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EPSTEIN-BARR VIRUS AND GENOMIC INSTABILITY- A NEW LOOK AT THE MECHANISMS OF VIRAL ONCOGENESIS

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Cover: EBV(+) metaphase chromosome illustrating the occurrence of telomere sister chromatid exchanges (T-SCEs).

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In the name of God the most beneficent, the merciful

This work is dedicated
To those who encouraged me to study
In particular my family
and
Those for whom I am studying

ABSTRACT

EBV is associated with a variety of lymphoid and epithelial malignancies but the mechanisms of oncogenesis are still not fully understood. The aim of the work described in this thesis was to assess whether induction of genomic instability, as defined by the accumulation of non-clonal genetic aberrations, could play a role in EBV oncogenesis and identify the viral protein(s) responsible for this phenotype. Cytogenetic analysis of a panel of EBV(+) and EBV(–) Burkitt`s lymphoma cell lines revealed a significant increase in dicentric chromosomes, chromosome fragments and chromatid gaps in EBV infected cells. EBV latency I, where only EBNA1 is expressed, was sufficient for this effect, whereas a stronger increase was observed in latency III suggesting the involvement of several latency proteins. Telomere analysis by fluorescent in situ hybridization (FISH) showed an increase in the prevalence of telomere fusion and double strand break fusion in dicentric chromosomes from EBV(+) cells pointing to telomere dysfunction and DNA double strand breaks (DSBs) as possible mechanisms by which EBV may promote genomic instability.

The significant increase of chromosomal aberrations in cells expressing latency I suggests a possible role for EBNA1 in the induction of genomic instability. This was confirmed by analyzing the occurrence of chromosomal aberrations, DSBs and engagement of DNA damage response (DDR) in B-cell lymphoma cell lines expressing constitutive or inducible EBNA1. EBNA1 expression correlated with a significant increase of reactive oxygen species (ROS) suggesting a possible role for oxidative stress, which was confirmed by the decrease of chromosome abnormalities in cells treated with ROS scavengers. EBNA1 was then shown to induce oxidative stress by transcriptional activation of the catalytic subunit of NADPH oxidise, NOX2.

Stable or conditional expression of EBNA1 was associated with the accumulation of telomere abnormalities, including loss and gain of telomere signals, telomere fusion and heterogeneous length of telomeres. This phenotype was coupled with the accumulation of extra-chromosomal telomeres, telomere dysfunction induced foci (TIFs), telomere-associated promyelocytic leukemia nuclear bodies (T-PNBs) and telomere-sister chromatid exchanges (T-SCEs), and with displacement of the shelterin protein TRF2 from telomeres. The induction of TIFs and T-PNBs was inhibited by treatment with scavengers of ROS that also promoted the re-localization of TRF2 at telomeres.

EBNA1 regulates virus replication and transcription, and participates in the remodeling of the cellular environment that accompanies EBV induced B-cell immortalization. We have profiled the transcriptional changes induced by short- and long-term expression of EBNA1 in the EBV negative B-cell lymphoma BJAB. Gene ontology analysis of forty seven genes that were consistently regulated independently on the time of EBNA1 expression revealed an unexpected enrichment of genes involved in the maintenance of chromatin architecture. The protein interaction network of the affected gene products suggests that EBNA1 may promote a broad rearrangement of the cellular transcription landscape by altering the expression of key components of chromatin remodeling complexes.

Collectively these studies highlight previously unrecognized mechanisms by which EBNA1 may promote malignant transformation and tumour progression through induction of oxidative stress and by promoting the epigenetic reprogramming of EBV infected cells.

Key Words: EBV, EBNA1, ROS, TIFs, T-PNBs, Burkitt`s Lymphoma, T-SCEs

LIST OF PUBLICATIONS

This thesis is based on the following articles and manuscript that will be referred to in the text by their roman numerals:

I. **Kamranvar SA**, Gruhne B, Szeles A, Masucci MG.
Epstein-Barr virus promotes genomic instability in Burkitt's lymphoma. *Oncogene. 2007 Aug 2; 26(35): 5115-23*

II. Gruhne B, Sompallae R, Marescotti D, **Kamranvar SA**, Gastaldello S, Masucci MG.
The Epstein-Barr virus nuclear antigen-1 promotes genomic instability via induction of reactive oxygen species.
Proc Natl Acad Sci. 2009 Feb 17, 106(7): 2313-2318

III. **Kamranvar SA**, Masucci MG.
The Epstein-Barr virus nuclear antigen EBNA-1 promotes telomere dysfunction via induction of oxidative stress.
Submitted for publication.

IV. Sompallae R, Callegari S, **Kamranvar SA**, Masucci MG.
Transcription profiling of Epstein-Barr virus nuclear antigen EBNA-1 expressing cells suggests targeting of chromatin remodeling complexes.
PLoS One. 2010 Aug 10; 5(8): e12052-

OTHER RELATED PUBLICATION

- Gruhne B, **Kamranvar SA,** Masucci MG, Sompallae R.
  EBV and genomic instability- A new look at the role of the virus in the pathogenesis of Burkitt`s lymphoma.
  Semin Cancer Biol. 2009 Dec; 19(6): 394-400

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LIST OF ABBREVIATIONS

ALT	Alternative lengthening of telomere
APBs	ALT associated promyelocytic leukemia nuclear bodies
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
AID	Activation induced cytidine deaminase
BART	BamHI A rightward transcript
BL	Burkitt`s lymphoma
BBF	Breakage bridge fusion
BER	Base excision repair
CTAR	C-terminal activator region
CIN	Chromosome instability
CSR	Class switch recombination
CGH	Comparative genomic hybridization
DS	Dyad symmetry
DSBR	Double strand break repair
DDR	DNA damage response
EBV	Epstein Barr virus
EBNA	EBV nuclear antigen
ECTR	Extra-chromosomal telomere repeat
EBER	EBV encoded RNA
FR	Family of repeat
hTERT	Human telomerase reverse transcriptase
HR	Homologous recombination
HD	Hodgkin's disease
HP1	Heterochromatin Protein 1
HJ	Holliday Junction
IM	Infectious mononucleosis
LOH	Loss of heterozygosity
LCL	Lymphoblastoid cell line
LMP	Latent membrane protein
MN	Micronuclei
MIN	Microsatellite instability
MMR	Mismatch repair
NPC	Nasopharyngeal carcinoma

NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NFKB	Nuclear Factor-kappa B
NADP	Nictinamide adenine dinucleotide phosphate
OriP	Origin of replication
ORC	Origin recognition complex
POT1	Protection of telomeres
PTLD	Post transplant lymphoproliferative disorder
PML-NB	Promyelocytic leukemia nuclear body
PTOP	POT1 and TIN2 organizing protein
RAP1	Repressor activator protein 1
RBP-JK	Recombination Signal-Binding Protein 1 for J-Kappa
ROS	Reactive oxygen species
SHM	Somatic hyper mutation
SSA	Single strand annealing
TRF	Telomere repeat binding factor
TPP1	TINT1, PTOP, PIP1
TERC	Telomerase RNA component
T-SCE	Telomere sister chromatid exchange
HHV4	Human Herpes Virus 4
TNFR	Tumor necrosis factor receptor
T-PNBs	Telomere-associated promyelocytic leukemia nuclear bodies
TIFs	Telomere dysfunction induced foci
TERRA	Telomeric repeat containing RNA
TIN2	TRF1 interacting nuclear factor 2
SSB	Single strand break
DSB	Double strand break

1 INTRODUCTION

1.1 EPESTIN-BARR VIRUS (EBV)

1.1.1 Virus structure

Epstein-Barr virus (EBV also called human herpesvirus-4, HHV4) is a human gamma herpes virus and the first discovered human tumour virus. EBV is widespread in almost all human populations, with more than 90% of adults being life-long carriers. The virion of EBV is about 120-300 nm in diameter and consists of a toroid shaped protein core wrapped with linear double stranded DNA in an icosahedral nucleocapsid with 162 capsomeres, an outer envelope with external glycoprotein spikes and a tegument protein between the nucleocapsid and envelope (Figure 1) (Kieff and Rickinson 2007).

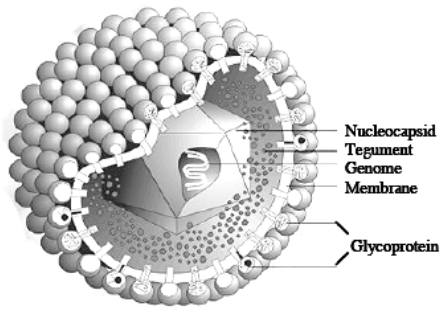

Figure 1: Schematic illustration of EBV virion

The EBV genome is a 184 kbp double-stranded DNA that encodes more than 85 genes (Kieff and Rickinson 2007). The DNA molecule includes 0.5 kbp tandem terminal direct repeats (TR) and internal repeat sequences (IRs) that serve to divide the genome into short and long unique sequence domains (Young and Murray 2003). When EBV infects a cell, the TRs join to form a covalently closed circular (episomal) DNA. The number of TRs is perpetuated from generation to generation during the episomal replication of viral genome in latently infected cells (Baumforth, Young et al. 1999). Although the EBV genome usually persists as an episome in latently infected cells, it can rarely integrate into the chromosomal DNA (Henderson, Ripley et al. 1983).

1.1.2 Virus genome replication

Two distinct forms of replication occur during the virus life cycle: lytic and latent. Lytic replication initiates from the origin of lytic replication, *oriLyt*, which is a region located in two separate sites of the viral genome within the promoter region of the BHLF1 and BHRF1 genes (Schepers, Pich et al. 1993). During the lytic phase, linear double stranded DNA genome is synthesized by rolling circle type of replication using

replication factors that are mostly encoded in the viral genome (Hammerschmidt and Sugden 1988). In contrast, latent replication occurs once per S-phase starting from the origin of replication of latent infection, *OriP*, which is a cis-acting element, located in the BamHI-C fragment of viral genome and confers the ability to replicate autonomously as a small plasmid in EBV infected cells. Replication of the EBV episome during latent infection is dependent on cellular replication machinery (Yates, Warren et al. 1984; Tsurumi, Fujita et al. 2005). The origin of viral replication in latent phase is not restricted to *OriP* and several other replication origins have been identified (Little and Schildkraut 1995).

1.1.3 Virus-Host cell interaction during primary infection

EBV binds to the CD21 and HLA antigen class II receptors of B lymphocyte through the most abundant outer envelope glycoprotein, gp350/220, and gp42, respectively, and enters the cell by endocytosis followed by low-pH dependent envelope and endosomal membrane fusion, which leads to release of the capsid into the cytoplasm. The EBV glycoprotein gH, gL, gB and also gp42 were shown to be necessary for envelope fusion (Hutt-Fletcher 2007). In contrast to B cells, entry of EBV into epithelial cells is poorly understood and is assumed to take place by fusion at the cell membrane (Miller and Hutt-Fletcher 1992). Binding of the virus to CD21 activates the Src family of tyrosine kinase and mobilizes calcium in B lymphocytes (Cheung and Dosch 1991). CD21 ligation also increases the activity of the nuclear factor-kappa B (NF-kB), which may promote the transcription of EBNAs at the initiation of EBV infection (Sugano, Chen et al. 1997). Once in the cytoplasm the capsid is likely to be transported on microtubules toward the nuclear pore where it disassembles, allowing the viral DNA to be imported into the nucleus (Kieff and Rickinson 2007).

1.1.4 Latent infection and proteins

The EBV genome persists during latency as a circular episome that is packaged in nucleosomal arrays and binds to metaphase chromosomes (Jankelevich, Kolman et al. 1992). The viral DNA is CpG methylated, which results in repression of the majority of viral genes and abrogation of virus synthesis in latent phase (Bergbauer, Kalla et al. 2010). The W promoter (Wp) is activated after circularization of viral genome, which launches a cascade of events leading to the expression of all the EBNA proteins and the two latent membrane proteins (LMPs) but 24–48h later, the promoter shifts to Cp that is the stronger EBV promoter in latent phase and is activated by EBNA2 (Rowe 1999; Palermo, Webb et al. 2008). EBNA LP and EBNA2 mRNAs are the first transcripts that can be detected only few hours after latent infection (Alfieri, Birkenbach et al. 1991). They drive resting B lymphocytes into the G1 phase of the cell cycle by binding

and inactivating cellular p53 and Rb tumour suppressor gene products (Szekely, Selivanova et al. 1993; Sinclair, Palmero et al. 1994).

The host and virus interaction can be divided into four different latency phenotypes, classified according to EBV gene expression pattern (Babcock, Hochberg et al. 2000). All latencies are found to express non-coding RNA (EBERs), BamHI-A rightward transcripts (BARTs) and microRNAs. In latency 0 infection, all viral antigens are suppressed whereas latency I infection expresses only EBNA1 from the Q promoter (Qp). Latency II infection expresses EBNA1 from Qp and the LMPs from promoters in the BamHI-N region of the genome whereas latency III infection expresses all the latent genes including EBNAs from one of two adjacent promoters, Cp and Wp, in the BamHI-C and BamHI-W regions of the genome, respectively and LMPs from their own promoters (Table 1) (Rowe 1999; Young and Rickinson 2004).

Latent Protein	Burkitt's Lymphoma (Latency I)	Hodgkin's Lymphoma, NPC, NK/T cell (Latency II)	Immunoblastoid Lymphoma, IM (Latency III)
EBNA1	+	+	+
EBNA2			+
EBNA3A			+
EBNA3B			+
EBNA3C			+
EBNALP			+
LMP1		+	+
LMP2A		+	+
LMP2B		+	+
EBERs	+	+	+
BARTs	+	+	+

Table 1: EBV gene expression patterns in latency programs

1.1.5 EBNA1

EBNA1 associates diffusely with mitotic chromosomes and is essential for persistence of the EBV episome in dividing cells (Ohno, Luka et al. 1977). EBNA1 enables the EBV episome to partition in progeny cells by binding to *OriP* and chromosomes. EBNA1 specifically binds to 20, 30-bp repeat elements in the FR region and with less avidly to 4 distinct repeat sites in DS (Figure 2) (Frappier and O'Donnell 1992). EBV lacking EBNA1 cannot establish stable latent infection (Lee, Diamond et al. 1999).

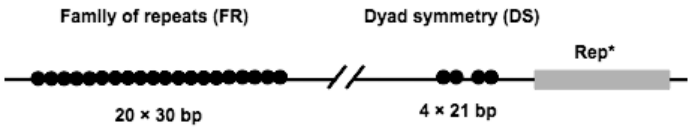

Figure 2: *OriP* containing FR and DS elements

EBNA1 is a multifunctional DNA binding protein that regulates transcription of viral and cellular genes. EBNA1 binds close to the transcriptional initiation sites of a large number of cellular genes such as HDAC3, CDC7 and MAP3K1 and regulates their expression (Lu, Wikramasinghe et al. 2010). EBNA1 enhances RNA pol III dependent EBER expression and induction of EBER associated cellular transcription factors, including TFIIIC, ATF-2 and c-Myc (Owen, O'Neil et al. 2010). EBNA1 interaction with binding sites downstream of the Qp, negatively regulates its own expression (Nonkwelo, Skinner et al. 1996). EBNA1 also acts as a transcriptional transactivator and upregulates transcription from the Cp and LMP1 promoters (Kieff and Rickinson 2007).

The EBNA1 protein is separated into amino- and carboxy-terminal domains by a glycine–alanine repeat (GAr) sequence that varies in size among different EBV isolates. The GA repeat inhibits EBNA1 degradation by the ubiquitin proteasome system (Levitskaya, Coram et al. 1995) preventing presentation of endogenous EBNA1 antigenic peptides through the MHC class I pathway (Levitskaya, Sharipo et al. 1997). Moreover, it was recently shown that the nascent GAr peptide targets the initiation of translation of any mRNA to which it is fused and prevents its MHC class I antigen presentation (Apcher, Daskalogianni et al. 2010). EBNA1 has two regions containing Arg-Gly (RGG) motifs that are termed linking regions (LR)-1 (amino acids 40 to 89) and -2 (amino acids 328 to 377) (Figure 3).

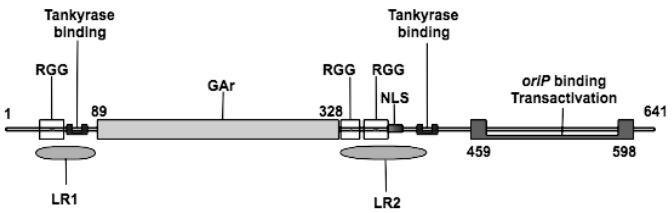

Figure 3: Schematic representation of DNA and protein interaction regions of EBNA1

LRs are involved in the metaphase chromosome tethering function (Marechal, Dehee et al. 1999), DNA replication, transcriptional activation (Mackey and Sugden 1999) and AT-rich DNA binding activity (AT-hook) (Sears, Ujihara et al. 2004) as well as RNA binding to G-rich and G-quadruplex structures (Snudden, Hearing et al. 1994; Norseen, Johnson et al. 2009). The RNA binding activity of EBNA1 is associated with the ORC recruitment at *OriP* that is essential for initiation of replication (Norseen, Thomae et al. 2008).

Several evidences imply that EBNA1 may play an important role in the development and progression of tumor. For instance, downregulation of EBNA1 was shown to reduce cell proliferation and survival (Hong, Murai et al. 2006; Yin and Flemington 2006). In contrast, expression of EBNA1 in cancer cells increases metastasis in nude mice through inhibition of the cell migration and metastasis suppressor Nm23-H1 (Murakami, Lan et al. 2005; Kaul, Murakami et al. 2007). Overexpression of a dominant negative EBNA1 mutant increases cell death in EBV positive Burkitt´s lymphoma cells indicating an anti-apoptotic role for EBNA1 (Kennedy, Komano et al. 2003). This role may be achieved by EBNA1 interaction with the ubiquitin specific protease 7 (USP7 or HAUSP), which blocks its binding to p53, thus resulting in p53 destabilization and inhibition of apoptosis (Saridakis, Sheng et al. 2005). EBNA1 has been also shown to disrupt promyelocytic leukemia nuclear bodies (PML-NBs) in nasopharyngeal carcinoma (NPC) cells through recruitment of Casein kinase 2 (CK2) to PML-NBs, phosphorylation and ubiquitin proteosome dependent degradation of the PML protein. EBNA1 mediated disruption of PML nuclear body promotes the survival of cells (Sivachandran, Sarkari et al. 2008; Sivachandran, Cao et al. 2010).

1.1.6 EBNA2

EBNA2 is a transcriptional activator of viral and cellular genes and is essential for the transforming potential of EBV (Kempkes, Spitkovsky et al. 1995). EBNA2 upregulates the transcription of cellular and viral genes such as CD21, CD23, c-Myc, c-FGR, the latent membrane proteins (LMP1-2) and induces transcriptional activation of the C promoter whereas downregulates transcription of the immunoglobulin heavy chain locus (Zimber-Strobl and Strobl 2001). EBNA2 does not bind directly to DNA but is tethered to EBNA2 responsive promoters through interaction with cellular sequence specific DNA binding proteins such as RBP-Jκ (Henkel, Ling et al. 1994) and PU.1 (Laux, Adam et al. 1994). In activating transcription through RBP-Jκ, EBNA2 mimics a constitutive Notch receptor signalling whose continuous activation can promote malignancy (Kohlhof, Hampel et al. 2009). The EBNA2 acidic domain interacts with TFIIB, TAF40, TFIIH, TBP, p100, p300/CBP and EBNA LP that in turn stimulates its transcriptional activity (Harada, Yalamanchili et al. 2001). In fact, EBNA2 initiates a cascade of transcriptional events that is broadened by the action of transcriptional effectors activated by target genes such as LMP1 or c-Myc. These transcriptional

effectors then, activate various cellular genes that are involved in the process of B cells transformation.

1.1.7 EBNA 3A, 3B and 3C

EBNA3A, 3B and 3C are transcriptional regulators that are encoded by three adjacent genes within the EBV genome. EBNA3A and EBNA3C are required for *in vitro* B-cell transformation, whereas EBNA3B is dispensable (Tomkinson, Robertson et al. 1993). The EBNA3 proteins are similar to EBNA2 in stably interacting with RBP-Jκ. Therefore, EBNA3s compete with EBNA2 and Notch for binding to RBP-Jκ and thereby regulate the transcriptional activity of EBNA2. Two to three fold overexpression of EBNA3A in LCL is sufficient to dissociate RBP-Jκ from the strong upregulatory effects of EBNA2, downregulation of c-Myc and eventually cell growth arrest (Cooper, Johannsen et al. 2003). EBNA3A and EBNA3C have repressive effects on the Cp promoter (Radkov, Bain et al. 1997; Cludts and Farrell 1998). EBNA3C has direct upregulatory effect on the CD21 promoter and down- or upregulatory effects on the viral LMP1 promoter (Lin, Johannsen et al. 2002). Furthermore, EBNA3C interferes with the regulation of the cell cycle progression by promoting the SCF^{SKP2} mediated degradation of p27 and pRb and cyclin A dependent kinase activity leading to cell cycle progression (Maruo, Wu et al. 2006). Degradation of p27 was shown to cause accumulation of bi- or multinucleated cells and abrogate the mitotic spindle checkpoint in NIH3T3 fibroblasts (Parker, Touitou et al. 2000).

1.1.8 EBNA LP

EBNA LP or EBNA5 is also called as leader protein because it is encoded by the 5` un-translated leader of the EBNA2 primary transcript (Wang, Petti et al. 1987). EBNA LP potentiates EBNA2 mediated transcription from the Cp and LMP1 promoters. It has minimal effects alone and requires EBNA2 for activation of specific promoters. EBNA LP forms a complex with a cellular anti-apoptotic protein BCL-2 or its EBV counterpart, BHRF1 and thus plays a role in the regulation of cell death by apoptosis in EBV infected cells (Matsuda, Nakajima et al. 2003). EBNA LP can also interact with the PML nuclear body associated protein SP100 and displaces SP100 along with HP1a from nuclear bodies (Szekely, Pokrovskaja et al. 1996).

1.1.9 LMP1

LMP1 is considered as a viral oncogene, and it is required for EBV associated B cell transformation and transforms NIH3T3 and Rat-1 fibroblasts (Wang, Liebowitz et al. 1985; Baichwal and Sugden 1988). LMP1 is an integral membrane protein that acts as a constitutively active tumour necrosis factor (TNF) receptor, similar to CD40 and hence

mimics the cellular growth signal that normally results from binding of the CD40 ligand (Gires, Zimber-Strobl et al. 1997). The carboxy-terminal tail of LMP1 contains two signalling domains known as carboxy-terminal activating regions (CTAR1 and CTAR2) that mediate signalling by directly interacting with TNFR-associated factors (TRAF) through CTAR1 and TNFR-associated death domain (TRADD) through CTAR2 (Mosialos, Birkenbach et al. 1995). Signalling through the CTAR domains activates a variety of signalling pathways, including NF-κB, MAPK kinase, c-Jun N-terminal kinase (JNK), and PI3K kinase through which LMP1 induces many of the changes associated with EBV infection of B lymphocytes (Devergne, Hatzivassiliou et al. 1996; Eliopoulos, Blake et al. 1999; Mainou, Everly et al. 2005). LMP1 promotes cell clumping, increased villous projections, intracellular free calcium, vimentin and IL-10 expression (Kieff and Rickinson 2007). LMP1 functionally activates homotypic adhesion through upregulation of intracellular adhesion molecule-1 (ICAM-1) and leukocyte function associated antigens (LFA-1 and -3). LMP1 increases cell surface markers, including HLA-II, CD21, CD23, CD39, CD40 and CD44 and downregulates CD10 and BCL-6 (Wang, Liebowitz et al. 1988). LMP1 also causes overexpression of proteins like BCL-2 and A20 that protects the infected cells from p53 mediated apoptosis (Fries, Miller et al. 1996; Wang, Rowe et al. 1996).

1.1.10 LMP2A, 2B

LMP2A and 2B are integral membrane proteins that differ in their N terminal domains. The N terminal cytoplasmic domain of LMP2A contains an immunoreceptor tyrosine-based activation motif that interferes with signal transduction through interaction with the B lymphocyte Src family of tyrosine kinases (Fruehling and Longnecker 1997). LMP2A inhibits signal transduction from surface immunoglobulin and blocks virus reactivation following surface immunoglobulin cross-linking (Miller, Lee et al. 1994). LMP2A allows surface immunoglobulin negative cells to colonize peripheral lymphoid organs in a transgenic mouse model suggesting that it may affect B-cell development and survival in the absence of normal B cell receptor signals (Caldwell, Wilson et al. 1998). It has been shown that LMP2A expression also leads to transcriptional repression of human telomerase reverse transcriptase (hTERT) in epithelial cells (Chen, Liu et al. 2005).

1.1.11 EBERs

EBER1 and 2 are non-polyadenylated, uncapped and non-coding RNAs that are expressed in all EBV infected cells with the exception of oral hairy leukoplakia lesions from AIDS patients and some EBV positive hepatocellular carcinomas (Rowe 1999; Sugawara, Mizugaki et al. 1999). EBER1 and EBER2 are transcribed by cellular RNA polymerase III and associated with interferon inducible protein kinase (PKR), which

exerts a potent antiviral activity. Binding of dsRNA to PKR activates its kinase activity resulting in inhibition of protein synthesis and induction of apoptosis of the viral infected cells. Binding of EBER1 to PKR blocks its activation and promotes resistance of the cell to Fas-mediated apoptosis (Nanbo, Yoshiyama et al. 2005). PKR activity is also regulated during progression of the cell cycle, suggesting a potential mechanism by which EBER RNAs may affect cell growth control (Zamanian-Daryoush, Der et al. 1999).

1.1.12 BARTs and MicroRNAs

BARTs are alternatively spliced and polyadenylated RNAs that are transcribed in the rightward direction from the BamHI-A region of the viral genome. BARTs are expressed in all EBV-associated tumors as well as in latently infected B cells. The antisense nature of the BARTs relative to the lytic genes of this region suggests that the BARTs might serve as regulatory RNAs to limit lytic gene expression and promote maintenance of the viral latency program (Karran, Gao et al. 1992). Analysis of the structure of individual BARTs has identified a number of potential open reading frames (ORFs) including BARF0, RK-BARF0, A73, and RPMS1 (Smith, de Jesus et al. 2000) There has been some evidence of immune response to the protein products of BART RNAs, but so far there is no evidence of protein expression in a natural EBV(+) carriers (Al-Mozaini, Bodelon et al. 2009).

MicroRNAs (miRNA) are small (~ 22 nt) non-coding RNAs derived from maturation of large primary transcripts and are able to regulate gene expression by mRNA cleavage or translational inhibition. EBV encodes two clusters of miRNAs expressed from the BHRF1 (BamHI fragment H rightward open reading frame 1) and BART regions. BHRF-1 gene codes for three precursors (miR-BHRF1-1, -2 and -3) with four mature miRNAs while the intronic regions of BART codes for twenty-two precursors (miR-BART1 to miR-BART22) with forty mature miRNAs (Chen, Chen et al. 2010). The expression pattern of EBV miRNAs depends on the type of cell line and pattern of latent gene expression. In NPC cell lines, BART miRNAs are robustly expressed, whereas they are less abundant in the majority of B cell derived cell lines. In contrast, BHRF1 miRNAs are not detectable in NPC cell lines, but are expressed in B cells (Cai, Schafer et al. 2006). Several functional targets have been identified for the EBV miRNAs. For instance, BART miRNAs have been shown to suppress the expression of LMP1 and modulate the NF-κB pathway (Lo, To et al. 2007). Moreover, the p53 upregulated modulator of apoptosis (PUMA) is downregulated by the miRNA BART5 (Choy, Siu et al. 2008).

1.1.13 The mechanism of EBV persistence

EBV is shed into and spreads via saliva. Entry is likely to occur in the lymphoepithelium of the Waldeyers ring at the back of mouth, where the virus infects resting naïve B cells and causes them to become blasts through expression of the growth program (Latency III). The epithelium is assumed to be the site of lytic infection that leads to amplification of the virus. In the infected B cells the EBV genome circularizes and transcription of the EBNA genes initiate at the W promoter (Wp). The relatively low level of EBNA2 expressed under this condition is sufficient to activate the resting B cells, which promotes activation of stronger C promoter (Cp) (Woisetschlaeger, Yandava et al. 1990), which sustains the expression of the Latency III program. This newly infected tonsillar B cells are thought to enter the follicle, where they switch to express the default program (Latency II) and undergo the germinal center (GC) differentiation process. The GC is the region where antigen-activated B cells undergo proliferation, class switch recombination (CSR), somatic hypermutation (SHM), antigen selection and affinity maturation (Leanderson, Kallberg et al. 1992; Liu and Arpin 1997).

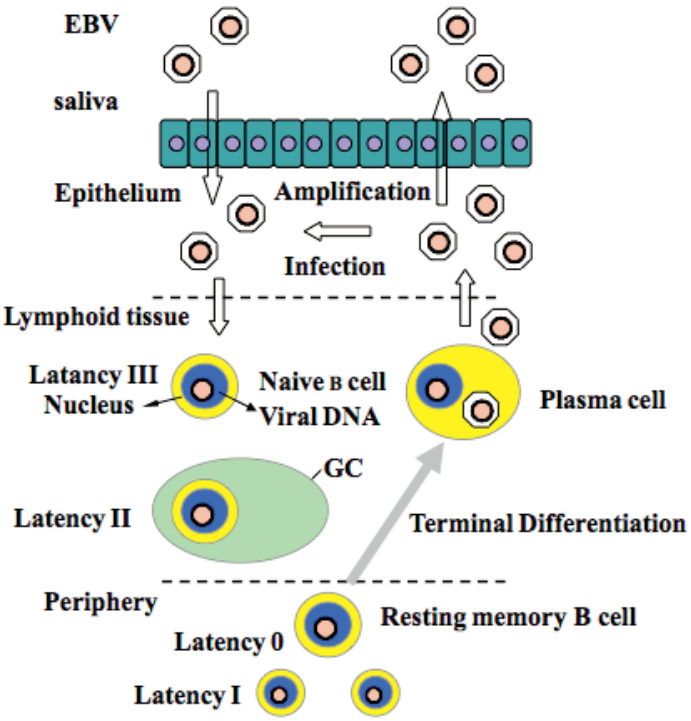

Figure 4: EBV life cycle and maintenance of persistent infection

In order to differentiate in the follicle, the newly infected cells must receive a signal to turn off EBNA2 and the growth program (latency III). This allows the infected cell to assume a GC phenotype and differentiate into memory cells. The default program (latency II) provides rescue or survival signals that allow the cells to exit the GC and enter the peripheral circulation as a resting memory B cells expressing latency 0 program or occasionally latency I program during cell division. The memory cells eventually return to the tonsil, where they undergo plasma cell differentiation, which triggers viral replication. The produced new virus may be shed into saliva or may infect other B cells (Figure 4) (Thorley-Lawson and Allday 2008) .

1.1.14 Reactivation of virus from latency

EBV reactivation is associated with the pattern of viral gene expression classified as immediate early, early and late genes (Figure 5). The expression of EBV immediate early genes, including BZLF1 and BRLF1 is consistent with the activation of transcriptional co-activators of the lytic cycle gene expression (Tsurumi, Fujita et al. 2005). Cells permissive for EBV replication exhibit characteristic herpes virus related cytopathic changes, including the formation of an intranuclear inclusion, margination of nuclear chromatin, assembly of capsids within the nucleus, budding of virus through the nuclear membrane and formation of cytoplasmic vesicles containing enveloped virus. Only a few cells within human B lymphocyte derived cell lines (Lymphoblastoid cell lines or LCL) are permissive for EBV replication while EBV positive Burkitt's lymphoma (BL) cell lines are less permissive than *in vitro* infected BL cells (Kieff and Rickinson 2007). Although the epithelium of the oropharynx is assumed to be the primary site for EBV replication, patients with B-cell-deficiencies show no evidence of even transient EBV infection in the throat, suggesting that acquisition of EBV by the naïve host dependents on initial B-cell infection (Faulkner, Burrows et al. 1999).

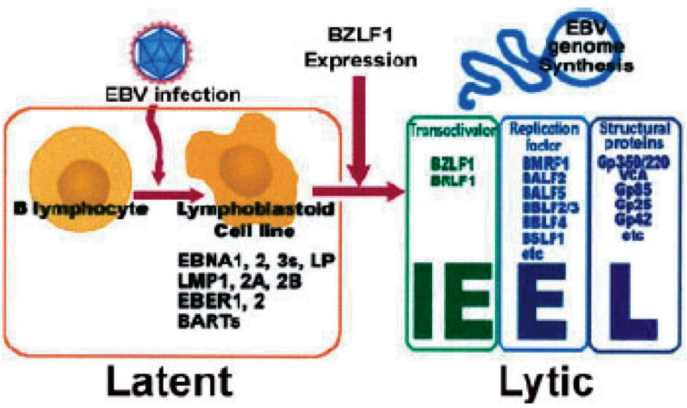

Figure 5: Switch from latent infection to lytic phase (Tsurumi, Fujita et al. 2005)

1.1.15 Mechanisms of EBV associated telomere homeostasis

The activation of telomerase is essential for cell immortalization and malignant transformation (Wai 2004). EBV employs three viral proteins, LMP1, LMP2A and EBNA2 to control the expression of the hTERT gene. LMP1 induces NF-κB leading to activation of the c-Myc gene that in turn stimulates the transcription of hTERT. NF-κB p65 may also facilitate translocation of hTERT into the nucleus through protein-protein interaction. In contrast, LMP2A is a negative regulator of the hTERT. LMP2A has been implicated in the Syk kinase dependent activation of ERK. ERK causes phosphorylation and activation of the transcription factor AP-1 that represses the expression of hTERT. EBNA2 induces transactivation of c-Myc through Notch signalling that mediates activation of the transcription factor CBF1 that in turn activates the hTERT gene. EBNA2 can also activate hTERT by transactivation of LMP1 (Reviewed by Liu, Cassar et al. 2006). Although activation of telomerase is a prerequisite for the long term maintenance of EBV transformed LCLs (Sugimoto, Ide et al. 2005), the upregulation of hTERT is a late event in B-lymphocytes immortalization and some LCLs were shown to maintain a constant telomere length for over 150 generations *in vitro* in the absence of detectable telomerase activity (Sugimoto, Ide et al. 1999). Furthermore, analysis of telomerase activity in a panel of EBV positive and negative B-lymphoma lines failed to demonstrate a consistent effect of EBV carriage (Mochida, Gotoh et al. 2005). Thus, EBV may utilize telomerase independent mechanisms for preserving telomere integrity.

1.1.16 EBV associated malignancies

EBV is linked to several malignant diseases, including Burkitt`s lymphoma (BL), nasopharyngeal carcinoma (NPC), Hodgkin's diseases (HD), a subset of gastric carcinoma and T cell lymphoma. EBV is also associated with lymphomas in immuno-compromised individuals such as post transplant lymphoproliferative diseases (PTLD), X-linked lymphoproliferative disorders and AIDS related lymphoproliferative disorders (Kieff and Rickinson 2007).

1.1.16.1 Burkitt`s Lymphoma (BL)

Burkitt's lymphoma cells carry chromosomal translocations between chromosome 8 and either chromosomes 14, 2, or 22 that juxtapose the c-Myc oncogene (chromosome 8) to the enhancer region of transcriptional active immunoglobulin heavy chain (chromosome 14) or light chain (chromosomes 2 or 22) loci resulting in deregulation of c-Myc expression (Zimonjic, Keck-Waggoner et al. 2001). The mechanism behind this translocation is not clearly known but activation-induced cytidine deaminase (AID) was shown to induce chromosome breaks during antibody class switch or somatic

hypermutation (Robbiani, Bothmer et al. 2008). In addition, overexpression of AID was shown to induce IgH:Myc translocations in murine B-cells stimulated with lipopolysaccharide (LPS) and Interleukin (IL)-4 (Ramiro, Jankovic et al. 2006) indicating that the translocations can be generated by activation of AID.
Three distinct forms of BL endemic, sporadic and HIV-associated have been described that differ in their geographic distribution, site of presentation, and EBV carriage but shares similar histological features and carry chromosomal translocations. The endemic form is associated with EBV and is predominant in the Africa equatorial belt. The other forms occur worldwide (Thorley-Lawson and Allday 2008; Bornkamm 2009). The role of EBV in the pathogenesis of BL is not clearly known but it was shown that infection of human B cells with Epstein-Barr virus results in activation-induced cytidine deaminase (AID) and polymerase-eta (pol-eta) gene expression that play important roles in the DNA-modifying processes involved in immunoglobulin gene class switch recombination and somatic hypermutation suggesting that the virus may promote oncogene translocation including the IgH:Myc in BL (Epeldegui, Hung et al. 2007).

1.1.17 EBV and tumorigenesis

Although EBV is associated with several malignant diseases, its contribution to tumorgenesis remains controversial. The first indication supporting the role of EBV in tumorgenesis is the clonal expansion of EBV positive tumor cells suggesting that the tumor is originated from an EBV(+) precursor whose selection may be promoted by the expression of viral proteins. The second indication is the capacity of the virus to transform human B cells *in vitro* (Pope, Horne et al. 1968) and predisposition of immuno-compromised patients for EBV mediated lymphomas that are aggressive and often fatal (Hopwood and Crawford 2000). Moreover, viral latent products can affect various aspects of malignant growth such as induction of proliferation and inhibition of apoptosis, deregulation of the cell cycle and induction of genomic instability.
However, there are counter arguments that do not supporting this scenario. For instance, EBV-associated malignancies can also exist as EBV(-) variants suggesting that they may originate from different precursors. EBV associated lymphoma in immuno-suppressed patients are frequently polyclonal and regress upon reconstitution of EBV specific immune responses (Cohen, Bollard et al. 2008). In addition, EBV(+) tumor cells are phenotypically different from LCL cells and do not express several of the viral proteins required for growth transformation.
Taken together, it is assumed that EBV may contribute to oncogenesis by the fixation of genetic and epigenetic changes that may promote cell growth through protecting the tumor cells from growth restriction.

1.2 GENOMIC INSTABILITY AND CANCER

Genomic instability, defined as the establishment of a mutator phenotype that promotes the fixation of multiple genetic changes required for the evolution of a pre-malignant cell clone to invasive cancers, is a hallmark of malignant transformation and tumor progression (Raptis and Bapat 2006). Two major types of genetic alterations are common in malignant cells, microsatellite instability (MIN), that is well characterized in colorectal and endometrial carcinoma and affects chromatin structure and gene expression (Modrich 1994), and chromosome instability (CIN), that is characterized by losses of entire or large portions of chromosomes, resulting in aneuploidy, translocations and loss of heterozygosity (LOH) (Honma 2005).

1.2.1 Chromosome instability (CIN)

Chromosome instability is characterized by the occurrence of clonal and non-clonal cytogenetic aberrations. Clonal chromosome aberrations such as reciprocal translocations, deletions, inversions and duplications can be transmitted to the progeny while non-clonal aberrations, including dicentric chromosomes, rings, fragments, satellite associations, double minutes and chromatid gaps are often lethal and are therefore generated *de novo* at each cell cycle (Figure 6).

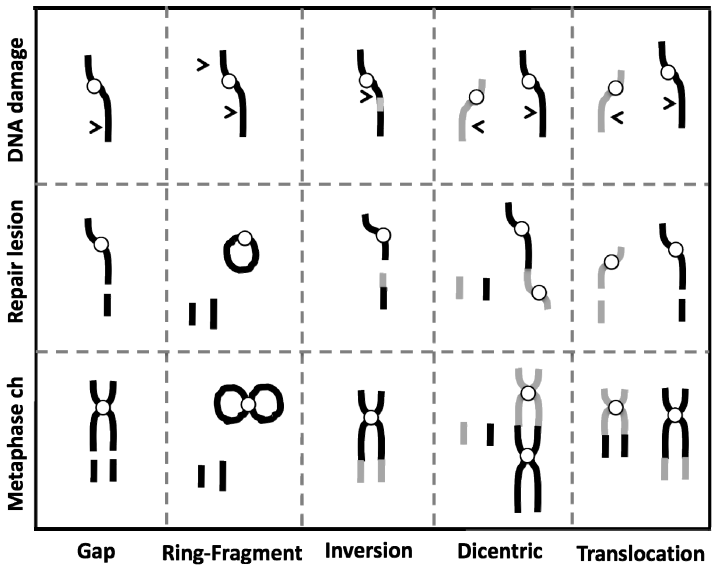

Figure 6: Origin of chromosome structural abnormalities

Dicentric chromosomes can initiate chromosomal instability via breakage bridge fusion (BBF) cycles in which two centromeres of a dicenteric chromosome are pulled to opposite poles during mitotic segregation and break (McClintock 1941). These breaks

can generate telomere free ends and new chromosome fusions, nonreciprocal translocations and genetic changes that contribute to genomic instability. Telomere dysfunction is also known as an important source of genomic instability (Cheung and Deng 2008).

1.2.2 EBV and Genomic Instability

Cytogenetic and molecular evidence support the involvement of genomic instability in the development of EBV-associated malignancies. Both clonal and non-clonal chromosomal aberrations have been observed in EBV carrying BLs and in other EBV associated tumors, including NPC, Hodgkin's disease (HD) and gastric carcinoma. The most frequent genomic instability in NPC is a nonrandom deletion of the short arm of chromosome 3 at loci 3p25 and 3p14 and induction of Micronuclei (MN) formation. Allelic losses were observed on chromosomes 1p, 3p, 9p, 9q, 11q, 13q, 14q, 16q, and 19q, and minimum deletion regions were also observed in primary NPC by LOH (loss of heterozygosity) and CGH (comparative genomic hybridization) analysis (Shao, Zeng et al. 2002). Analysis of genomic aberrations in gastric carcinomas has shown that gain in chromosome 11 and loss in 15q15 are more common in cases associated with EBV (Chan, Liu et al. 2002). The contribution of the virus to the type of aberrations has not been recognized but increasing evidence indicates that LMP1 and EBNA3C may play an important role in induction of genomic instability. LMP1 promotes genomic instability by inhibiting DNA repair through downregulation of ATM, failure to phosphorylate Chk2 and inactivation of the G2 checkpoint (Gruhne, Sompallae et al. 2009). The capacity of LMP1 to inhibit ATM in B cell lines is in line with the reported loss of ATM in EBV positive nasopharyngeal carcinoma (Bose, Yap et al. 2009). EBNA3C has also been shown to promote the accumulation of chromosomal abnormalities through disruption of the spindle checkpoint (Parker, Touitou et al. 2000).

1.3 DNA DAMAGE RESPONSE AND CANCER

Each cell in the human body receives plenty of DNA lesions per day. These lesions can block genome replication and transcription, and improper repair may lead to mutations and genomic aberrations that increase the risk of cancer (Kawanishi, Hiraku et al. 2006). Endogenous or exogenous DNA damaging events can induce DNA damage. Physiological processes such as DNA mismatch during DNA replication, abortive topoisomerase activity, hydrolytic reactions, non-enzymatic methylations and reactive oxygen species (ROS) are endogenous sources that can generate DNA lesions. In contrast, ultraviolet light, ionizing radiation and genotoxic chemical agents are exogenous sources of DNA damage (Jackson and Bartek 2009). The DNA damage response is executed through a series of steps. The initial step is detection of DNA

lesions by sensor proteins that recognize either the lesions themselves or chromatin alterations. Sensor proteins transmit the information to transducer proteins such as ATM and ATR, which control the damage response through the phosphorylation of effector proteins leading to modulation of numerous pathways involved in DNA repair (Barzilai and Yamamoto 2004). Histone H2AX, MDC1, BRCA1, 53BP1 and NBS1 are all targets for ATM- or ATR-mediated phosphorylation and involve in transmission of the DNA damage signal to downstream molecules such as Chk1 and Chk2. The Chk1 and Chk2 kinases phosphorylate effector proteins such as p53, CDC25A, and CDC25C and thereby delay cell cycle progression by activation of the G1–S, intra-S, or G2 cell cycle checkpoints. Thus, these DNA damage checkpoint mechanisms cooperate with DNA repair machinery to suppress genomic instability and cancer (Figure7) (Reviewed by Motoyama and Naka 2004).

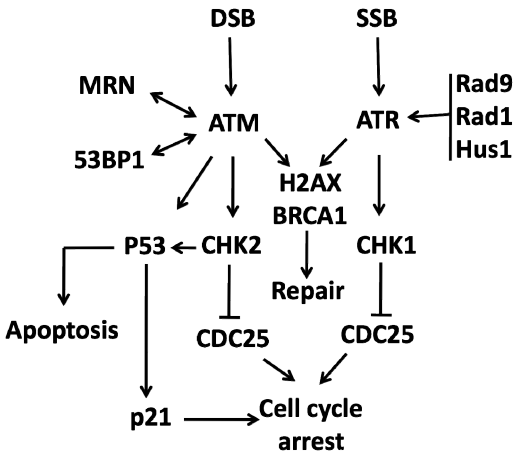

Figure 7: DNA damage and activation of checkpoint

The DNA damage response activates different types of repair responses including base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination (HR) and non-homologous end joining (NHEJ) that are activated by different types of DNA lesion (Jackson and Bartek 2009).

1.3.1 Double Strand Break Repair

The double strand breaks (DSBs) repair is important for cell survival, for maintenance of genomic integrity and for prevention of tumorgenesis. DSBs can be generated by either exogenous agents such as ionizing radiation and chemotherapeutic agents or endogenous mechanisms including class-switch processes and variable (diversity) joining [V(D)J] required for the maturation of B and T lymphocytes. Two mechanisms have been evolved for DSBs repair: homologous recombination (HR) and non-homologous end joining (NHEJ) (Li and Heyer 2008; Lieber, Lu et al. 2008).

1.3.1.1 Homologous Recombination (HR)

Homologous Recombination (HR) is a highly accurate repair pathway that utilizes a homologous template sequence to restore information lost at the site of a break (West 2003). It is predominantly active during the S and G2 phases of cell cycle when the sister chromatid is available as a template. HR is also used to restart stalled replication forks and to repair interstrand DNA crosslink. An initial step in HR is the generation of a 3`single stranded overhang followed by 5`to 3`resection of the DNA ends at the site of break by the MRN (Mre11-Rad50-Nbs1) complex. These single strand ends will be coated with RPA a single strand binding protein followed by recruitment of BRCA2, RAD51 and its paralogs including RAD51 (B, C, D) and XRCC2 that promote replacement of RPA and invasion of single strand into homologous duplex DNA. Following strand invasion, DNA synthesis elongates the invading strand using an intact strand as template. This causes displacement of the original strand and the formation of a Holliday Junction (HJ). The invading strand can be displaced and repaired by single strand annealing (SSA), which is also called synthesis-dependent strand annealing (SDSA) otherwise double HJs will be generated. Resolution of HJ can occur either with or without crossing over (Figure 8) (Penny A. Jeggo 2005). RAD51 and XRCC3 are proteins required for branch migration and HJ resolution (Liu, Masson et al. 2004).

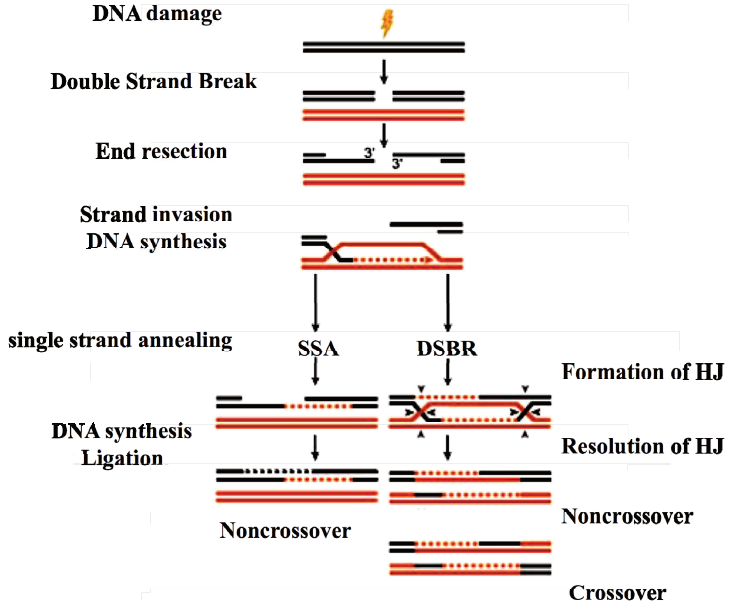

Figure 8: Pathways of DNA double-strand break repair by homologous recombination and.resolution of HJ (San Filippo, Sung et al. 2008)

1.3.1.2 Non-Homologous End Joining (NHEJ)

NHEJ is error prone mechanism in which two DNA ends simply rejoin. This process may result in the loss of nucleotides on each site of the break (Burma, Chen et al. 2006). NHEJ is active throughout of the cell cycle but is thought to be the dominating pathway in G1 when no sister chromatid is available as template for homologous recombination (Ferreira and Cooper 2004). NHEJ between chromosomes results in genomic instability, mitotic catastrophe and frequently cell death (Karlseder, Broccoli et al. 1999). NHEJ is critical not only for the repair of pathologic DSBs as in chromosomal translocations, but also for the repair of physiologic DSBs created during variable (diversity) joining [V(D)J] recombination and class switch recombination (CSR). Therefore, patients lacking normal NHEJ are sensitive to ionizing radiation (IR) and are also severely immunodeficient (Lieber 2010). Important factors involved in NHEJ are the Ku complex, a heterodimer composed of two subunits of Ku70 and Ku80, DNA dependent protein kinase (DNA-PK), XRCC4 and DNA ligase IV. Ku heterodimers encircle the DNA at the site of break and recruit DNA-PK. DNA-PK bridges the ends of the broken sites and allows DNA ligase 4 and its cofactor XRCC4 to carry out the rejoining of break ends (Jeggo 2005). Artemis is a nuclease that functions in end processing step prior to rejoining (Rassool 2003).

1.4 TELOMERES

The ends of linear chromosomes resemble naturally double strand break (DSBs) and must be protected to ensure the stable maintenance of the genome (Cesare and Reddel 2008). The linearity of DNA makes some problems in the termini including protection against nucleolytic attacks, imprecision of end replication and inappropriate DNA repair (McEachern, Krauskopf et al. 2000). The solution for these problems is provided by nucleoprotein structures called telomeres. They are composed of repetitive hexanucleotide sequences (TTAGGG)n of double stranded DNA terminating with a single stranded 3`overhang, which folds back into the double stranded telomere to form a t-loop structure (Tomaska and Nosek 2009). Telomeres are normally protected by a nucleoprotein DNA complex that contains proteins termed the shelterin complex including TRF1, TRF2, TIN2, TPP1 (PTOP), RAP1 and POT1. In vertebrates, double stranded telomeric repeats are directly bound by the homeodomain proteins TRF1 and TRF2, which recruit the TIN2 protein that via TPP1 can form a bridge with POT1, the protein that binds to the single stranded 3`overhang at the end of the G-rich strand (Figure 9) (de Lange 2005).

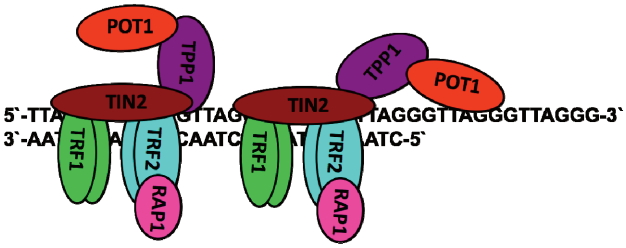

Figure 9: Structure of telomeres capped with shelterin complex

Telomeres also contain non-coding RNA referred to as Terra, which interacts with several telomere associated proteins including TRF1, TRF2 and subunits of the origin recognition complex (ORC), heterochromatin protein 1 (HP1) and histone H3 trimethyl K9 (H3 K9me3). Terra plays a crucial role in telomere structural maintenance and heterochromatin formation (Deng, Norseen et al. 2009).
Telomeres become shorter with each cell division because the DNA polymerase can only synthesize in 5`to 3` direction and so the 5`end of the lagging strand will be shortened when the terminal Okazaki`s fragment RNA primer is eliminated. This shortening has been proposed to be a mitotic clock that sets the limit of a cell life span and so the mechanism that regulates the number of cell division before entering senescence (Harley, Futcher et al. 1990; Harley 1991).

1.4.1 Telomere associated Genomic Instability

Telomere dysfunction is an important source of genomic instability (Cheung and Deng 2008). The chromosome ends become substrate for double strand break repair when the telomeres are damaged due to attrition of telomeres or when the shelterin complex is inhibited (de Lange 2005). When the length of telomeres falls below a critical threshold, a p53-dependent DNA damage response induces growth arrest, senescence and eventually apoptosis. In the absence of p53, dysfunctional telomeres become substrates of double stranded break (DSBs) repair that can mediate the generation of dicenteric chromosomes via non-homologous end joining (Batista and Artandi 2009). This type of telomere driven genomic instability leads to chromosome rearrangements through non-reciprocal translocations (Figure 10) (Mathieu, Pirzio et al. 2004).
Among the shelterin complex, TRF2 was shown to play an important role in the protection of telomeres against the activation of DSB repair. TRF2 is required for t-loop formation through telomere overhang processing by nuclease and inhibition of NHEJ pathway. Loss of TRF2 leads to a telomere deprotection phenotype that results in activation of the ATM kinase and accumulation of DNA damage response factors such as 53BP1, pH2AX and the Mre11 complex at chromosome ends and generation of telomere–telomere fusions via the NHEJ pathway (Celli and de Lange 2005). Although

the t-loops protect telomeres from NHEJ, they are at risk of being detected by homologous recombination if a Holiday junction is formed. It has been shown that telomeres undergo homologous recombination in cells that express a truncated form of TRF2. TRF2 can repress t-loop homologous recombination (Wang, Smogorzewska et al. 2004; Poulet, Buisson et al. 2009). Deletion of either TRF2, Rap1 or POT1 proteins are associated with activation of HR at telomeres which can be manifested by exchanges between sister telomeres (T-SCEs) (Kabir, Sfeir et al. 2010).

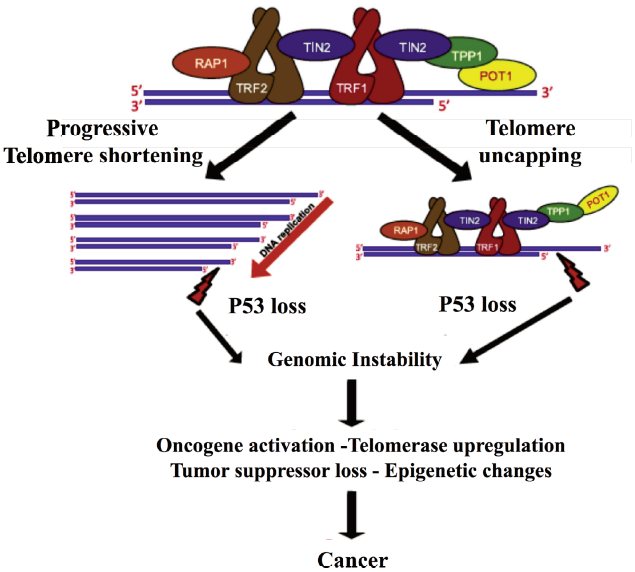

Figure 10. Illustration of telomere dysfunction, genomic instability and carcinogenesis (Batista and Artandi 2009)

Inhibition of either POT1 or TPP1 leads to a DNA damage response at telomeres due to displacement of these proteins from the telomere overhang. In contrast to depletion of TRF2, which activates ATM, removal of POT1 from telomeres initiates an ATR-dependent DNA damage response. (Guo, Deng et al. 2007; Hockemeyer, Palm et al. 2007). Depletion of Terra can also cause an increase in telomere dysfunction-induced foci and aberrations in metaphase telomeres (Deng, Norseen et al. 2009).

1.4.2 Telomerase dependent telomere elongation

Telomere shortening can be compensated by the activity of telomerase, a unique reverse transcriptase that adds TTAGGG repeats to the 3′ ends of telomere upon DNA synthesis (Blackburn, Chan et al. 2000). In human somatic cells telomerase is inactive but over 90% of cancer cells express high telomerase activity (Shay and Bacchetti 1997). The catalytic core of telomerase enzyme is composed of two subunits, a protein subunit with retrotranscriptase activity (hTERT) and a RNA subunit (TERC) with a sequence complementary to the telomere sequence which is used as a template to

synthesize telomeric sequences by reverse transcription at the telomeres (Feng, Funk et al. 1995). Telomerase is regulated at individual chromosome ends through telomere-binding proteins to mediate telomere length homoeostasis. For example, TRF1 is thought to be an inhibitor of telomere length through either emitting a negative signal to telomerase or binding to telomeres, thus inhibiting telomerase access to its substrate (van Steensel and de Lange 1997). The binding of TRF1 to telomeres is controlled by tankyrase, a telomeric PARP that modifies TRF1 by the addition of ADP-ribosylation polymers and thus inhibiting the capability of TRF1 to bind telomeric DNA and promoting telomere elongation (Donigian and de Lange 2007).

1.4.3 Alternative Lengthening of Telomeres

Telomeres can be maintained also by a homologous recombination based mechanism termed alternative lengthening of telomeres (ALT) that is active in ~15% of telomerase negative tumors (Cesare and Reddel 2008). There is evidence of co-existence of both telomere maintenance mechanisms in the same cell line (Johnson, Varkonyi et al. 2005). Moreover, it is known that ALT(+) primary tumors can cause telomerase positive secondary tumors and vice versa (Henson, Neumann et al. 2002). The main feature of ALT is a high degree of heterogeneity in telomere size, ranging from almost undetectable to extremely long telomeres, with more than 50 kb (Bryan, Englezou et al. 1995) and the presence of ALT associated promyelocytic leukemia (PML) nuclear bodies (APBs) containing telomeric DNA, telomere specific proteins (the shelterin complex) and other proteins involved in DNA replication, recombination and repair (Jiang, Zhong et al. 2009). Furthermore, ALT(+) cell lines contain a significant number of extra-chromosomal telomeric DNA repeats (ECTR) that can be linear or circular (Cesare and Griffith 2004). DNA-damaging agents such as H2O2 can promote the formation of APBs and ECTR in ALT(+) cells (Fasching, Neumann et al. 2007) indicating that ALT is a DNA damage response at telomeres. Another feature of ALT(+) cells is an increase in sister chromatid exchange (T-SCE) that is consequence of homologous recombination repair (Londono-Vallejo, Der-Sarkissian et al. 2004). The exact role of APBs in ALT is still unknown but APBs have been shown to contain recombination proteins that are required for telomere maintenance, so it is assumed that ALT activity occurs within these nuclear domains (Yeager, Neumann et al. 1999).

1.4.4 Telomere proteins in EBV episomal maintenance and replication

Episomal maintenance and DNA replication of EBV genome from the origin of replication (*OriP*) depend on the viral encoded *OriP* binding protein, EBNA1, and several cellular factors in particular, telomere associated proteins, including TRF1, and TRF2, the TRF2-interacting protein hRap1, telomere-associated poly (ADP-ribose)

polymerase (Tankyrase). These cellular proteins bind to the dyad symmetry (DS) element of *OriP* in an EBNA1-dependent manner and regulate DS-dependent DNA replication and *OriP* plasmid maintenance. The TRF binding site on *OriP* is three telomere like nonamer sites (TTAGGGTTA) flanked by EBNA1 binding sites that contributes to DNA replication efficiency and episome maintenance and RNA-dependent recruitment of the origin recognition complex (ORC) (Norseen, Thomae et al. 2008). In addition, telomere-associated poly-ADP ribose polymerase (TNKS) binds directly to EBNA1 through two RXXPDG-like motifs and downregulates *OriP* dependent replication and plasmid maintenance and this inhibition correlates with poly-ADP ribosylation (PARsylation) of EBNA1 (Deng, Lezina et al. 2002; Deng, Atanasiu et al. 2003; Deng, Atanasiu et al. 2005). PARP1 is also associated with *OriP* replication activity through PARsylation of EBNA1 and adjacently bound TRF2 that induce structural changes at DS element, thus reducing the DNA binding affinity of EBNA1 and functional recruitment of origin recognition complex 2 (ORC2) and mini-chromosome maintenance complex 3 (MCM3) and abrogates *OriP* dependent replication and maintenance of EBV genome (Tempera, Deng et al. 2010).

1.5 OXIDATIVE STRESS AND CANCER

1.5.1 Oxidative Stress

Oxidative Stress is a hallmark of many tumors and is caused by an imbalance between the generation and clearance of Reactive Oxygen Species (ROS) in the cells leading to induction of genomic instability and activation of signalling pathways required for tumorigenesis and metastasis (Figure 11) (Weinberg and Chandel 2009).

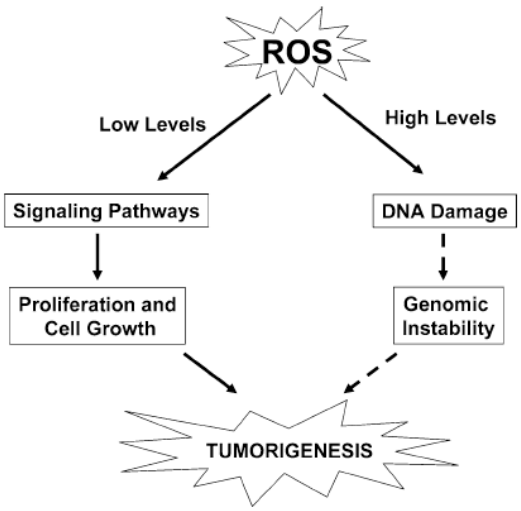

Figure 11: Contribution of ROS to tumorigenesis (Weinberg and Chandel 2009)

1.5.2 Reactive Oxygen Species (ROS)

Reduction of oxygen results in the production of reactive oxygen species (ROS) including superoxide (O2•-), hydrogen peroxide (H2O2) and hydroxyl radical (OH •). Superoxide dismutases (SODs) convert superoxide to hydrogen peroxide, which can be neutralized by glutathione, and catalase to water. Hydrogen peroxide is also converted to hydroxyl radicals through Fenton reaction (H202 + Fe2+ → HO· + OH- + Fe3+ and Fe3+ + H2O2 → Fe2+ + OOH· + H+) catalyzed by transition metals such as Fe2+, Cu2+, Cr(V) and Ni (Stohs and Bagchi 1995; Thannickal and Fanburg 2000; Leonard, Harris et al. 2004).

Cellular organelles that consume oxygen such as mitochondria and peroxisomes are endogenous sources of ROS. Complex I and III of the mitochondrial electron transport chain is involved in the majority of superoxide production in this organelle while superoxide anion can be also enzymatically produced by NADPH oxidase, xanthine oxidase, lipoxygenases and cylooxgenases (Figure 12) (Curtin, Donovan et al. 2002).

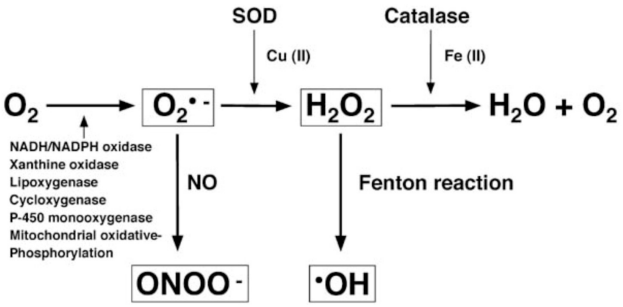

Figure 12: Intracellular generation of oxidant

Inflammatory cells including neutrophils, eosinophils and macrophages contribute to production of cellular ROS through NADPH oxidase. The NADPH oxidase is an enzymatic source of superoxidase, which catalyzes the production of superoxide from oxygen and NADPH. The NADPH oxidase family includes the NOX (NOX1-5), and DUOX1-2 family members and is composed of two membrane-bound components consisting of a catalytic NOX2 (also known as gp91phox) and p22phox subunits (Babior 1999). Ionizing radiation, environmental agents such as metal ions and phorbol esters and therapeutic agents are the external sources for production of ROS. Nitric oxide (NO) is also a reactive species and interacts with O2•- to generate the peroxynitrite anion (ONOO-) whose reactivity is roughly similar to OH• and NO2 (Valko, Rhodes et al. 2006).

ROS plays important roles in the modulation of gene expression. ROS can release calcium from intracellular stores, which results in the activation of kinases such as protein kinase C (PKC) that regulates a variety of cell functions (Wu 2006).

Furthermore, cellular oxidants are associated with activation of transcription factors such as Nrf2, mitogen-activated protein (MAP) kinase/AP-1 and NF-kB pathways as well as hypoxia inducible transcription factor α (HIF-1α) (Trachootham, Lu et al. 2008).

1.5.3 Oxidative Stress and Genomic Instability

The production of ROS is a major cause of DNA damage that leads to mutations. ROS can attack almost all cell components and can induce many types of DNA damage. Hydroxyl radical is the predominant ROS targeting DNA while peroxide, a precursor of hydroxyl radical is more likely involved in the formation of oxidized bases (Klaunig, Kamendulis et al.). According to the one estimation, approximately 5000 DNA single strand lesions (SSLs) are generated per nucleus by endogenous ROS during a single cell cycle (Vilenchik and Knudson 2003). The SSLs include single strand breaks, apurinic/apyrimidinic sites, 8-oxo-guanine, thymol glycol and some alkylation products. Approximately, 1% of these SSLs become converted to DNA double strand breaks (DSBs) at the time of DNA replication (Tanaka, Halicka et al. 2006).

1.5.4 Oxidative Stress and telomere dysfunction

There is some evidence suggesting that oxidative stress may promote telomere dysfunction. It has been shown that oxidative stress is associated with an increase of the rate of telomere shortening in human fibroblasts (von Zglinicki 2002). In addition, suppression of oxidative stress by antioxidative agents extends the replicative life span by reducing the rate of telomere shortening (Kashino, Kodama et al. 2003). Interestingly mitochondrial defect, which accelerates oxidative stress, was shown to cause telomere attrition and genomic instability (Liu, Trimarchi et al. 2002). Accumulation of breaks at telomere sequence after exposure to oxidative stress and the subsequent increase in 8-oxod-guanine content were reported at telomeres of human fibroblasts *in vitro* (Kawanishi and Oikawa 2004) suggesting that oxidative stress mediated 8-oxod-guanine conversion at telomeres may participate in the acceleration of telomere shortening. It has also been shown that 8-oxod-guanine lesion in telomeric DNA alters the telomere binding activity of TRF1 and TRF2 proteins thus promoting telomere dysfunction (Opresko, Fan et al. 2005).

2 AIMS

The overall aim of this work was to investigate possible mechanisms by which EBV may promote malignant transformation, in particular we wished to assess whether the induction of genomic instability may play a role in oncogenesis. To this end, we addressed the following specific aims:

- Investigate the occurrence of genomic instability in EBV positive and negative Burkitt`s lymphoma cell lines
- Identify the viral proteins involved in the induction of genomic instability
- Elucidate the molecular mechanisms by which the viral proteins induce genomic instability

3 METHODOLOGICAL CONSIDERATIONS

Full details of the experimental methods used in this work are available in the appended papers. This section discusses important features of the methodologies chosen for crucial experiments.

3.1 DETECTION OF GENOMIC INSTABILITY

Genomic instability in tumors has three major manifestations: (1) aneuploidy, characterized by the gain or loss of entire chromosomes, is found in the majority of solid tumors. This arises from defects of mitosis, aberrant centrosome duplication or the formation of multipolar spindles that result in unequal segregation of chromosomes in daughter cells; (2) chromosomal instability (CIN), characterized by the occurrence of DNA insertions, deletions, translocations, amplifications, and others alterations involving DNA breakage. These alterations result in gain or loss of chromosome sections or single genes that are also known as loss of heterozygosity (LOH). Another example of CIN is the formation of micronuclei (MN), small nuclei-like bodies found outside of the nucleus. MN is formed as by-products of DNA damage and provides an easy assay to evaluate the cells ability to repair DNA damage and segregate chromosomes after exposure to specific mutagens; (3) Single or small nucleotide sequence changes involving base substitutions, deletions, or insertion of one or few nucleotides, arise from DNA replication errors and failure of mismatch repair. These can be detected as increased mutation rates in random selected areas of the genome, also know as micro satellite instability (MSI or MIN), and are observed in DNA replication error inherited syndromes and a small fraction of sporadic cancers (Lengauer, Kinzler et al. 1997; Dasika, Lin et al. 1999; Richards 2001).

In solid staining of metaphase nuclei, the non-banded chromosome preparations are grouped and classified based on their size, shape, position of their centromeres, and gross chromosome morphology. The scoring of the chromosome and chromatid breaks and gaps, secondary constrictions, dicentric chromosomes, ring chromosomes, double minutes, can be facilitated by solid staining. Analysis of metaphase chromosome is a simple method by which both numerical and non-clonal structure abnormalities can be assessed and this method provides a global information in a single assay (Bridge 2008).

3.2 TELOMERE ANALYSIS IN METAPHASE CHROMOSOMES

To examine whether telomeres are involved in EBV associated chromosome abnormality, telomere analysis of metaphase chromosomes was performed using quantitative fluorescence in situ hybridization (Q-FISH). The purpose of this assay was to quantify telomere length and telomere status in individual chromosomes from EBV(+) and EBV(-) cell lines. The traditional method for estimation of telomere length

in a population of cells is southern blotting using restriction enzymes for cleavage in sub-telomeric sites to obtain telomere restriction DNA fragments (TRFs) with a length of 2.5-4 kb. This method gives an estimation of the telomere lengths of the entire cell population and is a robust method but less accurate since sub-telomeric sequences, which may vary from case to case, contribute to the assay. In contrast, fluorescence in situ hybridization (FISH) directly labels the telomeric sequences in individual chromosomes and the results of telomere length acquired by this quantitative approach (Q-FISH) is consistent with TRF values in Southern blotting assay (Hultdin, Gronlund et al. 1998). In Q-FISH, peptide nucleic acid (PNA) that is a synthetic DNA with neutral backbone is used to quantify target telomere sequences in chromosomal DNA followed by microscopic image acquisition and fluorescence signal analysis. The PNA probes hybridize to target DNA with a high affinity, specificity, stability, reproducibility and lower background than their nucleic acids counterparts (Egholm, Buchardt et al. 1993; de Pauw, Verwoerd et al. 1998). Another advantage of using Q-FISH is the ability to detect telomere associated chromosome abnormality such as gain or loss of telomere, telomere fusion and double stranded break fusion. Moreover, heterogeneity of telomere length and extra-chromosomal telomere fragments that are caused by activation of DNA damage response can be detected only by this method. Telomere-sister chromatid exchange (T-SCE) is another application of Q-FISH that detects the occurrence of telomere sister chromatid exchange, which is the consequence of homologous recombination dependent repair at telomeres. Cells are grown in medium containing synthetic nucleosides analogue of thymidine (BrdU) and cytidine (BrdC) for a single cell cycle. These substituted telomeric DNA are then removed through exposure to UV light and incubation with exonuclease III. The remained single strand telomeric DNA is incubated sequentially with TAMRA-TelG 5′-[TTAGGG]3-3′ and FITC-TelC 5′-[CCCTAA]3-3′ probes to hybridize with lagging and leading telomere strand, respectively. A chromosome with combination of two telomeric DNA signals FITC (green) and TAMRA (Red) in both sister chromatids is scored as T-SCE positive.

3.3 DETECTION OF DNA DAMAGE

Several methods have been developed to examine the DNA damage response. A routine method to detect DNA damage is immuno-fluorescence using specific antibody against DNA repair associated proteins such as pH2AX, 53BP1 and pATM. An early event after induction of DSBs is the phosphorylation of H2AX on serine 139 at the site of damage mediated by the phosphoinositide 3-kinase-like kinases such as ATM or DNA-PK (Foster and Downs 2005). The local formation of pH2AX allows microscopically detection of distinct H2AX fluorescent foci that most likely represent a single DSB (Pilch, Sedelnikova et al. 2003). Moreover, the intensity of fluorescence signals most likely has direct correlation with the amount of DNA damage.

An alternative method to detect the DNA strand break is the comet assay that is based on the occurrence of short DNA fragments that are transported by electric force resulting in a comet like shape. The size of the comets is directly proportional to the DNA damage in the cells.

3.4 IF-FISH FOR DETECTION OF TIF_S AND $T\text{-}PNB_S$

Dysfunctional telomeres mimic DSB that can activate the DDR resulting in the formation of DNA damage foci at telomeres. Activation of DDR at telomeres can be manifested by the occurrence of Telomere Dysfunction Induced Foci (TIFs). The combination of FISH and immunofluorescence (IF-FISH) provides a highly specific method for detection of TIFs and telomere protein interaction. This method can be also used to detect the formation of T-PNBs, which shows colocalization of PML body with telomere and is a characteristic feature of activation of homologous recombination repair at telomeres. Telomeric PNA probe and specific monoclonal antibodies against PML, 53BP1 and pH2AX were used to detect described foci in the cells. In IF-FISH method immuno-fluorescence (IF) is a step prior to fluorescent in situ hybridization (FISH).

3.5 QUANTIFICATION OF TELOMERE ASSOCIATED TRF2

TRF2 is an important protein in the shelterin complex that is involved in telomere integrity and protects the telomeres from being recognized as DSB by the DNA repair system. TRF2 binds specifically to duplex arrays of TTAGGG repeats at telomeres. Disruption of TRF2 can induce activation of ATM and p53 dependent apoptosis in human tumor cells suggesting that the amount of TRF2 at telomeres can be important for telomere function (Kim, Beausejour et al. 2004). To examine whether TRF2 is displaced in EBNA1 expressing cells, we performed quantitative IF-FISH using a specific antibody to detect TRF2 protein at telomeres. The presence of unbound TRF2 in the nucleus can interfere with the results of assay and makes the quantitative analysis more difficult. In order to remove unbound TRF2 from nucleus, the cells were permeabilized prior to fixation with 0.1% Triton X-100 for 5 minutes. The images were captured using the same acquisition set and analyzed with the Volocity Software© which provides a possibility to analyze a large number of events and allows to measure fluorescence intensity of signals inside a small nuclear spots. Telomeres (red fluorescence) were identified in hundred nuclei and the average intensity of red (telomere) and green (TRF2) fluorescence within each selected area was measured. Approximately one hundred single cell images containing almost 2300 telomere-TRF2 spots were evaluated in this analysis.

3.6 QUANTIFICATION OF REACTIVE OXYGEN SPECIES

A FACS-based method was used to measure endogenous level of reactive oxygen species (ROS). The colorless probe 2,7-dichlorodihydrofluorescein diacetate (DCFDA) is a cell permeable, non-fluorescent precursor of DCF that can be used as an intracellular probe for oxidative stress. It has many advantages over other techniques including being very easy to use and extremely sensitive to changes in the redox state of a cell. Intracellular esterases cleave DCFDA at the two ester bonds, producing a relatively polar and cell membrane impermeable product, H2DCF. This non-fluorescent molecule accumulates intracellularly and subsequent oxidation yields the highly fluorescent product DCF. The redox state of the cells can be monitored through detecting the increase in fluorescence intensity (Royall and Ischiropoulos 1993).

4 RESULTS AND DISCUSSION

4.1 EBV ENHANCES CHROMOSOME ABERRATION IN BL CELL LINES (PAPER I)

Cytogenetic analysis of a panel of EBV(–) and EBV(+) BL cell lines, including B95-8 converted and EBV genome loss variants revealed a significant increase of chromosome aberration in EBV infected cells. Various types of chromosome abnormality were detected in 4.6% and 19.4% of EBV(-) and EBV(+) BL lines, respectively. The frequency of dicenteric chromosome, chromatid gap and chromosome fragment were higher in EBV(+) cells whereas ring chromosome, satellite association and double minutes occurred almost with the same frequency in both group of cell lines. Comparison of paired cell lines with the same origin, including EBV(-) BL41 and BJAB cell lines versus their EBV converted sublines, BL41-E95-A and BJAB-B95-8 and originally EBV(+) Akata and Oma cl.6 versus EBV genome loss variants Akata and Oma cl.4 and the EBV reinfected Akata JSWT subline indicated an increase in the frequency of chromosome aberrations following EBV conversion and decrease upon loss of the viral genome, confirming the dependence of chromosome abnormalities on the presence of EBV.

Telomere analysis revealed an increase in the prevalence of telomere fusion (T2) and double strand break fusion (T0) in dicentric chromosomes from EBV(+) cell lines whereas most of the dicentric chromosomes in EBV(-) cell lines had four telomeres at the fusion site (T4). Comparison of telomere size in metaphase plates from EBV(+) and EBV(-) cell lines demonstrated that EBV positive cells possess a telomerase independent broad distribution of telomere size.

It is widely accepted that unrepaired or misrepaired DNA double strand breaks (DSBs) lead to the formation of chromosome aberrations (Iliakis, Wang et al. 2004). Phosphorylation of H2AX is an early event in DNA damage response and DSB repair. Immunofluorescence analysis using pH2AX specific antibody showed a strong fluorescence in EBV(+) cells versus EBV(-) cell lines. A significant increase of fluorescence was induced in all cell lines upon treatment with the DNA damaging agent confirming that the mechanisms leading to detection of DSBs and H2AX phosphorylation are intact.

Deregulated c-Myc expression has been shown to generate genomic instability by initiating gene amplification, gene rearrangements, and karyotypic instability (Mai and Mushinski 2003). To investigate whether EBV is sufficient for induction of genomic instability in the absence of c-Myc overexpression, the presence of pH2AX was checked in the P493-6 cell line that carries a P3HR1 virus with an estrogen driven EBNA2 and conditional c-Myc gene. In the presence of estrogen and tetracycline the cell line reexpresss a latency III gene expression program (P493-LCL), whereas withdrawal of the drugs results in switch to a latency I like BL phenotype with high expression of c-Myc (P493-BL). Significantly higher level of pH2AX was detected in

P493-LCL cell line, whereas a further increase was observed upon downregulation of EBNA2 and LMP1 and upregulation of c-Myc in P493-BL cell line.

The results presented in this paper provide a new insights into the pathogenesis of EBV(+) Burkitt`s Lymphoma (BL). Random chromosomal aberrations were previously described in BL cells, and were attributed to the capacity of c-Myc to induce genomic instability (Potter and Marcu 1997). Our data support this role of c-Myc as unstable chromosomal aberrations were detected in EBV(-) BL cells. However, the defects were between 3 and 10 times more frequent in EBV(+) cells and a distinct role of the virus was confirmed by comparison of paired cell lines that do or do not carry EBV following *in vitro* conversion or spontaneous loss of the viral genome and by P493-6 cell line. The non-transmissible chromosome aberrations that we reported in BL cell line, are also frequent in other EBV associated malignancies suggesting that induction of genomic instability may be common mechanism by which EBV contributes to tumor progression. Abnormal metaphases were more frequent in cells expressing latency I compared with EBV(-) cells and further increase in cells expressing latency III, suggesting that different viral products may be critical for the induction of the genomic instability. EBNA3C and LMP1 have been assumed to promote genomic instability through inhibition of cell cycle checkpoints and DNA repair, respectively but these viral proteins are not or rarely expressed in EBV(+) BL tumors suggesting that other viral products must cause DNA damage in cells expressing latency I. Comparison of the type of chromosomal aberrations detected in EBV(-) and EBV(+) cells suggests the involvement of DNA breakage and telomere dysfunction as two possible mechanisms by which EBV may promote genomic instability.

4.2 EBNA1 PROMOTES GENOMIC INSTABILITY VIA PRODUCTION OF REACTIVE OXYGEN SPECIES (ROS) (PAPER II)

The significant increase of chromosomal aberrations in latency I suggests a possible role for EBNA1 in the induction of genomic instability. We investigated this possibility in a panel of EBV(-) B lymphoma cell lines with stable or inducible expression of EBNA1. A 3- to 4-fold increase of metaphases with chromosomal aberrations was observed in stable EBNA1 expressing sublines and a similar increase was induced upon removal of tetracycline in inducible EBNA1 expressing cell lines. To investigate whether this phenotype is associated with induction of DNA damage, the comet assay was performed. A reproducible increase of comet length representing DNA double strand break was detected in stable EBNA1 expressing cell lines and inducible EBNA1 expressing cell line upon removal of tetracycline. EBNA1 associated DNA double strand breaks were involved in the phosphorylation dependent activation of ATM and its downstream target H2AX as shown by immuno-fluorescence.

DNA damage can be induced by exogenous and endogenous genotoxic stimuli (Slupphaug, Kavli et al. 2003). One of the major endogenous sources of DNA damage is excessive production of ROS (De Bont and van Larebeke 2004). EBV infection was shown to be associated with elevated level of ROS (Cerimele, Battle et al. 2005) suggesting that EBNA1 may be involved in the induction of DNA damage through production of ROS.

We demonstrated that the level of ROS is 10 times higher in stable or inducible EBNA1 expressing sublines of BJAB, and this was associated with enhanced activation of the DNA damage response, assessed by phosphorylation of H2AX, and increased chromosome abnormality. Treating the cells with ROS scavengers could reverse this effect.

Production of ROS may be caused by enhanced oxidative metabolism or by deregulation of enzymes involved in their production and neutralization to nontoxic compounds (D'Autreaux and Toledano 2007). By extracting the expression levels of genes involved in ROS metabolism from a collection of 24 gene expression datasets, derived from 18 EBV(+) and EBV(-) B cell lines available in public databases, we found several ROS associated genes which were differentially regulated in EBV(+) cell lines. Among them only NOX2 (catalytic subunit of NADPH oxidase) mRNA was strongly upregulated in EBV(+) cell lines. The up-regulation of NOX2 was confirmed by detection of mRNA and protein level in the described EBNA1 expressing cell lines. We tested whether EBNA1 may directly regulate NOX2 expression using a NOX2-Luc reporter plasmid. Co-expression of NOX2-Luc with EBNA1 resulted in a dose dependent increase of luciferase activity supporting the possibility that EBNA1 may directly regulate the expression of NOX2. Inhibition of the NADPH oxidase by chemical inhibitors or NOX2 specific RNAi blocked the production of ROS and diminished the induction of DDR suggesting that NOX2 is responsible for the production of ROS and consequent induction of genomic instability in EBNA1 expressing B cells.

Our finding in this paper demonstrates that expression of EBNA1 alone is sufficient to initiate a cascade of events leading to the generation of chromosome aberration and double strand DNA breaks. Earlier studies have reported elevated levels of ROS in EBV infected cells, attributing this effect to virion mediated triggering of the CR2 receptor (Kim, Park et al. 2006), induction of IL-10 by the viral EBV encoded untranslated RNAs (EBER) (Cerimele, Battle et al. 2005), or induction of lipoxygenases by unknown viral genes (Belfiore, Natoni et al. 2007). Although EBV might have evolved different strategies for inducing ROS during different phases of the infection, the selective upregulation of NOX2 and activation of the NADPH oxidase due to expression of EBNA1 suggest a pivotal role of ROS in growth transformation by interfering with signalling cascades regulating cell survival and death. Thus, EBNA1 mediated production of ROS may promote B cell transformation by initiating signalling cascades that are further activated by other viral proteins.

4.3 EBNA1 PROMOTES TELOMERE DYSFUNCTION VIA INDUCTION OF OXIDATIVE STRESS (PAPER III)

Telomere analysis in EBV(+) and EBV(-) BL cell lines revealed defects in the number and length of telomeres and increased occurrence of telomere fused (T2) dicentric chromosomes in EBV infected cells. To investigate whether EBV induces telomere dysfunction, the telomere analysis was performed in a large panel of paired EBV(+) and EBV(-) cell lines. While four distinguishable telomere signals of similar intensities were detected in the majority of chromosomes from the EBV(-) cell lines, approximately 40% of the chromosomes in EBV carrying cell lines showed abnormality in the number of telomeres such as gain or loss of telomeres, telomeres of widely different intensity and fused telomeres. Quantification of the intensity of telomeres in individual chromosomes revealed a broad distribution of telomere length in all EBV(+) cell lines. In contrast, a narrow distribution of signal intensity was regularly observed in the EBV(-) cell lines.

This phenotype was reproducible only in stable and inducible EBNA1 expressing cell lines four weeks after withdrawal of doxycycline, while expression of EBNA3A, EBNA3B and LMP1 had no effect suggesting that EBNA1 is responsible for the induction of telomere abnormalities. This was coupled with a significant increase of extra-chromosomal telomeric DNA in the stable and inducible EBNA1 expressing cell lines. Dysfunctional telomeres mimic double strand DNA breaks that lead to the activation of the DDR via either homologous recombination (HR) or non-homologous end joining (NHEJ). To investigate the occurrence of telomere dysfunction, IF-FISH analysis was performed in EBNA1 expressing cell lines. The results of this analysis demonstrated a significant increase of telomere dysfunction induced foci (TIFs), accumulation of pH2AX and 53BP1 at telomeres, and telomere associated PML nuclear body (T-PNB), co-localization of PML with telomeres, accompanied with induction of telomere sister chromatid exchange (T-SCEs) in the EBNA1 expressing cell lines.

Dysfunctional telomeres or uncapped telomeres can be generated through shortening of telomeres or alteration of telomere binding proteins. Quantification of TRF2 proteins within the individual telomeres of EBNA1(+) and (-) Ramos cell lines revealed a significant decrease in the intensity of TRF2 signals associated with the telomeres. Moreover, several telomeres appeared to lack any associated TRF2 fluorescence suggesting that EBNA1 may induce uncapping of telomeres through displacement of TRF2.

EBNA1 induces DNA damage through excessive production of ROS. We therefore asked whether the effect of EBNA1 on telomeres could be associated with the induction of ROS. For this purpose, the cells were treated with ROS scavenger (Ebselen) for one week to reduce the level of ROS in minimum level. Reduction of ROS was correlated with a significantly decrease in the occurrence of TIFs and T-PNB.

We then asked whether the capacity of ROS scavenger to inhibit the signs of telomere dysfunction is also associated with changes in the localization of telomeric TRF2. To this end, telomeres and TRF2 were quantified after treatment with ROS scavenger in EBNA1(+) and (-) Ramos cell lines. Treatment with Ebselen didn't affect the distribution of telomere signals whereas the intensity of telomere associated TRF2 fluoresence was significantly increased in EBNA1(+) cell lines.

In this paper, we have documented the occurrence of telomere dysfunction in EBV infected cells and we have identified EBNA1 as the viral protein causing this phenotype via induction of oxidative stress-dependent telomere uncapping. The effect of EBNA1 on the accumulation of TIFs containing the DNA damage response proteins was paralleled with the displacement of TRF2 from telomeres, which supports the involvement of telomere de-protection in the telomere dysfunction phenotype induced by EBNA1. The formation of TIFs and T-PNBs in EBNA1 positive cells was inhibited when the endogenous level of ROS was decreased by treatment with Ebselen. Most importantly, this was associated with relocalization of TRF2 at telomeres, establishing a direct correlation between the oxidative stress induced by EBNA1 and telomere uncapping. The telomere abnormalities associated with EBNA1 expression are characteristic features of ALT that maintains telomere homeostasis in a subset of malignant cells with poor or absent telomerase activity. The previous reports demonstrating that EBV conversion does not alter the activity of telomerase in B lymphoma lines (Mochida, Gotoh et al. 2005). The possibility that EBV may activate telomerase independent mechanisms for maintenance of telomere homeostasis is line with the observations that the average length of telomeres remains relatively constant during the early phases of EBV-induced growth transformation, whereas a robust upregulation of telomerase activity is only detected after several weeks or months in culture. Furthermore, some EBV immortalized LCLs overcome the growth crisis observed after approximately 150 passages *in vitro* in the absence of detectable telomerase activity (Sugimoto, Ide et al. 1999). Conceivably, the capacity of EBV to activate ALT could rescue the infected cells from replicative senescence during the early phase of B-cell immortalization.

4.4 EBNA1 REGULATES THE EXPRESSION OF GENES INVOLVED IN CHROMATIN REMODELING (PAPER IV)

EBNA1 can regulate the transcription of viral and cellular genes. EBNA1 binds to specific sites in the Qp promoter and autoregulates EBNA1 expression in different latency types (Sample, Henson et al. 1992). Moreover by binding to *OriP*, EBNA1 positively regulates the Cp promoter leading to transcription of six EBNA genes in cells expressing latency III (Sugden and Warren 1989; Gahn and Sugden 1995). EBNA1 can also bind to cellular DNA, although with a lower affinity compared to viral DNA and regulates cellular gene expression (Horner, Lewis et al. 1995). The role of EBNA1 on cellular transcription may also play an important role in EBV associated

malignancies. To investigate the effect of EBNA1 on cellular transcription, stable and inducible EBNA1 expressing cell lines were used to identify early and late effect of EBNA1 on the regulation of cellular gene expression. Three hundred and nineteen cellular genes and five thousand nine hundred twenty one genes were differentially regulated short and long time after EBNA1 expression, respectively while four thousand three hundred eighty three genes were regulated in stable EBNA1 expressing cells. A significant number of regulated genes had EBNA1 binding sites in their promoter. In addition, EBNA1 can indirectly regulate genes that are annotated as transcription regulators (Songin, Jesko et al.).

The analysis of commonly EBNA1 regulated genes revealed a downregulation of chromatin maintenance complex such as SMARCB1, SMARCA4 and SMARCD2 subunits of SWI/SNF chromatin remodeling complex suggesting that EBNA1 may promote the transcriptional activation through downregulation of chromatin remodelers.

5 CONCLUDING REMARKS AND FUTURE PRESPECTIVES

The results described in this thesis provide new insights into the mechanisms by which EBV may contribute to the pathogenesis of Burkitt`s Lymphoma and other EBV associated malignancies.

We have shown that EBV promotes genomic instability, which plays an essential role in oncogenesis, by inducing the accumulation of mutations that lead to the activation of oncogenes and inactivation of tumor suppressor genes. Different signs of genomic instability have been observed in all EBV associated malignancies suggesting that genomic instability may be a common strategy by which EBV contributes to tumor progression. Genomic instability due to EBV infection is associated with the expression of viral proteins and EBNA1 appears to be sufficient for induction of genomic instability.

We have shown that EBNA1 induces genomic instability through production of ROS. Production of ROS has been also shown in primary EBV infected cells as well as other EBV positive tumor cells. EBNA1 specifically induces the accumulation of ROS through upregulation of the NADPH oxidase indicating the importance of sustained production of ROS in virus-infected cells. ROS regulates many signalling pathways involved in growth transformation. Growth transformation of B-lymphocytes is essential for the establishment of persistent EBV infection and colonization of the B cell compartment. Reciprocal translocation of c-Myc to Ig locus is a characteristic feature of Burkitt`s Lymphoma leading to deregulation of c-Myc oncogene. V(D)J recombination and activation induced cytidine deaminase (AID) are mechanisms promoting translocations in Burkitt`s Lymphoma which also activated in EBV infected B cells (Epeldegui, Hung et al. 2007). Moreover, higher mutation rates in V(D)J sequences were observed in EBV(+) compared to EBV(-) BLs. It remains to be elucidated whether the effect of EBNA1 on the levels of ROS may play a role in c-Myc translocation and growth transformation.

We have shown that the oxidative stress induced by EBNA1 is also associated with telomere uncapping through displacement of the shelterin protein TRF2. Telomere dysfunction may give rise to many of the chromosomal abnormality observed in EBV(+) cell lines. Telomere dysfunction mimics DSB and triggers double strand break repair pathways including NHEJ or HR, which results in the formation of dicentric chromosomes and heterogeneous length of telomeres, respectively. Dicenteric chromosome can generate other chromosomal abnormalities due to break fusion bridge cycle whereas telomere heterogeneity may be a sign of elongation of telomeres that is required for cell proliferation through activation of homologous recombination at

telomeres. Telomerase negative tumor cells can maintain telomere length and continuously proliferate in the absence of telomerase dependent extension of telomeres. We observed signs of activation of HR pathway in telomeres of EBV(+) cells but it is not clear whether this activation interferes with telomere homeostasis. Primary EBV infected LCLs can proliferate until 150 PD in the absence of detectable telomerase suggesting that EBV may regulate telomere homeostasis through other mechanism than telomerase dependent telomere elongation in primary infected cells. EBV may prevent shortening of telomeres through EBNA1 dependent activation of HR at telomeres.
Transcription profiling of inducible and stable EBNA1 expressing cells indicated that EBNA1 may cause a downregulation of chromatin maintenance complex, which can play key roles in many nuclear processes such as recombination and repair by controlling the level of chromatin compaction (euchromatin and heterochromatin), and the accessibility of DNA to DNA repair factors.

Collectively the findings described in this thesis have generated new knowledge concerning the mechanisms by which EBV encoded proteins that are expressed in malignant cells may contribute to oncogenesis. They also raise important questions, including for example the role of these potentially dangerous viral strategies in the infection process and establishment of latency, that require further investigations.

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