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Abnormal Crosstalk between Mesangial Cells and Podocytes in Idiopathic Membranous Nephropathy: ECM1 Rearrange Podocyte Cytoskeleton Via EGFR/FAK/Rac1 Axis

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Abstract

Background: Idiopathic membranous nephropathy (IMN) is an autoimmune disease of the kidney glomerulus. It was confirmed that the increase in ECM proteins causes the characteristic thickening of the GBM, however, the effect of ECM on podocytes is still unknown.

Methods: In the present study, we downloaded 4 microarray data from Gene Expression Omnibus (GEO). The differentially expressed genes (DEGs) were identified with R software. Immunofluorescence was used to evaluate relative proteins expression. CCK8 was performed to assess the cell proliferation in immortalized human podocytes. The active GTPase pull-down and detection kit was used to assay the expression level of active form of RAC1. Western blotting was performed to evaluate the expression level of relative proteins.

Results: We found that ECM1, acting as a differentially expressed gene, was upregulated in the mesangial cells of glomerulus from IMN patients. In order to find out the relationship between ECM1 and Rac1 on podocyte, we used the recombinant human ECM1 on human podocyte, we found that ECM1 rearranges the podocyte cytoskeleton via activating Rac1. Mechanically, ECM1enhanced activation of p-EGFR, meanwhile the

expression of p-FAK was upregulated, which results in the activation of Rac1. Consistently, we found rhECM1 treatment increase GTP-Rac1 expression in podocytes, and this change can be blocked by FAK inhibitor.

Conclusion: We concluded that secreted ECM1 by mesangial cells potentiated p-EGFR of podocyte, increasing the expression of p-FAK and activating Rac1, and thus rearrange the podocyte cytoskeleton, resulting in morphological changes of podocytes and massive proteinuria.

Keywords: Idiopathic membranous nephropathy; Extracellular matrix protein 1; Epidermal growth factor receptor; Focal adhesion kinase; Rac1

Introduction

Idiopathic Membranous Nephropathy (IMN) is an autoimmune mediated inflammatory disease of the kidney glomerulus, which is characterized by the accumulation of massive immune complexes in the glomerulus. The incidence of IMN is 5~10 patients per million population and is the most common cause of nephrotic syndrome in adults **[1,2].** Recent decades, great progress has been made in the treatment of IMN, with rituximab become the first line drugs in IMN **[3].** Moreover, a great deal of effort has been made to understand the molecular mechanism of IMN with the discovery of several antigens, such as Phospholipase A2 Receptor (PLA2R), Thrombospondin domain-containing 7A (THSD7A), Neural EGF-like-1 protein (NELL-1), Exostosin 1/Exostosin 2 (EXT1/EXT2), Neural Cell Adhesion Molecule 1 (NCAM1), Semaphorin 3B, and protocadherin 7(PCDH7) **[4-10].** However, the initiative mechanism of IMN is largely unknown.

It is believed that the morphological change of podocyte is the core reason led to massive proteinuria in IMN **[11,12].** The Rho GTPases, especially Rac1, a key determinant of actin polymerization, regulates various cellular processes including cell migration, adhesion, polarity, and division **[13].** Rho-GTPases polymerized to form actin filaments in various structures and change the shape of the cells via the effector after receiving signaling stimulus **[13].** Rac1 plays an essential role in actin lamellipodia induction and cell-matrix adhesion, and it cycles between GTP and GDP-loaded activity states **[13].** Recent study showed that overexpression of Rac1 plays an important role in podocyte FP effacement and proteinuria **[14].** Constitutive activity Rac1 expressed specifically in podocytes leads to rapid FP effacement **[15].** In contrast, podocyte-specific Rac1 deletion mitigates FP effacement induced by protamine **[16].** All these studies revealed that activation of Rac1 plays an essential role in podocyte morphology changes. Rac1 is regulated by FAK in podocyte **[17],** which plays an active role in various cellular behaviours by receiving various signaling inputs from integrins and some growth factor receptors, including Epidermal Growth Factor Receptor (EGFR) **[18].** The glomerular Extracellular Matrix (ECM) is a dynamic structure which functions as cell structural support and cell signaling component **[19].** Among ECM components, ECM protein-1 (ECM1) is particularly noteworthy. The ECM1 is a soluble protein that is involved in endochondral bone formation, angiogenesis, and tumor biology **[20].** Moreover, ECM1 may contribute to the metastasis of invasive breast cancer cells by regulating the cytoskeletal structure of actin **[21].** In a multi-dataset joint analysis, we found that ECM1, acting as a differentially expressed gene, is upregulated in glomerulus from IMN patients compared with healthy controls. However, the detailed molecular mechanisms involved in the ECM1-mediated signaling pathway in IMN are not yet clear.

Materials and Methods

Patients

All cases used in this study were defined by a kidney biopsy diagnosis of idiopathic MN and any suspected secondary cases due to drugs, malignancy, infection, or autoimmune disease were excluded. All patients' consents were obtained and approved by the Ethics Committee of The Second Affiliated Hospital of Nanchang University. The study protocol was reviewed and approved by an institutional review board.

Microarray data information

We used "membranous nephropathy" as the keyword to search the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo). We selected the expression profiling of the array: the attribute name was tissue, and the organisms were Homo sapiens, and we got 5 datasets including accession number GSE99340, GSE104948, GSE108113, GSE115857, and GSE133288. Among these 5 datasets, GSE99340 and GSE108113 are composed of tissues from glomeruli and tubulointerstitium from kidney biopsy, GSE104948 only contain glomeruli tissues, GSE133288 only contain tubulointerstitium tissues. Thus, we chose the datasets containing glomeruli tissues to further analysis, namely GSE99340 (glomerili from kidney biopsy), GSE108113 (glomerili from kidney biopsy), GSE104948, and GSE115857. Since all data in this study is obtained from public databases, the approval of the ethics committee is not necessary.

Identification of differentially expressed genes

The R software (Version 4.0.5) was performed to data processing, analysis, and mapping. The differential expressions between IMN and control groups were observed using Differentially Expressed Genes (DEGs) as variables. An adjusted p-value of <0.05 and |logFC| >1.0 were considered statistically significant. DEGs were visualized by the "limma" package. The venn map was drawn using the "Venn Diagram" package. The volcano map and box map were plotted using "The ggplot2" and "RColorBrewer" package to screen the differential expression.

Cell culture and treatment

In this study, the immortalized human podocytes were a friendly gift from Prof. Xianfeng Wu (Shanghai Sixth People's Hospital, Shanghai, China), and maintained in modified RPMI-1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 μg/mL) at 33°C with 5 % CO2 for 2-3 days [22]. Cells were maintained at 37 °C for 10 days without ITS to induce differentiation. Within 10–14 days of differentiation and maturation, the cells were ready for use in the experiments. Podocytes were divided into two groups: The control group, and the ECM1 group incubated with recombinant human ECM1 (purchased from R&D Systems, USA).

CCK8 assay

Podocytes were treated with different concentration of recombinant human ECM1 (rhECM1). After podocytes were adherence (12 h after cell plated), cell viability was tested at different times (6, 12, 18 and 24 hours). According to the manufacture protocol, 10% reconstituted CCK-8 reagent (C0039, beyotime, China) was added in 100 ul of the modified RPMI-1640 medium, then return to incubator at 37°C for 2 hours. The microplate reader (BioTek, USA) was used to detect the Optical Density (OD) value at 450 nm.

Western Blot

Podocytes were collected, and total proteins were extracted by RIPA lysis buffer (78510, Thermo Fisher, USA). Total protein (equal, 30 μg/lane) were separated by 10% SDS-PAGE, and transferred to a PVDF membrane

(LC2002, ThermoFisher, USA). Then the PVDF membrane was incubated at room temperature with 5% nonfat milk for 1 h. Finally, these PVDF membranes were incubated with primary antibody, rabbit monoclonal anti-phospho-FAK Ab (ab230813, 1:1000, Abcam, UK), rabbit monoclonal anti-FAK Ab (ab76496, 1:5000, Abcam, UK), rabbit monoclonal anti-phospho-EGFR (ab32086, 1:5000, Abcam, UK), rabbit monoclonal anti-EGFR ab32077, (1:5000, Abcam, UK), and rabbit monoclonal anti-GAPDH (ab181602, 1:10000, Abcam, UK) at 4°C overnight. Next day, the membranes were washed three times and then incubated with Goat anti-rabbit IgG (1:10000; Abcam, ab205718) or anti-mouse IgG (1:10000; Abcam, ab6789) at RT for 2 hours. The ECL reagent (WBKLS0100, Beijing Xinjingke Biotechnologies Co., Ltd, China) was used to detect the PVDF membranes. The Image J 3.0 (IBM, USA) was performed to analyze densitometry.

Active GTPase pull-down and detection

The active form of Rac1 was detected using an active RAC pull-down and detection kit (16118, ThermoFisher), which can selectively enrich and detect GTP-binding Rac1 GTPase by interacting with specific proteins in the PAK1 protein-binding domain. Briefly, cell extracts were incubated with Glutathione S-Transferase (GST) conjugated human PAK-PBD beads for 1 hour at 4℃。Beads were washed using washing buffer from the kit twice. Then beads were resuspended with Laemmli sample buffer and boiled for 3 min. After the Rac1-GTP was pull down, the protein samples were used for western blot analysis, and the expression of total RAC1 acts as control. The Image J 3.0 (IBM, USA) was used to quantitatively analyze the intensity of chemiluminescence bands.

Immunofluorescence

12 renal biopsy samples were obtained from May 2020 to August 2020 in Nephrology Department of Nanchang University Second Affiliated Hospital, including 9 IMN and 3 normal renal tissue samples **(Supplementary Table 1).**

Supplementary Table 1: Profiles and data of patients.

Patient no.	Group	Age	Sex	Serum Albumin (g/L)	Proteinuria (mg/24hours)
1	Control	49	M	44.6	124.3
2		36	M	47.1	89.7
3		63	F	43.4	101.5
4	IMN	61	M	28.4	11064.2
5		66	F	22.5	7682.8
6		34	M	20.7	21145.4
7		52	M	26.3	8631.7
8		44	F	20.2	14312.6
9		58	M	27.9	6123.8
10		67	M	25.5	9012.6
11		62	M	21.3	8319.3
12		59	M	29.4	8078.8

4 % paraformaldehyde was used to fix renal tissues, then renal tissues were embedded with paraffin and cut to 5~6 nm thick slice. After procession of dewaxing, gradient alcohol dehydration, antigen repaired and washed with Phosphate-Buffered Saline (PBS) three times, slides were incubated with the primary antibody, rabbit

monoclonal anti-ECM1 Ab (1:200, ab126629, Abcam, UK), 4°C for overnight. Then, the anti-FITC-conjugated secondary antibody was incubated with slices away from light for 1~2 hours at RT. Finally, images were obtained by fluorescence microscope. 4% paraformaldehyde was used to fix podocytes for 15 min at RT. Then, NaCl/Pi was used to wash cells. Podocytes were permeabilized with 0.2% Triton X-100, and incubated with Rac1 monoclonal antibody (rabbit anti-rabbit anti-Rac1, 1:20, ab155938, Abcam, UK) at 4°C for overnight. Cells were then incubated with Alexa 594-conjugated secondary antibody for 1 h at 37°C. were captured using fluorescence microscope.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software. Differences between two groups were analyzed using Two-sided Student's t-test. Using one-way ANOVA analysis of variance between multiple groups. Values were presented as the mean ± standard deviation. $P < 0.05$ was considered to indicate a statistically significant.

Results

Bioinformatics found that ECM1 expression was upregulated in the glomeruli

The gene matrix of 4 datasets were normalized and processed. In total, 138 DEGs were identified in GSE 99340 (76 upregulated, 62 downregulated), 73 DEGs were identified in GSE 104948 (39 upregulated, 34 downregulated), 356 DEGs were identified in GSE 108113 (116 upregulated, 240 downregulated), and 1422 DEGs were identified in GSE115857 (952 upregulated, 470 downregulated). 2 DEGs, namely ECM1 and HLX, were shared among these four datasets, as identified through Venn diagram analyses **(Figure 1).**

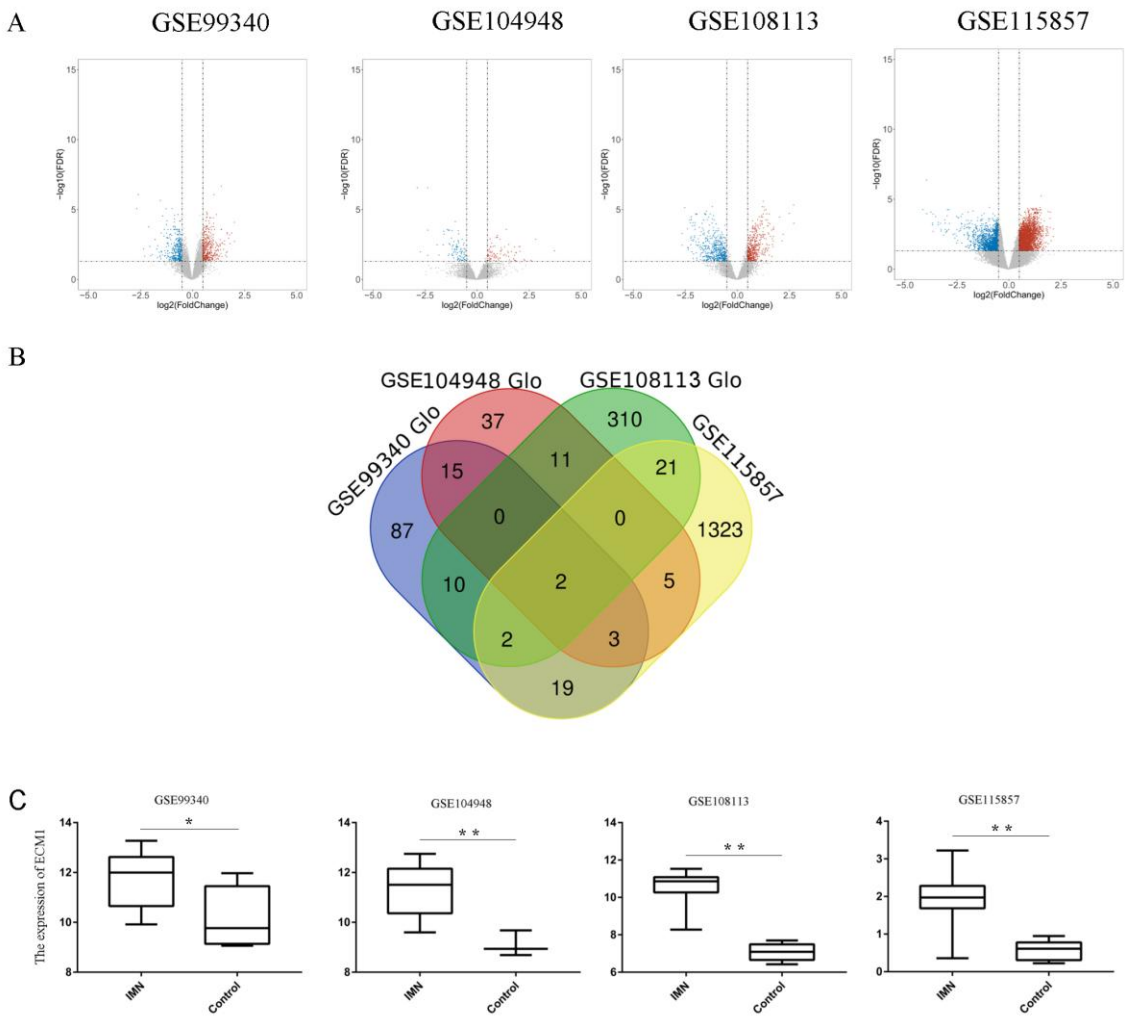

Figure 1: Detection of Differentially Expressed Genes (DEGs) in the GSE99340, GSE104948, GSE108113, and GSE115857. A: Volcano plots corresponding to these 4 datasets. B: Identification of shared DEGs with venn map. C: The expression of ECM1 in these 4 datasets.

Notably, ECM1 was upregulated in all datasets, while HLX was upregulated in GSE99340, GSE 104948, and GSE 108113, but downregulated in GSE115857. Thus, these data put together led our focus on ECM1 as the main candidate for further investigation in the context of IMN.

ECM1 was expressed in the mesangial cells of the glomeruli in IMN

To found out which cells dose ECM1 mainly expressed, the expression level of ECM1 in glomeruli from 10 IMN patients and 3 healthy controls was observed using immunofluorescence staining **(Figure 2).** The result showed that ECM1 is overexpressed in the mesangial cells of the glomeruli in IMN patients.

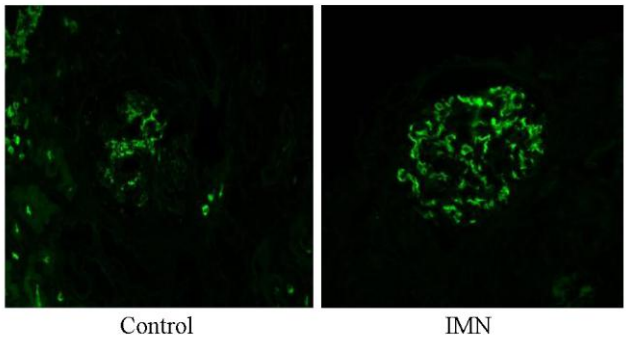

Figure 2: Immunofluorescence staining showed that the expression of ECM1 is mainly expressed in the mesangial cells of the glomeruli in IMN patients.

ECM1 promotes podocytes proliferation

We used a CCK8 kit to investigated whether ECM1 is capable of inducing podocyte proliferation, we treated podocytes with 15, 30, 60, and 120 ng/ml of rhECM1 for different times (6 hours, 12 hours, 18 hours, 24 hours), and saline was used as a control. As we expected, rhECM1 promotes podocytes proliferation within 12 hours as quantified by cell viability. And the most effective treatment was 60 ng/ml rhECM1 in 12 hours. After 12 hours, the effect of rhECM1 on podocytes was disappeared gradually **(Figure 3).** These data suggest that receptors may be saturated at these concentrations, rather than rhECM1 having a dose-dependent effect.

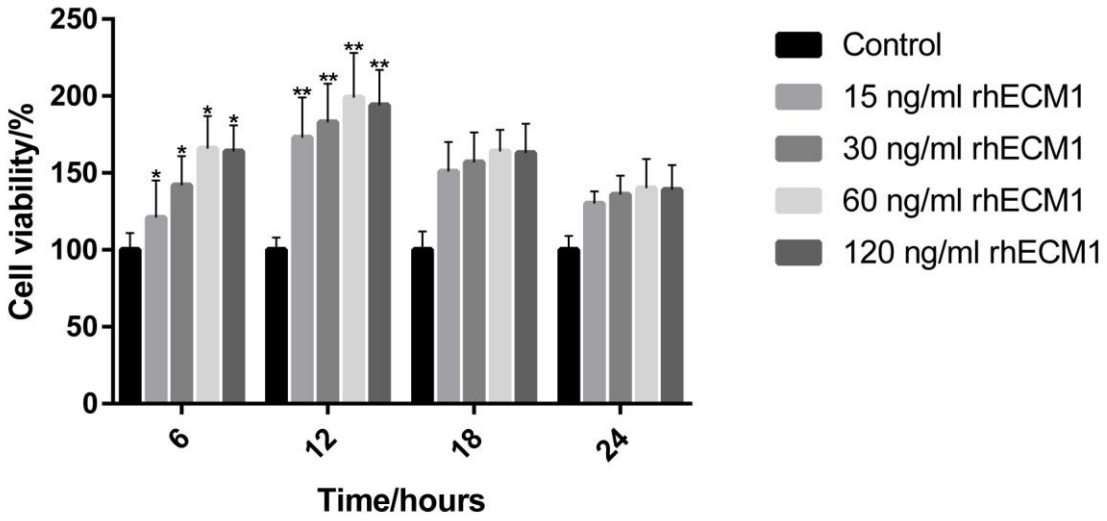

Figure 3: ECM1 upregulates cell viability of podocytes within 12 hours: After being incubated with different concentration of rhECM1 for different time, the viability of podocytes was determined by CCK8.

ECM1 induces podocytes cytoskeleton remodelling via EGFR/FAK/Rac1 axis

In cultured podocytes, rhECM1 induced cytoskeleton remodelling using immunofluorescence staining **(Figure 4A).** As EGFR/FAK/Rac1 pathway has been shown to play an active role in formation of lamellipodium and migration of cells **[17,18,23],** the expression level of EGFR, p-EGFR, FAK, p-FAK, and Rac1 was detected in

podocytes treated with rhCEM1. As shown in **Figure 4B,** ECM1enhanced activation of p-EGFR, meanwhile the expression of p-FAK was upregulated, which could result in the activation of Rac1. Consistently, using small GTPase pull-down assay and western blot, we revealed that the expression of GTP-Rac1 was increased in podocytes after ECM1 treatment, and FAK inhibitor could mitigate this change **(Figure 4C).** These data demonstrated that ECM1-induced podocyte cytoskeleton remodelling is induced via EGFR/FAK/Rac1 signaling.

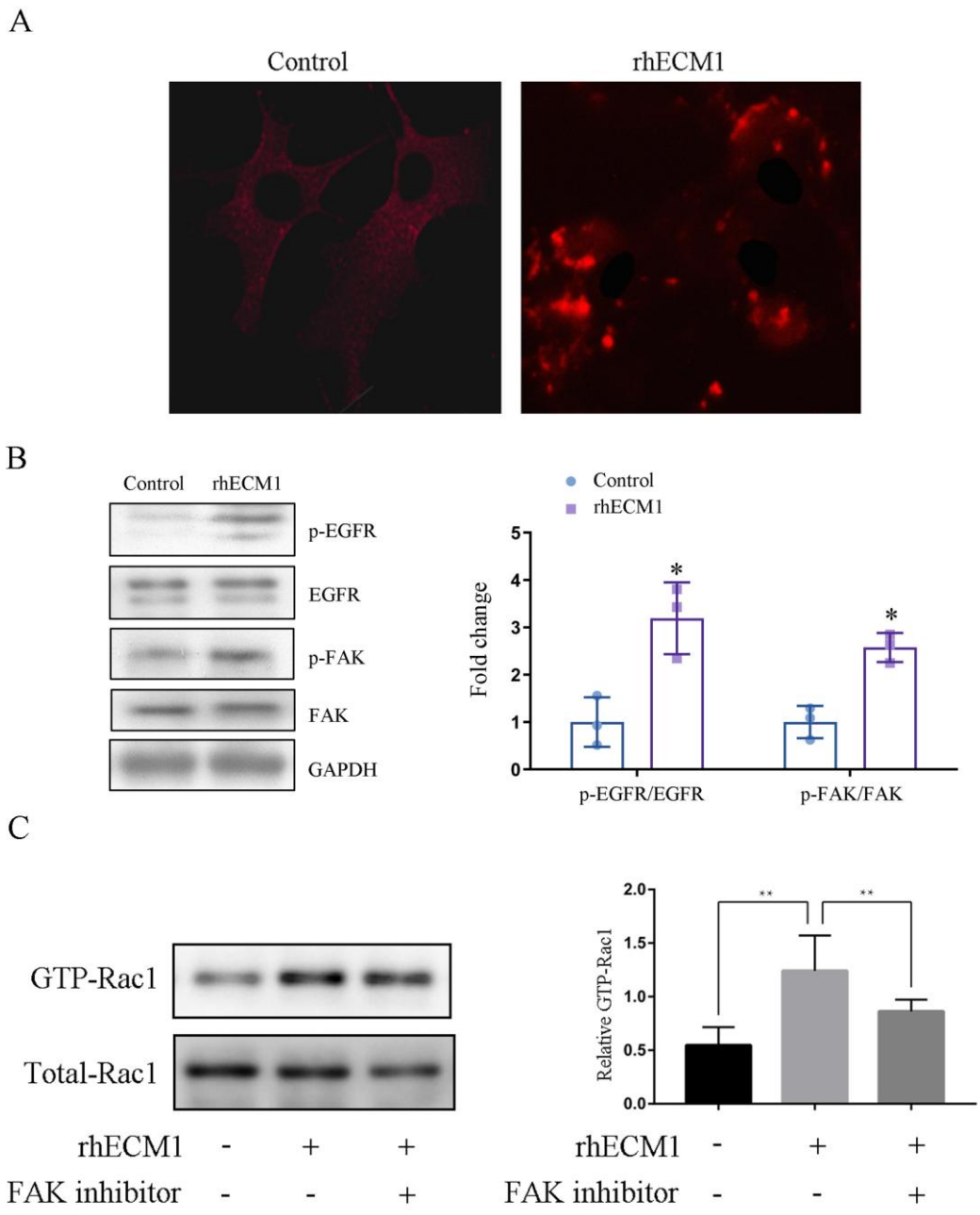

Figure 4: ECM1-induced podocyte cytoskeleton reorganization by the EGFR/FAK/Rac1 axis. A: rhECM1 treatment induced cytoskeleton remodelling in cultured podocytes using immunofluorescence staining. B: rhECM1 activates FAK by enhancing the expression of EGFR. C: rhECM1-induced Rac1 switch-on can be blocked by FAK inhibitor.

Discussion

In present study, we reported an abnormal crosstalk between mesangial cells and podocytes in IMN. In a multi-dataset joint analysis, ECM1 is upregulated in mesangial cells of glomeruli in IMN patients, which may act as a critical step to initiate proteinuria. As Rac1 is involved in podocyte foot process effacement and proteinuric nephropathies **[24],** we further analyze whether there is a regulatory relationship between ECM1 and Rac1. We fund that ECM1 rearranges the podocyte cytoskeleton via activating Rac1. Mechanically, ECM1enhanced activation of p-EGFR, meanwhile the expression of p-FAK was upregulated, which results in the activation of Rac1. Consistently, using active GTPase pull-down and detection kit and immunoblotting, we revealed that the expression of GTP-Rac1 was increased in podocytes after ECM1 treatment, and FAK inhibitor could mitigate this change. Thus, we concluded that secreted ECM1 by mesangial cells activates EGRF/FAK/Rac1/cytoskeletal signaling in podocyte results in the morphological changes of podocytes, eventually contributing to massive peoteinuria in IMN.

IMN is an immune-mediated disease caused by the deposition of IgG and complement components in the subepithelial layer of capillary wall of the glomeruli. Numerous studies confirmed that podocyte injury leads to morphological changes characterized by the expansion and contraction of the foot process by remodelling its cytoskeletal structure in IMN **[25,26].** It has been shown that in nephrotic syndrome, the main contributor to the podocytes FP effacement is the overactivation of Rac-1 **[27].** Moreover, Hui Zhang et al. **[28]** revealed that overactivation of Rac1 disturb the intricate structure of podocyte foot processes, leading to proteinuria in the passive heymann nephritis model. Furthermore, glucocorticoid therapy in nephrotic syndrome has been used for decades, and a recent study found that Rac1 activity was reduced with steroids treatment in podocyte **[29].** In consistent with previous study, our present study demonstrated that overexpression of Rac-1 in podocyte results in the morphological changes of podocytes, eventually contributing to massive peoteinuria in IMN. The ECM1 is a soluble protein that is involved in endochondral bone formation, angiogenesis, and tumor biology **[20].** It is believed that ECM1, as a target, may induce a strong immune response rather than self-tolerance **[30].** Moreover, ECM1 is a more attractive immunotherapeutic target **[30].** However, the role of ECM1 in kidney diseases is little studied. Flavia et al. **[31]** reported that several ECM associated proteins, including ECM1, that may be important for development of glomerular damage in IgAN. Besides, the morphologic change of MN is the thickening basement membranes with spike-like extensions of ECM, which consists of normal GBM constituents **[32,33].** The increase in ECM proteins lead to the characteristic thickening of the GBM **[34].** In IMN podocyte model, Torbohm I et al. **[35]** found markedly upregulated production of ECM, and molecular studies have confirmed that the gene expression of ECM was increased **[36,37].** The accumulation of ECM was occurred in the presence of increased production of matrix-degrading proteases, suggesting that there must be an imbalance existed between the production of proteases and protease inhibitors in MN **[34].** Consistently, we found that ECM1 is upregulated in mesangial cell of glomeruli in IMN patients. Moreover, as we know that normal podocytes are terminally differentiated and quiescent cells. In experimental MN study, low level of proliferation of podocytes was observed **[38].** Our study revealed that ECM1 is capable of promoting proliferation of podocytes, which may act as a critical step to initiate proteinuria. Mechanically, secreted ECM1

from mesangial cells activates EGRF/FAK/Rac1/cytoskeletal signaling in podocyte results in the morphological changes of podocytes **(Figure 5),** eventually contributing to massive proteinuria in IMN.

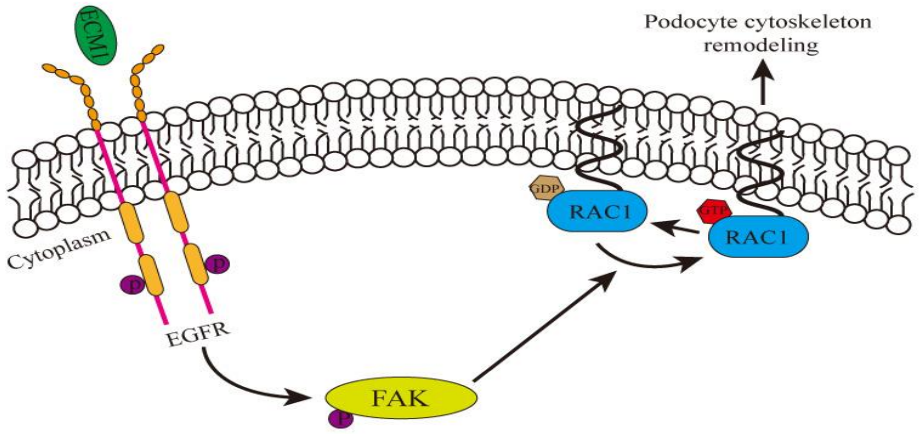

Figure 5: Podocyte cytoskeleton remodelling.

Conclusion

The present study supports the important role for Rac1 in the morphology of podocyte. In particular, overexpression of ECM1 in mesangial cells appears to be initiative step in IMN, which gives rise to proteinuria via activating EGRF/FAK/Rac1/cytoskeletal signaling in podocyte resulting in the morphological changes of podocyte. These results suggest that ECM1 inhibitor may keep Rac1 from activation, and protect podocytes from injury in IMN.

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Author Information

Weiping Tu and Xiangdong Fang outlined the manuscript, conceived the project and wrote the manuscript. Ben Ke and Fuli Luo designed and performed data analysis, drafted the manuscript with additional input from all authors and wrote the manuscript. Jianling Song and Wen Shen performed CCK8 and western blot. Wei Qiao and Jingjing Huang performed immunohistochemical staining and small GTPase pull-down assays. Ben Ke and Wen Shen contributed to the literature search and data collection. All authors read and approved the final manuscript.

Ethical Approval

Ethics approval and consent to participate.

The study is in accordance with the Declaration of Helsinki and was approved by the Bioethics Committee of The Second Affiliated Hospital to Nanchang University (IRB approval no.: The Second Affiliated Hospital to Nanchang University, 111)). All patients are unrelated. All patients signed an informed consent for participation prior to their inclusion into the study.

Consent for Publication

Not applicable.

Conflict of Interest

The authors have declared that no conflict of interest exists.

Data Availability Statement

Most data in this experiment were from the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/).

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