

Epigenetic Alterations in Epstein-Barr Virus-Associated Diseases

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Abstract

Latent Epstein-Barr virus genomes undergo epigenetic modifications which are dependent on the respective tissue type and cellular phenotype. These define distinct viral epigenotypes corresponding with latent viral gene expression profiles. Viral Latent Membrane Proteins 1 and 2A can induce cellular DNA methyltransferases, thereby influencing the methylation status of the viral and cellular genomes. Therefore, not only the viral genomes carry epigenetic modifications, but also the cellular genomes adopt major epigenetic alterations upon EBV infection. The distinct cellular epigenotypes of EBV-infected cells differ from the epigenotypes of their normal counterparts. In Burkitt lymphoma (BL), nasopharyngeal carcinoma (NPC) and EBV-associated gastric carcinoma (EBVaGC) significant changes in the host cell methylome with a strong tendency towards CpG island hypermethylation are observed. Hypermethylated genes unique for EBVaGC suggest the existence of an EBV-specific “epigenetic signature”. Contrary to the primary malignancies carrying latent EBV genomes, lymphoblastoid cells (LCs) established by EBV infection of peripheral B cells *in vitro* are characterized by a massive genome-wide demethylation and a significant decrease and redistribution of heterochromatic histone marks. Establishing complete epigenomes of the diverse EBV-associated malignancies shall clarify their similarities and differences and further clarify the contribution of EBV to the pathogenesis, especially for the epithelial malignancies, NPC and EBVaGC.

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Keywords

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3.1 Introduction

Epstein-Barr virus (EBV) is one of the eight currently known human pathogenic herpesviruses, together with herpes simplex virus (HSV) -1 and -2, varicella zoster virus (VZV), cytomegalovirus (CMV), human herpesvirus (HHV) -6, HHV-7, and HHV-8, also called Kaposi sarcoma-associated herpesvirus (KSHV). EBV was the first human tumor virus to be discovered, by electron microscopy in cultivated cells derived from Burkitt lymphoma (BL) biopsies (Epstein et al. 1964). Shortly after, seroepidemiology linked EBV with endemic BL (Henle and Henle 1966), the stunning ability of EBV to induce B cell growth (Henle et al. 1967; Moore et al. 1967) and to immortalize and growth-transform B cells (Pope et al. 1968) was recognized, and EBV was identified as the agent causing infectious mononucleosis (Henle et al. 1968). Localizing EBV to the malignant cells of anaplastic carcinomas of the nasopharynx (NPC), but not to the plenty of infiltrating lymphocytes, installed EBV as the first epitheliotropic human tumor virus (Wolf et al. 1973), a decade before high-risk papillomaviruses were identified in cervical carcinoma biopsies (Durst et al. 1983).

Meanwhile, EBV was also detected in between 30 and 40 % of classical Hodgkin lymphoma subtypes world-wide (Weiss et al. 1987, 1989), but close to 100 % of Hodgkin lymphoma in severely immunosuppressed hosts, and in a subset of gastric carcinomas (Burke et al. 1990; Shibata et al. 1991), encompassing approximately 8–10 % of this world-wide occurring sporadic tumor. Beyond BL, NPC and gastric carcinoma, EBV was also associated with T or natural killer (NK) cell lymphomas (Harabuchi et al. 1990), leiomyosarcoma (McClain et al. 1995), and post-transplant lymphoproliferative disease (Hamilton-Dutoit et al. 1993). All in all, EBV is probably co-responsible for about 200,000 new cases of

cancer per year corresponding to between 1 and 2 % of all human neoplastic disease (Munz 2014). Furthermore, ever since elevated anti-EBV antibody levels were found in patients with systemic lupus erythematosus (Evans et al. 1971) and multiple sclerosis (Sumaya et al. 1980), EBV has been incriminated as a trigger of autoimmune diseases (reviewed in Niller et al. 2009, 2011). Therefore, the discovery of EBV, more than 50 years ago, has enormously stimulated tumor virology and cancer research in general, and high hopes have been pinned to it that EBV might turn out as a Rosetta stone for understanding multistep carcinogenesis, and also for better understanding the role of viruses in immunopathological disorders (de The 1984, 1985).

Because after primary infection EBV genomes persist in host cell nuclei as circular chromatin-structured mini-chromosomes in a latent mode, EBV has served as a very prolific model system for the development of large scale sequencing technology (Baer et al. 1984), and for studying the cellular mechanisms of DNA replication (Yates et al. 1985), and transcriptional and epigenetic regulation of gene expression (Li and Minarovits 2003). Altogether, EBV is one of the most comprehensively, if not the best-studied of all viruses. Therefore, we dedicate a separate book chapter to the epigenetic alterations in EBV-associated tumors. EBV gene expression is highly dependent on epigenetic regulatory mechanisms. In diverse cell types carrying latent viral genomes, genetically identical viral genomes bear different epigenetic marks, thereby leading to specific patterns of viral latent gene expression and establishing distinct viral epigenotypes (Minarovits 2006). However, also the host cell genomes of EBV-infected cancer cells carry distinct epigenetic marks differing from each other in dependence of the respective tumor type and differing from the epigenetic profiles of their

healthy counterparts (Niller et al. 2009). Before we review the current state of epigenetic alterations in selected EBV-associated lymphoid and epithelial malignancies, we give a brief description of the biology and clinical aspects of EBV infection, the viral latency gene products with an emphasis on their epigenetic impact, the establishment of latency in memory B cells, and the different epigenotypes that EBV genomes may assume.

3.2 Physiological Course of EBV Infection

EBV is the only known human member of the subfamily *Gammaherpesvirinae* that belongs to the genus *Lymphocryptovirus*, the name reflecting the viral property of homing to and “hiding” in the B lymphocyte compartment. Worldwide, primary EBV infection usually occurs very early in life with no or little symptoms so that at the age of 10 years most people have turned seropositive. In Europe and the USA, primary infection of up to 50 % of the population occurs later in life, i.e. in adolescence or early adulthood. In this case, symptomatic infection may ensue in up to 50 % of the infected youths, with the more severe cases presenting as infectious mononucleosis, also named kissing disease, typically including pharyngitis, tonsillitis, fever, malaise, lymphadenopathy. The disease is often accompanied with splenomegaly, a minority also with hepatomegaly, and characteristically with more than 50 % of atypical mononuclear cells in the blood smear which can be mistaken for leukemia, and from which the name mononucleosis is derived. In rare and extreme cases of mononucleosis, splenic rupture or fulminant hepatitis with liver failure may occur (reviewed in Longnecker et al. 2013).

Contrary to VZV, the virus causing chickenpox, the infectivity of EBV is not high enough to make airborne infection the regular way of transmission. Thus, EBV is transmitted by intimate contact, usually through saliva, mostly from asymptomatic mothers which are occasionally shedding the virus due to intermittent lytic replication from their tonsillar epithelia and their

salivary glands, to their infants, or between adolescents. The virus is assumed to initially enter B cells at the surface of the tonsils or the oropharyngeal lymphoid tissue of Waldeyer’s ring, through engagement of its major envelope glycoprotein gp350/220, with the CD21 surface receptor which is also known as the CR2 receptor for the complement component C3d (Fingeroth et al. 1984). Tonsillar epithelial cells most likely serve as helpful enhancers for initial high-level virus production and occasional virus shedding later-on, as they can take up the virus through direct cell to cell contact and sustain an efficient lytic viral replication more easily than lymphoid cells (Borza and Hutt-Fletcher 2002; Hadinoto et al. 2009).

In the course of primary infection, the lytic replication cycle is triggered by the expression of two major viral immediate early genes BRLF1 and BZLF1 which in turn activate transcription of the viral early proteins whose expression leads to viral DNA replication, and the sequential expression of viral late genes which mainly code for virus structural building blocks. During an incubation time of approximately 4 weeks, the lytic replication cycle leads to a massive local virus production and shedding within the oropharyngeal lymphoid tissue, with the virus spreading systemically to the blood stream. Within lymphoid tissues, naïve, germinal center and memory B cells are infected. A high percentage of the circulating blood memory B cell pool is infected in acute mononucleosis which may, in extreme cases, affect up to 50 % of circulating memory B cells. Contrary to naïve B cells within the lymphoid tissue, naïve B cells in the blood stream remain largely uninfected (Hochberg et al. 2004). In the course of mononucleosis the immune system mounts an efficient defense through both its cellular and humoral arms. The vigorous immune response more so than the cytolytic viral damage leads to the well-known clinical symptoms of mononucleosis.

When primary infection is finally overcome, the virus remains latent in a minority of 1–10 in a million of peripheral B cells, where no viral gene expression occurs at all, or only a very restricted set of viral latency genes is occasionally

expressed upon cell division (Qu and Rowe 1992; Miyashita et al. 1997; Hochberg et al. 2004). After primary infection, the latent virus persists invisibly to the immune system in the healthy host, and the lytic replication cycle is restricted to lymphoid surface or salivary gland epithelia to which the immune system has only limited access (reviewed in Longnecker et al. 2013).

3.3 EBV Latency Types and Tumors

In EBV-associated tumor biopsies and EBV-infected cultured cell lines, the gene expression patterns of latent viral genomes depend largely on the host cell phenotype or stage of B cell development. Based on the activity status of the C promoter (Cp), we discern two major latency groups, **Cp-on and Cp-off latency**. When the major B lymphoid-specific promoter Cp is on, the full set of latency gene products, including all six EBNA (Epstein-Barr nuclear antigen) proteins, two latent membrane proteins, the multiply spliced BART transcripts, the non-translated EBER1 and -2 RNAs and three clusters of BHRF1 and BART microRNAs, are expressed. This expression pattern is commonly called **latency type III**. Except in BL cell lines where genetic alterations have caused permanent growth and which have undergone an *in vitro* switch to latency type III, cells in latency III have been immortalized by EBV, and are driven to permanent lymphoblastoid growth. When undergoing growth-transformation, the complete EBNA-transcription program is first directed by the W promoter (Wp) which becomes progressively methylated, before upstream Cp takes over the transcriptional control of the EBNA genes (Woisetschlaeger et al. 1990; Jansson et al. 1992; Tierney et al. 2000).

The astonishing ability and efficiency of EBV to immortalize and growth-transform B cells from the peripheral blood (Pope et al. 1968; Nilsson et al. 1971) *in vitro* has captured the interest of tumor virologists since its discovery and has also dominated EBV research and tumor virology until a decade ago. However, this ability

is not to be mistaken for bona fide oncogenesis, because the specific viral contribution to growth-transformation and BL-oncogenesis appear to be rather distinct (Niller et al. 2004b). In order to identify the impact of EBV-induced transformation on the methylome of primary human B cells, Hansen et al. (2014) performed whole genome bisulfite sequencing and gene expression analysis of uninfected quiescent B-cells, B-cells activated by CD40 Ligand (CD40L) plus interleukin 4 (IL-4) for 3 weeks, and EBV-infected B cells obtained from the same donors 3 and 6 weeks after the infection. Surprisingly, compared to normal B cells, large scale hypomethylated blocks comprising two thirds of the cellular genome were induced 6 weeks after EBV infection, but not by B cell activation or 3 weeks after infection. These hypomethylated blocks significantly overlap with the positions of lamina-associated domains (LADs) and large organized chromatin lysine (K9)-modified regions (LOCKs), previously described in lung and pulmonary fibroblasts (Guelen et al. 2008; Wen et al. 2009). Comparison with the data obtained from the ENCODE project on the tier 1 lymphoblastoid cell line (LCL) GM12878 suggested, that they may also be enriched in H3K27me3, while depleted of all transcription factors. Furthermore, these regions were associated with gene expression hypervariability, and largely corresponded to hypomethylated blocks previously described in human cancer (Hansen et al. 2011). These results suggest, that the formation of hypomethylated blocks may play a central role in the EBV induced transformation of B cells (Hansen et al. 2014). Accordingly, EBV-induced transformation led to a genome-wide decrease and redistribution of heterochromatic marks (Hernando et al. 2014). Thus, the physiologically activated B-cell appears to be highly different from the growth-transformed B-lymphoblast. EBV-converted LCs can only expand in the absence of an anti-EBV directed immune response, i.e. if taken into *in vitro* culture or if the immune system is defunct, e.g. in XLP (X chromosome-linked lymphoproliferation) patients or in severely immune suppressed patients who suffer early-onset post-transplant lymphoproliferative disease (PTLD).

LCLs which can be EBV-transformed *in vitro* from the white blood cells of healthy donors, are assumed to correspond to early onset PTLD cells. Therefore, early onset PTLD does not constitute a primarily malignant tumor, because it may be resolved, if immune control kicks back in early enough. However, it may turn monoclonal in the course and regularly become malignant, if growing too long out of immune control. In support of a molecular distinction between BL and PTLD tumors, the molecular signatures of viral and cellular protein and microRNA expression indicated that the respective contribution of EBV to both tumor types was different in each case (Navari et al. 2014).

A special latency type, called **latency IIb** has recently been highlighted by the secondary *in vitro* infection of chronic lymphocytic B cell leukemia (B-CLL) cells which exhibited EBNA2, but not LMP1 expression, which use Wp or both Wp and Cp, and which do not proliferate (Rasul et al. 2014). Secondarily EBV-infected B-CLL cells have also been found *in vivo* (Lewin et al. 1991). Latency IIb has also been observed in mononucleosis (Niedobitek et al. 1997; Kurth et al. 2000), in PTLD disorders (Oudejans et al. 1995; Brink et al. 1997), and in a unique case of a threefold mixed-latency type endemic BL with type IIb expression from a single chromosomally integrated viral genome (Kelly et al. 2006). Most likely latency IIb represents a transitional stage which may have got frozen on the path to full-blown growth-transformation exhibiting Cp-on latency III, through constraints imposed on viral gene expression by the cellular phenotype, e.g. secondarily infected CLL cells. Cells exhibiting latency IIb may normally get deleted by the healthy immune response, after primary infection is resolved (reviewed in Klein and Nagy 2010; Klein et al. 2013).

When Cp does not become switched on, expression of the key viral replication protein EBNA1 is governed by the Q promoter (Qp) instead of Cp, and the key immortalizing EBNA2 protein and the other four EBNA proteins are not expressed. Cells from EBV-infected germinal center B cells (**latency I or II**) or memory B cells (**latency 0**), and also from EBV-associated tumor

Table 3.1 Host cell-dependent expression of latent Epstein-Barr virus proteins. Key viral latency proteins or so-called oncoproteins are differentially expressed in EBV-associated malignancies and transformed cell lines. Depending on the specific viral gene expression patterns, specific viral latency classes are defined

Protein	Cell phenotype and latency type				
	Cp-off latency group		Cp-on latency		
	BL	HL	NPC	GC	LCL
	Type I	Type II	Type IIa	Type Ib	Type III
EBNA1	+	+	+	+	+
EBNA2	-	-	-	-	+
LMP1	-	+	+/-	-	+
LMP2A	-	+	+	+/-	+
BART mRNAs	+	+	+	+	+
BARF1	-	-	+	+	-
BART microRNAs	+	+	++	++	+
BHRF1 microRNAs	-	-	-	-	++

Abbreviations: *BL* Burkitt lymphoma, *HL* Hodgkin lymphoma, *NPC* nasopharyngeal carcinoma, *GC* gastric carcinoma, *LCL* lymphoblastoid cell line, probably corresponding to early-onset PTLD (post-transplant lymphoproliferative disease), *EBNA* Epstein-Barr virus nuclear antigen, *LMP* latent membrane protein, *BART* BamHI A region transcripts, *BARF1* BamHI A rightward frame 1, *BHRF1* BamHI H right frame

biopsies, e.g. Burkitt lymphoma (latency I), Hodgkin lymphoma (latency II), NPC (latency II, +/- LMP1, +BARF1 expression) and gastric carcinoma (latency I, +/- LMP2A, +BARF1, +BARF0 expression), exhibit **Cp-off expression patterns** (Table 3.1). In addition, the two epithelial cancers highly express the BART, but not the BHRF1 microRNAs (Kim et al. 2007; Zhu et al. 2009; Cancer Genome Atlas Research Network 2014). Cp-off tumors constitute primarily malignant lymphomas or cancers which do not require a grossly defunct immune system in order to develop, but may arise in the face of a more or less functional or, in the case of BL or Hodgkin lymphoma, an even hyperstimulated, yet possibly imbalanced immune system, while EBV-associated epithelial cancers can, in addition, be viewed as molecular accidents by which the virus entered and succeeded to persist in a non-physiological target cell.

The fundamental difference between Cp-off and Cp-on latency types is further underscored by the fact that both NPC and EBVaGC are not characterized by a massive genome demethylation, as EBV-immortalized cells are, but they belong to the group of CIMP cancers, i.e. they exhibit a CpG Island Methylator Phenotype. The importance of immune hyperstimulation for explaining the pathogenesis of endemic BL has, to our knowledge for the first time, been brought to attention and discussed by Bornkamm, Klein, and Lenoir in 1987 (Klein 1987; Lenoir and Bornkamm 1987). The possibility of a complex temporary composite between immune suppression and hyperstimulation in the same patient, i.e. in patients with HIV, solid organ transplant, parasitic infestation or holoendemic malaria, and the occasional confusion which may arise from this complex overlay has been clarified (Niller et al. 2004b). Recently, the discussion has been revisited under the aspect of novel epidemiological data, i.e. analysing BL incidence in equatorial Africa with a combination of both holoendemic malaria and HIV (Rickinson 2014). The disproportionate immune hyperstimulation may be provided by antigens of *Plasmodium falciparum* or through other parasitic infections (Araujo et al. 1996, 1999; Torgbor et al. 2014), together with hotspots of arboviral outbreaks, or by early-phase HIV-infection in the case of HIV-associated BL (Kalter et al. 1985; van den Bosch and Lloyd 2000; van den Bosch 2004). An immune response skewed from Th1 towards Th2 helper cells may further contribute to increase BL risk (Lubega 2007; van den Bosch 2012).

3.4 EBV Latency Gene Products

For a more extensive description of viral latency gene products, we refer to recent reviews (Niller et al. 2011; Tsao et al. 2015) and a textbook chapter (Longnecker et al. 2013), and for a quick overview to Table 3.1. In short, the **EBER** genes code for two small abundant nuclear RNAs. As a matter of routine, they serve histo-pathologists to identify EBV-infected cells by in situ hybridization. EBERs complex with the La autoantigen

of systemic lupus and with the ribosomal protein L22, and bind dsRNA dependent protein kinase R, thereby blocking the pro-apoptotic interferon α dependent signaling pathway (Clarke et al. 1991). The EBERs exert tumorigenic and anti-apoptotic functions in addition to blocking dsRNA-PKR (Ruf et al. 2000). A significant amount of La-complexed EBERs can be secreted by infected cells which leads to TLR3 (Toll-like receptor) signaling in neighbouring cells. EBER-induced innate immune responses may be associated with EBV-induced autoimmune pathology (Iwakiri et al. 2009). Interestingly, although the EBERs are non-translated RNAs, both contain at least one hypothetical full length open reading frame, the significance of which has so far not been experimentally addressed (Niller et al. 2004c).

EBNA1 is the only viral transcription and replication protein required for oriP replication and nuclear maintenance through the nuclear matrix attachment region (Sugden and Warren 1989; Jankelevich et al. 1992; White et al. 2001) encompassing oriP. Within the viral genome, EBNA1 binds oriP and the latency promoter Q_p, and EBNA1-binding sites within the cellular genome have been described as well (O'Neil et al. 2008; Canaan et al. 2009; Dresang et al. 2009; d'Herouel et al. 2010; Lu et al. 2010, 2011). EBNA1-bound oriP also acts as a long-distance enhancer within the viral genome for the mutually exclusive control of the viral latency promoters Cp and Q_p and for additional viral promoters (Gahn and Sugden 1995; Zetterberg et al. 2004). EBV-specific epigenetic alterations may be due to viral gene products interacting with cis-acting regulatory elements and with cellular epigenetic regulators (Table 3.2). As oriP and Q_p are constitutively unmethylated, EBNA1 keeps its binding sites in the viral genome free of CpG methylation (Lin et al. 2000; Salamon et al. 2000, 2001). EBNA1 may even serve as a Bookmarking protein which keeps cellular genes marked for activation throughout the cell cycle (Niller and Minarovits 2012). In gastric carcinoma cell lines, EBNA1 by itself kept the bidirectional promoter for the tumor suppressor gastrokine genes GKN1 and GKN2 methylation-

Table 3.2 Epigenetic modifiers encoded by EBV latent genomes and their potential actions

Latency product	Epigenetic mechanism	Putative outcome
EBNA1	Binding to EBNA1 binding sites; bookmarking, may act as pioneer factor	Hypomethylation, gene activation
EBNA2	Histone acetylation	Promoter activation
EBNA3A (EBNA3)	PRC2 recruitment	Promoter repression
EBNA3C (EBNA6)	PRC2 recruitment HDAC1/2 recruitment	Promoter repression
EBNA-LP (EBNA5)	HDAC4 displacement	Coactivation
LMP1	DNMT1,-3A, -3B upregulation	Promoter silencing
	Bmi-1 (PRC1-part) upregulation	Promoter silencing/activation
	KDM6B upregulation	Promoter activation via H3K27me3 demethylation
	miR-155 upregulation	KDM3A downregulation Promoter silencing (via H3K9me2)
LMP2A	DNMT1,-3B upregulation	Promoter silencing
	miR-155 upregulation	KDM3A downregulation Promoter silencing (via H3K9me2)

free (Lu et al. 2014). Several transcriptional activators have been shown to cooperate with EBNA1 in activating cellular genes. In EBNA1 expressing B cell lymphoma cells, EBNA1 and Sp1 coactivated the antiapoptotic survivin promoter (Lu et al. 2011). The ability of EBNA1 to induce transcriptional activation may also be connected to its binding to Brd4, a bromodomain protein which preferentially interacts with acetylated chromatin (Lin et al. 2008). Further, nucleosome assembly protein NAP1 supports EBNA1 replicative and transcriptional functions (Wang and Frappier 2009). EBNA1 may play a larger chromatin modifying role than previously thought. It attracts a histone deubiquitylating complex to oriP (Sarkari et al. 2009), and interacts with multiple nuclear proteins, among them high mobility group (HMG) B2 (Jourdan et al. 2012), and HMGA proteins (Coppotelli et al. 2013).

EBNA2 is considered the main transforming viral protein (Hammerschmidt and Sugden 1989) which acts as major transactivator of promoters via indirect binding to RBP-Jκ/CBF1-recognition sequences involved in immortalizing and growth-transforming B cells *in vitro* (Ling et al. 1993). Besides CBF1, additional cellular transcription factors binding to sites for PU.1, AUF1, and CRE tether EBNA2 to its transcriptional targets

(Johannsen et al. 1995; Fuentes-Panana et al. 2000). Specific targets of EBNA2 are viral promoters Cp, LMP2Ap and LMP1p, and promoters for B cell activation markers which push the resting B cell towards entering the cell cycle (reviewed in Gyory and Minarovits 2005). Although EBNA2 activity overlaps with Notch signaling, it governs its own extensive cellular transcriptional program and epigenetic reprogramming via recruitment of histone acetyl transferases (HAT) p300, CBP and PCAF to drive B cell proliferation (Wang et al. 2000; Zhao et al. 2011b). Further, EBNA2 interacts with human SNF-SWI-homolog chromatin modifier proteins in order to open up chromatin conformation (Wu et al. 1996).

EBNA-LP (EBNA5 in the Stockholm nomenclature) is a protein containing the repetitive units of the internal viral W-repeat. During the course of growth-transformation, it is the first viral latency protein to be expressed and it greatly enhances transformation efficiency, as it serves as a transcriptional coactivator for EBNA2 (Mannick et al. 1991). Because the W-repeat is frequently recombined during lytic viral replication, EBNA-LP exhibits a considerable size variation (Finke et al. 1987). EBNA2 coactivation through EBNA-LP is mediated by displacing the PML-nuclear body protein Sp100 from PML-NBs (Ling

et al. 2005) and by displacing HDAC4 and HDAC5 from EBNA2-bound promoters (Portal et al. 2006).

Among viral latency proteins, the three members of the **EBNA3** family, EBNA3A (EBNA3), EBNA3B (EBNA4), and EBNA3C (EBNA6) dominate the cytotoxic immune response against EBV (reviewed in Taylor et al. 2015). The three genes are part of the multiply spliced EBNA transcription unit, are arranged in tandem and show distant homology to each other. EBNA3C is the best-studied of the three proteins. While EBNA3A and -3C are required for EBV-mediated growth-transformation of primary B cells (Tomkinson et al. 1993), EBNA3B is not (Tomkinson and Kieff 1992). By competing with EBNA2 for promoter-bound CBF1 they modulate the transcriptional effects of EBNA2 (Johannsen et al. 1996). Like EBNA2, EBNA3C interacts also with the cellular histone acetyl transferase (HAT) p300, thereby downmodulating the EBNA2 effect (Cotter and Robertson 2000). Furthermore, EBNA3C forms a complex with HDACs 1 and 2, prothymosin α , and the corepressors mSin3A and NCoR to down-regulate transcription (Knight et al. 2003). EBNA3C-regulated genes overlap significantly with EBNA2-, EBNA3A-, and EBNA3B-regulated genes (White et al. 2010; Zhao et al. 2011a; McClellan et al. 2013). The requirement for continuous EBNA3A and -3C expression for transformed B cell growth was explained with their binding of the transcriptional co-repressor CtBP, via which cell cycle checkpoint control proteins p16INK4A, p14ARF are epigenetically down-regulated through the deposition of inhibitory histone modifications on their promoters (Skalska et al. 2010; Maruo et al. 2011). Recruitment of PRC2 and deposition of the repressive histone mark H3K27me3 on the promoter of pro-apoptotic Bcl-2 family member Bim is dependent on EBNA3A and -3C, too (Paschos et al. 2012).

The latent membrane protein **LMP1** is an integral membrane protein containing six transmembrane domains which can mimic a constitutively active TNF receptor and, like EBNA2, is essential for the growth-transformation of primary B cells. Its expression is governed by its

terminal repeat promoter L1-TRp, while the bidirectional promoter LMP1p for LMP1 and LMP2B, is mainly active in growth-transformed B cells. In the germinal center, LMP1 signaling may replace and overlap with the CD40 signal transduction pathway (Uchida et al. 1999). Therefore, LMP1 may contribute to apoptosis resistance during the physiologically vulnerable and apoptosis-prone phase of the GC reaction, in case the contact between the B cell and the T helper cell is lost. However, LMP1 is not only expressed on EBV-transformed B cells and Hodgkin lymphoma (HL) cells, but variably also on the surface of NPC cells. In NPC cells, LMP1 induces the level of DNMTs 1, -3A and -3B leading to the epigenetic repression of several tumor suppressor genes (Tsai et al. 2002, 2006; Seo et al. 2008). In GC cells, however, and in GC-derived EBV-positive HL cells, only DNMT3A was upregulated by LMP1, while the other two DNMTs were downregulated (Leonard et al. 2011). In Hodgkin lymphoma cell lines, LMP1 induces NF- κ B signaling which has broad effects on the transcriptional expression profile of the HL cell. One of the induced genes is the PRC1 component Bmi-1 which contributes to the survival of HL cells through its influence on transcriptional regulation of many genes (Dutton et al. 2007). Independently of NF- κ B, in GC and HL cells LMP1 induces KDM6B (JMJD3), an eraser enzyme removing the methyl groups of H3K27me3 repressive marks (Anderton et al. 2011). LMP1 induces several microRNAs, miR-155 and miR-146a (Motsch et al. 2007), miR-29b (Anastasiadou et al. 2010), and miR-10b (Li et al. 2010), thereby leading to the downregulation of several cellular genes or promoting metastasis. In NPC cells, LMP1 cooperates with LMP2A in upregulating miR-155 which on its part down-regulates KDM3A (JMJD1A) expression, a histone demethylase for removing the repressive H3K9me2 mark (Du et al. 2011). In epithelial cells, LMP1 induces epithelial-mesenchymal transition (EMT), and LMP1 expression is associated with metastasis of clinical NPC tumors (Horikawa et al. 2007).

The other latent membrane protein **LMP2A** containing 12 transmembrane domains mimics a

constitutively active B cell receptor (Merchant et al. 2000; Mancao and Hammerschmidt 2007), and it is not as essential for the growth-transformation of primary B cells as LMP1 or EBNA2. Its expression is governed by the LMP2Ap and, in the case of the shorter splice variant in LCs, LMP2B, by the bidirectional promoter LMP1p for both LMP1 and LMP2B. The LMP2A mRNA can only be transcribed after an initial circularization of the viral genome upon infection (Laux et al. 1988). In the GC reaction, LMP2A showing strong homology to the cellular B cell receptor may replace, overlap and interfere with the B cell receptor signal transduction pathway. Therefore, LMP2A may contribute to apoptosis resistance during the GC reaction, in case the contact between the B cell and the antigen presenting cell is lost. In EBV-associated gastric carcinoma cells (EBVaGC) which do not express LMP1, LMP2A may induce DNMT1 or DNMT3B via STAT3 phosphorylation eventually leading to the widespread methylation of tumor suppressor genes, like PTEN (Hino et al. 2009) and a multitude of additional genes (Zhao et al. 2013b). In epithelial cells, LMP2A has transforming potential (Scholle et al. 2000; Fukuda and Longnecker 2007) and induces EMT (Kong et al. 2010).

The multiply spliced **BART RNAs**, also called CST (complementary strand transcripts), are transcribed in all viral latency forms, but at particularly high levels in NPC cells and also in EBVaGC (Smith 2001; Cancer Genome Atlas Research Network 2014). The BARTs contain reading frames potentially coding for proteins named BARF0, RK-BARF0, A73, and RPMS1 and introns encoding a set of 40 **BART microRNAs** in two clusters (Pfeffer et al. 2004). The reading frames have been expressed *in vitro* and their products analyzed for interactions and function. The proteins may modulate EBNA2 transcriptional regulation and modify Notch signaling pathways, if the proteins are actually expressed *in vivo* (Smith et al. 2000; van Beek et al. 2003; Thornburg et al. 2004). The BART microRNAs are preferentially expressed in epithelial cells (Cai et al. 2006) and in NPC and EBVaGC cell lines and tumor tissues (Kim et al. 2007; Zhu

et al. 2009; Cancer Genome Atlas Research Network 2014). Many viral and cellular target genes have been identified in microRNA targetome studies (Dolken et al. 2010; Riley et al. 2012; Skalsky et al. 2012). Distinct BART microRNAs modulate viral LMP1 and LMP2A expression (Lo et al. 2007; Lung et al. 2009), expression of viral lytic genes BALF5, BRLF1, and BZLF1 (Barth et al. 2008; Jung et al. 2014), downregulate the expression of cellular pro-apoptotic genes PUMA, Bim and BID (Choy et al. 2008; Marquitz et al. 2011), genes involved in immune recognition (Nachmani et al. 2009; Dolken et al. 2010), tumor suppressor genes DICE1 and CDH1 (Lei et al. 2013; Hsu et al. 2014), and the epithelial cell-specific suppressor of metastasis NDRG1 in NPC cells (Kanda et al. 2015).

BARF1, a transforming gene product from the viral BamHI A region which was previously counted among the lytic genes, is expressed from latent viral genomes in the EBV-associated epithelial cancers, NPC and EBVaGC (Decaussin et al. 2000; zur Hausen et al. 2000; Seto et al. 2005). BARF1 shows homology with the human colony stimulating factor 1 receptor (CSF1R), the gene product of the *c-Fms* proto-oncogene (Strockbine et al. 1998) and appears to modulate the host immune response to EBV infection (reviewed in Takada 2012).

The **BHRF1** cluster of four microRNAs is mostly expressed in latency type III, but not in epithelial cells (Pfeffer et al. 2004; Cai et al. 2006; Kim et al. 2007). During the early phase of B cell infection, BHRF1 microRNAs may induce apoptosis resistance and cell proliferation (Seto et al. 2010). In primary lymphomas exhibiting latency type III, miR-BHRF1-3 may contribute to the downregulation of the T cell attracting chemokine CXCL11 (Xia et al. 2008).

3.5 Establishing EBV Latency in Memory B Cells

Ground-breaking work has established the blood memory B cell as the site of permanent EBV latency *in vivo* (Miyashita et al. 1997; Babcock

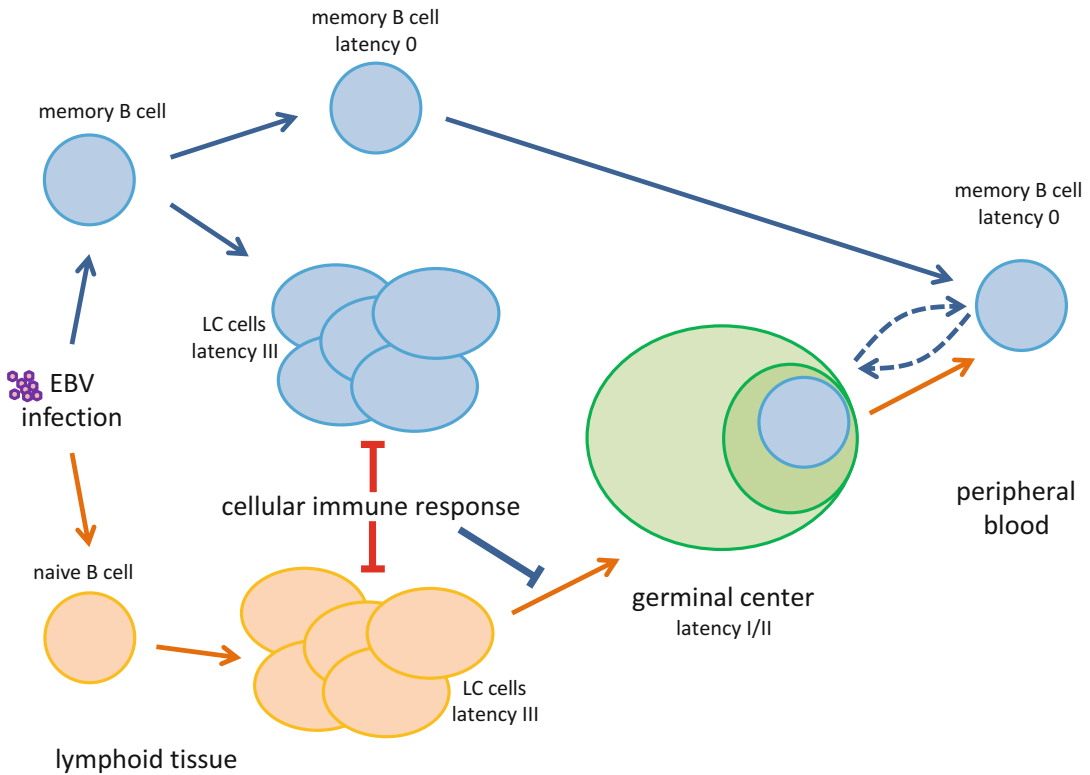


Fig. 3.1 Establishment of EBV latency in memory B cells of the peripheral blood. The traditional model (*below, brown arrows*) posits that upon EBV infection of naïve B cells in the tonsils, they become growth-transformed cells exhibiting a viral latency type III gene expression pattern, then some of them undergo a germinal center passage, where they switch from latency type III to latency type I or II, either by a promoter switch from Cp to Qp or by deletion of the EBNA2 reading frame, upon which they end up as memory B cells (Thorley-Lawson 2001). The alternative model (*above, blue arrows*) posits that upon EBV infection of memory B cells in the tonsils,

they circularize the incoming viral genome, don't become growth-transformed, and remain in latency types 0, I, or II. All naïve or memory B cells which express the highly immunogenic latency type III pattern are extinguished by the cellular immune response. Generally, EBV-infected cells exhibiting latency III tend to avoid GC reactions. However, in cases of immune hyperstimulation, EBV-infected memory B cells may regularly enter or re-enter GC reactions (Araujo et al. 1999) where molecular accidents, like the Burkitt-translocation may occur (Niller et al. 2003; Rossi and Bonetti 2004)

et al. 1998). However, there is some uncertainty on which path permanent viral latency is established. The traditional view is certainly that upon EBV infection of naïve cells in the tonsils, the expanded and growth-transformed infected B cells undergo a germinal center passage, upon which they end up as memory B cells (Fig. 3.1). According to this model, the viral latency gene expression pattern would change in concordance with the B cell phenotype or the developmental stage on its path through the germinal center reaction. An implication of this model is that the EBV-transformed lymphoblast is the direct

precursor of the BL cell. Specifically, a switch of the cellular phenotype accompanied by a switch in the expression profile from the extensive latency III pattern of growth-transformed lymphoblasts to the restricted latency I or 0 patterns of GC or memory cells, respectively, is required by this model (Hawkins et al. 2013), reviewed in (Rickinson 2014). Although such a latency switch has not been observed *in vivo*, a phenotypic switch of LCs, not accompanied by a latency type switch, could be engineered *in vitro* through overexpression of c-Myc in conditionally EBNA2-expressing cells (Polack et al. 1996;

Pajic et al. 2001). Partial latency type switches could be reconstructed *in vitro* by addition of IL-21 or by coculture of LCs with CD4+ T cells secreting soluble CD40L (Kis et al. 2010; Nagy et al. 2012). Furthermore, EBV superinfection of BL cells exhibiting a viral latency type I of their endogenous viral genomes silenced EBNA2 expression of the incoming viral genomes after an initial phase of Wp-on or Cp-on latency (Hughes et al. 2011, 2012).

Another view, first proposed more than 10 years ago, posits that the EBV transformed LC is fundamentally different from the physiologically antigen-activated B cell which undergoes a GC reaction (Fig. 3.1). In this model, the LC, whose epigenome was recently described by Hansen et al. (2014) is not the direct precursor of the BL cell, because EBV-transformed LCs exhibiting a latency class III expression pattern are mostly extinguished by the vigorous immune response which is mounted in the course of mononucleosis or also during asymptomatic primary infection (Niller et al. 2003, 2004a, b). Thus, a prior LC stage is not required for BL oncogenesis. Here, BL oncogenesis depends on c-Myc binding to its recognition site in the locus control region (LCR) of the viral genome which expresses latency I or II functions, but not on prior EBNA2 expression. A minority of BLs which exhibit a Wp-directed gene expression pattern close to latency type III, but without expressing EBNA2 (Kelly et al. 2002), then likely rose from a secondary latency type I to III switch in a pre-existing incipient BL tumor, made possible by bouts of immune suppression due to severe malaria tropica (Whittle et al. 1984). EBNA1 expression in this subset of BL tumors is governed by Wp instead of Cp or Qp, which may signify that an imminent EBV-driven growth-transformation was at its onset abrogated by deletion of EBNA2, before fully growth-transformed cells arose which would have been exterminated by the immune response (Kelly et al. 2002). EBNA2 suppression in BL cells is then a tertiary event, achieved either through deletion of the EBNA2 reading frame in the minority of cases, or through extirpation of all BL cells that arrived at the highly immunogenic LC phenotype in the majority of BL cases, after

the patients re-gained immune control (Kelly et al. 2002; Niller et al. 2004b). Cp-on latency type III as it occurs in LCs *in vitro* and in early-onset PTLDs *in vivo* is mostly regarded as a form of viral latency (Rowe et al. 1987). Latency III, however, may also be viewed as an alternative viral activation or re-activation form alongside the lytic replication cycle, but not as a form of latency in the strict sense, for which only Cp-off latency types 0, I or II would qualify. Thus, latency type III has been also named the **B cell growth-program** (Thorley-Lawson 2001). This notion is supported by the observation that upon demethylating treatment of a latency type I BL cell culture, lytic cycle transcription and the growth-program were activated in distinct cell subsets (Masucci et al. 1989).

The ability of EBV to sustain two distinct paths to viral activation, both lytic cycle and growth-program, seems to be due to the architectural intricacy of the EBV genome, in contrast to the HSV and CMV genomes (for review see Niller et al. 2007). How frequently the two distinct activation paths may overlap in one single cell subset (Ma et al. 2012), or how frequently the growth program may follow upon the lytic cycle program (Kalla and Hammerschmidt 2012) in EBV-associated malignancies, remains to be established.

Upon *in vitro* infection of isolated peripheral blood B cells, linear EBV genomes are circularized to become covalently closed circular (ccc) extrachromosomal episomes as early as 16 h post infection. Infected G0 cells do not need to pass through G1 or to enter S phase for EBV genomes to become circularized, however, a single G0 to early-G1 transition is sufficient for circularization (Hurley and Thorley-Lawson 1988). Interestingly, in already activated or cycling cells, incoming EBV was subject to a linear chromosomal integration, but not to circularization (Hurley et al. 1991), reviewed in (Morissette and Flamand 2010). Circularization occurs, before EBV-infected B cells express activation markers and start proliferating, and well before the viral episomes are amplified later-on in the course of growth-transformation (latency III) which would be the natural course of events *in vitro* (Hurley

and Thorley-Lawson 1988). The possibility of establishing viral latency *in vitro* before cellular activation and proliferation take place reflects on the *in vivo* situation during primary infection. During mononucleosis, several distinct patterns of latency gene expression are observed, including latency III (Niedobitek et al. 1997) which is the most prominent, due to the ease of *in vitro* culture and because growth-transformed B cells overwhelm the organism, if unchecked by the immune system. And indeed, the rare carriers of X chromosome-linked lymphoproliferative (XLP) disease, are overwhelmed by growth-transformed B cells after primary infection, due to a mutant *SH2D1A* gene coding for SLAM-associated protein (SAP) which is important for immune cell signaling, and mononucleosis may end deadly (Coffey et al. 1998), reviewed in (Latour and Veillette 2003). However, if kept at bay through an ever increasing immune response, infected B cells are basically able to establish EBV latency, even if they do not complete a cell cycle, or if they cycle only transiently and become resting again, and if they never reach a Cp-governed LC stage exhibiting latency III.

Correspondingly, during the systemic spread of EBV from Waldeyer's ring to the blood stream, many naïve, as well as resting cells, memory cells and germinal center cells are directly infected, but infected naïve cells do not expand or participate in GC reactions (Kurth et al. 2000), and the expanding EBV-infected GC and memory cells in tonsils of mononucleosis patients do not participate in GC reactions either (Kurth et al. 2003). This observation was confirmed by Araujo et al. (1999) who did not find any EBV-infected cells in GC reactions in a series of excised tonsils from children in Berlin (Araujo et al. 1999). Thus, under normal immune conditions, EBV infected cells do not even seem to regularly reach the germinal center. However, in tonsils from Northeast Brazil where children suffered a high load of parasitic infections at the time (Araujo et al. 1996), a GC passage of EBV-infected cells and the expansion of EBV-positive GC clones was indeed observed. However, the hyperstimulated GC environment permitted only latency type I or occasional type II viral gene expression,

but not latency type III viral gene expression (Araujo et al. 1999). Because the viral growth-program which depends on EBNA2 expression is inhibitory for AID expression which is the key enzyme of the germinal center reaction, latency III is apparently incompatible with a physiological GC reaction (Panagopoulos et al. 2004; Tobollik et al. 2006; Boccellato et al. 2007). More recently, this was supported by Roughan et al. (2010) who found that EBV-infected cells in non-IM tonsils from persistently EBV-infected carriers which expressed GC markers did not express the latency III protein EBNA2 and did not expand in physiological GC reactions which would yield 100s or 1000s of cells, but achieved only abortive reactions of less than ten cells (Roughan et al. 2010). The general "avoidance behaviour" of GC reactions by EBV-infected cells is also in accordance with the observation made in transgenic mice that LMP1 inhibits GC formation (Uchida et al. 1999). If a regular GC cell suffers a *c-Myc*-translocation or becomes a Reed-Sternberg cell, it is designated for apoptosis. However, if such a "crashed" B cell is EBV-infected, it may have a bigger than remote chance to survive the GC reaction and become the founder cell of a hematological malignancy, like a BL or a Hodgkin lymphoma. This is then due to the viral or cellular anti-apoptotic functions induced by the latent virus or exerted by its gene products (Niller et al. 2003; Rossi and Bonetti 2004).

Altogether, after Hansen et al. (2014) described the massive demethylation taking place during EBV-transformation of peripheral blood B cells to the growth-program (Hansen et al. 2014), the evidence for the establishment of latency through direct infection of memory B cells (Kurth et al. 2000) and for the origin of BL without a general detour through an LC stage (Niller et al. 2003, 2004a, b; Rossi and Bonetti 2004), is further solidifying (Niller et al. 2014b). A point which still needs clarification is whether the cellular epigenomes and gene expression profiles of early-onset PTLDs are actually the same as or as close to the molecular profiles of the *in vitro* EBV-transformed LC (Hansen et al. 2014) as is generally assumed. A complete bisulfite

sequence map of early-onset PTLD genomes would be very helpful to clarify this point. Furthermore, complete methylomes of EBV-associated malignancies besides the endemic BL cell line Daudi (Kreck et al. 2013) are desiderata.

3.6 Epigenetic Regulation of EBV Latency Promoters

Cell-type specific and developmental stage-dependent viral gene expression patterns are achieved by the repertoires of viral and cellular transcription factors present in the respective cell types and by diverse epigenetic mechanisms acting on the major viral latency promoters thereby defining distinct viral epigenotypes (Minarovits 2006; Fernandez et al. 2009; Takacs et al. 2009; Arvey et al. 2013). Before the growth-program of Cp-on latency is established upon *in vitro* infection, Wp is governing latency III gene expression at first. However, Wp becomes gradually switched off by CpG-methylation, while Cp takes over (Tierney et al. 2000; Elliott et al. 2004). In *in vitro* EBV-infected tonsillar GC B cells, LMP1-induced DNMT3A binding to Wp correlates with the promoter methylation status (Leonard et al. 2011). In Cp-on latency, unmethylated Cp is located to an acetylation island, while in Cp-off latency, heterochromatic histone marks are deposited on the methylated Cp (Fejer et al. 2008). In this case, among the EBNA proteins, solely replication protein EBNA1 is expressed from Qp. In Cp-on latency, Qp is suppressed by heterochromatic histone marks and binding of repressor proteins, but not by CpG-methylation (Zhang and Pagano 1999; Salamon et al. 2001; Fejer et al. 2008). CTCF binding closely upstream contributes to the protection of Qp from methylation which stays constitutively unmethylated in all latency types (Tempera et al. 2010). The LMP promoters are also regulated by epigenetic modifications (reviewed in Takacs et al. 2009, 2010). LMP1p activity correlates with its methylation status, but not with transcription factor occupancy (Minarovits et al. 1994; Salamon et al. 2001; Takacs et al. 2001). LMP2Ap activity again is co-regulated by its methylation status, histone marks

and transcription factor occupancy (Salamon et al. 2003; Gerle et al. 2007). The EBER genes closely upstream of constitutively unmethylated oriP (Salamon et al. 2000) are also constitutively unmethylated and expressed in all latency types (Minarovits et al. 1992), but their promoters are sensitive to CpG-methylation which prevents binding of transcription factors c-Myc and ATF (Niller et al. 2003; Banati et al. 2008).

Recently, the chromatin boundary protein CCCTC-binding factor (CTCF) has come into the focus of viral latency studies. CTCF mRNA and protein was expressed at higher levels in BL cells than in LCs, and it contributed to EBNA2 repression by downregulating Cp via its binding site between oriP and Cp (Chau et al. 2006). A knockdown of CTCF in BL cells did not lead to the activation of Cp, and a switch to latency III was not observed. However, deletion of that CTCF-binding site led to a prolonged transcription from Cp of incoming viral genomes in one superinfected EBV-positive BL cell line. Thus, CTCF was suggested to contribute, among other factors, to Cp silencing (Hughes et al. 2012). Contrary, Salamon et al. (2009) did not find a correlation between CTCF binding and Cp or Qp activity in several cell lines and, by genomic footprinting found hints for additional proteins besides CTCF playing a role in determining viral latency types (Salamon et al. 2009). CTCF binding at Qp may not serve a repressive function, but as a chromatin boundary separating methylated and unmethylated regions (Tempera et al. 2010). Holdorf et al. (2011) profiled the occupancy of CTCF, RAD21 (a cohesin component), and RNA polymerase II on the EBV episomes in Raji cells, a latency III type BL cell line. They found very prominent RNA pol II binding in the middle of oriP and at a couple of other locations which coincided with known promoter sequences. Raji is a BL cell line which does not use Wp or Cp, but a so far unknown promoter, in order to express a latency III transcriptional pattern (Woisetschlaeger et al. 1989; Walls and Perricaudet 1991). Thus, this large peak of RNA pol II binding within oriP may constitute the missing latency III promoter for the EBNA transcripts in Raji cells (Fig. 3.2). Further, they found CTCF sites not only at active

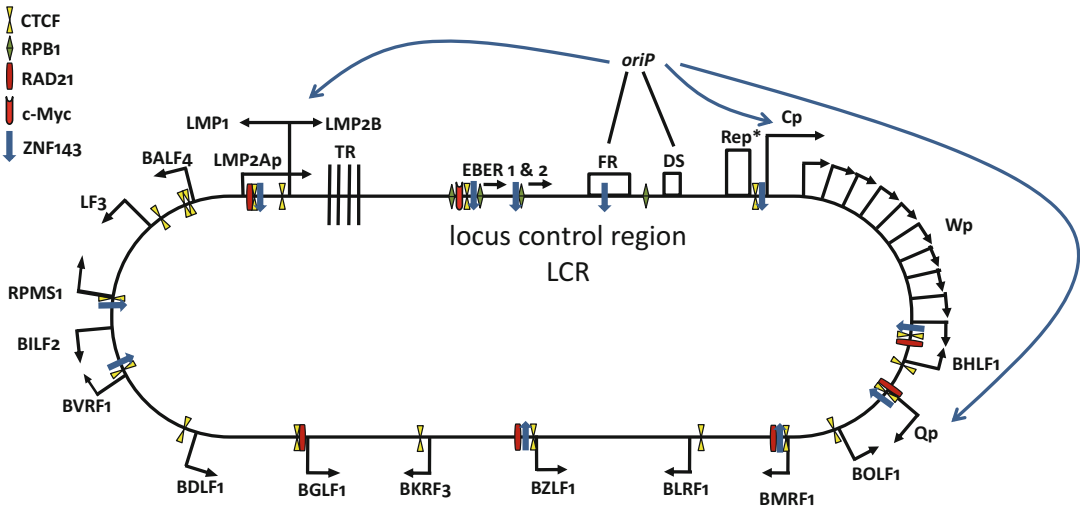


Fig. 3.2 Binding of CTCF, cohesin, RAD21, ZNF143, c-Myc to the latent EBV genome. The EBV genome (not drawn to scale) is depicted as an episome which was circularized at the terminal repeats (TR) immediately after infection. Binding sites for CTCF, cohesin, and RAD21 (a subunit of the RNA pol II complex) were determined by Holdorf et al. (2011) in Raji cells, the binding sites for CTCF, cohesin, RAD21 and the transcriptional activator ZNF143 were determined by Arvey et al. (2012, 2013) mainly in a metaanalysis from EBV-transformed lymphoblastoid cell lines. The binding site for c-Myc at the EBER locus was determined by Niller et al. (2003) in several cell lines, among them Raji. The locus control region encom-

passing the EBER locus and the origin of plasmid replication (oriP) with its elements FR (family of repeats) and DS (dyad symmetry) is crucial for regulating the latency type (Tempera et al. 2011). In latency type I, the LCR loops to and activates Qp, in latency III it loops to and activates Cp, and also acts as a transcriptional enhancer for the bidirectional LMP promoter for LMP1 and LMP2B (latent membrane proteins). Rep* is a sequence adding to oriP function. Cp, the repetitive Wps, and Qp are alternative latent promoters for the EBNA (Epstein-Barr nuclear antigen) transcripts. Lytic cycle promoters around the viral genome are labeled according to the EBV mapping nomenclature

and inactive latency control elements, but also at lytic promoters suggesting several distinct functions of CTCF, like repression, activation, or chromatin insulation, depending on the context of additional binding proteins. Some of the CTCF sites overlapped with cohesin binding, but not RNA pol II binding (Holdorf et al. 2011).

A recent metaanalysis was performed on data sets which were extracted from public databases containing total genomic chromatin data from large scale deep-seq projects that included EBV-transformed LCs. Thereby, a total of 19 CTCF binding sites was found around the EBV genome, mostly located to important regulatory regions (Arvey et al. 2012, 2013). One major role of CTCF may be to support distinct three-dimensional conformations of viral genomes depending on the latency type. In latency I, the locus control region (LCR) of EBV containing oriP looped to Qp, while in latency III the LCR was contacting

Cp. Chromatin loop formation and maintenance between the LCR and other regions of the viral genome was dependent on intact CTCF binding sites (Tempera et al. 2011). However, how the selectivity of the looping is achieved remains to be established. One possibility is certainly that cohesin may play a distinguishing role. Thus, the knockdown of cohesin subunits RAD21 and SMC1 led to the loss of an LCR-LMP-region loop in LCs (Arvey et al. 2012). This is in concordance with the immunoglobulin H locus, where cohesin binding was associated with multiple chromatin looping and recombinatorial activity (Degner et al. 2009), and with KSHV, where chromatin looping was facilitated by CTCF-cohesin interactions (Kang et al. 2011). Remarkably, the viral LCR exhibited a partial non-specific stickiness for protein (Arvey et al. 2013), probably reflecting on the nuclear matrix attachment function of the LCR (Jankelevich et al. 1992).

Therefore, it is almost certain that many more factors than just CTCF and cohesin are involved in specific chromatin looping and viral 3D structure accompanying the regulation of viral latency (Fig. 3.2). For example binding of ZNF143, a most intriguing zinc-finger protein which mediates promoter looping in the cellular chromatin by directly binding to promoters and indirectly to CTCF-cohesin complexes (Bailey et al. 2015), was found just upstream of the EBERs and at a few additional locations of the viral genome, in each case colocalized with CTCF (Arvey et al. 2012). At the same location of the LCR, a binding site for c-Myc had been found in Raji cells by *in vivo* footprinting and chromatin immune precipitation which likely plays a crucial role in the origin of endemic BL tumors in the GC reaction (Niller et al. 2003, 2004a, b).

3.7 Epigenetic Profiles of Cellular Genomes in EBV-Associated Neoplasms

Cancers in general exhibit epigenomic profiles which are different from that of their healthy cellular counterparts (Baylin and Jones 2011) and from each other (Costello et al. 2000). As a rule, cancer cells carry stretches of hypomethylated DNA throughout their genomes, but exhibit also cancer type-associated hypermethylations at specific CpG islands which are frequently overlapping with tissue-specific regulatory elements and with developmentally regulated PRC-binding elements (Esteller 2007a, b; Baylin and Jones 2011). Thus, also EBV-associated neoplasms differ regarding their transcriptomes and, as far as partial or complete CpG-methylation maps have been established, also regarding their epigenomes. Furthermore, the EBV-infected counterparts of a specific tumor type exhibit transcriptomes and epigenetic profiles different from their EBV-negative counterparts. Lymphomas and epithelial cancers are two major groups of EBV-associated malignancies, whose epigenomes need to be individually studied in order to learn more about their individual modes of pathogenesis.

B cells transformed to the LC phenotype through EBV infection exhibit a massive hypomethylation affecting two thirds of their genome and one third of all genes in a complete genomic bisulfite sequence analysis. A total of almost 3000 small differentially methylated regions (DMRs), mostly located in the vicinity of promoters, amounting to a total of 1 MB, were also observed in LCs, approximately half of them hyper- and hypomethylated (Hansen et al. 2014). Hypomethylation of around 250 genes was observed in LCs using a 27K chip, but no hypermethylation (Hernando et al. 2013). Demethylation of most promoters associated with the transformation process was associated with binding of B cell specific transcription factors, half of them binding NF- κ B (Hernando et al. 2013). Correspondingly, a major part of the epigenetic reprogramming in LCs and a set of different **DLBCLs** (Diffuse Large B cell Lymphomas) was due to NF- κ B signaling (Vento-Tormo et al. 2014). Hypomethylated genes are involved in cellular proliferation, B cell signaling, chemotaxis, cell adhesion, immune response and inflammation (Hernando et al. 2013). An earlier study found hypomethylated genes involved in cell cycling, transcriptional regulation and the immune response (Caliskan et al. 2011). Parallel to demethylation, EBV transformation leads to a major redistribution and decrease of heterochromatic histone marks (Hernando et al. 2014).

A focused analysis of the p16INK4A and Bim loci found heterochromatic histone marks which were deposited through PRC2 mediated by EBNA3A and -3C (Skalska et al. 2010; Paschos et al. 2012). A major finding of both Hernando et al. (2013) and Hansen et al. (2014) is the clear distinction of epigenomes between EBV-transformed LCs, on the one hand, and CD40L/IL4-activated B cells, on the other hand. The methylation profile of activated B cells was very close to that of quiescent B cells, while that of LCs had drifted far away (Hansen et al. 2014). Correspondingly, the heterochromatin profile of transformed LCs could also be achieved through infection with EBNA2- or LMP1-deleted viruses, but not through CD40L/IL4-stimulation of B cells to proliferate. Thus, the transformation trait

could be clearly dissected from mere proliferation of activated B cells in those experiments (Hernando et al. 2013). This is a most intriguing observation which certainly deserves further in-depth analysis.

The epigenetic profile of BL tumors seems to considerably differ from that of LCs. So far, there is only one completed bisulfite sequence map of an EBV-positive endemic BL cell line, Daudi (Kreck et al. 2013). Sixty nine percent of all genomic CpG dinucleotides were methylated, and more than 90 % of 969 genes which are hypermethylated in mature aggressive B cell lymphomas including BL, were also silenced by methylation in Daudi cells (Martin-Subero et al. 2009b; Kreck et al. 2013). Nearly 60 % of hypermethylated genes in BL tumors were target genes of PRC2 in embryonic stem cells (Martin-Subero et al. 2009a). PRC2-targeted hypermethylation does probably not depend on EBV infection. Possibly, BLs are derived from precursor cells with stem-cell features (Martin-Subero et al. 2009b). In addition, c-Myc hyperactivity in BL tumors may contribute to their “stemness phenotype” (Takahashi et al. 2007). The overall tendency of BL is towards a high methylation profile, contrary to the hypomethylation-prone profile of LCs. A point which needs further clarification is whether primary BL tumors exhibit the same epigenetic profile as the cell line Daudi. Also in this case complete bisulfite maps would be helpful.

The epigenetic profiles of both NPC and EBV-associated GC suggest a CpG Island (CGI) Methylator Phenotype (CIMP) for both epithelial cancers (reviewed in Niller et al. 2007, 2012, 2014a, c; Kaneda et al. 2012; Lo et al. 2012). EBV gene expression patterns in NPC and GC belong mostly to latency I (or II) with a variable expression of LMP1 in the case of NPC, and with expression of BARF1, BARF0 and a variable expression of LMP2A in addition to EBNA1 and the BART microRNAs in the case of GC (Sugiura et al. 1996), reviewed in (Niller et al. 2012). Because EBV is usually monoclonal in malignant tissues, a causal role for the virus is assumed (Raab-Traub and Flynn 1986). Furthermore, a rather peculiar case of a very rapidly developing

EBV-positive GC in an immune suppressed patient suggests a direct role for EBV in GC carcinogenesis (Au et al. 2005). However, the sequential order of carcinogenesis in both epithelial cancer types remains to be elucidated. Chromosomal aberrations have been found in NPC precursor lesions, i.e. in high grade dysplasia and carcinoma in situ, however, virus-infected normal epithelia have so far not been found for both NPC and GC (Kaneda et al. 2012; Lo et al. 2012).

Nevertheless, it is very likely that EBV infection is causal for a major epigenetic disruption of epithelial cells on their path to malignancy. One mechanism by which EBV causes the hypermethylation in **EBVaGC**, may be that LMP2A, in the cases where it is expressed, activates DNMT1 and DNMT3B expression which on their part lead to a global but non-random CGI-methylation in EBVaGC (Hino et al. 2009; Zhao et al. 2013b). How target specificity is achieved remains unclear at the moment. Candidate gene studies have reported a series of genes significantly more frequently hypermethylated in EBVaGC, but not in the other GC subtypes, e.g. *CDH1* (Wu et al. 2000; Sudo et al. 2004), *p16INK4A*, *PTEN*, *RASSF1A*, *GSTP1*, *MGMT*, *MINT2* (Kang et al. 2002), *p14ARF*, *p15INK4B*, *p16INK4A* (Chong et al. 2003), *p14ARF*, *p16INK4A* (Sakuma et al. 2004), *p73* (Ushiku et al. 2007), *HOXA10* (Kang et al. 2008). EBVaGC and microsatellite instability were mutually exclusive (Chang et al. 2003), which was reflected in constitutively unmethylated *MLH1* and *MSH2* genes in EBVaGC (Kang et al. 2002; Vo et al. 2002). An epigenetic field of cancerization, like in *Helicobacter pylori*-associated GC (Ushijima 2007), was not observed in the surrounding mucosa of EBVaGC (Enomoto et al. 2007; Ushiku et al. 2007). Further tumor suppressor genes *WNT5A* (Liu et al. 2013) and *SSTR1* (Zhao et al. 2013a) were found to be hypermethylated in EBV-positive GCs (reviewed in Niller et al. 2009, 2014a). Further, hypermethylated promoters of *p16INK4A*, *FHIT*, *CRBP1*, *WWOX*, and *DLC1*, *HOXA11* have recently been reported or confirmed (Cui et al. 2015; He et al. 2015).

Candidate gene approaches were extended through genome wide screens. A remarkable genome-wide methylation analysis of 51 GC samples yielded three different GC epigenotypes, two low- and high-methylation epigenotypes in EBV-negative GCs, and one very high-methylation epigenotype of EBV-positive GCs. In the EBV-infected subtype, a surplus of 270 genes was methylated, the DNA repair gene *MLH1* was regularly unmethylated, and, contrary to the EBV-negative hypermethylated subtype, PRC target genes of embryonic stem cells were not enriched. Furthermore, EBV infection of an EBV-negative GC cell line led to an increase of DNA methylation which resembled the methylation patterns of the EBV-infected very high-methylation GC epigenotype. Thus, in addition to the methylation mechanisms of other GC subtypes which may mostly be due to long-lasting *Helicobacter pylori* infection, additional mechanisms of aberrant methylation are assumed to act in the EBV-infected epigenotype. Hypermethylation could so far not simply be attributed to one of the latent gene products (Matsusaka et al. 2011). Consistent with the results of Matsusaka et al. (2011), a comparison of cultured GC cells yielded 886 genes which became hypermethylated in EBV-infected subclones, but not in uninfected subclones. Many of those genes are involved in tumor suppression pathways (Zhao et al. 2013b).

Two recent genome-wide studies of genetic and epigenetic alterations, one on 98 GC samples of different subtypes in comparison with 31 normal tissue samples (Wang et al. 2014), and the other one on 295 primary gastric adenocarcinomas including 9 % of EBVaGC (Cancer Genome Atlas Research Network 2014), confirmed the very high genomic CIMP status of EBVaGC (Toyota et al. 1999; Matsusaka et al. 2011). EBVaGC turns out to exhibit the highest CIMP status of all tumor types studied so far by The Cancer Genome Atlas Research Network (TCGA, reviewed in Li et al. 2014b). A list of 526 genes epigenetically silenced in EBVaGC (Cancer Genome Atlas Research Network 2014) and a list of 90 genes epigenetically silenced in different subtypes of GC (Wang et al. 2014) is

available in the Supplementary Material of the respective papers. Interestingly, there is an intersection of 36 genes between the two lists of TCGA and Wang et al. (2014), an intersection of eleven genes between TCGA and Matsusaka et al. (2011), and only one gene between Wang et al. (2014) and Matsusaka et al. (2011), but no single gene is present on all three lists.

The epigenetic analysis of **undifferentiated NPC** is somewhat lagging behind compared to that of EBVaGC. Like EBVaGC, also NPC belongs to the group of CIMP-cancers. In the cases of NPC, where LMP1 is expressed, LMP1 may induce the expression of all three DNMTs and therefore play a role in the frequent hypermethylation of tumor suppressor genes in the tumor tissue (Tsai et al. 2002, 2006), reviewed in (Niller et al. 2009, 2012, 2014a, c; Lo et al. 2012). A comparative analysis of the cellular methylation status and LMP1 expression between NPC tissues and normal nasopharyngeal tissue showed that LMP1 expression correlated with the degree of methylation of CGIs at ten pre-selected tumor suppressor genes (Challouf et al. 2012).

Candidate gene approaches have identified a number of genes frequently hypermethylated in NPC tissue in comparison with healthy nasopharyngeal mucosal tissue, e.g. *p16INK4A* (Lo et al. 1996), *CDH1* and *CTNNB1* (Zheng et al. 1999), *RASSF1A* (Lo et al. 2001), *EDNRB* (Lo et al. 2002), *TSLC1* (Hui et al. 2003), *ZMYND10/BLU* (Qiu et al. 2004), *CHFR* (Cheung et al. 2005), *GADD45G* (Ying et al. 2005), *PCDH10* (Ying et al. 2006), *LTF* (Yi et al. 2006; Zhou et al. 2008), *DLC1* (Peng et al. 2006), *RASAL1* (Jin et al. 2007a), *ADAMTS18* (Jin et al. 2007b), *CCNA1* (Yanatatsaneejit et al. 2008), *ADAMTS9* (Lung et al. 2008), *DLEC1* (Ayadi et al. 2008), *IRF8* (Lee et al. 2008), *OPCML* (Cui et al. 2008), *BRD7* (Liu et al. 2008), *ZNF382* (Cheng et al. 2010), *PCDH8* (He et al. 2012), *RRAD* (Mo et al. 2012). CGI hypermethylation may have practical consequences for diagnostics and therapy (reviewed in Niller et al. 2009, 2012, c; Li et al. 2011, 2014b; Lo et al. 2012). Tumor suppressor or marker genes frequently methylated in NPCs may allow the use of methylation sensitive PCR (MSP) for the early detection of NPCs in high

risk groups besides high levels of anti-EBV-IgA in serum samples, e.g. *RASSF1A*, *p16INK4A*, *WIF1*, *CHFR* and *RIZ1* (Hutajulu et al. 2011), *CACNA2D3* (Wong et al. 2013), *CDK10* (You et al. 2013), *ASS1* (Lan et al. 2014), *CYB5R2* (Xiao et al. 2014). In addition, epigenetic alterations acquired by tumor cells during chemotherapy may play a role in the development of drug resistance and may allow predictions on the prognosis of EBVaGC. Hypermethylation of *RARB2* (Kwong et al. 2002; Seo et al. 2008), *CRBP1* and *CRBP4* (Kwong et al. 2005b), and *RARRES/TIG1* (Sriuranpong et al. 2004; Kwong et al. 2005a) prevent retinoic acid-mediated growth inhibition. High levels of H3K27me3 (Cai et al. 2011) and EZH2 (Hwang et al. 2012) may indicate the overall level of tumor suppressor gene silencing. Hypermethylation at *DLCL1*, *CHFR*, and *PEG10*, and hypomethylation at *ABCC5*, *ERBB2*, and *GSTP1* may predict taxol resistance of NPC tumors (Zheng et al. 2012).

Beyond the candidate gene analyses, genome-wide methylation analyses were performed on several NPC tumor samples and cell lines in comparison with normal tissue and non-transformed nasopharyngeal cell lines by chromatin immunoprecipitation combined with promoter microarray hybridization (MeDIP-chip) (Li et al. 2014a). In this study, an overall high methylation level of CGIs was found, and many previously reported genes were confirmed as methylated in NPC tumors and cell lines. Two newly found genes, *PAX5* and *SLIT2*, were selected and again confirmed as methylated in tumors and cell lines. Several genes from the Wnt pathway, i.e. *SFRP*, *DACT*, and *DKK* family members were selected and examined for their methylation status and tumor suppressor function in NPC tumors and cell lines. Several members of these gene groups were indeed more frequently hypermethylated in NPC derived material than in normal nasopharyngeal cells and exhibited a tumor suppressor function in a colony formation assay. Remarkably, while in NPC tumors, the top affected pathway was the Wnt signaling pathway, the MAPK signaling pathway was the mostly affected pathway of NPC cell lines (Li et al. 2014a). This raises a note of caution in dealing with cell lines instead

of tumor samples, because their epigenotype may have been altered in the course of long-term *in vitro* culture. On the other hand, if not microdissected, tumor samples are composed of distinct tissue types, e.g. cancer cells and infiltrating immune cells which make their epigenotype a composite of distinct epigenotypes.

Until very recently there was only a surprisingly small subset of four known hypermethylated gene loci which EBVaGC and NPC had in common, i.e. *PTPRG*, *ASS1*, *GSTP1*, and *MIPOL1* (for a recent review and a detailed comparison of hypermethylated genes in EBVaGC versus NPC see (Niller et al. 2014a), and Table 3.3). However,

Table 3.3 List of genes hypermethylated both in EBV-associated gastric carcinoma and nasopharyngeal carcinoma

Hypermethylated in NPC and EBVaGC	Chromosomal location
<i>PTPRG</i>	03p14.2
<i>SLC6A20</i>	03p21.31
<i>MARCH1</i>	04q32.3
<i>OXCT1</i>	05p13.1
<i>B3GALT4</i>	06p21.32
<i>TNXB</i>	06p21.33
<i>LY6G5C</i>	06p21.33
<i>PPP1R18</i>	06p21.33
<i>GNL1</i>	06p21.33
<i>TRIM31</i>	06p21.3
<i>GRB10</i>	07p12.1
<i>SCRNI</i>	07p14.3
<i>SGCE</i>	07q21.3
<i>ATP6V0E2</i>	07q36.1
<i>ASS1</i>	09q34.11
<i>NEUROG3</i>	10q22.1
<i>PRKCDBP</i>	11p15.4
<i>ZNF214</i>	11p15.4
<i>GSTP1</i>	11q13.2
<i>CHFR</i>	12q24.33
<i>MIPOL1</i>	14q13.3
<i>ZNF701</i>	19q13.41
<i>ZNF542P</i>	19q13.43
<i>GATA5</i>	20q13.33

References for individual genes in the list are given in Matsusaka et al. (2011), Li et al. (2011, 2014a), Lo et al. (2013), Niller et al. (2014a), Wang et al. (2014), Cancer Genome Atlas Research Network (2014), and Dai et al. (2015)

a comprehensive 450K-chip methylome analysis of 60 NPC tumor samples in comparison with other tumor methylomes already established by The Cancer Genome Atlas (TCGA) Research consortium increased the overlap of methylated genes between EBVaGC and NPC (Dai et al. 2015). Dai et al. showed that also NPC belongs to the highest methylated of all cancers, described additional genes hypermethylated both in EBVaGC and NPC, confirmed a significant overlap of de novo methylated genes with bivalent chromatin markers H3K4me3 and H3K27me3 in all examined cancer types, and found a remarkably strong peak of hypermethylation at chromosomal position 6p21.3 for both EBVaGC and NPC, and a weaker, but significant methylation-peak at 6p21.3 also for prostate cancer (Dai et al. 2015). Chromosomal region 6p21.3 contains several genes apparently involved in tumorigenesis and importantly contains the human leukocyte antigen (HLA) genes which have earlier been shown to be strongly linked with NPC risk (Tse et al. 2009; Tang et al. 2012). The currently known intersection of hypermethylated genes in both EBVaGC and NPC is shown in Table 3.3, which illustrates the accent on chromosomal region 6p21.3. In-depth comparative analysis of complete genomic bisulfite sequence maps which have to be established yet for early-onset PTLTD, NPC and EBVaGC, and the genome-wide analysis of histone marks may further reveal the overlaps and differences between EBV-associated tumors and thereby illuminate the specific role of EBV in the patho-epigenetics of both EBV-associated carcinomas. Furthermore, analysing the function of all newly described hypermethylated genes is of importance for understanding EBV-associated cancerogenesis.

3.8 Conclusion

The sequence-, promoter- or locus-specificity of the mechanisms targeting hypermethylation or hypomethylation to viral and cellular promoters in EBV-infected cells is most intriguing and needs further clarification. It should be sorted out which mechanisms are involved in the establishment of

widespread hypomethylated blocks during the outgrowth of LCs, despite the expression of LMP1 and LMP2A which are capable of inducing cellular DNMTs. The mechanisms targeting hypermethylation at PRC target genes of embryonic stem cells in lymphomas, and targeting hypermethylation at non-PRC target genes in the highly methylated CIMP-cancer EBVaGC may clarify the reason for the apparently EBV-specific “epigenetic signature”. Further in-depth analyses of the overlaps and differences in the methylomes of EBV-associated malignancies and functional analyses of the hypermethylated genes are required to sort out the specific contribution of EBV in each case. Epigenetic marks and genes differentially methylated in EBV-associated neoplasms may also have a diagnostic and prognostic value. Because epigenetic changes are principally reversible, drugs which affect the epigenotype of an EBV-associated tumor may be exploited for cancer treatment.

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