Studies on DNA Methylation (Epigenetics), 2018

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The Research Group: Dr.rer.nat. Stefanie Weber has received her doctorate in 2013 from the University of Cologne for research performed in the Institute for Clinical and Molecular Virology, FAU Erlangen-Nürnberg. Since then, she has continued postdoctoral work in the same Institute on the role of DNA methylation in the genomes of viruses and mammalian cells.

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Principal Investigator: Walter Doerfler (W.D.) is Professor emeritus, University of Cologne and Guest Professor (2002- ) Institute for Clinical and Molecular Virology, FAU Erlangen-Nürnberg. After Medical School in Munich (LMU, MD 1959) and an internship at Mercer Hospital, Trenton, N.J., W.D. did postdoctoral work at the Max-Planck-Institute for Biochemistry in Munich (1961-1963) and the Department of Biochemistry at Stanford University Medical School (1963-1966). W.D. held faculty positions at Rockefeller University in New York City, N.Y., USA (Assistant-, Associate-, and Adjunct-Professor, 1966-1978) and at the Institute of Genetics in Cologne (Professor, 1972-2002). He was guest professor at Uppsala (1971/72, 2002, 2006, 2007, 2009) as well as at Stanford (1978, 1993), Princeton (1986, 1999), and Vanderbilt (2006) Universities. W.D. was the speaker of SFB 74 (1978-1988) and of SFB 274 (1988-2000) of the DFG. In 1994, members of SFB’s 274 and 288 in Cologne played an important role in the foundation of the Center for Molecular Medicine Cologne (CMMC). Since 2002, W.D. has continued basic research in molecular genetics as guest professor in the Institute for Clinical and Molecular Virology, University Erlangen-Nürnberg.

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Interests of the Research Group: The sequence-specific methylation of cytosine residues mainly, but not exclusively, of CpG dinucleotides in eukaryotic DNA is one of the powerful signals for the modification of genetic functions, also in mammalian DNA. Our laboratory has been one of the first (1979-1983) to document the importance of promoter methylation in long-term gene silencing. Most likely, this effect on promoter function is enacted by altering specific DNA-protein interactions in the promoter region. In the current report, the results of the following research projects will be described:

(i) We have identified a distinct DNA methylation boundary in the 5'-upstream region of the human FMR1 (fragile X mental retardation 1) gene promoter. This boundary region is capable of binding nuclear proteins whose nature we currently try to identify. Loss of the boundary and expansion of CpG methylation into the promoter region of the gene is associated with the fragile X syndrome (FXS), one of the most frequent causes of mental retardation in humans. In rare cases of the FXS, in so called high functioning males, the boundary is preserved, in the presence of a full expansion of the CGG repeat which is conventionally thought to be the major cause for FXS. Hence CGG expansion and promoter methylation are not necessarily linked, an observation which argues for the overall importance of promoter methylation in FXS causation. We also report on a loss of CpG methylation in the far upstream region of the promoter in cells which carry foreign DNA in the episomal (Epstein Barr Virus genomes) or integrated (telomerase gene) configuration. (See also section 3/iii).

(ii) DNA viral genomes integrated into their host genome often become extensively de novo methylated. However, HIV1 proviral DNA in the genomes of peripheral mononuclear blood cells (PMBC’s) in HIV1-infected individuals frequently remain unmethylated. Interestingly, we have identified one long-term non-progressor in whose PBMC’s, which were studied over an 11-year period, varying levels of proviral CpG methylation were observed. Apparently, de novo methylation of HIV1 proviral genomes might be subject to complex and multiple regulatory mechanisms which remain to be investigated.

(iii) We have adduced evidence in several biological systems (viral, human FMR1 promoter region) that alterations in cellular DNA methylation and transcription profiles in mammalian genomes in the wake of insertions of foreign DNA are a general phenomenon. These alterations might play a role in (viral) oncogenesis and are possibly instrumental during evolution as a consequence of multiple retroviral DNA insertions into ancient genomes. Over evolutionary times, these alterations of transcription profiles might have led to novel phenotypes.

(iv) We have also shown that the genome of African Swine Fever Virus does not become de novo methylated after the infection of monkey Vero cells. This study is part of a series of investigations on the methylation status of different DNA viruses in mammalian cells.
Lastly, we are currently determining to what extent the methylation profiles of human cells become altered upon the productive infection with human adenovirus type 12 (Ad12). The data adduced so far show very marked alterations of CpG methylation at numerous sites in the human genome starting as early as 12 h post infection. The Ad12 genomes remain unmethylated during the entire infection cycle.

Since July 2002, our laboratory has been located in the Institute for Clinical and Molecular Virology, FAU Erlangen Medical School and maintains close ties to the Institute of Genetics, University of Cologne.

Research Projects

1. Structure and possible function of the methylation boundary in the upstream region of the FMR1 promoter

![Figure 1 - The CpG methylation boundary 5'-upstream from the FMR1 promoter.](image)

DNA samples were extracted from (A) telomerase gene-transformed fibroblasts of a non-FXS male individual with the boundary located at CpG 75. (B) non-transformed PBMC’s from an FXS patient. These data document the methylation boundary and its loss in FXS individuals. In (A) and (B), the CpG dinucleotide positions were not depicted according to scale as in (C) but were compressed and immediately juxtaposed to each other. (C) Map of the 5'-upstream region of the FMR1 gene drawn to scale. The nucleotide numbers 9,500 to 15,500 refer to NC_000023: 146,786,201 to 146,840,303 Homo sapiens FMR1 region. The numbers 1 to 104 designate the CpG dinucleotides in the region - ○/□ unmethylated, ●■ methylated. Other symbols are as follows: Arrow – site of transcriptional initiation; blue - CGG repeat; yellow - CTCF binding sequences; green - FMR1 promoter; orange – origin of DNA replication. On this map, the boundary has been located to CpG pair 65. The limits of the range of CpG’s determined by bisulfite sequencing were demarcated by red numbering (20 to 104). This figure was taken from Naumann et al., 2014.
1.1 Safeguard against methylation spreading into the promoter area

The human genome segment upstream of the FMR1 (fragile X mental retardation 1) gene (Xq27.3) contains several genetic signals (see legend to Figure 1), among them a DNA methylation boundary which is located 65 to 75 CpG’s upstream of the CGG repeat. In fragile X syndrome (FXS), the boundary is lost, and the promoter is inactivated by methylation spreading. We have documented boundary stability in spite of critical expansions of the CGG trinucleotide repeat in male or female premutation, in female full mutation carriers and in infrequently found high functioning males (HFMs) (Figure 2D, E). HFMs carry a full CGG repeat expansion but exhibit an unmethylated promoter and lack the FXS phenotype. The boundary is also stable in Turner (45, X) females. A CTCF-binding site is located slightly upstream of the methylation boundary (Figure 1C) and carries a unique G to A polymorphism (SNP) which occurs 3.6 times more frequently in genomes with CGG expansions. In CGG expansions, the CTCF-site does not harbor additional mutations. A methylation boundary is also present in the human genome segment upstream of the huntingtin (HTT) promoter (4p16.3) and is stable both in normal and Huntington disease chromosomes. Hence, the vicinity of an expanded repeat does not per se compromise methylation boundaries which might have an important function as promoter safeguards.

1.2 Foreign DNA in human HCT116 cells alters the methylation profile far upstream of the methylation boundary in the FMR1 promoter region

In FXS individuals with an expanded CGG repeat, a signal akin to foreign DNA, and often in cells transgenomic for foreign DNA, like the episomal Epstein-Barr Virus (EBV) genome or the transgenomic telomerase gene, the large number of previously methylated CpG’s in the far upstream region of the boundary is about fourfold decreased (Naumann et al., 2014). This finding is an additional case in point for the alterations of DNA methylation profiles in trans upon the introduction of foreign DNA into mammalian cells (see section 3).

1.3 Proteins binding to the boundary region of the FMR1 upstream promoter region

We have now set out to isolate the proteins which can bind to the methylation boundary in the upstream region of the FMR1 promoter. In collaboration with Marcus Krüger’s laboratory, CECAD Research Center University of Cologne, quantitative high-resolution mass spectrometry will be employed to identify these proteins and their function.


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Figure 2 - The CpG methylation boundary in the 5’-upstream FMR1 region remains intact in borderline cases of CGG trinucleotide repeat expansions. DNA samples were analyzed by bisulfite sequencing and were derived from (A) un-transformed PBMC’s from a premutation male (n = 113 +/-1). (B) DNA from un-transformed PBMC’s of a premutation female (n = 109 +/-1). Between CpG’s 20 and 65, 45 % of the CpG’s were methylated in this female premutation genome. (C) DNA from un-transformed PBMC’s from a full mutation female (n = > 200) with 59 % of the CpG’s methylated; (D) DNA from EBV-transformed PBMC’s from a high functioning male (n = ~ 400); (E) DNA from telomerase gene-transformed fibroblasts from a high functioning male (n = ~ 330). (F) Map as described in the legend to Figure 1C. This figure was taken from Naumann et al., 2014.

2. Genetic and epigenetic studies on HIV-1 proviral genomes in HIV-1 infected individuals with a wide spectrum of infection modes

Unmethylated HIV-1 proviral genomes in HIV-1 infected individuals and an exception

Integrated DNA from various viruses often becomes methylated de novo and transcriptionally inactivated. We therefore investigated CpG methylation profiles in 55 of the 94 CpG’s (58.5%) in HIV-1 proviral genomes including ten CpG’s in each LTR and additional CpG’s in portions of the gag, env, nef, rev, and tat genes. We analyzed 33 DNA samples from PBMC’s of 23 subjects representing a broad spectrum of HIV-1 disease or infection. In 22 of 23 HIV-1-infected individuals, there were only unmethylated CpG’s regardless of infection status. In one long term non-progressor, however, methylation of proviral DNA varied between 0 and 75% over an 11-year period although the CD4+ counts of this individual remained stable. Hence levels of proviral DNA methylation can fluctuate. The preponderance of unmethylated CpG’s suggests that proviral methylation is probably not a major factor in regulating HIV-1 proviral activity in PBMC’s of infected individuals.

3. Destabilization of the human epigenome: consequences of foreign DNA insertions

Based on the study of human adenovirus type 12 (Ad12) as an oncogenic DNA virus, the fate of foreign DNA in mammalian systems and the epigenetic consequences of foreign DNA insertions have been a long-term interest in this laboratory. Foreign DNA which emanates from a panoply of sources is ubiquitous and abundant in our environment. Research about the fate of this very stable and biologically potent molecule in the environment is a medically highly relevant topic. How can DNA interact with and be taken up by living cells, how frequently is it integrated in the invaded cell’s genome, and what are the consequences of these interactions for cell survival and genetic integrity? In studies on the integrated state of Ad12 DNA in Ad12-transformed hamster cells, we discovered that the CpG methylation profiles in some of their endogenous retrotransposon sequences and in several cellular genes were increased. This augmented methylation persisted in revertants of the transformed cells that had lost all Ad12 genomes (“hit and run” mechanism) (Heller et al., 1995). Moreover, alterations of DNA methylation and transcription profiles were documented in Ad12 DNA- and in bacteriophage λ DNA-transgenomic cells.

- We have adduced evidence in several systems that epigenetic effects in mammalian genomes due to the insertion of foreign DNA are a general phenomenon. These alterations of methylation and transcription profiles might play a role in (viral) oncogenesis and are possibly instrumental during evolution as a consequence of multiple retroviral DNA insertions into ancient genomes.
- To examine the general significance of these observations, we designed a model system for proof of principle assessment. Human cells from cell line HCT116 were rendered transgenomic by transfecting a 5.6 kbp bacterial plasmid and selecting cell clones with foreign plasmids stably integrated, most likely at different genomic sites in different cell clones.
- In five non-transgenomic HCT116 control clones without the plasmid, transcription and methylation patterns proved similar, most frequently identical, among individual cell clones. This finding opened the possibility for comparisons of these patterns between non-transgenomic and transgenomic clones.
- In 4.7% of the 28,869 human gene segments analyzed, the transcriptional activities were upregulated (907 genes) or downregulated (436 genes) in plasmid-transgenomic cell clones in comparison to control clones (Figure 3A). A significant gene set enrichment was found in 43 canonical pathways. Frequent upregulations were noted in small nucleolar RNA genes that regulate RNA metabolism and in genes involved in signaling pathways.
- Genome-wide methylation profiling was performed for 361,983 CpG sites. In comparisons of methylation levels in five transgenomic versus four non-transgenomic cell clones, 3791 CpG’s were differentially methylated, 1504 CpG’s were hyper- and 2287 were hypo-methylated (Figure 3B).
- Thus, the epigenetic effects in the wake of foreign DNA integration events can be considered a general effect also in human cells. We still lack insights into the role of transgenome size, gene or
CG content or copy number. The mechanism(s) underlying the observed epigenetic alterations are unknown. Extent and location of alterations in genome activities and CpG methylation might depend on the site(s) of foreign DNA insertion.

- We note that genome manipulations in general have assumed a major role in molecular biology and medicine:
  - Epigenetic factors in (viral) oncogenesis;
  - Thoughts on epigenetics and evolution;
  - Experimental approaches using genome manipulations;
  - Transgenic and transgenomic cells and organisms in all biological systems;
  - Gene therapeutic regimens;
  - Induced embryonic stem cells;
  - Knock-out or knock-in experiments;
  - Applications of the CRISPR-Cas9 technology.

The consequences of cellular genome manipulations for epigenetic stability have so far received unwarrantedly limited attention. Before drawing far-reaching conclusions from work with cells or organisms with manipulated genomes, critical considerations for and careful analyses of their epigenomic stability will prove prudent. With previous and current research described here, we have barely scratched the surface of the problem but are now poised to ask more precise questions and will now pursue more far-reaching questions by using the Ad12 system as a versatile model organism and guide.

**Thoughts on epigenetics & evolution.** During evolution, both genome stability and flexibility must have been decisive factors which were in constant competition with each other. Since foreign DNA is readily inserted into established genomes, this competition continues to be relevant in today’s biology. The presence of ancient retroviral and retrotansposon elements in the human genome attests to the long evolutionary history of retroviral insertions into genomes at evolutionary times. The ancient, now degenerate retroviral genomes, continue to be transcribed with unknown function; active viral genomes are not produced. Each insertion in evolutionary times added novel genetic information. However, more importantly, each impact upon the insertion of foreign DNA into an ancestral genome had epigenetic consequences in that it altered methylation and transcription profiles and could have led to the generation of completely new cell types. Depending on the environmental conditions then prevalent, the novel cell types were either eliminated or had gained an evolutionary advantage, survived and contributed to the development of present day (human) genomes. Hence, the most significant contribution of the insertion of ancient foreign genomes would have been the generation of new (epi)-genetic profiles and the rise of new cell types.

As a corollary to these studies, we have investigated whether the alterations in transcriptional and methylation profiles had affected also repetitive genome elements like the HERV and LINE-1.2 sequences in the same transgenomic HCT116 cell clones which had exhibited epigenetic alterations in other parts of the human genome. Such differences were not found. Apparently, in
the cell clones selected for this investigation the HERV and LINE elements had not responded to foreign DNA insertions. In addition, our present study provided a survey of the CpG modifications in the human endogenous viral sequences HERV-K, HERV-W, HERV-E and in LINE-1.2 whose methylation levels ranged between 60 and 98%. At least some of these elements were transcribed into RNA as determined by reverse transcription and PCR. Obviously, there are enough unmethylated control sequences to facilitate transcription of at least some of the tested elements into RNA.


p-value) on the y-axis for the comparison of four non-transgenomic against five pC1-5.6 transgenomic cell clones of all 361,983 CpG’s interrogated. Hyper-methylated CpG’s in transgenomic cell clones were displayed in red and hypo-methylated CpG’s in blue (Δβ value ≥0.2, adjusted p-value < 0.05; n=3,791 CpG’s). This Figure and its legends were taken from Weber et al., 2015.

4. Intracellular African swine fever virus DNA remains un-methylated in infected Vero cells

African swine fever virus (ASFV) is an important animal pathogen in many countries. In basic research on numerous different viruses, profound analyses of the molecular genetics of the virus have proved of paramount importance to understand its biological and medical characteristics. In this report, we describe the analysis of the methylation status of ASFV DNA after the infection of monkey Vero cells in culture. By applying the bisulfite sequencing technique, the gold standard in work on DNA methylation, no evidence was found for the presence of methylated cytosine residues in the BA71V strain of the ASFV genome in Vero cells. A selection of genome sites spread across the entire genome was analyzed; 5-methylcytosine (5-mC) residues were not found. The graph (Figure 4) presents a map of the 170,101 base pair genome of the BA71V strain in which the bisulfite-analyzed genome segments and the viral genes therein have been indicated. The strings of open symbols (◊) lined up by brackets to the investigated genome sections symbolically depict unmethylated cytosine residues. These genome segments have remained unmethylated throughout virus infection. We have analyzed 7.25% of all the methylation-receptive CpG sites and 8.55% of the genes in the ASFV genome. Although unlikely, we cannot rule out the possibility that pockets of methylated sequences might exist somewhere in the genome.

![Graph showing the unmethylated status of ASFV DNA in Vero cells.](image)

**Figure 4.** African Swine Fever Virus (ASFV) DNA is not de novo methylated in monkey Vero cells. ASFV BA71V DNA in Vero cells, DNA methylation profiles at 2, 6, 14, 24 h p. i. in selected parts of the viral genome as determined by the bisulfite sequencing technique.

5. Global methylation changes in human genomic DNA early after adenovirus type 12 (Ad12) infection

Sequence-specific CpG methylation of eukaryotic promoters has been identified as an important epigenetic signal for the silencing of gene activities. Beyond promoter signaling, cellular methylation patterns may have additional unknown functions and determine cell type also via their impact on cellular gene regulation. The productive infection of cells with DNA viruses leads to the reprogramming of cellular transcriptional programs, efficient virus replication, and cell death. We are presently studying the consequences of Ad12 infection for the stability of cellular DNA methylation patterns in productively infected human cells. The Ad12 genomes remain unmethylated throughout the Ad12 productive infection cycle, as determined by bisulfite sequencing and restriction enzyme analyses. Within the first 12 h after the productive infection of A549 human cells with Ad12, the levels of global DNA methylation in the recipient cell genomes become significantly altered. Bisulfite sequencing of > 800,000 CpG dinucleotides in the cellular genomes of Ad12-infected human cells has revealed that many cellular CpG dinucleotides exhibit an increased, others a decreased profile of DNA methylation.


Collaborations

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Present and Recent Funding

1. Institute for Clinical and Molecular Virology, FAU Erlangen-Nürnberg, Medical School, 2002 -
3. NIH grant # 2 UOI AI035004-2, subcontract from WIHS – Erlangen University, 2013.
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Selected Publications 2007 – 2018


W. Doerfler. Discoveries in molecular genetics with the adenovirus 12 system: Integration of


**Dissertations**

Between **1967 and 2013**, Walter Doerfler has guided **81 doctoral students** at different times at Rockefeller University in New York City (1967-1976), at the Institute of Genetics in Köln (1972-2002), and at the Institute for Clinical and Molecular Virology (2002-2013).

**Selected Publications 1968 to 1999: Integration of Foreign DNA and DNA Methylation**


Cologne Spring Meeting 1981: DNA Methylation and Gene Activity – First International Meeting on DNA Methylation
Weissenburg Symposia (Weissenburg in Bayern, Germany)

Weissenburg Symposium 2001: Medicine and Molecular Biology

Second Weissenburg Symposium 2004: DNA Methylation, an Important Genetic Signal

Third Weissenburg Symposium 2007: Medicine at the Interface between Science and Ethics

Fourth Weissenburg Symposium 2011: Epigenetics and the Control of Genetic Activity.


Organizer and Speaker at the Annual Meeting of the American Association for the Advancement of Science (AAAS), 12-16 February, 2009 in Chicago, IL, USA: Epigenetics: Mechanisms and Impact on Biomedicine.

Organizer and Speaker at the Annual Meeting of the American Association for the Advancement of Science (AAAS), 18-22 February, 2010 in San Diego, CA, USA: Science and Divinity – Genetics and Ethics.

Co-organizer with Andrew Feinberg of Symposium on Epigenetics: Methylating the Mind. Annual Meeting of the American Society of Human Genetics (ASHG), 02-06 November 2010 in Washington, DC, USA.

Co-organizer 34th Ernst-Klenk Symposium in Molecular Medicine – Epigenetics: Basic principles and clinical applications. 04-06 October 2018, Cologne, Germany.

Datenschutzerklärung: hier