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Frontiers in Research: Chronic Kidney Diseases: The pivotal role of pericytes in kidney fibrosis

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SUMMARY

1. Kidney pericytes were recently identified as collagen-Iα1 producing cells in healthy kidney, but the developmental, physiological and pathological roles of kidney pericytes remain poorly understood. Pericytes are stromal-derived cells that envelop, and have intimate connections with adjacent capillary endothelial cells (ECs). Recent studies in eye and brain have revealed that pericytes are crucial for angiogenesis, vascular stability and vessel integrity.
2. In response to kidney injury, pericytes promptly migrate away from the capillary wall into the interstitial space. Here, pericytes are activated and differentiate into scar-forming myofibroblasts. In the absence of pericytes, peritubular capillaries are destabilized leading to vascular regression. Consequently, capillary loss and fibrosis following kidney injury are intimately linked and hinge centrally around pericyte detachment from ECs.
3. Kinetic mathematical modeling demonstrated that pericytes are the major source of myofibroblasts in fibrotic kidney. Comprehensive genetic fate mapping studies of nephron epithelia or kidney stroma has demonstrated that epithelial cells do not migrate outside of epithelial compartment to become myofibroblasts rather that interstitial pericytes are progenitors of scar-forming myofibroblasts. Bidirectional signaling between pericytes and ECs is necessary for pericyte detachment from peritubular capillaries.
4. In the present review, we summarize the pathologically vital roles of kidney pericytes in fibrosis including our new findings. The study of kidney pericytes and endothelial-pericyte crosstalk will identify novel therapeutic targets for currently incurable chronic kidney diseases.

Keywords

capillary rarefaction; chronic kidney diseases; endothelial cells; epithelial-to-mesenchymal transition (EMT); fibrosis; kidney pericytes; kidney injury; myofibroblasts; peritubular capillary

INTRODUCTION

Chronic kidney disease (CKD) is a common pathway of injury responses initiated by many different diseases. It is characterized by a progressive loss of renal function, atrophy of tubules, inflammation (recruitment of leukocytes), microvascular rarefaction and deposition of extracellular matrix (ECM) in the interstitial space between nephrons, leading to

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widespread tissue fibrosis. The extent and severity of fibrosis most strongly predicts functional deterioration. Regardless of initiating events, interstitial fibrosis is a remarkably consistent process characterized by expansion of cells known as myofibroblasts, that are not observed in healthy kidney. Myofibroblasts are fibroblast-like cells of the interstitial space expressing smooth muscle proteins and are the principle effector cells responsible for excess deposition of interstitial ECM under pathological conditions. The origin of myofibroblasts has been intensely investigated for the past twenty years. Clarifying their origin, functions and mechanisms of proliferative expansion will lead to development of new therapies for currently incurable progressive CKD. In exciting new studies the kidney pericyte, a cell-type that has been neglected by the nephrology community, has been identified as the major source of myofibroblasts, and for the first time has uncovered a comprehensive understanding of the link between organ fibrosis and microvascular rarefaction. The present review focuses on (1) the structure of pericytes, (2) the contribution of pericytes to development of kidney fibrosis and (3) the origins of myofibroblasts.

PERICYTE STRUCTURE

Pericytes are defined as extensively branched cells located on the abluminal side of endothelial cells (ECs) in microvasculature (capillaries, precapillary arterioles, postcapillary venules and collecting venules)[1], partially embedded within the microvascular basement membrane (BM), and incompletely enveloping ECs with which they establish specific and focal contacts. Pericytes are cells of mesenchymal origin and may be distinguished from other vascular mural cells such as perivascular fibroblasts and vascular smooth muscle cells (vSMCs), which surround larger blood vesseles and do not make contact with ECs[2, 3]. Pericytes in kidney were first unequivocally identified in 1983 using electron microscopy[4]. Kidney pericytes are sheathed, fully or in part, with microvascular BM and thus fulfill the general anatomical definition of pericytes. The sheathing is a duplication of microvascular BM, which is often found to be incomplete between pericytes and ECs hereby enabling direct intercellular crosstalk to occur (Figure 1). These gaps in BM frequently coincide with adhesion plaques and variants of pericyte projections, which are believed to be sites of cell-cell signaling. Those signaling sites are called ‘peg and socket’ junctions[2, 4], and may contain tight-, gap- and adherence junctions[2] (Figure 1). The ratio of pericyte to EC is 1:1 in the retina, 1:2.5 in the kidney, indicating that single kidney pericyte partially surrounds and has contacts with multiple ECs and that not all of the kidney pericytes are completely within microvascular BM. Pericytes are widely believed to contribute direclty to capillary BM synthesis including laminins and collagens[5]. In contrast to pericytes, vSMCs are segregated from adjacent ECs by the internal elastic lamina in the vascular wall[2, 3]. In kidney, some pericytes span from peritubular capillary (PTC) to the tubule with processes abutting tubular BM and may have capability of signaling bilaterally with ECs as well as tubular epithelia[3, 6].

Pericytes should also be distinguished from mesangial cells and podocytes of the kidney glomerulus. The ECs of glomerular capillary loops have intimate connections both with podocytes and mesangial cells which are reported to be essential for physiological angiogenesis, maintenance of glomerular capillary[7–9] and filtration barrier, suggesting that these glomerular cells are specialized types of pericytes.

Fibroblasts are spindle-shaped cells of mesenchymal origin, embedded in ECM and recognized as a cell type that synthesizes ECM including collagen. They share developmental origins with pericytes and share many markers. Pericytes and fibroblasts are distinguished by anatomical connections with ECs through cell processes within capillary BM (Figure 1), because fibroblasts do not directly interact with ECs. Currently there are no definitive molecular markers that distinguish fibroblasts from pericytes, although pericytes are typically rich in vascular growth factors including vascular endothelial growth factors

and angiopoietins. Our own studies which have combined modern mouse genetics with high quality electron microscopy suggest that although other investigators have previously reported fibroblasts in normal kidney[10, 11], the vast majoriy of these cells are in fact pericytes[12–14].

PERICYTE FUNCTION

For more than 100 years since pericytes were described[15, 16], pericytes have been considered to be contractile cells regulating vascular tone and blood flow, because anatomically the processes of pericytes envelope ECs and pericytes express contractile microfilaments (actin, myosin) and intermediate filaments (desmin, vimentin)[17–19]. Definitive functional studies have proved that pericytes control capillary diameter in whole retina and cerebellar slices[20]. Emerging data, however, indicate that pericytes are much more heterogeneous and potent than previously believed. For example, two groups, independenly, showed that pericytes regulate the formation of tight junction and transcytosis in brain capillary ECs and absolute pericyte coverage is essential for EC integrity in blood-brain bareer during development[21, 22]. In addition, pericytes in other tissue beds have been shown both to stabilize capillaries and prevent capillary regression and rarefaction[23–28]. The molecular mechanisms by which pericytes mediate vascular stabilization are poorly studied and heterogeneity in pericyte populations within tissues and across different tissue beds complicates understanding these mechanisms. Such heterogeneity, combined with developmental studies indicate that pericytes are derived from local tissue–specified mesenchyme rather than a single progenitor.

Functional *ex vivo* experiments using three dimensional (3D) collagen gel assays have begun to dissect the impact of pericyte coverage on integrity of ECs in microvasculature[25]. Such assays may serve as useful tools to uncover the molecular mechanisms responsible for microvascular loss following acute kidney injury and in CKD. Intriguingly, recent studies have indicated similarities between pericytes and mesenchymal stem cells (MSCs)[29, 30]. Pericytes isolated from multiple human organs have been shown to possess osteogenic, chondrogenic and adipogenic potentials and express MSC markers[29]. Indeed, bone marrow MSCs attach to microvascular tubes and stabilize vessels, as do pericytes, therefore MSCs may be pericytes of bone marrow[31].

THE ROLE OF PERICYTES IN KIDNEY FIBROSIS

In 2008, pericytes and perivascular fibroblasts were identified as the primary source of interstitial myofibroblasts in the fibrotic kidney[12]. The investigators used transgenic mice expressing green fluorescent protein (GFP) in cells producing the collagen type I, α1 (*Coll1a1*) transcript (*Coll-GFP* mice). In addition to podocytes and perivascular fibroblasts, they detected GFP signaling in cells lining peritubular capillaries, where GFP^+ cells are located in subendothelial region in direct apposition with $CD31^+$ ECs and exhibit intimate areas of adhesion with ECs suggestive of ‘peg and socket’ junctions. From these characteristics they concluded peritubular GFP^+ cells are kidney pericytes[12]. In post-natal kidneys (P12), these GFP^+ cells expressed typical pericyte markers:platelet-derived growth factor receptor-β (PDGFR-β), CD44, CD90, NG2, CD73 and αsmooth muscle actin (αSMA). However, many pericyte markers were downregulated as the kidney matured and kidney pericytes became less active. When *Coll-GFP* mice were subjected to kidney injury induced by unilateral ureteral ligation (UUO), a robust and standardized model for inducing inflammatory fibrosis, *Coll1a1* producing pericytes: (1) promptly reacvtivated genes associated with post-natal pericytes and myofibroblasts; (2) detached themselves from PTC and migrated away from capillaries into the interstitial space; (3) up-regulated collagen production and other matrix proteins (Figure 1)[12, 13]. These events occurred in advance of

pericyte proliferation. Using a mathematical model to study the expansion of interstitial *Coll1a1* producing pericytes by analysis of cell number and cell cycle *in vivo*, the study indicated that the proliferation of the initial population of pericytes that detached themselves from capillaries in response to kidney injury could sufficiently explain the appearance of myofibroblasts observed in progressive fibrosis of the kidney[12]. Strikingly, although kidney was remarkably fibrotic, no tubular epithelia expressed *Coll1a1* transcripts. These findings did not support an epithelial contribution to collagen producing myofibroblasts.

THE ORIGIN OF MYOFIBROBLASTS IN KIDNEY

To definitively test whether tubular epithelia might be myofibroblast precursors, all renal epithelial cells or stroma-derived interstitial cells were permanently fate mapped using a Cre/LoxP system in the kidney at an early stage of organ development[32]. All nephron epithelia were genetically labeled during embryogenesis using *Six2-Cre* transgenic mice and *HoxB7-Cre* transgenic mice and all interstitial but not epithelial cells were labeled using *FoxD1-Cre* knock-in mice. Six2 is expressed in cap mesenchyme that is fated to become all non-ureteric bud derived, nephron epithelia including podocytes, proximal tubules, loop of Henle, and distal tubules, but not any interstitial population[33, 34]. HoxB7 expression is restricted developmentally to the ureteric bud, which becomes collecting duct epithelia and ureteral epithelia of adult kidney[35]. FoxD1 (also known as Bf-2), a forkhead family transcription factor, is expressed in the nephrogenetic interstitium (stroma), which overlies cap mesenchyme ($Six2^+$ layer) in the developing kidney (Figure 2). $FoxD1^+$ cells are fated to develop into pericytes, perivascular fibroblasts, vSMCs and mesangial cells[32].

By expressing the *Six2-Cre* or *HoxB7-Cre* alleles in *Rosa26 reporter* (Rs26R) (with bacterial LacZ enzyme as fate marker) or *Z/Red reporter* (with DsRed as fluorescent fate marker) mice, bigenic mice were generated that permitted fate tracing of the entire kidney epithelia. These bigenic mice were subjected to two different models of severe interstitial fibrotic disease, namely UUO and unilateral ischemia-reperfusion injury (IRI). Despite close inspection of these adult kidneys with fibrosis, no cells expressing the epithelial fate markers were detected in the expanded interstitial space of kidneys in either bigenic mice. Furthermore, fate $marker^+$ cells were not observed in any αSMA expressing myofibroblasts or fibloblast specific protein-1 (FSP-1 or S100A4) expressing interstitial cells[32]. But, in contrast, when proximal tubule cells were isolated from kidneys of *Six2-Cre; Z/Red* bigenic mice, cultured *in vitro* and exposed to transforming growth factor (TGF) β1, tubule cells coexpressed fate marker and αSMA or FSP-1 (S100A4) and lost E-cadherin expression, indicating that tubular epithelia acquired some features of mesenchymal cells and myofibroblasts *in vitro* but not *in vivo*. This phenomenon *in vitro* is called ‘epithelial to mesenchymal transition (EMT)’ and has supported a hypothesis that tubular epithelia are one of the major sources of myofibroblasts *in vivo*. This inconsistency between *in vivo* and *in vitro* findings indicated that although phenotypic changes reported to represent EMT can occur *in vitro*, the EMT process does not directly contribute to the interstitial myofibroblast pool (renal fibrosis) *in vivo* and that myofibroblasts are not derived from tubular epithelia.

To further verify that $FoxD1^+$ stroma-derived pericytes are the major source of myofibroblasts *in vivo*, kidney stromal cells, but not ECs, were labeled permanently during nephrogenesis of *FoxD1-Cre; Rs26R* bigenic mice. In these mouse kidneys, all mesangial cells, vSMCs and many interstitial cells were identified by LacZ staining. $LacZ^+$ interstitial cells were also positive for PDGFR-β and CD73 (i.e. the same as GFP^+ cells in *Coll-GFP* mice above) but negative forαSMA, endothelial and macrophage markers. After injury to the kidney of *FoxD1-Cre; Rs26R* mice, there was marked expansion of $LacZ^+$ cells exclusively in the interstitium of the kidney and $LacZ^+$ cells exclusively expressed αSMA. Furthermore, nearly 100% of αSMA^+ interstitial cells were positive for LacZ (Figure 3A-D). This

experiment provided strong support for a model in which the FoxD1$^+$ stroma-derived interstitial cell population, selectively expands in fibrotic kidneys and becomes myofibroblasts[32]. Although FoxD1 was not detected in normal adult kidney, following injury to adult kidney the *FoxD1* gene was reactivated, compromising the ability to distinguish proliferation and differentiation of previously labeled cells from new activation of Cre in an unlabeled cell type (i.e. myofibroblast from another source) with new expression of LacZ fate reporter. To circumvent this issue, an inducible CreER^{T2} driven at the *FoxD1* locus in *Rs26R* mice was used[32]. The CreER^{T2} is a tamoxifen-inducible form of Cre recombinase. Tamoxifen is required to bind to the CreER^{T2} in order to translocate the CreER^{T2} to the nucleus where this inducible Cre can recombine genomic DNA to activate the reporter. Mouse embryos were exposed transiently *in utero* to tamoxifen during early nephrogenesis. This exposure triggers the translocation of the CreER^{T2} protein to the nucleus of FoxD1 expressing kidney progenitor cells making them transiently permissive for recombination at the *Rs26R* locus. A cohort of 20% of all FoxD1$^+$ stroma-derived cells was labeled with a single dose of tamoxifen (Figure 3E-G). Because tamoxifen is toxic for embryos at high dose, multiple injections to label more than 20% of FoxD1$^+$ cells were not feasible. After injury to adult kidneys of *FoxD1-Cre;Rs26R* mice that had been exposed to tamoxifen during nephrogenesis, the cohort of LacZ-labeled, FoxD1$^+$ stroma-derived, pericytes expanded 15-fold, all acquired αSMA expression, and represented 20% of the total myofibroblast pool. These findings provided unequivocal genetic fate mapping evidence that FoxD1$^+$ stroma-derived pericytes are the myofibroblast progenitor (Figure 3E-G). Subsequent to these studies four other groups published genetical fate mapping results of tubular epithelia in fibrotic kidney[36–39] and all of them failed to find any epithelial cells migrating outside the epithelial compartment or transitioning into myofibroblasts during fibrosis.

THE ROLE OF ENDOTHELIAL/PERICYTE CROSSTALK IN MICROVASCULAR RAREFACTION

In mouse kidneys, pericytes promptly migrate away from the capillary wall into the interstitium within 9 hours after injury[12, 13]. After pericyte detachment from capillaries, pericytes are activated and contribute to fibrosis as myofibroblasts. However the injured, proliferating endothelium is now devoid of the close contacts with pericytes and the crosstalk between endothelia and pericytes that is vital for vessel stability. In other organs vasculature that lacks pericytes is unstable, prone to aneurysm, hemorrhage or drop-out (rarefaction). In recent studies, Lin and colleagues show that blockade of vascular endothelial growth factor receptor 2 on ECs following injury to the kidney abrogated pericyte detachment and attenuated both PTC loss and fibrosis during progressive kidney injury induced by UUO or IRI[13]. Conversely blockade of PDGFR-β on pericytes following kidney injury also prevented pericyte detachment and attenuated both PTC loss and fibrosis in response to injury[13]. This study indicates that bidirectional signaling between pericytes and ECs is necessary for pericyte detachment and that in the kidney, pericyte detachment is responsible for both capillary rarefaction and fibrosis. Therefore, targeting crosstalk between these two types of cells may provide a novel therapeutic opportunity to treat acute and chronic kidney injuries.

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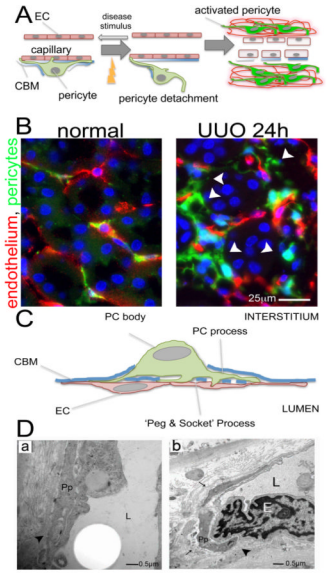

Figure 1. Pericytes and pericyte detachment in kidney injury

(A-B) Schematic (A) and fluorescence images (B) from *Coll-GFP* mice of pericytes (PCs, green) and capillary endothelial cells (ECs, immunostained for CD31 as red) in normal kidney and 24 hours after unilateral ureteral obstruction (UUO). In response to kidney injury, PCs detach themselves from ECs, spread, migrate (arrowheads in B) and increase collagen expression (become more green). Progression of this process (A) leads to unstable vasculature, capillary loss and interstitial matrix expansion. (C–D) Schematic (C) and electron microscopy images (human sample) (D) of PC-interaction with EC in normal kidney. PC processes are enveloped in capillary basement membrane (CBM) (arrows) where intimate connections and cell:cell signaling occurs known as ‘peg and socket’ junctions (arrowheads). L=capillary lumen, E=EC, Pp=pericyte process.

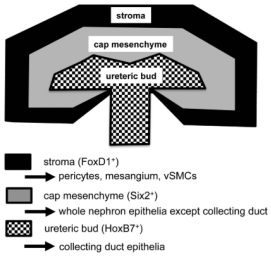

Figure 2. Schematic of mouse kidney progenitor cells in the metanephric mesenchyme (embryonic day 10.5–15.5)

$Six2^+$ cap mesenchyme (gray) is induced by invading uteteric bud (check), to differentiate into all cells in the nephron epithelia except collecting duct. Cortical and medullary interstitium but not endothelium or resident dentritic cells/macrophages, and also mesangial cells and vSMCs are derived from $FoxD1^+$ stroma (black) overlying the $Six2^+$ cap mesenchyme.

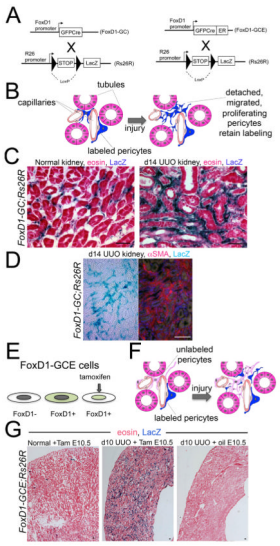

Figure 3. Fate mapping using Cre/LoxP and tamoxifen sensitive Cre/LoxP systems indicates that FoxD1$^+$ stroma-derived kidney interstitial cells (pericytes and perivascular fibroblasts) are the precursors of myofibroblasts

(A) Schematic showing *FoxD1-GFP-Cre;Rosa26 Reporter* (*FoxD1-GC;Rs26R*) or *FoxD1-GFP-Cre-ER^{T2};Rosa26 Reporter* (*FoxD1-GCE;Rs26R*) alleles. The *Rosa26* endogenous promoter drives permanent expression of LacZ in all cells where Cre recombinase-mediated excision of a STOP sequence in genomic DNA has occurred. (B-D) Schematic (B), bright–field photomicrographs (C), fluorescence micrographs (split panel) (D) of normal (left panels in B and C) and diseased (right panels in B and C) kidneys from *FoxD1-GC;Rs26R* adult mice. FoxD1$^+$ stroma-derived cells (LacZ$^+$, blue) are Coll1a1$^+$, CD73$^+$, PDGFR-β$^+$, αSMA$^-$, F4/80$^-$ (macrophage marker) and CD31$^-$ (endothelial marker) in normal kidney (not shown). Note perivascular location in normal kidney and marked interstitial expansion in day 14 UUO kidneys (d14 UUO) of blue FoxD1$^+$ stroma-derived cells (C). All of these LacZ$^+$ cells acquireαSMA expression (red) (D). (E) Schematic of a hypothetical cell from *FoxD1-GCE* mice. The GFP-Cre-ER^{T2} fusion protein (green) is able to recombine genomic DNA at LoxP sites only when it is in the nucleus. This can occur only when the endogenous *FoxD1* gene is active during early embryonic kidney development and the estrogen receptor (ER) agonist, tamoxifen, is applied exogenously. (F–G) Schematic (F) and bright-field images (G) of normal and diseased kidneys from *FoxD1-GCE;Rs26R* adult mice showing FoxD1$^+$ stromal-derived cells (blue). Note only mice that were exposed to tamoxifen (Tam) *in utero* on embryonic day 10.5 (E10.5) have a cohort (20% of total) of labeled FoxD1$^+$ stroma-derived cells (blue), which are Coll1a1$^+$, CD73$^+$, PDGFR-β$^+$, αSMA$^-$, F4/80$^-$ and CD31$^-$ in normal kidney (not shown). After d10 UUO there is marked expansion of this 20% of labeled FoxD1$^+$ stroma-derived cells (LacZ$^+$, blue) which all acquire αSMA (not shown) (note some images are taken from the reference 32).