Chapter 8

Summary

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Kidney disease is a common disorder that affects up to 10% of the population and this number is rapidly rising. Chronic kidney diseases occur when disorders such as diabetes, hypertension, inflammation or toxic substances eventually lead to kidney failure. The patient then requires dialysis or kidney transplantation, procedures that are either not successful in the long term (dialysis), or not always available (transplantation). As MicroRNAs (miRNAs) seem to be important in virtually all biological processes it is no longer a question if miRNAs are involved in renal injury and repair but what critical pathways they modulate. Therefore, miRNAs could serve as attractive targets for intervention. In this thesis, studies are described in which roles of miRNA-155, -126 and -132 in the context of kidney functioning are investigated.

Chapter 1 provides a general background on the prevalence of kidney disease and describes the kidney and its function. In addition, evolution and nephrogenesis of the kidney is discussed, providing background for studies described in this thesis. Furthermore, the biogenesis, function and mode of action of miRNAs is described, followed by a short overview of the relevance of miRNAs for kidney functioning.

In **Chapter 2** we investigated the role of miRNAs, miR-155 in particular, in endothelial to mesenchymal transition (EndoMT), which has been proposed to be involved in the loss of microvascular capillaries in the pathophysiology of fibrosis and organ failure. In EndoMT, endothelial cells (EC) undergo a mesenchymal transition associated with the loss of cell-cell contacts and the acquisition of a synthetic, contractile phenotype. In a TGF-β dependent *in vitro* model for EndoMT, we identified miRNAs that were differentially expressed in normoxic and hypoxic conditions. These studies identified miR-155 to be significantly upregulated in EndoMT, an effect that was enhanced under hypoxia, which further augments EndoMT. Silencing of miR-155 directly increased RhoA expression and activity in endothelial cells and affected phosphorylation of downstream LIMK. In contrast, overexpression of miR-155 counteracted RhoA function. Using a selective Rho kinase inhibitor, we could partly suppress EndoMT, strengthening the notion that RhoA plays a central role in EndoMT. Forced overexpression of miR-155 completely suppressed EndoMT, as evidenced by the maintenance of EC characteristics and blocking the acquisition of a mesenchymal phenotype, as compared to control cells. Our data demonstrate that miRNA-155 functions as a negative

regulator of RhoA signaling in TGF-β-induced endothelial to mesenchymal transition.

We previously showed that miR-126 plays a role in angiogenesis. Based on the seed sequence, miR-126 can also be predicted to regulate vasculogenesis by modulating the endothelial expression of stromal cell-derived factor-1 (SDF-1). In **Chapter 3** this was investigated. Using miR-reporter constructs, we first validated that miR-126 inhibits SDF-1 expression in endothelial cells *in vitro*. Next, we investigated the potential relevance of this observation with respect to the mobilization of progenitor cells. For this, we studied the migration of human $CD34^+$ progenitor cells towards chemotactic factors present in endothelial cell-conditioned medium. Antagomir-induced silencing of miR-126 elevated SDF-1 expression by human umbilical vein endothelial cells and enhanced migration of the $CD34^+$ cells. In a murine model of hind limb ischemia, a striking increase in the number of circulating $Sca\text{-}1^+/Lin^-$ progenitor cells in antagomir-126-treated mice was observed as compared to scramblemir-treated controls. Immunohistochemical staining of capillaries in the post-ischemic gastrocnemius muscle of miR-126-silenced mice revealed elevated SDF-1 expressing CD31-positive capillaries, whereas a mobilizing effect of miR-126 inhibition was not detected in healthy control animals. In conclusion, miR-126 can regulate the expression of SDF-1 in endothelial cells. In the context of an ischemic event, systemic silencing of miR-126 leads to the mobilization of $Sca\text{-}1^+/Lin^-$ progenitor cells into the peripheral circulation, potentially in response to elevated SDF-1 expression by endothelial cells present in the ischemic tissue.

Ischemia/reperfusion injury (IRI) is a central phenomenon in kidney transplantation. Managing the effects of renal IRI is crucial to short-and long-term graft survival following kidney transplantation. The peritubular capillary network may well be the rate limiting factor in the recovery of IRI. Following our findings that microRNA-126 plays a central role in maintaining vascular integrity, in **Chapter 4** we investigated if overexpression of microRNA-126 in bone marrow cells can aid in the recovery after IRI. Using a lentiviral construct we overexpressed miR-126 in bone marrow cells. Subsequently these cells were injected into lethally irradiated mice. Eight weeks after reconstitution of the bone marrow the mice underwent renal bilateral IRI. We found functional protection against IRI as a result of overexpression of miR-126 as demonstrated by decreased urea levels. In addition, damage markers Kim-1 and NGAL were decreased. This protection was associated with increased capillary density and increased influx of bone marrow derived

cells. We demonstrated that an elevated amount of endothelial cells was bone marrow derived that matched the observation of increased circulating hematopoietic stem and progenitor cells. We show that miR-126 attenuated CXCR4 expression on these cells, suggesting these cells would have a lower propensity to be retained in the BM. We confirmed the vascular role of hematopoietic miR-126 in a matrigel assay, where we show that overexpression of miR-126 results in increased neovascularization.

These findings suggest that over expression of miR-126 in the hematopoietic compartment protects against renal ischemia/reperfusion injury through stimulation of hematopoietic progenitor cell mobilization that leads to an improvement of the renal microvasculature.

In **Chapter 5** we investigated the role of miRNAs in fibrosis with a focus on the formation of myofibrobalsts. Lineage analysis has shown that during nephrogenesis, FoxD1-positive mesenchymal cells give rise to adult interstitial pericytes. These FoxD1-derivative interstitial cells expand and differentiate into smooth muscle actin (α-SMA) positive myofibroblasts during renal fibrosis, accounting for a large majority of myofibroblasts, which are responsible for scar formation in fibrotic kidney disease. MicroRNAs (miRNAs) involved in this differentiation could serve as a target to decrease myofibroblast formation in fibrotic kidney disease. To identify differentially expressed microRNAs, renal fibrosis was induced in FoxD1-GC;Z/Red mice by unilateral ureteric obstruction (UUO) and miRNAs were profiled in FoxD1-derivative interstitial cells (dsRed positive) that were isolated using FACS sorting from fibrotic versus healthy kidneys. MiR-132 was amongst the most highly up regulated microRNAs in these cells in fibrotic kidneys. *In vitro* we demonstrated that silencing miR-132 results in reduction of myofibroblast marker α-SMA and increased levels of its established target Sirt1 and downstream Cox2.

In vivo silencing of miR-132 in the UUO induced fibrosis model resulted in a ~35% decrease in renal collagen deposition after 10 days as compared to scramblemir controls, while no difference was observed yet after 5 days. In addition, immunohistochemical analyses demonstrate that the number of interstitial α-SMA positive cells is similarly decreased, which is confirmed by both western blot and qRT-PCR analyses. However, no difference is observed in capillary density. Surprisingly, silencing miR-132 is associated with reduced levels of Sirt-1, indicating this is not the responsible mechanism.

However, we demonstrated that miR-132 silencing has anti-proliferative effects, suggesting miR-132 plays an important role in the proliferation of myofibroblasts.

In **Chapter 6** we describe a role for miR-132 in diuresis. The collecting duct principal cells of our kidneys are critical in this maintenance of blood water levels, as binding of vasopressin (anti-diuretic hormone) to its V2-receptor and the subsequent translocation of AQP2 water channels to the apical membrane fine-tunes the water balance. Cyclooxygenase-2 (Cox2) produces prostaglandins such as PGE2, which are known to counteract renal vasopressin action in principal cells by inducing the internalization and lysosomal degradation of Aquaporin-2. Using miR-reporter constructs we identified microRNA-132 to directly target Cox2. Silencing of miR-132 *in vitro* in collecting duct cells as well as in fibroblast cells resulted in upregulation of Cox2 expression. Silencing of miR-132 *in vivo* in mice resulted in increased renal PGE2 production, as determined by urinary PGE2 levels, in combination with an attenuated AVP response. Subsequently, translocation of AQP2 to the apical membrane in collecting duct cells was disturbed. This ultimately led to acute diuresis and severe weight loss. These data demonstrate an essential role for miR-132 in water homeostasis.

Finally, in **Chapter 7**, all studies described in this thesis are discussed and put in perspective with respect to present knowledge.