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TGF-β-induced angiogenesis is a key driver of DKD pathogenesis

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Glomerular endothelial dysfunction and neoangiogenesis have long been implicated in the pathogenesis of diabetic kidney disease (DKD). However, specific molecular pathways contributing to these processes in the early stages of DKD are not well understood. To assess the molecular changes associated with glomerular endothelial cell (GEC) injury in early DKD, we recently performed transcriptomic profiling of isolated GECs from streptozotocin-induced diabetic mice with endothelial nitric oxide synthase (eNOS) deficiency. Analysis of differentially expressed genes indeed indicated that angiogenesis and endothelial proliferation pathways were significantly upregulated in GECs of diabetic mice and that leucine-rich alpha-2 glycoprotein 1 (LRG1) was among the top upregulated genes. LRG1 is a secreted glycoprotein belonging to the leucine-rich repeat (LRR) protein family, whose expression is increased in plasma and urine of patients with a variety of cancer malignancies. Notably, a recent finding by Wang *et al.* demonstrated that LRG1 mediates the pro-angiogenic effects through enhancement of endothelial TGF-β1/ALK1 signaling in the murine models of ocular disease. However, its role in the kidney, particularly in the setting of DKD, was not known.

TGF-β regulates many aspects of endothelial functions including cell proliferation in early diabetic kidneys resulting in glomerular hypertrophy, but also the induction of apoptosis in microvascular endothelial cells. These contrasting actions of TGF-β on endothelial cells are regulated by differential activation of two Type 1 receptors: the ubiquitously expressed ALK5 and predominantly EC-restricted ALK1 receptors. ALK5 activation induces the Smad2/3 activation to block EC proliferation, migration, and angiogenesis. In contrast, ALK1 activation induces the Smad1/5/8 activation to promote EC proliferation, migration, and tube formation, resulting in neo-angiogenesis. This led us to speculate that the function of LRG1 would promote diabetes-induced glomerular angiogenesis via ALK1-induced signal transduction in GECs.

Analysis of its expression in the kidney by in situ mRNA hybridization analysis in mouse and human kidneys confirmed its predominant expression in the GECs and further indicated its expression in the tubulointerstitium. Consistent with the bulk RNA-seq of isolated mouse GECs and our recent single-cell RNA-seq of isolated mouse glomeruli, its expression was markedly increased in the diabetic kidneys, particularly in the glomeruli. To determine the role of LRG1 in DKD, we next examined the effects of LRG1 loss using the global *Lrg1*-null (*Lrg1*$^{-/-}$) mice. *Lrg1*$^{-/-}$ mice were grossly normal, as reported previously. Because *Lrg1*$^{-/-}$ mice were in the relatively DKD-resistant C57BL/6J background, unilateral nephrectomy (UNx) was performed prior to diabetes induction in mice to aggravate the ensuing diabetic kidney injury. Diabetes (DM) was induced by low-dose streptozotocin injections in *Lrg1*$^{-/-}$ mice and in *Lrg1*$^{+/+}$ littermate controls (+STZ) three weeks post-UNx. Citrate buffer-injected uni-nephrectomized mice served as nondiabetic controls (-STZ), and all mice were euthanized at 12 weeks post diabetes (DM)-induction. The extent of hyperglycemia, body weight loss, and blood pressure were similar between diabetic *Lrg1*$^{+/+}$ and *Lrg1*$^{-/-}$ mice compared to non-diabetic mice. Histological analysis showed that the development of glomerular hypertrophy and mesangial expansion was significantly blunted in diabetic *Lrg1*$^{-/-}$ mice compared to diabetic *Lrg1*$^{+/+}$ mice. The extent of albuminuria was markedly attenuated in diabetic *Lrg1*$^{-/-}$ mice. Podocyte foot process effacement and loss were also reduced in diabetic *Lrg1*$^{-/-}$ mice in comparison to diabetic *Lrg1*$^{+/+}$ mice. These results indicated that diabetic glomerulopathy is markedly attenuated by the loss of LRG1.

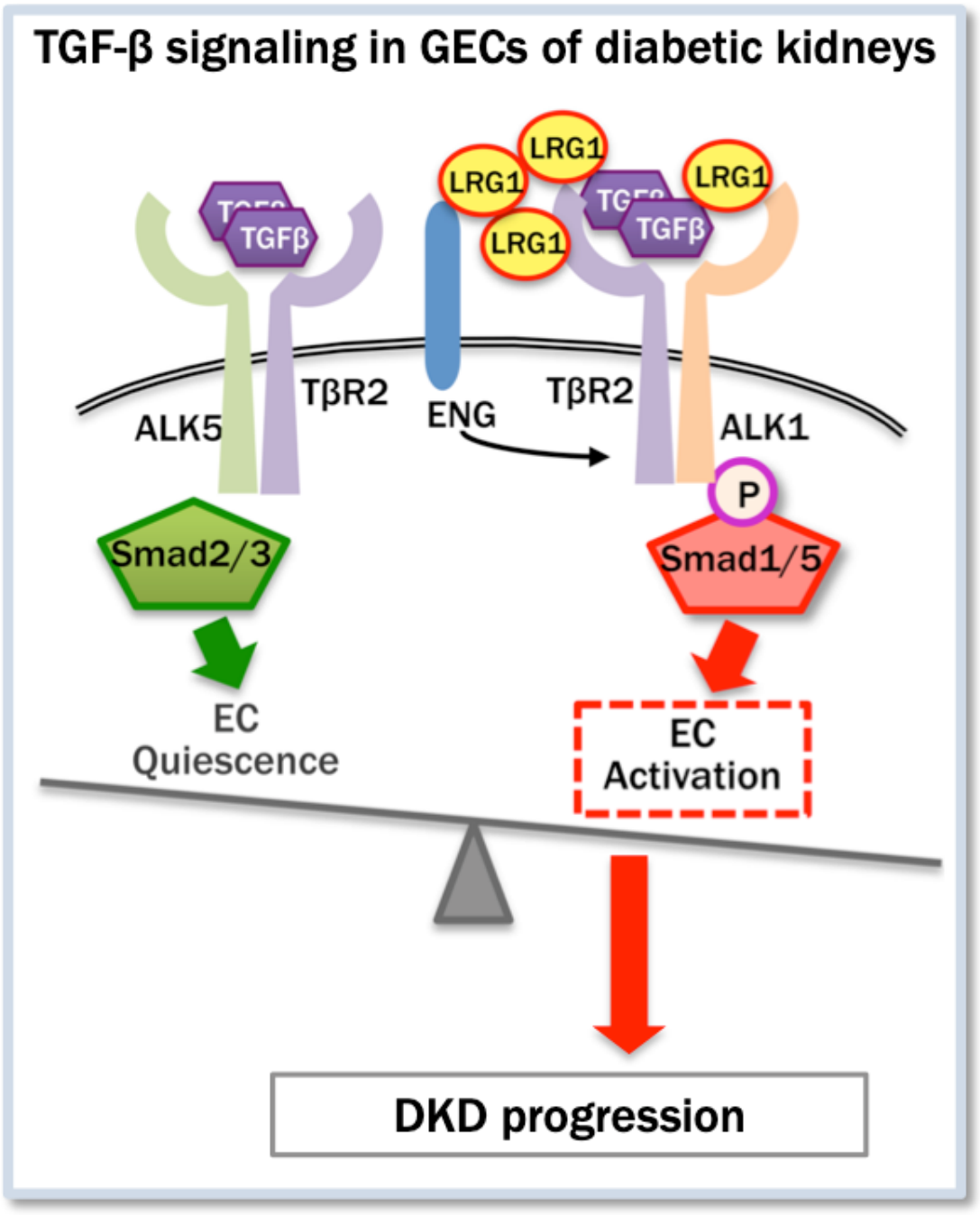
As LRG1 was previously shown to be pro-angiogenic in the retinal vasculature, we next examined

whether LRG1 ablation would alter the DM-induced angiogenesis in vivo. Directed in vivo angiogenesis assay (DIVAA) as well as CD31 immunofluorescence indicated increased angiogenesis in the diabetic mice, which was attenuated with the loss of LRG1. To quantitate the change in the number of GECs in the context of LRG1 ablation in early diabetic kidneys, *Lrg1*$^{+/+}$ and *Lrg1*$^{-/-}$ mice were crossed with Flk1-H2B-EYFP transgenic mice, which expresses a nuclear enhanced yellow fluorescent protein (EYFP) driven by *Flk1* promoter. Diabetes was similarly induced with low-dose STZ injections 3 weeks after UNx. Indeed, the number of EYFP+ cells in the diabetic Lrg1$^{+/+}$;EYFP mouse glomeruli was found to be increased at 8 weeks post-DM, but this increase was mitigated in the diabetic Lrg1$^{-/-}$;EYFP mouse glomeruli. We further confirmed that the observed attenuation of diabetic glomerulopathy in the *Lrg1*$^{-/-}$ mice was associated with dampened Smad1/5/8 activation in the GECs, strongly indicating that LRG1 is a critical mediator of TGF-β/ALK1-mediated angiogenesis in the diabetic kidneys.

As LRG1 is a secreted glycoprotein elevated in human and mouse diabetic kidneys, we next examined whether plasma LRG1 levels in DKD patients are associated with renal outcome. We measured LRG1 on plasma samples from a sub-cohort of 871 participants with type 2 diabetes in the Bio*Me* Biobank Cohort at the Icahn School of Medicine at Mount Sinai. Plasma LRG1 was not significantly correlated with eGFR (r= -0.02, p= NS) or albuminuria (r=0.06, p = NS) at baseline at the time of enrollment into the Bio*Me* Biobank Program. Of the 871 patients analyzed, 121 (13.8%) experienced the composite endpoint, as defined by 40% sustained eGFR decline or ESRD during a median follow-up of 4.5 [IQR 3.3-6.1] years. Notably, participants with renal endpoint had higher levels of LRG1 (80.74 vs. 53.79 ng/ml). The event rates for the composite renal endpoints of 40% sustained decline in eGFR or ESRD were 7.5% for the lowest tertile, 12.7% for the middle tertile, and 21.3% for the top tertile of LRG1, which yielded an adjusted HR of 1.6 (95% CI 0.9-2.7) and 2.7 (95% CI 1.6-4.4) for the middle and top tertiles, respectively vs. the bottom tertile. For each doubling in LRG1, the adjusted HR was 1.3 (95% CI 1.1-1.4).

Taken together, our results reveal the role of LRG1 as a pathogenic mediator of DKD progression via increased TGF-β-induced glomerular angiogenesis and as a potential risk factor in DKD progression.

Summary Figure


TGF-β signaling in GECs of diabetic kidneys
LRG1
LRG1
LRG1
LRG1
TGFβ
TGFβ
TβR2
TβR2
ALK5
ENG
ALK1
P
Smad2/3
Smad1/5
EC
Quiescence
EC
Activation
DKD progression