Low-dose hydralazine prevents fibrosis in a murine model of acute kidney injury–to–chronic kidney disease progression

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Acute kidney injury (AKI) and progressive chronic kidney disease (CKD) are intrinsically tied syndromes. In this regard, the acutely injured kidney often does not achieve its full regenerative capacity and AKI directly transitions into progressive CKD associated with tubulointerstitial fibrosis. Underlying mechanisms of such AKI-to-CKD progression are still incompletely understood and specific therapeutic interventions are still elusive. Because epigenetic modifications play a role in maintaining tissue fibrosis, we used a murine model of ischemia-reperfusion injury to determine whether aberrant promoter methylation of *RASAL1* contributes causally to the switch between physiological regeneration and tubulointerstitial fibrogenesis, a hallmark of AKI-to-CKD progression. It is known that the antihypertensive drug hydralazine has demethylating activity, and that its optimum demethylating activity occurs at concentrations below blood pressure–lowering doses. Administration of low-dose hydralazine effectively induced expression of hydroxylase TET3, which catalyzed *RASAL1* hydroxymethylation and subsequent *RASAL1* promoter demethylation. Hydralazine-induced CpG promoter demethylation subsequently attenuated renal fibrosis and preserved excretory renal function independent of its blood pressure–lowering effects. In comparison, *RASAL1* demethylation and inhibition of tubulointerstitial fibrosis was not detected upon administration of the angiotensin-converting enzyme inhibitor Ramipril in this model. Thus, *RASAL1* promoter methylation and subsequent transcriptional RASAL1 suppression plays a causal role in AKI-to-CKD progression.

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Acute kidney injury (AKI) and chronic progressive kidney disease (CKD) are principal problems in nephrology.[1–3] Although both were long considered as strictly distinct pathologies, it is becoming increasingly clear that they are closely related because CKD predisposes for AKI and because AKI is a prominent risk factor to develop CKD.[1,3] AKI-to-CKD progression is a complex process that is still poorly understood.[1,3,4] Although the kidney in principle possesses a unique capacity to repair itself even after severe acute injury, such regenerative capacity often is not fully realized, culminating in initiation of tubulointerstitial fibrosis, the hallmark pathway of chronic progressive kidney disease.[5–7] Similar to any wound repair, regeneration of acute kidney injury is associated with fibroblast activation.[8] However, although activated fibroblasts return to their quiescent state upon adequate renal regeneration (or upon wound closure), such reversal of fibroblast activation is not achieved in self-contained fibrogenesis and fibroblasts maintain their activated state independent of further external stimuli.[9,10] We and other investigators previously have shown that epigenetic modifications play a causal role in maintaining such fibrotic fibroblast activation.[11–14]

Epigenetics in general are chromatin modifications that stably alter gene transcription and can be passed on over several mitotic generations.[15] So-called CpG promoter methylation (also known as DNA methylation and CpG island methylation) is a prototypical epigenetic mechanism that refers to the addition of methyl groups to the C5 position at cytosine nucleotides.[15,16] Once such methylation occurs in cytosine clusters (so-called CpG islands) within promoter regions, transcription of affected genes is suppressed.[17] Promoter methylation plays an essential role in cell differentiation during development (preventing cells from reverting into an undifferentiated state or differentiating into phenotypes of different lineage),[18] but also plays a causal role in pathologies such as cancer (in which methylation can delete tumor-suppressor genes similar to mutations, causally causing cancer),[19] and, as discussed later, in fibrogenesis.[11,20–23] Renal fibrosis, similar to fibrosis in any other organ, is associated with robust changes in methylation patterns.[24–27] We previously identified *RASAL1*, encoding for Ras-Gap–like protein-1 and hydrolyzing active Ras–guanosine triphosphate

to inactive Ras–guanosine diphosphate, to be methylated consistently in fibrotic fibroblasts.[11] We further showed that *RASAL1* methylation and subsequent transcriptional silencing of *RASAL1* causally contributes to sustained fibroblast activation (by increasing intrinsic Ras-guanosine triphosphate levels, similar to cancer cells).[11] We further discovered that *RASAL1* is hypermethylated consistently in murine models of chronic progressive fibrosis (including unilateral ureteral obstruction, subtotal nephrectomy, and nephrotoxic serum nephritis),[28,29] but not in a murine model of fully reversible ischemia-reperfusion injury.[11] Furthermore, we discovered that therapeutic *RASAL1* demethylation *in vivo* could be achieved either by administration of the prototypical demethylating drug 5'-azacytidine (which is in clinical use for treatment of refractory myelodysplastic syndrome despite its substantial side effects),[11] but also by administration of hydralazine (and its derivate dihydralazine), and such *RASAL1* demethylation correlated with ameliorated fibrosis.[28]

Hydralazine was first approved by the U.S. Food and Drug Administration as an anti-hypertensive drug in 1952.[30] Today, it has its place in clinical practice as a third-line antihypertensive regimen in patients with complicated hypertension, as an antihypertensive in intensive care unit settings, as a second-line therapeutic for chronic heart failure (when use of inhibitors of the renin-angiotensin-aldosterone system [RAAS] is not warranted), or because of its safety profile in pregnancy hypertension.[30–35] Although the biological mechanism through which hydralazine exerts vasodilation are diverse and still not fully understood, it has long been known that hydralazine also possesses demethylating activity, and that its optimum demethylating activity already is realized at concentrations below blood pressure–lowering doses (low-dose hydralazine).[36,37] Because of its demethylating activity, low-dose hydralazine currently is undergoing clinical testing in various solid tumors.[27,36,37] We previously showed that low-dose hydralazine was equally effective in demethylating *RASAL1* within renal fibroblasts and in attenuating experimental renal fibrogenesis in murine models of unilateral ureter obstruction and folic acid–induced nephropathy as 5'-azacytidine.[28]

Here, we aimed to explore if aberrant promoter methylation of *RASAL1* contributes causally to the shift from regeneration to fibrosis that is associated with AKI-to-CKD progression upon acute kidney injury, and, if so, whether such fibrotic response could be prevented by administration of low-dose hydralazine. We show that fibrogenesis ensuing after severe ischemia-reperfusion injury (IRI) is associated with *RASAL1* promoter methylation and transcriptional silencing, whereas moderate IRI followed by full recovery is not.[38,39] We provide evidence that rescue of RASAL1 expression in R*ASAL1*-transgenic mice inhibits renal fibrogenesis upon severe IRI, supporting that loss of RASAL1 causally contributes to AKI-to-CKD progression. We further show that administration of low-dose hydralazine during acute kidney injury at a dose of 5 mg/kg normalizes aberrant *RASAL1* promoter methylation and effectively prevents AKI-to-CKD progression and renal fibrogenesis upon severe IRI. In contrast, administration of the angiotensin-converting enzyme inhibitor (ACEi) ramipril during acute kidney injury had no demethylating activity on renal fibroblasts and no beneficial effects on fibrogenesis or AKI-to-CKD progression in this model.

RESULTS

AKI-to-CKD progression upon ischemia-reperfusion injury is associated with *Rasal1* promoter methylation

Previously, we established that *Rasal1* promoter methylation contributed causally to the progression of tubulointerstitial fibrosis in multiple murine models of chronic kidney disease (unilateral ureteral obstruction, folic acid nephropathy, and nephrotoxic serum nephritis),[11,28,29] whereas *Rasal1* was not methylated in a rodent model of fully reversible ischemia-reperfusion injury.[11] Based on these findings, we hypothesized that *Rasal1* promoter methylation could be causally involved in switching AKI recovery to AKI-to-CKD fates. To test this hypothesis, we aimed to use models allowing for direct comparison of involved pathomechanisms owing to comparable insults and injury modes. For this purpose, we decided to use 2 mouse models of ischemia-reperfusion injury: a rodent model of IRI with effective recovery from injury (moderate IRI) and a mouse model leading to tubulointerstitial fibrosis (severe IRI) after 42 days (Figure 1a–g).[38-40] Such severe IRI was associated with impaired recovery from tubular injury (Figure 1a and b), increase of the relative interstitial volume (Figure 1a and c), accelerated deposition of type I collagen (Collagen-1) (Figure 1a and d), accumulation of α-smooth muscle actin (α-SMA)-positive myofibroblasts (Figure 1a and e), increased intrarenal mRNA expression levels of *Collagen-1a1* (Figure 1f) and *Acta2* (encoding α-SMA) (Figure 1g), and accumulation of proliferating Ki67-positive interstitial cells (Figure 1a and h) at 42 days after renal injury. In the moderate IRI model correlating with completed tubular regeneration, proliferative activity of tubular epithelial cells (as assessed by immunolabeling with proliferation marker Ki67) had ceased after 42 days, correlating with completed tubular regeneration (Figure 1a and i). Incomplete tubular regeneration after 42 days in the severe IRI model correlated with sustained Ki67 immunolabeling of tubular epithelial cells, indicating ongoing response to injury and prolonged repair (Figure 1a and i), in line with previous findings.[38,41] AKI-to-CKD progression with tubulointerstitial fibrogenesis upon severe IRI was associated with increased *Rasal1* promoter methylation (Figure 1j and k) and consecutive loss of intrarenal *Rasal1* mRNA expression levels (Figure 1l), whereas *Rasal1* methylation and transcriptional suppression was not observed upon moderate IRI associated with full regeneration (Figure 1j–l). Loss of intrarenal Rasal1 during AKI-to-CKD progression was confirmed, especially in interstitial compartments (Figure 1m and n), in line with previous findings that loss of Rasal1 is involved in determining fibroblast activation in the kidney.[11] In summary, tubulointerstitial fibrosis in response to severe