ShcA expression in podocytes is dispensable for glomerular development but its upregulation is associated with kidney disease

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Abstract: Background: ShcA (SHC1) is a phosphotyrosine adaptor protein which plays broad signaling roles within the cell. Systemic loss of ShcA during embryogenesis is lethal, while its aberrant expression contributes to disease. We recently demonstrated that ShcA is highly expressed during glomerular development and that it is upregulated within podocytes in experimental kidney injury and chronic kidney disease. The objective of this study was to analyze the *in vivo* role of ShcA in podocytes. Methods: We selectively deleted all three isoforms of ShcA from mouse kidney podocytes using the Cre/lox system driven by the podocyte-specific podocin promoter (Nphs2). Immunostaining of kidney sections was used to confirm ShcA deletion in podocytes. Coomassie blue staining of protein gels was used to detect urinary albumin. Light and electron microscopy were used to assess glomerular morphology. Transcript levels of SHC1 in human renal disease were assessed using the Nephroseq database. Results: Mice lacking podocyte ShcA were born at the expected Mendelian frequency and did not display overt renal impairment or changes in podocyte architecture beyond one year of age. In parallel, we correlated increased ShcA mRNA expression in the human kidney with proteinuria and reduced glomerular filtration rate. Conclusion: Our studies reveal that ShcA is dispensable for normal kidney function, but its upregulation is associated with disease.

Keywords: ShcA, glomerulus, podocyte, knockout mice, Nephroseq

Introduction

The integrity of the kidney’s glomerular filtration barrier relies on the coordination of its three layers: an inner fenestrated endothelium, an intervening glomerular basement membrane (GBM) and an outer layer of specialized actin-rich epithelial cells known as podocytes [1]. Between neighbouring podocytes lies the slit diaphragm, a unique cell-cell junction that acts as a filtration sieve. Nephrin contributes to the core of the slit diaphragm and acts as a signaling scaffold via several tyrosine-based motifs embedded in its short cytosolic tail [2]. Once phosphorylated by Src family kinases, Src homology (SH) 2 domain-containing adaptor proteins Nck1/2, p85/PI3K and ShcA can be recruited to nephrin, where they mediate a variety of signaling cascades that contribute to actin polymerization, cell survival and barrier turnover [2]. ShcA is the best-characterized member of the Shc family of adaptor proteins [3]. It consists of an N-terminal phosphotyrosine binding (PTB) domain and a C-terminal SH2 domain (Figure 1A), which flank a central collagen homology (CH) 1 region containing three phosphorylatable tyrosine residues and an α-adaptin binding motif, which enables ShcA’s trafficking function [4]. Splicing and alternative translation start sites produce the 66, 52 and 46 kDa isoforms of ShcA [5] (Figure 1A and 1B). The p46 and p52 isoforms are well established activators of the Ras/mitogen-activated protein kinase (MAPK) pathway via their recruitment of Grb2 to phosphorylated CH1 tyrosines [3]. The p66 isoform, which contains an extended CH2 region, is uniquely involved in sensing and mediating oxidative stress [6], and its role in the progression of multiple kidney diseases has been well defined [7]. Accordingly, global depletion of p66 ShcA is protective against some
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forms of experimental kidney disease [8-10]. However, the specific role of ShcA in podocytes has yet to be explored in vivo.

We recently characterized the interaction between ShcA and nephrin within podocytes, and identified a novel role for ShcA in promoting nephrin endocytosis [11]. Furthermore, we profiled the glomerular expression of ShcA, and determined that all ShcA isoform levels are higher during development than in the mature kidney, and that ShcA expression is elevated in podocytes in chronic kidney disease (CKD) [11]. Here, we have analyzed the in vivo requirement for ShcA in podocytes. As global knockout of all ShcA isoforms results in embryonic lethality prior to podocyte development [12], we used the Cre/lox system to generate podocyte-specific ShcA conditional knockout mice. We also probed an online transcriptomic database to correlate ShcA expression with several clinical indicators of human kidney disease.

Materials and methods

Generation of podocyte-specific ShcA conditional knockout mice

Shc1<sup>flx/flx</sup> mice [13] were crossed to mice expressing Cre recombinase under the control of the podocyte-specific podocin (Nphs2) promoter (Pod-Cre), which is expressed at embryonic day 14 [14]. The Shc1<sup>flx</sup> conditional allele contains loxP sites flanking exon 2, which harbors the start codons for p66, p52 and p46 ShcA, thereby allowing Cre-mediated excision of all 3 ShcA isoforms. Pod-Cre<sup>+</sup>, Shc1<sup>flx/flx</sup> mice were
subsequently crossed to the Shc1<sup>wt/Δex2/3</sup> line [12], wherein the Δ2/3 allele contains a deletion of the first two coding exons (exons 2 and 3), disrupting systemic expression of all ShcA isoforms. Mice were bred for several generations to produce Pod-Cre<sup>+</sup>, shc1<sup>fx/Δex2/3</sup> animals. Animals were sourced from existing colonies at Mount Sinai Hospital (Toronto, ON) and maintained on a mixed ICR/129 background under standard housing conditions with free access to food and water. Genotyping was performed by PCR on tail biopsies as described previously [13]. Mice were euthanized by CO<sub>2</sub> inhalation. For Pod-Cre<sup>+</sup>, Shc1<sup>fx/fx</sup> genotype, a total of 35 animals were obtained and for Pod-Cre<sup>+</sup>, shc1<sup>fx/Δex2/3</sup>, a total of 16 animals were obtained. Both male and female animals were analyzed. All procedures were carried out in accordance with guidelines established by the Canadian Council on Animal Care and approved by the Animal Care Committee of Mount Sinai Hospital (Toronto, Ontario, Canada).

**Evaluation of proteinuria**

Spot urine samples were passively collected from mice. Urine dipsticks (Chemstrip 5, Roche Diagnostics) were used to detect the presence or absence of protein. Alternately, 2 µL of urine was diluted in 2 × SDS loading buffer, separated by 10% SDS-PAGE and stained with Coomassie brilliant blue to assess urinary albuminuria.

**Histological and ultrastructural analyses**

Periodic Acid-Schiff (PAS) staining and electron microscopy (EM) of isolated kidneys were performed at the Advanced Bioimaging Centre at Mount Sinai Hospital, as described previously [15]. Immunohistochemical staining was performed using commercially available mouse anti-ShcA antibody (sc-967, Santa Cruz Biotechnology) with the Vector Stain ABC Elite kit and counterstained with nuclear fast red following manufacturer protocols.

**Nephroseq analysis**

Human ShcA expression data were downloaded from 4 independent datasets (Ju et al. [16], Sampson et al. [17], Reich et al. [18] and from the European Renal cDNA Bank (ERCB) cohort [19]) and analyzed using the Nephroseq data mining platform (www.nephroseq.org, 2017; University of Michigan, Ann Arbor, MI).

**Results**

**ShcA deletion in developing podocytes does not affect glomerular function or ultrastructure**

We and others have previously identified ShcA as a binding partner for nephrin [11, 20], thus we first examined the in vivo requirement for ShcA expression in podocytes. To generate podocyte-specific ShcA conditional knockout mice, we intercrossed Shc1<sup>fx/fx</sup> animals targeting all three ShcA isoforms (Figure 1A and 1B) [13] with those harbouring Cre under the control of the podocyte-specific podocin promoter (Nphs2) [14]. To ensure maximal loss of ShcA protein in the event of poor excision of the floxed allele, these mice were subsequently bred with the Shc1<sup>wt/Δex2/3</sup> line [12] to generate compound floxed/null animals. Pod-Cre<sup>+</sup>, shc1<sup>fx/fx</sup> and Pod-Cre<sup>+</sup>, shc1<sup>fx/Δex2/3</sup> animals showed comparable phenotypes, and are hereafter collectively referred to as ShcA Pod-cKO mice. Littermates with and without Nphs2-Cre served as controls (Figure 1C). Immunostaining of kidney sections from ShcA Pod-cKO and control mice using ShcA antibodies confirmed the absence of ShcA expression in podocytes of ShcA Pod-cKO animals (Figure 1D). To monitor glomerular function over time, spot urine samples were collected from mice monthly. The presence of protein in the urine was scored by dipstick analysis, and further analyzed using SDS-PAGE. No significant proteinuria was observed in ShcA Pod-cKO animals compared to littermate controls, even with aging beyond one year (Figure 2A). Urine samples from previously characterized Nck Pod-cKO mice [15], which were generated and housed in parallel, were included as positive controls. Histological analysis of glomerular morphology revealed no overt differences in ShcA Pod-cKO animals compared to controls (Figure 2B). Similarly, ultrastructural analysis showed that podocytes retained their regular arrangement of foot processes along the GBM in ShcA Pod-cKO mice, and the GBM was unchanged compared to controls (Figure 2C). Altogether, ShcA Pod-cKO mice appear to develop and age normally.

**Upregulation of ShcA is correlated with decreased GFR and proteinuria in humans**

We have recently shown that both ShcA transcript and protein levels are upregulated in human proteinuric kidney diseases [11], and here we sought to extend this analysis to
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explore the potential relationship between ShcA and clinical indicators of CKD. To this end, we used the Nephroseq gene expression database and analysis platform to compare ShcA expression with glomerular filtration rate (GFR) and proteinuria. We uncovered evidence that increased ShcA expression within the tubulointerstitium was strongly correlated with decreased GFR, both broadly within individuals identified to have CKD, and more specifically in subsets of patients with focal segmental glomerulosclerosis (FSGS), lupus nephritis (LN) and diabetic nephropathy (DN) (Table 1). Similar correlations between ShcA overexpression within the glomerulus and decreased GFR were observed, although as is the case in most of the datasets available, low patient numbers limited the statistical significance. In addition, we found that ShcA overexpression was correlated with increased proteinuria in both the glomerulus and tubulointerstitium in IgA nephropathy (IgAN), LN and minimal change disease (MCD) (Table 2). Taken together, elevated ShcA is positively associated with biomarkers of CKD.

Discussion

Here we demonstrate that loss of ShcA expression in podocytes does not lead to proteinuria or abnormal renal ultrastructure, indicating its non-essential role during development and throughout aging. By contrast, we show that upregulation of ShcA correlates with clinical markers of advanced kidney disease.

Figure 2. ShcA podocyte-specific conditional knockout (cKO) mice do not develop proteinuria or significant ultrastructural changes. A. Coomassie-stained SDS-PAGE gel of urine collected from 3 representative control and cKO animals at 8 months of age. Urinary albumin is absent in control and ShcA cKO mice, but present in Nck cKO reference animals. B. Light microscopy analysis of periodic acid Schiff (PAS)-stained sections of kidneys from ShcA control and cKO animals at 8 months of age demonstrates comparable glomerular morphology. Scale bar 10 µm. Magnification 40 x. C. Transmission electron microscopy (TEM) analysis of kidneys in control and cKO animals at 8 months of age shows normal podocyte foot process organization (arrows) and intact glomerular basement membrane. Scale bar 2 µm. Magnification 6,500 x.
Coupled with our previous findings, these results suggest that controlled low-level expression of ShcA is required to maintain glomerular function.

Podocyte structure and function are highly dependent on organization of the actin cytoskeleton [21]. ShcA plays a central role in actin dynamics, as ShcA null fibroblasts show defects in actin patterning and cell spreading [12] and it is a key component of adhesion complexes [22]. Moreover, previous studies have demonstrated requirements for ShcA in the development of many tissues including the cardiovascular [12, 23], neural [24], skeletal [13] and immune [25] systems. Similar systemic requirements exist for Nck1/2 phosphotyrosine adaptors [26-29]; however, their deletion within podocytes during development [15] or in adulthood [30] results in massive proteinuria and complete collapse of the podocyte actin cytoskeleton. Nck and ShcA both participate in nephrin signaling and endocytosis [2, 31] and they are recruited to 3 similar tyrosine-based motifs on nephrin’s cytoplasmic tail [11, 15] where they induce nephrin phosphorylation through modulation of Fyn kinase [11, 32]. Phosphorylation of these tyrosine residues is critical for maintenance of podocyte function, as we recently demonstrated using the nephrin-Y3F mouse model [33]. Given the prior implication of ShcA in actin organization and nephrin signaling, the lack of a phenotype in ShcA Pod-cKO mice is somewhat surprising. Nonetheless, it is in line with a previous report demonstrating that podocyte-specific deletion of Grb2, which functions immediately downstream of ShcA, is similarly viable and that Grb2 expression is not required to restore podocyte function after kidney injury [34]. Our findings instead suggest that the nephrin-Nck signaling axis may preferentially govern podocyte cytoarchitecture during development and in adulthood, when ShcA expression is low [11]. By contrast, aberrant ShcA upregulation during disease may perturb nephrin signaling.

**Table 1. Correlation of ShcA expression with glomerular filtration rate (GFR) in chronic kidney disease patients**

<table>
<thead>
<tr>
<th>Disease</th>
<th>Dataset</th>
<th>Tissue Type</th>
<th>R value</th>
<th>#Patients Measured</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Chronic Kidney Disease Samples</td>
<td>Ju CKD</td>
<td>Glom</td>
<td>-0.361</td>
<td>192</td>
<td>NA (low R)1</td>
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<tr>
<td></td>
<td></td>
<td>TubInt</td>
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<td>Glom</td>
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<td>0.088</td>
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<tr>
<td></td>
<td></td>
<td>TubInt</td>
<td>-0.722</td>
<td>17</td>
<td>0.002</td>
</tr>
<tr>
<td>Lupus Nephritis</td>
<td>ERCB Lupus</td>
<td>Glom</td>
<td>-0.568</td>
<td>9</td>
<td>0.111</td>
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<td></td>
<td></td>
<td>TubInt</td>
<td>-0.954</td>
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<td>8.75e-4</td>
</tr>
<tr>
<td>Diabetic Nephropathy</td>
<td>ERCB Nephrotic Syndrome</td>
<td>Glom</td>
<td>not assessed</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TubInt</td>
<td>-0.781</td>
<td>8</td>
<td>0.022</td>
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</tbody>
</table>

Only datasets with R values >0.5 are included. Modification of Diet in Renal Disease (MDRD) GFR values reported. Low GFR was correlated to a greater extent with heightened ShcA expression within the tubulointerstitium (TubInt) than within the glomerulus (Glom). *Datasets not analyzed.

**Table 2. Correlation of ShcA expression with proteinuria in kidney disease patients**

<table>
<thead>
<tr>
<th>Disease</th>
<th>Dataset</th>
<th>Tissue Type</th>
<th>R value</th>
<th>#Patients Measured</th>
<th>P value</th>
</tr>
</thead>
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<td>Reich IgAN</td>
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<td>Lupus Nephritis</td>
<td>ERCB Lupus</td>
<td>Glom</td>
<td>0.631</td>
<td>8</td>
<td>0.093</td>
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<td>TubInt</td>
<td>0.671</td>
<td>7</td>
<td>0.099</td>
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<td>Minimal Change Disease</td>
<td>Sampson Nephrotic Syndrome</td>
<td>Glom</td>
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<td>7</td>
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<td>11</td>
<td>NA (low R)1</td>
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</table>

Only datasets with R values >0.5 were included. Enhanced proteinuria was correlated with heightened ShcA expression within both the tubulointerstitium (TubInt) and the glomerulus (Glom). *Datasets not analyzed.
and promote its endocytosis, ultimately disrupting the integrity of the glomerular filtration barrier. ShcA is thus aligned with other negative regulators of podocyte function, such as Crk1/2/L, which is likewise recruited to the slit diaphragm downstream of nephrin tyrosine phosphorylation, and has been shown to engage in detrimental signaling pathways during disease [35-37].

While loss of ShcA is dispensable for establishment and maintenance of the glomerular filtration barrier, gain of ShcA expression is associated with podocyte injury. Similar to our observations in MCD and FSGS [11], enhanced ShcA protein staining has been reported in both the glomerulus and tubulointerstitium of IgAN patients [38]. ShcA expression has also been correlated with disease severity in patients with LN, with a greater percentage of stage III and IV patients showing increased ShcA staining within podocytes than in healthy controls [39]. The Nephroseq database supports these reports, with our analysis suggesting that upregulation of ShcA in FSGS, LN, DN and, more broadly, CKD patients correlates with decreased GFR, a marker of renal function. Further, proteinuria was elevated in IgAN, LN and DN patients with high levels of ShcA, collectively identifying ShcA expression as a potential risk factor in many forms of CKD. Future studies are required, however, to clarify the contributions of glomerular and tubular ShcA in disease pathogenesis, and to determine the specific contributions of the p66 and/or p46/52 ShcA isoforms in these mechanisms [40].

Limitations and future prospects

It should be noted that a prior line of ShcA Pod-cKO mice was established using Cre recombinase under control of the nephrin (Nphs1) promoter [41]. Early generations of these mice developed proteinuria by 2 months of age, as well as alterations in podocyte ultrastructure (N. Jones, unpublished observations). However, this phenotype did not persist in later generations. As we were unable to reproduce this finding with Nphs2-Cre, which was bred in parallel to produce Nck Pod-cKO mice with a consistent and highly penetrant phenotype [15], we hypothesize that our earlier results were due to an unexpected toxicity of Nphs1-Cre. We also acknowledge that, as this breeding colony is no longer being maintained, it is not possible to induce injury in ShcA Pod-cKO mice, as has been described in other podocyte-specific knockout models with no basal phenotype [34, 35, 37, 42, 43], or to undertake detailed molecular characterization of podocytes isolated from these mice. Despite this limitation, given the repeated findings that ShcA is upregulated in CKD patients, it may be of more relevance in the future to assess pathogenesis in a mouse model of ShcA overexpression.

For Nephroseq data, we reported only results that reached our significance threshold with R-values greater than 0.5. Several other datasets within the Nephroseq collection did not show significant correlation between ShcA expression and either protection or susceptibility to disease phenotypes, contributed in part by the low number of patient samples in datasets. ShcA’s categorization as a kidney disease risk factor is expected to continue being refined as additional datasets are added to the Nephroseq collection.

In conclusion, our results demonstrate that the ShcA adaptor protein is dispensable for normal kidney function, but its upregulation is associated with disease. Future studies will be needed to assess whether ShcA can serve as a biomarker of podocyte injury.

Acknowledgements

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Disclosure of conflict of interest

None.

Abbreviations

CKD, Chronic kidney disease; cKO, Conditional knockout; DN, Diabetic nephropathy; FSGS, Focal segmental glomerulosclerosis; GBM, Glo-
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merular basement membrane; GFR, Glomerular filtration rate; IgAN, IgA nephropathy; LN, lupus nephritis; MCD, minimal change disease.

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