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14q32 Noncoding RNAs in vascular remodelling

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Part III

Chapter 7

General Discussion and Future Perspectives

General discussion

A large noncoding RNA gene cluster located on the 14th chromosome in human includes the largest known microRNA cluster, a snoRNA cluster and three long noncoding RNAs. MicroRNAs and snoRNAs of the 14q32 locus are known to play different roles in cardiovascular disease and vascular remodelling. The aim of this thesis was to elucidate the differential expression of 14q32 microRNAs and snoRNAs in the vasculature and to identify regulators of 14q32 microRNAs in vascular remodelling. It is known that microRNAs can change in vascular remodelling processes. However, it was not uncovered whether expression of 14q32 microRNAs differs between different locations in the vasculature. Moreover, 14q32 microRNAs are regulators of vascular remodelling processes, but can also be regulated. The mechanisms of action of 14q32 microRNA regulators and the possibility to use these regulators in targeting vascular remodelling were investigated in this thesis.

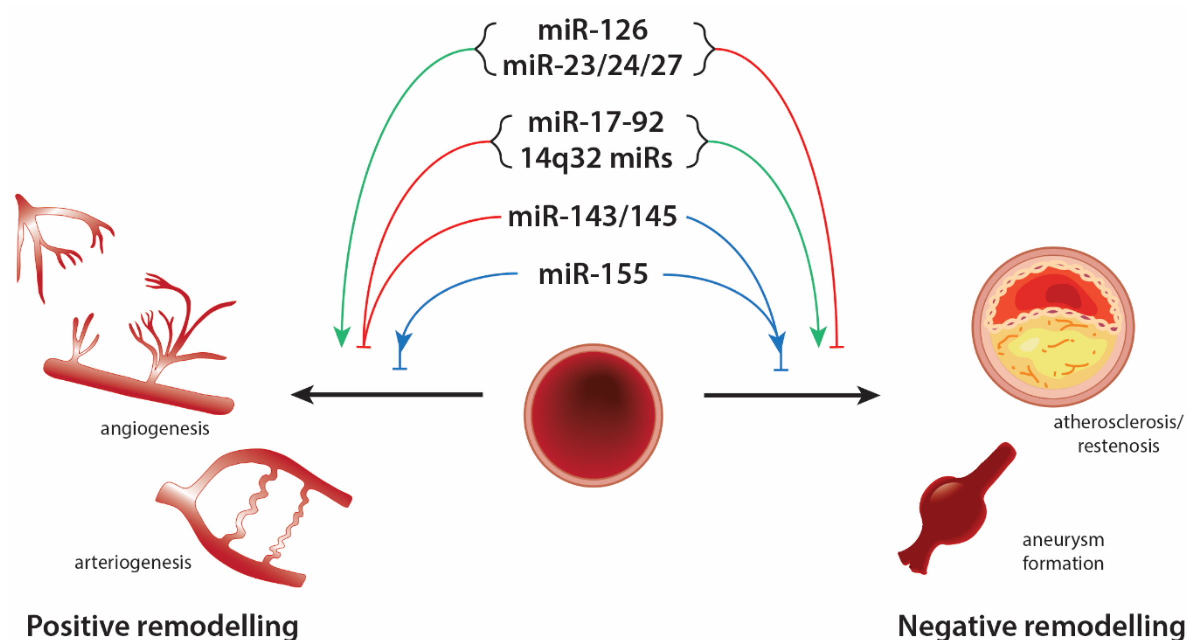

Figure 1 Graphical representation of microRNA clusters in vascular remodelling. MiR-126 and miR-23/24/27 induce positive remodelling and inhibit negative remodelling, whereas miR-17-92 and 14q32 microRNAs inhibit positive remodelling and stimulate negative remodelling. The effect of miR-143/145 and miR-155 on negative remodelling is inconclusive, but miR143/145 inhibits positive remodelling. Figure from *Welten & Goossens et al. Cardiovasc Res. 2016.*

In **Chapter 2**, we focused on microRNAs that are involved in both positive vascular remodelling (neovascularization) and negative vascular remodelling (atherosclerosis, restenosis and aneurysm formation). The individual microRNAs miR-126 and miR-155 as well as microRNA gene clusters miR-17/92, miR-23-24-27, miR-143/145 and 14q32 microRNAs were reviewed. Their multifactorial nature was highlighted as they play a role in

multiple vascular remodelling processes. MiR-126 and the miR-23/24/27 family was shown to stimulate positive vascular remodelling and inhibit negative remodelling at the same time. MiR-17/92, miR-143/145 and the 14q32 microRNA cluster, on the other hand, inhibit positive remodelling and induce negative remodelling. MiR-155 was described to play a role in all vascular remodelling processes, but could not be determined as specific inhibitor or inducer of remodelling processes. As different microRNAs or microRNA families affect vascular remodelling similarly or oppositely, it could be suggested that they reinforce each other, however, this needs to be studied further. This chapter provides openings for new therapeutic opportunities and more research is needed to identify the efficacy of promising microRNAs or microRNA clusters in treatment of cardiovascular disease. Findings of **Chapter 2** are summarized in Figure 1.

14q32 ncRNA expression

The human 14q32 ncRNA cluster is known to include 41 snoRNAs. In mice, the number of snoRNAs has not yet been established. The function of snoRNAs in general in cardiovascular disease[1, 2] and the function of 14q32 microRNAs in cardiovascular disease[3-6] was studied. However, the role of 14q32 snoRNAs in cardiovascular disease had not yet been elucidated. In **Chapter 3**, several lines of evidence are found to support 14q32 snoRNAs to be highly important regulators in cardiovascular disease. Firstly, single nucleotide polymorphisms (SNPs) that are present in genes encoding for 14q32 snoRNAs, were associated with heart failure, independently of microRNAs and long noncoding RNAs. Furthermore, in human blood vessel samples, both healthy and diseased, 14q32 snoRNAs were measured and showed higher expression in the head and neck area than in the lower limbs. Moreover, five out of seven measured 14q32 snoRNAs were upregulated in failed human vein grafts compared to the naïve vein grafts. In mice a similar upregulation of snoRNA expression was observed in vein graft disease. Next, in the STEMI cohort blood samples collected during ST-Elevation Myocardial Infarction (STEMI). After STEMI, SNORD113-2 in peripheral blood was changed 4 days after the myocardial infarct compared to 30 days after the event. This implies that 14q32 snoRNAs are affected in various forms of cardiovascular disease. Additionally, functional *in vitro* assays showed that upregulation of snoRNAs in murine cells led to a decreased cell migration in two of five measured snoRNAs and Fibrillarin, a methyltransferase that acts as a snoRNA-guided 2’O-ribose-methylation agent of target ncRNAs, was bound to 14q32 snoRNAs. Taken together, this study demonstrates the importance of 14q32 snoRNAs in cardiovascular disease and vascular remodelling. Further studies into finding snoRNA targets and mechanistic pathways of 14q32 snoRNAs are required to fully understand the role of snoRNAs in vascular processes.

More was already known about 14q32 microRNAs in cardiovascular disease. Inhibition of 14q32 microRNAs decreases negative vascular remodelling processes and improves positive vascular remodelling[3-6]. As it was not known what 'normal' expression of these microRNAs is, how they change under pathophysiological conditions and whether 14q32 microRNAs have vascular location-specific expression patterns, we aimed to make a vascular map of microRNA expression. This study is described in **Chapter 4** for which a biobank was compiled of vascular tissue samples originating from most location in the vasculature. Surplus vascular tissue samples were discarded during surgery and microRNA expression levels were measured. As for 14q32 snoRNAs, microRNA expression was shown to be highly vascular location specific. We could therefore conclude that microRNA expression really shows vascular fingerprints. Highest expression was present in the lower limb vessels, whereas vessels in the neck area that could suffer from the same vascular disease, namely atherosclerosis, showed lowest expression for all microRNAs. Furthermore, all 14q32 microRNAs showed higher expression in arteries than in veins. Within a blood vessel wall, highest microRNA expression was present in the smooth muscle cell layer. This is a layer that is prominently present in the arterial wall, but smooth muscle cells are less abundant in the venous vessel wall. Endothelial cells had lowest expression for all microRNAs. 14q32 microRNA expression did not correlate with age, nor with sex, but showed inverse expression of target genes that were known to play a role in cardiovascular disease. Malignancies tended to increase vascular microRNA expression which was already shown within tumor cells itself. While compiling the miRMap biobank, even though we succeeded in including many different vessels, we realized that parts of the vasculature are missing to complete the atlas. For example, lung vessels, heart tissue, intracranial vessels and caval vein are not included as these tissues are not taken out during surgery of living patients. However, it would be interesting to include these samples as cardiovascular disease like thromboembolic events occur in the brain and lung vessels.

Interestingly, expression in atheroprone locations as the lower limb vessels and the neck vessels is opposing for 14q32 snoRNAs (**Chapter 3**) and microRNAs (**Chapter 4**). Whereas both ncRNAs showed increased cell migration upon downregulation. Given these differences in expression patterns, the question remains what the association is between microRNAs and snoRNAs encoded in the same locus. Both ncRNAs could antagonize each other, like was seen between lncRNAs and other ncRNAs as microRNAs and snoRNAs[7]. Another possibility is that, for example, in a healthy vessel microRNAs are residing and, therefore, not active during low expression in the neck vessels, whereas the snoRNAs are

highly expressed and actively repressing vascular remodelling. In that way, they cooperate in maintaining integrity of the vessel wall. The exact mechanism of interaction between 14q32 microRNAs and snoRNAs has to be investigated. Summarizing, the first part of this thesis emphasizes that expression is regulated individually for each 14q32 ncRNA and is highly vascular disease and location specific.

Expression regulation

DNA methylation along the 14q32 locus was proposed as a possible gene expression regulator and, therefore, a regulator of 14q32 microRNA expression. In **Chapter 4** 14q32 DNA methylation was measured throughout the human vasculature. In comparison of arteries and veins of patients with either PAD or CAD, differences between naïve VSMs harvested before implantation as coronary bypass, lower limb veins of patients with PAD and critically ischemic lower limb veins were observed. Especially in patients with severe ischemia, for which lower limb amputation was indicated, 14q32 DNA methylation was extremely high in the IG-DMR-CG4-2, MEG3-DMR-1B and MEG8-DMR-1H. Furthermore, in a murine ischemia model and a vein graft disease model, we observed remodelling-specific 14q32 DNA methylation changes. In vein graft remodelling, during active remodelling at two weeks after grafting[8, 9], DNA methylation was changed. At four weeks this process is more or less stabilized and 14q32 DNA methylation level returned to the native status. This was observed for DNMTs as well. In hindlimb ischemia, DNA methylation increased over time within the different DMRs between ischemic and control hindlimbs. However, as microRNAs and DNA methylation vary between vascular remodelling statuses, it was assessed whether these two correlate directly. This was not the case for any microRNA or DMR along the 14q32 locus nor for primary microRNAs and DNA methylation and not for (primary) microRNAs and DNMT expression. Therefore, 14q32 DNA methylation is not directly linked to 14q32 microRNA expression throughout the human vasculature. This was previously claimed by Aavik et al.[10] for atherosclerotic plaques. This difference could be explained by the fact that we looked at individual methylation site changes using restriction enzyme digestion followed by qPCR and not looked at a more global level, using bisulfite sequencing. DNA methylation is not only known as a gene expression regulator, but was also described as a regulator of alternative splicing[11]. However, the extend in which 14q32 DNA methylation acts in this process in cardiovascular disease, has to be investigated. Taken together, 14q32 DNA methylation cannot be considered as regulator of 14q32 microRNA expression in cardiovascular disease, but is associated with vascular remodelling status independently of 14q32 microRNAs.

Another possible 14q32 microRNA regulator discussed in this thesis is myostatin. As described in **Chapter 5**, myostatin is a negative regulator of muscle cell proliferation and of 14q32 microRNA expression. This is due to the ability of myostatin to bind the callipyge locus, another name for the 14q32 locus, and thereby affects microRNA expression. Therefore, we decided to study the effects of inhibiting 14q32 microRNA expression by myostatin administration. It is interesting that previous studies found that microRNA expression of the whole locus was affected by myostatin, because we observed in **Chapter 4** that expression of the 14q32 microRNAs along the locus differs from microRNA to microRNA. We observed indeed a downregulation of 14q32 microRNA expression upon myostatin administration in a dose-specific way. Next to the effect of myostatin on microRNA expression, the functional hypothesis was that myostatin, as negative muscle cell regulator, could inhibit vascular smooth muscle cell proliferation, one of the main features of post-interventional restenosis. Proliferation was indeed inhibited both *in vitro* and *in vivo*. However, myostatin did not have a clear consistent effect on the other cell type that is involved in restenosis, i.e. macrophages. Moreover, 14q32 microRNA expression in macrophages was not consistently changed either. As myostatin only has an effect on VSMCs, but not on macrophages, no clinical effect was found. This emphasizes that for targeting restenosis, compounds are needed that target both inflammation and VSMC proliferation. Findings of **Chapter 5** are summarized in Figure 2.

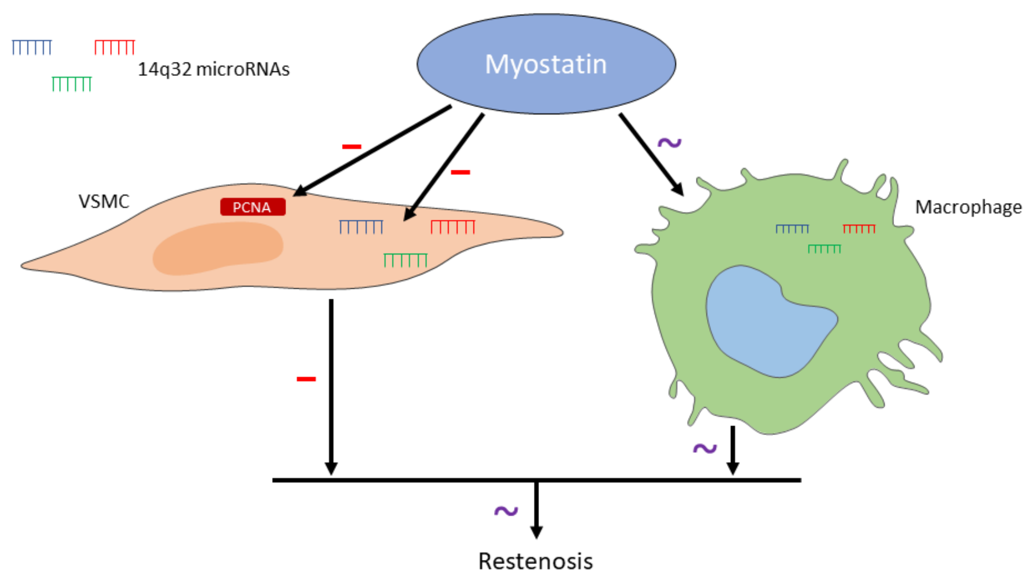

Figure 2 Graphical representation of the function of myostatin in restenosis.

The last possible regulator of 14q32 microRNA expression that is discussed in this thesis, is on RNA binding protein (RBP). We investigated cold-inducible RNA binding protein (CIRBP) in detail. It was shown previously that this RBP is able to bind 14q32 precursor microRNAs of

miR-329 and miR-495 to enhance processing into mature microRNAs[12]. **Chapter 6** investigates the *in vitro* effects of hypothermia and knockdown of CIRBP on total CIRPB, its splice variants, antisense long noncoding RNA and the target microRNAs. The target microRNAs of CIRBP, miR-495 but not miR-329, were downregulated in CIRBP knockdown. They were not altered in CIRBP overexpression, suggesting that CIRBP is needed for processing, but is not a rate limiting factor under normothermic conditions. Furthermore, the proangiogenic features of CIRBP knockdown, especially splice variant 1 of CIRBP, CIRBP-SV1, were assessed and it was shown that this condition had better migration and tube formation capabilities. Moreover, the antisense long noncoding RNA of CIRBP, CIRBP-AS1, seemed to react similarly to hypothermia and CIRBP knockdown as CIRBP itself. Therefore, CIRBP-AS1 was knocked down and this showed a knockdown of CIRBP as well. Again, CIRBP-SV1 was most prominently affected. Furthermore, endothelial cell migration increased upon CIRBP-AS1 knockdown. However, it remains unclear whether CIRBP affects CIRBP-AS1 or vice versa and whether they collaborate or reinforce each other in angiogenesis. This still needs to be elucidated. Taken together, CIRBP is a promising target to stimulate post-ischemic neovascularization, like another RBP, MEF2A, was shown to act on 14q32 microRNAs to stimulate neovascularization[5]. HADHB in a previous study was shown to have the same 14q32 microRNA targets as CIRBP[12] and it is interesting to assess the effect of HADHB knockdown on angiogenesis and its target microRNAs. Moreover, other RBPs that target microRNAs[13] have to be investigated for binding to 14q32 microRNAs to link them to specific vascular remodelling processes. Given this, it is interesting to target RBPs to affect microRNAs and thus regulating vascular remodelling processes. Thereby, off target effects of direct microRNA manipulation are avoided, as microRNAs have specific expression patterns for each vessel type. This was shown in **Chapter 4**. However, it remains to be investigated whether RBPs only bind microRNAs under specific conditions or stress factors or that this binding and affecting process is always present. Findings of **Chapter 6** are summarized in Figure 3.

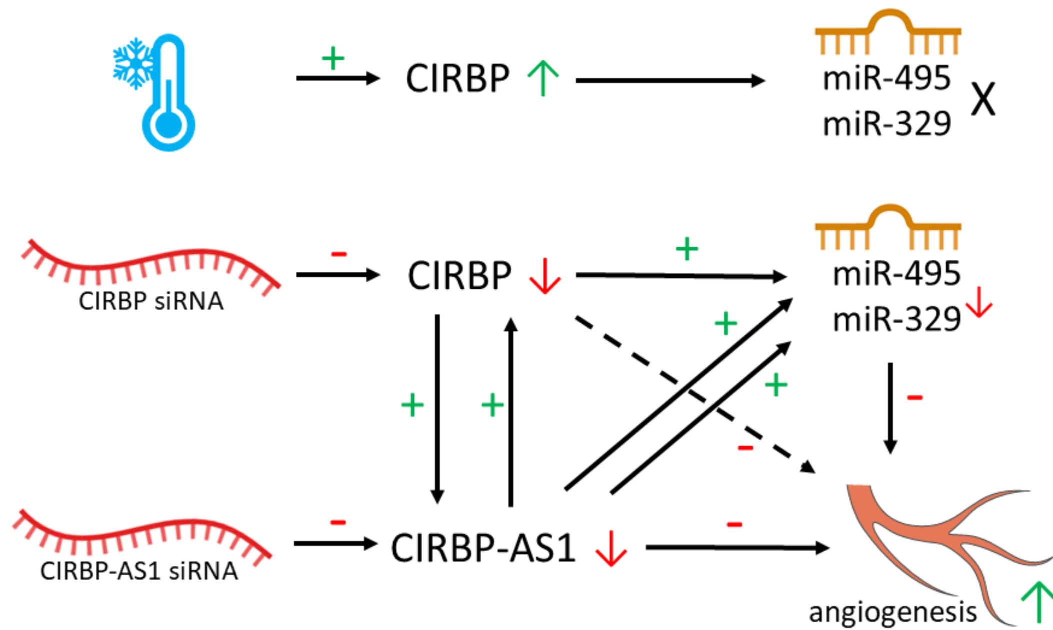

Figure 3 Graphical representation of CIRBP in angiogenesis.

Future perspectives

This thesis has uncovered that 14q32 noncoding RNA expression differs highly throughout the human vasculature. Moreover, we have discussed several potential 14q32 microRNA regulators. However, still numerous hurdles have to be taken before this knowledge can be applied in clinical practice.

As we now know that 14q32 noncoding RNAs have location-specific expression fingerprints, it would be interesting to investigate the possibility to target these noncoding RNAs locally. Systemic side effects of noncoding RNAs are avoided in this strategy. In future clinical practice it would be ideal to treat patients suffering from cardiovascular disease by targeting, for example, a microRNA or snoRNA that is only present in the diseased vessel. It would even be more interesting to use microRNAs or snoRNAs as guide RNAs to bring a specific substance to particular cells and once it is in the cell, release a DNA methylation changing substance to affect methylation that is specific for the remodelling process, as DNA methylation is vascular remodelling process-specific. Thereby, systemic DNA methylation treatments that are mostly toxic and used in the field of oncology, are avoided. Steps that have to be taken, are investigation of off-target effects of noncoding RNA treatment in different cell types and vascular locations. Identifying these effects, results in finding an optimal therapeutic window.

In this thesis, restenosis is emphasized to involve both VSMC migration and -proliferation and inflammatory reactions, mainly by macrophage influx. These factors are equally important to be targeted in restenosis prevention or treatment. Therefore, to overcome post-interventional vascular remodelling, both VSMCs and macrophages should be targeted. Myostatin acts on local VSMCs and local microRNAs in VSMCs, but a regulator of macrophages should be added to decrease restenosis. Preferably, again only local anti-inflammatory compounds should be considered, as systemic immune suppressing agents are not favorable.

CIRBP is a promising target for increasing angiogenesis and it is even more interesting as downregulation of CIRBP-AS1 also targets CIRBP and angiogenesis. It has to be elucidated in further research what the actual interaction is between CIRBP and CIRBP-AS1. Furthermore, future research will point out whether CIRBP knockout *in vivo* results in increased blood flow recovery in hindlimb ischemia. Does CIRBP knockout increase neovessel formation around the occluded vessel in this model and ultimately in PAD? The final aim is to target CIRBP in human PAD or CAD. Moreover, in this thesis, we found that miR-495 is affected by CIRBP knockdown, but it remains to be determined whether this is the key factor in promoting angiogenesis or that CIRBP has another main target in stimulating neovascularization. A remaining question is whether CIRPB-AS1 knockdown affects angiogenesis via 14q32 microRNA expression or via which other pathway CIRBP-AS1 knockdown stimulates neovascularization. In finding answers to these questions CIRBP will turn out as potential clinical target or not.

The ultimate goal in noncoding RNA research in vascular remodelling is to find a therapeutic option to prevent, stop or even diminish cardiovascular disease and the subsequent burden on patients. This aim could either be accomplished by direct or indirect targeting of noncoding RNAs.
In conclusion, this thesis provides novel insights in the differential expression of 14q32 noncoding RNAs in the human vasculature. Experimental studies have identified possible regulators of 14q32 microRNA expression in vascular remodelling processes as restenosis and neovascularization. With many steps to take, ncRNA therapies in cardiovascular disease are still far away, but hopefully in the future it is possible to prescribe these therapies to patients that are currently not treated optimally.

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