

The long noncoding RNA LOC105374325 causes podocyte injury in individuals with focal segmental glomerulosclerosis

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Focal segmental glomerulosclerosis (FSGS) is a common kidney disease that results in nephrotic syndrome. FSGS arises from dysfunction and apoptosis of podocytes in the glomerulus of the kidney, leading to podocytopathy. The molecular mechanisms underlying podocyte apoptosis remain incompletely understood. Using an array of gene expression profiling, PCR, and *in situ* hybridization assay, we found here that the levels of the long noncoding RNA LOC105374325 were elevated in the renal podocytes of individuals with FSGS. We also observed that the microRNAs miR-34c and miR-196a/b down-regulated the expression of the apoptosis regulators BCL2-associated X, apoptosis regulator (Bax), and BCL2 antagonist/killer 1 (Bak) in podocytes. Competitive binding between LOC105374325 and miR-34c or miR-196a/b increased Bax and Bak levels and caused podocyte apoptosis. Of note, the mitogen-activated protein kinase P38 and the transcription factor CCAAT enhancer-binding protein β (C/EBP β) up-regulated LOC105374325 expression. P38 inhibition or C/EBP β silencing decreased LOC105374325 levels and inhibited apoptosis in adriamycin-treated podocytes. LOC105374325 overexpression decreased miR-34c and miR-196a/b levels, increased Bax and Bak levels, and induced proteinuria and focal segmental lesions in mice. In conclusion, activation of the P38/C/EBP β pathway stimulates the expression of LOC105374325, which, in turn, increases Bax and Bak levels and causes apoptosis by competitively binding to miR-34c and miR-196a/b in the podocytes of individuals with FSGS.

Focal segmental glomerulosclerosis (FSGS)⁴ is a common pathological type of nephrotic syndrome in which the sclerosis lesion involves <50% of the total number of glomeruli and <50% of the glomerular capillary surface. FSGS accounts for 40% of the cases of nephrotic syndrome in adults (1). The degree of proteinuria is an independent risk factor for renal function decline in patients (2, 3). Under normal conditions, the podocyte slit diaphragm serves as an important domain contributing to the permeability barrier of the glomerulus. Podocyte loss is involved in the pathogenesis of FSGS, and apoptosis is the major cause of reduced podocyte number, leading to proteinuria and glomerulosclerosis (4).

Bax and its homolog Bak are the key regulators of the mitochondrial pathway of apoptosis. Under conditions of cell stress, Bax and Bak accumulate on the mitochondrial surface, where they oligomerize and mediate cytochrome *c* release, thereby leading to cell death. A previous study showed an increased level of Bax expression and apoptosis in the glomerular tissues of FSGS patients (5). Nevertheless, the mechanisms underlying the expression of Bax and podocyte apoptosis remain incompletely understood.

Long noncoding RNAs (lncRNAs) are longer than 200 nucleotides and have no protein-encoding capacity (6). Several lines of evidence suggest that lncRNAs are involved in the pathogenesis of podocyte injury. Hu *et al.* (7) reported that the level of the lncRNA MALAT1 was elevated in diabetic nephropathy and was involved in high glucose-induced podocyte injury via its interplay with β -catenin. Long *et al.* (8) reported that Tug1 regulates mitochondrial function in podocytes by the epigenetic targeting of the transcription factor peroxisome proliferator-activated receptor γ coactivator 1 α . Ling *et al.* (9) showed that lncRNA ENSRNOG00000037522 is involved in the podocyte epithelial-mesenchymal transition in diabetic rats.

In this study, we performed a transcriptome analysis of glomerular tissues in 5 FSGS patients and 5 controls. Among the differentially expressed lncRNAs, the level of LOC105374325 showed the most significant increase in the glomerular tissues

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This article contains Figs. S1 and S2 and Tables S1 and S2.

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⁴ The abbreviations used are: FSGS, focal segmental glomerulosclerosis; lncRNAs, long noncoding RNAs; ISH, *in situ* hybridization; ADR, adriamycin; IHC, immunohistochemical; miR, microRNA; ASO, antisense oligonucleotide; GSK3 β , glycogen synthase kinase 3 β ; ERK, extracellular signal-regulated kinase; IL, interleukin; C/EBP, CCAAT enhancer-binding protein; MAPK, mitogen-activated protein kinase; CMV, cytomegalovirus; STAT, signal transducers and activators of transcription; ANOVA, analysis of variance.

Role of lncRNA LOC105374325 in podocytes of FSGS

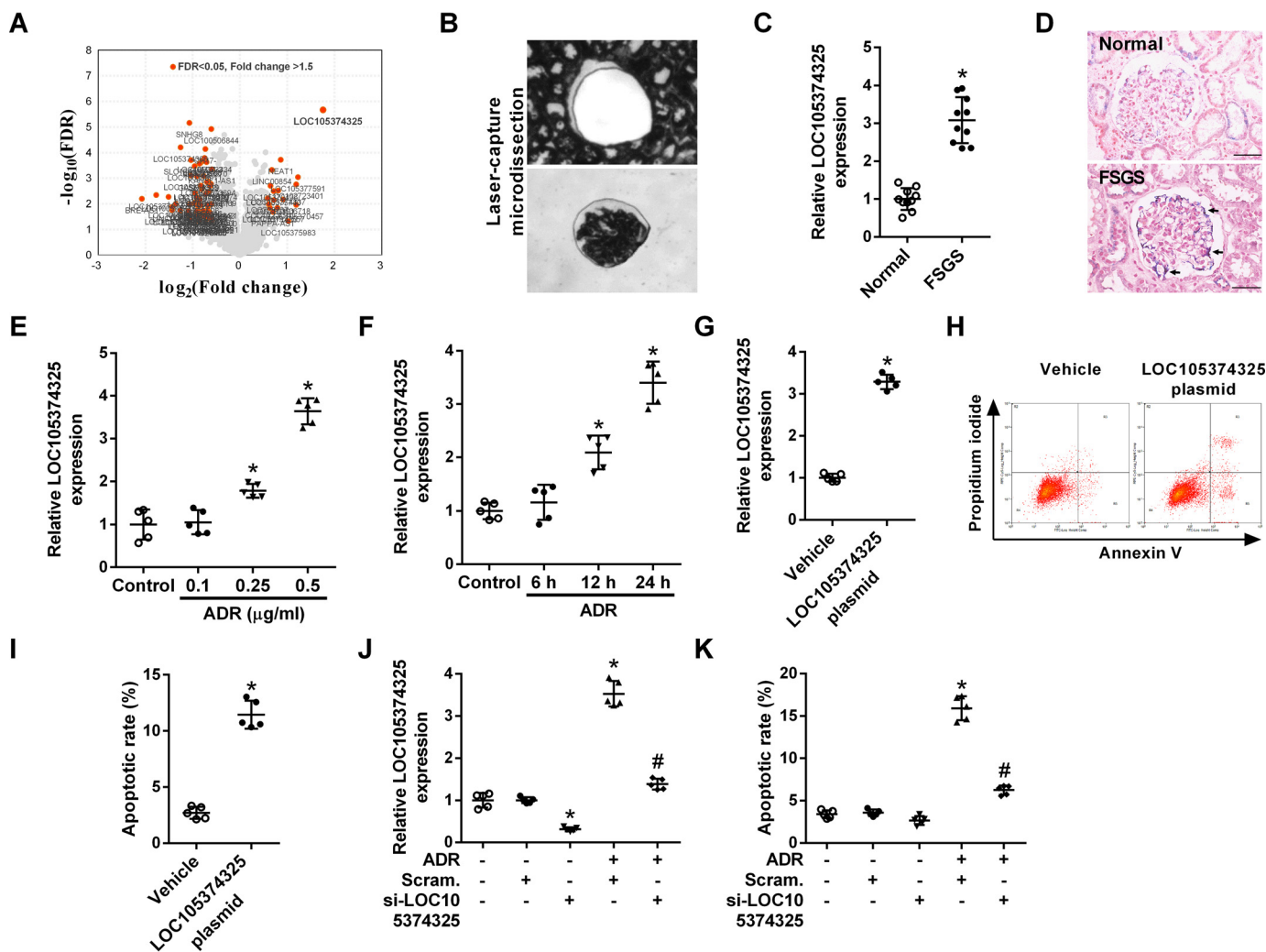


Figure 1. Change of LOC105374325 level in renal podocytes of FSGS patients. A, volcano plot of differentially expressed lncRNAs in glomerular tissues of FSGS patients, with cutoff values of fold-change >1.5 and false discovery rate <0.05 ($n = 5$); B, isolation of glomerular tissues by laser capture microdissection; C, level of LOC105374325 in glomerular tissues of FSGS patients ($n = 10$, validation cohort); D, ISH analysis of LOC105374325 in glomerular tissues of FSGS patients and normal controls ($n = 5$); E, level of LOC105374325 in podocytes treated with different doses of ADR for 24 h ($n = 5$); F, level of LOC105374325 in podocytes treated with ADR (0.5 $\mu\text{g}/\text{ml}$) for different times ($n = 5$); G, level of LOC105374325 in podocytes transfected with LOC105374325 plasmid ($n = 5$); H, flow cytometric analysis of apoptotic cells in podocytes transfected with LOC105374325 plasmid ($n = 5$); I, quantitative analysis of apoptotic cells in podocytes transfected with LOC105374325 plasmid ($n = 5$); J, level of LOC105374325 in podocytes treated with ADR and LOC105374325 siRNA ($n = 5$); K, flow cytometric analysis of apoptotic cells in podocytes treated with ADR and LOC105374325 siRNA ($n = 5$). For statistical analysis, a two-tailed Student's *t* test was used for C, G, and I, and one-way ANOVA with Tukey's post hoc test was used for E, F, J, and K. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with podocytes treated with ADR. Bar, 20 μm .

of FSGS patients. Whether the up-regulated LOC105374325 leads to the increase of Bax expression and podocyte injury remains unclear. We conducted both *in vitro* and *in vivo* experiments to investigate the role of LOC105374325 in podocyte injury in FSGS patients.

Results

LncRNA LOC105374325 is up-expressed in renal podocytes of FSGS patients

Renal glomerular tissues from 5 FSGS patients and 5 controls were microdissected, and an Affymetrix HTA 2.0 microarray was used to perform a global analysis of the gene expression pattern in the tissues. Among the differentially expressed lncRNAs, the level of LOC105374325 showed the most significant increase in the glomerular tissues of FSGS patients (Fig. 1A). RT-PCR analysis of another set of patients confirmed that the level of LOC105374325 was increased in the glomerular

tissues of FSGS patients (Fig. 1, B and C). *In situ* hybridization (ISH) staining showed that LOC105374325 expression was up-regulated in renal podocytes of FSGS patients (Fig. 1D).

Treatment with adriamycin (ADR) in cultured human podocytes increased the expression of LOC105374325 in both a dose- and time-dependent manner (Fig. 1, E and F). The level of LOC105374325 was significantly increased in podocytes treated with 0.5 $\mu\text{g}/\text{ml}$ of ADR for 24 h. Overexpression of LOC105374325 alone induced the cell apoptosis in cultured podocytes (Fig. 1, G–I). Conversely, silence of LOC105374325 decreased the cell apoptosis in podocytes treated with ADR (Fig. 1, J and K).

LOC105374325 increases the expression of Bax and Bak in podocytes

Apoptosis antibody array analysis indicated that the level of Bax and Bak were obviously increased in the glomerular tissues

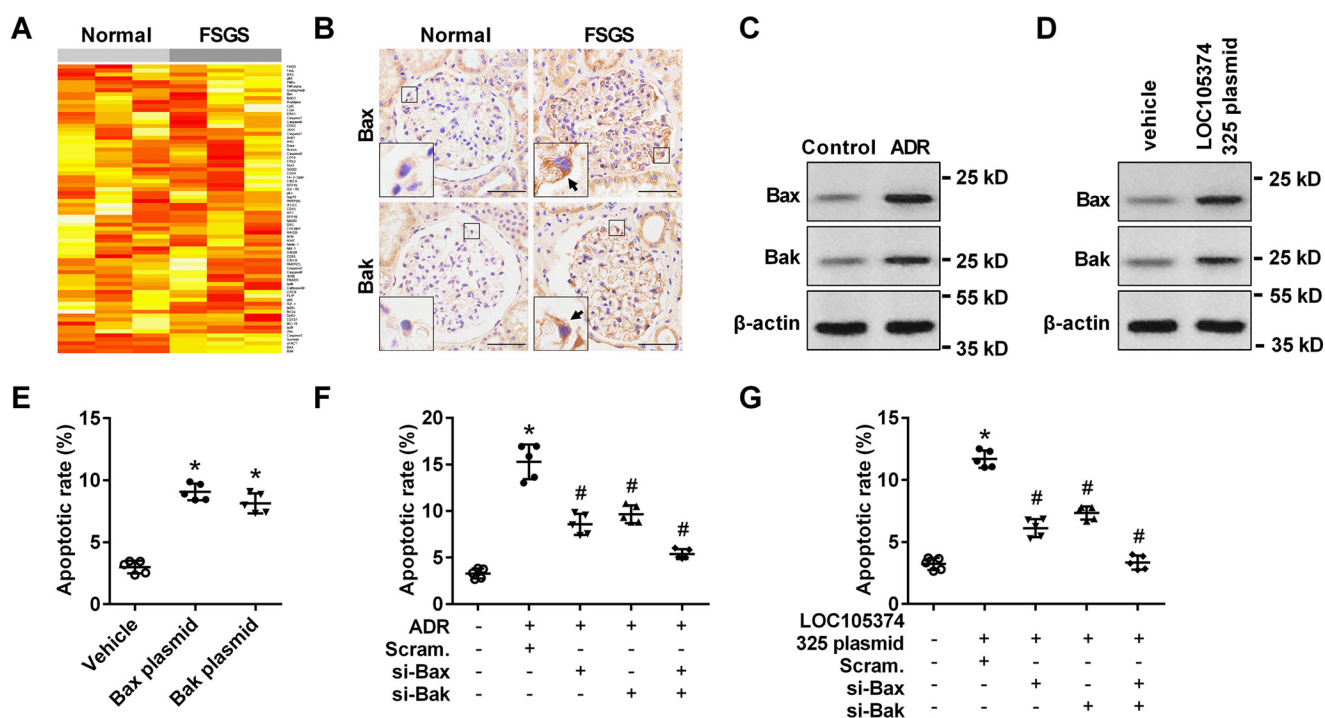


Figure 2. Effect of LOC105374325 on the expression of Bax and Bak in podocytes. A, apoptosis antibody array analysis of glomerular tissues of FSGS patients ($n = 3$); B, IHC analysis of Bax and Bak in glomerular tissues of FSGS patients ($n = 5$); C, Western blot analysis of Bax and Bak in podocytes treated with ADR ($n = 3$); D, Western blot analysis of Bax and Bak in podocytes transfected with LOC105374325 plasmid ($n = 3$); E, flow cytometric analysis of apoptotic cells in podocytes transfected with Bax or Bak plasmid ($n = 5$); F, flow cytometric analysis of apoptotic cells in podocytes treated with ADR, Bax siRNA, and Bak siRNA ($n = 5$); G, flow cytometric analysis of apoptotic cells in podocytes transfected with LOC105374325 plasmid, Bax siRNA, and Bak siRNA ($n = 5$). For statistical analysis, one-way ANOVA with Tukey's post hoc test was used for E–G. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with podocytes treated with ADR or transfected with LOC105374325 plasmid. Bar, 20 μm .

of FSGS patients (Fig. 2A). Bax and its homolog Bak are the key regulators of the mitochondrial pathway of apoptosis. On cell stress, Bax and Bak accumulate on the mitochondrial surface where they oligomerize and mediate cytochrome *c* release, leading to cell death. Immunohistochemical (IHC) analysis showed that, the level of Bax and Bak were increased in the glomerular podocytes of FSGS patients, compared with normal controls (Fig. 2B). Treatment with ADR or overexpression of LOC105374325 increased the expression of Bax and Bak in podocytes *in vitro* (Fig. 2, C and D).

Overexpression of Bax or Bak induced the cell apoptosis in podocytes, and mimics the effect of ADR treatment or LOC105374325 overexpression (Fig. 2E). Conversely, silence of Bax or Bak decreased the cell apoptosis in both ADR-treated podocytes and LOC105374325-overexpressed podocytes (Fig. 2, F and G).

LOC105374325 up-regulates the expression of Bax by competitive binding of miR-34c in podocytes

Although the protein levels of Bax and Bak were increased in glomerular tissues of FSGS patients, PCR analysis showed that the mRNA levels of Bax and Bak were not changed in the glomerular tissues of FSGS patients or in the podocytes transfected with LOC105374325 plasmid (Fig. 3, A and B), suggesting a post-transcriptional mechanism involved in the regulation of Bax and Bak in podocytes of FSGS.

MiRNA PCR array analysis showed that the level of miR-34c and miR-196a/b were significantly decreased in podocytes transfected with the LOC105374325 plasmid (Fig. 3C). The absolute levels of LOC105374325, miR-34c, and miR-196a/b were quanti-

fied by the standard curve method. The levels of LOC105374325, miR-34c, and miR-196a/b were comparable in podocytes, and overexpression of LOC105374325 decreased the absolute level of miR-34c and miR-196a/b in podocytes (Fig. S1).

Computational analysis detected a miR-34c-binding sequence in Bax mRNA 3'UTR (Fig. 3D). Luciferase reporter analysis showed that miR-34c mimics significantly inhibited the reporter activity of Bax 3'UTR-Luc, and site-directed mutations rescued the miR-34c-mediated inhibition of Bax 3'UTR-Luc (Fig. 3E). Transfection with miR-34c mimics also decreased the level of Bax in podocytes treated with ADR or transfected with LOC105374325 plasmid (Fig. 3, F and G). Conversely, transfection with the miR-34c antisense oligonucleotide (ASO) increased the level of Bax in podocytes (Fig. 3H).

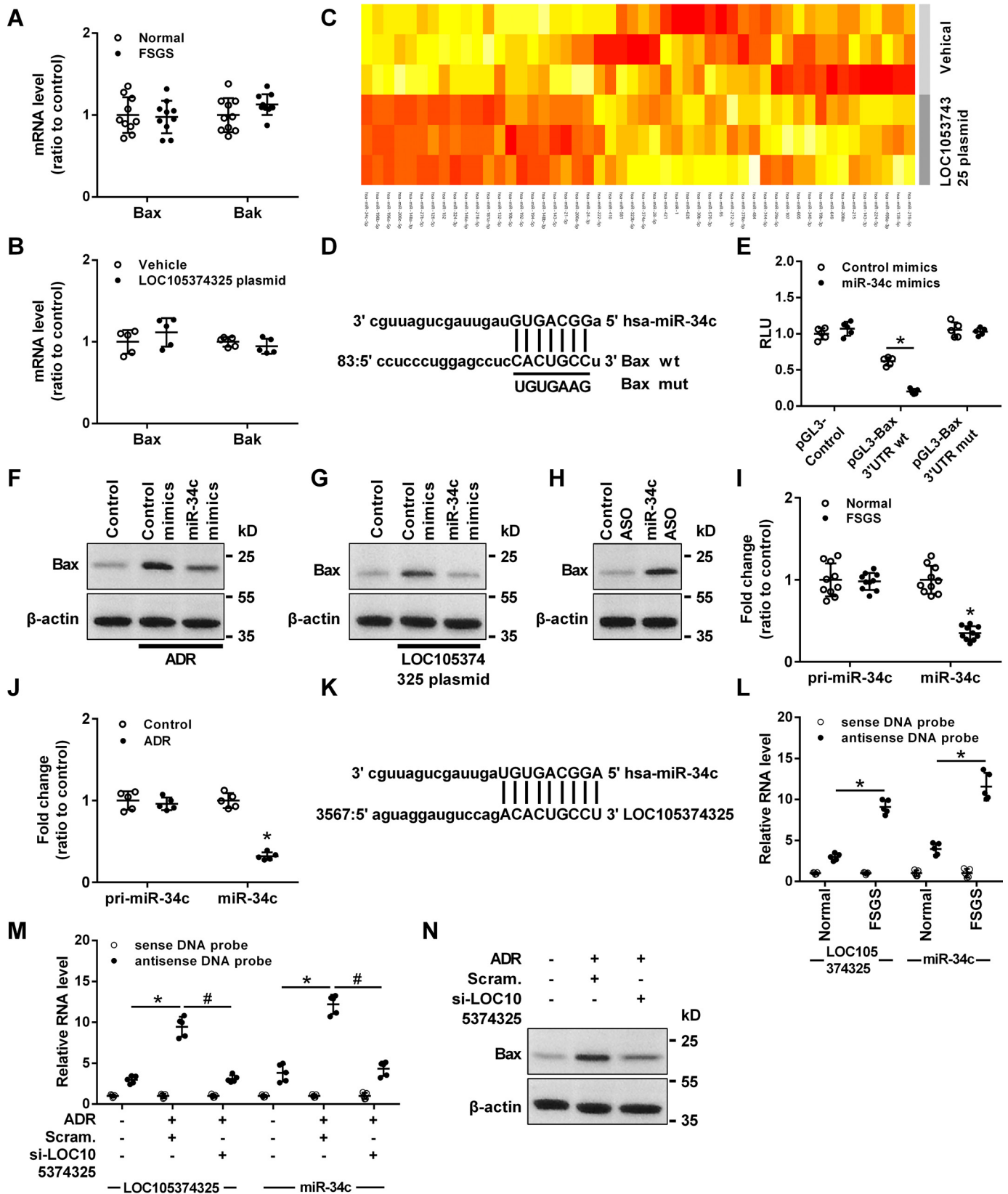
RT-PCR analysis showed that the level of miR-34c but not pri-miR-34c was decreased in the glomerular tissues of FSGS patients and in ADR-treated podocytes (Fig. 3, I and J). Bioinformatic analysis with RNAHybrid software showed that LOC105374325 contains the response element of miR-34c (Fig. 3K). RNA pulldown and RT-PCR analysis confirmed that miR-34c bound to LOC105374325 in glomerular tissues, and formation of the LOC105374325–miR-34c complex was increased in the tissues of FSGS patients compared with normal controls (Fig. 3L). Treatment with ADR increased the formation of the LOC105374325–miR-34c complex in podocytes, which was prevented by LOC105374325 siRNA (Fig. 3M). Silence of LOC105374325 also decreased the level of Bax in ADR-treated podocytes (Fig. 3N).

Role of *lncRNA LOC105374325* in podocytes of FSGS

LOC105374325 up-regulates the expression of *Bak* by competitive binding of *miR-196a/b* in podocytes

Computational analysis detected a *miR-196a/b*-binding sequence in *Bak* mRNA 3'UTR (Fig. 4A). *MiR-196a/b* mimics

significantly inhibited the reporter activity of *Bak* 3'UTR-Luc, and site-directed mutations rescued the *miR-196a/b*-mediated inhibition of *Bak* 3'UTR-Luc (Fig. 4B). Transfection with *miR-196a/b* mimics also decreased the level of *Bak* in podocytes



treated with ADR or transfected with LOC105374325 plasmid (Fig. 4, C and D). Conversely, transfection with miR-196a/b ASO increased the level of Bak in cultured podocytes (Fig. 4E).

RT-PCR analysis showed that the level of miR-196a and miR-196b but not pri-miR-196a or pri-miR-196b was decreased in the glomerular tissues of FSGS patients and in ADR-treated podocytes (Fig. 4, F and G). Bioinformatic analysis with RNA-Hybrid software showed that, LOC105374325 also contains the response element of miR-196a/b (Fig. 4H). RNA pulldown and RT-PCR analysis confirmed that miR-196a/b bound to LOC105374325 in glomerular tissues, and the formation of LOC105374325–miR-196a/b complex was increased in the tissues of FSGS patients compared with normal controls (Fig. 4, I and J). Treatment with ADR increased the formation of LOC105374325–miR-196a/b complex in podocytes, which was prevented by LOC105374325 siRNA (Fig. 4, K and L). Silence of LOC105374325 also decreased the level of Bak in ADR-treated podocytes (Fig. 4M).

ADR treatment induces the expression of LOC105374325 by activating p38 MAPK pathway in podocytes

Phosphokinase array analysis showed that p38, ERK, AKT, and GSK3 β pathways were activated in ADR-treated podocytes (Fig. 5A). The p38 inhibitor SB203580 but not the AKT inhibitor MK2206, the ERK inhibitor PD098059, or the GSK3 β inhibitor TDZD-8 prevented the increase of LOC105374325 in podocytes treated with ADR (Fig. 5B). The p38 inhibitor SB203580 also suppressed formation of LOC105374325–miR-34c and LOC105374325–miR-196a/b complexes, decreased the level of Bax and Bak, and inhibited cell apoptosis in podocytes treated with ADR (Fig. 5, C–F).

Level of p-p38 was increased in renal podocytes of FSGS patients and in podocytes treated with ADR (Fig. 5, G and H). Overexpression of LOC105374325 alone did not change the level of p-p38 in podocytes (Fig. 5I). Conversely, treatment with the p38 activator U-46619 increased the level of LOC105374325, promoted formation of LOC105374325–miR-34c and LOC105374325–miR-196a/b complexes, increased the level of Bax and Bak, and caused cell apoptosis in podocytes (Fig. 5, J–N).

Transcription factor C/EBP β regulates the expression of LOC105374325 in podocytes

The PROMO (TRANSFAC 8.3) software was applied to analyze the sequence of the LOC105374325 promoter region. Among the predicted transcription factors, 7 factors have been reported to be the downstream substrates of p38 MAPK

(Fig. 6A) (10). Knockdown of C/EBP β but not other factors significantly inhibited the expression of LOC105374325 in podocytes treated with ADR (Fig. 6B). The phosphorylation of C/EBP β was increased in podocytes treated with ADR, and inhibition of p38 prevented the ADR-induced phosphorylation of C/EBP β (Fig. 6C).

Chromatin immunoprecipitation (ChIP) analysis confirmed that C/EBP β bound to the promoter region of LOC105374325 in podocytes, which was enhanced by ADR treatment (Fig. 6D). Overexpression of C/EBP β increased the expression of the luciferase reporter construct containing the binding sequence, and site-directed mutations rescued the C/EBP β -mediated up-regulation of the LOC105374325 promoter-luciferase reporter plasmid (Fig. 6, E and F). Overexpression of C/EBP β also increased the level of LOC105374325, promoted the formation of LOC105374325–miR-34c and LOC105374325–miR-196a/b complexes, increased the level of Bax and Bak, and caused cell apoptosis in podocytes (Fig. 6, G–K). Conversely, knockdown of C/EBP β suppressed the formation of LOC105374325–miR-34c and LOC105374325–miR-196a/b complexes, decreased the level of Bax and Bak, and inhibited cell apoptosis in ADR-treated podocytes (Fig. 6, L–O).

Overexpression of LOC105374325 causes podocyte injury in mice

The miR-34c–binding site in Bax 3'UTR and the miR-196a/b–binding site in Bak 3'UTR were conserved in mouse, but LOC105374325 was not conserved in mouse (Fig. S2). To analyze the pathogenic role of LOC105374325 *in vivo*, we elevated renal LOC105374325 expression by a hydrodynamic-based delivery of LOC105374325-expressing plasmid in mice. After 4 weeks of treatment, LOC105374325 was obviously expressed in the podocyte of mice (Fig. 7, A and B). Mice overexpressing LOC105374325 developed proteinuria and focal segmental lesions (Fig. 7, C and D).

Overexpression of LOC105374325 did not change the level of pri-miR-34c, pri-miR-196a, or pri-miR-196b, instead it decreased the level of miR-34c and miR-196a/b in the glomerular tissues of mice (Fig. 7, E and F). RNA pulldown and RT-PCR analysis showed that the formation of LOC105374325–miR-34c and LOC105374325–miR-196a/b complexes was increased in the glomerular tissues of mice overexpressing LOC105374325 (Fig. 7, G and H). Meanwhile, overexpression of LOC105374325 increased the expression of Bax and Bak in the podocytes of mice (Fig. 7I). As a result, the apoptotic podocytes were obviously increased in the mice overexpressing LOC105374325 (Fig. 7J).

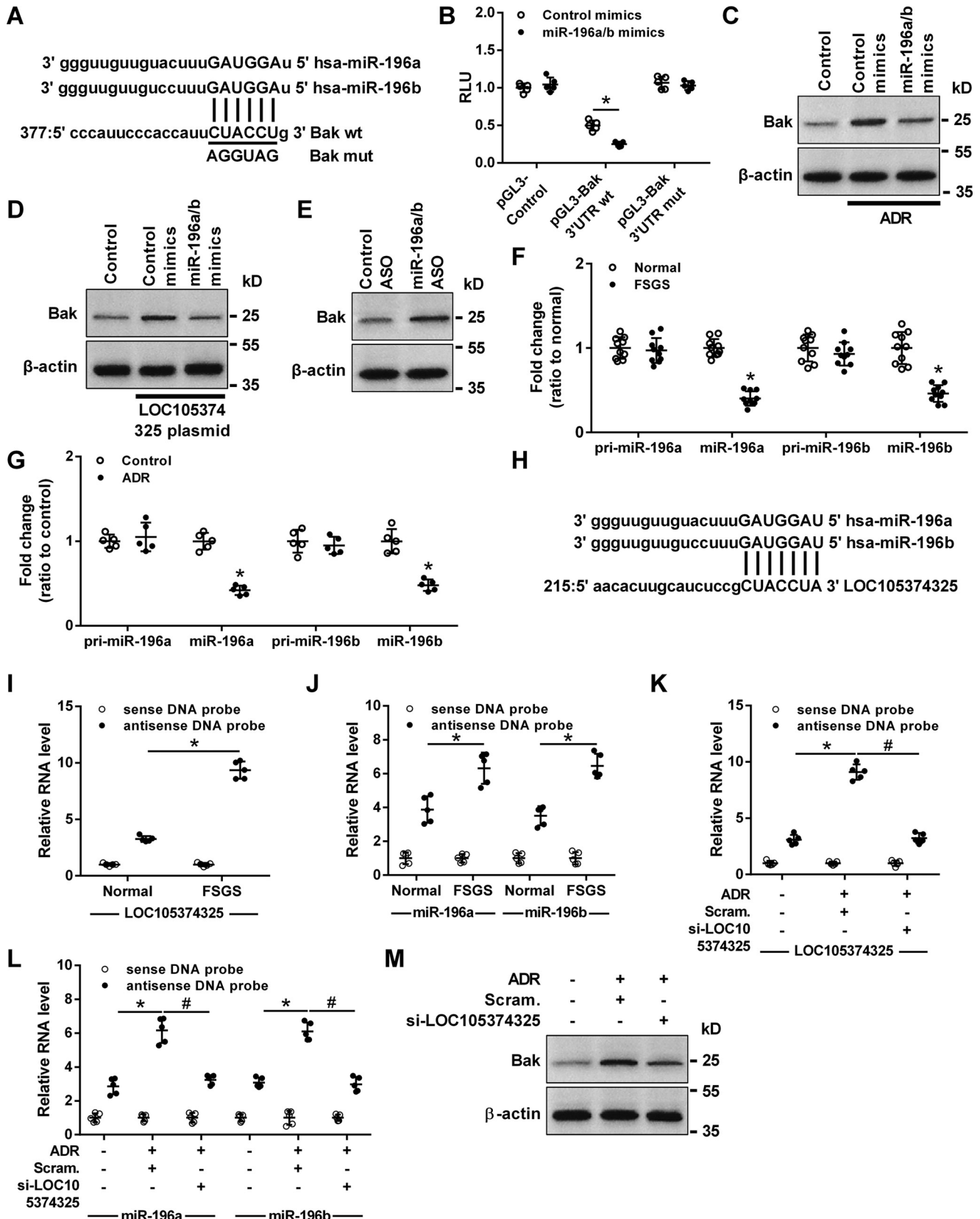
Figure 3. Effect of the binding between LOC105374325 and miR-34c on the expression of Bax in podocytes. A, level of Bax and Bak mRNA in glomerular tissues of FSGS patients ($n = 10$); B, level of Bax and Bak mRNA in podocytes transfected with LOC105374325 plasmid ($n = 5$); C, miRNA PCR array analysis of podocytes transfected with LOC105374325 plasmid ($n = 3$); D, the binding site in the 3'UTR of Bax mRNA targeted by miR-34c; E, normalized luciferase activity of reporter constructs containing the 3'UTR of Bax or mutant 3'UTR of Bax in cells cotransfected with miR-34c mimics ($n = 5$); F, level of Bax protein in podocytes treated with ADR and miR-34c mimics ($n = 3$); G, level of Bax protein in podocytes transfected with LOC105374325 plasmid and miR-34c mimics ($n = 3$); H, level of Bax protein in podocytes transfected with miR-34c ASO ($n = 3$); I, level of pri-miR-34c and miR-34c in glomerular tissues of FSGS patients ($n = 10$); J, level of pri-miR-34c and miR-34c in podocytes treated with ADR ($n = 5$); K, the binding site in LOC105374325 targeted by miR-34c; L, RNA pulldown and RT-PCR analysis of the LOC105374325–miR-34c complex in glomerular tissues of FSGS patients ($n = 5$); M, RNA pulldown and RT-PCR analysis of the LOC105374325–miR-34c complex in podocytes treated with ADR and LOC105374325 siRNA ($n = 5$); N, level of Bax protein in podocytes treated with ADR and LOC105374325 siRNA ($n = 3$). For statistical analysis, a two-tailed Student's *t* test was used for A, B, E, I, and J, and one-way ANOVA with Tukey's post hoc test was used for L and M. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with podocytes treated with ADR.

Role of *lncRNA LOC105374325* in podocytes of FSGS

Discussion

FSGS accounts for ~40% of nephrotic syndrome in adults (1). Most FSGS patients have massive nonselective proteinuria

and severe podocyte injury at disease onset (11). The prognosis of FSGS is poor, with a renal survival rate of only 43.8% at 10 years after renal biopsy (3). Podocyte loss is the central patho-



logical mechanism underlying glomerular sclerosis in FSGS patients (12). Although several signaling pathways have been implicated in podocyte loss, the mechanisms underlying podocyte apoptosis in FSGS patients remain incompletely understood (13, 14).

lncRNAs have been shown to play important roles in renal disease (15). Puthanveetil *et al.* (16) reported that the lncRNA MALAT1 regulated the glucose-induced up-regulation of inflammatory mediators IL-6 and tumor necrosis factor α through the activation of SAA3 in endothelial cells. Sun *et al.* (17) reported that the lncRNA Erbb4-IR promoted renal fibrosis by suppressing miR-29b in db/db mice. To date, the role of lncRNAs in podocyte injury in FSGS patients has not been revealed. We performed a glomerular transcriptome analysis and found that the level of LOC105374325 showed the most significant increase in FSGS patients, and the overexpression of LOC105374325-induced apoptosis in podocytes.

Bax and its homolog Bak are key regulators of the mitochondrial pathway of apoptosis. Aviles *et al.* (18) showed that the level of pro-apoptotic Bax and cFLIP were elevated following IL-2 exposure in podocytes. Lei *et al.* (19) reported that high glucose treatment caused the up-regulation of Bax and Bak and the down-regulation of Bcl-2 in podocytes. Burlaka *et al.* (20) reported that albumin uptake in renal epithelial cells was accompanied by the mitochondrial accumulation of Bax, the down-regulation of Bcl-xL and mitochondrial membrane depolarization. Aberrant increased expression of Bax and decreased expression of Bcl-2 have been observed in glomerular tissues of ADR-treated mice (21). We found that the level of Bax and Bak protein, but not of mRNA, were increased in the renal podocytes of FSGS patients, suggesting that a posttranscriptional mechanism is involved in the regulation of Bax and Bak in the podocytes of FSGS.

Competing endogenous RNA is an important mechanism by which lncRNA participates in the post-transcriptional regulation of gene expression (15, 22). We found that the level of miR-34c and miR-196a/b were most significantly decreased in LOC105374325-overexpressed podocytes, and miR-34c and miR-196a/b were predicted and confirmed to regulate the expression of Bax and Bak in podocytes. The levels of miR-34c and miR-196a/b have been shown to be enriched in renal tissues by Solexa sequencing (23). Li *et al.* (24) reported that the level of miR-34c in differentiated podocytes was 187 times higher than those in undifferentiated podocytes. Liu *et al.* (25) reported that miR-34c was down-regulated in high glucose-treated podocytes, and the overexpression of miR-34c inhibited high glucose-induced podocyte apoptosis. Our previous studies showed that the urinary miR-196a level was correlated with disease activity in FSGS patients, and miR-196a/b mitigated

renal fibrosis by targeting transforming growth factor- β receptor 2 in the tubular cells of FSGS (23, 26, 27). These results indicated the important role of miR-34c and miR-196a/b in maintaining renal homeostasis. The relationship between miR-34c or miR-196a/b and apoptosis has also been indicated in other types of cells. Catuogno *et al.* (28) showed that miR-34c protected lung cancer cells from paclitaxel-induced apoptosis. Wang *et al.* (29) reported that miR-196b inhibited late apoptosis in pancreatic cancer cells. Cao *et al.* (30) reported that overexpression of miR-196b enhanced viability and inhibited apoptosis of human HeLa and 293T cells. We found that the response elements of miR-34c and miR-196a/b existed in the sequence of LOC105374325. The overexpression of LOC105374325 decreased the level of miR-34c and miR-196a/b, increased the level of Bax and Bak, and caused podocyte apoptosis in mice.

The activation of p38 MAPK has been reported to be involved in podocyte apoptosis by independent studies. Koshikawa *et al.* (31) showed that the enhanced phosphorylation of p38 was detected in the nuclei of podocytes in biopsy specimens from FSGS patients. Bao *et al.* (32) showed that treatment with puromycin aminonucleoside induced the activation of p38 in podocytes. Liu *et al.* (33) reported that the inhibition of p38 reduced Bax expression and attenuated apoptosis in podocytes treated with high glucose. Van Laethem *et al.* (34) reported that the activation of p38 was required for Bax translocation to mitochondria, cytochrome *c* release, and apoptosis in human keratinocytes exposed to UVB irradiation. We confirmed that the activation of p38 phosphorylated C/EBP β , which bound to the promoter and increased the expression of LOC105374325 in podocytes. Chen *et al.* (35) reported that the level of C/EBP β was increased in renal tissues after ischemia/reperfusion-induced injury. Liu *et al.* (36) reported that C/EBP β was up-regulated in heme-induced endothelial cell apoptosis. Chen *et al.* (37) showed that the overexpression of C/EBP β abrogated the effect of atorvastatin on increasing Bcl-2/Bax, whereas the early and late apoptosis rate was increased in cardiomyocytes. Silencing C/EBP β decreased the level of LOC105374325, suppressed the formation of LOC105374325–miR-34c and LOC105374325–miR-196a/b complexes, decreased the level of Bax and Bak, and inhibited apoptosis in podocytes treated with ADR. In conclusion, activation of the p38/C/EBP β pathway induces the expression of LOC105374325 in podocytes, and LOC105374325 increases the level of Bax and Bak and causes cell apoptosis by the competitive binding of miR-34c and miR-196a/b in the podocytes of FSGS patients.

Figure 4. Effect of the binding between LOC105374325 and miR-196a/b on the expression of Bak in podocytes. A, the binding site in the 3'UTR of Bak mRNA targeted by miR-196a/b; B, normalized luciferase activity of reporter constructs containing the 3'UTR of Bak or mutant 3'UTR of Bak in cells cotransfected with miR-196a/b mimics ($n = 5$); C, level of Bak protein in podocytes treated with ADR and miR-196a/b mimics ($n = 3$); D, level of Bak protein in podocytes transfected with LOC105374325 plasmid and miR-196a/b mimics ($n = 3$); E, level of Bak protein in podocytes transfected with miR-196a/b ASO ($n = 3$); F, level of pri-miR-196a, miR-196a, pri-miR-196b, and miR-196b in glomerular tissues of FSGS patients ($n = 10$); G, level of pri-miR-196a, miR-196a, pri-miR-196b, and miR-196b in podocytes treated with ADR ($n = 5$); H, the binding site in LOC105374325 targeted by miR-196a/b; I and J, RNA pull-down and RT-PCR analysis of the LOC105374325–miR-196a/b complex in glomerular tissues of FSGS patients ($n = 5$); K and L, RNA pull-down and RT-PCR analysis of the LOC105374325–miR-196a/b complex in podocytes treated with ADR and LOC105374325 siRNA ($n = 5$); M, level of Bak protein in podocytes treated with ADR and LOC105374325 siRNA ($n = 3$). For statistical analysis, a two-tailed Student's *t* test was used for B, F, and G, and one-way ANOVA with Tukey's post hoc test was used for I–L. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with podocytes treated with ADR.

Role of lncRNA LOC105374325 in podocytes of FSGS

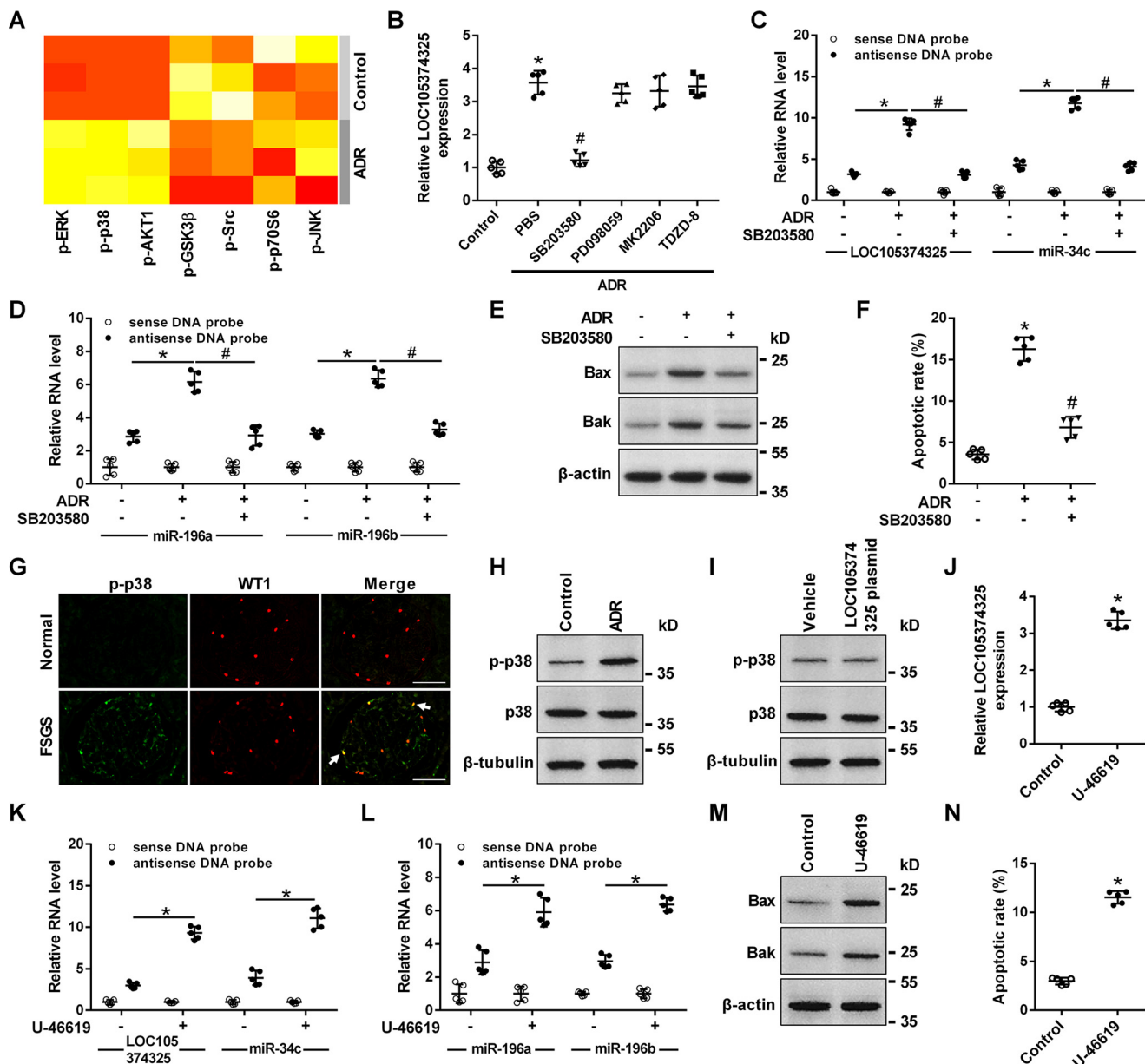


Figure 5. Role of p38 MAPK in the expression of LOC105374325 in podocytes. A, human phosphokinase array analysis in podocytes treated with ADR ($n = 3$); B, level of LOC105374325 in podocytes treated with ADR, SB203580, PD098059, MK2206, and TDZD-8 ($n = 5$); C and D, RNA pulldown and RT-PCR analysis of the LOC105374325–miR-34c and LOC105374325–miR-196a/b complexes in podocytes treated with ADR and SB203580 ($n = 5$); E, level of Bax and Bak protein in podocytes treated with ADR and SB203580 ($n = 3$); F, flow cytometric analysis of apoptotic cells in podocytes treated with ADR and SB203580 ($n = 5$); G, immunofluorescence staining of p-p38 in glomerular tissues of FSGS patients and normal controls ($n = 5$); H, level of p-p38 in podocytes treated with ADR ($n = 3$); I, level of p-p38 in podocytes transfected with LOC105374325 plasmid ($n = 3$); J, level of LOC105374325 in podocytes treated with U-46619 ($n = 5$); K and L, RNA pulldown and RT-PCR analysis of the LOC105374325–miR-34c and LOC105374325–miR-196a/b complexes in podocytes treated with U-46619 ($n = 5$); M, level of Bax and Bak protein in podocytes treated with U-46619 ($n = 3$); N, flow cytometric analysis of apoptotic cells in podocytes treated with U-46619 ($n = 5$). For statistical analysis, a two-tailed Student's *t* test was used for J and N, and one-way ANOVA with Tukey's post hoc test was used for B–D, F, K, and L. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with podocytes treated with ADR. Bar, 20 μm .

Materials and methods

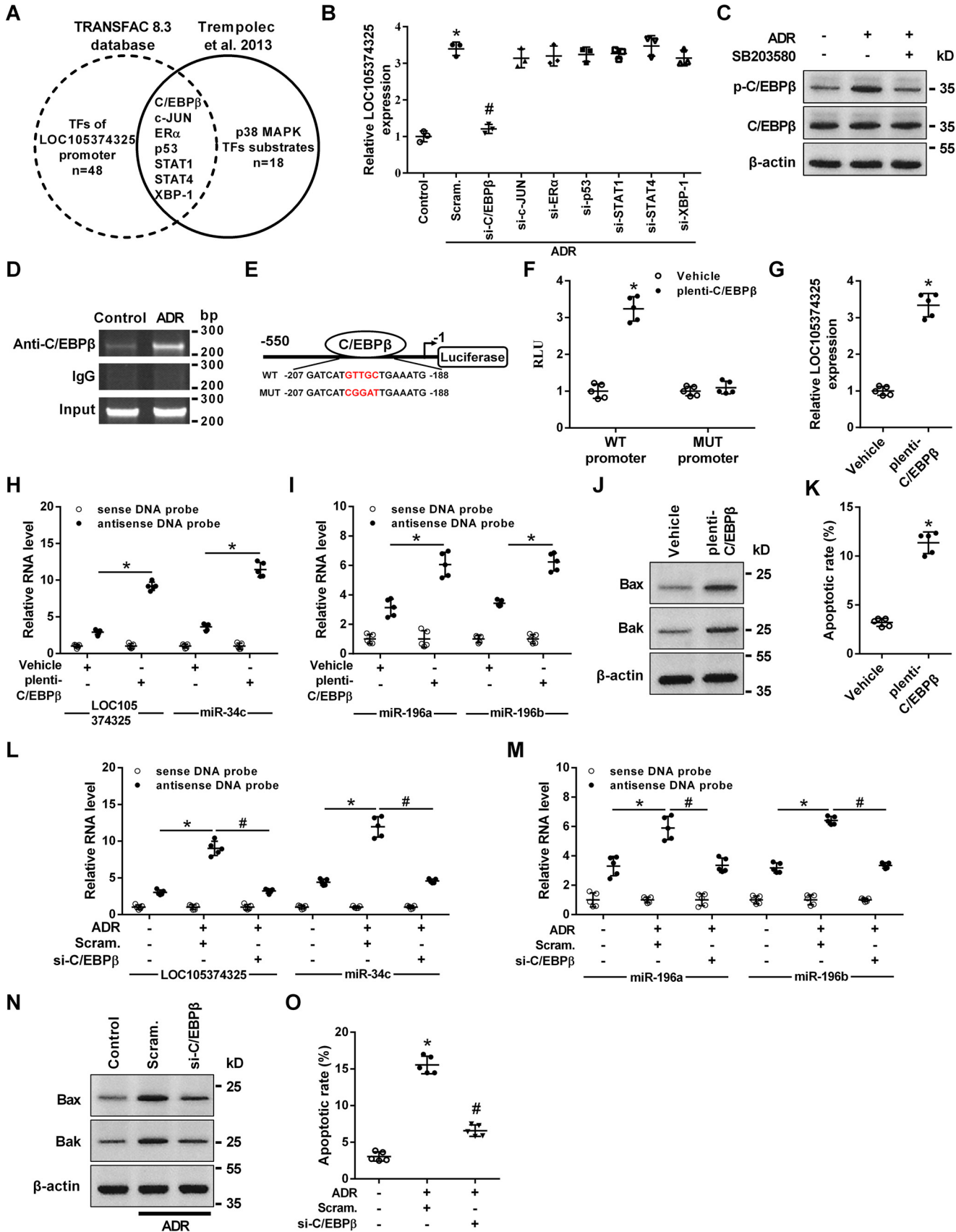
Patients and control subjects

Five FSGS patients were recruited for glomerular transcriptome analysis (Table 1). Another 10 FSGS patients were enrolled for the confirmation study. Control tissues were obtained from the unaffected portion of surgical nephrectomies, and were confirmed to be normal through light microscope analysis. The study was carried out in accordance with the principles of the Declaration of Helsinki and was approved

by the ethics committees of Jinling Hospital. Renal specimens were kept in Renal Biobank of National Clinical Research Center of Kidney Diseases at Jinling Hospital. Informed consent has been obtained from each participant.

Glomerulus isolation

For array analysis, pink-colored, spherical-shaped glomeruli were manually isolated under a stereomicroscope using 2 dissection needle holders in RNeasy lysis buffer at 4 °C. RNA quality and



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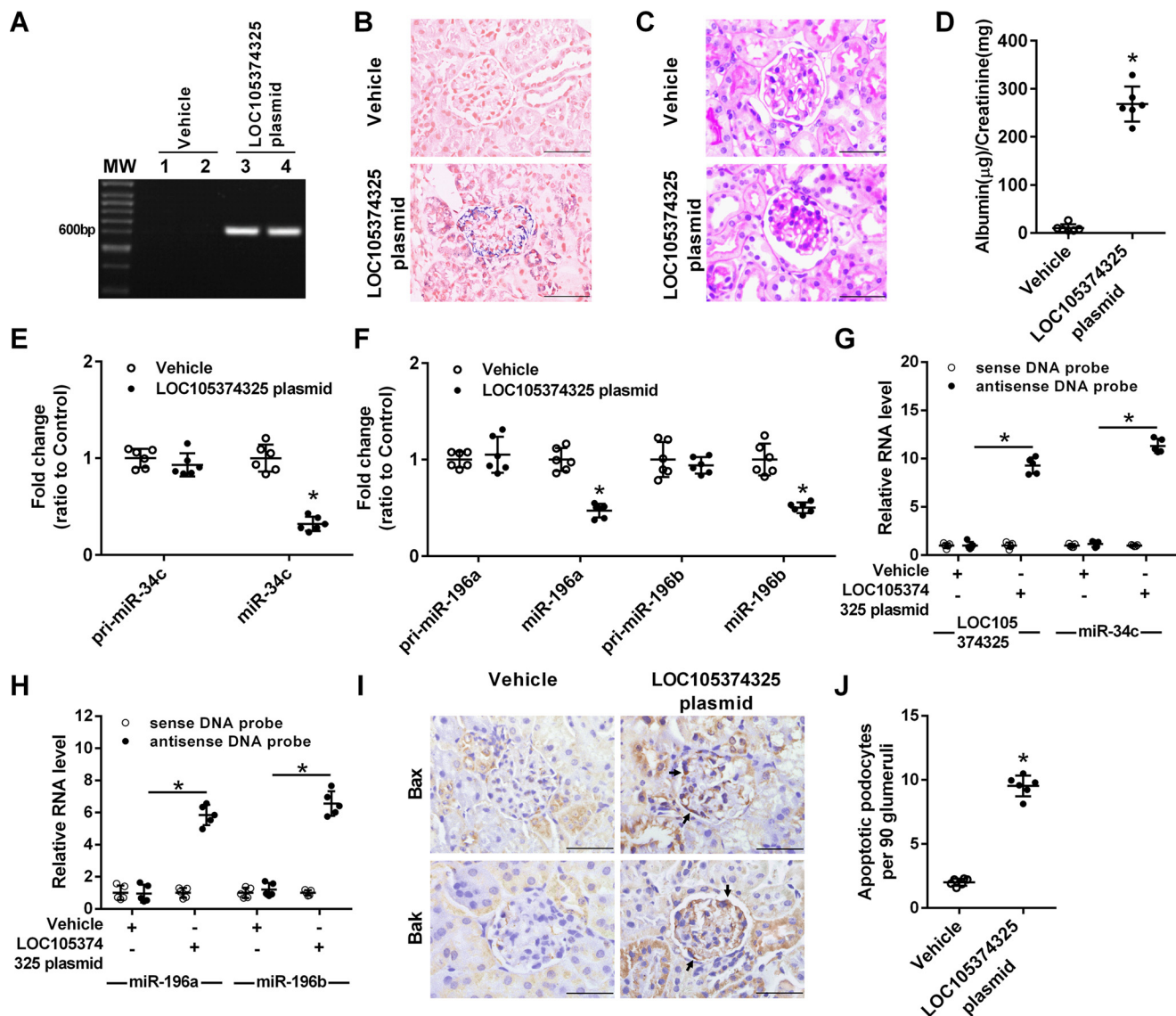


Figure 7. Effect of exogenous LOC105374325 expression on podocyte injury in mice. A, PCR analysis of LOC105374325 in glomerular tissues of mice treated with control plasmid or LOC105374325-expressing plasmid; B, ISH analysis of LOC105374325 in glomerular tissues of mice ($n = 6$); C, periodic acid-Schiff staining of renal sections in mice ($n = 6$); D, urinary albumin excretion in mice ($n = 6$); E, level of pri-miR-34c and miR-34c in glomerular tissues of mice ($n = 6$); F, level of pri-miR-196a, miR-196a, pri-miR-196b, and miR-196b in glomerular tissues of mice ($n = 6$); G and H, RNA pull-down and RT-PCR analysis of the LOC105374325-miR-34c and LOC105374325-miR-196a/b complexes in glomerular tissues of mice ($n = 6$); I, IHC analysis of Bax and Bak in glomerular tissues of mice ($n = 6$); J, apoptotic podocytes in glomerular tissues of mice ($n = 6$). For statistical analysis, a two-tailed Student's *t* test was used for D, E, F, and J, and one-way ANOVA with Tukey's post hoc test was used for G and H. *, $p < 0.05$ compared with vehicle control mice. Bar, 20 μm .

quantity were determined using the Laboratory-on-Chip Total RNA Pico Kit (Agilent BioAnalyzer, Waldbronn, Germany). For PCR analysis, glomeruli were isolated by a laser capture microdissection system (Leica Microsystems AG, Wetzlar,

Germany). Approximately 50 glomerular cross-sections were captured from each case (38). The expression ratio of WT1 in laser-captured glomerular *versus* tubulointerstitial tissues was 53.5.

Figure 6. Role of C/EBP β in the expression of LOC105374325 in podocytes. A, prediction of transcription factors binding to LOC105374325 promoter region; B, level of LOC105374325 in podocytes treated with ADR, C/EBP β siRNA, c-JUN siRNA, ER α siRNA, p53 siRNA, STAT1 siRNA, STAT4 siRNA, and XBP-1 siRNA ($n = 3$); C, level of C/EBP β and p-C/EBP β protein in podocytes treated with ADR and SB203580 ($n = 3$); D, ChIP analysis of the binding between C/EBP β and LOC105374325 promoter in podocytes treated with ADR ($n = 3$); E, schematic of the constructed LOC105374325 promoter-luciferase reporter plasmids; F, normalized luciferase activity of reporter constructs in podocytes cotransfected with C/EBP β plasmid ($n = 5$); G, level of LOC105374325 in podocytes transfected with C/EBP β plasmid ($n = 5$); H and I, RNA pull-down and RT-PCR analysis of the LOC105374325-miR-34c and LOC105374325-miR-196a/b complexes in podocytes transfected with C/EBP β plasmid ($n = 5$); J, level of Bax and Bak protein in podocytes transfected with C/EBP β plasmid ($n = 3$); K, flow cytometric analysis of apoptotic cells in podocytes transfected with C/EBP β plasmid ($n = 5$); L and M, RNA pull-down and RT-PCR analysis of the LOC105374325-miR-34c and LOC105374325-miR-196a/b complexes in podocytes transfected with C/EBP β plasmid ($n = 5$); N, level of Bax and Bak protein in podocytes treated with ADR and C/EBP β siRNA ($n = 3$); O, flow cytometric analysis of apoptotic cells in podocytes treated with ADR and C/EBP β siRNA ($n = 5$). For statistical analysis, a two-tailed Student's *t* test was used for F, G, and K, and one-way ANOVA with Tukey's post hoc test was used for B, H, I, L, M, and O. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with podocytes treated with ADR.

Table 1
Demographic characteristics in the discovery and validation cohorts

Group	Number	Age	Gender	Disease course
		years	% female	months
Discovery cohort				
Normal	5	34.6 ± 7.3	20	
FSGS ^a	5	32.2 ± 15.7	20	6.0 (0.7–18.0)
Validation cohort				
Normal	10	38.5 ± 6.1	40	
FSGS	10	35.1 ± 16.6	40	5.6 (0.9–11.6)

^a FSGS, focal segmental glomerulosclerosis.

Gene expression profile analysis

The Affymetrix HTA2.0 microarrays were used for the global profiling of human lncRNAs in microdissected glomerular tissues. The accession number for the microarray data reported in this paper is GEO accession number GSE121233.

RT-PCR analysis

Total RNA was extracted using miRNeasy Mini Kit (217004, Qiagen, CA). Reverse transcription was carried out with miScript II RT Kit (218161, Qiagen). QuantiTect SYBR Green PCR Master Mix (204143, Qiagen) was used for gene expression level measurement. The primers for PCR analysis were listed in Table S1. The levels of miR-34c, miR-196a, and miR-196b were quantified by the TaqMan miRNA assay (Applied Biosystems, Foster City, CA) with small nuclear RNA U6 as an endogenous control (38).

In situ hybridization analysis of LOC105374325

Paraffin tissue sections were deparaffinized with xylene, rehydrated with ethanol dilution series and treated with 15 μg/ml of proteinase K (03508811103, Roche Applied Science, Switzerland) at 37 °C for 15 min. Then slides were fixed in 4% paraformaldehyde and hybridized with 5' digoxin-labeled LOC105374325 probe (Exiqon, Vedbaek, Denmark) at 55 °C overnight. After washing, slides were treated with blocking buffer for 30 min. Slides were then incubated with anti-digoxin-AP in blocking buffer for 1 h. LOC105374325 was visualized in a staining reaction with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution (39–41).

Podocyte culture and treatment

Immortalized temperature-sensitive human podocytes (gift from Dr. M Saleem) were propagated at permissive temperature (33 °C) in RPMI 1640 medium supplemented with 10% FBS and insulin/transferrin/selenium (42). Cells were then allowed to differentiate for 2 weeks under nonpermissive conditions at 37 °C in the same medium. Podocytes were treated with ADR (0.5 μg/ml) for 24 h. For intervention studies, 10 μM p38 MAPK inhibitor SB203580 (sc-3533, Santa Cruz), 10 μM Akt inhibitor MK2206 (sc-364537, Santa Cruz), 50 μM ERK inhibitor PD98059 (sc-3532, Santa Cruz), 5 μM GSK3β inhibitor TDZD-8 (sc-221692, Santa Cruz), or 1 μM U-46619 (sc-201242, Santa Cruz) was given 30 min before treatments. To infect podocytes with plenti-CMV-LOC105374325, plenti-CMV-Bax, plenti-CMV-Bak, or plenti-CMV-C/EBPβ plasmid, the lentiviral stock was mixed with Polybrene (1 μg/ml) and added to podocytes. BAX siRNA (sc-29212), BAK siRNA (sc-29786), C/EBPβ siRNA (sc-44251), c-JUN siRNA (sc-29223), ERα siRNA (sc-29305), p53 siRNA (sc-29435), STAT1

siRNA (sc-44123), STAT4 siRNA (sc-36568), and XBP-1 siRNA (sc-38627) were bought from Santa Cruz Biotechnology (Dallas, TX). LOC105374325 siRNA were bought from Thermo Fisher (4390771, Carlsbad, CA). Transfection of siRNA, miRNA mimics, or miRNA ASO was conducted with Lipofectamine 2000.

Apoptosis analysis with flow cytometry

Detection of apoptotic podocytes was performed using FITC Annexin V Apoptosis Detection Kit I (556547, Biosciences, San Jose, CA).

Apoptosis antibody array analysis

Apoptosis antibody array featured highly specific and well-characterized antibodies related to apoptosis (APP069, Full Moon BioSystems). The level of apoptosis-related proteins in glomerular tissues were analyzed with the apoptosis antibody array according to the manufacturer's protocol.

Immunohistochemical staining

Paraffin-embedded sections were deparaffinized, rehydrated, and stained using Super Sensitive™ IHC Detection System kit (BD5001, Bioworld, MN). Endogenous peroxidase was blocked with 0.3% hydrogen peroxide for 30 min. The sections were incubated for 1 h with primary antibody. Negative controls were obtained by omission of the primary antibody from the staining procedure.

Western blot analysis

Tissues or cells were lysed in RIPA buffer supplemented with protease inhibitors. Protein concentrations were determined using a bicinchoninic acid protein assay kit (BCA1, Sigma) (43). Samples with equal amounts of total protein (30 μg) were fractionated on 10% SDS-polyacrylamide gels and analyzed by Western immunoblot (44). The antibodies were listed in Table S2.

MiRNA PCR array assay

MiRNAs of LOC105374325-overexpressed podocytes were converted to cDNA using the miRNA first-strand kit. The level of miRNAs were detected using the Human miFinder miScript miRNA PCR Array (MIHS-3001, Exiqon, Vedbaek, Denmark) (38).

Luciferase assays

Podocytes were transiently cotransfected with 0.1 μg of the reporter constructs, 0.02 μg of the *Renilla* construct, and 50 nM synthetic miRNA mimics. The firefly and *Renilla* luciferase activity were determined using the Dual-Luciferase Reporter Assay System (E1910, Promega, Madison, WI). Values were normalized with *Renilla* luciferase (38, 45).

Biotin pulldown assay

Tissue or cells were lysed in 10% glycerol, 20 mM Tris (pH 8), 0.2 mM EDTA, 0.5% Nonidet P-40, 0.5 M KCl, 1 mM DTT (46). The biotinylated DNA probe complementary to LOC105374325 (100 pmol) was incubated with Dynabeads M-280 Streptavidin (11205D, Invitrogen) at room temperature for 10 min to generate probe-coated beads according to

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the manufacturer's protocol. Then, tissue or cell lysates were incubated with the probe-coated beads, and the RNA complexes bound to these beads were extracted for RT-PCR analysis (47, 48).

Human phosphokinase array analysis

The relative phosphorylation level of AKT1(Ser-473), ERK-(Thr-202/Tyr-204), GSK3 β (Ser-9), JNK(Thr-183/Tyr-185), p38(Thr-180/Tyr-182), p70-S6(Thr-421/Ser-424), and Src-(Tyr-416) in ADR-treated podocytes were measured by human phosphokinase array (Phospho RTK4, Labospace, Milano, Italy).

ChIP analysis of C/EBP β DNA binding

ChIP assay was performed with the ChIP-IT Express Magnetic Chromatin Immunoprecipitation kit (53008, Active Motif, Carlsbad, CA) and anti-C/EBP β or IgG antibodies (38).

Overexpression of LOC105374325 in mice

Animal studies were approved by the Institutional Animal Care and Use Committee of Jinling Hospital. We obtained the male C57BL/6 mice (8 weeks) from the Model Animal Research Center of Nanjing University (Nanjing, China). LOC105374325 was not conserved in mouse. For introducing exogenous LOC105374325 in the kidney of mice, mice were administrated LOC105374325 expression plasmid by a hydrodynamic-based gene transfer technique (23). Briefly, we mixed LOC105374325-expressing plasmid (20 μ g) into \sim 2.6 ml of TransIT-EE Hydrodynamic Delivery Solution (Mirus, Madison, WI). Then we injected the mixture into mice via the tail vein in 5 s. Each mouse received injections once a week for a total of 4 weeks. Urine samples were collected in metabolic cages, and the albumin and creatinine levels were measured using Albuwell M and Creatinine Companion Kits (1101 Exocell, Philadelphia, PA).

Isolation of mouse glomeruli

Mice glomeruli were isolated by the magnetic bead-based isolation technique (49–51). Mice were anesthetized and the kidney was perfused with 5 ml of saline containing 8×10^7 Dynabeads M-450 (DynaL Biotech ASA, Oslo, Norway). The kidneys were then cut into 1-mm³ pieces and then digested in a digestion buffer containing 1 mg/ml of collagenase A for 30 min at 37 °C. The tissue was then pressed gently through a 100- μ m cell strainer (Falcon, Bedford, MA) and washed with ice-cold sterilized PBS. Glomeruli-containing Dynabeads were gathered using a magnetic particle concentrator.

Analysis of podocyte apoptosis in renal tissue

Apoptotic cell death in kidney sections was detected by a FITC-labeled *in situ* TUNEL assay (11684795910, Roche Molecular Biochemicals, Mannheim, Germany) following the manufacturer's protocol. The cells labeled by 4',6-diamidino-2-phenylindole, WT1, and TUNEL were counted as apoptotic podocytes.

Statistics

All of the data are expressed as the mean \pm S.D. The data from multiple groups were analyzed with a one-way ANOVA

followed by a Tukey's post hoc test. Data from two groups were compared by t tests. *p* values <0.05 were considered significant.

Author contributions—S. H., R. H., J. S., X. Z., W. Q., and C. Z. investigation; S. H., R. H., J. S., X. Z., W. Q., and C. Z. methodology; S. H. and H. B. writing-original draft; S. H., H. B., and Z. L. writing-review and editing; H. B. conceptualization; H. B. and Z. L. funding acquisition; H. B. and Z. L. project administration.

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