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Usp2-69 overexpression slows down the progression of rat *anti*-Thy1.1 nephritis

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ABSTRACT

Mesangial proliferative glomerulonephritis is characterized by proliferation of mesangial cells (MCs) and transforming growth factor-β (TGF-β)-dependent stimulation of abnormal extracellular matrix (ECM) accumulation. We previously showed that Decorin––a leucine-rich proteoglycan inhibiting the progression of glomerulonephritis and glomerular sclerosis––can be degraded by the ubiquitin-proteasome pathway and deubiquitinated and stabilized by ubiquitin-specific processing protease 2-69(Usp2-69). Usp2-69 is highly expressed in the kidney and has been implicated in the regulation of cell proliferation and apoptosis. However, its role in mesangial proliferative glomerulonephritis remains unclear. Here, we explored the effect of Usp2-69 on MC proliferation and ECM deposition by transfecting Usp2-69 plasmid into rat *anti*-Thy1.1 nephritis model and into cultured MCs, as well as detected Usp2-69 and Decorin in rat *anti*-Thy1.1 nephritis model by western blot. Overexpressing Usp2-69 at the early stage, but not advanced stage, of *anti*-Thy1.1 nephritis alleviated cell proliferation and ECM deposition, which was shown by decreased Ki-67, Collagen IV and Fibronectin detected by immunohistochemistry. Overexpression also increased Decorin and decreased TGF-β1 and Collagen IV both *in vitro* and *in vivo*. In conclusion, our findings suggest that Usp2–69 overexpression alleviates the progression of rat *anti*-Thy1.1 nephritis and, therefore, that exogenous plasmid injection *via* the renal artery enhanced by electrotransfer technology could be a promising avenue for glomerular disease research.
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1. Introduction

Proliferation of mesangial cells (MCs) and abnormal accumulation of extracellular matrix (ECM) in mesangium are the common pathological characteristics in many mesangial proliferative glomerulonephritises such as IgA nephropathy (IgAN), mesangial proliferative glomerulonephritis and lupus nephritis. These two detrimental responses are implicated in the progression of further nephron damage and glomerular sclerosis, ultimately leading to end-stage renal disease. During the course of disease progression, activated proliferating MCs releases a large number of proinflammatory and profibrotic cytokines, including transforming growth factor-β (TGF-β), tumor necrosis factor (TNF), interleukin-6 (IL-6), and angiotensin II (Schlondorff, 1996; Schlondorff and Banas, 2009). TGF-β is a major cytokine involved in glomerular sclerosis (Gupta et al., 2000), by inducing ECM such as fibronectin-1 and Collagen IV (Col IV) secreted by MCs in an autocrine fashion through its downstream pathways Smads (Schiller et al., 2004) and MAPKs (Chin et al., 2001).

Decorin, a leucine-rich proteoglycan secreted by MCs in glomeruli, is capable of neutralizing TGF-β1 signaling either directly by physical interaction with TGF-β1 and its three receptors (leucine-rich repeats receptors), or indirectly by regulating other modulators of TGF-β1 such as: fibrillin-1 and myostatin, thus antagonizing the biological activities of TGF-β1 in inflammation, fibrosis and cell growth (Border et al., 1992; Schaefer, 2011). Our previous study further observed overexpression of Decorin markedly reduced the protein level of TGF-β1 in cultured MCs (Wu et al., 2008). *In vivo* experiments showed that either transferring Decorin plasmid into rat skeletal muscle by using HVJ-liposome method or transferring Decorin-transfected cloned MCs into rat glomeruli by left renal artery injection of the rat *anti*-Thy1.1 glomerulonephritis model, gradually increased the protein level of Decorin in the kidney which remarkably reduced TGF-β1 expression and ECM

Abbreviation: Usp2-69, Ubiquitin-specific processing protease 2-69; ECM, Extracelluar matrix; MC, Mesangial cell; Col IV, Collagen IV; TGF-β, transforming growth factor-β; TNF, tumor necrosis factor; IL-6, interleukin-6; PDGF-BB, platelet-derived growth factor-BB; PCNA, proliferating cell nuclear antigen; H&E, hematoxylin-eosin; PAS, periodic acid Schiff; SD rats, Sprague-Dawley rats; FFPE, Formalin-Fixed and Parrffin-Embedded; α-SMA, α-smooth muscle actin; HA, hemagglutinin; GFP, green fluorescent protein.

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accumulation, thus exhibiting a therapeutic effect on the progression of glomerulonephritis and glomerular sclerosis (Border et al., 1992; Huijun et al., 2005; Isaka et al., 1996; Sun et al., 2012). However, clinical application of Decorin is hampered by limited approaches to modulate its *de novo* synthesis.

Recently, we reported that Decorin could be degraded by ubiquitin-proteasome pathway and deubiquitinated and stabilized by ubiquitin-specific processing protease 2–69 (Usp2–69), which is highly expressed in the kidney (Gousseva and Baker, 2003; Luo et al., 2015; Wu et al., 2010). As enzymes can be easily drug-targeted, either by inducers (also known as activators) or inhibitors which alter the metabolic activity of enzymes by binding to the catalytic domain or regulate their active repressors (Feely, 1990; Robertson, 2005), we thus aimed to explore the direct effect of Usp2–69, the deubiquitinating enzyme of Decorin, in glomerulonephritis featured by MCs proliferation and ECM deposition.

In this study, we observed the relationship between Usp2–69 and TGF-β1 protein expression and ECM accumulation, and examined the effect of exogenous Usp2–69 on TGF-β1 protein expression and ECM accumulation by using rat *anti*-Thy1.1 nephritis model *in vivo* and cultured MCs treated with cytokines including TNF-α, TGF-β and platelet-derived growth factor-BB (PDGF-BB) *in vitro*, to explore the potential significance of Usp2-69 in the progression of mesangial proliferative glomerulonephritis.

2. Materials and methods

2.1. Ethics statement

Permission on performing animal experiment for research purposes (No.20120413-007) was approved by the Ethics Committee of Shanghai Medical College, Fudan University, China. All procedures were carried out according to the approved guidelines.

2.2. Antibodies

Antibodies used in this study included Decorin (R&D Systems), Usp2-69, Collagen IV, proliferating cell nuclear antigen (PCNA), α-smooth muscle actin (α-SMA), Ki-67 (Abcam), Fibronectin (Calbiochem), hemagglutinin (HA), green fluorescent protein (GFP) (Cell Signaling Technology), β-actin(Sigma-Aldrich), TGF-β1, Peroxidase conjugated goat anti-mouse IgG and goat anti-rabbit IgG antibody (ProteinTech Group). The catalog number was shown in Table S1.

2.3. Cell culture and treatment

Immortalized rat mesangial cells (CRL2573, ATCC, USA) and mesangial cells stably transfected with Decorin plasmid were cultured in DMEM (Hyclone, USA) and 10% fetal bovine serum (Gibco, Australia) at 37 °C in a humidified atmosphere containing 5% CO2/95% air as previously described (Wu et al., 2010). MCs or MC/Decorin, seeded in six-well plates at 70–80% confluence, were treated with TGF-β1 (Sigma Aldrich, MO, USA) at 10 ng/ml, TNF-α (Sigma Aldrich, MO, USA) at 10 ng/ml, or PDGF-BB (Sigma Aldrich, MO, USA) at 25 ng/ml for 0, 3, 6, 12, and 24 h, or by *anti*-ox-7 antibody (Santa Cruz, *CA*, USA) at 0.5, 1.25, 2.5, 5 μg/ml, supplemented with normal rat serum 10 μl/ml, for 6 h. The mouse IgG (Bioworld, MN, USA) was used as control.

2.4. Protein isolation from MC and western blot analysis

The harvest of the pellet of MCs using cell lysis buffer was as described (Wu et al., 2010). Lysates (40 μg), measured by BCA Protein Assay Reagent kit (Piece, IL, USA) were used for western blot as described (Wu et al., 2010).

2.5. Proliferation analyses by flow cytometry

MCs, treated by *anti*-ox-7 antibody or mouse IgG as above, were harvested and fixed in 1 ml citrate buffer. Analyses of the phases of the cells were as described (Sun et al., 2012).

2.6. Anti-Thy1.1 nephritis and electroporation-mediated plasmid transfer via renal artery

The male Sprague-Dawley rats (SD rats) (Selack, China) weighing 145–155 g were housed in our facilities providing free access to water and regular chew. *Anti*-Thy1.1 nephritis was induced by single *i.v.* tail injection of ox-7 antibody (Santa Cruz, CA, USA) (250 μg/150 g of body weight). At the 2nd, 4th, or 6th day of injection, the Usp2-69 plasmids, mediated by electroporation, were transferred to glomeruli. Briefly, the rats were anesthetized, and the aorta, left renal artery and the left kidney were exposed. The renal vein and the abdominal aorta, both proximal and distal to the renal artery, were clamped. The 0.45 mm infusion needle, connected to the syringe, punctured the aorta and then branched into the renal artery. The kidney was perfused with 500 μl BSS, and Usp2-69 plasmid solution (250 μg in 500 μl TE buffer) was infused into the kidney. Afterwards, the kidney was sandwiched by the oval tweezer-type electrodes (Xinzhi, China, WJ-2005), and electric pulses were delivered with 6 pulses of 100 V, one pulse per second with duration of 900 ms between pulses. After transfection, the needle was removed, the puncture fixed with gelatin sponge and the clamps removed.

The rats were anesthetized and sacrificed at day 5, 8 since ox-7 antibody injection. Both kidneys were removed for the preparation of Formalin-Fixed and Parrffin-Embedded (FFPE) samples and protein isolation. The FFPE samples were stained with hematoxylin-eosin (H&E) (Baso, China) and periodic acid Schiff (PAS) staining (Baso, China).

2.7. Protein isolation from the glomeruli and western blot analysis

The glomeruli of the kidney were isolated in a sieving method as described (Wu et al., 2013). The glomeruli were homogenized in the lysis buffer in the presence of protease inhibitor cocktail and vortexed every 10 min for 7 times on ice, which was followed by centrifugation. The lysate measurement and the blotting was the same as above.

2.8. Immunohistochemisty

The sections were dewaxed and hydrated. Endogenous peroxidase was removed using 3% H_2O_2. The antigen was retrieved in citrate buffer. The sections were blocked by 5% normal sheep serum, incubated with primary antibody at 4 °C overnight (Col IV: 1:500; Ki-67: 1:200; α-SMA:1:200; FN: 1:50; PCNA: 1:100), and secondary antibody for 1 h in 37 °C. The expression was detected by diaminobenzidine and hematoxylin was used as the nuclear counterstain.

2.9. Transient transfection

The pRK5-USP2-69-HA plasmid was amplified in *E. coli* DH5α and the authenticity was verified by sequencing. MCs (1×10^6) were seeded in six-well plates. At 70–80% confluence the cells were transfected using plasmid (10 μg) and 10 μl Lipofectamine 2000 (Life technologies, MD, USA) at 37 °C for 6 h, followed by adding 2 ml 15% fetal bovine serum/DMEM medium and incubating for 48 h at 37 °C.

2.10. Statistical analysis

The experiments were all repeated for at least three times in independent biological replicates. We selected 25 different glomeruli from each slide randomly and evaluated of staining results. The positive

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