universität wien

DIPLOMARBEIT

MODULATION OF EPIGENETIC CONTROL OF ESTROGEN RECEPTOR - α EXPRESSION AND TUMOR SUPPRESSOR GENES BY SPECIFIED BIOACTIVE FOOD COMPOUNDS

Einfluß bestimmter bioaktiver Pflanzeninhaltstoffe auf die epigenetische Regulation des Östrogen-rezeptors- α und von Tumorsuppressorgenen

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BSP	bisulfit sequencing PCR
cDNA	complementary DNA
CGI	CpG islands
DNMT	DNA methyltransferase
EGCG	epigallocatechin-3-gallate
CDK	cyclin dependent kinase
HAT	histone acetyltransferase
HDAC	histone deacetylase
ESR1	estrogen receptor alpha
MBD	methyl-CpG-binding domain
miRNA	microRNA
MSP	methylation specific PCR
PCR	polymerase chain reaction
Rb	retinoblastoma protein
SAM	S-adenosylmethionine
SIRT	sirtuin
SNP	single nucleotide polymorphism

1 INTRODUCTION

Dietary habits have long been suspected to take part in the development of widespread multifactorial illnesses like cardiovascular disease and cancer. Modification of lifestyle to include daily intake of plant-based food containing anticancer and antiinflammatory phytochemicals represents a promising approach to prevent their development. Vegetable diet contains plenty of "bioactive compounds", extra-nutritional constituents that typically occur in small quantities and exhibit capacity to influence molecular events. Since it has been documented that deficiency of certain nutrients may induce inadequate gene expression and failure in genomic integrity (Cheng 2009), nutritional science has investigated a plethora of different food components playing a role in regulating the genomic machinery.

Cancer growth and metastasis are due to coordinated change in expression of a set of genes, for which genetic alterations can not solely be blamed. It is becoming apparent that a lot of observed alterations in gene expression are generated by epigenetic modifications (Szyf 2005). Epigentic modifications are potenially reversible, opposed to DNA mutations and therefore are covetable targets for potential chemoprevention strategies and therapy. Since epigenetic alterations can be early incidences in carcinogenesis, reversal of inaccurate epigenetic patterns is aimed to affront pre-neoplastic cells before final carcinogenic changes occur (Myzak and Dashwood 2006).

Epigenetic influences of several bioactive food compounds might be mechanisms of disease prevention by not only being able to have gene-nutrient interaction but also by modifying methylation marks in specific gene regions or global DNA methylation, linking nutrition with modulation of gene expression (Ross and Poirier 2002). The impact of bioactive food components on genespecific DNA methylation is less clearly understood than their effect on global DNA methylation (Davis and Uthus 2004). Nevertheless understanding DNA methylation patterns of disease asscoiated genes through interaction with nutrients is elementary to explain pathophysiological processes responsible for the appearence or absence of certain ailments and to eventually evolve into prevention and therapeutic strategies for risk populations.

2 SCIENTIFIC BACKGROUND

2.1 The Mammalian Epigenome

Genetics alone cannot explain human variation and disease. Even though possessing the same DNA sequence, monozygotic twins and cloned animals display different phenotypes, occurence and penetrance of illnesses. The epigenetics field of research tries to find partial explanation for these phenomena. The term epigenetics describes mechanisms inducing changes in gene expression or phenotype not caused by alterations in the underlying DNA sequence, which are established during development or somatic cell prolieferation and transmitted through mitosis. Compared to the genome, which is almost identical in different cell types and consistent throughout life, the epigenome is varying between different cell types as well as over the course of a lifetime (Szyf 2005).

The Human Epigenome Project aims to uncover the missing link between genetics, disease and enviroment by investigating the regulation of gene expression through epigenetic remodelling of chromaine strucure by identifiying and interpreting genome –wide DNA methylation patterns of all human genes in all major tissues. Further mechanisms to configurate chromatin architecture and change gene expression are histone modification and micro RNAs (Holliday 2006).

2.1.1 DNA methylation

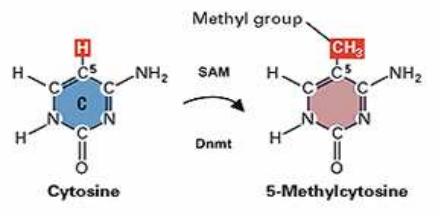

Figure 1: DNA Methylation (www.hgu.mrc.ac.uk)

The epigenetic modification most thoroughly investivated is DNA methylation. In eukaryotic organisms the adherence of a methyl group to the DNA occurs specifically in the carbon -5 position of cytosines followed by guanosine, in so-called CpG dinucleotides and might have evolved both as a protective mechanism against parasitic DNA elements and as a regulatory machinery in more complex genomes. DNA methylation

has been implicated in appropriate embryonic development, X-chromosome inactivation and control of monoallelic expression of genes via genomic imprinting. Cytosines in CpG arrangements are receptive to methylation, facilitating discrimination between newly synthesized and parental DNA strands and eventually supporting DNA proofreading after duplication (Fatemi, Hermann et al. 2002; Esteller 2008). CpG dinucleotides make up even less than they statistically should in the entire genome.

This rarity of CpGs might be due to the mutability of 5-methylcytosine, being prone to turn into thymines after spontaneous deamination. The same mechanism seems to be responsible for the inactivation of retroviruses by changing the virus sequences (Morgan, Santos et al. 2005).

In mammals the majority of CpGs is methylated and throughout a large part of the genome CpGs are sparsely spread but concentrated in short stretches or clusters, so called CpG islands (CGI). CGIs are of between 0.5 kb and 2 kb length and commonly span the promoter region of ubiquitiously expressed “housekeeping” genes and the 3´ or 5´ regions of a number of tissue specific genes. Alltogether CpGs occur in about half of all human genes. Approximately 80% of the CpG dinucleotides outside of CGIs are heavily methlyated, in contrast those in promoter regions are usually unmethylated. The bulk of CpGs though are located at low density within transposable elements and repeat sequences (Davis and Uthus 2004; Agrawal, Murphy et al. 2007; Hinshelwood, Melki et al. 2009).

CpG methylation is known to mostly repress gene transciption, as methylated DNA can hinder binding of transcriptional factors or attrac protein complexes, so called methyl-CpG-binding domain proteins (MBDs), promoting histone modification and therefore leading to chromatin compaction and gene silencing, whereas unmethylated CGIs are associated with transcriptionally active loosened chromatin. DNA methyltransferases catalyze transferation of methylgroups to carbone 5 of the cytosine ring, by using S-adenosylmethionine (SAM) as a methyl donor (Grafi, Zemach et al. 2007). The DNA methylation pattern has to be duplicated together with the genetic information at any cell mitosis. Recopying DNA methylation is mediated by DNA methyltransferase-1 (DNMT1), which is incorporated in the DNA replication complex and preferentially

methylates CpGs on hemimethylated DNA strands (Bestor 2000). Since aberrant methylation patterns are transmitted through mitosis, abnormal methylation might undergoe clonal expansion, changing gene expression and lead to phenotypic changes comparable to a mutation (Johnson and Belshaw 2008). DNA methyltransferases 3a and 3b are said to be responsible for *de novo* methylation, which is especially distinctive during embryogenesis whilst first cleavage divisions. Here DNA methylation is reconstituted after an almost absolute demethylation. But *de novo* methylation also appears later in development and actually in mature adult cells in order to silence aquired proviral DNA or to change the cell´s developmental program. Data suggests that DNMT1 also participates in *de novo* methylation. *De novo-activity* is stimulated by binding of methylated DNA to an allosteric site in the enzyme´s N- terminal domain, appearing to cooperate with DNMT3a, which initiates methylation processes by delivering methyl groups to one region of the DNA, leading to recruitment of DNMT1, which proceeds methylating the whole DNA domain. All three methyltransferases are essential for development and viability, as knockout mutations in any of these genes lead to fetal or postnatal death in mice (Fatemi, Hermann et al. 2002).

2.1.2 Histone modification

Chromatine in the eukaryotic nucleus is a complex of DNA and histones, which are octamers of two of each H2A, H2B, H3 and H4 and 147 base pairs of DNA. An additional histone, H1 serves as an anchor to fasten the DNA to the nucleosome. Acetylation, biotinylation, methylation, phosphorylation ubiquitination, SUMOylation, glycosylation, carbonylation and ADP ribosylation are processes to posttranslationally modify the core histone´s protruding N-terminal tail and thus change chromatin stucture. Combinations of these modifications determine interactions between histone and DNA as well as between chromatine and regulatory proteins. The “histone code” hypothesis declares a given modification of a certain histone residue to be a prerequisite for a succesive modification on the same or adjacent histone, leading to altered DNA configuration and extending the information potential of the genetic code.

Alterations in the configuration of chromatin structure are connected to changes in gene expression with euchromatine, densly packed and transcriptionally inactive and loose heterochromatine, representing transcriptionally active regions, as regulatory proteins and transcription factors are able to bind to the DNA. Proteins containing bromo- and chromodomains have been reported to bind to acetylated- and methylated lysines respectively. Heterochromatine protein -1 (HP1) for instance is needed for the formation of heterochromatin. It possesses chromodomains cooperating with a trimethylated H3K9 (histone 3-lysine 9) and attracting SUV39H1, a histone H3K9 methylase that subsequently methylates the neighbours nucleosome H3 ending at lysine 9 to cause HP1 binding and further distribution of heterochromatic regions (Yoo and Jones 2006; Delage and Dashwood 2008).

Acetylation is one of the most popular histone modifications. Transfer of acetyl-coenzyme A as a donor group to lysine residues of histones H4 and H3 is catalyzed by histone acetyltransferases (HATs). Histone acetylation is associated with transcriptional activation, since alteration in histone- DNA binding, due to lysine loosing a positive charge, leads to a more incoherent DNA configuration and facilitate binding of transcription factors (Turner 2002).

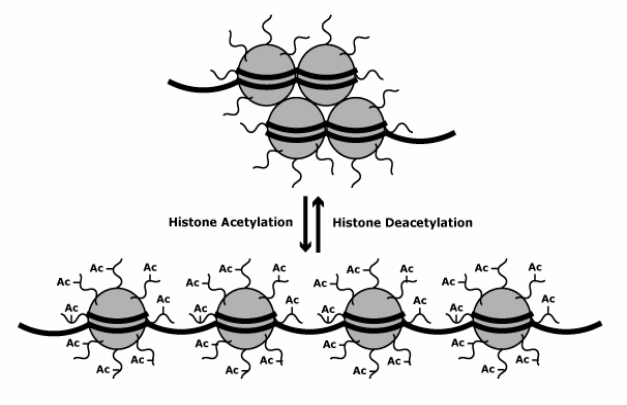

Figure 2: Histone Acetylation (Huang 2009)

Balance between enzymatic activity of HATs and histone deacetylases (HDACs) determines the extend of histone acetylation. Four families of HDACs are well known and classified due to their function and homology to yeast *Saccharomyetes cerevisiae*: Class I (HDAC 1-3,8) is mainly found in the nucleus and class II (HCAC 4-7,9) can shuttle signal dependently between nucleus and cytoplasm. Class I and II are selectively inhibitable by trichostatin, an organic compound that serves as an antifungal antibiotic.

Class IV is an atypical category based only on DNA sequence homology to the other classes. Solely class III (sirtuins 1-7), named after yeast protein Sir2 (silent information regulator 2) due to their analogy, is structured differently to class I,II and IV HDACs and also alters in the enzymatic mechanism, as sirtuins use NAD+ as a cofactor (Villar-Garea and Esteller 2004). SIRT1 was found out to participate in basal cellular processes, such as antiapoptosis, cellular senescence, aging and longevity (Kikuno, Shiina et al. 2008).

Another well investigated mechanism is histone methylation, which is linked to both transcriptional activation and repression, depending on the residue it is attached to. Lysine and arginine residues are targets for histone methyltransferases (HMTs) and histone demethylases (HDMs), wich mono-, di- and trimethylate lysine or monomethylate arginine histone tails (Turner 2002).

H3K4 methylation for instance is known to be an activating and H3K9 as well as trimethylation of H4K20 to be an inactivating marker. Mono- and dimethylated H3K9 are present in transcriptionally inacitve, euchromatic regions compared to trimethylated H3K9, commonly found in pericentromeric heterochromatin.

Nevertheless, methylated H3K9 has even been found in region of active transcription. Accordingly, further studies are necessary to discover the full extend of these precise mechanisms (Laird 2005).

2.1.3 Micro RNAs

First micro RNAs (miRNAs), lin-4 and let-7 were identified 1993 in *Caenorhabditis elegans* as fundamental development regulators. By now a multitude of miRNAs were discovered to play crucial roles in several biological pathways, affecting gene expression on the posttransciptional level. They are highly conserved, of 18 - 24 nucleotide length and regulate cognate mRNAs by blocking through basepairbinding or by degradation. Target mRNAs are attacked usually at the partially complementary sites in the 3´untranslated region (He and Hannon 2004). Genes for miRNAs are arranged in the genome and biosynthesis is initiated with transciption by an RNA

polymerase II into capped and polyadenylated primary (pri-) miRNAs which form loops. RNAse III, so called Drosha, and the double stranded RNA binding protein DGCR8 (DiGeorge syndrome critical region) form a microprocessor complex, generating a ~ 70nt hairpin precursor (pre-) miRNA. Nuclear export factor exportin 5 transportes pre-miRNAs from nucleus to the cytoplasm, where they are digested by another double strand specific ribonuclease, Dicer, into an imperfect 2nt 3´overhanging, 18-24 nucleotide miRNA duplex. The duplex is incorporated into the RISC complex (RNA – induced silencing complex) preferably at its weakest base pairing at the 5´ terminus. Argonaute proteins, also being included in the complex, remove one strand of the miRNA duplex while the other one remains solidly bound to the RISC complex (Gregory, Chendrimada et al. 2005). RISC complex is conducted by mature miRNA to the target RNA, provoking its degradation through imperfect complementary miRNA-mRNA base pairing, or its translational silencing (He and Hannon 2004). MiRNA induced mRNA degradation is presumed to take place in so called p-bodies and the exact mechanism of miRNA induced disabled translation remains unexplained, since initiation as well as translation seem to be concerned (Liu, Valencia-Sanchez et al. 2005).

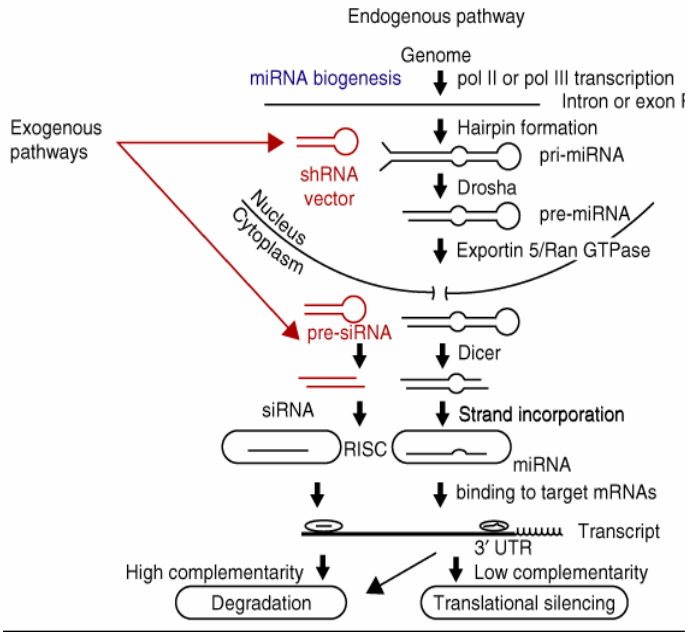

Figure 3: Biogenesis of microRNAs (www.nature.com)

On the contrary, evidence also indicate miRNAs to enhance translation of mRNAs. MicroRNAs oscillate between repression and activation depending on the stage of cell cycle. They inhibit translation in proliferating cells and induce activation in G_1/G_0 arrest (Vasudevan, Tong et al. 2007).

Next to micro RNAs three additional types of small RNAs have been found in animals: small interfering RNAs (siRNAs), repeat associated small interfering RNAs and Piwi-interacting RNAs (piRNAs). SiRNAs, genereated out of double stranded RNA and originated from viruses or overlapping transrcipts, match perfectly to their target mRNA and trigger degradation. Rasi RNAs were explored in *Drosophila* and emerge from antisense strands of genomic repeats or retrotransposons. PiRNAs are a germline specific class of small RNAs, existing in vertebrates and operating by attaching to Piwi proteins, a subfamily of Argonaute proteins.

MiRNAs are expressed tissue specifically during development, hinting at miRNAs to be eventually involved in differentiating and maintaining tissue identity (Kloosterman and Plasterk 2006). More than 500 human miRNA genes have already been identified (Sevignani, Calin et al. 2006) and the human genome is assumed to encode roughly a 1000, whereas the function of most mammalian miRNAs is not known. Each miRNA is said to have hundreds of evolutionary conserved targets and even more unconserved ones, which gives reason to speculate about 30% of the genome to be regulated by miRNAs (Davis and Ross 2008). For cells producing transcripts instead of proteins is energetically less costly and might be the reason why regulation at the RNA level is more efficient than on protein level (Sevignani, Calin et al. 2006).

2.2 Cancer Epigenetics

Cancer cells can be discribed to be immature, grow abnormally and generally lead to uncontrolled proliferation, sometimes loose attachment and metastazise.

Scientists investigating the principles of why and how organisms develop cancer, until recently focused studying a diversity of genomic and genetic alterations like point mutation, deletion, amplification and translocation. These findings built the groundwork for identifying oncogens and tumor suppressor genes. Nowadays, ascertainment of the importance epigenetic influences have on the generation of neoplasia has led to extensive efforts to study epigenetic modifications in the neoplastic cells. A majority of research has concentrated on the role DNA methylation

has on genexpression under physiological and pathological conditions. Aberrant DNA methylation is not only involved in tumorigenic events but also in several other ailments like cardiovascular diseases, the metabolic syndrome and neurological diseases. Genetic syndromes also contain epigenetic components. The Rett syndrome for example is caused by mutation in the MeCP2 gene (methyl-CpG-binding protein) and patients with ICF (immunodeficiency, centromer instability and facial anomalie) suffer from a mutation in DNMT3b. Cells` DNA of lupus patients were detected to be hypomethylated and methylation of the fragile X mental retardation-1 (FMR) gene is involved in the same titeled disorder (Robertson and Wolffe 2000; Esteller 2008).

2.2.1 Epigenetic patterns in cancer

Neoplastic developments involve aberrant regulation of multiple genes. In cancer cells fundamental epigenetic changes arise, causing aberrant gene expression patterns and leading to uncontrolled cell cycle progession and cell growth.

DNA methylation in cancer cells

In healthy cells, majority of the CpGs in genes or promoter regions are unmethylated, allowing genes to be transcribed when stimulated by appropriate transcription factors. In certain cases gene promoters can be methylated as a part of the common developmental process. Genomic or parental imprinting early in the germline requires a compact chromatine state and DNA hypermethylation in one allel of for instance growth-related genes, to ensure monoallelic expression. Similar gene switch off mechanism is observed in X-chromosome inactivation in women. Tissue specific expression pattern can also to some extend be attributed to DNA methylation (Esteller 2008).

Two major events happen in the DNA methylation landscape of neoplastic cells:

On the one hand the entire genome becomes hypomethylated, on the other hand intense hypermetylation occurs mainly in promoters of cancer related genes (Davis and Uthus 2004). Genome hypomethylation is an early event in neoplastic

development and assembles during all cancerogenic steps. Its extent correlates with the degree of tumor aggressiveness (Esteller 2008). Cancer progression might be due to loss of imprinting induced by global hypomethylation, reactivating silenced transposable elements and endogenous retroviruses. Transposable elements might also endorse genomic mutations and unregular chromosomal recombinations, furthermore hypomethylation could cause chromosomal instability (Davis and Uthus 2004). Hypomethylation of centromeric sequences usually occurs in neoplastic cells, eventually generating aneuploidy (Esteller 2008). Abnormal CpG hypermethylation commomly occurs in the promoter region of tumor suppressor genes, leading to inhibited expression of genes affecting virtually all cellular functions, such as cell cycle ($p16^{INK4a}$, $p15^{INK4}$, Rb), hormonal response (ESR1), apoptosis (DAPK, APAF-1), carcinogen metabolism (GSTP1), DNA repair (hMLH1, BRCA1) and cell adherence (CDH1 and 3, E-cadherin) (Davis and Uthus 2004). It has also been reported, that exonic CpG islands of $p16^{INK4a}$, $p15^{INK4}$ were more susceptible to *de novo* methylation than promoter CpG islands. Methylation might be seeded in exonic regions, wherefrom it could spread to other islands, including promoter regions. Subsequently, cell selection due to growth or multiplication advantage, emerged from spread of methylation into and inactivation of certain promoter regions, could generate the development of a specific type of cancer (Nguyen, Liang et al. 2001). Particular methylation inactivated genes are reported to be strongly tissue specific. Thus, a tissue specific profile of CpG hypermethylation exists in human cancer, potentially serving as biomarker in the detection of malignant disease with unknown origin (Davis and Uthus 2004). Colorectal cancers and adenomas for instance have been reported to show what was termed to be a “CpG island methylator phenotype”, a status characterized by coincidantal hypermethylation of a number of defined genes (Hinshelwood, Melki et al. 2009).

Reasons for global hypomethylation and loci specific DNA hypermethylation remain unclear. Several mechanisms are discussed: Overexpression of DNMT3b4, an inactive splice variant of DNMT3b, correlates with the degree of DNA hypomethylation on pericentromeric satellite regions of preneoplastic and neoplastic tissues. Moreover,

DNMT1 deficiency in APC mutant gene carrying *Min mice* caused a prevention of tumor development (Laird, Jackson-Grusby et al. 1995).

DNA methyltransferases have been shown to bind with higher affinity to broken DNA strands, abasic sites and uracil than they bind to their cognate hemimethylated DNA strand sites, in analogy to their ancestral function as DNA repair enzymes. It has been hypothezised that DNA lesions, which apppear frequently in human tumorigenic DNA, may be required for the disruption of normal DNA methylation patterns (Davis and Uthus 2004).

Histone modification in cancer cells

Changes in DNA methylation are closely associated with those in histone modification. Similarly to DNA methylation, disarranged global genome histone patterns, with both loss and gain of histone lysine acetylation and methylation have been reported in transformed cells. Primary event of gene silencing seems to be loss of lysine acetlyation whereas enhanced histone methylation occurs afterwards. Thus HDACs are appointed to be one of the major targets for therapy. Diminished acetylation does not exclusivley lead to gene silencing as it can also result in degraded DNA repair, escalating the risk of evolving neoplastic cells. HDA1, a class II HDAC homologue in *Caenorhabditis elegans*, participates in controlling cancer related extracellular as well as tissue specific genes and abnormal telomer elongation might be caused by loss of H3-K9 methylation in mice (Yoo and Jones 2006). The majority of aberrant histone modifications due to inappropriate targeting of one or more histone modifying enzymes were discovered at individual gene promoters. E2F transcription factor, for example, recruits retinoblastoma protein (Rb), a tumor suppressor, to its target genes and Rb in turn attracts HDAC1, leading to transcriptional silencing of genes such as Cyclin E, participating in cancerogenic pathyways (Brehm, Miska et al. 1998). Studies using chromatin immunoprecipitation (ChIP), a method determining sites of protein – DNA interaction, found $p16^{INK4A}$ tumor suppressor silencing to be associated with hypermethylation of H3K9 and hypomethylation of H3K4 (Meng, Zhu et al. 2007).

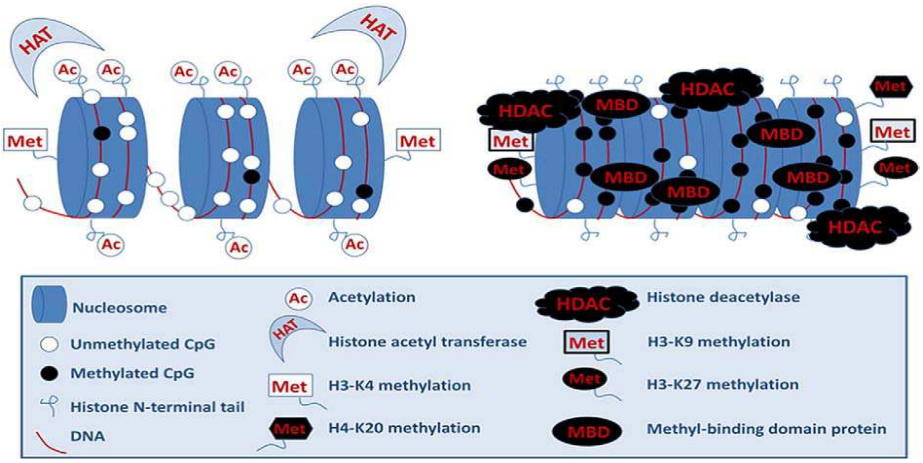

Figure 4: DNA methylation and histone modification in normal and cancer cells (Kristensen, Nielsen et al. 2009)

MicroRNA expression in cancer cells

Abberant expression profile of the miRNome has been documented to correlate with several human diseases including neurodegenerative illnesses and diabetes. As miRNAs regulate cellular processes like apoptosis, proliferation and differentiation they are also involved in the pathophysiology of all types of human cancer. (Sevignani, Calin et al. 2006). The reason for miRNAs abnormal regulation in cancer cells might be their prevalent location at fragile sites, sites of loss of heterozygosity, amplification and common breakpoint regions, which are susceptible to genetic alterations in cancer cells, leading to altered miRNA expression in cancer (Davis and Ross 2008). Furthermore loss of expression of several miRNAs could be due to CpG hypermethlation on their regulatory region, as demethylating agents are able to induce reexpression of for example miR-127 and downregulation of its putative target proto – oncogen BCL-6. In contrary, epigenetically silenced miR-124a causes activation of Cyclin -D -kinase 6, an oncogenic factor and subsequent phosphorylation of cell cycle regulator Rb and cell cycle progression (Esteller 2008). Some data reveal that downregulation of certain subsets of miRNAs occures commonly in cancerous cells, leading to the suggestion, that they might function as potential tumor suppressors or participate in tumor suppressor signaling pathways (Davis and Ross 2008; Esteller

2008). P53, being one of the most frequently mutated genes, transcriptionally regulates levels of miR-34 through direct binding to the promoter region. In addition, DNA damage can induce activation of miR-34 in wild type animals but not in p53 knock outs. Ras oncogen overexpression, present in 15-30% of all cancers, seems to be regulated by miRNA. Studies conducted in lung cancer patients showed, that low level of let-7 correlated with poorer prognosis after surgery, as let-7 was demonstrated to target a number of genes involved cell cycle regulation (Zhang, Gao et al. 2009).

2.2.2 Epigenetic biomarker and diagnostics

CGI methylation aberration occurs early and is functionally operative in carcinogenesis, therefore it offers potential biomarkers to assess an individual's risk of having or developing neoplasia (Belshaw, Elliott et al. 2008). Several genes have the potential to serve as DNA methylation biomarkers for early cancer diagnostics or for predicting prognosis and response to therapy.

Being a stable molecule, as opposed to RNA, DNA can simply be extracted from tissues and easily accessible body fluids like blood, sputum or urine, potentially allowing early recognition and diagnosis of tumors and screening of high-risk populations. Circulating tumor derived material has been detected to reflect methylation pattern of different types of primary tumors. $P16^{INK4a}$ promoter methylation, in the sputum of smokers for instance, has been assessed to be a biomarker for early detection of lung cancer. Futhermore, isolation of DNA, carrying the methylation information out of formalin fixed, paraffin embedded (FFPE) tissue, displaying the bulk of clinical archived tissue, and subsequent PCR technique can be conducted without any difficulty. Moreover, sample handling for subsequent methylation analysis is not as elaborat as for cDNA or protein expression assays. Methylation signals are easier detectable in comparison to loss of heterozygosity or changes in gene expression, which are delicate to discover when plenty of normal DNA is “disturbing”. Since measurement of DNA methylation via mulitplex PCRs and DNA methylation microarrays enables detection of multiple genes all at once, identification of cancer tissue specifc DNA methylation profiles was highly simplified (Paluszczak and Baer-Dubowska 2006; Kristensen, Nielsen et al. 2009).

Measuring promoter methylation of $p16^{INK4b}$, $p15^{INK4a}$, RASSF1A and ESR1 has been determined to be a epigenetic biomarker in a noninvasive approach to early detection of hepatocellular carcinoma and non small lung cancers in high-risk patient population. Methylated DNA sequences in serum correspond to cancer-related epigentic events in occult tumors, suggesting that DNA sequences shed from tumors into blood might represent a potentially valuable biomarker for preclinical detection of neoplastic events. In some cases methylated allels could even be detected years before clinical diagnosis (Rivenbark and Coleman 2007; Suga, Miyajima et al. 2008). Thus DNA methylation measurement has also been shown to have predicitve abilitys. Methylation of $p15^{INK4b}$´s and estrogen receptor´s promoter site for instance forecasts poor prognosis in patients with early stage myelodysplastic syndrome and hypermethylation of E-cadherine was negativley correlated with disease free survival in oral toungue carcinoma as well as with early recurrance in gastric stromal tumors. Methylation detection might also have promising potential in the molecular monitoring of disease after therapy. DNA hypermethylation has been associated with the prediction of response to chemotherapy. Two established hypermethylation markers of clinical response to therapy are largely debated: MGMT and missmatch repair gene hMLH1. Hypermethylation of MGMT can be seen ambivalently, since in early tumor stages diminished expression affects cells´ defense against carcinogenic DNA alkylating agents whereas hypermethylation in present cancer conditions response to alkylating drugs. Loss of mismatch repair often caused by hMLH1 promoter hypermethylation is associated with resistance to clinically fundamental drugs. Methyltransferase inhibitor 5 aza-2´-deoxy azacytydine dependent demethylation of hMLH1 reversed resistance to cisplatine, carboplatin and several more chemotherapeutics (Paluszczak and Baer-Dubowska 2006; Kristensen, Nielsen et al. 2009). Levels of several HDACs have also been recommended to eventually serve as potential biomarkers and detection of extent of histone acetylation might be a good parameter to measure clinical response to HDAC inhibitors (Stimson and La Thangue 2009).

MiRNAs are more stable than mRNAs, giving the possiblity to quantitativley recover them from formalin- fixed paraffinembedded tissues (Davis and Ross 2008). Large scale miRNA microarrays have been used to determine and compare miRNA expression profiles in healthy cells and tissues to those in cancers, determining distinct miRNA patterns to be associated with certain tumor types. Researchers even claime to more successfully classify poorly differentiated tumours, by the use of miRNA expression profile than by messenger RNA profiles, underlining the capability of miRNA profiling in cancer diagnosis and therapy planning (Lu, Getz et al. 2005).

2.2.3 Epigenetic therapy

The insight in aberrant epigenetic patterns in malignant cells and potential reversibility of epigenetic modifications leads to an extensive quest for new drugs, offering a promising opportunity for novel cancer treatment.

DNA methylation inhibitors / DMNT inhibitors

DNA methylation inhibitors can be classified into nucleoside analogues and non nucleoside analogues. Nucleoside analogues have a modified cytosine ring, which is affixed to a ribose or deoxyribose residual. They are processed by kinases transforming nucleosides into nucleotides to integrate them into DNA or RNA. Methylation of those altered cytosines is unfeasible, as modifications at carbone 5 leads to the formation of a covalent complex preventing the release of DNMTs. 5 –azacytidine (azacitidine) and 5- aza-2´ deoxycytidine (deacatabine) have been the first “epi-drugs” to be synthesized and are potent DNA methylation inhibitors at micromolar concentration. These agents have been thoroughly investigated for treatment of haematological diseases and have been approved for treatment of myelodysplasia. Unfortunately, short half lives in aqueous solutions, their instability even in neutral pHs and their cytotoxicity complicates their delivery. Anyhow, tumor suppressor gene demethylation already takes place at low, not cytotoxic concentrations, allowing elongated treatment duration to ensure persistent demethylating effects (Yoo and Jones 2006; Kristensen, Nielsen et al. 2009).

In comparison to azacitidine, further nucleoside analogues like 5-fluoro-2´deoxycytidine and zebularine are much more stable and less cytotoxic (Cheng, Yoo et al. 2004). A handful of non-nucleoside analogues have been described to inhibit DNA methylation but not many of them reached clinical trials. These molecules suppress DNA methylation by directly binding to DNMTs catatlytic region, without being incorporated into the DNA. RG108 was designed to accurately fit into the catalytic pocket of human DMNT1 and suppresses its activity, whereas MG98 prevents translation of DNMT1 by binding to its mRNA. Anticancerous effects of these substances have been observed in several solid cancers in phase I studies. The demethylating effects of epigallocatechin-3-gallate, hydralazine and procainamide have been compared to deacatabine in a study, drawing the conclusion that deacatabines, and accordingly the potential of nucleoside analogues in general, is far more effective (Chuang, Yoo et al. 2005). MicroRNA expression seems also to be enhanced by DNMT inhibitors subsequently modyfing the epigenome independent from DNA methylation (Kristensen, Nielsen et al. 2009). Natural sponge *Pseudo-ceratina pupurea* components, so called psammaplins, are a group of bromo tyrosine derivates inhibiting both: DNA methyltransferases and histone deacetylases (Yoo and Jones 2006).

HDAC inhibitors

Since different diseases have been associated with abnomal expression of different HDAC isoforms, many drugs with HDAC suppressing effects have been described and plenty of them are participating in ongoing clinical trials. Most substances function through interference with HDAC´s catalytic domain, blocking substrate recognition and inducing gene expression. With many existing enzymes implying the highly conserved catalytic domain of class I,II and IV HDACs, it is hard to design isoform specific inhibitory drugs. Most of the listed substrates target class I and II HDACs , which are zinc- dependent. HDAC inhibitors are very diverse in origin and structure. Based on chemical properties they can be divided into different categories. Sodium butyrate and valproic acid belong to the group of short chain fatty acids. Valproat has been reported

to degrade HDAC2 in a proteasome and potentially reduce breast cancer tumor growth and metastazation in rodents and a phenylbutyrate derived HDAC inhibitor named OSU-HDAC42 demonstrated promising effects on ovarian and hepatocellular cells by inducing apoptosis as well as prevention of tumor progression to less differentiated prostatatic intraepithelial tumors in mice (Kristensen, Nielsen et al. 2009).

Hydroxamic acids form another class of HDAC inhibitors comprising trichostatin, vorinostat, panobinostat and belinostat. Their mutuality is the zinc-binding group, which is analogous to N-acetylated lysines acetyl group, chelating zink ions in their cylindrical pocket (Butler and Kozikowski 2008).

Trichostatin A is a very potent HDAC inhibitor having many antitumorigenic effects. But due to its severe side effects it is not applied in clinical trials. Belinostat has shown good antitumorigenic effects in ovarian xenograft tumors and suppressed bladder and prostatate cancer growth in vivo and in vitro, partially by enhanced expression of $p21^{WAF1}$. Vorinostat is utilized in combination with other chemotherapeuticals in class I, II and III clinical tirals. Nevertheless a noted problem among hydroxyamic acids is, that they are unselective to HDAC isoforms. Cyclic peptide romidepsin (FK228), being activated due to reduction of its disulfide bond, displays a further class of HDAC inhibitors and has been reported to function against human tumor xenografts and murine tumors. Benzamide, entinostat (MS – 274) and MGCD- 0103 for instance, exibit 2´aminoanilide residues, acting as weak zinc-chelating group, and thus leading to interference with HDACs catalytic domain. In the end, several microarray studies have demonstrated that HDAC inhibitors generally concern only a small section of the transcriptome (Kristensen, Nielsen et al. 2009). The amplitude of interactions HDACs have with non-histone proteins such as transctiption factors, DNA repair enzymes, chaperone proteins, structural and signal transduction proteins makes it difficult to exactly predict effects and to generate HDAC inhibiors, which are able to reactiavate targeted genes without unwanted outcomes (Su, Altucci et al. 2008).

Finally synergistic effects of DNMT and HDAC inhibitors in combined treatment strategies have been observed in various studies on different cancer cell cultures and rodent studies (Kristensen, Nielsen et al. 2009). As “epi-drugs” impact is transient and

with discontinuity of treatment, aberrant epigenetic patterns can return and malignant cell population is able to reappear. An advantage of epigenetic drugs is that most of them function at low doses, showing hardly any cytotoxic effect and therefore being applicable over a certain time (Yoo and Jones 2006).

2.2.4 Environmental and nutritional influences on DNA Methylation and cancer susceptibility

The health of organisms and their offspring is modifiable by interactions between enviroment, nutrition and the genome. Since epigenetic events are receptive for modification they might explane how environmental factors, such as diet, modify cancer risk.

Statistical evidence demonstrates that our ancestors life circumstances, auch as food availability impacts life expectancy of subsequent generations. This longevity effect was attributed to epigenetic inheritance (Kaati, Bygren et al. 2007).

Changes in dietary habits in Japan for instance, altered the incidence of several cancer´s appereance, providing strong evidence for the importance of diet as a enviromental factor in the aetiology of diseases (Hinshelwood, Melki et al. 2009).

A multitude of biologically active food compounds have been described to impact DNA methylation directly and by enzyme activity interference, some of which are discussed in this study and indirectly by metabolic processes associatd with energy metabolism (Johnson and Belshaw 2008). Most remarkable evidence to show nutritions influence on the epigenome of mammals came from studies with mice possessing the *agouti viable yellow gene*, with its normal funktion of confering the wild type coat colour. Dominant mutations in this gene have been discovered to cause coat colour change to yellow, together with systemic phenomena as obesity, non-insulin-dependent-diabetic like constitution and susceptibility to various types of cancer. Mutation in the *agouti viable yellow* mouse (A^{vy}) bases on a intracisternal A particle (IAP) which is a retroviral insert, having been included into the DNA in an antisense orientation, directly 5´of the first coding exon of the gene. Methylation status of the 5´ long terminal repeat within the inserted IAP alters the gene expression. It is acting like a wild type allel in a

methylated state and when unmethylated, the gene is expressed ubiquitously leading to the full extend of the agouti syndrome. An intermediate state of methylation entails a speckeled appearence. Thus the coat colour stands for the methylation status of the allele (Wolff, Kodell et al. 1998).

Researchers availed themselves of this model, showing that feeding a diet rich in methyl-donors, as for example folic acid, to pregnant females can lead to alterations in the offspring`s agouti gene. There is good evidence that this alteration is due to a change in the epigenetic state and might be passed to a consecutive generation via female germline. Even exposure to methyl donors in utero modifies epigenetic marks of developing germ cells. These modifications seem to be maintained through gametogenesis, fertilisation and embryonic development of the following generation (Johnson and Belshaw 2008).

Figure 5: Agouti mice (Jirtle RL 2009)

Food components, participating in the one-carbon metabolism, including vitamine B_{12}, vitamine B_6, folate and methionine provide methyl groups for the biochemical pathway of methylation processes. A carbon unit from serine or glycine is delivered to tetrahydrofolate to transfer it into 5,10-methylenetetrahydrofolate. Vitamin B_6 is a transferenzymes co-facto. 5,10-methylenetetrahydrofolate is utilized to produce thymidine, formyltetrahydrofolate for purines and 5-methyltetrahydrofolate to methylate homocystein to form methionine, catalyzed by the vitamine B_{12} dependent enzyme methionine synthase. An ATP-dependant adenosylation of methionine generates S-adenosylmethionine (SAM), which is the methyl group donor for more than 80 biological methylations, inlcuding DNA, RNA and protein. A lack of dietary folate depletes plasma SAM pool, causing enhanced concentration of S-adenosylhomocystein, as the equilibirum of the interconversion favors SAH synthesis, leading to SAH- level dependent inhibition of the DNA methyltransferase (Dolinoy, Weidman et al. 2006).

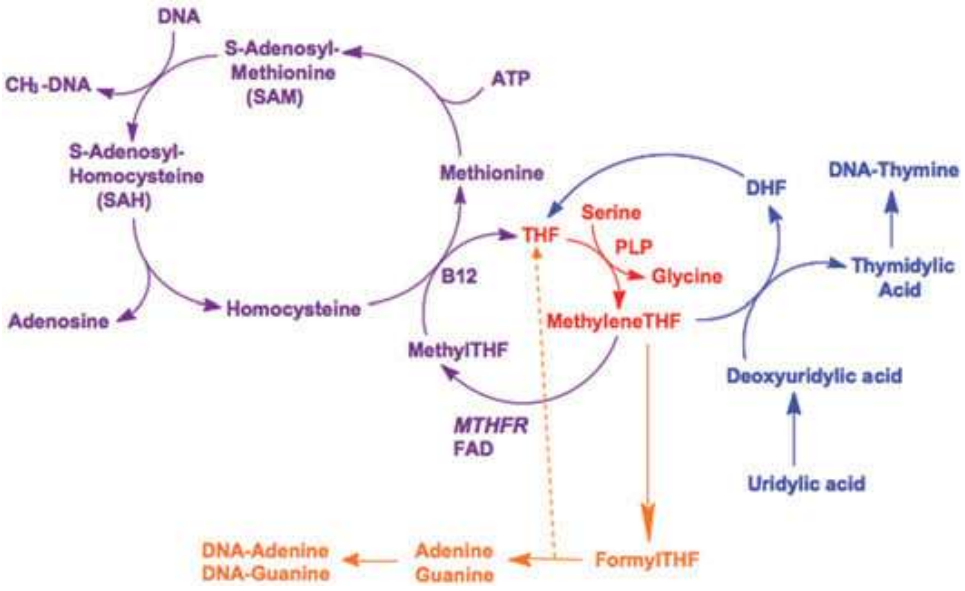

Figure 6: Folate metabolism, SAM supply
(Miller, Borowsky et al. 2008)

The SAM:SAH ration thus seems to be a limiting determinant for the transmethylation capacity of a system. Diminished dietary folate induced inhibition of DNA methylation appears to be associated with increased cancer susceptibility. Epidemiological data present, that low folate status is linked to higher risk in developing colorectal cancer, as tumor suppressor gene p53 or E-cadherine for instance also undergoe hypomethylation. Alkohol consumption affects folate metabolism and enhances cancer susceptibility in oral cavity, pharynx, esophagus and liver. DNMT mRNA levels in sperm were reported to be altered by chronic exposure to alcohol, therefore interrupting parental genomic imprinting. This effect is assumed to be responsible for paternal impact of alcohol on abnormal fetal development and growth (Davis and Uthus 2004).

Polymorphisms in major DNA methylation regulating enzymes, mainly in the methylentetrahydrofolat reductase (MTHFR) gene, affect genome wide and gene specific DNA methylation. Most common gene variants are C677T, which has been associated with a reduced risk of developing colon cancer but an increased risk of developing breast cancer and A1298C possessing reduced enzyme activity that causes deficiency in methyl group donors (Johnson and Belshaw 2008).

Several more bioactive food components impacting DNA methylation, such as selenium, vitamin A and genistein, have been suggested to alter cancer susceptibility. High levels of retinoic acid and zinc deficiency induced DNA hypomethylation in rat livers, due to diminished utilisation of methyl groups from SAM (Davis and Uthus 2004). Selenium has been noted to correlate inversly with homocystein levels in humans eventually influencing methylation reactions through the SAM:SAH ratio and a deficiency induced hypomethylation has been observed in Caco-2 cells as well as in the liver and colon of rats. This might be due to direct interaction between the DNA methyltransferase and selenium, as increased selenium concentrations have been shown to suppress DNMT1 actvity and expression (Johnson and Belshaw 2008). Cadmium is an DNMT inhibitor as shown in animal studies. This effect is likely to be due to cadmium´s, and also zink´s propertie as a metal possibly binding to the cystein residue of the DNMT acitve center. All things considererd there might be an optimal level for dietary components contributing an unobstructed DNA methylation process, as for instance an absence as well as an excess of arsenic was demontsrated to entail global hypomethylation in rat liver (Davis and Uthus 2004).

A diet rich in plant-derived foods conceals a variety of biologically active compounds also named phytochemicals, a large part of which are polypheols affect epigenetic modifications. Some of them, which were used in these experiments, are discribed below.

An impressing example of how enviroment impacts epigenetic patterns was shown by Weaver et al. demonstrating how maternal care can affect DNA methylation state of the glucocorticoid receptor (GR) gene promoter in the hippocampus of the offsping. Ratmother´s nusing modified histone acetylation, DNA methylation and binding of the transcription factor NGFI-A to the GR promoter, altering its expression and hypo-thalamic-pituitary-adrenal response to stress. This effect was shown for 900 further genes and these changes were modifiable with HDAC inhibitors or methyl donors (Weaver, Meaney et al. 2006).

Nuclear receptor estrogen receptor alpha (ESR1) expression is associated with alterations in rats maternal behaviore. Enhanced ESR1 promoter methylation came

along with less licking and grooming, in comparison to low level methylation rats. Aging has been reported to have an elemantary impact on DNA methylation in several cell types. Studies conducted with monozygotic twins provide evidences, that the major contributors to these age related modifications on the epigenetic pattern are enviroment and lifestyle (Johnson and Belshaw 2008). Genomic DNA hypomethylation and gene specific promoter DNA hypermethylation, dependent on the type of tissue, is associated with aging. ESR1 hypermethylation has been shown to be altered in human colon, heart, vessels, and prostatate tissue. In aged human colonic mucosa ESR1 expression was discovered to be diminished due to promoter hypermethylation, which is analogical to earliest epigenetic alterations predisposing to cancerogenesis. (Issa, Ottaviano et al. 1994).

Many more genes responsible for tumor suppression, cell cycle regulation, apoptosis, detoxification and lipid metabolism have been shown to be affected by aging dependent promoter DNA methylation. Comparing young and old mice, $p16^{INK4a}$ promoter methylation enhanced concurrent with age and treatment with dietary folate. But $p16^{INK4a}$ was also found to be hypermethylated in aged human liver and stomach tissue (Kim, Friso et al. 2009).

Different possible mechanisms have been proposed to be responsible for age – associated changes in genomic DNA methylation. DNMT1 declines continuously from birth to age, eventually leading to passive decreased doublication of methylation patterns during mitosis (Vertino, Issa et al. 1994). Furthermore aging implicates raised homocystein levels, which might cause disruption of methyltransfer as S-adenosyl-homocystein inhibits DNA methyltransferase (Yi, Melnyk et al. 2000). Enhanced homocystein levels are associated with an age related decrease in sex hormones. Because of decreased folate intake and changed folate bioavailability folate status also declines during aging. Deficiency in zink and selenium might also contribute to DNA hypomethylation by modifing one carbone metabolism. Finally, unlike global hypomethylation due to decreased DNMT1 activity, gene specific promoter hypermethylation was detected during aging is possibly induced by enhanced *de novo* methylation through other DNMTs. DNMT3b for instance was found to be more active

in aged and immortalized cells. Another assumption is, that heterochromatine, being highly methylated, might spread to euchromatine over boundary elements by aging. However, aging processes have been suggested to not only be associated with DNA methylation, but other epigenetic phenomena of chromatin remodelling appears to also be involved (Kim, Friso et al. 2009).

2.3 Examined genes and substances

Gene selection based on studies suggesting, that two distinct types of promoter methylation occur in cancer cells. One is suggested to involve gene methylation in cells as a function of age (ESR1) and the second one mainly in neoplastic cells ($p16^{INK4a}$ and $p15^{INK4b}$) (Hinshelwood, Melki et al. 2009). Each of the three genes is involved in the regulation of the cell cycle through directly or indirectly influencing cyclin dependent kinases (CDKs) (Foster, Henley et al. 2001; Chim, Wong et al. 2003).

2.3.1 The ESR1 gene

The estrogen receptor alpha (ESR1) plays an important role in physiological processes but is also known to be involved in many pathologies as it regulates growth and differentiation of several tissue types. ESR1 appertains to the superfamily of nuclear receptors and family of steroid receptors, acting as a ligand- inducible transcription factor. Ligand bound ESR1 works as a key-transcription factor in various molecular pathways and adequate modulation of ESR1 expression is crucial to determine growth potential. A variety of hormones, growth factors and other agents implicate multiple cellular systems in the control or ESR1 expression (Pinzone, Stevenson et al. 2004). Estrogen has at least two courses of action. The nonclassical pathway of estrogen action leads to the activation of the MAPK (Mitogen-Activated Protein Kinase) cascade and other cell proliferating effects (Wong, McNally et al. 2002). The classical pathway depends on immediate interaction with its receptor, mediating gene transcription through binding palindromic ERE (Estrogen Response Elements) or interacting with transcription factors influencing their activity. ESR1 mediates cell cycle progression

through transcriptionally regulating estrogen-responsive genes, such as Cyclin-D1 (Foster, Henley et al. 2001), a critical mitogen-regulated cell cycle control element, affecting activation of Rb (Retinoblastoma Protein) (Lamb, Ladha et al. 2000).

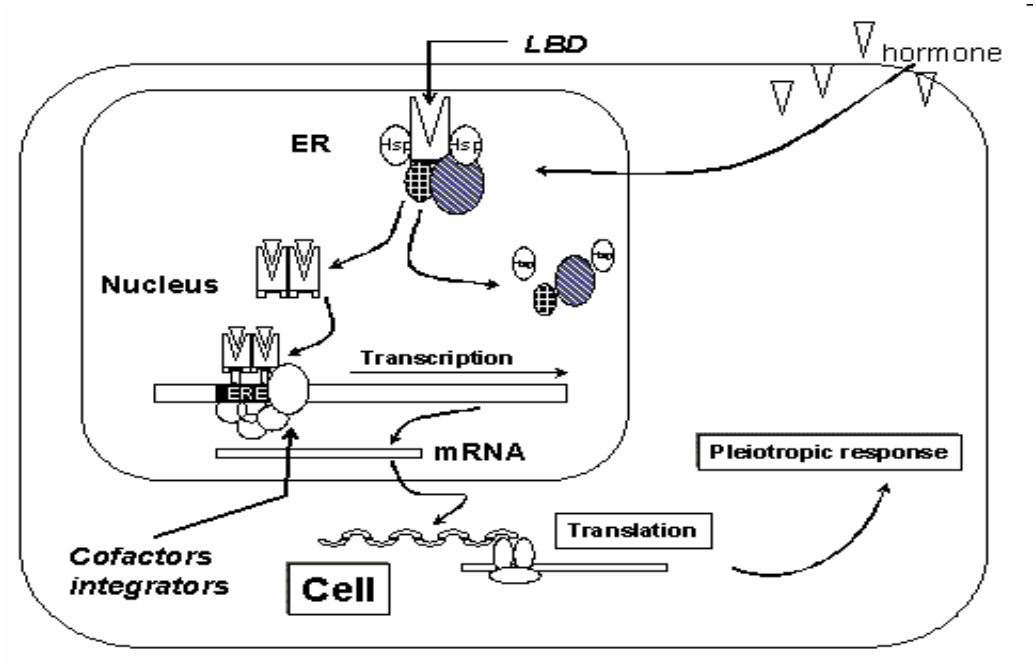

Figure 7: Estrogen receptor mechanism
(www.andrology.be)

Approximately two-thirds of cases of breast cancers express ESR1 protein and with lacking gene expression these cancers seldomly respond to endocrine therapy and are associated with worse clinical outcome. Hence deprivation of ESR1 gene expression leads to acquirement of hormone resistance. ESR1 negative breast cancers show no significant genetic alterations in the ESR1 gene, such as insertion, deletion, rearrangement or point mutation. Loss of gene expression is currently explained by either epigenetic modifications or lack of *trans*-acting factors (Yoshida, Eguchi et al. 2000). ESR1s expression is known to be epigenetically disregulated in several diseases comprising breast, prostatate and colon cancer but may also play a role in atherogenesis and development of heart disease (Post, Goldschmidt-Clermont et al. 1999; Li, Shiina et al. 2004; Macaluso, Montanari et al. 2007), as it has been demonstrated to modulate expression of proteins involved in the regulation of plasma lipid metabolism, such as apolipoprotein E and LDL receptor (Klos, Boerwinkle et al.

2008). Variants (SNPs) of the ESR1 gene have been associated with components of the metabolic syndrome, including obesity, HDL-cholesterol, blood pressure and type 2 diabetes (Gallagher, Langefeld et al. 2007).

The ESR1 gene on chromosome 6q25 has a typical CpG island in its promoter and first exon that contains several methylation- sensitive restriction ezyme sites. In the ESR1 promoter of normal, not neoplastic human colon mucosa, methylation of several CpG sites were found to be direrctly related to the age of examined individuals. Thus ESR1 CpG island methylation arises as a direct funktion of age in normal colonic mucosa and is present in virtually all colonic neoplasias. In cultured colon cancer cells, methylation dependent loss of ESR1 expression resulted in deregulated growth, linking CpG methylation during physiological aging to the development of neoplasia (Issa, Ottaviano et al. 1994). Similar results were found in vascular tissue with age dependent methylation associated inactivation of the ESR1 gene, propably playing a role in the aging of the vascular system and its consequences (Post, Goldschmidt-Clermont et al. 1999). Estrogen is known to be neuroprotective. Ischemia induction in the cerebral cortex of female rats resulted in a repression of DNA methylation and MeCP2 adhesion in the ESR1 promoter. MECP2 usually interacts with the ESR1 gene to maintain methylation and suppress gene expression (Westberry, Prewitt et al. 2008).

ESR1 silencing due to hypermethylation is associated with binding of specific methyl-binding proteins, DMNTs and HDACs. Inhibition of HDAC acitvity through Trichosatin A lead to an accumulation of hyperacetylated core histones. HDAC and DNMT inhibitors modulate histone methylation at K3-K9 and H3K4 to allow an open transcription active chromatine configuration needed for reactivation of silenced ESR1 transcription (Pinzone, Stevenson et al. 2004; Sharma, Blum et al. 2005).

Trans active factors also seem to play an important role in the epigenetic modulation of ESR1. The presence of a specific pRb2/p130 multimulecular complex on the ESR1 promoter strongly correlates with the methylation status of this gene, eventually by cooperating with DNA methyltransferases in maintaining methylation patterns of the ESR1 gene (Macaluso, Montanari et al. 2007).

2.3.2 Tumor suppressor genes $p16^{INK4a}$ and $p15^{INK4b}$

The mammalian cell cycle system is controlled by CDKs, catalyzing the addition of phosphate groups to particular serines or threonines in order to alter protein´s attributes. CDKs are activated through subunits termed cyclins. During cell cycle the amount of CDKs persists at the same level whereas the number of cyclins oscillates. Reacting on antiproliferative and growth deprecating signals, CDKs associate with inhibitory subunits, CDK inhibitors (CDKi) (Chim, Wong et al. 2003).
ESR1 contributes to cell cycle progression by inducing transcription of Cyclin –D1 (Foster, Henley et al. 2001), a cell cycle control element, which associates with CDK 4 or CDK 6 (Lamb, Ladha et al. 2000). Among the group of the CDK - inhibitors are tumor suppressor genes $p16^{INK4a}$ and $p15^{INK4b}$ belonging to the INK4 family and specifically binding to CDK 4 and 6 leading to pause of cell cycle progression in the G1 phase. The INK family members share similar functional domains, ankyrin repeats for protein-protein interactions, which allow them to compete with Cyclin D for CDK 4 and 6 (Chim, Wong et al. 2003).

In the setting of $p16^{INK4a}$ and $p15^{INK4b}$ inactivity, CDK 4/6 is able to couple with Cyclin D, leading to phosphorylation of Rb, which sequesters the transcription-factor E2F, stimulating entry into the S phase (Ishiguro, Takahata et al. 2006).

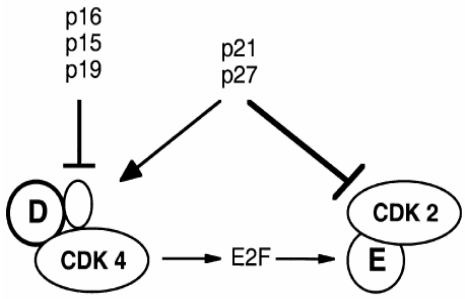

Figure 8: CDK-inhibitors (Blagosklonny 2009)

Even though $p16^{INK4a}$ and $p15^{INK4b}$ are located close to each other on chromosome 9p21 and show high homology in the amino acid sequence, transcriptional activation systems were suggested to be different. $P15^{INK4b}$ induction during TGF-β triggered growth inhibition was reported to be associated with downregulation of protooncogen myc, whereas another study found $p16^{INK4a}$ transcription to be regulated by junB, a transcription factor inhibiting cell cycle progression. Moreover, induction of both $p16^{INK4a}$ and $p15^{INK4b}$ expression were reported to be modulated by the RAS/Raf/MEK/MAPK pathway in cell models. MAPK

members mediate cell prolifeation, stress response and growth suppression due to a variety of agonists (Wen-Sheng 2003; Ishiguro, Takahata et al. 2006).

$P16^{INK4a}$ and $p15^{INK4b}$ are known to undergo genetic alterations such as point mutations, deletions or loss of heterozygosity in the course of cancer development but also methylation dependent inactivation of the $p16^{INK4a}$ promoter has been found in various neoplasias, such as colorectal, lung and breast carcinomas. $P15^{INK4b}$ is well documented in hematolocical malignangy, but less in solid tumors (Ishiguro, Takahata et al. 2006).

Both tumor suppressor genes have a promoter and an exonic CpG island. In a study with several cancer tissues invariable hypermethylation of exon 2 has been detected for either, $p16^{INK4a}$ and $p15^{INK4b}$, whereas promoter methylation was specific for the cancer type. This might be explained by nonpromoter CpG islands to be somehow less protected against de novo methylation than those in the 5´region of a gene, maybe due to promoter CpG protection from DNA methyltransferases through steric hindrance by transcription-initiation complexes and various transcription factors. Differences in chromatine structure at exonic CpG islands could be another explanation. It has been demonstrated for instance, that suppression of poly (adenosine diphospate ribosyl-)ation of histone H1 induces *de novo* methylaton in transfected foreign DNA, inducing chromatin compaction and hypermethylation of endogenous islands (Nguyen, Liang et al. 2001). Several studies on hepatocellular carcinoma have demonstrated methylated $p16^{INK4a}$ and $p15^{INK4b}$ to be detectable in the patients serum at the time of cancer diagnosis. Accordingly, methylation of $p16^{INK4a}$ and $p15^{INK4b}$ could serve as an potentially valuable biomarker for early preclinical, modestly invasive detection of malignant lesions (Wong, Lo et al. 1999; Wong, Lo et al. 2000).

2.3.3 Substances

Since epigenetic modifications are potentially reversible, compared to genetic mutations, they display attractive targets for therapeutic or dietary support of pharmacological intervention.

Substances used in this study are all bioactive food components or result of micobiobial processing, aside from zebularine, serving as a reference for DNA methylation inhibition.

Folic acid

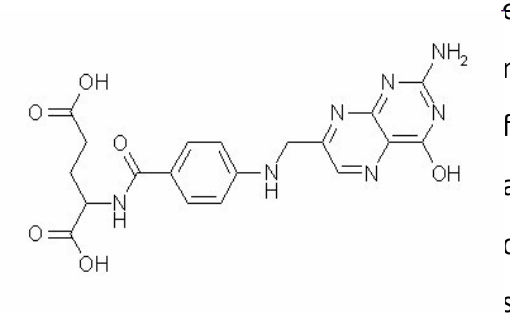

Figure 9: Folic acid structure

Being a water soluble vitamine, folate is essential for numerous cellular processes. 5,10-methylene-tetrahydrofolate can be transformed to 10-formyltetrahydrofolate, which is a purine and it is necessary for the conversion of deoxyuridine to thymidylate for DNA synthesis and repair. Folate deficiency can cause uracil mis-incorporation and DNA repair mechanisms cut misplaced uracil, whithout having newly synthesized thymidylate for replacement. Thus DNA becomes fragile, provoking strand breaks. This predisposes to tumorigenic processes.

Fundamental for epigenetic procedures is the conversion of 5,10 methylene-tetrahydrofolate to 5-methyltetrahydrofolate. It is needed for the transfomation of homocysteine into methionine, since it serves as methyl donor. ATP therafter activates methionine to S-adenosylmethionine (SAM), which in turn is an universal methyl donor, dispensing methyl groups in a variety of reactions. Low SAM levels due to a lack of folate supply suppresses methylation reactions, including DNA and histone methylation (Miller, Borowsky et al. 2008). Effects of folate deficiency and supplementation on DNA methylation are gene and site specific and seem to depend on degree and duration of folate depletion, cell and tissue type and kind of organ (Kim 2005).

Genistein

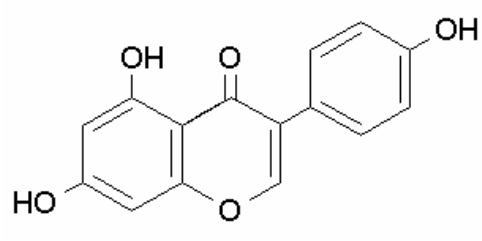

Figure 10: Genistein structure

Genistein is a natural isoflavone found in soybean products. Incidence of breast and prostate cancer is significantly higher in the US or Europe than Asia. Researchers assume, that this might be due to different dietary patterns, with asians consuming a diet traditionally high in soy products. Supporting this supposition epidemiological studies detected an inverse association with the risk of developing benign fibrocystic conditions or different cancer types and comparatively high levels of soy isoflavone consumption.

Due to genisteins hydrophobic property it is assumed to be taken up by cells not being processed and biologically activated before. Since genistein exhibits structural homology it competes with 17β-estradiol in estrogen receptor binding assays, therefore offering potential mechanism in the prevention of hormone related cancers. High plasma concentrations of genistein correlated with antimetastatic acitvity in vitro. Next to its anticancerogenic properties including increase of sensitivity of cancer cells to radiotherapy, chemotherapeutical and cytotoxic drugs, genistein lowers the incidence of cardiovascular diseases (Banerjee, Li et al. 2008). Thanks to genisteins antioxidative effect, it neutralizes free radicals, protecting cells against reactive oxygen induced expression of stress response related genes (Ruiz-Larrea, Mohan et al. 1997).

Genistein inhibits protein-tyrosine kinase, a mediatior of growth factor effects. Also HER-2 protooncogene phosphorylation is inhibited by genistein leading to delayed HER-2 overexpressing tumor growth. Genisteins antiproliferative and proapoptotic properties might also be due to its ability to inhibit 5 alpha reductase and protein histidine kinase (Banerjee, Li et al. 2008). However there is growing evidence, that genistein might also be a risk factor under certain conditions, since recent reports suggest genistein to have genotoxic effects as it inhibits topoisomerase I and II , causing DNA breakage and genetic lesions, such as chromatid exchanges and chromosomal translocation in peripheral lymphocytes (Kim, Yang et al. 2008).

Genistein is able to eventually induce apoptosis by preventing activation of nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NFkB) via AKT signaling mediation. Akt is a serine/threonine protein kinase and a crucial part of cellular survival pathway. It works downstream of phosphatidylinositol 3-kinase (PI3K) in response to mitogen or growth stimulation and its activation modulates the activity of several transcription factors such as fork head transcription factor (FOXO) and NFkB. FOXO proteins operate downstream of PTEN (phosphatase and tensin homolog) tumor suppressor, that was demonstrated to be induced by genistein and to negativley modulate Akt activity. NFkB is constitutionally activated in several tumor types and modulates gene expression of proliferative, apoptotic, inflammatory and immune response proteins. Sirtuines regulate the acitvity of several transcription factors and coregulators, like p53, NFkB, FOXO1, 3a and PPARγ. Genisteins epigenetic properties of cell growth inhibiton and apoptose induction seem to be due to inacitvation of the AKT-pathway via inhibition of PI3K and increased mRNA levels of PTEN, tumor suppressor gene p53, and FOXO3a. NF-κB´s DNA-binding activity is decreased by inhibited nuclear translocation. SIRT1 expression decreased followed by enhanced histone acetylation of H3K9 in gene promoter regions of PTEN and FOXO3a, resulting in heightened gene expression (Kikuno, Shiina et al. 2008).

Genistein was also reported to down-regulate telomerase activity by inhibtion of hTERT (human telomerase reverse transcriptase) transcription, which is the catalytic subunit of the human telomerase enzyme, contributing to apoptosis of cancer cells. Genistein is assumed to inhibit expression by epigenetic mechanisms as the three major DNA methyltransferases (DNMT1, 3a and 3b) were also decreased in genistein-treated cells and genistein induced chromatin structure remodeling of the *hTERT* promoter by increasing trimethyl-H3K9 but decreasing dimethyl-H3K4 (Li, Liu et al. 2009).

Butyrate

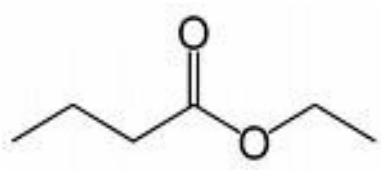

Figure 11: Butyrate structure

Microbial metabolism of dietary fibers in the lower gastrointestinal tract generates butyrate, a short chain fatty acid. Butyrate has been reported to impart several health benefits including anti-neoplasic properties (Davie 2003; Waldecker, Kautenburger et al. 2008). The effect of butyrate on epigenetic patterns of cancer cells seems to be twofold: Primarily butyrate possesses the ability to non specificly inhibit HDACs. Reactivation of tumor suppressor genes like $p21^{Waf1/Cip1}$ (CDKN1A) and down-regulation of Cyclin D, both playing an important role in the cell cycle, was reported be due to butyrate treatment, inducing growth arrest and apoptosis. This effect might additionally be modulated by butyrates effects on nonhistone proteins, such as transcription factors, or on noneuchromatic target (Rada-Iglesias, Enroth et al. 2007). Furthermore its ability to induce apoptosis might be due to pro-caspase-3 cleavage, transforming it into its active form whereas additional intermediate steps seem to be involved, as pharmacologically blockage of protein syntheses prevents butyrate from mediating apoptosis (Ruemmele, Dionne et al. 1999).

Secondly, in addition to butyrates HDAC inhibitoring activity, it seems to reverse aberrant DNA methylation. But the mechanism by wich butyrate impairs DNA methylation patterns is presently unclear. Surprisingly, decrease in DNA methylation was independent of excessive DNA synthesis, although usually, in order to originate unmethylated DNA by DNMTs, a new strand needs to be synthesized. Thus butyrate seems to stimulate active demethylation. A feasible explanation for active promoter demethylation might be direct enzymatic removal of methylgroups from 5-methylcytosines or excision and replacement of methylated cytosine through base excision repair mechanisms. The demethylating impact of butyrate only influences a limited number of loci but does not cause genome wide demethylation (Spurling, Suhl et al. 2008).

Butyrates ability to influence DNA methylation patterns might be associated with its HDAC inhibition property, as CpG islands are significantly more frequent in

deacetylated regions, pointing towards a mechanistic link between DNA methylation and histone acetylation. Until today the question whether deacetylation is the cause or consequence of gene silencing has not been resolved (Rada-Iglesias, Enroth et al. 2007). Another explanation to this phenomenom might be existence of a butyrate response element in the promoter regions of the affected genes, representing only 2% of all mammalian genes. Trancription factors Sp and Sp3, located within butyrate response element binding sites, can activate or repress transcription. $P21^{Waf1/Cip1}$ for instance contains six Sp1-binding sites (Davie 2003).

Butyrates ability to modify the epigenetic program might be supported by a combination with another epigenetically active agent. A combination of butyrate with genistein caused a better gene reactivation than one agent alone (Spurling, Suhl et al. 2008). Similar to butyrate together with resveratrol, enhancing butyrate's effect on the induction of $p21^{Waf1/Cip}$ in Caco-2 cells (Wolter and Stein 2002).

EGCG

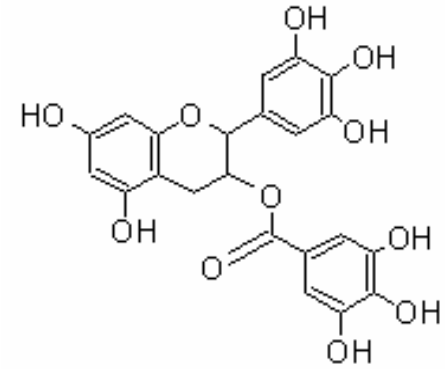

Figure 12: EGCG structure

One of the major polyphenols found in green tea is Epigallocatechin-3-gallate. As EGCG is able to inhibit cancer cell growth, induce apoptosis and suppress tumor angiogenesis, it presumably mediates its antitumorigenic properites via several different transactions (Berletch, Liu et al. 2008): Beside its antioxidant, radical scavenging property, EGCG was found to have an anti NF-kB transactivation activity in a broad range of human malignancies, for instance in colon and breast cancer, as well as in chronic inflammation. Blockage of NFkB signal transduction pathway by EGCG leads to suppression of nitric oxide synthase (NOS2) production, which plays a role in cancer development.

EGCG exhibits efficient anti-HAT activity, showing global specificity for the majority of HAT enzymes, eventually representing the mechanism of NFkB inhibition. EGCG-induced hypoacetylation of p65, a subunit of NF-kB, might cause an exchange between HDACs and HATs in the promoter region of a NF-kB– regulated genes (Choi, Jung et al. 2009).

EGCG treatment was shown to ablate histone acetylation and decrease promoter methylation in the hTERT promoter, whereas an increased promoter binding of E2F-1, a hTERT repressor, could be detected. This lead to dimished cell proliferation and provoked apoptosis. The decrease in promoter methylation might be due to inhibition of DNMT1 (Berletch, Liu et al. 2008), as SAM methylates EGCG on two methylation sites and therefore is converted into SAH. With SAM being substrate of DNMTs EGCG inhibits DNMT1 competitivley, leading to an decease in DNA methylation (Fang, Chen et al. 2007).

Resveratrol

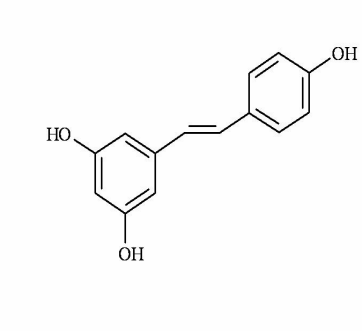

Figure 13: Resveratrol structure

Epidemiological studies in the early 1970´s, correlated red wine consumption with decreased appearance of cardiovascular disease and so created the `frech paradox´, which was resolved with the identification of antioxidants such as resveratrol, which is mainly present in grape skin and peanuts. Resveratrol was found to interact with lipoproteins, decrease oxidation of low density lipoproteins (LDL) and inhibit platelet aggregation as well as polymorphonuclear cell activation, which are known to generate reactive oxygen species. Additionally, resveratrol induces vasorelaxation and impaired expression of adhesion molecules, thus decreasing thrombogenic potential (Signorelli and Ghidoni 2005).

Resveratrol has been suggested to be a potential calorie restriction mimetic. It slows aging by promoting conformational changes and therefore activate sirtuins, especially SIRT 1 (Borra, Smith et al. 2005). A recent study showed resveratrol to stimulate

mitochondrial function and to obviate metabolic disorders in mice, which were fed a high-fat diet. This effect was mediated by SIRT1-dependent activation of PGC1α, a key regulator of energy metabolism (Delage and Dashwood 2008).

Resveratrol can be considered a selecive estrogen modulator, positivley influencing estrogen receptor expressing and non expressing human tumors, as it is similar to synthetic estrogen diethylsilbestrol, activating hormone receptor mediated gene expression. In contrast to its estrogenic properties it also displays an antiestrogenic effect via triggering pathways that inhibit estrogen mediated outcomes like proliferation, tumor transfomation and progression. Binding to Erβ, it inhibits expression of ESR1 and regulates androgen receptor (Signorelli and Ghidoni 2005).

Resveratrols antineoplastic properties base on blocking three stages of carcinogenesis: initation, promotion and progression. It participates in the regulation of proteins responsible for DNA synthesis and cell cycle, such as p53 and Rb/E2F, cyclins, cyclin-dependent kinases (CDKs) and their inhibitors. Furthermore, resveratrol affects the activity of transcriptional factors influencing proliferation and stress responses, such as NF-kB and AP1. These processes might be mediated by mitogen-activated protein kinases (MAPKs) and tyrosine kinases, entailing the modulation of survival and apoptotic factors as well as enzymes involved in carcinogenesis such as cyclooxygenases (COXs), nitric oxide synthase (NOS) and phase I and II enzymes (Signorelli and Ghidoni 2005).

Activated SIRT1 mediates RelA/p54 deacetylation, inhibiting TNF α- induced NF-kB transcription and sensitizing cells to apoptosis (Yeung, Hoberg et al. 2004), whereas SIRT 1 also deacetylates and inactivates p53 in a balanced equilibrium with the actions of HDAC1 eventually blocking apoptosis and promoting cell survival. Expression of NF-kB and p53 activating acetyltransferase p300 can also be increased by resveratrol.

Other studies found out, that resveratrol does not inhibit but increase TNF α induction of NFkB due to SIRT1s pharmacological blockage, in contrast SIRT1 overexpression inverts activation of NFkB, possibly mediated by p300. Thus, resveratrol modulates the two gene transcription factors p300 and SIRT1, triggering opposite pathways of

apoptosis and proliferation, with p300 acivating and SIRT1 inhibiting proliferative NFkB and apoptotic p53 (Signorelli and Ghidoni 2005).

Zebularine

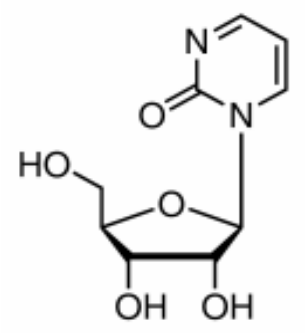

Figure 14: Zebularine structure

Zebularine serves as a reference DNMT inhibitor in this study. Originally synthesized as a cytidine deaminase inhibitor, zebularine structurally corresponds to cytidine and was found to be a potent inhibitor of DNA cytosine methyltransferase. These findings promote zebularine to be a auspicious anticancer agent as it preferetially targets tumorigenic cells. Since its mRNA seems to be unaffected in a study with LD419 fibroblasts, DNMT inhibiton seems to function on protein level by covalent complexing of the DNMT enzymes (Billam, Sobolewski et al. 2009). DMNT1, being mainly associated with maintenance methylation, seems to be primarily affected, followed by DMNT3b and DNMT3a. Zebularine was found to function in vitro and in experimental animal studies after oral application. Clinical trials were already conducted, mainly on acute leukemias and myelodisplasia. As mentioned above, two further drugs acting in a similar way, 5- azacytidine and 5-aza-2´deoxycytidine were found to inhibit DNA methylation but are unfortunately limited due to their instability. Zebularine, on the other hand, is highly stable at neutral pH- and nontoxic in adequate doses. Gene silencing by DNA remethylation of tumor suppressor genes is a conversant issue for demethylating drugs, requiring a constant application. Due to its minimal cytotoxicity, zebularine can be given uninterrupted in low doses over a longer period to provide continous demethylation. As shown in human bladder tumor cells, zebularine caused de-methylation as well as reactivation of a hypermethylated promoter and therefore silenced $p16^{INK4a}$ and $p15^{INK4b}$ in an AML cell line (Yoo, Cheng et al. 2004; Marquez, Kelley et al. 2005).

3 OBJECTIVES

The nuclear hormone receptor estrogen receptor α (ESR1) stimulates cellular growth through ligand-dependent induction of genes involved in cell proliferation, possibly leading to carcinogenesis. Moreover, ESR1 plays a role in the development of arthereosclerosis, as it impacts plasmalipid levels and certain ESR1 SNPs seem to be associated with the metabolic syndrome and obesity. The tumor suppressor proteins $p16^{INK4a}$ and $p15^{INK4b}$ (the INK4 proteins) participate in the regulation of the mammalian cell division cycle to ensure balanced cell proliferation and are frequently inactivated in human cancers. Numerous studies have shown ESR1, $p16^{INK4a}$ and $p15^{INK4b}$ to be regulated epigenetically via DNA CpG promoter methylation. Unlike many other genomic alterations that occur during carcinogenesis, DNA methylation is potentially reversible via the use of preventive or therapeutic agents. A multitude of bioactive food components have been detected to epigenetically impact the methylation state of genes involved in the pathways of complex diseases. To correlate hormonal and epigenetic activities of selected bioactive food components we analyzed the effect of butyrate, resveratrol, genistein, EGCG, folic acid and reference DNMT inhibitor zebularine on the promoter methylation state of ESR1, $p16^{INK4a}$ and $p15^{INK4b}$ in Caco-2 cells. Results of this study should contribute to the insight how food components might interfere with hormonal signaling and epigenetic pathways of cancer development.

4 MATERIALS AND METHODS

4.1 Cell culture conditions and treatment

For this essay the human Caco-2 cell line, an immortalized line of heterogeneous epithelial colorectal adenocarcinoma cells (Shah, Jogani et al. 2006), were grown at 37°C in a humidified atmosphere (5% CO2), in six well tissue culture plates and cultured in RPIM 1640 media, supplemented with 10% heat inactivated fetal bovine serum, 4mM glutamine and 100 units of streptomycin and penicillin respectively. Cells mature as confluent monolayer and a 48-h treatment was carried out after exactly 2 weeks of cell gowth. Cell treatments were performed in dublicates and pooled for the analysis. Table 1 lists agents and agent´s concentration used for the cell treatment.
Cells were washed twice with cooled PBS (4°C) before nucleotide extraction.

Substance	Concentration
Zebularine	100 µmol/l
Folic acid	200 µmol/l
Genistein	200 µmol/l
EGCG	200 µmol/l
Butyrate	200 µmol/l
Resveratrol	10 µmol/l

Table 1: Substances and concentrations of cell treatment

4.2 PCR and real time PCR

Principle of PCR

Principle of Polymerase chain reaction (PCR) is to amplify small regions of a single or double stranded DNA by an enzymatic reaction. Specific parts of the original DNA molecule are replicated by a DNA polymerase enzyme, doubling the number of DNA molecules.

Following components are required for the reaction:

- Deoxy-nucleotidetriphophats (dNTPs), for synthesizing new DNA fragments
- DNA-Polymerase enzyme (*Taq* polymerase) from *Thermus aquaticus*
- Buffer and $MgCl_2$, serving as polymerase cofaktor
- Primers, being short oligonucleotides reqired as start signal for the DNA-Polymerase
- Fluorescent reporter for detection (only in real time PCR)

A three-step reaction exponentially increases the amount of DNA fragments :
The reaction is performed by temperature cycling. High temperatures (90°C to 95°) are needed to separate strands of the DNA double helix (**Denaturation**) then temperature is lowered (50°C to 70°C) to let primers bind to the template (**Annealing**) and finally the temperature is set to the enzyms optimum (72°C), building the DNA strand by extending primers through dNTPs incorporation (**Elongation**).

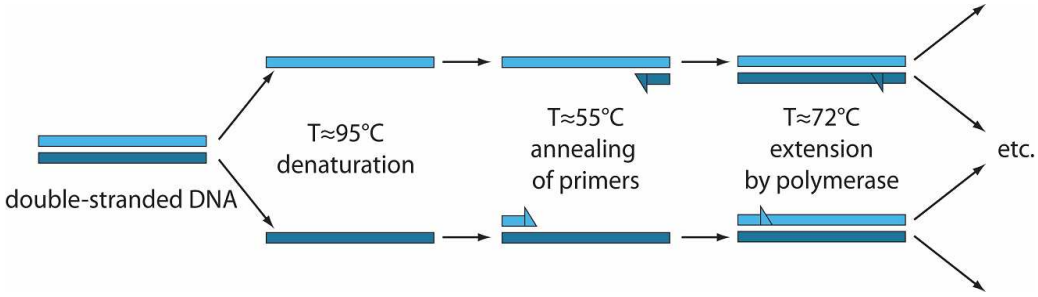

Figure 15: PCR temperature cycle

For one PCR run usually 30 to 40 cycles of denaturation, annealing and elongation are conducted. For optimal reaction conditions concentration of $MgCl_2$, which builds complexes with dNTPs and stables primer annealing on the template, should be approximately 1,5 mM and of deoxynucleotides approximately 400 µM. Primer concentration between 0.1 µM and 1 µM of each primer, depending on DNA target lentgh are needed.

Reqired degradation temperature and duration can change with length and sequence, as a G-C base pair contains three hydrogen bonds being more stable than an A-T base pair with only two hydrogen bonds.

Anneling temperature is based upon primer-constitution. It should be some degrees lower than than melting temperature of the two primers. Primers should have a length beween 18 and 30 nucleotides and be designed to have similar melting temperatures, not build self or cross dimers, hairpins or other secondary structures. GC content should be between 40 and 60%. Self annealing can be prevented by choosing primers that are not complementary especially at the 3´ends.

Primer melting temperature depends on DNA-DNA hybrid stability and is critical in determining temperture for the annealing (Ta). Too high T_a will produce low PCR yield because of insufficient primer-template hybridization, whereby too low T_a may possibly cause non-specific products through a high number of mismatched base pairs. Mismatch tolerance has the strongest effect on impact on PCR specificity, followed by primer length, template size and product size limit.

Calculation of annealing temperature :

$T_a = 0.3 \times T_m(\text{primer}) + 0.7\ T_m\ (\text{product}) - 14.9$

T_m(primer) = Melting Temperature of the primers,

T_m(product) = Melting temperature of the product (Rychlik, Spencer et al. 1990)

The amount of DNA copies is doubled during each cycle and can be formulated as follows:

$$N = N_0 \times 2^n$$

N : number of DNA copies

N_0 : initial number of DNA-molecules

n : number of cycles

2 : optimal efficiency (every cycle 2 DNA molecules result out of one)

Real time PCR

Real time PCR technique is able to monitor the progress of a PCR reaction in real time, thus continous visualization of PCR amplification during every phase of the reaction is given compared to traditional end-point PCR using agarose gel after completion of PCR cycles to detect amplification outcome and product quantity.

Minimal amounts of nucleic acid are quantifiable, the technique is resource and time saving, very sensitive, reproducible and specific. The real time technique allows melting point analysis and mutltiplex PCR, enabling simultaneous amplification of many targets of interest in one reaction by using more than one pair of primers and detection systems.

Detection is based on increasing fluorescence signals, emitted by a reporter molecule, as the reaction proceeds. Fluorescent reporter molecules include dyes attaching to double-stranded DNA or sequence specific probes.

For the visualisation and quantitation of the reaction several real time PCR detection systems are available according to different requirements:

DNA-binding agents: DNA-binding agents like SYBR® Green which was utilized in this study, are double-stranded DNA binding dyes, to detect PCR product as it accumulates during PCR cycles. The resulting DNA-dye-complex emits a fluorescence signal. As more double stranded amplicons are produced in the elongation phase fluorescence signal multiplies.

Hydrolysis probe technique (Taq man®)*:* This systems uses a hydrolysis probe, which exploits 5´exonuclease acitvity of polymerases, that cleaves a labeled hybridization probe during extension phase of the PCR. At 3´and 5´ ends, probe is marked with a fluorescent dye (fluorochrome) and a quencher. The probe is desigend to anneal the single stranded DNA between the two primers. The quencher absorbs fluorescence signal as long as the probe is not cleaved. During elongation phase the probe is cleaved due to polymerases exonuclease activity. As fluorochrome and the quencher get separated the fluorescence can be detected.

Hybridization probe technique (molecular beacons) : Two probes are required for this system, one labeled with donor fluorochrome binding nearby the second probe, which is labeled with acceptor fluorochrome. With both probes annealing to the DNA, the emitted light of the donor will excite the acceptor emitting a fluorescence signal (FRET) which can be detected during the annealing and early elongation phase.

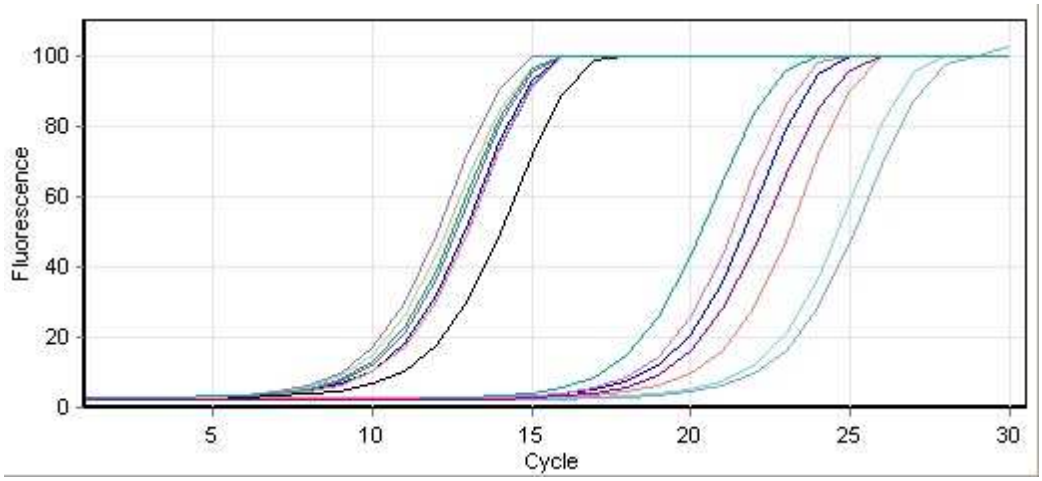

Figure 16: Real time PCR amplification curves for two products

After PCR amplification, real time machine can be programmed to perform a melt curve, in which the temperature is raised by a fraction of a degree and changes in fluorescence- signal is measured. (Derivative of fluorescence with respect to temperature-dF/dT; temperature-deg). At the melting point, double stranded DNA separates and the signal rapidly decreases. Primer-dimer artifact would give an additional peak with lower melting temperature.

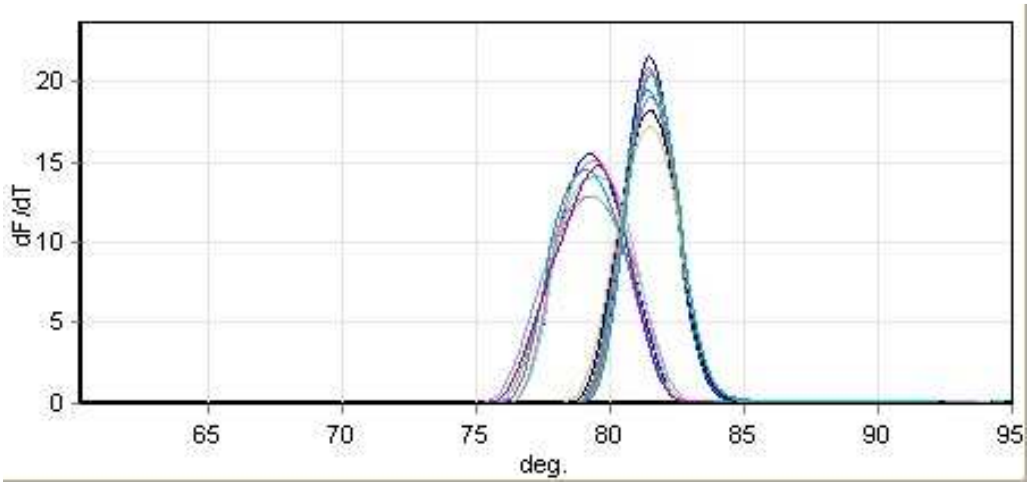

Figure 17: Real time PCR melt curves for two products

4.3 Gene expression quantification

Principle of gene expression detection

Messenger RNA as a inerstage of gene expression was extracted, converted into cDNA by reverse trancriptase reaction and quantified by real time PCR.

Procedure

mRNA extration: NucleoSpin RNA 2-Kit (Marcherey-Nagel) was applied according to manufactures instruction (protocol attached).

reverse transription: To convert mRNA into more stable cDNA, Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics) was applied.

Reaction mixes were compiled as follows:

reverse transcriptase reaction buffer	4 µl
deoxynucleotide mix	2 µl
protector RNase inhibitor	0,5 µl
anchored-oligo (Post, Goldschmidt-Clermont et al.)18 primer	1 µl
reverse transcriptase	0.5 µl
water	6 µl
RNA	6 µl
total volume	20 µl

Mixes were heated up to 50°C for 60 minutes to perform the transcription then samples were exposed to 85°C for 5 minutes for transcriptase inactivation.

Besides the anchored-oligo (Post, Goldschmidt-Clermont et al.) primers, consisting only of thymine bases and binding to the poly-A-tail of mRNAs, random hexamer primers, small primers of six base pairs, binding randomly to all mRNAs in the sample, or sequence specific primers which only allow transciption of specific genes or gen parts to be converted during reaction, can be used for the reverse transcription.

real time PCR: Primer sequences, reaction mixture composition and cycle condition were perfomed as listed below:

Primer	Sequence	Annealing temp	Concentration
ESR1 se	5´- TGG GCT TAC TGA CCA ACC TG -3´	60°C	5pmol/µl
ESR1 as	5´- CCT GAT CAT GGA GGG TCA AA -3´	60°C	5pmol/µl

Table 2: Primer conditions for ESR1 gene expression

Component	Volume
Sensi Mix	5µl
Primer	1µl
Sybr Green	0,2µl
Water	2,8µl
DNA	1µl
Total Volume	10µl

Table 3: Components and volumes of reaction mixture for ESR1 gene expression

Polymerase activation	10 min	95°C
Cycling	35 times	
Denaturation	45 sec	95°C
Annealing	60 sec	66°C
Elongation	60 sec	72°C
Melt curve	0,5°C every 5 sec	50°C - 95°C

Table 4: Cycling conditions for ESR1 gene expression

4.4 DNA methylation detection

Principle of DNA methylation detection

Total genomic DNA was extracted from the cells. The bisulfit conversion reaction is based on the reaction of bisulifite with cytosine in single stranded DNA which, in an aqueus milieu, are converted to uracil, whereas 5- methylcytosine remains unreacitve.

The modified DNA strands, now low in C content are amplified by the use of PCR amplification. To increase specifity for this experiments nested PCR was used to amplify converted DNA within two steps. For final detection bisulfit genomic sequencing (BSP) or methylation specific PCR (MSP) can be used.

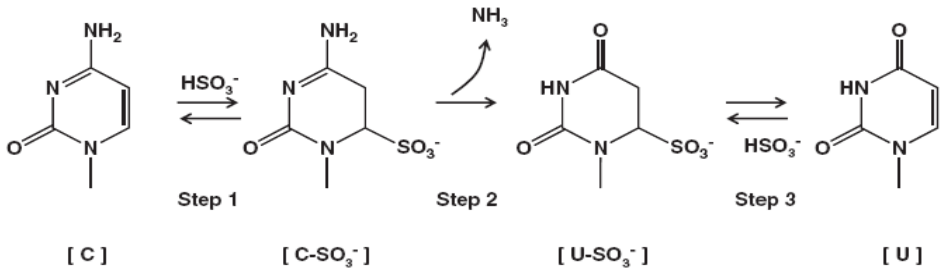

Figure 18: Bisulfite conversion
(Hayatsu 2008)

Bisulfit sequencing PCR (BSP)

Bisulfite genomic sequencing is the method of choice for the production of methylation maps with single-base resolution. The method contains selective deamination of cytosine to uracil by treatment with bisulfite and the subsequent purification and sequencing of generated PCR products. In contrast to cytosine, 5-methylcytosine does not react with bisulfite and can therefore be distinguished,
implying a C in CpG-site of interest interesting meaning that the C was methylated, whereas a T used to be a fomer unmethylated CpG.

Methylation specific PCR (MSP)

MSP entails initial modification of DNA by bisulfit conversion and subsequent amplification of the target DNA sequence by two sets of primers matching specificly for either methylated or unmethylated DNA .

<u>Procedure</u>

DNA extration: QIAamp DNA Mini Kit from Qiagen was applied according to manufactures instruction (protocol attached).

Bisulfit conversion: EpiTect Bisulfite Kit from Qiagen was applied according to manufactures instruction (protocol attached).

Methylation specific PCR: To increase specifity of MSP often nested PCR approaches are used. Nested PCR reduces contaminations in PCR products due to amplification of unintended primer binding.

Two sets of primers are used in two successive PCR runs. The second set intends to amplify a secondary target within the first run product. Step one external primer flanke the CpG- rich promoter region of the target gene and thus do not discriminate between methylated and unmethylated CpGs due to bisulfit treatment. First step was perfomed by conventional end point PCR and correct amplification was verified by agarose gel electrophoresis and gel red detection using 300-nm transillumination.

First step PCR products were diluted 1:1000 and subjected to second, internal MSP utilizing real time PCR and a set of two primer pairs, designed to distinguish bisulfite-induced modifications of unmethylated from methylated cytosines.

Primer conditions, reaction mixture composition and cycle condition were perfomed as listed below:

	sense	antisense	Ann.temp
External			
ESR1	GGYGAGGTGTATTTGGATAGTAGTAAGT	CRAACTCRAAAACACRCTATTAAATAAA	58°C
$p15^{INK4b}$	GAYGTYGGTTTTTTGGTTTAGTTGA	AACRCAACCRAACTCAAAACC	55°C
$p16^{INK4a}$	AGAAAGAGGAGGGGTTGGTTGG	ACRCCCRCACCTCCTCTACC	55°C
Internal Unme			
ESR1	TGTTGTTTATGAGTTTAATGTTGTGGTT	AAAAAAACCCCCCAAACCATT	58°C
$p15^{INK4b}$	GGTTGGTTTTTTATTTTGTTAGAGTGAGG	AACCACTCTAACCACAAAATACAAACAC	58°C
$p16^{INK4a}$	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	58°C
Internal Me			
ESR1	ACGAGTTTAACGTCGCGGTC	ACCCCCCAAACCGTTAAAAC	58°C
$p15^{INK4b}$	GGTTTTTTATTTTGTTAGAGCGAGGC	TAACCGCAAAATACGAACGCG	58°C
$p16^{INK4a}$	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	58°C

Table 5: Primer conditions for ESR1, $p15^{INK4b}$, $p16^{INK4a}$ MSP

Primer concentration: 5pmol/µl

	External PCR	Internal PCR
Mastermix / Sensimix	25µl	5µl
Primer	5µl	1µl
SybrGreen	/	0,2µl
Water	15µl	3µl
DNA	5µl	1µl
Total volume	50µl	10µl

Table 6: Components and volumes of reaction mixture for MSP, external and internal PCR

		External p16/p15	External ESR1
Polymerase activation	10 min	95°C	
Cycling	30 times		
Annealing	60 sec	55°C	58°C
Elongation	50 sec	72°C	
Melt curve	0,5°C every 5 sec 50°C - 95°C		

Table 7: Cycling conditions for MSP, external and internal PCR

4.5 Data analysis

Real-time PCR runs were analyzed with the Rotor-Gene Version 6.1 software belonging to the Corbett Rotor Gene 300 real time cycler. Results were evaluated by comparative quantitation of the Rotor Gene software. Analyse was based on fluorescence history of the reactions. The software uses a second derivative of raw amplification data and determines take off points of the reactions. The slope of the line from take off point until exponential amplification stops is used to calculate amplification efficiency.

Hypothetical concentrations were calculated and amplification efficiency was adjusted between individual samples. Gene expression was normalized to GAPDH as a reference gene, which was found to be stable expressed after cell treatments and gene expression as well as methylation results were calibrated relative to untreated cells as a control. Results were expressed in ratios. Average and the standard deviations were calculated with Microsoft Office Excel 2007. An *F-test* for homogeneity of variances

and a two tails t-test to determine the significance of the results were used. Results were regarded as significantly different at $p<0.05$.

5 RESULTS

5.1 ESR1 gene expression and promoter methylation

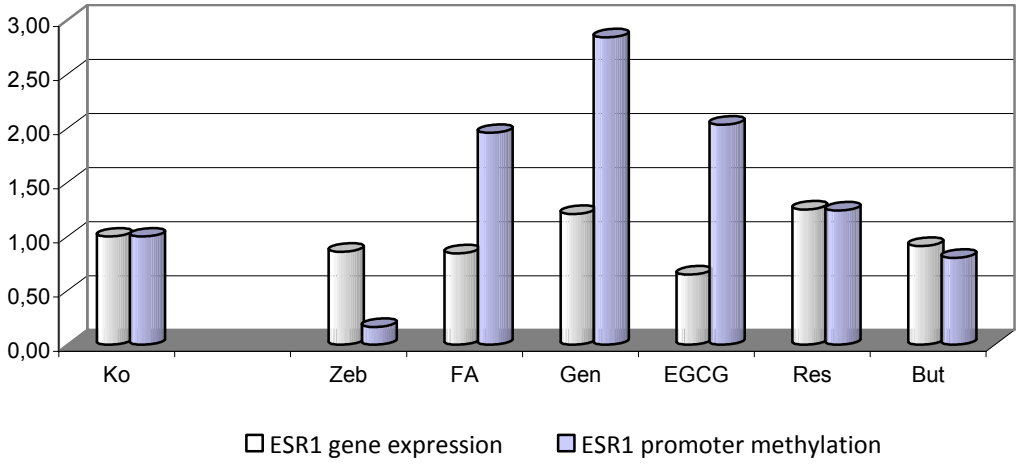

Figure 19: ESR1 gene expression and promoter methylation after 48h treatment with zebularine (Zeb), folic acid(FA), genistein (Gen), epigallocatechin-3- gallate (EGCG), resveratrol (Res) and butyrate (But)

Untreated Caco- 2 cells are predominantly methylated at the ESR1 pomoter site in a 56:1 ratio. A distinct effect on ESR1´s promoter region methylation has been detected after cell treatment with genistein, causing a clear 2,84-fold ± 0,2 ($p \leq 0,05$) hypermethylation compared to untreated cells, whereas nearly no change in mRNA expression could be seen. Treatment with methyl donor folic acid results in an almost doubled ESR1´s promoter methylation grade, 1,96-fold ± 0,05 ($p \leq 0,01$) correlating with decreased (0,85 ± 0,57) but no significant decrease in gene expression. A clear 2,03-fold ± 0,07 ($p \leq 0,05$) hypermethylation was also observed after cell exposure to EGCG and a slight but not significant decrease in gene expression could be seen (0,65 ± 0,02). Treatment with butyrate caused a diminished ESR1 expression (0,91 ± 0,15) and a significant decrease in methylation (0,48 ± 0,05; $p \leq 0,05$). Resveratrol marginally impacts ESR1 and $p16^{INK4a}$ promoter methylation and only a slight (1,25± 0,36) but not significant increase in ESR1 mRNA expression could be detected. Methyltransferase inhibitor zebularine distinctively diminished methylation in ESR1, $p16^{INK4a}$ and $p15^{INK4b}$.

Methylation at the ESR1 promoter site decreased significantly, 0,16 ± 0,01 (p≤ 0,01) but no significant change in mRNA expression (0,86 ± 0,20) could be observed.

5.2 $p16^{INK4a}$ and $p15^{INK4b}$ promoter methylation

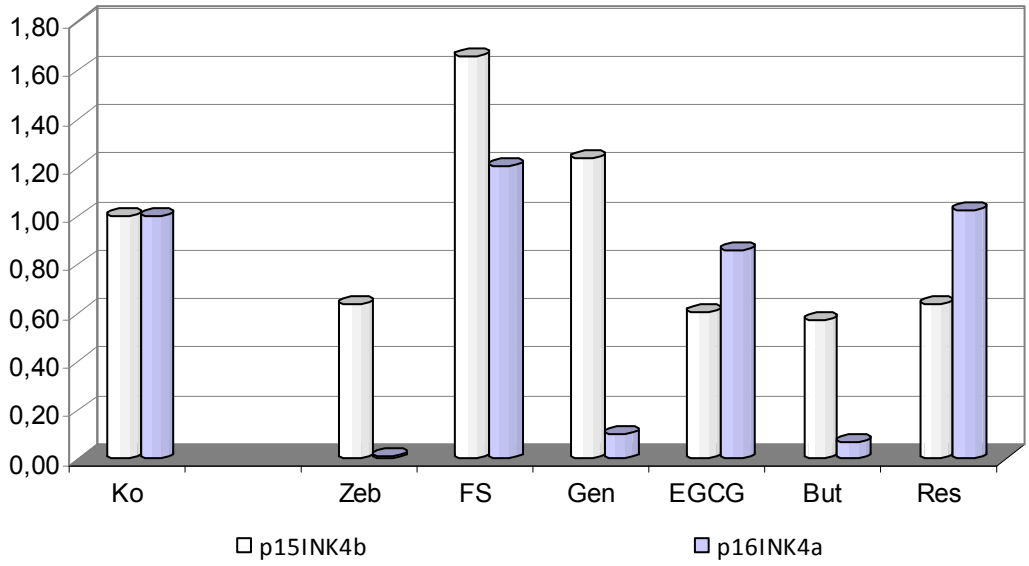

Figure 20: p15(INK4b) and p16 (INK4a) promoter methylation after 48h treatment with zebularine (Zeb), folic acid(FA), genistein (Gen), epigallo-catechin-3-gallate(EGCG), resveratrol (Res) and butyrate (But)

In untreated cells $p16^{INK4a}$ CpG rich promoter site is predominantly methylated in contrast to the $p15^{INK4b}$ promoter, that was found to be regularily unmethylated: the comparison methylated versus unmethylated DNA showed a ratio of 5,00E-03 for $p15^{INK4b}$ gene and 8,29 E03 for $p16^{INK4a}$.

Treatment with genistein caused a 1,24 fold ± 0,18 hypermethylation in the $p15^{INK4b}$ promoter site compared to untreated cells. In contrast $p16^{INK4a}$ promoter was found to be nearly completely demethylated. Exposure to folic acid enhanced $p16^{INK4a}$ methylation 1,66-fold ± 0,40 and also $p15^{INK4b}$ methylation grade but not as clearly as for $p16^{INK4a}$, 1,21 fold ± 0,27. EGCG treatment caused a significant reduction of $p15^{INK4b}$ (0,60 ± 0,01) (p≤ 0,05) and a slight decrease in $p16^{INK4a}$ (0,86 ± 0,41) promoter methylation. Exposure to butyrate significantly diminished both, $p16^{INK4a}$ and $p15^{INK4b}$

methylation state 6,92E-02 ± 0,04 (p≤ 0,05) and 0,57 ± 0,01 (p≤ 0,01) compared to untreated cells. Resveratrol had almoust no effect on $p16^{INK4a}$ promoter methylation but reduced methylation of $p15^{INK4b}$: 0,62 ± 0,03 (p≤ 0,01). Reference DNMT inhibitor zebularine explicitly diminished methylation in the $p16^{INK4a}$ and $p15^{INK4b}$ promoter region: 6,74E-03 ± 0,01 (p≤ 0,05) and 0,64± 0,02 (p≤ 0,01).

6 DISCUSSION

6.1 Methods

As the Caco-2 cell displays characteristics of mature enterocytes (Sambuy, De Angelis et al. 2005), this study appears to be a good model for estimating the direct epigenetic impact of bioactive food components on the intestine. For the incubation agent concentrations were selected assumed to have an impact on cells epigenetic machinery. As this study was conducted using only one concentration and durance of each substance, different extends of impact due to higher or lower concentrations or treatment duration were not assessed. Further studies with this bioactive food components should comprise different concentrations and incubation durances as well as combinations of DNMT ihibitors and histone modifying agents including subseqent detection of histone modifications. Furthermore, global DNA methylation should be assessed to estimate the impact of a substance on the complete methylation state. A good approach would be to estimate substances concentrations in normal diet and to compare them to concentrations actually having an impact on the epigenetic pattern.

6.2 ESR1 gene expression and promoter methylation

The nuclear receptor ESR1 functions as a transcription factor upon hormonal signals, enhancing the transcription of genes involved in the regulation of cell growth. Epigenetic disregulation of ESR1 expression and concurrent alterations in the integration of hormonal signaling has been associated with the development of several diseases, such as the metabolic syndrome and cancer.

Our results show untreated cells to be predominantly methylated at the ESR1 promoter site. Caco-2 cells are derived from human epithelial colorectal carcinoma cells. These results are in agreement with studies describing human adenomatous and colon cancer tissue to be hypermethylated at the ESR1 promoter site (Issa, Ottaviano et al. 1994; Hinshelwood, Melki et al. 2009). Methylation patterns of free DNA, found in the buffy coat of patients with several cancer types, coincide with that of cancer

suppressor genes in the tumors (Feng, Hu et al. 2009). In contrast to Caco-2 cells, methylation state of ESR1 promoter region in 13 healthy women's buffy coat DNA turned out to be nearly completely unmethylated (data not shown). These discrepancies in the methylation state in different tissues need to be considered in the evaluation of effects bioacitve food components have on methylation or de-methylation, as the original methylation state of the promoter seems to impact the degree of treatment induced methylation changes (quod vide results: $p15^{INK4a}$).

An elevated methylation level of the ESR1 promotor region after Caco-2 cells' exposure to folic acid correlated with a reduced gene expression. These findings support preliminary studies suggesting that the rate of ESR1 methylation in colonic tissue is modifiable by dietary folate (Belshaw, Elliott et al. 2005). Zhu and Williams hypothesized that development of ER-negative breast cancers, which are unsensitive to hormonal therapy, might result from methylation dependant inactivation of ESR1 transcription due to methyl deficient diets (Zhu and Williams 1998). Proving that hypothesis an epidemiological study associated higher folate intake with reduced risk of developing ER-negative breast cancer, assuming that adequate folate intake is particularly important for women with higher risk potential (Zhang, Hankinson et al. 2005).

Genistein regulates ESR1 gene expression in a very complex way, amongst others due to its estrogenic, epigenetic (Hong, Nakagawa et al. 2004) and tyrosine kinase inhibiting (Li, Liu et al. 2009) properties. Although promoter methylation increased after treatment, we observed an enhanced expression of ESR1. This might be due to a lowered HDAC SIRT1 expression, increasing histone acetylation of H3K9 in several gene promoter regions accompanied by changes in methylation state (Kikuno, Shiina et al. 2008). Similar observerations for genistein are being analyzed for IL-8 and will be presented elsewhere.

EGCG treatment caused, in contrast to results for $p15^{INK4a}$ and $p16^{INK4a}$, a twofold enhanced methylation of ESR1 promoter region and a correlating gene expression reduction by half. As EGCG was shown to be cancer cell growth inhibiting, these results

seem to be conclusive as $p15^{INK4a}$ and $p16^{INK4a}$ are cell cycle modulators and ESR1, activated by growth factors, acts as a potent mitogen for epithelial cells.

Butyrate has been proven to induce histone acetylation by inhibiting histone deacetylases, as well as growth arrest or apoptosis (Delage and Dashwood 2008). We detected a slight decrease in ESR1 expression after treatment with butyrate, as previously shown in breast cancer cells (deFazio, Chiew et al. 1992), accompanied by clearly diminished methylation. CpG islands are significantly more common in deacetylated regions. This finding points towards a mechanistic link between DNA methylation and histone acetylation although it is not yet clear if histone deacetylation is the reason or the consequence of gene silencing. It might also be possible that these epigenetic alterations are responses to stress caused by butyrate treatment (Rada-Iglesias, Enroth et al. 2007). Resveratrol, acting as a selective estrogen receptor modulator and modulator of SIRT1 HDAC activity, does seem to neither significantly affect methylation nor gene expression of ESR1. Studies working with Caco-2 cells investigated global DNA methylation after resveratrol treatment and obtained similar results. In this study only one concentration of 10 µmol/l and an incubation time of 48h were used. In contrast, investigations on ER-positive breast cancer cells showed resveratrol to induce methylation changes in a variety of CpG islands after treatment with 50 µmol/l for 36 hours (Wenyi Qin 2005).

Although exposure to well established reference DMNT inhibitor zebularine caused a virtually complete demethylation of the ESR1 promoter, almoust no effect on gene expression except for a slight decrease could be noted. As gene expression of ESR1 in this study does not always correlate with its promoter methylation state after treatment, expression could rather be influenced by CpG islands within exons of the gene or other epigenetic mechanisms, as shown by Zhou et al. They demonstrated reactivation of ESR1 gene by HDAC inhibitors to be whithout alteration in promoter DNA hypermethylation, suggesting expression of ESR1 to be regulated by histone modification or by a combination of DNA methylation and histone modifications (Zhou, Shaw et al. 2009). A recent study shows most methylation alterations in colon cancer to occur not in promoter regions but 2kb distant, which are termed “CpG island

shores". Methylation of these DNA sites was strongly related to gene expression (Irizarry, Ladd-Acosta et al. 2009). Moreover regulation of ESR1 also comprises trans–active factors such as TFAP2C inducing ESR1 transcription through an AP-2 regulatory region in the ESR1 promoter. Epigenetic chromatin modification could faciliate admission of trans active factors influencing gene transcription (Woodfield, Hitchler et al. 2009).

6.3 $p16^{INK4a}$ and $p15^{INK4b}$ promoter methylation

ESR1 contributes to cell cycle progression by inducing transcription of Cyclin –D1, which in association with CDK 4 or CDK 6 effects phosphorylation of Rb (Retino-blastoma) promoting proliferation (Lamb, Ladha et al. 2000). $P16^{INK4a}$ and $p15^{INK4b}$ are members of the INK 4 family, known to be CDK 4 and 6 inhibitors. Even though the $p15^{INK4b}$ gene indicates high homology to $p16^{INK4a}$ in the amino acid sequence, they seem to show differing patterns of transcriptional activation and epigenetic regulation (Ishiguro, Takahata et al. 2006). Generally $p15^{INK4b}$ is not as thoroughly investigated as $p16^{INK4a}$ and few data exist concerning the impact of bioactive food compounds on $p15^{INK4b}$ promoter methylation. A variety of cancers feature mutations in $p16^{INK4a}$ and $p15^{INK4b}$ as well as transcriptional inactivation in association with *de novo* methylation during neoplastic processes. $P16^{INK4a}$ methylation could be a prognostic marker for the metastatic potential of a tumor (Ng, Chung et al. 1997; Nakayama, Hibi et al. 2007). In untreated Caco-2 cells $p16^{INK4a}$ is prevailingly methylated in contrast to $p15^{INK4b}$ promoter region, which seems to be regularily unmethylated. These results agree with studies describing different promoter methylation patterns, distinguishing for instance leukaemias from colorectal cancer: $p15^{INK4b}$ promoter hypermethylation has been found only in leukemias, whereas $p16^{INK4a}$ promoter hypermethylation occured mainly in primary colonic tumors (Herman, Jen et al. 1996; Nguyen, Liang et al. 2001). Gene expression and promoter DNA methylation of $p16^{INK4a}$ in the colon of mice was shown to be dependent on age and folate intake (Fang, Chen et al. 2005). These results concur with this studies findings of folic acid treatment to broadly enhance

methylation in the $p16^{INK4a}$- and even more notedly in the $p15^{INK4b}$ - promoter region. Downregulation of DNMT3a and upregulation of caspase-3, an important component of the apoptotic cascade, was observed in the Caco-2 cell model, after 48h treatment with genisteine (data not shown). $p16^{INK4a}$ induction by methylation inhibitors in cancer cells was shown to lead to growth inhibition (Auerkari 2006). Our study showed genisteine to induce hypomethylation in the $p16^{INK4a}$ promoter region. Similar situations were found in benign and cancerogenic breast cells after exposure to genisteine: decrease in the expression of DNMTs leading to a hypomethylation of several genes associated with telomerase activity and cancer (Li, Liu et al. 2009). Hence genisteine seems to be able to induce re-expression of tumor suppressor genes and thus to interfere with cell cycle progression of neoplastic cells .

For EGCG we observed increased promoter methylation, confirming data suggesting that different concentrations of EGCG were able to inhibit the growth of malignant cell lines and increase the cell number in G(0)/G(1) phase or rather induce apoptosis in many tumors (Butt and Sultan 2009), presumably by impeding DNMT activity and reactivating methylation-silenced genes in cancer cells (Fang, Wang et al. 2003).

Treatment of Caco-2 cells with butyrate clearly decreased $p16^{INK4a}$ promoter methylation, possibly leading to its re-expression. Butyrate was reported to lead to cell cycle arrest and $p16^{INK4a}$ transcription (Schwartz, Avivi-Green et al. 1998). Upregulation of $p16^{INK4a}$ expression was demonstrated to be due to an increase of acetylation and degradation of methylation (Shen, Dai et al. 2008). Epigenetic regulation of $p16^{INK4a}$ appears to be composed of DNA methylation associated with histone modifications in nucleosome-free regions. Thus promoter methylation seems likely to be affected by HDAC inhibitors as changes in chromatine configuration can cause altered enzyme access to the DNA (Bachman, Park et al. 2003).

The DNMT inhibitor zebularine almost completely demethylated the $p16^{INK4a}$ promoter but had less effect on $p15^{INK4b}$, propably because $p15^{INK4b}$ already has a low methylation status in Caco-2 cells.

These results show specified bioactive food compounds to be able to modify CpG methylation in the promoter regions of ESR1, $p16^{INK4a}$ and $p15^{INK4b}$. However the degree of change in methylation state of the different genes is variable and often CpG methylation might cooperate with additional mechanisms such as histone acetylation in the regulation of gene expression. Modulation of the methylation of relevant CpGs of ESR1, $p16^{INK4a}$ and $p15^{INK4b}$ by food components might be a way to interfere with hormonal signaling in addition to pharmaceutical intervention. Inducing reexpression of tumor suppressor genes and impairing ESR1 mediated expression of blood lipid regulation might positivley intervene in complex diseases. ESR1 re-expression in ER-negative breast cancers could offer the possibility for using hormonal therapy.

7 SUMMARY / ZUSAMMENFASSUNG

SUMMARY

Epigenetics generally investigates heritable changes in gene expression that do not involve changes in the underlying DNA sequence. Epigenetic patterns are altered in cancer cells and have been studied extensivley, as they influence expression of genes regulating cell cycle or encoding hormone receptors, which respond to growth singnals. Epigenetic mechanisms are DNA methylation, histone modification and interference of small non coding RNAs, whereas especially DNA methylation was assessed accurately, detecting global DNA hypomethylation and gene specific hypermethylation as a characteristic of senescent and neoplastic cells. Opposed to many genomic alterations that emerge during carcinogenesis, DNA methylation is potentially reversible via the use of preventive or therapeutic agents, certain bioacitve food components amongst them.

ESR1 (estrogen receptor alpha) appertains to the superfamily of nuclear receptor and mediates estrogen effects through its function as ligand-inducible transcription factor. ESR1 promoter is known to be hypermethylated in ceveral types of cancer including breast and colon cancer. Tumor suppressor genes $P16^{INK4a}$ and $p15^{INK4b}$ are involved in the regulation of the mammalian cell cycle. $P16^{INK4a}$ and $p15^{INK4b}$ can undergo genetic alterations in neoplastic cells but also methylation dependent inactivation of the $p16^{INK4a}$ promoter has been found in various cancer types, such as colorectal, lung and breast carcinomas whereas $p15^{INK4b}$ is well documented in haematological malignancies. To integrate hormonal and epigenetic activities of selected bioactive food components, Caco- 2 cells were treated with folic acid, epigallocatechin-3-gallate (EGCG), genistein, butyrate, resveratrol and reference DNA methylation inhibitor zebularine and changes in the promoter methylation state of ESR1, $p16^{INK4a}$ and $p15^{INK4b}$ were assessed. Promoter methylation has been measured via bisulfit treatment and methylation specific nested PCR and aditionally gene expression of ESR1 has been quantified using real time PCR. Treatment with genistein, folate, EGCG,

and resveratrol increased and zebularine and butyrate decreased methylation of ESR1. Changes in gene expression sparsely responded to treatment, suggesting that CpG methylation might cooperate with additional mechanisms such as histone acetylation in the regulation of gene expression. $P15^{INK4b}$ methylation was enhanced by genistein and folate and diminished by zebularine, butyrate, EGCG, and resveratrol. Exposure to folate entailed $p16^{INK4a}$ hypermethylation whereas all other agents resulted in hypomethylation of this gene. These results suggest food ingredients to influence epigenetic modification of disease relevant gene expression and might be an effective approach to intervene in complex diseases with misregulated ESR1 signaling or cell cycle regulation.

ZUSAMMENFASSUNG

Epigenetik, die sich mit vererbbaren Zelleingenschaften befasst, welche die Genexpression beeinflussen ohne jedoch die DNA-Sequenz zu ändern, scheint auf grund ihres Ansprechens auf Umwelteinflüsse der Mittler zwischen Umwelt und Genom eines Organismus zu sein. In Krebszellen sind die epigenetischen Muster im Vergleich zu gesunden Zellen stark verändert und haben Einfluß haben auf die Expression verschiedener Tumorsuppressorgene und Hormonrezeptoren, welche den Zellzyklus kontrollieren beziehungsweise auf Wachstumssignale reagieren. Zu den epigenetischen Mechanismen zählen die DNA Methylierung, Histon Modifikationen und der Einfluß kleiner, nicht kodierender RNAs. Besonders die DNA Methylierung wurde bis dato ausführlich erforscht, wobei man feststellte, dass eine umfassende DNA Hypomethylierung und genspezifische Hypermethylierung charakteristisch für alternde und neoplastische Zellen ist. Im Gegensatz zu den meisten genetischen Veränderungen, welche während der Karzinogenese auftreten, kann die DNA Methylierung durch den Einfluß präventiver oder therapeutischer Substanzen modifiziert werden. Zu diesen Substanzen zählen unter anderem bestimmte bioaktive Nahrungsinhaltsstoffe.

Östrogenrezeptor alpha (ESR1) gehört zu der Superfamilie der nuklären Rezeptoren und ist durch seine Funktion als Liganden aktivierbarer Transkriptionsfaktor Mediator östrogener Einflüße auf die Zellen. In verschiedenen Krebsarten ist die Promoter Region von ESR1 hypermethyliert und dessen Expression somit fehlreguliert. Die Tumorsuppressorgene $p16^{INK4a}$ und $p15^{INK4b}$ sind Teil der Zellzyklus–Kontrolle. In Krebszellen sind diese genetisch verändert aber auch die promoter-methylierungsabhängige Inaktivierung von $p16^{INK4a}$ wurde in mehreren Krebsarten beobachtet, während selbiger Vorgang im $p15^{INK4b}$ Promoter vornehmlich in diversen Blutkrebsarten gefunden wurde.

Um hormonelle und epigenetische Vorgänge ausgewählter bioaktiver Nahrungs-bestandteile zu verbinden, wurden Caco-2 Zellen mit mit Folsäure, Epigallocatechin-3-gallate (EGCG), Genistein, Butyrat, Resveratrol und Referenz DNA Methylierungs Inhibitor Zebularin behandelt und deren Einfluß auf den Promoter Methyleriungsstatus des Östrogenrezeptor alpha (ESR1) und der beiden Tumorsuppressorgene $p16^{INK4a}$ und $p15^{INK4b}$ bestimmt.

Die Promotermethylierung wurde durch Bisulfit-Konversion und methylierungs-spezifischer nested PCR gemessen. Zusätzlich wurde mittels real-time PCR die Genexpression von ESR1 quantifiziert. Nach der Zellbehandlung mit Genistein, Folat, EGCG und Resveratrol konnte eine verstärkte ESR1 Promotermethylierung festgestellt werden, Zebularin und Butyrat verminderten diese. Die Genexpression korrelierte jedoch nur teilweise mit den Ergebnissen der Methylierung, was auf einen zuätzlichen epigenetischen Mechanismus wie Histonacetylierung in der Expressionsregulation von ESR1 schließen lässt.

$P15^{INK4b}$ Promotermethylierung konnte duch Genistein und Folat verstärkt werden und zeigte sich durch Butyrat, EGCG, Resveratrol und Zebularine vermindert. Die stärkste Reaktion zeigte die $p16^{INK4a}$ Methylierung nach Behandlung mit Folsäure wobei alle anderen Substanzen eine Demethylierung hervorriefen. Die Ergebnisse dieser Studie weisen darauf hin, dass spezifische Nahrungsinhaltsstoffe durchaus einen Einfluss auf den epigenetischen Status krankheitsrelevanter Gene haben können. Diese Erkenntnisse stellen mögliche Ansatzpunkte für eine Prävention und unterstützende

Therapie zusätzlich zur pharmakologischen Intervention bei komplexen Krankheiten mit fehlreguliertem ESR1-Signalweg oder fehlerhafter Zellzyklusregulation.

8 PROTOCOLS

8.1 RNeasy® Lipid Tissue Handbook (Qiagen)

1. If using the TissueLyser, add one stainless steel bead (5 mm mean diameter) per 2 ml microcentrifuge tube (not supplied). If working with tissues that are not stabilized in RNAlater RNA Stabilization Reagent, place the tubes on dry ice.
2. Excise the tissue sample from the animal or remove it from storage. Determine the amount of tissue. Do not use more than 100 mg. Proceed immediately to step 3. Weighing tissue is the most accurate way to determine the amount. If the tissue sample was stored in RNAlater RNA Stabilization Reagent, remove it from the reagent using forceps and be sure to remove any crystals that may have formed. RNA in harvested tissues is not protected until the tissues are treated with RNAlater RNA Stabilization Reagent, flash-frozen, or disrupted and homogenized in step 3. Frozen tissues should not be allowed to thaw during handling. The relevant procedures should be carried out as quickly as possible. 3. Disrupt the tissue and homogenize the lysate using either the TissueRupter (follow step 3a) or TissueLyser (follow step 3b). See “Disrupting and homogenizing starting material”, page 12, for more details on disruption and homogenization. Note: Incomplete homogenization leads to significantly reduced RNA yields and can cause clogging of the RNeasy spin column. Homogenization with the TissueRupter or TissueLyser generally results in higher RNA yields than with other methods.

3a. Disruption and homogenization using the TissueRuptor:

■ Place the tissue in a suitably sized vessel containing 1 ml QIAzol Lysis Reagent. Note: Use a suitably sized vessel with sufficient extra headspace to accommodate foaming, which may occur during homogenization. Generally, round-bottomed tubes allow more efficient disruption and homogenization than conical-bottomed tubes.

■ Place the tip of the disposable probe into the vessel and operate the TissueRuptor at full speed until the lysate is uniformly homogeneous (usually 20–40 s). Proceed to step 4. Note: To avoid damage to the TissueRuptor and disposable probe during operation, make sure the tip of the probe remains submerged in the buffer. Foaming may occur during homogenization, especially of brain tissue. If this occurs, let the homogenate stand at room temperature for 2–3 min until the foam subsides before continuing with the procedure.

3b. Disruption and homogenization using the TissueLyser:

■ Place the tissues in the tubes prepared in step 1.

■ If the tubes were stored on dry ice, place them at room temperature. Then immediately add 1 ml QIAzol Lysis Reagent per tube.

■ Place the tubes in the TissueLyser Adapter Set 2 x 24.

■ Operate the TissueLyser for 2 min at 20 Hz. The time depends on the tissue being processed and can be extended until the tissue is completely homogenized.

■ Rearrange the collection tubes so that the outermost tubes are innermost and the innermost tubes are outermost. Operate the TissueLyser for another 2 min at 20 Hz. Rearranging he tubes allows even homogenization and carefully pipet the lysates into new microcentrifuge tubes (not supplied). Proceed to step 4. Do not reuse the stainless steel beads.

4. Place the tube containing the homogenate on the benchtop at room temperature (15–25°C) for 5 min. This step promotes dissociation of nucleoprotein complexes.
5. Add 200 µl chloroform. Securely cap the tube containing the homogenate, and shake it vigorously for 15 s. Thorough mixing is important for subsequent phase separation.
6. Place the tube containing the homogenate on the benchtop at room temperature for 2–3 min.
7. Centrifuge at 12,000 x g for 15 min at 4°C. After centrifugation, heat the centrifuge to room temperature (15–25°C) if the same centrifuge will be used in the later steps of this procedure. After centrifugation, the sample separates into 3 phases: an upper, colorless, aqueous phase containing RNA; a white interphase; and a lower, red, organic phase. For tissues with an especially high fat content, an additional, clear phase may be visible below the red, organic phase. The volume of the aqueous phase should be approximately 600 µl.
8. Transfer the upper, aqueous phase to a new tube (not supplied). Add 1 volume (usually 600 µl) of 70% ethanol, and mix thoroughly by vortexing. Do not centrifuge. Proceed immediately to step 9. Note: The volume of lysate may be less than 600 µl due to loss during homogenization and centrifugation. Precipitates may be visible after addition of ethanol. Resuspend precipitates completely by vigorous shaking, and proceed immediately to step 9.
9. Transfer up to 700 µl of the sample to an RNeasy Mini spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at 8000 x g (10,000 rpm) at room temperature (15–25°C). Discard the flow-through. Reuse the collection tube in step 10.
10. Repeat step 9 using the remainder of the sample. Discard the flow-through. Reuse the collection tube in step 11. Optional: If performing optional on-column DNase digestion (see “Important points before starting”), follow steps C1–C4 (page 32) after performing this step.
11. Add 700 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at _8000 x g (_10,000 rpm) to wash the membrane. Discard the flow-through. Reuse the collection tube in step 12. After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely. Skip this step if performing optional on-column DNase digestion (page 32).
12. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 8000 x g (10,000 rpm) to wash the membrane. Discard the flow-through. Reuse the collection tube in step 13. Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use (see “Things to do before starting”, page 15).
13. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and

centrifuge for 2 min at 8000 x g (10,000 rpm) to wash the membrane. The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions. Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

14. Optional: Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min. Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column after step 13.
15. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid gently. To elute the RNA, centrifuge for 1 min at 8000 x g (10,000 rpm).
16. Repeat step 15 using another volume of RNase-free water, or using the eluate from step 15 (if high RNA concentration is required). Reuse the collection tube from step 15. If using the eluate from step 15, the RNA yield will be 15–30% less than that obtained using a second volume of RNase-free water, but the final RNA concentration will be higher.

8.2 QIAamp® DNA Mini and Blood Mini Handbook (Qiagen)

1. Pipet 20 µl QIAGEN Protease (or proteinase K) into the bottom of a 1.5 ml microcentrifuge tube.
2. Add 200 µl sample to the microcentrifuge tube. Use up to 200 µl whole blood, plasma, serum, buffy coat, or body fluids, or up to 5 x 106 lymphocytes in 200 µl PBS. If the sample volume is less than 200 µl, add the appropriate volume of PBS. QIAamp Mini spin columns copurify RNA and DNA when both are present in the sample. RNA may inhibit some downstream enzymatic reactions, but not PCR. If RNA-free genomic DNA is required, 4 µl of an RNase A stock solution (100 mg/ml) should be added to the sample before addition of Buffer AL. Note: It is possible to add QIAGEN Protease (or proteinase K) to samples that have already been dispensed into microcentrifuge tubes. In this case, it is important to ensure proper mixing after adding the enzyme.
3. Add 200 µl Buffer AL to the sample. Mix by pulse-vortexing for 15 s. In order to ensure efficient lysis, it is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution. If the sample volume is larger than 200 µl, increase the amount of QIAGEN Protease (or proteinase K) and Buffer AL proportionally; for example, a 400 µl sample will require 40 µl QIAGEN Protease (or proteinase K) and 400 µl Buffer AL. If sample volumes larger than 400 µl are required, use of QIAamp DNA Blood Midi or Maxi Kits is recommended; these can process up to 2 ml or up to 10 ml of sample, respectively. Note: Do not add QIAGEN Protease or proteinase K directly to Buffer AL.
4. Incubate at 56°C for 10 min. DNA yield reaches a maximum after lysis for 10 min at 56°C. Longer incubation times have no effect on yield or quality of the purified DNA.
5. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.
6. Add 200 µl ethanol (96–100%) to the sample, and mix again by pulse-vortexing for 15 s.

After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid. If the sample volume is greater than 200 µl, increase the amount of ethanol proportionally; for example, a 400 µl sample will require 400 µl of ethanol.

7. Carefully apply the mixture from step 6 to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate. Close each spin column in order to avoid aerosol formation during centrifugation. Centrifugation is performed at 6000 x g (8000 rpm) in order to reduce noise. Centrifugation at full speed will not affect the yield or purity of the DNA. If the lysate has not completely passed through the column after centrifugation, centrifuge again at higher speed until the QIAamp Mini spin column is empty. Note: When preparing DNA from buffy coat or lymphocytes, centrifugation at full speed is recommended to avoid clogging.
8. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection.
   tube containing the filtrate. It is not necessary to increase the volume of Buffer AW1 if the original sample volume is larger than 200 µl.
9. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min.
10. Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min. This step helps to eliminate the chance of possible Buffer AW2 carryover.
11. Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 µl Buffer AE or distilled water. Incubate at room temperature (15–25°C) for 1 min, and then centrifuge at 6000 x g (8000 rpm) for 1 min. Incubating the QIAamp Mini spin column loaded with Buffer AE or water for 5 min at room temperature before centrifugation generally increases DNA yield. A second elution step with a further 200 µl Buffer AE will increase yields by up to 15%. Volumes of more than 200 µl should not be eluted into a 1.5 ml microcentrifuge tube because the spin column will come into contact with the eluate, leading to possible aerosol formation during centrifugation. Elution with volumes of less than 200 µl increases the final DNA concentration in the eluate significantly, but slightly reduces the overall DNA yield (see Table 5, page 26). For samples containing less than 1 µg of DNA, elution in 50 µl Buffer AE or water is recommended. Eluting with 2 x 100 µl instead of 1 x 200 µl does not increase elution efficiency.

8.3 EpiTect® Bisulfite Handbook (Quiagen)

1. Thaw DNA to be used in the bisulfite reaction. Dissolve the required number of aliquots of Bisulfite Mix by adding 800 µl RNase-free water to each aliquot. Vortex until the Bisulfite Mix is completely dissolved. This can take up to 5 min.
   Note: If necessary, heat the Bisulfite Mix–RNase-free water solution to 60°C

and vortex again.
Note: Do not place dissolved Bisulfite Mix on ice

2. Prepare bisulfite reactions in 200 µl PCR tubes according to Table 2.
Add each component in the order listed.
Note: The combined volume of DNA solution and RNase-free water must total 20 µl

Table 2. Bisulfite Reaction Components

Component	Volume per reaction (µl)
DNA solution (1 ng – 2 µg)	Variable*(maximum 20)
RNase-free water	Variable*
Bisulfite Mix (dissolved), see step 1	85
DNA Protect Buffer	35
Total volume	**140**

* The combined volume of DNA solution and RNase-free water must total 20 µl

3. Close the PCR tubes and mix the bisulfite reactions thoroughly. Store tubes at room temperature.
Note: The DNA Protect Buffer should turn from green to blue after addition to DNA–Bisulfite Mix, indicating sufficient mixing and correct pH for the bisulfite conversion reaction.
4. Perform the bisulfite DNA conversion using a thermal cycler.
Program the thermal cycler according to Table 3.
The complete cycle should take approximately 5 h.
Note: It is important to choose the largest possible thermal cycler volume setting.

Table 3. Bisulfite Conversion Thermal Cycler Conditions

Step	Time	Temperature (°C)
Denaturation	5 min	99
Incubation	25 min	60
Denaturation	5 min	99
Incubation	85 min (1 h 25 min)	60
Denaturation	5 min	99
Incubation	175 min (2 h 55 min)	60
Hold	Indefinite	20

5. Place the PCR tubes containing the bisulfite reactions into the thermal cycler. Start the thermal cycling incubation.
Important: Since the bisulfite reaction is not overlaid with mineral oil, only thermal cyclers with heated lids are suitable for this procedure. It is important to use PCR tubes that close tightly.

Converted DNA can be left in the thermal cycler overnight without any loss of performance.

Cleanup of bisulfite converted DNA

6. Once the bisulfite conversion is complete, centrifuge the PCR tubes containing the bisulfite reactions briefly, then transfer the complete bisulfite reactions to clean 1.5 ml microcentrifuge tubes.
   Any precipitants in the solution will not affect the performance or yield of the reaction.
7. Add 560 µl freshly prepared Buffer BL containing 10 µg/ml carrier RNA (see "Things to do before starting", page 15). Mix the solution by vortexing and centrifuge briefly.
   Note: Carrier RNA is not necessary when using >100 ng DNA.
8. Place an EpiTect spin column and collection tube in a suitable rack. Transfer the whole mixture from step 7 into the EpiTect spin column.
9. Centrifuge the column at maximum speed for 1 min. Discard the flow-through, and place the spin column back into the collection tube.
10. Add 500 µl Buffer BW (wash buffer) to the spin column, and centrifuge at maximum speed for 1 min. Discard the flow-through, and place the spin column back into the collection tube.
11. Add 500 µl Buffer BD (desulfonation buffer) to the spin column, and incubate for 15 min at room temperature.
    If there are precipitates in Buffer BD, avoid transferring them to the spin column.
    Note: It is important to close the lid of the column before incubation.
12. Centrifuge the column at maximum speed for 1 min. Discard the flow-through, and place the spin column back into the collection tube.
13. Add 500 µl Buffer BW and centrifuge at maximum speed for 1 min. Discard the flow-through, and place the spin column back into the collection tube
14. Repeat step 13 once
15. Place the spin column into a new 2 ml collection tube, and centrifuge the spin column at maximum speed for 1 min to remove any residual liquid.
    Note: If the purified DNA is intended for use in real-time PCR, extend the centrifugation time to 5 min.
16. Place the spin column into a clean 1.5 ml microcentrifuge tube (not ovided). Add 20 µl Buffer EB to the center of the membrane. Elute the purified DNA by centrifugation for 1 min at approximately 15,000 x g (12,000 rpm).
    Note: To increase the yield of DNA in the eluate, transfer the spin column to a new 1.5.ml microcentrifuge tube, add an additional 20 µl Buffer EB to the center of the membrane, and centrifuge for 1 min at maximum speed. Combine both eluates.

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10 PUBLICATIONS

10.1 Draft paper

Epigenetic control of estrogen receptor alpha expression and tumor suppressor genes is modulated by bioactive food compounds

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ABSTRACT

Hormonal regulation and epigenetic control of estrogen receptor alpha (ESR1) is involved in the pathogenesis of metabolic disorders and various cancers. To integrate hormonal and epigenetic activities of selected bioactive food components, the effect of butyrate, resveratrol, genistein, EGCG, folic acid and reference DNMT inhibitor zebularine on the promoter methylation state of ESR1, $p16^{INK4a}$ as well as $p15^{INK4b}$ was analyzed in Caco-2 cells. Gene expression of ESR1 was measured by real time PCR and methylation of ESR1, $p16^{INK4a}$ and $p15^{INK4b}$ promoter CpG sites were determined by bisulfit conversion and methylation-specific PCR. Treatment with genistein, folate and EGCG increased, zebularine and butyrate diminished methylation of ESR1. Changes in promoter methylation partly corresponded with altered ESR1 gene expression, suggesting implication of additional epigenetic pathways such as histone modification, especially for genistein and butyrate. In contrast to $p15^{INK4b}$, $p16^{INK4a}$ promoter region was highly methylated in untreated cells, whereas folic acid increased methylation of both tumor suppressor genes. $p16^{INK4a}$ methylation was significantly reduced by butyrate and $p16^{INK4a}$ methylation by EGCG and butyrate.
These results endorse dietary concepts to support therapys, which modulate ESR1 signaling or reexpression of silenced ESR1 in hormone mediated complex diseases.

INTRODUCTION

Aberrant methylation of CpG islands located in the promoter region of genes is fairly established as an important mechanism leading to abnormal gene activation and has been described in a multitude of cancer relevant genes [1]. Cell cycle and cellular growth are under epigenetic control of tumor suppressor genes like $p16^{INK4a}$ and $p15^{INK4b}$, regulating cyclin dependant kinases [2]. Also hormonal signals are epigenetically regulated via nuclear receptors such as estrogen receptor alpha (ESR1). ESR1 is known to be overexpressed in ER-positive breast cancers and is a target in hormonal therapy [2]. The receptor is believed to contribute to tumorigenesis trough stimulatory effects on proliferation of mammary cells [3]. Elevated levels of ESR1 in benign breast epithelium appear to indicate an increased risk of developing breast cancer [4]. Moreover, ESR1 plays a role in the development of arthereosklerosis, as it has been demonstrated to modulate expression of proteins involved in the regulation of plasma lipid metabolism, such as apolipoprotein E and LDL receptor. [5]. Variants of the ESR1 gene have been associated with components of the metabolic syndrome, including obesity, blood levels of HDL-cholesterol, high blood pressure and type 2 diabetes [6]. The nonclassical pathway of estrogen action leads to the activation of the MAPK (Mitogen-Activated Protein Kinase) cascade and other cell proliferation effects [8]. The classical pathway depends on direct interaction with its receptor, mediating gene transcription through binding palindromic ERE (Estrogen Response Elements) or interact with transcription factors influencing their activity. ESR1 mediates cell cycle progression through transcriptionally regulating estrogen-responsive genes, such as Cyclin D1 [9], a critical mitogenregulated cell cycle control element, affecting activation of Rb (Retinoblastoma Protein) [6].
Transcriptional regulation of ESR1 involves both, epigenetic mechanisms, including CpG methylation as well as histone H3 deacetylation and trans-active factors such as TFAP2 inducing ESR1 transcription. Epigenetic chromatin modification might faciliate the access of trans–active factors to enhance or rather

decrease transcription [7].
Local remodeling of ESR1 promoter chromatin structure by a specific Rb– related protein complex strongly correlates with its susceptibility to specific DNA methylation. Methylation associated inactivation of the ESR1 gene in ageing -colorectal mucosa could be one of the earliest events in the onset of sporadic colorectal tumorgenesis [8]. In comparison, advanced colorectal tumors frequently display epigenetical inactivation of $p16^{INK4a}$ [9]. ESR1 transcriptionally regulates cell cycle modulators such as Cyclin –D1, which causes the initial inactivation of Rb in association with cyclin dependent kinase 4, thereby promoting proliferation. Tumor-suppressor -proteins $p16^{INK4a}$ and $p15^{INK4b}$ participate in cell growth regulation by inhibiting CDK4 and CDK6. *De novo* CpG island hypermethylation has also been found to be associated with transcriptional silencing of the $p16^{INK4a}$ and $p15^{INK4b}$ genes in a number of human neoplasia [10].
Diet is known to influence the development of complex diseases such as cancer and the metabolic syndrome. Evidence that epigenetic properties of bioacitve food components might contribute to this effect becomes more compelling [11]:
Epigallocatechin-3-gallate (EGCG), the major polyphenol from green tea, is known to inhibit DNMT1 [16] and significantly reduce acetylation of histones due to its function as histone- acetyltransferase (HAT) inhibitor [17]. Genistein, the most abundant isoflavone found in soybean has been demonstrated to restore methylation patterns and gene expression of tumor suppressor genes in neoplastic cultured cells, partially through direct inhibition of DNA methyltransferases [18]. These activities were associated with inhibited cell growth and induction of apoptosis [19] and might be indirectly due to a modulation of NFkB and fork head transkription factors [13]. Resveratrol, described as a possible mimetic of the dietary restriction effect, might mediate its effects through maintainance of DNA methylation patterns through SIRT-1, a class III histone deacetylase, mediating epigenetic processes [12]. Folic acid regulates intracellular levels of SAM (S-adenosyl methionine), the methyl donor for DNA methyltransferases, modulating gene stabiltiy and gene expression through altered methylation patterns, both within CpG islands and on single CpGs in the genome. Butyrate is a short chain fatty acid produced by the gastrointestinal microbiota through the breakdown of non-digestible fibers [13]. Its epigenetic effects mainly base on non-competitive inhibition of HDACs, especially class I and II, leading to global histone hyperacetylation [14]. An up-regulation of tumor suppressor CDK1A gene after treatment with butyrate has been reported with an attendant histone promoter acetylation, potentially explaining its growth arrest and apoptotic as well as anti-tumorigenic properties [24]. Zebularine, an orally administerable and well established DNA methyltransferase inhibitor, served as a reference in this study. Thaler et al. demonstrated a decrease in methylation and a concommittant increase of MnSOD expression in the Caco-2 cell line after treatment with zebularine [15]. To find out if and to what extend bioactive compounds in diet might affect epigenetic modification of response to estrogenic signals and cancer development, we analyzed the influence various bioactive food components have on promoter CpG methylation of ESR1, $p15^{INK4b}$ and $p16^{INK4a}$.

MATERIALS AND METHODS

Cell line and culture conditions

Human Caco-2 cells (colon carcinoma cell line) were grown at 37°C in a humidified atmosphere (5% CO2), in six well tissue culture plates and cultured in RPIM 1640 medium, supplemented with 10% heat inactivated fetal bovine serum, 4mM glutamine and 100 units of streptomycin and penicillin respecively. 48-h treatment was carried out after 14 days of cell gowth. Cell treatments were performed in dublicates and pooled for the analysis.
Agents concentration: butyrat 5 mmol/l, genistein 200 µmol/l, folic acid 200 µmol/l, EGCG 100 µmol/l, Resveratrol 10 µmol/ml, Zebularine: 100 µmol/l;
Cells were washed twice with cooled PBS (4°C) before nucleotide extraction.

Genomic DNA isolation and bisulfite treatment

DNA was isolated from cells using QIAamp DNA Mini Kit (Qiagen) and bisulfite conversion for detection of unmethylated cytosines was performed using the Epitect Bisulfite Kit (Qiagen). Both kits were used according to manufacturer's instructions.

CpG methylation analysis

Methylation status of the promoter regions of ESR1 and tumor suppressor genes was determined by methylation-specific PCR (MSP), further modified as a nested approach in order to increase sensitivity. External primers flanke the CpG- rich promoter regions of the target gene not distinguishing methylated from unmethylated nucleotides. PCR products of the external PCR were diluted 1:1000 and used as templates in the internal PCR. Two pairs of internal primers are required to recognize bisulfit induced alterations in nucleotide sequences of unmethylated cytosines.
Table I lists the primer sequences and PCR product sizes for each MSP approach.
External PCR was performed in a 50µl reaction volume, without addition of SybrGreen using the Promega PCR Master Mix (Wisconsin, USA). Internal PCR was performed a 10µl reaction volume using 5 µl Promega PCR Master Mix (Wisconsin, USA) containing 0,5µmol/l $MgCl_2$, 0,2 µl SybrGreen as detection dye, 1 µl template and a primer concentration of 5pmol/ l.
Thermo cycling conditions: 95°C for 5 min, 35 cycles or denaturation - 95°C for 30 sec, annealing 60 sec at a specific temperature (ESR1: 58°C external and internal step; p15,p16: 55°C external step, 58°C internal step) ,elongation 60 sec at 72°C . Melt curve analysis was performed 50-95°C – 0,5°C each step.

EXTERNAL	5´ PRIMER	3` PRIMER	PRODUCT SIZE (bp)
ESR1	GGYGAGGTGTATTTGGATAGTAGTAAGTT	CRAACTCRAAAACACRCTATTAAATAAAA	204
$p15^{INK4b}$	GAYGTYGGTTTTTTGGTTTAGTTGA	AACRCAACCRAACTCAAAACC	139
$p16^{INK4a}$	AGAAAGAGGAGGGGTTGGTTGG	ACRCCCRCACCTCCTCTACC	193
INTERNAL UNMETH.			
ESR1	TGTTGTTTATGAGTTTAATGTTGTGGTT	AAAAAAACCCCCCAAACCATT	124
$p15^{INK4b}$	GGTTGGTTTTTTATTTTGTTAGAGTGAGGT	AACCACTCTAACCACAAAATACAAACACA	80
$p16^{INK4a}$	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	151
INTERNAL METH.			
ESR1	ACGAGTTTAACGTCGCGGTC	ACCCCCCAAACCGTTAAAAC	110
$p15^{INK4b}$	GGTTTTTTATTTTGTTAGAGCGAGGC	TAACCGCAAAATACGAACGCG	68
$p16^{INK4a}$	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	150

Table I: Primers for ESR1, $p15_{INK4b}$, $p16_{INK4a}$ CpG methylation analysis [39]

Gene expression analysis

Total mRNA was extracted from cells using NucleoSpin RNA II (Macherey-Nagel, Germany) according to the manufacturer's instructions and converted into cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Germany). Quantification was performed by SybrGreen real-time PCR using the Corbett Rotor-Gene 3000.
The target mRNA expression was normalized to the GAPDH mRNA expression and results compared to untreated cells. The primers for gene expression are characterized in table II. Thermo-cycling conditions: 95°C for 10 minutes followed by cycles at 95°C for 30 sec, further 60 sec 60°C and 30 sec at 72°C. All reactions were set-up in a 10 µl final volume using the SensiMix DNA Kit (Quantace, London GB). Optimum reaction conditions were obtained with 5 µl 2x PCR master mix, a primer concentration of 5pmol/ µl and 0,2 µl Sybr-green.

	5´ PRIMER	3´ PRIMER
ESR1 EXPRESSION	TGG GCT TAC TGA CCA ACC TG	CCT GAT CAT GGA GGG TCA AA

Table II: Primers for ESR1 gene expression analysis

Data and statistical analyzis

Real-time PCR runs were analyzed with the Rotor-Gene software Version 6.1 applying comparative quantitation. Hypothetical concentrations were calculated and corrected for amplification efficiencies. Gene expression results were normalized to GAPDH, which was found to be stably expressed. Gene expression and methylation results were calibrated relative to untreated cells and expressed in ratios. Mean and standard deviations were calculated using Microsoft Office Excel 2007. Homogeneity of variances and signficance of results were tested applying the *F-test* and a two tails T-test, also in Microsoft Office Excel 2007. Results were regarded as significantly different at $p<0,05$.

RESULTS

ESR1 gene expression and promoter methylation

Untreated Caco- 2 cells are predominantly methylated at the ESR1 pomoter site in a 56:1 ratio. A distinct effect on ESR1´s promoter region methylation has been detected after cell treatment with genistein, causing a clear 2,84-fold ± 0,2 ($p\leq 0,05$) hypermethylation compared to untreated cells, whereas nearly no change in mRNA expression could be seen. Treatment with methyl donor folic acid results in an almost doubled ESR1´s promoter methylation grade, 1,96-fold ± 0,05 ($p\leq 0,01$) correlating with decreased (0,85 ± 0,57) but no significant decrease in gene expression. A clear 2,03-fold ± 0,07 ($p\leq 0,05$) hypermethylation was also observed after cell exposure to EGCG and a slight but not significant decrease in gene expression could be seen (0,65 ± 0,02). Treatment with butyrate caused a diminished ESR1 expression (0,91 ± 0,15) and a significant decrease in methylation (0,48 ± 0,05; $p\leq 0,05$). Resveratrol marginally impacts any observed gene´s promoter methylation state and only a slight (1,25± 0,36) but not significant increase in ESR1 mRNA expression could be detected. Methyltransferase inhibitor zebularine distinctively diminished methylation in ESR1, $p16^{INK4a}$ and $p15^{INK4b}$. Methylation at the ESR1 promoter site decreased significantly, 0,16 ± 0,01 ($p\leq 0,01$) but no significant change in mRNA expression (0,86 ± 0,20) could be observed.

$p15^{INK4b}$ and $p16^{INK4a}$ promoter methylation

In untreated cells $p16^{INK4a}$ CpG rich promoter site is predominantly methylated in contrast to the $p15^{INK4b}$ promoter region that was found to be regularily unmethylated: the comparison methylated versus unmethylated DNA showed a ratio of 5,00E-03 for $p15^{INK4b}$ gene and 8,29 E03 for $p16^{INK4a}$.

Treatment with genistein caused a 1,24- fold ± 0,18 hypermethylation in the $p15^{INK4b}$ promoter site compared to untreated cells. In contrast $p16^{INK4a}$ promoter was found to be nearly completely demethylated. Exposure to folic acid enhanced $p16^{INK4a}$ methylation 1,66-fold ± 0,40 and also $p15^{INK4b}$`s methylation grade but not as clearly as for $p16^{INK4a}$, 1,21- fold ± 0,27. EGCG treatment caused a significant reduction of $p15^{INK4b}$ (0,60 ± 0,01) ($p\leq 0,05$) and a slight decrease in $p16^{INK4a}$ (0,86 ± 0,41) promoter methylation. Exposure to butyrate significantly diminished both, $p16^{INK4a}$ and $p15^{INK4b}$ methylation state 6,92E-02 ± 0,04 ($p\leq 0,05$) and 0,57 ± 0,01 ($p\leq 0,01$) compared to untreated cells. Resveratrol had almoust no effect on $p16^{INK4a}$ promoter methylation but reduced methylation of $p15^{INK4b}$: 0,62 ± 0,03 ($p\leq 0,01$). Reference DNMT inhibitor zebularine explicitly diminished methylation in the $p16^{INK4a}$ and $p15^{INK4b}$ promoter region: 6,74E-03 ± 0,01 ($p\leq 0,05$) and 0,64± 0,02 ($p\leq 0,01$).

DISCUSSION

ESR1 Promoter Methylation and Gene expression

The nuclear receptor ESR1 functions as a transcription factor upon hormonal signals, enhancing the transcription of genes involved in the regulation of cell growth. Epigenetic disregulation of ESR1 expression and concurrent alteration in the integration of hormonal signaling has been associated with the development of several diseases, such as the metabolic syndrome and cancer.
The Caco-2 cell model was chosen as a model for the human gastrointestinal tract, due to its similar properties compared to mature enterocytes [18].
Our results show untreated cells to be predominantly methylated at the ESR1 promoter site. Caco-2 cells are derived from human epithelial colorectal carcinoma cells. These results are in agreement with studies describing human adenomatous and colon cancer tissue to be hypermethylated at the ESR1 promoter site [8, 16]. Methylation patterns of free DNA, found in the buffy coat of patients with several cancer types, coincide with that of cancer suppressor genes in the tumors [17]. In contrast to Caco-2 cells, methylation state of ESR1 promoter region in 13 healthy women's buffy coat DNA turned out to be nearly completely unmethylated (data not shown). These differences of the methylation state in different tissues need to be considered in the evaluation of effects bioacitve food components have on methylation or demethylation, as the original methylation state of the promoter seems to impact the degree of treatment induced methylation changes (quod vide results: $p15^{INK4a}$).
An elevated methylation level of the ESR1 promotor region after Caco-2 cells' exposure to folic acid correlated with a reduced gene expression. These findings support preliminary studies suggesting that the rate of ESR1 methylation in colonic tissue is modifiable by dietary folate [18]. Zhu and Williams hypothesized that development of ER-negative breast cancers, which are unsensitive to hormonal therapy, might result from methylation dependant inactivation of ESR1 transcription due to methyl deficient diets [19]. Proving that hypothesis an epidemiological study associated higher folate intake with reduced risk of developing ER-negative breast cancer, assuming that adequate folate intake is particularly important for women with higher risk potential [20].
Genistein regulates ESR1 gene expression in a very complex way, amongst others due to its estrogenic, epigenetic [21] and tyrosine kinase inhibiting [22] properties. Although promoter methylation increased after treatment, we observed an enhanced expression of ESR1. This might be due to a lowered HDAC SIRT1 expression, increasing histone acetylation of H3K9 in several gene promoter regions accompanied by changes in methylation state [23]. Similar observerations for genistein are being analyzed for IL-8 and will be presented elsewhere.
EGCG treatment caused, in contrast to results for $p15^{INK4a}$ and $p16^{INK4a}$, a twofold enhanced methylation of ESR1 promoter region and a correlating gene expression reduction by half. As EGCG was shown to be cancer cell growth inhibiting, these results seem to be conclusive as $p15^{INK4a}$ and $p16^{INK4a}$ are cell cycle modulators and ESR1, activated by growth factors, acts as a potent mitogen for epithelial cells [24].
Butyrate has been proven to induce histone acetylation by inhibiting histone deacetylases, as well as growth arrest or apoptosis [25]. We detected a slight decrease in ESR1 expression after treatment with butyrate, as previously shown in breast cancer cells [26], accompanied by clearly diminished methylation. CpG islands are significantly more common in deacetylated regions. This finding points towards a mechanistic link between DNA methylation and histone acetylation although it is not yet clear if histone deacetylation is the reason or the consequence of gene silencing. It might also be possible that these epigenetic alterations are responses to stress caused by butyrate treatment [27]. Resveratrol, acting as a selective estrogen receptor modulator and modulator of SIRT1 HDAC activity, does seem to neither significantly affect methylation nor gene expression of ESR1. Studies working with Caco-2 cells investigated global DNA methylation after resveratrol treatment and obtained similar results. In this study only one concentration of 10 µmol/l and an incubation time of 48h were used. In contrast, investigations on ER-positive breast cancer cells showed resveratrol to induce methylation changes in a variety of CpG islands after treatment with 50 µmol/l for 36 hours [28].
Although exposure to well established reference DMNT inhibitor zebularine caused a virtually complete demethylation of the ESR1 promoter region, almoust no effect on gene expression except for a slight decrease could be noted. As gene expression of ESR1 in this study does not always correlate with its promoter methylation status after treatment, expression could rather be influenced by CpG islands

within exons of the gene or other epigenetic mechanisms, as shown by Zhou et al. They demonstrated reactivation of ESR1 gene by HDAC inhibitors to be whithout alteration in promoter DNA hypermethylation, suggesting expression of ESR1 to be regulated by histone modification or by a combination of DNA methylation and histone modifications [2]. A recent study shows most methylation alterations in colon cancer to occur not in promoter regions but 2kb distant, which are termed “CpG island shores”. Methylation of these DNA sites was strongly related to gene expression [29]. Moreover regulation of ESR1 also comprises trans–active factors such as TFAP2C inducing ESR1 transcription through an AP-2 regulatory region in the ESR1 promoter. Epigenetic chromatin modification could faciliate admission of trans active factors influencing gene transcription [7].

$p15^{INK4b}$ and $p16^{INK4a}$ promoter methylation

ESR1 contributes to cell cycle progression by inducing transcription of Cyclin –D1 [9], cell cycle control element, which, in association with CDK 4 or CDK 6 effects phosphorylation of pRb (Retinoblastoma) promoting proliferation [6]. $P16_{INK4a}$ and $p15_{INK4b}$ are members of the INK 4 family, known to be CDK 4 and 6 inhibitors. Even though the $p15_{INK4b}$ gene indicates high homology to $p16_{INK4a}$ in the amino acid sequence, they seem to show differing patterns of transcriptional activation and epigenetic regulation [30]. Generally $p15_{INK4b}$ is not as thoroughly investigated as $p16_{INK4a}$ and few data exist concerning the impact of bioactive food compounds on $p15_{INK4b}$ promoter methylation. A variety of cancers feature mutations in $p16_{INK4a}$ and $p15_{INK4b}$ as well as transcriptional inactivation in association with *de novo* methylation during neoplastic processes, leading to uncontrolled cell cycle progression. $P16_{INK4a}$ methylation could be a prognostic marker for the metastatic potential of a tumor [10, 31]. In untreated Caco-2 cells $p16_{INK4a}$ is prevailingly methylated in contrast to $p15_{INK4b}$ promoter region, which seems to be regularily unmethylated. These results agree with studies describing different promoter methylation patterns, distinguishing for instance leukaemias from colorectal cancer: $p15_{INK4b}$ promoter hypermethylation has been found only in leukemias, whereas $p16_{INK4a}$ promoter hypermethylation occured mainly in primary colonic tumors [1, 32]. Gene expression and promoter DNA methylation of $p16_{INK4a}$ in the colon of mice was shown to be dependent on age and folate intake [33]. These results concur with our findings of folic acid treatment to broadly enhance methylation in the $p16_{INK4a}$- and even more notedly in the $p15_{INK4b}$ - promoter region. In our Caco-2 cell model we observed downregulation of DNMT3a and upregulation of caspase-3, an important component of the apoptotic cascade after 48h treatment with genisteine (data not shown). $p16_{INK4a}$ induction by methylation inhibitors in cancer cells was shown to lead to growth inhibition [34]. Our study showed genisteine to induced hypomethylation in the $p16_{INK4a}$ promoter region. Similar situations were found in benign and cancerogenic breast cells after exposure to genisteine: decrease in the expression of DNMTs leading to a hypomethylation of several genes associated with telomerase activity and cancer [22]. Hence genisteine seems to be able to induce reexpression of tumor suppressor genes and thus to interfere with cell cycle progression of neoplastic cells [19].

For EGCG we observed increased promoter methylation, confirming data suggesting that different concentrations of EGCG were able to inhibit the growth of malignant cell lines and increase the cell number in G(0)/G(1) phase or rather induce apoptosis in many tumors [35], presumably by impeding DNMT activity and reactivating methylation-silenced genes in cancer cells [36]

Treatment of Caco-2 cells with butyrate clearly decreased $p16_{INK4a}$ promoter methylation, possibly leading to its reexpression. Butyrate was reported to lead to cell cycle arrest and $p16_{INK4a}$ transcription [37]. Upregulation of $p16_{INK4a}$ expression was demonstrated to be due to an increase of acetylation and degradation of methylation [38]. Epigenetic regulation of $p16_{INK4a}$ seems to be composed of DNA methylation associated with histone modifications in nucleosome-free regions. Thus promoter methylation seems likely to be affected by HDAC inhibitors as changes in chromatine configuration can cause altered enzyme access to the DNA.

The DNMT inhibitor zebularine almost completely demethylated the $p16_{INK4a}$ promoter but had less effect on $p15_{INK4b}$, propably because $p15_{INK4b}$ already has a low methylation status in Caco-2 cells.

Our results show specified bioactive food compounds to be able to modify CpG methylation in the promoter regions of ESR1, $p16_{INK4a}$ and $p15_{INK4b}$. However the degree of change in methylation state of the different genes is variable and often CpG methylation might cooperate with additional mechanisms such as histone acetylation in the regulation of gene expression. Modulation of the methylation of

relevant CpGs of ESR1, $p16_{INK4a}$ and $p15_{INK4b}$ by food components might be a way to interfere with hormonal signaling in addition to pharmaceutical intervention. This could be an effective approach to intervene in complex diseases with misregulated ESR1 signaling, for example by improvement of ESR1 mediated expression of blood lipid regulating proteins in metabolic syndrome. In ER-negative breast cancers, induction of ESR1 re-expression could offer the possibility for using hormonal therapy.

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10.2 Book chapter

for the book **"Epigenetics and Human Health, Linking hereditary, environmental and nutritional aspects"** (Editor: Dr. Alexander Haslberger)

INTERACTION OF HEREDITARY AND EPIGENETIC MECHANISMS IN THE REGULATION OF IMMUNE AND AGING RELEVANT GENE EXPRESSION

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Abstract Hereditary dispositions and environmental factors such as nutrition, natural and societal environment interact on human health. Diet compounds rise increasing interest due to their influence in co-regulating epigenetic gene expression. Nutrition and specific food ingredients have been shown to alter epigenetic markers such as DNA methylation or histone acetylation involving regulation of genes with relevance for fundamental mechanisms such as antioxidative control, cell cycle regulation or expression of immune mediators.

Hereditary dispositions

Interactions between genes and environment are not linear and often include direct and indirect cause of events. Many complex diseases are linked to various heritable dispositions like single nucleotide polymorphisms (SNP) or allelic translocations. Consequently they have become a main focus in modern biomedical research and also started to raise public interest. Single nucleotide polymorphisms are common rather than exceptions. SNP´s may determine the efficiency of gene transcription, gene translation or protein structure, leading to an altered amount of enzyme and/or –activity, thus influencing further metabolic pathways. A SNP can occur within coding sequences of genes, non-coding regions of genes or in intergenic regions. SNPs within a coding sequence do not necessarily change the functional efficiency of the protein (silent mutations) (Nothnagel, Ellinghaus et al. 2008) (Haiman and Stram 2008).

Upon completion of the human genome project it was confirmed that 99.9 percent of the human genome's 3 million base pairs are identical in any person. The remaining 0.1 percent, (mainly SNPs) are assumed to play a key role in congenital sensitivity to disease and drug side effects. They also appear to influence peoples' variable responses to pharmaceuticals or different penetrance of the same disease. The International HapMap (haplotype map) Project aimed to study the scope of these SNP variations in different population groups. Clusters of SNPs located on the same chromosome tend to be inherited in blocks. It is expected that the outcomes of this project will provide crucial tools that allow researchers to detect genetic variations with effects on health and disease. In so called genome-wide association studies, researchers compare genomes of individuals with known diseases to a control group of healthy ones in order to detect suspicious tag SNPs which might play a role in development/genesis or course of disease. (HapMap project, http://snp.cshl.org/)

A variety of functional SNPs on several genes, covering almost all aspects for cell viability, is well described in literature. In superoxide metabolism for example, an increase in superoxide radicals is given by the SNP (rs4880) regarding the mitochondrial Superoxide Dismutase (MnSOD) gene (Taufer, Peres et al. 2005). Several kinds of cancer (Kang, Lee et al. 2007), Alzheimer disease (Wiener, Perry et al. 2007), as well as an accelerated aging process are discussed to be related to this SNP. In opposite, three SNPs in the human forkhead box O3A gene (FOXO3A) were statistically significantly associated with longevity. Polymorphisms in this gene were indeed associated with the ability to attain exceptional old age, the FOXO3A association was considerably stronger in centenarians than in nonagenarians. In nutritional sciences, the methylenetetrahydrofolatereductase (MTHFR) gene is well known as it is essential for folic acid metabolism (Miyaki, Takahashi et al. 2008). The polymorphism C677T in the MTHFR gene is discussed to have an influence in several methylation pathways as for example in the DNA –methylation pathway.

So far, a multitude of ailments have been correlated with their respective SNPs. Yet, the predictability for the development of diseases from the analysis of SNPs still requires larger controlled studies. The present difficulty in utilizing SNPs analysis arises from missing the functional relevance of many SNPs. In addition, the mostly small and variable penetrance of single SNPs leads to statistical limitations and poor reproducibility in many clinical studies associating SNPs and diseases (Janssens, Gwinn et al. 2008).
The combination of an individual's relevant SNPs in addition to environmental influences may define his risk for developing diseases. Sets of candidate SNPs and experiences from the analysis of the work with biobanks need to be evaluated to understand gene – environment interactions (Vineis, Veglia et al. 2007).

THE EPIGENOME

Despite of the genetic code, mammalian cells contain an additional regulatory level which predominates over the DNA code: modification of gene expression by altering chromatin. Thus, due to different chromatin status, same genetic variants might be for example associated with opposite phenotypes depending on different environmental influences. New insights in research clarify the molecular pathways by which, amongst others, nutrition and lifestyle factors influence chromatin packaging and further health status. Indeed several studies clearly correlate the risk for developing multifactorial chronic diseases like cancer, diabetes or obesity with nutritional and lifestyle habits (Mann, Li et al. 1999; Johanning, Heimburger et al. 2002; Jirtle and Skinner 2007; Tsugane and Sasazuki 2007).
Measurable consequences of these influences are alterations in the expression of a growing number of genes, caused by remodeling of chromatin and DNA methylation. Methylation of cytosines in DNA, histone modifications as well as alterations in the expression level of micro RNA (miRNA) and short interference RNA (siRNA) are the mechanisms involved in chromatin remodelling. This topic is being studied by the fast evolving research area of epigenetics. The term "epigenome" is used to define a cell's overall epigenetic state. Epigenetic modifications can be stably passed over numerous cycles of cell division. Some epigenetic alterations can even be inherited from one generation to the next (Anway, Memon et al. 2006; Dolinoy, Weidman et al. 2006). Studies conducted by the Department of Community Medicine and Rehabilitation of the Umeå University in Sweden showed transgenerational effects due to nutritional habits during a child's slow growth period (SGP), the phase of the prepubertal peak in growth velocity. Clear correlations with descendants' risk of death from cardiovascular disease and diabetes were also seen (Kaati, Bygren et al. 2002). The finding that monozygotic twins are epigenetically indistinguishable early in life but, with age exhibit substantial differences in the epigenome, indicates that environmentally determined alterations in a cell's epigenetic markers might be responsible (Fraga, Ballestar et al. 2005).
During the development of germ cells and during early embryogenesis DNA is specifically methylated and these markers confer genome stability, imprinting of genes, totipotency, correct initiation of embryonic gene expression and early lineage development of the embryo (Morgan, Santos et al. 2005). According to experiments in the Agouti mouse model, early epigenetic programming is alterable through mothers' diet during pregnancy, leading to lifelong modification of selected genes for the offspring (Dolinoy, Weidman et al. 2007). Thorough knowledge of both epigenetic and classic genetic code is necessary in order to truly understand interactions between the environment and gene expression.

EPIGENETIC MECHANISMS

Epigenetic regulation includes DNA methylation, histone modifications and post-transcriptional alteration of gene expression based on microRNA interference.

DNA Methylation

Basic biological properties of segments such as gene density, replication timing and recombination are tightly linked to their guanine –cytosine (CG) content, therefore isochors (DNA fractions > 300 kb on average) of the genome can be classified accordingly (Costantini, Clay et al. 2006). CpG islands are defined as genomic regions with a GC percentage greater than 50% and with an observed/expected CpG(cytosine base followed by a guanine base) ratio greater than 60 %. In mammals, CpG islands typically are 200-3.000 base pairs long. CpGs are rare in vertebrate DNA due to the tendency of such

arrangements to methylate cytosines to 5-methylcytosines followed by a turn into thymines because of spontaneous deamination. The same mechanism seems to be responsible for the inactivation of retroviruses by changing the virus sequences [17].
The consequences are large DNA -regions low in GC and gene density, clearly visible on isochore maps as “genome deserts”. However, some regions escaped large scale methylation and determination during evolution and, therefore, show a high amount of GCs, which is generally parallel to a high CpG island and gene density (16). CpG islands mostly occur in these isochors, at or near the gene's transcriptional start site. Promoters of tissue-specific genes that are situated within CpG islands normally are largely unmethylated in expressing and non-expressing tissues (Bestor 2000). There are three known ways by which cytosine methylation can regulate gene expression: first 5-methylcytosine can inhibit or hinder the association of some transcriptional factors with their cognate DNA recognition sequences, second methyl-CpG-binding proteins (MBPs) bind to methylated cytosines mediating a repressive signal and third MBPs can interatct with chromatin forming proteins modifying surrounding chromatin, linking DNA methylation with chromatin modification (Klose and Bird 2006). Mostly DNA methylation cause a repression of mRNA gene expression, however, when CpG methylation blocks a repressor binding site within a gene promoter, this may induce a transcriptional activation as shown for Interleukin-8 in breast cancer (De Larco, Wuertz et al. 2003)
DNA -methylation at position five of CpG-cytosines is conducted by DNA methyltransferases (DNMT’s), which are expressed in most dividing cells (Schaefer, Ooi et al. 2007). Mammalian DNMT’s can be divided in two general classes. DNMT1 enzyme is responsible for the maintenance of global methylation patterns on DNA. It preferentially methylates CpGs on hemimethylated DNA (CpG methylation on one site of both DNA strands), therefore guaranteeing transfer of methylation marks through the cell cycle in eukaryotic cells. The DNMT1 enzyme is directly incorporated in the DNA replication complex. The de novo methyltransferases DNMT3a and DNMT3b establish methylation patterns at previously unmethylated CpGs. DNMT3L is a DNMT related enzyme which associates with DNMT3a/3b. It influences its enzymatic activity while lacking one of its own. Finally, for the DNMT2 enzyme, in mammals a biological function remains to be demonstrated (Bestor 2000; Schaefer, Ooi et al. 2007). Specific mutations in the DNMT3b gene were found in patients affected by the autosomal recessive immune disorder ICF.
Most DNMT’s contain a sex-specific germline promoter which is activated at specific stages during gametogenesis. Genomic methylation patterns are largely erased during proliferation and migration of primordial germ cells and reestablished in a sex-specific manner during gametogenesis, resulting in a high methylation of the genome. For viability, close regulation of the DNMT genes during these stages and during early embryogenesis is needed (Schaefer, Ooi et al. 2007). After fertilization a second phase of large epigenetic reprogramming takes place. Upon fertilization, a strong, presumably active DNA demethylation can be observed in the male pronucleus while the maternal genome is slowly and passively demethylated. Imprinted methylation is maintained for both the paternal and the maternal genome. DNA-demethylation occurs until the morula stage, after which de novo methylation is observed (Dean, Santos et al. 2003). See figure 1, adapted from Morgan et al 2005 and Dean et al 2003 (Dean, Santos et al. 2003; Morgan, Santos et al. 2005).
For targeting DNA de-novo methylation, three mechanisms are described. First, DNMT3 enzymes themselves might recognize DNA or chromatin via their conserved PWWP (relatively well-conserved Pro-Trp-Trp-Pro residues, present in all eukaryotes) domain. Mouse experiments show that this domain is necessary for targeting the enzyme to pericentromeric heterochromatin. In humans, mutations in the PWWP domain can cause ICF syndrome Immunodeficiency (Centromere instability and Facial anomalies syndrome). Second, by interaction of DNMT’s with site-specific transcriptional repressor proteins DNMT’s can be targeted to gene promoter regions. Third, in vitro studies have shown that introduction of double stranded RNA corresponding to the promoter region of the target gene leads to its de-novo DNA methylation and decreased gene expression, suggesting the existence of an RNAi mediated DNA methylation mechanism. However, further efforts are needed to clarify this pathway (Klose and Bird 2006).
Their catalytic role aside, DNMTs seem to mediate gene silencing by modifying chromatin via proteinprotein interactions. They biochemically interact with histone methyltransferases (HAT) and histone deacetylases (HDACs). As mentioned above, binding of MBP’s to methylated CpGs mediates

silencing of gene expression by the associaton with chromatin remodeling co-repressor complexes. Thus, under certain circumstances, gene silencing by DNA methylation may be attributed directly to chromatin modifications. So far, six different methyl-CpG- binding domains (MBD) are known for the MBPs: MBD1 to 4, MeCP2 and Kaiso. All of them mediate silencing of gene expression by chromatin remodeling (Clouaire and Stancheva 2008). For example, the MBD1 and a histone H3 methyltransferase enzyme (SetDB1) interact during the cell cycle, linking DNA methylation to rearrangement of chromatin by histone methylation (Klose and Bird 2006). Even if no active demethylation for DNA is known, gene silencing by DNA methylation is not irreversible. For example, Il-4 expression in undifferentiated T cells is silenced via binding of MBD2 on the methylated promoter of the gene (Hutchins, Mullen et al. 2002). After differentiation, TH2 cells express the transcription factor GATA-3 which competes with MBD2 for binding to the IL-4 promoter. In this case epigenetic factors impose a threshold to be overcome in order to achieve efficient gene expression. The binding of MBDs to DNA seems to be for the most domains sequence specific.
In order to achieve DNA methylation, often alterations at the chromatin level must occur before. As previously discussed, DNMTs interact with HATs and HDACs. Small modifications of histones by acetylation, methylation, phosphorylation and ubiquitation on certain amino acids can alter gene expression by chromatin remodeling. The effect of these small modifications on chromatin remodeling depends by type, amount and site of modification as well as the interactions as shown in table 1 (adapted from He and Lehming 2003 (He and Lehming 2003)).

Histone modifications

Some modifications, including acetylation and phosphorylation, are reversible and dynamic, others, such as methylation, are found to be more stable and involved in long term alterations (Cheung and Lau 2005; Shahbazian and Grunstein 2007).
As an attempt to organize the complexity of the different possible modifications, the establishment of a "histone code" is a major focus of epigenetic research. Its necessity is demonstrated in the following example: We know 24 methylation sites on a histone. The possibility of mono-, di-, or trimethylated lysine residues and mono- or dimethylated arginine side chains lead to $3x10_{11}$ different histone methylation states. The need to distinguish between short-term changes in histone modification associated with ongoing processes and changes that have long term effects still pose a problem to be solved (Turner 2000; Turner 2002). The significance of some histone modifications has been identified, especially regarding acetylation and deacetylation of histones (Shahbazian and Grunstein 2007). Histone acetylation is the major factor regulating the degree of chromatin folding, global loss of monoacetylation and trimethylation at histone H4, all of which are commonly seen in human tumor cells. Histone acetylation also regulates nucleosome assembly.
Over time, the relationship between DNA-methylation and histone modifications will become clearer (Cheung and Lau 2005; Klose and Bird 2006). Several factors are involved in the process of gene silencing by DNA-methylation. One prerequisite is attributed to the recruitment of HDACs through the methyl-DNA binding motifs, while methylation at Lys9 on H3 is another prerequisite. Methylation of both DNA and histones seem to have a reciprocative reinforcing effect in gene silencing. Histone variants like macro H2A, accumulated on the inactive X chromosome, have been reported to play a role in gene expression regulation. Their presence in the IL-8 promoter-region is connected to tissue related gene silencing of the IL-8 gene (30). The comprehension of a histone code will be an important step towards in understanding further mechanisms for epigenetic gene expression.

Histone Modification Effect

Histone	Modification	Effect
H1	Phosphorylation	Chromatin condensation, gene specific condensation and repression.
	Ubiquitination	Transcriptional activation
H2A	Acetylation	Transcriptional activation
	Ubiquitination	Elusive
H2B	Ubiquitination	Prerequisite of H3 methylation

	Phosphorylation	Chromatin condensation
	Acetylation	Chromatin remodeling
	Methylation	Chromatin stabilization
H3	Methylation (H3-K4, R17)	Transcriptional activation
	Methylation (H3-K9, K79)	Transcriptional repression
	Acetylation	Transcriptional activation
	Phosphorylation	Chromatin condensation; transcriptional activation
	Ubiquitination	Nucleosome loosening
H4	Acetylation	Transcriptional activation
	Methylation (H4-K20)	Transcriptional repression
	Methylation (H4-R3)	Transcriptional activation

Table 1: Consequence of histone modifications on chromatin

Through alteration of gene expression and destabilization of chromatin histone modifications can have an impact on the risk of cancer. Modified cancer pathogenesis is linked to varying activities of both HATs and HDACs. This mechanism is best described for acute promyelocytic leukemias, where a chromosomal translocation leads to inappropriate HDACs activity. A change in HDACs expression was also detected in other types of tumors. For example, in gastric cancer, esophageal squamous cell carcinoma, and prostate cancer an increased HADC1 expression is related to pathogenesis. In contrast, in colon cancer an overexpression of HDAC2 causes a decreased expression of the APC (adenomatous polyposis coli) tumor suppressor gene. Another way of influencing the expression of tumor suppressor genes are abnormal functions of HDACs, for instance by atypical targeting. These observations lead to the current exploration of HDAC inhibitors as anticancer therapeutics. The examined substances aim to shift several cell functions which are known to be down regulated in cancer cells. Among other presumed advantages of the approach show cancer cells' an increased sensitivity for HDAC inhibitors compared to normal cells, thus there may be a possibility to prevent or to decelerate the development of tumours by such substances. Overall, the broad range of function promises efficacy at all stages of tumorigenesis.
Not only HDACs but also HATs influence the risk of developing cancer. Especially in cancer of epithelial origin an overexpression or mutation of HAT genes has been detected. Some lines of lung, breast, and colorectal cancer have in common a mutation which inactivates a specific HAT.
Changes of the methylation status of histones have also been observed in some types of cancer. The loss of the trimethylated form of the lysine 20 residue of the H4 histone is characteristic for cancer cells. Also, the demethylation of lysine 9 on H3 histones increase the formation of B-cell lymphoma in mice significantly because of its links to chromatin silencing (Davis and Ross 2007).

Micro RNAs

In addition to DNA and Histone modifications, micro RNAs are part of the epigenetic gene expression regulatory complex. MicroRNAs are small non-coding RNAs that posttranscriptionally regulate the expression of complementary messenger RNAs and function as key controllers in a countless number of cellular processes, including proliferation, differentiation and apoptosis. Over the last few years, increasing evidence has indicated a substantial number of microRNA genes to be subjected to epigenetic alterations, resulting in aberrant patterns of expression upon the occurrence of cancer (Guil and Esteller 2009).

ENVIROMENTAL INFLUENCES

Early life conditions

By now, various findings showing the effects of environmental factors on the epigenetic regulation of gene expression derive largely from mouse experiments. Environmental factors influencing epigenetic gene regulation include more than epigenetically active natural food compounds such as vitamin B12 or genistein. Transgenerational effects are for example caused by environmental toxins such as the endocrine disruptor vinclozolin, a common fungicide used in vineyards. Vinclozolin were seen to disrupt epigenetically the germ line in mice. These effects were transferred through the male germ line for several generations. The described mechanism appears to be attributed to a heritable alteration of

epigenetic programming of DNA-methylation in the germ line, which alters the transcriptomes of developing organs, in this case the testis development (Anway, Memon et al. 2006).
Early mammalian development is a crucial period for establishing and maintaining epigenetic markers. Modifications of the epigenome are not limited to the fetal period but extended to the plastic phase of early life. Broad epigenetic reprogramming can be seen after fertilization to achieve totipotency of developing embryo cells, while methylation patterns associated with imprinting are sustained. Epigenetic modifications settled during fetal development are generally stable passed through cell division processes throughout a lifetime.
Prenatal exposure to famine for instance, as shown in an epidemiological study, is also likely to change the epigenetic status. Observed individuals showed less DNA methylation of imprinted IGF-2 gene than their unexposed same sexed siblings (Heijmans, Tobi et al. 2008). A further, nutrition-related epigenetic alteration have been demonstrated in the viable yellow Agouti (=Avy) mouse model (Dolinoy, Weidman et al. 2006). Maternal diet containing bisphenol A (BPA), an estrogenic monomer used in polycarbonate plastic production, significantly decreases offsprings methylation of the Avy gene promoter which induces reversal to a wild-type phenotype. Maternal nutritional supplementation with methyl-donors counteracts the effects from BPA, showing that simple dietary changes may protect against harmful epigenetical effects caused by environmental toxins.
Modified promoter methylation and, accordingly, modified gene expression of the hepatic glucocorticoid receptor and the peroxisome proliferator –activated receptor alpha (PPAR alpha), both important elements in carbohydrate and lipid metabolism regulation, can be seen in the offspring of rats fed a protein restricted diet. The selective methylation of PPAR alpha in the liver without consequences for the related transcription factor PPAR gamma demonstrates that maternal nutrition and behavior can also influence specific promoter regions rather than being associated with global DNA -methylation alteration. Such changes in gene expression and promoter methylation were also seen to be transmitted to the next generation without further nutritional challenge for the first generation (Gluckman, Lillycrop et al. 2007).
Finally, the elucidation of the impact of epigenetic modifications on behaviour and psychic health presents an intriguing challenge. Rodents experiments show that some epigenetic changes can be induced promptly after birth through mother's physical behaviour toward her newborn (Henry, Kabbaj et al. 1994; Darnaudery and Maccari 2008; Zuena, Mairesse et al. 2008) Licked and groomed newborns appear to grow up to be relatively brave and calm. In contrast, neglected newborns grow up to be nervous and hyperactive. The difference in their behaviour can be explained by analyzing specific regions in the brain. The hippocampus of both groups reveal different DNA-methylation patterns for specific genes agreeing with the difference in behaviour. This entails a better developing of the hippocampus in the licked newborns, possibly by releasing less of the stress hormone cortisol.
Furthermore, recent research results have demonstrated that complex epigenetic mechanisms have long-lasting effects in mature neurons and that they possibly play a vital role in the etiology of major psychoses, such as schizophrenia or bipolar disorder. Paternal age at conception is a strong risk factor for schizophrenia, explaining about a quarter of all cases. The possible mechanisms for the elevated risk may be de novo point mutations or defective epigenetic regulation of paternal genes. The risk might also be related to paternal toxic exposures, nutritional deficiencies, suboptimal DNA repair enzymes or other factors that influence the reliability of the transfer of genetic information in the constantly replicating male germ line.

Pollution and Toxins

There is now a mounting body of evidence that environmental exposures to toxins, particularly during early development, can induce epigenetic changes, which may be transmitted to subsequent generations or serve as basis for diseases developed later in life. Either way, epigenetic mechanisms will help interpreting toxicogenomic approaches (Reamon-Buettner, Mutschler et al. 2008).
Air pollution, for instance, particular matters and cigarette smoke, appear to have omnipresent toxicological influences on humans. Promotor hypermethylation in early tumorigenesis is likely to have a clinical importance, because dissentient promoter methylation in tumor suppressor genes has been detected in a large percentage of human lung cancers. Noticeably the p16 gene, which expresses an inhibitor of cyclin dependent kinase 4 and 6 consequently, interrupting the cell cycle progression is

methylated at its promoter region in 20-65% of the lung tumors. (Hayslip and Montero 2006) Smoking habits alter the extent of promoter methylation patterns. Increasing methylation of p16 could be seen with increasing smoking duration, packets per years and smoking when a juvenile, whereas methylation decreases gradually over time when a person quits smoking.
Similar effects could be seen in rodents' lung cancer, induced by particulate carcinogens carbon black and diesel exhaust, where the tumor cells showed an overmethylation in the p16 promoter region. Equal circumstances could be found in murine lung tumors, caused by cigarette smoke. Remarkable effects improvements could be achieved by treating rodents with histone deacetylation inhibitors in a lung cancer mouse model. A greater than 50 % reduction of tumor growth was seen in treated mice.
In addition to well-known carcinogens, air pollution components and particulate matter (PM10), nickel and beryllium compounds have also been shown to also have an impact on histone acetylation and/or altered DNA methylation patterns (Vineis, Hoek et al. 2006).

Diets

Dietary habits as well as a sedentary lifestyle clearly contribute to today's increasing number of chronic diseases. The influence played by numerous food compounds on the epigenetic machinery is of growing interest. The large number of established and probable epigenetic active compounds found in food will challenge the understanding of how diet may influence epigenetic gene expression. Studies of dietary effects on epigenetic gene regulation are still in their infancy, but first results are shown (Thaler, Karlic et al. 2008). Food contains compounds influencing both, DNA methylation and histone modifications. However, the consumption of some of these compounds can vary by season, dietary habits, age and environment.
Dietary compounds like vitamin B_{12} or folic acid are implicated in the regulation of the cytosine methylation pathway (Stempak 2002; Choi, Friso et al. 2004; Kim 2004). Vitamin B_{12} plays a central role as it acts as co-factor of the methionine synthetase, remethylating homocysteine to methionine. Methionine is further activated to S-adenosylmethionine (SAM), the methyl donor for DNA methylation. SAM converts to S-adenosylhomocysteine (Lyko, Ramsahoye et al.) after DNA-methylation. Reversible hydrolysis of SAH to homocysteine completes the cycle (figure 2, adapted from Kim 2004 (Kim 2004)). Folate, cholin or betaine are potent methyl-donors directly implicated in the DNA-methylation pathway. However, under conditions of vitamin B_{12} depletion, cellular folate accumulates in the methylfolate form due to activity of methionine synthetase being blocked, thereby creating a conditional form of folate deficiency. Deficiency in vitamin B_{12} leads also to an accumulation of serum homocysteine. Under conditions of elevated homocysteine concentrations, levels of the potent SAM–inhibitor SAH represses DNA methylation. The role of folate in DNA-methylation seems to be rather complex. Evidence from animal, human, and in vitro studies suggest that folate-dependent DNA methylation is highly complex, gene and site specific(Cravo, Fidalgo et al. 1994; Rampersaud, Kauwell et al. 2000; Kim, Baik et al. 2001; Kim 2004). These studies have shown that the extent and direction of changes in SAM and SAH in cell lines in response to folate deficiency are cell specific and that genomic-site and gene-specific DNA demethylation are not affected by the changes in SAM and SAH induced by folate depletion. Transgenerational studies on a rat model could furthermore demonstrate that folate deficiency during pregnancy impacts on methyl metabolism, but does not effect global DNA methylation in rat foeti (Maloney, Hay et al. 2007). A study analyzing the expression of the antioxidative enzyme mitochondrial superoxide dismutase (MnSOD) showed that this gene is expressed differently in vegetarians and omnivores due to different DNA-methylation patterns in the MnSOD promoter region: vegetarians expressed a significant higher amount of MnSOD, showing a low promoter methylation for this gene. Inversely, a lower MnSOD expression by a higher MnSOD promoter methylation was seen in omnivores (Thaler, Karlic et al. 2008). Several factors which may reduce DNA–methylation machinery in vegetarians are discussed. Studies analyzing the B-vitamin status of vegans and vegetarians compared with omnivores, showed generally a higher dietary supply of folate and a lower dietary supply of vitamin B_{12} (since vitamin B_{12} intake comes predominantly from animal food products) and methionine for vegetarians and vegans (Millet, Guilland et al. 1989; Majchrzak, Singer et al. 2006). Correlations between plasma folate and vitamin B_{12} values were found and study findings showed that vegetarians and vegans show higher plasma homocysteine concentrations (Majchrzak, Singer et al. 2006). Furthermore, DNA hypomethylation due to high homocysteine levels has been reported in vitro and in

vivo (Jamaluddin, Chen et al. 2007; Jiang, Sun et al. 2007). Inhibition of DNA methyltransferase 1 activity by 30% and reduced binding of methyl CpG binding protein 2 have also been seen in this context. A further study (Geisel, Schorr et al. 2005) comparing vegetarians and omnivores observed a reverse correlation between SAH concentrations and DNA global methylation levels in blood. However, this study was unable to correlate homocysteine concentrations with the degree of DNA global methylation and found no correlation between the degree of CpG methylation of the promoter of the p66Shc gene (involved in oxidative stress) and homocysteine, or SAM or SAH levels.

Other diet derived factors such as diallyl sulfide (Mathers 2006), an organosulfur compound found in garlic, genisteine, the main flavanoid in soy (Dolinoy, Weidman et al. 2006), vitamin D_3 or all-transretinoic-

acids (Krawczyk and Fabianowska-Majewska 2006) have been shown to influence DNA methylation by altering histones and chromatin structure. On the histone level, much more food compounds show effects on epigenetic gene regulation. Food compounds acting as histone modifiers are generally weak enzyme ligands and thus are needed in high concentrations to generate a consistent effect and therefore might subtly regulate gene expression (Dashwood, Myzak et al. 2006). A number of dietary agents are discussed to have a role in epigenetics as HDAC inhibitors. In the literature well described agents in this context are butyrate, diallyl disulfide or sulforaphane. Butyrate is the smallest known HDAC inhibitor, and inhibits histone acetylation at high micromolar or low millimolar concentrations in vitro, levels nonetheless considered to be achievable in the gastro intestinal tract by bacterial metabolism (Dashwood, Myzak et al. 2006). Intriguing examples for a HDAC inhibitor are conjugated linoleic acids (Hinshelwood, Melki et al.), which have anticancerogenic and antiatherogenic properties.

Interestingly, CLAs decreases Bcl-2 and induces p21, a known HDAC target. Effects of CLAs on HDACs are discussed. Regarding the short chain fatty acids (SCFs) resulting from the microbial fermentation of dietary fibers, acetate, propionate and butyrate are taken up by the colonic mucosa. Butyrate is the preferred energy source for colonocytes and is transported across the epithelium. Butyrate, and to a lesser extent other SCFs, influence gene expression and inflammation primarily through its action as an inhibitor of histone deacetylases. Therefore, diet compounds, toxins or medications interfering with the colonic fermentation may also modulate potential epigenetic effects generated by the microbial flora. By now, there is much work to be done to clarify the influence on histone modifications of a great number of dietary factors, alone or in combination and their tissue specific characteristics. A list of discussed dietary HDAC activity modulators is shown in table 2.

Evidences show that a diet rich in vegetables and fruits might prevent some kind of cancers (Research 2007). In this context, the role of flavonoids is often discussed. Flavonoids can act either in a pro- or antioxidative manner depending on their structure and characters. Because of the antioxidative properties of some flavonoids, oxidative damage of DNA can be prevented and cancerogenesis altered. Guarrera et al postulated recently that flavonoids might influence the gene expression of DNA repair genes, which could be a possible explanation for the decrease in tumour development (Guarrera, Sacerdote et al. 2007).

In many animal models caloric restriction has been shown to prevent the development of cancer, Thereby the role of sirtuins becomes clearer. In higher eukaryotes, HDACs are grouped in four classes (IIV).

Class I of HDACs are found exclusively in the nucleus, whereas class II of HDACs shuttle between nucleus and cytoplasm. Resveratrol activates the HDAC sirtuin 1 (SIRT1) a HDAC belonging to class III HDACs. Because of their homology to yeast sir2, class III HDACs belong to the sir2 family (Davis and Ross 2007). SIRT is an abbreviation for silent mating type information regulation two and there are seven groups in humans (Davis and Ross 2007). Sirtuins are nicotineamide adenine dinucleotide (NAD(+)) dependent deacetylases which exhibit a well-defined regression during aging that is dramatically reverted in transformed cells. .

Another group of diet related epigenetic active compounds comprise short chain fatty acids (SCFs) resulting from the fermentation of dietary fibers. Acetate, propionate and butyrate are taken up by the colonic mucosa. Butyrate is the preferred energy source for colonocytes and is transported across the epithelium. Butyrate, and to a lesser extent other SCFs, influence gene expression and inflammation primarily through its action as an inhibitor of histone deacetylases. Therefore, diets, toxins or medications interfering with the colonic fermentation, possibly by an interaction with the composition

of the gastro- intestinal microbiota, may modulate SCFs and their epigenetic effects.

Nutrition and the immune system

Expression of Interleukin 8 and the IL23/IL17 pathway is pivotal in the development of chronic inflammation such as Crohn's disease (CD) or inflammatory Bowel disease.(Li, Moran et al. 2004; McGovern, Rotter et al. 2009)

IL-8, a member of the CXC chemokine family, is an important activator and chemoattractant for neutrophils is primarily regulated at the transcriptional level. IL-8 was shown to be affected by methylation of several CpG islands upstream from the promoter region as well as by histone modification. Compounds, taking part in DNA methylation pathways and histone acetylation such as folic acid, vitamin B12, genistein, zebularine and valproate seem to influence epigenetic modification and gene expression of the IL-8 gene in human CACO-2 colon cancer cells.

By contrast to leukemic blasts, where IL-8 is activated by differentiation(Delaunay, Lecomte et al. 2008), immune activated transcription of IL-8 gene may be silenced after differentiation of cells from solid tumors such as breast cancer (Chavey, Muhlbauer et al. 2008) through histone deacetylation by elements located outside of the immediate 5´flanking region.

The commensal microflora regulates the local expansion of CD4 T cells producing proinflammatory cytokines including IL-17 (Th17 cells) in the colonic lamina propria. (Niess, Leithauser et al. 2008) TH cells, which produce IL-17 and IL-17F, two highly homologous cytokines whose genes are located in the same chromosomal region have been recently identified to promote tissue inflammation. In these cells Histone H3 acetylation and Lysine 4 tri-methylation were specifically associated with IL-17 and IL-17F gene promoters. (Akimzhanov, Yang et al. 2007)

As natural food compounds and diets have been shown to modify the epigenetic regulation of these expression of these mediators (Kikuno, Shiina et al. 2008; Romier, Van De Walle et al. 2008) dietary strategies might be a possibility in modulating inflammatory diseases.

Nutrition and aging

Hereditary disposition, environmental elements, individual lifestyle and nutritional factors are known to interact with the aging process. These impacts on the aging-process can be divided in intrinsic and extrinsic factors. Intrinsic aging (cellular aging) mainly depends on individual hereditary background. Extrinsic aging is generated by external factors like smoking, excessive alcohol consumption and poor nutrition. During aging, epigenetic changes occur, for instance global DNA demethylation decrease contrasting to a CpG island hypermethylation.Progressive loss of global methylation during aging caused by passive demethylation is probably due to increasing DNMT1 enzyme inefficiency. Epigenetic changes can be seen as important determinants of cellular senescence and organism aging. Sirtuins take part in various cellular developments like chromatin remodelling, mitosis, life-span duration and are furthermore involved in the regulation of gene expression, insulin secretion and DNA repair. Since NAD is one of the critical molecules involved in many metabolic pathways, sirtuins activity is NAD(+) dependent and sirtuins control the activity of many other proteins involved in cell growth, it was suggested that sirtuins are involved in the elongation of life-span mediated by caloric restriction. Indeed, in mammals, caloric restriction decreases the incidence or delays the onset of age-associated diseases including cardiovascular diseases, cancer, and osteoporosis as well as neurodegenerative diseases. SIRT1 is known to be down regulated in senescent cells and during aging. Transcription factors like p53, p73, NF-KB, E2F1, p300 and others are also shown to be SIRT1 substrates, suggesting an oncogenetic potential for SIRT 1. In an ongoing process more and more sirtuin inhibitors are being discovered. Recent detections are sirtinol, cambinol, dihydrocoumarin and indole, coumarin and indoles being natural food compounds. Coumarin can be found in dates, dihydrocoumarin naturally in sweet clover and synthetically manufactured in foods and cosmetics. Indole is produced by intestinal bacteria as product of tryptophan degradation. Early findings about this novel class of HDACs are encouraging. Even more intensive research will be necessary to clarify connections between sirtuins, nutrition, aging and cancer genesis.

It has been suggested that sirtuins may be involved in the elongation of life-span mediated by caloric restriction transcription factors, such as p53, p73, NF-KB, E2F1, p300 and others are shown to be SIRT1

substrates. Currently sirtuin inhibitors from natural compounds are being discovered (Kwon, Owa et al. 1998; Akkermans, Kleerebezem et al. 2007). Intensive research will be necessary to clarify any connections between sirtuins, nutrition, aging and cancer genesis.

CONCLUSION

Epigenetic mechanisms contribute to the control of gene expression as a result of environmental signals due to their inherent malleability. Considerable evidence suggests that nutritional imbalance and metabolic disturbances during critical time windows of development, may have a persistent effect on the health of the concerned individulas and may even be transmitted to the next generation. For example, in addition to a "thrifty genotype" inheritance, individuals with obesity, type 2 diabetes, and metabolic syndrome with an increased risk of cardiovascular diseases may have suffered improper ”epigenetic programming" during their fetal/postnatal development due to maternal inadequate nutrition and metabolic disturbances and also during their lifetime, that could even be transmitted to the next generation(s), (Asim K Duttaroy, Evolution, Epigenetics, and Maternal Nutrition, Darwins Day Celebration, 2006).

The balance between genetic and epigenetic impacts in development of malignancy is therefore changing from childhood to later age. Whereas e.g. the majority of childhood tumors are associated with an inherited genetic or epigenetic (e.g., imprinted) burden, this balance shifts in favor of acquired epigenetic and genetic hits in tumors of adults and elderly (Haslberger, Varga et al. 2006) (Fig .3).

As predicted by Rupert Riedl working with the so-called “General Systems Theory” the evolution of complex adaptation requires a match between the functional relationships of the phenotypic characters and their genetic representation [60]. Regarding to Riedl “evolvability” results from such a match. If the epigenetic regulation of gene expression "imitates" the functional organization of the traits then the improvement by mutation and selection is facilitated. Riedl predicts that the evolution of the genetic representation of phenotypic characters tends to favor those representations which imitate the functional organization of the characters. Imitation means that complexes of functionally related characters shall be "coded" as developmentally integrated characters but coded independently of functionally distinct character complexes (Wagner and Laubichler 2004).

Epigenetic mechanisms thus appear as a machinery of recursive feedback which determines the average rate of change of characters at all levels from molecules to cells, organisms and their environment(Haslberger, Varga et al. 2006). A better description of role of the epigenetic machinery in the interaction between hereditary and environmental interactions will also add to our understanding of evolution and an improved, individual and preventive health care system.

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10.3 Abstracts for poster presentations

19th International Congress of Nutrition, Bangkok, Oct. 4-9 2009

REGULATION OF IL-8 AND IL-17 EXPRESSION BY BUTYRATE, FOLIC ACID, AND GENISTEIN INCLUDES DNA METHYLATIONAND HISTONE ACETYLATION

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IL-8 and IL-17 regulate interactions between immune cells and epithelia in the GI-tract, involving epigenetic mechanisms. Because of the epigenetic activities of the SCF (short chain fatty acid) butyrate, produced by intestinal microbiota, genistein and folic acid we analyzed effects on histone acetylation and DNA-methylation on interleukin-8 and -17 genes.

We analyzed gene expression by real time PCR, DNA-methylation by bisulfite sequencing PCR, and histone acetylation by Chromatin immunoprecipitation in the CACO-2 cell line model.

IL-8 and IL-17 expression was increased by genistein, butyrate, and folic acid. Folic acid stimulated the IL-8-DNA-methylation on the sites -1342 and -1412. Butyrate stimulated the histone acetylation on H3 lysine 9 at IL-8. Genistein and butyrate also stimulated apoptosis relevant caspase-3. Changes in butyrate producing GI-microbiota (*Clostridium* cluster IX and XIVa) were found due to nutrition.

As the levels of genistein, folic acid, and butyrate in the GI-tract can be influenced by nutrition, nutritional concepts may be feasible to interfere with IL-8 and IL-17 expression in inflammatory GI diseases.

19th International Congress of Nutrition, Bangkok, Oct. 4-9 2009

COMBINED HEDERITARY/GENETIC AND EPIGENETIC ASPECTS IN GENETIC TESTING OF COMPLEX DISEASES AND NUTRIGENETICS

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Genetic testing and preventive health care based on the use of Single Nucleotide Polymorphisms (SNPs) was criticized because of methodological errors and low penetrance. The potential need considering epigenetic aspects in the genetic analysis of complex diseases and nutritional disorders is being discussed. To evaluate epigenetic influences in genetic and nutrigenetic testing, interactions of SNPs and epigenetic DNA-methylation are analyzed.
Penetrance of selected SNPs with relevance to selected cancers are evaluated calculating odds ratios (OR) and p-values (p) in meta-analysis. In comparison, relevance of DNA-methylation is assessed.
Whereas penetrance of analyzed SNPs was usually found to be low, strong correlations of epigenetic DNA-methylation were found. For example, ORs of 1,5 ($p \approx 0.55$, after Bonferroni-correction) were found for the common A148T variant of the tumor-suppressor-gen p16 in colorectal cancer. In contrast, hypermethylation was found in about 30 % of sporadic colorectal cancer. Hypermethylation of tumor-suppressor-genes such as p16 is reversible by nutritional components such as Genistein in vitro.
These findings underline the importance of integrating hereditary genetic aspects in concepts for genetic and nutrigenetic analysis in public health care.

NuGOweek 2009, Montecatini Terme, Aug. 31-Sep. 3 2009

EPIGENETIC REGULATION OF IL-8 AND E-CADHERIN GENE EXPRESSION BY BUTYRATE, FOLIC ACID, AND GENISTEIN INCLUDES DNA-METHYLATION AND HISTONE ACETYLATION

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The expression of IL-8 and E-Cadherin regulates the interactions between immune cells and epithelia in the GI-tract involving epigenetic mechanisms. Because of the epigenetic activities of the SCF butyrate, produced by intestinal microbiota, as well as genistein and folic acid we analyzed effects on histone acetylation and DNA-methylation on the expression of IL-8 and E-Cadh.

Gene expression was analyzed by real time PCR, DNA-methylation by Methylation Specific PCR (MSP), bisulfite sequencing PCR (BSP) and histone acetylation by Chromatin immunoprecipitation in the CACO-2 cell line model.

IL-8 and E-Cadh expression was increased by genistein, butyrate, folic acid and the DNA methyltransferase inhibitor zebularine. Whereas zebularine decreased DNA methylation of E-Cadh and IL-8 (on the sites -1342 and -1412), butyrate enhanced the histone acetylation on H3 lysine 9 at

IL-8. Genistein and butyrate also stimulated the apoptose relevant caspase3. Changes in butyrate producing GI-microbiota (*Clostridium* cluster IX and XIVa) were found due to nutrition.

As the levels of genistein, folic acid, and butyrate in the GI-tract can be modulated by nutrition, nutritional concepts may be feasible to interfere with epigenetic mechanisms in inflammatory GI diseases.

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Poster
S. Englert, C. Berner, A.G. Haslberger : Combined hederitary/genetic and epigenetic aspects in genetic testing of compley diseases and nutrigenetics. International Congress of Nutrtion, Oct. 4-9 2009, Bangkok

Aumueller E., Berner C., Gnauck A., Klein P., Haslberger A.G.: Regulation of Il-8 and Il-17 expression by butyrate, folic acid, and genistein includes DNA methylation and histone acetylation. International Congress of Nutrtion, Oct. 4-9 2009, Bangkok
Aumueller E., Gnauck A., Berner C., Klein P., Haslberger A. G.: Epigenetic regulation of IL-8 and E-Cadherin gene expression by butyrate, folic acid, and genistein includes DNA methylation and histone acetylation. NuGOweek 2009, Aug. 31-Sep. 3, 2009, Montecatini Terme