14q32 Noncoding RNAs in vascular remodelling

Goossens, E.A.C.

Chapter 1

General Introduction

General introduction

Cardiovascular disease

Cardiovascular disease is the collective term for multiple diseases of both the heart and blood vessels. It is among the most frequently occurring diseases in the world with high morbidity and mortality rates, especially in the western world[1]. These rates increase with increasing welfare of countries due to risk factors that are more common in a modern lifestyle. Examples of risk factors for cardiovascular disease are intake of too much salt and fat, a sedentary lifestyle and a lack of physical activity. This results in hypertension, hypercholesterolemia, obesity and diabetes and could lead to cardiovascular diseases like peripheral arterial disease and myocardial infarction. Eventually, end stage peripheral arterial disease could lead to severely ischemic lower limbs that need to be amputated in a final stage to save the patient's life. After myocardial infarction, the ischemic heart tissue is not able to contract properly. This leads to heart failure, defined by the inability of the heart to pump sufficient blood through the whole body to supply all organs with enough oxygen and nutrients. To avoid end stage peripheral arterial disease and heart failure, therapeutic interventions are present, but nowadays still insufficient to completely save patients from suffering. Therefore, new therapeutic options are needed to decrease the burden of cardiovascular diseases.

A common factor in the various types of cardiovascular disease is that some form of vascular remodelling occurs. Vascular remodelling processes can be divided into negative and positive vascular remodelling. Negative remodelling processes include atherosclerosis, restenosis, aneurysm formation and remodelling after arteriovenous fistula formation, all disadvantageous for vascular health. Positive remodelling processes comprise angiogenesis and arteriogenesis, together called neovascularization and are beneficial adaptive processes.

Negative vascular remodelling

Atherosclerosis is the complex process of vessel wall thickening. In fact, it is the thickening of the intimal layer of the arterial wall. Atheroma formation starts with lipid accumulating cells that extravasate and form a fatty streak[2]. Among the extravasating cells, macrophages (foam cells) and T cells are the most common cell types[3]. Over time, cells accumulate in the intimal layer and smooth muscle cells will migrate from the medial layer to the intimal layer to form a large extracellular matrix and fibrous cap[4]. When the atheroma grows, the inner part can become necrotic and the fibrous cap loses stability. Such a plaque is called instable and, when an instable atherosclerotic plaque ruptures, a thrombus can attach to it in the lumen causing a partial or complete occlusion of the artery. The initial vessel wall thickening already

decreases lumen area and causes a reduced blood flow to downstream tissues. This results in deprivation of oxygen and nutrients to the tissues and in peripheral arterial disease a ruptured atherosclerotic plaque with arterial occlusion causes clinical features of pain, pallor, pulselessness, paresthesia, poikilothermia and paralysis (the six Ps). A ruptured atherosclerotic plaque in the carotid artery can lead to ischemic stroke as parts of the carotid thrombus can release from it and can obstruct the flow in the smaller downstream branching intracranial vessels. In the coronary artery, a thrombus leads to myocardial infarction. Ischemic myocardium results in dying myocardial cells with subsequent inability to contract and pump sufficient blood through the body. This lack of reaching adequate cardiac output is called heart failure and, more specifically, ischemic heart failure in case of myocardial infarction.

These acute clinical situations should be treated immediately to save ischemic tissues. Current invasive revascularization treatment options for peripheral arterial disease and coronary artery disease comprise endovascular interventions or bypass surgery. Endovascular therapies include balloon angioplasty with or without stent placement. This treatment is effective, however, the disadvantage of this therapy is the high risk of restenosis[5]. The second treatment option, bypass surgery, is less favorable in the acute situation, but recommended when endovascular therapies are not possible, not successful or the vessel shows recurrent occlusions. Bypasses can be made from the patients own arterial or venous vessels. The latter is more common and more easily available, but tends to show higher graft occlusion rates. This is called vein graft disease, the formation of an atheromatous plaque in the grafted vein[6].

During restenosis, previously stenosed arteries that were treated by an angioplasty procedure with or without stenting, are narrowed again. This secondary stenosis is triggered by manipulation of the occluded vessel. The response to this manipulation consists of two cellular components, namely an inflammatory cell reaction and a reaction by vascular smooth muscle cells (VSMCs). Inflammatory cells adhere to the injured vessel wall and cause an inflammatory reaction. Macrophages, like in atherosclerosis, are the most common cell type that invade into the vessel wall and even further increase the inflammatory reaction. VSMCs are the second cell type that is triggered by the manipulation of the occluded vessel wall. VSMCs start to proliferate and migrate from the medial layer into the intimal layer, where they form a neointimal layer. Furthermore, extracellular matrix remodelling accompanies these reactions, resulting in thickened vessel walls.

Positive vascular remodelling

Positive vascular remodelling comprise processes that are beneficial during vascular disease and includes angiogenesis and arteriogenesis, together called neovascularization. Angiogenesis is the formation of new capillary blood vessels into tissues where no existing blood vessels are located and is driven by hypoxia[7]. Arteriogenesis is the process of blood vessel maturation out of small pre-existing vessels by increased shear stress[8]. Both neovascularization processes are needed to restore blood flow to ischemic tissues in atherosclerosis or restenosis. Under physiological conditions neovascularization occurs, however, this is not sufficient to fully restore blood flow to downstream tissues and should be reinforced to form fully functional new blood vessels that function as bypass. Neovascularization is not always regarded as a positive vascular remodelling process. In the field of oncology, neovascularization to supply blood to the neoplasm is an undesirable process and possible therapeutic targets should be investigated for not affecting this neovascularization. This thesis will focus on neovascularization as treatment strategy for cardiovascular disease and thus as a positive remodelling process.

Taken together, new treatment options are needed for cardiovascular diseases either to stimulate the body to improve positive remodelling processes or to inhibit negative vascular remodelling processes. Since current interventional treatment options for cardiovascular disease are not completely optimal, the aim of research is to find new therapeutic options with higher efficacy. Genetic components of cardiovascular disease have been studied widely over the last decades. One of these genetic components is the field of noncoding RNAs (ncRNAs) and what is known, is yet far from complete.

Noncoding RNAs

Only a small proportion of the genome encodes for mRNA leading to protein production. The majority of the genome (>95%) is not protein-coding[9] and therefore called noncoding. Before the role of ncRNAs was discovered, this was called junk-DNA. NcRNAs are RNAs that are transcribed from DNA, but not further translated into proteins. However, as RNA it regulates other RNAs, like messenger RNA (mRNA), transfer RNA (tRNA) or ribosomal RNA (rRNA). Thereby, ncRNAs can affect ongoing cellular processes. All ncRNAs can be divided into small ncRNAs, including microRNAs and small nucleolar RNAs (snoRNAs), and long noncoding RNAs, based on their length in nucleotides. This thesis will focus on microRNAs and snoRNAs.

MicroRNAs

MicroRNAs are small ncRNAs of approximately 22 nucleotides in length and were first described in 2001[10-13]. They are transcribed by RNA polymerase II to primary microRNAs and subsequently processed by Drosha, a processing complex, into precursor microRNAs. These are transported from the nucleus to the cytosol and there the precursor microRNAs are cleaved into two mature microRNA strands by the enzyme Dicer[14, 15]. Mature microRNAs are loaded onto the RNA Induced Silencing Complex (RISC) and in this form able to bind complementary to the 3'-untranslated region of their target mRNA. By binding to a target mRNA, translation is inhibited. Therefore, microRNAs are called post-transcriptional regulators of gene expression. One microRNA can have up to several hundreds of target genes. If the target genes act in common physiological or pathophysiological processes and are targeted by one single microRNA, entire processes can be regulated. This has previously been shown for several non-cardiovascular[16, 17] and cardiovascular diseases[18, 19]. MicroRNAs tend to occur in clusters and have already shown tissue specific expression patterns[20].

Small nucleolar RNAs

As well as microRNAs, small nucleolar RNAs (snoRNAs) belong to the group of small ncRNAs, although they are longer than microRNAs with an average length of 60-200 nucleotides[21]. SnoRNAs can be divided into C/D-box snoRNAs and H/ACA-box snoRNAs based on their mechanism of action. C/D-box snoRNAs modulate target ncRNAs via 2'O-ribose-methylation, whereas H/ACA-box snoRNAs modulate other ncRNAs via pseudouridylation. Some snoRNAs have known cellular functions like in cholesterol trafficking[22] and in metabolic stress[23], but they also target other regulators like rRNAs[24] or regulate alternative splicing of mRNAs[25]. However, many snoRNAs do not have known target RNAs and are presumed to have non-canonical targets. Moreover, the role of snoRNAs in cardiovascular disease has not been elucidated and needs to be investigated in depth.

DNA methylation

DNA methylation is an epigenetic feature that is characterized by the addition of a methyl group to a cytosine nucleotide to form a 5-methylcytosine. DNA methylation is subject to mutagenic loss, but is conserved in CpG-islands that mainly are located in promotor regions of genes[26]. DNA methylation occurs under the influence of DNA methyltransferases (DNMTs).

DNMTs can be divided into de novo DNMTs DNMT3A and 3B[27, 28], that methylate unmethylated DNA, and maintenance DNMT DNTM1[29], that methylates hemi-methylated DNA or preserve preexisting methylation patterns during replication. DNA methylation is variable between individuals and that is why CpG-rich regions are called Differentially Methylated Regions (DMRs). DNA methylation status can change under the influence of pathophysiological conditions, like in cancer[30-34], but also in cardiovascular disease[35, 36]. DNA methylation acts mostly by inhibiting gene expression through prevention of transcription, but is also known to affect alternative splicing[37].

14q32 noncoding RNA cluster

NcRNAs and DNA methylation appear throughout the whole genome and are factors that influence and regulate gene expression. Previously published Reverse Target Prediction analysis[38, 39] analyzed genes that were known to act in cardiovascular disease, for microRNAs by which they were targeted. The result of this was that many microRNAs were located closely to each other. In fact, they were located in a large conserved gene cluster on the 14th chromosome in humans (14q32 locus)[40]. Besides 54 microRNAs, the human 14q32 locus contains 41 snoRNAs, 3 DMRs, 3 long noncoding RNAs, and 3 coding genes[38, 41]. The locus is shown in Figure 1. The human 14q32 locus is also known as DIO3-DLK1 locus, called after two coding genes located along this locus. The murine equivalent of this locus is located on the 12F1 location[40] and contains 61 microRNAs[38].

MicroRNAs of this cluster have been studied extensively in several experimental murine models. Our group has found that inhibition of miR-494 inhibits atherosclerotic plaque development and increases plaque stability[39], but inhibition of miR-495 decreases post-interventional restenosis in mice[42]. Furthermore, inhibition of miR-329, miR-487b, miR-494 and miR-495 also increases post-ischemic neovascularization in mice[38] and miR-487b plays a role in angiotensin II-induced aneurysm formation in rats[43]. Moreover, other groups also found that the 14q32 microRNA cluster is involved in many different vascular remodelling processes[41, 44, 45].

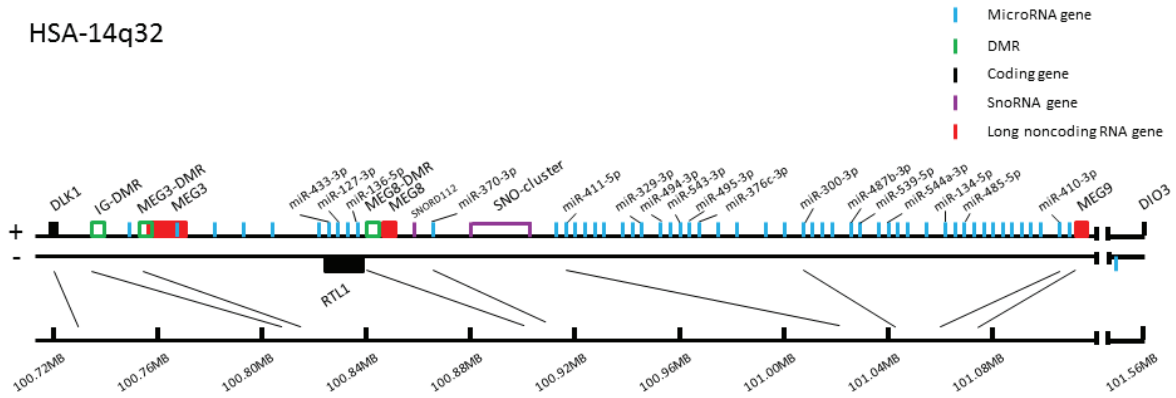

Figure 1 Schematic presentation of the human 14q32 locus. Protein coding genes are depicted in black, long noncoding RNA genes in red, microRNA genes important in vascular remodelling in blue, DMRs in green and snoRNA genes in purple.

NcRNAs of the 14q32 locus have functions in cardiovascular disease or vascular remodelling, but it is unknown whether all ncRNAs are located in all vessels, healthy or not, or that ncRNAs are vessel-specific and actually have their own "fingerprint". We know that parts of the human vasculature are more prone to different types of cardiovascular disease than others. Atherosclerosis occurs predominantly in larger arterial walls where disturbed flow is present[46], while arterial aneurysms occur in the aortic wall or intracranial artery walls[47, 48]. As we know that these processes can be regulated by different 14q32 ncRNAs, it is interesting to investigate whether 14q32 ncRNA expression is vascular location specific or even cell layer specific, like has been shown for non-14q32 microRNAs miR-126[49] and miR-145[19, 50].

Expression regulators of 14q32 microRNAs – (post-)transcriptional regulators

Previous studies have shown that microRNAs of the 14q32 cluster have multiple functions in vascular remodelling processes and that inhibiting them is beneficial, i.e. negative remodelling is inhibited and/or positive remodelling is stimulated. It is possible to directly interfere with microRNA expression by administrating microRNA-inhibiting antisense oligonucleotides[38, 39, 42] or microRNA mimics. However, it would be interesting to find factors by which 14q32 microRNA expression is regulated naturally and, thereby, finding targets to regulate complete physiological processes instantly. Myocyte Enhancer Factor 2A (MEF2A), Cold-Inducible RNA-Binding Protein (CIRBP) and Hydroxyacyl-Coenzyme A Dehydrogenase Trifunctional Multienzyme Complex Subunit Beta (HADHB) have already been shown to bind 14q32 microRNAs post-transcriptionally[51, 52] and more in-depth knowledge about the mechanism of action of RNA-binding proteins and possible transcriptional factors is needed.

DNA methylation

DNA methylation is one of the mechanisms that is known to regulate transcription. The three DMRs located along the human 14q32 locus are called Intergenic-DMR (IG-DMR), Maternally Expressed Gene 3-DMR (MEG3-DMR) and Maternally Expressed Gene 8-DMR (MEG8-DMR). IG-DMR is located between the DLK1-gene and the DIO3-gene, but upstream from all ncRNAs. MEG3-DMR is partially overlapping the beginning of the lncRNA MEG3-gene and MEG8-DMR is located just upstream of the lncRNA MEG8-gene. The murine DMRs are called Dlk1-DMR, IG-DMR and Glt2-DMR. DNA methylation of the 14q32 locus is known to change in several pathophysiological conditions[30, 32, 33, 53] and was also found to be changed in cardiovascular disease[54]. 14q32 DNA methylation has been described to associate with microRNA expression[31, 54] and it has to be assessed whether individual microRNAs also correlate with DNA methylation status within the DMRs. Furthermore, like microRNA expression, DNA methylation is disease specific and vascular diseases occur in different locations throughout the human vasculature. It could be hypothesized that DNA methylation is altered in a vascular location-specific and disease-specific manner.

Myostatin

Myostatin, also known as Growth Differentiation Factor-8 (GDF-8), is a member of the Transforming Growth Factor beta (TGF-β) superfamily and it is a protein that negatively regulates skeletal muscle cell proliferation and differentiation. Myostatin knockout in mice results in excessive skeletal muscle growth[55]. It is known that myostatin affects microRNA expression of the 14q32 locus, also called the callipyge locus. In fact, mutations in the myostatin gene or in the callipyge locus, that result in the inability of myostatin to bind to the locus, cause the typical callipyge phenotype. This phenotype is characterized by excessive muscle mass and that explains the origination of the name “callipyge”. In ancient Greek the word καλλος means beautiful and πυγη means buttocks. This phenotype is favorable in cattle. In mice, myostatin knockdown resulted in increased muscle mass and 14q32 microRNA upregulation[56], whereas myostatin addition led to muscle atrophy[57]. However, myostatin not only acts in skeletal muscle cells, but also in vascular smooth muscle cells (VSMCs)[58]. Exactly this is the cell type to target in restenosis and, more specifically, of which proliferation and hyperplasia has to be inhibited. Taken together, it could be hypothesized that myostatin administration causes 14q32 microRNA downregulation and, subsequently, restenosis is decreased. Together with the fact that a previous study showed that direct inhibition of 14q32 microRNA miR-495 caused decreased restenosis[42], this makes myostatin an even more promising target to inhibit restenosis via downregulation of the 14q32 locus in experimental restenosis. The effect of myostatin on macrophages has to be investigated, as this previous

study already found that miR-495 inhibition decreased influx of macrophages into the vessel wall in experimental restenosis.

Cold-Inducible RNA-Binding Protein

Cold-Inducible RNA-Binding Protein (CIRBP) is another 14q32 microRNA expression regulator and belongs to the post-transcriptional regulators. RNA-binding proteins (RBPs), as the name suggests, are proteins that are able to bind RNAs during post-transcriptional processing[59]. They do not only bind mRNAs, but also microRNAs[60]. CIRBP was shown to bind to two 14q32 precursor microRNAs, namely miR-329 and miR-495. By doing this, CIRBP induces processing into mature microRNAs[51]. As already described in 2014 by Welten et al[38], these microRNAs have an effect on post-ischemic neovascularization. By direct inhibition of these microRNAs with antisense oligonucleotides, translation of pro-angiogenic mRNAs is no longer inhibited, and therefore post-ischemic neovascularization is promoted upon microRNA inhibition. CIRBP is also known to be regulated under hypothermic conditions[61-65]. Exactly the symptom of cold extremities is one of the clinical features of peripheral arterial disease due to insufficient blood supply to peripheral tissues. In this situation neovascularization is needed and CIRBP has to be investigated as a potential target in promoting post-ischemic neovascularization.

Thesis outline

The aim of this thesis is to investigate the expression of 14q32 noncoding RNAs in the vasculature and to identify possible regulators of 14q32 microRNA expression in vascular remodelling.

The first part of this thesis focusses on the role of 14q32 microRNAs, snoRNAs and DNA methylation in cardiovascular diseases and their presence in the human vasculature.

Chapter 2 reviews individual microRNAs and microRNA clusters and families that act in various forms of cardiovascular disease. It highlights the multifactorial nature of vascular remodelling processes in which microRNAs are acting and regulating their targets.

Chapter 3 is the first study that describes the importance of 14q32 snoRNAs in cardiovascular disease. In this chapter we show in a GWAS that single nucleotide polymorphisms (SNPs) in the snoRNA cluster were significantly associated with heart failure independently of other SNPs along the cluster. This indicates an independent role of the 14q32 snoRNA cluster in cardiovascular disease. Furthermore, 14q32 snoRNA expression varied widely throughout the human vasculature and expression seemed to be highly vessel location specific. In failing human coronary bypasses compared to naïve equivalents, snoRNA expression differed and 14q32 snoRNA expression was upregulated in plasma samples of ST-Elevation Myocardial Infarction patients. Potential mechanisms of action of 14q32 snoRNAs had not been uncovered, but in this study an attempt was made to find snoRNA target regulators.

In **chapter 4** the findings on intervascular and vascular disease specific 14q32 microRNA expression and DNA methylation were reported. We found vessel- and disease specific microRNA expression and, even within a vessel wall, different cell layers varied in microRNA expression. 14q32 microRNA expression did not associate with 14q32 DNA methylation or DNMTs. However, we observed highly vascular disease specific DNA methylation patterns suggesting that DNA methylation of the 14q32 locus does not directly affect individual microRNA expression, but acts as independent regulator of vascular remodelling. We confirmed these findings in a murine model for vein-graft disease and in a tissue ischemia mouse model.

The second part of this thesis zooms in on the regulatory mechanisms of 14q32 microRNA expression in vascular remodelling.

Chapter 5 focusses on myostatin as negative regulator of muscle cell proliferation and possible transcriptional regulator of 14q32 microRNA expression in restenosis. In this chapter, we report that addition of myostatin both *in vitro* and *in vivo* downregulated 14q32 microRNA expression and decreased proliferation marker expression. However, it did not affect post-interventional restenosis in a murine model, as myostatin did not clearly affect macrophages inflammatory properties and its 14q32 microRNA expression. This highlights the importance of targeting both the vascular smooth muscle cell proliferation and the macrophage induced inflammation in the process of restenosis inhibition.

In **chapter 6** we describe cold-inducible RNA binding protein (CIRBP) as post-transcriptional regulator of 14q32 microRNAs miR-329 and miR-495 in *in vitro* angiogenesis. CIRBP was known to inhibit precursor to mature microRNA processing and by downregulating the mature microRNA by knockdown of CIRBP, both scratch wound healing and tube formation were increased. Moreover, during cold-stress, which is a main feature of peripheral tissue in peripheral arterial disease, CIRBP was upregulated, but microRNA expression did not alter. Moreover, specifically splice variant 1 changed during siRNA mediated knockdown of CIRBP and hypothermia, whereas the other splice variants were not shown to be affected. The antisense strand of CIRBP contains a long noncoding RNA, CIRBP-AS1, with a yet unknown function and this RNA was also shown to be affected similarly by CIRPB knockdown and hypothermia. Knockdown of CIRBP-AS1 led to CIRBP downregulation and increased scratch wound healing as well. Although the exact pathophysiological mechanisms are not completely understood yet, this provides new possible targets for improving neovascularization in peripheral arterial disease.

All results of the described studies in this thesis are summarized and discussed in **chapter 7.** This chapter also provides future perspectives of the research described in this thesis.

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