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Original Article

Distinct effects of ANGPT2 on gene expression of glomerular podocytes and mesangial cells

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Abstract: Glomerular diseases are the leading cause of chronic kidney diseases with the pathomechanisms largely unclear. ANGPT2 is known to regulate endothelial cell homeostasis through TEK/Tie2 and its dysregulation causes endothelial damage. Here, we found that ANGPT2 is upregulated in glomerular diseases and wondered whether it also acts on the other two glomerular cell types, podocytes and mesangial cells. We treated podocytes and mesangial cells in culture with ANGPT2 but didn't find changes in morphology and survival. RNA-seq analysis revealed that gene expression was altered in both podocytes and mesangial cells and that the differentially expressed genes in the two cell types were fundamentally different and enriched in distinct cellular processes and pathways according to GO and KEGG analyses. Mechanistically, the Ingenuity Pathway Analysis (IPA) analysis revealed that ERK and AKT were the most connected nodes in the networks of the regulated genes of both podocytes and mesangial cells, suggesting that ANGPT2 affected ERK and AKT in both cell types. Interestingly, immunoblotting showed that phosphorylated ERK and AKT were both increased in podocytes while decreased in mesangial cells by ANGPT2. We found that mesangial cells, but not podocytes, expressed TEK and ANGPT1, suggesting that ANGPT2 could antagonize ANGPT1-TEK-ERK axis in mesangial cells similarly to endothelial cells. We searched databases and found that integrin alpha(v) (ITGAV) is an ANGPT2 interacting protein and expressed in podocytes, suggesting that ITGAV mediates ANGPT2 effect on podocytes. In conclusion, increased ANGPT2 may be involved in glomerular injury by affecting podocytes and mesangial cells in addition to endothelial cells. The complexity of the effect of ANGPT2 in glomeruli may apply to other factors.

Keywords: ANGPT2, podocyte, mesangial cell, ERK, AKT, integrin

Introduction

Glomeruli are the apparatus in kidney, which filter the blood to remove waste molecules but retain cells and macromolecules in blood vessels. They are composed of three cell types, i.e., podocytes, endothelial cells and mesangial cells. There is a thin glomerular basement membrane (GBM) between the podocytes and endothelial cells [1]. Glomerular injury causes proteinuria and may further leads to chronic kidney diseases [2]. Studies have shown that glomerular injury involves interactions between the three types of glomerular cells [3].

Angiopoietins are a family of growth factors that play important roles in angiogenesis and other processes [4]. They bind to and activate one of the receptor tyrosine kinases (RTK), TEK/TIE2, which is specifically expressed in endothelial cells, to induce activation of ERK, AKT and other pathways [5]. ANGPT1 plays a crucial role in endothelial cell proliferation, migration, adhesion, cell spreading and survival, thereby regulating angiogenesis, vascular remodeling, and structural and functional stability of blood vessels [4, 6]. ANGPT2 antagonizes ANGPT1, resulting in optimal signaling under the physiological condition; and overexpression of ANGPT2 could disrupt the essential function of ANGPT1 in above processes, leading to abnormal angiogenesis, loosening of endothelial cell-matrix contacts, endothelial cell apoptosis, and vascular regression [5, 6]. ANGPT2 can act directly on TEK/TIE2 to induce

its tyrosine phosphorylation in the absence of ANGPT1, resulting in PI3K/Akt signaling and angiogenesis [7, 8]. Upregulation of ANGPT2 has been observed in multiple diseases and found to induce endothelial cell injury [9, 10]. Inhibition of ANGPT2 can alleviate the diseases [11, 12].

Recently, we performed single-cell RNA-sequencing analysis and found that ANGPT2 was expressed in all the mesangial cells that were sequenced [13], suggesting its possible role in glomerular endothelial homeostasis and the likely mechanism by antagonizing ANGPT1 to balance TEK signaling in the cells. We then found in a cohort of patients with diabetic nephropathy [14] that ANGPT2 was upregulated in the glomeruli and that its increase was correlated with disease severity. Nephroseq database searches revealed ANGPT2 upregulation in additional glomerular diseases. Although it has not been experimentally tested, increased ANGPT2 is presumed to contribute to endothelial injury and facilitate glomerular disease progression.

In the present study, we investigated whether increased ANGPT2 also acts on the other two glomerular cell types, podocytes and mesangial cells. This issue may be important because extensive crosstalk between glomerular cell types is believed to be critical for glomerular physiology and pathology [15]. Therefore, determination of simultaneous impacts of ANGPT2 on all glomerular cell types would provide additional insights into the role and mechanism of ANGPT2 in glomerular injury from the perspective of glomerular cell crosstalk.

To address this issue, an ideal approach would be to generate a glomerulus-specific or mesangial cell-specific ANGPT2 knockout mice to prevent ANGPT2 upregulation in glomerular disease models, followed by examination of phenotypes or gene expression each of the glomerular cell type. However, there is lack of a Cre transgene that can be specifically expressed in glomerular cells, making glomerulus-specific deletion of ANGPT2 impossible. Meanwhile, the global knockout of ANGPT2 in mice results in defects in vascular remodeling and early death at 2 weeks of age [16], preventing its use from testing our hypothesis. We therefore sought to use cultured cells to address the issue. We found that ANGPT2 induced different gene expression changes in podocytes and mesangial cells, and these two gene sets were enriched in distinct cellular and molecular processes and pathways.

Materials and methods

Database mining and bioinformatics analysis

We used single-cell RNA-seq data of mouse mesangial cells deposited in the Gene Expression Omnibus database with accession number of GSE92650. ANGPT2 expressions in human and mouse glomerular cell types were available in the KIT database (http://humphreyslab.com/SingleCell/) and the GSE123179 in the GEO database. To investigate ANGPT2 expression changes in glomerular diseases, we searched the database of Nephroseq (www.nephroseq.org).

Reagents

The information of the reagents used in the present study is as follows: Antibodies against ANGPT2 (Affinity), SYNPO (Santa Cruz), GAPDH (Proteintech), total ERK1/2, phospho-ERK1/2, total AKT and phospho-AKT (Cell Signaling Technologies); Reverse transcription kit (DRR-037A, Takara); Quantitative PCR kit (Thermo Fisher Scientific); RIPA cell lysis buffer, BCA protein quantification kit (Beyotime, Shanghai); RNA extraction kit (Takara); ANGPT2 (HY-P7510, MCE); Trizol (Invitrogen).

Human podocytes treatment and total RNA preparation

The immortalized human podocyte cell line was a gift from Dr. Saleem M's laboratory (University of Bristol, UK). The cells were cultured with RPMI1640 medium supplemented with 10% FBS, 1% Pen/Strep and 1% ITS (insulin-transferrin-selenium) (Gibco) at 33°C in 5% CO_2 incubator for growth, followed by trypsinization, replating and culture at 37°C for 10 days to obtain differentiated cells. Then the differentiated podocytes were either treated with 500 ng/ml recombinant ANGPT2 or left untreated with only vehicle for 24 hours. The cells were homogenized with Trizol and the total RNA was

extracted following the manual instructions. The RNA was then subject to RNA-sequencing.

Human mesangial cells treatment and total RNA preparation

The immortalized human mesangial cell line was purchased from the CELL SYSTEMS (WA, USA) and cultured with DMEM medium supplemented with 10% FBS at 37°C in 5% CO_2 incubator for growth. The cells were then treated with 500 ng/ml recombinant ANGPT2 or left untreated with only vehicle for 24 hours. The cells were homogenized with Trizol and the total RNA was extracted following the manual instructions. The RNA was then subject to RNA-seq.

Western blotting analysis

After ANGPT2 treatment, podocytes or mesangial cells were washed with ice cold PBS and then lysed with 70 μl of RIPA buffer containing proteinase inhibitors cocktail and phosphatase inhibitors (Roche). The lysates were incubated on ice and then centrifuged 12,000 g for 15 min at 4°C. The supernatant protein concentration was measured using BCA protein kit (Bio-Rad). After adding loading buffer, the samples were boiled at 98°C for 5 min, followed by 10% or 8% SDS-PAGE fractionation and PVDF membrane transfer using semi-dry transfer system (Beyotime). The blot was incubated with 5% milk in TBST solution (20 mM Tris-HCl, PH 7.14, 150 mM NaCl, 0.1% Tween-20) for blocking for 1 hour at room temperature, and then incubated with a primary antibody overnight at 4°C. After washed with TBST for 3 times, the blot was incubated with HRP-labeled secondary antibody for 1 hour at room temperature. After washed, ECL system (Millipore) was used to detect the protein.

RNA quantification and qualification

The amount and integrity of RNA samples were assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

Library preparation for transcriptome sequencing

mRNA was purified from total RNA by using poly-T oligo-attached magnetic beads. The detailed methods for library preparation for transcriptome sequencing are described in the Supplemental methods (Supplementary Information).

Sequencing data analyses

The detailed description of the methods and procedures for the quality control of the sequencing, reads mapping to the reference genome, and quantification of gene expression level can be found in the supplemental methods (Supplementary Information).

Bioinformatics analyses

Differential expression analysis and gene set enrichment analysis are described in the supplemental methods (Supplementary Information). Ingenuity Pathway Analysis (IPA) tool was used to determine the upstream regulators and the networks of the regulated genes following the procedure online.

Statistics

Data are presented as the mean ± SD. Differences between 2 groups were analyzed using a 2-tailed Student's t test and incorporated into GraphPad Prism 5 software (GraphPad Software). $P<0.05$ was considered statistically significant.

Data availability statement

The original RNA-seq data have been deposited with the Gene Expression Omnibus database (GEO) with accession numbers of GSE185292 for podocytes, and GSE185293 for mesangial cells.

Ethics statement

No animal study and human subjects were included in the present study. As such, no ethics statement is made.

Results

ANGPT2 is expressed in glomeruli

Single-cell RNA-seq has provided gene expression information at single-cell resolution, making gene expression profiles of different cell types in a complex tissue available by combin-

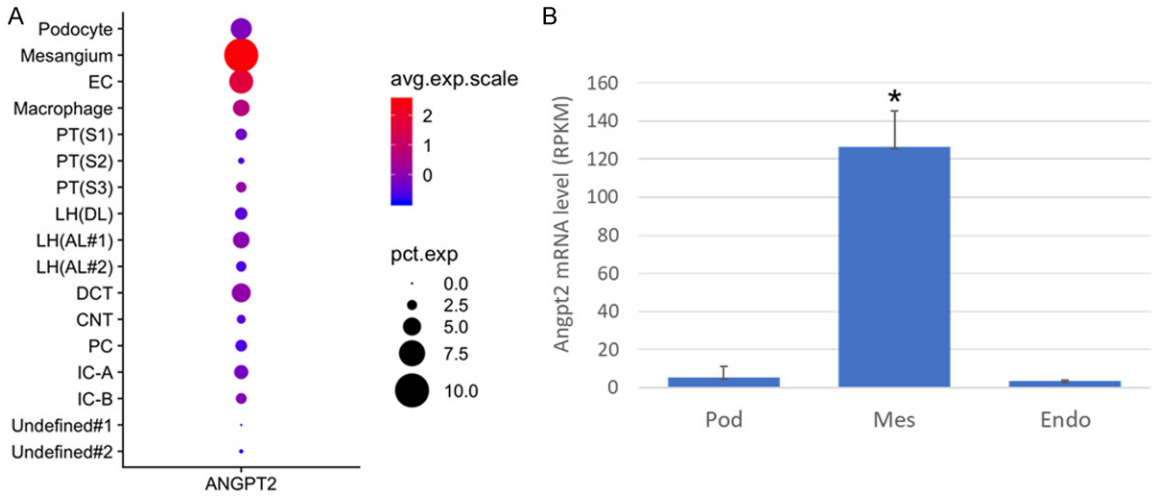

Figure 1. ANGPT2 mRNA expression in glomerular cells of human and mouse. A. The single-cell RNA-seq database search with KIT (http://humphreyslab.com/SingleCell/displaycharts.php) identified ANGPT2 mRNA expression in various kidney cell types, including glomerular cells (podocytes, mesangial cells and endothelial cells) and various tubular cell types. EC, endothelial cells; PT (S1), proximal tubular cells (segment 1); LH (DL), Loop of Henle (descending limb); LH (AL), Loop of Henle (ascending limb); DCT, distal convoluted tubule; PC, principal cells; IC, intercalated cells. B. GEO database search (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE123179) revealed ANGPT2 mRNA in podocytes (Pod), mesangial cells (Mes) and endothelial cells (Endo) from mice by bulk sequencing. N=3 for each group; *P=0.00046, mesangial cells vs. podocytes; P=0.00037, mesangial cells vs. endothelial cells.

ing the RNA-seq results of the same cell types. Alternatively, a specific cell type in a tissue can be labeled by a fluorescent protein, e.g., GFP or RFP, and isolated by fluorescent sorting for RNA-seq analysis. To determine ANGPT2 expression in human kidney cell types, we searched the kidney single-cell RNA-seq database, Kidney Interactive Transcriptomics (KIT) (http://humphreyslab.com/SingleCell/displaycharts.php) for ANGPT2 expression and found ANGPT2 mRNA in podocytes, mesangial cells and endothelial cells at levels significantly higher than other kidney cell types (**Figure 1A**). To determine ANGPT2 expression conservation in mice, we searched the GEO database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE123179), in which ANGPT2 mRNA levels in purified podocytes, mesangial cells and endothelial cells from mice were analyzed by bulk RNA-sequencing. Consistently, ANGPT2 was also expressed in the three glomerular cell types with the level in mesangial cells much higher than podocytes and endothelial cells (**Figure 1B**). To confirm ANGPT2 expression at protein level, we performed immunostaining of ANGPT2 in both human and mouse kidney tissues. As shown in **Figure 2**, ANGPT2 was detected in human glomeruli and localized in both podocytes (as shown by podocyte marker, SYNPO) and non-podocytes, consistent with the RNA expression. ANGPT2 was also detected in mouse glomeruli with a similar staining pattern (**Figure 2**).

ANGPT2 is upregulated in glomerular diseases

Next, we examined whether ANGPT2 expression is changed in glomerular diseases. We analyzed our microarray gene expression profiling data of glomeruli from control and diabetic patients (GSE96804) [14] and found that ANGPT2 was increased in glomeruli of the patients, and the increase correlated with the progression of diabetic nephropathy (**Figure 3**). We further searched the Nephroseq database and found ANGPT2 upregulation in several other glomerular diseases, including FSGS, lupus nephritis, and hypertension nephropathy (**Figure 4**). These results suggest that increased ANGPT2 may be involved in multiple glomerular diseases.

ANGPT2 induced differential gene expression regulation in podocytes and mesangial cells in vitro

We next tested whether ANGPT2 has any effect on podocytes and mesangial cells. We treated

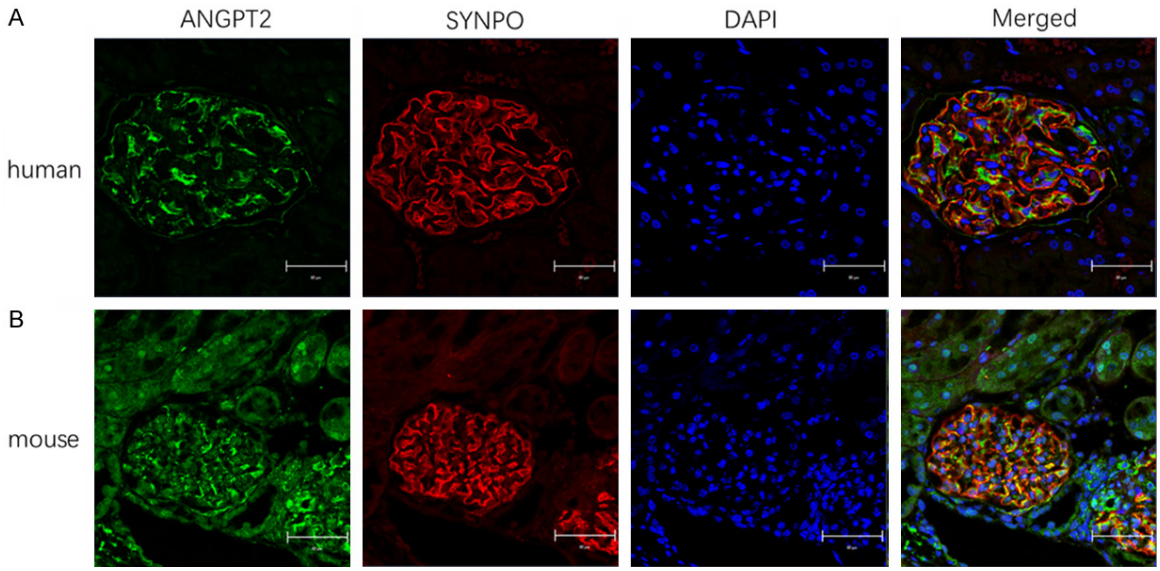

Figure 2. ANGPT2 protein expression in glomerular cells of human and mouse. A. Immunofluorescence staining of ANGPT2 in normal human glomeruli. Podocyte marker, SYNPO, was co-stained, thereby localizing ANGPT2 to podocytes and non-podocytes. B. Similar co-staining of ANGPT2 and SYNPO in mouse glomeruli showing ANGPT2 is present in both podocytes and non-podocytes in mice.

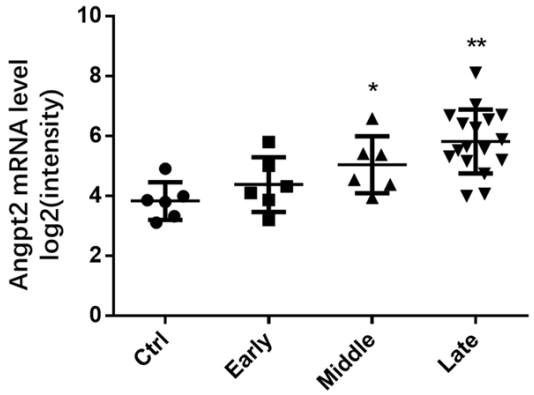

Figure 3. ANGPT2 expression in glomeruli was upregulated in patients with diabetic nephropathy. From the gene expression dataset of glomeruli from diabetic patients (GSE96804), the glomerular ANGPT2 levels in DN patients at different stages of the diseases were obtained. Early: urinary albumin <0.5 g/24 h with normal serum creatinine; middle: urinary albumin >0.5 g/24 h with normal serum creatinine; late: serum creatinine >1.24 mg/dL. *P<0.05, **P<0.01, significant difference vs. normal subjects (Ctrl).

podocytes and mesangial cells in culture with recombinant ANGPT2. However, we did not observe overt changes of the cells in morphology and survival after treatment of ANGPT2 even with prolonged time at high concentrations. We then decided to determine the effect of ANGPT2 on gene expression of the two cell types using RNA-sequencing.

We treated podocytes and mesangial cells with ANGPT2 for 24 h, followed by RNA harvest and RNA-sequencing. The results showed that there were a total of 340 genes that were regulated by ANGPT2 in podocytes, among which 140 were upregulated and 170 downregulated (fold change >2; P<0.05). In mesangial cells treated with ANGPT2, a total of 930 genes had altered expression, including 467 genes upregulated and 463 genes downregulated (fold change >2; P<0.05) (**Figure 5A**). When the upregulated genes from podocytes were compared with those from mesangial cells, we were surprised to find that there were few genes that were overlapped. This situation was the same for the downregulated genes (**Figure 5B**). These results indicated that ANGPT2 had fundamentally different effects on the gene expression of the two cell types.

We performed upstream regulators analyses for the ANGPT2-regulated genes in podocytes and mesangial cells using the bioinformatics tool, the Ingenuity Pathway Analysis (IPA). As expected, the upstream regulators of the two groups of regulated genes were completely different (Supplementary Table 1).

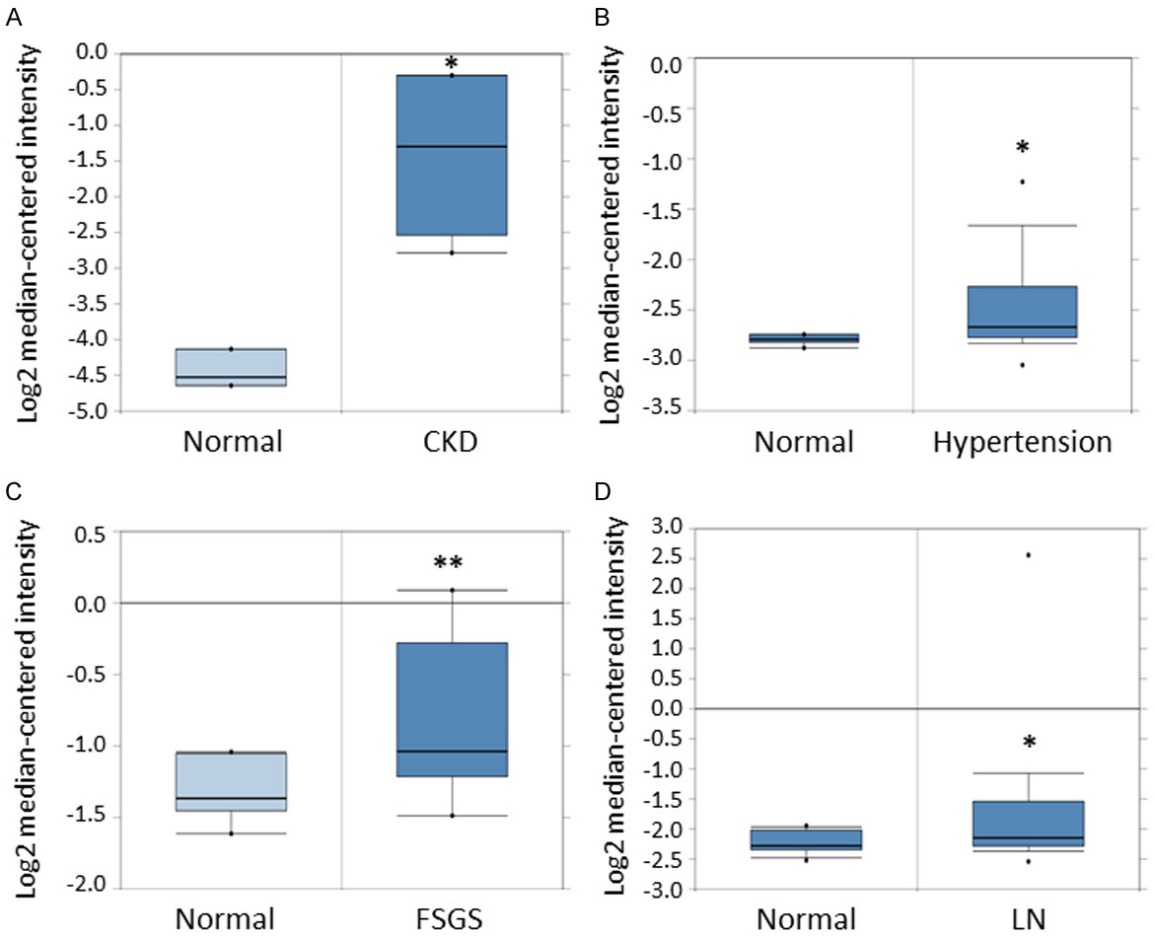

Figure 4. ANGPT2 expression in glomeruli was upregulated in patients with multiple glomerular diseases. A. Nephroseq searches identified ANGPT2 upregulation in glomeruli of patients with CKD (Normal n=3; CKD n=5; fold change 6.98, P=0.001). B. ANGPT2 upregulation in hypertension (Normal n=4; hypertension n=14; fold change 1.22, P=0.023). C. ANGPT2 upregulation in FSGS (Normal n=9; FSGS n=8; fold change 1.36, P=0.033). D. ANGPT2 in LN (Normal n=14; LN n=32; fold change 1.41, P=0.005). *P<0.05, significant vs. normal subjects.

GO and KEGG analyses showed functional differences of the regulated genes between podocytes and mesangial cells treated by ANGPT2

We further performed bioinformatic analysis of the regulated genes in ANGPT2-treated podocytes and mesangial cells. In the GO analyses, the enrichments of the regulated genes from podocytes were fundamentally different from those of mesangial cells (**Figure 6**). For example, for podocytes, we found top enrichments, exoribonuclease activity, transcription factor activity, and regulation of transmembrane transporter activity, etc., and they were not statistically significant; while for mesangial cells, various molecular or organelle binding activities were remarkable. In the KEGG analyses, the pathways enrichments of the podocyte genes were also different from those of mesangial cells (**Figure 7**); for example, podocytes had the enrichments of virus infection, cancer, and mineral absorption; while mesangial cells had ribosome, cell cycle, and oxidative phosphorylation, etc. In the Reactome analyses, the two groups of genes of podocytes and mesangial cells had completely different results (Supplementary Figure 1).

Bioinformatics analysis revealed altered activity of ERK and AKT in both ANGPT2-treated podocytes and mesangial cells

We then subjected the regulated genes to IPA analysis to identify the gene networks in

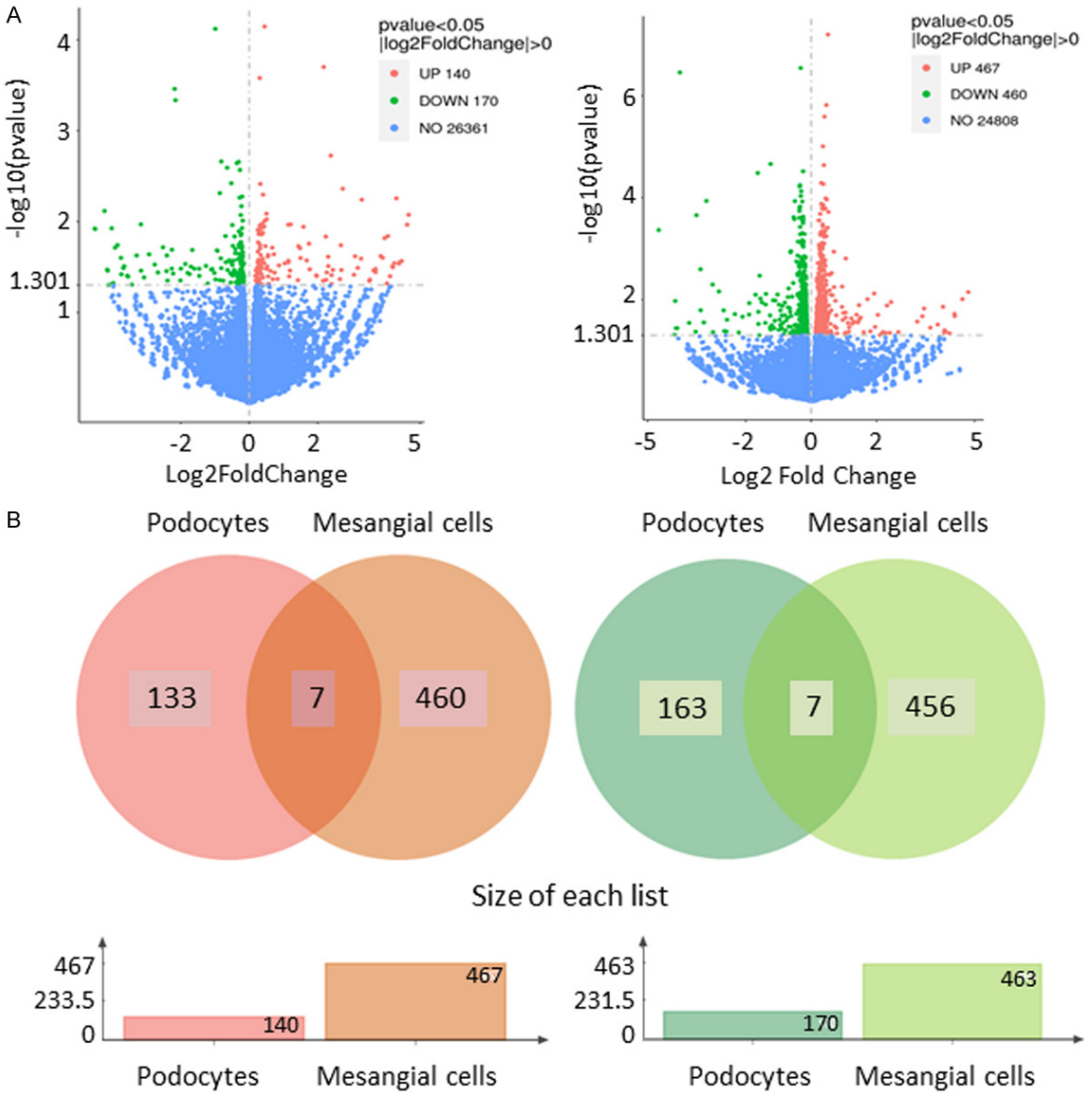

Figure 5. Gene expression regulation by ANGPT2 in podocytes and mesangial cells in culture. A. Volcano plot visualization of statistically significant gene expression changes in ANGPT2-treated podocytes (left) and mesangial cells (right) versus untreated cells, respectively. X-axis, log fold change; Y-axis, -log10 *P* value. B. Venn diagrams showing the huge difference in the list of genes regulated by ANGPT2 in podocytes vs. mesangial cells.

response to ANGPT2 and the key regulatory nodes in the networks. In both the top 1 networks of podocytes and mesangial cells, ERK was the most connected node (**Figure 8**). AKT was also found to be the most connected node in other top networks of podocytes and mesangial cells (Supplementary Figures 2, 3). These results suggest that ANGPT2 acts on podocytes and mesangial cells and regulates their gene expression partly via ERK and AKT.

To confirm the ANGPT2-induced alteration of ERK and AKT activities in podocytes and mesangial cells, we performed immunoblotting of phosphorylated ERK and AKT (pERK and pAKT) in the podocytes and mesangial cells treated with ANGPT2 for 24 h, at which time the RNA samples were collected for RNA-sequencing. The results showed that pERK level was increased in podocytes but decreased in mesangial cells after the ANGPT2 treatment

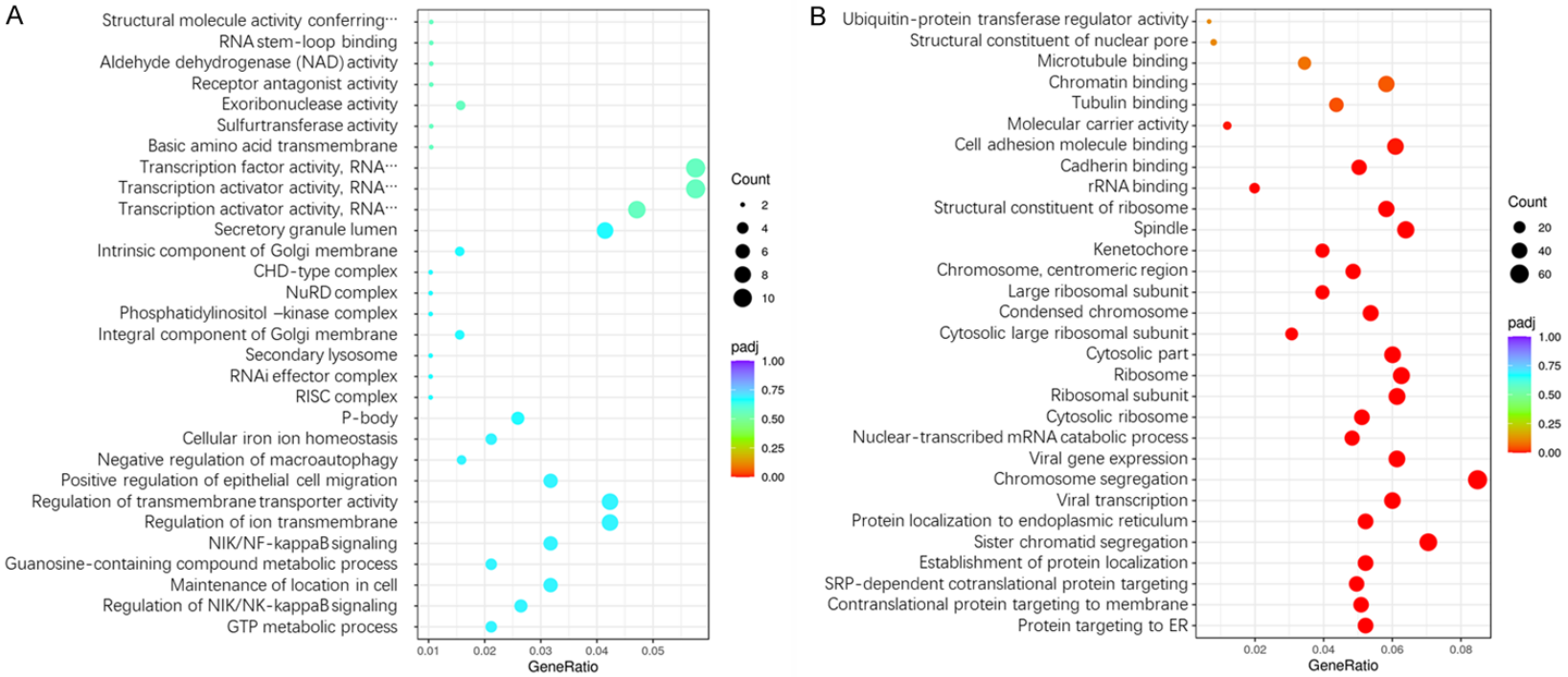

Figure 6. GO analysis shows fundamental differences in enrichments between the regulated genes in podocytes (A) and those in mesangial cells (B) treated with ANGPT2.

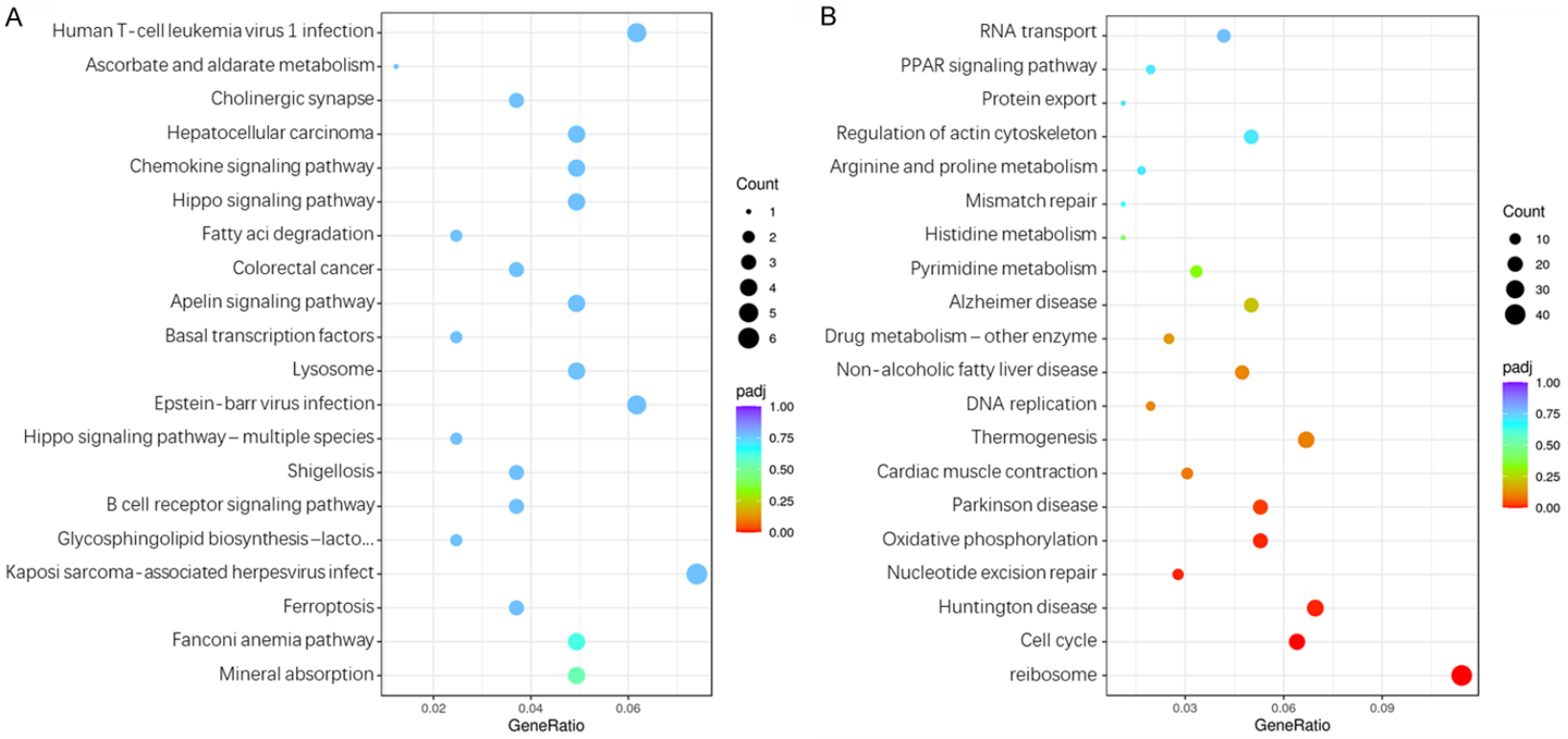

Figure 7. KEGG pathways analysis shows the difference in KEGG pathways between the group of genes regulated in podocytes (A) and that in mesangial cells (B) after treatment with ANGPT2.

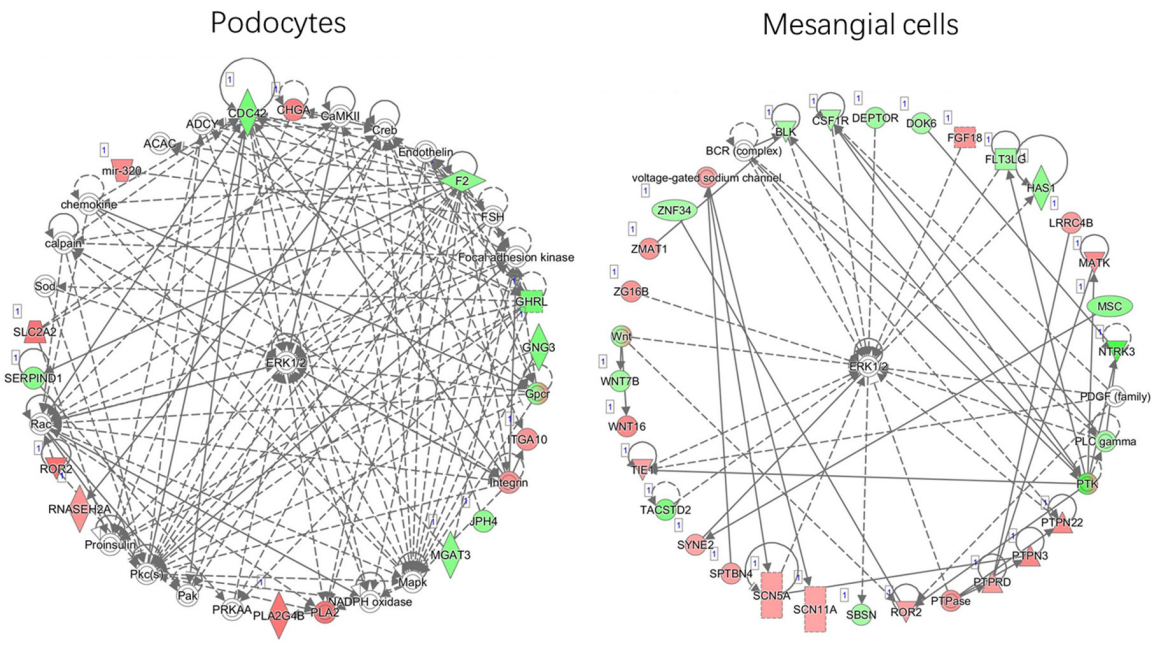

Figure 8. IPA network analysis revealed ERK as the most connected node in the top 1 network of podocytes (left) and mesangial cells (right).

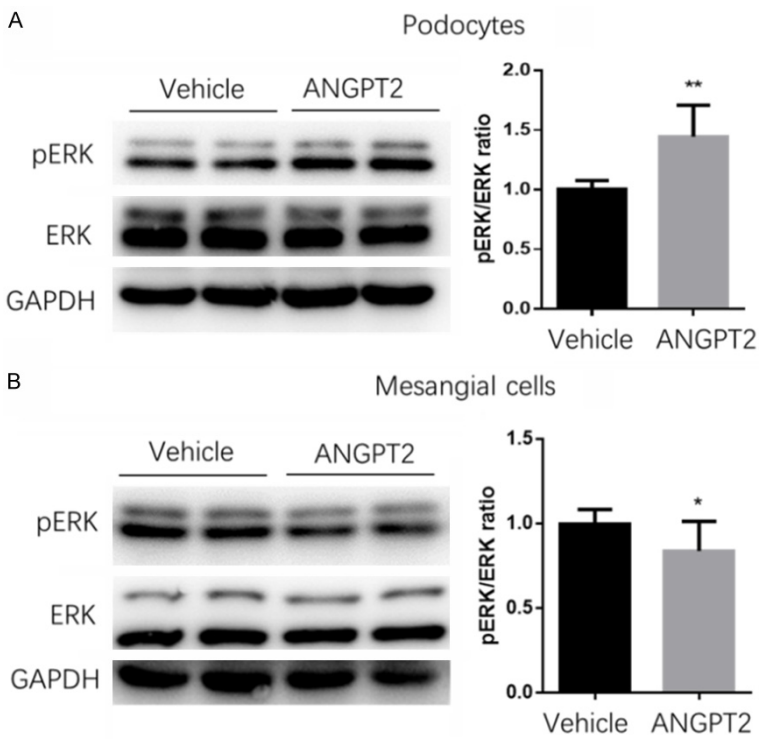

Figure 9. pERK was increased in ANGPT2-treated podocytes but decreased in mesangial cells in culture. ANGPT2 (500 ng/ml) was used to treat podocytes and mesangial cells for 24 h. The results represent the data from three independent experiments and are expressed as mean ± SD. *P<0.05; **P<0.01 vs. control.

(**Figure 9**); meanwhile, pAKT also increased in podocytes but decreased in mesangial cells (**Figure 10**). These results were consistent with the findings from the bioinformatics analyses, and might have explained why ANGPT2-induced gene expression changes in the two cell types were so different.

Mesangial cells, but not podocytes, expressed TEK and ANGPT1

To further explore potential mechanisms underlying differential responses of podocytes and mesangial cells to ANGPT2, we investigated whether ANGPT2 interacting proteins were expressed differentially in podocytes and mesangial cells, hoping that certain differentially expressed ANGPT2 interacting proteins would explain the distinct effects of

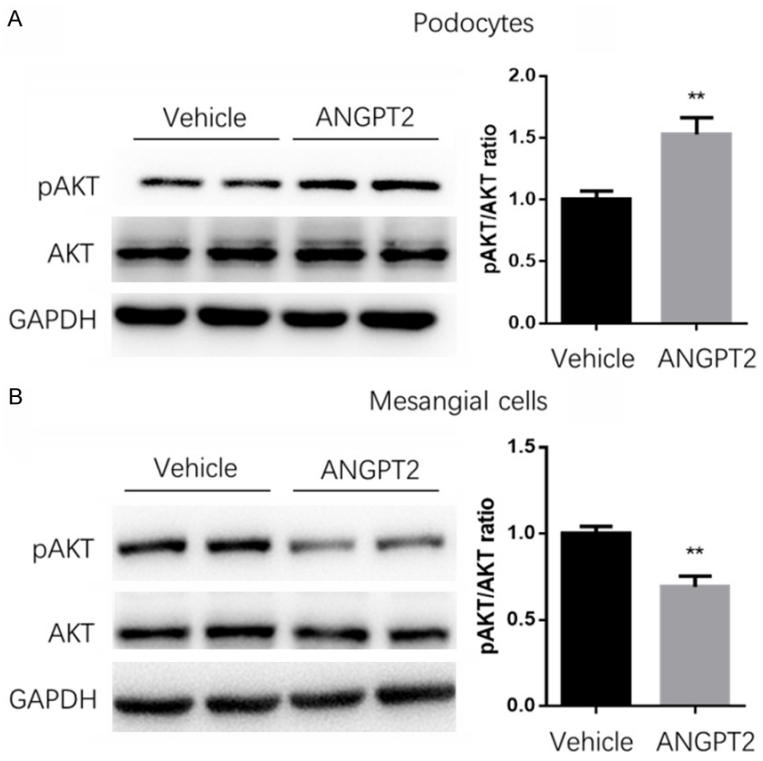

Figure 10. pAKT was increased in ANGPT2-treated podocytes but downregulated in mesangial cells in culture. Podocytes and mesangial cells were treated with 500 ng/ml ANGPT2 for 24 h and subject to immnunoblotting. The results represent the data from three independent experiments and are expressed as mean ± SD. $^{**}P<0.01$ vs. control.

ANGPT2 on the two cell types. We firstly searched NCBI database and obtained the list of ANGPT2 interacting proteins (Supplementary Table 2). We then acquired the expression information of the genes in podocytes and mesangial cells from our RNA-seq datasets of the two cell types (GSE185292; GSE185293). It was shown that the ANGPT2 interacting proteins had similar expression levels between podocytes and mesangial cells (**Table 1**) except for TEK and ANGPT1. The expressions of TEK and ANGPT2 in mesangial cells were 31.25- and 91.5-fold higher than podocytes, respectively, suggesting the presence of ANGPT1-TEK-ERK/AKT axis in mesangial cells but likely not podocytes. Thus, just like in endothelial cells, ANGPT2 may disrupt the axis by antagonizing ANGPT1, resulting in decreased activity of ERK and AKT.

Discussion

In the present study, we report ANGPT2 expression in normal glomeruli and its upregulation in diseased glomeruli. We then tested whether increased ANGPT2 also acts on podocytes and mesangial cells. Using cultured cells, we showed that ANGPT2 induced different gene expression regulation in podocytes and mesangial cells, and that it increased ERK and AKT activities in podocytes but decreased them in mesangial cells. Our study suggests that increased ANGPT2 may contribute to glomerular disease progression by affecting podocytes and mesangial cells, in addition to endothelial cells. We have also explored potential mechanisms of ANGPT2 actions on mesangial cells and podocytes. These results warrant further studies, particularly, with animal models.

We first examined ANGPT2 mRNA expression in glomeruli of both human and mouse. With the rapid expansion of single-cell RNA-sequencing data, glomerular gene expression has now been determined at the resolution of single cells and cell types [17-20]. This has greatly facilitated the mechanistic studies of glomerular physiology and pathology. To determine ANGPT2 expression precisely in human glomerular cell types, we searched the KIT database in which ANGPT2 expression in all three glomerular cell types are available. We found that ANGPT2 mRNA has the highest level in mesangial cells, followed by podocytes and endothelial cells; meanwhile, ANGPT2 expressions in other renal cell types, including tubular cells, are much lower. To investigate ANGPT2 expression in mouse kidney, we searched GEO database and found bulk RNA-sequencing of isolated podocytes, mesangial cells, and endothelial cells of mice by Dr. Potter's laboratory. According to the data, Angpt2 mRNA is mainly present in mesangial cells, while podocytes and endothelial cells express Angpt2 at much lower levels. Next, we performed immunostaining of ANGPT2 proteins in both human and mouse biopsies and observed, unexpectedly, that ANGPT2 protein in podocytes was compa-

Table 1. Expression of ANGPT2 interacting proteins in podocytes and mesangial cells

Gene	Tek	Angpt1	Anxa7	Cdkn1a	Csnk2b	Fbxo28	Hectd3	Iqcb1	Itga5	Itgav	Itgb1	Lyn	Vim	Zzef1
Podocytes	0.04	0.06	31.82	1463.30	8.25	7.52	15.63	7.72	43.13	10.43	159.26	4.11	237.07	2.97
Mesangial cells	1.25	5.49	23.97	517.71	5.30	6.16	13.37	3.35	73.63	8.47	234.54	6.54	395.90	2.52

rable to that of mesangial cells in abundance, suggesting that ANGPT2 mRNA has a higher translation efficiency in podocytes than mesangial cells, that ANGPT2 protein has a faster turnover in mesangial cells because of more efficient secretion, or that ANGPT2 protein is less stable in mesangial cells.

We analyzed our previously published glomerular gene expression data of patients with diabetic nephropathy at different stages (GSE96804) [14], and found continuous increase of ANGPT2 mRNA with the progression of diabetic nephropathy. We also searched Nephroseq data and found ANGPT2 upregulation in additional glomerular diseases. Given that ANGPT2 is known to be injurious to endothelial cells in other tissues [5, 6], these results suggest that ANGPT2 may be involved in glomerular disease progression. However, we wondered whether increased ANGPT2 has any effects on podocytes and mesangial cells, thereby influencing the disease progression.

Due to lack of Cre transgene that allows gene knockout in mesangial cells in animals, in the present study we had to test the hypothesis using cultured podocyte and mesangial cells. We found that recombinant ANGPT2 did not cause any overt changes of the cultured cells in prolonged and high dose treatment of ANGPT2. We then analyzed the effect of ANGPT2 on gene expression of podocytes and mesangial cells, and found a significant gene expression changes in both cell types. ANGPT2 treatment resulted in expression change of more genes in the mesangial cells than the podocytes. Interestingly, either the upregulated or the downregulated genes of the two cell types were almost completely different, indicating distinct effects of ANGPT2 on podocytes and mesangial cells. Consistent with this result, IPA analysis showed that the regulated genes in podocytes and mesangial cells had totally different upstream regulators.

GO, KEGG and Reactome analyses of the regulated genes of podocytes and mesangial cells treated with ANGPT2 showed distinct enrichments of molecular and cellular processes and signaling pathways, demonstrating that the regulated genes in podocytes and mesangial cells are functionally different, and confirming the distinct effects of ANGPT2 on the two cell types. Importantly, the bioinformatics analyses essentially did not show enrichment of injurious pathways or processes, e.g., cell death, apoptosis, mitochondrial dysfunction, etc., consistent with our observation that ANGPT2 did not cause gross cellular changes in culture. Although ANGPT2 does not cause apparent cellular changes, the resulting alteration in gene expression could reprogram the molecular processes in the cells thereby influencing the disease progression. This speculation needs to be investigated further, particularly, using appropriate animal models though they are not available currently.

To elucidate how ANGPT2 changed gene expression in podocytes and mesangial cells differently, we performed IPA network analysis and identified ERK and AKT as the most connected nodes in the top networks for both podocytes and mesangial cells, suggesting that ANGPT2 altered ERK and AKT activities in the two cell types. To prove the change of ERK and AKT in the cells, we performed immunoblotting of pERK and pAKT in podocytes and mesangial cells and found that ANGPT2 increased pERK and pAKT in podocytes but decreased them in mesangial cells. The opposite effects of ANGPT2 on podocytes and mesangial cells partly explain why ANGPT2-induced gene expression changes in the two cell types were almost completely different. As AKT is known to be crucial for podocyte survival [21] and mediate protective signaling, e.g., insulin [22], it is possible that ANGPT2 protects podocytes via AKT in glomerular disease development in contrast with endothelial cells which are damaged by ANGPT2 through interrupting ANGPT1-TEK signaling. This issue deserves further investigation.

Considering that ANGPT2 has opposite effects on ERK and AKT activities in podocytes and

mesangial cells, it is expected that some genes upregulated in podocytes would be present in the list of downregulated genes in mesangial cells, and *vice versa*. We then compared the 140 genes upregulated in podocytes with the 460 genes downregulated in mesangial cells, but did not find any common ones. We next compared the 170 genes downregulated in podocytes with the 467 genes upregulated in mesangial cells, and found few genes (C9orf40, TMEM158, SAPCD2, and STAG3L5P) that are shared by the two genes lists. However, literature search did not find associations of the genes with AKT or ERK signaling, suggesting that they are not the downstream targets of ERK and AKT and were overlapped in the two genes lists just by chance. This finding is a little surprising; however, it could be explained by that AKT and ERK signaling pathways govern the expression of different genes in podocytes and mesangial cells, thus functioning differently in the two cell types.

ANGPT2 has been shown to promote inflammation after myocardial infarction [23]. Consistently, we found several enrichments of "chemokine signaling pathway" and "virus infection" in ANGPT2-treated podocytes but not mesangial cells, suggesting that ANGPT2 could induce inflammatory reaction via podocytes. Given that increased ANGPT2 has been observed in glomeruli of patients with lupus nephritis, which is characterized by glomerular infiltration of immune cells, it is possible that increased ANGPT2 promotes inflammation and contributes to immune cell infiltration in inflammatory glomerular diseases, e.g., lupus nephritis. However, this speculation needs to be carefully proved.

To further dissect the mechanisms of ANGPT2 effects on gene expression of podocytes and mesangial cells, we searched for known and predicted interacting proteins of ANGPT2 and determined their expression in the two cell types, hoping that some of the interacting proteins are differentially expressed in the two cell types, thus potentially explaining the distinct effects of ANGPT2 on them. The search identified more than ten such interacting proteins (Supplementary Table 2), and further found that mesangial cells expressed much higher levels of TEK and ANGPT1 than podocytes (**Table 1**), suggesting that mesangial cells may also possess the ANGPT1-TEK-ERK/AKT axis just like endothelial cells, while podocytes essentially do not. These results raise the possibility that increased ANGPT2 could disrupt ANGPT1-TEK-ERK/AKT signaling and result in gene expression alteration in mesangial cells. For podocytes, this mechanism does not exist. This partly explains the difference in gene expression between podocytes and mesangial cells in response to ANGPT2 treatment.

Interestingly, there are several integrins among the ANGPT2 interacting proteins list, including ITGA5, ITGAV, and ITGB1 (**Table 1**). It has been shown that ANGPT2 can bind to integrins on endothelial cells to induce TEK-independent phosphorylation of the integrin adaptor protein FAK, resulting in RAC1 activation, migration, and sprouting angiogenesis [24]. As the signaling of FAK and Rho family of small GTPases play important roles in pathophysiology of podocytes [25-28] and integrin signaling has been shown to mediate podocyte injury [29, 30], it is possible that the increased ANGPT2 binds to the integrins on podocytes to induce injury. In fact, ITGAV (alpha(v)) has been shown to mediate podocyte injury [29, 31].

In conclusion, ANGPT2 is upregulated in glomerular diseases and the increased ANGPT2 may act on all three glomerular cell types to exert its effect on disease progression. ANGPT2 may be a potential target for the diagnosis and treatment of glomerular diseases.

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Disclosure of conflict of interest

None.

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Supplementary Information

Supplementary methods

Library preparation for transcriptome sequencing

mRNA was purified from total RNA by using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase, then use RNase H to degrade the RNA. Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and dNTP. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3' ends of DNA fragments, Adaptor with hairpin loop structure were ligated to prepare for hybridization. In order to select cDNA fragments of preferentially 370~420 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). After PCR amplification, the PCR product was purified by AMPure XP beads, and the library was finally obtained. In order to ensure the quality of the library, the library needs to be tested. After the construction of the library, the library was initially quantified by Qubit2.0 Fluorometer, then diluted to 1.5 ng/ul, and the insert size of the library is detected by Agilent 2100 bioanalyzer. After insert size meets the expectation, qRT-PCR is used to accurately quantify the effective concentration of the library (the effective concentration of the library is higher than that of 2 nM) to ensure the quality of the library.

Clustering and sequencing

After the individual libraries were qualified, they were pooled according to the effective concentration and the target amount of data off the machine, then being sequenced by the Illumina NovaSeq 6000. The end reading of 150 bp pairing was generated. As the principle of sequencing is to synthesize and sequence simultaneously, four fluorescent labeled dNTP, DNA polymerase and splice primers were added to the sequenced flow cell and amplified. When the sequence cluster extends the complementary chain, each dNTP labeled by fluorophore released the corresponding fluorescence. The sequencer captured the fluorescence signal and converted the optical signal into the sequencing peak to obtain the sequence information.

Quality control

The image data measured by the high-throughput sequencer were converted into sequence data (reads) by CASAVA base recognition. Raw data (raw reads) of fastq format were firstly processed through in-house perl scripts. In this step, clean data (clean reads) were obtained by removing reads containing adapter, reads containing N base and low-quality reads from raw data. At the same time, Q20, Q30 and GC content the clean data were calculated. All the downstream analyses were based on the clean data with high quality.

Reads mapping to the reference genome

Reference genome and gene model annotation files were downloaded from genome website directly. Index of the reference genome was built using Hisat2 (v2.0.5) and paired-end clean reads were aligned to the reference genome using Hisat2 (v2.0.5). We selected Hisat2 as the mapping tool for that Hisat2 can generate a database of splice junctions based on the gene model annotation file and thus a better mapping result than other non-splice mapping tools.

Quantification of gene expression level

Feature Counts (v1.5.0-p3) was used to count the reads numbers mapped to each gene. FPKM of each gene was calculated based on the length of the gene and reads count mapped to this gene. FPKM,

expected number of Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced, considers the effect of sequencing depth and gene length for the reads count at the same time, and is currently the most commonly used method for estimating gene expression levels.

Differential expression analysis

Differential expression analysis of the two groups of genes (control vs. ANGPT2) was performed using the DESeq2 R package (1.20.0). DESeq2 provide statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting *P*-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Padj ≤0.05 and log2 (fold change) ≥1 were set as the threshold for significantly differential expression.

Enrichment analysis of differentially expressed genes

Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented by the clusterProfiler R package (3.8.1), in which gene length bias was corrected. GO terms with corrected *P* value less than 0.05 were considered significantly enriched by differential expressed genes. KEGG is a database resource for understanding high-level functions and utilities of the biological system, such as the cell, the organism and the ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-through put experimental technologies (http://www.genome.jp/kegg/). We used clusterProfiler R package (3.8.1) to test the statistical enrichment of differential expression genes in KEGG pathways. The Reactome database brings together the various reactions and biological pathways of human model species. Reactome pathways with corrected *P* value less than 0.05 were considered significantly enriched by differential expressed genes. We used clusterProfiler R package (3.8.1) to test the statistical enrichment of differentially expressed genes in the Reactome pathway.

Gene set enrichment analysis

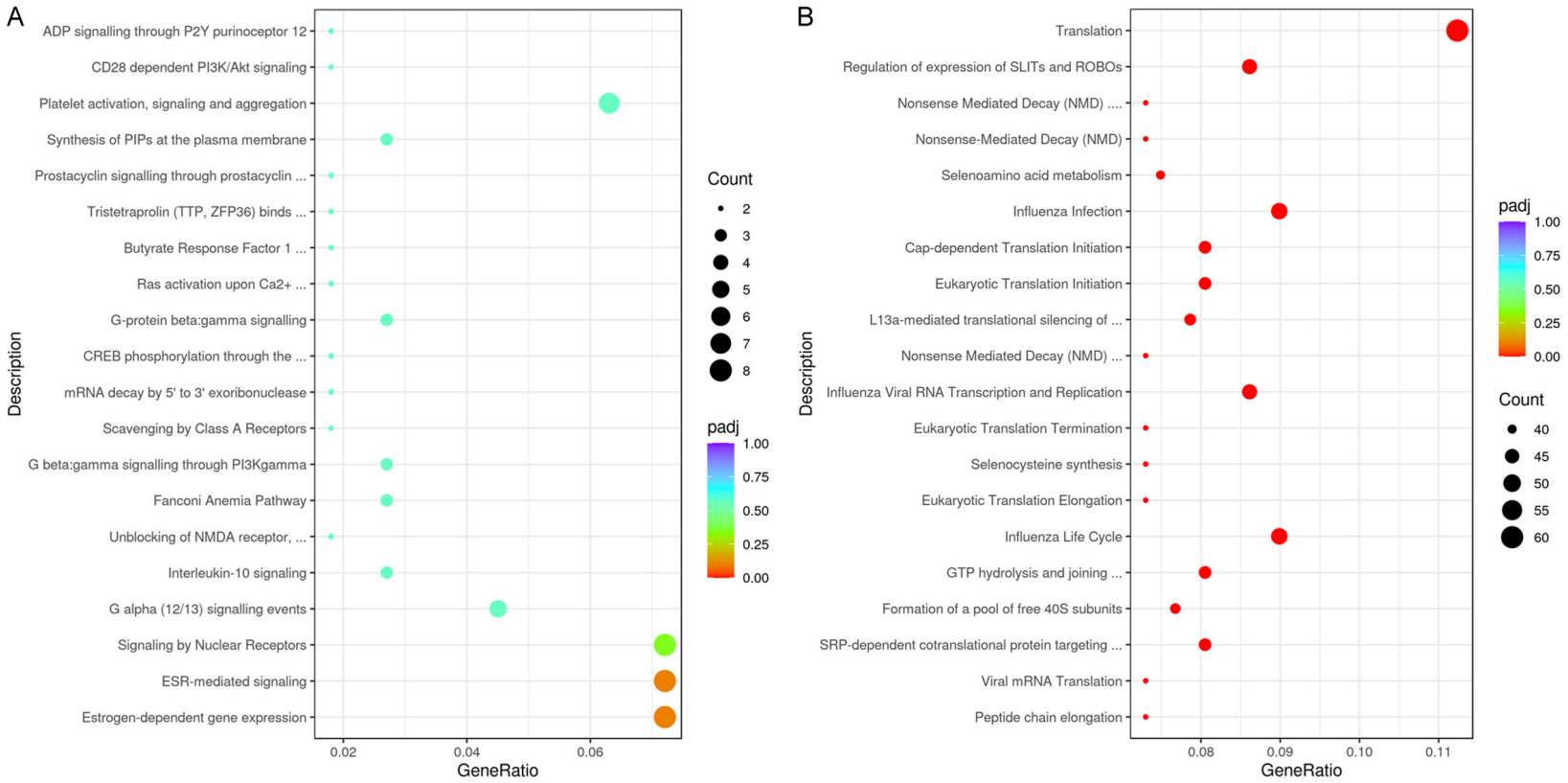
Gene Set Enrichment Analysis (GSEA) is a computational approach to determine if a pre-defined Gene Set can show a significant consistent difference between two biological states. The genes were ranked according to the degree of differential expression in the two samples, and then the predefined Gene Set were tested to see if they were enriched at the top or bottom of the list. Gene set enrichment analysis can include subtle expression changes. We use the local version of the GSEA analysis tool http://www.broadinstitute.org/gsea/index.jsp, GO, KEGG, and Reactome data sets were used for GSEA independently.

Ingenuity pathway analysis (IPA)

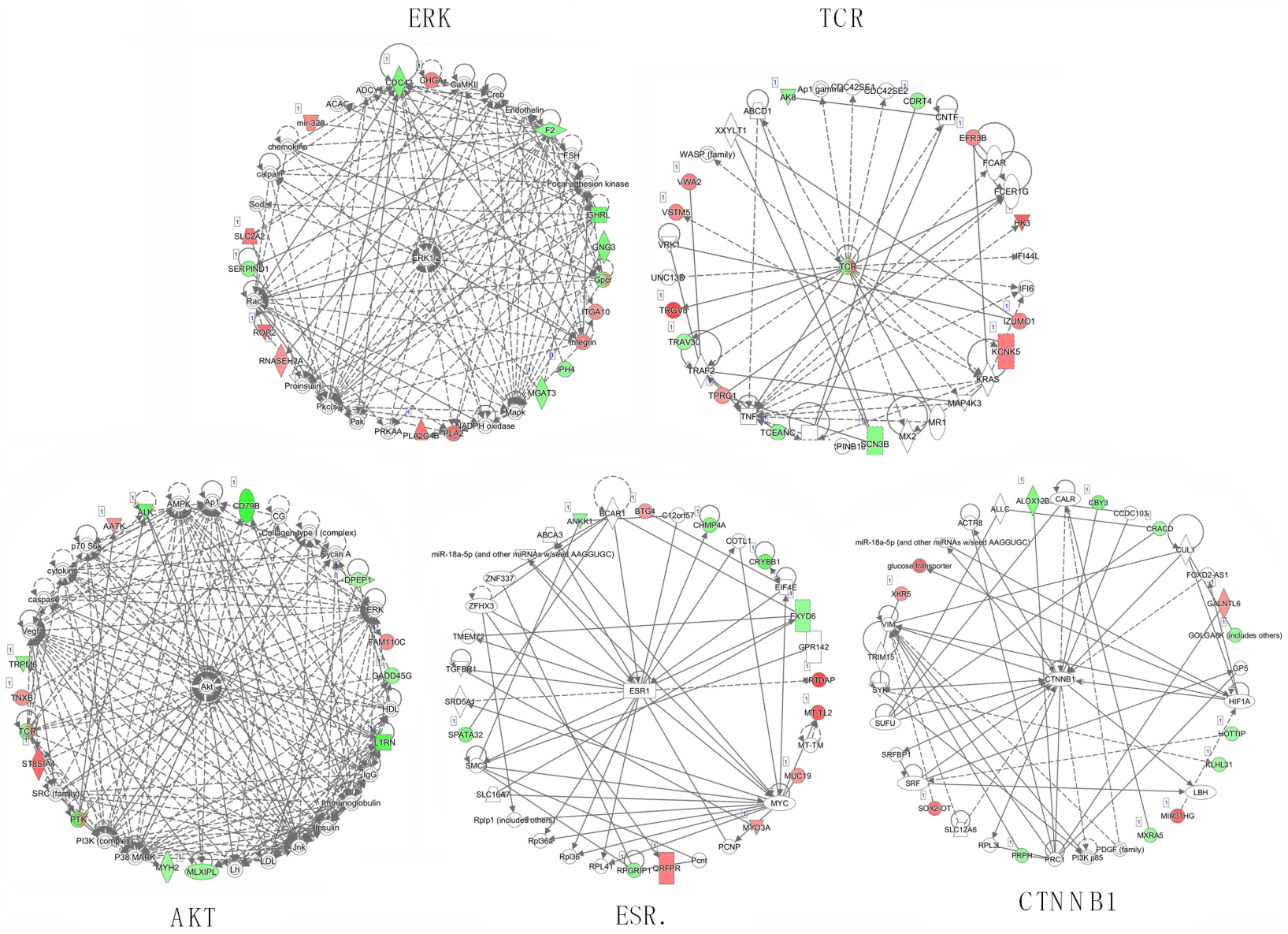
IPA tool was used to determine the upstream regulators and the networks of the regulated genes following the procedure online.

Supplementary Table 1. Upstream regulators of the ANGPT2-induced gene expression changes in podocytes and mesangial cells

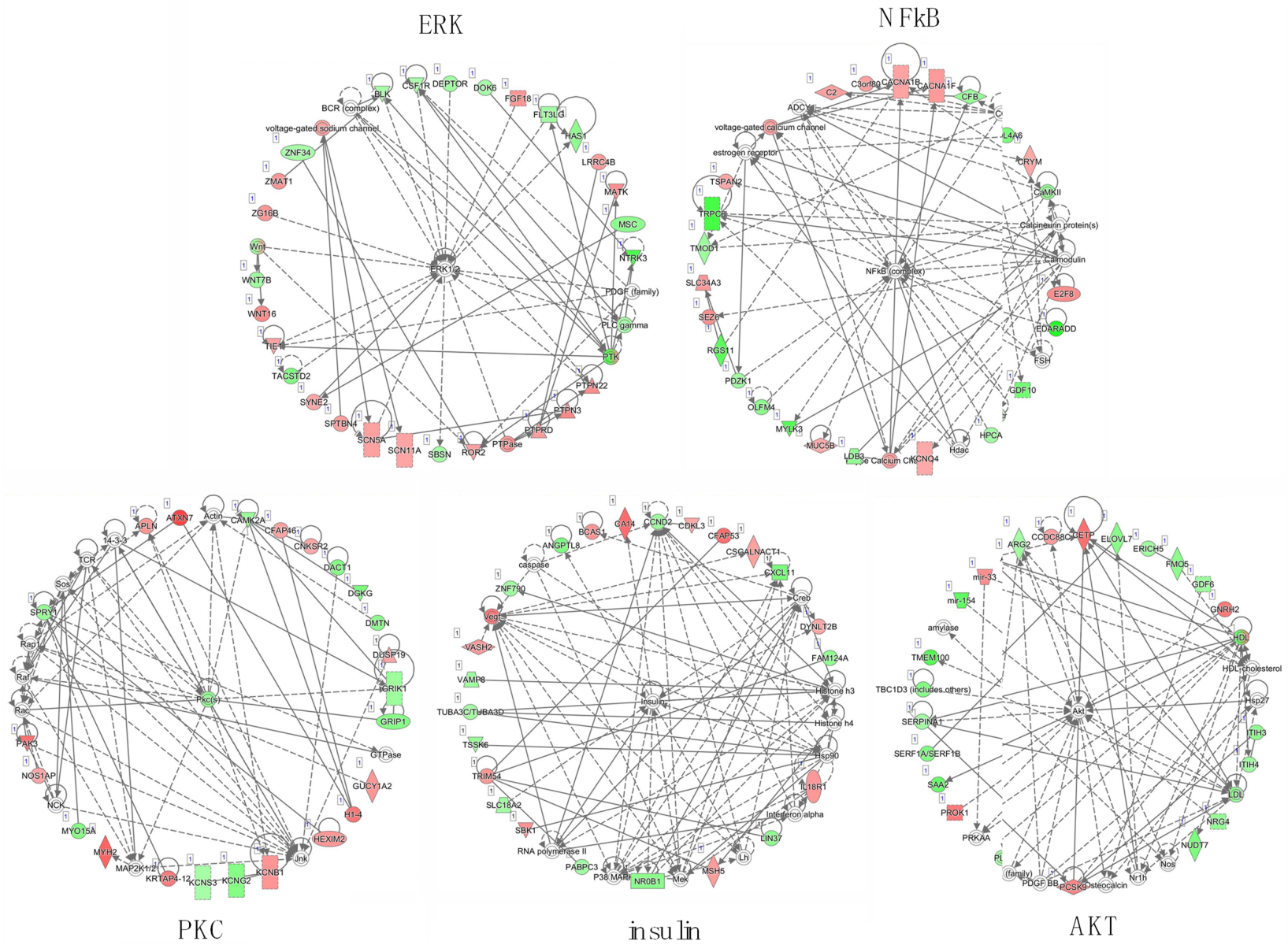
Podocytes		Mesangial cells	
Upstream Regulator	*p*-value	Upstream Regulator	*p*-value
3-aminotriazole	0.000328	RUNX1T1	0.000274
APOA1	0.0025	DICER1	0.000999
GW3965	0.00271	leucine-2-alanine enkephalin	0.00136
NEUROD1	0.00276	pimozide	0.00241
NKX2-2	0.00398	DSCAM	0.00332
RFX3	0.00398	zinc oxide	0.00333
GLIS3	0.00439	miR-219a-5p	0.00333
olesoxime	0.00473	CDK2AP1	0.00407
Cadherin	0.00473	naltrexone	0.00434
CHGB	0.00473	SIX4	0.00461
HS2ST1	0.00473	PRDM1	0.00675
FARP2	0.00473	filgrastim	0.00803
RHOU	0.00473	UGDH	0.00958
Scgb1b27 (includes others)	0.00473	TSHZ3	0.00977
pentagastrin	0.00473	NCKAP1L	0.0116
15-(S)-hydroperoxyeicosatetraenoic acid	0.00473	ELF2	0.0116
MMP2	0.00484	SNHG11	0.0138
MLX	0.00572	prostaglandin D2	0.0139
DIO2	0.00678	PTF1A	0.0139
P2RY14	0.00773	SMARCA5	0.0142
CA4	0.00773	2,2',3,5',6-pentachlorobiphenyl	0.0152
carbohydrate	0.00828	UBAP1	0.0152
CBFB	0.0087	EMC2	0.0152
Fudan-Yueyang-Ganoderma lucidum	0.00944	HDLBP	0.0152
GMNC	0.00944	TLR2/3/4	0.0152
SLIT3	0.00944	CCDC47	0.0152
SLC5A1	0.00944	EMC6	0.0152
MYH1	0.00944	SLC39A13	0.0152
MAP4K3	0.00944	IL34	0.0152
N-acetyl-lysyltyrosylcysteine-amide	0.00944	CHMP4C	0.0152



Supplementary Figure 1. Reactome analysis shows fundamental differences in reactome between in the regulated genes in podocytes and those in mesangial cells after ANGPT2 treatment.



Supplementary Figure 2. IPA analysis revealed top 5 networks and their most connected nodes in podocytes treated with Angpt2. The nodes include (1) ERK, (2) TCR, and (3) AKT, (4) ESR, and (5) CTNNB1.



Supplementary Figure 3. IPA analysis revealed top 5 networks and their most connected nodes in mesangial cells treated with Angpt2. The nodes include (1) ERK, (2) NFkB, (3) PKC, (4) insulin, and (5) AKT.

Supplementary Table 2. ANGPT2 interacting proteins found in the NCBI database

Gene	Interactant	Source	Description
TEK	NP_000450.1	BIND	Tie2 interacts with Ang2
ANGPT1	Q15389	HPRD	N/A
ANXA7	BioGRID:106807	BioGRID	Two-hybrid
CDKN1A	BioGRID:107460	BioGRID	Two-hybrid
CSNK2B	BioGRID:107843	BioGRID	Two-hybrid
FBXO28	BioGRID:116826	BioGRID	Affinity Capture-MS
HECTD3	BioGRID:122781	BioGRID	Affinity Capture-MS
IQCB1	BioGRID:115015	BioGRID	Affinity Capture-MS
ITGA5	BioGRID:109884	BioGRID	Affinity Capture-Western; Reconstituted Complex
ITGAV	BioGRID:109891	BioGRID	Affinity Capture-Western
ITGB1	BioGRID:109894	BioGRID	Affinity Capture-Western; Reconstituted Complex
LYN	BioGRID:110245	BioGRID	Affinity Capture-Luminescence
TEK	BioGRID:112869	BioGRID	Affinity Capture-Western; Reconstituted Complex
VIM	BioGRID:113272	BioGRID	Proximity Label-MS
ZZEF1	BioGRID:116757	BioGRID	Affinity Capture-MS