Chapter 3
Discoveries in Molecular Genetics with the Adenovirus 12 System: Integration of Viral DNA and Epigenetic Consequences

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Abstract Starting in the 1960s, the human adenovirus type 12 (Ad12) system has been used in my laboratory to investigate basic mechanisms in molecular biology and viral oncology. Ad12 replicates in human cells but undergoes a completely abortive cycle in Syrian hamster cells. Ad12 induces neuro-ectodermal tumors in newborn hamsters (*Mesocricetus auratus*). Each tumor cell or Ad12-transformed hamster cell carries multiple copies of integrated Ad12 DNA. Ad12 DNA usually integrates at one chromosomal site which is not specific since Ad12 DNA can integrate at many different locations in the hamster genome. Epigenetic research occupies a prominent role in tumor biology. We have been using the human Ad12 Syrian hamster cell system for the analysis of epigenetic alterations in Ad12-infected cells and in Ad12-induced hamster tumors. Virion or free intracellular Ad12 DNA remains unmethylated at CpG sites, whereas the integrated viral genomes become de novo methylated in specific patterns. Inverse correlations between promoter methylation and activity were described for the first time in this system and initiated active research in the field of DNA methylation and epigenetics. Today, promoter methylation has been recognized as an important factor in long-term genome silencing. We have also discovered that the insertion of foreign (Ad12, bacteriophage lambda, plasmid) DNA into mammalian genomes can lead to genome-wide alterations in methylation and transcription patterns in the recipient genomes. This concept has been verified recently in a pilot study with human cells which had been rendered transgenomic for a 5.6 kbp bacterial plasmid. Currently, we study epigenetic effects on cellular methylation and transcription patterns in Ad12-infected cells and in Ad12-induced hamster tumor cells. These epigenetic alterations are considered crucial elements in (viral) oncogenesis.

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3.1   Background on Interest in DNA Methylation and Epigenetic Effects

In the history of research on viruses and their interactions with host cells, unexpected observations have frequently drawn the investigator to the study of mechanisms far beyond the realms of virology. For several decades, molecular virology has thus succeeded in occupying a pioneering role in molecular genetics. In the late 1960s and the 1970s, my laboratory at Rockefeller University in New York City, NY, and later at the Institute of Genetics in Köln has been investigating the integration of human adenovirus type 12 (Ad12) DNA into the genome of hamster cells (Doerfler 1968, 1970; Groneberg et al. 1977; Sutter et al. 1978; Sutter and Doerfler 1980; Stabel et al. 1980; Hochstein et al. 2007). Ad12 had been shown to induce tumors at the site of virus inoculation into newborn hamsters (Trentin et al. 1962). This discovery had attracted my interest and led to detailed studies on the molecular biology of the DNA virus Ad12. In a 1970 publication in the Journal of Virology, I had raised the question of whether the integration of the Ad12 genome into the hamster genome was the decisive precondition (*conditio sine qua non*) for the oncogenic consequences of Ad12 infection (Doerfler 1970).

At the same time, we analyzed details of the structure and sequence of the adenovirus DNA molecule (Doerfler 1969; Sprengel et al. 1994), established the denaturation maps of Ad2 and Ad12 DNA (Doerfler and Kleinschmidt 1970; Doerfler et al. 1983), and demonstrated that the DNAs of Ad2 and Ad12 did not contain 5-methyl-deoxycytidine (5-mC) (Günthert et al. 1976; Wienhues and Doerfler 1985). One of these studies (Günthert et al. 1976) also demonstrated that the DNA of hamster cells transformed by Ad12 contains 3.11 and 3.14% 5-methylcytosine (cell lines HA12/7 and T637 cells, respectively), whereas the DNA from untransformed hamster cells (BHK21 cells) contains only 2.22% 5-methylcytosine. These results suggested early on that levels of DNA methylation in transformed or tumor cells were fundamentally altered (Günthert et al. 1976).

In the late 1970s and early 1980s, we used restriction enzyme analyses of cellular DNA with integrated Ad12 genomes in Ad12-transformed cells to elucidate the viral DNA integration patterns and to localize more precisely the site of foreign DNA insertion. In the course of this work, we recognized that the restriction endonuclease HpaII, which cuts unmethylated virion Ad12 DNA frequently to small fragments, did not cleave the integrated Ad12 DNA efficiently. In contrast,

the restriction endonuclease MspI, known to be methylation insensitive (Waalwijk and Flavell 1978), cut the integrated viral genome to small fragments (Sutter et al. 1978; Sutter and Doerfler 1980). HpaII and MspI are isoschizomers, i.e., restriction endonucleases which recognize the same 5′-CCGG-3′ sites, where HpaII is blocked by a 5-mC residue in the 3′-position, whereas MspI is refractory to this inhibition. These data documented that the integrated Ad12 genomes had become extensively de novo methylated. These thus roughly determined profiles of Ad12 DNA methylation were more precisely mapped by using the bisulfite sequencing technique in a later publication (Hochstein et al. 2007). We went on to show that there was an inverse correlation between the levels of methylation in the different regions (early versus late) of the integrated adenovirus genomes and their genetic activities (Sutter and Doerfler 1980; Vardimon et al. 1980). These data were the first to functionally relate genetic activity and promoter methylation in eukaryotic and viral genomes and became the basis for our interest in problems of DNA methylation which have been continuously pursued in different systems until today.

Here, I will refrain from describing all of our research on DNA methylation of the past 30 years, but instead refer the reader to recently published reviews on these topics (Doerfler 2011, 2012, 2016). Based on these earlier studies, we have continued to investigate the biological meaning of DNA methylation and the fifth nucleotide, 5-methyldeoxycytidine. Here are some of the key references to our work on DNA methylation: Sutter and Doerfler (1980), Vardimon et al. (1980), Doerfler (1983, 2011), Toth et al. (1989), Kochanek et al. (1990), Kochanek et al. (1993), Orend et al. (1995), Heller et al. (1995), Zeschnigk et al. (1997), Naumann et al. (2009), and Weber et al. (2015).

3.1.1 Introduction to the Adenovirus System

Epigenetic research has gradually occupied a prominent role also in virology. Ad12 offers the opportunity to study virus infections in a productive (human cells) and a completely abortive system (Syrian hamster cells). In addition, Ad12-induced hamster tumors allow epigenetic analyses in an efficient viral oncogenesis model. Ad12 tumorigenesis in hamsters was originally discovered by Trentin, Yabe, and Taylor in 1962. Ad12 induces undifferentiated neuro-ectodermal tumors in >90% of the inoculated and surviving newborn hamsters (*Mesocricetus aureatus*) within 3–6 weeks after inoculation (Hohlweg et al. 2003). A combination of important parameters, like high incidence and short latency between infection and tumorigenesis, coupled with Ad12 DNA integration in all tumor cells (Knoblauch et al. 1996; Hilger-Eversheim and Doerfler 1997) facilitates the molecular analysis of tumor induction by this DNA virus. My laboratory has been working on the molecular biology, genetics, and epigenetics of adenoviruses since 1966 (Doerfler 1968, 1969, 1970; Doerfler and Kleinschmidt 1970): molecular strategies of adenovirus types 2 (Ad2) and Ad12 (199 citations in PubMed); studies on foreign DNA integration (61 citations); and investigations on the role of DNA methylation in

epigenetic regulatory mechanisms in adenovirus infection and transformation (117 citations) as well as in human genetics (>70 citations). We were probably the first laboratory to initiate epigenetic investigations of an (adeno-) viral system (Günthert et al. 1976; Sutter and Doerfler 1980; Vardimon et al. 1980). A more detailed account of previous work has been summarized in Weber et al. (2016b) and will be briefly summarized in the following sections.

3.1.1.1   Ad12-Syrian Hamster Cells: The Abortive System

When viruses transcend their natural host range, which has been developing over evolutionary timescales, their impact on the noncanonical host can be catastrophic. Human Ad12 infecting hamster cells and leading to oncogenic transformation is a case in point and only one of several examples. The retrovirus HIV of originally simian origin or the coronavirus SARS of canine origin upon their adaptation to the human organism are examples of extraordinary medical importance. Hence the study of the interaction of human Ad12 with cells of the Syrian hamster has been considered of importance to understand this abortive interaction of oncogenic relevance at the molecular level.

One of the characteristics of the Ad12-hamster cell system is a strictly abortive infection cycle (review Hösel et al. 2003). The block of Ad12 replication lies before viral DNA replication (Doerfler 1969) and late gene transcription which cannot be detected (Ortin et al. 1976). Ad12 adsorption, cellular uptake, and transport of the viral DNA to the nucleus were less efficient in the nonpermissive hamster cells than in permissive human cells. However, many of the early functions of the Ad12 genome were expressed in BHK21 cells, though at a low level. In the downstream region of the major late promoter (MLP) of Ad12 DNA, a mitigator element of 33 nucleotide pairs in length was identified which contributed to the inactivity of the MLP in hamster cells and its markedly decreased activity in human cells (Zock and Doerfler 1990). The E1 functions of Ad2 or Ad5 were capable of partly complementing these Ad12 deficiencies in hamster cells in that Ad12 viral DNA replication and late gene transcription could proceed, e.g., in a BHK hamster cell line, BHK297-C131, which carried in an integrated form and constitutively expressed the E1 region of Ad5 DNA (Klimkait and Doerfler 1985). Nevertheless, the late Ad12 mRNAs, which were synthesized in this system and carried the authentic Ad12 nucleotide sequence, failed to be translated to structural viral proteins (Schiedner et al. 1994). Hence, infectious virions were not produced even in this partly complemented system. There appears to exist an additional translational block for late Ad12 mRNAs in hamster cells. We have further shown that the overexpression of the Ad12 preterminal protein (pTP) or of E1A genes facilitated the synthesis of full-length, authentic Ad12 DNA in Ad12-infected BHK21 hamster cells. Apparently, the Ad12 pTP had a hitherto unknown function in eliciting full cycles of Ad12 DNA replication even in nonpermissive BHK21 cells when sufficient levels of Ad12 pTP were produced (Hösel et al. 2001). The amounts of Ad12 DNA in the nuclei or cytoplasm of the complemented hamster

cells were about 2 orders of magnitude [2 h postinfection (p.i.)] and 4–5 orders of magnitude (48 h p. i.) lower than in permissive human cells. Cell line BHK21-hCAR is transgenic for and expresses the human coxsackie and adenovirus receptor (hCAR) gene. Nuclear uptake of Ad12 DNA in BHK21-hCAR cells was markedly increased compared to that in naïve BHK21 cells. Ad12 elicited a cytopathic effect in BHK21-hCAR cells but not in BHK21 cells. Quantitative PCR or [^{3}H]-thymidine labeling followed by zone velocity sedimentation however failed to detect Ad12 DNA replication in BHK21 or BHK21-hCAR cells. Newly assembled Ad12 virions could not be detected. Thus, the block in Ad12 DNA replication in hamster cells was not released by the hCAR-enhanced nuclear import of Ad12 DNA (Hochstein et al. 2008).

We pursue the possibility that the completely abortive infection cycle of Ad12 in hamster cells ensures the survival of Ad12-induced hamster tumor cells which all carry multiple copies of genomically integrated Ad12 DNA. In this way, the viral genomes are immortalized and expanded into a huge number of tumor cells. Moreover, the totally abortive cycle of Ad12 in Syrian hamster cells is one of the decisive preconditions for the ability of Ad12 to induce tumor cells and tumors in Syrian hamsters, since Ad12-infected cells are capable of surviving virus infection, in contrast to the Ad12-human cell system in which all infected cells are killed (Doerfler 1991).

3.1.2 Foreign DNA in the Environment

DNA, free or embedded in tissue remnants, abounds all over the living world. These DNA molecules might still reside in complexes with specific proteins and RNAs attached. Their stability is variable and dependent on numerous, unidentified environmental factors. DNA fragments with free termini and DNA protein complexes belong to the most stable and aggressive molecules in nature and represent versatile recombination partners which can target the genomes of recipient cells. The pathways of DNA from cellular remnants in the environment to the nucleus of successfully entered cells and their genomes are poorly understood. DNA carried by viruses is well equipped for entry into specialized cells. In an earlier study on the fate of adenovirus type 2 (Ad2) DNA added directly to a culture of actively replicating human KB cells, between 3 and 9% of this DNA became associated with the cells in a DNase-resistant form (Groneberg et al. 1975). At 24 hr after the addition of DNA to the culture medium, 70% of the cell-associated DNA was found in the nucleus, and this DNA had been endonucleolytically fragmented. Direct penetration of the cytoplasmic membrane, perhaps during cell division, appeared the most likely mechanism for DNA uptake in these experiments. I suspect that there are less haphazardly acting mechanisms via DNA-sensitive receptors on the cell's surface.

3.1.3 Biology of Ad12-Induced Hamster Tumors

Histological and Immuno-histochemical Properties (Hohlweg et al. 2003) The following is a quote from this article in which the histopathological analysis was performed by Reinhardt Büttner, now in Cologne.

> Independent of location and size, the histology of all Ad12-induced tumors revealed small, rounded, uniformly stained cells with a large hyperchromatic nucleus and granular chromatin, numerous mitotic cells, and Homer–Wright rosette structures characteristic of primitive neuro-ectodermal tumors (Fig. 3.1). Immuno-histochemical assays for the expression of tissue-specific markers identified vimentin, synaptophysin, and neuronal-specific enolase in all tumors. The presence of the latter two proteins was compatible with the neuronal origin of the Ad12-induced tumors, whereas vimentin was typical for their mesenchymal derivation. Tests for additional tissue markers proved marginally positive (S-100, glia fiber protein) or negative (Table 3.1).

Figure 3.1 and Table 3.1 were taken from Hohlweg et al. (2003).
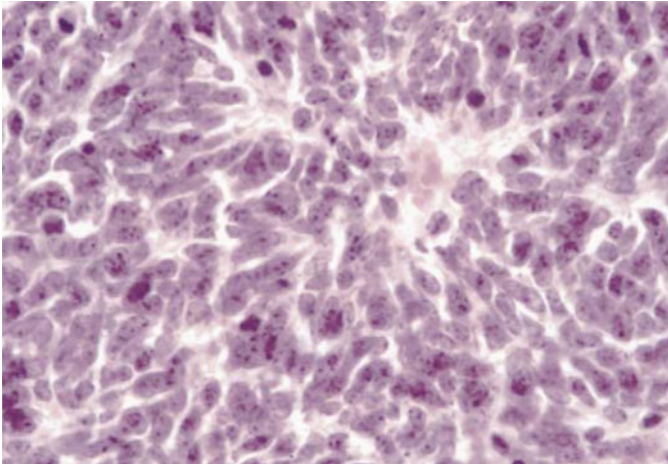
Fig. 3.1 Histological section of an Ad12-induced hamster tumor stained with hematoxylin and eosin

Table 3.1 Immuno-histological properties of Ad12-induced hamster tumors

Immuno-histological staining
Cytokeratin −
Vimentin +
Neuronal-specific enolase (NSE) +
Synaptophysin (+)
S-100a (+)
Gliafiber protein (+)
Ewing sarcoma marker: MIC2/CD99 −
Chromogranin A −

3.1.4 Characteristics of Chromosomally Integrated Adenovirus DNA

- Ad12-transformed cells or Ad12-induced hamster tumor cells carry up to >30 copies of viral genomes chromosomally integrated by covalent bonds between viral and cellular DNAs (Stabel et al. 1980; Hilger-Eversheim and Doerfler 1997; Knoblauch et al. 1996; Hohlweg et al. 2003; Hochstein et al. 2007).
- Although multiple copies of viral DNA are integrated, there is most frequently only one site of Ad12 DNA insertion on the chromosomes. In one study, 59/60 Ad12-induced tumors showed only one chromosomal site of Ad12 DNA integration as detected by fluorescent in situ hybridization (FISH) (Hilger-Eversheim and Doerfler 1997). The integratiion site was different in each tumor.
- In different Ad12-transformed cells or Ad12-induced tumor cells, viral DNA integration occurred at different sites in the cellular genome. There is no evidence for a specific cellular site of viral DNA insertion (Deuring et al. 1981b; Doerfler et al. 1983; Knoblauch et al. 1996; Hilger-Eversheim and Doerfler 1997).
- At the sites of recombination between the Ad12 and the cellular genomes, short or patchy nucleotide sequence homologies were frequently observed (Gahlmann et al. 1982; Stabel and Doerfler 1982). Moreover, the cellular pre-integration sites of viral DNA insertion often showed transcriptional activity and presumably an open chromatin structure (Schulz et al. 1987; Hochstein et al. 2007).
- Most of the integrated Ad12 genomes appeared to be intact, but fragmented genomes were also observed (Stabel et al. 1980; Hochstein et al. 2007). At the site of foreign DNA integration, cellular DNA sequences have been found to be completely conserved (Gahlmann and Doerfler 1983). In other instances, some of the abutting cellular DNA sequences were deleted.
- Recombination of Ad12 DNA with a cloned hamster cell DNA fragment, which had previously been identified as an integration site of Ad12 DNA, could be elicited also in a cell-free system by using high-salt nuclear extracts from hamster cells (Jessberger et al. 1989; Tatzelt et al. 1993).
- There is evidence that adenovirus DNA can recombine with cellular DNA also in human cells productively infected with adenoviruses (Burger and Doerfler 1974; Schick et al. 1976). An integrated state of viral DNA is difficult to prove since human cells productively infected with adenoviruses do not survive the infection. However, the discovery of a symmetric recombinant between Ad12 DNA and human cellular DNA has documented that Ad12 DNA does in fact recombine with cellular DNA even in productively infected cells (Deuring et al. 1981a; Deuring and Doerfler 1983).
- All of the aforementioned characteristics of Ad12 DNA integration in hamster cells have been confirmed in studies on the integration of replication-deficient adenovirus vector genomes in mouse cells (Stephen et al. 2010). In this system, adenoviral infection is compatible with cell survival due to the replication deficiency of the adenoviral vector genomes used. The results described in this system confirmed all our earlier findings in the Ad12 hamster cell system and in human cells productively infected with Ad12.

3.1.5   The Consequences of Inserting Foreign DNA into Established Mammalian Genomes

We have studied the consequences of foreign DNA insertions into the mammalian genome, namely (i) the de novo methylation of the transgenomes and (ii) alterations in the epigenetic stability of the recipient genomes. Several independently investigated mammalian systems with integrates of adenovirus DNA, bacteriophage lambda DNA, plasmid DNA, EBV DNA, or the telomerase gene as well as expansions of a CGG repeat were investigated (Heller et al. 1995; Remus et al. 1999; Müller et al. 2001; Naumann et al. 2014; Weber et al. 2015, 2016a).

3.1.5.1   Hypermethylation of Integrated Ad12 DNA, the Transgenome

As mentioned above, the virion genomes of Ad12 and Ad2 lack 5-methyldeoxycytidine nucleotides (Günthert et al. 1976). Free intracellular adenovirus DNA also remains unmethylated (Wienhues and Doerfler 1985; Kämmer and Doerfler 1995). In contrast, the integrated form of Ad12 DNA in Ad12-transformed hamster cells or in Ad12-induced hamster tumor cells becomes hyper-methylated in specific patterns (Sutter et al. 1978; Sutter and Doerfler 1980; Orend et al. 1995; Hochstein et al. 2007). De novo methylation appeared to be initiated at certain regional sites and extend from there by spreading (Toth et al. 1989; Orend et al. 1995).

3.1.5.2   Promoter CpG Methylation and Promoter Silencing

In 1979/1980, we documented an inverse correlation between adenoviral gene expression and CpG methylation of integrated adenovirus DNA in Ad12- and Ad2-transformed hamster cells (Sutter and Doerfler 1980; Vardimon et al. 1980; Doerfler 1983). These data were extended to activity studies of promoter-indicator gene constructs by using adenoviral promoters in order to document that pre-methylated promoters led to gene silencing, whereas unmethylated promoters allowed gene transcription (Vardimon et al. 1982; Kruczek and Doerfler 1983; Langner et al. 1984, 1986; Knebel and Doerfler 1986; Munnes et al. 1998). The silencing effect of promoter methylation could be partly or completely reversed by the expression of a viral trans-activator/ enhancer sequence, e.g., of the E1 proteins of Ad2 (Langner et al. 1986; Weisshaar et al. 1988), or by the close vicinity of a strong viral promoter/enhancer element, e.g., that of the human cytomegalovirus (Knebel-Mörsdorf et al. 1988). Today, it has been generally appreciated that many factors collaborate in regulating eukaryotic promoter function. Nevertheless, promoter methylation remains one of the dominant and experimentally convenient factors when studying the long-term silencing of gene activities.

3.1.5.3   Foreign DNA Integration into Mammalian Genomes Leads to Alterations in Methylation and Transcription Patterns

Genome-Wide Increases in DNA Methylation in Ad12-Transformed Cells—Stability of Changes Even After the Loss of All Viral Genomes: A "Hit-and-Run" Mechanism

The Ad12-transformed cell line T637 originated from BHK21 hamster cells following the infection of these cells with Ad12 and the selection of cells which expressed early Ad12 gene products (Strohl et al. 1970). In comparison to the levels of DNA methylation in the ~900 copies of the retrotransposon intracisternal A particle (IAP) genomes in the parent BHK21 cells, methylation of the IAP sequences in T637 cells, which are transgenomic for Ad12 DNA, was very markedly increased as detected by Southern blot hybridization (Heller et al. 1995). The extent of methylation augmentation in the T637 cell genome suggested that alterations in CpG methylation involved the entire cellular genome, since IAP sequences are distributed over many hamster chromosomes, frequently on their short arms (Heller et al. 1995; Meyer zu Altenschildesche et al. 1996). Methylation in other parts of the T637 genome—including single copy genes—was also enhanced (Heller et al. 1995). These hyper-methylation patterns persisted in TR3 cells, a revertant of T637 cells (Groneberg et al. 1978; Groneberg and Doerfler 1979) which had lost all Ad12 DNA sequences.[1] Hence, the effects of foreign DNA insertions on cellular CpG methylation patterns were not dependent on the continued presence of the originally causative insertion of foreign (Ad12) genomes. This mechanism has the characteristics of a "hit-and-run" event. Since we consider the genome-wide methylation effects of Ad12 integration as crucial to the transformation and its oncogenic consequences (Doerfler 1995, 2000, 2011, 2012), the much debated possibility of a "hit-and-run" mechanism in viral oncogenesis is again raised by these results and has to be considered highly relevant.

Alterations of Cellular DNA Methylation and Transcription Patterns Are Also Elicited in Bacteriophage Lambda or Bacterial Plasmid Transgenomic Cells

Alterations of CpG DNA methylation patterns were also observed in BHK21 cells transgenomic for bacteriophage lambda or bacterial plasmid DNA (Heller et al. 1995). Alterations of DNA methylation in the lambda DNA trans-genomic cells were documented in more detail by bisulfite sequencing of a subsegment of the IAP transposon DNA in these cells (Remus et al. 1999). Bisulfite sequencing (Frommer

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[1] By using the very sensitive PCR technique, which was not available in 1979, the revertant cell line TR3 of the Ad12-transformed hamster cell line T637 (Groneberg et al. 1978; Groneberg and Doerfler 1979) has recently been shown to be completely devoid of any Ad12 genome segments (S. Weber and W. Doerfler, unpublished studies).

et al. 1992; Clark et al. 1994) provides a positive display of all 5-mC residues in a DNA sequence. The inserted bacteriophage lambda DNA became also de novo methylated. There was no evidence that the increase in IAP DNA methylation might have preexisted in some of the non-transgenomic BHK21 cell clones (Remus et al. 1999).

A wide array of cellular DNA segments and genes was analyzed in hamster cells transgenomic for Ad12 or bacteriophage lambda DNA (Müller et al. 2001) for alterations in their transcriptional profiles as well by using the techniques of methylation-sensitive representational difference analysis (MS-RDA) (Ushijima et al. 1997) and suppressive subtractive hybridization. The data demonstrated that the insertion of foreign (Ad12 or bacteriophage lambda) DNA into an established mammalian genome can lead to extensive alterations also in cellular DNA transcription patterns (Müller et al. 2001).

Alterations of CpG Methylation Patterns Way Upstream of the FMR1 Boundary in Human Cells Immortalized by Epstein–Barr Virus (EBV) or by Transformation with the Telomerase Gene

We had previously investigated CpG methylation patterns in the human FMR1 segment (Genç et al. 2000; Gray et al. 2007) and in the human Prader–Willi region on chromosome 15q11-13 (Zeschnigk et al. 1997; Schumacher et al. 1998). The genome segment upstream of the FMR1 (fragile X mental retardation 1) gene on chromosome Xq27.3 contains several genetic signals (Naumann et al. 2009). Among them, we have described a DNA methylation boundary which is located 65–70 CpGs upstream of the CGG repeat in the gene's untranslated first exon and has been detected in any human (or mouse) cell type investigated (Naumann et al. 2009). In patients with the fragile X syndrome (FXS) (OMIM 300624), the methylation boundary is lost and, as a consequence, de novo methylation spreads downstream into the FMR1 promoter region and subsequently leads to promoter inactivation (Naumann et al. 2009). Loss of the FMR1 gene product is the cause for the FXS (for review O'Donnell and Warren 2002). This stable methylation boundary appears to help protect the promoter against the spreading of de novo methylation (Naumann et al. 2009, 2010). In cells transgenomic for EBV DNA or for the telomerase gene, the large number of normally methylated CpGs in the far-upstream region of the boundary is decreased about fourfold (Naumann et al. 2014). We have interpreted this marked decrease of DNA CpG methylation in a well-studied part of the human genome as a consequence of the introduction of foreign genomes (EBV or telomerase gene) into human cells (Naumann et al. 2014).

A Model System to Study the Epigenomic Destabilization in Human Cells
Transgenomic for a 5.6 kbp Bacterial Plasmid

We have recently described a model system to study the effects of foreign DNA
insertion into human cells in culture (Weber et al. 2015). Human cells from cell line
HCT116 were rendered transgenomic for a 5.6 kbp bacterial plasmid.
Transgenomic cell clones were selected with foreign plasmids stably integrated,
most likely at different genomic sites. In five non-transgenomic HCT116 control
clones, transcription and methylation patterns proved very similar among individ-
ual cell clones. These control data facilitated comparisons of these patterns between
non-transgenomic and transgenomic clones. In 4.7% of the 28.869 gene segments
analyzed, the transcriptional activities were upregulated (907 genes) or
downregulated (436 genes) (Weber et al. 2015) (Fig. 3.2a). Upregulations were
frequently found in small nucleolar RNA genes which 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 CpGs were differen-
tially methylated, 1504 CpGs were hyper-methylated, and 2287 were hypo-
methylated (Fig. 3.2b). These differential values, both for transcriptional activities
and methylation patterns, were statistically corrected for minute differences in
some of the 28.869 genome segments in non-transgenomic cell clones. The impor-
tance of transgenome size, CG or gene content, copy number, and the mechanism
(s) responsible for the observed epigenetic alterations have not yet been
investigated.

As a corollary to this earlier study (Weber et al. 2015), we have investigated
whether the alterations in transcriptional and methylation profiles had extended also
to repetitive genome elements like the HERV and LINE-1.2 sequences in the same
transgenomic HCT116 cell clones which had exhibited epigenetic alterations in the
above studied 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 (Weber et al 2016a). In
addition, this work provided a survey of the CpG modifications in the human
endogenous viral sequences HERV-K, HERV-W, and 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.

3.1.6 Résumé

Based on the study of human Ad12 as an oncogenic DNA virus, the fate of foreign
DNA in mammalian systems and the epigenetic consequences of foreign DNA
insertions in general have been a long-term interest in my laboratory (Doerfler et al.
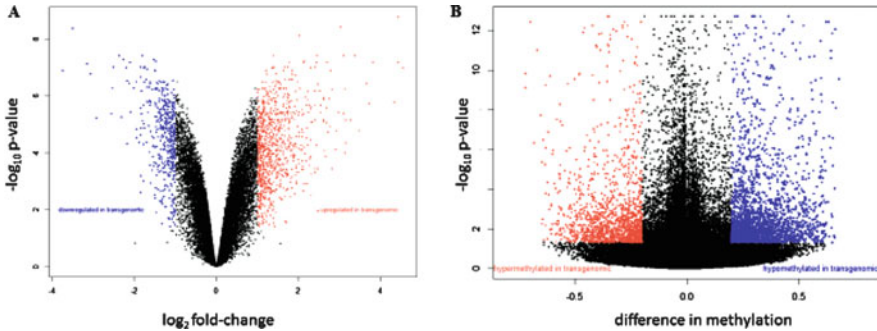
Fig. 3.2 Alterations in patterns of transcription (**a**) and methylation (**b**) in pC1-5.6 transgenomic HCT116 cell clones as compared to non-transgenomic cells. (**a**) Volcano plot displays non-standardized signals (log2 fold-change) on the *x*-axis against standardized signals ($-\log 10$ FDR-adjusted *p*-value) on the *y*-axis for the comparison of five non-transgenomic against seven transgenomic cell clones of all 28,869 genes analyzed. Upregulated genes in transgenomic cell clones were displayed in *red* and downregulated genes in *blue* (FC $\pm$ 2, adjusted *p*-values <0.05; n = 1343 genes). (**b**) Volcano plot displays differences in methylation on the *x*-axis against standardized methylation ($-\log 10$ FDR-adjusted *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 CpGs interrogated. Hyper-methylated CpGs in transgenomic cell clones were displayed in *red* and hypomethylated CpGs in blue ($\Delta\beta$ value ≥ 0.2, adjusted *p*-value <0.05; n = 3791 CpGs). This Figure and its legends were taken with permission from Weber et al. (2015)

1983; Weber et al. 2016b). 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 into the invaded cell's genome, and what are the consequences of these interactions for cell survival and genetic integrity—oncogenicity?

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). Moreover, alterations of DNA methylation and transcription profiles were documented in Ad12 DNA- and in bacteriophage λ DNA-transgenomic cells.

- I have previously hypothesized that epigenetic effects in mammalian genomes due to the insertion of foreign DNA are a general phenomenon (Doerfler 2012). 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 that were then selected for or against depending on environmental conditions during evolution (Doerfler 2016).

- 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 five non-transgenomic HCT116 control clones without the plasmid, transcription and methylation patterns proved similar, if not identical, among five 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 gene segments analyzed, the transcriptional activities were upregulated (907 genes) or downregulated (436 genes) in plasmid-transgenomic cell clones in comparison to control clones. 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 CpGs were differentially methylated, 1504 CpGs were hyper-methylated, and 2287 were hypo-methylated.
- Thus, the epigenetic effects in the wake of foreign DNA integration events can be considered a very significant effect also in human cells. We still lack insights into the role of transgenome size, gene or CG content, or copy number of the transgenome. 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.
- In the same cell clones studied as described above, differences in methylation and transcription profiles in some of the HERV and LINE-1.2 repetitive elements were not observed.
- We note that genome manipulations in general—work with transgenomic or knocked cells and organisms—have assumed a major role in molecular biology and medicine. 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. The Ad12 system has been a very reliable guide to this approach which has in due course been extended also to other types of foreign DNA molecules. We will now pursue more far-reaching questions and again use the Ad12 system as a versatile model organism and guide.

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