

# Chapter 1

General Introduction



**C**ardiovascular diseases (CVD) have long been the leading cause of mortality and disability in developed countries, and it is rapidly becoming the number one killer in developing countries.<sup>1</sup> Worldwide 16.7 million people die from CVD each year. The primary cause of CVD is atherosclerosis, which is a multi-factorial disorder occurring in the large and medium-sized arteries of the body. Atherosclerosis is a complex disease that starts in childhood and progresses throughout life. Major risk factors for developing atherosclerosis are high blood pressure, high blood cholesterol, smoking, diabetes, and inherited genetic disposition. Although scientific advances in basic, clinical, and population research have been phenomenal, the complications of atherosclerosis such as myocardial infarction, stroke and peripheral vascular disease still makes it a prevailing disease in the western society. In the beginning 90s promising lipid lowering therapies predicted a strong reduction in cardiovascular deaths for the upcoming years, however 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. This chapter describes the general aspects of the development of atherosclerosis, discusses the role of cell cycle and apoptosis genes in atherosclerosis development in greater detail, and concludes with the outline of the thesis.

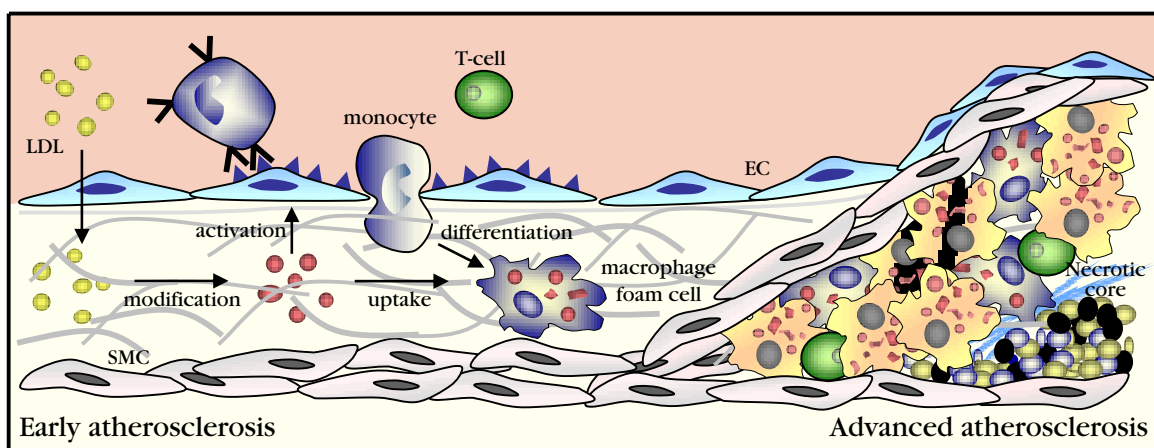
## **THE PATHOGENESIS OF ATHEROSCLEROSIS**

Atherosclerosis comes from the Greek words athero (meaning gruel or paste) and sclerosis (hardness). Although the knowledge on atherosclerosis has expanded extremely the past couple of decades, the exact mechanism of initiating events is still unclear. Generally atherosclerosis can be considered as a form of chronic inflammation resulting from interaction between modified lipoproteins, monocyte-derived macrophages, T-cells, and the normal cellular elements of the arterial wall. This inflammatory process ultimately leads to the development of complex atherosclerotic lesions.<sup>2</sup>

Elevated plasma cholesterol levels are unique in being sufficient to drive the development of atherosclerosis even in the absence of other known risk factors.<sup>3</sup> Cholesterol together with triglycerides are the most important lipids in the circulation and are indispensable to various cellular processes. Cholesterol is necessary for the synthesis of cellular membranes, steroid-hormones and bile, whereas triglycerides function as a major energy source for the body. Cholesterol and triglycerides are lipophilic and are therefore transported in water-soluble lipoprotein particles. These lipoproteins are divided into different groups according to their density and size: chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). Chylomicrons are responsible for transport of dietary lipids, on the other hand, VLDL, LDL and HDL function to transport endogenous lipids. Increased LDL and VLDL cholesterol levels are associated with increased risk of cardiovascular disease.<sup>4,5</sup> These increased levels of plasma LDL and VLDL result in retention of the lipoproteins in the vascular wall, where they get modified (i.e. oxidation, proteolysis and aggregation).<sup>4</sup> Retention of the modified

lipoproteins leads to activation of the endothelium and expression of adhesion molecules. Following, monocytes from the circulation are recruited and adhere to the injured vessel wall where they subendothelially differentiate into macrophages due to the presence of various growth factors and cytokines (including interleukin-1 (IL-1), tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), and interferon- $\gamma$  (IFN $\gamma$ )). Subsequently, the macrophages become lipid-laden foam cells due to the continuous uptake of modified lipoproteins. The presence of foam cells in the vascular wall is called the fatty streak, the first occurrence of atherosclerosis.<sup>6</sup>

Although the recruitment of monocytes to the vascular wall and their differentiation into macrophages may initially serve as a protecting factor, progressive accumulation of these macrophages and subsequent production of cytokines, chemokines, and metalloproteinases and the continuous presence of modified lipoproteins results in the formation of more complicated atherosclerotic lesions.<sup>3,7</sup> Transition of the fatty streak towards the advanced atherosclerotic lesion is characterized by migration of smooth muscle cells (SMCs) from the medial layer of the artery into the intimal or subendothelial layers. SMCs can proliferate and take up modified lipoproteins contributing to foam cell formation. More importantly, SMCs synthesize extracellular matrix proteins that lead to the formation of a fibrous cap. The lesion continues to grow by the entrance of new mononuclear cells from the blood, which enter at the shoulders of the lesion. This is accompanied by cell proliferation, extracellular matrix production and the accumulation of extracellular lipid.<sup>4</sup> Gradually, the lesion develops towards an atheromatous or fibrofatty plaque<sup>6</sup> in which the fibrous cap overlies a pool of smooth muscle cells, lipid-laden macrophages, T-lymphocytes, necrotic debris and cholesterol crystals (Figure 1). Although advanced atherosclerotic lesions can grow sufficiently large to block blood flow, the most important clinical complication is the formation of a so-called vulnerable atherosclerotic lesion with an occluding thrombus, resulting in acute ischemia. The clinical outcome of acute ischemia is dependent on the site of the thrombus in the body and can be for example gangrene of the limbs, myocardial infarction or stroke.<sup>4</sup>



**Figure 1.** Atherosclerotic lesion formation from early to advanced atherosclerosis. Indicated are adhesion, migration, uptake of modified LDL and differentiation of monocytes to macrophage foam cells. Smooth muscle cells migrate and proliferate to form a fibrous cap, overlying a pool of lipid-laden macrophages, T-cells, necrosis and cholesterol crystals. SMC, smooth muscle cell; EC, endothelial cell; LDL, low density lipoprotein.

## THE VULNERABLE ATHEROSCLEROTIC LESION

Every year over 19 million people worldwide are diagnosed with acute coronary syndromes (including: unstable angina, acute myocardial infarction, or sudden coronary death).<sup>1</sup> Most acute coronary syndromes are the consequence of the formation of an occluding thrombus at the site of the atherosclerotic lesion, which can arise from three different atherosclerotic lesion morphologies (rupture, erosion, and calcified nodules). Atherosclerotic lesion rupture is the most common type of complication, accounting for 60-75% of all cases. Pathological analysis of these ruptured lesions shows lesions with a necrotic core and an overlying thin disrupted fibrous cap heavily infiltrated by macrophages and T-lymphocytes. Activation of the clotting cascade results from contact of platelets with thrombogenic agents of the core of the lesion leading to the formation of a thrombus. Second, erosions account for 25-40% of all coronary thrombi. Erosions are characterized by a luminal thrombus on top of a proteoglycan-rich lesion containing mostly SMCs with few inflammatory cells. Loss or dysfunction of the luminal endothelial cells is the primary cause of the formation of a thrombus. If present at all, there is no contact of the necrotic core with the overlying thrombus since the overlying fibrous cap is still intact. Last, the calcified nodule accounts only for 2-7% of all coronary thrombi. These lesions are characterized by the presence of calcified plates along with bony nodules that protrude into the vessel lumen, which contains disrupted endothelium.<sup>8-11</sup>

The cellular composition of an atherosclerotic lesion is an important determinant of its stability. In general, lipid-poor lesions with a prominent presence of fibroblasts, SMCs and collagen are relatively stable and resistant to rupture. On the other hand, lesions rich in cholesterol-loaded macrophages and extracellular lipid deposits, covered by a thin SMC-rich cap, are relatively soft and considered to be vulnerable to rupture.<sup>12</sup>

On cellular level, macrophages play a prominent role in creating a vulnerable lesion. They release chemokines (i.e. MCP-1, MCP-4, and RANTES) that attract additional macrophages, T-cells and mast cells into the site. Together they produce a pool of enzymes, including the family of matrix metalloproteinases (MMPs), which contribute to the degradation of the cap matrix and increase plaque vulnerability through secretion of collagenases, gelatinases, and stromolysin.<sup>13,14</sup> Eventually, foam cells contribute to the ongoing growth of the necrotic core, which is considered as a lesion destabilizing factor.<sup>8</sup>

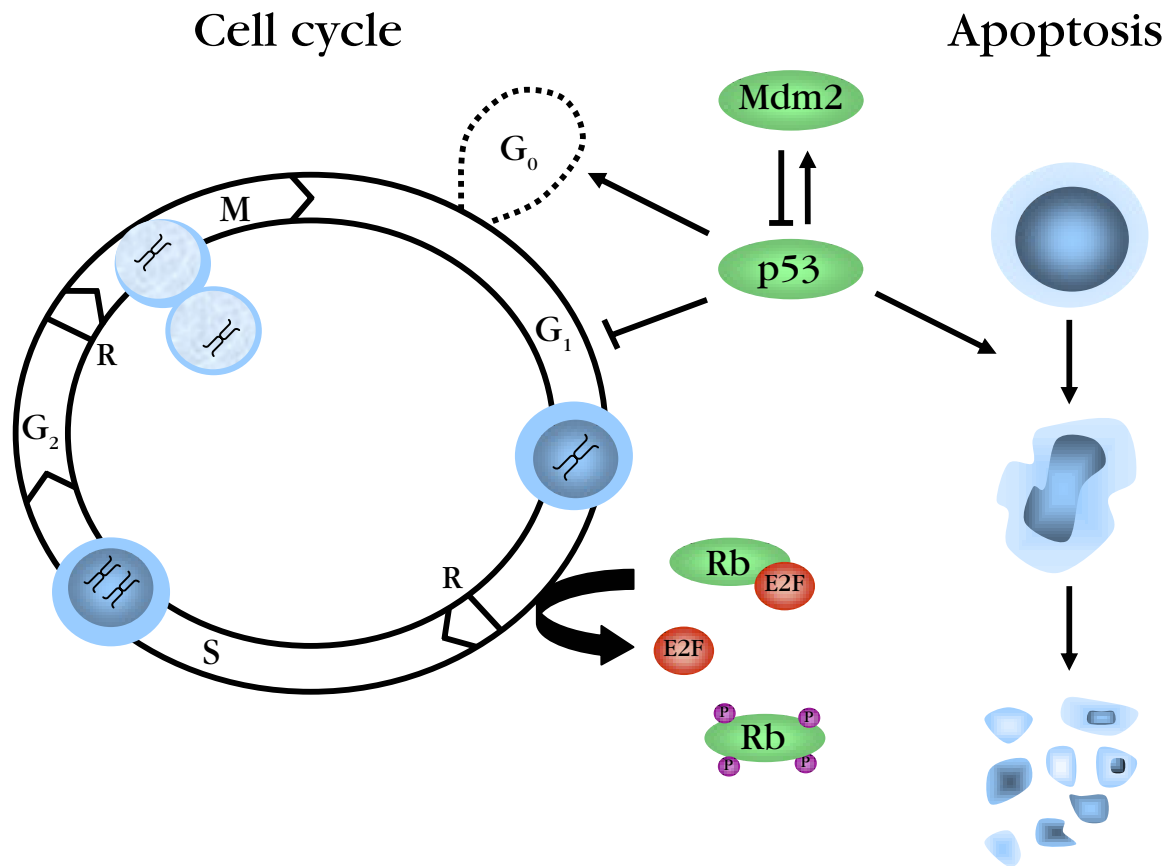
Vulnerability to lesion rupture is not only characterized by the morphology of the lesion. In addition to lesion vulnerability, the vulnerable patient is also characterized by vulnerable blood (i.e. increased levels of C-reactive protein (CRP) and interleukin-6 (IL-6) and increased blood thrombogenicity) and a vulnerable myocardium (i.e. ECG abnormalities). Therefore, improved identification and treatment of vulnerable patients is a goal of great importance since it would result in major decreases in cardiovascular disease, morbidity and mortality.<sup>9</sup>

## CELL PROLIFERATION AND CELL DEATH

Many physiological processes, including proper tissue development and homeostasis, require a delicate balance between cell gain (proliferation/cell cycle) and cell loss (apoptosis). All somatic cells proliferate via a mitotic process determined by progression through the cell cycle. Apoptosis (programmed cell death) occurs in a wide variety of physiological settings. Cell proliferation and apoptosis are coupled by cell-cycle regulators and apoptotic stimuli that affect both processes.<sup>15</sup> Normal cellular growth can be divided into five distinct phases (the cell cycle). The cell cycle is a conserved mechanism by which eukaryotic cells replicate themselves. Quiescent cells are found in the  $G_0$  phase of the cell cycle and remain in a state in which messenger RNA (mRNA) and protein syntheses are minimal. A cell may stay in this state for years, but can re-enter the cycle at the first gap ( $G_1$ ) phase when stimulated by growth factors. During  $G_1$  the cell synthesizes series of mRNAs and proteins that are necessary for the next phase, the DNA synthesis (S) phase. Following the S-phase the cell enters a second gap ( $G_2$ ) phase. During this phase the cell synthesizes additional mRNAs and proteins in preparation for cell division or mitosis (the M-phase), in which the cell divides into two daughter cells.<sup>16</sup> A number of checkpoints (restriction points) exist within the cell cycle to ensure that DNA synthesis and cell division proceed correctly. The two checkpoints occur at the  $G_1$ -S and the  $G_2$ -M transition. The checkpoints are also activated by DNA damage resulting in growth arrest and subsequent repair of the DNA damage. After damage repair, progression through the cell cycle resumes. If the damage cannot be repaired, the cell is eliminated through programmed cell death or apoptosis.<sup>17</sup> Thus, normal cellular proliferation is under tight regulations that control whether conditions are satisfactory for a particular cell to complete a round of division.

Apoptosis is a highly conserved mechanism by which eukaryotic cells commit suicide. It enables an organism to eliminate unwanted and defective cells through an orderly process of cellular disintegration avoiding undesirable inflammatory responses.<sup>18</sup> Apoptosis can be triggered by a wide variety of stimuli including DNA damage, oxidative stress, death receptor ligands, growth factor withdrawal, viral or bacterial infection, oncogenes, and irradiation.<sup>19</sup> Although the events inducing apoptosis may vary from cell to cell, there are basic features of a cell undergoing apoptosis: (1) cell shrinkage, (2) chromatin condensation, (3) DNA degradation, (4) protein fragmentation, (5) disassembly of organelles, and finally, (6) the collapse of cells into small apoptotic bodies that retain membrane integrity, which are removed by phagocytes. Apoptotic elimination of cells occurs during normal development and turnover, as well as in a variety of pathological conditions. Besides apoptosis, which is an active process, cells can also die as a part of a passive, degenerative, uncontrolled way of cell death, termed necrosis.

Necrosis represents a passive consequence of gross injury to the cell. It is morphologically different from apoptosis, and its physiological consequences are also very different from those of apoptosis.<sup>20</sup> Necrosis is characterised by cell swelling, loss of cytoplasmic membrane integrity, and mitochondrial damage. This leads to rapid depletion of energy levels, a breakdown of homeostatic control, cell membrane lysis, and release of intracellular contents, eventually resulting in an inflam-



**Figure 2.** Coupling of cell cycle and apoptosis. Coupling cell cycle (left panel) and apoptosis (right panel) guarantees safe development and maintains homeostasis in organisms. Genes involved in both pathways are Rb and p53 (together with its inhibitor Mdm2). R, restriction point; P, phosphorylation

matory response, with damage to the surrounding cells. Necrosis must clearly be distinguished from apoptosis where cell death results from energy dependent, metabolically active, endogenous cellular processes and where the dying cells do not elicit an inflammatory reaction. The factors contributing to necrosis are mostly extrinsic in nature and therefore necrosis is mostly occurring under pathological conditions.<sup>21</sup> In contrast to apoptosis which occurs both under pathological and physiological circumstances.

## CELL CYCLE AND APOPTOSIS GENES IN ATHEROSCLEROSIS

Cell proliferation and apoptosis are important processes in regulating macrophage and SMC numbers in the atherosclerotic lesion and thereby directly influence lesion stability.<sup>22</sup> Proliferating cells are present at all stages of atherosclerotic lesion development.<sup>23-27</sup> Although the ultimate signals that stimulate cell proliferation in the atherosclerotic lesions may be quite diverse, it is clear that cell proliferation is a crucial component of the atherogenic process.<sup>28</sup> Proliferation of SMCs contributes to atherosclerotic lesion stability. SMCs synthesize extracellular matrix proteins (i.e. collagen) that lead to the formation of a stable lesion covered by a fibrous cap. On the other hand, excessive SMC proliferation in restenosis is a direct complication of

surgical procedures such as balloon angioplasty or stent implantation used for the treatment of occluding atherosclerotic lesions.<sup>29</sup> Although peripheral macrophages are often considered mature (non-proliferating) cells, studies using combinations of different proliferation markers and macrophage-specific antibodies showed the presence of proliferating macrophages in the lesion.<sup>28,30,31</sup> Macrophage proliferation is often considered detrimental to the atherosclerotic lesion, because an increase in the number of macrophages might result in an enhanced production of growth factors, cytokines, chemokines and metalloproteinases. This array of proteins stimulates the formation of an advanced atherosclerotic lesion.

Apoptosis (programmed cell death) is increasingly observed as atherosclerotic lesions develop, although the exact mechanism and consequences of apoptosis in the development and progression of atherosclerosis are still controversial.<sup>32</sup> Factors inducing apoptosis comprise high concentrations of oxidized LDL, oxysterols, Tumor Necrosis Factor- $\alpha$  (TNF $\alpha$ ), Fas ligand, nitric oxide, growth factor withdrawal, hypoxia/ATP depletion and intracellular accumulation of unesterified (free) cholesterol (activating the Unfolded Protein Response (UPR) pathway).<sup>33-35</sup> Apoptotic cells within lesions are typically SMCs, macrophages and T-cells.<sup>36</sup> Increased SMC apoptosis has been detected in unstable compared with stable atherosclerotic lesions.<sup>32</sup> The loss of SMCs via apoptosis can be detrimental for plaque stability since most of the interstitial collagen fibers, which are important for the tensile strength of the fibrous cap, are produced by SMCs. In addition, apoptosis of SMCs is the basis for the generation of microparticles within the circulation, which act as potent procoagulant substrates both locally and systemically. The specific effect of macrophage apoptosis is even more controversial. Macrophages and macrophage apoptosis co-localize with sites of rupture, suggesting a direct causal role in rupture. Any reduction in macrophage numbers via apoptosis could improve plaque stability, due to less metalloproteinase activity and the decreased breakdown in collagen.<sup>37</sup> However, a decrease in macrophages would also reduce the scavenging of apoptotic SMCs and macrophages, allowing the cells to undergo secondary necrosis thereby increasing the inflammatory status and thrombogenicity of the lesion.<sup>32</sup> Hence, defining the exact role of proliferation and apoptosis in atherosclerosis will extend the knowledge on atherosclerotic lesion development and stability in general.

Cell proliferation and apoptosis are coupled by cell-cycle regulators and apoptotic stimuli that affect both processes.<sup>15</sup> Among these common cell-cycle regulators are Rb and p53 (and its inhibitor Mdm2).<sup>17</sup> The importance of these genes in maintaining homeostasis in embryonic and adult tissue becomes evident when concerning their roles in cancer development. Despite the more than 100 proto-oncogenes that have been identified, the pathways dominated by the two tumor suppressor genes Rb and p53 are the most frequently disrupted in cancer cells.<sup>38</sup> 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.<sup>39-42</sup> Moreover, the proposed important role for both proliferation and apoptosis in determining atherosclerotic lesion composition and stability also opened the new era on the research of these processes in atherosclerosis via key genes such as p53, Rb, Mdm2, TNF $\alpha$ , and FasL.



Recent studies in mice demonstrated that genes involved in regulating cell cycle and apoptosis play an important role in the progression of atherosclerotic lesions coinciding with changes in the cellular composition.<sup>43-53</sup> P53, a tumour suppressor protein, plays a pivotal role in the cellular response to a range of environmental and intracellular stress signals (i.e. agents which cause DNA strand breaks, ultraviolet radiation, hyper-proliferation and hypoxia).<sup>54</sup> Mutations in p53 occur in about half of the human cancers, resulting in loss of apoptotic function. P53 belongs to a small family of related proteins that includes two other members p63 and p73.<sup>55</sup> P53 is a potent transcription factor, predominantly acting in the G<sub>1</sub> phase of cell cycle progression, regulating multiple downstream genes implicated in cell cycle control, apoptosis, differentiation, and senescence. Depending on many different factors that are both intrinsic and extrinsic to the cell, p53 activation results in activation of one of the abovementioned pathways.<sup>56,57</sup> In most cases, induction of p53 leads to an irreversible inhibition of cell growth, most decisively by activating apoptosis. The extent of DNA damage and p53 protein levels, however, are factors that contribute to making a choice between life and death. Mice homozygously knock out for p53 appear normal in embryogenesis and shortly after birth but are prone to the spontaneous development of a variety of neoplasms by 6 months of age. Hence, the p53 gene is dispensable for embryonic development but is required for the protection against formation of tumours.<sup>58</sup>

Recent studies demonstrated that p53 plays an important role in the progression of atherosclerotic lesions in mice. P53 is upregulated after various conditions of cellular stress found in atherosclerotic lesions, including DNA damage, hypoxia, oxidative stress and stress caused by oxidized lipoproteins.<sup>59</sup> Deletion of the tumour suppressor gene p53 strongly exacerbated atherosclerosis in different atherosclerosis-susceptible mouse models. Whole body p53 inactivation in apolipoprotein E-deficient (apoE<sup>-/-</sup>) mice accelerated atherosclerosis by increased cellular proliferation.<sup>47</sup> In addition, hematopoietic inactivation of p53 via bone marrow transplantation in both APOE\*3-Leiden<sup>51</sup> and LDL receptor deficient (LDLR<sup>-/-</sup>)<sup>48</sup> mice confirmed the anti-atherogenic properties of the tumour suppressor gene. In addition to hematopoietic-derived p53, SMCs from human atherosclerotic lesions displayed increased sensitivity to p53-mediated apoptosis compared with normal SMCs.<sup>60</sup> Moreover, carotid artery lesions in apoE<sup>-/-</sup> mice treated locally with an adenovirus containing the p53 gene, displayed a phenotype that has been associated with increased vulnerability to plaque rupture.<sup>52</sup> Thus, abovementioned studies indicate an important role for p53 in atherosclerosis development.

P53 transcriptionally activates many target genes, one of which is its own inhibitor the murine double minute 2 (Mdm2) gene. Mdm2 was originally identified as an oncoprotein that binds to p53 and inhibits p53-mediated-transactivation. The human homologue of the Mdm2 gene is often found overexpressed in human cancers, particularly in breast tumours and carcinomas<sup>61-64</sup> and soft tissue sarcomas.<sup>65-73</sup> Mdm2 is an E3 ubiquitin ligase that mediates, together with enzymes E1 and E2, the ubiquitylation and proteasome-dependent degradation of p53.<sup>74</sup> Because Mdm2 inhibits p53 activity, it forms a negative feedback loop that tightly regulates p53 function. In turn, decreased p53 activity results in decreased Mdm2 to constitutive levels.<sup>75</sup> Mdm2 can also ubiquitinate itself and induce its own degradation.<sup>76,77</sup> *In*

*in vivo* experiments demonstrated the importance of the Mdm2/p53 interaction.<sup>78-81</sup> Mice lacking Mdm2 are early embryonic lethal and die before implantation. This phenotype is completely rescued by concomitant deletion of p53, suggesting that p53 overexpression resulted in the embryonic lethal phenotype.

Early studies showed co-expression of p53 and Mdm2 in human carotid artery atherosclerotic lesions.<sup>82</sup> It was speculated that the destiny of individual p53 and Mdm2-co-expressing cells, either to undergo p53-dependent apoptosis or to re-enter the cycle of cell proliferation, may depend on the relative ratios of the two proteins. It was only recently that gene expression analysis showed that pro-apoptotic genes (p53, amongst others) are significantly more expressed in lesions causing acute coronary syndromes, whereas anti-apoptotic genes (Mdm2, amongst others) are more transcribed in stable angina atherosclerotic lesions.<sup>83</sup> Homozygous deletion of Mdm2 is an ideal method to specifically overexpress p53.<sup>78-81</sup> However, to date the embryonic lethality following homozygous allelic Mdm2 deletion hampered studies on the role of Mdm2 (and thereby p53 overexpression) in atherosclerosis.

Another important cell cycle regulatory gene, next to p53 and Mdm2, is Retinoblastoma (Rb). It is the first tumour suppressor gene identified molecularly and plays an important role in inhibiting cell proliferation. In addition, Rb can also act as an anti-apoptotic factor. The gene has been named after its disease Retinoblastoma, a rare childhood cancer of the retina which is caused by Rb inactivation. Rb is a nuclear phosphoprotein that arrests cells during the G<sub>1</sub>-phase of the cell cycle by forming complexes with the members of the E2F transcription factor family. The E2F family of transcription factors has binding sites in the promoters of many of the genes that are involved in cell cycle progression.<sup>84,85</sup> In the cell, Rb is regulated via phosphorylation by cell cycle dependent kinases (CDKs) and cyclins, which, in turn, are inhibited by the cell cycle-inhibitor, p21. The identification of p21 as a p53 target gene implicated p53 in the upstream control and regulation of Rb.<sup>86,87</sup> Mutation or inactivation of both p53 and Rb have also been found in a variety of human tumours.<sup>38,88-90</sup> Mice homozygously knock out for Rb die at mid-gestation (E12-15) with defects in the haematopoietic system and impaired development of the central and peripheral nervous system resulting from massive cell death.<sup>91-93</sup>

Excessive proliferation of SMCs plays an important role in the pathobiology of different vascular occlusive diseases (i.e. atherosclerosis, (in-stent) restenosis, transplant vasculopathy). Therefore, earlier studies on the role of Rb in vascular diseases merely focussed on the role of Rb in SMC proliferation. Human plaque-derived SMCs show reduced proliferation and earlier senescence due to an increased ratio of the active form of Rb.<sup>94</sup> In addition, localized infection of the arterial wall with an adenovirus encoding a constitutively active non-phosphorylatable form of Rb significantly reduced medial vascular smooth muscle cell proliferation and restenosis in two animal models of balloon angioplasty.<sup>43</sup> Moreover, a phosphorylation-competent full-length and a truncated form of Rb inhibited vascular smooth muscle cell proliferation and neointima formation.<sup>50</sup> Although Rb is a key regulator of cell cycle progression in the G<sub>1</sub>-phase (thereby directly affecting proliferation), more recent studies suggest that Rb activation is also seen in other stages of the cell cycle and in response to stresses, including hypoxia and DNA damage.<sup>95,96</sup>

Abovementioned studies indicate that cell cycle and apoptosis are important

processes in regulating macrophage and SMC numbers in the atherosclerotic lesion and might thereby directly influence lesion composition and stability.

## **APOPTOSIS AND INFLAMMATORY GENES IN ATHEROSCLEROSIS**

The primary choice of cell death in atherosclerotic lesions is apoptosis. However, the harsh micro-environment, which is predominantly present in the growing complex atherosclerotic lesion, hampers the normal clearance of apoptotic bodies. Following, cumulative apoptotic bodies are ineffectively phagocytosed as a result of the presence of oxidized lipoproteins and lipids in the lesions or by the cholesterol-loaded state of the macrophage. It is a generally held concept that this situation precedes necrotic cell death as a result of the increased harmful content of the atherosclerotic lesion. Necrosis itself is more detrimental than apoptosis since necrosis by definition leads to an increased inflammatory status of the atherosclerotic lesion.<sup>97</sup>

Inflammation plays a key role in atherosclerosis. Immune cells dominate the early atherosclerotic lesions, their effector molecules accelerate progression of the lesions, and activation of inflammation can elicit acute coronary syndromes.<sup>98</sup> Besides macrophages also T- and B-lymphocytes have been reported to contribute to lesion development.<sup>99-103</sup> In addition, activated immune cells in the atherosclerotic lesion produce various inflammatory cytokines (interferon- $\gamma$  (IFN- $\gamma$ ), interleukin-1 (IL-1), and Tumor Necrosis Factor- $\alpha$  (TNF $\alpha$ )), which induce the production of substantial amounts of interleukin-6 (IL-6). IL-6, in turn, stimulates the production of large amounts of acute-phase reactants, including C-reactive protein (CRP), serum amyloid A (SAA) and fibrinogen.<sup>98</sup> Thus, the local inflammatory process in the atherosclerotic artery leads to increased systemic blood levels of inflammatory cytokines and other acute-phase reactants. Therefore, measurements of these systemic cytokines and acute-phase reactants are particularly useful for clinical diagnosis. One of the key regulators of inflammation is the transcription factor nuclear factor  $\kappa$ B (NF- $\kappa$ B). It controls transcription of many atherosclerosis-related genes, such as cytokines, chemokines, adhesion molecules, acute phase proteins, regulators of apoptosis, and cell proliferation. NF- $\kappa$ B plays an important role in directing both pro- and anti-inflammatory genes and also acts as a regulator of cell survival and proliferation in the atherosclerotic lesion.<sup>104</sup>

Different receptor-ligand couples play an important role in modulating both apoptotic and inflammatory processes. Tumor Necrosis Factor- $\alpha$  (TNF $\alpha$ ) and one of its receptors TNFReceptor-1 (TNFR1) belong to the tumour necrosis factor receptor gene superfamily. This family comprise the so called "death receptors" from which the receptor-ligand couples TNFR1-TNF $\alpha$  and Fas-FasL are best characterized.<sup>105</sup> Death receptors are cell surface receptors that transmit apoptosis signals initiated by specific death ligands (i.e. TNF $\alpha$  and FasL). These receptors can activate death caspases causing apoptosis of the cell. In addition to its role in apoptosis, TNF $\alpha$  is a pro-inflammatory cytokine that mediates key roles in acute and chronic inflammation, and infection.<sup>106</sup> Whereas binding of TNF $\alpha$  to TNFR1 (p55) activates responses associated with induction of adhesion molecule expression,<sup>107</sup> apoptosis,<sup>108</sup> and resistance to bacterial infection,<sup>109,110</sup> binding to TNFReceptor-2 (TNFR2, p75) activates induction of T cell proliferation,<sup>111</sup> induction of TNF $\alpha$ -mediated skin

tissue necrosis,<sup>112</sup> and modulation of TNF $\alpha$ -mediated pulmonary inflammation.<sup>113</sup> TNF $\alpha$  deficient mice develop normally, suggesting that TNF $\alpha$  does not have an irreplaceable role in prenatal tissue and organogenesis. However, TNF $\alpha$  deficient mice completely lack splenic primary B-follicles and cannot form organized follicular dendritic cell (FDC) networks and germinal centers.<sup>114,115</sup>

Although TNF $\alpha$  and its receptors are thought to be considerably important in a number of biological activities relevant to atherosclerosis, its function in atherogenesis remains unclear. Human association studies on TNF $\alpha$  polymorphisms are controversial varying from no,<sup>116-118</sup> weak<sup>119</sup> or strong<sup>120</sup> associations between different TNF $\alpha$  polymorphisms and coronary heart disease. Moreover, studies on the role of TNF $\alpha$  in atherosclerosis using several transgenic or knock out mouse models also yielded controversial results. TNF $\alpha$  ligand deficiency on a wild type C57BL/6 background showed variable effects varying from either no effect on early lesion development<sup>121</sup> to a reduction in atherosclerosis.<sup>122</sup> In addition, on the same background, TNFR1 deficiency did affect atherosclerosis formation, resulting in enhanced (early) lesion formation.<sup>123</sup> Overall, the abovementioned human and mouse studies demonstrate strong divergent results on the role of TNF $\alpha$  in atherosclerosis.

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that, upon ligand activation, form heterodimers with the nuclear receptor RXR and bind to specific DNA sequences thereby transcriptionally regulating gene expression. PPAR $\alpha$  and  $\gamma$  are the two main categories of these receptors, which are both characterized by their ability to influence cell apoptosis, inflammation, proliferation, differentiation, and as well lipid metabolism and glucose homeostasis.<sup>124</sup> PPARs are activated by ligands of physiological and pharmacological origin. PPAR $\alpha$  is activated by polyunsaturated fatty acids and oxidized derivatives and by drugs of the fibrate family (i.e. fenofibrate and gemfibrozil).<sup>125,126</sup> Fibrates are clinically used to treat patients with lipid disorders and have been shown to reduce cardiovascular mortality. Ligands of PPAR $\gamma$  include naturally occurring fatty acid derivatives, prostaglandin derivatives and antidiabetic thiazolidinediones (glitazones), such as troglitazone, rosiglitazone, and pioglitazone.<sup>127-129</sup> PPAR $\alpha$  and  $\gamma$  agonists have shown positive effects on lipid metabolism in animal models and in clinical practice,<sup>130-135</sup> moreover several PPAR $\gamma$  agonists improve insulin resistance in type 2 diabetes.<sup>136,137</sup> In atherosclerosis PPAR $\alpha$  and PPAR $\gamma$  activation results in reduction of atherogenic triglycerides and systemic plasma inflammatory proteins and raise HDL levels.<sup>138</sup> At a cellular level, PPAR $\alpha/\gamma$  agonists act on most cell types involved in atherosclerosis reducing their involvement in the tissue response associated with lesion development.

## MOUSE MODELS TO STUDY ATHEROSCLEROSIS

The mouse was generally regarded as a species that was resistant to the development of atherosclerosis. Therefore, early development of murine models for studying peripheral arterial disease focused on the identification of strains that were susceptible to atherosclerotic like lesion formation on high fat/high cholesterol diets. However, even the most sensitive strains (e.g. C57BL/6) required a diet high in cholesterol and the bile salt sodium cholate to develop atherosclerotic lesions. In the early 1990's the development of apolipoprotein E-deficient (apoE<sup>-/-</sup>) mice revolu-

tionized the use of murine models in the study of cardiovascular disease.<sup>139-141</sup> These animals show strong elevated plasma cholesterol levels and vascular lesions similar in appearance to those observed in humans. The atherosclerotic lesions develop in the aortic root, the coronary arteries, and in the entire aorta at branch points of the major arteries in a time dependent manner.<sup>139,142</sup> ApoE-deficient mice on a chow diet spontaneously develop atherosclerosis, but feeding the mice a high fat diet, induces a strong acceleration of this process. Currently, the ApoE-deficient mouse is the most widely used experimental mouse model for studying atherosclerosis.

The LDL-receptor-deficient (LDLR<sup>-/-</sup>) mouse, with its elevation in LDL levels, is also a useful model for studying atherosclerosis. In humans mutations in the gene for the LDL-receptor cause familial hypercholesterolemia, a major risk factor for developing atherosclerosis.<sup>143</sup> Homozygous LDLR-deficient mice show delayed clearance of VLDL and LDL from plasma.<sup>144</sup> In contrast to apoE-deficient mice, these mice do not manifest severe hypercholesterolemia on a chow diet and hence do not develop atherosclerosis. Upon feeding a high fat diet, plasma cholesterol levels increase strongly resulting in the formation of atherosclerotic lesions.<sup>145,146</sup>

In addition to the mouse models mentioned above, the APOE\*3-Leiden mouse model is also frequently used for atherosclerosis and lipoprotein research. APOE\*3-Leiden is a dominant negative mutant form of apolipoprotein E consisting of a tandem duplication of codons 120-126 in the apoE gene.<sup>147-149</sup> The introduction of this human mutation in a mouse resulted in a slight increase in cholesterol and triglyceride levels on a chow diet, whereas on a high fat diet cholesterol and triglyceride levels rose considerably.<sup>150,151</sup> In APOE\*3-Leiden mice the degree of hypercholesterolemia can easily be adjusted to any desired level by varying in dietary contents. Moreover, APOE\*3-Leiden mice have successfully been used in research on lipid-lowering and anti-atherosclerotic drugs and dietary supplements.<sup>152-156</sup>

Since the introduction of the abovementioned mouse models, the use of mice in atherosclerosis research has boomed over the last decade, driven by the development of knockout mice and transgenic animals. With these approaches, researchers have the tools to study the *in vivo* function of a specific gene product on an atherosclerotic background, while avoiding the difficulties associated with the use of antibodies or receptor agonists/antagonists (i.e. non-specificity, immunoreactivity, dosing and tachyphylaxis). Although, targeting specific genes of interest in atherosclerosis-susceptible mice elucidated the role of many genes in atherosclerosis, those genes that induce embryonic lethality associated with germline null alleles hampered the research on their role in atherogenesis. Site-specific recombinase (SSR) technology gives the opportunity to study the role of genes beyond the first required function of a gene, bypassing embryonic lethality associated with germline null alleles. SSR technology is a relatively new approach to induce gene deletion in a cell type of interest. The SSR Cre (an enzyme that causes recombination of the bacteriophage P1 genome) is able to recombine specific sequences of DNA with high fidelity without the need for cofactors. Therefore Cre has been used effectively to create gene deletions, insertions, inversions and exchanges in exogenous systems such as flies,<sup>157-159</sup> mammalian cell culture<sup>160,161</sup> and mice.<sup>162-164</sup> Cre recombines DNA at defined target sites, termed loxP sites, in actively dividing and post-mitotic cells, as well as in most tissue types. The activity of Cre involves DNA strand cleavage,

exchange and ligation.<sup>165</sup> The loxP sites consist of a 13 basepair (bp) palindromic sequence, or inverted repeats, separated by an 8 bp asymmetric core, or spacer sequence. Strand cleavage, exchange, and ligation occur within the spacers. Moreover, the development of ligand-regulated forms of Cre has added a temporal control to SSR activity to enable the induction of gene changes in late embryogenesis or in adult tissue. A successful strategy for inducing temporal SSR activity has involved fusing a mutant estrogen receptor (ER) ligand binding domain (LBD) to the C-terminus of Cre.<sup>166-170</sup> Currently, different ERs are available which do not bind endogenous  $\beta$ -estradiol but are only responsive to the synthetic estrogen antagonist 4-hydroxy-tamoxifen (4-OHT). Two mouse lines are required for conditional gene deletion. A conventional transgenic mouse line with Cre expression in a specific tissue or cell type (“Cre-expressing mouse”), and a mouse strain that embodies a target gene (endogenous gene or transgene) flanked by two loxP sites (“floxed mouse”). Recombination (excision and consequently inactivation of the target gene) occurs only in those cells expressing Cre recombinase, leaving the target gene active in all other cells and tissues which do not express Cre. Cell proliferation and apoptosis are central themes in the cancer research field. As a result many “floxed-mice” are available aiming at different genes related to cell proliferation and apoptosis.<sup>171-173</sup> However, whereas Cre-loxP models are commonly used in cancer research,<sup>172-176</sup> the use of this system is still relatively new in the atherosclerosis research field.<sup>177-180</sup>

## OUTLINE OF THIS THESIS

The general aim of the research presented in this thesis is to evaluate the role of different cell cycle and apoptosis genes in atherosclerosis. The knowledge on the role of cell cycle and apoptosis genes in atherosclerosis has become increasingly important over the last few years. To analyze these processes in greater detail in atherosclerosis development several key genes are studied (p53, Rb and Mdm2). To this end, we generated and analysed several mouse models. Because germline null alleles of the cell cycle genes of our interest (e.g. p53, Rb and Mdm2) lead to either formation of tumours after the age of 6 months (p53) or to embryonic lethality (Rb and Mdm2) we chose to use site-specific recombinase (SSR) technology, as described above. In addition, SSR technology gives the opportunity to study these genes in one single cell type of interest. To obtain cell type specificity, aiming at the two central cell types in atherosclerotic lesions, we used the lysozyme myeloid-Cre (LysMCre)<sup>181</sup> and the smooth muscle cell-Cre (SM-CreER<sup>T2</sup>(ki))<sup>182</sup> mouse model for targeting macrophages and SMCs, respectively.

The tumour suppressor gene p53 has been shown to inhibit cell proliferation and stimulate apoptosis in many cell types. In **chapter 2** we address the role of macrophage p53 in atherosclerosis. A macrophage specific knock out for p53 demonstrated that p53 is major regulator of foam cell death in atherosclerotic lesions. To further extend the studies on cell cycle genes we targeted macrophage Retinoblastoma using a similar approach. Rb plays a pivotal role in regulating cell proliferation and apoptosis. **Chapter 3** describes that macrophage Rb plays a crucial role in atherosclerotic lesion development. Although SSR technology is a rather novel approach to study genes of interest, some genes turn out to be so vitally important

in maintaining homeostasis in adult tissues that SSR is not the solution to study the gene of interest in atherosclerosis. **Chapter 4** describes the mechanisms behind lethality induced via conditional deletion of Mdm2 in SMCs. Although lethality in this mouse model hampered studies on the role of SMC-Mdm2 in atherosclerosis, the mouse model showed that Mdm2 prevents accumulation of active p53 in quiescent SMCs and thereby the induction of p53-mediated necrotic cell death *in vivo*. Atherosclerosis, being a disease of the large and medium sized arteries also gives the advantage of proper accessibility for treatment. New technologies focusing on conditional, temporal and spatial gene deletion in atherosclerosis resulted, in addition to cell-type and time specificity, also in place specificity, which is described in **chapter 5**. TNF $\alpha$  is a protein often primarily described as a pro-inflammatory cytokine. On the contrary in **chapter 6** we demonstrate, using conventional whole body deletion of TNF $\alpha$  on an APOE\*3-Leiden background, that TNF $\alpha$  is also a strong regulator of cell death in atherosclerotic lesions. Finally, **chapter 7** describes the effects of pharmacological regulation of PPAR $\alpha$  and  $\gamma$  on atherosclerosis development in APOE\*3-Leiden mice. These receptors influence cell inflammation, proliferation, differentiation, apoptosis and lipid and glucose homeostasis. **Chapter 8** discusses the results of these studies and the use of the SSR technology in the atherosclerosis research field, concluding with the implications for future research.

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