Bioinformatics Analysis of Proteomic Profiles During the Process of Anti-Thy1 Nephritis*

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Anti-Thy1 nephritis is a well-established experimental mesangial proliferative nephritis model. Exploring the molecular mechanisms of pathophysiology in anti-Thy1 nephritis may elucidate the pathogenesis of mesangial proliferation. We examined the roles and acting mechanisms of differentially expressed proteins (DEPs) by bioinformatics analysis of glomeruli proteomic profiles during the course of anti-Thy1 nephritis. In total, 108 DEPs were found by two-dimensional fluorescence difference gel electrophoresis (2D-DIGE), and 40 DEPs were identified by matrix-assisted laser desorption ionization/time of flight and liquid chromatography-MS. DEPs were classified into five clusters (Clusters 1–5), according to their expression trends using Cluster 3.0 software, involved in regulating biological processes such as stress response, cell proliferation, apoptosis, energy metabolism, transport, and the actin cytoskeleton. The expression patterns of ten DEPs, distributed across five clusters, including AKR1A1, AGAT, ATPI6V1B2, HIBADH, MDH1, MPST, NIT2, PRDX6, PSMB7, and TPI1, were validated by Western blotting. Based on Western blotting and immunohistochemistry, we also found that the DEP FHL2, which was primarily expressed in the mesangial region, was down-regulated on days 3 and 5, and up-regulated on day 10. In vitro, we found that FHL2 overexpression induced mesangial cell proliferation by increasing the number of S-phase cells and decreasing G2/M-phase cells, whereas inhibiting FHL2 had the opposite effect. This study explored novel DEPs and their expression patterns during anti-Thy1 nephritis, and elucidated FHL2’s effect on mesangial cell proliferation. These results will contribute to our understanding of the pathogenesis of mesangial proliferation. Molecular & Cellular Proteomics 11: 10.1074/mcp.M111.008755, 1–13, 2012.

Mesangial proliferative glomerulonephritis (MesPGN)\(^1\) is the most common chronic kidney disease and is characterized by pathological changes in mesangial cell proliferation and extracellular matrix accumulation. As an important cause of end-stage renal disease, MesPGN has been a major focus of renal disease research. Examining the molecular mechanisms of MesPGN may elucidate its pathogenesis and thereby facilitate the development of clinical treatments. Animal models, such as rat anti-Thy1 nephritis, provide a useful tool to explore these mechanisms.

Anti-Thy1 nephritis is a well-established model of mesangial proliferative glomerulonephritis with two major physiological phases (1): the mesangial proliferative phase (e.g. 5 and 7 days after anti-Thy1 antibody injection) and the recovery phase (e.g. 10 and 14 days after anti-Thy1 antibody injection). Biological functions such as stress (2), cytokine action (3, 4), cell proliferation (5, 6), and apoptosis (7, 8) mediate these changes in pathology during the process of anti-Thy1 nephritis. Many proteins have been reported to be involved in regulating these biological functions. For example, Porst (9) reported that fibrillin-1 may regulate mesangial cell proliferation and migration in anti-Thy1 nephritis. Sasaki et al. (6) showed that Galectin-3 modulates rat mesangial cell proliferation and matrix synthesis during experimental glomerulonephritis. However, these studies were limited to a relatively small number of proteins, and it is therefore necessary to examine additional proteins involved in the regulation of the biological functions in anti-Thy1 nephritis. New and powerful tools, such as proteomics, should be used to explore these proteins on a larger scale, as such research could improve current understanding of the molecular processes of anti-Thy1 nephritis.

Nazeer et al. (10) used two-dimensional electrophoresis (2DE) techniques to study differences in the proteomic profiles between control and anti-Thy1 nephritis at day 7 and found 16 differentially expressed proteins, providing new insight into the mechanisms of anti-Thy1 nephritis. However,

\(^1\) The abbreviations used are: MesPGN, mesangial proliferative glomerulonephritis; DEP, differentially expressed protein; 2D-DIGE, two-dimensional fluorescence difference gel electrophoresis; PCNA, proliferative cell nuclear antigen; DTT, dithiotreitol.
the study examined only day-7 proteins (within the proliferation phase), whereas other time points such as days 10 and 14, which are within the resolution phase, were not analyzed. Here, we examined the different expressed proteins (DEPs) of anti-Thy1 nephritis, including both the proliferative and recovery phases, using 2D fluorescence difference gel electrophoresis (2D-DIGE), which has higher sensitivity and is more reproducible than traditional 2D methods.
Among the DEPs, four and a half LIM domain protein 2 (FHL2), whose expression was localized to the mesangial region, was down-regulated on day 3 and 5, and up-regulated on day 10 in anti-Thy1 nephritis, which suggests that the changes in FHL2 expression may be associated with mesangial cell proliferation. It was previously reported that FHL2 has multiple functions associated with cell proliferation. Overexpression of FHL2 inhibits the growth of specific cells, such as colon cancer (11) and hepatoma cells (12), and FHL2 may also promote cell proliferation in mouse fibroblast (13) and glioblastoma cells (14). However, FHL2’s effect on mesangial cell proliferation is not known. In the present study, we explored whether FHL2 induces mesangial cell proliferation by mediating the cell cycle, and hypothesized a role for FHL2 in activating mesangial proliferation during anti-Thy1 nephritis.

**EXPERIMENTAL PROCEDURES**

**Anti-Thy1 Nephritis Model**—Anti-Thy1 nephritis was induced in 30 male Wistar rats (200 g) by a single intravenous injection (2.5 mg/kg) of the monoclonal anti-Thy1 antibody. Six rats that served as controls were injected with the same volume of phosphate buffered saline. Anti-Thy1-treated animals were sacrificed on days 3, 5, 7, 10, and 14 following injection (six rats per time point), and the control group was sacrificed at day 0. Glomeruli from the renal cortex of each rat (>90% purity) were isolated by selective sieving as described previously (15). Isolated glomeruli were snap frozen and stored at −80 °C for further analyses as described below.

**Glomerular Histology and Immunohistochemical Staining**—Renal tissues for light microscopy were fixed in 10% buffered formalin and embedded in paraffin. Sections (4 μm) were stained with periodic acid-Schiff reagent and counterstained with hematoxylin. Examination of proliferating cell nuclear antigen (PCNA) and FHL2 expression was performed using the indirect immunoperoxidase technique on paraffin-embedded sections as described previously (16).

**2D-DIGE and Imaging**

**Minimal Fluorescent-Dye Labeling**—Three rats were randomly selected from each group for gel electrophoretic analysis. Glomerular protein from each rat in each group (three rats) was separately analyzed on a 2-D gel. The proteins from the glomeruli were isolated by lysis buffer (30 mmol/L Tris, 7 mol/L Urea, 2 mol/L Thiourea, 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate (CHAPS, pH 8.5). The pH of the proteins was adjusted to 8.5 by 50 mmol/L NaOH, and the concentration was adjusted to 5 mg/ml with lysis buffer. Equal amounts of proteins from the 18 samples were pooled together as the internal standard. A 50 μg proteins was labeled with 400 pmol of Cy3, Cy5, or Cy2 (Cy2 was used to label the internal standard) on ice for 30 min and then quenched with 1 μl lysine (10 mmol/L).

**2-DE**—The 50 μg Cy3- and Cy5-labeled samples from different groups were combined before mixing with 50 μg of Cy2-labeled internal standard. An equal volume of 2× sample buffer (7 mol/L Urea, 2 mol/L Thiourea, 2% (w/v) CHAPS, 130 mmol/L dithiothreitol (DTT), 1% IPG buffer) was then added to the sample, which was topped up to a total volume of 450 μl with rehydration buffer (7 mol/L Urea, 2 mol/L Thiourea, 2% (w/v) CHAPS, 65 mmol/L DTT, 0.5% IPG buffer). Samples were applied to 24-cm pH 3–11 (NL) IPG strips, and isoelct-
tric focusing was performed using the IPGphor IEF system. The isoelectric focusing program was set as follows: 30 V for 12 h, Grd 200 V for 6 h, Grd 500 V for 3 h, Grd 10,000 V for 1 h, and step 10000 V 64000 V h. The IPG strips were equilibrated in equilibration buffer A (50 mmol/L Tris-HCl, pH 8.8, 6 mol/L urea, 30% glycerol, 2% SDS, 10 mg/ml DTT) for 15 min at room temperature followed by equilibration.

**TABLE I**

| Spot no. | NCBI no. | Name of identified protein | Symbol | Theoretical molecular mass (Da) | Theoretical isoelectric point (pI) | One-way ANOVA p value | Mascot scores | Peptides matched | Peptides unmatched | Coverage (%) |
|----------|-----------|---------------------------|--------|--------------------------------|-----------------------------------|-----------------------|----------------|----------------|-----------------|-------------|
| 1        | NP_058771.1 | Acetyl-Coenzyme A Acetyltransferase 1 | ACAT1  | 45009                           | 8.92                              | 4.30E-03              | 192            | 24              | 15              | 58          |
| 2        | NP_112293.1 | Acetyltransferase 1-Arginine:Glycine Amidotransferase | AGAT  | 48724                           | 7.17                              | 1.90E-03              | 151            | 22              | 40              | 52          |
| 3        | NP_114023.1 | Alanine-Glyoxylate Aminotransferase 2 | AGT2  | 57689                           | 8.46                              | 2.90E-03              | 139            | 11              | 2               | 30          |
| 4        | NP_058831.1 | Adenylosuccinate Kinase 3-Like 1 Ado-Keto Reductase | AK3L1 | 25301                           | 7.79                              | 2.50E-03              | 65             | 10              | 45              | 48          |
| 5        | NP_112262.1 | Adenylosuccinate Kinase 3-Like 1 Ado-Keto Reductase | AKR1A | 36761                           | 6.84                              | 3.90E-03              | 86             | 7               | 3               | 21          |
| 6        | NP_00110788 | Alpha 2, H+ Transporting, Lysosomal V1 Subunit A | ATP6V1A | 65847                           | 5.42                              | 1.20E-03              | 154            | 13              | 2               | 23          |
| 7        | NP_062164.1 | Carbonic Anhydrase II Capping Protein (Actin Filament) Muscle Z-Line, Alpha 2 | CA2  | 29267                           | 6.89                              | 6.70E-05              | 68             | 6               | 8               | 26          |
| 8        | NP_031630 | Carbonic Anhydrase II Capping Protein (Actin Filament) Muscle Z-Line, Alpha 2 | CAP2A | 33118                           | 5.57                              | 4.60E-02              | 137            | 14              | 28              | 61          |

**TABLE II**

| Spot no. | NCBI no. | Name of identified protein | Symbol | Theoretical molecular mass (Da) | Theoretical isoelectric point (pI) | One-way ANOVA p value | Mascot scores | Peptides matched | Peptides unmatched | Coverage (%) |
|----------|-----------|---------------------------|--------|--------------------------------|-----------------------------------|-----------------------|----------------|----------------|-----------------|-------------|
| 1         | NP_063970.1 | Annexin A2 | ANXA2  | 38678                           | 7.55                              | 1.70E-03              | 98             | 14              | 11              | 32          |
| 2         | NP_476561 | Vacuolar H+ ATPase B2 | ATP6V1B2 | 56550                           | 5.67                              | 5.90E-04              | 100            | 25              | 15              | 15          |
| 3         | NP_001005903 | Capping Protein (Actin Filament) | CAP2B | 30628                           | 5.88                              | 2.50E-03              | 66             | 11              | 56              | 46          |
| 4         | NP_03029988 | Catechol-O-Methyltransferase | COMT  | 31024                           | 6.9                               | 7.70E-03              | 76             | 10              | 19              | 45          |
| 5         | NP_445743.2 | Enolase | EH1    | 24909                           | 8.02                              | 1.20E-02              | 126            | 12              | 4               | 34          |
| 6         | NP_446028 | Peroxiredoxin 6 | PRDX6  | 24860                           | 5.64                              | 8.20E-03              | 65             | 5               | 4               | 22          |
| 7         | NP_476489.1 | Septin 2 | SEPT2  | 41737                           | 6.15                              | 6.60E-05              | 71             | 11              | 18              | 39          |
| 8         | NP_001094200 | Septin 2 | SEPT2  | 47042                           | 7.57                              | 8.70E-03              | 222            | 29              | 31              | 53          |
| 9         | NP_001029298 | Thioredoxin | TRX  | 31676                           | 5.56                              | 4.00E-05              | 111            | 8               | 3               | 45          |

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buffer B (50 mmol/L Tris-HCl, pH 8.8, 6 mol/L urea, 30% glycerol, 2% SDS, 25 mg/ml iodoacetamid) for another 15 min incubation at room temperature. IPG strips were placed on top of 12% homogeneous polyacrylamide gels that had been precast with low fluorescence glass plates using an Ettan DALT six-gel caster. Strips were overlaid with 0.9% agarose in 1× running buffer containing bromphenol blue and were run for 12–16 h (5 W per gel, overnight) at 15 °C in an Ettan DALT six electrophoresis system. After the run was completed, the 2D gels were scanned with a Typhoon 9400 imager.

**Data Analysis**—Analysis of 2D-DIGE was performed using DeCyder 6.0 software (GE Healthcare) according to the manufacturer’s recommendation. Briefly, the DeCyder biological variation analysis module was used to detect spots (the estimated number of spots was 2500) and simultaneously match all 27 protein spot maps from nine gels. All matches were also confirmed manually. Standard deviation of a protein spot data in each group was calculated according to the differences among each of the three rats. A one-way analysis of variance (ANOVA) was used for statistical analysis of the protein spot data, and a p value of <0.05 indicated that a protein spot was significantly changed across the six groups.

**Protein Identification**

**In-gel Digestion**—Spot picking was carried out with preparative gels. Two-dimensional electrophoresis was performed as described under “2D-DIGE and Imaging” except that the IPG strips were loaded with 500–1000 μg of protein, and gels were stained with silver stain and Coomassie Brilliant Blue. Protein spots of interest were excised and destained with 100 mM sodium thiosulfate, 30 mmol/L ferricyanatum Kalium, and 25 mmol/L ammonium bicarbonate, 50% acetonitrile. The destained gels were incubated in 10 mmol/L DTT at 56 °C for 1 h and in 55 mmol/L IAM at room temperature for 45 min in the dark. Gels were then dried completely by centrifugal lyophilization and digested overnight at 37 °C with gentle agitation using 0.05 μg/μl of sequence grade-modified trypsin (Promega, Madison, WI). The reaction was stopped by 2% trifluoroacetic acid.

**MALDI-TOF Analysis**—A 1-μl aliquot of tryptic peptide was mixed with 1 μl of matrix (4-hydroxy-α-cyanocinnamic acid in 30% acetonitrile, 0.1% trifluoroacetic acid) before spotting on the target plate. MS analyses were performed on an AXIMA matrix-assisted laser desorption ionization/time of flight (MALDI-TOF) instrument (Shimadzu Corp., Kyoto, Japan). Peptide mass maps were acquired in the positive reflection mode, averaging 1500 laser shots per MALDI-TOF spectrum by KOMPACT V2.4.0 software. The mass spectra were analyzed using MASCOT 1.2.0 software and searched against the NCBI rattus database (released April 2009, 68457 sequences). Peptide tolerances were set at 200 p.p.m. and maximum missed cleavage was set as 2 with fixed modifications: carbamidomethyl (C)/variable: oxidation (M). Known contaminant ions (keratin) were excluded. PMF scores (probability-based MOWSE score) >60 were considered statistically significant (p values < 0.05). The molecular weight and pl values of most proteins were consistent with the gel regions from which the spots were excised.

**Liquid Chromatography-MS (LC-MS) Analysis**—The tryptic peptides were extracted with 5% formic acid/50% CH3CN, and the analysis was performed using an LTQ instrument (Thermo) supplied with C18 capillary columns. Mobile phase A was 0.1% formic acid and mobile phase B was 0.1% formic acid in acetonitrile. The program was set as follows: L: 100% (0 min)–100% A (5 min)–5% B (5.1 min)–65% B (60 min)–100% B (75 min)–100% B (85 min), Flow rate: 200–800 nl/min. The peak list was generated by Xcalibur version 2.0 SR2 software and the data were analyzed using SEQUEST V27 software and searched against the NCBI rattus database (released March 2009, 14820 sequences). Scoring criteria were set as follows: Del CN

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**Table III**

| Cluster | TransAMs | Oxido-reductases | Enzymes (27) | Others (9) |
|---------|----------|----------------|-------------|-----------|
| 1       | ACAT1, AGAT, AGT2 | PRDX3, HIBADH, AKR1A, CAS1 | FAHD2A, NIT2, ATP6V1A, Atp6v1b2 | ECHS1 SUCLA2 HSPD1, SPI6 |
| 2       | AK3L1, COMT, PGK1 | DLD PSMA3 CA2 SUCLG2 | TP11 | |
| 3       | ECH1 | CAP2B, CAPZA2, SEP12, FH12, PDLCM1 | | |
| 4       | ECH1 | | | |
| 5       | GLYAT | | | |
DEPs were classified into five clusters according to their expression patterns. The colored graph displays the five DEP clusters (red: up-regulated, green: down-regulated), and the curve graph shows the expression of DEPs in each cluster (mean ± std) during the process of anti-Thy1. Cluster 1 showed decreased expression starting on days 5 and/or 7, and expression continued to decline on days 10 and 14. Cluster 2 showed up-regulation on day 5 and down-regulation on days 7, 10, and 14. Cluster 3 showed down-regulation on days 3, 5, 7, and 10 and recovery on day 14. Cluster 4 showed down-regulation on days 3 and/or 5 and up-regulation on days 10 and/or 14. Cluster 5 showed up-regulation on days 5, 7, and/or 10 and recovery by day 14.
value \geq 0.1, \text{xCorr} \geq 1.9$ for charge state 1$^+$; $\geq 2.5$ for charge state 2$^+$; and $\geq 3.75$ for charge state 3$^+$.

Hierarchical Cluster Analysis—Hierarchical cluster analyses were carried out using the Cluster program 3.0, and the results were compiled using Eisensoftware-TreeView. The proteins in a given cluster exhibited similar expression profiles in a synergistic manner.

Western Blotting—Renal glomeruli and cellular protein were isolated using RIPA (containing 50 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 0.5% deoxycholate, 1% Nonidet P-40, 0.1% SDS, 1 mm phenylmethanesulfon fluoride, and a variety of protease inhibitors). AKR1A1, AGAT, PRDX6, MDH1, PSMB7, and HIBADH antibodies were purchased from ABCAM (Cambridge, MA); ATP6V1B2, MPST, TP1, cyclin D1 and FHL2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The Nit2 antibody was produced by our laboratory. \text{\textbeta-}actin (Sigma) was used as the control. Approximately 80 \mu g of protein were loaded for 12% SDS-PAGE electrophoresis. The membrane was incubated in primary antibodies at suitable concentrations. The blots were developed with ECL reagent (Santa Cruz Biotechnologies) according to the manufacturer’s instructions and exposed to x-ray film. The protein bands were quantified using Quantity One software (Bio-Rad, Hercules, CA).

Primary Mesangial Cell (MC) Culture—Primary MCs were cultured as reported previously (17). Briefly, glomeruli were isolated from Wistar rats weighing 200 g using a stainless-steel sieve. Isolated glomeruli were cultured in RPMI 1640 with 15% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 \mu g/ml streptomycin at 37 °C. First passage MCs were grown from glomeruli 5–7 days later. MCs were identified by detecting MC markers such as desmin, vimentin, \text{\textalpha-}SMA et al. MCs from passages 10 to 15 were used in our study.

Cell Transfection—Cells at 70–80% confluency in six-well plates were prepared for plasmid transfection. The pcDNA-FHL2 plasmid was a gift from Professor Qinong Ye. Plasmid (2 g/ml) and 4 \mu ml Jetprime (Polyplus-transfection) were applied for 8 h at 37 °C, then replaced with fresh medium (RPMI 1640, 15% fetal bovine serum) for 16 h. For siRNA transfection, 100 nm siRNA were transfected into 3 × 10^6 cells in six-well plates using lipofectamine (Invitrogen, Carlsbad, CA), according to the manufacturer’s reverse transfection protocol. The siFHL2 target sequence was 5’-GCAAGGACTTGTCCTACAA-3’ (18). The cell number was determined for detecting cell proliferation prior to harvesting. FHL2 expression in cells was detected by Western blotting, as described previously.

Determination of the Cell Cycle Phase by PI Staining—Cells were harvested and fixed in 75% ethanol for 24 h at 4 °C. The nuclei were stained with 50 \mu g/ml propidium iodide (PI) in phosphate-buffered saline containing 200 \mu g/ml DNase-free RNase, and the DNA content was analyzed by flow cytometry using FACS Calibur (BD, USA). The percentage of cells in each phase of the cell cycle was determined by the Modfit LT program (Verity Software House, Topsham, ME).

Results

Mesangial Proliferation and Recovery Were Two Major Phases During Anti-Thy1 Nephritis—Following intravenous injection of anti-Thy1 antibodies, a partial complement-dependent mesangiolysis appeared on day 3; obvious mesangial cell proliferation (more than three MCs in a single mesangial region) and extracellular matrix accumulation occurred on day 5 and peaked on day 7. Recovery from glomerular injury began on day 10 and the number of MCs showed an obvious decrease as extracellular matrix accumulation attenuated at

| Cluster | DEPs regulated biological functions such as stress, cell proliferation, apoptosis, etc. | Stress | Apoptosis | Actin cytoskeleton regulation | Transport | Energy metabolism |
|---------|------------------------------------------------------------------------------------|--------|-----------|-------------------------------|----------|-----------------|
| 1       | AGAT, PRDX6, MDH1, HSPD1                                                          | AGAT,  |          |                                |          |                 |
|         |                                                                                   | PRDX6, |          |                                |          |                 |
|         |                                                                                   | MDH1,  |          |                                |          |                 |
| 2       | CA2, COMT, DLD, PGK1, AK3L1                                                        | CA2,   |          |                                |          |                 |
|         |                                                                                   | COMT,  |          |                                |          |                 |
|         |                                                                                   | DLD,   |          |                                |          |                 |
|         |                                                                                   | PGK1,  |          |                                |          |                 |
| 3       | MPST, HSPA8, PRDX6                                                                | MPST,  |          |                                |          |                 |
|         |                                                                                   | HSPA8, |          |                                |          |                 |
|         |                                                                                   | PRDX6, |          |                                |          |                 |
| 4       | CAPZB, CAPZA2, SEPT2, FHL2                                                         | CAPZB, |          |                                |          |                 |
|         |                                                                                   | CAPZA2,|          |                                |          |                 |
|         |                                                                                   | SEPT2, |          |                                |          |                 |
| 5       | ERP29                                                                               | ERP29  |          |                                |          |                 |
day 14 (Fig. 1). Detection of PCNA expression reflects the state of the cell cycle. High levels of PCNA are consistent with onset of S-phase, whereas low levels are consistent with nonproliferative cells in G1-phase. We found that there were higher levels of PCNA at every time point during anti-Thy1 nephritis as compared with the control (0 d) (Fig. 2), which suggests that the cell cycle remained active from days 3 to 14. PCNA expression levels had a unique pattern during anti-Thy1 nephritis. PCNA increased on days 3 and 5, peaked on day 7, and decreased from days 10 to 14. This suggests that cell cycle activity increased from days 3 to 7, and subsequently gradually decreased from days 10 to 14. Thus, our results imply that mesangial proliferation and recovery are the two major phases during the process of anti-Thy1 nephritis.

Forty DEPs, Identified by 2D-DIGE, Were Classified Into Five Clusters According to Their Expression Patterns During Anti-Thy1 Nephritis—We analyzed the proteomic profiles of each group by 2D-DIGE, and the Cy2, Cy3, and Cy5 channels of each gel were individually imaged (Figs. 3A, 3B, 3C). In total, 108 proteins were significantly changed (p < 0.05), and 40 proteins were identified by MALDI-TOF (Table I) and LC-MS (Table II). The corresponding sites of these DEPs, including identified proteins in DIGE profiles, are provided in Fig. 3D. The DEPs contained proteins such as enzymes, cytoskeletal proteins, and heat-shock proteins (Table III). The enzymes included oxidoreductases (PRDX3, PRDX6, DLD, HIBADH, MDH1, AKR1A, CAS1), transferases (ACAT1, AGAT, AGT2, GLYAT, MPST, COMT, AK3L1, Pgk1), ligases (SUCLA2, SUCLG2), hydrolases (FAHD2A, NIT2, ATP6V1A, ATP6V1B2, PSMA3, PSMB7), isomerases (TPI1), and lyases (CA2, ECHS1, ECH1). Cytoskeletal-related proteins included CAPZA, CAPZB, SEPT2, ANXA2, FHL2, and PDLIM1. The heat-shock proteins HSPD1 and HSPA8 were identified along with other types of proteins such as NDRG1, SPI6, TOLLIP, and ERP29.
Cluster analysis was performed to investigate the expression trends of DEPs during anti-Thy1 nephritis. In total, 40 DEPs were classified into five clusters based on their expression signals (Fig. 4 and Table III). Proteins in Cluster 1 showed decreased expression starting on day 5 and/or 7 and continued to decline on days 10 and 14. Proteins in Cluster 2 were up-regulated on day 5 and down-regulated at days 7, 10, and 14. Cluster 3 proteins were down-regulated on days 3, 5, 7, and 10 and showed recovered expression on day 14. Cluster 4 proteins were down-regulated on days 3 and/or 5 and up-regulated on days 10 and/or 14. Cluster 5 proteins were up-regulated on days 5, 7, and/or 10 and recovered by day 14. All DEPs identified in our study have known roles in processes such as the stress response, cell proliferation, apoptosis, energy metabolism, transport, and actin cytoskeleton regulation, suggesting that these processes may also be operative in different phases of anti-Thy1 nephritis (Tables III and IV). We harvested the glomeruli from three rats in each group, pooled the protein, and evaluated the expression of DEPs distributed across five clusters including AKR1A1, AGAT, ATP6V1B2, HIBADH, MDH1, MPST, NIT2, PRDX6, PSMB7, and TPI1 (Fig. 5). Based on Western blotting, we found that the expression patterns of all 10 proteins were altered at one or several time points as compared with the control. Western blotting and DIGE revealed similar expression patterns for eight DEPs (less than two time points showed significant differences), except for AKR1A1 and PRDX6 (three time points showed significant differences).

**Down-regulation of FHL2 Expression in MCs on Days 3 and 5, and Up-regulation on Day 10 During Anti-Thy1 Nephritis—**

FHL2 (a member of the LIM protein family) was closely associated with cell proliferation. It is known that FHL2 expression is altered when detected using DIGE. We used Western blotting and immunohistochemistry to monitor changes in FHL2 expression during anti-Thy1 nephritis using independent glomeruli samples in each group. Both methods showed that FHL2 expression in the glomeruli mesangial region was significantly down-regulated on days 3 and 5 and up-regulated on day 10. This suggests that changes in FHL2 expression may be related to cellular processes such as proliferation in MCs.

**Overexpression of FHL2 Induces MC Proliferation by Increasing the Percentage of S Phase Cells, Whereas Inhibition of FHL2 Has the Opposite Effect—**

We wanted to evaluate the effects of FHL2 overexpression on cells. To accomplish this, we used Western blotting to examine FHL2 protein expression in cells transfected with the pcDNA-FHL2 plasmid (FHL2 group). Cells transfected with the pcDNA plasmid were used as a control. We found that FHL2 and cyclin D1 significantly
increased in the FHL2 group as compared with the control at 24 h post-transfection (Fig. 7A). Next, we compared the number of cells in each group. More cells were present in the FHL2 group than in the control group at 24 h post-infection (1.9-fold increase, Fig. 7B). We monitored the cell cycle distribution in two groups using a flow cytometric DNA array. Overexpression of FHL2 increased the percentage of S phase cells and decreased the percentage of G0/G1 and G2-M phase cells significantly (Fig. 7C).

Next, we used siRNA transfection to inhibit FHL2 expression in MCs. We found that siFHL2 transfection completely inhibited FHL2 expression and suppressed cyclin D1 expression 48 h post-transfection, as detected by Western blotting (Fig. 8A). Fewer cells were present in the siFHL2-transfected group than in the control group (siCon) at 24 h post-transfection (0.5-fold decrease, Fig. 8B). The flow cytometric DNA array revealed that siFHL2 significantly decreased the percentage of S phase cells and increased the percentage of G2-M phase cells at 48 h post-transfection (Fig. 8C). These results confirm that overexpression of FHL2 induces MC proliferation by increasing the percentage of S phase cells, whereas siFHL2 has the opposite effect.

**DISCUSSION**

In this study, we bioinformatically analyzed anti-Thy1 nephritis and explored the effect of FHL2 on MC proliferation. In our classical mesangial proliferative model, anti-Thy1 nephritis induced obvious MC proliferation and recovery of proliferation. In our study, we determined that anti-Thy1 nephritis has two distinct mesangial phases: proliferation (days 5 to 7) and recovery (days 10 to 14) (Fig. 1). We also applied the PCNA stain to determine the state of the cell cycle during anti-Thy1 nephritis (Fig. 2) and found that cell cycle activation in MCs increased on days 5 to 7, and declined gradually from days 10 to 14, consistent with the proliferation process of MCs. However, the higher PCNA-positive level on day 3, suggesting activation of the cell cycle, did not correlate with pathological change in days (only partial mesangiolysis appeared with no mesangial proliferation). At this time, we also found that positive regulation cyclin proteins (such as cyclin D1 and E) were increased (data not shown), suggesting that cell cycle activation may begin on day 3. Based on these observations, we hypothesize that cell cycle activation on day 3 may trigger subsequent cell proliferation (cell cycle activation occurs prior
to an increase in cell number). The relationship between cell cycle activation and mesangiolysis, however, requires further investigation.

We found that DEPs consisted mainly of enzymes and cytoskeletal components, which is supported by previous findings (10, 19, 20). According to the recent literature, DEP enzymes mediate biological functions such as energy and lipid metabolism and transport, by way of their catalytic activity. For example, ACAT1 (21, 22) and ECHS1 (23, 24) may be involved in lipid metabolism, whereas ATP6V1A and ATP6V1B2 mediate H^+/H1001 transport (25, 26). PRDX3 (27, 28) and PRDX6 (29, 30) have antioxidant effects, regulating apoptosis and cell damage by way of DLD (31). SUCLG2, SUCLA2, and TPI1 (32) play a role in ATP and/or GTP metabolism. Another major DEP component are the cytoskeletal proteins, which may be involved in actin cytoskeleton regulation, according to recent reports. For example, CAPZA2 and CPAZB function in actin filament regulation (33), SEPT2 (34) plays a role in stabilizing or maintaining actin bundles, while FHL2 (35) and PDLIM1 (36) may bind actin-based cytoskeletons. Other than functioning as enzymes or cytoskeleton modulators, DEPs such as heat shock proteins (HSPA8, HSPD1) and toll-related proteins (TOLLIP) may function during stress or inflammatory responses. To corroborate the expression patterns of specific DEPs, we selected 10 proteins from five distinct clusters, representing different general expression trends (Fig. 4), and monitored any expression changes during the process of anti-Thy1 nephritis by Western blotting. We found that Western blotting and DIGE detected similar DEP expression patterns from 8 of the 10 proteins evaluated (Fig. 5). However, it should be mentioned that the actual function of these DEP expression changes during anti-Thy1 nephritis is still uncertain, despite the fact that their biological functions are well characterized. Thus, we chose a specific DEP (FHL2) and evaluated its effect on MC proliferation and hypothesized a role for FHL2 in anti-Thy1 nephritis. FHL2 is an important LIM domain protein and is involved in regulating cell processes such as proliferation, apoptosis, and signal transduction. We found that expression of FHL2 in the mesangial region was significantly altered during the process of anti-Thy1 nephritis when detected by immunohistochemistry and Western blotting (Fig. 6), suggesting that FHL2 may be involved in mediating cell processes such as proliferation in MCs during anti-Thy1 nephritis. Therefore, we investigated FHL2’s effect on

**Fig. 8. Inhibition of FHL2 suppressed MC proliferation by decreasing the number of cells in S phase.**

A, siFHL2 transfection completely inhibited expression of FHL2 and suppressed cyclin D1 expression at 48 h post-transfection, when detected by Western blotting. B, There were fewer cells in the siFHL2-transfected group than the siCon group at 72 h post-transfection, based on cell counts (0.5 fold). C, Flow cytometric DNA arrays revealed that siFHL2 significantly decreased the percentage of S phase cells and increased the percentage of G2-M phase cells at 48 h post-transfection. *p < 0.05 versus siCon-transfected team.
MC proliferation in vitro. In this study, we found that overexpression of FHL2 induced MC proliferation by increasing the number of S phase cells and decreasing the number of G2/M cells, whereas inhibition of FHL2 had the opposite effect. Moreover, we also found that cyclin D1 was up-regulated in FHL2 overexpressed cells, and down-regulated in FHL2 inhibited cells, suggesting that cyclin D1 is involved in the proliferative effect of FHL2. The specific effects of FHL2 on proliferation depend on the cell type by various mechanisms. For example, FHL2 has pro-proliferative effects on breast cells, mouse embryonic fibroblasts, and U2OS osteosarcoma cells by interacting with cyclins (p21Cip1/Waf1, cyclin D), signaling pathways (beta-catenin, MAPK signal pathway), and transcription factors (E4F1) (13, 37–39). FHL2 inhibits cell growth in liver cancer cells, human neuroblastoma cells, prostate cancer cells, or cardiac myocytes by interacting with transcription factors (Id2), signaling pathways (TGF beta), and enzymes (SK1) (12, 18, 40). In MC, FHL2 promote proliferation probably by activating cyclin D1. However, other mechanisms, such as signal transduction involved in FHL2 regulation on MC proliferation are still unknown.

FHL2 modulation of MC proliferation was involved in mesangial proliferation during anti-Thy1 nephritis. Interestingly, we found that on days 3 and 5, when cell cycle activation in MCs began (PCNA results shown), down-regulation of FHL2 antagonized cell cycle activity. Other cell cycle inhibitory factors may exist during anti-Thy1 nephritis. For example, up-regulation of p-p38 MAP kinase on day 3 (41) and up-regulation of p53, p21, and bax (data not shown) on days 5 and 7 we found that on days 3 and 5, when cell cycle activation in MC proliferation depend on the cell type by various mechanisms. For example, FHL2 has pro-proliferative effects on breast cells, mouse embryonic fibroblasts, and U2OS osteosarcoma cells by interacting with cyclins (p21Cip1/Waf1, cyclin D), signaling pathways (beta-catenin, MAPK signal pathway), and transcription factors (E4F1) (13, 37–39). FHL2 inhibits cell growth in liver cancer cells, human neuroblastoma cells, prostate cancer cells, or cardiac myocytes by interacting with transcription factors (Id2), signaling pathways (TGF beta), and enzymes (SK1) (12, 18, 40). In MC, FHL2 promote proliferation probably by activating cyclin D1. However, other mechanisms, such as signal transduction involved in FHL2 regulation on MC proliferation are still unknown.

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