Involvement of β-PIX in Protamine Sulfate Induced Loss of Stress Fibers in Renal Podocytes

Master Thesis in Medicine

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ABSTRACT

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Chronic kidney disease (CKD) is a major cost to the healthcare systems with poor treatment options causing low quality of life among patients. Most CKDs are caused by podocyte damage. Podocyte morphology and stability in the glomerular filtration barrier is controlled by Rho GTPases (RhoA, Rac1 and Cdc42). The Rac1 activating Guanine Nucleotide Exchange Factor (GEF) β-PIX seems to be involved in renal physiology and pathogenesis. Protamine sulfate (PS) has been shown to disrupt the glomerular filtration barrier by up regulating Rac1 and resulting in podocyte stress fiber loss. Here we aimed to investigate the involvement of β-PIX in PS induced stress fiber rearrangement. By using a lentiviral approach to knockdown β-PIX in immortalized mouse podocytes and treating them with PS (600ug/ml) for 1 h we could show that podocyte β-PIX knockdown prevented stress fiber loss when compared to PS treated Scr shRNA (control) infected podocytes, which lost their stress fibers. This data indicate that β-PIX is involved in PS induced podocyte stress fiber loss, probably due to its Rac1 activating properties and demonstrates the importance of β-PIX in renal pathogenesis.

Key Words: Chronic kidney disease, glomerular filtration barrier, podocyte, Rho GTPases, β-pix, protamine sulfate

INTRODUCTION

In 2002 chronic kidney disease (CKD) was defined by the US National Kidney Foundation Kidney Disease Outcomes Quality Initiative (K/DOQI) clinical practice guidelines as kidney damage or glomerular filtration rate (GFR) < 60 mL/min/1.73m^2 for ≥ 3 months [1]. There are 5 stages (1-5, from mild to severe) of CKD depending on the level of proteinuria and the GFR [2]. Patients with CKD at stage 3-5 are at higher risk than others to develop end stage renal disease (ESRD) and thereby become in need of dialysis and transplant [3]. Unfortunately, these poor treatment options of ESRD cause a low quality of life [4]. They are also together with the treatment of CKD a major cost to the health care systems [5]. The prevalence of CKD was higher in the United States in 1999-2004 than it was in 1988-1994, which was correlated with the prevalence of cardiovascular risk factors [6]. Thus, developing new treatment therapies is essential.

Proteinuria appears when there is a dysfunction in the glomerulus, which is a highly specialized structure in the kidney cortex that enables the selective ultrafiltration of plasma and retains essential proteins in the blood [7]. The glomerular filtration barrier (blood to urine barrier), which is size- and charge-selective, consists of the capillary fenestrated endothelium, the glomerular basement membrane (GBM) and the podocytes (unique kidney cells) [8]. The podocytes have a cell body from which major processes (MPs) arise and split into foot processes (FPs) that form a characteristic interdigitating pattern with FPs from other podocytes on the outer aspect of the GBM, which generates filtration slits bridged by filtration diaphragms, a construction that is critical for the filtration barrier [9]. Podocyte FP effacement is observed in glomerular dysfunction [10]. Since the submembranous actin

cytoskeleton has a central role in maintaining the stability and the morphology of the podocyte's cell body, MPs and FPs it is of major importance to study the actin dynamics in order to find new treatment targets in CKDs [11].

All eukaryotic cells, including the podocytes, express the Rho family of small guanosine triphosphatases (Rho GTPases: RhoA, Rac1 and Cdc42), which are known to be involved in actin dynamic signal transduction pathways in the cells. The Rho GTPases are regulated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) [12]. GEFs activate Rho GTPases by exchanging GDP to GTP, which enables interaction of Rho GTPases with target proteins, and GAPs inactivate Rho GTPases by accelerating the GTPase activity of Rho GTPases [12]. RhoA controls the contractile actin and myosin filaments, Rac1 controls the protrusions of lamellipodia and Cdc42 controls the extensions of filopodia [13].

Mouse and human genetic studies suggest that several proteins involved in regulating the podocyte actin cytoskeleton are significant in stabilizing and maintaining an intact glomerular filtration barrier [14]. It has been shown that synaptopodin is vital for a stable podocyte actin cytoskeleton by blocking ubiquitination (the inactivation of a protein by the attachment of ubiquitin to it) of RhoA and consequently observing the induction of stress fibers [15]. When the podocyte synaptopodin gene was silenced the loss of stress fibers and the formation of filopodia was notable due to ubiquitination of RhoA [15]. Rho GDIα is known to be involved in the activation of Rho GTPases by shuttling them from the cytosol to the membrane where the Rho GTPases get activated by GEFs. Silencing of Rho GDIα in mice resulted in the predominance of active Rac1 signaling resulting in massive proteinuria [16].

Moreover, transient receptor potential canonical types 5 and 6 (TRPC5 and TRPC6) have been identified as regulators of cell migration in fibroblasts and podocytes [17]. Rac1 is activated by TRPC5-mediated Ca^{2+} influx and induces cell migration, while RhoA is activated by TRPC6-mediated Ca^{2+} influx and inhibits cell migration [17]. Furthermore, it has been shown that protamine sulfate (PS) perfusion of the kidney induces a concentration dependent increase in cytosolic Ca^{2+} activity in the podocyte causing podocyte FP effacement [18]. All these findings strengthen the importance of Rho GTPases as podocyte actin cytoskeleton regulators and emphasize the importance of actin regulatory mechanisms in the podocyte in renal pathogenesis.

β-PIX (p21-activated kinase (PAK)-interacting exchange factor β), also known as COOL1, K1AA0142, P50BP, P85, P85SPR, PAK3 and PixB (HUGO Gene Nomenclature Committee) [19], is a Rac1 and/or Cdc42 activating GEF encoded by the ARHGEF7 gene in chromosome 13 in humans, and the β_1-PIX isoform which is predominantly expressed in the kidney seems to be significant in renal pathology and physiology [20]. Western Blotting with antibodies against β-PIX have demonstrated the expression of β-PIX in several different kidney cells and immunohistochemistry has showed strong β-PIX staining in the glomeruli [21]. β-PIX and other PIX isoforms all have the diffuse B-cell lymphoma homology (DH) domain, which is required for their GEF activity on the Rho GTPases, the flanking Pleckstrin homology (PH) domain, which is required for binding to phosphatidylinositol lipids and proteins with heterotrimeric G-protein βγ-subunits [22], and a N-terminal SH3 domain in common [23]. The β_1-PIX isoform has a coiled-coil structure at the C-terminus that is involved in its peripheral cell localization and PIX dimerization, and thereby differs from the β_2-PIX isoform, which has a serine rich C-terminus instead [24].

Overexpression of β_1-PIX in HeLa cells promotes the formation of membrane ruffles and microvillus-like structures, an event that cannot be seen when overexpressing β_2-PIX [24].

Since Rac1 has been shown to be involved in renal pathogenesis by disturbing the filtration barrier and disrupting podocyte stress fibers we aimed to elucidate the role of the Rac1 activating protein β-PIX in podocyte stress fiber regulation. PS disrupts the glomerular filtration barrier by up regulating the Rho GTPase Rac1 resulting in podocyte stress fiber loss [25], a study that was extended here to investigate the role of β-PIX as a Rac1 GEF in PS induced podocyte stress fiber rearrangement. In vitro podocyte β-PIX knockdown, using β-PIX shRNA, prevented PS induced podocyte stress fiber loss. This indicates that β-PIX is involved in PS induced podocyte stress fiber loss, probably due to its Rac1 activating properties, which demonstrates the importance of β-PIX in podocyte stress fiber regulation and thereby strengthens the urge to evaluate β-PIX in renal pathogenesis in order to develop novel target treatment therapies.

AIM

In order to find novel treatment targets and develop new treatment therapies in CKD it is crucial to elucidate the mechanism behind renal pathogenesis. Since the stability of the podocyte actin cytoskeleton is critical for maintaining an intact glomerular filtration barrier it is important to study actin regulatory proteins as the Rho GTPases (RhoA, Rac1 and Cdc42). This study is based on the hypothesis that β-PIX, a Rac1 activating GEF, is involved in PS induced rearrangement of podocyte stress fibers. It has previously been shown that PS disrupts the glomerular filtration barrier by up

regulating the Rho GTPase Rac1 and resulting in podocyte stress fiber loss. Here we aimed to investigate the role of β-PIX in PS induced podocyte stress fiber rearrangement. By podocyte β-PIX knockdown and PS treatment the role of β-PIX in PS induced podocyte stress fiber loss could be investigated.

MATERIAL AND METHODS

Culturing of E.coli containing lentiviral plasmids

Expanding the ampicillin resistant lentiviral plko.1 vector in *E.coli* was performed using petri dishes with growth medium containing ampicillin, to select for plko.1 containing bacteria. Five plko.1 plasmids containing different shRNA sequences in *E.coli* obtained from SigmaAldrich were cultured to perform plasmid purification. 2 g Tryptone, 1 g Yeast, 2 g NaCl, 3 g Bactor-Agar and 200 ml H_2O_{MQ} was added to a 250 ml bottle, mixed on a magnetic blender and sterilized by autoclavation. After cool down to approximately 55°C, 200ul ampicillin (0.1g/ml) was added to the solution, which was then poured out carefully into petri dishes to cover the bottoms. They were stored in 4 °C after solidification. Five different tubes of *E.coli* stored in –80°C were plated on five different agar plates respectively using bacterial loops. The agar plates were incubated upside-down at 37°C over night. Colonies were noticed after 18h and the dishes were wrapped in parafilm to be stored upside-down in a cold room.

Plasmid DNA Isolation/ Plasmid purification

Plasmid *E.coli* DNA was purified from five different types of *E.coli,* which were cultured on Petri dishes, for upcoming lentiviral production. To purify plasmid DNA from *E.coli* cultures the QIAGEN Plasmid Maxi Prep Kit was used according to its

quick start protocol. *E.coli* were harvested by centrifugation at 4000 x g for 30 min at 4°C. Modifications to the protocol were performed by adding a piece of a kim wipe into the QIA filter cartridge to avoid clogging of the filter and DNA was concentrated by reducing the elution buffer (Buffer TE) to 700 ul of instead of 1 ml. After measuring the shRNA concentrations with the NanoDrop spectrophotometer, the tubes with the plasmids were stored in -20 °C.

Increase DNA plasmid concentrations

The desired DNA plasmid concentrations where not achieved by using the commercial bought β-PIX plko.1 bacterial stock. To increase the DNA concentrations 100ng of the β-PIX DNA that was obtained from purification of the commercial bacterial clones was retransformed into high efficiency NEB 5-α Competent *E.coli* (New England Biolabs Inc, Land) according to the 5 Minute Transformation Protocol. 1ul of each β-PIX DNA was transferred to eppendorf tubes containing the NEB 5-α Competent *E.coli* respectively and Heat Chock was performed by putting the tubes in a 42°C water bath for 30s. Thereafter they were put on ice for 2 min. Later on 1ml of SOC-medium was added to each tube and the retransformed bacteria was smeared out on Petri dishes buy transferring 25ul of each tube to Petri dishes and shaking them with pearls. They were incubated upside-down in 37°C over night.

Podocyte culture

Podocytes were cultured in Collagen Type I coated flasks and dishes for the infection experiments. To promote proliferation podocytes were cultured in 33°C in RPMI supplemented with 10% fetal bovine serum (FBS), 1% Penicillin/Streptomycin (P/S) and 30u of IFN-γ (100u/uL). The podocytes were observed in microscope every day

and their media was refreshed every other day until they were 80-95% confluent and ready for calculation and splitting. Podocytes were seeded at a density of cells/cm^2, IFN-γ was removed, and podocytes were thermo shifted to 37°C to induce differentiation by undergoing growth arrest. On the 11^{th} day after thermo shifting they were infected with β-PIX lentivirus to knockdown β-PIX.

Human Embryonic Kidney (HEK293T) - cell culture

HEK293T cells were cultured for DNA plasmid transfection and lentiviral production of β-PIX shRNAs and scrambled shRNA (Scr shRNA). HEK293T cells stored in -80°C were thawed up in a 37°C water bath and resolved in DMEM media supplemented with 10% FBS and 1% P/S and plated on a T75 flask for incubation in 37°C. The cells were observed in microscope every day and their media was refreshed every other day until they were 80-95% confluent and ready for splitting. After 2 passages they were ready for transfection with the shRNAs and helper plasmids to produce more lentivirus.

HEK-cell transfection for lentivirus production

HEK293T cells were used for transfection to produce lentivirus from the purified DNA plasmids. HEK293T cells in antibiotic free DMEM media (transfection-media) were transfected at 60-80% confluence in 10 cm dishes with pLKO.1 plasmid, pMDG DR8.91 (packaging plasmid) and pCMV-VSV-G (envelope plasmid) at a ratio 3:2:1 in OPTIMEM/Fugene). 18 hours after transfection transfection-media was substituted with regular DMEM supplemented with 10% FBS and 1% P/S. On the 3^{rd} day of transfection additional 10 ml regular media was added to the cells resulting in a total volume of media reaching 20 ml. On the 4^{th} day following transfection the HEK-cells

were filtered through 0.45 mm filter, batched up in 2.5-3 ml portions and stored in -80°C.

Podocyte infection

Podocytes were infected with β-PIX shRNA to knockdown β-PIX. Scr shRNA infection was used as control. Podocytes at passage 11 were infected in 10 cm plates 11 days after thermo shift. The media was changed to polybrene (4ug/ml) containing RPMI (4ml/plate) supplemented with 10% FBS and 1% P/S. shRNAs 1-5 and Scr shRNA stored in -80°C were dissolved quickly and 2 ml of each shRNA was added to the six 10 cm plates respectively. The plates were put in 37°C incubator. On the 2nd day of podocyte infection the infection media was changed to regular RPMI media supplemented with 10% FBS and 1% P/S and put in 37°C incubator. On the 4th day of infection the media was refreshed and the plates were put in 37°C incubator. Protein was harvested on the 5th day of infection.

Protein harvest

On the 5th day following podocyte infection protein harvest was performed for SDS-PAGE and Western Blot to evaluate the most efficient β-PIX knockdown lentivirus. Lysis buffer was prepared by adding 10ml Chaps buffer, one tablet Complete mini (Roche) and 100ul phosphatase inhibitor (SigmaAldrich) dissolved in 37°C water bath into a 15ml falcon tube and vortexing it. It was kept in an ice bucket with ice while the media in the six 10 cm plates with infected podocytes were vacuumed and washed with 1xPBS (8ml/plate) twice. 300ul Lysis buffer was added to each plate on ice. The plates were scrapped and the lysed podocytes were collected in six eppendorf tubes respectively, which were then put on ice for 10 min and afterwards centrifuged

in 4°C at maximum speed for 10 min. 60uL of the supernatant of each eppendorf tube was collected and added to new eppendorf tubes that were put in -20°C. Additional 300ul of the supernatant of each eppendorf tube was collected and added to new eppendorf tubes. 107ul Laemmli buffer and 21uL 2-mercaptoethanol (ME) was added to each eppendorf tube containing 300uL supernatant, which were then shaked in 95°C for 5 min and afterwards got a short spin in the centrifuge to later on be stored in -80°C.

Protein assay

To determine protein concentrations of podocyte lysates and calculate SDS-PAGE and Western Blot loading volume of each sample the PierceTM BCA Protein Assay Kit (Thermo SCIENTIFIC) was used. Diluted Albumin (BSA) Standards were prepared, according to the dilution scheme for Standard Test Tube and Microplate Procedure in the kit, and stored in -80°C. BCA Working Reagent (WR) was prepared and Microplate Procedure was done. The loading volume of each sample was calculated for the SDS-PAGE and Western Blot when the mean concentration results of the unknowns were received.

SDS-PAGE and Western Blot (WB)

To evaluate the most efficient β-PIX knockdown lentivirus SDS-PAGE and WB probing for β-PIX and GAPDH (as control) was performed according to the SDS-PAGE and Western Blot protocol. Gels were prepared for two WBs, one probing for β-PIX and one for GAPDH. Since the molecular weight of the β-PIX molecule is 81kDa 10% separating gels and 3% stacking gels were prepared. The WB lanes were loaded with the same amounts of protein. The SDS-PAGE and WB was run on

15mA/gel until an optimal separation could be achieved. The primary antibody for probing β-PIX (Cool1/betaPix Rabbit (Ref: 04/2014, 4515S, Cell signaling Technology)) was diluted in blocking solution (5% non-fat dry milk powder in TBST) 3:1000. The secondary antibody for probing β-PIX (Anti Rabbit IgG, HRP Conjugate, Ref: W401B, Promega) was diluted in blocking solution (5% non-fat dry milk powder in TBST) 1:1000. The primary antibody for probing GAPDH (Anti-GAPDH Mouse mAb, Cat# CB 1001 (Calbiochem)) was diluted in blocking solution (5% non-fat dry milk powder in TBST) 1:5000. The secondary antibody for probing GAPDH (Anti-Mouse IgG, HRP Conjugate, Ref: W402B, Promega) was diluted in blocking solution (5% non-fat dry milk powder in TBST) 1:10000. Enhanced chemiluminescence (ECL) mixture and enhancer was used to easily get bands on the films. After 7 min of film exposure bands could be seen on the film and the most efficient β-PIX knockdown lentivirus could be detected for future use in podocyte β-PIX knockdown.

β-PIX *knockdown and protamine sulfate (PS) treatment*

The most efficient β-PIX shRNA was used to knockdown β-PIX in podocytes that were going to be treated with PS afterwards to see if there would be stress fiber loss or not. Podocytes plated on five 3,5 cm plates at passage 11 were used for infection on the 7th day after thermo shift from 33°C to 37°C. Two plates were infected with Scr shRNA; two with the most efficient β-PIX shRNA and one was left untreated (WT) as control to see that the Scr shRNA was not toxic. Infection media was prepared for ten plates: 6ml RPMI supplemented with 10% FBS and 1% P/S and 3.2uL of polybrene was added to a 15mL falcon tube and vortexed. The media was changed to infection-media (600uL/plate). Scr shRNA and β-PIX shRNA stored in -80°C were thawed up. 200uL Scr shRNA was added to two plates respectively and

200uL β-PIX shRNA5 was added to two other plates respectively. All five plates were put in 37°C incubator. On the 2nd day of infection the infection media was changed to regular RPMI (2mL/plate) supplemented with 10% FBS and 1% P/S and put in 37°C incubator. PS-treatment is known to induce podocyte stress fiber loss and was therefore used here to see if it would induce stress fiber loss even in β-PIX knockdown podocytes. On the 4th day of infection, podocytes were treated with PS (600ug/ml) for 1 h. 0.2g PS was dissolved in 20ml $H2O_{MQ}$. The PS-solution was sterilized by filtration with a 10ml syringe twice. 600ug/ml of the PS-solution was added to one of the Scr shRNA infected plates and to one of the β-PIX shRNA infected plates respectively. They were put in 37°C for 60 min. The podocytes were checked every 15 min to make sure that they did not collapse.

Actin cytoskeleton assessment

To reveal the podocyte actin cytoskeleton, Rhodamine Phalloidin staining was used. The media in all five plates was vacuumed off and the plates were washed with 1xPBS (1ml/plate) twice. The podocytes were fixed with fixing solution (1ml/plate) for 10 min. The fixing solution was vacuumed off and each plate was washed with 1xPBS for three min three times. 0.3% Triton solution was applied to each plate, which were then put in a bigger plate and incubated in 4°C for 5 min. Meanwhile, Rhodamine Phalloidin was prepared in 1xPBS 1:700 on ice. The Triton solution was vacuumed and the plates were rinsed with 1xPBS (1ml/plate) once before they were washed with 1xPBS (1ml/plate) for three min three times. Afterwards, Rhodamine Phalloidin was applied to the five plates (1ml/plate), which were incubated in room temperature in a dark chamber with aluminum foil for 30 min. Later on, the five plates were rinsed with 1xPBS (1ml/plate) once and washed with 1xPBS (1ml/plate)

for three min three times each. In the end one drop of Mountig media was put on to the center of each plate and a circular cover glass was put on top of the drop (this was done one by one). The plates were put in a bigger plate covered with aluminum foil and stored in room temperature.

Fluorescence confocal microscopy

To analyze stress fiber-containing podocytes a Zeiss upright confocal microscope was used. Pictures of the podocytes in each five plates (10 pic/plate) were taken. The pictures were then used to calculate stress fiber containing cells. >50 cells were calculated in each plate. Podocytes with phalloidine positive lines that spanned continuously through the cell body were considered as stress fiber-containing podocytes.

DATA COLLECTION PROCEDURES

NanoDrop spectrophotometer was used to calculate the plasmid concentrations after plasmid purification.

To calculate the Western Blot loading volumes of the proteins that were harvested after podocyte infection with shRNAs the PierceTM BCA Protein Assay Kit (Thermo SCIENTIFIC) was used for protein concentration measurements. The weakest band on the WB was considered as the one with least β-PIX since the WB was probed for β-PIX. The β-PIX shRNA that was used to knockdown β-PIX in the plate with the podocytes from which those proteins were harvested was the most efficient β-PIX knockdown lentivirus. GAPDH was used as control.

10 pictures per plate of each five plates with as many cells as possible in every picture were taken with fluorescent confocal microscopy. All cells were calculated in a non-blinded fashion and the amount of stress fiber-containing cells in each plate was divided with the total amount of cells in each plate to get the proportions. The data was inserted in Excel to make a staple diagram showing the proportions of stress fiber containing cells in each five plates.

ETHICS

The experiments were performed in vitro without any human tissues.

RESULTS

β-PIX shRNA efficiency

First it was desired to investigate which one of the five β-PIX shRNAs that was the most efficient to reduce β-PIX levels to be able to determine which one to use in our experiments. Probing WB membranes using β-PIX and GAPDH antibodies showed that β-PIX shRNA #5 was the most efficient β-PIX knockdown lentivirus, thus it was determined that it would be used in podocyte β-PIX knockdown experiments (Figure 1.). The sequence of the most efficient β-PIX knockdown shRNA is as follows: CCGGGATACGGTGTATGCGTTAAAGCTCGAGCTTTAACGCATACACCGTA TCTTTTTG.

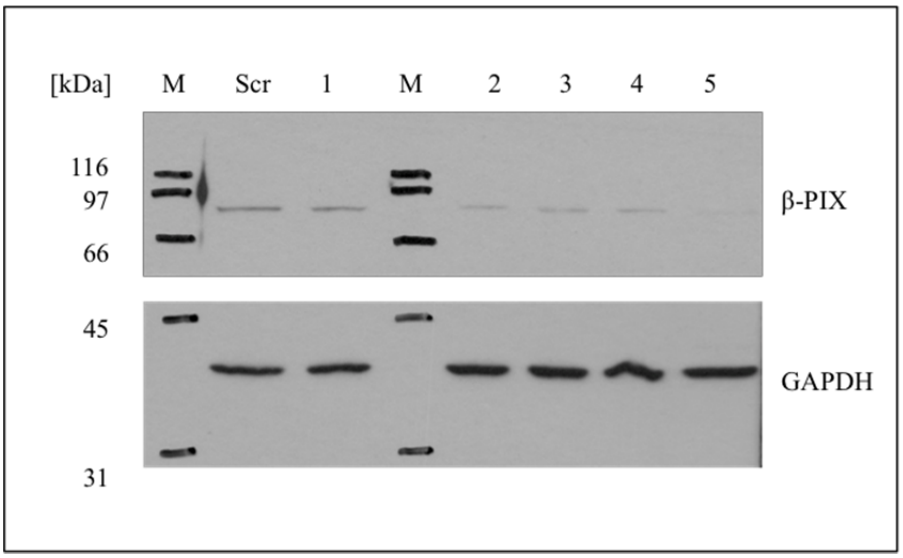

Figure 1. shRNA #5 was the most efficient β-PIX knockdown lentivirus
SDS-PAGE and Western Blot probed for β-PIX (81 kDa) showed that β-PIX shRNA #5 was the most efficient β-PIX knockdown lentivirus compared to control (Scr). GAPDH (37 kDa) served as a loading control.

β-PIX promotes PS-induced podocyte stress fiber loss

The Scr shRNA was not toxic to the podocytes (Figure 2.), which can be seen when comparing pictures of untreated podocytes (WT) and pictures of Scr shRNA infected podocytes. β-PIX knockdown podocyte stress fibers were thicker than other podocytes (Figure 3.). β-PIX knockdown podocytes were prevented from stress fiber loss when treated with PS compared to PS treated Scr shRNA infected podocytes (Figure 3.). The proportions of podocytes containing stress fibers continuously throughout the cell body were: WT 83.7%, Scr 81.7%, Scr + PS 10.8%, β-PIX 92.2% and β-PIX + PS 82.6%, telling us that β-PIX knockdown prevents podocyte stress fiber loss, thus β-PIX promotes PS-induced podocyte stress fiber loss (Figure 4.).

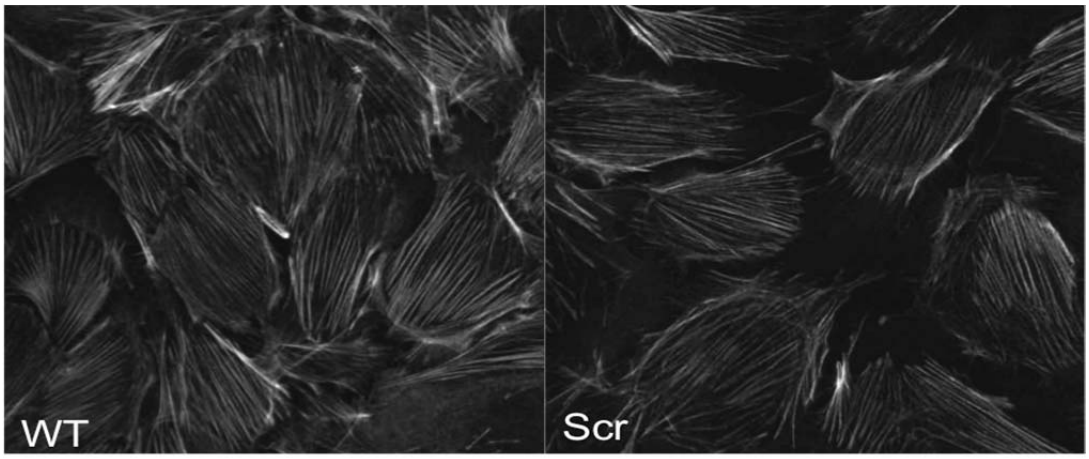

Figure 2. Scrambled shRNA (Scr) was not toxic to the differentiated podocytes
The plate with differentiated podocytes that were not infected with lentivirus or treated with PS served as control (WT) to the plate with differentiated podocytes that were infected with Scrambled shRNA (Scr), which did not loose stress fibers, demonstrating that the Scr shRNA was not toxic to the differentiated podocytes.

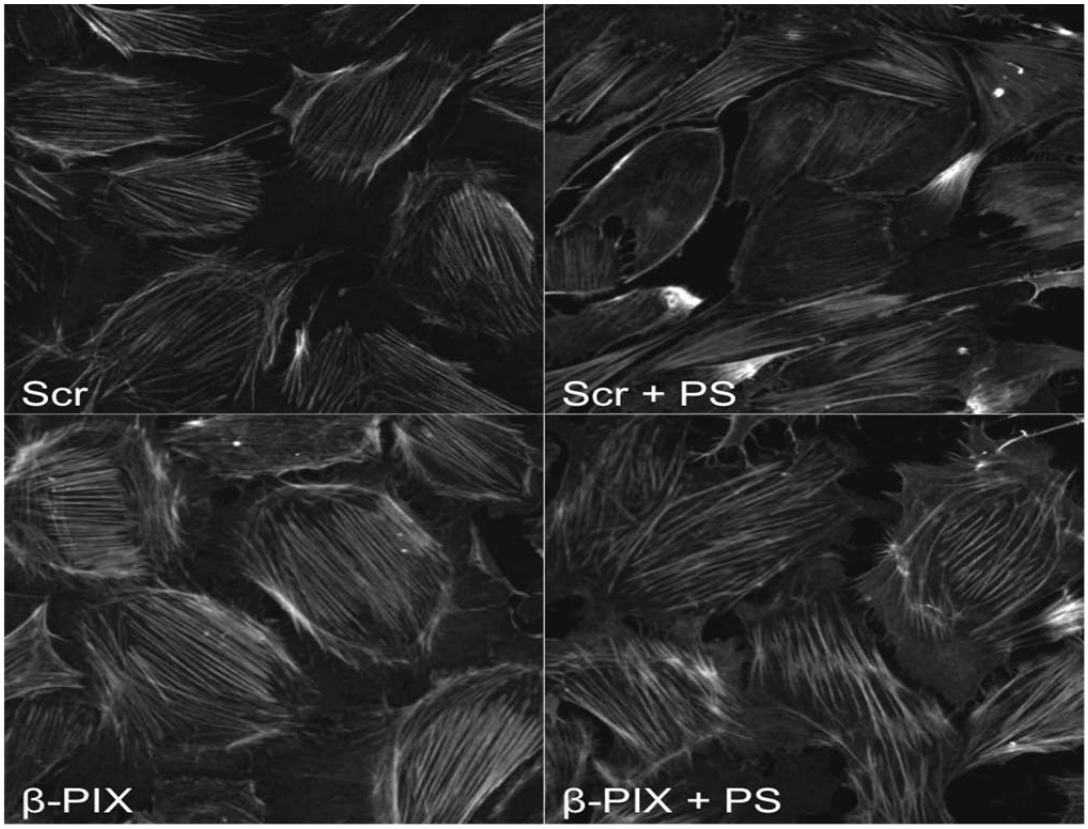

Figure 3. Differentiated β-PIX knockdown podocytes are protected from stress fiber loss when treated with PS
The plate that was infected with Scr shRNA and treated with PS (Scr + PS) contained differentiated podocytes with stress fiber loss, whereas there was no stress fiber loss in the plate that was infected with Scr shRNA (Scr), the plate that was infected with β-PIX shRNA and the plates that was infected with β-PIX shRNA and treated with PS (β-PIX + PS), demonstrating that differentiated β-PIX knockdown podocytes are protected from stress fiber loss when treated with PS.

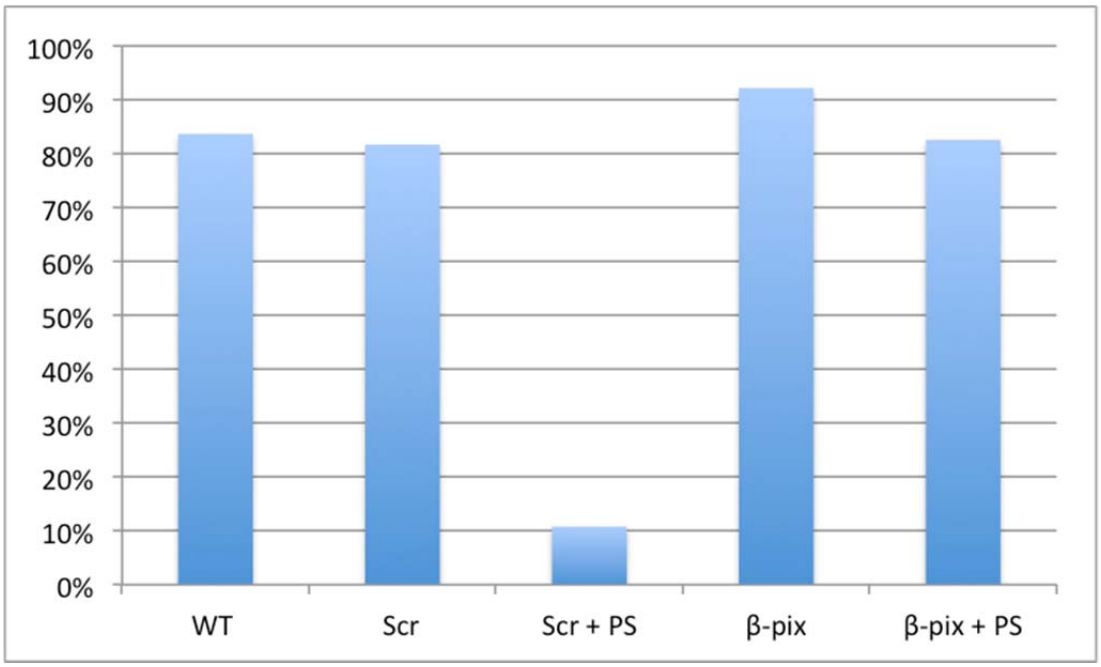

Figure 4. The proportion of stress fiber containing podocytes in each plate
Stress fiber containing podocytes were calculated in fluorescent confocal microscopy pictures (10/plate). Podocytes that contained stress fibers continuously through the cell body where counted as stress fiber containing podocytes, whereas the others where counted as podocytes that had lost their stress fibers. Proportions of stress fiber containing cells: WT 83.7%, Scr 81.7%, Scr + PS 10.8%, β-PIX 92.2% and β-PIX + PS 82.6%.

DISCUSSION

The findings of this study suggest that β-PIX knockdown prevents podocyte stress fiber loss when treated with PS, thus β-PIX promotes PS-induced podocyte stress fiber loss, probably due to its Rac1 activating properties. As mentioned, we extended the study where Schaldecker, T., et al showed that PS induces podocyte stress fiber loss by up regulating the Rho GTPase Rac1 which in turn results in disruption of the glomerular filtration barrier [25]. Thus the novel results in our study are complementary to the results of the previous study.

The Scr shRNA was not toxic to the podocytes (compared to the untreated ones), supporting the thought of PS as the stress fiber loss inducer in the plate with Scr shRNA infected podocytes, which is a methodological strength in this study. Also, the β-PIX shRNA that was considered to be the most efficient one in podocyte β-PIX knockdown is described to have a β-PIX knockdown efficiency of 80% by sigmaAldrich, which is consistent with the results from this study. The stress fiber containing podocyte quantifications were not done in a blinded fashion, which contributes to a weakness in the methodological design. Also, to be sure that the experiment results are valid and reliable it needs to be repeated at least twice.

If same results can be obtained when repeating the experiment twice and it can be concluded that β-PIX promotes PS-induced podocyte stress fiber loss, the next step would be to investigate in which step of the Rac1 activating signaling cellular pathway β-PIX acts and how. Recently a study showed the relation between the proto-oncogene Vav2 and β-PIX in cell spreading and migration; it seems like EGF stimulation leads to VaV2 regulation of PKL and β-PIX to focal adhesions [26]. Hence, it would be interesting to examine how VaV2 regulates β-PIX, if β-PIX can activate Rac1 independently of VaV2 or not and if there are other proteins that can activate Rac1 independently of β-PIX or not.

Since the β-PIX antibody was not specific to the β-PIX isoforms in this study, and overexpression of β_1-PIX but not β_2-PIX in HeLa cells has been shown to promote membrane ruffle and microvillus like structure formations previously, it would also be interesting to further investigate the role of these two isoforms in Rac1 activation [24].

CONCLUSIONS

Before any conclusions can be drawn the study has to be repeated at least twice and the stress fiber containing podocyte quantification has to be done in a blinded fashion. The data indicate that β-PIX promotes PS- induced podocyte stress fiber loss. If same results can be obtained when repeating the experiment twice it can be determined that β-PIX is important in podocyte stress fiber regulation, probably due to its Rac1 activating properties, which strengthens the urge to evaluate β-PIX in mechanisms behind renal pathogenesis. By investigating the cellular signaling pathways that activate Rac1 and thereby lead to disruption of the glomerular filtration barrier new treatment targets and thereby new treatment therapies in CKD can be developed.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Vid kronisk njursjukdom läcker viktiga proteiner i blodet ut i urinen när blodplasman filtreras i njurarna för att filtrationsbarriären är rubbad. Detta är ett globalt hälsoproblem med få behandlingsmöjligheter; dialys alternativt njurtransplantation, vilket orsakar låg livskvalitet hos patienter samt är mycket kostsamt för samhället.

För att skapa nya behandlingsmetoder bör man inrikta forskningen på att undersöka den komponent som är av störst vikt i filtrationsbarriären vid njurskada. Studier har visat att olika njursjukdomar som resulterar i läckage av essentiella proteiner i urinen har det gemensamt att podocyterna i filtrationsbarriären är skadade.

Podocyter är unika njurceller med utskott som utgår ifrån cellkroppen och omger blodkärlen i filtrationsbarriären. I cellkroppen finns det s.k. aktinfilamentet som utgör

själva cellskelettet. Tidigare studier antyder att aktinfilamentet har en viktig roll för podocytens form och stabilitet i dess läge. Aktinfilamentet kontrolleras av s.k. Rho GTPaser (RhoA, Rac1 och Cdc42) som är proteiner som uttrycks inuti cellerna.

β-PIX är ett protein med förmågan att aktivera Rho GTPasen Rac1 som kontrollerar lamellipodia utskott. Dessa utskott utgår från cellkroppen och innehåller förlängningar av aktinfilamentet. I en tidigare studie har man visat att protamin sulfat (PS) uppreglerar Rac1 så att podocyterna förlorar sina aktinfilament vilket leder till att filtrationsbarriären rubbas och protein läcker ut från blodet till urinen.

Genom att undersöka olika proteiners roll i regleringen av podocytens aktinfilament önskar man kunna komma närmare det nyckelprotein och eller/ den huvudmekanism som är viktig vid podocytskada, för att kunna utveckla nya behandlingsmetoder som är riktade mot dessa. I denna studie undersöktes hur PS påverkar podocytens aktinfilament vid avsaknad av β-PIX.

För att nedreglera β-PIX uttrycket i podocyterna producerades β-PIX kodande lentivirus som man infekterade podocyterna med. Därefter utsatte man podocyter med nedreglerade β-PIX och podocyter utan nedreglerade β-PIX för PS i ca 1 h. Sedan jämförde man deras aktinfilament med varandra genom att ta bilder med konfokal mikroskopi där man kunde urskilja aktinfilament som röda fibrer genom cellkroppen. Det man kunde se var att de podocyter som man nedreglerade β-PIX uttrycket hos hade kvar sina aktinfilament medan de som hade kvar β-PIX uttryck förlorade sina aktinfilament.

Denna studie visar att β-PIX är involverad i förlusten av aktinfilament orsakad av PS, troligen genom β-PIX Rac1 aktiverande förmåga. Alltså är β-PIX av stor vikt i regleringen av aktinfilament hos podocyter. Detta är en god anledning att utvärdera β-PIXs roll i utvecklingen av njursjukdom.

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FIGURES

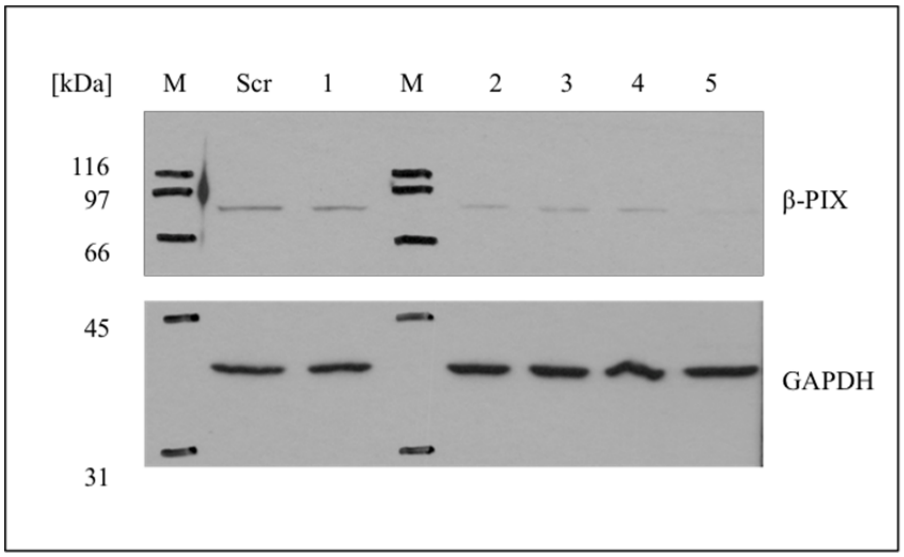

Figure 1. shRNA #5 was the most efficient β-PIX knockdown lentivirus
SDS-PAGE and Western Blot probed for β-PIX (81 kDa) showed that β-PIX shRNA #5 was the most efficient β-PIX knockdown lentivirus compared to control (Scr). GAPDH (37 kDa) served as a loading control.

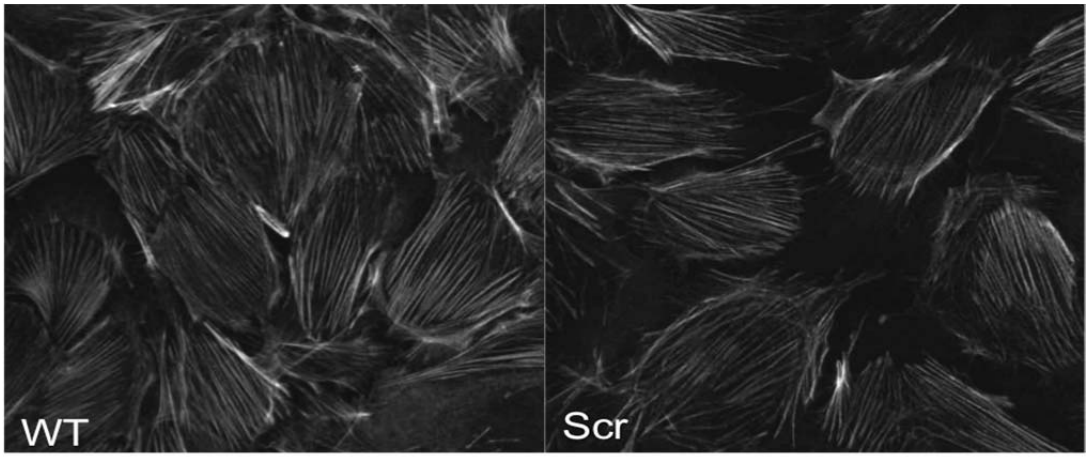

Figure 2. Scrambled shRNA (Scr) was not toxic to the differentiated podocytes
The plate with differentiated podocytes that were not infected with lentivirus or treated with PS served as control (WT) to the plate with differentiated podocytes that were infected with Scrambled shRNA (Scr), which did not loose stress fibers, demonstrating that the Scr shRNA was not toxic to the differentiated podocytes.

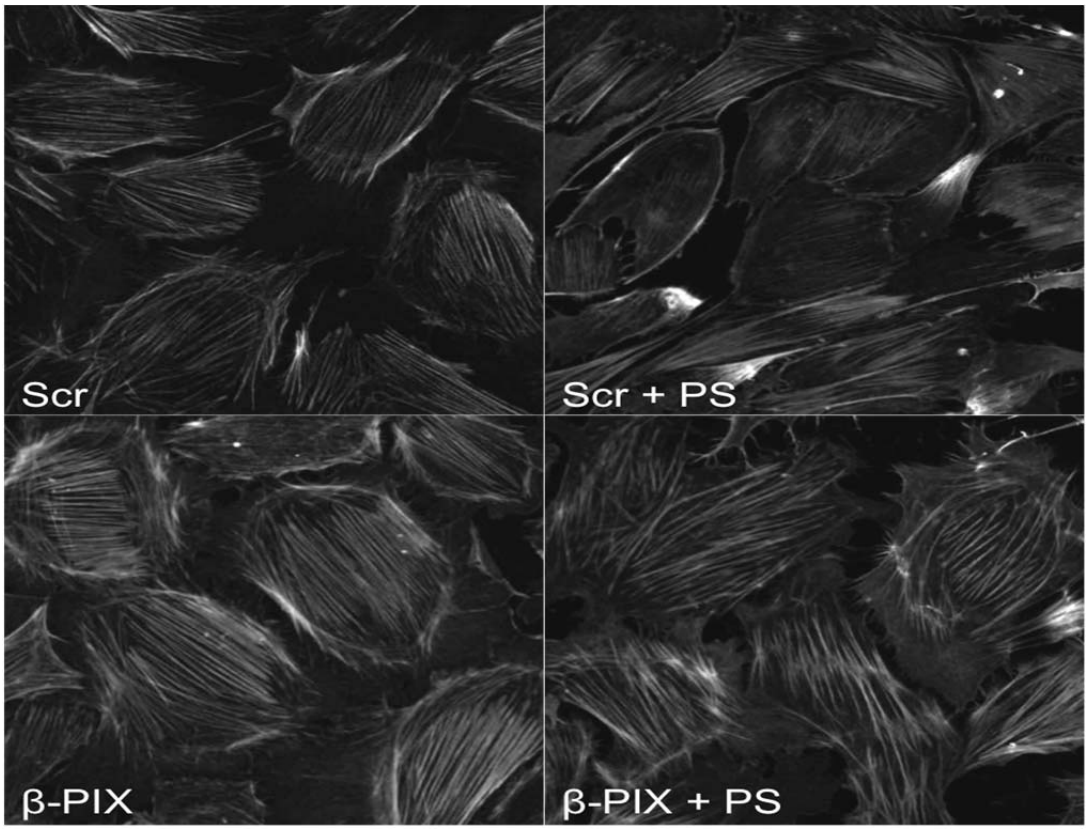

Figure 3. Differentiated β-PIX knockdown podocytes are protected from stress fiber loss when treated with PS
The plate that was infected with Scr shRNA and treated with PS (Scr + PS) contained differentiated podocytes with stress fiber loss, whereas there was no stress fiber loss in the plate that was infected with Scr shRNA (Scr), the plate that was infected with β-PIX shRNA and the plates that was infected with β-PIX shRNA and treated with PS (β-PIX + PS), demonstrating that differentiated β-PIX knockdown podocytes are protected from stress fiber loss when treated with PS.

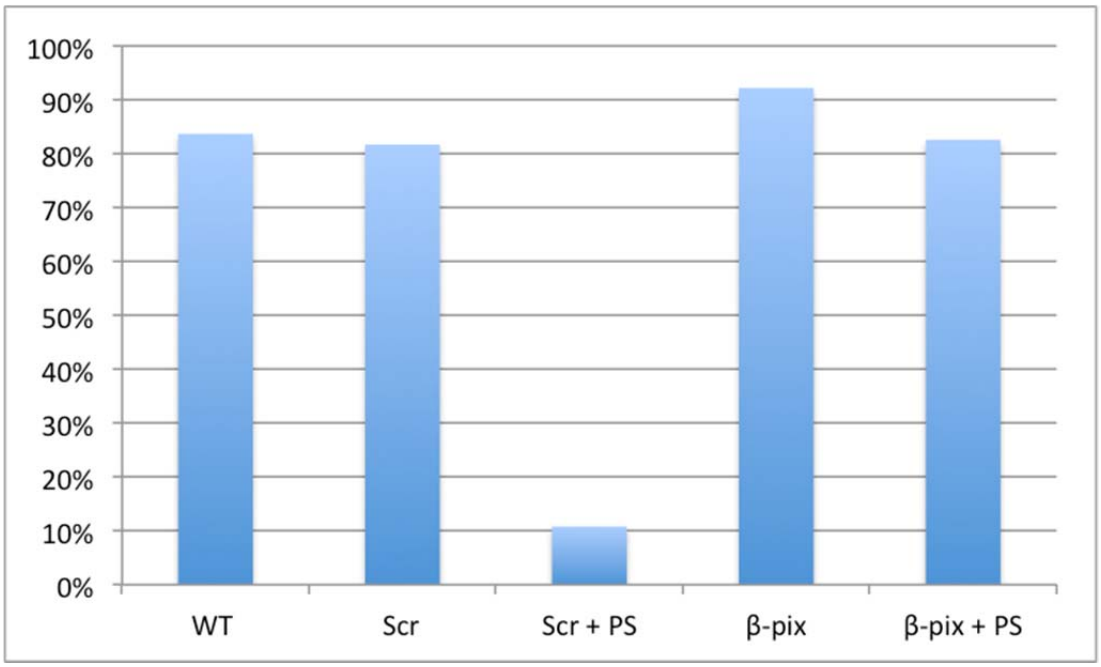

Figure 4. The proportion of stress fiber containing podocytes in each plate
Stress fiber containing podocytes were calculated in fluorescent confocal microscopy pictures (10/plate). Podocytes that contained stress fibers continuously through the cell body where counted as stress fiber containing podocytes, whereas the others where counted as podocytes that had lost their stress fibers. Proportions of stress fiber containing cells: WT 83.7%, Scr 81.7%, Scr + PS 10.8%, β-PIX 92.2% and β-PIX + PS 82.6%.