Antagonism of profibrotic microRNA-21 improves outcome of murine chronic renal allograft dysfunction

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Chronic renal allograft dysfunction (CAD) is a major limiting factor of long-term graft survival. It is characterized by interstitial fibrosis and tubular atrophy. The underlying pathomechanisms are incompletely understood. MicroRNAs are powerful regulators of gene expression and may have an impact on various diseases by direct mRNA decay or translational inhibition. A murine model of allogenic kidney transplantation was used resulting in CAD at 6 weeks after kidney transplantation. We identified fibrosis-associated miR-21a-5p by whole miRNAome expression analysis to be among the most highly upregulated miRNAs. *In vitro* in renal fibroblasts, miR-21a-5p was transcriptionally activated by interleukin 6–induced signal transducer and activator of transcription 3. Co-culture of LPS-activated macrophages with renal fibroblasts increased expression levels of miR-21a-5p and markers of fibrosis and inflammation. In addition, mature miR-21a-5p was secreted by macrophages in small vesicles, which were internalized by renal fibroblasts, thereby promoting profibrotic and proinflammatory effects. Notch2 receptor was identified as a potential target of miR-21a-5p and validated by luciferase gene reporter assays. Therapeutic silencing of miR-21a-5p in mice after allogenic kidney transplantation resulted in an amelioration of CAD, as indicated by a reduction in fibrosis development, inflammatory cell influx, tissue injury and BANFF lesion scoring. In a life-supporting model, miR-21a-5p antagonism had beneficial effects on kidney function. miR-21a-5p silencing may therefore be a viable therapeutic option in the treatment of patients following kidney transplantation to halt the development of CAD.

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Chronic allograft dysfunction (CAD) represents a major limiting factor of long-term survival after kidney transplantation. It has been shown to be associated with progressive interstitial fibrosis, tubular atrophy (IF/TA), and graft dysfunction, ultimately culminating in graft loss.[1,2] The main underlying pathophysiological causes of CAD include recurrent renal diseases and immunologic events such as acute rejection episodes.[2] Acute rejection can be either antibody- or T-cell–mediated, whereas chronic rejection is driven by infiltrated macrophages, which are associated with fibrosis development, leading to CAD.[3] However, the underlying pathomechanisms are not completely understood. The development of highly effective immunosuppressive agents over the past 20 years has significantly improved the 1-year survival of kidney grafts; however, the overall survival rate has remained the same.[4] Further, long-term treatment with immunosuppressive drugs such as calcineurin inhibitors is accompanied with severe nephrotoxicity, thereby contributing to CAD.[5] As up to 30% of patients listed for renal transplantation are patients with chronic allograft failure, the development of new treatment strategies to prevent fibrotic remodeling is of utmost importance.[1,2]

Recently, microRNAs (miRNAs) have come into focus as powerful epigenetic regulators of gene expression. MiRNAs are small, non-coding RNAs that cause the repression of target genes through the posttranscriptional degradation of mRNA and/or translational inhibition of protein expression.[6] MiRNA antagonists (anti-miRs) are available to specifically cleave mature miRNAs, thereby silencing their cellular effects and thus, they serve as powerful novel therapeutics for various diseases.[7–9] Several fibrosis-associated miRNAs have recently been identified in kidney disease.[10] In a previous study, miR-21a-5p was found to be significantly higher in

biopsies of renal transplant patients with interstitial fibrosis and tubular atrophy (IF/TA) than in biopsies of transplant patients without signs of IF/TA, as assessed using deep sequencing.[11] In the present study, using a model of CAD in allogenic mouse kidney transplantation, we found that miR-21a-5p was one of the most upregulated miRNAs. The underlying cellular up- and downstream mechanisms were assessed to develop a potential therapeutic approach with regard to pharmacologic silencing of miR-21a-5p in mice subjected to kidney transplantation.

RESULTS

Renal miR-21a-5p is upregulated after kidney transplantation in mice

To identify miRNAs critically involved in the development of CAD, we performed a miRNA microarray analysis of healthy and transplanted mice kidneys at 6 weeks after transplantation. The experimental setup of mouse allogeneic kidney transplantation (left kidneys of male C57Bl/6N mice into female Balb/c mice) is schematically shown in Figure 1a. This model ensures minor and major histocompatibility mismatching and results in allograft fibrosis within 6 weeks, as shown previously.[12,13] Isogeneic control transplantations showed no signs of fibrosis or injury development (Supplementary Figure S1A and B). Using whole miRNAome expression analysis, miR-21a-5p was identified as one of the most deregulated miRNAs in transplanted kidneys when compared with those in control kidneys (Figure 1b). The other differentially expressed miRNAs are stated in Supplementary Table S1. The results were validated by quantitative real-time polymerase chain reaction (qRT-PCR) using highly specific miR-21a-5p TaqMan primers (Figure 1c and Supplementary Tables S2 and S3). miR-21a-5p was not differentially expressed between the 2 mouse strains Balb/c and C57BL/6N (Supplementary Figure S1C).

Crosstalk between infiltrating inflammatory cells and resident renal cells is mediated by interleukin-6

In mouse models of CAD with associated IF/TA, macrophages have been shown to accumulate in the damaged kidney.[14] Moreover, the presence of macrophages in an early biopsy has been reported to be predictive of IF/TA development in humans.[15] Macrophages may also contribute to IF/TA by expressing transforming growth factor-β (TGF-β) and promoting matrix deposition through fibroblast activation or the promotion of inflammatory cytokines or reactive oxygen species. miR-21a-5p has previously been shown to activate cardiac fibroblasts.[8] We therefore investigated the potential crosstalk between monocytes and/or macrophages and renal fibroblasts in order to identify the underlying mechanisms of fibrosis development related to miR-21a-5p. The miR-21a-5p promotor expresses a putative binding site for the transcription factor signal transducer and activator of transcription 3 (STAT3).[16] STAT3 is activated by the proinflammatory cytokine interleukin 6 (IL-6), which can be secreted by macrophages. We hypothesized that infiltrated macrophages secrete high levels of IL-6, which activates miR-21a-5p expression in resident renal fibroblasts, thereby leading to a profibrotic remodeling of the transplant.

Macrophages were activated by lipopolysaccharide (LPS) (scheme in Figure 1d), which resulted in a significant time-dependent upregulation of IL-6 mRNA (Figure 1e) as well as secretion of IL-6 in the cell culture supernatant (Figure 1f), peaking at 24 hours. To investigate the crosstalk between infiltrated macrophages and renal fibroblasts, we performed a coculture experiment, in which macrophages were cultured at the top and fibroblasts at the bottom of a transwell system (scheme in Figure 1g, expression levels of miR-21a-5p and IL-6 are shown in Supplementary Figure S2A). Coculture of renal fibroblasts with LPS-activated macrophages for 24 hours increased the expression levels of miR-21a-5p, IL-6, and connective tissue growth factor (CTGF) in the fibroblasts compared to the levels on coculture with unstimulated macrophages (Figure 1h and i). In addition, primary miR-21a-5p expression was significantly increased in renal fibroblasts, indicating that miR-21a-5p upregulation is related to transcriptional activation (Figure 1h). To assess the role of IL-6 in this crosstalk, fibroblasts were treated with recombinant IL-6, which resulted in increased expression levels of miR-21a-5p, pri-miR-21a-5p, CTGF, and IL-6 (Figure 1j and k). Furthermore, electrophoretic mobility shift assay (EMSA) demonstrated IL-6-induced activation of STAT3 in renal fibroblasts (Supplementary Figure S2B).

Secreted vesicles from infiltrating macrophages transfer miR-21a-5p to fibroblasts

A previous study showed that miRNAs might be shuttled between cells through vesicle transfer.[17] Secreted miRNA-enriched small vesicles are subsequently internalized by recipient cells, thereby influencing their genetic programming. We hypothesized that increased levels of fibroblast miR-21a-5p in our coculture model may be mediated by not only miR-21a-5p transcription related to IL-6 secretion and uptake in fibroblasts but also direct transfer of vesicles enriched with mature miR-21a-5p.

Cultured macrophages were activated by LPS (scheme in Figure 2a), which resulted in increased expression levels of miR-21a-5p and IL-6 (Supplementary Figure S3A). Moreover, macrophages secreted small vesicles (<200 nm), as assessed by electron microscopy (Figure 2b) and the expressions of specific vesicle markers, such as flotillin-1 and CD81, on western blot analysis (Supplementary Figure S3B). The number of small vesicles was comparable between control and LPS-activated macrophages (Figure 2b). The expression of mature miR-21a-5p in isolated vesicles was significantly higher in LPS-activated macrophages (referred to as LPS vesicles) than in control cells (referred to as ctrl vesicles) (Figure 2c). The treatment of cultured renal fibroblasts with isolated vesicles (scheme in Figure 2d) resulted in time-dependent uptake of small vesicles (Figure 2e and Supplementary Movie S1). Uptake of LPS vesicles in renal