Glomerular Diseases

Review Article

Glomerular Dis 2021;1:265–276
DOI: 10.1159/000518404

Received: April 23, 2021
Accepted: July 8, 2021
Published online: July 14, 2021

Deconvolution of Focal Segmental Glomerulosclerosis Pathophysiology Using Transcriptomics Techniques

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Keywords

Transcriptomics · Single-cell transcriptomics · Focal segmental glomerulosclerosis · Focal segmental glomerulosclerosis · Biomarker

Abstract

Background: Focal segmental glomerulosclerosis is a histopathological pattern of renal injury and comprises a heterogeneous group of clinical conditions with different pathophysiology, clinical course, prognosis, and treatment. Nevertheless, subtype differentiation in clinical practice often remains challenging, and we currently lack reliable diagnostic, prognostic, and therapeutic biomarkers. The advent of new transcriptomics techniques in kidney research poses great potential in the identification of gene expression biomarkers that can be applied in clinical practice. ***Summary:*** Transcriptomics techniques have been completely revolutionized in the last 2 decades, with the evolution from low-throughput reverse-transcription polymerase chain reaction and in situ hybridization techniques to microarrays and next-generation sequencing techniques, including RNA-sequencing and single-cell transcriptomics. The integration of human gene expression profiles with functional in vitro and in vivo experiments provides a deeper mechanistic insight into the candidate genes, which enable the development of novel-targeted therapies. The correlation of gene expression profiles with clinical outcomes of large patient cohorts allows for the development of clinically applicable biomarkers that can aid in diagnosis and predict prognosis and therapy response. Finally, the integration of transcriptomics with other "omics" modalities creates a holistic view on disease pathophysiology. ***Key Messages:*** New transcriptomics techniques allow high-throughput gene expression profiling of patients with focal segmental glomerulosclerosis (FSGS). The integration with clinical outcomes and fundamental mechanistic studies enables the discovery of new clinically useful biomarkers that will finally improve the clinical outcome of patients with FSGS.

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Introduction

Focal segmental glomerulosclerosis (FSGS) is a renal histopathological pattern of injury defined by the presence of segmental sclerosis in some glomeruli (i.e., "focal") on light microscopy, and is therefore not a speci-

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fied disease entity [1]. FSGS encompasses a notoriously heterogeneous group of clinical conditions, all characterized by podocyte damage and subsequent glomerular loss as the central pathophysiological event. This clinically results in proteinuria, nephrotic syndrome, and progressive kidney function loss [1]. FSGS can be subdivided in primary, secondary, and genetic forms with different outcomes and therapeutic options [1]. Primary FSGS is caused by an unidentified circulating permeability factor that causes sudden and generalized injury to podocytes, the visceral glomerular epithelial cells, and is typically treated with immunosuppressive therapy or plasmapheresis [1]. Primary FSGS is a severe disease with a grim prognosis: about 20–40% of patients will likely develop end-stage kidney disease (ESKD) after a mean follow-up period of 3–6 years [2, 3]. Patients that receive a kidney transplant have a high risk of disease recurrence (20–40%) which can already occur a few days after transplantation [4]. Secondary FSGS results from glomerular hypertension, in which glomeruli are subjected to abnormal stress ("maladaptive" FSGS) [1]; the treatment of the maladaptive form is centered around unloading the pressure on the glomeruli with renin-angiotensin system blockade [5]. In comparison with primary FSGS, prognosis is considerably better with fewer patients progressing to ESKD [1, 3]. The genetic forms of FSGS result from mutations in the genes that encode for podocyte proteins (e.g., nephrin, podocin, CD2-associated protein, and α-actinin-4) and are typically resistant to therapy [1]. Although FSGS remains a rare disease with current incidence rates in the USA, of 3.2 cases per 100,000 person-years, this rate has doubled over the past 10 years [5].

Despite the heterogeneous etiology and prognosis of the different FSGS subtypes, the first clinical and histopathological presentation may be very similar [1]. Moreover, to date, there are no validated serums, urine, or histopathological biomarkers that reliably discriminate primary from secondary and genetic forms. It is therefore often challenging to identify the correct FSGS subtype in daily clinical practice [1]. Importantly, a misclassification will lead to inappropriate and therefore ineffective and potentially harmful therapy. We lack prognostic biomarkers, despite the fact that some patients rapidly progress to ESKD, while others remain relatively stable on antiproteinuric therapy. Next, to date, no markers reliably predict whether the patient will benefit from immunosuppressive treatments, such as corticosteroids. And finally, we currently lack targeted therapies to treat FSGS patients because the underlying pathophysiological mechanisms are incompletely understood.

Recently, "omics" technologies have revolutionized kidney research, in which the whole genome, epigenome, transcriptome, and proteome of disease entities are studied, instead of only a few candidate disease targets [6, 7]. Transcriptomics techniques can detect differential gene expression in disease versus healthy states which translates in altered protein expression. Transcriptomics techniques have undergone a true revolution from low-throughput reverse transcription polymerase chain reaction (RT-PCR) and in situ hybridization (ISH) techniques that detect only a few expressed genes to the current extremely high-throughput and performant single-cell and single-nucleus RNA-sequencing techniques, which can be validated with multiplex assays or used in a multimodal approach which simultaneously analyses other "omics" from the same single cells [7]. Therefore, analysis of the transcriptome of patients with different subtypes of FSGS poses great potential to identify the underlying pathophysiology and candidate biomarkers that can ultimately improve clinical care.

This review provides an introduction in transcriptomics analysis of disease models and human patients with FSGS. Next, we describe how fundamental and clinical approaches can translate preliminary gene expression profiles into candidate tissue, urine, or blood biomarkers. The ideal biomarker correlates with clinical outcomes so that it can be used in clinical diagnosis, prognosis, and prediction of treatment susceptibility, while a fundamental insight of the underlying disease pathway will enable the development of novel targeted treatments.

Elucidating Pathophysiology through Gene Expression Analysis

The transcriptome refers to the type and quantity of all RNA-based molecules that are present in the cell in a (patho-) physiological condition [8]. These RNA molecules consist of protein-coding messenger RNA (mRNA), and non-protein-coding RNA, including microRNA, ribosomal RNA, and transfer RNA [9]. Most human cells express ~11,000–13,000 genes, of which approximately ~8,000 genes are ubiquitously expressed among different tissues and ~3,000–5,000 expressed genes exhibit a cell type-specific pattern [10, 11]. This differential gene expression results in a different phenotype, representing distinct cell populations with their own behavior both in healthy and disease states. To un-

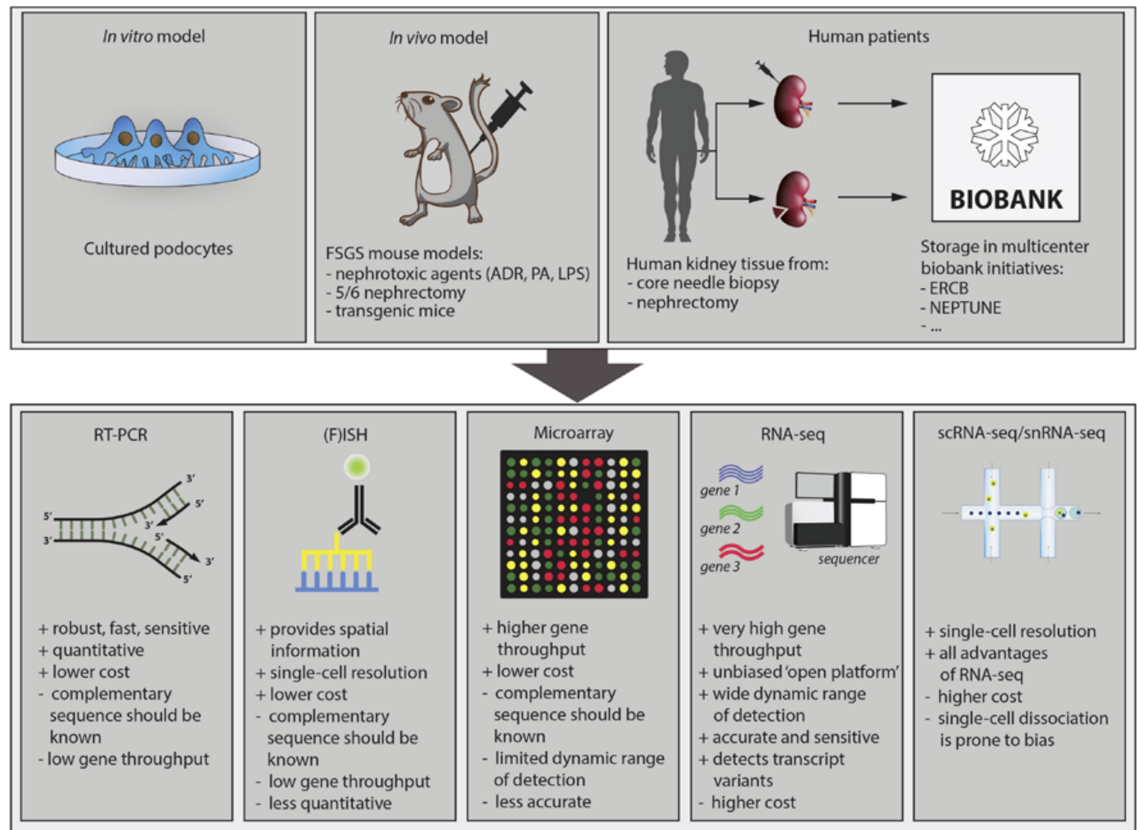

Fig. 1. Overview of transcriptomics analysis of disease models and human patients with FSGS. ADR, adriamycin; PA, puromycin aminonucleoside; LPS, lipopolysaccharide; ERCB, The Consortium of European Renal cDNA Bank; NEPTUNE, Nephrotic Syndrome Study Network; RT-PCR, reverse transcription polymerase chain reaction; (F)ISH, (fluorescence) in situ hybridization; scRNA-seq, single-cell RNA-sequencing; snRNA-seq, single-nucleus RNA-sequencing; (+), advantage; (–), disadvantage; FSGS, focal segmental glomerulosclerosis.

derstand cell responses in different diseases, gene expression (transcriptome), and protein expression (proteome) can both be studied [12]. Although both fields have been completely revolutionized in the past decade with transcriptome and proteome studies at the single-cell level [7], this review focuses on the transcriptome as a tool to decipher the pathophysiology of kidney diseases and more specifically FSGS. Although the potential of (single-cell) transcriptomics in the analysis of kidney diseases is already established [6, 13–15], the study of glomerular diseases is still lagging behind, with most gene expression data still originating from older and lower throughput techniques. This is certainly true for FSGS, as this is a rare glomerulopathy, which makes the acquisition and analysis of sufficient tissue material and rigorous validation of candidate transcriptional biomarkers quite challenging.

In vitro and in vivo Models of FSGS

As the acquisition of kidney tissue from patients with FSGS is difficult, many research groups have used experimental in vitro and in vivo models to study the disease (Fig. 1). Several functional studies have used cultured human podocytes to study podocyte injury and cytoskeleton morphology in vitro, by modifying candidate genes implicated in cytoskeleton regulation [16–18], glomerular fibrosis [19], podocyte apoptosis [20], or by exposure to nephrotoxic agents (e.g., doxorubicin) [16]. However, the transcriptional profile and protein expression of in vitro podocytes differ substantially from podocytes in their native glomerular micro-environment, with loss of lineage-specific gene expression and phenotype, which limits the translation into human clinical practice [21, 22]. Alternatively, various in vivo FSGS animal models have been

used, created by the use of nephrotoxic agents, (partial) nephrectomy, or genetic engineering (for a comprehensive overview, see Yang et al. [23]). In mice or rats, glomerular damage and FSGS-lesions can be induced by using toxic podocyte-damaging agents, including doxorubicin/Adriamycin [16, 18, 24–29], puromycin aminonucleoside [18], and lipopolysaccharide [27, 29]. Resection of 5/6 of functional renal mass by nephrectomy (5/6 nephrectomy model) simulates human secondary FSGS due to a reduction in the number of functioning nephrons [23]. Genetic engineering of FSGS models include knockout mice for genes such as podocin (NPHS2) [30], COL4A3 (which is also associated with Alport syndrome) [31], C2DAP [32], and knock in of ACTN4 mutations [33, 34] or TRPC6 mutations [35], which are all associated with familial or sporadic genetic FSGS in humans [1]. Additionally, new candidate target genes (e.g., *SRGAP1* [16]and *NEBL* [18]) can be genetically altered in in vivo models to validate the role of these genes in disease pathophysiology [16–18, 25].

The Challenge of Obtaining Human Tissue of FSGS Patients

Candidate transcriptional disease targets or biomarkers identified in animal studies should be validated in human kidney tissue before clinical relevance can be claimed. In general, human kidney tissue for research is only available as 2 types of biopsy specimens: core needle biopsies and nephrectomy tissue (Fig. 1). While core needle biopsies are frequently performed in clinical practice, they yield little tissue material. More tissue can be acquired through partial or complete nephrectomy, but this is seldomly performed in patients with glomerular disease as the main indication is a renal mass. As a consequence, tissue from patients with FSGS is generally only available as core needle biopsies. While the identification and validation of disease targets or transcriptional tissue biomarkers require many samples, this is generally not feasible in a single-center setting. To combat this shortcoming, many different multicenter initiatives and biobanks have been initiated. The Consortium of European Renal cDNA Bank (ERCB) – Kroener-Fresenius Biopsy Bank was founded in the early year 2000 and is a collaboration of European research centers that collects kidney biopsy tissue for gene expression analysis in a predefined preservation and processing protocol [36], together with clinical information [6]. A second initiative, the Nephrotic Syndrome Study Network (NEPTUNE), is a multicenter collaborative consortium which unites research centers across the USA and Canada to focus on translational research in patients with minimal change disease, FSGS, and membranous nephropathy [37]. NEPTUNE has been running a prospective observational trial since 2010, which includes a biopsy cohort and a pediatric nonbiopsy cohort and collects clinical data together with biological samples, including an additional research biopsy core during clinically indicated kidney biopsy in participants of the biopsy cohort [37]. The biopsy cohort has currently included >500 patients [38]. A more recent initiative, cure glomerulonephritis (CureGN), is also a multicenter initiative and prospective observational trial which aims to collect clinicopathological data from a total of 2,400 patients with biopsy-proven minimal change disease, FSGS, membranous nephropathy, or IgA nephropathy (including IgA vasculitis) across research centers in the USA, Canada, and Europe since 2014 [38]. Contrary to the biological samples obtained in the NEPTUNE trial, this study only collects blood and urine samples and no additional kidney biopsy tissue. CureGN is designed to include new patients, but also to complement and extend follow-up of NEPTUNE participants, which will allow better correlation of their molecular profile with long-term clinical outcome data [38]. The cohort identified in CureGN will also be used for validation studies of identified candidate blood and/or urine biomarkers [38]. Many other smaller registries exist, which can also function as independent validation cohorts [38]. In conclusion, these large registries allow the compilation of sufficient patients with FSGS to study their molecular transcriptomic profile in kidney tissue, blood, and urine, and allow correlation with histopathological and long-term clinical outcome data to identify and validate new biomarkers that can be applied in clinical practice.

How to Study the Transcriptional Profile of Human FSGS Patients

The aforementioned multicenter initiatives are very promising to the research field of transcriptomic profiling in FSGS. Indeed, several studies have already used samples from FSGS patients derived from ERCB [22, 39–44] or NEPTUNE [41, 42, 44, 45]. Most of them used microarrays, although recently next-generation sequencing techniques, such as bulk RNA-seq have also been used [44, 46]. However, the first single-cell transcriptomics study on kidney tissue of FSGS patients is yet to be published. Published transcriptomics datasets of kidney dis-

ease, including FSGS are also easily accessible online, at different repositories or through the search engine and data mining tool "Nephroseq" (available at www.nephroseq.org, formerly known as "Nephromine" [47]). These datasets can be used to validate a candidate transcriptional biomarker or signature that was identified in in vivo or in vitro experiments [16, 18, 20, 31, 48–50] or in an independent human FSGS cohort. Vice versa, these datasets can first be "mined" for potential disease targets or biomarkers, which could subsequently be studied in in vivo or in vitro models (typically FSGS mouse or rat models or cultured podocytes) or validated with transcriptomics or proteomics (e.g., immunohistochemistry) in independent cohorts to elucidate their role in disease pathophysiology [25]. Here, we systematically outline the major findings of the transcriptomics studies that have analyzed the gene expression profile of the kidney tissue in human patients with FSGS (Fig. 1; see online suppl. Tables 1, 2; see www.karger.com/doi/10.1159/000518404 for all online suppl. material).

Studies Using RT-PCR and ISH

The earliest techniques to study gene expression include RT-PCR and in situ hybridization. In these low-throughput techniques, only a few target genes can be studied, and oligonucleotide sequences of the targeted transcripts should be known in order to choose the correct PCR primers or hybridization probes, respectively. Indeed, the first studies that analyzed gene expression in human kidney biopsies of FSGS patients with these techniques studied only a few candidate genes (online suppl. Table 1). Studies were done on snap-frozen or formalin-fixed paraffin-embedded (FFPE) tissue and mostly involved analysis of unprocessed biopsy cores, while some used microdissected glomeruli [51–54]. Upregulated genes in FSGS patients were primarily involved in inflammation (e.g., leukotriene metabolism [55], leukocyte infiltration [56, 57], upregulation of nuclear factor-κB [58], cytotoxic T-cell mediators [59]), and cell proliferation (e.g., upregulation of proto-oncogens [60]) or fibrosis (e.g., genes involved in the TGFβ-pathway [59, 61]). Downregulated genes were primarily involved in the podocyte slit diaphragms (e.g., nephrin [51, 54] and podocalyxin [54]). These studies identified important pathways involved in FSGS pathophysiology that were the focus of later research (e.g., TGFβ-pathway, apoptosis, and gene regulation of podocyte-specific proteins). Later transcriptomics studies have used more high-throughput methods (microarray and RNA-seq), but still utilize RT-PCR and ISH to validate the expression of candidate marker genes or use ISH to spatially localize gene expression.

Studies Using Microarrays

Similar to PCR and ISH, microarrays also depend upon existing knowledge of the oligonucleotide sequence of the transcripts of interest [8]. In this technology, fluorescently labeled cDNA is created from RNA from the sample and is subsequently hybridized to thousands of gene-specific complementary oligonucleotide probe-sets that are aligned on a high-density microarray [6, 8, 62]. When compared to RT-PCR and ISH, microarrays have a much higher throughput, as they simultaneously detect thousands of genes and enable the identification of gene expression profiles or "signatures" [62]. As freezing of tissue is a conventional way of RNA preservation, either snap-frozen (stored at −80°C) or tissue conserved in RNase inhibitor (stored at −20°C, implemented in the protocol of ERCB [36]) was used in microarray studies [6]. One study compared microdissected glomeruli from FFPE kidney tissue to frozen tissue. Despite reduced RNA quality in FFPE tissue, gene expression profiles of both sample types were highly correlated, making FFPE tissue a reasonable alternative to frozen tissue [63]. The first microarray studies were exploratory and identified a general FSGS tissue fingerprint of many differentially expressed genes (online suppl. Table 2) [63–65]. These differentially expressed genes were primarily involved in TGF-β signaling and fibrosis [63–65], apoptosis [64], and podocyte-specific genes involved in the slit diaphragms and possibly foot process effacement [63–65]. With this approach, it is challenging to elucidate the pathophysiological role or specificity of certain significantly up- or downregulated genes. Therefore, later studies were more hypothesis-driven, implementing transcriptomics in a few different approaches.

In a first approach, candidate genes or gene signatures from a large transcriptomics dataset are explored in depth, with correlation in animal models and in vitro experiments to gain fundamental mechanistic insight into the underlying pathophysiology (Fig. 2) [25, 39, 40, 43, 66, 67]. For example, 2 research groups found upregulation of genes involved in the mammalian target of rapamycin complex 1 (mTORC1) signaling pathway and parietal epithelial cell activation in kidney tissue of FSGS patients, which is associated with podocyte hypertrophy and glomerulosclerosis [43, 68]. Both groups created a transgenic mouse model with mTORC1 hyperactivation in podocytes, which resulted in excessive podocyte hypertrophy, podocyte loss, and development of FSGS-lesions

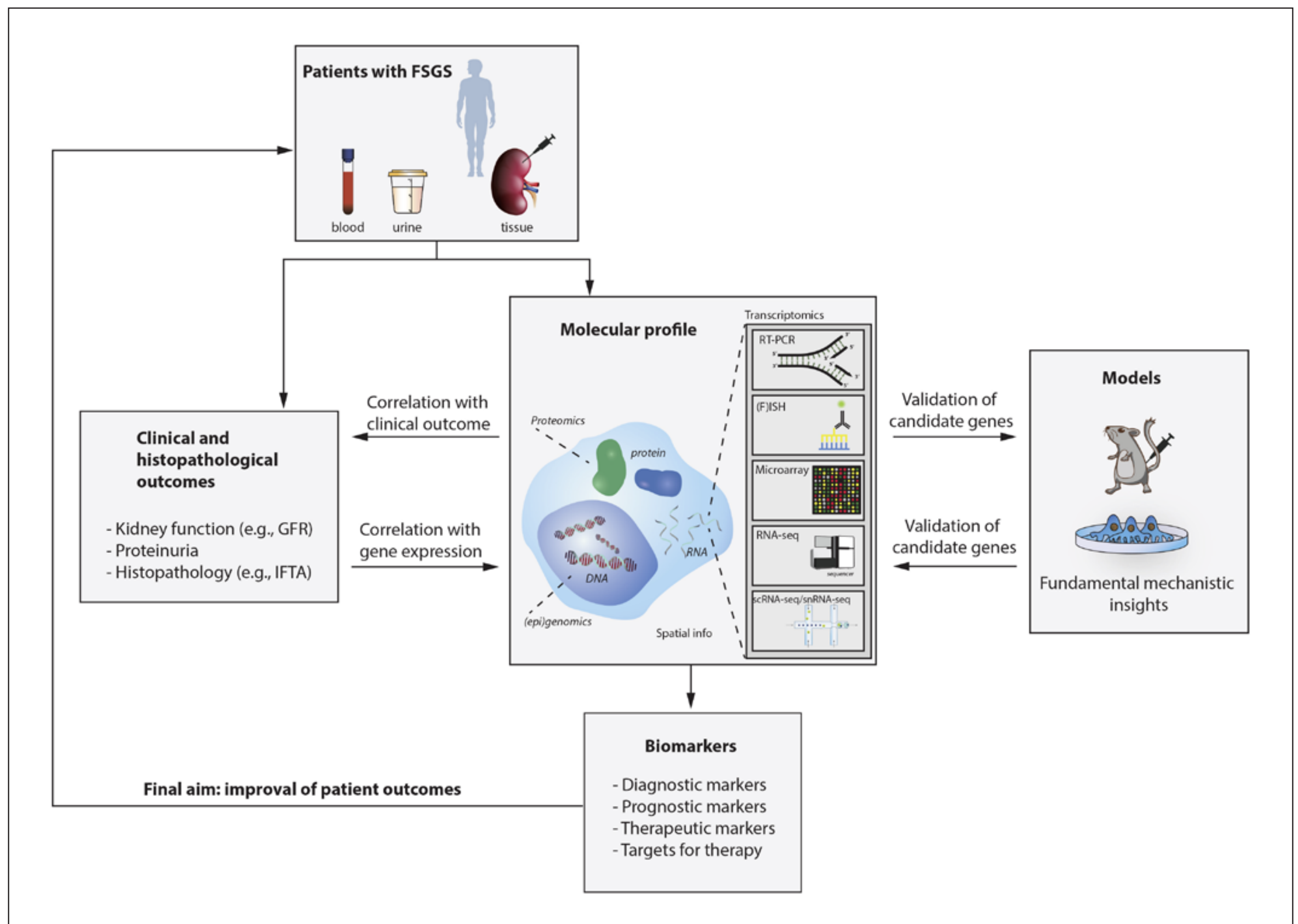

Fig. 2. Overview of fundamental and clinical approaches to identify FSGS biomarkers. Kidney tissue, blood, and urine derived from FSGS patients can be used for molecular profiling of the transcriptome and other (single-cell) omics including the (epi-) genome, proteome, and spatial information. The correlation of this molecular profile with clinicopathologicals outcomes and fundamental mechanistic insights from FSGS disease models yields clinically useful biomarkers and disease targets for novel targeted therapies. GFR, glomerular filtration rate; IFTA, interstitial fibrosis and tubular atrophy; FSGS, focal segmental glomerulosclerosis; RT-PCR, reverse transcription polymerase chain reaction; (F)ISH, (fluorescence) in situ hybridization; scRNA-seq, single-cell RNA-sequencing; snRNA-seq, single-nucleus RNA-sequencing.

[43, 68]. Surprisingly, however, complete genetic podocyte-specific mTORC1-inhibition in a mouse model with active podocyte loss also resulted in disease progression, while partial genetic mTORC1-inhibition resulted in amelioration of disease progression, which suggests that moderate mTORC-inhibition might be beneficial [68]. Both research groups also used pharmacological mTORC1-inhibition in FSGS mouse models with active podocyte loss, which yielded conflicting results. The first study found a protective role of pharmacological mTORC1-inhibition with amelioration of disease progression [68], while the second study found exacerbated disease [43]. Therefore, it is hypothesized that mTOR-mediated podocyte hypertrophy is initially an adaptive protective response, until a critical threshold is reached, and hypertrophy itself becomes maladaptive and leads to podocyte detachment [43]. This might suggest that mTOR-inhibition in FSGS has a therapeutic window and might be beneficial in these cases where podocyte hypertrophy is no longer protective but leads to podocyte loss [43, 68]. This hypothesis remains to be validated and future studies should focus on early versus late introduction of mTOR-inhibition and the use of different inhibitors and dosing strategies. Other mechanistic studies that an-

alyzed possible human disease targets in in vivo models identified versican 1 and RARRES1 as possible tissue biomarkers in FSGS patients [25, 67]. Versican 1 (a tubular cell-derived extracellular matrix protein) is upregulated in tubular cells of FSGS patients, which leads to fibroblast activation, collagen synthesis, and also correlates with renal function decline and fibrosis [67]. Upregulation of RARRES1 (a retinoic acid-related gene largely restricted to podocytes) in FSGS patients contributes to podocyte apoptosis and is also correlated with renal function decline [25]. One final example nicely illustrates how mechanistic insights into differentially expressed genes may indeed point to new possible targeted treatments. The research group of Wilkening et al. [69] found upregulation of both C-C motif chemokine ligand 2 (CCL2) and its receptor C-C chemokine receptor type 2 (CCR2) in glomeruli of patients with FSGS, which play an important role in the pathophysiology of glomerulosclerosis through CCL2-mediated recruitment of CCR2-positive kidney macrophages. Next, CCL2 was shown to be also upregulated in glomeruli of mice that were treated with adriamycin and developed glomerulosclerotic lesions. Finally, transgenic mice deficient in CCR2 expression were treated with adriamycin and developed significantly less albuminuria, less inflammation, and less glomerulosclerosis than wild-type mice, proposing CCR2 inhibitors as a potential therapy in FSGS. In fact, currently 3 trials are running that will evaluate the effect of CCR2 inhibitors in human patients with FSGS [70].

Vice versa, gene expression from in vivo and in vitro studies can be correlated with published transcriptomics datasets in humans (Fig. 2) [16, 18, 20, 31, 48–50]. For example, the gene expression signature of a transgenic rat model of FSGS based on podocyte-specific genetic mTORC1 inhibition correlated with gene expression of human FSGS patients [50]. Another study found a CKD progression gene expression signature in COL4A3$^{-/-}$ knockout mice, and these upregulated mouse signature genes significantly overlapped with upregulated genes in human FSGS patients [31]. This study also identified vorinostat, a lysine deacetylase inhibitor, as a candidate drug treatment to prevent CKD progression, which again illustrates the potential of these mechanistic studies in identifying new possible targeted treatments [31].

In an alternative more clinical approach, a validated gene expression signature that is highly correlated with clinical outcomes can be used as a diagnostic or prognostic tissue biomarker, even if the underlying pathophysiology of all the differentially expressed genes is incompletely understood (Fig. 2) [42, 71, 72]. For example, 1 study compared the gene expression profile of steroid-sensitive FSGS versus steroid-resistant FSGS [72]. Ideally, such a "steroid-resistance" signature should additionally be validated in larger cohorts to provide a prognostic transcriptional tissue biomarker, although the exact pathophysiology of all involved genes is not necessarily known. As upregulated JAK-STAT signaling is involved in many glomerular diseases, another research group created a STAT1 activity score which comprised the composite expression of 17 genes [42]. Kidney tissue of FSGS patients showed higher scores than healthy patients, and higher scores correlated with worse kidney function at baseline, increased proteinuria, and a worse prognosis, which makes this STAT1 score a possible prognostic tool [42].

Ideally, both fundamental and clinical approaches are combined, yielding a tissue, urine, or blood biomarker of which the underlying pathophysiology is understood and can be targeted with new treatments, while also correlating with clinical outcomes so that it can be used in clinical diagnosis, prognosis, and prediction of treatment susceptibility. One study identified epidermal growth factor (EGF) as such a promising biomarker for the "regenerative functional reserve" of kidney tubules in CKD, as intrarenal EGF transcripts and urinary EGF protein concentrations correlated with kidney function at time of biopsy and higher values were associated with slower renal function decline [41]. This study first identified intrarenal transcripts that correlate with kidney function at time of biopsy, which identified EGF as such a potential candidate because it shows kidney-specific expression in tubular cells and previous studies had already shown the anti-apoptotic and pro-proliferative effects of EGF on tubular cells [41]. Next, they confirmed that urinary EGF protein concentrations correlated with intrarenal EGF transcription and with renal function at time of biopsy and inversely correlated with renal function decline and interstitial fibrosis and tubular atrophy (IFTA) on biopsy, making this a potential noninvasive urinary biomarker to identify high-risk CKD patients with a poor prognosis [41].

Studies Using RNA-Seq

RNA-seq does not rely on probes that hybridize with known sequences of the transcripts of interest, as opposed to RT-PCR, ISH, and microarrays. It is therefore an unbiased way of analysis, in which pre-existing knowledge of the oligonucleotide sequence of transcripts is not necessary [62]. Indeed, RNA-seq is considered an "open detection platform" as it enables the detection of both known and new transcripts [62]. RNA-seq is also able to

Glomerular Dis 2021;1:265–276
DOI: 10.1159/000518404

detect small oligonucleotide changes and transcript variants and has a wider detection range of gene expression levels than microarrays, as it is more sensitive and does not have the problem of "saturation" at high transcript levels [8, 11, 62]. Up until now, only 2 studies that use RNA-seq on kidney tissue of human FSGS patients have been published (online suppl. Table 2) [44, 46]. The first study performed small RNA-seq to detect the differential expression of microRNAs in FSGS patients [46]. MicroRNAs are small noncoding RNA molecules that regulate gene expression on the post-transcriptional level by pairing with their target mRNA and promoting mRNA degradation or blockade of translation [73]. This study found upregulation of *miR-21-5p* in kidney tissue of FSGS patients, which suppresses *PTEN*, eventually leading to collagen deposition and fibrosis [46]. In the second study, Menon et al. [44] proposed the expression of *A2M*, which encodes for α-2-macroglobulin and is a target gene of STAT1, as new prognostic biomarker in FSGS, and exemplifies how large transcriptomics datasets can be boiled down to 1 clinically relevant candidate tissue biomarker. This study first performed single-cell RNA-sequencing (scRNA-seq) of healthy kidney biopsy tissue. Next, they identified 78 genes that were exclusively expressed in glomerular endothelial cells (GECs) and created a composite GEC score which was determined by the expression of these target genes. They interrogated bulk RNA-seq datasets of microdissected glomeruli of different glomerular diseases and found that FSGS patients had the highest GEC score. Next, they divided the FSGS patients in 2 groups: 1 group with a relatively lower GEC score versus another group with a higher score. The differential gene expression of group 1 versus group 2 was compared and yielded *A2M* as a significantly differentially expressed target gene with higher expression in the second group. Finally, higher *A2M* expression correlated with worse prognosis, proposing *A2M* expression as a possible prognostic tissue biomarker in FSGS [44].

The Power of Single-Cell Transcriptomics

Microarrays and bulk RNA-seq generate a "transcriptional average" of a group of cells, thereby losing information on the transcriptional profile of individual cells, which is undesirable in the analysis of the human kidney that consists of a highly specialized and differentiated cell population [14]. This problem is illustrated by the fact that several bulk transcriptomics studies of human FSGS patients have found downregulated podocyte-gene expression [27, 51, 54, 63, 65, 71]: it remains unclear whether this represents true podocyte downregulation of these genes, or whether this observation is confounded by the fact that less podocytes are present in the sample due to podocyte loss in FSGS. To study the gene expression of individual cell populations, cell-sorting techniques, such as fluorescence- or magnetic-activated cell sorting can be used to enrich for a target cell population, in combination with "bulk" transcriptomics techniques. However, this approach also averages the transcriptional profile of the sorted cell population and still does not reach single-cell resolution. Some computational techniques have been used to discover cell lineage-specific transcripts from microarray datasets, but again are unable to elucidate gene expression on the single-cell level [22]. In contrast, ISH techniques do reach single-cell resolution, but conventional low-throughput techniques can only profile a few genes per cell and are therefore not capable of detecting complex gene expression signatures.

Recently, scRNA-seq and single-nucleus RNA sequencing (snRNA-seq) have been developed, which are powerful new high-throughput techniques that are able to profile the gene expression of thousands of individual cells while retaining single-cell resolution [13, 74]. In these techniques, biopsy tissue is mechanically and enzymatically dissociated into a viable single-cell suspension or single-nucleus suspension [75]. These individual cells or nuclei are captured with microfluidic devices into single-cell reaction vessels [75]. Most popular techniques are droplet-based (e.g., 10× Genomics Chromium Single-Cell Gene Expression Solution [76], Drop-seq [77], and inDrops [78]), in which single cells or nuclei are co-encapsulated with barcoded oligonucleotide primers and enzymes in partitioning oil, although nanowells (e.g., Seq-Well [79], STRT-seq-2i [80]), or chip-based integrated fluidic circuits (compatible with the C1 system by Fluidigm [81]) are used as well [75]. Cells or nuclei are subsequently lysed and mRNA is captured with oligonucleotide primers. Next, these transcripts are reverse transcribed with incorporation of barcoded single-cell information, amplified, and sequenced. Downstream bioinformatic analysis is subsequently able to identify different cell types and cell states in a mixed population of cells, without prior knowledge of the identified cell types and markers [13, 82]. ScRNA-seq and snRNA-seq have already been applied to healthy human kidneys [44, 83–86], and kidney diseases, including glomerular disease (lupus nephritis [87–89], IgA nephropathy [90], diabetic kidney disease [91]), and renal allografts [44, 74, 92]. Single-cell transcriptomics would be very useful in FSGS patients to identify the gene expression of individual glomerular cells and therefore solve the aforementioned

problem of whether downregulated podocyte genes in FSGS represent true downregulation on the single-podocyte level.

Although very promising, some technical challenges arise when using single-cell transcriptomics to study glomerular diseases, as glomerular cells and especially podocytes are not easily identified in these single-cell transcriptomics experiments [93, 94]. Indeed, kidney tissue of patients with FSGS is generally only available as core needle biopsies, which yields low cell amounts as starting material [74]. To date, only 3 scRNA-seq studies have been able to isolate podocytes from core needle biopsies, and absolute cell counts were very low [44, 90, 95]. This might be especially problematic for FSGS patients in which podocyte damage, hypertrophy, and detachment are all hallmarks of disease [43], which potentially makes the isolation and identification of these abnormal podocytes in single-cell transcriptomics experiments more difficult. Fortunately, other cells from the glomerular micro-environment, such as glomerular endothelial cells are more abundantly identified in scRNA-seq experiments [44]. Furthermore, microarrays from the tubulointerstitial compartments of FSGS patients have also shown differential gene expression [28, 42, 66], which can also be further explored on the single-cell level. When compared to scRNA-seq, snRNA-seq might be superior in detecting podocytes because it uses stronger dissociation techniques which are more efficient in isolating difficult-to-dissociate cell types [94, 96]. Indeed, all snRNA-seq studies on human kidneys were successful in identifying podocytes [74, 86, 91]. However, to date, no study has applied snRNA-seq method to core needle biopsies of human kidneys, which is the only sample type available in FSGS patients [94]. The first single-cell transcriptomics studies on kidney tissue of FSGS patients are highly anticipated and will have to show whether current experimental pipelines are efficient enough to identify affected podocytes in FSGS. Ideally, patients with primary, secondary, and genetic FSGS should be included and their gene expression compared, to help elucidate the differences in pathophysiology and guide identification of candidate diagnostic, prognostic, and therapeutic markers in the different subtypes.

Recently, 3 studies have performed scRNA-seq on individual cells present in the urine [87, 95, 97] of which 1 study identified urinary podocytes, whose transcriptome was highly correlated with kidney podocytes [97]. This technique might be of interest in FSGS, as affected and detached podocytes might be detectable in the urine with this technique. However, the viability of these damaged and detached podocytes might be lower, and the transcriptional signature of FSGS might be obscured by transcriptional changes induced by the long travel of these individual cells through the entire nephron and urinary tract. Nevertheless, analysis of urinary cells, including podocytes might help in the deconvolution of disease pathophysiology and might be a means to discover non-invasive urinary biomarkers.

Finally, many new (single-cell) "omics" technologies enable the measurement of different cell modalities, including the genome, epigenome, proteome, and spatial context [7]. Some assays combine the measurement of several cell modalities in 1 pipeline, including the measurement of single-cell transcriptomes together with cell surface proteins (e.g., CITE-seq [98]), chromatin accessibility (e.g., sci-CAR [99], which combines snRNA-seq with sciATAC-seq [100]) or spatial context (e.g., spatially resolved transcriptomics with Slide-seq [101] and 10× Genomics Visium Spatial Gene Expression Solution [94, 102]. In conclusion, the field of single-cell "omics" is rapidly evolving and will create new insights into the underlying pathophysiology of FSGS and related glomerular diseases.

Conclusion

The field of transcriptomics has evolved massively since the first microarray study on kidney tissue of FSGS patients in 2004 [64]. Many multicenter initiatives have compiled large and well-defined FSGS cohorts, and recent next-generation sequencing and single-cell (multi-) omics technologies have enabled integrative analysis of disease pathophysiology at the single-cell level. These conditions set the stage for the development of diagnostic, prognostic, and therapeutic biomarkers for FSGS patients, as well as more targeted therapies. We envision a near future in which RNA or protein biomarkers are used on kidney tissue, blood, or urine of FSGS patients to reliably differentiate them into primary, secondary, or genetic subtypes, predict their risk for rapid disease progression and their responsiveness to therapies. Finally, a deep understanding of FSGS disease pathophysiology will enable the development of targeted therapies, which will ultimately improve patient outcomes.

Acknowledgment

We would like to thank Albert Herelixka for designing the figures included in this publication.

Conflict of Interest Statement

The authors declare no conflict of interest.

Funding Sources

Dries Deleersnijder holds a PhD scholarship from the Special Research Fund KU Leuven, Belgium (Grant No. DB/19/010/BM). Ben Sprangers is a senior clinical investigator of the Research Foundation Flanders (F.W.O.) (Grant No. 1842919N).

Author Contributions

D.D., B.S., and A.H.V.C. were responsible for the conception and design of the review. D.D. was responsible for data acquisition. D.D., B.S., and A.H.V.C. were responsible for analysis and interpretation of the data. D.D., B.S., and A.H.V.C. drafted the work and revised it critically for important intellectual content. D.D., B.S., and A.H.V.C. approved the published version of the manuscript. D.D., B.S., and A.H.V.C. agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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