Epigenetic regulation of gene expression in the vascular endothelium

Mia Magnusson

UNIVERSITY OF GOTHENBURG

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mia.magnusson@gu.se

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ABSTRACT

Epigenetic mechanisms, such as DNA methylation and histone modifications, constitute one way for a cell or an organism to respond to changes in the surrounding environment. While histone modifications are recognized to be quite dynamic, DNA methylation has been considered a more stable, or long-term, modification.

Ischaemic heart disease and stroke are major causes of morbidity and mortality in the Western world. In the majority of cases, these conditions are caused by intra-arterial clot formation, which can occur because the components of the haemostatic system are out of balance. This can be caused by either genetic or life-style issues. With this thesis, I have focused on epigenetic regulation of genes in endothelial cells, specifically the *PLAT* gene which encodes the key fibrinolytic enzyme tissue-type plasminogen activator (t-PA).

In Study I, we found that the expression from *PLAT* was induced when endothelial cells were treated with the histone deacetylase inhibitor valproic acid (VPA), and that this indeed was associated with increased acetylation levels around the t-PA promoter. In patients, a defective t-PA expression results in an increased risk of suffering from myocardial infarctions, and the findings in Study I open up for a new possible treatment regimen.

In Study II and III, we used sub-culturing of primary human umbilical vein endothelial cells (HUVECs) as a model of environmental challenge to study how this affects the DNA methylation level, around the t-PA gene (Study II) as well as genome-wide (Study III). In Study II, we found that the DNA methylation level decreased in the t-PA enhancer, but not in the promoter nor in the region immediately upstream of the promoter. This enhancer demethylation was in strong negative correlation with an increase in t-PA gene expression. Thus, methylation in the t-PA enhancer may constitute a previously unrecognized way to regulate the expression of this essential fibrinolytic enzyme.

In Study III, we went on to examine how sub-culturing of HUVECs changed the genome-wide methylation level. We discovered that to passage 4, almost 2% of the investigated sites showed dynamic methylation, mostly displaying decreasing levels. The majority of the differentially methylated sites (DMSs) were annotated as “enhancer”. In addition, we found that several gene ontology terms were highly enriched for among the genes with DMSs situated in their enhancers. Taken together, this indicates that the demethylation process was not random, and that it occured quite fast.

We suggest that the fibrinolytic enzyme t-PA is dynamically regulated on a transcriptional level by both histone acetylation and DNA methylation, which is important in order for the production of this key enzyme to be able to be rapidly modified locally. We also propose that DNA methylation in endothelial cells is more dynamic than previously recognized, as high levels rapidly can be erased.

Keywords: epigenetics, histone acetylation, DNA methylation, t-PA, *PLAT*, HUVECs, valproic acid, gene expression, enhancers

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LIST OF ORIGINAL PAPERS

This thesis is based on the following papers, referred to in the text by their Roman numerals:

I Larsson P, Ulfhammer E, Magnusson M, Bergh N, Lunke S, El-Osta A, Medcalf RL, Svensson PA, Karlsson L, Jern S. Role of Histone Acetylation in the Stimulatory Effect of Valproic Acid on Vascular Endothelial Tissue-Type Plasminogen Activator Expression.
PLoS One. February 2012;7(2): e31573.

II Magnusson M, Lu EX, Larsson P, Ulfhammer E, Bergh N, Carén H, Jern S. Dynamic Enhancer Methylation - A Previously Unrecognized Switch for Tissue-Type Plasminogen Activator Expression.
PLoS One. October 28, 2015;10(10):e0141805.

III Magnusson M, Larsson P, Lu EX, Bergh N, Carén H, Jern S. Rapid and specific hypomethylation of enhancers in endothelial cells during adaptation to cell culturing.
Submitted.

OTHER RELEVANT PUBLICATIONS NOT INCLUDED IN THIS THESIS

Ulfhammer E, Larsson P, Magnusson M, Karlsson L, Bergh N, Jern S. Dependence of Proximal GC Boxes and Binding Transcription Factors in the Regulation of Basal and Valproic Acid-Induced Expression of t-PA.
Int J Vasc Med. 2016;2016:7928681.

Larsson P, Bergh N, Lu E, Ulfhammer E, Magnusson M, Wåhlander K, Karlsson L, Jern S. Histone deacetylase inhibitors stimulate tissue-type plasminogen activator production in vascular endothelial cells.
J Thromb Thrombolysis. 2013 Feb;35(2):185-92.

van der Pals J, Götberg MI, Götberg M, Hultén LM, Magnusson M, Jern S, Erlinge D. Hypothermia in cardiogenic shock reduces systemic t-PA release.
J Thromb Thrombolysis. 2011 Jul;32(1):72-81.

SAMMANFATTNING PÅ SVENSKA

Epigenetik är den del av genetiken vilken behandlar förändringar i genuttryck som inte beror på att själva DNA-sekvensen ändrats. De två främsta epigenetiska mekanismerna anses vara histonmodifieringar och DNA-metylering. Vi vet att yttre faktorer, exempelvis förändringar i den omgivande miljön, kan medföra modifieringar av de epigenetiska mekanismerna. Dessa modifieringar är ofta ärftliga och kan föras vidare när en ny cell bildas. De kan också ärvas mellan generationer, och på så vis kan miljöfaktorer komma att påverka hur våra gener uttrycks under en lång tid framöver.

Hjärt- och kärlsjukdomar, som till exempel hjärtinfarkt, utgör idag den ledande dödsorsaken i västvärlden. En hjärtinfarkt beror på att en blodpropp helt eller delvis täpper till de kärl som förser hjärtat med syre, vilket medför att en del av hjärtat drabbas av syrebrist och riskerar att skadas. I kroppen finns ett system som syftar till att bilda blodproppar för att stoppa akut blödning (koagulationssystemet), medan ytterligare ett system ser till att blodpropparna löses upp när skadan är läkt och proppen fullgjort sin uppgift (det fibrinolytiska systemet). Dessa två system måste vara i balans för att vi ska må bra. Vävnadsspecifik plasminogenaktivator (t-PA) är en mycket viktig del av det fibrinolytiska systemet, och man vet att den som har för lite t-PA har en ökad tendens att bilda blodproppar, vilket har till följd att risken för att drabbas av exempelvis hjärtinfarkt ökas flerfaldigt. Man vet också att både ärftliga och livsstilsberoende faktorer kan leda till minskade t-PA-nivåer. Bland de livsstilsberoende faktorerna finns bland annat övervikt, rökning och högt blodtryck.

Med denna avhandling avsåg jag dels att utforska om (och hur) t-PA-genen regleras av de epigenetiska modifieringarna histonacetylering och DNA-metylering, och dels försöka ta reda på hur snabbt DNA-metyleringen i de endotelceller som kantar blodkärlsväggen egentligen förändras när cellerna utsätts för en drastisk miljöförändring (cellodling). I delstudie ett såg vi att mer t-PA bildades efter att odlade endotelceller behandlats med valproinsyra (VPA), och att detta berodde på att histonacetyleringsnivåerna runt genen ökade. Med tanke på att VPA redan ges till patienter skulle detta kunna vara en möjlig väg att stärka det kroppsegna försvaret mot blodproppar. I delstudie två tittade vi istället på hur DNA-metyleringsnivåerna ser ut runt t-PA-genen. Vi fann då att metyleringen specifikt i t-PA-enhancern, ett område som man sedan tidigare känner till är viktigt för förmågan att bilda t-PA, snabbt sänktes när endotelcellerna odlades. Detta var också starkt kopplat till att mer t-PA producerades. Med denna studie kunde vi för första gången visa att t-PA-genen regleras av DNA-metylering. Då DNA-metylering traditionellt har ansetts vara en stabil mekanism ville vi, som en direkt konsekvens av resultaten från delstudie två, analysera hur påverkbar DNA-metyleringen generellt är i endotelceller. Vi fann att den totala metyleringen sjönk flera procent när endotelcellerna odlades och att det, precis som för t-PA-genen, framförallt var i enhancrar som metyleringen ändrades. Detta indikerar att demetyleringsprocessen inte sker slumpvis, och att den går snabbare än man tidigare trott.

Sammantaget behandlar avhandlingen epigentisk reglering (histonacetylering och DNA-metylering) av t-PA-genen, samt innehåller en analys av generell DNA-metylering i endotelceller för att undersöka hur dynamisk metyleringen är i en differentierad celltyp.

CONTENTS

ABBREVIATIONS

A	Adenine
ANOVA	Analysis of variance
BER	Base-excision repair
BSP	Bisulphite sequencing
C	Cytosine
ChIP	Chromtin immunoprecipitation
CpG	Cytosine-phosphate-guanine
DMSs	Differentially methylated sites
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
ELISA	Enzyme-linked immunosorbent assay
G	Guanine
GO	Gene ontology
HAT	Histone acetyl transferase
HCAEC	Human coronary artery endothelial cell
HDAC	Histone deacetylase
HUVEC	Human umbilical vein endothelial cell
PAI-1	Plasminogen activator inhibitor -1
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SAM	S-adenyl methionine
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
T	Thymine
TET	Ten-eleven translocation
TIS	Transcription initiation site
t-PA	Tissue-type plasminogen activator
VPA	Valproic acid
VPM	Valpromide
5-hmC	5-hydroxymethylcytosine
5-mC	5-methylcytosine

INTRODUCTION

The complex interplay between an organism and its surrounding environment has been important in the development and evolution of all life. The ability to adjust and adapt, which is determined by genetic constitution and physiologic tolerance, determines the persistence of a species [1]. Environmental changes can be drastic alterations to the external environment, which an organism has to be able to adjust to in order to survive. However, environmental changes can also occur within the organism itself, such as the force of increased blood flow on the endothelial cells that line the blood vessel walls.

Environmental adaptation can result in ***genetic*** changes, through the shifting of allele frequencies within a population (even with relatively short generation times). This may occur because individuals less suited to the environment are more unlikely to reproduce than individuals that are better suited, as stated by Charles Darwin in the mid-19th century [2]. In contrast, a changed environment may also induce ***physiological*** alterations, which occur rapidly. One such example is the activation of intracellular signal transduction pathways, which can help the cells cope with an altered environment. Such changes are swift, and readily reversible. However, a changed environment may also cause alterations that are more long-lasting, while not as permanent as genetic changes. Those are chemical alterations to the DNA or the chromatin structure, so called ***epigenetic*** modifications, which can be inherited through successive rounds of replication and even between generations. In that way, epigenetic mechanisms can alter gene expression states in a more long-term manner. Already in the late 18th century, the French biologist Jean-Baptiste Lamarck formulated a theory of how an organism can pass on characteristics that have been acquired during a lifetime to its offspring. This theory, called Lamarckism, was dismissed by many leading scientists. However, with the rise of epigenetics, we may well be able to prove Lamarck *partially* right, almost two hundred years after his death [3].

Basic genetics

Historical background

Genetics is defined as the study of heredity [4]. Although heritability has been recognized for a long time, the history of modern genetics started in the 19th century with the work of the Augustinian monk Gregor Mendel (1822-1884). He performed hybridization experiments with garden peas to study the inheritance of distinct traits such as colour of the flower and height of the plant. Mendel, who was trained in physics and mathematics, concluded that the traits were inherited in units – now referred to as genes [5]. In 1910, Morgan showed that the genes are located on specific chromosomes (reviewed in [6]) and in 1953, Watson and Crick could demonstrate the molecular structure of deoxyribonucleic acid (DNA) [7].

Current view - DNA and genes

Today, we know that eukaryotic DNA (that of animals, plants, fungi, and protists) consists of four bases: adenine (A), thymine (T), cytosine (C), and guanine (G). A and

T, and C and G form complementary base pairs which are linked together by hydrogen bonds. In addition, each nucleotide (consisting of a sugar residue, a phosphate group, and a base) is connected to the neighbouring nucleotide via covalent phosphodiester bonds, thus making up two DNA strands which are complementary and run in opposite directions. The strands twist around each other, together forming a double helix. Each cell contains approximately two meters of DNA, compacted and organised by protein structures called histones. The histones and the DNA are further organised into nucleosomes, each consisting of eight histone proteins with about 146 base pairs of DNA twisted around them. DNA and associated proteins are referred to as chromatin. The chromatin is further systematised into 23 chromosome pairs which harbour approximately 25,000 genes [8, 9].

The classical view of a gene is that it can be translated into a protein. Since the late seventies, we have known that a gene is constituted of both exons and introns; while exons code for the amino acids which make up the actual protein, introns are non-coding elements that get spliced off [10, 11]. The gene regulatory region includes a promoter situated 5' of the transcription start site, but also elements located farther away, such as enhancers and silencers. However, in recent years, the definition of a gene has become more complex. Part of the reason for this is the discovery of alternative splicing, which means that the exons may be put together in different orders, in turn yielding different proteins [12, 13]. There is also evidence of overlapping genes, and genes within other genes [14, 15]. In addition, we now know that plenty of the RNAs encoded by the human genome not are translated into proteins – instead, microRNAs and other RNA molecules may have functions themselves, for example in controlling cellular processes [16]. (For reviews, see [17, 18]).

The central dogma of molecular biology

The flow of genetic material from DNA to RNA to polypeptide is often referred to as the central dogma of molecular biology [19, 20]. ***Replication*** is the process in which new DNA is synthesized. In short, the DNA helix is unwound by a helicase, which enables the binding of a polymerase and subsequent synthesis of new daughter strands. ***Transcription*** is the term used for the transfer of information stored within the DNA into messenger RNA (mRNA), a process which is governed by RNA polymerases and transcription factors. ***Translation*** describes the process in which the information encoded by the mRNA is used to create a protein. This is performed by the ribosome, and takes in eukaryotic cells most often place in the cytoplasm (unlike replication and transcription, which occur within the cell nucleus) (Figure 1).

Epigenetics

Historical background

Whether the environment has the ability to affect gene expression remained a matter of debate for several decades until in 1956, Conrad Waddington described how environmentally acquired characteristics can be inherited in a population [21]. Subsequently, he gave the phenomenon a name: epigenetics, which is Greek for outside, or above, genetics [22, 23]. Epigenetic mechanisms include histone modifications, DNA

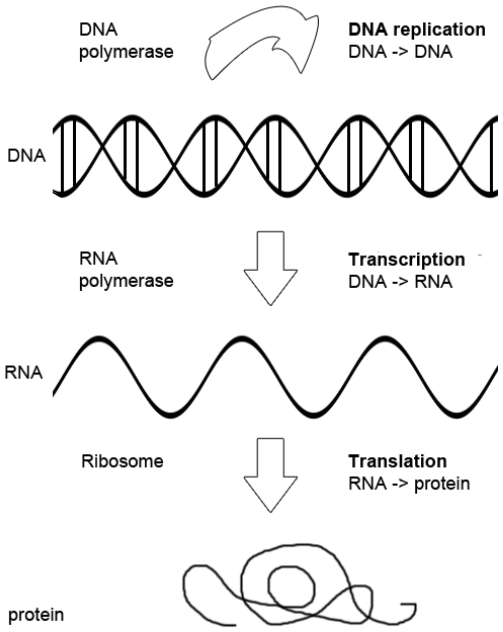


Figure 1. The central dogma of molecular biology. The flow of DNA to RNA to polypeptide (protein).

methylation, and non-coding RNAs [24, 25]. However, over the years, epigenetic research has come to focus mainly on histone modifications and DNA methylation [26].

Post-translational modifications of histones were first studied in the 1960s, when Allfrey described that acetylation, methylation, and phosphorylation of lysine residues seemed to be actively involved in transcription control [27]. In 1997, the structure of the nucleosome was determined with X-ray, indicating that basic amino N-tails protrude from each nucleosome and make contact with adjacent nucleosomes [28].

The first report on a biological role for DNA methylation came in 1969, when Griffith and Mahler proposed that it was involved in long term memory formation [29]. In 1975, two papers independently described that DNA methylation is important in the switching of genes on and off, and in cell differentiation [30, 31].

Current view - Histone modifications

The complexity of the many histone modifications are just beginning to be uncovered, however we now understand that they are essential to numerous biological processes that involve the expression of DNA.

Histone acetylation is recognized to be quite dynamic, being regulated by the opposing actions of two families of enzymes: the histone acetyltransferases (HATs) and the

histone deacetylases (HDACs). HATs catalyse the transfer of acetyl groups to histone lysine residues, which leads to a neutralization of the positively charged lysine. This results in weakened interactions between histones and DNA, and hence a more "permissive" chromatin state which allows the exposure of transcription factor binding sites. HDACs, in contrast, can reverse lysine acetylation, thus restoring the positive charge which leads to compaction of the chromatin (for a review, see [32]).

However, HDAC modifications do not only work through changing the electrostatic interactions between histones and DNA. According to the "histone code hypothesis", the regulatory information contained within the particular combinations of histone marks is "read" by a specific protein (or protein complex) that regulates gene expression accordingly [33, 34].

Among the other histone modifications are methylation, phosphorylation, ubiquitylation, and sumoylation, which however are not covered by this thesis.

Current view - DNA methylation

Today, DNA methylation has been extensively studied; in February 2016, a search on PubMed (http://www.ncbi.nlm.nih.gov/pubmed) returned over 50,000 results. DNA methylation is known to be essential to all mammalian development, and has been recognized as a main contributor to the stability of gene expression states.

The emerging picture is that the function of DNA methylation varies with the context in which it is found. DNA methylation in promoter regions represses gene expression (either by recruiting methyl binding proteins that are involved in gene repression, or by directly inhibiting the binding of transcription factors to DNA [35, 36]), while methylation in gene bodies instead is associated with a higher level of gene expression [37-39]. Gene body methylation may also have an impact on splicing. (For reviews, see [40, 41]).

In mammals, DNA methylation is a post-replicational modification, which primarily can be found on cytosines in the context of CpG dinucleotides (a cytosine residue situated upstream of a guanine). It is maintained by a family of DNA methyltransferases (DNMTs) that transfer the methyl group from the methyl donor s-adenosylmethionine (SAM) to the fifth carbon of the cytosine residue [42]. In a simplified version, DNMT1 manages maintenance methylation, while DNMT3a and DNMT3b are *de novo* methyltransferases that set up methylation patterns in early development [41].

Indeed, DNA methylation plays an important role during early embryonic development. Within hours of fertilization, a wave of demethylation strips the paternal genome of methylation, and by the time of implantation, the genome becomes *de novo* methylated [43-45]. However, while dynamic DNA methylation has been described in the developmental context, as well as during differentiation [46] and linage commitment [47], differentiated cells have traditionally been considered to display stable methylation levels.

Passive demethylation has been recognized for quite some time, but despite that evidence has pointed toward the existence also of an active demethylation process, the search for demethylases has been long one. Recently, it was discovered that the ten-eleven translocation (TET) enzyme family can oxidize 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) [48-50], which may be diluted in a passive manner through successive rounds of replication [51], or further oxidized and subsequently actively excised through the base excision repair (BER) pathway [48]. However, it is very likely that additional, not yet identified, pathways of demethylation exist [52].

DNA methylation and histone modifications interact through methyl binding proteins such as MBDs, UHRF proteins, and MeCP2. Both MBDs and UHRF proteins are known to interact with methylated DNA and histones to reinforce gene repression [53-57], and MeCP2 recruits histone deacetylases which further contribute to the repression of gene transcription [53, 58, 59]. Thus, it seems that DNA methylation and histone modifications work closely together to regulate gene expression (Figure 2).

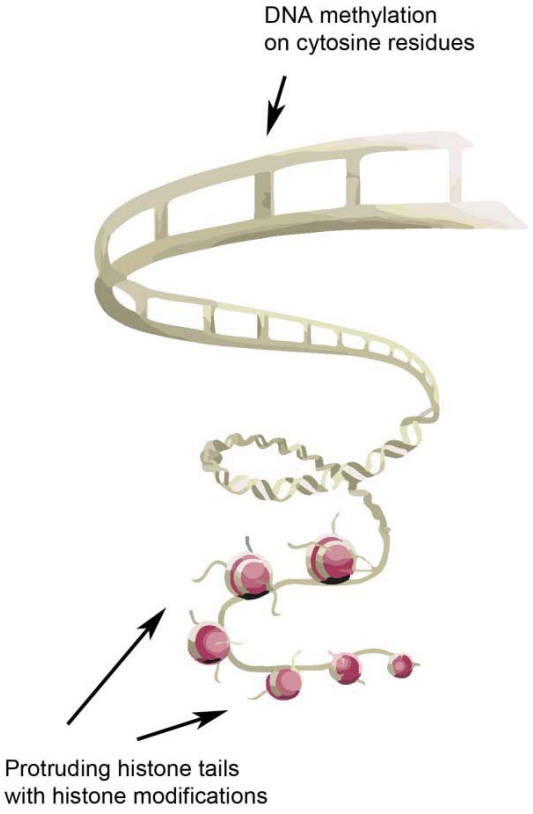


Figure 2. Histone modifications and DNA methylation. While DNA methylation is found on the single base-level, the histones and associated DNA look like “beads on a string”.

Coagulation and fibrinolysis

Historical background

Coagulation is an evolutionarily conserved biological process crucial to each organism with a blood flow, as it has the ability to limit blood loss in case of an injury or a wound. As early as 400 B.C. the father of medicine, Hippocrates, noted that blood that was removed from the body congealed as it cooled. This so-called cooling theory prevailed until 1627, when Mercuralis observed that clots in veins could form at body temperature [60].

The clot dissolving process was first described in the early 19th century when scientists found that blood did not coagulate after sudden deaths caused by for example electricity, or if animals "are run very hard, and killed in such a state" [61]. In the late 19th century, this clot dissolving process was given the name "fibrinolysis" [62].

Current view – coagulation and fibrinolysis

Upon vascular injury, sub-endothelial matrix proteins such as collagen, von Willebrand factor, and fibronectin are exposed. This leads to the activation of circulating platelets, which ultimately results in activated thrombin converting soluble fibrinogen into insoluble fibrin. The result of this process is haemostasis - the restriction of blood flow through the damaged area.

However, in order not to compromise the blood flow through the vessel lumen, the size of the thrombus must be restricted. This is performed mainly through the actions of tissue-type plasminogen activator (t-PA). t-PA catalyses the conversion of inactive plasminogen, which circulates in plasma, into active plasmin. Plasmin, in turn, degrades the fibrin strands, which limits clot expansion and eventually leads to clot dissolution (reviewed in [63]).

Tissue-type plasminogen activator (t-PA)

The t-PA gene
The t-PA gene, *PLAT*, is situated on chromosome 8 at p11.21 and consists of 14 exons encoding 527-530 amino acids. The end product, the t-PA protein, is a serine protease with a molecular weight of approximately 71,000 daltons [64-67].

In the t-PA promoter, two differential start sites have been identified: one TATA-dependent and one TATA-independent. However, in all cell types tested, the TATA-independent initiation site is the predominant one. In endothelial cells, the transcription rate from the TATA-independent start site is approximately ten-fold higher [68, 69]. Therefore, in this thesis, the positions in the t-PA gene and its regulatory region are given relative the TATA-independent (major) initiation site.

The t-PA enhancer was identified in 1995 by Bulens and co-workers. They discovered a region several thousand bases upstream of the t-PA start site that mediated the t-PA response to retinoic acid [70]. Subsequently, a 900 bp minimal enhancer spanning between -7.1 and -8.0 kb relative the t-PA transcription start site was defined [71].

Transcriptional regulation

Several regulatory elements can be found in the t-PA promoter as well as in the enhancer (Figure 3). In the text below, the positions are given according to the original publications (in relation to the major initiation site). However, it is worth noting that according to updated genome assemblies, all positions can be remapped a few bases.

In the promoter, three GC boxes (GC box I, II, and III) have been identified. It is known that TATA-independent promoters often are driven by transcription factors, including the Sp-family members, that assemble at GC-boxes [72]. Indeed, GC-box II (bp -71 to -65) and III (bp -48 to -42) in the t-PA promoter have been shown to bind Sp1 [69, 73, 74], and t-PA gene expression levels have been found to correlate with transcription factor binding to GC box III [75]. A CRE-like site (bp -222 to -214), which binds transcription factors of the AP-1 and CREB/ATF-families, has also been shown to be important for both basal and induced expression from the t-PA promoter in endothelial cells [69]. In addition, a consensus site for the binding of NF1 has been described (bp -202 to -187) [73].

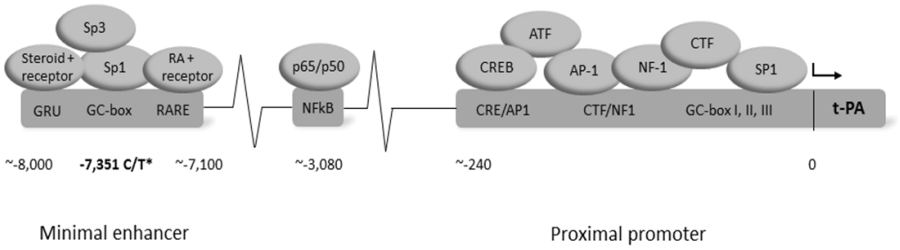


Figure 3. The t-PA regulatory region. The t-PA proximal promoter and minimal enhancer, with binding sites and transcription factors depicted. Using updated genome assemblies will shift all positions slightly, and thus the positions in Figure 3 are given as approximations. *= the -7,351 C/T enhancer polymorphism is denoted according to its original mapping, but has been remapped to -7,355.

Regulatory elements located further upstream of the t-PA promoter include a κB-element (bp -3,081 to -3,072) [76], as well as the t-PA enhancer. The enhancer can be activated by glucocorticoids, progesterone, androgens, and mineralocorticoids, i.e. by all classical steroid hormones except for oestrogen (through the GRE elements situated at bp -7,501, -7,703, -7,942, and -7,960). In addition, the enhancer region contains a retinoic acid response element (RARE) localised at -7,319 to -7,303 [71], as well as an Sp1-binding GC box which is involved in both basal and induced t-PA expression (bp -7,355 to -7,346) [74]. This GC box is the place for the single nucleotide polymorphism (SNP) -7,351 C/T, which previously was identified by our group [77]. The T allele, which breaks up a CpG site, has been found to convey a lower t-PA release *in vivo* [78].

t-PA release and functional inhibition

The endothelial cells that line the blood vessel walls are the main source of t-PA. They can release t-PA in two ways: either in a constitutive or in an acute, regulated manner. While constitutive release occurs in order to maintain a basal t-PA level in the blood,

regulated release has the ability to drastically increase local levels of biologically active t-PA. Regulated release can be initiated as a response to substances originating from activated platelets, or to products that are produced during the process of coagulation [79].

However, most of the t-PA in the circulation is present in an inactive form, as serine protease inhibitors (serpines) are available in excess concentrations in plasma [63, 80]. The principal inhibitor of t-PA is plasminogen activator inhibitor-1 (PAI-1), which forms stable 1:1 stoichiometric complexes with t-PA [81, 82]. This reaction is highly efficient, and hence the relative amount of free (un-bound) t-PA in plasma is small [83]. However, only free t-PA has fibrinolytic activity. The concentration of free t-PA in the blood is determined partly by the inhibition through PAI-1, but also by the rate of t-PA secretion from the endothelial cells and the hepatic clearance of t-PA [84]. In addition, t-PA needs fibrin for its activation; without fibrin, t-PA is a poor initiator of fibrinolysis [67, 85]. These factors ensure that plasmin activation is restricted to fibrin-containing areas, so that random plasmin activation does not occur in plasma.

Role of t-PA outside the fibrinolytic system

Besides functioning as an activator of intravascular fibrinolysis, t-PA also plays an important role in the central nervous system, where it contributes to synaptic plasticity and learning [86-88]. In the peripheral nervous system, t-PA protects from demyelination by removing fibrin deposits near the damaged nerves [89]. In addition, t-PA is possibly a contributor to angiogenesis (reviewed in [90]).

Impairment of t-PA synthesis

t-PA expression can be genetically impaired. For several decades, it has been known that familial decreased capacity for acute t-PA release leads to early-onset thrombotic events, mainly in the venous circulation [91-94]. In addition, we know that individuals carrying the T allele of the -7,351 C/T enhancer polymorphism has a reduced ability for transcription factor binding to the GC box in which the polymorphism is situated. Indeed, the T allele conveys a lower capacity for t-PA expression and release and, as expected, individuals carrying this allele have an increased risk of suffering from myocardial infarctions [95, 96]. This demonstrates that sufficient t-PA production is important in maintaining vascular patency also on the arterial side of the circulation.

t-PA expression can also be functionally impaired; several risk factors associated with atherothrombotic disease have been shown to reduce the capacity for regulated release. One example is hypertension [83, 97, 98], which is associated with decreased capacity for t-PA release at least in part as a consequence of increased mechanical stress on the endothelium [99, 100]. Overweight/obesity, especially in combination with a sedentary lifestyle, is also associated with reduced t-PA release [101-103]. Cigarette smoking reduces t-PA release capacity in the forearm [104-106] as well as in the coronary circulation [107]. Atherosclerotic coronary artery disease and coronary atherosclerotic burden have also been found to be associated with reduced coronary artery t-PA release [107, 108].

Epigenetic regulation of t-PA

Despite that classical gene regulation has been well studied, not much is known about epigenetic regulation of the t-PA gene. At the time of onset of this thesis work, very little information was available regarding epigenetic regulation of the t-PA gene. A few previous studies had reported that the t-PA gene may be sensitive to changes in histone acetylation status, and that short chain fatty acids cause an increase in the transcription level of the t-PA gene [109-111]. This was important to investigate further, as it is essential to understand more about how the gene encoding this essential fibrinolytic enzyme is regulated. In the future, a therapy including HDAC inhibitors could even help restore a defective endogenous t-PA production.

Only one study has (briefly) investigated the DNA methylation pattern in the t-PA promoter [111] but no study has examined the methylation pattern in the enhancer. Thus, this called for further investigation.

HUVECs and methylation

For experimental purposes, umbilical veins are the most common source of primary human endothelial cells; HUVECs are readily available, free of degenerative pathological processes, and often more physiologically relevant than cell lines. Endothelial cells in culture are widely used as a model for studying the biology of haemostasis, inflammation, and blood vessel wall dynamics [112, 113]. In a wider perspective, cell culturing could be viewed as a model of extreme environmental change or challenge; quiescent endothelium from a blood vessel is placed in a foreign environment, where it is stimulated to divide until it covers the plastic surface. One way for the cells to adapt to the new surroundings, and to be able to grow, could be through changing the DNA methylation level at specific features and/or genes. Still, there are no studies where the immediate effects of cell culturing on the methylome have been studied. This would be valuable to know, partly because it would give a clue as to how reliable a model cell culturing is, but mainly because it would indicate how stable, or dynamic, DNA methylation really is.

AIMS

To examine epigenetic gene regulation in endothelial cells, we investigated:

- if the expression of the key fibrinolytic enzyme t-PA is regulated by histone modifications (specifically histone acetylation) (Study I)

- if the DNA methylation level in the t-PA gene regulatory region is affected during the sub-culturing of endothelial cells (which may function as an environmental challenge) (Study II)

- if the genome-wide DNA methylation level is affected when endothelial cells are sub-cultured, and specifically which subset of genes and genomic regions that are affected (Study III)

MATERIALS AND METHODS

Cell culture

Human umbilical vein endothelial cells (HUVECs) were used in all studies in this thesis. In addition, certain experiments in Study I were verified in human coronary artery endothelial cells (HCAECs). While HCAECs were purchased from Lonza (Basel, Switzerland), HUVECs were extracted from fresh umbilical cords obtained from the Sahlgrenska University hospital delivery ward. The HUVECs were removed with collagenase treatment as described by Jaffe *et al* [114]; in short, the umbilical vein was catheterized under sterile conditions, and blood remaining inside was removed with the flushing of lukewarm PBS. After incubation with 0.1% collagenase followed by gentle manipulation of the umbilical cord, the HUVECs could be extracted.

HUVECs were maintained in complete endothelial cell culture medium (EGM-2 from Lonza), supplemented with 2% foetal bovine serum and growth factors. HCAECs were grown in EGM-2 medium supplemented with foetal bovine serum to a final concentration of 5%. The cells were maintained at 37°C in a humidified 5% CO_2-incubator, and sub-cultured by trypzination when at approximately 90-100% confluency. HUVECs were grown at the most to passage 4, while experiments with HCAEs were performed in passage 5.

Experimental design – Study I

This study aimed at determining the effect of valproic acid (VPA) on the expression of t-PA, and further to examine if the observed increase in expression seemed to be related to the HDAC-inhibitory function of VPA. The HUVECs were seeded in plastic culture flasks or plates, and grown to confluence. VPA (Sigma-Aldrich, St Louis, MO, USA) was diluted in EGM-2 medium to a 0.3 M stock solution, and kept at -70°C. Valpromide (VPM) from Alfa Aesar (Karlsruhe, Germany) was diluted in DMSO to a stock concentration of 1.5 M, and stored at -20°C. Confluent cells were subsequently exposed to optimal concentrations of VPA or VPM for up to 72 h (with fresh medium and new VPA/VPM added every 24 h), after which cells and conditioned media were harvested. All cell culture experiments were performed in duplicate, on at least three individuals (unless otherwise stated).

Experimental design – Study II and III

These studies aimed at determining the DNA methylation status in the t-PA gene regulatory region (Study II) as well as genome-wide (Study III) in primary (non-cultured) and cultured endothelial cells.

Immediately after the HUVECs were extracted, potentially contaminating cell types were removed by cell sorting with MACS magnetic beads (Milteny Biotech, Bergisch Gladbach, Germany) directed against the endothelial cell surface antigen CD105. One fraction of the sorted cells was subsequently extracted for methylation analysis, while

the rest of the cells were placed in culture (as previously, the cells were grown in plastic culture flasks). At each passaging, one cell fraction was extracted for methylation analysis, while the rest were placed back in culture.

For mRNA experiments, HUVECs were collected either from the same individuals or from additional individuals (depending on the amount of material available). Cells for mRNA analysis were seeded according to the same procedure, and sub-cultured at corresponding time-points.

Analyses – Principles and methods

Methylation analyses

Bisulphite sequencing PCR (BSP)

Principle

Bisulphite sequencing PCR (BSP) was used to analyse methylation status in the t-PA gene regulatory region (Study II). BSP, which was developed by Frommer *et al*, relies on the ability of sodium bisulphite to convert unmethylated cytosines to uracils while methylated cytosines remain unaffected [115, 116]. In this method, the bisulphite converted DNA is subjected to conventional PCR in order to amplify the desired fragment, followed by Sanger sequencing of the amplicon. During the PCR reaction, the uracils will be replaced by thymines, and thus the sequencing result will reveal whether a specific cytosine is methylated (displayed as cytosine) or unmethylated (displayed as a thymine). However, when the entire PCR product is sequenced, the result for each site will be an average of all cells included in the analysis. Thus, the result can be anywhere between 0 and 100% methylation.

Because of the limitations inherent to the Sanger sequencing technology, such as the different fluorochromes coupled to the different bases having unequal intensities, a correct estimation of methylation status cannot be obtained by simply measuring the peak heights in the raw data from the sequencing reaction. Instead, in each PCR reaction, we chose to co-amplify an unmethylated and a methylated control sample as well as 30/70, 50/50, and 70/30 mixtures of the two, which subsequently are sequenced along with the samples. This serves as a standard curve which allows a compensation for the above mentioned problem, as well as a control for selective amplification of unmethylated or methylated fragments during the PCR reactions. Methylation status is assigned semi-quantitatively after visual comparison between the sample and the standard curve.

Method

DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer´s instructions. Up to 500 ng of DNA was subsequently bisulphite-converted with the EZ DNA methylation kit (Zymo Research Corporation, Irvine, USA) according to the protocol provided.

BSP primers were designed with the MethPrimer software (www.urogene.org), or used as published by Dunoyer-Geindre [111]. 15-40 ng of the bisulphite-treated DNA

was subjected to 35 cycles of PCR amplification. Each fragment was first amplified with a set of outer primers, followed by amplification with a set of inner primers. This so called nested PCR can be used in order to enhance the specificity of the PCR reaction. In addition, fragment sizes were verified on 2% agarose (Sigma-Aldrich, St. Louis, MO, USA) gels supplemented with GelRed Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA). The PCR products were subsequently sequenced at Genomics Core Facility at the University of Gothenburg (Gothenburg, Sweden), and at GATC Biotech (Constance, Germany), and analysed in Applied Biosystems SeqScape Software 3 (Life Technologies, Carlsbad, CA, USA).

Pyrosequencing

Principle

Pyrosequencing was used in Study II in order to verify the original method (BSP) for methylation analysis. This is a sequencing strategy that relies on the detection of pyrophosphate (PPi) release upon nucleotide incorporation. During the sequencing process, only one of the four nucleotides is available at the time, and the intensity of the light that is emitted during the PPi release determines the number of identical nucleotides in a row. The method was developed by Ronaghi and Nyrén in the 1990's [117, 118], and has been widely used for methylation analysis [119].

The pyrosequencing template is generated by PCR amplification of the desired region. One of the PCR primers is biotinylated, which results in biotin-labelled fragments that subsequently are captured by streptavidin-coated sepharose-beads. The PCR-products are then heated to obtain single-stranded fragments, and sequenced using a specific sequencing primer.

Method

Primary, passage 0, and passage 4 HUVECs from four individuals, included also in the original methylation analysis, were analysed in two selected CpG sites in the t-PA enhancer using pyrosequencing. The PCR reaction was run using the PyroMark PCR Kit (Qiagen) according to protocol. The sequencing was performed according to the PyroMark Q24 Advanced and PyroMark Q24 Advanced CpG Reagents Handbook (Qiagen), and run on a PyroMark Q24 system. The pyrosequencing assay was evaluated with a standard curve of methylated/unmethylated template.

Hydroxymethylation

Principle

In Study III, genome-wide hydroxymethylation was quantified with the Quest 5-hmC DNA ELISA kit (Zymo Research). This is a sandwich-based ELISA approach, in which genome-wide hydroxymethylation is detected with antibodies in a microtiter plate. First, 5-hydroxymethylcytosine polyclonal antibody is coated to the bottom of the wells. Then, 100 ng of denatured DNA is added to each well, and DNA containing 5-hmC is trapped by the antibody. Subsequently, anti-DNA HRP antibody, which recognizes the bound DNA, is added to the wells. The addition of HRP developer produces a greenish-blue colour in the wells containing 5-hmC, which can be quantified in an ELISA plate reader. A control set of DNA with known percentage of 5-hmC is

included in the kit, and run along on the plate. To be able to quantify the percentage of 5-hmC in the samples, a standard curve which the samples can be compared to is generated from the controls.

Method
The level of hydroxymethylation in primary, passage 0, and passage 4 HUVECs from five individuals was quantified according to the instructions from the manufacturer (Zymo Research). All samples were analysed in duplicate. The plate was allowed to incubate for an hour, after which it was read in a plate reader at 405 nm wavelength.

Methylation array

Principle
In Study III, the Infinium HumanMethylation450 microarray (450K) (Illumina, San Diego, CA, USA) was used to determine genome-wide DNA methylation status. This is one of the most comprehensive microarray platforms available for genome-wide methylation analysis in humans. It includes 485,577 CpG sites, which cover 99% of RefSeq genes and 96% of CpG islands. The CpG sites are classified into epigenetically important genomic regions such as CpG islands, shores (0-2 kb from CpG island), shelves (2-4 kb from CpG island), and open sea (>4 kb from nearest island), as well as directly transcriptionally related features such as enhancer elements and the area around the transcription start site (TSS) [120-122].

The 450K platform combines Infinium I and II probes, which both are around 50 bases long but detect methylation by slightly different mechanisms. The Infinium I technology uses two probes for each CpG site; one corresponding to the methylated and the other to the unmethylated sequence. The last base is normally the one that matches the cytosine, and thus is the variable position. The Infinium II technology relies on the single probe two-colour approach, which utilizes that red and green fluorescently labelled single base extension occurs differentially at thymine and cytosine of bisulphite-converted DNA. The signal intensities are used to determine the methylation level [121].

Method
DNA from primary, passage 0, and passage 4 HUVECs from six individuals was extracted with the DNA Mini Kit (Qiagen), bisulphite-converted using the EZ DNA methylation kit (Zymo Research) and sent to SciLifeLab, Uppsala University, Uppsala, Sweden, for analysis on the 450K array. The hybridisation to the array was performed according to standard Illumina protocols. At least 500 ng of each sample was used.

Data analysis and normalisation (BMIQ) was done using the programming language R with the package ChAMP [123]. The methylation in each possible site was presented as a β-value, defined as the ratio of the methylation.

The differentially methylated sites were subsequently analysed for gene ontology terms with the DAVID database (https://david.ncifcrf.gov/), and visualised with a venn diagram in the Venny database (http://bioinfogp.cnb.csic.es/tools/venny/).

Gene expression analyses

Real-time RT-PCR

Principle

Real-time RT-PCR was used to quantify the levels of mRNA transcripts as a measurement of transcription and gene activity (Study I-III). In this method, the mRNA inside the cells is purified and reverse-transcribed into cDNA. In the probe-based real-time RT-PCR method that we used, the transcript of interest is amplified in a PCR reaction containing a dual-labelled probe. When the fluorescently labelled probe hybridizes to its target sequence, the Taq-polymerase cleaves the reporter dye from the probe thus releasing the reporter dye into the solution. The increase in dye emission is monitored in real time, and the threshold cycle (C_T) is analysed. C_T is defined as the cycle number at which the reporter fluorescence reaches a fixed threshold level. There is a linear relationship between the C_T value and the log-value of the initial target copy number [124]. To obtain a relative expression value for the gene of interest (the target gene), the difference in C_T value between the target gene and a reference gene (which must be validated to remain stably expressed) in the analysed sample is compared to a control sample. This can be done using the comparative C_T method [125].

Method

Total RNA was prepared using RNeasy Mini Kit (Qiagen) according to the manufacturer´s protocol, and genomic DNA was removed using RNAse free DNase (Qiagen). RNA was transcribed into cDNA using the High-capacity RNA-to-cDNA Kit (Life Technologies, Carlsbad, CA, USA). mRNA levels were analysed with real-time RT-PCR on an Applied Biosystems 7500 Fast Real-Time PCR system using TaqMan reagents from Life Technologies. t-PA was detected with Gene Expression Assay Hs00938315_m1 (Life Technologies). Hypoxanthine phosphoribosyl transferase (HPRT) (Study I) and glucoronidase beta (GUSB) (Study II and III) were used as endogenous reference genes.

Gene expression microarray

Principle

The effect of VPA on genome-wide gene expression in HUVECs was determined by microarray analysis (Study I). The principle of this method is that thousands of DNA probes are attached to a solid surface in an ordered fashion. The Affymetrix Human Gene 1.0 ST (Affymetrix, Santa Clara, CA, USA) which was used in our study contains 764,885 oligonucleotide probes representing 28,869 genes. The method is a "whole-transcript expression analysis", meaning that the probes (on average 26 per gene) span the entire genes, as opposed to previous arrays which have been 3' based [126].

Purified RNA from samples (in our case VPA-treated) and controls (non-treated) is converted into cDNA in a two-step model, which amplifies the original RNA sample. The cDNA is then fragmented, biotin-labelled, and hybridised onto the array, one sample per array. After washing, the binding of biotinylated cDNA to the probes is detected by the addition of streptavidin coupled to a fluorescent dye. The intensity of the fluorescent signal for each probe is proportional to the relative expression level of the corresponding gene.

Method
Gene expression in VPA-treated and untreated HUVECs from four individuals was analysed using the Human Gene 1.0 ST microarray (Affymetrix). Target preparation and hybridisation to the microarray were performed according to standard Affymetrix protocols at Uppsala Array Platform (Uppsala, Sweden). Raw data were analysed using the RMA (robust multi-array average) method implemented in the Affymetrix software Expression Console. Probe sets with a log2 ratio of above +1 or below -1 with a significantly changed expression ($p<0.05$, false discovery rate (FDR) adjusted p-value) were classified as regulated.

Gene ontology analysis (to extract haemostasis genes) was performed with the AmiGO database (http://amigo.geneontology.org/amigo).

Short interfering RNA transfections

Principle
To determine the relevance of the nine class I, IIa, and IV HDACs in basal and VPA-stimulated t-PA expression, each one of those HDAC enzymes was independently depleted with short interfering RNA (siRNA) (Study I). siRNA transfection is used to specifically but transiently silence the expression of a target gene, thereby shutting down the production of the protein of interest. In brief, short double-stranded oligonucleotides (21-23 nucleotides long) are introduced into the cell through transfection. The siRNA oligonucleotides subsequently associate with the RNA induced silencing complex (RISC), and guides RISC to the complementary mRNA transcripts. There, RISC cleaves and destroys the mRNA molecules, thus resulting in a knock-down of the production of the specific protein.

Method
siRNA specific for class I, IIa, and IV HDACs were obtained from Dharmacon (Thermo Fisher Scientific, Lafayette, CO, US). The day before transfection, HUVECs were plated in 24-well plates and maintained in EGM-2 medium without antibiotics. The following day, siRNA was combined with DharmaFECT 4 transfection reagent (Dharmacon) in OptiMEM medium (Invitrogen) and added to the cells. After additional 48 h, the cells were treated with either VPA or control medium. 24 h later, the cells were harvested and mRNA was extracted. To determine target mRNA reduction as well as t-PA mRNA expression, real-time RT-PCR was run. The results from the transfections were used only when target reduction was found to be over 80%.

Protein analyses

Enzyme-linked Immunosorbent Assay (ELISA)

Principle
ELISA was used to quantify t-PA antigen after various stimulations (Study I). This is a method that uses antibodies and colour change to detect a certain antigen. In the sandwich-ELISA approach that we used, samples and standards are analysed in a microtiter plate where the wells have been coated with an antibody against the antigen of interest (which often is a protein). After the antigen in the samples and standards has been allowed to bind to the antibody, another antibody conjugated with an enzyme,

often peroxidase, is added. Subsequently, the wells are washed to remove excess antibody, and a peroxidase substrate is added. The peroxidase enzyme converts the substrate to a coloured product, where the intensity of the colour is directly proportional to the amount of protein present in the sample. This can be quantified by spectrophotometry in a plate reader.

Method
Conditioned medium from cell cultures was collected and centrifuged to remove cell debris, and concentrations of t-PA antigen were determined using the commercially available Trini-Lize t-PA antigen ELISA (Trinity Biotech, Bray, Ireland) according to the manufacturer's protocol.

Western Blot

Principle
Western blot was used to detect levels of pan-acetylated as well as total H3 and H4 protein in Study I. Western blot is an analytical technique used to detect specific proteins in a sample. First, the cells to be analysed must be homogenised and centrifuged in order to extract the protein fraction. The proteins are subsequently separated (based on molecular mass) on a denaturing SDS-PAGE gel, and blotted onto a membrane. After blocking (which prevents unspecific binding), a dilute solution of the primary antibody is incubated with the membrane. When excess primary antibody has been washed away, the membrane is exposed to a secondary antibody which is directed against a species-specific portion of the primary antibody. The secondary antibody can be linked to a peroxidase enzyme, and upon the addition of a substrate, a chemiluminescent signal is emitted which can be detected by an imaging system.

Method
Membranes were incubated with primary antibodies to pan-acetylated histone H3 and H4 as well as to total histone H3 and H4. Proteins were detected according to the standard protocols and visualized using chemiluminescence.

Chromatin Immunoprecipitation (ChIP)

Principle
Chromatin Immunoprecipitation (ChIP) was used to investigate the acetylation status of the histones associated with the t-PA promoter (Study I). The principle behind ChIP is that it enables the study of DNA-protein interactions in the living cell (i.e. with an intact chromatin structure), which is in contrast to methods like electrophoretic mobility shift assay (EMSA) that are used for protein-DNA interaction studies but does not take chromatin conformation into account. In ChIP, the DNA-protein interactions are fixed with formaldehyde, which is added directly to the living cells. Subsequently, the fixed chromatin is sheared to an optimal fragment length with sonication. Antibodies against the protein or modification of interest are then added, and those that have bound protein (and attached DNA) are extracted using protein-A-coated magnetic beads. After extensive washing, the DNA fragments are liberated by protease degradation of the bound protein/antibody. Subsequently, the enriched DNA is purified, and the region of interest amplified using real-time RT-PCR. Quantification can be performed by comparing the precipitated material to two controls: one input control

(representing chromatin that has not been subjected to immunoprecipitation) and one no-antibody control (representing chromatin that has been subjected to the immunoprecipitation procedure, but without antibody added). The real-time RT-PCR signal is directly proportional to the amount of specific protein bound to the region of interest, and relative protein binding is expressed as percent of input of DNA corrected for background binding.

Method
Confluent HUVECs were stimulated with VPA or control medium for 24 h. After formaldehyde fixation and washing, the chromatin was sheared to a length of 100-500 bp (according to protocol in Study I). Each immunoprecipitation reaction was performed on 1 ug of sheared DNA. The antibodies used were directed against pan-acetylated histone H3 (K9, 14, 18, 23, and 27) (Active Motif, Carlsbad, CA, USA), and pan-acetylated histone H4 (K5, 8, 12, and 16) (Merck Millipore, Darmstadt, Germany). The following mono-lysine acetylation modifications were also detected, using a specific antibody for each modification: acH3K9, acH3K14, acH3K18, acH3K23, acH3K27, and acH4K5, acH4K8, acH4K12, and acH4K16 (Active Motif and Merck Millipore). Isolated DNA fragments were quantified with real-time RT-PCR with SYBR green detection.

Statistics

In this thesis, data are presented as mean and standard error of the mean (SEM). Statistical evaluations were performed either using a paired Student's t-test, or with analysis of variance (ANOVA). P-values of less than 0.05 were considered significant. All relevant comparisons are specified in the figure legends.

RESULTS AND DISCUSSION

Study I

With Study I, our aim was to start exploring epigenetic regulation of t-PA, which until then had been largely overlooked. However, two older studies suggested that the t-PA gene may be sensitive to changes in histone acetylation status [109, 110]. In Study I, we explored how HDAC inhibition by valproic acid (VPA) affected t-PA expression in cultured HUVECs. In individuals with low t-PA production, caused by genetic or life-style factors, a pharmacological restoration of the endogenous t-PA production and thus the capacity for t-PA release would be desirable. Because VPA already is in clinical use for another indication, this may open up for a possible treatment regimen, for the first time enabling a restoration of an impaired endogenous t-PA production.

FINDING 1: THE CLINICALLY USED HDACI VPA IS A STIMULATOR OF T-PA EXPRESSION IN ENDOTHELIAL CELLS

To examine the effect of valproic acid (VPA) on t-PA expression in HUVECs, HUVEC cultures were incubated with VPA for 24 h. This caused a significant concentration-dependent increase in t-PA mRNA expression, evident already at concentrations as low as 0.3 mM and reaching a maximum at 3-4 mM. At high VPA concentrations, t-PA mRNA level increased 9-fold after 24 h, and the amount of t-PA protein released into the medium was comparably (8-fold) induced, as determined by ELISA analysis (Figure 4 A,B).

To verify that this effect was present also in a more representative endothelial cell type, human coronary artery endothelial cells (HCAECs) were incubated with VPA. This revealed a similar response pattern as in HUVECs after 24 h (with an 8 and 5-fold increase in mRNA and protein, respectively) (Figure 4 C,D).

In order to investigate the temporal response pattern, HUVECs were exposed to a clinically relevant concentration (1 mM) as well as to the maximum *in vitro* tolerated concentration (4 mM) of VPA for up to 72 h, and t-PA mRNA levels were quantified. For the 1 mM dose, a ~4-fold steady-state induction was reached after 12 h, which remained throughout the period studied (<72 h). The response pattern for the higher dose (4 mM) was somewhat different with an initial transient peak induction of about 12-fold at 24 h, which gradually leveled-off to about 4-fold at 72 h (Figure 4 E).

Thus, we found that VPA markedly increased t-PA mRNA expression and protein secretion in two different endothelial cell types in a dose-dependent manner. Importantly, this was evident at low, clinically relevant concentrations with a significant increase already at 0.3 mM, and reaching a maximum of approximately 10-fold induction at 3-4 mM.

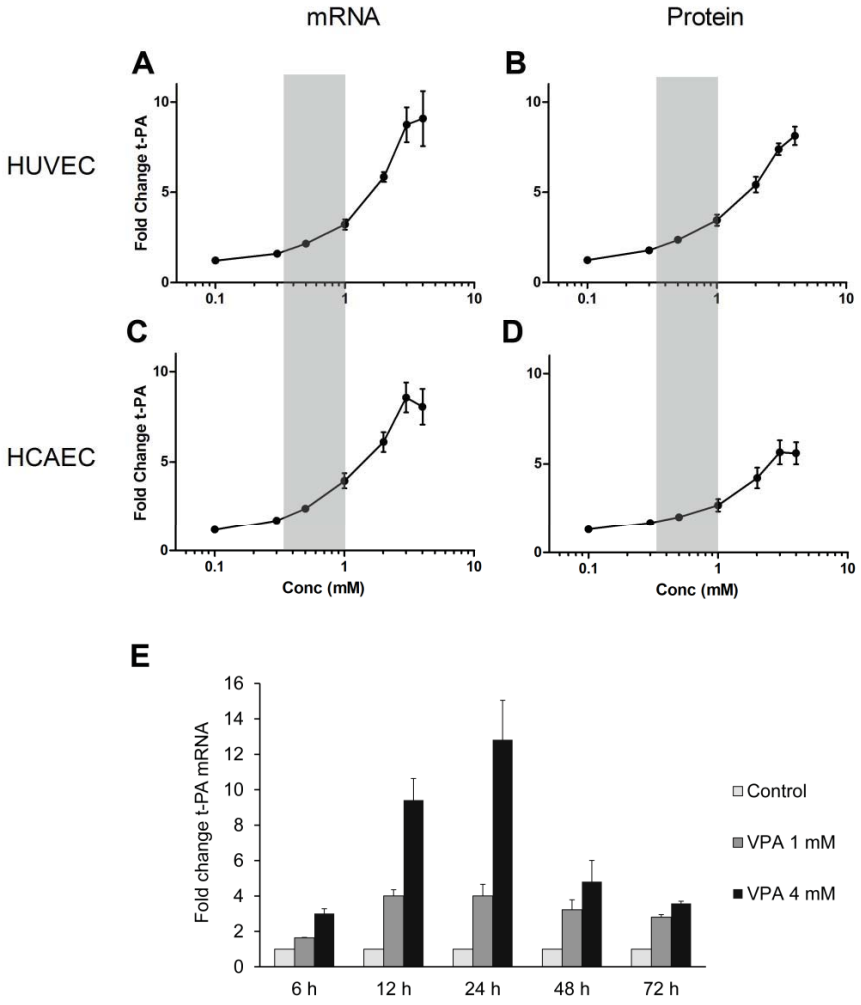


Figure 4. Effects of valproic acid (VPA) on mRNA and protein expression in HUVEC and HCAEC. HUVECs (**A** and **B**) and HCAECs (**C** and **D**) were exposed to different concentrations (0.1-4 mM) of VPA for 24 h. mRNA (**A** and **C**) was quantified with real-time RT-PCR and secreted t-PA protein (**B** and **D**) in conditioned media by ELISA. Values are expressed as fold change over control cells. The shaded areas show the plasma concentration range of VPA achieved after clinical VPA treatment. **E**. HUVECs were treated with 1 mM or 4 mM of VPA and t-PA mRNA quantified after 6, 12, 24, 48, and 72 h. The results show mean values ± SEM of three independent experiments (n=3) performed in duplicate. For Figure 4 E statistical significance was tested using 2-way ANOVA for repeated measures: p(dose)<0.01, p(time)<0.01 and p(dose x time)<0.001.

FINDING 2: THE HDAC INHIBITORY ACTIVITY OF VPA APPEARS TO BE IMPORTANT FOR THE INDUCTION OF T-PA

To confirm that VPA acts as an HDAC inhibitor in endothelial cells, western blot assays with antibodies to acetylated as well as to total histone H3 and H4 were performed. This revealed an increase of global acetylated histone H3 and H4 after VPA treatment, whereas no induction of total histone proteins could be detected (Figure 5 B). Additional experiments revealed that the VPA analogue valpromide (VPM), a substance lacking HDAC inhibitory activity [127, 128], failed to induce t-PA in endothelial cells (Figure 5 A). Taken together, these experiments suggested that the HDAC inhibitory effect of VPA is responsible for the increase in t-PA expression.

To investigate whether the effect of VPA on t-PA expression is mediated through a specific HDAC enzyme, the class I and IIa HDACs (which VPA has been reported to inhibit) as well as the only class IV HDAC were independently depleted with siRNA. Depletion of two of the class I HDACs (HDAC3 and 8) caused modest elevations of basal t-PA levels, while depletion of three HDACs belonging to class I (HDAC3) and IIa (HDAC5 and 7) partially reduced the VPA response (data not shown). This indicates that no single HDAC enzyme mediates the VPA effect, but that VPA instead may work through several different HDAC enzymes.

To investigate the effect of VPA on histone acetylation status specifically at the t-PA promoter region, ChIP-analyses were performed using antibodies to pan-acetylated histone H3 and H4, and primers flanking the major t-PA transcription initiation site. This showed a significant 2-fold increase of both acetylated H3 and H4 associated with the region surrounding the major t-PA transcription start site after VPA treatment (Figure 6 A, B), thus indicating that histone acetylation in the t-PA promoter region is increased after treatment with VPA.

Recent accumulating evidence points to the existence of a histone code that is recognized and interpreted by effector proteins with chromatin-modifying activities [33]. There are also data implying that certain specific modifications directly influence higher-order chromatin structure and compaction. Thus, to obtain more detailed information, we analysed which specific lysine residues in histone H3 and H4 that were affected. This was performed by ChIP analysis, using separate antibodies to the specific lysine modifications. We found a significant increase in acetylation of lysines 9, 18, 23, and 27 on histone H3 as well as lysines 8 and 16 on histone H4. Acetylation of H3K14 was undetectable, whereas H4K5 and K12 acetylation was not significantly changed after VPA treatment (Figure 6 C, D). Interestingly, acetylation of H4K16 has been reported to have a strong influence on higher-order chromatin structure [129]. Taken together, this may indicate that the chromatin in the t-PA promoter area is less tightly compacted after treatment with VPA.

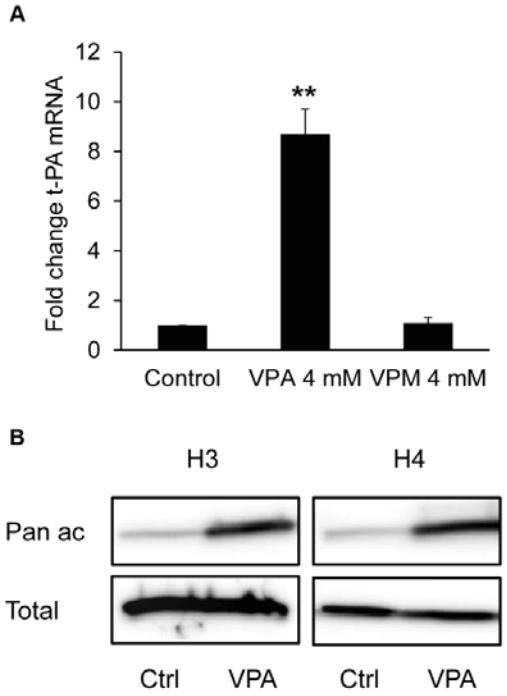


Figure 5. Dependence of HDAC-inhibitory activity for t-PA induction and effect of VPA on global histone acetylation. A. t-PA mRNA expression in HUVECs stimulated with 4 mM VPA or 4 mM VPM (a structural amide analogue of VPA which is reported to lack HDAC inhibitory activity) for 24 h. The results are presented as mean values ± SEM of five independent experiments performed in duplicate. **$p<0.01$. **B.** HUVECs were exposed to 3 mM VPA for 24 h after which cells were harvested in Laemmli sample buffer for analysis of global acetylation of histone H3 and H4 by Western blotting. Data are representative of three independent experiments.

These results further supported the hypothesis that the HDACi activity of VPA causes the increase in transcription. This is also in accordance with the recently published study by Kruithof and coworkers, who noted increased acetylation of histones associated with the t-PA regulatory region 1 kb upstream of the major transcription start site after treatment of HUVECs with the HDACi TSA or MS-275 [111].

Considering that VPA increased global histone acetylation in endothelial cells, and that acetylation is regarded as a permissive modification, one possibility was that hyperacetylation might have caused a generalized increase in gene expression and that, accordingly, the induction of t-PA expression may be non-specific. In order to investigate this, gene expression microarray analysis was performed on HUVECs af-

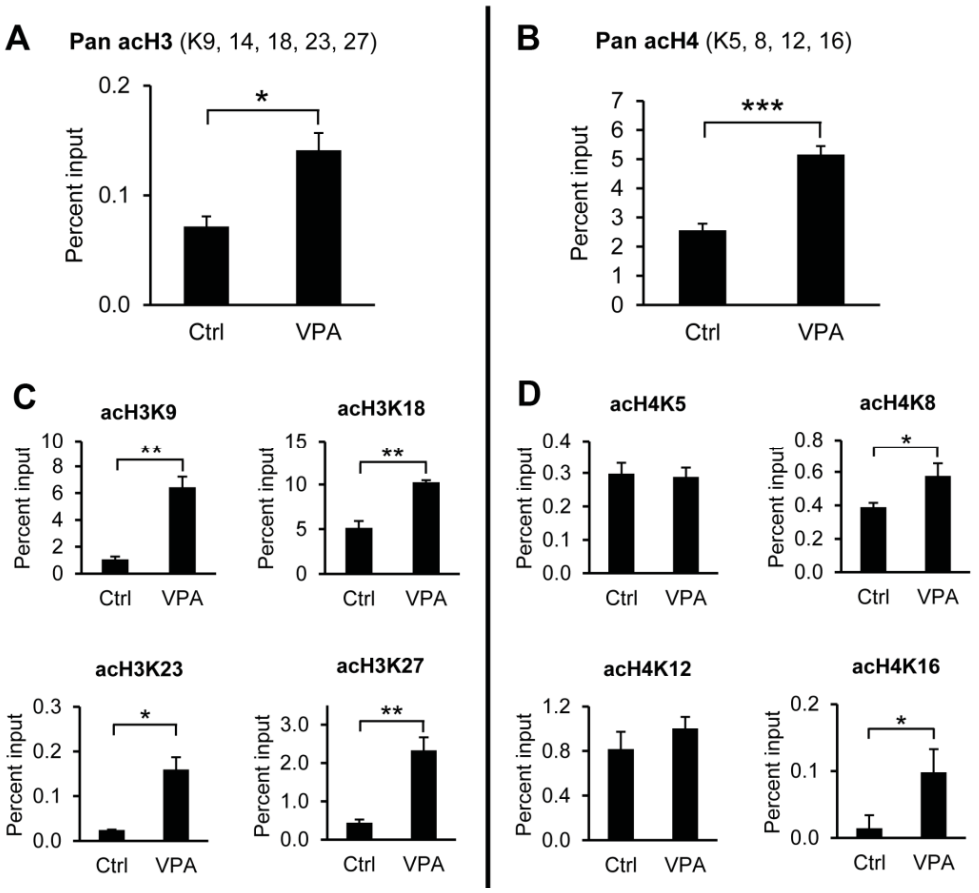


Figure 6. Chromatin Immunoprecipitation (ChIP) for acetylated histones in the t-PA promoter. HUVECs were exposed to 3 mM of VPA for 24 h after which cells were fixed and chromatin harvested. ChIP-analyses for acetylated histone H3 and H4 were performed with real-time RT-PCR primers flanking the major t-PA transcription start site. Data are presented as percent input corrected for background binding and mean values ± SEM of four to five independent experiments are shown. **A.** ChIP for pan-acetylated histone H3 (n=4). **B.** ChIP for pan-acetylated H4 (n=5). **C.** ChIP for specific histone H3 acetylation. Antibodies for monoacetylated acH3K9, acH3K18, acH3K23 and acH3K27 were used (n=4). **D.** ChIP for specific histone H4 acetylation. Antibodies for monoacetylated acH4K5, acH4K8, acH4K12 and acH4K16 were used. n=5. *p<0.05, **p<0.01, ***p<0.001.

ter exposure to maximal concentration (4 mM) of VPA for 24 h. This revealed that on a global scale, only 2.6% of the genes were affected by this treatment. Out of these, not all were up-regulated, but 37% were actually suppressed (data not shown). This indicates that HDAC inhibition not always is a stimulatory mechanism but may also reduce gene expression, perhaps via up-regulation of regulatory proteins that are repressors of transcription or mediators of RNA instability [130]. In line with

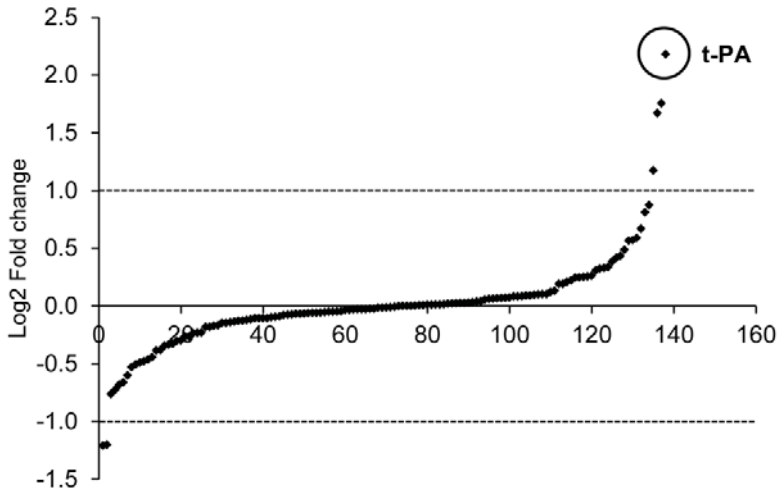


Figure 7. Effect of VPA on the mRNA expression of haemostatic genes. HUVECs (from four individuals) were stimulated with 4 mM VPA for 24 h and mRNA from treated and untreated cells were analyzed using the Human Gene 1.0 ST microarray. Haemostasis genes (a total of 138 genes) were identified using the Amigo database and plotted in the order of increasing log2 fold change. Probe sets with a log2 ratio above +1 or below -1 and a significantly changed expression ($p<0.05$, FDR adjusted p-value) were classified as regulated. The dot representing t-PA is highlighted (log Fold Change 2.18). The other regulated haemostasis genes are: Tissue factor pathway inhibitor 2 (TFPI2) (log Fold Change 1.18), signal peptide CUB domain EGF-like 1 (SCUBE1) (log Fold Change 1.67), coagulation factor II (thrombin) receptor-like 2 (F2RL2) (log Fold Change 1.75), P-selectin (log Fold Change -1.21) and fermitin family homolog 3 (drosophila) (FERMT3) (log Fold Change -1.20).

our findings, previous array studies with other HDAC inhibitors in various cell types have indicated that only a small number of genes, about 2-5%, are in fact affected by HDAC inhibitors [131, 132].

When looking more specifically at genes involved in haemostatic pathways, the expression levels of the majority were not significantly affected by VPA. Of the 138 genes annotated as being involved in haemostasis by the AmiGO database (http://amigo.geneontology.org/amigo), only four were up-regulated and two suppressed. Indeed, the haemostatic gene that was most strongly regulated by VPA was t-PA (Figure 7). In addition, the relative lack of effect of VPA on other haemostatic genes like plasminogen activator inhibitor-1 (PAI-1), urokinase plasminogen activator (u-PA), and von Willebrand (vWF) factor in the array was confirmed by real-time PCR. Maximal doses of VPA only caused a minor, approximately 30%, increase of both PAI-1 and u-PA, and a 30% decrease of vWF transcript (data not shown).

Of note, it has been suggested that genes that are dependent on the Sp1 transcription factor often are negatively regulated by HDACs and greatly induced by HDAC-inhib-

itors [133-136]. Interestingly, the t-PA promoter contains three Sp1 binding GC-boxes reported to be crucial for constitutive t-PA expression [73, 75]. Sp1 has been shown to recruit cofactors with both HAT and HDAC activities [137-139], and it is possible that HDAC inhibition affects the balance of these activities in this region hence altering gene expression. As HDACs also are known to de-acetylate many non-histone proteins including Sp1, it is also possible that HDAC inhibition results in acetylation of the Sp1 protein itself potentially changing its DNA binding affinity or protein-protein interactions [139].

In Study I, the effect of VPA on t-PA production *in vitro* was established. However, it remained to be determined whether VPA could be used clinically for stimulation of endogenous fibrinolytic capacity *in vivo*. VPA has been extensively used in epilepsy treatment and its profile of adverse effects is well known; the majority of side effects are mild, reversible, and occurring mainly at high plasma concentrations [140]. More severe adverse effects may occur in young children (hepatotoxicity) and pregnant women (teratogenicity), but these groups are rarely considered for cardiovascular prevention. Interestingly, concentrations comparable to those in the lower therapeutic plasma concentration range (0.3-0.5 mM) caused a significant 2-3 fold increase of t-PA synthesis. Unfortunately, endothelial t-PA release is difficult to assess *in vivo* since it requires arterial cannulation with local organ measurement due to the rapid degradation of t-PA by the liver [83]. If, however, a similar enhancement of t-PA production could be obtained in patients treated with VPA, this could theoretically reduce the risk of acute atherothrombotic disorders.

In a series of follow-up studies by our group, the effect of VPA on fibrinolytic parameters *in vivo* was investigated. In a porcine *in vivo*-model, VPA was found to increase regulated t-PA release, whereas basal t-PA and PAI-1 levels remained unaffected [141]. Subsequently, treating healthy human volunteers with VPA was found not to affect regulated t-PA release, but to lower both t-PA and PAI-1 baseline levels [142]. In an additional study on subjects with coronary disease, VPA showed no effect on baseline t-PA levels, while PAI-1 levels were decreased. The ability for regulated t-PA release, on the other hand, was found to be increased after treatment with VPA [143, 144]. Taken together, it seems that VPA through one or several mechanisms favours a pro-fibrinolytic state, which very well could lead to an improved thrombosis defence. Indeed, Olesen *et al* recently reported a 40% reduced risk of myocardial infarction in a Danish nation-wide study of epileptic patients treated with VPA [145]. Even though VPA has been reported to affect several aspects of the haemostatic system [140], it is conceivable that a substantial part of this reduced risk could be attributable to enhanced t-PA production.

The findings in Study I are novel in several ways. Firstly, we were able to show that VPA induces an increase in t-PA expression, which is present both on the gene and protein level. Secondly, we could show that the HDAC inhibitory effect indeed was involved in this. Thirdly, for the first time, this opens up for a possible treatment regimen of patients with a defective t-PA production – VPA is already in clinical use, and is generally well-tolerated.

Study II

Besides histone modifications, DNA methylation is an important epigenetic mechanism which allows an organism, or even a cell, to respond to the surrounding environment. We wanted to investigate if HUVECs modify their methylation pattern as a response to the environmental change they are subjected to when placed in culture. As an indication of the stability of the DNA methylation pattern in the t-PA enhancer and promoter, the levels of methylation in these regions were compared between primary (non-cultured), passage 0 (4-5 days in culture), and passage 4 (15-17 days in culture) HUVECs from the same subjects. Study II aimed at determining the methylation status of the t-PA gene regulatory region (promoter and enhancer), while Study III aimed at investigating genome-wide methylation dynamics.

FINDING 3: T-PA ENHANCER METHYLATION DECLINES DURING CELL CULTURING, WHILE PROMOTER AND UPSTREAM PROMOTER REGION METHYLATION IS STABLE

To assess the methylation levels in primary, p.0, and p.4 HUVECs, bisulphite sequencing PCR followed by Sanger sequencing of the PCR products were used.

We found that a gradual but rapid demethylation of the t-PA enhancer occurred when HUVECs were cultured. In the primary cells, the average methylation level was 30-40%, but in passage 4, hardly any methylation could be detected. In contrast, the t-PA promoter was found to be unmethylated in primary HUVECs, and remained so as the cells were cultured. The region immediately upstream of the promoter was fully methylated in primary HUVECs, and as the cells were cultured, the methylation level also in that region remained unaffected (Figure 8 and Figure 9). Thus, dynamic methylation appears to be restricted to the enhancer.

Traditionally, DNA methylation has been perceived as a stable modification responsible for long-term repression of gene expression [146, 147]. However, recently, there have been reports of a more dynamic CpG methylation that can be affected by e.g. long-term culturing [148], and that, in pluripotent undifferentiated cells, can change depending on the culture condition [149]. Interestingly, the former study found these dynamic CpG sites to be co-localized with transcription factor binding sites, and specifically with enhancers.

Still, to our knowledge, there are no previous studies where methylation levels have been compared between non-cultured cells and cultured cells at low passages. Instead, cells at higher passages have been used as representatives of the primary cell type and the consensus view has indeed been that methylation changes are unlikely to have occurred in early passage cells [111]. In one study, it is stated that primary cell lines are suitable to use as models for understanding tissue-specific regulation of DNA

methylation [150]. Surprisingly, our findings indicate that cell culture may alter methylation levels faster than previously anticipated. Therefore, we believe that it cannot and should not be assumed that DNA methylation levels are stable even between cells at low passages.

One could perhaps argue that one explanation to the rapid demethylation could be that a cell fraction might have been unmethylated in the t-PA enhancer initially, and that this subpopulation was selected for during the culturing process. In that case, cell culturing would not have induced a demethylation event, but rather the selection of a certain subpopulation. This scenario, however, is highly unlikely as at least one of the enhancer CpG sites (-7,373) was completely methylated in the primary state in more than half of the individuals. If an unmethylated subpopulation still had existed in the primary cells, this population would not only have been too small to detect, but would also have been unable to grow to constitute as much as half of the total cell population after only a few days in culture.

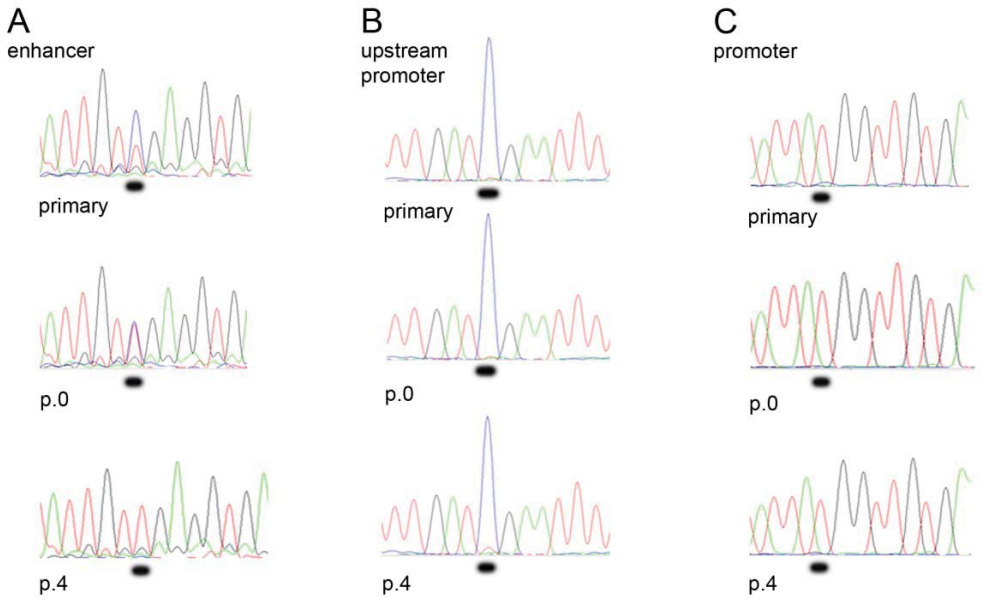


Figure 8. Gradual demethylation occurs in the enhancer but not in the promoter regions during cell culturing. Chromatogram showing primary, passage 0, and passage 4 HUVECs sequenced in the (**A**) enhancer, (**B**) upstream promoter, and (**C**) proximal promoter regions. The position of the original cytosine residue is underlined. The blue peak corresponds to cytosine (methylated) and the red peak to thymine (unmethylated).

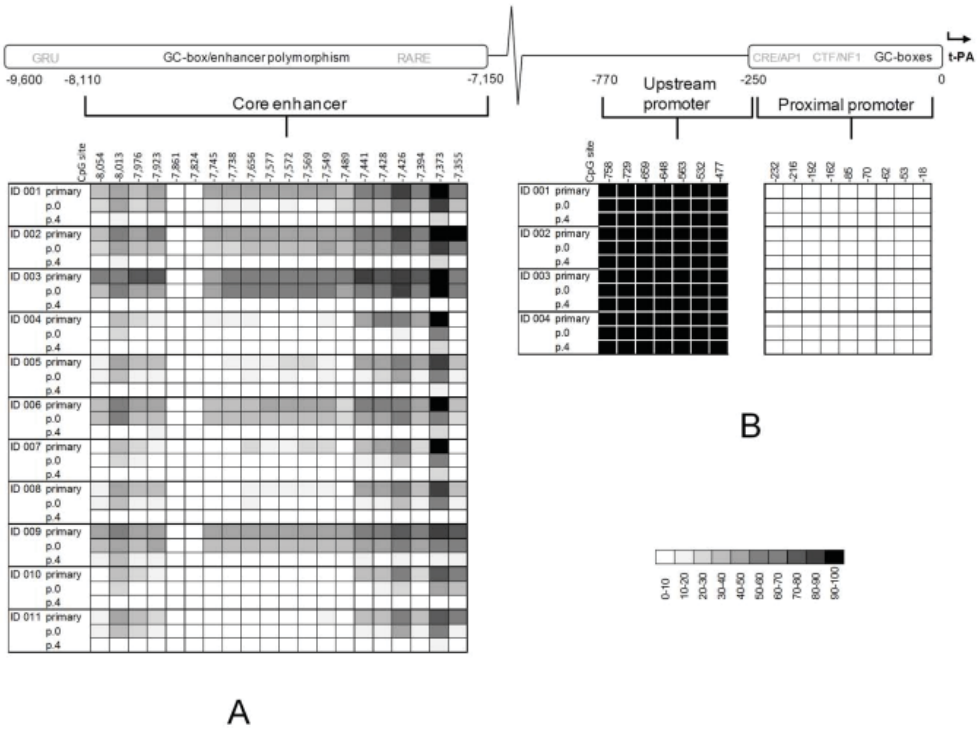


Figure 9. DNA methylation in t-PA enhancer, upstream promoter, and proximal promoter regions in primary, p.0, and p.4 HUVECs. (A) The methylation level in the t-PA enhancer in primary, passage 0, and passage 4 HUVECs from 11 subjects (ID 001-011) as determined by direct sequencing of bisulphite-treated PCR-amplified DNA. All the CpG dinucleotides in the core enhancer, stretching from -7,150 to -8,110, were included in the analysis. The most 3´ enhancer CpG site, -7,355, corresponds to the t-PA -7,351 C/T enhancer polymorphism (which, using updated genome assemblies, has been remapped to -7,355 relative the major transcription initiation site (TIS)). (**B**) The methylation levels in the upstream promoter and promoter regions from four subjects (ID 001-004). The 9 CpG dinucleotides in the t-PA proximal promoter, stretching from TIS to -240, were analysed, along with the 7 CpG dinucleotides in the region immediately upstream of the promoter (from -240 to -760).

FINDING 4: T-PA ENHANCER DEMETHYLATION CORRELATES WITH ELEVATED GENE EXPRESSION

To examine the effect of sub-culturing on t-PA gene expression, real-time RT-PCR was run on primary – p.4 HUVECs. We found an increased t-PA expression in all passaged cells (p.0 – p.4) compared to primary. To p.0, the t-PA expression was increased by a factor of approximately 25 (Figure 10). This was in strong negative correlation with the change in enhancer methylation level observed between primary and p.0 HUVECs (Figure 11).

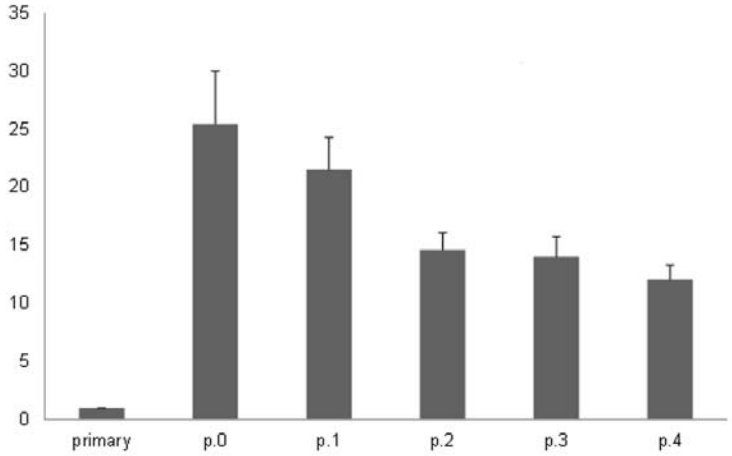


Figure 10. t-PA gene expression in primary and cultured HUVECs. Relative mRNA expression of tissue-type plasminogen activator (t-PA) in non-cultured HUVECs and p.0-4 HUVECs from the same subjects as determined by real-time RT-PCR. Seven of the 11 subjects from the methylation analysis (ID 005-011) were included also in this analysis (total n=12). p<0,001 (one-way ANOVA).

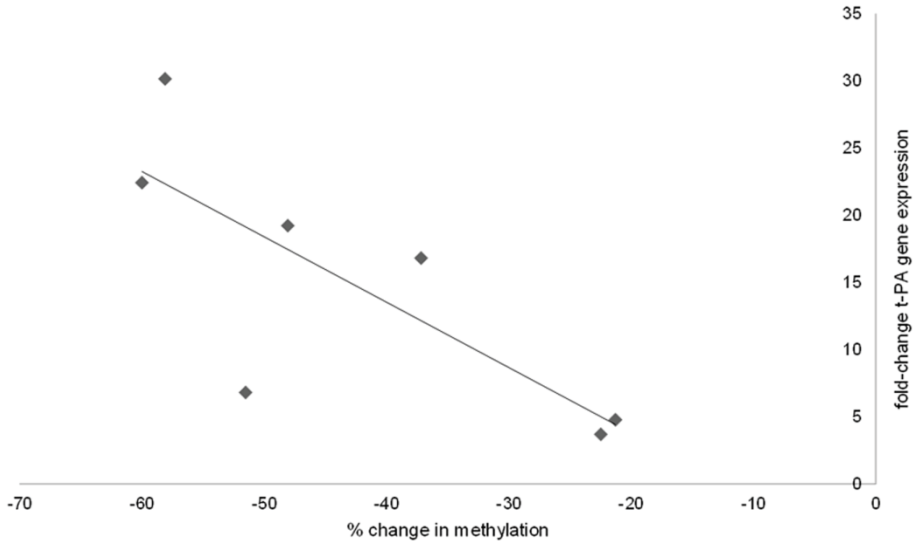


Figure 11. Correlation between decrease in methylation levels and increase in t-PA expression. A correlation analysis was performed on change in methylation and change in gene expression between primary and p.0 HUVECs. After rank transformation of the data, a Spearman's correlation test gave a correlation coefficient of -0.86. The seven individuals (ID 005-011) for which both methylation and gene expression data were available were included in the analysis (n=7, p<0.05). The figure depicts crude data.

Previously, promoter methylation in particular has been considered closely connected to gene expression (reviewed in [151]), and the two previous studies examining DNA methylation in relation to the t-PA gene have indeed evaluated only the promoter methylation level [111, 152]. Enhancer methylation has only recently gained attention. One study found changed gene expression to be more closely correlated with altered enhancer methylation than with altered promoter methylation [153]. That study suggested that, for some genes, enhancer methylation may serve as a main determinant of gene transcription levels.

The t-PA enhancer is well-established and known to be essential for gene expression. Of note, experiments with transgenic mice harbouring different lengths of the t-PA regulatory region fused to a lacZ reporter gene have revealed that the t-PA enhancer region seems to control tissue-specific expression of t-PA [154]. Given this established importance of the t-PA enhancer for gene expression levels, it is not surprising to find that t-PA may belong to the category of genes where enhancer methylation governs gene expression.

Individuals homozygous for the C allele of the t-PA -7,351 C/T enhancer polymorphism have twice the t-PA release rate compared to those that carry the T allele [78]. Indeed, the enhancer polymorphism has been shown to be functional at the transcriptional level, and the C allele has an approximately 10-fold greater binding affinity for the Sp1 and Sp3 proteins compared to the T allele [155]. In our study, two of the subjects were genotyped as homozygous for the T allele; those are ID 004 and ID 007, which are the only two individuals in which the C situated at -7,355 (corresponding to the -7,351 C/T enhancer polymorphism) appears unmethylated in primary HUVECs (because, in reality, it is a T). The rest of the individuals were genotyped as CC and CT. In the present study, each individual served as its own control (i.e. the primary HUVECs were used as starting point), and we never examined the absolute expression levels but rather the change in expression. Only one of the individuals homozygous for the T allele (ID 007) was included in the correlation analysis, and this individual behaved in the same manner as the others. A considerably larger material would be required in order to be able to determine whether cell culturing has genotype-specific effects on t-PA gene expression in this setting.

While the binding affinity of the Sp-proteins is affected by the transition of C to T (which changes a central position in the GC box to which they bind), Sp1 has been shown to be insensitive to the methylation status of the C within the GC box [156]. In contrast, the binding affinity of Sp1/Sp3 has been reported to be affected by the methylation status of adjacent CpG sites, as hypermethylation around the GC box has been found to reduce Sp1/Sp3 binding [157]. Against this background, it is possible that the enhancer demethylation observed in our study may have enabled increased binding of Sp1/Sp3, which in turn could have contributed to the observed increase in t-PA expression.

The findings in Study II are novel in two ways. Firstly, they indicate that DNA methylation during cell culturing may be a more dynamic modification than previously recognized, as high methylation levels in the t-PA enhancer can be completely and

stably erased after just a few days. Secondly, we found that the demethylation event was specific, as it occured only in the t-PA enhancer and not in the promoter nor in the region immediately upstream of the promoter. We therefore hypothesize that methylation of the t-PA enhancer acts as a previously unrecognized switch that can be used to turn on t-PA transcription in response to external stimuli.

Study III

As a direct consequence of the findings in Study II, we set out to analyse genome-wide methylation dynamics in HUVECs during the first stages of cell culture.

FINDING 5: ALMOST 2% OF CPG SITES DISPLAY ALTERED METHYLATION DURING THE EARLY STAGES OF CELL CULTURING

To investigate the effect of sub-culturing on the genome-wide DNA methylation level, the Illumina Infinium HumanMethylation450 microarray (450K) was run on primary, p.0, and p.4 HUVECs. We found the overall methylation to be bimodally distributed (as previously reported for other cell types [46, 158, 159]), with most of the sites being either methylated or unmethylated. This pattern was the same in all three passages analysed. However, scatter plots of absolute methylation (β-values) between primary and p.0 HUVECs, and primary and p.4 HUVECs, showed that while the methylation pattern was quite conserved to passage 0 (R^2=0.9946), it showed greater variability to passage 4 (R^2=0.9815) (Figure 12).

Using a 17% change in methylation together with an adjusted p-value of less than 0.05 as cut-off, as previously used in other studies [158, 160, 161], we found that 0.4% of the sites changed methylation level between primary and p.0 HUVECs, while 0.8% changed methylation between p.0 and p.4. Between primary and p.4 HUVECs, 1.8% of the sites changed methylation level, out of which approximately 80% became demethylated, while the rest of the sites gained methylation.

Despite a seemingly modest percentage, 1.8% is equal to almost 10,000 sites. Indeed, it is comparable to the number of differentially methylated sites (DMSs) occurring during granulopoesis; using 17% cut off for alteration in methylation (the same as in the present study), Rönnerblad *et al* found 10 156 sites to be dynamic and undergo primarily hypomethylation when analysed with the 450K array [158]. Of note is that in another setting, the methylation status of single CpG sites in enhancers have been shown to have strong effects on the transcriptional competence [162].

It has previously been reported that abnormal or dynamic methylation can be found in cancer cell lines [163] as well as in cultured embryonic stem cells [164, 165], and there have also been a few previous reports of hypomethylation during the sub-culturing of a more differentiated cell type (fibroblast) [166, 167]. Recently, Nestor *et al* noted a 4-8% reduction of methylation in mouse embryonic fibroblasts after 7-9 days of culture, which was suggested by the authors to probably reflect loss of

hydroxymethylation, while the overall level of methylated cytosine was conserved during culturing. However, they found that no functional categories were enriched for among the genes losing 5-hydroxymethylcytosine (5-hmC) in culture, and also no restriction to particular genomic compartments, which led them to conclude that the loss of 5-hmC was general in nature [168].

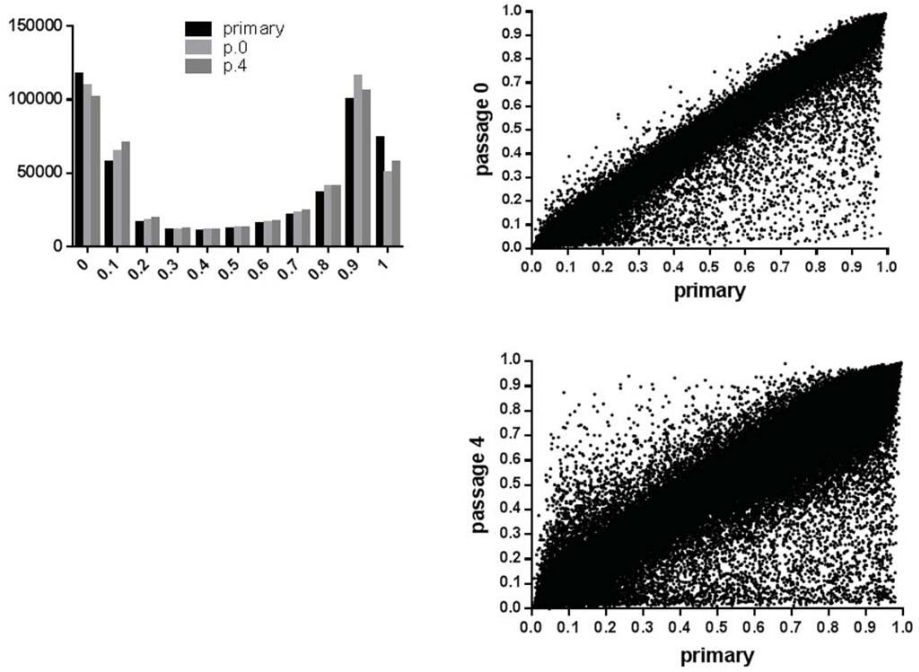


Figure 12. Absolute methylation is bimodal in shape. A. Dispersion of absolute methylation (β-values) obtained by the 450K array, all CpG sites included. The level of methylation was classified between 0 (no methylation) and 1 (mostly methylated). **B**. Scatter plot showing the correlation of the β-values between primary and p.0 HUVECs. *C*. Scatter plot showing the correlation of the β-values between primary and p.4 HUVECs.

To investigate if the decreased methylation in our study was constituted mainly of declining hydroxymethylation levels, a genome-wide 5-hmC analysis using an ELISA-based approach was performed. We found that the 5-hmC level rapidly decreased already to passage 0 (4-5 days in culture) (Figure 13), in contrast to the loss in 5-mC methylation which occurred gradually and was most prominent to passage 4. Thus, we have no reason to believe that the observed demethylation in our study was constituted specifically of hydroxymethylation; instead, it seems that the overall level of methylated cytosine decreases during the culturing of HUVECs.

To instead investigate whether the observed demethylation was caused by altered transcription levels of components of the methylation or demethylation machineries, mRNA analysis was performed to determine the relative levels of DNA methyl-

transferases (DNMTs) and ten-eleven translocation enzymes (TETs). This revealed that *DNMT1* and *DNMT3B* both increased in expression, indicating that there is no lack in DNMTs which could be responsible for passive demethylation. The TETs, on the other hand, both decreased (*TET1*) and increased (*TET3*) in expression (data not shown). Thus, there is no clear indication that changes in expression levels of any single part of the methylation or demethylation machineries is responsible for the observed demethylation.

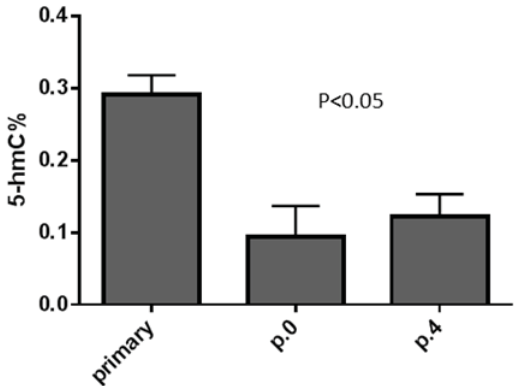


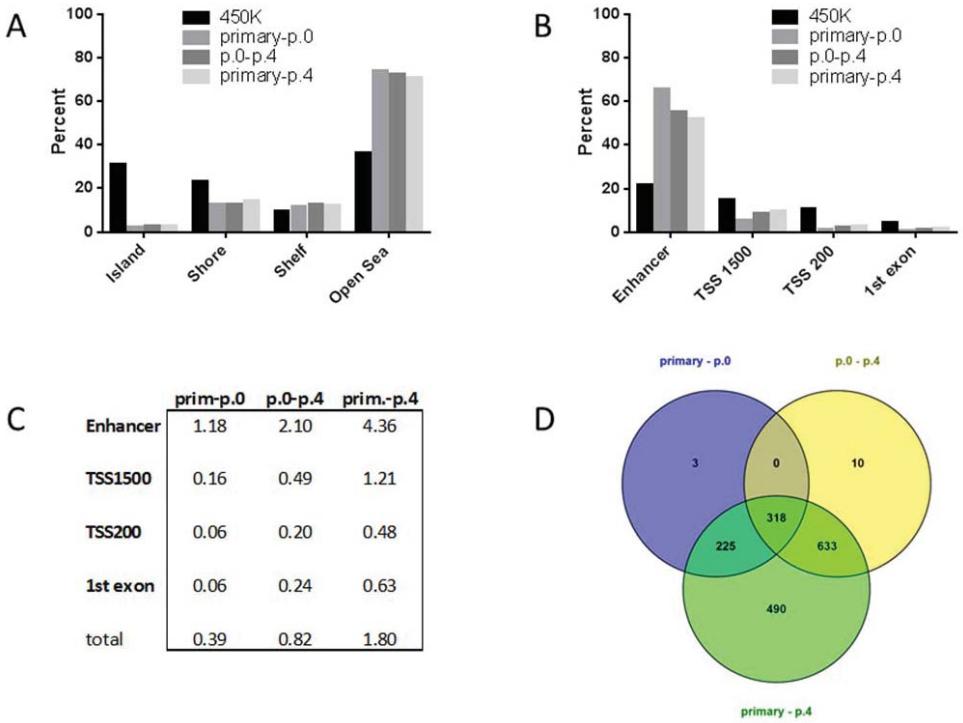
Figure 13. The DNA hydroxymethylation level rapidly decreases when cells are placed in culture. The hydroxymethylation analysis revealed a rapid reduction in the level of genome-wide 5-hmC already to passage 0 (n=5).

FINDING 6: DYNAMIC METHYLATION IS PREDOMINANTLY LOCATED TO ENHANCER ELEMENTS

To analyse in which features the DMSs were located, we used the annotation obtained from the 450K array. We found the DMSs to be located to the open sea region, and more specifically to enhancer elements (Figure 14 A, B, C). This is in contrast to the findings of several previous studies, which instead have reported dynamic methylation to be situated in CpG island shores [148, 169].

Using a venn analysis diagram (Venny 2.1; http://bioinfogp.cnb.csic.es/tools/venny/) to visualise the genes with enhancer DMSs, we found a substantial overlap between the three datasets (primary-p.0, p.0-p4, and primary-p.4), thus indicating that the shift in methylation may be a targeted process (Figure 14 D). Gene ontology (GO) analysis (performed in David Bioinformatics Resources 6.7 (https://david.ncifcrf.gov/)) could confirm this, as several terms, for example related to angiogenesis and blood vessel development, were highly enriched for among these genes (data not shown).

We were not surprised to find that the most variable methylation was located outside of the promoter regions; many promoters contain CpG islands that are known to be constitutively unmethylated. In addition, Sp1 binding sites, which often can be found



	prim-p.0	p.0-p.4	prim.-p.4
Enhancer	1.18	2.10	4.36
TSS1500	0.16	0.49	1.21
TSS200	0.06	0.20	0.48
1st exon	0.06	0.24	0.63
total	0.39	0.82	1.80

Figure 14. The DMSs occur in the open sea region, in sites annotated as enhancer. A. Relative distribution of CpG sites annotated as shore, shelf, and open sea in the DMS between primary and p.0, p.0 and p.4, and primary and p.4 HUVECs, presented as percent of the total number of DMSs in the relevant dataset (y axis). The distribution of CpG sites in the same features on the 450K array is displayed as percent of the total number of sites. **B.** The distribution of DMSs in genomic regions, calculated as percent of the total number of changed sites in the corresponding datasets. The relative amount of sites on the 450K array is calculated as percent of the total number of sites. **C.** Percent of sites annotated as enhancer, TSS1500, TSS200, and 1st exon with altered methylation during cell culturing, compared to percentage of total DMSs. **D.** Venn diagram (produced in Venny 2.0, http://bioinfogp.cnb.csic.es/tools/venny/) showing overlap in the DMSs between the three datasets (primary-p.0, p.0-p.4, and primary-p.4).

at promoters, have been shown to induce demethylation of DNA [170]. Thus, promoter regions may not be expected to display dynamic methylation levels in response to environmental challenge. Interestingly, it has previously been reported that cell type specific correlations between DNA methylation and gene expression often allow a link between distal regulatory elements, like enhancers, and their target genes [171].

Many previous studies have focused on genomic areas with high CpG content, primarily promoter regions. Indeed, many of the available methodologies for genome-wide methylation analysis are biased toward regions with higher-than-average CpG content. For example, the reduced representation bisulfite sequencing (RRBS) method enriches for CpG islands [150, 172], and the Infinium HumanMethylation27 BeadChip, which has been used in many previous studies, was designed to primarily cover promoter regions [173]. Also its successor the 450K array was, despite having a much

larger coverage, designed with an emphasis on CpG islands [120]. It is well established that hypermethylation of promoter (and CpG island) regions frequently occur in cancer; in fact, this is found in virtually all human neoplasms (reviewed in [174]). Thus, many of the available methods for methylation analysis may be well suited for the cancer field, while not always quite appropriate for other areas of methylation research. It is even possible that many studies of methylation dynamics during development or cell culturing have underestimated the effect on the methylome. Indeed, it is likely that instead using a method which focuses on enhancer sites would have led this study, along with several others, to discover more substantial and conclusive patterns in the dynamics of the DNA methylome.

FINDING 7: *HDAC9* EXPRESSION IS STRONGLY UP-REGULATED BY CELL CULTURING

The 91 genes with the most significant methylation change in a site annotated as enhancer were selected for gene expression analysis, which was performed using a TaqMan array card. We found the expression of approximately half of these genes to be changed, with *HDAC9* having the most altered expression; *HDAC9* expression was 413 times up-regulated between primary and p.4 HUVECs. Subsequently, separate *HDAC9* real-time RT-PCR analysis could confirm this result as well as determine that the expression gradually increased over the passages (Figure 15).

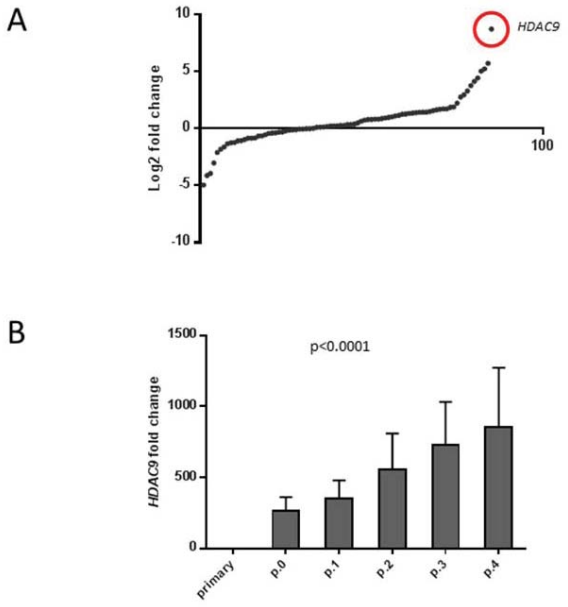


Figure 15. *HDAC9* is the most up-regulated gene with enhancer DMS. A. The genes with the most changed enhancer methylation (all of which displayed hypomethylation) were analysed with gene expression analysis on a TaqMan array. The fold change data was log2 transformed and plotted from the most down-regulated gene to the most up-regulated (n=6). **B**. Separate gene expression analysis of *HDAC9* (n=4).

Since endothelial cells are the model system in this study, and in line with our finding of enrichment of GO-terms associated with vasculature development (including angiogenesis), it is worth noting that HDAC9 recently has been discovered to promote angiogenesis [175]. In addition, HDAC9 has also been found to be closely associated with large vessel stroke [176] and coronary artery disease [177]. The precise mechanism behind this is not known, but it has been shown that high levels of *HDAC9* mRNA markedly increase the risk of stroke, most likely mediated by atherosclerosis [178, 179]. Targeting HDAC9 has been proposed as a possible strategy to prevent the progression of atherosclerosis [178, 180] but little is known about the regulation of *HDAC9* expression, and no previous study has identified variable methylation of the *HDAC9* region to be important for its level of expression. In this study, we can show that declining DNA methylation levels in the *HDAC9* enhancer and dramatic increases in *HDAC9* gene expression occur during the early stages of cell culturing. Indeed, it is plausible that the same mechanisms may be used by cells also in more physiologic settings where the endothelium is challenged, such as in atherogenic areas or areas of endothelial injury. Clearly, this calls for further investigation.

The findings in Study III are novel in several ways. Firstly, this study shows that DNA methylation can be more dynamic than previously recognized, and that low passage cells not always are suitable as representatives of the "primary" cell type. In addition, our results indicate that it is of significance not only whether a methylation change is positive or negative, but also in which feature it is observed, as this will indicate if the methylation changes are random (evenly distributed), or seem to be part of a targeted process. This should also be considered when choosing method to examine methylation status, as many that are available today are biased toward areas with high CG content, primarily promoter regions.

SUMMARY

In summary, this thesis presents seven major findings:

- the clinically used HDACi VPA is an important stimulator of t-PA expression in endothelial cells (Study I)

- the HDAC inhibitory activity of VPA appears to be important for the induction of t-PA (Study I)

- t-PA enhancer methylation declines during cell culturing, while promoter and upstream promoter region methylation is stable (Study II)

- t-PA enhancer demethylation correlates with elevated gene expression (Study II)

- almost 2% of CpG sites display altered methylation during the early stages of cell culturing (Study III)

- dynamic methylation is predominantly located to enhancer elements (Study III)

- *HDAC9* expression is strongly up-regulated by cell culturing (Study III)

Taken together, this thesis includes an examination of epigenetic regulation (histone acetylation and DNA methylation) of the t-PA gene, as well as an analysis of how dynamic genome-wide DNA methylation is during the early stages of cell culturing. It was concluded that the t-PA gene (*PLAT*) is regulated on a transcriptional level by changes in histone acetylation level, as well as DNA methylation status in the gene enhancer. Further, we could show that t-PA was not the only gene which was affected by cell culturing; almost 2% of the CpG sites that we examined changed methylation until passage 4. Thus, the early stages of cell culturing have a seemingly large impact on the genome-wide DNA methylation status.

CONCLUDING DISCUSSION

Study I and II focus on t-PA, a key enzyme in the haemostatic system (which involves both the formation and the resolution of blood clots). The components that contribute to the formation of clots, or coagulation, circulate in the blood at constant concentrations. In contrast, several of the factors of the fibrinolytic system, which lyse the blood clots, must be produced and released locally, as high levels in the circulating blood can be dangerous. Thus, the fibrinolytic system must be dynamic, and able to quickly respond to changes in the surrounding environment that indicate for example that a clot is being formed. It is plausible that the fibrinolytic system is sensitive to epigenetic mechanisms, and as such interesting to study in order to learn more about epigenetic gene regulation. However, more studies on the matter are required.

In all three studies, we used cultured HUVECs as a model system. However, in Study II and III, sub-culturing of HUVECs served as a model of extreme environmental challenge, which helped us study changes in DNA methylation patterns during adaptation to cell culturing. We hypothesized that this well may resemble more natural processes like wound-healing or similar situations when the endothelium rapidly has to go from a quiescent state into a state of proliferation and reorganisation. In these studies, we found that DNA methylation changes can occur faster than previously anticipated. Thus, in conclusion, we could show that endothelial cells are able to rapidly respond to the drastic environmental challenge exerted by cell culturing, and that one way to do so is through changing the DNA methylation pattern at specific genomic features. However, it remains to be established whether this happens also in more physiologic settings, for example during healing of vascular injuries, or during angiogenesis.

Taken together, we can be sure that that our genomes are able to adapt to the environment and regulate gene expression through epigenetic mechanisms such as histone modifications and DNA methylation. Epigenetics, which is a rapidly expanding field, holds the key to the ability to adapt to different conditions, external as well as internal.

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