From Department of Microbiology, Tumor and Cell Biology
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THE ROLE OF EBNA BINDING PROTEINS IN CELL TRANSFORMATION

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ABSTRACT

Epstein-Barr virus (EBV) infects majority of the human population and maintains sub-clinical infection. However, under certain conditions it is associated with several B-cell malignancies, such as Burkitt lymphoma, Hodgkin's lymphoma etc. Moreover, EBV also plays a causative role in acquired immunodeficiency syndrome (AIDS) associated lymphomas and post-transplant lymphoproliferative disease (PTLD). EBV maintains latent infection and expresses a particular set of proteins that are necessary for host cell proliferation. Studying function of EBV latent proteins could help us to understand the mechanisms underlying EBV induced B-cell transformation.

EBV transformed B cells, i.e. lymphoblastoid cell lines (LCLs) is a well-established *in vitro* model system to study the molecular mechanisms of B-cell transformation. In the present work, we have identified vitamin D receptor (VDR) as a binding partner of EBNA3. We showed that EBNA3 can block the VDR mediated gene transactivation and protects B-cells from vitamin D3 induced growth arrest/ apoptosis. We have observed that hypoxia inducible factor 1 alpha (HIF1α) is stabilized in LCLs at normoxic conditions. HIF1α is not hydroxylated and therefore it is not degraded in LCLs. We have shown that prolylhydroxylases 1 and 2 (PHD1 and 2) that are responsible for hydroxylation of HIF1α, form complexes with EBNA5 and EBNA3, respectively. Due to this binding catalytic activity of PHDs is blocked, resulting in inhibition of HIF1α hydroxylation and subsequent degradation. Stabilized HIF1α is transcriptionally active and induces genes that are involved in glycolysis. Moreover, LCLs have high levels of pyruvate and lactate in contrast to mitogen activated B cells, indicating induction of aerobic glycolysis or Warburg effect.

We have shown that mitochondrial ribosomal protein MRPS18-2 (S18-2), an EBNA6 binding protein, can immortalize rat embryonic fibroblasts (REFs). These immortalized cells express stem cell markers like SSEA1, Sox2, Oct3/4 and have the characteristics of embryonic stem cells. S18-2 also immortalized the adult rat skin fibroblasts (RSFs). Moreover, single clones from immortalized REFs and RSFs resulted in tumors in SCID mice.

This thesis work reveals three different aspects of EBV induced B-cell transformation, i.e. protection from vitamin D3 induced apoptosis, metabolic adaptation required for proliferation and hijacking functions of novel protein MRPS18-2 for immortalization.

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I. Yenamandra SP, Hellman U, Kempkes B, **Darekar SD**, Petermann S, Sculley T, Klein G, Kashuba E. (2010)
"Epstein-Barr virus encoded EBNA-3 binds to vitamin D receptor and blocks activation of its target genes."
Cell Mol Life Sci, 2010. 67(24): p. 4249-56.

II. **Darekar SD**, Georgiou K, Yurchenko M, Yenamandra SP, Chachami G, Simos G, Klein G, Kashuba E. (2012)
Epstein-Barr virus immortalization of human B-cells leads to stabilization of hypoxia-induced factor 1 alpha, congruent with the Warburg effect.
PLoS One, 2012. 7(7): p. e42072.

III. Kashuba E, Pavan Yenamandra S, **Darekar SD**, Yurchenko M, Kashuba V, Klein G, Szekely L. (2009)
MRPS18-2 protein immortalizes primary rat embryonic fibroblasts and endows them with stem cell-like properties.
Proc Natl Acad Sci U S A, 2009. 106(47): p. 19866-71.

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RELATED PUBLICATIONS

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LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
ATP	Adenosine triphosphate
BART	BamH1-A rightward transcripts
BL	Burkitt lymphoma
CMV	Cytomegalovirus
EBER	Epstein-Barr virus–encoded small RNA
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
GFP	Green fluorescent protein
GLUT	Glucose transporter
GST	Glutathione-S-transferase
HD	Hodgkin’s lymphoma
HIF	Hypoxia inducible factor
HK	Hexokinase
HPV	Human papilloma virus
IM	Infectious mononucleosis
KSHV	Kaposi's sarcoma-associated herpesvirus
LCL	Lymphoblastoid cell line
LDHA	Lactate dehydrogenase A
LMP	Latent membrane protein
MRP	Mitochondrial ribosomal protein
NADP	Nicotinamide adenine dinucleotide phosphate
NPC	Nasopharyngeal carcinoma
PDK1	Pyruvate dehydrogenase kinase 1
PHD	Prolyl hydroxylase
PKM	Pyruvate kinase muscle isozyme
PTLD	Post-transplant lymphoproliferative disease
REF	Rat embryonic fibroblasts

RSF	Rat skin fibroblasts
RSV	Rouse sarcoma virus
SCID	Severe combined immunodeficiency
TCA	Tri-carboxylic acid
VDR	Vitamin D receptor
VHL	Von Hippel-Lindau

1 BACKGROUND

Cancer is a major public health concern and the second leading cause of death resulting in 7.6 million deaths every year worldwide [1]. Disease of cancer was recognized for thousands of years and some of the earliest evidence of human bone cancer was found in 4 million–year-old fossilized hominid remains; nasopharyngeal carcinomas and osteogenic sarcomas were seen in ancient Egyptian mummies. The written records for the human cancer are found in Babylonian Code of Hammurabi (1750 BCE), ancient Egyptian papyri (1600 BCE), the Chinese Rites of the Zhou Dynasty (1100–400 BCE), and the ancient Indian Ramayana manuscript (500 BCE)[2]. Hippocrates is credited for the origin of word cancer. He used the terms carcinos and carcinoma, which means crab in Greek, to describe appearance of tumors. These Greek terms were then translated to Latin cancer by Roman physician Celsus.

Scientific oncology advanced in the 19th century and with the development of microscopy, cancer could be studied at microscopic level. Cellular theory of cancer was established and in the same century infection was considered as an etiology for the cancer. Vilhelm Ellermann and Olaf Bang, two Danish scientists reported in 1908, that cell free filtrate of chicken leukemia passed on the disease in other chickens [3]. However, leukemia was not considered as cancerous at that time and so causative virus was not considered to be a tumor virus. Three years later, in 1911, Payton Rouse at the Rockefeller Institute reported another avian malignancy- a sarcoma developing in Plymouth Rock fowls could be transmittable to healthy chickens by cell free filtrates from tumor cells. Unlike leukemia, the avian sarcoma was shown to be genuine cancer by Rouse [4]. Thus Rouse sarcoma virus (RSV), as it is called now, became the first known tumor virus. In 1964, Epstein-Barr virus (EBV) was discovered in malignant cells of Burkitt lymphoma and is considered to be the first known human tumor viruses amongst many linked afterwards.

Today we know that viruses are etiological agents of about 15 to 20 % of all cancers worldwide. Apart from pathogenesis of cancer, they have also served as an important discovery tools in tumor biology and have led to identification of many genes and cellular pathways involved in carcinogenesis. Both RNA (RSV, hepatitis C virus (HCV) etc) and DNA tumor viruses (EBV, human papilloma virus (HPV) etc) have been instrumental in development of current concept of tumor biology as well as identification of normal cellular growth pathways. They encode relatively fewer genes, induce tumors in animals and transform cells in culture and hence, are simple model

system to study [5]. Studying the first tumor virus has led to the development of the concept of oncogene and also discovery of the first oncogene, v-src [6]. On the other hand, DNA tumor viruses, like Epstein-Barr virus or human papilloma virus, have their own oncogenes of viral origin. p53 tumor suppressor protein was discovered with the help of DNA tumor virus SV40 (Simian virus 40) large T oncoprotein [7]. Often viral oncoproteins, like large T antigen of SV40, E6 and E7 of HPV, by physical interaction with cellular proteins block their function or perturb cellular pathways, leading to cell survival and that may result in oncogenic transformation of the host cell. Study of such viral oncoproteins and their interacting cellular proteins have immensely contributed and will continue to add in understanding of molecular mechanisms behind cellular transformation.

In this doctoral thesis, we aimed to elucidate Epstein-Barr virus (EBV) strategies to induce cell transformation using EBV immortalized B-cell line, lymphoblastoid cell line (LCLs). We have shown that one of the viral proteins, EBNA3, via its binding with vitamin D receptor (VDR), blocks the activation of VDR-responsive genes and protects LCLs against vitamin-D3-induced growth arrest and/or apoptosis [8]. We have observed that in LCLs, HIF1α protein, which generally is degraded at the normal conditions, is stabilized and transcriptionally active. This finding has implication in metabolism in LCLs as HIF1α induces aerobic glycolysis, i.e. Warburg effect [9]. Overexpression of MRPS18-2 protein, that binds to Epstein-Barr virus nuclear antigen 6 (EBNA6), can immortalize the primary rat embryonic fibroblasts and these cells show stem cells like characteristics [10, 11].

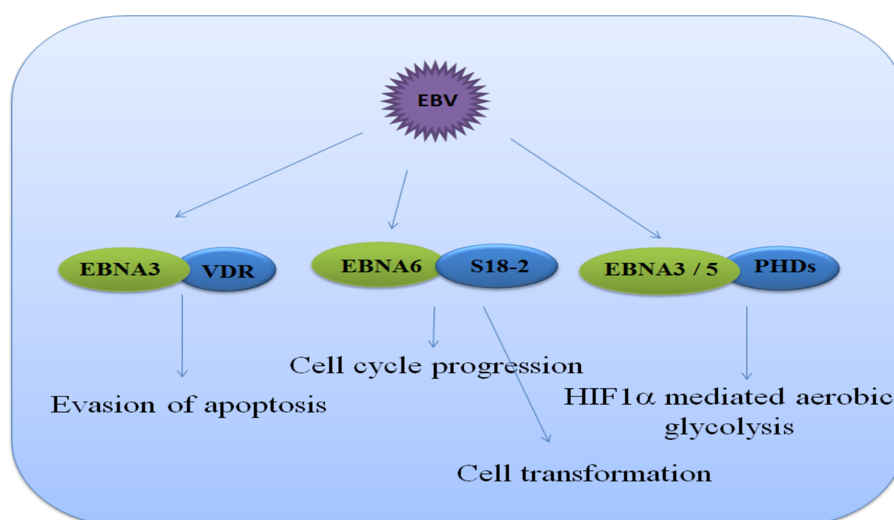

Figure 1: EBNA binding cellular proteins studied in this doctoral work and their functions. VDR- vitamin D receptor; PHDs- Prolyl hydroxylases.

1.1 EPSTEIN-BARR VIRUS BIOLOGY

In 1958, British surgeon Denis Burkitt described a tumor common in sub-saharan Africa which was later termed as Burkitt lymphoma (BL) [12]. It was initially linked to arthropod borne infectious agent, but in 1964, Epstein and co-workers identified herpes virus like particle with the help of electron microscope in a cell line established from BL biopsy. This virus was named as Epstein-Bar virus or human herpes virus 4 [13]. EBV is the first discovered human tumor virus and is linked to many lymphoid malignancies, such as Burkitt lymphoma, Hodgkins lymphoma, T-cell lymphomas, post-transplant lymphoproliferative disease (PTLD) and also some epithelial malignancies such as nasopharyngeal carcinoma (NPC) and gastric carcinoma [14].

Epstein-Barr virus is a sole member of human *lymphocryptovirus* or gamma 1 herpes virus subfamily. EBV genome is composed of linear double stranded DNA of 184 kilo base pairs flanked with terminal repeats (TR) on both sides. It encodes about 84 genes although EBV shows restricted gene expression pattern during latent infection in EBV associated malignancies [14]. Virus has co-evolved with the primate host for millions of years and it is a very efficient virus infecting over 90% of world's adult human population.

1.1.1 EBV latency programs

EBV maintains latent infection in humans and shows different latency programs, which varies from full growth proliferative type III to the more restricted type II, type I or type 0. The type III latency program comprises six nuclear antigens EBNA 1-6, three latent membrane proteins LMP1, LMP2A and LMP2B plus two types of non-coding viral RNAs, EBERs (EBV-encoded non coding, non polyadenylated RNAs) and BARTs (BamH1-A rightward transcripts). This program is expressed by *in vitro* EBV infected B-cell lines, *in vivo* upon EBV infection of naive tonsillar B-cells [15], in posttransplant lymphoproliferative disease and in primary central nervous system lymphoma which arises in AIDS patients [16, 17]. In type II latency only EBNA1 and three latent membrane proteins are expressed and it is seen *in vitro* in EBV infected epithelial cells and *in vivo* in nasopharyngeal carcinoma and B-cell Hodgkin lymphoma. While in type I latency, just EBNA1 is expressed and is found in Burkitt lymphoma and in cell lines derived from Burkitt lymphoma. Type 0 may exists in EBV genome carrying circulating memory B-cells where lack of gene expression allows escape from T cell surveillance [18].

Table 1: Expression of latent proteins by EBV in different conditions.

Latency type	Latent genes and transcripts expressed	Disease / condition
0	No latent protein expressed	Memory B-cells
I	EBNA1,EBERs, BARTs	Burkitt lymphoma
II	EBNA1, LAMP1, LMP2A, LMP2B, EBERs and BARTs	Nasopharyngeal carcinoma; Hodgkin's disease, Gastric carcinoma
III	EBNA1-6, LMP1, LMP2A, LMP2B, EBERs and BARTs	Infectious mononucleosis; Posttransplant lymphomas, AIDS-related lymphomas, Lymphoblastoid cell lines

1.1.2 Function of the latent proteins

1.1.2.1 EBNA1

EBNA1 is the only EBV latent protein consistently expressed in all virally associated tumors. It is DNA binding nuclear phosphoprotein which recognizes plasmid replication origin, oriP which is necessary for replication and maintenance of episomal EBV genome [19, 20]. EBNA1 can also bind to viral promoters and negatively regulate its own expression [21]. EBNA1 plays a critical role in survival of Burkitt lymphoma cells by inhibiting apoptosis [22].

1.1.2.2 EBNA2

EBNA2 is one of the first viral protein expressed in EBV infected B-cells and is essential for B-cell transformation [23]. EBV strain P3HR-1, with deletion of *EBNA2* gene is unable to transform B-cells *in vitro*. Restoration of EBNA2 in this strain transforms B-cells, this indicates the important role of EBNA2 in cell transformation [24, 25]. EBNA2 activates transcription of cellular genes like CD21, CD23 and viral genes such as LMP1, LMP2, EBNA3 family proteins, EBNA1, 2 and 5 [26]. It does not bind directly to the DNA but form complex with ubiquitous DNA-binding protein, RBP-Jk [27, 28]. EBNA2 can replace the intracellular part of Notch receptor and functionally mimic activated Notch [29]. In co-operation with EBNA5, EBNA2 induces progression of B-cells from G_0 to G_1 [23].

1.1.2.3 EBNA3 family

EBNA3, 4 and 6 (also known as EBNA3A, 3B, and 3C) are the family of nuclear proteins tandemly arranged at the middle of the nuclear genome. They have slight similarity in amino acid sequence, similar gene structure and are transcribed from

the common promoter [30]. EBNA3 and 6 are essential for the B-cell transformation while EBNA4 is dispensable [31]. This family of proteins have been shown to inhibit the transactivation activity of the EBNA2 protein by competitively binding with RBP-Jk and disrupting its binding with cognate Jk sequence and EBNA2 [27, 32-34]. EBNA6 has been shown to co-operate with (Ha-) ras in co-transfection assays to immortalize rat embryo fibroblasts (REFs) [35]. This family of proteins has been shown to disrupt the G_2/M cell cycle checkpoint [36].

1.1.2.4 EBNA5

EBNA5, also known as *EBNALP*, is one of the first viral gene expressed after B-cell infection along with *EBNA2* [37]. It encodes a protein of variable size depending upon the number of *Bam*HI repeats contained by a particular EBV isolate [38-40]. It is necessary for efficient transformation and clonal outgrowth of B-cell *in vitro.* EBNA5 stabilizes the level of p53 tumor suppressor by binding to protein MDM2 and forming tri-molecular complex EBNA5-MDM2-p53 which could result into inhibition of transacriptional activity of p53 [41].

1.1.2.5 LMP1

LMP1 is the major oncoprotein of EBV proven by its ability to transform rodent fibroblasts, being necessary for the B-cell transformation *in vitro* and tumorigenic ability in nude mice [26, 42, 43]. It has pleotropic effect on cells upon EBV infection resulting in increased homotypic adhesion, upregulation of B-cell activation markers [44], upregulation of anti-apoptotic proteins [45, 46], and stimulates the secretion of cytokines like IL-6, IL-8 [47]. LMP1 functions as constitutively activated receptor of tumor necrosis factor receptor (TNFR) superfamily, thus inducing downstream signaling cascade in ligand independent manner [48]. *In vivo* LMP1 can partially mimic functional properties of CD40, resulting in growth and differentiation responses in B-cells [49].

1.1.2.6 LMP2

LMP2A and LMP2B are encoded by LMP2 gene which is formed by joining of the terminal repeats in circularized EBV episome as found in latently infected cells. Both the genes are not essential for the immortalization of B-cell *in vitro* [50] but it has been suggested that they play a role in viral persistence [18]. It has been shown in *in vivo* study that LMP2A can provide survival signals to B-cells in absence

of B-cell receptor (BCR), which otherwise would result in cell death [51]. This finding has been significant in the context of Hodgkins lymphoma which lacks functional B-cell receptor and expresses LMP2A constitutively [52].

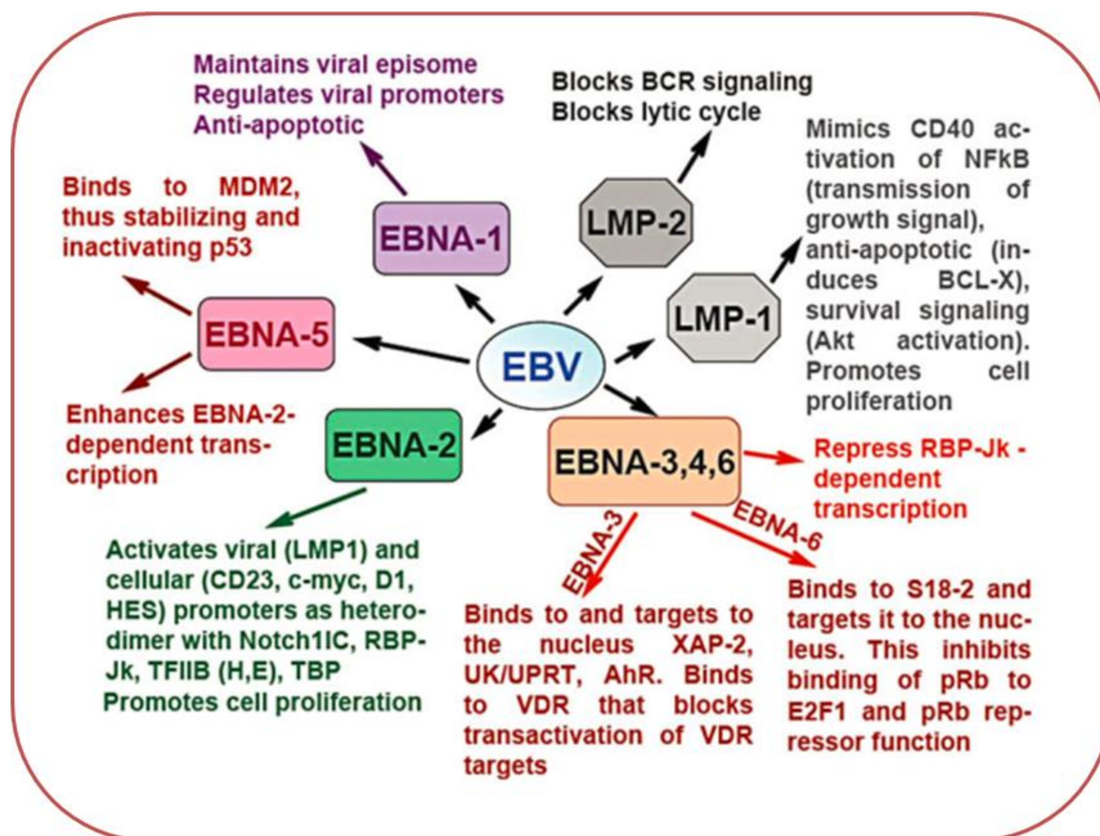

Figure 2: Epstein-Barr virus modulation of cellular proteins. (Adapted from [53]). Scheme shows studies of viral latent proteins and cellular pathways modified by them.

1.1.3 EBV infection *in vivo*

Although EBV has been detected in tumors of B, T and epithelial cells, in healthy individuals it is mainly restricted to B-cell compartment. In healthy carriers 1-50 per million B-cells are infected with EBV [54]. *In vivo* EBV follows the natural B-cell biology to establish and maintain lifelong persistence in B-cell population. Most primary EBV infections occur in early childhood and they are usually asymptomatic. After primary infection EBV persists in the infected host who becomes a lifelong asymptomatic carrier. If the primary infection is delayed to adolescence EBV causes debilitating self-limiting syndrome known as infectious mononucleosis (IM). In infected person EBV is constantly or intermittently shed into the saliva and transmission occurs by close oral contact with the uninfected individuals [55], hence IM also called as kissing disease. Virus replicates in the epithelium of oropharynx and EBV infection of the B lymphocyte is believed to occur in the oropharyngeal lymphoid

organs. EBV interacts with CD21 receptor via its glycoprotein gp350. This follows with rapid proliferation and expansion of EBV positive B-cells which is brought under control by cytotoxic T cells (CTLs). Naïve tonsillar B-cells are the population believed to express type III growth proliferative genes upon EBV infection. These EBV infected cells express CD80, a marker of activated proliferative lymphoblast [15]. These infected B-cells follow the natural B-cell activation biology similar to the activation of B-cells by an antigen. Activated B-cells expand into germinal centers and expression of LMP1 and LMP2A viral proteins which mimic the CD40 and BCR signaling respectively, provide the B-cell survival signals (otherwise result into apoptosis) in germinal centers [56]. B-cells then exit germinal centers as memory B-cells and these EBV infected cells transiently express EBNA1 but otherwise do not express any viral proteins and escape immune surveillance [18]. According to Thorley-Lawson model, germinal center B-cells can also exit and differentiate into plasma cells and these cells are the site of viral lytic reactivation [57]. Primary EBV infection induces both humoral and cellular immune response and antibodies are also produced against lytic as well as latent antigens. Despite immune mechanism, EBV persist latently in peripheral memory B-cell compartment and there is low level production of infectious virus in to saliva [55].

1.1.4 EBV infection *in vitro*

Henle and co-workers in 1967 found that virus can infect and immortalize B-cells *in vitro* transforming them in to lymphoblastoid cell line (LCL) [58, 59]. LCLs can also be generated by culturing peripheral blood lymphocytes from chronic virus carriers, provided the depletion of T cells with cyclosporine A [60]. This is associated with dramatic change in phenotype and behavior of cells in culture. The cell changes its morphology from the round small size to large irregular, develop villipodia and grow in tight clumps. These phenotypic changes are associated with entry into cell cycle and continuous proliferation. LCLs express type III latency genes that is sufficient to drive the cell proliferation and maintenance of the EBV genome. For initiation of immortalization, expression of only EBNA1, 2, 3, 5, 6 and LMP1 are required [24, 31, 61, 62], and EBNA2 and LMP1 are also required for the maintenance of this state [63, 64]. LCLs also show expression of B-cell activation markers CD23, CD30, CD39 and CD70 which are absent or expressed at very low levels on resting B-cells [65]. After the discovery that LCLs can be obtained by the EBV infection of B cells *in vitro*, this system became a model for B-cell transformation and it is believed that study on it can

explain EBV associated oncogenicity. EBV encoded latent nuclear antigens interact with the host cellular proteins and hijack their normal functions to promote the cell growth. Studying these protein-protein interactions has been instrumental in our understanding of various mechanisms behind the B-cell transformation by EBV.

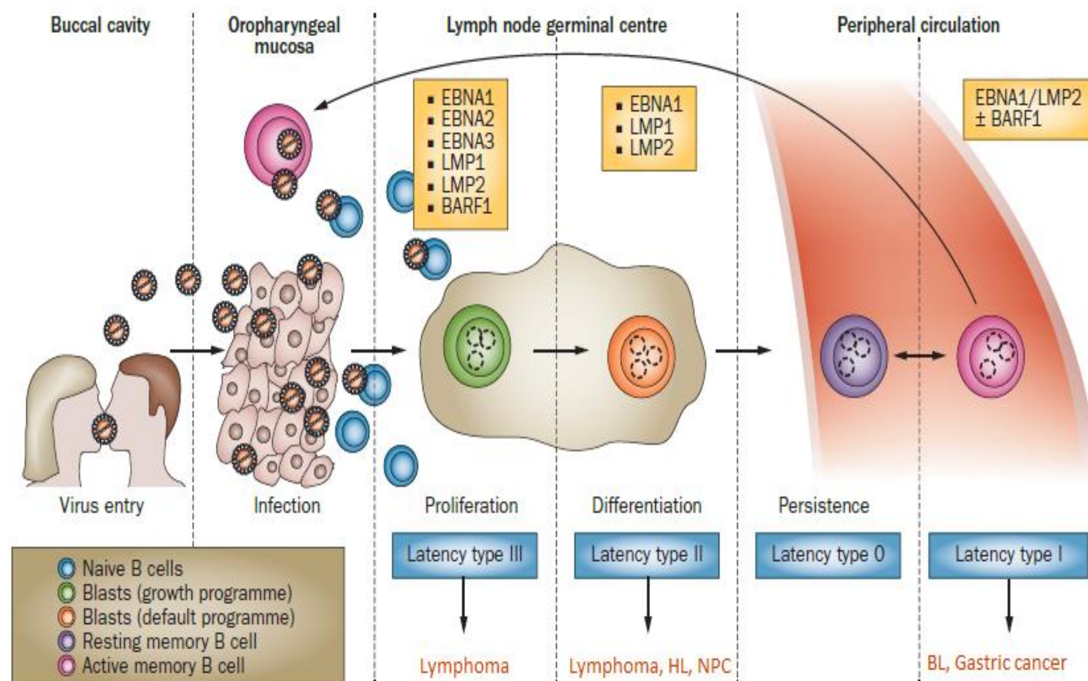

Figure 3: EBV life cycle, latency types and associated lymphoma. BL- Burkitt lymphoma; HL- Hodgkin's lymphoma; NPC- nasopharyngeal carcinoma. (adapted from [66])

1.1.5 EBV and cancer

EBV prevalence is much higher compared to associated tumors. Many of the EBV associated malignancies are geographically restricted, some occur in immunodefficient people while some requires other genetic alterations. However, many evidences support that EBV plays central role in associated malignancies. First its strong association with variety of malignancies and causal role in PTLD and AIDS associated lymphoma, second its ability to induce B-cell lymphoma in non-human primates, third to transform primary human B-cells in culture and finally because of its causal role in infectious mononucleosis which could lead to Hodgkin's lymphoma [67]. Here we go through some of the EBV associated malignancies and pathogenic role of EBV in each one.

1.1.6 EBV associated lymphomas

1.1.6.1 Burkitt lymphoma

Burkitt lymphoma (BL) is common childhood tumor in equatorial Africa, also called as endemic form. Incidence elsewhere in the world is called sporadic from. There are geographical and ethnic variations as well as genetic factors associated with BL. EBV association is strongest in endemic BL (96% of cases) which shares epidemiological characteristics with the holoendemic malarial infection, which generally believed to explain geographical difference in the EBV association. Incidence of EBV associated BLs are rare (15%) in North America and Western Europe even in African population. While with intermediate incidence of around 50-70% of EBV associated BLs are found in North Africa and South America and Russia (discussed in [68]). BL also occurs in about 30-40% of HIV positive people [14] in the early stages of disease where immunosuppression is minimal [69]. Irrespective of the form and EBV association, all BL cells show one of the three chromosomal translocation (8:14, 8:2 or 8:22) that places c-Myc regulation under the control of the immunoglobulin locus resulting in constitutive activation of the c-Myc oncogene [70]. Every third case of BL shows p53 mutation in small stretch of 33 amino acids (codons 213 to 248) [71, 72] and there are genetic alterations in tumor suppressor retinoblastoma-like 2 (RB2) in most of endemic cases and some sporadic cases [73]. BL cells shows latency I viral gene expression program with only EBNA1 protein expressed along with EBERs and BamH1A transcripts [30].

Since only EBNA1 nuclear protein is expressed in BL cells, the role of virus in pathogenesis of BL has been debated. EBERs have been shown to induce expression of anti-apoptotic gene bcl-2 [74], induce interleukin 10 and facilitate resistance to interferon-α [75, 76]. BL cells phenotype matches with the centroblast, the antigen activated cells in lymphnodes which form germinal centers. These proliferating centroblasts undergo hypermutations and the recombinase genes *RAG1* and *RAG2* play important role in this process. EBNA1 has been shown to upregulate these genes which might subsequently lead to genetic mutations and c-Myc translocation [77]. EBER expression is positively regulated by *MYC*. Thus expression of EBERs by centroblasts (which have acquired a c-Myc translocation and its constitutive activity) counteract pro-apoptotic effect of c-Myc [78]. Loss of EBV in Akata BL cells is associated with loss of tumorigenicity and re-establishment of type I latency can regain tumorigenicity in these cells [79].

1.1.6.2 Hodgkin's lymphoma

Hodgkin's lymphoma (HL) comprises about 20% of all lymphomas in western world and it involves children in developing world and young adults in westernized society. HL is characterized by the disruption of normal lymph node architecture and the presence of 1-2% of malignant Hodgkin-Reed Sternberg (HRS) cells. Based on histology and immunophenotype of HRS cells, classical form of Hodgkin lymphoma is distinguished from a lymphocyte predominant form [80]. Lymphocyte predominant form is always EBV negative, while approximately 40% cases of classical HL are EBV positive. Association is higher in Latin America where nearly in all cases EBV is detected [81]. EBV is linked with HL because there is threefold increased risk of HL with history of IM [82] and HRS cells are also found in some cases of IM [83]. There is an altered pattern of serum antibodies to EBV before tumor development [84] and clonal EBV DNA was detected in HRS cells in 40% of HL [85]. EBV positive HL express restricted form of latency, with EBNA1, LMP1 and LMP2A protein expression, along with EBER and BamH1A transcripts [86].

1.1.6.3 Lymphoma in immunosuppressed patients

T- cell immunocompromised individuals are at high risk of developing post-transplant lymphoproliferative disease (PTLD) and it occur in 10 % of transplant patients and rarely in other immune-compromised patients [87]. High dose immunosuppressive therapies along with the primary EBV infection are the predisposing factors for PTLD. Suppression of T-cells allows uncontrolled proliferation of EBV transformed B-cells. Recipients who are EBV negative prior to transplant and undergo infection while immunosuppressed are at higher risk of developing disease. In such cases PTLD develop early after transplantation [88] and these tumors are polyclonal or oligoclonal. Almost all of the early developing tumors are EBV positive and express latency III viral expression program [89]. Post-transplant lymphoma can also develop many years after transplantation. EBV association in late onset PTLDs can fall below 50% and these tumors are monoclonal. Some of them express latency III gene expression program while others are EBNA2 and LMP1 negative or EBNA2 negative but LMP1 positive for a proportion of cells [90, 91]. Lymphomas are also very common in AIDS patients particularly at the end stages of disease when there is intense immunodeficiency. Almost all of these tumors are EBV positive and brain is the most common site of tumor [92].

1.1.7 EBV associated epithelial malignancies

1.1.7.1 Nasopharyngeal carcinoma

Nasopharyngeal carcinoma (NPC) originates from the squamous epithelial cells and classified depending on the degree of differentiation of tumor cells. Undifferentiated form of NPC shows most consistent association with EBV and it comprises around 100% of total cases of NPCs. This form is characterized by the undifferentiated carcinoma cells with heavy lymphocyte infiltration, while EBV present latently only in carcinoma cells.

Like BL, NPC also shows geographical restricted pattern with very high incidence in southern China accounting 20% of all adult cancers. In geographical regions of Canton and Hong Kong NPC accounts for 25-30 cases per 100 000 individuals, while it is very rare in Europe and North America with the incidence rate of less than 1 per 100 000 individuals [93]. NPC also shows very high incidence in Inuit in North America and Greenland and also in Malaysians, Dyaks, Indonesians, Filipinos and Vietnamese. In other parts of the world it has very low incidence.

NPC was linked to EBV based on serological grounds in 1966 [94] and confirmation was made with the demonstration of EBV DNA in all malignant epithelial cells [95]. Irrespective of geographical location, 100% of undifferentiated form of NPC is EBV associated [96]. EBV in NPC shows latency II gene expression pattern with EBNA1, LMP1, LMP2A and LMP2B protein expression along with EBER and BamH1A transcripts [97]. LMP1 is assumed to contribute largely in NPC malignancy by inhibiting differentiation of tumor cells in culture [42] and by inducing tumorigenic phenotype in animals [98].

1.1.7.2 Gastric carcinoma

Gastric carcinoma is the second most common carcinoma in the world with EBV association that varies between 4-18%. EBV association also related to the histology and 80% of the lymphoepithelioma types are EBV positive while none of the intestinal and diffuse types are [99]. EBV viral DNA is found as episomal and is clonal. EBV expresses EBNA1 only or EBNA1 protein along with LMP2A mRNA, EBER and BamH1A transcripts are also expressed [100].

1.2 APOPTOSIS AND CANCER

Apoptosis or programmed cell death is a mechanism responsible for cell death that involves genetic regulation. Apoptosis contributes to various processes, such as normal cell turnover, development and functioning of immune system and embryonic development. Defects in apoptosis can result in various disorders like neurodegenerative diseases, autoimmune disorders, ischemic damage and cancer. It is characterized by distinct morphological changes like cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation [101, 102]. The central processes involved in apoptosis are conserved, and most of our knowledge about them has emerged from studies done in nematode *Caenorhabditis elegans* [103].

Evasion of apoptosis is one of the important mechanisms that cancer cells exhibit during the process of carcinogenesis. Cancer cells can express antiapoptotic proteins like Bcl-2 or down-regulate pro-apoptotic proteins, such as Bax, tipping the balance towards cell survival/ proliferation. Certain B-cell lymphomas such as chronic lymphocytic leukemia, overexpress Bcl-2 which gives strong evidence of implication of apoptosis in cancer [104]. Tumor cells also show evasion of immune surveillance to suppress apoptosis. They show reduced response to death receptor pathway mediated apoptosis by FasL. Evasion of apoptosis is also brought about by the expression of non-functional Fas receptor or secretion of high levels of soluble form of Fas receptor [105, 106]. Some tumor cells counteract tumor infiltrating lymphocytes (TIL) by Fas ligand mediated apoptotic depletion of TILs [107]. Another way of deregulating apoptosis is alteration in cell signaling pathways involved in cell cycle progression and/or growth arrest. *TP53* is a tumor suppressor gene which is mutated in approximately 50 percent of all human cancers. Cells expressing wt p53, in response to DNA damage, can cause the growth arrest at G_1/S phase of cell cycle. If the damage is irreparable, cells undergo apoptosis. p53 protein regulates the expression of many genes involved in apoptosis like Bcl-2 and Bax [108].

Mechanism of apoptosis is sophisticated and is energy dependent. There are two main pathways involved in apoptosis: intrinsic or mitochondrial pathway and extrinsic or death receptor pathway. These two pathways are interconnected and they can affect each other [109]. Both these pathways involve aspartyl-specific proteases (caspases) with proteolytic activity that cleaves proteins at specific aspartic acid residues.

Intrinsic pathway results from a variety of non-receptor mediated stimuli, resulting in intracellular signaling leading to changes in inner mitochondrial membrane. It results in release of two groups of pro-apoptotic proteins from the mitochondrial intermembrane space into cytosol [110]. First group made up of cytochrome c, Smac/DIABLO, and the serine protease HtrA2/Omi. Second group comprises of AIF, endonuclease G and CAD. Members of the Bcl-2 family of proteins control and regulate the mitochondrial apoptotic events. They can be both anti-apoptotic or pro-apoptotic and they work via changing the mitochondrial membrane permeability, thus controlling the release of cytochrome C. There are a total of 25 genes included in Bcl-2 family and the tumor suppressor protein p53 plays an important role in transcriptional regulation of these proteins.

Extrinsic pathway initiates the apoptotic process by transmembrane receptor mediated interactions involving death receptors of tumor necrosis factor (TNF) receptor subfamily [111]. TNF receptor superfamily contains cysteine rich extracellular domain and cytoplasmic death domain [112]. Death domain plays key role in transmitting signals from cell surface to intracellular signaling pathways leading to apoptosis. Caspase 8 is activated then by the death domains in the intra-cytoplasmic portion of these receptors and initiates the caspase cascade.

There is another mechanism of apoptosis brought about by cytotoxic T cells mediated via perforin-granzyme dependent cell death, involving granzyme A and granzyme B. Granzyme B is caspase dependent, while granzyme A executes apoptosis via caspase independent single stranded DNA damage pathway [113].

1.2.1 Viruses and apoptosis

In addition to its role in tissue homeostasis and surveillance against tumor cells, apoptosis is also an essential component of the cell response to injury, such as viral infection. To inhibit virus production and release of progeny virus, infected cell tend to undergo programmed cell death. Conversely, viruses have evolved to overcome the host cell apoptosis by exploitation of host cellular machinery. Viral responses are intended to function in a way that they will prevent premature death of host cell during lytic infection and/ or transform host cell while latently infecting them. Viruses, however, can also use other mechanisms to overcome hindering effects of apoptosis. They can multiply quickly before effective immune response is mounted or in another strategy, called cryptic infection virus remains undetected after infection by host [114, 115].

Viruses adopt different mechanisms to inhibit the apoptosis. They can disrupt the apoptosis by inhibition of tumor suppressor protein p53, as in case of SV40 large T binds to p53 and block its functions [7, 116]; papillomavirus E6 and adenovirus E1B-55K proteins promote degradation of p53 [117-120]. Some tumor associated viruses, like EBV and Kaposi's sarcoma-associated herpesvirus (KSHV), express Bcl-2 homologues BHRF1 and KSbcl-2, respectively. These Bcl-2 protein homologues show anti-apoptotic function and contribute to tumor progression [121, 122]. Adenovirus E1B-19K is similar to Bcl-2 protein in sequence and function and is also a distinctive example of mimicking Bcl-2 [123, 124]. Many viruses target and modulate the activity of TNFR signaling. They express TNFR orthologs [125], can neutralize TNF by soluble decoy receptor [126] and can loss [127] or enhance the expression of death receptors [128]. Some viruses interfere with the activation of caspases by expressing proteins to protect the host cell from apoptosis [129]. Interferon (IFN) plays important part in the host response to infection and it does that by inducing cell death via FADD protein dependent apoptotic pathway. IFN can also arrest viral life cycle at various steps, thus reducing the viral replication and spread. Some tumor viruses also target IFNs and their responsive genes to affect IFN mediated antiviral action [130]. However, for the host cells successful evasion of apoptosis by viruses is harmful and may result into oncogenic transformation of host cell.

1.2.2 EBV and apoptosis

Many EBV latent proteins are involved in the evasion of apoptosis of host cell. LMP1 latent protein is a major factor in EBV mediated evasion of apoptosis. LMP1 mimics the constitutively active CD40/ TNF receptor and recruits several TNF receptor associated factors at its C terminal activating domains (TRAF), turning on TRAFs/NF-κB, MAPK and JAK/STAT signal transduction cascades [131]. Activation of NF-κB pathway results in up-regulation of anti-apoptotic gene products, such as A20 and cIAPs [132]. NF-κB activation also result in inhibition of the BAX promoter activity [133] and induction of anti-apoptotic BFL-1 [134]. LMP1 is also shown to confer resistance to TGFβ mediated anti-proliferative effect by inducing the expression of Id1 [135].

EBV encodes two viral BCL-2 homologues, BHRF1 and BALF1, which are expressed maximally during the early stages of infection but do not required in latently infected cells. However, they prevent the infected cells from apoptosis during

early stages of infection [136]. BHRF-1 can directly bind with pro-apoptotic protein PUMA and interferes with its function. Moreover, EBV encodes the microRNA, miR-BART5 [137] and induce cellular microRNA miR-155 [138], which can target PUMA transcript. BIK mediated apoptosis can be inhibited by overexpressing BHRF-1 [139].

Another EBV latent membrane protein, LMP2A, functions like a B-cell receptor (BCR) by associating with Syk and the Src family of tyrosine kinases [50]. Generally these kinases are recruited to BCR following the antigen binding and results into its activation and downstream events that leads to B-cell survival [140]. Moreover, LMP2A can induce the expression of anti-apoptotic protein BCL-2 and BCL-X_L via activation of NF-κB [141] and RAS/PI3K/AKT pathway [142], respectively. EBV nuclear antigens have also been shown to inhibit apoptosis. EBNA2 can transactivate the anti-apoptotic BFL-1 gene [143]. EBNA3 and EBNA6 cooperatively repress the expression of the pro-apoptotic protein BIM [144]. In EBV immortalized LCLs, EBNA5 froms tri-molecular protein complex with MDM2 and p53, which inhibits degradation of p53, but abolishes its transactivational function [41].

1.2.3 Vitamin D

The role of Vitamin D is more than a micronutrient and is, actually, precursor of a steroid hormone. Biologically most active form of vitamin D is $1\alpha,25(OH)_2D_3$, also known as calcitriol. Its widely accepted physiological role is to maintain calcium homeostasis in body and is vital for normal bone mineralization [145]. Dietary vitamin D is present in two forms: vitamin D3 (cholecalciferol) that is animal source, while vitamin D2 (ergocalciferol) is a plant source. However, precursor of vitamin D3, 7-dehydrocholesterol [7-DHC] or provitamn D3, is present in human skin. 7-dehydrocholesterol is converted to cholecalciferol on exposure to UVB radiations from Sun. Vitamin D from diet or skin gets into the circulation by binding to vitamin D-binding protein (DBP). In liver vitamin D is hydroxylated to form the prohormone 25-hydroxy vitamin D [25(OH)D] by the enzyme 25-hydroxylase (CYP27A1). 25(OH)D is subsequently converted to calcitirol in kidney by the enzyme 1α-hydroxylase (CYP27B1).

Calcitriol exerts its effect by binding to specific nuclear vitamin D receptor (VDR) to regulate target gene expression. VDR is a nuclear transcription factor that belongs to steroid hormone receptor superfamily, and acts to regulate its responsive genes in a ligand dependent manner [146]. VDR is expressed in virtually all

tissues, including heart, muscle, breast, kidney, bone, intestine, colon, prostate, brain, osteoblasts, and cells of immune system [147].

Calitriol binds to VDR in cytoplasms and this complex is then transported to nucleus by importin α. In nucleus calcitriol-VDR heterodimerizes with the retinoid X receptor (RXR) and its ligand (9 cis-retinoic acid). This heterodimer then binds to DNA at vitamin D response element (VDRE) in the promoter region of target genes [148]. Calcitriol binding induces phosphorylation and conformational changes to VDR which causes the release of co-repressors. Conformational changes also promote the binding of stimulatory factors, including chromatin modifiers to VDR by repositioning the VDR activation function 2 (AF-2) domains [148].

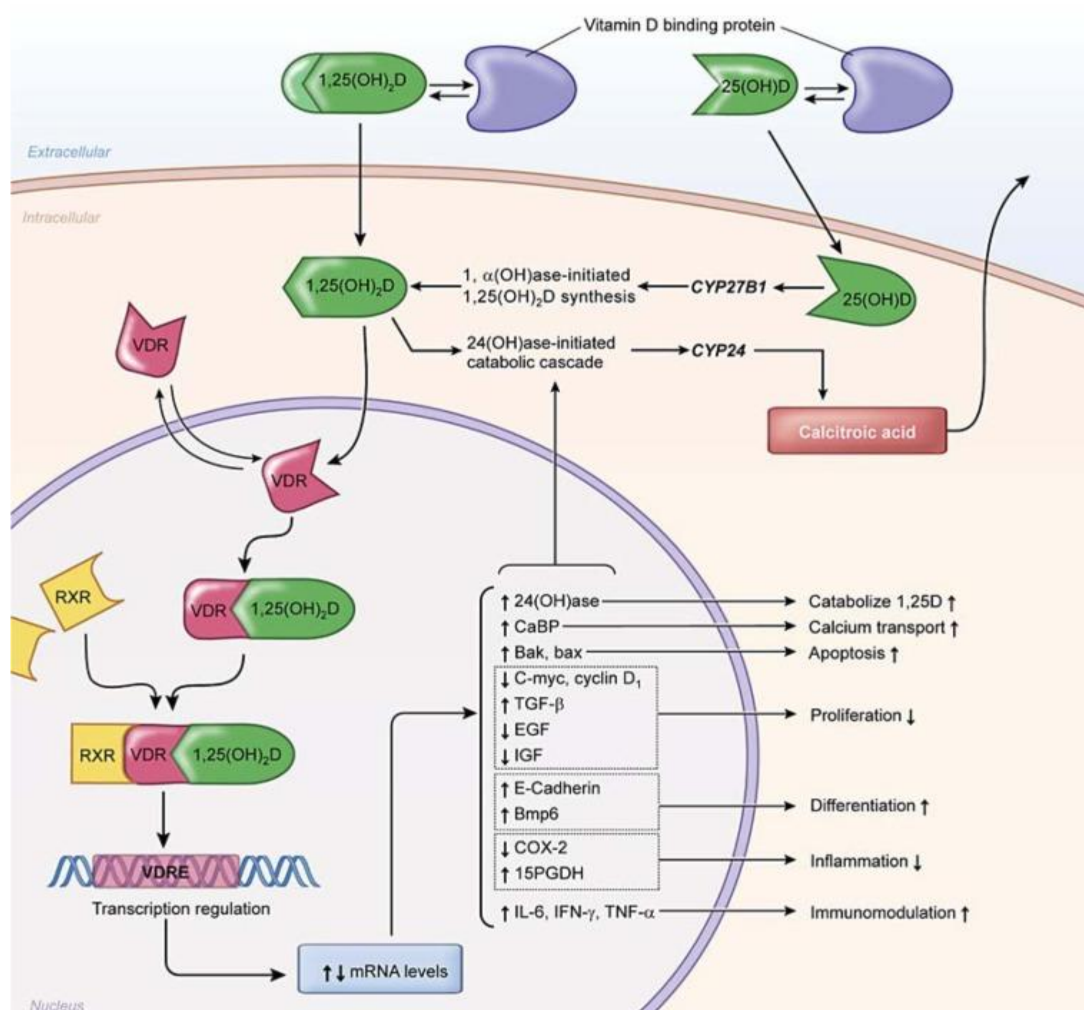

Figure 4: Mechanism of Vitamin D genomic action and its effect on cancerogenesis. 1α,25$(OH)_2D_3$ binds to vitamin D receptor (VDR) in cytoplasm and this complex is translocated to nucleus. Here VDR heterodimerizes with retinoid X receptor (RXR) and binds to vitamin D responsive element (VDRE) to induce expression of target genes. These genes are involved in apoptosis, differentiation, cell cycle arrest, nflammation and immunomodulation. (Adapted from [149]).

1.3 WARBURG EFFECT

One of the distinguishing features of cancer cells from normal cells is their altered metabolism. Cancer cells change their metabolic profile to cope up with continuous requirement of cell survival and proliferation. The best and oldest known metabolic change is increased glycolysis in cancer cells. Otto Warburg in his seminal findings observed that cancer cells even in the presence of ample oxygen prefer glycolysis over oxidative phosphorylation for the energy production [150]. This phenomenon of increased glycolysis at oxygen rich condition is known as aerobic glycolysis or Warburg effect. This feature of cancer cells is also exploited clinically for the imaging technique; positron emission tomography, using the glucose analogue tracer fluorodeoxyglucose (FDG-PET) [151, 152]. In addition to increased glycolysis cancer cells also have other metabolic changes, like increased gluconeogenesis, glutaminolytic activity, pentose phosphate pathway activity etc.

1.3.1 Glucose metabolism

Glucose derived from break down of carbohydrates or by gluconeogenesis is transported into the cells by specific glucose transporters. Glucose gets converted to pyruvate by a fundamental biochemical process called glycolysis and this results in production of net two ATP molecules. At oxygen rich conditions, pyruvate enters the mitochondria and is converted to acetyl-CoA by the enzyme pyruvate dehydrogenase (PDH). Citrate synthase catalyzes condensation reaction involving Acetyl CoA and oxaloacetate to produce citrate. Citrate subsequently is oxidized in tri-carboxylic acid (TCA) cycle to generate reducing agent NADH, electrons and CO_2. Electrons from reducing agent passed through electron transport chain (ETC) to final electron acceptor oxygen. ETC produces another 34 ATP molecules making total 36 molecules of ATP produced from one glucose molecule. Only at the anaerobic conditions normal cells produce more lactate from pyruvate when oxygen is not available for final electron acceptor in ETC. In contrast, proliferating or cancer cells show increased glycolysis and produce more lactate from pyruvate, regardless of the availability of oxygen. Lactate produced by cells in glycolysis is transported out of the cell by monocarboxylate transporters (MCTs).

Alternatively, glucose can be metabolized by Pentose phosphate pathway (PPP). PPP maintains intracellular nicotinamide adenine dinucleotide phosphate

(NADPH), which is critical for cell survival during rapid cell growth and also produces ribose sugar for nucleic acid synthesis.

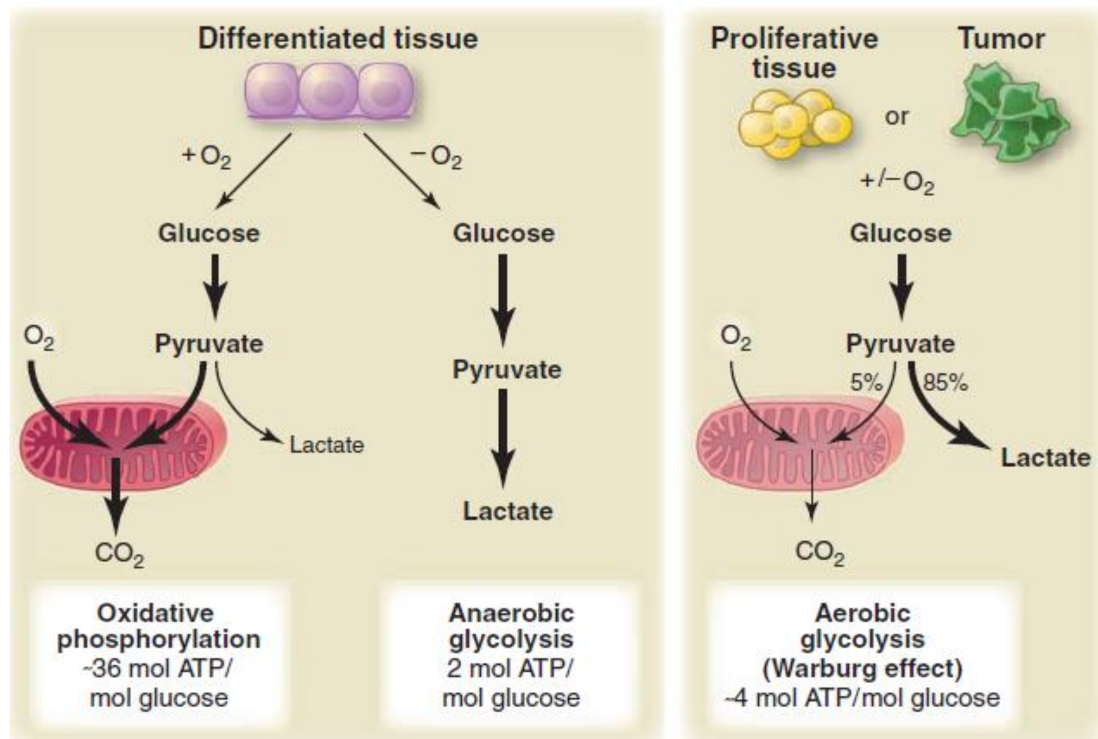

Figure 5: Schematic representation of difference in glucose metabolism between normal cell and cancer cell. Differentiated cells showed different metabolism at aerobic or anaerobic conditions. They prefer mitochondrial respiration at aerobic conditions to produce 36 molecules of ATP per glucose molecule. In anaerobic conditions they induce glycolysis and produce 2 molecules of ATP per glucose molecule. However, cancer cells prefer glycolysis irrespective of the availability of oxygen. (Adapted from [153]).

1.3.2 Proliferative advantages of glycolysis

Glycolysis is less efficient way to produce ATPs than oxidative phosphorylation (OXPHOS), even though, cancer cells prefer it. Otto Warburg proposed that increased aerobic glycolysis is because of impairment in the oxidative metabolism [114]. Many studies in tumor cells later showed that they do not have defects in oxidative metabolism [115]. So, why proliferating cells show Warburg effect?

Cancer cells are continually exposed to nutrient supply in circulating blood and ATP may never be a limiting factor in these cells [153, 154]. Cells, exhibiting aerobic glycolysis also show high ratio of ATP/ADP, no matter how often they divide [155]. Although ATP production from the glycolysis is low, but if glycolytic flux is high, percentage of ATP produced from glycolysis is higher than that

of OXPHOS [150, 156]. This is because of high rate of ATP production during glycolysis [157], and it is advantageous for the proliferating cells.

Another explanation is that premalignant tumors expand and outgrow the diffusion limits of blood supply, leading to hypoxic tumor microenvironment. Hypoxia puts limitation on the oxidative metabolism and tumor cells depend on glycolysis for the energy production. Such tumors do produce vascularization by angiogenesis but it is disorganized and may not deliver blood effectively and do not completely escape cells from hypoxia [158]. Oxygen levels in tumor fluctuate spatiotemporally and it results into selection of tumor cell population, which shows constitutively induced glycolysis.

It has also been debated that tumors with increased aerobic glycolysis produce lactate and excrete it into the tumor microenvironment resulting, in its acidification. This facilitates tumor invasion via destruction of extracellular matrix and normal cell population while tumor cells get adapted to this changes [158]. Acidosis formed by lactic acid also shown to suppress anticancer immune effectors [159].

Glycolysis also functions to support the synthesis of biomolecules like nucleotides, amino acids and lipids. Glucose metabolism can provide the precursors to build the macromolecules essential for cell division and this is important for the generation of daughter cell [160]. NADPH produced during PPP is also required for neutralizing reactive oxygen species (ROS) related cellular damage.

1.3.3 Hypoxia inducible factor 1

In eukaryotic cells hypoxia inducible factor (HIF) plays an essential role in cellular oxygen homeostasis [161-163]. As name suggests, this protein is stabilized at the hypoxic conditions which are caused by mismatch between oxygen delivery and its consumption. HIF induces transcription of more than 60 proteins, which are responsible for oxygen delivery and utilization at low availability of oxygen. This is achieved by reduced consumption of oxygen (by inducing glycolysis) or facilitating delivery of oxygen (via erythropoiesis or angiogenesis).

Hypoxia inducible factor 1 (HIF1) is heterodimeric DNA binding transcription factor that belongs to the bHLH-PAS (Per/Arnt/Sim) family and consists of two subunits- HIF1α and HIF1β [164]. HIF1α is oxygen regulated subunit, while HIF1β, which is also called as aryl hydrocarbon receptor nuclear translocator (ARNT), is constitutively expressed. At normoxic conditions HIF1α is hydroxylated on

proline 402 and/ or proline 564 within its oxygen dependent domain (ODD). Hydroxylation is catalyzed by the enzymes prolyl hydroxylase domain proteins (PHD), which uses O_2 and α-ketoglutarate as substrates [165]. The hydroxylated HIF1α is recognized then by the von Hippel-Lindau tumor suppressor protein (VHL) complexed with elongins B and C. VHL is an E3 ubiquitin ligase which targets HIF1α for the proteosomal degradation [166, 167]. In hypoxia, when internal partial pressure of oxygen is less than 10-15 mm Hg in context of tumors [168, 169], PHD cannot hydroxylate HIF1α due to lack of oxygen. Thus HIF1α cannot be recognized by the VHL tumor suppressor, avoids proteosomal degradation and becomes stabilized.

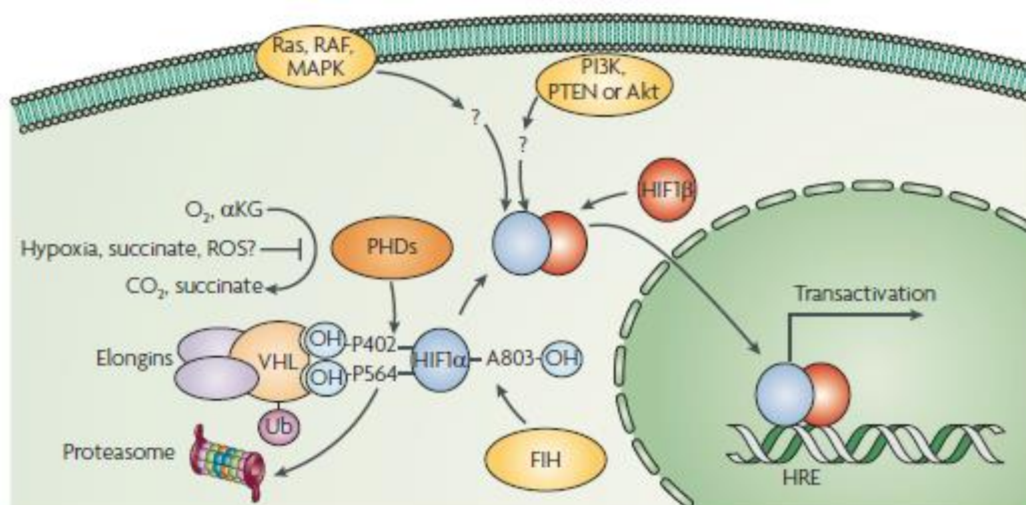

Figure 6: Mechanism of HIF1α stabilization. (Adapted from [170])

1.3.4 HIF activators at aerobic condition

HIF1α plays an important role in cancer progression at the hypoxic conditions. However, all cancers are not hypoxic, but HIF1α still plays important role in them. In some cases, oncogenic signaling results into HIF1α stabilization in O_2 independent manner. Expression of HRAS-v12 leads to accumulation of HIF1α at hypoxic as well as normoxic conditions [171], while expression of SRC [172] and eRBB2 [173] results into increased level of HIF1α at normoxic conditions. Activation of Ras-MAPK (mitogen activated protein kinase) [174] and PI3K pathways [175] can also lead to stabilization of HIF1α protein at oxygen rich conditions. Effect of PI3K induced HIF1α accumulation could be due to direct activation of PI3K or its downstream kinase Akt or loss of inhibitor PTEN tumor suppressor. HIF1α protein translation increases due to Akt dependent activation of mammalian target of rapamycin (mTOR) [176].

Alteration in mitochondrial metabolism also results in stabilization of HIF1α. TCA cycle enzymes provide positive feedback mechanism to stabilize HIF1α. Loss of function of succinate dehydrogenase (SDH) and fumarate hydratase (FH) results in the accumulation of substrate for these enzymes, i.e. succinate and fumerate, respectively. They can leak into cytosol and compete with α-ketogluterate for the binding with PHDs (responsible for hydroxylation of HIF1α), inhibiting catalytic activity of PHDs. It results into the inhibition of hydroxylation and proteosomal degradation of HIF1α protein [177-180]. Another TCA cycle enzyme, isocitrate dehydrogenase (IDH), in contrast, limits the availability of α-ketogluterate required for activity of PHDs [181]. Mitochondrial reactive oxygen species (ROS), generated during electron transport chain, can also signal to stabilize the level of HIF1α [182-184]. Hydrogen peroxide (H_2O_2) in particular oxidizes Fe^{2+} to Fe^{3+}. Fe^{2+} can act as co-activator for PHDs for the hydroxylation of HIF1α but $Fe3^{+}$ can't. Due to decrease of Fe^{2+}, PHDs cannot hydroxylate HIF1α and HIF1α escapes proteosomal degradation and is stabilized [185].

1.3.5 HIF1 regulation of glycolysis

HIF1α regulates glycolysis in several ways. The largest group of genes regulated by HIF1α is involved in glycolysis [186-188]. Intake of glucose from blood into cells requires specific glucose transporters. There are 13 different glucose transporters. However, HIF1α induces the expression of GLUT1 and GLUT3, which import the major portion of intracellular glucose [189]. After the intake, glucose is phosphporylated to glucose-6-phosphate by the enzyme hexokinase (HK). Glucose-6-phosphate can also enter pentose phosphate pathway, which is another pathway of glucose metabolism that is involved in the synthesis of glycogen or structural component of glycoproteins. However, HIF1α induces HKI and HKII and also many other enzymes involved in Embeden mayerhoff pathway (EMP) (Glycolysis), which channels glucose via EMP. Once the glucose is phosphorylated, it can't escape the cell. Phosphorylated glucose molecule is converted to pyruvate via series of enzymatic processes. Almost all the enzymes, that are necessary for this process, are regulated by HIF1α [161, 190].

Pyruvate can be converted to acetyl coenzyme A (AcCoA) by pyruvate dehydrogenase (PDH) and after that enters into tricarboxylic acid (TCA) cycle. Pyruvate can also be converted to lactate by lactate dehydrogenase A (LDHA) and

secreted out of cell. HIF1α induces the expression of pyruvate dehydrogenase kinase 1 (PDK1), which phosphorylates and inactivates the catalytic domain of the PDH [191]. This inhibits entry of pyruvate into the TCA cycle in mitochondria and attenuates oxidative phosphorylation [192]. This can result into accumulation of pyruvate which is harmful to the cell. HIF1α induces LDHA to convert pyruvate to lactate [193], and also monocarboxylate transporter 4 (MCT4), to transfer the lactate into extracellular space [194].

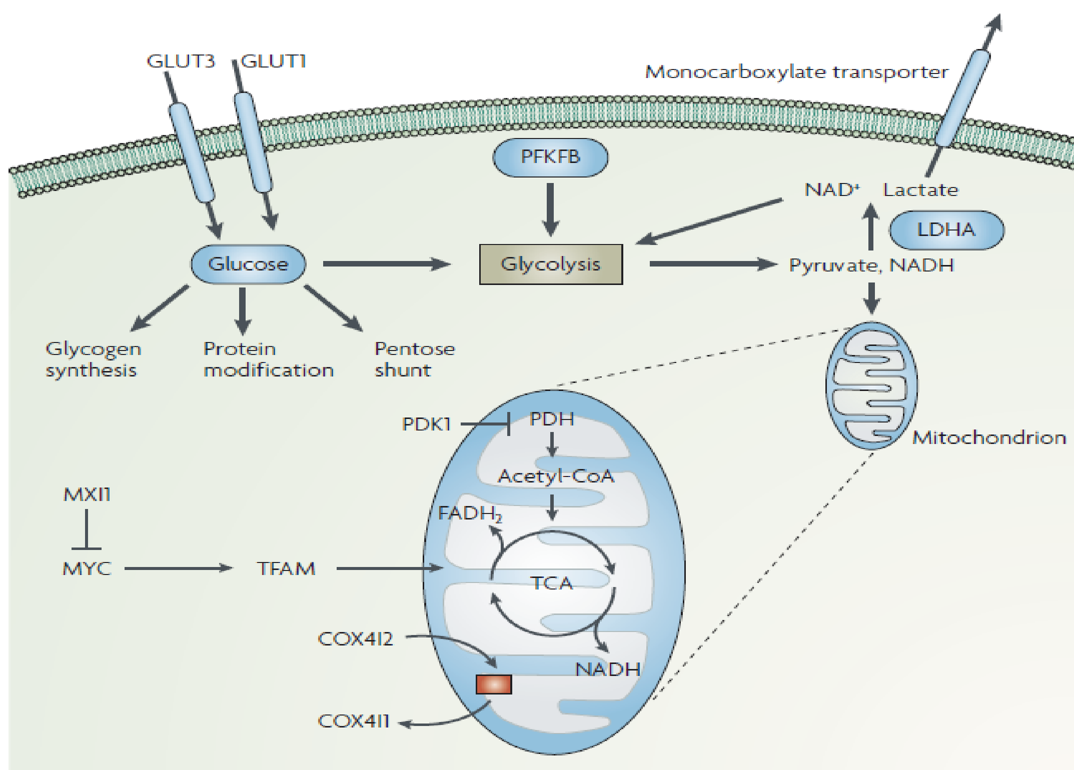

Figure 7: Regulation of glycolysis by HIF1α. HIF1 activation leads to increase in glycolysis and a decrease in oxidative phosphorylation. HIF1 stimulates increased uptake of Glucose by glucose transporters (GLUT) that is then metabolized by increased expression of glycolytic enzymes. Pyruvate produced as a result of glycolysis will then be converted to lactate by lactate dehydrogenase A (LDHA) and excreted from the cell by monocarboxylate transporters 4 (MCT4). Pyruvate dehydrogenase kinase 1 (PDK1) decreases flow of pyruvate to mitochondria and there is switch of more efficient isoform from cytochrome oxidase subunit 4 isoform 1 (COX4I1) to COX4I2 subunit. (Adapted from [170])

1.3.6 Important HIF1 regulated molecules in glycolysis

Hexokinase (HK) is responsible for the ATP dependent phosphorylation of glucose to glucose-6-phosphate. HK gene family consists of 4 isoforms, HK I-IV. HK II is highly expressed during early carcinogenic events in liver and pancreatic cells; HK I is upregulated at lesser extent and HK IV is downregulated [195-198]. HK I and II can directly interact with specific voltage-dependent anion channels (VDACs) in mitochondria, and this results in inhibition of VDACs function, i.e. shuttling of ATPs

between mitochondrial matrix and cytosolic compartment. Secondly, it also influences on the interaction of BAX/BAK with VDAC; hence, inhibiting apoptosis. Accumulated ATP is harnessed by HK for the phosphorylation of glucose, thus committing glucose through glycolysis [199].

Proliferating cells need energy as well as macromolecules for the cell division. Pyruvate kinase (PK) seems to play an important role in this context. In the final step of glycolysis it converts phosphoenolpyruvate to pyruvate, producing ATP molecules. There are 4 forms of PK (L, R, M1 and M2) which are expressed from two genes. PKM1 and PKM2 are isoforms from the same gene; PKM2 is the predominant form of the PK in cancer cells. M2 isoform is expressed in embryonic tissue and it is rapidly replaced by the M1 isoform during the developmental stages; in cancer cells M2 isoform is re-expressed [200]. PKM2 activity is depended on its quaternary structure and its tetrameric form is active one [201]. PKM2 is allosterically activated by fructose-1,6-bisphosphate (FBP) [202]. PKM2 has less catalytic activity than M1 isoform and PKM2 promotes both aerobic glycolysis and anabolic metabolism [155]. Low activity of PKM2 is advantageous for proliferating cells, because it allows glycolytic intermediate to be used for biosynthetic pathway. mTOR induces PKM2 via HIF1 [203] and PKM2 is shown to upregulate HIF1activity by direct binding [204], thus, creating positive feedback loop between HIF1 and PKM2.

Table 2: HIF1 transactivating genes that regulate glucose metabolism.

HIF1 responsive genes	Role in metabolism
GLUT-I , GLUT-III	Glucose entry into the cell
HK	Phosphorylation of glucose.
PGI, PFK1, aldolase, TPI, GAPDH, PGK, PGM, enolase, PK, PFKFB1–4	Glycolysis
LDHA	Conversion of pyruvate to lactate.
MCT4	Removal of lactate from the cell
PDK1, MXI1	Decreased mitochondrial activity
COX4I2, LON protease	Increased O2 consumption in hypoxia

COX4I2, cytochrome oxidase isoform 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLUT, glucose transporter; HIF, hypoxia-inducible factor; HK, hexokinase; LDHA, lactate dehydrogenase A; McT, monocarboxylate trasporter; MXI, max interactor; PDK, pyruvate dehydrogenase kinase; PFK, phosphofructokinase; PFKFB, 6-phospho-2-kinase/fructose 2,6 bisphosphatase; PGK, phosphoglycerate kinase; PGI, phosphoglucose isomerase; PGM, phosphoglycerate mutase; PK, pyruvate kinase; TPI, triosephosphate isomerase.

1.4 MITOCHONDRIAL RIBOSOME

Mitochondria are considered to be descendent of eubacteria that incorporated into pre-eukaryotic cell during evolution by endosymbiosis [205]. They have their own DNA and ribosome for translation. Mitochondrial ribosomes are responsible for the translation of all the 13 mRNAs encoded by mitochondrial DNA, and translated proteins are essential for the oxidative phosphorylation system. Mammalian mitochondrial ribosome (55S) differs from that of cytoplasmic (80S) and bacterial ribosome (70S). It is made up of large (39S) subunits and small (28S) subunits. Ancestral mitochondrial ribosome (70S) has been remodeled during the evolution to current mitoribosome (55S) and has lost half of its rRNA which are replaced by proteins (about 80) [206]. These extra proteins which are not homologous to bacterial or cytoplasmic ribosome seem to have extra-ribosomal function. For instance, MRPS29, a GTP binding protein implicated to be involved in apoptosis [207]. The small subunit of the mitochondrial ribosome contains 30 proteins. Large subunit is made up of 48 proteins making 78 proteins in mitochondrial ribosome [208]. All of the mitochondrial ribosomal proteins (MRP) are encoded by nuclear genome, translated in cytoplasmic ribosome and imported into the mitochondria. At least 1500 gene products are translated and migrated to the mitochondria [108, 109].

1.4.1 Mitochondrial ribosomal protein S18-2

Mitochondrial ribosomal S18-2 protein belongs to S18 family proteins which contain three variants in mammals, MRPS18-1 (AAD34129), MRPS18-2 (NP_054765) and MRPS18-3 (NP_060605), also termed as MRPS18C, B and A respectively. Three variants of S18 homologues are also found in *D. melanogaster* and mouse, while two corresponding to MRPS18-1 and MRPS18-2 are found in *C. elegans* mitochondrial ribosomes [209]. *E. coli* contains one form while other prokaryotes like *Streptomyces coelicolor* and *Mycobacterium tuberculosis* contains two variants of this protein. Like *E. coli*, yeast also contains one form which is closer to S18-1. Amino acid sequence alignment amongst three forms shows a conserved region in the central part of the proteins. Primary sequence of human variants is more related to prokaryotic homologue than to each other. S18-1 gene is mapped on the chromosome 4 at 4q21.23 while genes for other two are located on chromosome 6, MRPS18-2 at 6p21.3 and MRPS18-3 at 6p21.1.

2 AIMS

The goal of the thesis is to elucidate the role of some EBNA binding cellular proteins in cell transformation.

The specific aims were:

- How does EBV counteract Vitamine D induced apoptosis in B cells?
- Study on the role of HIF1α in the EBV induced B-cell transformation.
- To explore the role of MRPS18-2 in cell transformation, using rat embryonic and terminally differentiated skin fibroblasts.

3 RESULTS AND DISCUSSION

3.1.1 Vitamin D in apoptosis

Calcitriol ($1\alpha,25(OH)_2D_3$) exert anti-tumor effect by regulating few key mediators of the apoptotic pathway. They can repress the expression of anti-apoptotic proteins or upregulate pro-apoptotic proteins. In MCF7 breast carcinoma cells and HL-60 leukemia cells calcitriol downregulates Bcl-2 expression. In prostate cancer, colorectal adenoma and carcinoma cells calcitriol induces the expression of BAX and BAK [210]. Calcitriol destabilizes telomerase reverse transcriptase (TERT) mRNA, thus promoting apoptosis due to downregulation of telomerase activity and telomere erosion [211]. In LNCaP human prostate cancer cells, calcitriol induces apoptosis with simultaneous downregulation of antiapoptotic proteins Bcl-2 and Bcl-Xs. Overexpression of Bcl-2 in these cells blocks calcitriol induced apoptosis and reduces calcitriol induced growth inhibition [212].

Pre-treatment of calcitriol can sensitize BCa (breast cancer cell line) cells for apoptosis. They are more susceptible to apoptosis triggered by reactive oxygen species (ROS). Caspase dependent and independent pathways are induced causing mitochondrial membrane damage with subsequent release of cytochrome C [213]. Calcitriol can also increase cytotoxicity of TNFα on the same cells [214]. Calcitriol increases the intracellular level of calcium by activating calcium entry from extracellular spaces and by depleting calcium stores in endoplasmic reticulum. It is detrimental to MCF7 cells, which do not express calbindin-D, that buffers intracellular calcium levels, resulting in activation of mu-calpain and caspase-12 and subsequent apoptosis [215]. Calcitriol can activate mitogen activated protein kinase (MAPK) pathways; p38 and JNK. Activation of these pathways play a role in calcitriol induced apoptosis in BCa cells [216]. Promoter of PTEN tumor suppressor gene contains VDRE. Upregulation of PTEN via VDR can attenuate PI3 kinase pathway, resulting in apoptosis in gastric cancer cells [217].

In a previous work of our group, on expression of nuclear receptors in EBV transformed B-cells, it was found that VDR is expressed at lower levels in LCLs compared to naive and mitogen activated B cells [218]. It has been also shown that VDR is constitutively expressed at mRNA and protein level in B-cells and there is a functional block for the lα,25-(OH),D3 mediated gene regulation in these cells [219].

EBV has been known to protect infected B-cells from apoptosis via its latent encoded proteins [220]. EBNA3 family proteins inhibit apoptosis of Burkitt lymphoma cells infected with EBNA2 deleted virus [221]. They can suppress the transcription of *BIM,* resulting in anti-apoptotic effect [144]. EBNA3 was also shown to regulate the function of another nuclear receptor, aryl hydrocarbon receptor (AhR) thus shown to influence the transcription of AhR responsive genes upon its ligand binding, protecting cells from apoptosis [222].

3.1.2 EBNA3 binds to VDR

Using mass spectrometry, we aimed to find EBNA3 binding proteins after immunoprecipitations. To achieve higher expression of EBNA3, GFP-EBNA3 was overexpressed in MCF7 cell lines. GFP-EBNA3 containing protein complexes were collected by immunoprecipitations using anti-GFP antibody. Few specific bands on SDS PAGE which were absent in negative control were analyzed by mass spectrometry. We have found SW1/SNF proteins on EBNA3, and one of the subunit of this complex was VDR. VDR could be immunoprecipitated with anti-EBNA3 antibody from LCL cell lysate. This interaction was confirmed with reverse immunoprecipitaions with anti-VDR antibody and detection of EBNA3 by western blotting (Fig. 1, Paper I).

After we found that endogenous EBNA3 and VDR were captured in one immunocomplex, we investigated the expression of VDR responsive genes. It was measured in the isogenic lymphoblastoid cell lines, expressing EBNA3 (BK-LCL) and cells where *EBNA3* gene was replaced by neomycine gene (ΔEBNA3-BK-LCL). The VDR responsive genes (*C-FOS, CYCLIN C, CYP24A1, GADD45A*, and *P21*) were expressed at low level in BK-LCL compared to ΔEBNA3-BK-LCL (Fig. 4A, Paper I). Same isogenic cell lines were treated with VDR ligand 1-alpha-25-dihydroxyvitamin-D3 (1,25(OH)2D_3) and expression of VDR responsive genes were measured. 1,25(OH)2D_3, treated BK-LCL did show higher expression of VDR responsive genes than untreated cells, however, this induction is lower than 1,25(OH)2D_3 treated ΔEBNA3-BK-LCL cells (Fig. 4B, Paper I). In another set of experiment, EBV negative BL DG75 sub-lines that express either EBNA5 (DG75-EBNA5) or EBNA3 (DG75-EBNA3) were studied. After treatment with 1,25(OH)2D_3, genes such as *CYCLIN C, CYP24A1, GADD45A* and *P21,* showed up-regulation in DG75-EBNA5 cells compared to untreated cells, while 1,25(OH)2D_3 treatment did not induce

responsive genes induction in DG75-EBNA3 cells (Fig. 4C, Paper I) . These results demonstrate that EBNA3 plays a crucial role in repressing VDR responsive genes expression in LCLs.

3.1.3 EBNA3 counteracts vitamin D_3-induced cell death

As many VDR responsive gens are pro-apoptotic and they were repressed by EBNA3, we wanted to see the physiological relevance of binding between EBNA3 and VDR. To do that, the proliferation rate of freshly established LCLs, BK-LCL and ΔEBNA3-BK-LCL were assessed. Cells were cultured in medium that contained 1.10^{-7} M of 1,25(OH)2D_3 and in the ordinary medium. Untreated ΔEBNA3-BK-LCL proliferated slower than untreated LCL and BK-LCL, which proves that EBNA3 is necessary for efficient transformation. Treatment of ΔEBNA3-BK-LCL with 1,25(OH)2D_3 decreased the proliferation rate significantly compared with untreated cells while LCL and BK-LCL did not show any difference upon treatment. This shows that in absence of EBNA3 cells are vulnerable to 1,25(OH)2D_3 mediated cell death and EBNA3 protects LCLs from 1,25(OH)2D_3 induced cell death (Fig. 5A, Paper I) .

Annexin V assay showed that more than 90 % of the ΔEBNA3-BK-LCL cells were apoptotic upon treatment with 1,25(OH)2D_3. 1,25(OH)2D_3 was used to treat cells at the higher levels compared with physiological levels but still EBNA3 could save LCL from 1,25(OH)2D_3 induced cell death (Fig. 5B, Paper I).

3.2 VIRUSES AND GLYCOLYSIS

Few studies done in 1970s and before showed that the virally transformed cells, such as transformed chick embryo cells by RSV [223], feline fibroblasts transformed by Feline leukemia virus and HEp-2 cells [224] and monkey kidney cells transformed by poliomyelitis virus [225] has increased glycolysis. More recently, there was work done on metabolic changes upon infection by tumor viruses. In a global approach to study the effect of cytomegalovirus (CMV) infection on host cell metabolism, 63 different intracellular metabolites were measured at different time points after infection, using liquid chromatography-tandem mass spectrometry. It was observed that with the progression of infection, metabolites that are involved in glycolysis, the citric acid cycle, and pyrimidine nucleotide biosynthesis increased. This increase was also confirmed by transcriptional up-regulation of enzymes involved in glycolysis and citric acid cycle [226]. In another metabolomics approach to check the host cell metabolic changes after infection of endothelial cells with Kaposi sarcoma herpes virus (KSHV), one third of the 194 different biochemicals were found to be altered. The number of metabolic pathways altered in KSHV infected cells was similar to cancer cells. This concerns amino acid metabolism and many glycolytic intermediates like 3-phosphoglycerate, 2-phosphoglycerate and phosphoenolpyruvate (PEP). The pentose phosphate pathway intermediates like ribose 5-phosphate, ribulose 5-phosphate and/or xylulose 5-phosphate were elevated significantly in KSHV infected samples. Metabolites involved in *de novo* fatty acid synthesis were significantly increased in KSHV infected cells. Inhibition of fatty acid synthesis resulted in induction of apoptosis in infected cells [227]. E7 oncoprotein encoded by Human papilloma virus (HPV) interacts with the glycolytic enzyme PKM2 which catalyzes the final step of the glycolysis, converting phosphoenolpyruvate to pyruvate. PKM2 is activated when it forms a tetramer, while interaction with E7 stabilizes it in dimeric inactivated form. So, this could be in favor of the glucose molecule to be used via pentose phosphate pathway that gives biosynthetic advantages to the cell [228].

3.2.1 HIF1α is stabilized in lymphoblastoid cells (LCLs)

In our study we have found that HIF1α transcription factor is stabilized in EBV immortalized lymphoblastoid cells at the normal cell culture conditions. Concurrent with this, there is no change in the mRNA expression level between LCLs

and activated B-cells (Fig. 1, Paper II). This indicates that HIF1α is regulated at protein level rather than at the RNA level. We further checked if any of EBNAs play a role in stabilization of HIF1α. By GST pull down assays, EBNA3 but not EBNA 5 and 6 are found to be associated with the HIF1α. This interaction was also confirmed by the immunoprecipitation with EBNA3 antibody immobilized on CNBr beads (Fig. 2A and B, Paper II).

We further investigated the cellular distribution of the HIF1α, EBNAs and HIF1α hydroxylating enzymes, prolyl hydroxylases (PHDs 1 and 2). HIF1α was found to be nuclear, while it was almost absent in the nucleus in mitogen activated B-cells. This was also confirmed by immunoblotting, using subcellular fractions, that showed higher HIF1α levels in nuclear fraction of LCL (Fig. 4, Paper II). HIF1α was found to be co-localized with EBNA3 and partially with EBNA5 in MCF7 breast carcinoma cell line when EBNAs were overexpressed after transient transfection. In the same system, PHDs 1 and 2 which are mainly cytoplasmic could be localized to the nucleus. PHD1 co-localized with HIF1α and EBNA5, while PHD2 was found to be co-localized with HIF1α and EBNA3 (Fig. 2 C and D, Paper II). We next performed GST pull down assays and verified that PHD1 form complex with EBNA5, while PHD2 with EBNA3 (Fig. 3, Paper II). These results indicate that stabilization of HIF1α in LCLs could be due to impairment of function of HIF1α hydroxylating enzymes, PHDs. This interaction may impair the catalytic activity of PHDs due to steric hindrance.

3.2.2 HIF1α induces aerobic glycolysis (Warburg effect) in LCLs

In spite being in complex with EBNA3, HIF1α was found to be transcriptionally active. Quantitative PCR was performed to check the expression of HIF1α responsive genes in LCLs, freshly EBV infected cells and the mitogen activated B-cells. Many HIF1α responsive genes were up-regulated in LCLs and freshly EBV infected B-cells compared to mitogen activated B-cells. Mitogen activated B cells, treated with hypoxia mimicking compound, which stabilizes HIF1α, showed up-regulation of responsive genes. This is in contrast to LCLs and freshly EBV infected cell in which HIF1α responsive genes were expressed at higher level under all conditions.

LCLs do not experience hypoxia in culture conditions but they do stabilize the protein responsible for dealing with hypoxic situation. So, we looked for other functions of HIF1α. It has also been shown to modulate cellular metabolism by

inducing genes involved in glycolysis and attenuating the mitochondrial oxidative metabolism. We examined whether HIF1α plays any role in glycolysis in LCLs or not. We found that at mRNA level, HIF1α activate many genes which induce glycolysis in LCLs that exhibit Warburg effect (Fig. 5, Paper II). GLUT1 (glucose transporter), which imports glucose to cell, and enzymes like PFKL (phosphofructokinase), HK1 (hexokinase) and PKLR (pyruvate kinase), PGK1 (phosphoglycerate kinase), which are involved in glycolysis process, were up-regulated in LCLs. LDHA (lactate dehydrogenase), which converts pyruvate to lactate, and MCT4, (monocarboxylate transporter) which exports lactate out of the cell, were also induced. PDK1 (pyruvate dehydrogenase kinase), which stops pyruvate entry into mitochondria by inhibiting the catalytic activity of the enzyme pyruvate dehydrogenase (PDH) [191] was also up-regulated. However, production of reactive oxygen species (ROS) in LCLs was observed to be very high (Fig. 6B, Paper II), indicating that even though PDK1 inhibit entry of pyruvate (that fuels mitochondrial respiration), mitochondrial respiration is not completely abolished in LCLs.

Moreover, the end product of glycolysis, i.e. pyruvate, was produced 5 times more in LCLs, than in activated B-cells and freshly EBV infected cells. Enzymatic activity of lactate dehydrogenase A (LDHA), which converts pyruvate to lactate, was also extremely up-regulated in LCLs that was also revealed by the lactate measurement from the medium of the cells. LCLs showed 10 fold more lactate production compared to the mitogen activated and freshly EBV infected B-cells (Fig. 6A, Paper II). Thus, up-regulation of glycolysis with increased lactate production in LCL shows that they exhibit Warburg effect and HIF1α plays an important role in this phenomenon.

3.3 MRPS18B (S18-2) CAN INDUCE CELL TRANSFORMATION

3.3.1 Overexpression of S18–2 immortalizes REFs

S18- 2 was first cloned from CD34+ hematopoietic stem/progenitor cells [229]. It serves as a bridge between EBNA6 and RB in EBV transformed B-cells. EBNA6 binds to S18-2 and targets it to the nucleus, thus promoting its binding to RB. S18-2 binds to RB at the same pocket where RB has binding site for E2F1. Thus, S18-2 competes with E2F1 for binding with RB and EBNA6 expressing cells shows increased level of free E2F1 compared to EBNA6 null cells [230]. This promotes the cell cycle progression from G_1 to S phase.

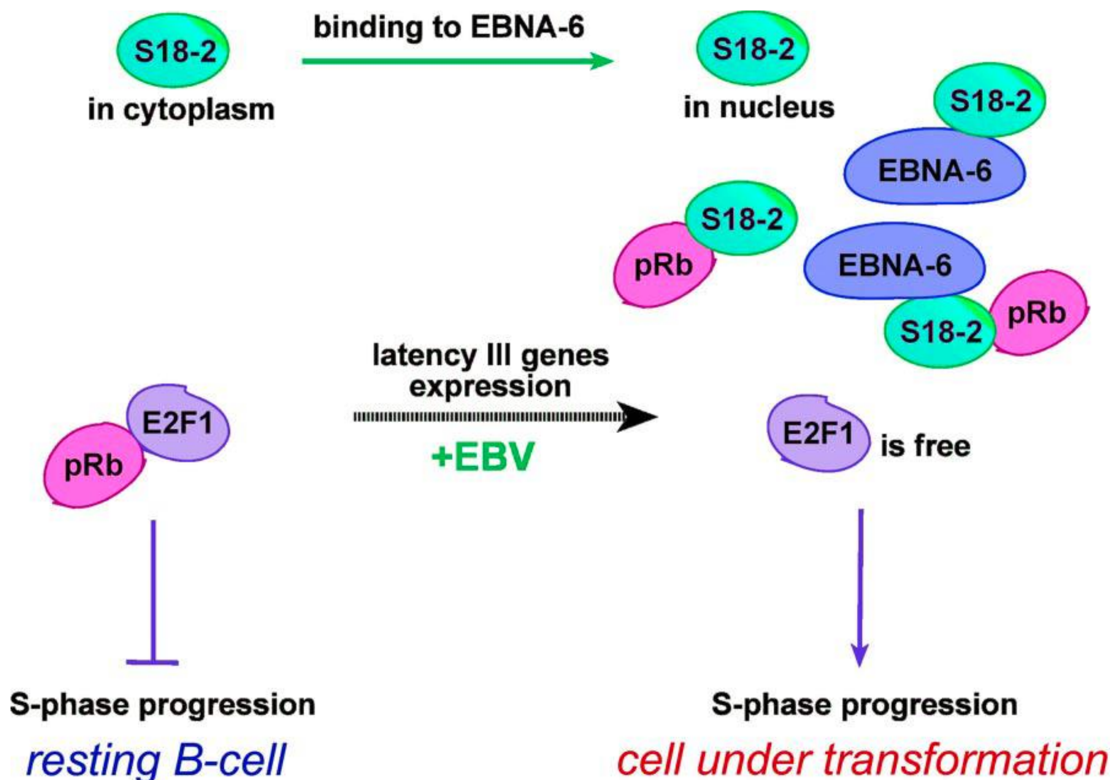

Figure 8: Mechanism of S18-2 mediated cell cycle progression in EBV infected B-cells. In resting B cells pRb is in bound state with E2F1, while S18-2 is present in cytoplasm. After EBV infection EBNA-6 binds and translocates S18-2 to nucleus. In nucleus S18-2 competes with E2F1 for the binding to pRb. This results in increased amount of free E2F1 and cell enters into S-phase of the cell cycle. (adapted from [230])

It was shown earlier that EBNA6 is an immortalizing onco-protein and can co-operate with Ha-Ras in transformation of REFs [35]. As EBNA6 uses S18-2 to induce S-phase entry we thought that it might be possible that S18-2 has its own role in cell transformation. To study this hypothesis, we decided to check whether S18-2 can co-operate with Ha-Ras or EBNA6 in classical rat embryonic fibroblasts (REFs)

transformation assay. GFP fused S18-2, carrying neomycin resistance gene, was overexpressed alone or in combination with Ha-Ras or EBNA6 in REFs. Transfected cells were cultured on G418 selection medium for 2 weeks. Surprisingly, S18-2 alone was able to induce massive transformation of REFs. Large colonies were formed in petri dishes, where un-transfected control cells died in a week on selection medium. This result was also obtained by transfecting cells with c-myc-tagged S18–2 (MTS18–2) and pBabe-S18–2 (in triplicate). Clones from GFP-S18-2 and pBabe-S18–2 transformed cells were maintained and observed for more than 20 months in culture. To verify that for transformation S18-2 protein is needed, mutants lacking ATG start codon and mixture of 4 different small interfering RNA oligos designed to antagonize S18-2 mRNA were transfected along with non-specific SiRNA as a control. Cell death occurred after 48 hours of transfection concurrent with the decrease in S18-2 protein level while control siRNA had no such effect.

3.3.2 S18–2-immortalized cells show embryonic stem cell-like properties

S18-2 immortalized cells termed as 18IM, grew as large multilayered foci in culture and resembled stem cells growth. 18IM cells, when grown in bacterial petri dish, formed large compact aggregates, reminiscent embryoid bodies (Fig.1E, Paper III). These embryoid bodies grew normally like 18IM cells when cultured back at the ordinary conditions. However, c-myc and mutated Ha-ras transformed REFs (MR) formed clumps in bacterial petri dish and did not form embroyid bodies (Fig.1F, Paper III). RT- PCR data showed loss of mesenchymal differentiation genes like vimentin and FUT4 in 18IM cells but not in REFs. Embryonic stem cell specific genes like Sox2 and Oct 3/4 were upregulated in 18IM cells. Further we tested 18IM cells for the expression of stem cell markers by immunostainings. 30-40% of 18IM cell showed strong staining for stage specific embryonic antigen 1 (SSEA-1), in contrast to REFs and MR. Immunostaining data also confirmed that vimentin and smooth muscle actin (mesodermal markers) were lost by 18IM cells (Fig.2A and C, Paper III). Strikingly, it was found in available microarray data that S18-2 is expressed in embryonic stem cells at the higher levels than in differentiated tissues.

3.3.3 18IM cells can differentiate *in vitro* and *in vivo*

Some of the cells from the confluent 18IM cells detached from the surface. These cells were collected on cytospin slides. They had large cytoplasmic

vacuoles and were positive for the Oil Red O staining specific for the fat cells. No such cells were detected in control REF or MR cells. Moreover, rat specific MHC class II and ectoderm specific beta-III-tubulin-expressing cells were also found in 18IM culture. Cells obtained from re-culturing of embryoid bodies also showed expression of ectoderm specific pan-keratin (Fig.2A,B,C and D, Paper III). This indicates that 18IM cells could differentiate into cells from other lineages and expressed endoderm and ectoderm specific markers.

18IM grew in soft agar with high efficiency, a characteristic of transformed cells. To study the tumorigenic ability of 18IM cells *in vivo*, 5 millions of cells were inoculated in 5 SCID mice subcutaneously. Small tumors of maximum volume of approximately 20 mm^3 appeared at the site of inoculation in 9-13 days. However, after 3-4 weeks they decreased in volume to 2-3 mm^3 in three mice while disappeared in other two. Hematoxylin and eosin staining of the paraffin embedded samples revealed morphologically heterogenous anaplastic cells. Single large neoplastic cells with prominent nucleoli and cytoplasmic vacuoles were also observed. Rat cells could be distinguished by the absence of Hoechst positive pericentromeric heterochromatin foci present in mouse cells and fraction of such rat cells show pan-keratin staining in cytoplasmic filaments. Expression of pan-keratin showed that 18IM cells have trans-differentiated *in vivo* (Fig.4, Paper III).

3.3.4 Expression profile of 18IM and REFs

The gene expression profile of 18IM cells were compared with REFs by microarray technique. Genes, showing more than two fold difference with FDR-corrected P-value more than 0.05, were chosen for further studies. More than 4000 genes found to be differentially expressed and 19 cellular pathways altered in 18IM cells (Fig.1, Paper IV). Many genes involved in self-renewal pathways like WNT, BMP, β-catenin (Ctnn-b), Bmpr3 (BMP receptor), Smad1/5/8, Stat3, Spp1, Sox11, and Sh3glb1 were up-regulated while genes involved in mesenchymal differentiation like Runx2, Hoxc8, Creb, and Tlx2 were downregulated. Also many genes involved in stem cell development and proliferation were differently expressed (Table 1, Paper IV).

15 genes from microarray data, which were differentially expressed and may play a role in stem cell renewal or in carcinogenesis, were validated by quantitative PCR. As 18IM cells show stem cell like characteristics, genes involved in experimental induction of pluripotency in fibroblasts were also checked by Q-PCR. Oct4, Nanog, and Sox2 were found to be up-regulated while C-myc and Klf4 did not

show induction in 18IM cells (Fig.2, Paper IV). Notably, C-myc was found to be expressed at higher level at protein level in 18IM cells. Western blotting data showed loss of vimentin and smooth muscle actin from 18IM cells, while Sox2 was overexpressed at the protein level (Fig.3, Paper IV). This confirms the previous RT-PCR study observations. Moreover, 18IM cells continued to express the embryonic stem cell marker SSEA-1 even after 2 years in culture.

De-methylation of the CpG islands in the promoter region has been shown to be the mechanism behind expression of the genes involved in induction of pluripotency [231]. So, we investigated the promoter methylation of the 6 genes playing the role in this process. Oct4, Nanog, Sox2, Lin-28, C-Myc and Klf4. They were analysed by the treatment of cells with sodium bisulfite and subsequent sequencing of the amplified fragments from genomic DNA was performed. 8 of the clones for each gene from 18IM and REFs were analysed but none of the genes showed substantial difference in promoter methylation pattern.

3.3.5 Experiments on newly generated clones

Three new cell lines were generated, two by overexpression of GFP-S18-2 and one by another construct pBabe-S18-2, which gives lower expression of the protein compared to GFP construct. Three clones namely, clone-10 and 12 from GFP-S18 -2 and clone-6 from pBabe vector were followed in culture for a year. Clones10 and 12, like 18IM cells, formed foci with dense center while clone-6 with lower expression of S18-2 did not form compact foci and show high degree of cellular heterogeneity in culture. None of the clones showed tumor formation in SCID mice two months after inoculation. Cloning in soft agar gave colonies from clone-10 and 12 but not from clone-6.

All the clones showed expression of c-myc at protein level. They did not lose smooth muscle actin expression, unlike 18IM cells. However, clone-10 and 12 did show Oct4 and Sox2 upregulation at the protein level. Clone-6, which gives lower expression of the S18-2 protein did not show Oct4 expression or induction of Sox2 as high as other clones. Clone-6 also showed expression of mesodermal marker vimentin, unlike 18IM and clone10 and 12 (Fig.5, Paper IV). This indicates that level of expression of S18-2 might be responsible for determining the degree of differentiation of the cells.

Moreover, inhibition of protein synthesis in zebra fish by introduction of the S18-2 specific morpholino resulted in embryonic lethality while simultaneous

introduction of *in vitro* transcribed S18-2 mRNA permitted the normal development of the zebra fish.

3.3.6 Immortalization of primary rat skin fibroblasts (RSFs) by overexpression of MRPS18-2

18IM cells formed embryoid bodies and expressed some stem cell markers with loss of mesodermal markers. However, they were derived from rat embryonic fibroblasts and hence, it might be possible that primary culture contained some of the stem cell from rat embryo, i.e. there was a mix population where some cells might have stem cell like characters. Therefore, we decided to investigate whether S18-2 will immortalize fibroblasts derived from adult tissue of rat or not. To do this, we transfected rat skin fibroblasts with GFP-S18-2 and selected cells with G418. After 3 weeks clones were selected and the fastest growing clones, i.e 3, 6, 13 and 17, were expanded in culture flask from petri dish. These cells showed loss of contact inhibiton and anchorage independent growth, they were maintained in culture over the period of 20 months and passed 200 passages. So, the S18-2 protein could indeed immortalize the rat skin fibroblasts. However, they did not form embryoid bodies in bacterial petri dish. Further we studied a gene expression pattern and expression of differentiation markers.

Quantitative PCR studies showed that genes involved in induced pluripotency, i.e. Sox2, Oct4, Klf4, and c-Myc, were upregulated compared to primary RSFs. Transcription factor Tcea3 and elongation factor Eef1a2 which were extremely up-regulated in 18IM cells were expressed at lower level in immortalized RSFs (Fig.2, Manuscript). Western blotting showed that immortalized clones of RSFs did not lose expression of vimentin and smooth muscle actin while they upregulate Oct4, Sox2 and c-Myc. Surprisingly, E-cadherine, specific for epithelial cells, showed induction in all clones from RSFs and in 18IM cells as well (Fig.3 and 4, Manuscript). Cell cycle distribution showed more cells in S-phase or G_2-M phase of immortalized REFs and RSFs compared to parental primary cells. Immortalized clones (and 18IM) cells also showed much higher activity of telomerase enzyme compared to parental cells ((Fig.6 and 7, Manuscript)).

3.3.7 Study of the tumorigenicity of immortalized cells in SCID mice

Newly generated clones from rat embryonic fibroblasts and rat skin fibroblast were tested for tumorigenicity. Around 2 million cells of each cell line were

injected subcutaneously in SCID mice and observed for the tumor growth. To our surprise, Clone 10 from immortalized REFs and clone 6 from immortalized RSFs gave rise to tumor. Tumors could be palpable after 2 months after inoculation from clone-10 of REFs and 3 months after inoculation from clone 6 of RSFs (Table 1, Manuscript). Mice became cachexic after 5 months and they were sacrificed. Tumors were characterized as aggressive invasive fibrosarcomas (Fig.5, Manuscript). They showed feature of mesothelial tumor with expression of both mesenchymal (smooth muscle actin, partially vimentin) and epithelial (E-cadherin) cell markers. Notably, previous attempts to induce tumors in SCID mice by 18IM or clones from immortalized REFs failed. We transplanted cells in 5 mice and we did not observe tumor growth. It might be because mice were not observed for long enough period till the development of tumor.

It was reported that tumorigenic conversion of rat embryonic fibroblasts require activation of at least two cooperating oncogenes [232]. Subsequent studies in same model showed that one activated oncogene can induce tumors without requirement of second mutation [233, 234]. Activation of ras alone could transform the cells but it was weak in immortalizing cells. C-myc alone could immortalize cells potently but failed to induce transformed characteristics efficiently. So, conclusion from this study was to induce naturally occurring tumors, activation of multiple oncogenes are required. Here, in our study, low S18-2 expressing cells i.e. clones derived from pBabe transformed cells did not give tumors in SCID mice nor did they form foci in culture. However, overexpression of GFP-S18-2 could immortalize REFs as well as transform them as revealed by formation of foci in culture, growth in soft agar and their ability to give tumors in SCID mice. In result, as S18-2 can alone immortalize and transform cells we can suggest that S18-2 is a putative oncogene.

4 CONCLUDING REMARKS

In result of present work, we have identified new EBV strategies in evasion of apoptosis and alteration of cell metabolism to promote the cell transformation. Vitamin D receptor (VDR) mediated apoptosis is blocked due to binding of VDR with EBNA3 that results in subsequent inactivation of transcriptional activity of VDR. According to our data, vitamin D_3 shall be given with caution to patients with lymphoproliferative disease.

Under the aerobic culture conditions HIF1α is stabilized in LCLs and is transcriptionally active. EBNA3 and EBNA5 by binding to prolyl hydroxylases 1 (PHD1) and 2 (PHD2), respectively, block the hydroxylation of HIF1α that inhibits its degradation on proteasomal machinery. HIF1α favors glycolysis over oxidative phosphorylation in LCLs, probably, to support the biosynthetic needs of the rapidly proliferating cells. Our finding makes HIF1α the new putative target for anti-cancer therapy in patients with lymphoproliferative diseases. Efforts should also be given to block the pathways in cellular metabolism orchestrated by HIF1α. Status of HIF1α should also be studied in Burkitt lymphoma with special attention on the role of EBV.

My doctoral work resulted in identification of new cellular functions of mitochondrial ribosomal protein MRPS18-2 (S18-2), the EBNA6 binding protein. S18-2 can transform rat embryonic fibroblasts (REFs) as well as adult skin fibroblasts (RSF) in culture. Strikingly, established cell line from REFs and RSFs can give tumors in SCID mice. Thus, S18-2 protein is a putative oncoprotein and its role in oncogenic transformation of cells should be further elucidated.

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