Chapter 7

General discussion

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Chronic kidney diseases occur when disorders such as diabetes, chronic inflammation or toxic substances eventually lead to kidney failure and fibrosis deteriorating the structure of the organ. Patients with failing kidneys require dialysis or kidney transplantation, procedures that are either not successful in the long term (dialysis), or not always available (transplantation). As microRNAs (miRNAs) have been shown to be important in virtually all biological processes,it is no longer the question *if* miRNAs are involved in renal injury and repair but, instead, the focus is now on *what* critical pathways they modulate. Moreover, the fact that miRNAs can coordinate multiple genes, that are often functionally related, makes them attractive targets for intervention. In this thesis, we have provided compelling evidence for a regulatory role of miRNA-155, -126 and -132 in the pathophysiology of the kidney.

In **Chapter 2** we describe that miR-155 plays a rate-limiting role in endothelial to mesenchymal transition (EndoMT). EndoMT was originally recognized as a process in the developing embryo. Endothelial cells (ECs), lining the endocardium can dedifferentiate to cellularize the endocardial cushions in the development of functional heart valves[1]. This process is reminiscent to the formation of the tubular system in the kidney through mesenchymal to epithelial transitions during nephrogenesis[2,3] as described in the introduction of this thesis. During EndoMT, ECs loose their expression of endothelial cell markers, delaminate from the surrounding monolayer, and migrate into the cardiac jelly[4]. TGF-β signaling has been described to drive this process[5,6]. Next to the physiological role of EndoMT in embryogenesis, ECs that undergo EndoMT develop into myofibroblast-like cells and are thought to contribute to the development of renal fibrosis[7-9]. The analogue of EndoMT for epithelial cells, epithelial to mesenchymal transition (EMT), has even more often been described as a source of profibrotic myofibroblasts[10]. However, the exact origin of myofibroblasts has long been the subject of controversy. Genetic labeling studies in murine models of kidney fibrosis clearly demonstrated that no double-staining of labeled tubular epithelial cells with markers of activated fibroblast such as α-SMA and FSP-1 occurs, thus defying the existence of EMT[11]. The evidence for endothelial-to-mesenchymal transformation is built on genetic labeling of endothelial cells using ambigious markers like Tie-2[7]. Here clear double-staining of Tie-2 labeled cells and myofibroblast markers has been demonstrated. However Tie-2 is also expressed on myeloid cells and notably on pericytes. It is therefore very intriguing that studies

using sophisticated models of cell fate tracing demonstrated that pericytes and perivascular fibroblasts and not epithelial cells are the primary source of myofibroblasts in kidney fibrosis[11,12]. This could mean that the processes of EMT and EndoMT do not contribute quantitatively to the development of tissue fibrosis. However, a change in phenotype of these cells could be profibrotic through other mechanisms. For instance, EndoMT results in the loss of capillaries. Along the same lines, loss of pericyte–endothelial cross-talk in response to renal injury causes microvascular rarefaction and interstitial fibrosis, emphasizing the role of both the pericyte and the endothelial cell in this process[13]. The detachment of pericytes from endothelium under pathological conditions and differentiation into myofibroblasts leads to pericyte deficiency at the microvascular interstitial interface, resulting in unstable microvasculature and hence rarefaction, ultimately leading to nephron ischemia and loss[14].

As pericytes were demonstrated to constitute the primary source of myofibroblasts in kidney fibrosis, we investigated whether pericyte miRNAs play a rate-limiting role in myofibroblast formation. In **Chapter 5**, we used the same FoxD1-based murine lineage tracing model that was used to demonstrate the dominant role of pericytes in myofibroblast formation[11], and applied the ureter obstruction model to induce renal fibrosis. Following FACS sorting to isolate (differentiated) pericytes we showed a 21-fold upregulation of miR-132 in these FoxD1-derivative interstitial cells in obstructed kidneys versus contralateral kidneys. In total kidney extracts, we only found a 3-fold increase. This suggests that the increase in miR-132 is pericyte-myofibroblast specific. Silencing of miR-132 in this renal fibrosis model using antagomir-based inhibition resulted in attenuation of renal fibrosis as demonstrated by decreased collagen deposition and α-SMA expression. The number of α-SMA positive cells was decreased, suggesting less differentiation towards myofibroblasts by pericytes since it has been shown that virtually 100% of α-SMA positive interstitial cells are coming from FoxD1-positive progenitors[11]. In addition, *in vitro* antagonism of miR-132 in 3T3 mouse fibroblasts strongly decreased proliferation as determined by a thymidine incorporation assay, supporting an anti-proliferative role of miR-132 in pericytes/myofibroblasts.

Antagomir-based inhibition of miRNA function was used throughout this thesis. **Chapters 2, 3, 5 and 6** describe studies that involve miRNA silencing through intravenous injections of antagomirs, chemically modified cholesterol conjugated antisense oligonucleotides, which bind to its complementary miRNA, thereby blocking its function[15]. Antagomir administration almost

completely abrogates miRNA expression as demonstrated for example in **Chapters 5**, where, as long as ten days after injection this inhibition was observed. The data described in this thesis illustrates the potential therapeutic use of antagomir-based approaches for silencing of miRNAs. Indeed, a similar chemistry for antagonizing miRNA function is now investigated in humans in a phase II clinical trial for treating Hepatitis C[16]. Results of this study so far demonstrated that the drug was safe and well tolerated and resulted in decreased viral titers[17]. However, only few miRNAs are as tissue specific as miR-122 is for the liver[18], as most miRNAs are present in multiple cell types and tissues[19] and mostly have several targets and can affect very different pathways[20,21]. This makes the use of miRNA inhibitors challenging, as it could theoretically have multiple off-target effects. On the other hand, miRNAs could coordinately target multiple related genes within a certain pathway[22], thereby making them potentially even more attractive candidates to therapeutically target.

That miRNAs can have pleiotropic effects is demonstrated in this thesis. First, in **Chapters 3 and 4**, we describe an increase in circulating hematopoietic progenitor cells that results from both systemic silencing of miR-126 (**Chapter 3**) as well as from overexpression in the hematopoietic compartment (**Chapter 4**). This indicates that a single miRNA can work in opposite ways depending on cell type and the micro-environmental context and suggests that miR-126 could serve as a biological switch, with the response magnitude of biological pathways being dependent on cell type, the context and source of the external stimulus, and underlines the importance of miR-126 in vascular homeostasis. A striking example of the pleiotropic actions of miRNAs is presented in **Chapter 5** and **7.** MiR-132 has been described to be present in pericytes and to be upregulated in these cells during renal fibrosis. When we silenced this miRNA using antagomir-132 we could suppress the formation of myofibroblasts and reduce the development of renal fibrosis. In **Chapter 6**, using the same antagomir-132, we observed a completely different role for this miRNA. Here, we describe miR-132 to play an essential role in Aquaporin-2 mediated water reabsorption in the collecting duct through a prostaglandin-E2 and vasopressin dependent mechanism. This illustrates the difficulties that can be encountered using miRNA based therapeutics, as antagomir-132 used to suppress fibrosis development, could concomitantly affect water reabsorption and thereby have harmful side-effects. In fact, in our fibrosis study described in **Chapter 5** we observed weight loss 24 hour after injection of the antagomir-132, while scramblemir-treated mice did not lose weight. This is probably due to the polyuretic effects of miR-132

described in **Chapter 6**. Although hypothetical at this point, in both chapters, Cox2 can be a target of mir-132 that contributes to the observed findings. As described above, in **Chapter 6** miR-132 silencing increased Cox2 levels and elevated renal prostaglandin-E2 levels and potentially explain the observed polyuretic effects. In **Chapter 5**, augmented prostaglandin-E2 levels due to Cox2 increase in interstitial cells could reduce renal fibrosis by protecting these cells against apoptosis and oxidative stress[23].

In **Chapters 3 and 4** we focus on the role of miR-126 in vascular homeostasis. We previously collected data supporting a role for miR-126 in the angiogenic response induced by ischemia using a murine unilateral hind limb ischemia model[24]. Using this model we were able to assess the hypoxia induced angiogenic response in the gastrocnemius calf muscle[25] and found that the mice treated with antagomir-126 showed a strongly reduced capillary density in this muscle as compared to the scramblemir-treated control mice. However, *in vitro* experiments to assess the effects of antagomir-126 silencing in human umbilical vein endothelial cells (HUVEC) revealed functional impairments. The effects of miR-126 on angiogenesis likely involve mechanisms operational in and on endothelial cells in the *in vivo* context, possibly through its interaction with pericytes, as results described in **Chapter 4** suggest that miR-126 also influences pericyte incorporation in the vasculature.
Other groups also showed that targeted deletion of miR-126 in mice and zebrafish impairs angiogenesis, likely through upregulation of Sprouty-related EVH1 domain-containing protein (Spred-1) and phosphoinositol-3 kinase regulatory subunit 2 (PIK3R2) expression[26,27], which are actively involved in the negative regulation of vascular endothelial growth factor (VEGF) receptor and CXCR4 signaling[28] and are both predicted targets of miR-126[29]. Consequently, low levels of miR-126 would be associated with elevated levels of Spred-1 or PIK3R2 and repressed angiogenic signaling, which makes them likely targets to be associated with the decreased ischemia-induced angiogenesis as demonstrated in our studies. This mechanism could also apply to our findings in **Chapter 4**. Here we demonstrate that the angiogenic potential of miR-126 may reach beyond its presence in endothelial cells, as miR-126 was previously considered to be endothelial cell specific[30,31]. Recently, miR-126 has also been identified in platelets, epithelial cells and hematopoietic cells[32-34]. We over-expressed miR-126, together with a dsRed gene, in bone marrow cells and subsequently performed bone marrow transplantation. Following the restoration of the hematopoietic system, we induced renal ischemia reperfusion injury, and in separate mice we inserted angiogenic matrigel plugs supplemented with SDF-1 and VEGF in the flank

of mice. We demonstrate a protective role for miR-126 in renal ischemia reperfusion injury and improvement in capillary density in the kidney. In combination with higher numbers of bone marrow derived endothelial cells, as determined by dsRed and CD31 co-staining, this suggests protection of renal function through increased vasculogenesis or preserved vascular integrity by endothelial progenitor cells (EPC). Interestingly, it is known that circulating EPC are markedly reduced in renal failure[35] and kidney graft function was shown to determine EPC number in renal transplant recipients[36]. Of particular interest is that immunosuppressants, already at sub-clinical concentrations, appear to induce rapid cell death of EPC in early stages of their differentiation[37,38]. Another important player in our study could be the vesicles derived from EPCs that have been shown to protect against renal IRI through transfer of miR-126[39]. Therefore, overexpression of miR-126 in the hematopoietic compartment could potentially contribute to protection against renal IRI by transfer of miR-126 via microvesicles. Together these observations emphasize that augmenting microvascular endothelial repair through miR-126 modulation may well have therapeutic implications in renal disease.

To validate the role of hematopoietic miR-126 in vascular integrity, we performed a matrigel assay to determine whether miR-126 could contribute to neovascularization. We found an almost two fold increase in vascularization of the plugs. This confirms the angiogenic role of miR-126 we described previously[24]. Furthermore, MECA32+dsRed+ cells were incorporated in the vasculature, again implying a role for EPCs. This data supports our findings from the IRI experiment that hematopoietic miR-126 functions, at least partly, by stimulating vascularization.
The observed increase in vascularization of the matrigel plug however, could also be due to increased recruitment of leukocyte subsets. For instance, extravasation of monocytes into a foreign body can results in differentiation into macrophages that then can support angiogenesis[40,41]. Subsequently, macrophages can contribute to angiogenesis by producing angiogenic factors like VEGF and TGF-β[42,43] or facilitating anastomosis of the forming vessels[44].
In addition to bone marrow-derived EC, in the matrigel plug we observed double staining for dsRed and pericyte marker NG-2. This suggests that also pericytes could originate from bone marrow. Support for this notion is emerging in literature, where the role of bone marrow derived pericytes is increasingly discussed[45,46].

In our study described in **Chapter 4**, detailed flow cytometric analyses of the circulating hematopoietic cells in the mice that overexpressed miR-126 in the hematopoietic compartment, demonstrated selective mobilization of Lin-/Sca-1+/cKit+ (LSK) and Lin-/Sca-1+/Flk+ (LSF) cells in response to elevated SDF-1 expression by the ischemic kidney. SDF-1 expression is induced after renal IRI[47] and can augment the homing of bone marrow-mobilized progenitor cells to sites of injury[48-50]. A possible explanation for this selective mobilization could be that, in the BM, only the LSK cells displayed lower CXCR4 expression while the lineage-positive leukocytes expressed increased levels of CXCR4 and therefore would have a higher propensity to be retained in the BM[51]. Several studies have described a regulatory role for miR-126 in SDF-1/CXCR4signaling. MiR-126 was shown to target SDF-1 directly[52,53] but also indirectly via targeting regulator of G protein signaling 16 (RGS16), a negative regulator of CXCR4function[54]. Silencing of RGS16 is thought to stimulate an autoregulatory feedback loop that increases the production of SDF-1. Silencing of RGS16 by miR-126 could provide a mechanism for the elevated renal epithelial SDF-1 expression observed in mice that overexpressed miR-126.

In conclusion, the data described in this thesis illustrates the importance of miRNAs in (renal) biology. It also demonstrates the dynamic interplay between different miRNAs within the vascular system. Specifically, we describe roles for miR-155, miR-126 and miR-132 in vascular stability and differentiation of cells of the vascular system, i.e. endothelial cells and pericytes. MiR-126 is involved in neovascularization and maintaining vascular integrity, while miR-155 plays a role in differentiation of endothelial cells towards a mesenchymal-like cell, thereby potentially leaving the capillaries unstable. The same is true for miR-132, but then at the level of pericytes. The differentiation of these cells can result in detachment from the capillary thereby destabilizing it. In addition, it is reported that miR-132 in pericyte progenitor cells plays an important role in angiogenesis in the context of cardiac fibrosis[55]. Also, tumor endothelial cells were described to contain high levels of miR-132 which is involved in pathological (tumor) angiogenesis[56]. This emphasizes again the spectrum of activities of a single miRNA. These data show that all three miRNAs are involved in renal (patho)physiology and maintaining vascular integrity and further emphasizes the importance of the cellular context of miRNA function.

Future perspectives/Considerations

As has been shown in this thesis, miRNAs can have pleiotropic effects. To fully understand a single miRNA's diverse functions, one needs to comprehensively study a miRNA of interest in several models, different (micro)environments and a variety of cell and organ types. For example, profiling can be done on total kidney tissue from injury models but the interplay of so many different cell types including the inflammatory cells make it essential to employ models of lineage tracing to reveal the true role of the individual players as its roles may be contradictory in different cells types. On the other hand, to fully understand a biological process, one also needs to study the interplay of different miRNAs involved in the same process. Although the studies described in this thesis provide an important step towards better understanding, extensive follow up research is needed to fully comprehend renal miRNA biology.

Furthermore, we can learn from other organs. For example, in animal models of cardiac fibrosis loss of miR-29a function in fibroblasts was related to the excessive production of extracellular matrix proteins[57]. Also, upregulation of miR-21 in fibroblasts of the failing heart appears to enhance fibroblast survival and cardiac fibrosis[58]. In fact, also in renal fibrosis models, these miRNAs have been shown to be important modulators of fibrosis[59,60].

In addition, the mechanisms underlying the regulation of miRNA expression itself need more investigation. If we can identify how miRNA expression is controlled, this provides us with the opportunity to take over control and modulate this expression.

In conclusion, identifying the role of miRNA involvement in all aspects and layers of modulation within renal functioning is a vast task and challenge, but one that will lead to the generation of novel therapeutic approaches to maintain and improve renal function after injury.

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