Cell cycle and apoptosis genes in atherosclerosis

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Chapter 8

General Discussion

Cardiovascular diseases (CVD) are the leading cause of mortality and disability in the industrialized world, and it is rapidly becoming the number one killer in developing countries.[1] Atherosclerosis is the primary cause of cardiovascular disease, a multi-factorial disorder occurring in the large and medium-sized arteries of the body. Although in the beginning 90s promising lipid lowering therapies predicted a strong reduction in cardiovascular deaths for the upcoming years, in westernized societies it still accounts for 40% of the total number of annual deaths, indicating that treatment of atherosclerosis goes beyond lipid lowering solely. In addition to lipid accumulation, continuous cell proliferation (cell cycle) and cell death (apoptosis) processes are thought to play a central role in the development of atherosclerotic lesions. Proliferation and apoptosis are important processes in regulating macrophage and smooth muscle cell (SMC) numbers in the atherosclerotic lesion and may thereby directly influence lesion composition and stability. The research described in this thesis was designed to identify the role of cell cycle and apoptosis genes in atherosclerosis. The major conclusions concerning the studies on cell cycle and apoptosis genes in atherosclerosis and the importance of site-specific recombinase (SSR) technology in atherosclerosis research are discussed in this chapter, concluding with the potential implications for future research.

CELL CYCLE AND APOPTOSIS GENES IN ATHEROSCLEROSIS

Many physiological processes, including proper tissue development and homeostasis, require a delicate balance between cell proliferation and apoptosis. Cell proliferation and apoptosis are linked by cell-cycle regulators and apoptotic stimuli that affect both processes.[2] Among these common cell-cycle regulators are Rb, p53, and its inhibitor Mdm2. The importance of these genes in maintaining homeostasis becomes evident if one considers that despite the identification of more than 100 proto-oncogenes, the pathways dominated by the two tumor suppressor genes Rb and p53 are the most frequently disrupted in cancer.[3] The unique role of these cell cycle and apoptosis genes in cancer puts a special interest for a role of these genes in atherosclerosis. Not the least because recently a series of shared molecular pathways have emerged that have in common a significant role in the pathogenesis and progression of both cancer and atherosclerosis.[4,5]

Initial *in vitro* studies demonstrated a central role for p53 in human plaque vascular smooth muscle cells (VSMCs). These plaque-derived VSMCs showed an increased sensitivity to p53-mediated apoptosis.[6] Moreover, plaque VSMCs displayed slower rates of cell proliferation and earlier senescence due to a higher ratio of active Rb.[7] Furthermore, p53 was shown to be able to mediate apoptosis of these cells through Fas transport from cytoplasmatic stores.[8] These studies were initial indications that p53 (and downstream targets) play a potentially important role in determining atherosclerotic lesion composition and stability, and thereby opened a new era of research on these processes in atherosclerosis via key genes such as p53, Rb, and Mdm2.

Following, to unravel molecular mechanisms underlying the role of p53 in atherosclerosis murine studies were performed. These studies started using conventional knock out mice crossbred to different atherosclerosis-susceptible back-

grounds (i.e. apoE$^{-/-}$, LDLR$^{-/-}$ and APOE*3-Leiden mice) and gradually became more cell-type specific using bone marrow transplantations. Initial studies started with whole body deficiency of p53[9], followed by hematopoietic inactivation of p53 via bone marrow transplantations.[10,11] Whole body and hematopoietic p53 deficiency indicated strong anti-atherogenic properties of the tumour suppressor gene. In **chapter 2** we completed the research on the role of p53 in the development of atherosclerosis by studying macrophage p53 using LysMCre^{+} p53$^{loxP/loxP}$ apoE$^{-/-}$ mice. Whole body inactivation of p53 can induce severe side-effects interfering with the atherosclerotic process.[12] In addition, hematopoietic inactivation using bone marrow transplantation studies[10,11] targets a diverse spectrum of bone marrow derived cells. Cells originating from bone marrow are macrophages, T- and B-cells, SMCs,[13] and endothelial cells.[14,15] With the use of the LysMCre^{+} p53$^{loxP/loxP}$ apoE$^{-/-}$ mouse model we scaled our research down to one single cell-type (macrophages) and in addition prevented development of severe side-effects as a result of whole body p53 deletion. This mouse model allowed us to define that macrophage p53 plays a minimal role in atherosclerotic lesion size but has a unique role in inducing foam cell apoptosis, preventing lesional necrosis and thereby affects lesion composition and progression.

Both p53 and Rb are potent inhibitors of cell cycle progression. In contrast to the comprehensive studies on the role of p53 in the development of atherosclerosis, we were the first group showing a role for (macrophage) Rb in the development of atherosclerosis (**chapter 3**). Under identical experimental conditions macrophage Rb deficiency showed to have more pronounced effects on atherosclerotic lesion size than macrophage p53 deficiency. This difference might be ascribed to the extent of successful gene deletion. However, a more plausible explanation could be that each individual gene plays its role in different processes. Rb is important in controlling the progression of the cell cycle from G1-phase to S-phase, principally by binding to and inactivating the E2F transcription factors, and in addition acts as an anti-apoptotic factor.[16] Deletion of macrophage Rb showed that this gene has strong anti-atherogenic properties. On the other hand, p53, next to regulating proliferation and apoptosis, also is a potent transcription factor inducing the expression of many downstream target genes. These target genes can be divided in the following sub-categories: (1) genes involved in cell cycle control, (2) genes involved in apoptosis, (3) genes involved in DNA repair, (4) genes involved in angiogenesis, (5) genes involved in cellular stress response. Although currently around 60 target genes have been identified, it is predicted that the human genome contains 200-300 p53 target genes.[17] Thus, p53 targets many genes amongst which several might have either pro- or anti-atherosclerotic effects, giving a possible explanation for the less pronounced effects on atherosclerotic lesion size. Hence, as a result of the multi-targeted nature of p53, definition of macrophage p53 as an anti-atherosclerotic gene is more complex.

How do the different studies on cell cycle and apoptosis genes as described in this thesis and by others expand our knowledge on the role of these two processes in atherosclerosis? From literature it is know that SMC apoptosis can selectively weaken the fibrous cap, thereby accelerating the process towards plaque rupture.[18,19] Human plaque-derived smooth muscle cells displayed increased rates of

spontaneous apoptosis and high susceptibility to p53-mediated apoptosis.[6,20] In addition, adenovirus-induced p53[21] or FasL[22] overexpression in murine carotid artery lesions resulted in increased apoptosis and in a phenotype that has been associated with increased vulnerability to plaque rupture, whereas lesion size was unaffected in both studies. In **chapter 2** we showed that macrophage p53 deletion induced a decrease in macrophage apoptosis resulting in differences in lesion composition and again leaving atherosclerotic lesion size unaffected. Concerning earlier studies on proliferation in atherosclerosis it was shown that human plaque-derived SMCs show reduced proliferation and earlier senescence due to an increased ratio of the active form of Rb.[7] In addition, localized infection of the arterial wall with an adenovirus encoding a constitutively active non-phosphorylatable form of Rb[23] or a phosphorylation-competent full-length and a truncated form of Rb significantly[24] inhibited vascular smooth muscle cell proliferation and neointima formation in different animal models of balloon angioplasty. Moreover, mouse studies showed that (hematopoietic) inactivation of p27, a cyclin-CDK regulating cell cycle inhibitor, resulted in an increase in lesional proliferation, thereby exacerbating atherosclerosis in apoE$^{-/-}$ mice.[25,26] Finally, in **chapter 3** we demonstrated that increased macrophage proliferation (via macrophage Rb deletion) also enhanced atherosclerotic lesion size. Summarizing, we can conclude that lesional apoptosis has primarily qualitative effects on atherosclerotic lesion development (affecting lesion composition and stability), whereas lesional proliferation has primarily quantitative effects on atherosclerotic lesion development (affecting lesion size).

Analysis of proliferation and cell death (either apoptosis or necrosis) form the common denominator in the different chapters of this thesis (**chapters 2, 3, 4, and 6**). Both in **chapter 2 and 6** we showed that a reduction in apoptosis, either via deletion of macrophage p53 (**chapter 2**) or via active TNFα (**chapter 6**), resulted in enhanced death via necrosis, thereby stimulating the formation of advanced atherosclerotic lesions. Moreover, our earlier studies on the role of hematopoietic p53 in the development also showed this similar trend (B.J.M. van Vlijmen and L.S.M. Boesten et al.).[10] Apoptosis often precedes necrosis in the formation of an advanced atherosclerotic lesion and thereby results in the release of cellular contents from dying cells.This may lead one to suggest that apoptosis itself strongly contributes to lesion pathology, by leading to the release of harmful molecules and finally the formation of a necrotic core.[27,28] However, our current data challenge this traditional point of view that apoptosis is harmful and suggest that apoptosis itself is a direct protective factor in the development of atherosclerosis.

Our studies led to the following hypothesis behind cell death in atherosclerotic lesions:

In early lesions foam cells preferentially die quickly via a relatively clean apoptotic death followed by phagocytosis and disposal of apoptotic bodies.This process limits the number of cells in early lesions.[27] However, it is the harsh microenvironment in the growing lesion that hampers the normal clearance of apoptotic bodies. Following, these accumulating apoptotic bodies are ineffectively phagocytosed, partly as a result of cytoplasmic overload of macrophages and competition among oxidized red blood cells, oxidized LDL and apoptotic bodies for the same receptor(s) on the macrophages,[29] thereby promoting the inflammatory status of the lesion. Eventu-

ally, it is this increase in inflammatory status that promotes (secondary) necrosis[27] and thereby the formation of an advanced atherosclerotic lesion. Necrosis of foam cells may be more slowly but is more detrimental since necrosis itself leads to the release of pro-inflammatory and pro-thrombotic substances. The increase in the inflammatory status of the lesion goes beyond processes involved in the apoptotic machinery. Thus, foam cell apoptosis is in principal a beneficial process, leading to a reduction in the production of cytokines, chemokines, and metalloproteinases, thereby reducing lesion pathology. However, it is the complicated atherosclerotic environment that restricts proper execution of apoptosis.

In addition to its role in regulating apoptosis and necrosis in atherosclerosis (as described above) TNFα is more often described as a strong pro-inflammatory cytokine in different diseases.[30,31] However, to date, studies on the role of TNFα in atherosclerosis yielded controversial results. TNFα deficiency on a wild type C57BL/6 background did not affect early lesion development.[32] On the contrary, another research group demonstrated that TNFα-deficiency, also on a C57BL/6 background, reduced atherosclerosis.[33] A direct anti-atherosclerotic effect of TNFα deficiency could not be concluded from these experiments because they also showed an unexpected TNFα-mediated effect on atherogenic lipoproteins. In addition, **chapter 6** describes a subtle role for TNFα in the development of advanced atherosclerosis. In accordance with the data on early atherosclerosis of Schreyer et al.[32] we do not demonstrate an effect of TNFα deficiency on the size of atherosclerotic lesions. TNFα deficiency solely results in less advanced lesions as a result of a shift in cell death towards apoptosis at the expense of necrosis. None of the three abovementioned studies showed an effect of TNFα deficiency on either systemic or local inflammatory parameters. This brings to doubt the generally held concept that TNFα has strong pro-inflammatory properties in atherosclerosis development. Therefore, we conclude that the pro-inflammatory properties of TNFα play a minor role in the development of atherosclerosis. Hence, the primary effects of TNFα on atherosclerosis development are at the level of cell death regulation.

TNFα and one of its receptors TNFR1 belong to the tumour necrosis factor receptor gene superfamily. This family comprises the so called "death receptors" from which the receptor-ligand couples Fas-FasL and TNFR1-TNFα are best characterized.[34] Death receptors are cell surface receptors that transmit apoptosis signals initiated by specific death ligands (i.e. TNFα and FasL). These receptors can activate death caspases within seconds of ligand binding, causing apoptosis of the cell within hours. Different vascular studies aiming at the role of Fas-FasL in atherosclerosis demonstrated that this couple inhibited the infiltration of inflammatory cells, thereby inhibiting the progression of the disease.[35-38] However, our study on the role of FasL in pre-existing lesions in apoE$^{-/-}$ deficient mice, showed that FasL expression increased apoptosis in the SMC-rich caps of the lesions, thereby remodelling the lesions towards a more vulnerable phenotype (A.S.M. Zadelaar and L.S.M. Boesten et al.).[22] Taken together, these two studies indicate that the actions at the level of atherosclerosis development of these two ligands of the death receptor family (TNFα and FasL), are merely attributable to their activity at the level of cell death (both apoptosis and necrosis), and not at the level of inflammation.

MOUSE MODELS TO STUDY CELL CYCLE AND APOPTOSIS GENES IN ATHEROSCLEROSIS

Throughout this thesis we made use of state-of-the-art SSR mouse models to study the role of cell cycle and apoptosis genes in atherosclerosis. In this part of the discussion the advantages and disadvantages of the different mouse model technologies applied in atherosclerosis research will be discussed.

Conventional whole body knock out models can be designed to introduce the desired genetic changes into the germ line, thereby affecting all tissues during the entire lifespan of the resulting mouse. However, several limitations of the conventional germ line gene-targeting approach hamper analysis of target genes. As the mutation will be already present in the first developing cell, an embryonic lethal phenotype might be provoked (i.e. Rb and Mdm2-germline null alleles), precluding any further functional analysis during embryogenesis and/or adulthood. In addition, pleiotropic effects as a compensatory reaction to the introduced germ line mutation are often observed. Moreover, when the gene of interest has a wide expression pattern, its inactivation might induce a highly complicated accumulative phenotype involving multiple tissues. Hypomorphic mutations, which is a mutation that reduces, but does not completely eliminate, the function of a gene, could partly be a solution to these problems. Mice carrying for example one hypomorphic Mdm2 allele (*mdm2*^{puro}) and a known *mdm2* null allele showed that Mdm2 is critical for regulating p53 under homeostatic conditions.[39] However, it still might be important to completely delete a gene at a specific developmental time point or during a particular stage in disease.[40] Thus, although conventional whole body knock out mice boomed our knowledge on multiple genes under physiological and pathological conditions, the abovementioned limitations activated researchers to search for alternative approaches to study genes. Concerning the atherosclerosis research field several different approaches have been applied the last couple of years.

Adenovirus vectors can be used to efficiently overexpress a gene of interest *in vivo*.[41-43] Replicative deficient adenoviruses, in which the E1 genes have been replaced with an appropriate transgene and transcriptional regulatory element(s), can be used to efficiently infect most replicating and nonreplicating cell types *in vivo*, lacking the ability to regenerate infectious progeny after an initial injection into mice. Compared with the production of knock out/transgenic animals, adenoviruses are more convenient and less expensive to prepare. Moreover, they can be used alone and in combinations to rapidly produce large numbers of animals expressing one or more transgenes. Finally, they ultimately may have potential applications for human gene therapy. Adenovirus vectors injected intravenously home to the liver and a single intravenous injection of mice results in the selective transduction of 10% to 100% of the hepatocytes in these animals. This also directly shows the disadvantage of using adenoviruses. *In vivo* atherosclerosis research using adenoviruses gives the opportunity to study genes associated with the liver (mainly lipoprotein-related genes), which has been successfully performed for several genes including: apolipoprotein A-I (APOA-I),[44] secreted macrophage scavenger receptor-AI (SR-AI),[45] and plasma phospholipids transfer protein (PLTP).[46] However, when one wants to study a gene in a specific cell type in the vessel wall or at the site of

the lesion, intravenously injected adenoviruses can't be used. Von der Thusen et al.[21] and Zadelaar et al.[22] anticipated on this limitation by locally incubating a collar-induced carotid artery lesion using an adenovirus encoding p53 or FasL, respectively. Using this approach, solely the endothelial and smooth muscle cells are affected (leaving macrophages and other lesional cells unaffected). Again, this can either be an advantage or a disadvantage, depending on the type of cell one wants to target. Thus adenoviruses are perfect tools to systemically target liver associated genes or locally target EC and SMC associated genes in carotid artery lesions.

Bone marrow transplantation (BMT) studies opened the field for cell-type specific research in atherosclerosis research.[47] In combination with the different mouse models for atherosclerosis (i.e. apoE$^{-/-}$, LDLR$^{-/-}$ and APOE*3-Leiden mice) this approach enabled researchers to study macrophage-specific genes. Although BMT studies extended our knowledge on the role of multiple genes in atherosclerosis, years of research also revealed the disadvantages of using this technique in atherosclerosis research. Graft-versus-host disease is the most common technical problem associated with BMT. In addition, the radiation used for the recipient mice to remove its bone marrow often makes the mice seriously ill requiring a long recovering period, with often many premature deaths. However, a careful titration of the applied radiation for each single mouse strain prevents illness in the recipient mice. Although often claimed as a cell-type specific approach, BMT targets many cell types. Next to macrophages, also smooth muscle cells, endothelial cells, T-cells and B-cells develop from bone marrow progenitor cells.[13-15,46] Thus, earlier published studies using BMT analyzed the role of a gene of interest in all the abovementioned cell types. Therefore, concerning BMT studies in atherosclerosis, the term "macrophage-derived" is currently being replaced by "hematopoietic-derived".

Alternative mouse models for atherosclerosis research also include conditional "gain of function" and knock-in mouse models. Next to inactivation of a gene ("loss of function") also activation of a gene ("gain of function") can be achieved. Transcriptional transactivation, used to activate transgenes in gain of function experiments, is more widely used than DNA recombination, because the latter is irreversible.[48] Tetracycline-dependent regulatory systems are most often used for transcription transactivation systems ("Tet-on" and "Tet-off"). These systems use a chimeric transactivator to control transcription of the gene of interest from a silent promoter. Depending on the system used target genes are expressed in presence or absence of the inducer doxycyclin with impressive induction levels, reaching in some tissues five orders of magnitude.[40,49] Alternatively, knock-in experiments are used to place a transgene (either cDNA or a reporter construct) under the transcriptional control of an endogenous gene. The most widely used knock-in strategy is the replacement of a gene by a reporter gene (e.g. LacZ or GFP) to monitor its expression patters during development, in adult mice or during a disease (atherosclerosis), both in a spatial and temporal matter.[49] The APOE2 knock-in mouse model is a clear example of the use of knock-in techniques in lipid research. In the APOE2 knock-in mouse, the endogenous mouse ApoE gene has been replaced by the human Apolipoprotein E2 (APOE2) gene, a relatively common recessive allele, which is the main cause of type III hyperlipidemia in humans.[50]

Mouse models expressing conditionally regulated genes (pioneering work from

the laboratory of Klaus Rajewsky[51]) initiated a new era for all scientific areas. Using the site-specific recombinase (SSR) technology, those genes that induce embryonic lethality associated with germline null alleles, could now be studied in a wide variety of diseases. In the following decade, from the introduction of the SSR technique, there has been a tremendous expansion in the number of Cre-expressing and floxed mice. Currently an excellent list of all Cre-expressing and floxed mice has been established and is available on: http://www.mshri.on.ca/nagy. In addition, with the introduction of the spatiotemporally controlled genes (deletion of a gene at a time point of interest) shifted the research question from: "What is the role of this gene in this disease?" towards "At what particular stage does this gene play a role in this disease?". Although SSR techniques were first applied in developmental and cancer research, the use of these systems is now more often adapted in research on many different diseases (i.e. atherosclerosis[52-55], diabetes[56], and multiple sclerosis[57]).

However, any new technique also brings its limitations. When considering the first practical limitations, one encounters that Cre recombinase can cause chromosomal rearrangements/aberrations and is speculated to be involved in causing cell cycle arrest.[58-61] Applying the SSR technique in the atherosclerosis research field particularly brings extensive breeding work before atherosclerosis experiments can be performed. In addition to combining the Cre-expressing mouse with the floxed-mouse, an atherosclerosis-susceptible background (i.e. apoE$^{-/-}$, LDLR$^{-/-}$ or APOE*3-Leiden mice) also needs to be introduced. At least two years of breeding and genotypic analysis are required to achieve a triple homozygous mouse line. Thereafter, atherosclerosis experiments can be initiated. Concerning these time-consuming breedings, a time reducing approach has been described by Kanters et al., who first combined the Cre-expressing and floxed mouse lines and subsequently performed a BMT onto an atherosclerotic-susceptible background. [55]

Another potential drawback is that the targeting constructs for both Cre-expression and the floxed-gene might be present on the same chromosome. As a consequence, homozygous floxed-embryos are not formed or die in utero in a very early stage (these topics have not been addressed in the current thesis). We experienced this unfortunate practical problem while breeding the LysMCre mouse strain[62] (lysozyme M gene: chromosome 10, genome coordinates: 116966783-116971716) with Mmd2$^{loxP/loxP}$ mouse strain[63] (Mdm2 gene: chromosome 10, genome coordinates: 117379898-117401709). With both targeting constructs present on the same chromosome, only 410 kb apart, homozygous LysMCre^{+} Mdm2$^{loxP/loxP}$ ApoE$^{-/-}$ mice were not formed, resulting in the birth of heterozygous LysMCre^{+} Mdm2$^{loxP/+}$ ApoE$^{-/-}$ mice only. Although atherosclerosis experiments were performed using these heterozygous LysMCre^{+} Mdm2$^{loxP/+}$ ApoE$^{-/-}$ mice, as described in **chapter 2 and 3** for p53 and Rb respectively, heterozygous deletion of macrophage Mdm2 did not affect atherosclerosis development. Additional Western blot analysis on macrophages from LysMCre^{+} Mdm2$^{loxP/+}$ mice did not show a (partial) upregulation of p53, indicating that one functional Mdm2 allele is sufficient to keep p53 levels in constraint. These coincidental practical shortcomings hampered studies on Mdm2 deletion (and thereby p53 overexpression) in lesional macrophages. Hence, *in vivo* modulation of macrophage Mdm2 is not conceivable with the current tools available (LysMCre mice and Mdm2$^{loxP/loxP}$ mice).

Next to targeting macrophages (**chapters 2 and 3**) we also aimed at targeting the other central cell type in atherosclerotic lesions: SMCs. To this end we crossbred $p53^{loxP/loxP}$,[64] $Rb^{loxP/loxP}$,[65] $Mdm2^{loxP/loxP}$,[63] mice, and the rosa26[66] reporter mouse line with the SM-CreER^{T2}(ki)[67] mouse line. Although all four mouse lines were viable, we were unable to reproduce the data of Kuhbandner et al.[67] using the SM-CreER^{T2}(ki)/rosa26 mouse model. The data described in **chapters 4 and 5** define the difficulties which hampered studies on SMC-p53, -Rb and -Mdm2 in atherosclerosis. Opposite to the published study of Kuhbandner et al.[67] we demonstrated only a 2-8% gene deletion efficiency in the vasculature of SM-CreER^{T2}(ki)/rosa26 mice both after systemic and local application of tamoxifen and 4-hydroxytamoxifen, respectively (**chapter 5**). This is much less efficient than the reported 60% gene deletion in the vasculature by Kuhbandner et al. In addition, the data described in **chapter 4** show a phenotype after inducible SMC-specific Mdm2 deletion only in the gastro-intestinal (GI) tract, inducing lethality in adult mice, leaving the vasculature unaffected. Although not described in this thesis we performed multiple optimization procedures to increase the number of cells displaying gene deletion (β-Gal positive cells) in the vasculature of SM-CreER^{T2}(ki)/rosa26 mice, however all attempts proved to be unsuccessful. Combination of these data forced us to decide to no longer use the SM-CreER^{T2}(ki) mouse model for research on gene deletion (p53, Rb and Mdm2) in the vasculature.

Various factors might have contributed to the failure of this model in atherosclerosis research. Overestimation of the degree of gene deletion in the vasculature, as a consequence of the choice of the reporter mouse model by Kuhbandner et al,[67] could be an underlying cause. In addition, SM22 promoter activity might have been affected or even reduced at our sampling time points. Although studies using a non-inducible SM22-Cre transgenic mouse line argue against this point of view[52,68] showing successful gene deletion from birth on using the SM22 promotor. Finally, the mixed background or differences in accessibility of the loxP sites between the vasculature and the gastro-intestinal tract (GI-tract) for the Cre enzyme might have attributed to the low efficiency of gene deletion in the vasculature using the SM-CreER^{T2}(ki) mouse model. Which of these options, or a combination of them, is the cause of the limitation of the SM-CreER^{T2}(ki) mouse model concerning studies in the vasculature, remains subject to speculation. In this light, it is worthwhile to mention that 5 years after the publication of Kuhbandner et al. no data have been published using the SM-CreER^{T2}(ki) mouse model, although various research groups attempted to introduce this mouse model in their research lines. In this thesis we show that the SM-CreER^{T2}(ki) mouse model is not suitable for research concerning the vasculature but can be efficiently applied for research focussing at gene deletion in the GI-tract.

FUTURE PERSPECTIVES

The findings on cell cycle and apoptosis genes described in this thesis may provide a possible starting point for pharmacological intervention or further specialized application of Cre-loxP models in atherosclerosis, as discussed below.

Stimulation of p53 and/or Rb may prove beneficial in inhibition of atheroscle-

rosis development. However, one should consider that both proteins are of vital importance in all cells present in the body. Therefore, systemic pharmacological modulation of these genes is not applicable for the treatment of atherosclerosis. In contrast, local pharmacological modulation of these genes might prove efficacious in the treatment of atherosclerosis. Local pharmacological treatment is currently often used in treatment of occluding atherosclerotic lesion and (in-stent) restenosis after PTCA or placement of a stent.[69-72] To treat these unregulated proliferative diseases, the use of drug-eluting stents has emerged as a highly promising local approach.[73] The different drugs (i.e. rapamycin, paclitaxel) used in these drug-eluting stents successfully target cell cycle genes (p53 and Rb, amongst others). This local approach gives an unique opportunity to locally target p53 and Rb, since activation of these genes both at the level of SMCs[23,24,74,75] and macrophages (this thesis) is shown to be beneficial for inhibition of vascular disease. In addition, one could envision that stents coated with a combination of pharmacological compounds targeting cell cycle, apoptosis and inflammatory genes may prove increasingly efficacious in the treatment of cardiovascular disease.

The Cre-loxP system induced major advancements in many scientific areas. Future research will focus on the refinement of these techniques. The need for transgenic mouse lines that tissue-specifically and inducibly express Cre-recombinase in the appropriate cell type will increase. Designing a truly macrophage-specific Cre-expressing mouse line will further improve state of the art atherosclerosis research, as the currently available LysMcre mouse line targets both macrophages and granulocytes.[62] In addition, both research on atherosclerosis and restenosis will thrive on the development of a well-functioning inducible SMC-specific Cre-expressing mouse line. Following, multiple genes involved in lesion stability and rupture may be analysed by the combination of this inducible SMC-specific mouse model and local application of a 4-hydoxytamoxifen loaded perivascular delivery device.[76] With the introduction of Cre-loxP models in atherosclerosis the opportunities to determine the contribution of each single gene in the disease process are within reach. Understanding the contribution of each gene/pathway in this disease may yield novel (pharmacological) ways to interfere in atherosclerosis development.

Taking the complex pathogenesis of cardiovascular diseases into account, targeting a single gene or process, although it might be an attractive candidate, may prove to be inadequate therapeutically. Although current (lipid-lowering) treatments for atherosclerosis show considerable progress, combinatorial therapies will prove most efficacious. Despite the considerable difficulties involved, the use of combinatorial therapy aiming at (1) lifestyle interventions (i.e. food and physical habits), (2) lipid therapies (i.e. statins, fibrates), (3) inflammation (i.e. regulation by PPARs) and (4) targeting cell cycle and apoptosis genes on lesional or cellular level, might prove the most effective way to reduce the burden of atherosclerosis.

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