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Cell-cycle regulatory proteins in the podocyte in collapsing glomerulopathy in children

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Podocyte is a terminally committed cell in G₁ arrest of cell cycle, and is unable to overcome G₁/S transition phase in children with minimal change disease (MCD) and classic focal segmental glomerulosclerosis (FSGS), in contrast to dysregulated proliferative phenotype of idiopathic collapsing glomerulopathy (CGN) in adults. Forty-two kidney biopsies, MCD (14), FSGS (12), CGN (4), and normal (CON) (12), were evaluated by immunohistochemistry using dual staining for expression of p27, p21, and p57, and cyclins D and A, in podocytes of children with CGN. On light microscopy, all podocytes expressed p27, whereas p21 and p57 expression was seen in a portion of podocytes in normal kidney biopsies. Cyclin D was expressed in a small percentage of podocytes. Cyclin A expression was absent in normal biopsies. The staining for p27 decreased significantly, in order, from normal (100%) to MCD (45.8%) to CGN (24.2%) to FSGS (16.6%). p21 staining was significantly decreased from normal (69.8%) to CGN (15.5%) to MCD (2.2%) to FSGS (0.6%), and the difference between CGN and MCD and FSGS was also significant. There was no significant difference in staining of p57. Cyclin D staining was significantly increased in CGN (26.8%) compared to normal (7.2%), MCD (1.6%), and FSGS (0.0%), and the difference between CGN and MCD and FSGS was also significant. *De novo* cyclin A staining was only observed in children with CGN. Thus, p27 and p21 but not p57 was decreased in CGN, as in FSGS when compared to normal. Both cyclins D and A staining were increased in CGN. The staining pattern in CGN would suggest that podocyte is able to overcome G₁/S transition phase, and has a proliferative phenotype. We propose, based on the significant contrast observed in podocytes injury response between CGN (proliferative) and classic FSGS (non-proliferative), that CGN not be considered as a morphological variant of FSGS.

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KEYWORDS: cyclin A; cyclin D; p21; p27; p57; idiopathic collapsing glomerulopathy

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The cell-cycle process is a highly organized process that allows the cell to proliferate under both physiological and pathological conditions. The process ensures that DNA replication occurs only once and that the DNA replication is completed before mitosis occurs in each cycle.^{1–3} This highly organized cell-cycle process is regulated by a number of cell-cycle regulatory proteins: (a) positive cell-cycle regulatory proteins, such as 'cyclins' and 'cyclin-dependent kinase (CDK)', which aid in the progression and completion of cell cycle and (b) negative cell-cycle regulatory proteins, 'cyclin kinase inhibitors', which inhibit cell-cycle process. Cells can also exit from cell cycle at any phase by undergoing apoptosis (cell death), senescence (permanently growth arrested cells), terminally committed specialized cells (cells can proliferate under appropriate stimuli), or become uncontrolled as in neoplasia.³ Mature podocytes (or visceral glomerular epithelial cells) in the glomerulus are regarded as terminally committed specialized cells that normally express negative cell-cycle regulatory proteins p27, p21, and p57, and positive cell-cycle regulatory proteins cyclin D but not cyclin A, cyclin B1, or Ki-67.^{4,5}

The characteristic findings in idiopathic nephrotic syndrome of childhood in podocytes are effacement of foot processes, apical displacement of slit diaphragms, and detachment from glomerular basement membrane.^{6–8} Our findings from earlier studies have led us to propose that podocyte injury clinically manifests as proteinuria and, at the molecular level, podocyte proteins, such as synaptopodin, glomerular epithelial protein 1 (GLEPP-1), and nephrin, become disorganized. We showed a decrease in synaptopodin, GLEPP-1, and nephrin expression in children with minimal change disease (MCD) and focal segmental glomerulosclerosis (FSGS).^{9,10} The common disease entities found on renal biopsy in idiopathic nephrotic syndrome of childhood include MCD, diffuse mesangial hypercellularity (DMH), and FSGS, which together constitute ~90% of idiopathic nephrotic syndrome of childhood.¹¹ FSGS is classified into the following major categories: classic FSGS (also known as FSGS, not otherwise specified), tip lesion variant of FSGS, cellular variant of FSGS, and collapsing variant of FSGS. Based on morphological grounds, the distinction between the cellular and collapsing variants of FSGS can be extremely difficult. In the cellular variant of

FSGS, hyperplastic podocytes form a pseudocrescent without attachment to Bowman’s capsule. Collapsing FSGS is characterized by focal segmental or global glomerular capillary collapse, with wrinkling and folding of basement membrane, and with overlying hyperplasia and hypertrophy of podocytes. Both the cellular and collapsing variants of FSGS present with severe proteinuria, hypoalbuminemia, higher serum creatinine levels at presentation, shorter time course from presentation to biopsy, and progression to end-stage renal disease.¹²

The podocyte is a unique glomerular cell because its growth response to injury differs from the mesangial or endothelial cell by virtue of its apparent inability to proliferate.³ This raises the question of how a podocyte responds to injury. In adult subjects with cellular and collapsing variants of FSGS and with HIV-associated nephropathy (HIVAN), Shankland *et al.*¹³ found decreased p27 and p57 expression and *de novo* expression for p21 in podocytes. In collapsing FSGS, Barisoni *et al.*¹⁴ reported a decrease in p27, p57, and cyclin D1 expression along with the expression for cyclin A and Ki-67 in podocytes. We found that podocytes in children with MCD, DMH, and classic FSGS downregulate the expression of cyclin kinase inhibitors such as p21 and p27 but not p57, and do not upregulate cyclins D and A that are needed to overcome the G₁/S transition and to move the cell forward in the cell cycle.⁴ Thus, the podocyte remains trapped in the G₁ arrest phase and has a non-proliferative phenotype, in contrast to the dysregulated proliferative podocyte phenotype seen in adults with cellular and collapsing variants of FSGS.^{13,14} In the current study, we evaluated the expression of cell-cycle regulatory proteins p27, p57, p21, and cyclins D and A in podocytes from children with idiopathic collapsing glomerulopathy (CGN). We found that the podocyte injury response in CGN in children is consistent with a proliferative phenotype, in contrast to children with MCD and classic

FSGS. This raises the question as to whether CGN should be considered a separate disease entity rather than a morphologic variant of FSGS.

RESULTS

The mean ± s.d. for age in years for normal (8.4 ± 5.3) was not significantly different from CGN (10.7 ± 7.0, *p* = 0.38), MCD (5.4 ± 3.2, *p* = 0.09), or FSGS (9.1 ± 4.0, *p* = 0.73). Children with MCD were younger than children with CGN (*p* = 0.045) and FSGS (*p* = 0.047). The kidney biopsies in children with proteinuria were performed for the evaluation of associated hematuria in five (16.7%), steroid-sensitive frequently relapsing/steroid-dependent nephrotic syndrome in 12 (40%), and steroid-resistant nephrotic syndrome in 13 (43.3%). The subjects included 19 boys and 11 girls. There were 21 Caucasian, six African-American, and three other race children in the study. In the control group, there were six boys and six girls, of which 11 were Caucasian and one African-American.

The total number of glomeruli evaluated in each section was 25.6 ± 17.4 (range 5–67) for control, 20.3 ± 13.7 (range 5–50) for MCD, 19.7 ± 15.3 (range 6–52) for FSGS, and 11.0 ± 4.1 (range 5–14) for CGN. The total podocyte count per glomerulus for normal (22.7 ± 2.9) was not different from MCD (19.6 ± 3.1, *p* = 0.1) but was decreased in FSGS (17.3 ± 3.5, *p* = 0.002), and CGN (13.7 ± 4.3, *p* ≤ 0.001).

Table 1 summarizes the results for the expression of cell-cycle regulatory proteins p27, p21, p57, and cyclins D and A. By light microscopical examination, all podocytes within all glomeruli expressed p27 in normal biopsies. The percent of positive glomeruli and podocytes expressing p27 decreased in biopsies from CGN, MCD, and FSGS (Table 1 and Figure 1). The decrease in p27 expression in podocytes reached statistical difference between normal and CGN as well as MCD and FSGS (Table 1). There was no statistical difference between the expression in CGN and MCD or FSGS (Table 1).

Table 1 | Expression of p27, p21, p57, and cyclin D in podocytes of normal and children with CGN, MCD, and FSGS.

	Percent-positive glomeruli				Net percent-positive podocytes			
	Normal (n=12)	CGN (n=4)	MCD (n=14)	FSGS (n=12)	Normal (n=12)	CGN (n=4)	MCD (n=14)	FSGS (n=12)
p27	100.0 ± 0.0	76.5 ± 37.4	66.9 ± 39.4	39.7 ± 35.2	100.0 ± 0.0	24.2 ± 19.3	45.8 ± 31.5	16.6 ± 18.8
Normal versus (p-value)	—	0.579	0.054	<0.001	—	<0.001	<0.001	<0.001
CGN versus (p-value)	—	—	0.95	0.202	—	—	0.310	0.931
p21	100.0 ± 0.0	58.6 ± 39.4	9.2 ± 15.1	2.8 ± 6.6	69.8 ± 9.9	15.5 ± 18.4	2.2 ± 4.3	0.6 ± 1.6
Normal versus (p-value)	—	0.001	<0.001	<0.001	—	<0.001	<0.001	<0.001
CGN versus (p-value)	—	—	<0.001	<0.001	—	—	0.019	0.009
p57	100.0 ± 0.0	76.5 ± 37.4	99.0 ± 2.0	96.3 ± 7.0	55.7 ± 14.3	55.5 ± 26.6	44.7 ± 18.7	45.0 ± 18.1
Normal versus (p-value)	—	0.006	0.997	0.869	—	1.000	0.453	0.506
CGN versus (p-value)	—	—	0.007	0.023	—	—	0.728	0.754
Cyclin D	36.2 ± 37.5	76.5 ± 37.4	8.3 ± 14.8	0.0 ± 0.0	7.2 ± 9.4	26.8 ± 13.3	1.6 ± 3.6	0.0 ± 0.0
Normal versus (p-value)	—	0.029	0.035	0.005	—	<0.001	0.187	0.065
CGN versus (p-value)	—	—	<0.001	<0.001	—	—	<0.001	<0.001
Cyclin A	0.0 ± 0.0	43.6 ± 28.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	10.3 ± 6.7	0.0 ± 0.0	0.0 ± 0.0
Normal versus (p-value)	—	<0.001	1.000	1.000	—	<0.001	1.000	1.000
CGN versus (p-value)	—	—	<0.001	<0.001	—	—	<0.001	<0.001

Abbreviations: CGN, collapsing glomerulopathy; FSGS, focal segmental glomerulosclerosis; MCD, minimal change disease) The results are expressed as mean ± s.d.

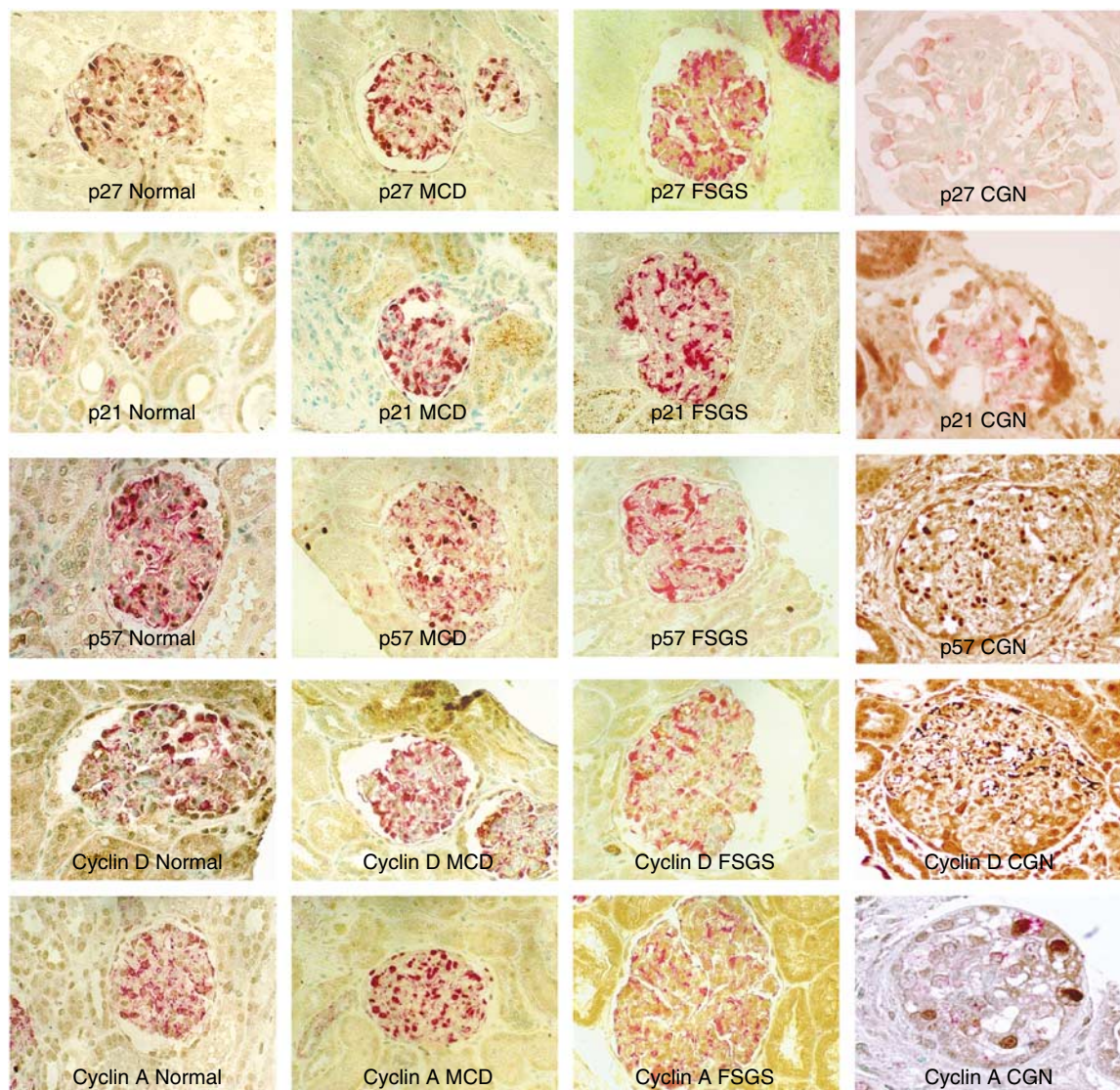


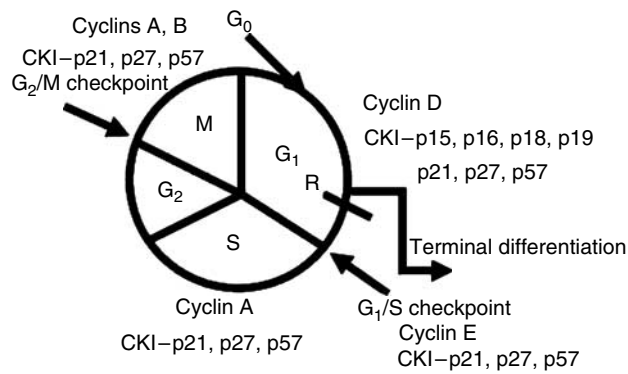
Figure 1 | The photomicrograph shows the staining of p27, p21, p57, and cyclins D and A in podocytes of normal and children with MCD, classic FSGS, and CGN in kidney tissue. The podocyte is identified by WT-1 staining (stained red with Vector red) and the cell-cycle regulatory protein is stained brown-black with diaminobenzidine with 3% cobalt. Only podocytes that showed homogeneous staining for cell-cycle regulatory proteins above the background were accepted as positive staining. The staining for p27 decreased in order from normal to MCD to CGN to FSGS. p21 staining was markedly decreased in children with MCD, CGN, or FSGS compared to normal. p57 staining remained unchanged between normal, MCD, FSGS, and CGN. Cyclin D expression was increased in CGN and similar between normal, MCD, and FSGS. Cyclin A expression was only observed in CGN and absent in normal, MCD, or FSGS.

Thus, p27 expression in podocytes decreased in biopsies with CGN when compared to normal, but was similar to that seen in biopsies from MCD and FSGS.

In normal biopsies, podocytes expressing p21 were observed in all glomeruli; however, in a given glomerulus not all podocytes expressed p21 (Table 1 and Figure 1). The percent of positive glomeruli and positive podocytes expressing p21 were significantly reduced in biopsies from CGN, MCD, and FSGS when compared to normal and this decrease was statistically significant (Table 1 and Figure 1). The p21 expression, although decreased, was better preserved in biopsy from CGN where it was overall higher than in biopsies from MCD and FSGS (Table 1).

In normal biopsies, all glomeruli had podocytes that expressed p57 protein, but like p21, not all podocytes in the glomeruli expressed p57 (Table 1 and Figure 1). Although the number of glomeruli-expressing p57 was decreased in CGN, there was no statistically significant difference in the expression within podocytes in normal versus CGN, MCD, and FSGS (Table 1).

By light microscopy, cyclin D was predominantly observed in endothelial and mesangial cells in the glomeruli, although a small percentage of glomeruli and podocytes did express cyclin D in normal biopsies (Table 1 and Figure 1). Cyclin D expression was increased in CGN, whereas it decreased in biopsies from MCD and FSGS when compared



	Normal	Classic FSGS	CGN
p27	Present	Decreased	Decreased
p21	Present	Decreased	Decreased
p57	Present	Present	Present
Cyclin D	Present	Decreased	Increased
Cyclin A	Absent	Absent	Present
Phenotype	Terminally differentiated	Non-proliferative	Proliferative

Figure 2 | Schematic diagram of cell-cycle phases, checkpoints, and restriction point (R) in a eukaryotic cell showing major cyclin and cyclin kinase inhibitors (CKI). The cell-cycle arrest can occur at G₁/S or G₂/M checkpoint. The podocyte is a terminally differentiated cell that normally expresses low level of cyclin D and CKIs' p27, p21, and p57. The podocyte in MCD and FSGS remain trapped in G₁/S phase as suggested by a decrease in CKIs' p27 and p21, but not p57, and no increase in cyclins D or A. On the other hand, podocyte in CGN shows a decrease in CKIs' p27 and p21, but not p57, and increase in cyclins D and A.

to normal. There was a significant increase in cyclin D expression in podocytes from CGN when compared to normal, MCD, and FSGS. There was no significant difference in cyclin D expression between normal and biopsies from MCD and FSGS. Thus, the staining for cyclin D was markedly increased in CGN as compared to normal, MCD, and FSGS.

Light microscopical evaluation for cyclin A showed that it was not expressed in the podocytes of normal, MCD, and FSGS (Table 1 and Figure 1). Cyclin A expression was only observed in biopsies from CGN.

In summary, the normal podocyte, a terminally committed cell, shows expression of cyclin kinase inhibitors p27, p21, and p57, as well as cyclin D but not cyclin A. In MCD and FSGS, following podocyte injury, the podocyte remains trapped in the G₁/S cell-cycle phase, as manifested by decreased staining of cyclin kinase inhibitors p27 and p21, but not p57, and no increased staining of cyclins D and A. On the other hand, podocytes following injury in CGN are able to overcome the G₁/S cell-cycle restriction step, and can proliferate, as observed by decreased staining of p27 and p21, but not p57, and increased staining of cyclins D and A (Figure 2).

DISCUSSION

In recent years, cell-cycle regulatory proteins have become an area of intense research in order to understand the changes that occur in various renal diseases. The roles of these proteins in renal diseases have been addressed in several reviews.¹⁻³ In adult subjects with cellular or collapsing variant of FSGS and HIVAN, podocytes were observed to have decreased expression of p27, p57, and cyclin D, and increased expression of p21, cyclin A, and Ki-67, suggesting a dysregulated podocyte phenotype characterized by de-differentiation and proliferation.^{13,14} In contrast, Nagata *et al.*¹⁹ has contested that the proliferating epithelial cells in collapsing FSGS are not podocytes but instead are parietal epithelial cells. To overcome the issue of podocyte versus parietal epithelial cell type, we identified podocyte by WT-1 expression, based on the observation of Mundlos *et al.*¹⁸ that WT-1 protein is exclusively expressed in podocytes. This technique then allowed us to study cell-cycle regulatory proteins expressed solely in podocytes by excluding other resident cells of the glomerulus. The expression pattern of the cell-cycle regulatory proteins p27, p21, p57, and cyclins D and A were similar from control tissue (normal) obtained from children who had normal renal biopsies for loin pain-hematuria syndrome and from nephrectomy specimens from children with WT (unpublished observation), hence we used them as a single control group.

We had observed that podocytes in children with MCD, DMH, and classic FSGS showed decreased expression of cyclin kinase inhibitors such as p21 and p27, but not p57, and do not show increased expression of cyclins D and A that are needed to overcome the G₁/S transition in order to move the cell forward in the cell-cycle process.⁴ Therefore, the podocyte remains trapped in the G₁ arrest phase and has a non-proliferative phenotype. This dysregulated podocyte phenotype observed in classic FSGS was different than that described in adults with either cellular or collapsing variant of FSGS.^{13,14} Hence, we studied the cell-cycle regulatory proteins p27, p57, p21, and cyclins D and A expression in podocytes in children with CGN to evaluate if the podocyte injury response is similar or different in CGN from classic FSGS.

p27 is believed to play a role in differentiation of cells, maintain podocytes in quiescence by cell-cycle arrest in G₁ phase, and to modulate apoptosis and cell-cycle exit response to antimutagenic cues.^{5,20} In our earlier study, we observed a significant decrease in p27 expression in podocytes in children with idiopathic nephrotic syndrome, decreasing in order from normal to MCD to DMH to FSGS.⁴ Similarly, in adults, p27 is reported to be decreased in cellular and collapsing variant of FSGS and HIVAN.¹³ p27 levels are generally high in most quiescent cells but decrease during proliferation.²¹ Thus, it was not surprising to find decreased p27 expression in CGN in the current study as such a decrease would be anticipated if the podocyte is to de-differentiate and proliferate.

The p21 protein plays an important role at the G₁ checkpoint.²² p21 is rapidly induced in response to physiological and chemical inducers of differentiation, and the p21 gene is a candidate gene linking differentiation signals to G₁ arrest in multiple cell lines.²³ In our earlier study, we had shown p21 to be expressed in normal podocytes and to decrease significantly in children with MCD, DMH, and FSGS.⁴ Interestingly, p21 exists predominantly as quaternary complexes of p21/proliferating cell nuclear antigen/cyclin/CDK, which can be both catalytically active and inactive depending on the stoichiometric ratio of p21 to the kinase subunits.^{24,25} Thus, at a low stoichiometric ratio, p21 facilitates kinase complex assembly and promotes kinase activation. Therefore, it was not unexpected to find a decrease p21 expression in our study, if the podocyte is to de-differentiate and proliferate in CGN. Our finding is in contrast to Shankland *et al.*¹³ who did not detect p21 in normal tissue but instead found *de novo* expression of p21 in adults with cellular and collapsing variants of FSGS and HIVAN. The antibody used in the current study is clone 187, produced by immunization with full length p21 of human origin. The antibody used by Shankland *et al.*¹³ was from clone SX 118, which is raised against a purified recombinant human p21-GST fusion protein and only recognizes the proliferating cell nuclear antigen-binding domain of p21. Based on what we know about the structure of p21 and the epitope recognition sites of the antibodies used in the two studies, we speculate that in CGN, as the podocyte de-differentiates and develops a proliferative phenotype, the cryptic proliferating cell nuclear antigen-binding domain of p21 is freed up from the quaternary complex, which is recognized by the antibody. This would make the discrepant findings of p21 expression observed in our study and that observed by Shankland *et al.*¹³ congruent.

p57 plays a role in the differentiation of cells and overexpression of p57 arrests the cell in G₁ phase.^{26,27} p57 is reported to be decreased in cellular and collapsing variant of FSGS and HIVAN.^{13,14} We had not observed any difference in p57 expression in podocytes from normal biopsies or those with MCD, DMH, or FSGS.⁴ There was a decrease in the number of glomeruli expressing p57, but the net podocyte expression of p57 was not significantly different between CGN and normal, MCD, and FSGS. p57 is believed to be a tumor suppressor protein based on its association with numerous malignancies and has a reduced affinity for cyclin D-CDK6 complex compared to p27.²⁷ Our findings suggest that p57 may play a role in podocyte differentiation, but it may not be a critical player in podocyte proliferation.

Cyclin D is required for cell proliferation and G₁/S transition. It works through inactivation of the retinoblastoma protein.³ In our study, cyclin D was predominantly expressed in endothelial and mesangial cells of the glomerulus in normal biopsies, MCD and FSGS. In our earlier study, we did not observe any difference in cyclin D expression in podocytes from normal biopsies and those with MCD, DMH, and FSGS.⁴ Cyclin D was markedly upregulated in podocytes

in CGN. There was almost a twofold increase in the number of glomeruli expressing cyclin D and a fourfold increase in podocytes expressing cyclin D. Cyclin D is important for G₀/G₁ and G₁/S transition in the cell-cycle process. An increase in cyclin D levels would support a proliferative phenotype of podocytes in CGN in contrast to a non-proliferative phenotype in classic FSGS.

Cyclin A increases in late G₁, peaks in both S and G₂ phases, and is required for the onset of DNA synthesis.³ In our earlier study, we did not observe any expression of cyclin A in podocytes from normal biopsies and those with MCD, DMH, and FSGS.⁴ In children with CGN, there was *de novo* expression of cyclin A and this has also been previously observed by Barisoni *et al.*¹⁴ in adults with collapsing and cellular variants of FSGS and HIVAN. *De novo* expression of cyclin A would support that the podocyte has crossed the G₁/S restriction point and moved to S and G₂ phase of cell cycle. This again supports a proliferative phenotype of podocytes in CGN in contrast to a non-proliferative phenotype in classic FSGS.

There is an ongoing debate in the literature whether it is the parietal epithelial cell or the podocyte that is proliferating in the cellular variant or collapsing variant of FSGS. In our study, we used WT-1 as a surrogate podocyte marker; hence, the cell-cycle regulatory proteins changes observed by us in CGN are localized to podocytes, and not the parietal epithelial cell (which lack WT-1 expression). The staining pattern of increased cyclins D and A, and decreased staining in p27 and p21 would support a proliferative phenotype for the podocyte in CGN, in contrast to classic FSGS. In CGN, there is the possibility of podocytes becoming de-differentiated to the extent that they lose WT-1 staining or are completely lost from the glomerulus. The observed pattern of staining for cell-cycle regulatory proteins in podocytes between CGN and classic FSGS are markedly different. In CGN, we observed an increased staining of cyclin D and *de novo* staining of cyclin A, which represent markers for cell-cycle phase beyond G₁/S in CGN, in contrast to the absent or decreased staining for cyclins D and A in classic FSGS. Thus, even if we underestimated the percentage of positive podocytes because of the de-differentiated podocytes lacking WT-1 staining, our results would still support that the podocytes have a proliferative phenotype in CGN in contrast to classic FSGS. If podocyte loss was responsible for the quantitative differences in cell-cycle regulatory proteins in CGN, then we should have seen a further decrease staining of cell-cycle regulatory proteins in CGN. On the contrary, we found increased staining of cyclin D, cyclin A, and p21 in podocytes. In addition, the expression of p27 in CGN was higher than in FSGS, which had a higher percentage of positive podocyte count. Thus, loss of podocytes from the glomerulus or de-differentiation of podocytes to the extent of losing WT-1 staining should not alter the results significantly. Could our observed quantitative differences in cell-cycle regulatory proteins in CGN be age dependent or due to a small sample size? With regard to age-dependent differences,

there was no significant difference between the ages of the normal group and children with CGN, MCD, and FSGS; therefore, we cannot attribute the observed changes in cell-cycle regulatory proteins in podocytes to the age of the child. CGN is a rare entity in pediatric age group. In our own series of 34 children with FSGS from 1984 to 1995 seen in the midwest (USA), we did not find a single child with either cellular or collapsing variant of FSGS.¹¹ This study is an extension of our earlier work carried out in collaboration with The University of North Carolina at Chapel Hill who had previously described a small case series of children with CGN, and provided these clinical samples.²⁸ Although the sample size is small ($n=4$), the robust qualitative changes seen in both cyclins D and A expression in podocytes support our result of a de-differentiated and proliferative podocyte phenotype. We can cautiously speculate that inclusion of a larger sample size would likely not alter our conclusions. There was no statistically significant difference between the expression of podocyte cell-cycle regulatory proteins and clinical response to steroids, nor was there a statistically significant difference in the expression of cell-cycle regulatory proteins between Caucasian and African-American children (data not shown). The study does have its limitations owing to a small sample size of CGN, use of archived human tissue, and a semiquantitative analysis approach; however, despite these limitations, the changes are sufficiently uniform to justify our findings.

In conclusion, we observed p27, p57, and p21 to be present in normal kidneys, which keeps the podocyte differentiated and quiescent in G₁ arrest. In MCD and classic FSGS, we found podocytes to have decreased staining for p21 and p27, but not p57, and failure to have increased staining for cyclins D and A (marker of 'S' and 'G2' phase) that are needed for proliferation. Thus, the podocyte remains trapped in G₁ arrest phase. In contrast, in CGN, there is decreased staining of p27 and p21, but not p57, and increased staining for cyclins D and A to support a proliferative podocyte phenotype.

Our current study was a follow-up on our earlier study where we had failed to demonstrate a proliferative phenotype in children with classic FSGS compared to reports in adults of

a de-differentiated and proliferative podocyte phenotype in cellular and/or collapsing FSGS. We had speculated that the pathogenic mechanisms that underlie classic FSGS are different from CGN. In the current study, we demonstrated that proliferation is a characteristic feature of the podocyte in CGN, unlike the non-proliferative phenotype of classic FSGS. Although both classic FSGS and CGN result from a yet undefined immunological podocyte injury, the cellular response of podocytes to the injury is strikingly different in CGN and classic FSGS. Therefore, this raises the question as to whether CGN should be considered as a continuum of FSGS with a more severe podocyte injury. Given the distinct differences in the podocyte response of the two disease entities, we would propose that CGN be considered as a separate podocyte disease (podocytopathy) rather than a morphological variant of FSGS.

MATERIALS AND METHODS

Kidney biopsies from children were included in the study only if the histology was consistent with MCD or FSGS as described by the International Study of Kidney Disease in Children¹⁵ and CGN as described by Valeri *et al.*¹⁶ and Detwiler *et al.*¹⁷ Control tissue (normal) was obtained from children who had a normal renal biopsy following evaluation for loin pain-hematuria syndrome ($n=7$) and from nephrectomy specimens from children with Wilm's tumor ($n=5$). A total of 42 kidney biopsies were investigated: MCD ($n=14$), FSGS ($n=12$), CGN ($n=4$), and normal ($n=12$). The biopsy tissue for CGN came from The University of North Carolina at Chapel Hill while the rest were from Children's Mercy Hospital. Patient demographics including age, sex, and race were noted at the time of biopsy. The sections were examined by immunohistochemistry using a dual staining method as described by Srivastava *et al.*⁴ with appropriate negative and positive controls (Table 2).

Kidney biopsies stained for Wilm's tumor-1 (WT-1) and one of the cell-cycle regulatory proteins were first examined by light microscopy. Podocytes were identified in the biopsies by WT-1 staining as it is exclusively expressed in the podocytes in glomeruli.¹⁸ On light microscopy, a qualitative evaluation of each glomerulus was carried out for the expression of: (1) WT-1 and each of the cell-cycle regulatory proteins (i.e. podocytes expressing the protein under study), (2) WT-1 protein only (i.e. podocytes not expressing the protein under study), and (3) only the cell-cycle regulatory

Table 2 | Antibodies used in the dual staining immunohistochemistry

Antibody to	Source	Company	Dilution	Universal Dako's LSAB+ with	Chromogen
WT-1 (M3561)	Mouse monoclonal	Dako Corp., Carpinteria, CA, USA	1:100	Streptavidin-biotin conjugated to alkaline phosphatase	Vector red
p27 (sc-1641)	Mouse monoclonal	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA	1:50	Streptavidin-biotin conjugated to horseradish peroxidase	Diaminobenzidine with 3% cobalt
P21 (sc-817)	Mouse monoclonal	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA	1:100	Streptavidin-biotin conjugated to horseradish peroxidase	Diaminobenzidine with 3% cobalt
p57 (sc-1040G)	Goat polyclonal	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA	1:50	Streptavidin-biotin conjugated to horseradish peroxidase	Diaminobenzidine with 3% cobalt
Cyclin D1 (sc-8396)	Mouse monoclonal	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA	1:50	Streptavidin-biotin conjugated to horseradish peroxidase	Diaminobenzidine with 3% cobalt
Cyclin A (sc-751)	Rabbit polyclonal	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA	1:100	Streptavidin-biotin conjugated to horseradish peroxidase	Diaminobenzidine with 3% cobalt

protein but not WT-1 (non-podocyte glomerular cells expressing the protein under study). Following an initial qualitative assessment, a semiquantitative analysis for cell-cycle regulatory protein expression was performed. First, the percentage of glomeruli that stained positive for cell-cycle regulatory protein in the biopsy section was obtained. Next, in glomeruli that stained positive for cell-cycle regulatory protein under study, the percentage of podocytes that express the cell-cycle regulatory protein was obtained. The product of the above two values gave the proportion of total podocytes that expressed the cell-cycle regulatory protein in each section, and are shown as net percent-positive podocytes. Statistical analysis was performed using univariate analysis of variance followed by Tukey's honestly significant difference test.

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