Epigenetic modifications in vascular disease and regenerative medicine

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Erklärung

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From far away to us you came
to improve your ken and knowledge
it was your dream and inner aim
to graduate and end the college

foreign country, different manner
the start wasn’t easy, not at all
but still faithful under your banner
you always followed your own call

persistent and beyond the doubt
you went on with lifted head
in hope of fortune looking out
high spirits around did you spread

scientist with heart and soul
all the time you have been
challenging the others goal
to see more beyond the scene

always open, ready to aid
to explain, improve the others
never mind the price you paid
as you’d help your own brothers

So I esteemed to work with you
and helped you to find your fate
‘cause you’re the one to have a clue.
And now here you stand to pass the gate


dedicated
by
J.P.
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1 INTRODUCTION

1.1 Epigenetics

An epigenetic trait is a stable inheritable phenotype resulting from a changing of chromosome structure without alterations in the DNA sequence [1]. Main epigenetic mechanisms include DNA methylation and various histone modifications, such as e.g. methylation, acetylation or phosphorylation (Figure 1).

Epigenetic regulation of gene activity is a fundamental mechanism that occurs in all eukaryotic cells – in animals, humans, plants – and is important for development, tissue regeneration, and maintaining of cell phenotype. Defects in epigenetic modulation of gene activity have already been connected to cancer and other serious diseases [2-5]. Thus, understanding of epigenetics may lead to new diagnostic methods and discovery of novel therapeutic targets. This knowledge can be also useful in regenerative medicine by e.g. targeted cell reprogramming.

![Figure 1. Schematic overview of epigenetic mechanisms according to Rodenhiser et al. [6]. Condensation of the chromatin plays an important role in regulation of gene transcription. Open chromatin together with unmethylation of the cytosine leads to “switching on” the gene transcription; the “switching off” of the transcription is therefore associated with a condensed/folded chromatin and methylated cytosines.]

1.1.1 DNA methylation and DNMTs

DNA methylation consists of the addition of a methyl group to the 5th position of the cytosine pyrimidine ring by DNA methyltransferase (DNMT) enzymes (Figure 2) [7]. This
unique pyrimidine “5-methylcytosine” continues to pair with guanine. In humans and other mammals, DNA methylation is exclusively restricted to CpG dinucleotides. It is catalyzed by three different DNMTs encoded by different genes on distinct chromosomes: *DNMT1*, *DNMT3A*, and *DNMT3B*. *DNMT1* maintains DNA methylation and is responsible for the propagation of DNA methylation pattern during replication (mitotic cell division). It is present at the replication fork and is very specific for the hemi-methylated DNA. *DNMT1* guides the methylation of CpG dinucleotides on the new DNA strand according to the methylation status of the complementary template strand. *DNMT3A* and *DNMT3B* are catalyzing *de novo* methylation and are important in the establishment of the methylation patterns in the early embryo state and also during development.

*DNMT3A* and *DNMT3B* were shown not only to correlate with changes in histone modifications [8], but also, more importantly, to play essential roles in *de novo* methylation. In mice, deletion of *DNMT3B* reduces the methylation of CpG islands on repetitive sequences such as LINE1 and Satα and activates gene expression on inactive X chromosome [8, 9].

![Figure 2](image1.png)

**Figure 2.** Mechanism of DNA methylation [10]. DNA methylation has a specific pattern for cells in different stages of development and differentiation. This process is supported by three main enzymes: *DNMT1*, *DNMT3A*, and *DNMT3B*. Maintenance of the established methylation is provided in part by *DNMT1* and *DNMT3B*. *De novo* methylation is supported by *DNMT3A* and *DNMT3B*.

Approximately 70% to 90% of CpG dinucleotides, representing 3% to 6% of all cytosine, are methylated in healthy somatic cells [11]. Regarding gene regulation, DNA methylation is a repressive mark associated with transcriptional silencing. It has been shown to be involved in different cellular functions, including repetitive sequences, X chromosome inactivation, mammalian embryonic development, and lineage specification. It is also linked to a number of human diseases, mostly cancer [12]. More and more studies are emphasizing the importance of DNA methylation in other diseases such as atherosclerosis or diabetes [13, 14].
DNA methylation can affect gene expression in two general ways. First, DNA methylation itself can inhibit the binding of transcription factors to the methylated DNA sequence. This mechanism is relevant for many transcription regulators, for example MYC [15], activator protein-2 [16], or HIF-1α (hypoxia-inducible factor-1α) [17]. The second mechanism involves a family of methyl-CpG-binding proteins that have been described as being able to recognize methyl residues in the DNA of mammals. This family includes four proteins containing a homologous methyl-binding domain (MBD1, MBD2, MBD3, MBD4, and MeCP2) and a non-homologous methyl binding protein named KAISO [18]. These proteins can directly repress transcription, prevent the binding of activating transactors, or recruit enzymes that catalyze histone posttranslational modifications and chromatin remodeling complexes, which in turn alter the structure of chromatin and promote transcriptional repression [11, 12]. The study of Chan et al. [19] demonstrated an important role in DNA methylation during transcriptional silencing of the human iNOS promoter in non-responsive human endothelial cells. In addition, another study showed that DNA methylation is not only responsible for iNOS transcription silencing, but also for posttranslational modifications of histones [20]. Thus, DNA and histone methylation were found to be important in the transcriptional silencing of iNOS in cultured human endothelial cells. Hence, deregulation of these epigenetic modifications may lead to aberrant iNOS expression and consequently to atherosclerosis.

1.1.2 Hydroxymethylation, TET1

Recently, TET1 was found to be involved in the oxidation process of 5-azacytidine into 5-hydroxymethylcytosine (5hmC) [21]. Other studies have detected 5hmC in embryonic stem cells (ESCs) and in mouse cerebellum. This led to the hypothesis that 5hmC is a short-lived intermediate in the removal of 5-methylcytosine (5mC) [21, 22] and is involved in the epigenetic network with an important role in epigenetic reprogramming and regulation of tissue specific gene expression [23]. Moreover it has been suggested that high levels of Tet1 are associated with a pluripotent state of the cells [24].

The further steps leading to demethylation include oxidation of 5mC to 5-formylcytosine and 5-carboxylcytosine. The ten-eleven translocation (TET) enzyme family was shown to have three members: TET1, TET2, and TET3. All three have been shown in vitro and in vivo to oxidize 5mC to 5hmC [25, 26] and also that the presence of 5hmC is depending on the presence of 5mC (Figure 3). This suggests that this is the only way for the synthesis of genomic 5hmC [27, 28]. TET1 depletion in mouse ESCs e.g. led to the accumulation of 5mC both globally [29] and at specific genomic regions, such as LINE1 retrotransposons and transcription factor binding sites [19].
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Figure 3. Cytosine methylation and demethylation. Methylation is catalyzed by DNMT enzymes. The recently discovered TET1 protein is a 2-oxoglutarate and Fe II – dependent- dioxigenase that catalyzes the formation of 5hmC, which may represent a critical step in active oxidative DNA demethylation or may itself comprise a new epigenetic mark.

Regarding the binding domain and the exact mechanism of TETs, there are couple of contradictory studies, and it is not yet clear whether they share the same binding domain with DNMT1 [29-31] or whether this mechanism is preventing the DNMT1 activity during replication.

1.1.3 Histone modifications – methylation and acetylation

Nucleosomes, the main components of chromatin, consist of histones. These proteins have positively charged amino-terminal tails that are exposed on the outside of nucleosomes. Histones are modified at many sites, with more than 60 different residues detected by mass spectrometry or with the help of specific antibodies [32]. Modifications of the histone tails include methylation, acetylation, phosphorylation, ubiquitination, SUMOylation, citrullination, and ADP-ribosylation. Acetylation of the lysine at the N terminal end of the histone H3 (like K9, K14, K18, and K23) and H4 (such as K5, K8, K12, and K16) as well as methylation of lysine residues in H3 (K4, K9, K27, K36, and K79) and H4 (K20) are the most relevant epigenetic modifications that have been identified until now (Table 1) [33]. They are carried out by specific enzymes, methyltransferases and acetyltransferases. The adding of an acetyl group has a major effect on lysine as it neutralizes the positive charge. This reduces electrostatic attraction between the histone and the negatively charged DNA backbone, loosening the chromatin structure; highly acetylated histones form more accessible chromatin and tend to be associated with active transcription. Histones that are methylated at certain residues can act epigenetically to repress or activate gene expression. Methylation of histones is catalyzed by histone methyltransferases (HMTs), which use S-adenosylmethionine (SAM) as a cofactor in nearly the same way as histone acetylases (HATs) utilize acetyl-coenzyme A as their cofactor [34]. Eukaryotic genomes are conveniently described as transcriptionally active (euchromatin) or transcriptionally silent (heterochromatin). Due to the fact that only very few demethylases were identified so far, it is believed that methylation is a process that is more stable than acetylation. Furthermore,
lysine residues in H3 and H4 tails appear to be targets for either acetylation or methylation, but not both at the same time [34].

An additional complexity of the histone modification processes comes from the fact that methylation at lysine or arginine may be mono-, di-, or tri-methyl for lysine and mono-, or di- (symmetric or asymmetric) for arginine [32].

It is to mention that the plethora of histone modifications cannot happen at the same time and on the same histone. The exact timing depends on the signaling conditions within the cell. Furthermore, acetylation of histones is associated with euchromatin, in contrast histone methylation can have opposite roles depending on the site and degree of methylation. As an example, H3K4 methylation was shown to be responsible for active transcription, while the methylation of H3K9 is involved in gene silencing.

<table>
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<tr>
<th>Histone-Modifying Enzymes</th>
<th>Proposed Function</th>
<th>Reference</th>
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<tr>
<td>Set1 (S. cerevisiae)</td>
<td>permissive euchromatin (di-Me)</td>
<td>[35]</td>
</tr>
<tr>
<td>Set7/9 (vertebrates)</td>
<td>transcriptional activation (tri-Me)</td>
<td>[36]</td>
</tr>
<tr>
<td>MLL</td>
<td>transcriptional activation</td>
<td>[37]</td>
</tr>
<tr>
<td>Suv39h,Clr4</td>
<td>transcriptional silencing (tri-Me)</td>
<td>[38, 39]</td>
</tr>
<tr>
<td>G9a</td>
<td>transcriptional repression genomic imprinting</td>
<td>[40]</td>
</tr>
<tr>
<td>SETDB1</td>
<td>transcriptional repression (tri-Me)</td>
<td>[41]</td>
</tr>
<tr>
<td>Ezh2</td>
<td>transcriptional silencing X inactivation (tri-Me)</td>
<td>[42]</td>
</tr>
<tr>
<td>G9a</td>
<td>transcriptional silencing</td>
<td>[40]</td>
</tr>
<tr>
<td>Set2</td>
<td>transcriptional activation (elongation)</td>
<td>[43]</td>
</tr>
<tr>
<td>Dot1</td>
<td>euchromatin transcriptional activation (elongation) checkpoint response</td>
<td>[44-46]</td>
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In summary, gene function and cell phenotype can be influenced not only by variation in the gene sequence but also by the epigenetic programming of gene expression. Epigenetic changes can be modified by pharmacological factors [47]. Several epigenetic drugs are already in different stages of clinical trials for cancer treatment [48, 49] and psychiatric disease [50].

1.1.4 Histone methyltransferases and their role

Histone methyltransferases are enzymes that are facilitating the transfer of the methyl group (–CH3) to a specific site of proteins or nucleic acids. Theses enzymes catalyze the transfer of the methyl group from SAM on the side-chain nitrogen of lysine or arginine to histones, resulting in a methylated biomolecule and S-adenosyl-L-homocysteine (SAH) – as a byproduct.
Protein methyltransferases (PMTs) can be classified into two families – protein lysine methyltransferases (PKMTs) and protein arginine methyltransferases (PRMTs). Mono-, di- or tri-methylation of the lysine can occur (*Figure 4*). They are distinguished by the amino acid that accepts the methyl group and by the conserved sequences of their respective catalytic domains. Among the many PKMTs that have been identified so far, some of them have been validated for their methyltransferase activity [51].

![Figure 4. Schematic view of methylation at lysine residue by protein lysine methyltransferases (PKMTs). Some PKMTs add just a single methyl group, resulting in a mono-methylated product, whereas some others produce di- or tri-methylated lysine modifications.](image)

Several proteins responsible for methylation have been characterized and all but one of these enzymes contains a SET domain. The exception is the *DOT1* family of methyltransferases, members of which methylate K79 in the globular region of histone H3 [52]. The SET domain was first recognized as a conserved sequence in three *Drosophila melanogaster* proteins: a modifier of position-effect variegation, suppressor of variegation 3-9 (Su(var)3-9), the polycomb-group (Pc-G) gene enhancer of zeste [E(z)], and the trithorax-group chromatin regulator trithorax (Trx). The SET domain, which is approximately 130 amino acids long, was characterized in 1998 and SET-domain proteins have been found in all eukaryotic organisms studied so far [52]. Seven main families of the SET-domain proteins are known: SUV39, SET1, SET2, EZ, RIZ, SMYD, and SUV4-20 families. Table 2 summarizes the most relevant histone methyltransferases of the SET domain PKMTs.

The *SUV39* family has been characterized very thoroughly. Members of this family, human SUV39H1, were the first SET-domain protein lysine methyltransferases identified. These proteins are specifically involved in the methylation of H3K9 [38]. SUV39H1 is similar to SUV39H2 with up to 55%, depending on species [52]. The members of the SUV39 family discussed above (H1 and H2) are involved in the methylation of histone residues within both, euchromatin and heterochromatin. In contrast, another member of the same family, G9a, is the predominant histone H3K9 methyltransferase in mammalian euchromatin [52, 53]. G9a and SUV39H1 belong to the same family of SET-domain proteins and both have pre-SET and post-SET domains surrounding the SET domain.
### Table 2. Selected histone methyltransferases and their targets according to Albert et al. [54].

<table>
<thead>
<tr>
<th>Name</th>
<th>Histone target</th>
<th>Transcriptional activity</th>
<th>Transcriptional repression</th>
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<tr>
<td>MLL</td>
<td>H3K4me1/2/3</td>
<td>H3K4me2/3</td>
<td>-</td>
</tr>
<tr>
<td>MLL2</td>
<td>H3K4me1/2/3</td>
<td>H3K4me2/3</td>
<td>-</td>
</tr>
<tr>
<td>SETD1A</td>
<td>H3K4me1/2/3</td>
<td>H3K4me2/3</td>
<td>-</td>
</tr>
<tr>
<td>SETD1B</td>
<td>H3K4me1/2/3</td>
<td>H3K4me2/3</td>
<td>-</td>
</tr>
<tr>
<td>SUV39H1</td>
<td>H3K9me2/3</td>
<td>-</td>
<td>H3K9me2/3</td>
</tr>
<tr>
<td>SUV39H2</td>
<td>H3K9me2/3</td>
<td>-</td>
<td>H3K9me2/3</td>
</tr>
<tr>
<td>EHMT2 (G9a)</td>
<td>H3K9me1</td>
<td>H3K9me1</td>
<td>H3K9me2</td>
</tr>
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<td>EZH1</td>
<td>H3K27me2/3</td>
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<td>EZH2</td>
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</tr>
<tr>
<td>DOT1L</td>
<td>H3K79me1/2</td>
<td>H3K79me1/2</td>
<td>-</td>
</tr>
<tr>
<td>SET2[55]</td>
<td>H3K36me2</td>
<td>H3K36me2</td>
<td>-</td>
</tr>
</tbody>
</table>

**ESET** (also called **SETDB1**), which predominantly methylates H3K9 in transcriptionally silent euchromatin [56], has been also found to play a crucial role in post-implantation development and in methylation of H3K36 and H4K20 [57]. This protein was also found to be structurally similar with **SETD1A** [52].

**MLL1**, also member of the SET1 family, is often implicated in leukemia as a result of aberrant *Hox* gene activation mediated by histone H3K4 methylation [58]. **EZH2** acts mainly as a gene silencer; it performs this role by the addition of three methyl groups to Lysine 27 of histone 3, a modification leading to chromatin condensation [42]. EZH2 is frequently overexpressed in a wide variety of tumors and its up-regulation correlates with advanced stages of disease and poor prognosis. Knockdown of *EZH2* inhibits growth of myeloma and prostate cancer cells [59, 60], whereas *EZH2* overexpression promotes colony formation, anchorage-independent growth and cell invasion [60-62] as well as xenograft tumor growth. This methyltransferase interacts also with HDAC1 and HDAC2 in order to repress transcription during embryonic ectodermal development [63]. Taken together, these results suggest that EZH2 could be a driving oncogene. Important is to underline that the H3K27 methyltransferase EZH2 is essential for normal embryonic development [64] It has been found highly expressed in a large number of primary tumors and several data suggest that tumors invasion is dependent on its expression, as reviewed by Albert *et al.* [54].

Histone acetylation was first reported in 1964 by Allfrey *et al.* [65]. Since then it was shown that acetylation of lysine is reversible and highly dynamic through the opposing actions of two families of enzymes, histone acetyltransferases (HAT) and histone deacetylases (HDACs) [66]. The HATs utilize acetyl CoA as cofactor and catalyse the transfer of an acetyl group to the ε-amino group of lysine side chains. The result is neutralization of the positive charge of the lysine, action that has the potential of weakening the interactions between histones and DNA. These enzymes modify multiple sites within the histone N-terminal tails [66]. HDAC enzymes have opposite effects of HATs and reverse
lysine acetylation, an action that restores the positive charge of the lysine. This process potentially stabilizes the local chromatin architecture and is consistent with HDACs being mainly transcriptional repressors. There are four classes of HDAC [67] and the following are the most representative for each class: class I (HDAC 1, 2, 3, 8), class II (HDAC 4, 5, 6, 7, 9), class III (Sirtuins, SIRT1, 2, 3, 4, 5, 6, 7), and class IV (HDAC 11). Class III requires a specific cofactor for its activity, NAD+. Generally, HDACs have relatively low substrate specificity by themselves, a single enzyme being capable of deacetylating multiple sites within histones. The problem of enzyme recruitment and specificity is further complicated by the fact that the enzymes are typically present in multiple distinct complexes, often with other HDAC family members. For examples, HDAC1 is found together with HDAC2 within the NuRD, Sin3a, and Co-REST complexes [68]. Therefore, it is difficult to determine which activity (specific HDAC and/or combined) is responsible for a specific effect. Nevertheless, in certain cases it is possible to determine, which enzyme is required for a given process, as it has been shown that HDAC1, but not HDAC2, controls embryonic stem cell differentiation [69].
1.2 Epigenetic changes in vascular disease

Non-communicable diseases (NCDs), classified by World Health Organization (WHO) into heart disease, stroke, cancer, chronic respiratory diseases, and diabetes, are the leading cause of mortality in the world. Common, risk factors that underlie the major NCDs, include tobacco, harmful use of alcohol, unhealthy diet, insufficient physical activity, overweight/obesity, raised blood pressure, raised blood sugar, and raised cholesterol. Of the 57 million global deaths in 2008, 36 million (63%) were due to NCDs [70]. In 2011, a report published by WHO states that cardiovascular diseases (CVDs) are the primary cause of death globally; more people die annually from CVDs than from any other cause. An estimated 17.3 million people died from CVDs in 2008, representing 30% of all global deaths. Of these, an estimated 7.3 million were due to coronary heart disease and 6.2 million were due to stroke. In the year 2010, CVDs were estimated to have become the leading cause of death all countries [71]. According to WHO, by 2030 almost 23.6 million people will die from CVDs annually, mainly from heart disease or stroke, both having as common cause the atherosclerotic plaque, diseases that are supposed to remain the single leading causes of death [70].

1.2.1 Atherosclerosis is the leading cause of stroke and heart attack

Atherosclerosis (AS, also known as arteriosclerotic vascular disease, ASVD) is characterized by a gradual thickening and hardening of the vessel wall as a result of the accumulation of fatty acids and cholesterol (Figure 5). Inflammation, together with endothelial dysfunction, is a key event in the formation of lipid-laden foam cells, the initiation and development of AS.

Sudden arterial plaque rupture causes the formation of a thrombus that rapidly slow or stop blood flow, thus leading to death of the tissues supplied by the artery (infarction) in approximately 5 min. One of the usual infarctions is the heart attack and it involves the occlusion of the coronary artery by plaque, leading to infarction of the myocardium (Figure 6).
Atherosclerosis changes within the vessel wall of the carotid artery can lead to plaque vulnerability, which constitutes the main reason for carotid-related ischemic events [72]. If this process is happening in an artery supplying the brain, the consequence is an ischemic stroke, frequently followed by decease or permanent disabilities. Due to the lack of blood flow in certain regions of the brain responsible for sensitive or motor functions, certain activities, such as walking or speaking, may be impaired (Figure 7).

1.2.2 Stages of atherosclerosis – histological classification

Atherosclerosis is commonly described as a chronic inflammatory disease of the vessel wall, characterized by lipid accumulation, inflammation, and extensive degradation of extracellular matrix (ECM) components [73].

The initiation step for atherosclerosis is thought to be endothelium dysfunction, possibly triggered by the oxidized low-density lipoprotein (ox-LDL) (Figure 8). Ox-LDL is
often associated with proteoglycans in sub-endothelium. Activation of endothelial cells triggers an increased expression of cytokines and chemokines, enhancement of the permeability of endothelial cell layer, and increased expression of adhesion molecules. Monocytes and T-lymphocytes are attracted by chemokines and connected to endothelial cells.

Figure 8. Schematic view of processes leading to atherosclerosis. Inflammation together with endothelial dysfunction are key events in the formation of lipid-laden foam cells and the initiation and development of atherosclerosis.

Subsequently, the inflammatory cells are infiltrating the sub-endothelium of the vascular wall and initiate the inflammatory reaction leading to “fatty streak” – the type I lesion. At this stage, there are minimal histological changes consisting in small groups (double in number compared with the normal intima) of macrophages and macrophage foam cells (macrophages containing lipid droplets).

The foam cells are cholesterol engorged monocyte-derived macrophages, and are dominant type of immune cells found within the lesions. Macrophages are taking up the ox-LDL, but are not able to digest it sufficiently, resulting in the formation of foam cells. It is still uncertain, whether the fatty streaks are the precursors of more advanced lesions [74, 75]. If the inflammatory factors are not removed, the endothelial dysfunction continues and within the type II lesion there are visible more lipid-laden cells than in the initial lesion; within n the smooth muscle cells (SMCs) appear lipid droplets but their number is smaller than the number of macrophage-derived foam cells. The number of intimal SMCs in fatty streaks is similar to the number of SMCs in normal intima, but an enhanced number of proliferating SMCs is observed.
Intimal macrophage foam cells accumulate in the deep part of the proteoglycan layer and fill the space up to the level of the endothelial cells (ECs). Extracellular space contains small quantities of thinly dispersed lipid droplets and vesicular particles that vary in size and are visible only by electron microscopy. The inflammation at this level is characterized by the presence of few T-lymphocytes and numerous macrophages. Regarding the ECs, it has been observed their loss of orientation according to blood flow, a rounding of the cells, an increase in stigmata and stomata, an increase in stress fiber content, the formation of multinuclear cells. Activated ECs have an increased permeability of endothelium layer. With the progression to type III lesion (preatheroma) the lipid droplets accumulate massively in the extracellular matrix and SMCs start to become involved in intima thickening. Cholesterol starts to be rarely present in the lesion and there is a higher amount of fatty acid and fatty streaks than in the previous stages [76]. The first types are often described as early stage of atherosclerosis (Figure 9).

Within the advanced stages of atherosclerosis other cell types become involved. The first three types of lesions are not being considered clinically relevant. However, once started, the reaction tends to amplify itself. The first advanced lesion with a potential clinical importance is the type IV lesion (atheroma) (Figure 10) [76].

\[ \text{Figure 9. Early stages of atherosclerosis.} \]

\[ \text{Figure 10. Atherosclerotic lesion type IV.} \]
This type is characterized by a massive aggregate of extracellular lipid (a lipid core) that occupies an extensive but well defined region of intima. Smooth muscle cells (SMCs) within the lipid core are dispersed, have elongated shape and unusually thick basement membrane. Calcified areas are often found within the lipid cores. The part of the adaptive thickening between the lipid core and EC surface contains macrophages and SMCs with or without lipid droplets, T-lymphocytes and mast cells. In addition to the components of atheroma, a thick layer composed of newly formed layer of proliferating SMCs and collagen (a fibrotic cap) is located in the region between the lipid core and the endothelial cell layer at the arterial lumen, this process being specific for type V lesion (fibro-atheroma) [75]. Advanced stages of atherosclerosis are summarized in Figure 11.

Within this lesion type, the primary proteoglycan-rich layer between lipid core and endothelial surface contains substantial more fibrotic collagenous material. Granulation tissue and capillaries at the lateral and luminal margins (shoulders) of the lipid core may be larger than in the atheroma stage. Reparative connective tissue forms in and around regions of intima with large accumulation of extracellular lipid (lipid core); new tissue consists of substantial increase of collagen and synthetic SMCs; lymphocytes, macrophages, and plasma cells are frequently associated with the capillaries, and micro-hemorrhages may be present around them. Several cores separated by fibrous connective tissue may start to form together with additional lipid cores, varying in localization, size and shape. Further progression in the atheroma will lead to the type VI lesion (complicated atheroma). In the type of lesion V, thrombotic deposits and/or marked hemorrhage that accelerate growth and complexity of the lesions are visible. Erosion or ulceration of the lesion surface due to shearing fissures are main cause of the hemorrhage into lesions, thrombotic deposits, therefore this stage is perhaps the most clinically relevant. Type VII (calcified atheroma) of the lesion is similar with the type V but with calcium deposits that replace the accumulated remnants of dead cells and extracellular lipid. Type VIII lesions (fibrotic lesion) are the
lesions consisting almost entirely of scar collagen. Within this type of lesion the lipid components are no longer present [75].

**1.2.3 Role of epigenetics in vascular disease**

The major causes of cardiovascular disease are tobacco use, physical inactivity, an unhealthy diet, and harmful use of alcohol. Current recommendations for treatment of CVD aim to reduce risk factors [72].

The role of epigenetics in chronic diseases, such as atherosclerosis, consists in more than just acute reaction to the environmental insult (exposure) – it represents a long term change of the gene expression in the cells of the vascular wall. This means that cells are responsive and they are changed epigenetically during lifetime, suggesting that diet – gene and environment–gene interactions are among the key processes in the disease history. This hypothesis is supported by many studies that are connecting nutrition [77-79], smoking [80, 81] and environmental changes [82] with alternations in epigenetics. An interesting study of Baccarelli et al. showed that pollution from traffic, an environmental challenge associated with increased risk of CVD, affected DNA methylation in a study group in USA [83, 84]. The focus of these studies was on repetitive sequences LINE-1 and Alu1. A correlation has been found between the exposure to carbon dioxide and hypomethylation and a significant demethylation effect was observed for LINE-1. These data suggest that DNA might reveal a link between exposure to pollutants and the development of CVD. LINE-1 demethylation has been also detected in vascular smooth muscle cells exposed to homocysteine – a very well characterized molecule with a role in epigenetics [85].

Hyperhomocysteinemia is an accepted risk factor for CVD, supported by a large amount of studies [86, 87]. High levels of homocysteine promote the development of atherosclerotic lesions by inducing aberrant DNA methylation in both vascular smooth muscle and monocytes [85, 88]. There are several of other findings that have confounded the issue. It can be assumed that homocysteine inhibition of DNMTs would promote an overall hypomethylation. However some data suggest that hypermethylation predomnates at specific loci, including extracellular superoxide dismutase, estrogen receptor α, and endothelial NO synthase [87]. Other reports, utilizing peripheral blood lymphocytes from patients with coronary artery disease, have produced conflicting results, suggesting that global methylation may be increased or decreased in different individuals [89, 90]. Collectively, the data support a role for altered DNA methylation secondary to homocysteine overload, but to date it has been difficult to specify how these alterations may correlate with disease pathogenesis.
INTRODUCTION

DNA methylation is a major epigenetic modification regulating gene expression, silencing repetitive DNA elements, and maintaining chromosomal structure. Animal model experiments on mice proved the principle that DNA hypomethylation is a pre-step in the onset of atherosclerosis [91]. A recent study of Castillo-Diaz et al. [92] on human atherosclerotic arteries sustains the hypothesis that aberrant DNA methylation plays a role in the critical regulatory genes for induction of pro-atherogenic cellular phenotype. One of the theories that describe possible mechanisms for some cancers seem to be facilitated by so called epimutations - changes not in the DNA itself, but in the pattern of methylation, with inactivation of genes that play a role in organism defense against cancer [93]. This approach is supported by the study of Hiltunen et al. [13], who describes that hypomethylation in atherosclerotic lesions is present at the same level as in malignant tumors, process which affects the smooth muscle cell proliferation and gene expression in artherosclerosis. Portions of DNA can be inactivated by covalently attaching methyl groups, which can interfere with the binding of transcriptional enzymes, and can also be signals to recruit enzymes that modify associated histones. DNA methylation is not maintained during replication, but methyl groups are added after each cycle of cell replication [94, 95].

The methylation of DNA is sustained by a family of methyltransferases (DNMT1, DNMT3A, and DNMT3B). A study on mouse embryonic fibroblasts deficient in DNMT1 were reported to have a decrease in the global DNA methylation status correlated with cell type-specific changes in gene expression that disturb several pathways, including expression of imprinted genes, cell-cycle control, growth factor/receptor signal transduction, and mobilization of retro elements [96]. The role of DNA methylation and DNMTs in cancer and developmental studies has been extensively examined. However the functions of DNMTs in cardiovascular diseases and atherosclerosis are not yet understood.

It is currently recognized that the epigenetic modifications of the genome play a major role in disease development, linking the environmental insults with gene regulation. Recent research has focused on how modification of DNA by methylation, and histone modification by acetylation, methylation, phosphorylation and/or SUMOylation may be targeted therapeutically [97]. Mouse models of hypercholesterolemia have demonstrated that HDAC7 plays a role in disease progression through repression of the cholesterol-metabolizing enzyme CYP27A1. Importantly, treatment of these animals with HDAC inhibitors significantly lowered serum cholesterol through enhanced expression of CYP27A1 and subsequently increased production of bile acids [98]. Statins, which are commonly employed as anti-cholesterol pharmaceuticals, have also been implicated in HDAC regulation, though there is doubt about how they interact with gene expression. In order to prove the complex relationship between HDAC regulation and atherosclerosis, Dje N'Guessan et al. [99]
demonstrated that ox-LDL reduces HDAC levels and modulates signaling pathways that are partially rescued with statin treatment. This result brings forward the utility of HDAC inhibition in CVD and highlights a portion of complex communication between metabolic and epigenetic pathways that takes place in CVD.

There is significant evidence supporting complex regulatory networks spanning metabolic and epigenetic processes in CVD. While many of the specific interactions remain to be identified, the data are so far promising. Continued research in this field is critically important given the large number of patients who are affected by CVD. Epigenetic-based therapies to target the underlying mechanisms of CVD will hopefully prove to be valuable treatments in the future [100].

1.2.4 Aim of the study – epigenetic changes in vascular disease

Chromatin is a flexible structure experiencing dynamic epigenetic changes through the whole life. The most interesting feature of epigenetics is that it can be affected by environmental interactions, giving the organism a feedback from surrounding conditions. Emerging evidences implicate a spectrum of epigenetic changes in the pathophysiology of atherosclerosis [13, 87, 94]. Genetic and epigenetic studies must be integrated to find new targets for atherosclerosis therapy and to completely understand the cause of this vascular disease. Since epigenetic modifications are potentially reversible, there is a possibility directed therapies targeted at specific modifications of the epigenome may have favorable effects on cardiovascular system of the patients suffering from atherosclerosis.

The aim of the current study was to evaluate possible alterations in DNA and histone modifications in carotid artery in concordance with the progression of atherosclerosis. In the present work several points should be addressed with regard to the above mention topic in an attempt to answer following questions:

1. Are there any changes in global DNA methylation in concordance with the progression of atherosclerosis in carotid artery?
2. Are there any changes in the genome wide methylation in DNA found in serum between patients with high grade carotid artery stenosis and healthy subjects?
3. What molecular mechanism is involved in the changes of DNA methylation? In this context, the expression of DNA-methyltransferases is examined.
4. Are there any changes in histone methylation in carotid atherosclerotic lesions compared to healthy individuals? In this case the study concentrate on two methylations in histone H3 and H4 at lysine K4 and K9, which possess opposite effect on gene activity. Methylation of H3K4 increases gene activity, in contrast methylation of H3K9 leads to gene silencing.
5. What molecular mechanisms are responsible for changes in histone H3K4 and H3K9 in atherosclerotic plaques? In this context, the expression of histone methyltransferases is evaluated.

6. Can the modification of H3K4 and H3K9 be associated with certain type of cells, important in AS, such as SMCs or inflammatory cells?
1.3 Epigenetic changes in regenerative medicine

One of the main efforts in medical treatment is the regeneration of organ function caused by injuries or diseases. Nowadays, this medical field has become a separate entity and it carries the name of “regenerative medicine”. Regenerative medicine is formed of more subdomains, all having the same purpose, namely the repair of dysfunctional cells, tissues or organs. These subdomains of regenerative medicine are reconstructive surgery, transplantation surgery, tissue engineering, and gene and cell therapy.

The 20th century has brought notable medical developments such as blood transfusion and organ transplantation. Organs were at first transplanted only between twins thanks to the immunological compatibility. Later, due to the discovery of immunosuppressive medication, allogeneic organ transplantation became a widespread surgical technique. Beginning with the 1990s, triggered by the pressing need for transplant organs, research has taken a great interest in tissue engineering. By using biocompatible materials with or without biological component, tissue engineered constructs can be used to repair and accelerate the healing of various injuries. Tissue engineering uses mostly biodegradable scaffolds that are slowly resorbed after implantation and replaced by the extracellular matrix (ECM) of the body for the purpose of building tissue grafts. The biological parts of the construct are generally cells. They contribute to the biocompatibility and integration into the host. The ideal cells applicable to the clinical setting are likely to possess the following characteristics:

1. Unlimited ability to renew symmetrically to provide abundant numbers of cells;
2. Ability to form all functional tissue of the body;
3. Compatibility with patient’s immune system.

Embryonic stem cells appear to have the characteristics 1 and 2 but not 3. Somatic stem cells can be harvested from the patient (satisfying the 3rd characteristic), but are developmentally restricted and therefore do not possess the characteristics 1 and 2 [101].

Different strategies have been developed to reprogram somatic cells. The most remarkable studies are from Takahashi et al. [102] and Yu et al. [103]. These research groups were able to reprogram fibroblasts into cells with the characteristics of embryonic stem cells through viral transfection of a combination defined factors, which have been acknowledged to be key “stemness” factors for achieving pluripotency: OCT4, KLF4, CMYC, NANOG, and SOX2. In this way, all three characteristics for an ideal regenerative cell, called induced pluripotent stem cells (iPSCs), as described above, can be fulfilled: the compatibility with the patient.
Induced pluripotent stem cells have, however, a major limitation for their use in clinical applications due to their oncogenic potential. The OCT4, KLF4, CMYC, NANOG, and SOX2 markers have been intensely studied and are known to be critically involved in self-renewal of undifferentiated embryonic stem cells. OCT4, also known as POU5F, is a transcription factor (TF) used for identification of stem cells, as well as cancer stem cells. KLF4, Kruppel-like factor 4, is a part of the KLF family that has been mainly studied for its involvement in cell proliferation, differentiation and survival, both in cancer and iPSCs. KLF4 is known to act as a transcriptional activator or repressor depending on the promoter and/or cooperation with other TFs. CMYC, whose persistent overexpression is associated with cancer, is also a TF. It has been suggested that the expression of CMYC regulated the expression of 15% of all genes [104]. It acts also by recruitment of HATs, proving its role in regulation of chromatin structure [105]. NANOG is believed to be a key TF in maintaining pluripotency, although Yamanaka et al. [106] proved that it is also possible to induce embryonic stem cells without this marker, making NANOG dispensable. NANOG expression is also associated with tumors and is used for diagnostic of germinoma (a type of germ cell tumor). SOX2, a TF that promotes the differentiation of ECs into neuronal ectoderm germ layer, has been shown to inhibit the differentiation into mesoderm and to have a key role in development of mammalian embryos [107]. However, its overexpression in lung carcinoma classified this TF as a key oncogene in lung squamous cell carcinoma with a role epithelial differentiation in tumor progression [108].

Adult mesenchymal stem cells have been used in many tissue engineering studies involving repair of various tissues, such as blood vessels, bone, cartilage, or skin [109-111]. These cells can be isolated from different organs of the patient himself and grown in vitro until a sufficient number of cells is obtain, after which they can be seeded onto the scaffold and implanted in vivo. In this way, the immune reactions to the construct are minimal and there are no ethical issues like those caused by the use of embryonic stem cells. Inside the body, the cells will proliferate and produce ECM that would lead to the incorporation of the construct into the host.

1.3.1 Reprogramming of mesenchymal stem cells (MSCs) through epigenetics

There have been studies concerning the differentiation of adult stem cells into cells of other phenotypes, e.g. endothelial features, mostly using mesenchymal stem cells (MSCs) [111, 112]. Bone marrow-derived MSCs (BM-MSCs) are able to differentiate into various cell lineages of bone, cartilage, adipose, myocardial, and even neuronal tissue [113-115] and into endothelial cells [115]. The disadvantages of BM-MSCs are, however, the invasive
harvesting procedure, which carries a high risk for the donor and the differentiation process lasts a long period of time. Therefore, other possible sources of such cells with less traumatizing intervention are necessary.

Adipose tissue represents an abundant and accessible source of adult stem cells, the adipose-derived mesenchymal stem cells (adMSCs). These cells can differentiate into a number of mesodermal lineages of ostogenic, chondrogenic, or adipogenic origin [116]. They have already been shown to have many characteristics in common with the BM-MSCs, including their proliferation potential and their capacity of differentiation.

Recently, Colazzo et al. studied the ability of adMSCs to differentiate into ECs [111]. Furthermore, Fischer and colleagues have shown that autologous adMSCs are able to support a vascular graft in vivo and that further work should be done to improve the differentiation process [117]. So, finding a possibility to increase the differentiation capacity of this type of cells is desired.

During development of the human body a process of differentiation of the embryonic stem cells occurs [118]. During differentiation adult cells arise, which constitute the tissues and organs of the adult organism, through a gradual inhibition of genes, until those only specific for a certain type of tissue are being expressed and transcribed. Consequently, all cells in one organism have the same genotype, but different phenotypes, according to which genes are active in each tissue. Phenotype differences are given by distinct epigenetic patterns, which involve the degree of chromatin folding and DNA methylation [119, 120]. Thus, targeted epigenetic changes, can make silenced genes available for transcription and facilitate desired reprogramming of the cell [121].

1.3.2 Epigenetic modifying drugs and cell reprogramming

During the last decades, studies of chromatin modifications have revealed their essential role in regulation of gene expression [122-124]. Epigenetic reprogramming may also allow re-establishment of the pluripotent state in already differentiated cells. Some studies have already shown that it is possible to use endogenously expressed pluripotency genes to generate stem-cell-like phenotype [125]. However, to achieve a successful reprogramming, DNA methylation status, histone modification, and chromatin structure need to be transferred into a state similar to that of the embryonic state (Figure 12) [126]. Expression of specific genes such as POU5F1a, KLF4, NANO, and CMYC induces a sequence of epigenetic events, which trigger chromatin modifications and changes in the DNA methylation. If a somatic cell is induced with all these factors, its phenotype transforms to a partially reprogrammed state [127]. Thus, appropriate changes in the chromatin of
somatic cells may consequently lead to the induction of pluripotency or, better said, to the de-differentiation state of the cells. At this point, a proper protocol is necessary to increase gene expression specific for the desired cell type.

![Figure 12. A schematic diagram of epigenetic modifications affecting cell reprogramming. The degree of DNA methylation inversely correlates with the ability of cells to regain their potential to differentiate. An appropriate alteration of epigenetics in somatic cells consequently leads to the increase in the expression of pluripotency related genes. Expression of these genes correlates with a higher reprogramming capability.](image)

Genes normally expressed during embryogenesis (e.g. pluripotency related genes) are silent in somatic donor cells. For gaining the reprogramming capacity, these genes must be turned on again. A possible approach might be the use of chemical modifying drugs that are able to interfere with epigenetic pattern of the treated cells, to inhibit either DNA methylation or different histone methyltransferases or deacetylases. For this purpose, DNA methylation pattern and chromatin structure must be reorganized accordingly, to resemble that of undifferentiated nuclei [128].

Several drugs such as 5-azacytidine (AZA), BIX-01294(BIX), and valproic acid (VPA) are known to have an effect on DNA methylation and/or histone modification.

### 1.3.2.1 5-azacytidine, inhibitor of global DNA methylation

Global inhibitors of DNA methylation are already used as research tools. Among these are demethylating agents as procainamide [129], zebularine [130], tea polyphenol (-)-epigallocatechin-3-gallate [131], and RG108 [130]. One of the most potent demethylating agents is 5-azacytidine (Figure 13) [132].
Azacitidine or 5-azacytidine, commercially also known as Vidaza, is a chemical analogue of cytosine, used in the treatment of myelodysplastic syndrome. It is known as a strong demethylating agent, an effect that has an important role in cellular reprogramming. 5-azacytidine-5-monophosphate inhibits orotidine-5-phosphate decarboxylase, thus blocking the de novo pyrimidine synthesis [133]. Azacytidine appears to restore normal growth and differentiation of bone marrow cells by causing hypomethylation of DNA and directing cytotoxicity on abnormal hematopoietic cells in the bone marrow. Hypomethylation may trigger the normal function of genes that regulate differentiation and proliferation [134].

![Chemical structure of 5-azacytidine](image)

**Figure 13.** Chemical structure of 5-azacytidine - (4-amino-1-beta-d-ribofuranosyl-1,3,5-triazin-2(1h)-one). The DNA methyltransferase inhibitor 5–azacytidine impedes the DNA methylation and may therefore confer gene activation [122, 123].

The incorporation of AZA into DNA *in vitro* prevents DNA methylation [135]. Due to its inhibition effects on the DNA methylation, it has already been used in differentiation protocols of adMSCs or embryonic stem cells into hepatic lineage [136-138]. Furthermore, it has been reported that exposure of bone marrow-derived MSCs to AZA induced a myocyte-resembling phenotype with enhanced response of calcium channels, which has potential applications in ameliorating muscle loss after myocardial ischemia [139]. AZA is unstable in aqueous solutions, with a 10% loss of the product in 2-3 h at RT in lactated Ringer's solution [140]. In addition, in the presence of AZA the cytosine methylation is reduced already after one replication cycle and a part of DNA becomes only hemi-methylated. Full double stranded demethylation requires two replication cycles and occurs in 50% of the dividing cells [141]. Therefore, the use of this substance must be adapted to the intended type of cell population.

### 1.3.2.2 BIX-01294, a small molecule able to inhibit the G9a methyltransferase

Acetylation of lysine at the N-tail of histone H3 (K9, K14, K18, and K23) and H4 (K5, K8, K12, and K16) and methylation of lysine residue in H3 (as K4, K9, K27, K36, and K79) and H4 (K2) are the most relevant histone modifications that have been identified until now [33].
Many studies have been performed in order to understand the involvement of H3K4 and H4K9 methylation in cancer, embryogenesis, and cell reprogramming. One of the best described modifications is the methylation of lysine 9 of histone H3 (H3K9) [142].

After Jackson et al. showed that H3K9me2 histone modification is most likely the sign of gene silencing in Arabidopsis thaliana [143], it has been proposed and later confirmed that there is a crosstalk between DNA methylation, absence of H3K4 methylation, and the presence of H3K9 methylation, although the exact mechanism is still unclear [144, 145]. This correlation may be caused in part by DNA methyltransferases specifically recognizing histone modifications. For instance, the de novo DNA methyltransferase, Dnmt3A and its cofactor Dnmt3L recognize unmethylated H3K4 via an ADD domain [146, 147]. Moreover, G9a Suv39h1/2 and Setdb1 (all H3K9 methyltransferases), Ezh2 (H3K27 methyltransferase), and heterochromatin protein 1 (HP1), have been implicated to recruit DNA methyltransferases in cancer cells and therefore to modify the DNA methylation pattern [148].

Recently, Kubicek et al. showed that BIX-01294 is a specific inhibitor of histone methyltransferase G9a, which is responsible for methylation of H3K9 (Figure 14) [124]. By using this chemical compound the epigenetic repression status of several genes could be altered. Furthermore, BIX-01294 seems to compensate the lack of Oct4a expression in mouse cells, which are not able to express it natively [125].

**Figure 14.** Chemical structure of BIX-1294 - (N-(1-benzylpiperidin-4-yl)-6, 7-dimethoxy-2-(4-methyl-1, 4-diazeplan-1-yl) quinazolin-4-amine) [149]. BIX is a small molecule with the capacity to specifically inhibit the G9a methyltransferase.

BIX modulates H3K9me2 levels in mammalian cells and potentiates induction of pluripotent stem cells from somatic cells in vitro [125]. BIX-01294 occupies the histone peptide binding site and in this way inhibiting the action of G9a [150]. The study of Feldman
et al. [151] confirmed that H3K9 methylation promotes by the SET-containing protein G9a is inhibiting the Oct3/4 re-expression, preventing the reprogramming of the mouse cells. Therefore, using this chemical compound, the epigenetic repression status of several genes, including POU5F1, could be altered and cellular reprogramming might be achieved.

### 1.3.2.3 Valproic acid as a histone deacetylase inhibitor

Acetyltransferases (HATs) are enzymes responsible for the transfer of acetyl group to lysine residues and histone deacetylases (HDACs) coordinate their removal. Acetylation neutralizes the positive amino acid charge decreasing the affinity for DNA. The histone tail dislodges from the nucleosome increasing accessibility of transcription factors, which leads to gene expression [152].

![Chemical structure of valproic acid (2-propylpentanoic acid).](image)

Valproic acid (VPA) is a simple fatty acid (2-propylpentanoic acid) that has a clinical use as anti-convulsive and mood stabilizing drug used in epilepsy and bipolar disorders (Figure 15). The mechanism of VPA includes the neurotransmitter GABA. By inhibiting the GABA-transaminase, it is increasing GABA concentration, therefore enhancing neurotransmission of GABA [153]. In addition, VPA has an inhibiting effect on voltage-gated Na⁺ channels. The mechanisms of action of VPA in neuropsychiatric disorders are far from fully understood. Moreover, it has been shown that VPA (and its metabolites) can bind covalently and irreversibly to various tissue proteins [13], resulting in alternation of their conformation [154]. VPA also induced changes in the expression of multiple genes, mediated at least partially through the direct inhibition of HDAC [155-157].

Valproic acid, in addition to selectively inhibiting the catalytic activity of class I HDACs, induces proteasomal degradation of HDAC2, in contrast to other inhibitors such as trichostatin A (TSA) [158]. As an inhibitor of histone deacetylases, VPA plays also an important role in the regulation of gene expression [124] and might be thus a promising effector molecule for epigenetic reprogramming.

### 1.3.3 Adipose tissue more than a fat storage

Adipose tissue is a loose connective tissue located in different places of the body. It is found beneath the skin (subcutaneous fat), around internal organs (visceral fat), in bone marrow (yellow bone marrow), and in breast tissue. The majority of the adipose tissue is
found directly underneath the dermis, but is also found intra-abdominally, as omental fat pads and surrounding several organs of the body.

Humans and other mammals have two main adipose tissue types: white adipose tissue (WAT) and brown adipose tissue (BAT) [159]. The distinction between them is given by the color and the appearance of the adipose cells. The cells from the WAT contains only one fat vacuole, whereas the BAT adipose cells contain multiple vacuoles of fat and many mitochondria [160], with its brown color presumably coming from the relatively high cytochrome c content and better vascularization [159].

WAT is mainly considered to be storage for excess energy acquired from food intake [161]. It makes up as much as 20% of the body weight in men and 30% in women [162]. It is mostly found subcutaneously and intra-abdominally between the abdominal muscles and the visceral organs, where it is forming fat deposits, such as the Omentum majus. Recently, research has shown that WAT has also important endocrine functions and is releasing the so-called “adipokines”, which regulate the immune response, blood pressure, angiogenesis, hemostasis, bone metabolism, and thyroid function [163].

1.3.4 **Cellular components of adipose tissue and relevant CD markers expression pattern**

Adipocytes are found in both types of adipose tissues, brown and white, but they have different functions and even different phenotypes (*Figure 16*). The cells originating from BAT have the main function in thermogenesis and present several smaller adipose droplets together with multiple mitochondria. By contrast, WAT cells are mainly regulating the energy balance and possess one large lipid vacuole [164]. These cells belong to a completely independent cell lineage and recent reports suggest that at least some precursors for white fat cells are derived from mural cells associated with blood vessels [165, 166]. White adipose tissue also contains progenitors of multiple mesodermal cell lineages and is an attractive source of autologous cells for transplantation [116]. An MSC population was found in human WAT, which can be driven to differentiate into adipocytes, osteoblasts, and chondrocytes. Whether all mesenchymal stem cells found in adipose tissue are adult mesenchymal stem cells or only a subpopulation, it is a matter of further investigation. There have been studies that are showing differences in the cell population with a regard not only to the tissue type – brown or white, but also to age, gender or body mass index (BMI) of the patients [167-172]. Similarities were shown between human WAT stroma vascular cells and bone marrow stromal cells [173]. There are studies that define, confirm, establish, and validate both the in
situ and in vitro links between adult human mesenchymal stem cells (MSCs) and perivascular cells, referred to as pericytes [174, 175].

![Figure 16. Different origins and properties of brown and white adipose tissue adipocytes. (A) Brown adipose tissue originating from myoblasts positive precursors; (B) White adipose tissue with origins from pericytes. Modified from Seale et al. [166].](image)

Overall yield of cells from adipose tissue is influenced by donor age, obesity and/or anatomical site of the sampling [169-171]. Although intensive research work has been performed on progenitor cells from adipose tissue, the mesenchymal stem cells population remains a cell population difficult to characterize. Mostly, this cell population mixture is named “adipose-derived mesenchymal stem cells” [176], but are often called adipose tissue mesenchymal stem cells [177], multi-lineage cells from adipose tissue [178], or adipose tissue derived stromal cells [179].

There are two main procedures to harvest adipose tissue, namely liposuction and plain excision during procedures like abdominoplasty or breast reduction. It is still controversial, which of these cell sources provide a better cell population. Some studies state that liposuction aspirates represent a better source compared with the excised one [180] and others claim the contrary [181].

It also has been shown that, in vitro, markers of MSCs are progressively decreased in their expression at higher passages and in this context also their osteogenic potential [182]. For reliable experiments it is recommendable to characterize the cell population, to establish a standard isolation procedure and to work with a homogenous cell population.

Tissue regeneration and recovery in the adult body depend on self-renewal and differentiation of stem and progenitor cells. MSCs have the ability to differentiate into various
cell types and have already been isolated from the stromal fraction of bone marrow, dental tissue or adipose tissue [183]. Studies that are comparing the different types of cell populations have found many similarities regarding their differentiation repertoire, their adherence on the plastic property, or cell phenotype and morphology. Further similarities have been found concerning growth kinetics, expansion capacity, and expression of surface proteins. A basic characterization of mesenchymal stem cell populations points out the presence of CD105, CD90, CD73 [184], and absence of C14, CD34 or CD45 [185].

1.3.5 Source of mesenchymal stem cells and their differentiation potential

Adipose tissue is considered an alternative source of MSCs compared to bone, BM-MSCs, since it can be obtained by a less invasive method and in larger quantities. Isolation of MSCs from adipose tissue has a high success rate with a considerably high yield via a quick and simple liposuction procedure with minimal downtime and without the need for prolonged cell culturing.

Adipose tissue is derived from the mesoderm and is one of the tissues that contain high fraction of mesenchymal stem cells [186]. AdMSCs are being increasingly considered as a readily available source of adult stem cells. It has been suggested that from 5 g of adipose tissue up to 35 million cells can be generated [136]. Therefore, this tissue can be regarded as a suitable source of potential stem cells for autologous cell therapies and regenerative medicine. It was recently shown that adMSC can undergo a process of differentiation towards the endothelial cell lineage [117].

1.3.6 Regenerative medicine and vascular disease

The surgical treatment of CVD often requires interventions, such as coronary artery or peripheral bypass, procedures that involve placement of vascular grafts usually originating from the patient’s own blood vessels. These grafts are currently the most successful types of implants [187], but their availability is limited due to their utilization in other procedures or because they are often diseased.

For replacement of blood vessels over 6 mm in diameter, synthetic grafts can be used, produced for example from polytetrafluoroethylene (PTFE) [188]. This synthetic implant is able to replace vessel function and to improve the life expectancy of the patient but has also plenty of drawbacks. There is a high risk of infection, stenosis of the anastomosis, propensity for occlusion due to thrombosis, and calcification [189]. The artificial vascular
grafts cannot fully reproduce the biologically sophisticated functions of native vessels. Additionally, they require anticoagulants to control the risk of thromboembolism, while the allograft and bio-prosthetics undergo calcification, and structure deterioration [190]. The undeniable advantages of natural vessels are the capability of growth and adaptation to various physiologic conditions [191]. The ideal properties of a tissue engineered vascular graft would be, among others, resistance to infections, cellular components that would allow the vessel to grow and renew, and non-trombogenicity. In the case of smaller diameter conduits (<6 mm), the use of the PTFE grafts is highly limited due to the currently very low patency rates of less than 25% after 3 years and the obligatory anticoagulant medication [192].

Given the high and increasing number of surgical interventions that require prosthetic transplants and the limited availability of autologous vessels to which there can be added, the poor performance of the synthetic alternatives, it is a big challenge for vascular surgeons and scientists to develop a new generation of biocompatible blood vessel substitutes [193].

1.3.7 Biological grafts

Biological vascular grafts can originate from different sources. The most successful vascular grafts are the ones harvested from the patient himself, named autografts, because risks of incompatibility are eliminated. Because of the very little availability of autografting material, alternatives have been investigated, and these efforts were materialized by the introduction of allografts (grafts from human donors) or xenografts (grafts from other species). Nevertheless, these methods present the great disadvantage of triggering the immune response of the host and the patient has to stay under immunosuppressive medication the whole life.

The concept of tissue engineering has been successfully applied in the production of vascular grafts. The aim is to create fully biocompatible grafts by completely eliminating the synthetic component of the constructs, combined with their repopulation with cells, either in vivo or in vitro. Strategies to achieve this goal include the use of biodegradable scaffolds or of naturally-derived scaffolds. The biodegradable scaffolds are based on cells seeded on polymers that can be resorbed by the body after implantation [194]. The idea behind this approach is to provide support for cells until the scaffold is replaced by newly-formed tissue. Examples of biodegradable polymers are polyglycolic acid (PGA), poly-L-lactic acid (PLLA), or poly-ε-caprolactone (PCL). This type of scaffolds can be completely degraded through hydrolysis and have a very good resistance to physiologic blood pressure. Scaffolds for vascular grafts can be derived from naturally-occurring polymers, with collagen being one of
the most abundant structural proteins in the human body [195] and a major component of the extracellular matrix of tissues, including that of blood vessels. Tubular constructs can be produced from collagen gels, which can be seeded with cells and implanted at the injured site [196]. Despite their potential and subsequent improvements, these constructs have low mechanical strength [197]. In 1998, L’Heureux introduced the sheet-based tissue engineering [198]. This method comprised of rolling sheets of smooth muscle cells grown in vitro onto a cylindrical support and culturing them until the sheets combine to each other. The construct is then devitalized by dehydration and the remaining ECM secreted by the cells is reseeded with endothelial cells. These scaffolds have good mechanical resistance but they were shown to be prone to intimal hyperplasia and aneurysm formation [199].

Another promising technique of obtaining biological scaffolds is decellularization [200]. Cells are being removed from xenogenic or allogenic blood vessels with the use of chemical, physical, or enzymatic agents. What remains is the ECM of the vessel, composed of collagen, fibronectin and glycoproteins, free of cellular antigens and cannot cause an immune response [201]. Onto this scaffold, similar to the above mentioned procedures, autologous cells are being seeded in order to coat the inner surface of the vessel [202]. A recent study has shown promising results, after this method was applied in vivo in a porcine model [189]. BM-MSCs and endothelial-like cells were seeded onto acellular arterial grafts, and implanted. The grafts showed lack of immune reaction, synthesis of autologous ECM, and in vivo remodeling of the construct.

1.3.8 Aim of the study - epigenetics in regenerative medicine

The need for biological tissue grafts is growing all over the world due to the obvious shortage of donors and the currently inadequate characteristics and limited applicability of the synthetic tissue substitutes. These issues have encouraged the research of autologous cells tissue engineering applications that could elude the above mentioned limitations.

This study focused on the possibility to epigenetically reprogram adult mesenchymal stem cells (MSCs), derived from adipose tissue (adMSCs) into cells with endothelial features. Autologous endothelial cells would be the best cell type used in coating artificial scaffolds prior to implantation, in order to reduce the risk of thrombosis and consequently the risk of cardiovascular events. The limitation of using native endothelial cells comes from their very limited availability. By contrast, adMSCs can be found in higher numbers in the patient’s own adipose tissue, and have already been shown to possess high replication potential and so, to be available in a sufficient number to be used in tissue engineering.
AdMSCs themselves do not possess any endothelial characteristics. However former studies have shown that it is possible to induce non-typical gene expression by means of viral transduction. But this approach has been challenged due to ethical reasons and its limited success rate. On the other hand, epigenetic reprogramming induces gene expression without manipulating of the genome. Therefore, treatment of adMSCs with epigenetic modifying drugs such as AZA, BIX or VPA could facilitate de-differentiation towards progenitor stem cells by increased expression of pluripotency-related genes. This step would be followed by triggering the expression of endothelial-specific genes, which could improve the differentiation of adMSCs into endothelial cells that would make them suitable for applications in e.g. vascular surgery.

The aim of the current study was therefore to evaluate the ability of adMSCs to increase their differentiation potential and consequently to differentiate into cells of endothelial characteristics. In this regard following items were to address:

1. Isolation and characterization of adMSCs from human adipose tissue to achieve homogenous cell population.
2. Treatment of adMSCs with epigenetic modifying drugs to induce an increase in the expression of pluripotency-related genes
3. To evaluate, whether epigenetic modifications have a positive effect on differentiation of adMSCs towards endothelial lineage.
2 MATERIALS AND METHODS

Collection of human tissue for research purposes was conducted according to the ethical guidelines of Technische Universität München, Klinikum rechts der Isar. The local ethics committee approved the study and written informed consent was given by all donors.

2.1 Cell culture experiments

2.1.1 Isolation of primary adipose derived mesenchymal stem cells (adMSCs)

Human adMSCs were isolated from abdominal subcutaneous adipose tissue obtained from the Department of Plastic Surgery (n = 68). Cell isolation was performed by modification of the method described previously (Figure 17) [203]. Briefly, the adipose tissue was cut into small pieces, placed into a 50 ml falcon tube, washed two times with Dulbecco’s phosphate buffered saline (PBS, PAA Laboratories GmbH, Cölbe, Germany) and centrifuged at 430 x g for 10 min w/o brakes. Then, the tissue was transferred into a new falcon tube and mixed with sterile collagenase solution (4 mg collagenase II in 5.5 ml PBS). Subsequently, the mixture was shaken at 37°C for 30 min until an emulsion was formed. Warm growth cell culture medium (DMEM with high glucose 4.5 g/l (Biochrom AG, Berlin, Germany) with 10% fetal bovine serum (FBS, PAA Laboratories GmbH), 1% L-glutamine (PAA Laboratories GmbH), 1% penicillin/streptomycin (PAA Laboratories GmbH)) was added to the suspension and centrifuged at 600 x g for 10 min. The remaining adipose tissue was removed, the cell pellet was re-suspended in culture medium, and the cells were plated in 175 cm² culture flasks. Growth medium was first changed after 24 h and then regularly every 3 days.
Figure 17. Isolation of adMSCs from adipose tissue.

After scaling down the adipose surgical material, the tissue was washed with PBS and digested in collagenase solution. Subsequent centrifugation separated adMSCs as a pellet at the bottom of the tube from the floating lipid layer (mature adipocytes) on the top. The supernatant was removed; the pellet was re-suspended in appropriate cell growth medium and subsequently seeded into culture flask to allow cells to expand.
2.1.2 *Cell culture*

2.1.2.1 *Culture of adipose-derived mesenchymal stem cells*

The cells were cultured in Dulbecco’s modified Eagle’s medium: DMEM with high glucose 4.5 g/l (Biochrom AG) supplemented with 10% FBS (PAA Laboratories GmbH), 5 ml penicillin/streptomycin (PAA Laboratories GmbH), and 2 mM L-glutamine (PAA Laboratories GmbH), and incubated at 37°C in 5% CO₂ atmosphere. To maintain the cells in culture, medium was changed every three days. AdMSCs were sub-cultured when they reached a confluence of 80%. Cell number and viability were determined by trypan blue exclusion method. For all experiments adMSCs were used in the third passage, except for the flow cytometry (fluorescence activated cell sorter - FACS analysis), where the cells were analyzed in each passage until the passage 3.

2.1.2.2 *Osteogenic differentiation*

The osteogenic differentiation was induced by cultivation of adMSCs in osteogenic differentiation medium. The medium was changed every 3 days. The potential of adMSCs to differentiate in osteoblast-like cells was evaluated at different time points, 7, 14, and 21 days. As positive control osteogenic progenitor cells were used. The human osteogenic progenitors were isolated from tubercular bone specimen of patients operated for hip joint replacement orthopaedic surgery. None of the donors had any disease with effect on the bone metabolism. Osteogenic differentiation media content was: 500 ml Alpha Medium (Biochrom AG), 50ml FBS (PAA Laboratories GmbH), 10 ml HEPES (Biochrom AG), 5 ml penicillin/streptomycin (PAA Laboratories GmbH), 5 ml L-Glutamine, 5 ml MEM Vitamins (Biochrom AG), dexamethasone 100 nM (Sigma-Aldrich, Munich, Germany), L-ascorbic acid 0.25mM (Sigma-Aldrich), Beta-Glycero-phosphate 10 mM (Sigma-Aldrich).

2.1.2.3 *Adipogenic differentiation*

Adipogenic differentiation was induced with appropriate adipogenic differentiation medium, as mentioned by Kern [204]. Medium was changed every 3 days. The potential of adMSCs to differentiate in adipocytes was evaluated 14 and 21 days after the start of the recommended protocol. Adipogenic differentiation medium comprised of 500 ml DMEM High Glucose (Biochrom AG), 50 ml FBS (PAA Laboratories GmbH), 5 ml penicillin/streptomycin (PAA Laboratories GmbH), 5 ml L-glutamine (PAA Laboratories GmbH), 5 ml Amphotericin B (PAA Laboratories GmbH), 1 mM dexamethasone (Sigma-Aldrich), 0.5 mM 3-isobutyl-1-methyl-xanthine (Sigma-Aldrich), 10 µg/ml recombinant human insulin (Sigma-Aldrich), and 100mM indomethacin (Sigma-Aldrich).
2.1.2.4 Endothelial differentiation

Endothelial differentiation medium (EM) was prepared by adding 5 ml penicillin/streptomycin (PAA Laboratories GmbH), 5 ml L-glutamine (PAA Laboratories GmbH), and 5 ml Amphotericin B (PAA Laboratories GmbH) in 500 ml endothelial medium (PAA Laboratories GmbH). The medium was changed every 3 days. The endothelial differentiation was evaluated after 7 and 14 days of cell cultivation under these differentiation conditions.

2.1.2.5 Cell line used as positive control

NTERA-2 (ACC DSMZ no. CRL-527) cell line were purchased from Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and cultivated under the recommended conditions [205].

HUVEC cells (C12200, Promocell GmbH, Heidelberg, Germany) used endothelial culture conditions, mentioned above.

Osteoblast progenitor cells have been isolated from trabecular bone specimens and cultures as previously described [206].

2.1.2.6 Cell propagation

Cells were seeded and cultivated in plastic culture flasks (175 cm²), Petri dishes (10 cm²), 6 well plates, 96-well plates, or 4-well chamber slides (all purchased from BD Bioscience, Heidelberg, Germany) depending on the experiments intended, as shown in Table 3.
Table 3. Cell cultivation condition used in the current study.

<table>
<thead>
<tr>
<th>Type of the seeding surface</th>
<th>Cell number at 70% confluence/well(flask)</th>
<th>Cells at full confluence/well(flask)</th>
<th>Trypsin / EDTA</th>
<th>Volume of the medium</th>
<th>Type of experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-175</td>
<td>1.5x10^6</td>
<td>2.5x10^6</td>
<td>5 ml</td>
<td>25 ml</td>
<td>Cell propagation</td>
</tr>
<tr>
<td>T-75</td>
<td>0.75x10^6</td>
<td>1.2x10^6</td>
<td>3 ml</td>
<td>15 ml</td>
<td>Cell propagation</td>
</tr>
<tr>
<td>Petri dish</td>
<td>0.75x10^6</td>
<td>1.2x10^6</td>
<td>2 ml</td>
<td>10 ml</td>
<td>RNA, DNA, protein extraction</td>
</tr>
<tr>
<td>6well plate</td>
<td>0.10x10^6</td>
<td>0.20x10^6</td>
<td>-</td>
<td>3 ml</td>
<td>Von Kossa ALP, and Oil RedO stain, cells for FACS analysis, ac-LDL uptake assay</td>
</tr>
<tr>
<td>96-well plate</td>
<td>0.005x10^6</td>
<td>0.01x10^6</td>
<td>-</td>
<td>0.100 ml</td>
<td>ALP assay, Oil Red O assay, MTT assay</td>
</tr>
<tr>
<td>4-well chamber slide</td>
<td>0.015x10^6</td>
<td>0.020x10^6</td>
<td>-</td>
<td>1 ml</td>
<td>ICC</td>
</tr>
</tbody>
</table>

2.1.2.7 Cell counting

The cell number was determined by using the Fuchs-Rosenthal counting chamber as shown in Figure 18. The Fuchs-Rosenthal consists of a thick glass slide with an H-shaped central area that is subdivided in two square counting areas. Each counting area is again divided in 16 areas of 1 mm\(^2\) each, which are sub-divided into 16 squares. It is generally recommended to count 16 areas of 1 mm\(^2\) each, preferably 8 in each chamber.

Procedure: a mixture of 10 µl Trypan Blue solution (Sigma-Aldrich) and 10 µl cell suspension was prepared in a 1.5 ml reaction tube. After 5 min of incubation, 10 µl of mixture was pipetted into the counting chambers of the haemocytometer and the cells visible in the 1 mm\(^2\) squares were counted in both counting areas after microscopic visualization in phase contrast field.
The cell number was calculated by using the following formula:

\[
\text{Cells per mL} = \text{average count per square} \times 5000 \times \text{dilution factor}
\]

2.1.3 Cell culture analysis

2.1.3.1 Cytochemistry

2.1.3.1.1 Von Kossa staining

This method uses a precipitation reaction in which silver ions react with phosphate, a component of hydroxyapatite (bone mineral) in the presence of acidic material. Photochemical degradation of silver phosphate occurs when exposed to light, and metallic silver is visualized as a dark-colored material. This technique is used for demonstrating deposits of calcium or calcium salt, so it is not specific for the calcium ions itself.

Procedure: Cells were fixed for 10 min with 4% formaldehyde (Apotheke des Klinikum rechts der Isar der TU München, Germany) at RT, washed for 10 min with distilled water, and then incubated with 1% silver nitrate solution (Sigma-Aldrich). The solution was added on the chamber slides and incubated under light exposure for 40 min at RT. Cells were then
carefully washed with distilled water (B. Braun Melsungen AG, Melsungen, Germany) and incubated for 5 min at RT in a solution of 5% sodium thiosulphate (Sigma-Aldrich) to remove the unreacted silver nitrate. Once the staining was finished the calcification of the matrix was evaluated microscopically and digitalized.

### 2.1.3.1.2 Alkaline phosphatase staining

5-bromo-4-chloro-3-indolyl-phosphate (BCIP) reaction produces a dark blue, precisely localized precipitate in the presence of alkaline phosphatase (ALP) and with the help of nitroblue tetrazolium (NBT) that is the most commonly used electron-transfer agent and co-precipitant for this reaction (Figure 19). BCIP is the ALP-substrate that reacts further after the dephosphorylating to give a dark-blue indigo-dye as an oxidation product. NBT serves herein as the oxidant and gives also a dark-blue dye. It intensifies thereby the color and makes the detection more sensitive.

**Figure 19.** Diagram of the NBT/BCIP reaction. When alkaline phosphatase removes the phosphate group of BCIP (5-bromo-4-chloro-3-indolyl-phosphate), the resulting molecules dimerize under oxidation conditions to give the blue precipitate (5, 5'-dibromo-4,4'-di dichloro-indigo). During the reaction with BCIP, NBT (nitroblue tetrazolium) is reduced to its colored form [207].

Procedure: Cells were fixed for 10 min with a fixation buffer (acetone- methanol ratio 1:1). Each time one tablet of NBT/BCIP (Roche, Mannheim, Germany) was dissolved in 10 ml distilled water. The cells were then covered with the solution for 5 min until a color became visible. Kaiser's mounting medium (Merck, Darmstadt, Germany) was applied and the stain was visualized under microscope.
2.1.3.1.3 Oil red O staining

Oil Red O staining is an assay performed to detect oil droplets produced by adipocytes. The mechanism of the staining of lipids is a function of the physical properties of the dye being more soluble in the lipid to be demonstrated than in the vehicular solvent. The Oil Red O is a polyazo dye (Figure 20).

![Figure 20. Oil Red O chemical structure (1-[(4-(Xylylazo) xylyl] azo)-2-naphthol 1-[2,5-Dimethyl-4-(2,5-dimethylphenylazo) phenylazo]-2-naphthol).]

Procedure: After fixing of the cells for 10 min with 4% formaldehyde, they were washed with tap water for 10 min and rinsed very briefly in 40\% isopropanol (Apotheke MRI). On the cells, 0.5\% Oil Red O solution in isopropanol was then added and incubated for 15 min at RT, after which the cells were washed briefly in 40\% isopropanol. Following this procedure the nuclei were counterstained with haematoxylin (Apotheke MRI) for 2 min. The cells were washed with tap water, covered with mounting medium consisting of Kaiser’s (hydrophilic mounting medium) (Merck) and glass cover slides. The staining was then analyzed under the microscope.

2.1.3.2 Assays

2.1.3.2.1 Viability assay

The viability assay is based on a colorimetric assay that measures cell viability and involves the reduction of yellow 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by the mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, dark purple formazan salt. This product is solubilized and measured spectrophotometrically at a wavelength of 570 nm. Since reduction of MTT can only occur in metabolically active cells, this assay is measuring the cell viability not their toxicity.
Procedure: AdMSCs were harvested by trypsinization, resuspended in culture medium, and plated in a 96-well plate. After 24 h, the cells were treated either with 5-azacytidine (Sigma-Aldrich), BIX-01294 (Sigma-Aldrich), or VPA (Sigma-Aldrich) for 24, 48 or 72 h. Following treatment, the medium was replaced by 0.5 mg/ml MTT in a water solution and the plate was incubated for 2 h at 37°C, in 5% CO₂. The MTT solubilization solution was prepared from 5 g sodium dodecyl sulphate (SDS) (Sigma-Aldrich), 49.7 ml dimethyl sulfoxide (DMSO) (Sigma-Aldrich), and 0.3 ml acetic acid (Merck). Solubilization solution (100 µl/well) was used to dissolve the formazan crystals. Absorbance was measured at 570 nm / 690 nm using a FLUOstar Omega fluorometer (BMG Labtech, Ortenberg, Germany).

2.1.3.2.2 Alkaline phosphatase activity assay

Alkaline phosphatase (ALP) is a hydrolytic enzyme acting optimally at alkaline pH. The activity of this enzyme can be quantified photometrically by measuring the p-nitrophenol amount produced in the reaction of p-nitrophenolphosphate substrate with ALP at alkaline pH (Figure 21).

![Reaction of p-nitrophenol with alkaline phosphatase in alkaline solution.](image)

Figure 21. Reaction of p-nitrophenol with alkaline phosphatase in alkaline solution.

Procedure: AdMSCs were plated in 96-well plates (5000 cells per well) and were cultured in osteogenic differentiation medium. As a negative control, adMSCs were cultivated in normal growth medium. As a positive control, osteoblast progenitor cells were cultivated in osteogenic differentiation medium. At selected time points (7, 14, and 21 days), ALP activity was measured. A standard curve with different concentration of p-nitrophenol (4-nitrophenol solution 10 mM, Sigma-Aldrich) was plotted. A substrate solution was prepared by adding 1.3
mg 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma-Aldrich) in an alkaline solution. The alkaline solution was prepared from 0.375 g glycine (Sigma-Aldrich), 1.211 g 2-Amino-2-(hydroxymethyl)-1,3-propanediol (or Trizma base, Sigma-Aldrich), 0.203 mg MgCl$_2$ (Sigma-Aldrich) in distilled water and adjusted to pH=10.5 with NaOH (Merck). After discarding and washing the cells with PBS, 100 µl substrate solution were added in each well and incubated at 37°C for 30 min. ASys Expert Plus plate reader (Omnilab, Bremen, Germany) was warmed up to 37°C and the kinetic measurement was performed at 405 nm in 5-minute intervals for 30 min.

2.1.3.2.3 Oil Red O quantification assay

By solubilizing the droplets stained with Oil Red O in isopropanol, this technique allows the quantification of adipogenic differentiation [208].

Procedure: For this assay, cells were seeded in 96-well plates and cultured in adipogenic differentiation medium. The cells from two study groups (elderly and young) were then stained with Oil Red O without counterstaining with haematoxylin; after washing with 40% isopropanol and distilled water, into each well was added 100 µl of 60% isopropanol and incubated for 15 min at RT on a shaker. The optical density of the solution was then measured at 520 nm with the spectrophotometer. In order to normalize the results to the protein amount, an additional assay was performed, namely Sulphorhodamine B (SRB assay).

2.1.3.2.4 Sulphorodamine B – protein measurement

The Sulforhodamine B (SRB) assay system allows the measurement of the total biomass of the cells by staining cellular proteins with the SRB [209]. The key component is the dye, Sulforhodamine B (or Acid Red 52). The cells are briefly washed, fixed, and stained with this dye. The incorporated compound is then liberated from the cells with a TRIS solution. An increase or decrease in the number of cells (total biomass) results in a concomitant change in the amount of dye incorporated by the cells.

Procedure: SRB solution was prepared in 1% acetic acid solution by adding 0.2 g Sulforhodamine B (Sigma-Aldrich) in 50 ml 1% acetic acid (Merck). After the Oil red O assay, cells were washed and into each well of a 96-well plate 50 µl SRB solution were added. After incubation for 30 min at RT on a shaker the SRB solution was removed; cells were washed carefully with 1% acetic acid five times, each time for 5 min, again on the shaker. Into each well were then added 100 µl of 10 mM TRIS aqueous solution and
incubated for 10 min at RT on the shaker. Measurement of the absorbance was performed at 595 nm (SRB solution) and 690 nm (other impurities or background absorbance) [209].

### 2.1.3.2.5 Ac-LDL uptake

Dil-Ac-LDL, acetylated low density lipoprotein labeled with 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, labels both vascular endothelial cells and macrophages. It can be used to identify and/or isolate these cells from mixed cells populations. When cells are labeled with Dil-Ac-LDL, the lipoprotein is degraded by lysosomal enzymes and the Dil fluorescent dye accumulates in the intracellular membranes. Labeling cell with Dil-Ac-LDL has no effect on cell viability. Non-endothelial cells (fibroblasts, smooth muscle cells, pericytes, epithelial cells) are not labeled [210].

Procedure: DiI labeled Ac-LDL was purchased from Bioquote Limited (York, UK). The cells were carefully washed with PBS and incubated with the Dil-Ac-LDL solution (10 µg/ml endothelial medium) for 4 h, at 37 °C, in 5% CO₂. Cells were carefully washed three times with PBS and afterward fixed in 4% formaldehyde for 10 min. The formaldehyde solution was removed, cells were washed with PBS, and cell nuclei were stained with Sybrgreen I dye (Sigma-Aldrich) for 15 min at RT, protected from light. Sybrgreen I excess was removed by washing with PBS three times. Cells were then analyzed by the use of a fluorescence microscope. The positive control comprised of HUVEC cells and the negative control of adMSCs that have been cultured under normal growth conditions.

### 2.2 Immunohistochemistry

The immunochemical procedures carried out in the present work were performed with the LSAB kit (DAKO, Hamburg, Germany) or APAAP kit (DAKO), following the instructions of the manufacturer. Each antibody was used at an optimal dilution and with an antigen retrieval method shown in Table 4, 5, and 6. Antibodies were diluted in an antibody diluent purchased from DAKO.

#### 2.2.1 LSAB method

Most of the immunochemical staining methods in use today are based on the high affinity that streptavidin (*Streptomyces avidinii*) and avidin (chicken egg) possess for biotin. The LSAB reagents are applied in the following order: primary antibody, biotinylated secondary antibody, streptavidin-enzyme conjugate. The color reaction is then developed with the appropriate substrate/chromogen.
Procedure: Prior to performing this staining method, TRIS buffer (1x) was prepared by diluting a stock solution (10x) in a distilled water. Stock of TRIS buffer (10x) was prepared by adding 60.5 g Trizma base (Sigma-Aldrich) and 90 g NaCl (Merck) in 1 l distilled water, and adjusting the pH to 7.6 with HCl (Apotheke MRI). Cells were fixed with acetone : methanol, ratio 1:1 (v/v), for 10 min. The fixation buffer was removed and cells were washed with 1x TRIS buffer. All following steps were performed at RT and according to the manufacturer instructions. Blocking of the peroxidase was performed with 0.3% hydrogen peroxide (Merck), followed by 1 h incubation with the appropriate antibody as presented in Table 4, 5, and 6. The samples were incubated with secondary antibody (solution A) for 25 min, followed by streptavidin-enzyme conjugate (solution B) for another 25 min. The chromogenic solution was prepared from 15 µl DAB+ (solution C) and 750 µl HRP substrate (solution D). Each step of the protocol was followed by washing in 1x TRIS buffer. After incubation for 3-5 min with the chromogen, samples were counterstained with haematoxylin for 30 seconds. Mounting medium used was Pertex (Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany).

2.2.2 APAAP method

This method is based on an indirect reaction. The staining sequence of this technique consists of the use of an unconjugated primary antibody, a secondary antibody, the soluble enzyme-anti-enzyme complex, and substrate solution. The primary antibody and the antibody of the enzyme immune complex must be from the same species. The secondary antibody must be directed against immunoglobulins of the species producing both the primary antibody and the enzyme immune complex. Soluble enzyme-anti-enzyme immune complex techniques were named after the particular enzyme immune complex they used.

Procedure: For this procedure TRIS buffer (1x) (see LSAB method) was used for washing the samples after each step of the following protocol. Cells have been fixed with a fixation buffer (acetone: methanol ratio 1:1) for 10 min. The fixation buffer was removed and cells were washed with 1x TRIS buffer. All following steps have been performed at RT and according to the manufacturer instructions. First antibody against the target protein was incubated with the samples for 1 h using optimized dilution for each antibody as shown in Table 4. The incubation with the secondary antibody (sol. A) for 25 min was followed by incubation with the APAAP immunocomplex (sol. B) for another 25 min. Meanwhile, the chromogen solution was prepared from 30 µl sol. C, 30 µl sol. D, 30 µl sol. E, and 750 µl sol. F (substrate solution). The chromogenic mix was incubated for 15 min, until the red color has been observed under the microscope. Haematoxylin counterstain followed for the nuclear stain, and mounting was done with hydrophobic mounting medium, Pertex (Leica Mikrosysteme Vertrieb GmbH).
### Table 4. Antibodies used for characterization of the adMSC population for ICC.

<table>
<thead>
<tr>
<th>Name</th>
<th>Reacts with</th>
<th>Clone</th>
<th>Isotype</th>
<th>Brand &amp; Cat. Nr.</th>
<th>Dilution</th>
<th>Detection System</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse anti vimentin</td>
<td>Human</td>
<td>Mouse</td>
<td>IgG</td>
<td>Dako</td>
<td>1:500</td>
<td>APAAP</td>
</tr>
<tr>
<td>Mouse anti Ki67</td>
<td>Human</td>
<td>Mouse</td>
<td>IgG</td>
<td>Chemicon International IC</td>
<td>1:200</td>
<td>APAAP</td>
</tr>
<tr>
<td>CD105 Ab-3, mouse anti-human</td>
<td>Human</td>
<td>SN6h</td>
<td>IgG</td>
<td>Neomarkers</td>
<td>1:500</td>
<td>LSAB</td>
</tr>
<tr>
<td>Monoclonal Mouse Anti-Human CD31, Endothelial Cell</td>
<td>Mouse</td>
<td>JC70A</td>
<td>IgG</td>
<td>DAKO (M0823)</td>
<td>1:50</td>
<td>LSAB</td>
</tr>
</tbody>
</table>

### Table 5. Antibodies used for detection of pluripotency markers.

<table>
<thead>
<tr>
<th>Name</th>
<th>Reacts with</th>
<th>Clone</th>
<th>Isotype</th>
<th>Brand &amp; Cat. Nr.</th>
<th>Dilution</th>
<th>Detection System</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCT4A(C30A3) Rabbit mAb</td>
<td>Human, Mouse</td>
<td>Rabbit</td>
<td>Rabbit IgG</td>
<td>Cell Signaling (#2840)</td>
<td>1:400</td>
<td>LSAB</td>
</tr>
<tr>
<td>Rabbit Polyclonal to OCT4</td>
<td>Human, Mouse</td>
<td>Polyclonal</td>
<td>Rabbit, IgG</td>
<td>Abcam (ab19857)</td>
<td>1:500</td>
<td>LSAB</td>
</tr>
<tr>
<td>Rabbit polyclonal to Nanog</td>
<td>Human, Mouse</td>
<td>Polyclonal</td>
<td>Rabbit IgG</td>
<td>Abcam (ab21624)</td>
<td>1:500</td>
<td>LSAB</td>
</tr>
<tr>
<td>Negative Control Rabbit Immunoglobulin Fraction (Normal)</td>
<td>-</td>
<td>-</td>
<td>Rabbit</td>
<td>DAKO (X0903)</td>
<td>1:500</td>
<td>LSAB</td>
</tr>
</tbody>
</table>

### Table 6. Antibodies used for characterization of the adMSCs differentiated into ECs.

<table>
<thead>
<tr>
<th>Name</th>
<th>Reacts with</th>
<th>Clone</th>
<th>Isotype</th>
<th>Brand &amp; Cat. Nr.</th>
<th>Dilution</th>
<th>Detection System</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flk-1/KDR/VEGFR2 Ab-1</td>
<td>Human</td>
<td>Rabbit</td>
<td>Rabbit</td>
<td>Thermo Scientific (RB-1526-P0)</td>
<td>1:200</td>
<td>LSAB</td>
</tr>
<tr>
<td>Monoclonal Mouse Anti-Human CD31, Endothelial Cell</td>
<td>Human</td>
<td>JC70A</td>
<td>IgG1</td>
<td>DAKO (M0823)</td>
<td>1:50</td>
<td>LSAB</td>
</tr>
<tr>
<td>Monoclonal Mouse Anti-Human Von Willebrand Factor</td>
<td>Human</td>
<td>F8/86</td>
<td>IgG1</td>
<td>DAKO (M0616)</td>
<td>1:400</td>
<td>LSAB</td>
</tr>
<tr>
<td>Mouse anti hVCAM-1 (CD106)</td>
<td>Human</td>
<td>Mouse IgG</td>
<td>IgG</td>
<td>R&amp;D Systems</td>
<td>1:500</td>
<td>LSAB</td>
</tr>
<tr>
<td>Negative Control Rabbit Immunoglobulin Fraction (Normal)</td>
<td>-</td>
<td>-</td>
<td>Rabbit</td>
<td>DAKO (X0903)</td>
<td>1:500</td>
<td>LSAB</td>
</tr>
<tr>
<td>Mouse IgG1 Negative Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>DAKO (X0931 )</td>
<td>1:50, 1:200, 1:400, 1:500</td>
<td>APAAP and LSAB</td>
</tr>
</tbody>
</table>

### 2.3 Flow cytometry (FACS analysis)

Flow cytometry analysis allows simultaneous measuring of relative cell size (FSC), cell granularity, internal complexity (SSC), and florescence intensity. The major components comprise a fluid system, an optical system with laser, reflector, and filter device. According to the florescence staining applied, wavelengths differ between 450 and 700 nm. FITC requires a laser wavelength of about 488 nm in the green range, APC has its optimum at 633 nm in the red spectrum, and PE requires laser with wavelength at 532 nm in the yellow spectrum.
For flow cytometry, adMSCs in passages 0, 1, 2, and 3 were used. The cells were detached from their culture plates with trypsin/EDTA and labeled by incubation at 4°C for 60 min with the following monoclonal antibodies: mouse anti-human FITC-labeled CD14, mouse anti-human FITC-labeled CD45 (Biozol Diagnostica Vertrieb GmbH, Eching, Germany), mouse anti-human PE-labeled CD105 (Biozol Diagnostica Vertrieb GmbH), and mouse anti-human APC-labeled CD90 (BioLegend, London, UK). Acquisition and analysis were performed on a FACS Canto II, BD Biosciences (San Jose, CA, USA) (Table 7). An isotype control was included in each experiment. Data processing and analysis were performed with the FlowJo software. All antibodies were used at a concentration of 0.5 µg/10^6 cells in a total volume of 100 µl.

Table 7. Antibodies used for FACS analysis.

<table>
<thead>
<tr>
<th>Name</th>
<th>Reacts with</th>
<th>Clone</th>
<th>Isotype</th>
<th>Brand &amp; Cat. Nr.</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse anti-human FITC-labeled CD14</td>
<td>Human, Mouse</td>
<td>mouse</td>
<td>mouse</td>
<td>Biozol, Germany</td>
<td>0.5 µg/10^6 cells in 100 µl.</td>
</tr>
<tr>
<td>mouse anti-human FITC-labeled CD45</td>
<td>Human, Mouse</td>
<td>mouse</td>
<td>mouse</td>
<td>Biozol, Germany</td>
<td>0.5 µg/10^6 cells in 100 µl.</td>
</tr>
<tr>
<td>mouse anti-human PE-labeled CD105</td>
<td>Human, Mouse</td>
<td>mouse</td>
<td>mouse</td>
<td>Biotech, USA</td>
<td>0.5 µg/10^6 cells in 100 µl.</td>
</tr>
<tr>
<td>mouse anti-human APC-labeled CD90</td>
<td>Human, Mouse</td>
<td>mouse</td>
<td>mouse</td>
<td>BioLegend, USA</td>
<td>0.5 µg/10^6 cells in 100 µl.</td>
</tr>
</tbody>
</table>

2.4 Tissue sampling, processing, and analysis

2.4.1 Study group, atherosclerotic plaque processing, and characterization

The study group included atherosclerotic plaque specimens from 26 patients with high grade carotid artery stenosis, who underwent carotid endarterectomy (CEA) (Figure 22). The specimens were taken from the biobank of the department of Vascular Surgery, selecting the entire range of atherosclerotic lesions from type II to VII (according to AHA classification [76, 211]). Control samples consisted of 10 healthy carotid vessels and were obtained from patients, who underwent trauma surgery. In addition, serum samples from 10 healthy volunteers were included as a control for blood analysis. All patients underwent neurological examination before and after the surgical intervention. The study was conducted according to the guidelines of the world medical association declaration of Helsinki [212]. All the patients gave their informed consent.
MATERIALS AND METHODS

Procedure: Carotid plaque was collected during the surgical procedure, segmented in blocks of 3-4 mm, fixed in 4% formaldehyde for 24 h, dehydrated, and embedded in paraffin. Segmenting was performed in the way to receive different stages of atherosclerosis plaques between II and VII. For a proper histological characterization, 3 µm sections were prepared from each sample followed by haematoxylin-eosin and elastica van Gieson staining. Histological classification was performed under the supervision of Dr. rer. nat. J. Pelisek and the technical assistant experienced in histology Fr. R. Hegenloh. Type II and III lesions were defined as an early stage of atherosclerosis, type V, VI, and VII as an advanced stage.

2.4.2 Decellularisation and recellularization

One of the processes used for creation of bio-scaffold for autologous vessels is decellularization. Through this process, the cells can be removed from tissue by variety of treatments (chemical, physic-chemical). What remains is a bio-scaffold consisting of the component of extracellular matrix such as collagens and proteoglycans. For this purpose, sodium dodecyl sulphate (SDS) is a detergent known to solubilize cell membrane and proteins without affecting the integrity of the bio-scaffold [213].

Procedure: A sterile 0.66% aqueous solution of SDS (Sigma-Aldrich) was used. Human saphenous vein was segmented in pieces of 2 cm; each was placed in a 15 ml tube filled with 7 ml SDS solution (decellularization solution). The tubes where then placed on a rotational-shaker and the decellularization solution was changed under sterile conditions after 30 min, 1 h, 6 h, 24 h, 48 h, and 96 h. The resulting bio-scaffolds were then washed three times under sterile conditions with PBS.

Cells were harvested with trypsin/EDTA (Biochrom AG), counted, and 0.75x10^6 were suspended in 2 ml endothelial medium. The scaffold was placed in a 10 cm² Petri dish.
without medium; the cell suspension was placed inside of lumen of the vessel (seeded) and incubated for 2.5 h at 37°C, 5% CO₂. As a next, endothelial medium was added and the cells were cultivated further on for 2 and 7 days.

2.4.3 **Histochemistry**

In order to use the atherosclerotic plaque material for histological or immunohistological staining, fresh tissues have been fixed for 24 h in 4% formaldehyde, followed by dehydration, embedding in paraffin, and sectioning in 3 µm thin slides. As an additional step in case of calcified tissue (advanced stage atherosclerosis plaque) the specimens were incubated in an aqueous solution saturated with EDTA for up to 7 days, dependent upon the extent of calcification. Prior the staining procedure all sectioned tissue slides were deparaffinised by incubation in xylene for 20 min, followed by isopropanol for 20 min, 99.8% ethanol for 10 min, 70% ethanol for 10 min, and distilled water for 10 min. All the materials used for histochemistry were purchased from Apotheke MRI.

2.4.3.1 **Haematoxylin – Eosin staining**

Following deparaffinization, nuclear staining was performed by incubating the slides with Mayer’s haematoxylin for 5 min at RT. The slides were then washed in tap water for 10 min, incubated with acidified eosin for 5 min, rinsed briefly in tap water and in increasing concentrations of alcohol (70% ethanol, 96% ethanol, 100% isopropanol) followed by xylene. The samples were mounted in hydrophobic mounting medium and covered with glass cover slides.

2.4.3.2 **Elastica van Giesson staining**

The Elastica van Gieson staining is a combination of Weigert’s haematoxylin, van Gieson’s picrofuchsin, and the resorcin-fuchsin solution that allows differential analysis of nuclei, connective tissue, muscle and elastic fibers.

Procedure: Weigert’s haematoxylin was prepared fresh by mixing Weigert’s solution A and Weigert’s solution B (ratio 1:1). The slides, previously dried at 56°C and deparaffinized, were incubated in Weigert’s haematoxylin for 10 min and washed in warm tap water for another 10 min. Next step consisted of staining of the cytoplasm by incubation with van Gieson solution for 3 min. This step was followed by washing in distilled water and increasing concentrations of alcohol (70% ethanol, 96% ethanol, isopropanol) followed by xylene. The samples mounted in hydrophobic mounting medium and covered with glass cover slides.
2.4.4 Immunohistochemistry

All stainings were performed on thin slices of tissue fixed with formaldehyde and embedded in paraffin (FFPE tissues). Depending on the target antigen, an antigen epitope retrieval step was employed (see Table 8 and 9).

Procedure: The tissue was sectioned into slices of 3 µm, mounted on glass slides, briefly dried at 56°C, and deparaffinized. The antigen-retrieval methods used were heat induced epitope retrieval (HIER) or proteolytic enzyme induced epitope retrieval (PIER). HIER consists of incubating the sectioned slides at 100°C under pressure for 7 min in 10 mM aqueous sodium citrate solution (Sigma-Aldrich), at pH 6.0. PIER consists of 20 min incubation with a 5 µg/ml protein kinase (Sigma-Aldrich). After epitope retrieval, the protocol followed the manufacturer’s instructions described above.

Table 8. Antibodies used for characterization of the atherosclerotic plaque.

<table>
<thead>
<tr>
<th>Name</th>
<th>Clone</th>
<th>Isotype</th>
<th>Brand &amp; Cat. Nr.</th>
<th>Dilution</th>
<th>Detection System</th>
<th>Antigen Retrieval Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoclonal Mouse Anti-Human CD31, Endothelial Cell</td>
<td>JC70A</td>
<td>IgG1</td>
<td>DAKO (M0823)</td>
<td>1:50</td>
<td>LSAB</td>
<td>HIER</td>
</tr>
<tr>
<td>Monoclonal Mouse Anti-Human Muscle Actin</td>
<td>HHF35</td>
<td>IgG1</td>
<td>DAKO (M0635)</td>
<td>1:200</td>
<td>APAAP</td>
<td>HIER</td>
</tr>
<tr>
<td>Monoclonal Mouse Anti-Human Von Willebrand Factor</td>
<td>F8/86</td>
<td>IgG1</td>
<td>DAKO (M0616)</td>
<td>1:400</td>
<td>APAAP</td>
<td>PIER</td>
</tr>
<tr>
<td>Monoclonal Mouse Anti-Human CD68</td>
<td>Kb1</td>
<td>IgG1</td>
<td>DAKO (M0184)</td>
<td>1:2000</td>
<td>LSAB</td>
<td>HIER</td>
</tr>
<tr>
<td>Monoclonal Mouse Anti-Human CD45, leucocyte Common Antigen</td>
<td>2B11+PD7/26</td>
<td>IgG1</td>
<td>DAKO (M0701)</td>
<td>1:200</td>
<td>LSAB</td>
<td>HIER</td>
</tr>
<tr>
<td>Mouse IgG1 Negative Control</td>
<td>-</td>
<td>-</td>
<td>DAKO (X0931)</td>
<td>1:50; 1:200; 1:400</td>
<td>APAAP and LSAB</td>
<td>PIER and PIER</td>
</tr>
</tbody>
</table>

Table 9. Antibodies used for detection of epigenetic changes at histone level.

<table>
<thead>
<tr>
<th>Name</th>
<th>Reacts with</th>
<th>Isotype</th>
<th>Brand &amp; Cat. Nr.</th>
<th>Dilution</th>
<th>Detection System</th>
<th>Antigen Retrieval Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Di-Methyl-Histone H3 (Lys4)(C64G9) Rabbit mAb</td>
<td>Human, Mouse, Rat, Monkey</td>
<td>Rabbit IgG</td>
<td>Cell Signaling (#9725)</td>
<td>1:3000</td>
<td>LSAB</td>
<td>PIER</td>
</tr>
<tr>
<td>Di-Methyl Histone H3(Lys9) Antibody</td>
<td>Human, Mouse, Rat, Monkey</td>
<td>Rabbit IgG</td>
<td>Cell Signaling (#9753)</td>
<td>1:200</td>
<td>LSAB</td>
<td>PIER</td>
</tr>
<tr>
<td>Negative Control Rabbit Immunoglobulin Fraction (Normal)</td>
<td>-</td>
<td>Rabbit</td>
<td>DAKO (X0903)</td>
<td>1:200</td>
<td>LSAB</td>
<td>PIER</td>
</tr>
</tbody>
</table>

2.4.5 Microscopy and digitalization
Digital micrographs were captured with a Zeiss AxioCam MRc digital camera attached to a Zeiss Axio Observer Z1 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany), equipped for light and fluorescence microscopy. The microscope was controlled by the AxioVision software, version 4.8.2 (Carl Zeiss Microscopy GmbH).

### 2.5 Gene expression analysis at mRNA level using PCR

#### 2.5.1 RNA extraction from cells

The RNA extraction method used in the present work is based on the reversible binding properties of silica-based columns and RNA. Cells are first lysed and RNases are inactivated. The cell lysates are then applied onto the columns to which total RNA binds, while cellular debris and DNA is digested with the peqGOLD DNase I enzyme and other contaminants are effectively washed out.

Procedure: RNA isolation was performed with peqGOLD Total RNA Kit purchased from PEQLAB GmbH (Erlangen, Germany). Cells were lysed with 400 µl lysis buffer and scraped off the 10 cm² culture dishes. Following steps were then performed according to manufacturer’s instructions. RNA was finally eluted in RNase-free water, the concentration and the quality measured with a Nanodrop 2000c spectrophotometer (PEQLAB GmbH), and stored at -80°C until further use.

#### 2.5.2 RNA extraction from FFPE tissue samples

RNA was extracted from FFPE tissue with the High Pure FFPE RNA Micro Kit (Roche Applied Science, Mannheim, Germany). Two tissue slices, each of 10 µm thickness, were placed into a 1.5 ml reaction tube, deparaffinized by adding 800 µl xylene, vortexed, and incubated at RT for 5 min. The xylene was then removed by 400 µl ethanol 98.6%, vortexed and centrifuged at 12000 rpm for 4 min at RT. The supernatant was carefully removed and 1000 µl ethanol 98.6% were added again and mixed through vortexing followed by centrifugation at 12000 rpm for 4 min at RT. The supernatant was carefully removed and extraction procedure was continued according to manufacturer’s instructions. RNA was eluted in RNase-free water, quantified spectrophotometrically, and stored at -80°C until further use.

#### 2.5.3 cDNA synthesis
The cDNA synthesis was performed with the RevertAid First Strand cDNA Synthesis Kit (Fermentas GmbH, St. Leon-Rot, Germany). The kit is using the M-MuLV Reverse Transcriptase that is active in a range between 42-50°C and oligo (dT)₁₈ primer.

Procedure: For each reaction up to 1 µg RNA in a total volume of 11 µl was used. To each RNA sample 1 µl oligo (dT)₁₈ primer was added, gently mixed, briefly centrifuged, and incubated at 65°C for 5 min. The sample was then chilled on ice for at least 2 min; meanwhile a working mix was prepared (4 µl of 5x reaction buffer, 2 µl of 10 mM dNTPs mix, 1 µl RNase inhibitor, and 1 µl M-MuLV Reverse transcriptase). The mix was added on the sample and incubated for 1 h at 43°C followed by 5 min at 70°C. At the end of the reaction the samples were diluted to a final concentration of 10 ng/µl. For real time PCR, 20 ng cDNA were used for each reaction.

2.5.4 SYBR Green-based real-time PCR

In conventional PCR, the amplified product, also named amplicon, is detected by an end-point analysis, running DNA on an agarose gel after the reaction is finished. In contrast, real-time PCR allows the accumulation of amplified product to be detected and measured as the reaction progresses in “real time” after each cycle. For this purpose, data are collected throughout the PCR process rather than at the end of the PCR. Real-time detection of PCR products is possible by including in the reaction a fluorescent molecule that reports an increase in the amount of DNA with a proportional increase in fluorescent signal. For this purpose, fluorescent DNA-binding dyes or labeled sequence specific primers or probes are employed. Specialized thermal cycling devices equipped with fluorescence detection modules are used to monitor the fluorescence as amplification occurs. The measured fluorescence reflects the amount of amplified product in each cycle. The biggest advantage of real-time PCR over conventional PCR is that it allows determining the starting template copy number with high sensitivity and accuracy. The quantitative RT-PCR reaction in this work was performed by two methods: the DNA-binding dye SYBR Green I or TaqMan hydrolysis probes.

SYBR Green I dye is a highly specific, double-stranded DNA (dsDNA) binding dye used to detect PCR product as it accumulates during PCR cycles. The melt-curve analysis should be used each time to distinguish specific PCR products from the nonspecific ones. The major drawback of DNA-binding dyes is that it binds non-specifically to every dsDNA in the reaction tube. A melt-curve is generated by increasing the temperature in small increments and monitoring the fluorescent signal at each step. As the dsDNA in the reaction denatures or melts, the fluorescence decreases. A characteristic peak at the amplicon’s
melting temperature (Tm, the temperature at which 50% of the base pairs of a DNA duplex are separated) distinguishes it from other products such as primer-dimers, which melt at different temperatures than the desired amplicon; two different peaks of the same samples with two different melting points thus mean two different amplicons, one of them unspecific (Figure 23). If the product is transferred onto an electrophoresis gel, two different bands are observed, corresponding to the two peaks in the melt-curve graph. In general to ensure reliable results, all primers have to be optimized and proved by the melting curve and gel electrophoresis.

Data analysis of the quantitative PCR (qPCR) consists in comparing the amplification curve in the exponential phase of different targets with the amplification curve of a housekeeping gene, which serves as internal control (Figure 24). The calculation method used in the present work was ΔCT using a reference gene, which is widely used and easy to understand. To compare target gene expression in two different samples, one was set as control-sample (e.g. the non-treated cells, or healthy tissue) and the following formula was used:

\[ \text{Relative expression} = 2^{\Delta CT (\text{GAPDH}) - CT (\text{target gene})} \]
Figure 24. Amplification plot for two different target genes; green and blue arrows indicate the CT value in the exponential phase for both targets.

Procedure: Transcript levels of pluripotency, endothelial, and angiogenic related genes were determined using the ready to use polymerase Sybr Fast qPCR Master Mix (PEQLAB GmbH). The cDNA template was equivalent to 20 ng of total RNA for each PCR reaction. Measurements were performed in triplicates; a non-template blank served as a negative control. Amplification curves and gene expression were normalized to the housekeeping gene GAPDH. Primers for the genes OCT4A, NANOG, KLF4, CMYC, VEGF, PDGF, VEGFR-2, ANG-1, and ANG-2 were designed by Primer3 software (version 0.4.0, Whitehead Institute for Biomedical Research, Cambridge, MA, USA), purchased from MWG Operon Eurofins (Ebersberg, Germany) and are summarized in Table 10. Primers for the genes SOX2, VCAM-1, Factor VIII, and PECAM-1 were purchased from Qiagen GmbH (Hilden, Germany) as ready-to-use primer mix as shown in the Table 10. PCR amplicons were confirmed to be specific by size (gel electrophoresis) and melt-curve analysis. The PCR program was set as follows: denaturation for 5 min at 95°C, amplification for 40 cycles. Each cycle comprised of 10 seconds at 95°C, 30 seconds at 60°C, and 10 seconds at 72°C. The device used was StepOnePlus (Life Technologies GmbH, Darmstadt, Germany).
**Table 10.** Primers used for the real time PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence forward (5'—3')</th>
<th>Sequence reverse (5'—3')</th>
<th>Amplicon (bp)</th>
<th>Gene bank Locus</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCT4a</td>
<td>TGGAGAAGGAGAAGCTGGAGC</td>
<td>TATCACCCCAACCGCCATCT</td>
<td>185</td>
<td>NM_002701</td>
</tr>
<tr>
<td>NANO6</td>
<td>ACCTTGCTGCCGTCTTGCTT</td>
<td>GCAAGCTTCCCAATCCCCCA</td>
<td>150</td>
<td>NM_024865</td>
</tr>
<tr>
<td>KLF4</td>
<td>AGTGCTGAGCAGCGAGGACTG</td>
<td>GGTATGGAGCCGCGGGACT</td>
<td>128</td>
<td>NM_004235</td>
</tr>
<tr>
<td>C-MYC</td>
<td>TGGGTCACACCCCTTCTCCCTT</td>
<td>TGAAGGCTCTGCTGTCGGGTT</td>
<td>149</td>
<td>NM_002467</td>
</tr>
<tr>
<td>VEGF</td>
<td>ATGAGGACACCGGCTGACCA</td>
<td>AGGCTCTAATCTCCAAGGC</td>
<td>126</td>
<td>NM_001025 366</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>ATGTGTGGAGGACTCCAGGAG</td>
<td>AGCTGACACATTGCCTTG</td>
<td>120</td>
<td>NM_002253</td>
</tr>
<tr>
<td>PDGF</td>
<td>TCAGGATGTAGATGGACTG</td>
<td>GAAAGGACACAGGAAGAG</td>
<td>126</td>
<td>NM_002607</td>
</tr>
<tr>
<td>ANG-1</td>
<td>GAGGACACGAGATGTGGTCTG</td>
<td>CGGCGCTTAGTGGCTCACTCCT</td>
<td>101</td>
<td>NM_139290</td>
</tr>
<tr>
<td>ANG-2</td>
<td>AAAAGCTGACACAGCCTCCA</td>
<td>ACTGGCTGTCTCTTCCCCAGCA</td>
<td>90</td>
<td>NM_001147</td>
</tr>
</tbody>
</table>

GapdH, QuantiTect Primer Assay Hs_GAPDH_2SG 119 NM_002046

VCAM-1, QuantiTect Primer Assay Hs_VCAM1_1_SG 106 NM_001078

Factor VIII, QuantiTect Primer Assay Hs_F8_1_SG 120 NM_000132

PECAM-1, QuantiTect Primer Assay Hs_PCAM1_1_SG 144 NM_000442

SOX2, QuantiTect Primer Assay Hs_SOX2_1_SG 64 NM_003106

DNMT1, QuantiTect Primer Assay Hs_DNMT1_1_SG 93 NM_001130 823

DNMT3A, QuantiTect Primer Assay Hs_DNMT3A_1_SG 144 NM_022552

DNMT3B, QuantiTect Primer Assay Hs_DNMT3B_1_SG 128 NM_001207 055

MLL1, QuantiTect Primer Assay Hs_C17orf49_1_SG 99 NM_001142 798

SETD1A, QuantiTect Primer Assay Hs_KIAA0339_1_SG 74 NM_014712

SETD1B, QuantiTect Primer Assay Hs_SETD1B_1_SG 110 NM_001145 415

SUV39H1, QuantiTect Primer Assay Hs_SUV39H1_1_SG 108 NM_003173

EHMT2, QuantiTect Primer Assay Hs_EHMT2_1_SG 97 NM_006709

### 2.6 Epigenetic analysis of methylated DNA using PCR

In the present work, DNA methylation status of three repetitive elements (LINE1, SATα, and Alu1) was analyzed by MethyLight, TaqMan-based real-time PCR assay. DNA isolated from cells was bisulfite converted, and then the amplification of LINE1, SATα, and Alu1 was performed by real-time PCR as described previously [214], using the methylated Alu1 sequence for normalization and the set of primers and probes from Table 11.

**Table 11.** Sequences of the primers for MethyLight assay according to Weisenberger et al. [214].

<table>
<thead>
<tr>
<th>Reaction ID</th>
<th>Sequence forward (5’ to 3’)</th>
<th>Sequence reverse (5’ to 3’)</th>
<th>Probe (5’ to 3’*)</th>
<th>Amplico n (bp)</th>
<th>Gene bank Locus</th>
</tr>
</thead>
<tbody>
<tr>
<td>LINE-M1</td>
<td>GGACGTATTTGGAAAAA</td>
<td>AATCTCGCGATACG</td>
<td>FAM-</td>
<td>80</td>
<td>X52235</td>
</tr>
</tbody>
</table>
2.6.1 DNA isolation from cells

PeqGOLD Tissue DNA Mini Kit (PEQLAB GmbH), QIAamp DNA FFPE Tissue Kit (Qiagen GmbH), and QIAamp DNA Blood Mini Kit (Qiagen GmbH,) were used to extract the genomic DNA from cells, from FFPE tissue, and from serum, respectively. These kits take advantage of the reversible binding properties of a silica-based material, combined with the speed of mini-column spin technology. The buffers provided with the columns allow genomic DNA to bind to the matrix. Samples were first homogenized, lysed under denaturing conditions, and then applied to the DNA Columns, where the DNA is effectively bound to the silica membrane. Cellular debris, proteins, and other contaminants were washed away by specific buffers. The high quality DNA was finally eluted in Elution Buffer.

Procedure: DNA from cultured cells was isolated with PeqGOLD Tissue DNA Mini Kit. The cells were lysed with 400 µl lysis buffer and scraped from the 10 cm² dishes. The lysate was placed in 1.5 ml reaction tubes and the procedure was followed according to manufacturer's instructions. The final concentration of the isolated genomic DNA was measured with a Nanodrop 2000c spectrophotometer (PEQLAB GmbH) and the DNA solution was stored at -20°C until bisulfite conversion was performed.

2.6.2 DNA isolation from FFPE tissue and serum

DNA was isolated with the QIAamp DNA FFPE Tissue Kit. First, 4 slices of 10 µm thickness were cut from the carotid tissue samples and placed in a 1.5 ml tubes. The protocol was followed as described in the manufacturer’s instructions. DNA concentration was measured with the spectrophotometer and stored at -20°C until bisulfite conversion was performed.

Fresh blood was collected, centrifuged and serum was stored at -80°C until DNA was further used. DNA was isolated with the QIAamp DNA Blood Mini Kit, according to manufacturer’s instructions using 200 µl of serum for each extraction procedure.
2.6.3 Bisulfite conversion

Incubation of the target DNA with sodium bisulfite results in conversion of unmethylated cytosine residues into uracil, leaving the methylated cytosines unchanged. Therefore, bisulfite treatment leads to changes in DNA sequence for methylated in contrast to unmethylated DNA as shown in Table 12.

| Table 12. Example of the effect of bisulfite conversion on unmethylated cytosines. |
|-----------------------------------------------|-----------------------------------------------|
| **Original sequence** | **After bisulfite treatment** |

The critical step for correct determination of a methylation pattern is the complete conversion of unmethylated cytosines. For this purpose, commercially available kits were used. Sodium bisulfite conversion of DNA from cells, FFPE material, or serum was in all cases performed by using EpiTect Bisulfite Kit (48) (Qiagen GmbH).

The EpiTect Bisulfite procedure comprises of the following steps: bisulfite mediated conversion of unmethylated cytosines; binding of the converted single-stranded DNA to the membrane of an EpiTect spin column; desulfonation of membrane-bound DNA; removal of desulfonation agent; elution of the converted DNA from the spin column. The eluted DNA was used for the analysis of DNA methylation by MethyLight assay, a Taqman based real time PCR.

Procedure: DNA was diluted to 500 ng DNA in 20 µl of elution buffer for each bisulfite conversion reaction. According to the manufacturer, bisulfite mix was added to the DNA solution. To this mixture, DNA protect buffer was added, well mixed, and placed in the thermal cycler with a program shown in Table 13.

| Table 13. Thermal cycler conditions for bisulfite conversion reaction. |
|--------------------------|--------------------------|
| **Step** | **Temperature** | **Time** |
| Denaturation | 95°C | 5 min |
| Incubation | 60°C | 25 min |
| Denaturation | 95°C | 5 min |
After bisulfite conversion was completed, the tubes were briefly centrifuged, and the content transferred into a new 1.5 ml reaction tube. The next steps were performed according to instructions provided with the kit. Finally, bisulfite converted DNA was diluted to 10 ng/µl and aliquots were stored at -80°C until the Methylight assay was performed. For each PCR reaction 20 ng bisulfite converted DNA was used.

### 2.6.4 TaqMan-based real time PCR

TaqMan procedure specifically detects the target gene sequence, so nonspecific products do not affect the accuracy of quantification this being the main advantage over DNA binding dyes. TaqMan assays employ a sequence-specific, fluorescently labeled oligonucleotide probe called the TaqMan probe, in addition to the sequence-specific primers. The probe contains a fluorescent reporter at the 5’ end and a quencher at the 3’ end. When intact, the fluorescence of the reporter is quenched due to its proximity to the quencher. During the combined annealing/extension step of the amplification reaction, the probe hybridizes to the target and the dsDNA-specific 5’→3’ exonuclease activity of nuclease (thermostable polymerases) cleaves off the reporter. As a result, the reporter is separated from the quencher, and the resulting fluorescence signal is proportional to the amount of amplified product in the sample.

Procedure: For the determination of global DNA methylation a TaqMan-based methylation specific real-time PCR (Methylight) system was used. Methylation of LINE1 (Metabion International AG, Germany) and Satα (MWG Operon Eurofins) was determined using the TaqMan Universal PCR Master Mix, No Amp Erase UNG (Roche). As reference for input of bisulfite converted DNA a methylation independent ALU1 (Metabion International AG, Martinsried, Germany) TaqMan system was used. For each real time PCR amplification the template was equivalent to 10 ng of bisulfite converted DNA and measurements were performed in triplicates. Fully methylated bisulfite converted DNA from the EpiTect PCR Control DNA Set (Qiagen GmbH) was used as 100% methylated control. Primers were optimized for StepOnePlus thermocycler. Quantification was normalized to the ALU1 gene within the log-linear phase of the amplification curve obtained for each probe/primer set using the ΔCT method using a reference gene.

### 2.7 Expression analysis on protein level using Western blot
Western Blot, also known as immunoblotting or protein blotting, is a method used to detect specific proteins in a complex mixture extracted from cells or tissue. The Western blotting procedure relies upon three key elements (Figure 25): 1. sample preparation and separation of the protein mixture by size using gel electrophoresis; 2. transfer of separated proteins to a membrane; and 3. detection of a target protein by appropriate antibodies. Once detected, the target protein is visualized as a band on a blotting membrane, X-Ray film, or an imaging system.

**Figure 25. Schematic overview of the Western Blot method.**

### 2.7.1 Sample preparation from cells

All chemical substances for this assay were purchased from Sigma-Aldrich, if not otherwise mentioned. Protein isolation from cells was made with RIPA (Radio Immuno Precipitation Assay) buffer. The content of the buffer was: 50 mM TRIS-HCl (Merk) (pH=7.5); 150 mM NaCl; 1% TRITON X-100; 0.1% SDS (w/v); 0.5% Natriumdeoxdesoxycholat (w/v) in distilled water. For optimal reproducibility all steps were carried out on ice. For protein isolation from the cells, they were cultivated in 10 cm² dish (0.5x10⁶ cells). The procedure was as follows: medium was removed, cells were washed once with sterile PBS (PAA Laboratories GmbH), 1 ml of RIPA buffer was added, and incubated 10 min at -20°C. The cell debris was collected with a cell scraper and the lysate was transferred into a 1.5 ml reaction tube, followed by centrifugation for 20 min at +4°C. After centrifugation the supernatant containing the proteins was collected in a new tube, the protein concentration was measured; aliquots were made and stored at -20°C until further use.

### 2.7.2 Sample preparation from tissue
Histone isolation from fresh carotid tissue was performed with EpiSeeker Histone Extraction kit (Abcam, Cambridge, UK), according to its instructions. Briefly, samples were homogenized with a pre-lysis buffer, and centrifuged at +4°C at 10000 rpm 1 min. The supernatant was removed and the tissue pellet was resuspended in lysis buffer, incubated on ice for 30 min, followed by a centrifugation step by 12000 rpm at +4°C for 5 min. The supernatant was collected in a new tube and supplemented with Balance-DTT buffer. The protein concentration was measured with BCA assay and stored at -20°C until further use.

Samples were diluted in RIPA buffer to a final concentration of 1 µg/µl. From each sample 20 µl were added into 1.5 ml reaction tubes, together with 5 µl Laemmli buffer 5x (6 ml 1M TRIS-HCl pH=6.8; 10 ml 99% Glycerol; 200 µl 500 mM EDTA; 2 g SDS; 10 mg Bromphenol Blue). Samples were heated for 5 min at 99°C for denaturation of proteins and incubated on ice.

2.7.3 Protein separation by polyacrylamide gel electrophoresis (PAGE)

The electrophoresis gel is composed of 2 gel types with a different concentration in acrylamide: the stacking gel and the separation gel (Figure 26).

![Figure 26. Arrangement of the electrophoresis gel.](image)

Depending on the target protein, different acrylamide concentrations for the separation gel were used as described in Table 14. For the stacking gel the composition is described in Table 15.

Table 14. Separation gel recipes.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume (ml) for 1 gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRIS-HCl 1.5M(pH=6.8)</td>
<td>2.5</td>
</tr>
<tr>
<td>Distilled water</td>
<td>2.3</td>
</tr>
<tr>
<td>H3K4me2</td>
<td>2.5</td>
</tr>
<tr>
<td>H3K9me2</td>
<td>2.5</td>
</tr>
<tr>
<td>OCT4A</td>
<td>2.5</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>5.3</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>5.3</td>
</tr>
</tbody>
</table>
### MATERIALS AND METHODS

| Acrylamide (29:1) | 5 | 3.3 | 2 |
| SDS 10% | 0.1 | 0.1 | 0.1 |
| Ammonium persulphate (APS) 10% | 0.1 | 0.1 | 0.1 |
| N,N,N’,N’-Tetramethylethylenediamine (TEMED) | 0.005 | 0.005 | 0.005 |

**Table 15. Stacking gel recipe.**

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume (ml) for 4ml (1 gel)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRIS-HCl 0.5M (pH=6.8)</td>
<td>1.25</td>
</tr>
<tr>
<td>Distilled water</td>
<td>2.7</td>
</tr>
<tr>
<td>Acrylamide (29:1)</td>
<td>0.7</td>
</tr>
<tr>
<td>SDS 10%</td>
<td>0.05</td>
</tr>
<tr>
<td>APS 10%</td>
<td>0.05</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.005</td>
</tr>
</tbody>
</table>

#### 2.7.4 Protein transfer to PVDF membrane

After the electrophoresis was finished, the procedure was continued by protein transfer onto a PVDF membrane using semi-dry system. The scheme of this procedure is shown in **Figure 27**.

![Assembling of the transfer sandwich.](image)

**Figure 27. Assembling of the transfer sandwich.**

An aqueous solution of the transfer buffer was prepared by adding following substances/solutions to 900 ml distilled water (B. Braun Melsungen AG): 3.03 g Trizma base, 14.4 g glycine, and 100 ml methanol (Merck). The filter papers and acrylamide gel were soaked in transfer buffer; the PVDF membrane was shortly activated in methanol and incubated in transfer buffer for 30 min. The assembling of the system was made as described in **Figure 27**. The semi-dry transfer device was set to 10V for 30 min followed by other 5 min at 15V.

#### 2.7.5 Protein detection
The PVDF membrane containing the proteins was then incubated in blocking buffer (5% non-fat skimmed milk powder (Biomol GmbH, Hamburg, Germany) in TBS-T) for 1 h at RT on a shaker. TBS-T was prepared from 900 ml distilled water to which following solution were added: 100 ml TBS buffer, and 1 ml Tween 20. TBS buffer was prepared by adding 24.2 g Trizma base and 80 g NaCl (Merck) to 1000 ml distilled water and adjusting the pH value to 7.6 with HCl 2N (Apotheke MRI). Each antibody was diluted in a blocking buffer and incubated overnight at +4˚C as described in Table 16, 17 and 18.

The next day the membrane was washed in TBS-T solution for 30 min to remove the excess of the first antibody. The secondary antibody solution was made in the same blocking buffer and added to the PVDF membrane, which was then incubated for 1 h at RT.

Table 16. Antibodies used for characterization of the angiogenesis in adMSCs.

<table>
<thead>
<tr>
<th>Name</th>
<th>Reacts with</th>
<th>Clone/Made in</th>
<th>Brand &amp; Cat. Nr.</th>
<th>Dilution</th>
<th>Size (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse anti hVACM-1(CD106)</td>
<td>Human</td>
<td>Mouse IgG</td>
<td>R&amp;D Systems</td>
<td>1:100</td>
<td>110</td>
</tr>
<tr>
<td>Fk-1 antibody(VEGFR-2)</td>
<td>Human</td>
<td>Mouse IgG</td>
<td>R&amp;D Systems</td>
<td>1:100</td>
<td>170</td>
</tr>
<tr>
<td>Anti-GAPDH antibody [6C5]</td>
<td>Rat, Rabbit, Chicken, Human,</td>
<td>6C5</td>
<td>Abcam (ab8245)</td>
<td>1:1000</td>
<td>36</td>
</tr>
<tr>
<td>Peroxidase-Labeled Affinity Purified Antibody to Mouse IgG (H+L)(Human Adsorbed)</td>
<td>Mouse</td>
<td>Goat</td>
<td>KLP (074-1806)</td>
<td>1:5000</td>
<td>-</td>
</tr>
<tr>
<td>Peroxidase-Labeled Affinity Purified Antibody to Rabbit IgG (H+L)(Human Adsorbed)</td>
<td>Rabbit</td>
<td>Goat</td>
<td>KLP (074-1516)</td>
<td>1:5000</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 17. Antibodies used for detection of pluripotency markers.

<table>
<thead>
<tr>
<th>Name</th>
<th>Reacts with</th>
<th>Clone/Made in</th>
<th>Brand &amp; Cat. Nr.</th>
<th>Dilution</th>
<th>Size (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCT4A(C30A3) Rabbit mAb</td>
<td>Human, Mouse</td>
<td>C30A3</td>
<td>Cell Signaling (#2840)</td>
<td>1:500</td>
<td>48</td>
</tr>
<tr>
<td>Rabbit polyclonal to Nanog</td>
<td>Human, Mouse</td>
<td>rabbit</td>
<td>Abcam (ab21624)</td>
<td>1:500 - 1:50</td>
<td>38</td>
</tr>
<tr>
<td>Anti-GAPDH antibody [6C5]</td>
<td>Rat, Rabbit, Chicken, Human,</td>
<td>6C5</td>
<td>Abcam (ab8245)</td>
<td>1:1000</td>
<td>36</td>
</tr>
<tr>
<td>Peroxidase-Labeled Affinity Purified Antibody to Rabbit IgG (H+L)(Human Adsorbed)</td>
<td>Rabbit</td>
<td>Goat</td>
<td>KLP (074-1516)</td>
<td>1:5000</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 18. Antibodies used for detection of di-methylation at K4 and K9 sites of histone H3.

<table>
<thead>
<tr>
<th>Name</th>
<th>Reacts with</th>
<th>Clone/Made in</th>
<th>Brand &amp; Cat. Nr.</th>
<th>Dilution</th>
<th>Size(kDa)</th>
</tr>
</thead>
</table>
The PVDF membrane was treated for 30 min with TBS-T then submersed in a working solution (Solution A/Solution B, ratio 1/1) of SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific, Bonn, Germany) for 5 min. The excess reagent was drained and the blot was covered with clear plastic wrap. The membrane was placed in a film cassette with the protein side facing up. In the dark, a FUJI Medical X-Ray Film (FUJIFILM Europe GmbH, Düsseldorf, Germany) was placed on top of the membrane and various exposure times were used to achieve optimal results. The film was then developed with Medical Film Processor (Konica Minolta model, SRX-101A, Konica Minolta Medical and Graphic Imaging Europe GmbH, Munich, Germany).

2.7.6 **Protein quantification**

In order to quantify the amount of each targeted protein, developed films were digitized using a color image scanner (HP Scanjet 3800). The bands on the films with the same exposure time were compared to each other after a previous normalization to a housekeeping gene for GAPDH. The intensity quantification was made with ImageJ software (National Institutes of Health, Bethesda, USA).

2.8 **Statistical analysis**

Data are presented as mean ± SEM. Statistical analysis was performed with GraphPad Prism (GraphPad Software Inc, San Diego, USA). For single comparison Mann-Whitney U test was used. The significance of differences with more than two samples was evaluated using Kruskal-Wallis test, followed by Dunns post-test for multiple comparisons: * for p<0.05; ** for p<0.01; *** for p<0.001 were considered statistically significant.
3 RESULTS

3.1 Epigenetic changes in vascular disease

3.1.1 Histological characterization of specimens

The stage of atherosclerosis (AS) was classified as described in the section Materials and Methods. Tissue specimens were divided into the following groups: control (healthy carotid artery - Figure 28), early stage of AS (II-III, AHA classification - Figure 29), and advanced stage of AS (V-VII, AHA classification - Figure 30).

Figure 28. Example of a control vessel. (A) H&E representative staining and (B) elastica von Gieson representative staining. Bar on the overview pictures represents 1 mm.

The early stage of AS type II and III was recognized by markedly enlarged intima compared with control vessels, with scattered yellow-colored fatty streaks, enhanced intracellular lipid accumulation, and increased number of macrophages and macrophage-derived foam cells. Furthermore, an increased amount of collagen fibers was observed (Figure 29).
Type V lesion was characterized by massive aggregates of extracellular lipids forming necrotic or lipid core with a fibrous cap that was either thick (stable plaque) or thin (<200 µm, unstable plaque) [215]. Moreover, increased inflammation and a higher amount of leukocytes, macrophages, and thrombotic deposits and/or marked hemorrhage were visible. Type VII lesion had calcium deposits that partially replaced the Type VI lipid/necrotic core. (Figure 30 and Figure 31)

In this manner 20 early and 20 advanced stage lesions were chosen, in each case early and advanced stages of atherosclerosis from the same patient and same atherosclerotic plaque.
3.1.2 Global DNA methylation

3.1.2.1 Global DNA methylation in carotid tissue samples

The results of global DNA methylation from carotid specimens adjacent to different types of atherosclerotic lesions, determined by histology, are summarized in Figure 32. The extent of global DNA methylation correlated negatively with the degree of atherosclerosis. In early AS, the methylation of LINE1 decreased to 62.3% (p<0.01) compared with the DNA
methylation in healthy control vessels. Further decrease in DNA methylation to 32.4% (p<0.001) was observed in advanced stage of atherosclerosis, again compared with the healthy controls. Similar results were found also for the repetitive sequence Satα, with significant reduction in DNA methylation to 73.2% (p<0.01) in early stage and to 39.3% (p<0.001) in advanced stage of AS, compared with healthy vessels (Figure 32).

![Figure 32](image1.png)

**Figure 32.** Global DNA methylation status is decreased with the progression of atherosclerosis. The graphs represent the quantitative evaluation after setting the methylation in the control group as 100%.

### 3.1.2.2 Global DNA methylation in serum

Interesting results were also observed following the analysis of blood serum samples. For both repetitive DNA sequences LINE1 and SATα, significant reduction in DNA methylation was found in patients with high grade carotid artery stenosis compared with healthy individuals. The global DNA methylation was reduced to 36.9% (p<0.001) for LINE1 and to 67.5% (p<0.001) for SATα, normalized to the DNA methylation in healthy volunteers (Figure 33).

![Figure 33](image2.png)

**Figure 33.** Global DNA methylation of free DNA extracted from blood serum. DNA methylation is significantly decreased in patients with advanced atherosclerosis. The graphs represent the quantitative evaluation after setting the methylation in the control group as 100%.
3.1.2.3 Expression of DNA methyltransferases and demethylase at mRNA level

At mRNA level the expression of DNA methyltransferases $DNMT1$, $DNMT3a$, $DNMT3b$, and demethylase $TET1$ were determined by quantitative SYBR-Green based real time PCR. In all cases, normalization to the expression of housekeeping gene $GAPDH$ was performed. $DMNT1$, a methyltransferase responsible for the maintenance of DNA methylation, showed a significant decrease in expression in AS (Figure 34). The differences between early and advanced stage of AS were not statistically significant ($p=0.4701$). $DNMT1$ expression in early stage was decreased to 53.2% ($p=0.0393$), and in advanced stage to 27.2% ($p=0.0393$), in comparison with the control healthy tissue.

![Figure 34](image)

*Figure 34. Expression of DNMT1 decreased with the severity of atherosclerosis. The graphs represent the quantitative evaluation after setting the control group as 100%.*

$TET1$, an enzyme involved in the demethylation process, responsible for the intermediate form 5-hydroxymethylation, showed an increased expression level that corresponded with the progression of AS (Figure 35). An increased by 13.2-fold, ($p=0.7441$) was observed in the early stage of AS, and by 44.1-fold ($p=0.4350$) in the advanced stage. However, due to broad distribution of the values the postulated significance was not achieved, compared with the expression of $TET1$ in control tissue samples.
De novo methylation process is supported by two enzymes, DNMT3A and DNMT3B, the latter having also a role in maintaining the DNA methylation pattern (Figure 36). The expression of DNMT3A was detected neither in control tissue nor in atherosclerotic lesions. In contrast, DNMT3B expression was found in both healthy and atherosclerotic samples. Decrease in expression of DNMT3B was observed in AS, compared to healthy tissue, both in early and advanced stage, with the tendency of further reduction in DNMT3B expression with the progression of AS. In early stage the expression decreased to 46.0% (p=0.9048), and in advanced stage to 31.0% (p=0.6095), in comparison with the healthy control tissue (Figure 36).

3.1.3 Immunohistochemical analysis of histone methylation

One of the objectives of the present study was to analyze the histone, methylation, especially di-methylation on histone H3 at lysine 4 and at lysine 9 (H3K4 and H3K9), and to
evaluate, whether any changes in this epigenetic modification are associated with certain type of cells within atherosclerotic plaques in comparison with healthy vessel tissue.

3.1.3.1 Histone methylation in smooth muscle cells

The first staining was performed for smooth muscle cells (SMCs) (Figure 37). Smooth muscle (SM) actin clearly identified SMCs within the control tissue as well in early and advanced atherosclerosis. Consecutive slides stained for SM-actin were used for detection of H3K4 di-methylation. In the control tissue, not all SMCs were positive for H3K4me2 staining. In contrast, both early and advanced stage of atherosclerosis showed an increase in the number of positive cells. No further differences were observed between early and advanced atherosclerosis (Figure 37).
Figure 37. Representative stainings of consecutive slides for smooth muscle cells and H3K4me2. Red color represents the positive cells for smooth muscle, whereas brown color represents cells positive for H3K4me2. Samples were counterstained with haematoxylin (blue color). The bar in the overview image represents 1 mm.

Similar consecutive staining as described before was performed for SMCs and H3K9me2. The results revealed that only a small fraction of cells was positive for H3K9me2 and SM-actin (Figure 38). No differences were observed between the control vessels, early, or advanced stage of atherosclerosis.
3.1.3.2 Histone methylation in inflammatory cells

Consecutive slices of FFPE tissue samples from each study group were stained for the inflammatory marker CD45 and for H3K4me2 (Figure 39). CD45 is a leucocyte common antigen present on all blood cells except erythrocytes. It is expressed especially in B- and T-lymphocytes.

Figure 38. Representative stainings of consecutive slides for smooth muscle cells and H3K9me2. Red color represents the positive cells for smooth muscle, whereas brown color represents the cells positive for H3K9me2. Samples were counterstained with haematoxylin (blue color). The bar in the overview image represents 1 mm.
In general, in a healthy vessel, no significant amount of inflammatory cells, comprising CD45 or CD68 antigens, is present. In agreement, no CD45 positive cells were found in the control tissue of the selected study group. For this reason, images of the stained control healthy vessels were omitted in Figures 39-42. In contrary to the control vessel, in early and especially advanced stage of atherosclerosis marked accumulation of inflammatory cells, positive for CD45, was observed. Interestingly, all these cells were negative for H3K4me2 (Figure 39).

Figure 39. Examples of consecutive staining for CD45 positive inflammatory cells and for histone H3 di-methylation of lysine K4 in early (first row) and advanced (second row) stage of atherosclerosis. Brown color represents the positive cells for different markers: CD45 – first column, H3K4me2 – second column. Blue color shows the nuclear counterstain by haematoxylin. The bar in the overview image represents 1 mm.

The same staining procedure as for H3K4 was performed also for H3K9 (Figure 40). In contrast to the negative staining for H3K4me2 in inflammatory cells, CD45 positive cells were found to be positive for H3K9me2. No differences were observed between early and advanced stage of AS. Albeit, increased intensity and more cells were seen in advanced atherosclerotic plaques.
**Figure 40.** Examples of consecutive staining for CD45 positive inflammatory cells and for histone H3 di-methylation of lysine K9 in early (first row) and advanced (second row) stage of atherosclerosis. Brown color represents the positive cells for different markers: CD45 – first column, H3K9me2 – second column. Blue color shows the nuclear counterstain by haematoxilin. The bar in the overview image represents 1 mm.

Another group of inflammatory cells that markedly contribute to plaque progression are CD68 positive cells, comprising monocytes, macrophages, and macrophage-derived foam cells. Therefore, consecutive staining for CD68 and histone di-methylation of H3K4 or H3K9 was performed (*Figure 41 and 42*). Only few CD68 positive cells were observed in early stage of atherosclerosis, according to their morphology, mainly monocytes and macrophages. In contrast, advanced stage of atherosclerosis comprised predominantly lipid-laden macrophages and macrophage-derived foam cells.

After examination of the corresponding regions for CD68 and H3K4, only a small fraction of CD68 positive cells was found to be also positive for H3K4me2 in both early and advanced stage (*Figure 41*). Furthermore, no differences were observed between early and advanced atherosclerosis.
**RESULTS**

*Figure 41.* Examples of consecutive staining for CD68-positive inflammatory cells (monocytes, macrophages, and macrophage-derived foam cells) and di-methylation of histone H3 at lysine K4 in early (first row) and advanced (second row) of atherosclerosis. Brown color represents positive cells for CD68 – first column, and for H3K4me2 – second column. Blue color shows the nuclear counterstain by haematoxylin. The bar in the overview image represents 1 mm.

In contrast to the staining for H3K4me2, markedly more CD68 positive cells in atherosclerotic lesions were positive also for H3K9me2 staining (*Figure 42*). In both, early and advanced stage of atherosclerosis, almost all monocytes, macrophages, and macrophage-derived foam cells were methylated on histone H3 at the lysine K9.
Figure 42. Examples of consecutive staining for CD68-positive inflammatory cells (monocytes, macrophages, and macrophage-derived foam cells) and di-methylation of histone H3 at lysine K9 in early (first row) and advanced (second row) of atherosclerosis. Brown color represents positive cells for CD68 – first column, and for H3K9me2 – second column. Blue color shows the nuclear counterstain by haematoxylin. The bar in the overview image represents 1 mm.

3.1.4 Analysis of histone methylation at protein level

For possible quantification of histone methylation at the protein level, western blot analysis was performed in a similar way as for the immunohistochemistry as described above. The extraction of histones was made from fresh tissue of carotid arteries, including again three study groups: healthy tissue, early, and advanced stage of AS. Each group consisted of 9 samples. For quantification, the ImageJ software was used and samples were normalized to the amount of histone H3. Furthermore, the state of methylation in a healthy tissue was set as 100%. The extent of H3K4 and H3K9 di-methylation (H3K4me2 and H3K9me2) is shown in Figure 43. Significant reduction of histone H3 methylation at K4 and K9 was observed in both early and advanced stages of AS. In carotid plaque from early stage of AS, a decrease to 28.5% (p<0.01) was found compared with healthy control tissue. In advanced AS, a significant reduction of H3K4 di-methylation to 39.3% (p<0.05) was seen. With regard to H3K9me2, a significant decrease to 12.0% (p<0.05) and to 25.2% (p<0.01) was observed for early stage and advanced stage of AS, respectively. In both cases, for
H3K4 and H3K9, no significant differences in histone di-methylation were observed between early and advanced of AS.

Figure 43. Western blot analysis for di-methylation of histone H3 at lysine 4 (H3K4) and lysine 9 (H3K9). A statistically significant decrease for both H3K4me2 and H3K9me2 was observed with the progression of the AS. The expression of each modification in the control group was set as 100%.

3.1.5 Analysis of histone methyltransferases (HMTs)

3.1.5.1 HMTs responsible for H3K4 methylation

Finally, to constitute the changes in histone methylation pattern in atherosclerotic lesions, the expression of methyltransferases, responsible for the transfer of methyl group to H3K4, was analyzed by quantitative SYBR-Green based RT-PCR (Figure 44). The analyzed methyltransferases were MLL1, SET1A, and SET1B. Their expression was evaluated at the mRNA level on tissue samples extracted from FFPE tissues. From all three HMTs, only the expression of MLL1 could be detected. Interestingly, MLL1 expression increased continuously with the progression of atherosclerosis, in early stage lesions by 2.63-fold (p=0.7658) and in advanced stage by 2.09-fold (p=0.5800), however without the postulated significance. For SETD1A as well as for SETD1B no expression was observed (Figure 44).
Figure 44. Quantification of the expression of methyltransferase MLL1, SETD1a, and SETD1b responsible for H3K4 methylation. In contrast to the expression of MLL1, which continuously increased with the progression of atherosclerosis, no expression was observed for SETD1a and SETD1b. Expression of the control group was set as 100%.

3.1.5.2 HMTs responsible for H3K9 methylation

Similar to results described before, the expression of methyltransferases responsible for the transfer of methyl group to H3K9 was evaluated (Figure 45). The expression of SUV39H1 and EHMT2 (G9a), responsible for the corresponding methylation, was analyzed at mRNA level from FFPE tissue samples as for H3K4. No expression was detected for both enzymes.

Figure 45. Quantification of the expression of methyltransferase SUV39H1 and EHMT2 responsible for H3K9 methylation. No detectable expression was observed for both enzymes.
3.2 RESULTS - Epigenetic changes in regenerative medicine

3.2.1 Isolation and characterization of the adipose derived mesenchymal stem cells (adMSCs)

Mesenchyme is a type of undifferentiated loose connective tissue derived mostly from mesoderm. Mesenchyme is characterized morphologically by a prominent ground substance matrix containing a loose aggregate of reticular fibrils and unspecialized cells. The cells are capable of developing into connective tissue, such as bone, cartilage, and adipose tissue. Adipose tissue contains a high amount of mesenchymal stem cells that can be easily isolated. These cells have been described to have high proliferative capacity and also a potential to differentiate into osteoblast, chondrocytes or adipocytes, if they are cultivated under appropriate conditions.

3.2.1.1 Analysis of cell morphology and phenotype

Mesenchymal stem cells (MSCs) have been classified as a heterogeneous population in terms of morphology, proliferation capacity, and differentiation potential. They display different phenotypes: large flattened cells, thin star-shaped cells, and fibroblast-like cells (spindle shape) as already described by Muraglia et al. [216] (Figure 46). By examining the cell isolated from adipose tissue, it was observed that MCSs are characterized morphologically by small cell body with few long and thin cell processes. The cell body contains round nucleus with large nucleolous, which is surrounded by finely scattered chromatin particles. Cells isolated from human adipose tissue in this study displayed the typical features of MSCs, as shown in Figure 46.

![Figure 46. Phenotype of cultured human adMSCs. Three phenotypes were observed in adMSC colonies: (A) spindle shape, (B) large flattened cells, and (C) star shaped cells as described by Muraglia et al [216].](image)

The population of freshly isolated adMSCs is in general heterogeneous. Therefore, to assure reproducibility of the following experiments, it was necessary to further characterize the cell population over the passages. Following cell isolation and their cultivation until
passage 3, flow cytometry analysis revealed mainly homogenous cell population with a characteristic expression pattern of the known MSCs markers CD90 and CD105, while being negative for hematopoietic marker CD14 and CD45 (Figure 47). This assay was performed in every passage up to the passage 3 for all CD markers mentioned. Based on the cell characterization, passage 3 was selected for all further experiments due to the best proliferation/homogeny ratio. The expression of MSC markers in passage three was as follows: CD90 and CD105 - 86% and 96%, respectively, CD14 and CD45 - 1.88% and 1.06%, respectively. The markers CD14 and CD45 showed a decrease to less than 2%, while CD90 and CD105 showed an increase for up to 96% in the third passage (for detailed results see Table 19 and Figure 48)

Figure 47. FACS histograms for CD14, CD45, CD90, and CD105 used for characterization of adMSCs population. Cells were screened for (A) CD14, (B) CD45, (C) CD105, and (D) CD90 and their corresponding isotype controls. Each representative antibody is represented by the blue histograms, and the isotype control is red colored.

Table 19. Summary of adMSC characterization by FACS analysis.

<table>
<thead>
<tr>
<th>Passage number</th>
<th>CD14 positive cells (%)</th>
<th>CD45 positive cells (%)</th>
<th>CD90 positive cells (%)</th>
<th>CD105 positive cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Passage 0</td>
<td>8.4%</td>
<td>5.7%</td>
<td>73.72%</td>
<td>44.3%</td>
</tr>
<tr>
<td>Passage 1</td>
<td>3.4%</td>
<td>1.5%</td>
<td>77.7%</td>
<td>47%</td>
</tr>
<tr>
<td>Passage 2</td>
<td>3.12%</td>
<td>1.26%</td>
<td>81%</td>
<td>50%</td>
</tr>
<tr>
<td>Passage 3</td>
<td>1.88%</td>
<td>1.06%</td>
<td>86%</td>
<td>96.17%</td>
</tr>
</tbody>
</table>
3.2.1.2 Immunocytological characterization

Cells isolated from adipose tissue were first cultivated under normal growth conditions. In the third passage they were screened for the known mesenchymal markers vimentin and endoglin (Figure 49). Vimentin is the cytoskeletal component responsible for maintaining cell integrity, and endoglin, also known as CD105, is a glycoprotein part of the transforming growth factor receptor complex that binds several members of the TGFβ superfamily. The cells were proven to be homogenous with regard to these markers, with all cells positive for both antigens.

![Figure 49. AdMSC characterisation – vimentin and endoglin - ICC. (A) Red cells positive for the mesenchymal marker – vimentin (red); (B) CD105 - endoglin, is present on the membrane of all cells (brown); counter stain with Meyer's haematoxylin (nuclei are stained in blue).](image)

3.2.2 Analysis of cell proliferation

Ki-67, a marker of actively dividing cells, was used to determine the fraction of proliferating cells within the given population. Proliferating adMSCs in passage 3 were visualized by ICC staining. Cells were in average positive for Ki-67 at a rate of 52%, as shown in Figure 50 (quantification was performed with ImageJ software).
RESULTS

**Figure 50.** Immunocytochemistry for the Ki-67 antigen on adipose-derived mesenchymal stem cells (A) adMSCs. In third passage, red stain represents the proliferating cells, positive for Ki-67; (B) Negative control IgG.

### 3.2.3 Osteogenic and adipogenic differentiation

Since adMSCs are mesenchymal stem cells, they should have the capacity to undergo osteogenic, adipogenic, and chondrogenic differentiation. To prove their differentiation potential and their multipotent characteristics, the cells were seeded and cultivated in appropriate differentiation media.

#### 3.2.3.1 Osteogenic differentiation

Osteogenic differentiation was assessed after 7, 14, and 21 days of cultivation in osteogenic differentiation medium by analyzing the presence of the alkaline phosphatase (ALP) enzyme, deposits of collagen III, and mineralized matrix.

ALP is a marker known to be necessary for mineralization of bone tissue, present in cells of the osteoblastic lineage. The majority of isolated adMSCs cultivated under osteogenic conditions were positive for ALP and the results were similar to that of human osteoblast progenitor cells, compared with normal growth media (*Figure 51*).
Type-III collagen is a protein that is found in bone, cartilage, tendon and some other connective tissues. Therefore, adMSCs, cultivated in osteogenic medium, were tested also for their ability to synthesize collagen type III. Staining of differentiated adMSCs against collagen III revealed an increase in the production of the protein over the differentiation period, whereas under normal growth conditions the cells were not producing collagen type III (Figure 52).

Beside ALP activity and the production of extracellular matrix (ECM) during osteogenic differentiation, osteoblasts also produce mineralized matrix. For this reason, the matrix mineralization was tested using von Kossa staining after 7, 14, and 21 days, in order to prove the capacity of adMSCs to differentiate into osteogenic lineage (Figure 53).
staining confirmed the ability of adMSCs to deposit calcium phosphate within the cell. The deposition was observed in direct progression with the differentiation time.

### 3.2.3.2 Adipogenic differentiation

As mentioned above, adMSCs also possess the ability to differentiate into the adipogenic lineage. The differentiation potential of the cells was tested by Oil Red O staining. The lipid droplets appeared after 2-3 days in adipogenic medium and their accumulation was equivalent to an adipocyte phenotype. Furthermore, the staining revealed large lipid vacuoles after 14 days of culture (Figure 54).

**Figure 53.** Von Kossa stain of mineralized deposits (black color) in cultures of adMSCs that underwent osteogenic differentiation for (A) 7 days, (B) 14 days, and (C) 21 days; (D) undifferentiated adMSCs; (E) human osteoblast progenitor cells cultivated for 7 days in osteogenic medium.

**Figure 54.** Adipogenic differentiation of adMSCs. Morphological analysis of adMSCs cells under (A) adipogenic conditions for 14 days in comparison with (B) normal growth conditions; bar represents 100 µm.
3.2.4 **Effect of the epigenetic modifying drugs on adMSCs**

3.2.4.1 **Detection of the optimal concentration of epigenetic modifying drugs for adMSCs**

To find the optimal non-toxic concentration of the epigenetic modifying drugs used in this study, different amount of 5-azacytidine (AZA), BIX-01294 (BIX), and valproic acid (VPA) were used and cell viability assay (MTT) was applied.

3.2.4.2 **Effect of AZA on viability of adMSCs**

The viability of cells treated with five different concentrations of AZA (2.5, 5, 10, 20, and 40 µM) and compared with non-treated cells. Cell viability was not significantly affected by the tested concentrations independent of the different time point used (24, 48, and 72 h) (Figure 55). For this reason the two lowest AZA concentrations, 2.5 and 5 µM, were selected for further experiments.

![Figure 55](image)

*Figure 55. Viability of adMSCs treated with five different concentrations of 5-azacytidine (AZA) for different time points: (A) 24 h, (B) 48 h, and (C) 72 h. Viability of the non-treated cells was set as 100%.*

3.2.4.3 **Effect of BIX on viability of adMSCs**

The tested concentrations of BIX were 0.625, 1.25, 2.5, 5, and 10 µM. As for AZA, the viability of the cells was measured 24, 48, and 72 h following treatment (Figure 56). In comparison with non-treated cells, a statistically significant decrease in cell viability was observed after exposing the cells to concentrations of 5µM and 10 µM BIX for all three time points. Following decrease in cell viability was observed using BIX: After 24 h and at the concentration of 5 µM to 44% (p<0.001), at 10 µM to 5% (p<0.0001); after 48 h at 5 µM to 8.5% (p<0.0001), and at 10 µM to 0.5% (p<0.0001); after 72 h at 2.5 µM to 52% (p<0.05), at 5 µM to 6% (p<0.0001), and at 10 µM to 3% (p<0.0001). Thus, for the next experiments BIX concentration of 0.625 and 1.25 µM were selected.
3.2.4.4 Effect of VPA on viability of adMSCs

Valproic acid was tested on adMSCs at concentrations of 100, 200, 400 and 800 µM, and 1.2 mM at three different time points, 24, 48, and 72 h (Figure 57). Compared with non-treated cells, significant decrease in cell viability was observed after 48 h at 100 µM to 53.8%, at 400µM to 56.5%, at 800µM to 50.66%, and at 1.2 mM to 54.1% (for all p<0.05); after 72 h significant decrease in viability was observed only for 1.2 mM to 43.21% (p<0.05). Consequently, for all future experiments the concentrations of 100 and 200 µM were chosen.

Finally, also combination of these chemical compounds was tested and compared with the non-treated cells for the above mentioned time points (24, 48, and 72 h). Based on the previous results for each of the compound, following concentrations were chosen: AZA 5 µM, BIX 1.25 µM, and VPA 200 µM). In addition several of these compounds and their selected concentration were tested. Various combinations were tested to verify the viability of the cells as follow: AZA and BIX; AZA and VPA, BIX and VPA, AZA, BIX, and VPA. No significant differences were observed after the treatment independent of the combination or time point used (Figure 58).
RESULTS

Figure 58. MTT assay after treatment with a combination of AZA, BIX, and VPA for a time period of (A) 24 h, (B) 48 h, and (C) 72 h. Viability of the non-treated cells is set as 100%.

3.2.5 Effect of epigenetic modifying drugs on global DNA methylation

Global DNA methylation was assessed as described in section Materials and methods. All three chemical compounds described above are known for their ability to modify epigenetic pattern; However, only AZA has been shown to have a direct effect on DNA methylation [136]. Changes in DNA methylation pattern have been already associated with the reprogramming capacity of the cells. Consequently, epigenetic drugs might also improve the reprogramming capacity of adMSCs used in this study. Optimized non-toxic concentrations of AZA (2.5µM and 5µM), BIX (0.625µM and 1.25µM), and VPA (100µM and 200µM) were used for cell treatment at three different time points (24, 48, and 72 h) as already tested before to determine the optimal concentration of these chemical compounds on global DNA methylation. The results are summarized in Figure 59.

Following treatment of adMSCs with AZA for 24 hours no significant changes in DNA methylation pattern were observed. In contrast, global DNA methylation was decreased to 40.3% (p<0.001) using 5 µM AZA for 48 h, whereas 2.5 µM AZA for 72 h led to a decrease to
71.6% (p<0.05), compared with non-treated cells. In similar way, BIX treatment demonstrated significant changes in DNA methylation first after 48 hours of treatment. Using the higher non-toxic concentration of 1.25 µM, global DNA methylation was reduced to 49.80% (p<0.0001). Treatment of adMSCs with BIX for 72 hours led to a significant decrease in global DNA methylation for both concentrations applied. Using 0.625 µM the reduction went to 68.30% (p<0.05), by 1.25 µM to 68.35% (p<0.05), respectively. Treatment with valproic acid did not lead to any decrease in the global DNA methylation, but interestingly to a slight increase, even though not statistically significant (Figure 59).

3.2.6 Effect of epigenetic modifying drugs on the expression of pluripotency-related genes

OCT4A, NANOG, SOX2, KLF4, and CMYC were shown to induce pluripotency, if they are all expressed at the same time in human and mouse fibroblasts. A link between cell reprogramming and epigenetics connects these pluripotency markers with the chromatin remodeling. Thus, to analyse a possible effect of epigenetic modifying drugs on the differentiation potential of adMSCs, expression levels of the above mentioned genes were analyzed following treatment. The expression of the pluripotency-related markers was measured by real time PCR and quantified with the ΔCT method after normalization of each sample to housekeeping gene GAPDH. AdMSCs were treated again with 5µM AZA, 1.25 µM BIX, or 200 µM VPA for 48 h and non-treated cells were used as a control; their gene expression was set as 100%.

AZA and VPA had the tendency to increase the expression of OCT4A for up to 143.54% and 133.15%, respectively (p=0.0705 for both), statistical analysis showed however no significant differences. Only BIX treatment was able to significantly increase the expression of OCT4A up to 233.77% (p=0.0112) (Figure 60A). SOX2 expression significantly increased to 263.36% (p=0.0195) after BIX treatment, while AZA or VPA did not have any significant effects (Figure 60B). For NANOG expression only BIX led to a significant increase to 152.93% (p=0.0357). Both AZA and VPA had a tendency to increase the expression of this marker to 147% and 142%, respectively (p=0.09 for both), but without significance (Figure 60C).
RESULTS

Expression of the other pluripotency markers KLF4 and CMYC did not significantly change after the treatment of adMSCs with none of these epigenetic modifying drugs (Figure 61 A and B).

In addition, a combination AZA, BIX, and VPA was tested to verify, whether the expression of pluripotency-related genes could be further increased. Combinations of each of the two substances and even all three did not lead to any significant increase in the expression of these genes compared to single treatment and was therefore not considered for further experiments.

3.2.7 Differentiation of adMSCs into endothelial cells

After proving the hypothesis that epigenetic modifying drugs, namely BIX and AZA, are able to modify the global DNA methylation, and also that BIX was able to significantly increase the expression of pluripotency related genes at mRNA level, the next step was to evaluate, whether the reprogramming capacity was increased as well. Therefore, adMSCs were pre-treated with AZA, BIX, and VPA for 48 h at the optimal non-toxic concentrations as described above and then the cells were cultured in endothelial medium for up to 14 days (Figure 62). Cells were microscopically analyzed after being stained for VCAM-1, von Willebrand Factor, VEGFR-2, and PECAM-1. In order to quantify the expression of the relevant endothelial cell markers (VEGFR-2, VCAM-1, PECAM-1, F8) and angiogenesis markers (VEGF, PDGF, ANG-1, and ANG-2), qPCR analysis was performed.
3.2.7.1 **Evaluation of endothelial and angiogenic markers by immunocytochemistry**

During cultivation of adMSCs under endothelial conditions, the morphology of adMSCs changed with the time towards that of endothelial cells, showing the characteristic cobblestone-like shape. These changes were observed mostly in the group of epigenetic treated cells (*Figure 63*).

To further characterize the differentiation process, ICC was performed after 7 days under endothelial cultivation conditions. The cells were stained against VCAM-1 (*Figure 64*), vWF (*Figure 65*), VEGFR-2 (*Figure 66*), and PECAM-1 (*Figure 67*). The increased number of positive cells in the group that received BIX treatment confirmed that BIX had the highest potential to increase the reprogramming capacity of adMSCs to differentiate into cells with endothelial characteristics for all endothelial markers used.
RESULTS

**Figure 64.** VCAM-1 staining of adMSCs after 7 days under endothelial conditions. (A) AZA, (B) BIX, (C) VPA for 48 h treatment; (D) non-differentiating growth conditions (DMEM) and (E) adMSCs in endothelial medium without any epigenetic drug treatment.

**Figure 65.** Staining for von Willebrand Factor antigen after 7 days under endothelial conditions. (A) AZA, (B) BIX, (C) VPA for 48 h treatment; (D) non-differentiating growth conditions (DMEM) and (E) adMSCs in endothelial medium without any epigenetic drug treatment.
RESULTS

Figure 66. Staining for the antigen VEGFR-2 after 7 days under endothelial conditions. (A) AZA, (B) BIX, (C) VPA for 48 h treatment; (D) non-differentiating growth conditions (DMEM) and (E) adMSCs in endothelial medium without any epigenetic drug treatment.

Figure 67. Staining for the PECAM-1 antigen after 7 days under endothelial conditions. (A) AZA, (B) BIX, (C) VPA for 48 h treatment; (D) non-differentiating growth conditions (DMEM) and (E) adMSCs in endothelial medium without any epigenetic drug treatment.
3.2.7.2 Evaluation of endothelial and angiogenesis markers at mRNA level

Since the most changes were observed in the group adMSCs that received a treatment with BIX before endothelial differentiation, the next experiments were performed only on these cells. *Figure 68* shows the protocol followed for the endothelial differentiation.

![Figure 68. Endothelial differentiation protocol for further characterization of the differentiation process at mRNA and protein level.](image)

The effect of BIX on the differentiation capacity of adMSCs into endothelial cells was determined analyzing the expression of genes activated in an endothelial lineage or involved in neovascularisation: PECAM-1, VCAM-1, F8, VEGFR-2, VEGF, PDGF, ANG-1 and ANG-2. Two experimental groups of cells were compared: one group was cultured only in endothelial medium (EM), the other group was treated with BIX for 48 h before the endothelial medium was added (BIX plus EM). Gene expression was analyzed in adMSCs cultured for 7 and 14 days under endothelial conditions, and was normalized to GAPDH, with the EM group set as 100%. The results are presented in *Figure 69, 70, 71, and 72.*

- **PECAM-1** expression was increased by 2.91-fold (p=0.0500) after 7 days, and by 2.11-fold (p=0.0008) after 14 days of differentiation. As for **VCAM-1**, the increase was by 2.22-fold (p=0.0500) after 7 days and by 4.86-fold (p=0.0185) after 14 days of differentiation (*Figure 69*). Each of the increase was statistically significant.

![Figure 69. Relative expression of PECAM-1 and VCAM-1 in the "BIX plus EM" group of cells after 7 and 14 days of endothelial medium; red line represents the expression of the same genes in the "EM" group of cells that was set as 100%.](image)

- Von Willebrand factor gene expression was increased by 4.02-fold (p=0.0104) after 7 days and by 36-fold (p=0.0382) after 14 days in the “BIX plus EM” group. In the same group the expression of VEGFR-2 was increased by 2.17-fold (p=0.0844) and by 4.53-fold (p=0.0235) after 7 and 14 days, respectively (*Figure 70*).
Expression of VEGF and PDGF was also analyzed. After 7 days under endothelial conditions, an increase of VEGF expression by 4.68-fold (p=0.0851) and by 2.34-fold (p=0.0131) after 14 days was observed. As for PDGF, similar increases were found by 2.78-fold (p=0.3655) after 7 days and by 5.21-fold (p=0.0017) after 14 days.

In the “BIX plus EM” group significant changes in the expression of ANG-1 and -2 after 7 and 14 days were observed. Ang-1 expression was increased by 4.2-fold (p=0.0498) after 7 days and by 6.52-fold (p=0.0080) after 14 days, respectively. In contrast, expression of Ang-2 after 7 days under endothelial differentiation conditions presented a decrease by 0.40-fold (p=0.0498) and after 14 days by 0.64-fold (p=0.0498) in comparison with the EM group (Figure 72).
RESULTS

Figure 72. Ang-1 and Ang-2 gene expression in the "BIX plus EM" group of cells after 7 and respectively 14 days in endothelial medium; red line represents the expression of the same genes in the "EM" group of cells that was set as 100%.

3.2.7.3 Evaluation of endothelial and angiogenesis markers at protein level

In addition, to evaluate whether the increase in expression of endothelial and angiogenic factors take place also at protein level, selected markers specific for ECs showing significant differences in gene expression as shown above, VCAM-1 and VEGFR-2, were verified by western blot.

After quantification, results were normalized to the expression of GAPDH from the same samples. The amount of VCAM-1 protein was increased by 1.15-fold (p=0.0271) and by 2.04-fold (p=0.0286) after 7 and 14 days, respectively, compared with cells without BIX pre-treatment. The total amount of VEGFR-2 protein was increased after 7 days by 2.95-fold (p=0.0937) and after 14 days by 1.94-fold (p<0.0001) (Figure 73).

Figure 73. Western blot for VCAM-1 and VEGFR-2 in “BIX plus EM”. (A) VCAM-1 and (B) VEGFR-2 expression at protein level in the "BIX plus EM" group of cells after 7 and respectively 14 days of endothelial medium; red line represents the expression of the same genes in the “EM” group of cells that was set as 100% (C) representative blot for VCAM-1 and related GAPDH; (D) representative blot for VEGFR-2 and related GAPDH.
3.2.7.4 Analysis of endothelial cell functionality

One of the typical tests for verifying the functionality of endothelial cells is the acLDL uptake assay. AcLDL is labeled with a carbocynine salt that emits fluorescent light at 488 nm (red). Since only endothelial cells and macrophages are able to metabolize acLDL, the red color will be observed only after metabolization of acLDL in this type of cells.

After adMSCs were cultivated in normal growth medium no cellular uptake was observed. In “EM” group of cells, low metabolism of labeled acLDL was observed (Figure 49). In contrast, cells in the group “BIX pus EM” presented the highest capacity to take up acLDL, (Figure 74).

![Figure 74. AcLDL uptake into AdMSCs after 7 days under different cultivation conditions: DMEM (normal growth medium); EM (endothelial medium); EM plus BIX (AdMSCs have received BIX pre-treatment followed by cultivation in endothelial medium). Fluorescence staining for AcLDL labeled with DiI (red color), nuclear counterstaining with SybrGreen I Dye (green color).](image)

3.2.8 Autologous vessel for tissue engineering

3.2.8.1 Preparation of collagen based scaffold by decellularization of blood vessel

Decellularization of a collagen based scaffold can be done with several methods (physically, chemically, or enzymatic methods or these combined). In the present work, to obtain the acellular vessel scaffold, the chemical decellularization method was used. The acellular scaffold was obtained after optimization of the decellularization process with 0.66% SDS. Four time points were considered in order to establish the optimal protocol: 1, 24, 48, and 96 hours of treatment. Saphenous vein was washed with 0.66% SDS for the established time duration. The concentration of 0.66% SDS was previously established to be the lowest concentration that removed all the cellular components.

After treatment of the saphenous vein with 0.66% SDS at the different time points for up to 96 h, H&E staining was performed and the vessel was analyzed by light microscopy. Treatment of the vessel for 48 hours was considered to be sufficient to achieve complete cell removal (Figure 75).
Figure 75. H&E staining of the saphenous vein after treatment with SDS at different time points. (A) control vessel - not decellularized; (B) 1 h washing with 0.66% SDS; (C) 24 h washing with 0.66% SDS; (D) 48 h washing with 0.66% SDS; (E) 96 h washing with 0.66% SDS; blue color is representing the nuclear stain; original magnification 200x.

Consequently, these conditions were used for the following experiments. As shown in Figure 76, the macroscopic structure and form the biological scaffold was unaffected and retained the shape and size of the original vessel.

Figure 76. Macroscopic view of the collagen-based scaffold after decellularization process. The biological scaffold maintained the shape and the size of the original vessel.

3.2.8.2 Attachment of differentiated adMSCs on the decellularized blood vessel

Following the optimization of the decellularization protocol, the next step was to evaluate, whether endothelial cells, differentiated from adMSCs after BIX treatment, were able to attach to the previously established biological scaffold.

Various time points were tested for the optimal seeding time. The majority of cells attached already after 2.5 h in a Petri dish, therefore the same incubation time was assumed also for the attachment on the biological scaffold.
After the differentiated adMSCs were seeded onto the decellularized scaffold, the cell behavior was followed for up to 7 days. The scaffold and the cells were prepared as follows: cells have been suspended in small volume of growth medium and seeded into the lumen of the previously obtained matrix. The vessel segments were incubated in 5%CO₂ at 37°C for 2.5 h. Thereafter, the EM was added, and after 2 and 7 days of culture the constructs were histologically analyzed.

The staining applied verified the cell attachment. Two days following seeding, cells started to form a monolayer at the surface of the scaffold. In addition, some cells migrated into the deeper layers. After 7 days, a higher cell density was observed on the surface of the scaffold compared with the first time point. However, some cells seemed to detach from the scaffold (Figure 77).

![Figure 77. Van Gieson’s staining of biological scaffold. (A) decellularized vessel; (B) 2 days after new cells were seeded; (C) 7 days after new cells were seeded. Lower part corresponding pictures are with a higher magnification. Black color represents the nuclear staining.](image)

### 3.2.9 The influence of donor age on the properties of adMSCs

By regular examination of the morphology of adMSCs in culture, differences were noticed between different cell populations. Since the isolation procedure and cultivation were standardized, the differences were assumed to be associated with origin of the adipose tissue from which the cells were isolated. The most plausible explanation was the different age of the donors, the adipose tissue was isolated. For this purpose, 13 donors were divided
into two groups, namely young (less than 50 years old) and elderly (more than 50 years old) as described in Table 20. The osteogenic and adipogenic differentiation potential of adMSCs were verified again in the third passage.

Table 20. Study groups for the cells originating from elderly and young donors.

<table>
<thead>
<tr>
<th>Mean age (years)</th>
<th>Patient gender</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>69±13.5 (60-88)</td>
<td>3 women 4 men</td>
<td>elderly</td>
</tr>
<tr>
<td>39±14 (21-49)</td>
<td>4 women 2 men</td>
<td>young</td>
</tr>
</tbody>
</table>

3.2.9.1 Osteogenic and adipogenic differentiation

3.2.9.1.1 Osteogenic differentiation

Following osteogenic differentiation for 7, 14, and 21 days, the two groups were examined for the presence of alkaline phosphatase (ALP). Quantitative assays revealed a 1.8-, 2.4-, and 2.1-fold (p<0.05) increase in ALP activity (Figure 78 A) in the groups of cells isolated from younger individuals at 7, 14, and 21 days of differentiation, respectively, compared to cells from elderly donors. Differences between the groups were observed also after staining for ALP (Figure 78 B, C, and D).

![ALP activity graph](image)

*Figure 78. Alkaline phosphatase activity and staining after osteogenic differentiation of adMSCs from elderly and young donors. (A) ALP activity measured after 7, 14, and 21 days of cultivation in osteogenic differentiation medium; representative ALP staining for cells originating from elderly individuals (B), young individuals (C), and human osteoblast progenitor cells (D) cultivated under osteogenic conditions for 7 days – dark color represents the positive cells.*
3.2.9.1.2 Adipogenic differentiation

Following adipogenic differentiation for 14 and 21 days, adMSCs from the two groups of donors were examined by Oil Red O staining (Figure 79) and quantified accordingly (Figure 80). Under adipogenic conditions, adMSCs changed their shape to round or squared and produced lipid droplets. After the staining of the droplets with Oil Red O and using typical light microscopy technique, it was observed that cells originating from younger individuals produced droplets larger in diameter than cells from elderly donors. On the other side, there were more droplets in the cells from the elderly group than in the young group. Moreover, all cells belonging to the elderly group contained lipid droplets. In contrast, in younger individuals also cells without droplets were found.

![Figure 79](image)

**Figure 79.** Oil Red O staining in adMSCs under adipogenic differentiation conditions. AdMSCs from elderly (A) and young (B) donors, after 14 days under adipogenic conditions; scale bars represent 100 µm, magnification 200x.

After solubilizing the lipid droplets in isopropanol, spectrophotometric quantification revealed no significant differences between populations from young and elderly donors after 14 and 21 days under adipogenic conditions. Nevertheless, production of oil droplets was increased up to 11-fold (p<0.0001) at day 21 compared to day 14 for both groups.

![Figure 80](image)

**Figure 80.** Quantification of the adipogenic differentiation of adMSCs from young and elderly donors - Oil Red O quantification assay showed an 11-fold increase in lipid production after 21-days reference point compared to the 14-days point. No significant differences were observed between the young and elderly donors.
3.2.9.2 Analysis of cell proliferation

Ki67 is a nuclear protein marker for cellular proliferation. It can be exclusively detected within the cell nucleus during all active phases of the cell cycle (G₁, S, G₂, and mitosis), but is absent in resting cells (G₀). Both study groups were stained for Ki67 to evaluate possible differences in cell proliferation.

For cell populations originating from elderly donors, 29.04% were positive for Ki67 antigen (Figure 81). Regarding the cell population originating from younger donors, 66.26% were positive for this marker of proliferation. Comparing these two study groups, younger donors demonstrated significant higher proliferation by 2.28-fold (p=0.0022) than elderly cell population (Figure 82).

Figure 81. Proliferating cells in the comparison groups – elderly and young. Staining for Ki67 antigen in adMSCs from elderly (A) and young (B) donors. Red color represents the positive cells.

Figure 82. Quantification of proliferation rate in young and elderly donors. Ki-67 staining was analyzed by the ImageJ software.
3.2.9.3 Analysis of global DNA methylation

DNA was isolated from both study groups, elderly and young donors and the DNA methylation was evaluated by the MethylLight assay. After normalization to the control DNA (completely methylated DNA) that was set as 100%, it was observed that in cells from elderly donors the global DNA methylation was 32.36% (p<0.05) and that from young donors was 26.28% (p<0.05) compared to control (Figure 83). In elderly group it was observed an increase by 1.23-fold (p=0.0186) in global DNA methylation compared with the group of young donors.

![Graph showing global DNA methylation in AdMSCs from young and elderly donors](#)

*Figure 83. Global DNA methylation in adMSCs from young and elderly donors. DNA-methylation was significantly lower in cells isolated from young patients.*

3.2.9.4 Expression of pluripotency-related genes

Since the global DNA methylation status was reduced in cells from young donors, the next step was to verify expression of pluripotency associated genes at mRNA level. The expression of OCT4A, NANOG, SOX2, KLF4, and CMYC was assessed using SybrGreen-based real time PCR and normalized to the expression for the housekeeping gene GAPDH (Figure 84).

In cells from young donors, expressions of genes NANOG and SOX2 were significantly increased by 7.97-fold (p=0.0415) and 20-fold (p=0.0415), respectively, compared with the elderly group. OCT4A, KLF4, and CMYC showed a tendency to increase their expression in the cells from young donors by up to 11.74-fold (p=0.3992), 2.48-fold (p=0.0603), and 1.08-fold, respectively (p=0.1524), again compared to the elderly donors, however without statistical significance.
Figure 84. Expression of pluripotency related genes in adMSCs originating from elderly and young donors. Statistically significant differences were observed for NANOG and SOX2 expression. Both markers had increased expression in the cells from young donors.
4 DISCUSSION

4.1 Epigenetics and vascular disease – atherosclerosis

4.1.1 DNA methylation in early and advanced atherosclerotic plaque and serum

Chromatin is a flexible structure undergoing dynamic changes throughout the whole life. These heritable alternations of chromatin - leading to changes in gene expression or phenotype without influencing the underlying DNA sequence - are called epigenetics. The most interesting feature of epigenetics is that it can be affected by environmental interactions, giving the organism a feedback from surrounding conditions. Emerging evidences implicate a spectrum of epigenetic changes in the pathophysiology of atherosclerosis.

The results of the present study showed significant changes in the epigenetic pattern in advanced atherosclerosis, especially DNA- and histone-methylation, which was the aim of the current thesis. Furthermore, epigenetic changes correlated with the severity of atherosclerosis in carotid lesions. Thus, results confirmed the important role of epigenetics in atherosclerosis.

Characterization of carotid AS plaques had shown the classical morphology of atherosclerosis lesions, as described by Herbert Stary in accordance with AHA [75, 76]. Following plaque classification two study groups were built: early and advanced stage of atherosclerosis. The early stage comprised lesions type I, II, and III. For these stages, histopathology showed typical morphology of the AS plaque with macrophages infiltration and intimal thickening [76]. For the advanced stage lesions of type V, VI, and VII were chosen. The typical morphology for each lesion type observed was lipid core (type V) accompanied by thrombotic deposits (type VI), and necrotic core with calcified lesions (VII) [75]. Type IV, the first potential clinical lesion, characterized by a massive aggregate of extracellular lipid (a lipid core) without any fibrotic cap, thrombosis or hemorrhage, was excluded from the current study because very few patients, who underwent CEA in the Department of Vascular Surgery, had this type of lesion. In addition, type VIII, consisting entirely or almost entirely of scar collagen with minimal or absent lipid deposition and small vessel lumen, was also excluded from the study. As mentioned for type IV, type VIII was also
seldom in the biobank of the Department for Vascular and Endovascular Surgery, from which the plaques were selected.

DNA methylation can affect gene expression by preventing the access of transcription factors to the CpG nucleotides. A decreased level of methylation especially in the promoter region of a gene is usually acknowledged as transcriptional active sites, while the hypermethylation status is associated with transcriptionally silent state of gene activity. So far, very little is known about the changes in DNA methylation in atherosclerotic lesions and almost nothing in carotid plaques. Therefore, at first, the global DNA methylation status was analyzed. For this purpose three repetitive elements (LINE1, Satα, and Alu1) were selected. They are acknowledged as a surrogate marker for estimating global methylation levels [214]. Repetitive elements represent a large percentage of the human genome; approximately 45% of the genome is represented by interspersed repeats from transposable elements and tandem of simple sequences (DNA satellites) or complex sequences [214].

In the current work, DNA methylation was investigated by the method of Weisenberger et al. [214], who validated MethylLight, a Taqman-based real time PCR assay based on methylation of DNA repetitive elements. The results showed that the hypomethylation of DNA was significantly associated with the severity of atherosclerosis in the analyzed carotid plaques. Compared with healthy vessels, global DNA methylation diminished to 60% in early stages of atherosclerosis and was further reduced to 30% in advanced atherosclerotic lesions. Consequently, the level of DNA methylation in the repetitive DNA sequences LINE1 and SATα might also serve as an epigenetic marker of progression of atherosclerotic lesions. These results were confirmed by blood analysis, with significantly reduced methylation of DNA from serum of patients with advanced carotid artery stenosis, compared to healthy individuals. These results confirm the work of Castillo-Diaz and colleagues [92], who observed extensive DNA demethylation in human coronary samples of atherosclerosis. Interestingly, these data were in discordance with the work of Stenvinkel and colleagues [217], who found global DNA hypermethylation in blood samples of patients with cardiovascular disease. However, Stenvinkel included patients with chronic kidney disease (CKD) and analyzed, in contrast to the present study, peripheral blood leucocytes. CKD is frequently associated with chronic inflammation, which has been already suggested to be associated with general genome hypomethylation but selective gene hypermethylation and silencing [218]. These discrepancies confirm the importance of epigenetic changes in various diseases. Mechanistically, loss of DNA methylation may have several causes. For example, it could be a consequence of abnormal cellular proliferation, a local deficiency of methyl donors, or both. Early studies have suggested that e.g. certain
premalignant conditions may lead to decreased tissue folate content and, thus, a local deficiency in methyl donors [219].

### 4.1.2 DNA methyltransferases in atherosclerotic lesions

Methylation of DNA is provided by adding a methyl group at the nucleotide, mainly at the promoter site. The methylation of DNA is directed by a family of DNA methyltransferases (DNMTs) with DNMT1, DNMT3A, and DNMT3B as only functional known members. The role of DNA methylation and DNMTs in cancer and developmental studies has been extensively examined. In contrast, the expression pattern of DNMTs in atherosclerosis is so far unknown. The results from the DNA methylation experiments in this study demonstrated significant hypomethylation in advanced atherosclerotic lesions. The next logical explanation for these changes would be alternations in the expression of DNMTs.

The results reported in this work showed that the expression of DMNT1 decreases with the progression of AS, which was in accordance with the study of Hiltunen et al. [13]. DNMT1 is a methyltransferase responsible to maintain methylation of DNA within the cell. Thus, a decrease in the expression of DNMT1 might be one of the principal causes in the reduction of the level of global DNA methylation found in the carotid tissue and in serum from patients with high graded carotid artery stenosis. DNMT1 is constitutively expressed and methylates newly replicated DNA only when the template nucleotides are methylated. Consequently, the function of DNMT1 is to maintain global methylation after replication of DNA. Our results suggest however the function of DNMT1 also in the maintenance of DNA methylation, which would explain the significant demethylation in atherosclerosis.

In agreement with results obtained for global DNA methylation and decreased expression of DNMT1, it was found that the expression of methylcytosine dioxygenase TET1 (Ten-eleven translocation 1) increases with the progression of atherosclerosis. This protein is an enzyme involved in the demethylation process, responsible for the intermediate form 5-hidroxymethylation. It catalyzes the conversion of the modified genomic base 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC). It initiates a process leading to cytosine demethylation through deamination into 5-hydroxymethyluracil (5hmU) and subsequent replacement by unmethylated cytosine by the base excision repair system playing an important role in transcriptional regulation [220]. By controlling the levels of 5mC and 5hmC, it may regulate gene expression [21, 22, 221]. In conclusion, if DNMT1 does not function properly, it does not add methyl groups to maintain methylation. In addition, if the
Demethylase TET1 has an increased function, the results are that the genome wide demethylation will be induced.

*De novo* methylation process is provided with the help of DNMT3A and DNMT3B. Notably, DNMT3A expression was not found in the analyzed samples. DNMT3B expression level was found to be decreased in the atherosclerotic tissue, again corresponding with AS progression. The expression of DNMT3B was however up to 1000-fold lower than the expression of DNMT1. These results are in agreement with the study of Spin et al. [222] and indicated a significant downregulation of DNMT3A and 3B in e.g. SMCs during de-differentiation. In contrast, the work of Yiden et al [85] correlated genome wide hypomethylation induced in *vitro* in vascular smooth muscle cells (VSMCs) by homocysteine (Hcy) with increased levels of DNMT3A and DNMT3B. Since in the current work the level of DNMT3B is decreased, it might be possible that Hcy is not the primary trigger of AS, but an intermediary step in the progression of atherosclerosis. Nevertheless, the increased levels of Hcy may contribute, together with oxidative stress, apoptosis and inflammation to the progression of AS.

4.1.3 Correlation of methylated K4 and K9 of histone H3 with smooth muscle cells, inflammatory cells, and the progress of atherosclerosis

Based on the obtained results it was concluded that global DNA methylation might be used as a potential marker for the progression of AS. However, DNA methylation is just a part of the vast epigenetic apparatus. Transcription factors can bind more easily the hypomethylated promoter region of a gene, but only if this area of genome is accessible. Histone modifications play an important role in chromatin remodeling and changes to the histone tails control the state of the chromatin. Especially histone methylation is a highly interesting modification that, depending on the site of methylation, can lead either to gene activation or silencing. Furthermore, there is clear evidence that DNA and histone methylations are tightly controlled [223]. The histone H3 with an unmethylated K4 is required for DNA methylation [224]. In addition, methylation of histone H3 on K9 participates in DNA methylation as well, mediated by DNMT1 [120]. Because de-methylation seems to be the most important modification of H3K4 and H3K9 [225], this modification was analyzed in the present study in order to find out whether it corresponds with the progression of atherosclerosis and whether these changes can be associated with a certain type of cells, e.g. smooth muscle cells or inflammatory cells.
By staining of consecutive slices it was possible to connect the presence of smooth muscle cells in all stages of atherosclerosis and control tissue with the H3K4me2 positive cells. Interesting was to observe that with the progression of AS the smooth muscle cells were weaker stained in comparison with the control tissue. The reason for this is currently unknown and must be further investigated.

In contrast to skeletal and cardiac myocytes, mature vascular smooth muscle cells (VSMC) have the capability to modulate their phenotype towards an earlier state (de-differentiate) in response to multiple environmental signals, particularly under stimuli associated with vascular injury and diseases. These “de-differentiated” VSMCs present down-regulated markers and contractile proteins. Under this phenotype the cell may migrate, proliferate, produce extracellular matrix (ECM), and remodeling factors [222]. In the current study, VSMCs positive for H3K4me2 and their decreasing expression with the progression of atherosclerosis correlates with the phenotype changes in a vascular injury that leads to AS. Since H3K4 methylation is a reversible modification [226], the de-differentiated VSMCs could be, under favorable conditions, induced to re-differentiate into normal contractile state, and methylation of H3K4 may be regarded as a potential target for drug therapy in AS.

Regarding histone H3K9 methylation, VSMCs in control tissue were almost all negative. In AS samples these cells were increasingly positive with the progression of the disease. H3K9me2 is a marker known to be acting as a suppressor of transcriptional activity, by involvement in the suppression of contractile proteins of the normal VSMCs. This hypothesis is reinforced by the study of Lockman et al. [227], where the overexpression of histone demethylase was associated with decreased expression of H3K9me2 and with increased expression of transgelin. The other way around, the increased expression of H3K9me2 is associated with down-regulation of transgelin in normal VSMCs. By contrast, another study [228] showed that in vitro demethylation of the K9 lysine may trigger conversion to the transcriptionally viable euchromatin during SMC differentiation. The exact
mechanism is far from being understood due to high complexity of the H3K9me2 modification involvement in many different pathways.

The epigenetic modifications of the inflammatory cells within AS plaque must not be ignored. Consecutive tissue sections from the investigated samples were stained for CD45, CD68, and H3K4me2, and matching areas were compared. It was observed that almost all CD45-positive cells and all CD68-positive cells, both in early and advanced stage, were slightly positive for H3K4me2. Additionally, by western blot analysis of the AS samples, a significant decrease in the expression of H3K4me2 was observed with the progression of AS. Di-methylation of H3K4 has been reported as a boundary marker, preventing the spread of “repressive modifications” into the active regions, or directing modifying activities to the locus in a developmentally appropriate manner [229]. Histone H3K4 di-methylation has been shown to enhance the NF-kB-dependent expression of inflammatory genes in macrophages [230]. Correlations between increased H3K4 methylation and induction of transcription are also observed at most, but not all, loci where transcription is induced. Thus, H3K4 methylation may be used to control chromatin activity [231].

Nearly all CD45 positive cells were also positive for H3K9me2 in the immunohistological analysis of corresponding areas. Additionally, the western blot analysis of the tissue samples confirmed these results, amount of H3K9me2 positive cells increased with the progression of AS and with accumulation of inflammatory cells. The study of Tachibana concludes that H3K9me2 is dispensable in the B-cell lineage and/or other differentiated cells and that B cells require minimal levels of H3K9me2 [53]. Therefore, not surprisingly, the CD68 positive cells presented same characteristics for these two histone markers, as shown in the present work. Tissue- and/or stage-specificity of leucocyte development are controlled at many genetic and epigenetic levels beyond the simple presence of H3K9me2. More important might be that hypoxia is influencing the H3K9me2 with a concomitant loss of a specific histone demethylase activity that is followed by increased H3K9 methylation [232]. In the context of AS, it is already well recognized that hypoxia plays an important role in the progression of this disease [233].

In conclusion, methylation of H3K4me2 might be associated with changes in the phenotype of VSMCs and the methylation of H3K9me2 seems to be associated with inflammatory cells in atherosclerotic lesions. The western blot analysis of fresh samples, in controls, early and advanced stage AS, revealed interestingly a decrease in the di-methylation of both H3K4 and H3K9. A possible explanation for the discrepancy between IHC and western blot might be the involvement of other epigenetic mechanisms, such as histone acetylation/deacetylation. DNA methylation and histone deacetylation play also an
important role in the regulation of the H3K4me2. A recent study on T cells showed that AZA or trichostatin A (histone deacetylase inhibitor) were able to also affect di-methylation of K4 beside their known effects on DNA methylation and histone deacetylation [225]. Such results demonstrate that the processes of DNA methylation, histone methylation, and histone acetylation are connected and more complex than currently thought.

4.1.4 Gene expression of histone methyltransferases

Methylation of histones is accomplished by histone methyltransferases (HMTs). HMTs are enzymes, which catalyze the transfer of one to three methyl groups from S-adenosyl methionine to lysine or arginine residues of histone proteins. Responsible for adding a methyl group to the H3K4 site are HMTs containing the human SET1 (hSET1) domain. Among them are MLL1, SETD1A and SETD1B. The expression analysis showed that only MLL1 increased continuously with the progression of AS.

The MLL1 gene product is required for control of vertebrate HOX gene expression and thus involved in many developmental processes [234]. It is a candidate for establishing the patterns of H3K4 methylation. MLL1 has H3K4 methyltransferase activity [58, 235], but is unable to generate tri-methylation of H3K4 in vitro [235]. Furthermore, MLL1 has links to B-cell development, as translocations in the MLL1 gene can cause malignancy in pre-B cells suggesting the involvement of H3K4 methylation pattern in malignancy [236, 237]. In addition, it has been shown that MLL1 is involved in tumor angiogenesis by recruiting and sustaining of the blood vessels, but without a direct connection to H3K4me2 [224]. There is so far no data linking MLL1 expression, methylation of H3K4, and atherosclerosis. The mutual connection might be the inflammation process. MLL1 was suggested to be recruited to its target genes by activated NF-κB and regulates their transcription [238]. In the present work, immunohistological analysis of the inflammatory cells and western blot analysis showed a decrease in the expression of the H3K4me2 with the progression of AS, but the MLL1 expression was increased. This suggests that other processes are involved in AS, and that the mechanisms are more complex involving also other methyltransferases.

The histone methyltransferase hSET1 has been shown to be differentially over-expressed in the malignant cells [239]. SETD1A and SETD1B are both components of the HMT complexes able to mono-, di-, or tri-methylate H3K4, and have non-redundant roles [239]. In carotid tissue affected by AS, as well as in control tissue, the expressions of SETD1A and SETD1B were undetectable. This would suggest that these two methyltransferases are not playing an important role in the vascular wall. SETD1B was reported to methylate H3K4 only if H3K9 was not already methylated [240] and so affect the
methylation of H3K9. Moreover, *SETD1B* was shown to be down-regulated at the beginning of VSMC de-differentiation [53, 56]. In agreement with the presented results, methylation at K9 might occur ahead of the methylation of K4, thus being hindered by the first process.

HMTs responsible for di-methylation of K9 are SUV39H1 and EHMT2 (also known as G9a). Their expression was not detected in this study, independent of the tissue analyzed. During the de-differentiation process of the VSMCs, *EHMT2* (*G9a*) was shown to be down-regulated in the first stages of the differentiation and up-regulated in the late phase. In B lymphocytes, G9a serves as the major H3K9 di-methyltransferase, but is not the only HMT involved in this process since the global H3K9me2 was reduced in the absence of G9a but not absent [53, 241].

### 4.1.5 Conclusion

In summary, genomic hypomethylation occurs with the progression of atherosclerotic lesions. This is in a direct correlation with the failure to function of the methyltransferase DNMT1. In agreement with the increased expression of *TET1*, associated with a demethylation at the nucleotide level, the DNA is globally hypomethylated. Thus, the synergetic effect of DNMT1 and TET1 might be the main mechanism leading to DNA hypomethylation. As for histone modifications, the other component of the epigenetic code, di-methylation of H3K4 and H3K9 decreases with the progression of the atherosclerosis. Furthermore, H3K4me2 was mainly observed to exist in vascular smooth muscle cells and H3K9me2 was associated with inflammatory cells. Since the expression of *SETD1A* and -B was not found in atherosclerotic lesions, it can be concluded that they do not play a major role in the methylation of H3K4. MLL1 seems to have a compensatory mechanism to methylate H3K4. However, other mechanisms might also have implications in maintaining this modification. Methyltransferases SUV39H1 and G9a were not found to be involved in the methylation of the H3K9 in atherosclerosis. In conclusion, the presented results have brought new insights to understanding the epigenetic changes that occur within vascular disorders, especially atherosclerosis.

### 4.1.6 Future directions

The necessity to understand human epigenome has already been well recognized. Much effort has been addressed to associate changes in epigenetics with cancer progression and malignancy. In contrast, the cardiovascular field has not yet experienced such attention. The first results achieved in this field, as also confirmed by data of the current study, are promising and can contribute to better understand the mechanisms leading to
progression of atherosclerosis and other CVDs [242-245]. A particularly challenging task specific to atherosclerosis will be to define the epigenome of every cell type participating in the vascular lesion – VSMCs, macrophages, immune cells, and endothelial cells at different stages of the disease. A next task would be to incorporate these data into clinical studies, addressing the level of CVDs risk associated with specific epigenome. It is likely that epigenetic changes imposed by environmental and nutritional factors may contribute to the disease progression and could also serve as target to improve future therapeutic strategies.
4.2 Epigenetics and regenerative medicine

Vascular grafts are currently under a continuous development and so far they cannot reproduce the biologically sophisticated functions of native vessels. Currently available synthetic vascular grafts require a pre-treatment with anticoagulants due to the need to control thromboembolism, and the bio-prosthetics undergo structure deterioration and calcification [190]. Also for soft and hard tissue there remains the challenge of reconstruction, implantation, and plastic surgery. Nowadays, there is a high need for a suitable reconstruction of vessels (e.g. carotid artery), soft tissue (e.g. skin, liver, lung), and hard tissue (e.g. bone) defects resulting after atherosclerosis plaque removal, deep burns, trauma, or tumor resection. The biggest challenge of tissue engineering is to find a suitable cell or tissue application that is provided with a vascular network, which has the purpose to sustain and support the functions of the tissue.

4.2.1 AdMSC population characterization

Mesenchymal stem cells, compared to somatic cells, show attributes like plasticity, adherence and have a high proliferation rate, thus being suitable for induction of pluripotency. Until now it was considered that the main source of MSCs is the bone marrow, from which the cells can be isolated for experimental and clinical use. However, it was recently discovered that MSCs can also be obtained successfully from other sources such as adipose tissue [246]. In contrast to the bone marrow, the adMSCs are easily available. They can be isolated from larger quantities of adipose tissue, thus yielding higher amounts of cells.

Proper isolation of adMSCs is necessary to gain a homogenous cell population for reliable experimental data. At present there is still a lack of standardized methods for isolation of these cells [246]. Furthermore, there are still no definitive cell surface markers for a clear identification of adMSCs. Therefore, prior to treating the adMSCs with epigenetic drugs, the cells isolated from adipose tissue in this study were characterized appropriately. With regard to the intended experiments, different passages were tested concerning cell proliferation, viability, and commonly used negative and positive markers of this cell population. AdMSCs possess a non-hematopoietic phenotype and should be therefore negative for the hematopoietic markers CD14 and CD45. This cell characteristic was confirmed by FACS analysis revealing less than 2% hematopoietic cells in the isolated adMSC population. Positive markers for adMSCs include cell-surface molecules CD90 and CD105. CD90 (THY-1) is a GPI-anchored cell-surface protein and is used as a marker of stem cells [247]. CD105 (ENDOGLIN) is a part of the TGF-beta receptor complex and has been found on endothelial cells, activated monocytes/macrophages, fibroblasts, smooth
DISCUSSION

muscle cells, and also on mesenchymal stem cells [248]. The isolated adMSCs were highly homogenous for these markers, with more than 85% positive cells.

Antigen Ki-67 is a nuclear protein that is associated with cellular proliferation and ribosomal RNA transcription [249]. The cell population isolated from adipose tissue presented more than 50% proliferating cells. However, it is to note that adMSCs from different donors varied markedly in their proliferative state.

Moreover, the studied adMSCs were positive for the intermediate filament vimentin, which is known to be expressed in mesenchymal cells. Vimentin, together with tubuline-based microtubules and actin microfilaments are forming the cellular cytoskeleton. This marker is generally used as developmental marker for cells and tissues [250].

The best characteristics concerning proliferation, viability, homogeneity, and structural composition of adMSCs were found for passage three. Therefore, this passage was used for the whole experimental design.

4.2.2 **Differentiation potential as part of cell characterization**

Another feature of MSCs is their differentiation potential. They should be capable to achieve adipogenic, osteogenic, and chondrogenic characteristics using proper cultivation conditions. In perfect agreement with other studies [251], adMSCs used in the present work showed the ability to differentiate towards the adipogenic and osteogenic lineages.

4.2.3 **Epigenetic changes produced by epigenetic modifying drugs led to increase in expression of pluripotency related genes**

So far, several approaches have been used to generate stem-like cells from adult human somatic cells. In this study a novel strategy was followed to induce pluripotency by altering epigenetics pattern using epigenetic-modifying drugs 5-azacytidine (AZA), BIX-01294 (BIX), and valproic acid (VPA). These chemical compounds have been already used on cancer cells in order to modify their epigenetic pattern, with benefits for the clinical outcome [252].

Human adMSCs were treated with increasing concentrations of all three epigenetic drugs to find the optimal sub-toxic dosage. With the exception of AZA, the other epigenetic-modifying drugs BIX and VPA achieve their best effect on epigenetics using their maximal sub-toxic concentrations. Using AZA no significant toxicity was observed up to a concentration of 40 µM, independent of the tested treatment time. However, maximal
reduction in the global DNA methylation was achieved at 2.5 and 5 µM after 48 hours. Therefore, these conditions were also used for the experiments in this study. In contrast to BIX and VPA, AZA is unstable in aqueous solution [141], which could explain the low toxic effect of AZA on adMSCs in present experiments.

AZA is a chemical analogue of cytidine and is incorporated into DNA during cell replication. The incorporation inhibits methyltransferase activity, thereby causing DNA demethylation. AZA concentrations of 5, 10 and 50 µM showed already a significant reduction of DNA methylation on mouse embryonic fibroblasts without any changes in the relation between cell growth and cell viability [253]. These data confirm the present experimental results with no toxic effect of AZA on adMSCs for up to 40 µM [151]. Using AZA, the results demonstrated significant reduction in DNA methylation by 50%, compared with non-treated controls. However, regarding expression of the pluripotency markers analyzed in this study, such as POU5F1, NANOG, KLF4, SOX2 and CMYC, no significant changes at mRNA levels were observed for KLF4 and CMYC following treatment of adMSCs with AZA. In case of POU5F1, an increase in expression for up to 1.84-fold was found. Following BIX treatment significant reduction in global DNA-methylation for up to 70% was observed. These were interesting results because BIX-01294 influences especially histone methylation at the position H3K9 by inhibiting G9a-histone methyltransferase [5, 31]. BIX was already tested on different cells such as mouse embryonic stem cells and HeLa cells with a concentration of 4.1 µM, leading to the most efficient demethylation at the histone level [124]. One could suppose that G9a-histone methyltransferases also influence the expression of DNMTs (dinucleotide methyltransferases) as described [148, 254], and may therefore change DNA methylation [255]. Also, the G9a-histomethyltransferase was reported to indirectly mediate the expression of POU5F1 during cell differentiation influencing both global DNA methylation and expression of pluripotency genes. The presented results confirmed this presumption, observing not only significant reduction in DNA methylation but also a significant increase in the expression of pluripotency genes POU5F1, NANOG, and CMYC by 5.41-, 3.04-, and 2.39-fold respectively. These data presume that the specific inhibition of histone methyl tranferases (HMTs) by BIX is more involved in the activation of the specific pluripotency genes than in inhibition of DNMTs (by AZA). However, similar to the effect of AZA, the expression profile of the pluripotency genes strongly depended on the individual donors.

In contrast to AZA and BIX, no significant changes in DNA methylation were observed following treatment of adMSCs with VPA. The possible explanation is that VPA, although inhibiting histone deacetylases (HDAC), does not influence DNMTs or HMTs, which are known to positively regulate gene expression. The choice for using VPA was based on the
supposition that there is a common regulation mechanism between HDACs and HMTs [252]. Histone acetylation plays an important role in the regulation of gene expression. A study in mouse models demonstrated that only a subset of genes (7%) is deregulated by HDACs [256]. In addition, some studies have shown that VPA can also be used as a differentiating agent [157]. The results in the present study led to the assumption that the expression of long interspersed nuclear elements \textit{LINE1} is not regulated by HDACs and that in adMSCs VPA may induce a more undifferentiated state by up-regulation of stemness modulating genes.

Pluripotent embryonal carcinomas are good potential models to study the mechanisms that control differentiation during embryogenesis, or even as positive control for specific pluripotency related factors [257]. The pluripotent human embryonal carcinoma cell line NTERA-2 clone D1, also known as NT2/D1, is a sub clone derived from the parent line NTERA-2. These cells have been cited to be positive for all pluripotency related markers verified also in the present study: POU5F1, NANOG, SOX2, KLF4, and CMYC [258]. Therefore mRNA from these cells was used as positive control for the quantitative expression analysis.

Interestingly, \textit{KLF4} and \textit{CMYC} native expressions in adMSCs were 1000- to 2000-fold higher than native expressions of \textit{POU4F1} and \textit{NANOG}, and were comparable with the expression levels in NTERA-2 cell line used as positive control. This is probably due to the feature of adMSCs, which are not yet fully differentiated, to have high proliferative potential and therefore possess some properties of stem cells [259]. The CMYC protein is a transcription factor that activates expression of a great number of genes involved in the control of DNA replication [260]. Thus, CMYC activation may result in cell proliferation and growth. KLF4 plays an important role in regulation of cell growth, differentiation and embryogenesis [261]. Generally, KLF4 has an inhibitory effect on cell proliferation in adult tissue [262]. However, KLF4 has also been shown to cooperate with CMYC to influence stem cell self-renewal [262]. In pluripotent cells, however, KLF4 may suppress apoptosis induced by CMYC. The CMYC in turn may neutralize the cytostatic effect of KLF4 by suppressing P21 [258]. Thus, the balance between KLF4 and CMYC might be essential in the establishment of an immortalized state of induced pluripotent stem cells.

Interesting is the fact that both \textit{CMYC} and \textit{KLF4} expressions were not affected by BIX, which led to the hypothesis that G9a does not affect the expression of these two oncogenes, or they are regulated by an independent mechanism.
Again, as already mentioned above, these results strongly depended on the individual donor. Moreover, no correlation was observed between the expression of the pluripotency genes and patient characteristics, such as demographic data and known accompanying diseases. The only link seemed to be the amount of proliferating cells, which corresponds with the age of each donor.

4.2.4 **Epigenetic changes induced by BIX improve the endothelial differentiation**

So far, several approaches have been used to generate endothelial cells from mesenchymal stem cells. In the present study a novel strategy was followed, by which the potential of adMSCs to differentiate into cells with endothelial features was significantly improved.

The hypothesis was based on the assumption that modifying the epigenetic pattern with the epigenetic modifying drug BIX, the most effective epigenetic compound found in the present study, allows the cells to enter a pre-differentiation state and from this point they could be directed to different cell types under appropriate cell culture conditions. To verify this theory, cells were divided into two groups: “EM” – cells cultivated in endothelial medium, and “EM plus BIX” – cells that received a pre-treatment with BIX, and then were cultivated in endothelial medium.

To evaluate the capacity of adMSCs to differentiate into ECs were analyzed known vascular-specific markers. VEGF is a critical regulator of vascular formation that binds to VEGFR-2. It initiates the formation of immature vessels by vasculogenesis or angiogenic sprouting by cell mitogenesis and migration. In the current study, increased levels of VEGF were found both after 7 and 14 days in the BIX plus EM group.

Other factors necessary for vascular formation are angiopoetins [263]. ANG-1 is required for further remodeling and maturation of the initially immature vessels. In the present findings this marker was significantly increased after 14 days in “BIX plus EM” group. ANG-2 is the antagonist of ANG-1, binding to the same receptor – TIE-2. The results showed a significant decrease of ANG-2 in BIX-treated cells, decrease that is naturally occurring during endothelial differentiation and new blood vessel formation.

Active endothelial cells express also VCAM-1 during angiogenesis [264]. This is why the cells were further characterized for VCAM-1 expression and localization. As expected, following pretreatment of adMSCs with BIX, expression of this endothelial marker was significantly increased as well.
PDGF is involved in the maturation of nascent vessels and in the recruitment of pericytes and smooth-muscle cells that coat the new vessels. It is produced by smooth-muscle cells, activated macrophages, and endothelial cells [265]. In comparison to the non-treated cells, the expression of PDGF was increased in the “EM plus BIX” group.

Von Willebrand factor (vWF) is a glycoprotein present in blood plasma and produced constitutively in endothelial cells by the Weibel-Palade bodies [266]. The primary function of vWF is to bind other proteins, especially FACTOR VIII (F8), and is important in platelet adhesion to injured sites. Factor VIII is bound to vWF while inactive in circulation, and is released from vWF by the action of thrombin. F8 and vWF are often used as specific markers for endothelial cells. The differentiated adMSCs were positive for vWF, which is usual for all endothelial cells, and by quantification of the expression of the F8 gene it has been observed that BIX treatment induced a significant increase in expression of this marker. In a previous study by Collazo et al. [111] was shown that 7 days of differentiation was enough to increase the expression of vWF, but not of VCAM-1. In the present work it is shown an improvement of VCAM-1, F8, and also VEGFR-2 expression after 7 and even more after 14 days of differentiation, if the cells received BIX as a pre-treatment. Furthermore, the cells treated with epigenetic drug BIX were positive also for the CD31 (PECAM-1) marker. In contrast, undifferentiated cells showed no specific staining for these markers.

The functionality of the endothelial cells was investigated with the ac-LDL uptake assay. Dil-Ac-LDL is taken up via the “scavenger” cell pathway of LDL metabolism and is characteristic for endothelial cells [267, 268]. Results showed that the cells of “BIX plus EM” group are able to metabolize the ac-LDL. The assay performed on cells of the “EM” group was also positive, but with clearly fewer positive cells. These results are in agreement with the above described data regarding the expression of endothelial related genes in both groups.

In summary, BIX treatment led to an overall increase in expression of endothelial and angiogenic markers, facilitating the differentiation process of adMSCs into endothelial cells.

4.2.5 Endothelial cells differentiated from BIX pre-treated adMSCs for vascular grafts

Nowadays there are several vascular grafts used for bypasses. Unfortunately the grafts manufactured from synthetic material possess low patency rates, with high risks of trombogenicity, occlusion, infection, and lack of self-healing in comparison with the autologous grafts. First, decellularization of vessels has been investigated as a promising
solution in replacing the synthetic scaffolds [269]. Furthermore, the epigenetically modified adMSCs were tested after differentiation towards endothelial cells for their capacity to attach to decellularized saphenous vein. Two days after being seeded onto the grafts, cells displayed good attachment and adaptation to the new environment at the surface of the scaffolds. After 7 days in culture, there were an increased number of cells on the construct that were organized in multiple layers. These results were in agreement with previous, where autologous cells were seeded onto a similar scaffold [270, 271]. These first results confirmed the usefulness of natural decellularized scaffolds and the possibility to seed autologous differentiated endothelial cells. Further studies must be performed, to see whether the endothelial characteristics are kept under these conditions.

4.2.6 Cell proliferation, DNA methylation, and pluripotency markers related to donor age

Finally, a special concern was dedicated to the source of the mesenchymal stem cells. A high variation in the gene expression and global DNA methylation was observed in adMSCs. However, no correlation was found between the demographic data, gender or medical history of the donors. Until now, several studies have reported variances in the differentiation potential of adult stem cells originating from donors with different ages [272, 273]. In accordance with these studies an increase in the osteogenic differentiation potential was observed also in the adMSCs of the “young donors” group. No differences were found for the adipogenic differentiation. Thus, the adult stem cells originating from younger donors show an increase in the reprogramming capacity towards osteogenic lineage. Additionally in the present study was found an increased number of Ki67 positive adMSCs in the group of “young donors”. Currently there are only few studies that have investigated the amount of proliferating adMSCs originating from young and elderly donors [171, 274]. The difference in the number of the proliferating cells may be partly responsible for the variability in the differentiation potential of adMSCs.

Furthermore, analyzed cells from younger donors had a decreased global DNA methylation level compared with cells from elderly donors. DNA methylation is one of the main components of epigenetics. An alteration in its pattern is associated with a change in the reprogramming capacity of the cells, a decreased level being associated with an increased transcription activity. This hypothesis is supported by the study of Hupkes et al. [275], where a decreased global DNA methylation, and subsequently a change in the epigenetic pattern, directs the differentiation of mouse myoblasts from muscle to bone cells. Similarly, in the present work, the decreased level of DNA methylation in adMSCs from younger donors presented an increased differentiation potential into osteoblasts.
Nevertheless, no studies have found so far any connection between global DNA methylation status or expression of pluripotency related genes and the age of the donors in adMSCs. Somatic cells have already been reprogrammed into pluripotent stem cells by introducing a combination of several transcription factors (TFs), such as Oct3/4, Sox2, Klf4 and c-Myc [276]. Thus, an increased expression in these TFs is associated with an increased reprogramming capacity. In the present study the decreased level of genomic methylation is in agreement with the statistically significant increased expression of pluripotency related genes NANOG and SOX2, in the cells from the “young donors” group. The promoters of pluripotency genes such as nanog and Oct4 have been showed that are stably silenced by DNA methylation in somatic cells in mice [277], which likely interferes with transcription factor binding and gene activation during reprogramming. Similarly, this process might occur in adMSCs from elderly donors, where the reprogramming genes might be silenced through DNA methylation, therefore they possess a decreased differentiation potential in comparison with the cells from younger donors.

In summary, proper epigenetic changes might reverse the effect of aging on cells from elderly patients. These cells could then be used with a greater success rate in cell therapies based on autologous transplantation.

### 4.2.7 Conclusion

For future therapeutic developments of autologous and allogenic pluripotent stem cells, adMSCs are a promising cell source that can be easily harvested. Furthermore, a chemical approach for targeted epigenetics might be a feasible and safe method to improve pluripotency of somatic cells. In the present study, the epigenetic-modifying drugs AZA and BIX were able not only to significantly reduce global DNA methylation in adMSCs but also to increase the expression of genes for pluripotency such as POU5F1 and NANOG. Thus, reactivation of the native pluripotency genes by epigenetic changes using chemical compounds might represent a promising approach in future regenerative medicine. Furthermore, due to epigenetic changes, BIX was able to enhance the differentiation of adMSCs into cells with endothelial features. The treatment with the epigenetic modifying drug BIX is also able to decrease global DNA methylation. These epigenetic changes were responsible for the increase of the expression of the pluripotency related genes. Subsequently, expression of endothelial and angiogenic markers was increased. Further studies are necessary to investigate the feasibility of applying these cells in vivo, by e.g. producing autologous vascular grafts.

### 4.2.8 Future directions
In order to better understand and manipulate the epigenetic pattern, the present study has to be continued by analysis of other histone modifications, improvements of the differentiation capacity of adMSC, examination of other epigenetic processes such as ATP-dependent chromatin remodeling and DNA methylation at specific promoter regions in adMSCs, nuclear packaging and localization of gene loci of specific cell types (e.g. endothelial cells) in adMSCs. The inherent plasticity of the adMSC lineage has presented remarkable insights and unique challenges regarding the understanding of the control of cellular differentiation. This might bring research closer to optimization of differentiation towards particular tissues that are required in regenerative medicine. Cells that are epigenetically modified to change from one lineage to another, without insertion of foreign nucleic material, could be a safe and ethically approved way to an almost unlimited choice of tissue engineering applications.
5 SUMMARY

The present work evaluated two aspects of epigenetics: involvement in vascular disease and applicability in regenerative medicine.

5.1 Epigenetics and vascular disease

Epigenetic regulation of gene activity is a fundamental mechanism that occurs in all eukaryotic cells and is indispensable for development, tissue regeneration or maintaining of cell phenotype. Defects in epigenetics have already been connected to various diseases. DNA and histone methylation significantly contribute to the regulation of gene expression. So far, no sufficient data are available about epigenetic modifications in atherosclerotic lesions.

In the current study, carotid plaques of patients with high grade carotid artery stenosis in early (type II-III, n=20) and advanced (type V-VII, n=20) stage of atherosclerosis (AS), as well as 10 control vessels, and 10 healthy individuals were included. Carotid lesions were characterized by histology and immunohistochemistry (IHC). Global DNA methylation was analyzed by Methylight assay. Expressions of DNA methyltransferases (DNMT1, DNMT3A, and DNMT3B), demethylase TET1, and histone methyltransferases (HMT) MLL1, SED1A, and SETD1B for H3K4 and SUV39H1, and G9a for H3K9 were analyzed by quantitative real time PCR. Methylation of histone H3 at lysine K4 and K9 in atherosclerotic plaques was evaluated by western blot and by IHC in correlation with smooth muscle cells and inflammatory cells.

Global DNA methylation in carotid plaque decreased continuously with the progression of AS and correlated significantly with the genome-wide hypomethylation of free DNA from blood serum in patients with carotid artery stenosis. Expression of DNMT1 was decreased, DNMT3A and DNMT3A exhibited very low expression within AS. In contrast, TET1 expression was found to increase with the progression of the disease. Tissue slices stained for smooth muscle cells were found also to be positive for H3K4 and the intensity of staining decreased with the progression of AS. Smooth muscle cells were found mostly negative for H3K9 in all study groups. Inflammatory cells, positive for CD45 and CD68, were only slightly stained for H3K4, both in early and advanced stage of AS. The amount of inflammatory cells positive for H3K9 increased with the severity of AS. Western blot analysis revealed decreased level of methylation for H3K4 and H3K9, negatively corresponding with the progression of AS. Regarding HMTs responsible for methylation of lysine K4 analyzed in
this study, expression of *MLL1* increased in correlation with the severity of AS. *SETD1A* and *SETD1B* were not expressed in the analyzed samples. HMTs responsible for methylation of lysine K9, *SUV39H1* and *G9a*, showed no expression in AS specimens or in controls.

In conclusion, the level of DNA methylation and histone methylation was found to be significantly associated with the severity of atherosclerosis in patients with carotid artery stenosis. Thus, epigenetic changes seem to play a substantial role during atherosclerotic plaque progression.

### 5.2 Epigenetics and regenerative medicine

Chromatin remodeling plays an essential role in regulation of gene transcription. Thus, appropriate changes in the chromatin of somatic cells may lead to the induction of pluripotency. The aim of the present study was to evaluate the effect of epigenetic drugs 5-azacytidine (AZA), BIX-01294 (BIX), and valproic acid (VPA) on DNA methylation and expression of pluripotency genes *OCT4A*, *NANOG*, *KLF4*, *SOX2*, and *CMYC* in human adipose-derived mesenchymal stem cells (adMSCs) and to evaluate their potential to differentiate into cells with endothelial characteristics.

AdMSCs were isolated from human abdominal adipose tissue and characterized morphologically, by FACS analysis, and regarding their osteogenic and adipogenic differentiation potential. Viability assay (MTT) was used to determine the optimal dosage of epigenetic modifying drugs on adMSCs. Global DNA methylation was determined by MethyLight assay, and expression of pluripotency and endothelial genes by SYBRgreen-based real-time PCR. Immunocytochemistry and western blot were used in order to characterize the differentiated cells into endothelial lineage analyzing various endothelial and angiogenic factors such as vWF, VCAM-1, PECAM, VEGFR-2, Ang-1, and -2.

The optimal population of adMSCs with the best homogenous properties was found in passage three of culture, with 86% of cells positive for CD90, CD105 and 98% negative for CD14, CD45. The optimal sub-toxic concentrations of AZA, BIX, and VPA were observed after 48 hours of treatment using 2.5, 1.25, and 100 µM, respectively. Global DNA methylation status of adMSCs treated with the epigenetic modifying drugs was significantly reduced by BIX and AZA but was not affected by VPA. Pluripotency related genes *OCT4A*, *SOX2*, and *NANOG* showed significant increased expressions after treatment with BIX. AdMSCs cultured under endothelial conditions and pre-treated with BIX were observed to undergo changes in the phenotype towards endothelial cells. Up to 20% of cells were positive for endothelial and angiogenic markers tested in this study. The functionality of the
adMSC-derived endothelial cells was verified by their capacity to take up ac-LDL. Experiments concerning autologous grafts showed that decellularized native saphenous veins remained stable and adMSCs-derived endothelial cells were able to attach to this scaffold.

In conclusion, epigenetic modifying drugs AZA and BIX were able to significantly reduce global DNA methylation. Furthermore, BIX led also to a significant increase in the expression of genes for pluripotency and following differentiation the expression of endothelial and angiogenic genes. These adMSC-derived endothelial cells showed all characteristics of an endothelial lineage. Thus, targeted epigenetic modification using appropriate chemical compounds might represent a promising and feasible approach in regenerative medicine.
6 REFERENCES


70. *Cardiovascular diseases (CVDs)* 2011, World Health Organization.


REFERENCES


REFERENCES


7 APPENDIX

7.1 Abbreviations

Official gene symbols in this work were mentioned according to the Guidelines for Human Gene Nomenclature found at http://www.GeneNames.org/Guidelines.html.

5hmC 5-hydroxymethylcytosine
5mC 5-methylcytosine
acetylCoA acetyl Coenzyme A
adMSCs adipose derived mesenchymal stem cells
AHA American Heart Association
ALP alkaline phosphatase
Alu1 DNA repetitive sequence
ANG-1 Angiopoetin 1
ANG-2 Angiopoetin 2
APAAP Alkaline Phosphatase-Anti Alkaline Phosphatase
APC allophycocyanin
AS atherosclerosis
ASVD artherosclerotic vascular disease
AZA 5-azacytidine
BAT brown adipose tissue
BCIP 5,5'-dibromo-4,4'-dichloro-indigo phosphate
BIX BIX-01294
BM-MSCs bone marrow mesenchymal stem cells
CEA carotid endarterectomy
CMYC transcription factor essential for achieving pluripotency
CVDs cardiovascular diseases
Dil 1,1'-dioctadecyl – 3,3',3',3'-tetramethyl-indocarbocyanine perchlorate
DiL-Ac-LDL acetylated low density protein labeled with DiL
DMEM Dulbecco's modified Eagle medium
DMSO dimethyl sulfoxide
DNMT1 DNA Methyl Transferase 1, maintenance of the DNA methylation
DNMT3A DNA Methyl Transferase 3A, de novo methylation
DNMT3B DNA Methyl Transferase 3B, maintenance of the DNA methylation and de novo methylation
DNMTs DNA methyltransferases
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>dsDNS</td>
<td>double stranded DNA</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>ECs</td>
<td>endothelial cells</td>
</tr>
<tr>
<td>EHMT2</td>
<td>methyltransferase able to methylate lysine 9 of the histone H3</td>
</tr>
<tr>
<td>EM</td>
<td>endothelial medium</td>
</tr>
<tr>
<td>ESC</td>
<td>embryonic stem cells</td>
</tr>
<tr>
<td>F8</td>
<td>Factor VIII, role in blood clotting</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorter</td>
</tr>
<tr>
<td>FFPE tissue</td>
<td>formaldehyde fixed paraffin embedded tissue</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>G9a</td>
<td>methyltransferase able to methylate lysine 9 of the histone H3</td>
</tr>
<tr>
<td>GAPDH</td>
<td>housekeeping gene, Glyceraldehyde-3-Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>H&amp;E staining</td>
<td>Haematoxylin - eosin staining</td>
</tr>
<tr>
<td>HATs</td>
<td>histone acetyltransferases</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylases</td>
</tr>
<tr>
<td>HIER</td>
<td>heat induced epitope retrieval</td>
</tr>
<tr>
<td>HMTs</td>
<td>histone methyltransferases</td>
</tr>
<tr>
<td>ICC</td>
<td>immunocytochemistry</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>iPSCs</td>
<td>induced pluripotent stem cells</td>
</tr>
<tr>
<td>KLF4</td>
<td>transcription factor essential for achieving pluripotency</td>
</tr>
<tr>
<td>LINE1</td>
<td>long interspersed nuclear element 1, repetitive sequence</td>
</tr>
<tr>
<td>LSAB</td>
<td>Labeled Streptavidin-Biotin</td>
</tr>
<tr>
<td>MLL1</td>
<td>methyltransferase able to methylate lysine 4 of the histone H3</td>
</tr>
<tr>
<td>MSCs</td>
<td>mesenchymal stem cells</td>
</tr>
<tr>
<td>NAD+</td>
<td>nicotinamide adenin dinucleotide</td>
</tr>
<tr>
<td>NANOG</td>
<td>transcription factor essential for achieving pluripotency</td>
</tr>
<tr>
<td>NBT</td>
<td>nitroblue tetrazolinum</td>
</tr>
<tr>
<td>NCDs</td>
<td>non communicable diseases</td>
</tr>
<tr>
<td>OCT4A</td>
<td>transcription factor essential for achieving pluripotency</td>
</tr>
<tr>
<td>ox-LDL</td>
<td>oxidized low-density lipoprotein</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-Derived Growth Factor</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerytrin</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>Platelet Endothelial Cell Adhesion Molecule 1</td>
</tr>
<tr>
<td>PIER</td>
<td>proteolytic enzyme induced epitope retrieval</td>
</tr>
<tr>
<td>PKMTs</td>
<td>protein lysine methyltransferases</td>
</tr>
</tbody>
</table>
PMTs  protein methyltransferases
PRMTs  protein arginine methyltransferases
PTFE  polytetrafluoroethylene
PVDF membrane  polyvinylidene difluoride membrane
qPCR  quantitative PCR
RT  room temperature
RT-PCR  reverse transcriptase polymerase chain reaction
SAH  S-adenosyl-L-homocysteine
SAM  S-adenosyl-methionine
SATα  repetitive sequence
SDS  sodium dodecyl sulphate
SETD1A  methyltransferase able to methylate lysine 4 of the histone H3
SETD1B  methyltransferase able to methylate lysine 4, and 9 of the histone H3
SMCs  smooth muscle cells
SOX2  transcription factor essential for achieving pluripotency
TET  enzyme with demethylating function
TF  transcription factor
VCAM-1  Vascular Cell Adhesion Molecule 1
VEGF  Vascular Endothelial Growth Factor
VEGFR-2  Vascular Endothelial Growth Factor Receptor-2
VPA  valproic acid
VSMCs  vascular smooth muscle cells
WAT  white adipose tissue
WHO  World Health Organization


7.2 Publications

7.2.1 Original articles


7.2.2 Oral presentations


7.2.3 Poster presentations


Culmes M, Pelisek J., Schmitt M., Wagner E., Nüssler AK., Eckstein HH. (2011) Inhibition of G9a Methyltransferase is positively influencing the reprogramming capacity of adipose derived mesenchymal stem cells into cells with endothelial features, Rediscovering pluripotency: from teratocarcinomas to embryonic stem cells, Cardiff, UK.


Culmes M., Grabher-Meier V., Hegenloh R., Wagner E., Eckstein HH., Pelisek J. (2012) Targeted epigenetic changes induce a higher reprogramming capacity of adipose derived mesenchymal stem cells to differentiate into cells of endothelial features, INTERACT Symposium, Munich, Germany.

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