Wnt5a, whereas Wnt3a protein abundance was not changed as measured by Western blot assay in livers (Fig. 4, A and B) and kidneys (Fig. 4, C and D) from mice injected with Wnt5a shRNA compared with those injected with pcDNA6.2.

We then investigated whether down-regulation of Wnt5a could attenuate kidney fibrosis. A mouse model of kidney fibrosis induced by UUO or IRI was used (Fig. 4, E and F). At day 14 after UUO, marked tubular atrophy and interstitial fibrosis were detected in mice injected with pcDNA6.2, whereas in mice injected with Wnt5a shRNA plasmid, tubular damage and interstitial fibrosis were remarkably attenuated (Fig. 4, G and H). Immunofluorescence staining and Western blotting showed that FN and α-SMA expression was remarkably increased in kidneys from mice injected with pcDNA6.2 after UUO, whereas FN and α-SMA expression was much less in mice injected with Wnt5a shRNA (Fig. 4, I, J, M, and N). Similarly, at day 28 after IRI, kidney injury, fibrotic area, total collagen content, and FN and α-SMA expression were largely attenuated in mice injected with Wnt5a shRNA compared with those injected with pcDNA6.2 (Fig. 4, K, L, and TO). Collectively, we concluded that down-regulation of Wnt5a ameliorates kidney fibrosis after UUO or IRI in mice.
Wnt5a promotes kidney fibrosis
Down-regulation of Wnt5a inhibits macrophage accumulation and M2 polarization in the UUO or IRI kidneys

Macrophage accumulation and activation have been reported to play a crucial role in kidney fibrosis. Previous studies also reported that Wnt5a can promote cell migration (29). The above data showed that Wnt5a could stimulate macrophage activation and M2 polarization. We then wanted to know whether Wnt5a down-regulation can decrease macrophage accumulation in the fibrotic kidneys after UUO or IRI. We stained kidney tissues with antibody against F4/80 to identify macrophages. At day 14 after UUO or day 28 after IRI, macrophage accumulation was remarkably increased in the UUO or IRI kidneys from mice injected with pcDNA6.2, whereas it was much less in those from mice injected with Wnt5a shRNA (Fig. 5, A and B). We then sorted macrophages from the UUO or IRI kidneys and examined the abundance for Arg-1 and Mr, the markers for M2 polarized macrophages. Western blot results showed that Arg-1 and Mr were largely decreased in macrophages from mice injected with Wnt5a shRNA compared with those injected with pcDNA6.2 (Fig. 5, C and E). Moreover, immunofluorescence staining demonstrated that Mr was largely decreased in macrophages from mice injected with Wnt5a shRNA after UUO (Fig. 5D) or IRI (Fig. 5F) compared with those injected with pcDNA6.2. Consistently, mRNA abundance for M2 macrophage markers, including Arg-1, Mr, Fizz1, and Ym1, was markedly reduced in macrophages from mice injected with Wnt5a shRNA plasmid compared with those injected with pcDNA6.2 after UUO or IRI (Fig. 5, G and H). Flow cytometry analysis for CD11b and CD206 further demonstrated that down-regulation of Wnt5a could inhibit macrophage M2 polarization within the UUO or IRI kidneys (Fig. 5, I and J). Therefore, we conclude that knockdown of Wnt5a diminishes macrophage M2 polarization in the kidneys after UUO or IRI.

Down-regulation of Wnt5a reduces Yap/Taz expression in macrophages from the fibrotic kidneys

In cultured macrophages, we found that Wnt5a plus TGFβ1 could up-regulate Yap/Taz expression, which mediates Wnt5a-exacerbated macrophage M2 polarization. We then detected Yap/Taz expression in macrophages from UUO or IRI kidneys. A Western blot assay showed that Yap/Taz expression was induced in macrophages from mice injected with pcDNA6.2, whereas it was much less in mice injected with Wnt5a shRNA after UUO or IRI (Fig. 6, A and C). In addition, the mRNA abundance for Yap/Taz target genes, including Ankrd1 and Ctgf, in macrophages was largely elevated from mice injected with pcDNA6.2 but was much less in those from mice injected with Wnt5a shRNA after UUO or IRI (Fig. 6, B and D). Immunofluorescence staining for Yap/Taz and F4/80 further confirmed the reduction of Yap/Taz in macrophages from mice injected with Wnt5a shRNA compared with those injected with pcDNA6.2 after UUO (Fig. 6E). Therefore, these results suggest that knockdown of Wnt5a may down-regulate macrophage Yap/Taz expression and diminish macrophage M2 polarization in the kidneys after UUO or IRI.

Ablation of Taz in macrophages attenuates macrophage M2 polarization and kidney fibrosis in mice with UUO nephropathy

To further decipher the role and mechanisms for Taz induction in macrophage M2 polarization and kidney fibrosis, we generated a mouse model with inducible macrophage Taz deletion with a Cre-LoxP system (Fig. 7, A and B). Mice with macrophage ablation of Taz were generated by intraperitoneal injection of tamoxifen for 5 consecutive days in Csf1r-Cre+, Tazf/f mice and named as Mac-Taz−/−. The same gender with genotype Csf1r-Cre−, Tazf/f littermates were injected with tamoxifen and named as Mac-Taz+/+ (Fig. 7C). Western blot analysis demonstrated the ablation of Taz in macrophages from Mac-Taz−/− mice (Fig. 7, D and E). Kidney histology was comparable between the knockout and control littermates, whereas in the UUO kidneys from the knockouts, interstitial fibrotic area and total collagen content were much less compared with their control littermates (Fig. 7, F and G). Moreover, FN or α-SMA abundance was largely decreased in the UUO kidneys from Mac-Taz−/− kidneys compared with that from Mac-Taz+/+ kidneys (Fig. 7, H and I).

We then wanted to know whether ablation of Taz inhibits macrophage accumulation and M2 polarization in the UUO kidneys. The number of F4/80-positive macrophages was largely decreased in the UUO kidneys from mice with macrophage Taz deletion compared with their control littermates (Fig. 8A). The mRNA abundance for Arg-1, Fizz1, and chitinase 3–like 3/Ym1 was markedly reduced in macrophages from Mac-Taz−/− kidneys after UUO but was much less in macrophages from Mac-Taz+/− kidneys (Fig. 8B).

Figure 4. Knocking down Wnt5a ameliorates UUO nephropathy in mice. A and B, Western blot assay (A) and real-time qRT-PCR analysis (B) showing the expression of Wnt3a and Wnt5a in the mouse livers at 16 h after Wnt5a shRNA plasmid injection compared with those injected with pcDNA6.2. Numbers 1–3 indicate each individual animal within each group. *, p < 0.05 versus mice injected with pcDNA6.2, n = 3–4. Error bars represent S.E. C and D, Western blot assay (C) and real-time qRT-PCR analysis (D) showing the expression of Wnt3a and Wnt5a in the mouse kidneys at 16 h after Wnt5a shRNA plasmid injection. Numbers 1–3 indicate each individual animal within each group. *, p < 0.05 versus mice injected with pcDNA6.2, n = 3–4. Error bars represent S.E. E and F, strategies for Wnt5a shRNA plasmid injection and UUO (E) or IRI (F) surgery in mice. G, periodic acid–Schiff (PAS), Masson’s trichrome, and Sirius Red staining of kidney sections from various groups as indicated. Scale bar, 50 μm. H, graphic presentation showing the fibrotic area and total collagen content in the kidneys from various groups as indicated. *, p < 0.05 versus contralateral kidneys from mice injected with pcDNA6.2, n = 6; #, p < 0.05 versus UUO kidneys from mice injected with pcDNA6.2, n = 6. Error bars represent S.E. I, periodic acid–Schiff (PAS), Masson’s trichrome, and Sirius Red staining for kidney sections from various groups as indicated. Scale bar, 50 μm. J, graphic presentation showing the fibrotic area and total collagen content in kidneys among groups as indicated. *, p < 0.05 versus contralateral kidneys from mice injected with pcDNA6.2, n = 6; #, p < 0.05 versus kidneys from mice injected with pcDNA6.2 after IRI, n = 6. Error bars represent S.E. K and M, representative micrographs of immunostaining for FN and α-SMA in the UUO kidneys (K) or IRI kidneys (M). Scale bar, 50 μm. L and N, Western blot assay (left) and quantitative analysis (right) for FN and α-SMA in the UUO (L) or IRI (N) kidneys among groups as indicated. Numbers 1–3 indicate each individual animal within a given group. *, p < 0.05 versus contralateral kidneys from mice injected with pcDNA6.2, n = 4; #, p < 0.05 versus UUO or IRI kidneys from mice injected with pcDNA6.2, respectively, n = 4. Error bars represent S.E. CTL, control.
Macrophages from the fibrotic kidneys were sorted with CD115 microbeads, and Western blot analysis showed that Arg-1 abundance in macrophages enriched from Mac-Taz\(^{+/+}\) UUO kidneys was much less compared with those from Mac-Taz\(^{−/−}\) mice (Fig. 8C). Additionally, immunostaining results showed that deletion of Taz in macrophages could inhibit macrophage M2 polarization (Fig. 8D). Therefore, it can be concluded that ablation of Taz in macrophages attenuates macrophage accumulation, M2 polarization, and kidney fibrosis after UUO in mice.
Figure 6. Down-regulation of Wnt5a mitigates macrophage Yap/Taz expression in the fibrotic kidneys. A and C, Western blot assay and quantitative analysis for Yap/Taz in macrophages sorted from UUO (A) or IRI (C) kidneys with CD115 microbeads. Numbers 1–3 indicate individual samples that were pooled from two individual animals within the same group. *, p < 0.05 versus contralateral kidneys from mice injected with pcDNA6.2, n = 3; #, p < 0.05 versus UUO or IRI kidneys from mice injected with pcDNA6.2, n = 3. Error bars represent S.E. B and D, real-time qRT-PCR analysis showing the mRNA abundance for Ankrd1 and Ctgf in macrophages sorted from UUO (B) or IRI (D) kidneys with CD115 microbeads. Each sample was pooled from two individual animals within the same group. *, p < 0.05 versus contralateral kidneys from mice injected with pcDNA6.2, n = 3; #, p < 0.05 versus mouse UUO or IRI kidneys after pcDNA6.2 injection, n = 3. Error bars represent S.E. E, representative coimmunostaining images for F4/80 and Yap/Taz in UUO or IRI kidneys among groups as indicated. White arrows indicate costaining-positive macrophages. Scale bar, 50 μm. CTL, control.

Discussion

In this study, we report that Wnt5a could exacerbate TGFβ1-induced macrophage M2 polarization and contribute to kidney fibrosis. Additionally, we demonstrate that Yap/Taz induction mediated Wnt5a plus TGFβ1–promoted macrophage M2 polarization.

The Wnt family members are crucial players in regulating cellular and organ function. Previous studies demonstrated that Wnt/β-catenin signaling activation promotes kidney fibrosis through stimulating fibroblast activation and macrophage M2 polarization (8, 30). Among all the Wnt family members, Wnt5a is a pleiotropic cytokine that regulates the development of various organs and postnatal cellular function. Wnt5a may promote inflammation and fibrosis in multiple organs and tissues (31). In this study, we report that Wnt5a is crucial for promoting kidney fibrosis. Wnt5a has been reported to be involved in cell migration, invasion, and polarity (32). Under proinflammatory conditions, Wnt5a induces immunosuppressive macrophage activation (33). In this study, we found that down-regulation of Wnt5a could attenuate macrophage accumulation and M2 polarization. In cultured macrophages, Wnt5a could enhance TGFβ1-induced macrophage M2 polarization. Therefore, we conclude that Wnt5a may promote kidney fibrosis through stimulating macrophage activation and M2 polarization.

Alternative (M2) macrophage activation is known to be driven by multiple cytokines and transcriptional factors (26, 34–36). Our published studies reported that Wnt/β-catenin signaling modulates macrophage M2 polarization through Stat3 activation (8). Although Wnt5a is considered as a noncanonical Wnt family member, studies have shown that it can activate β-catenin in dendritic cells as well as several other cell types (37). Here, we found that Wnt5a could down-regulate β-catenin expression and inhibit β-catenin signaling activation in cultured macrophages, which suggests that β-catenin signaling may not mediate Wnt5a-exacerbated macrophage M2 polarization. Stat3 and Stat6 signaling are two major pathways mediating macrophage M2 polarization. In this study, we found that TGFβ1 alone could stimulate Stat3 but not Stat6 phosphorylation, whereas Wnt5a plus TGFβ1 could not enhance Stat3 or Stat6 phosphorylation. Thus, we can conclude that Wnt5a exacerbates TGFβ1-stimulated macrophage M2 polarization through Yap/Taz induction based on the following reasons. First, Wnt5a plus TGFβ1 could largely up-regulate Yap/Taz expression in both Raw 264.7 cells and BMMs. Second, suppression of Yap/Taz activation could markedly inhibit Wnt5a plus TGFβ1–stimulated macrophage M2 polarization. Third, overexpression of exogenous Yap could mimic the effect of Wnt5a in exacerbating TGFβ1–stimulated macrophage M2 polarization. Fourth, in animal models, down-regulating Wnt5a expression resulted in less macrophage Yap/Taz induction or macrophage M2 polarization.

Yap and Taz are transcriptional coregulators that bind primarily to enhancer elements by using Tead factors as DNA-
The Hippo pathway and its essential effectors Yap and Taz are necessary for fibroblast activation and tissue fibrosis (39–42). In the UUO model, Yap/Taz display nuclear translocation, and verteporfin, a chemical that inhibits Yap–Taz interaction and Yap transcriptional activity, reduces renal fibrosis (23). In this study, we found that ablation of Taz in macrophages could inhibit Wnt5a plus TGFβ1–induced macrophage M2 polarization. Moreover, inducible ablation of

Figure 7. Ablation of Taz in macrophages attenuates UUO nephropathy in mice. A, breeding strategy for generating Csf1r-Cre WT/−, Taz WT/− mice. B, PCR genotyping. 1, Csf1r-Cre WT/−, Taz WT/−; 2, Csf1r-Cre WT/−, Taz fl/fl; 3, Csf1r-Cre WT/−, Taz WT/−; 4, Csf1r-Cre WT/−, Taz WT/−. C, strategy for tamoxifen injection and UUO surgery in mice. D and E, Western blot assay (D) and quantitative analysis (E) showing the reduction of Taz in macrophages from Csf1r-Cre WT/−, Taz fl/fl mice after UUO. F, periodic acid–Schiff (PAS), Masson’s trichrome, and Sirius Red staining for kidney sections among groups as indicated. G, graphic presentation showing the fibrotic area and total collagen content in kidneys among groups as indicated. *, p < 0.05 versus Taz WT/− contralateral kidneys, n = 5–6. Error bars represent S.E. H, representative micrographs of immunostaining for FN and α-SMA in UUO kidneys. Scale bar, 50 μm. I, Western blot assay and quantitative analysis for FN and α-SMA in kidneys among groups as indicated. Numbers 1–3 indicate individual animal within a given group. *, p < 0.05 versus Taz WT/− contralateral kidneys, n = 5–6. #, p < 0.05 versus Taz WT/− UUO kidneys at day 14 after UUO, n = 5–6. Error bars represent S.E. CTL, control.
Taz in macrophages could largely inhibit macrophage M2 polarization and kidney fibrosis after UUO or IRI. Thus, it can be concluded that Wnt5a and TGFβ1 may promote macrophage M2 polarization and kidney fibrosis through converging into Yap/Taz induction. It is of note that the previous studies reported that Yap and Taz bind Smad2/3 through the coiled-coil region, and this interaction may dictate the subcellular localization of Smad2/3 (43, 44). However, more investigation is still needed to decipher the mechanisms for Yap/Taz activation in regulating TGFβ1-stimulated macrophage M2 polarization.

In summary, our study demonstrated that Wnt5a promotes macrophage M2 polarization via regulating Yap/Taz activity and contributes to kidney fibrosis after UUO or IRI in mice. Targeting Wnt5a/Yap/Taz in macrophages may represent a new therapeutic strategy for protecting against kidney fibrosis in patients with chronic kidney disease.

**Experimental procedures**

**Mice and animal models**

Male C57BL/6 mice weighing ~18–20 g were acquired from the specific pathogen-free laboratory animal center of Nanjing Medical University. Mice were sacrificed, and kidneys were harvested at different time points after UUO. For the kidney IRI model, the left renal pedicle of the mouse was clamped for 35 min. The right kidneys were not removed. Mice were sacrificed, and kidneys were harvested at different time points after IRI.

Mice expressing tamoxifen-inducible MerCreMer fusion protein under the control of macrophage-specific mouse Csf1r promoter (019098; FVB-Tg(Csf1r-Cre/Esr1)1)) were ordered from The Jackson Laboratory (Bar Harbor, ME). FVB-Tg(Csf1r-Cre/Esr1)1 mice were crossed with C57BL/6J mice for eight generations to obtain Csf1r-Cre transgenic mice on a C57BL/6J background. Homozygous Taz floxed mice were kindly provided by Dr. Randy L. Johnson from MD Anderson Cancer Center. By mating Taz floxed mice with Csf1r-Cre/Esr1 transgenic mice, mice that were heterozygous for the Taz floxed allele were generated (genotype, Csf1r-Cre+/−, Tazfl/wt). These mice were cross-bred with homozygous Taz floxed mice (genotype, Tazfl/fl) to generate offspring with different littersmates. Csf1r-Cre+/−, Tazfl/wt; Csf1r-Cre+/−, Tazfl/fl; Csf1r-Cre−/−; Tazfl/wt; Csf1r-Cre−/−; Tazfl/fl; and Csf1r-Cre−/−; Tazfl/fl mice were used in the study. Genotyping was performed by PCR assay using DNA extracted from the mouse tail. Csf1r-Cre+/−, Tazfl/wt mice and Csf1r-Cre−/−, Tazfl/fl control littermates were intraperitoneally injected with tamoxifen (T5648, Sigma-Aldrich) at 25 mg/kg for 5 consecutive days, and 2 days after the last injection, the mice were subjected to UUO operation. All animals were housed in the specific pathogen-free laboratory animal center of Nanjing Medical University according to the guidelines of the Institutional Animal Care and Use Committee at Nanjing Medical University.

**Plasmids**

shRNA specific for mouse Wnt5a gene was ordered from Ruizhen, Nanjing, China. BLOCK-iT Pol II mir RNAi Expression Vector kits were used (catalog number K4935, Invitrogen). The target sequence of the murine Wnt5a is GAGTTTCTGTAACGCCCTAGAGAA. The mouse Wnt5a-specific shRNA was cloned into pcDNA6.2. Mice on the C57BL/6J background were injected with Wnt5a shRNA in the tail vein at 1 mg/kg to induce endogenous Wnt5a down-regulation. For the UUO model, Wnt5a shRNA plasmid was injected at 1 day before the surgery and 7 days after the surgery. For the IRI model, Wnt5a shRNA plasmid was injected at 7 and 14 days after the surgery.

3XFlag pCMV5-TOPO TAZ(S89A) was a gift from Jeff Wrana (Addgene plasmid 24815) (43). BMMs were seeded on
Wnt5a promotes kidney fibrosis

12-well culture plates to 90–95% confluence in complete medium containing 10% fetal bovine serum and 10 ng/ml macrophage colony-stimulating factor. BMMs were transfected with pTAZ or empty vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instruction.

Western blot assay

BMMs and cultured Raw 264.7 cells were lysed in 1× SDS sample buffer. The kidneys were lysed with radioimmune precipitation assay buffer containing 1% Nonidet P-40, 0.1% SDS, 100 mg/ml phenylmethylsulfonyl fluoride, 1% protease inhibitor mixture, and 1% phosphatase I and II inhibitor mixture (Sigma-Aldrich) on ice. The supernatants were collected after centrifugation at 13,000 × g at 4 °C for 30 min. Protein concentration was determined by bicinechonic acid protein assay (BCA Protein Assay kit, Pierce Thermo Scientific) according to the manufacturer’s instructions. An equal amount of protein was loaded for SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The primary antibodies used were anti-FN (catalog number F3648, Sigma-Aldrich; 1:100,000), anti-α-SMA (catalog number A5228, Sigma-Aldrich; 1:50,000), anti-Arg-1 (catalog number 9819, Cell Signaling Technology; 1:1000), anti-MR (catalog number ab64693, Abcam; 1:1000), anti-Stat3 (catalog number 4904, Cell Signaling Technology; 1:1000), anti-p-Stat3 (Tyr-705) (catalog number 9145, Cell Signaling Technology; 1:1000), anti-p-Stat6 (Tyr-641) (catalog number 56554, Cell Signaling Technology; 1:1000), anti-Stat6 (catalog number ab32520, Abcam; 1:1000), anti-β-Actin (catalog number sc47778, Santa Cruz Biotechnology; 1:1000), anti-Yap/Taz (catalog number 8418, Cell Signaling Technology; 1:1000), and anti-Wnt5a/b (catalog number 2530, Cell Signaling Technology; 1:1000). Quantification was performed by measuring the intensity of the signals with the aid of the National Institutes of Health ImageJ software package.

Real-time qRT-PCR assay

Total RNA was extracted using TRIzol reagent (catalog number 15596018, Invitrogen) according to the manufacturer’s instructions. cDNA was synthesized using 1 μg of total RNA, ReverTra Ace (catalog number R111-02, Vazyme, Nanjing, China), and oligo(dT)12–18 primers according to the manufacturer’s protocol. Gene expression was measured by real-time qRT-PCR (catalog number Q141-02, Vazyme) and a 7300 real-time PCR system (Applied Biosystems, Foster City, CA). Gapdh was detected as an internal control. The primers are listed in Table 1. The relative amount of mRNA or gene to internal control was calculated using the equation 2ΔCT in which ΔCT = CTgene − CTcontrol.

Histology and immunohistochemical staining

Mouse kidney sample were fixed in 10% neutral formalin and embedded in paraffin. 3-μm-thick sections were stained with periodic acid–Schiff, Masson’s trichrome, and Sirius Red. Paraffin-embedded kidney sections were deparaffinized, hydrated, and subjected to antigen retrieval, and endogenous peroxidase activity was quenched by 3% H₂O₂. Sections were then blocked with 10% normal donkey serum followed by incubating with anti-Wnt5a/b (catalog number 2530, Cell Signaling Technolog-
column (Miltenyi Biotech, Bergisch-Gladbach, Germany) according to the manufacturer’s instruction.

**Flow cytometry**

Briefly, after perfusion with cold 1× PBS, kidneys was removed, minced into fragments, and digested with 1 mg/ml collagenase (catalog number 17018-029, Gibco) and 0.1 mg/ml DNase for 1 h at 37 °C with intermittent agitation. Kidney fragments were passed through a 40-μm mesh (Falcon, BD Biosciences), and ~1 × 10^7 cells were incubated in 2.5 μg/ml Fc blocking solution, centrifuged (800 × g, 10 min, 8 °C), and suspended with FACS buffer. Approximately 1 × 10^6 cells were stained for 20 min at room temperature with antibodies, including anti-CD45-FITC (catalog number 553310, BD Biosciences), anti-CD11b-allophycocyanin (catalog number 561018, BD Biosciences), and anti-CD206-phycerothyrin (catalog number 141705, BioLegend); washed; and suspended in FACS buffer. The suspensions were washed twice with FACS buffer, suspended in FACS buffer, and analyzed on a BD Canto II flow cytometer with FlowJo software.

**Statistical analyses**

All data examined are presented as mean ± S.E. Statistical analyses of the data were performed using SigmaStat software (Jandel Scientific Software, San Rafael, CA). Comparison between groups was made using one-way analysis of variance followed by the Student–Newman–Keuls test. Paired or unpaired t test was used to compare two groups. A p value of 0.05 or lower was considered statistically significant.

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