Tongxinluo ameliorates renal structure and function by regulating miR-21-induced epithelial-to-mesenchymal transition in diabetic nephropathy

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1Metabolic Disease Center, School of Traditional Chinese Medicine, Capital Medical University, Beijing, China; 2Department of Endocrinology, Tongren Hospital, Capital Medical University, Beijing, China; 3Department of Endocrinology and Metabolism, Capital Medical University, Beijing, China; and 4Department of Pathophysiology, Capital Medical University, Beijing, China

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Wang JY, Gao YB, Zhang N, Zou DW, Xu LP, Zhu ZY, Li JY, Zhou SN, Cui FQ, Zeng XJ, Geng JG, Yang JK. Tongxinluo ameliorates renal structure and function by regulating miR-21-induced epithelial-to-mesenchymal transition in diabetic nephropathy. Am J Physiol Renal Physiol 306: F486–F495, 2014. First published December 26, 2013; doi:10.1152/ajprenal.00528.2013.—Diabetic nephropathy (DN) is one of the most important diabetic microangiopathies. The epithelial-to-mesenchymal transition (EMT) plays an important role in DN. The physiological role of microRNA-21 (miR-21) was closely linked to EMT. However, remained elusive whether tongxinluo (TXL) ameliorated renal structure and function by regulating miR-21-induced EMT in DN. This study aimed to determine the effect of TXL on miR-21-induced renal tubular EMT and to explore the relationship between miR-21 and TGF-β1/smads signals. Real-time RT-PCR, cell transfection, in situ hybridization (ISH), and laser confocal microscopy were used, respectively. Here, we revealed that TXL dose dependently lowered miR-21 expression in tissue, serum, and cells. Overexpression of miR-21 can enhance α-smooth muscle actin (a-SMA) expression and decrease E-cadherin expression by up-regulating smad3/p-smad3 expression and downregulating smad7 expression. Interestingly, TXL also increased E-cadherin expression and decreased α-SMA expression by regulating miR-21 expression. More importantly, TXL decreased collagen IV, fibronectin, glomerular basement membrane, glomerular area, and the albumin/creatinine ratio, whereas it increased the creatinine clearance ratio. The results demonstrated that TXL ameliorated renal structure and function by regulating miR-21-induced EMT, which was one of the mechanisms to protect against DN, and that miR-21 may be one of the therapeutic targets for TXL in DN.

tongxinluo (TXL); microRNA; epithelial-to-mesenchymal transition (EMT); diabetic nephropathy

Diabetic nephropathy (DN) is associated with microvascular diseases and can also ultimately lead to end-stage renal disease and the need for dialysis and/or kidney transplantation (25). Epithelial-to-mesenchymal transition (EMT) is an essential process in diabetic nephropathy, not only in the pathogenesis of renal interstitial fibrosis but also glomerulosclerosis (17, 24). Loss of E-cadherin (E-CA) and gain in α-smooth muscle actin (α-SMA) expression are the key hallmarks of EMT (4).

MicroRNAs (miRs) are small noncoding RNAs that bind to 3′-untranslated region (UTR) of the target mRNAs, resulting in repression of target gene expression by translational inhibition or degradation of target mRNAs. Recent reports have shown that miRs play an essential role in EMT of various diseases, including kidney disease (28). MiR-30 inhibits transforming growth factor (TGF)-β1-induced EMT by targeting Smad4 (33). MiR-134 inhibits EMT by targeting FOXM1 in non-small cell lung cancer cells (13). Reexpression of miR-21 contributes to migration and invasion by inducing EMT in MCF-7 cells (6). MiR-21 regulates EMT phenotype in breast cancer stem cells (7). miR-21 promotes fibrogenic EMT of epicardial mesothelial cells involving programmed cell death4 and sprouty-1 (2). TGF-β1-induced EMT is partly mediated by microRNA-21 (11). TGF-β1/smads-mediated upregulation of miR-21 promotes EMT and renal fibrosis (45). These findings strongly suggested that miR-21 was not only closely related to EMT but also closely linked with TGF-β1/smads signals. However, whether miR-21 affected EMT by regulating TGF-β1/smads signals remained unclear in diabetic nephropathy.

With the progression of modern technology, more and more herbal compound extracts are being authenticated, standardized, and administered successfully in clinical practice. Tongxinluo (TXL) is extracted, concentrated, and freeze-dried from a group of herbal medicines, such as Panax ginseng and Paeonia lactiflora Pall. Numerous studies have showed that TXL has many potentially therapeutic values in various diseases such as heart disease, cerebral disease, and including kidney disease (35, 37, 39, 46). For example, TXL can improve cardiac function and cardiomyopathy in diabetic rats. TXL can inhibit the coronary arterial hyperplasia by mediating IL-1β (5). TXL reduces myocardial no-reflow and ischemia-reperfusion injury by stimulating the phosphorylation of endothelial nitric oxide synthase (eNOS) via the PKA pathway (15). TXL displays its anti-inflammatory action by inhibiting TNF-α (10). TXL might protect vascular endothelium by decreasing ET1 and increasing NO expression (31). Furthermore, Panax ginseng reduced heart remodeling by downregulating TGF-β1/smads3 in human chymase transgenic mice (34). Panax ginseng possesses a protective effect on the kidney of diabetic rats by...
inhibiting TGF-β1 protein and enhancing smad7 (29). More importantly, ginsenoside Rg1, a major active component isolated from Panax ginseng, inhibits renal tubular EMT and renal interstitial fibrosis by blocking TGF-β1 expression (36). Panoniflorin (PF), the key active constituent of P. lactiflora Pallas, positively prevented the progression of renal fibrosis by inhibiting TGF-β1 expression in unilateral ureteral obstruction (UUO) mice (41). These pharmacological effects demonstrated TXL plays a positive role in protecting against various diseases by inhibiting TGF-β1 expression and promoting miR-21 maturation by inhibiting target smad7. TXL ameliorated renal structure and function by regulating miR-21-induced EMT in diabetic nephropathy.

In the present study, our aim was to determine whether TXL affects renal structure and function and to explore the relationship between miR-21 and TGF-β1/smads signals in diabetic nephropathy. This study demonstrated that miR-21 and TGF-β1/smads3 formed a double-positive feedback loop to enhance renal tubular EMT by inhibiting target smad7. TXL ameliorated renal structure and function by regulating miR-21-induced EMT, which was one of the mechanisms to protect against DN, and miR-21 may be one of the novel therapeutic targets for TXL in diabetic nephropathy.

MATERIALS AND METHODS

Components and preparation of TXL. TXL superfine powder was provided by Shijiazhuang Yiling Pharmaceutical (Hebei, China), which was registered in the State Food and Drug Administration in China. The herbal drugs were authenticated and standardized on marker compounds according to the Chinese Pharmacopoeia 2005. TXL contains 12 medicinal components, which were ground to superfine powder with a diameter ≤10 μm by a micronizer. To reduce the dose variability of TXL among different batches, the species, origin, harvest time, medicinal parts, and concocted methods for each component were strictly standardized. Moreover, HPLC, high-performance capillary electrophoresis, and gas chromatography were applied to quantify the components of the TXL (43).

Cell culture. To explore the effect of TXL on miR-21 expression and the markers of renal tubular EMT, TXL superfine powder was dissolved in serum-free DMEM/F12. The solution was sonicated for 1 h followed by centrifugation at 3,500 rpm for 10 min. The supernatant was filtered using a 0.22-μm microfilter. Meanwhile, the precipitate was heated and dried at 60°C to calculate the practical performance capillary electrophoresis, and gas chromatography were applied to manifest nuclei. The sections were double stained with fast red staining being positive. The sections were double stained with fast red.

Real-time RT-PCR analysis. Total RNA from tissue and cells was isolated using TRIzol reagent (Invitrogen) to obtain both miR and mRNA. For analysis of miR-21 expression in tissue, cells, and serum, real-time PCR primers were designed as described previously (27). Relative expression was calculated using the 2−Δ△CT method (23) and normalized to the expression of U6 RNA. The relative expression for E-cadherin, α-SMA, smad3, smad7, fibronectin (FN), and collagen IV (col-IV) was normalized to the expression of β-actin. Primers for real-time PCR are as follows: miR-21, forward primer (F): 5'-cagctcaggggggaggctagc-3'; reverse primer (R): 5'-cagctcaggggggaggctagc-3'; E-cadherin, F: 5'-tctcagggaggagctgtcgg-3'; R: 5'-gcttgagtcgagagagctc-3'; α-SMA, F: 5'-ATCAAGGAAACAGCTGTTATGTAG-3'; R: 5'-gtaaagatcgccatgtaggcctc-3'; smad3, F: 5'-gcttgagtcgagagagctc-3'; smad7, F: 5'-agcttgagtcgagagagctc-3'; and colIagen V, F: 5'-tctcagggaggggggaggctagc-3'; R: 5'-accccttcttcagggaggctagc-3'. All real-time PCR was performed in triplicate, and the data are presented as means ± SD.

In situ hybridization. Four-micrometer sections of FFPE kidney tissue were used to assess the effect of TXL on miR-21 expression at 24 wk of age. Double-labeled digoxigenin LNA-miR-21 probes were purchased (Exiqon). The sequence of double-digoxigenin-labeled specific miR-21 probes are 5'-3'SDIGN/TCAACACATGCTGTAACGCTA3D/NN or Dig-conjugated control probes with scrambled sequence (Exiqon), and the protocol used to detect endogenous miRs was the single-day in situ hybridization method (Exiqon) per the manufacturer’s recommendations (21). A scrambled probe was used as a negative control probe (Exiqon), with light blue cytoplasmic staining being positive. The sections were double stained with fast red to manifest nuclei.

miR-21 transfection experiments. For transfection experiments, HKCs were seeded at a density of 2 × 10^4 cells/cm² in serum-free DMEM/F12, with the addition of a transfection agent and miR control lentivirus vector (miR-control group), pre-miR-21 lentivirus vector (pre-miR-21 group or miR-21 overexpression group), and HKCs without transfection were used as a blank group (blank control group). After 12-h transfection, the medium was changed and the HKCs were treated with 25 mmol/l glucose plus TXL as the TXL treatment (TXL) group.

In vivo studies. To explore the effect of TXL on miR-21 expression and renal tubular EMT in diabetic nephropathy, we utilized a polygenic KK-Ay mice model suitable for human type 2 diabetes mellitus, and its renal lesions closely resembled human DN (9). Male C57BL/6J and KK-Ay mice (8 wk of age) were from the Chinese Academy of Medical Sciences (Beijing, China). C57BL/6J mice (control group) were fed by common forage, and KK-Ay mice were fed by research diets for 4 wk, when their random blood glucose (RBG) was ≥300 mg/dl (16.7 mmol/l) and urine albumin/creatinine ratio (ACR) was ≥300 μg/mg. KK-Ay mice were considered as having diabetic nephropathy (DN). C57BL/6J mice treated with vehicle (normal saline, NS, gavage) was classified as the normal control group (n = 10), KK-Ay mice treated with vehicle (normal saline, NS, gavage) were utilized as the diabetic nephropathy control group (DN control group, n = 10), and KK-Ay mice treated with TXL solution (TXL superfine powder, 0.75 g·kg^-1·day^-1, n = 10, gavage) were utilized as DN with TXL group (TXL group) for 12 wk. Renal tissue from each mouse was also divided into two parts; one was immediately frozen in liquid nitrogen for Western blotting and RT-PCR, and the other was fixed with 4% paraformaldehyde for in situ hybridization and immunohistochemical staining, respectively. The study protocol was approved by the Institutional Animal Care and Use Committee at Capital Medical University.
To determine whether TXL affected miR-21 expression, miR-21 expression was examined by RT-PCR in vitro. miR-21 expression was unchanged at the concentration of 250–500 μg/ml (Fig. 1A), whereas miR-21 expression was significantly different in the TXL group compared with the DN control group (Fig. 1, B–D). Taken together, these results demonstrated that TXL lowered miR-21 expression both in vivo and in vitro.

Effect of TXL on miR-21 overexpression-induced renal tubular EMT. To determine whether TXL has an effect on miR-21-induced EMT in vitro and in vivo, we performed the following cell transfection experiments. First, HKCs were treated with 350 μg/ml TXL for 72 h. Then, a pre-miR-21 lentivirus vector was transfected into HKCs. Second, we transfected the pre-miR-21 lentivirus vector into HKCs, and the transfected cells were treated with 350 μg/ml TXL for 72 h. The results showed that miR-21 overexpression (pre-miR-21) enhanced α-SMA mRNA and decreased E-cadherin mRNA expression (P < 0.05). Furthermore, TXL alleviated the change in α-SMA and E-cadherin mRNA induced by pre-miR-21, and the protective effects of TXL were reversed by pre-miR-21 (Fig. 2A, P < 0.05). Next, α-SMA and E-cadherin protein were examined by immunocytochemistry and Western blotting. The results showed that miR-21 overexpression obviously enhanced α-SMA protein and decreased E-cadherin protein (Fig. 2, B–D, P < 0.05). Interestingly, the immunocytochemistry results of a-SMA and E-cadherin were consistent with the results of RT-PCR and Western blotting (P < 0.05).

Next, to test whether TXL affected the markers of EMT in vivo, α-SMA and E-cadherin mRNA and protein were also examined by RT-PCR and immunohistochemistry in kidneys. The results showed that the increase in α-SMA and the decrease in E-cadherin were significantly different in the DN control group compared with NC group (Fig. 2, E and F, P < 0.05). After the treatment of TXL (0.75 g·kg⁻¹·day⁻¹, gavage) for 12 wk, α-SMA was significantly decreased and E-cadherin was significantly increased in the TXL group compared with the DN control group (Fig. 2, E and F, P < 0.05). Taken together, our data demonstrated that miR-21 overexpression promoted renal tubular EMT and TXL can inhibit miR-21-induced EMT.

Effect of miR-21 overexpression on TGF-β1/smads3/smads7 expression and TXL intervention. The TGF-β1/smads signal pathway plays an important role in EMT, accumulating evidences demonstrated that smad3 promoted renal tubular EMT. Smad7 inhibited EMT in diabetic nephropathy, suggesting that smad3 and smad7 have an antagonistic effect on EMT (14). To determine that TXL has effects on the TGF-β1/smads signal pathway in vitro, we used RT-PCR to examine TGF-β1/smads3 and smad7 mRNA expression. The results showed that TXL directly reduced TGF-β1/smads3 mRNA and increased smad7 mRNA expression in vitro (Fig. 3A, P < 0.05). Meanwhile, TXL can further decrease TGF-β1/smads3 mRNA and increase.
smad7 mRNA expression induced by miR-21 overexpression. Next, to investigate whether miR-21 overexpression affects smad3 and smad7 mRNA and protein and TXL intervention in vitro, first according to bioinformatics microRNA targetscan (http://www.targetscan.org/), we found that smad7 may be a potential target of miR-21 (Fig. 3B). Second, we performed the function of miR-21 overexpression experiment. The results of Western blotting showed that miR-21 overexpression enhanced smad3/p-smad3 protein expression and decreased smad7 protein expression compared with the miR control group and blank group (Fig. 3, C and D, P < 0.05). More importantly, TXL inhibited smad3/p-smad3 and smad7 protein expression induced by miR-21 overexpression in vitro (Fig. 3, C and D, P < 0.05). In vivo, to further testify whether TXL affected smad3/p-smad3 and smad7 expression, we used RT-PCR and immunohistochemistry to examine smad3/p-smad3 and smad7 expression. The results of RT-PCR showed that smad3 was increased and smad7 was decreased in the DN control group compared with the normal control group, and TXL significantly decreased smad3 and increased smad7 mRNA expression (Fig. 3E, P < 0.05). Additionally, immunohistochemistry showed that smad3 and smad7 were mainly distributed in the cytoplasm of renal tubular epithelial cells, whereas, p-smad3 was expressed and distributed in the nuclei. Interestingly, the mean optical density of smad3/p-smad3 and smad7 were consistent with the result of RT-PCR (Fig. 3, F and G, P < 0.05). Taken together, our data exhibited that miR-21 overexpression could upregulate TGF-β1/sm ad3 and downregulate smad7 expression and that TXL could inhibit the alteration of TGF-β1/sm ad3 and smad7 induced by miR-21 overexpression both in vitro and in vivo.

Effect of TXL on col-IV and FN in vitro and in vivo. One key feature of DN was the accumulation of extracellular matrix (ECM) proteins such as col-IV and FN (19). EMT was the main reason to lead to the deposition of ECM and renal fibrosis. To determine whether TXL affects col-IV and FN in vitro, col-IV and FN mRNA and protein were examined by RT-PCR, Western blotting, and immunocytochemistry, respectively. The results of RT-PCR showed that col-IV and FN mRNA were remarkably increased in the HG group compared with the NC group (Fig. 4A), TXL decreased the expression of col-IV and FN mRNA (Fig. 4A). Meanwhile, the results of Western blotting and immunocytochemistry showed that col-IV and FN proteins were also strikingly increased in the HG group compared with the NC group, TXL also decreased the expression of col-IV and FN protein (Fig. 4, B–E). Interestingly, the results of immunocytochemistry were consistent with the results of Western blotting. In vivo, we further observed the effect of TXL on col-IV and FN mRNA and protein. We found that TXL significantly decreased the expression of col-IV and FN mRNA and protein (Fig. 4, F and H, P < 0.05). Furthermore, the results of col-IV and FN mRNA and protein in vivo were consistent with the results in vitro. Together, our results suggested that TXL can alleviate the excessive deposition of col-IV and FN.

Effect of TXL on renal morphology and function. The most key characteristics of DN are glomerular basement membrane (GBM) thickening and mesangial matrix hyperplasia, leading to the loss of renal function (20). GBM and glomerular area (GA) were the sensitive morphological markers of DN (22). The ACR has been demonstrated to be a good clinical predictor
of renal lesions in DN (30). The creatinine clearance ratio (Cr) is generally considered as the marker of renal function, reflecting renal filtration function. To evaluate the effect of TXL on morphological and functional changes at 24 wk of age, renal morphology was observed by light and electronic microscopy (Fig. 5A). GBM (85 ± 20.6 nm) was normal, and the glomerular foot process was slender and tidy in the normal control group. In contrast, GBM (145 ± 20.6 nm) thickened, foot process partly fused, the structure was disordered, GA (53.2 ± 4.4 vs. 38.9 ± 5.6 μm²) was enlarged, ACR (1,530 ± 28.9 vs. 35 ± 5.6 μg/mg) increased, and Cr (3.213 ± 0.143 vs. 4.369 ± 0.80 ml·min⁻¹·kg⁻¹) decreased (Fig. 5, B–E, P < 0.05) in the DN control group. Next, we evaluated the effect of TXL on GBM, GA, ACR, and Cr after treatment with TXL (0.75 g·kg⁻¹·day⁻¹, gavage) for 12 wk compared with the DN control group. GBM was decreased from 145 to 120 nm, GA was reduced from 53.2 to 45.9 μm², ACR was obviously decreased from 1,500 to 1,020 μg/mg, and Cr was slightly but significantly increased from 3.213 to 3.907 ml·min⁻¹·kg⁻¹ in the TXL treatment group. Together, our results suggested that TXL ameliorated renal structure and function.

DISCUSSION

DN is the leading cause of end-stage kidney disease. Although hyperglycemia, hypertension, hemodynamic and metabolic factors, hereditary factors, or family history are...
involved in the pathogenesis of DN, the exact cause of DN is currently unknown. EMT plays an important role in the development and progress of DN (18). Numerous research showed that miR-21 was closely linked to EMT in different fields (2, 6, 7). Recent research showed that miR-21 was a key therapeutic target for renal injury in a mouse model of type 2 diabetes (44). Our previous experiment showed that miR-21 was primarily located in renal tubular epithelial cells and participated in the mechanism of DN (32). However, it remained unclear whether TXL affected miR-21 expression and miR-21-induced EMT. Our in situ hybridization and/or RT-PCR results exhibited that miR-21 expression was significantly enhanced in HKCs, serum, and renal tissue in hyperglycemic conditions. Interestingly, miR-21 overexpression enhanced \(\alpha\)-SMA expression and decreased E-cadherin expression. More importantly, TXL not only inhibited miR-21 expression, but also alleviated the changes in \(\alpha\)-SMA and E-cadherin in vivo and in vitro. The most possible molecular mechanism was that hyperglycemia induced the increase in TGF-\(\beta\)/Smad3, expression further promoting miR-21 expression (3) and that TXL can down-regulate TGF-\(\beta\) expression further, leading to the decrease

Fig. 3. Effect of TXL on transforming growth factor (TGF)-\(\beta\)/smad3 and smad7 expression and TXL intervention. A: miR-21 overexpression increased TGF-\(\beta\)/smad3 mRNA expression and decreased smad7 mRNA expression (\(P < 0.05\)), and TXL can inhibit TGF-\(\beta\)/smad3 and smad7 expression induced by miR-21 overexpression in vitro (\(P < 0.05\)). B: miR-21 binding region of the 3′-untranslated region (UTR) of smad7. C: representative band of smad3/p-smad3 and smad7 by Western blotting in vitro. D: comparison of the grey value of p-smad3, smad3, and smad7 by Western blotting in vitro. E: real-time quantitative PCR showed that smad3 mRNA was significantly increased and smad7 mRNA was significantly decreased in the DN control group compared with the NC group (\(P < 0.05\)). TXL markedly decreased smad3 mRNA and increased smad7 mRNA expression (\(P < 0.05\)). F: representative immunohistochemical photograph for smad3/p-smad3 and smad7. G: mean optical density (MOD) value of smad3/p-smad3 and smad7 (\(P < 0.05\)).
in miR-21 expression (29, 34). Taken together, we speculated that TXL might have pleiotropic effects on miR-21 expression and miR-21-induced EMT both indirectly and directly, suggesting that miR-21 may be one of the therapeutic targets of TXL for protection from DN.

Accumulating evidence demonstrated that TGF-β1/smad3 and miR-21 promoted EMT (8, 40), and smad7 prevented renal tubular EMT by inhibiting smad3 phosphorylation (12, 14). More interestingly, smad7 was a potential target of miR-21, which can lead to smad7 degradation by binding to
smad7 mRNA (26). All this research showed that miR-21 and TGF-β1/smads existed in a complex regulation relationship. However, how miR-21 regulated the TGF-β1/smads signal and whether TXL affected smad3/p-smad3 and smad7 expression remain unclear. In our experiment, miR-21 overexpression decreased smad7 mRNA and proteins and enhanced smad3/p-smad3 mRNA and proteins, suggesting that miR-21 overexpression directly downregulated smad7 expression and indirectly upregulated smad3/p-smad3. Additionally, in vivo immunohistochemistry showed that smad3 and smad7 were mainly distributed in the cytoplasm of renal tubular epithelia cells, whereas, p-smad3 was expressed and distributed in the nuclei, suggesting that smad3 phosphorylated into p-smad3 and then entered the nucleus to promote miR-21. This suggested maturation and regulation of gene transcription related to EMT, in turn suggesting that miR-21 and TGF-β1/smads formed a double-positive feedback loop to enhance renal tubular EMT by inhibiting smad7. More importantly, TXL can downregulate smad3/p-smad3 and upregulate smad7 expression in vitro and in vivo, consistent with the previous experiment (29). Thus we concluded that TXL ameliorated renal structure and function by inhibiting miR-21 expression and miR-21-induced EMT in DN.

In summary, our study suggested that miR-21 and TGF-β1/smads formed a double-positive feedback loop to enhance renal tubular EMT by inhibiting target smad7. TXL ameliorated renal structure and function by regulating miR-21-induced EMT, which was one of the mechanisms to protect against DN, and miR-21 may be one of the novel therapeutic targets for TXL in DN.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: J.W., Y.G., and J.Y. provided conception and design of research; J.W., N.Z., D.Z., J.-y.L., S.-n.Z., and F.-q.C. performed experiments; J.W., Y.G., and L.-p.X. interpreted results of experiments; J.W. drafted manuscript; Y.G., D.Z., Z.-y.Z., X.-j.Z., and J.-g.G. analyzed data; Y.G. and S.-n.Z. edited and revised manuscript; Y.G. approved final version of manuscript.

Fig. 5. Effect of TXL on renal morphology and function. A: representative photograph for hematoxylin-eosin (HE), periodic acid-Schiff (PAS), and electron microscopy (EM). Foot process partly fused and structure was disordered in the DN control group. After the treatment with TXL, the morphological change was obviously improved. B: the glomerular basement membrane (GBM) was obviously thickened in the DN control group. TXL reduced GBM thickness (P < 0.05). C: glomerular area (GA) was enlarged in DN control group. TXL can decrease GA (P < 0.05). D: albumin/creatinine ratio (ACR) was increased in DN control group (P < 0.05). TXL decreased ACR (P < 0.05). E: creatinine clearance ratio (Ccr) was decreased in the DN control group (P < 0.05). After the treatment with TXL, Ccr was slightly but significantly increased. Light microscopy, ×400. EM, ×20,000.
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