The Role of Cell Cycle Proteins in Glomerular Disease

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Although initially identified and characterized as regulators of the cell cycle and hence proliferation, an extended role for cell cycle proteins has been appreciated more recently in a number of physiologic and pathologic processes, including development, differentiation, hypertrophy, and apoptosis. Their precise contribution to the cellular response to injury appears to be dependent on both the cell type and the nature of the initiating injury. The glomerulus offers a remarkable situation in which to study the cell cycle proteins, as each of the 3 major resident cell types (the mesangial cell, podocyte, and glomerular endothelial cell) has a specific pattern of cell cycle protein expression when quiescent and responds uniquely after injury. Defining their roles may lead to potential therapeutic strategies in glomerular disease.

THE TYPICAL clinical classification of glomerular diseases is division into those associated with either the nephritic or nephrotic syndromes. However, it may be more relevant to the underlying pathophysiology to consider the principal resident cell type injured. Thus, glomerular diseases may be classified as a consequence of injury primarily to the mesangial cells, podocytes, or glomerular endothelial cells. Depending on the cell type and form of injury, glomerular cells may respond by undergoing proliferation, hypertrophy, apoptosis, or necrosis. Each response to injury is central to the development of the histologic and clinical hallmarks of different glomerular diseases. A common denominator of these cellular events is that they are all ultimately regulated at the level of the cell cycle by specific cell cycle regulatory proteins. Although originally described and named for their role as coordinators of cell cycle progression and proliferation,[1] recent research has described broader involvement for cell cycle regulatory proteins in numerous other fundamental biologic processes, including development, differentiation, hypertrophy, and apoptosis.

The control of the cell cycle in the glomerulus is particularly intriguing given the contrasting responses of the 3 major resident cell types to injury. We provide a brief background on specific cell cycle regulatory proteins and discuss their role in glomerular diseases, considering the responses of the mesangial cell, podocyte, and glomerular endothelium.

CELL CYCLE AND CELL CYCLE REGULATORY PROTEINS

Cyclins and Cyclin-Dependent Kinases: Positive Regulators of the Cell Cycle

The cell cycle is divided into distinct phases, each representing a different function (Fig 1), and each being regulated by specific proteins (Fig 2).[1] After mitogenic stimuli, quiescent cells (G_0) enter the cell cycle (early G_1). Cells pass through a restriction point in late G_1, beyond which they are typically unresponsive to extracellular cues, and then are committed to complete the cell cycle despite the withdrawal of the initiating stimulus. DNA synthesis occurs in S phase. Cells then progress through a second phase of growth (G_2), in preparation for mitosis (M phase). Ultimately, cell division follows during cytokinesis. Our current understanding suggests there are at least 2 checkpoints, at G_1/S and G_2/M, where cell cycle progression may be arrested.

The progress of a somatic cell through the cell cycle is dependent on the activity of the cyclin-dependent kinases (CDKs), a family of serine/threonine kinases that phosphorylate downstream targets, to ultimately induce DNA synthesis.[2] Although the protein levels of the CDK catalytic subunits typically remain constant throughout the cell cycle, they are only functional after the binding of their specific partners, termed *cyclins*. Cyclins are unstable proteins that are expressed sequentially and degraded throughout the cell cycle, and activate their partner CDK by inducing conformational changes. Originally described for their

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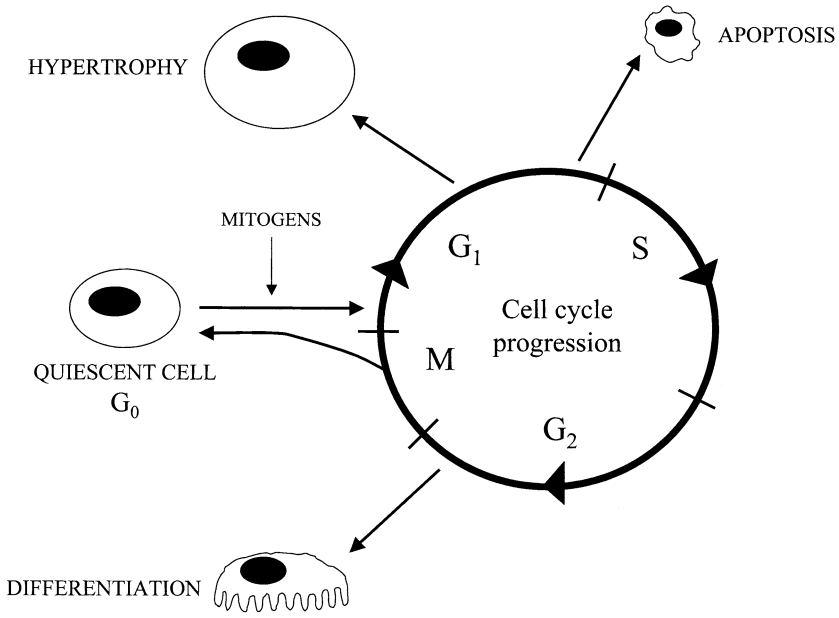

Fig 1. The cell cycle and potential outcomes of cell cycle exit.

fluctuation during the cell cycle,[3] members of the cyclin family are now defined by the presence of a conserved 100 amino acid cyclin box, which binds their complementary CDK. Cyclins are ubiquitinated by the ubiquitin protein ligases anaphase promoting complex (APC) and Skp1p-Cdc53p/cullin-F-box protein and degraded by the 26S proteasome.[4] In addition, the binding of inhibitors and accessory proteins, subcellular localization, and both inhibitory and activating phosphorylations influence the functional activity of the CDK-cyclin complex.[5]

The cell cycle is initiated during G_1 by the mitogen-driven induction of cyclin D.[6-8] The requirement for mitogen signaling prevents the cell from autonomous cycling. Depending on the cell type, 3 isoforms of cyclin D have been described (D1, D2, and D3). Receptor-activated Ras signaling pathways lead to accumulation of cyclin D by 3 mechanisms: gene transcription, assembly, and stabilization of the cyclin D–CDK complex.[9] Signaling through extracellular signal-related protein kinases both induces cyclin D transcription and promotes assembly of cyclin D CDK.[10,11] Glycogen synthase kinase 3β phosphorylates cyclin D1 specifically on threonine-286 (Thr-286), allowing proteasomal degradation. The activity of glycogen synthase kinase 3β is inhibited by a separate Ras signaling pathway involving phosphatidylinositol 3-kinase and protein kinase B, hence the rate of turnover of cyclin D1 also is mitogen dependent.[12] Although ectopic expression of cyclin D is insufficient to drive cell cycle progression, constitutive activation of the cyclin D pathway can reduce the reliance of the cell on mitogenic stimulation and lower the threshold for oncogenic transformation.[13] Cyclin D interacts with CDK4 and CDK6. The cyclin D–CDK4/6 complex enters the cell

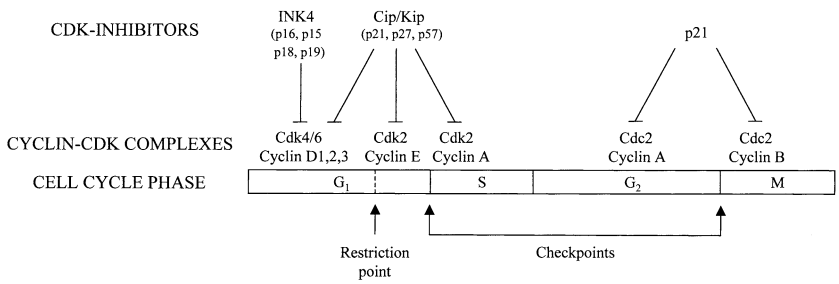

Fig 2. Positive and negative regulators of the cell cycle.

nucleus and is phosphorylated by CDK-activating kinase.[14] CDKs 2, 4, and 6, and cdc2 are phosphorylated by CDK-activating kinase on a conserved activating threonine residue, resulting in a structural reorganization of the activation center or T loop and allowing the phosphate group to interact with several other residues within the catalytic cleft.

Active cyclin D–dependent kinases phosphorylate the retinoblastoma protein (Rb), which in quiescent cells has a growth-inhibitory effect.[15,16] In its hypophosphorylated state, Rb suppresses the transcription of several genes whose proteins are required for DNA synthesis, including the E2F transcription factors. On phosphorylation of Rb, the E2Fs are released from inhibition, leading to the transcription of cyclins E and A. Cyclin E binds CDK2 and further phosphorylates Rb.[17,18] This releases the cell from the mitogen dependency of cyclin D–CDK4/6 complexes. Cyclin E–CDK2 activity is maximal at the G_1/S transition, beyond which cyclin E is replaced by cyclin A.

The entry to mitosis is controlled by cyclin B Cdc2.[19,20] Cell cycle regulated transcription of cyclin B begins at the end of the S phase. Phosphorylation on Thr-161 by CDK-activating kinase parallels cyclin B binding to Cdc2.[21] During G_2, cyclin B–Cdc2 complexes are maintained in an inactive state by phosphorylation on 2 inhibitory sites, Thr-14 and tyrosine 15 (Tyr-15). Phosphorylation on Tyr-15 is mediated by the nuclear Wee1 kinases,[22] that on Thr-14 is mediated by the membrane-bound Myt1.[23] In late G_2 phase, both Thr14 and Tyr15 are dephosphorylated by Cdc25, thus activating cyclin B Cdc2 and initiating mitosis.[24] Inappropriate triggering of mitosis is also prevented by the translocation of cyclin B to the cytoplasm by the nuclear export factor CRM1 (exportin 1) during S and G_2 phases.[25] Phosphorylation of cyclin B is thought to promote nuclear import at the G_2/M transition.[26] Cyclin B Cdc2 phosphorylates numerous downstream targets responsible for the structural reorganization of the cell to enable mitosis. Exit from mitosis is regulated by the APC ubiquitination of cyclin A and B at the end of metaphase.

CDK Inhibitors: Negative Regulators of the Cell Cycle

Two classes of CDK inhibitors have been described—the INK4 proteins, originally named for their ability to **in**hibit CD**K4**, and the Cip/Kip family.[27,28] The INK4 family comprises 4 proteins, namely $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$. Structurally these proteins are made up of multiple ankyrin repeats and bind only to the catalytic subunits CDK4 and CDK6, causing their dissociation from cyclin D.

The second class of CDK inhibitors is the Cip/Kip family and includes $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$, which share a conserved N-terminal CDK-binding domain.[28] They are capable of binding a wider range of targets and can variably effect the activities of cyclin D–, E–, A–, and B–dependent kinases.[27,29,30] In contrast to the INK4 family, the Cip/Kip proteins do not dissociate cyclin-CDK complexes. Although potent inhibitors of cyclin E– and A–dependent CDK2, and to a lesser extent Cdc2, the Cip/Kip proteins also have recently been characterized, paradoxically, as positive regulators of the cyclin D–dependent kinases.[31]

$p21^{Cip1}$ was the first member of the family to be identified,[32-34] and is usually present at a low level in quiescent cells. As the cell enters the replicative cycle, $p21^{Cip1}$ levels increase, displace INK4 proteins from binding to CDK 4/6, and promote the assembly of cyclin D–CDK complexes.[35,36] This stabilizes the active complex and additionally provides a nuclear localization signal, enhancing nuclear import of cyclin D.[31] Furthermore, $p21^{Cip1}$ blocks the interaction between cyclin D1 and the exportin CRM1, leading to increased nuclear levels of active cyclin D.[37] The transcription of $p21^{Cip1}$ is increased by both p53-dependent and -independent pathways.[38,39] The inhibitory role of $p21^{Cip1}$ becomes dominant later in the cell cycle, probably owing to the higher concentration of $p21^{Cip1}$, and levels also are increased in senescent cells.[40]

In contrast to $p21^{Cip1}$, the level of $p27^{Kip1}$ is usually high in quiescent cells, where its primary role is as an inhibitor of cell division.[41,42] Whereas $p21^{Cip1}$ is a principal mediator of the p53-dependent G_1 arrest that occurs after DNA damage,[43] $p27^{Kip1}$ appears to be responsible primarily for mediating extracellular antiproliferative signals.[41,42] The levels and activity of $p27^{Kip1}$ are regulated posttranscriptionally by changes in the rates of translation, ubiquitination, and phosphorylation.[44,45] As cyclin D levels increase in response to mitogens, both $p21^{Cip1}$ and $p27^{Kip1}$ are sequestered by cyclin D–CDK complexes and therefore are unable to inhibit CDK2.[27]

$p21^{Cip1}$ and $p27^{Kip1}$ appear to perform a number of other functions within the cell, dependent on their subcellular localization.[46] In the majority of cell types studied, both proteins protect from apoptosis, and for $p21^{Cip1}$ this appears to be associated with cytoplasmic translocation. A role in the regulation of cell motility also has been described. Synthetic peptides derived from $p21^{Cip1}$ dissociate the integrin receptor from focal adhesion contacts and thus inhibit cell spreading mediated by integrin signaling.[47] Cytoplasmic $p21^{Cip1}$ inhibits Rho kinase in neurites and prevents the formation of stress fibers.[48] In the nucleus, several studies suggest that the Cip/Kip proteins function as transcriptional cofactors.[46]

The most recently identified member of the family, $p57^{Kip2}$, was cloned in 1995.[49,50] Although tissue expression of $p21^{Cip1}$ and $p27^{Kip1}$ is widespread, that of $p57^{Kip2}$ is restricted to placenta, muscle, heart, brain, lung, and kidney. In addition to the CDK inhibitory domain and putative C terminal nuclear localization signal, $p57^{Kip2}$ also has proline-rich domain containing a consensus extracellular signal-related protein kinase phosphorylation site and an acidic domain, the functions of which are not known.[49,50] A role for $p57^{Kip2}$ in the cell cycle exit that accompanies terminal differentiation has been suggested.

Despite their structural similarities, knock-out studies have shown divergent roles for the 3 Cip/Kip CDK inhibitors. $p21^{Cip1}$ and $p27^{Kip1}$ are not essential for normal embryogenesis,[51-53] however, lack of $p57^{Kip2}$ results in profound developmental abnormalities.[54-56] Most $p57^{Kip2}$ null mice die shortly after birth and have severe cleft palates, abdominal wall and gastrointestinal tract defects, and abnormal skeletal ossification. Unlike adult $p21^{Cip1}-/-$ mice,[51,57] $p27^{Kip1}-/-$ mice are larger than wild-type animals and have hyperplasia of organs that usually express high levels of $p27^{Kip1}$, such as the thymus, spleen, adrenal and pituitary glands, testes, and ovaries.[52,53] In contrast, less than 10% of $p57^{Kip2}-/-$ mice survive the weaning period and are much smaller than their wild-type littermates.[56] The glomerular development of these mice appears normal.

Checkpoint Regulation

Checkpoints during the cell cycle prevent progression if DNA is damaged or incorrectly replicated.[19,58] Mammalian cells can arrest in G_1, S, or G_2 phase, allowing either DNA repair or apoptosis. The molecular events that detect damaged DNA remain elusive but lead to the activation of PIK-related proteins including ataxia-telangiectasia mutated (ATM) and AT and Rad3-related (ATR) kinases. Two checkpoint kinases, Chk1 and Chk2, link ATM and ATR to downstream targets such as Cdc25 and p53 to cause cell cycle arrest.

THE ROLE OF CELL CYCLE PROTEINS IN GLOMERULAR DISEASE

Diseases of the Mesangial Cell

Mesangial Cell Proliferation

Mesangial cell proliferation characterizes many forms of glomerular disease, including immunoglobulin A nephropathy, lupus nephritis, and diabetes.[59,60] The significance of mesangial cell proliferation and the accompanying accumulation of extracellular matrix have been shown in a variety of experimental models of glomerular disease.[61,62] Interventions to reduce mesangial proliferation, such as complement depletion,[63,64] heparin infusion,[65] blocking the action of the cytokines platelet-derived growth factor[66,67] and basic fibroblast growth factor,[62] and inhibiting their specific intracellular signaling pathways with phosphodiesterase inhibitors,[68] also reduces matrix expansion and subsequent glomerulosclerosis. Thus, the mechanisms regulating mesangial cell proliferation are of critical importance.

Role of CDK2 in mesangial cell proliferation. The Thy1 model of experimental mesangial proliferative glomerulonephritis, induced in rats by an antibody directed against the mesangial Thy1 antigen, has provided an opportunity to study the regulation and consequences of mesangial cell proliferation in vivo.[69,70] The initial complement-dependent mesangiolysis is followed by a phase of marked mesangial proliferation, paralleled by an increase in extracellular matrix accumulation and a decline in renal function. This model is useful because not only may the fluctuations of cell cycle proteins during proliferation be defined, but also the effect of their manipulation. Mesangial cell proliferation is associated with an increase in cyclins D1 and A, and their partners CDK4 and CDK2.[71]

CDK2 expression is absent in the normal rat glomerulus, in contrast to the majority of nonrenal cells in vitro where CDK2 is expressed constitu-

tively throughout the cell cycle.[71] Experimental mesangial proliferative glomerulonephritis is associated with increased CDK2 activity, measured by the histone H1 kinase assay on protein extracted from isolated glomeruli. CDK2 protein levels also are also increased in the remnant kidney model, a nonimmune glomerular disease associated with mesangial proliferation.[72] Finally, CDK2 protein and kinase activity increase in cultured mesangial cells in response to growth factors.[73,74] Taken together, these studies show that in contrast to most nonrenal cells, CDK2 protein is in low abundance in quiescent mesangial cells, and its levels and activity increase after injury.

To determine the role of increased CDK2 activity in inflammatory disease, we inhibited CDK2 activity in Thy1 rats with the purine analogue, roscovitine.[75] Our results showed that roscovitine given either immediately after disease induction or once mesangial proliferation was established both significantly reduced mesangial cell proliferation, indicating a role for CDK2 in the perpetuation of inflammatory injury. Of interest was that inhibiting CDK2 activity caused a marked reduction in glomerular extracellular matrix proteins (collagen IV, laminin, and fibronectin) and an improvement in renal function compared with controls. These results suggest that inhibiting CDK2 may be a potential therapeutic target in glomerular diseases characterized by proliferation.

Role of CDK inhibitors in mesangial cell proliferation. The CDK-inhibitor $p27^{Kip1}$ is expressed constitutively in quiescent mesangial cells both in vitro[76] and in vivo,[71] whereas $p21^{Cip1}$ and $p57^{Kip2}$ are essentially absent.[71,77] In cultured mesangial cells, proliferation induced by mitogenic growth factors reduces $p27^{Kip1}$ levels.[76] Mesangial cells derived from $p27^{Kip1}-/-$ mice have augmented proliferation in response to mitogens,[78] and decreasing $p27^{Kip1}$ levels with antisense has a similar effect in rat mesangial cells.[76]

Complement (C5b-9)-induced injury in the Thy1 model is associated with a marked decrease in $p27^{Kip1}$ levels.[71] However, there is de novo synthesis of $p21^{Cip1}$ in the resolution phase of the disease, coincident with a decrease in proliferation. To explore further the role of $p27^{Kip1}$ in inflammatory disease, we induced experimental glomerulonephritis in $p27^{Kip1}-/-$ mice.[79] Our results showed a marked increase in the onset and magnitude of glomerular cell proliferation and cellularity in nephritic $p27^{Kip1}-/-$ mice compared with control nephritic $p27^{Kip1}+/+$ mice. Moreover, this was associated with increased extracellular matrix proteins and a decline in renal function. To show that this result was not specific to glomerular cells or immune-mediated injury, we also obstructed a ureter by ligation to induce nonimmune injury to tubuloepithelial cells.[79] Our results showed that tubuloepithelial proliferation was increased in obstructed $p27^{Kip1}-/-$ mice compared with obstructed $p27^{Kip1}+/+$ mice. In summary, these studies showed that renal cell proliferation is regulated by the CDK inhibitor $p27^{Kip1}$, supporting a role for $p27^{Kip1}$ in controlling the threshold at which proliferation occurs.

Mesangial Cell Apoptosis

Although a characteristic response to mesangial cell injury is proliferation, apoptosis often is increased simultaneously.[80] Studies have shown that apoptosis is a vital mechanism required to normalize cell number in the reparative phase of injury.[81] However, the cellular pathways linking these opposing responses remain unclear. We reasoned that many cells undergoing apoptosis have also entered the cell cycle, before exiting by apoptosis. Thus, we predicted that cell cycle proteins have a critical role in both these processes.

Evidence to support this hypothesis was the observation that the resolution phase of Thy1 mesangial proliferation in the rat is characterized by mesangial cell apoptosis, a process that peaks when the levels of $p27^{Kip1}$ are at their lowest.[71] Considering glomerulonephritis and ureteral obstruction in $p27^{Kip1}-/-$ mice, in addition to the increase in glomerular cell proliferation after injury as described earlier, there was a marked increase in glomerular cell apoptosis in $p27^{Kip1}-/-$ mice compared with wild-type disease controls.[79] Moreover, apoptosis was also increased in $p27^{Kip1}-/-$ mesangial cells in culture after growth factor deprivation or cycloheximide, and reconstituting $p27^{Kip1}$ levels by transfection rescued cells from apoptosis.[78] In rat mesangial cells, apoptosis was also increased after treatment with anti-$p27^{Kip1}$ antisense oligonucleotides.[78] Taken together, these results confirm a role for $p27^{Kip1}$ beyond the regulation of proliferation in that it also protects both renal and nonrenal cells from apoptosis.

A clue to a possible mechanism whereby $p27^{Kip1}$ protects cells from apoptosis was the increase in CDK2 activity in $p27^{Kip1}-/-$ mesangial cells when deprived of growth factors.[78] The increase was due specifically to cyclin A-CDK2, and not cyclin E-CDK2. Moreover, inhibition of cyclin A-CDK2 activity by roscovitine or a dominant-negative mutant reduced apoptosis in mesangial cells and fibroblasts. In apoptotic $p27^{Kip1}-/-$ mesangial cells, CDK2 was bound to cyclin A, without a preceding increase in cyclin E-CDK2 activity. We suggest that uncoupling of CDK2 activity from the scheduled sequence of cell cycle protein expression may lead to an inappropriate and premature initiation of G_1/S phase transition, causing the cell to respond by undergoing apoptosis.

How might CDK2 control the growth and apoptotic fate of cells? Apoptosis typically begins in the cytoplasm, whereas DNA synthesis and mitosis are nuclear events. Accordingly, we tested the hypothesis that the subcellular localization of CDK2 determines if cells undergo apoptosis or proliferation.[82] As expected, CDK2 protein was cytoplasmic in quiescent, and nuclear in proliferating, mesangial cells. However, in proliferating cells injured by an apoptotic stimulus, CDK2 localized to the cytoplasm, and was no longer nuclear. Our results also showed that cyclin A, and not cyclin E, colocalized to the cytoplasm with CDK2 in apoptotic cells, to form an active cyclin A–CDK2 complex. The translocation of CDK2 is not p53 dependent, and inhibiting the nuclear localization signal has no effect. That inhibiting CDK2 decreased apoptosis provides further support for a critical role for cytoplasmic CDK2 in triggering programmed cell death. Thus, the subcellular localization of active CDK2 determines the fate of a cell: when nuclear, cells proliferate; when cytoplasmic, cells die by apoptosis. The mechanism by which nuclear CDK2 is translocated to the cytoplasm remains to be elucidated. These studies support the paradigm that specific cell cycle regulatory proteins have a role in glomerular disease beyond the regulation of proliferation.

Mesangial Hypertrophy: The Hallmark of Diabetic Nephropathy

Diabetes mellitus is now the most frequent cause of end-stage renal failure in Western countries.[83] The hallmarks of glomerular involvement are similar in both type I and type II diabetes and include mesangial cell hypertrophy and podocyte loss.[84-86] On entry into G_1, cells undergo a physiologic increase in protein synthesis before the DNA synthesis of S phase, and therefore one mechanism underlying cellular hypertrophy is arrest of the cell cycle at the G_1/S checkpoint. The increase in cell protein content is not matched by a concomitant increase in DNA. Research has concentrated principally on the ability of hyperglycemia to induce mesangial cell hypertrophy, however, it has been shown in aging rhesus monkeys that glomerular enlargement and other structural changes of diabetic nephropathy begin in the prediabetic hyperinsulinemic phase.[87]

In vitro culture of mesangial cells in high glucose media causes cell cycle entry and a biphasic growth response.[88] After initial proliferation, the cells arrest in G_1 phase and there is progressive hypertrophy. Both kidney and glomerular hypertrophy induced by hyperglycemia are associated with an early and sustained increase in expression of cyclin D1 and activation of CDK4.[89] An arrested cell cycle suggests a role for the CDK inhibitors in mediating hypertrophy, and indeed high glucose increases the levels of both $p21^{Cip1}$[90] and $p27^{Kip1}$[91] in cultured mesangial cells. This is mediated by a number of factors including glucose itself, transforming growth factor β (TGF-β), which then acts in an autocrine fashion[92,93] and connective tissue growth factor.[94] High glucose directly stimulates the transcription of $p21^{Cip1}$,[95] and activates MAP kinases, which prolong the half-life of $p27^{Kip1}$ by phosphorylation on serine residues.[96] Lowering $p21^{Cip1}$ (unpublished observations) or $p27^{Kip1}$[91] levels with antisense oligonucleotides reduces the hypertrophic effects of high glucose. Moreover, hypertrophy is not induced by high glucose in $p21^{Cip1}-/-$ (unpublished observations) and $p27^{Kip1}-/-$[97] mesangial cells in vitro. Indeed, high glucose increases proliferation in $p27^{Kip1}-/-$ mesangial cells, whereas it arrests cell cycle progression in $p27^{Kip1}+/+$ mesangial cells.[97] Reconstituting $p27^{Kip1}$ levels in $p27^{Kip1}-/-$ mesangial cells by transfection restores the hypertrophic phenotype.[97] These studies show a compelling role for the CDK-inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ in mediating the hypertrophy induced by high glucose.

These in vitro findings have been confirmed in experimental models of both type I and type II

diabetic nephropathy. Considering type I diabetes, the glomerular protein levels of $p21^{Cip1}$ are increased in the streptozotocin-induced model in the mouse,[90] and both $p21^{Cip1}$ and $p27^{Kip1}$ levels are increased in the glomeruli of diabetic BBdp rats.[98] A similar increase was noted in glomeruli of *db/db* mice[95] and the Zucker diabetic fatty rat,[99] models of type II diabetic nephropathy. Diabetic $p21^{Cip1}-/-$ mice are protected from glomerular hypertrophy.[100] The importance of $p21^{Cip1}$ has also been shown in the development of glomerular hypertrophy and subsequent sclerosis after partial renal ablation. $p21^{Cip1}-/-$ mice develop glomerular hyperplasia rather than hypertrophy, with protection from the development of progressive renal failure.[101] Diabetic $p27^{Kip1}-/-$ mice have only mild mesangial expansion and no glomerular or renal hypertrophy compared with control diabetic $p27^{Kip1}+/+$ mice, despite up-regulation of glomerular TGF-β.[102] These results support a critical role for the CDK inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ in mediating the glomerular hypertrophy seen not only in association with diabetes but also with a reduction in nephron number.

The cytokine TGF-β has been shown to mediate progressive fibrosis in renal disease.[103-105] TGF-β also decreases proliferation in mesangial cells, an effect that appears to be independent of $p21^{Cip1}$ and $p27^{Kip1}$, and induces cell hypertrophy.[106-108] To determine the role of CDK inhibitors in mediating the hypertrophic effects of TGF-β, we used mesangial cells from single and double $-/-$ mice.[108] Compared with wild type, hypertrophy was reduced significantly in double $p21^{Cip1}/p27^{Kip1}-/-$ mesangial cells. A less marked reduction in hypertrophy was seen in the single $p21^{Cip1}-/-$ and $p27^{Kip1}-/-$ cells. These results show that although $p21^{Cip1}$ and $p27^{Kip1}$ each contribute to the hypertrophic action of TGF-β, the presence of both is required for maximal effect.

The expression of CDK inhibitors has also been explored in response to connective tissue growth factor, considered to be a principle mediator of the downstream effects of TGF-β. Abdel Wahab et al[94] showed that connective tissue growth factor is a hypertrophic factor for human mesangial cells, and that this hypertrophy is associated with the induction of $p15^{INK4b}$, $p21^{Cip1}$, and $p27^{Kip1}$, with the maintenance of pRb in a hypophosphorylated state.

Diseases of the Podocyte

Podocytes, also called "visceral glomerular epithelial cells," are highly specialized cells overlying the outer aspect of the glomerular basement membrane.[109] Their complex cytoarchitecture is believed to allow them to counteract the high perfusion pressure of the glomerular capillaries and prevent vascular distension. Additionally, their interdigitating foot processes are connected by the slit diaphragm, enabling the selective permeability of glomerular filtration. Many diverse causes of podocyte injury are associated with foot process effacement and disruption of the slit diaphragm, leading to the characteristic clinical finding of proteinuria.[110] In marked contrast to mesangial and glomerular endothelial cells, podocytes do not typically proliferate in vivo. After injury, podocyte number may become depleted, with the lack of proliferation resulting in areas of denuded basement membrane. This is thought to result in the development of scarring, known as focal glomerulosclerosis, and chronic renal impairment.[111,112] Thus, the mechanisms regulating podocyte proliferation are being recognized increasingly as essential to our understanding of progressive renal disease, irrespective of the initiating injury.

Podocyte Development

During glomerulogenesis, immature podocytes are capable of proliferation.[77,113] However, during the critical S-shaped body stage of glomerular development, podocytes exit the cell cycle to become terminally differentiated and quiescent, necessary requirements for normal function. Thus, in podocytes, proliferation and differentiation are closely linked closely, akin to neurons and cardiac myocytes. In both mouse[77] and human[113] glomerulogenesis, immunostaining for $p27^{Kip1}$ and $p57^{Kip2}$ is absent from proliferating podocytes during the S-shaped body stage. On cessation of proliferation, there is strong expression of both CDK inhibitors, so that $p27^{Kip1}$ and $p57^{Kip2}$ are expressed constitutively in mature podocytes. Although glomerular development and differentiation appear normal in $p27^{Kip1}-/-$ and $p57^{Kip2}-/-$ mice, E13.5 metanephroi from double $p27^{Kip1}/p57^{Kip2}-/-$ mice have significantly larger glomeruli than both wild-type and the single mutants.[114] This increase in size is associated with an increase in podocyte number, with apparently normal terminal differentiation (as determined by electron microscopy and staining

for WT-1 and synaptopodin), suggesting that $p27^{Kip1}$ and $p57^{Kip2}$ are involved synergistically in determining the final complement of podocytes.

Immune-Mediated Podocyte Injury

The passive Heyman nephritis (PHN) model, induced by the administration of an antibody reactive against the F×1A antigen on the rat podocyte, has many similarities to human membranous nephropathy.[115,116] In common with the Thy1 model of mesangial proliferative glomerulonephritis, PHN is complement (C5b-9) dependent. However, in contrast to the observed proliferation of mesangial cells after immune injury, podocyte number remains constant, and decreases with time, suggesting a role for the cell cycle inhibitors in experimental podocyte disease.

We began by asking if podocytes are capable of increasing the cyclins and CDKs required for proliferation. After C5b-9–induced injury in PHN rats, protein levels for cyclin A and CDK2 increase.[117] However, only a limited increase in DNA synthesis occurs, suggesting the presence of an inhibitor to cell cycle progression rather than a failure to engage the cell cycle per se. Indeed, the levels of the CDK inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ increase specifically in podocytes after the induction of PHN, and studies showed that these inhibitors complex with cyclin A-CDK2.[117] The increase in $p21^{Cip1}$ is attenuated by administering the mitogen basic fibroblast growth factor to PHN rats, and this augments the increase in podocyte DNA synthesis.

A key role for $p21^{Cip1}$ in limiting the proliferative response of podocytes has been shown in studies using $p21^{Cip1}$ knock-out mice.[118] The administration of an antiglomerular antibody to induce experimental podocyte injury caused podocyte dedifferentiation and proliferation in $p21^{Cip1-/-}$ mice compared with control mice receiving the same antibody. Glomerular extracellular matrix accumulation was also increased in $p21^{Cip1-/-}$ mice, and was associated with a significant decrease in renal function.

The role of $p57^{Kip2}$ remains enigmatic owing to the lack of a viable knockout. Podocyte $p57^{Kip2}$ protein levels are decreased in PHN rats, and in antiglomerular antibody disease in the mouse, loss of expression predominantly localizes to proliferating podocytes, as determined by proliferating cell nuclear antigen (PCNA) expression.[77]

We have considered that the inability of podocytes to proliferate may be owing primarily to impaired DNA synthesis. Our data show that the lack of proliferation after complement (C5b-9) induced podocyte injury also involves a defect in mitosis. When cultured podocytes are exposed to sublytic C5b-9 attack, the cell cycle is engaged. However, there is a delay or inhibition in entering mitosis. This focused our attention on the possibility of a block in the G_2/M transition, and mechanisms that regulate this checkpoint. Exposing cultured podocytes to antibody and a complement source caused a marked increase in the cell cycle inhibitor p53, which was accompanied by an increase in $p21^{Cip1119}$. An increase in p53 and $p21^{Cip1}$ in vivo was also shown in the PHN model of C5b-9–induced podocyte injury.[120] This raised the possibility of DNA damage, and indeed, we have shown that sublytic C5b-9 causes DNA damage in podocytes in vitro and in vivo.[120] Moreover, C5b-9 increased the levels of checkpoint kinase-1 and -2 protein levels, which have been shown to arrest cells at G_2/M. Further studies are needed to explore the role of DNA damage in glomerular disease.

Podocyte Proliferation

Although the vast majority of human podocyte diseases are not associated with proliferation, exceptions are idiopathic collapsing glomerulonephritis and human immunodeficiency virus–associated nephropathy.[121,122] In these diseases, there is increased expression of cyclin A and the proliferation marker Ki-67, with a marked reduction in $p27^{Kip1}$ and $p57^{Kip2}$. In vitro, human immunodeficiency virus 1 induces cyclin D1 expression and pRb phosphorylation in differentiated mouse podocytes, accompanied by increased proliferation and a loss of the differentiation markers synpatopodin and podocalyxin.[123,124] In contrast, all other diseases of podocytes in humans, including membranous nephropathy, minimal change disease, and focal segmental glomerulosclerosis, are not associated with a decrease in CDK inhibitor levels, and typical markers of proliferation are absent.[121] Taken together, these studies further support a critical role for the CDK inhibitors $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$ in determining the podocyte response to injury.

Table 1. Glomerular Expression of Cell Cycle Related Proteins

		Cyclin CDK			CDK Inhibitor			
	Experiment	Increased	Decreased	No Change	Increased	Decreased	No Change	Reference
Mesangial cell								
Proliferation	In vitro exposure to mitogens (platelet-derived growth factor, basic fibroblast growth factor)	D1, E, A CDK4, 2			p21	p27	p57	73, 74, 76
	Animal models							
	Thy 1	D1, E CDK4, 2			p15, p21	p27		71
	Remnant kidney	E CDK2		CDK4				72
Suppression of proliferation	In vitro exposure to anti-mitogens (TGF-β, SPARC, heparin)		D1, E, A CDK4, 2		p15, p21, p27			73, 92, 133
Hypertrophy	In vitro exposure							
	Glucose			CDK2	p21, p27			90, 91
	TGF-β				p16, p21, p27			98,134
	Connective tissue growth factor				p15, p21, p27			94
	Animal models							
	Type I diabetes (Streptozotocin mouse, BBdp rat)			E, A	p21, p27			90, 98
	Type II diabetes (*db*/*db* mouse, Zucker rat)			CDK2	p27			95, 99
Podocyte								
Apoptosis	Cytoplasmic location of CDK2							82
Immune injury	In vitro exposure to C5b-9				p21			119
	Animal models							
	PHN	A, CDK2			p21	p57		117,120
	Antiglomerular antibody	A, CDK2			p21, p27	p57		133
Proliferation	In vitro exposure to human immunodeficiency virus 1	D_1						124
	Human disease							
	Collapsing GN	A	D_1			p27, p57		121, 122
Response to stretch	In vitro		D, A, B		p21,p27,p57			128

Abbreviations: SPARC, secreted protein acidic and rich in cysteine; GN, glomerulonephritis.

Table 2. Data From Knock-Out Mice

	Experiment	Outcome	Reference
p21−/−	Mesangial cells exposed to high glucose in vitro	Resistant to hypertrophy	Unpublished data
	Animal models		
	Streptozotocin diabetes	Reduced glomerular hypertrophy	100
	Antiglomerular antibody	Increased podocyte proliferation	118
	Remnant kidney	Glomerular hyperplasia, not hypertrophy	101
p27−/−	Mesangial cells exposed to high glucose in vitro	Resistant to hypertrophy	97
	Mesangial cells deprived of growth factors	Increased apoptosis	78
	Animal models		
	Streptozotocin diabetes	Reduced glomerular hypertrophy	102
	Antiglomerular antibody	Increased mesangial cell proliferation and apoptosis	79
p57−/−	Unmanipulated animal	Normal glomerulogenesis in embryonic mice	54-56

Role of CDK Inhibitors in Podocytes Injured by Mechanical Stress

Independent of the site of initial injury, a common pathway to progressive glomerulosclerosis is an increase in intraglomerular pressure, also known as *glomerular hypertension*.[111,112,125] Lowering intraglomerular pressure reduces progression in a number of glomerular diseases, including diabetic nephropathy.[126] Glomerular hypertension is associated with glomerular hypertrophy, and the resultant mechanical stretch causes injury to all 3 glomerular cell types. Studies by others have shown that applying mechanical stretch to cultured mesangial cells increased proliferation.[127] In contrast, we have shown that mechanical stretch decreases podocyte proliferation.[128]

Our recent studies showed that stretch decreased the levels of cyclins D1, A, and B1, and Cdc2 in cultured mouse podocytes.[128] In contrast, there was an increase in the CDK inhibitors. Stretch caused an early increase in $p21^{Cip1}$, followed by an increase in $p27^{Kip1}$ at 24 hours and $p57^{Kip2}$ at 72 hours. In contrast to the growth arrest seen in wild-type cells exposed to stretch, $p21^{Cip1}-/-$ podocytes exposed to stretch continued to proliferate. These results suggest a role for CDK inhibitors in limiting the podocyte's proliferative capacity after stretch.

In vitro and in vivo data describing the expression of cell cycle–related proteins in mesangial cells and podocytes are summarized in Table 1 and the results of experiments with knock-out mice in Table 2.

Diseases of Glomerular Endothelial Cells

The endothelial cells of mature glomeruli are quiescent but retain the capacity to proliferate and form new capillaries after injury.[129] The degree of proliferation appears to be dependent on the local balance of pro-angiogenic factors, such as vascular endothelial growth factor and anti-angiogenic factors, such as thrombospondin-1. An inadequate proliferative response may lead to loss of the glomerular microvasculature and contribute to glomerulosclerosis and progressive renal impairment. The beneficial effects of administration of vascular endothelial growth factor have been shown in animal models of acute glomerulonephritis,[130-132] suggesting that amelioration of human diseases may be achieved by augmenting the reparative potential of the glomerular endothelial cells. However, the underlying role of individual cyclins, CDK, and CDK inhibitors in these cells remains unknown.

CONCLUSIONS

Our understanding of the function of specific cell cycle regulatory proteins in both the normal glomerulus and disease states is increasing, and a large body of literature points toward novel actions of specific cell cycle regulatory proteins beyond their classic role in the control of proliferation. The expression and function of particular cell cycle proteins may be specific to each type of glomerular cell, and may likely also depend on the form of cell injury. The apparently diverse effects of cell cycle regulatory proteins in glomerular cell proliferation,

apoptosis, hypertrophy, and differentiation makes them a particularly appealing target for future therapeutic interventions because their manipulation offers an opportunity to influence several potentially deleterious outcomes. The challenges that lie ahead include the development of strategies that will permit targeted intervention.

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