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UNDERSTANDING THE ROLE OF EPSTEIN-BARR VIRUS ENCODED RNA-1 (EBER-1) IN THE CELL PROLIFERATION

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This thesis is submitted in partial fulfillment of the requirements for the degree of Master of Medical Sciences (Microbiology and Immunology)

Under the Supervision of Professor Gulfaraz Khan

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Declaration of Original Work

I, Yasmeen Atta Ali Abdelmowla, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled “Understanding the Role of Epstein-Barr virus Encoded RNA-1(EBER-1) in the Cell Proliferation”, hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Prof. Gulfaraz Khan, in the College of Medicine and Health Sciences at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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Abstract

Epstein-Barr virus (EBV) is a human herpesvirus that infects and persists in > 90% of human adults, generally without causing disease. However, EBV has oncogenic properties and is associated with several malignancies of epithelial and lymphoid origin. The details of the molecular steps leading to these different malignancies are poorly understood. It is believed that some of the EBV latent gene products are involved. Two small EBV-encoded RNAs, referred to as EBER1 and EBER2 have been shown to be expressed in all forms of EBV latent infection. These non-polyadenylated and non-protein coding RNAs are by far the most abundant transcripts expressed in EBV infected cells (>10^6 copies), but their function remains unknown. Although, not directly involved in cell transformation, a number of studies have reported that these RNAs may be involved in inhibiting apoptosis and providing a proliferative advantage to EBV infected cells. The molecular mechanisms involved in these processes are unclear. The aim of this study was to investigate the role of EBER1 in cell proliferation. We prepared stable EBER1 transfectants of different cell types (epithelial, B-cell and T-cell), together with corresponding control cell lines transfected with the control plasmid only. After confirming that EBER1 was expressed in all EBER1 transfected cells, we investigated their proliferative properties using a range of different approaches and methodologies. Cell proliferation was measured by trypan blue exclusion assay, after subjecting the cells to either normal growth requirements or serum-deprived condition. ATP activity, as an indicator of cell proliferation, was measured using Glo-cell viability assay. Soft agar assay was used to determine the colony forming ability of EBER1 transfected cells. Molecular mechanism underlying EBER1 induced proliferation was assessed by Real-Time qPCR, immunocytochemistry and western blot for known proliferation markers, namely Ki67, PCNA and MCM2. Finally, in an attempt to understand the potential intracellular pathways that may be activated in EBER1 transfected cells, we examined for the expression of genes involved in cell proliferation using microarray methodology. The results from these investigations indicated that EBER1 transfected cells, compared to controls, had higher ATP activity, proliferated at significantly higher rate, expressed
higher levels of RNAs and proteins associated with cell proliferation and formed larger and more colonies in soft agar. Our study also suggested that EBER1 could be inducing some of these changes by triggering the production of pro-inflammatory cytokines and anti-apoptotic signals.

Keywords: EBV, Epstein-Barr virus encoded RNA-1 (EBER1), cell proliferation, Ki67, PCNA, MCM2.
فهم دور وآليات الحمض النووي الريبوي (EBER1) في تكاثر الخلايا

الفحص

فيروس "ابستين بار" هو الهرس البشري الذي يصيب ويقيم في أكثر من 90% من البشر البالغين، وبشكل عام من دون أن يتسبب في أمراض. وبالرغم من ذلك فإن له خصائص سرطانية ويشارك مع الكثير من الأورام اللمفاوية والظهارية. والتتفاصل الجزيئية المؤدية إلى هذه الأورام لا تزال غير مفهومة بشكل جيد. ويعتقد أن بعض المنتجات في المرحلة الكامنة لفيروس ابستين بار TEBER1 ينتجان في كل المراحل الكامنة لفيروس ابستين بار، يعتبر هذه الرناوات الغير مشفرة وغير المشبعة بالبروتين هي أكثر النسخ وفرة في الخلايا المصابة بفيروس ابستين بار (10^6 نسخة)، لكن وظائفهم إلى الآن غير معروفة. على الرغم من عدم مشاركتهم بشكل مباشر في تحول الخلايا، فقد أفاد عدد من الدراسات أن هذه الرناوات قد تشارك في تثبيط موت الخلايا وتوفير ميزة التكاثر للخلايا المصاببة. يعتقد أن بعض المنتجات في المرحلة الكامنة لهربس البشري الذي يصيب و يقيم في أكثر من 90% من البشر البالغين، ومثله في الخلايا المصابة بفيروس ابستين بار، يعتبر هذه الرناوات الغير مشفرة وغير المشبعة بالبروتين هي أكثر النسخ وفرة في الخلايا المصابة بفيروس ابستين بار (10^6 نسخة)، لكن وظائفهم إلى الآن غير معروفة. على الرغم من عدم مشاركتهم بشكل مباشر في تحول الخلايا، فقد أفاد عدد من الدراسات أن هذه الرناوات قد تشارك في تثبيط موت الخلايا وتوفير ميزة التكاثر للخلايا المصاببة. الأورام سرطانية والظهارية، إلا أن الأسباب الجزيئية المشاركية في هذه العمليات غير واضحة. 

الهدف من هذه الدراسة هو دراسة دور EBER1 في تكاثر الخلايا. أعدنا أنواع مختلفة من الخلايا المستقرة (الخلايا الظهارية والخلايا B والخلايا التائية) التي تحتوي على ال EBER1 برمية، بالمقابل مع خلايا التحكم التي تحتوي على البلازم فقط. بعد التأكد من التعبير الجيني لل EBER1 في جميع الخلايا المتميزة، درسنا خصائص التكاثر باستخدام مجموعة من الأساليب والمنهجيات المختلفة. تم قياس تكاثر الخلايا عن طريق اختبار trypan blue، بعد تعرض الخلايا لفترات تمتد إلى G0، مما يشير إلى تكاثر الخلايا، باستخدام cell viability assay في EBER1 لتحديد قدرة الخلايا الحاملة لل EBER1 في تكاثر بواسطة الزمن. تم تطبيق الآليات الجزيئية الكامنة وراء حسب الاتصالات البروتينية western blot، والتفاعل مع البروتينات المستقلة، والكيمياء الخلوية المناعية MCM2 و PCNA و Ki67. في محاولة من أجل المعرفة، تم قياس الطاقة، كمؤشر على تكاثر الخلايا، باستخدام cell viability assay في EBER1. تم استخدام تقنية رقائق الحمض النووي الدقيقة. أشارت نتائج هذه الدراسة إلى أن الخلايا
الحاملة لـ EBER1، مقارنة بالغير الحاملة لهذا الحمض، كانت ذات طاقة أعلى، وتتكاثرت بمعدل أعلى، وعبرت عن مستويات أعلى من الأحماض النووية الريبووزية والبروتينات المرتبطة بتكاثر الخلايا وشكلت مستعمرات أكبر. اقترحت دراستنا أيضًا أن EBER1 يمكن أن يحفز بعضًا من هذه التغييرات من خلال إطلاق cytokines المؤيدة للالتهابات والإشارات المضادة لموت الخلايا.

مفاهيم البحث الرئيسية: فيروس ابستين بار، الحمض النووي الريبوزي المشفر لفيروس ابستين بار MCM2، PCNA، Ki67، (EBER1)
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Dedication

To my beloved parents and family
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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>ABC</td>
<td>Avidin biotinylated peroxidase complex</td>
</tr>
<tr>
<td>APTES</td>
<td>3-aminopropyltriethoxysilane</td>
</tr>
<tr>
<td>ARE</td>
<td>AU-rich element</td>
</tr>
<tr>
<td>ATF2</td>
<td>Activating transcription factor 2</td>
</tr>
<tr>
<td>AUF1</td>
<td>AU-rich element binding factor 1</td>
</tr>
<tr>
<td>BARTs</td>
<td>BamHI rightward transcripts</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell antigen receptor</td>
</tr>
<tr>
<td>BL</td>
<td>Burkitt’s lymphoma</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CF/S</td>
<td>Complement-fixing soluble</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte Surveillance</td>
</tr>
<tr>
<td>DIG</td>
<td>Digoxigenin</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EBER</td>
<td>EBV-encoded RNA</td>
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<tr>
<td>EBNAs</td>
<td>EBV encoded nuclear antigens</td>
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<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>GC</td>
<td>Gastric carcinoma</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>HHV</td>
<td>Human herpes virus</td>
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<tr>
<td>HL</td>
<td>Hodgkin’s lymphoma</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
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<tr>
<td>HVP</td>
<td>Herpesvirus papio</td>
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<tr>
<td>IM</td>
<td>Infectious mononucleosis</td>
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<tr>
<td>ISH</td>
<td><em>In situ</em> hybridization</td>
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<tr>
<td>La</td>
<td>Lupus antigen</td>
</tr>
<tr>
<td>LCLs</td>
<td>Lymphoblastoid cell lines</td>
</tr>
<tr>
<td>LMPs</td>
<td>Latent membrane proteins</td>
</tr>
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<td>MCM2</td>
<td>Minichromosome Maintenance Protein 2</td>
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<td>MHC II</td>
<td>Major histocompatibility complex class II</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
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<tr>
<td>NPC</td>
<td>Nasopharyngeal carcinoma</td>
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<tr>
<td>ORFs</td>
<td>Open reading frames</td>
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<tr>
<td>OriP</td>
<td>Origin of replication</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PK</td>
<td>Proteinase k</td>
</tr>
<tr>
<td>PKR</td>
<td>Double-stranded RNA-dependent protein kinase R</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PTLD</td>
<td>Post-transplant lymphoproliferative disease</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative Reverse Transcriptase Polymerase Chain Reaction</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
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<td>-----------------------------------------------</td>
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<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
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<td>RNP</td>
<td>Ribonucleoprotein</td>
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<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SEMs</td>
<td>Standard errors of the means</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>TE</td>
<td>Tris EDTA</td>
</tr>
<tr>
<td>TERC</td>
<td>Human telomerase RNA</td>
</tr>
<tr>
<td>TFIIIC</td>
<td>Transcription factor for polymerase III C</td>
</tr>
<tr>
<td>TLR3</td>
<td>Toll-like receptor 3</td>
</tr>
<tr>
<td>VZV</td>
<td>Varicella zoster virus</td>
</tr>
<tr>
<td>VCA</td>
<td>Viral capsid antigen</td>
</tr>
<tr>
<td>VAs</td>
<td>Adenovirus associated RNAs</td>
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Chapter 1: Introduction

1.1 History and discovery of EBV

Epstein-Barr virus (EBV) was first discovered in 1964 by Anthony Epstein, Yvonne Barr and Burt Achong by isolating the virus from a Burkitt’s lymphoma (BL) case. The story began when Burkitt presented a case regarding a jaw lymphoma in a lecture that took place at Middlesex Hospital Medical School. Among the audience was Anthony Epstein who was a medical virologist and was working on chicken tumor viruses (Epstein & Eastwood, 1995). Epstein was fascinated with what he had witnessed and requested Burkitt for tumor samples so he could focus on the isolation of the agent which causes Burkitt’s lymphoma (Smith, 2012). However, the virus that was eventually isolated was not enough to assume that it had a hand in the pathogenesis of BL. Years of further research and experiments were done before concluding that EBV was indeed involved (Esau, 2017).

Werner and Gertrude Henle contributed the most in demonstrating and characterizing the oncogenic potential of EBV. Their seminal experiments showed that culturing of irradiated BL cells with healthy controlled leukocytes led to hematopoietic cell proliferation, highlighting the possibility of viral oncogenesis (Henle et al., 1967; Henle, 1968). They were also a part of the team that first published EBV DNA discovery from the cells taken from BL biopsies, which proved to be a strong evidence on EBV’s association with BL (Esau, 2017; zur Hausen et al., 1970).
1.2 Classification of EBV

EBV is a part of the Herpesviridae family of large DNA viruses. Herpesviridae are known to infect reptiles, mammals and birds. Viruses in this group are also known to cause lifelong infections in their host. Only 8 viruses out of 200 belonging to this family are responsible for causing infections in humans (Arvey et al., 2012). Human herpesviruses are classified into three groups: *Alphaherpesvirinae, Betaherpesvirinae* and *Gammaherpesvirinae*. This subdivision is based on biological properties and genomic sequence of the virus.

The Alpha group is known for having a short reproductive life cycle but spreads rapidly in its host. Viruses that belong to this group include Varicella-zoster virus (VZV) and human herpes simplex virus 1 and 2 (HSV-1 and -2). The Beta group however, has a longer reproductive cycle and grows slowly in host cells. This group consists of human herpes virus-6A, 6B, and 7 (HHV6 and HHV7) and cytomegalovirus (CMV). The last group, the Gammaherpes viruses, which include EBV and Human herpesvirus 8 (HHV-8) also known as Kaposi’s sarcoma have varying reproducing characteristics and replicate in plasma and epithelial cells (Ehlers et al., 2010). HHV-8 has been detected in non-AIDS-associated and AIDS-associated Kaposi’s sarcoma, some cases from AIDS-associated multicentric Castleman's disease and solid lymphoma, primary effusion lymphoma. Unlike other viruses, HHV-8 encodes several human homologues including regulatory genes (receptor of G-protein coupled, cyclin D etc) and cytokines (proteins of macrophage inflammatory, IL-6 and interferon-regulatory factors). These proteins have important roles in the HHV-8 pathogenesis (Rozen, Sathish, Li, & Yuan, 2008).
Two types of EBV viruses infect humans; EBV-1 (also called type A) and EBV-2 (called type B) (Walling, Brown, Etienne, Keitel, & Ling, 2003). These two types differ mainly in their geographic distribution. For instance, EBV-1 is dominant in the developed Western countries, whilst EBV-2 appears to be common in sub-Saharan Africa (Zimber et al., 1986). These two strains are observed as super infections in immuno-compromised patients (Walling et al., 2003). However, it is yet to be confirmed whether both strains can infect immunocompetent individuals. The reason behind the division of EBV into these two subtypes was due to certain genetic variations observed in the sequence of EBV encoded nuclear antigens (EBNAs) (Sample et al., 1990). The two subtypes are further divided into strains based on the EBV DNA sequence variation (Ruf, Rhyne, Yang, Cleveland, & Sample, 2000).

1.3 Structure of the viral genome

The EBV genome has a linear double-stranded DNA of ~172 kbp length encoding for > 85 genes (Fig. 1.1). Open-reading frames (ORFs) are named after the BamH1-restriction fragments where they are located. BARF1 ORF, for example, can be located in the BamHI-A restriction fragment, and is observed extending rightwards (Fig. 1.1). Several EBV ORFs are organized in two set of genes, lytic or latent genes. Lytic genes are further divided into immediate early, early and late genes. In EBV, lytic process starts off by activation of two immediate early viral transcription factors Rta (also named R–encoded by BRLF1) and Zta (also named EB1, Z, ZEBRA—encoded by BZLF1) (Hong et al., 2005). Therefore, early gene promoters are activated which play role to express proteins for viral DNA synthesis (Chevallier-Greco et al., 1986). Late genes are essential
for encoding structural proteins (Hong et al., 2005). Furthermore, some latent gene products are non-translated; including EBV-encoded RNA (EBER)-1 and -2 (Fields et al., 2007).

There is a string of 0.5-kbp direct terminal repeats in the viral genome, located at both ends in addition to 6-12 tandem repeats and 3kb internal direct repeats (IRs). These repeat sequences play a role in the division of the genome into both long and short unique sequence domains including most of the coding regions (Cheung & Kieff, 1982). These terminal repeats help in identifying EBV infected cells that are from the same progenitor. In other words, when a cell is infected with EBV, the viral DNA becomes circular episome with unique number of terminal repeats undergoing replication, and the new progeny of EBV would have the same number of terminal repeats as the parental genome (Raab-Traub & Flynn, 1986).
Figure 1.1: The EBV genome double-stranded DNA. (a) An electron micrograph of EBV in Burkitt’s lymphoma cell line. (b) Genome of EBV shown as circular double stranded DNA (~172 kbp long) which is believed to encode >85 genes (c) Open reading frames for viral latent genes in the EBV genome. The OriP is the site of DNA replication and episome maintenance. Image adapted from: (Young & Rickinson, 2004).
1.4 Host range and cell tropism

EBV is ubiquitous and humans are its natural host. However, EBV-like viruses have been detected in higher primates. Anti-EBV antibodies have been found in many primate species, which could be due to cross-reactive antibodies existing against their own species-specific EBV homologues (Kieff et al., 1979). EBV results in a long term infection in newborn marmosets thus indicating that the response of both humans and marmosets to EBV infection may be similar (Cox, Chang, Karran, Griffin, & Wedderburn, 1996).

EBV latent infection in lymphocytes can lead to proliferation of the newly infected cells (Young & Rickinson, 2004). EBV infection in B cells is caused by a certain interaction of the viral envelope glycoprotein gp350/220 and the cellular receptor of the c3d complement component, CR2 (CD21) (Fingeroth et al., 1984). Three viral glycoprotein mechanisms, gp42, gp85 and gp25 mediate the fusion of the envelope with the host-cell membrane (Li, Turk, & Hutt-Fletcher, 1995). The viral gp42 has the ability to bind with major histocompatibility complex class II (MHC II), and is used by EBV as a mediator of B lymphocyte infection (Li et al., 1997). EBV is also believed to have the ability to infect cells through CD21 independent mechanisms, albeit with a lower efficiency. Cells that lack CD21 (like epithelial cells) are also thought to be prone to EBV infection. Furthermore, a virus that lacks gp350/220 may also cause infection (Imai, Nishikawa, & Takada, 1998). Although EBV is primarily B cell tropic, in some cases it can infect T lymphocytes, as evident in NK-/T-cell lymphoma (Janz et al., 2000). Studies have also suggested that the infection in healthy EBV carriers is mainly B cell specific. However, the virus can also be found in epithelial cells. These cells are thought to have a
part in the replication which in turn spreads EBV infection. This has been observed in the
tumors of epithelial origin, such as nasopharyngeal and gastric carcinomas (Kieff, 1979).

The previous studies showed that the glycoprotein gp110 promotes the EBV’s
ability to infect human cells. Moreover, gp110 was shown to play a crucial role in the
infection of B-cells and non-B cells by membrane fusion during the virus entry. Higher
gp110 expression were shown to be more efficient in infecting epithelial or lymphoid
cells (Neuhierl, Feederle, Hammerschmidt, & Delecluse, 2002).

1.5 Viral life cycle and gene expression

1.5.1 EBV life cycle

EBV is a ubiquitous human virus and infects or persists in > 90% of human
throughout their lifetime (Münz & Moormann, 2008), mostly without causing illness.
Because of the lack of empirical evidences, many aspects of EBV life cycle remain
enigmatic. Nonetheless, several models are thought to explain the dynamics of EBV
infection in the normal host. EBV utilizes normal differentiation of B cell physiology and
expresses variety of latent genes to progress from the initial infection to long term
persistence within the memory pool of B cells in the immunocompetent host (Thorley-

Figure 1.2 shows the interaction taking place between EBV and its host. EBV
spreads through infected saliva, by the Waldeyer tonsillar ring epithelium located in the
oropharynx. One model suggests that lytic infection takes place in epithelial cells resulting
in the release of virus. When EBV infects naïve B cells underlying lymphoid tissues, the
virus activates transcription program (latency III). There are several growth program proteins that appear to regulate the proliferation of the infected cells. This process causes migration of the infected cells to the follicle initiating germinal center reaction, and leading to default transcription program (latency II). The default program grants both survival and recovery signals and leads to exit of the cell from the germinal center as memory B cell. The latency program (latency 0) comes in the resting memory B cells, where all viral protein expression is switched off. Occasionally, when these cells divide, only EBNA1 is expressed (latency I) or else the B cells homeostasis is maintained.

Eventually, after returning back to the tonsil, infected B cells might go through differentiation into plasma cells. This process triggers viral replication. As a result, the virus might spread out to other possible hosts through saliva and/or re-infect B cells within the same host (Thorley-Lawson & Allday, 2008; Young & Rickinson, 2004). In the case of primary EBV infection, a strong cellular immune response is provoked which controls the EBV infection. Thus, in immunocompetent host, a latent-antigen specific T-cell response eliminates the recently infected cells efficiently. In resting memory B cells, where there is no viral protein expression, somehow protected or hidden from the immune system, the virus persists for life in the host (Thorley-Lawson & Allday, 2008; Thorley-Lawson & Gross, 2004).

EBV life cycle is similar in all herpesviruses; it consists of a latent state and lytic stage in which the virus reactivates and replicates. The newly formed viral particle infects naïve B cell, either in the same host or it could spread to another host. When memory B cells differentiate to plasma cells upon antigen recognition, then a shift takes place from
latency to lytic phase (Kapatai & Murray, 2007; Laichalk & Thorley-Lawson, 2005). The activation of the cellular transcription factor XBP-1 which causes the differentiation, leads to the activation of EBV ZP promoter resulting in BZLF1 gene transcription, the master lytic regulator (Thorley-Lawson & Allday, 2008). The only EBV gene that responds to activation of B cell antigen receptor (BCR) signal transduction pathway is BZLF1 gene. It also possesses the ability to shift the virus from latency to lytic phase (Countryman et al., 2009). Upon the activation of the lytic cell, several viral genes transcription takes place; the genes are classified to immediate early, early and late genes (Altmann & Hammerschmidt, 2005).
Figure 1.2: A proposed model for the interaction between the host and EBV in vivo. 

a) During primary infection, EBV in saliva reaches the lymphoepithelial layers of the new host where it is thought to undergo lytic cycle or passes the epithelial barriers to infect the naïve B cells. This induces the growth program, in which infected cells differentiate into proliferating B blasts. In the germinal center of lymph node follicles, B cells differentiate into memory B cells (which shed the virus from immune system while in the blood circulation). 

b) During persistent infection, or latency 0 no viral proteins are expressed except for EBERs, which are expressed in all latent programs. In order to start the lytic cycle, infected B cells re-enter the tonsil to differentiate to plasma cells. Image adapted from: (Young & Rickinson, 2004).
1.5.2 EBV gene expression

Table 1.1 presents the different EBV transcription programs in infected B cells. Many factors affect EBV-encoded proteins expression, like the differentiation, the type and activation status of the infected cell etc. The program of growth-stimulation depends on the expression of six nuclear and three membrane proteins. Six of them are very important for proliferation-driving and activation effect of the virus.

EBNA-1 which is a virally encoded protein is essential to maintain the viral episomes. is expressed in various degrees in cells (Thorley-Lawson, 2005). EBV expresses two classes of non-coding small RNA (EBER) 1 & 2 in all forms of latency. They are highly structured RNAs of 167 & 172 nucleotides. EBER 1 & 2 expression is limited to cell nucleus, they could be found at $10^7$ copies in each cell, in addition to that, over 22 micro-RNAs are encoded to different degrees in latency, I, II, III (Cai et al., 2006; Grundhoff et al., 2006).

B cell infection in vitro, has a minimum of six nuclear antigens (EBNAs1, 2, 3A, 3B, 3C and -LP) and three latent membrane proteins (LMPs 1, 2A and 2B). All of these genes operate in an organized manner to cause the infected cells to go into latent infection, leading to permanent lymphoblastiod cells lines (LCLs) outgrowth (Fields et al., 2007). In in vivo, growth-transforming infections of B cells is seen in patients with infectious mononucleosis, undergoing primary EBV infection, and are moved to the germinal center (Niedobitek, Young, & Herbst, 1997). Transformation seems to be central to the process where EBV can colonizes the naïve host. Eventually, T cells surveillance contain these infected B cell pool. However, that does not occur with some infected cells having down-
regulated viral gene expression and entering the memory B cell pool as resting cells (Babcock et al., 2000; Münz & Moormann, 2008).

Table 1.1: The programs of normal B cells with EBV transcription. Adapted from: (Thorley-Lawson, 2005).

<table>
<thead>
<tr>
<th>Transcription program</th>
<th>Genes expressed</th>
<th>Infected normal B-cell type</th>
<th>Function</th>
<th>Infected tumor type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth</td>
<td>EBNA1, 2, 3a, 3b, 3c, LP, LMP1, LMP2a, and LMP2b.</td>
<td>Naïve.</td>
<td>Activate B cell.</td>
<td>Immunoblastic lymphoma.</td>
</tr>
<tr>
<td>Default</td>
<td>EBNA1, LMP1, and LMP2a.</td>
<td>Germinal center.</td>
<td>Differentiate activated B cell into memory.</td>
<td>Hodgkin's disease (HD).</td>
</tr>
<tr>
<td>Latency</td>
<td>None.</td>
<td>Peripheral memory.</td>
<td>Allow lifetime persistence.</td>
<td></td>
</tr>
<tr>
<td>EBNA1 only</td>
<td>EBNA1.</td>
<td>Dividing peripheral memory.</td>
<td>Allow virus in latency program cell to divide.</td>
<td>Burkitt's lymphoma.</td>
</tr>
<tr>
<td>Lytic</td>
<td>All lytic genes.</td>
<td>Plasma cell.</td>
<td>Replicate the virus in plasma cell.</td>
<td></td>
</tr>
</tbody>
</table>

1.5.3 Viral gene expression in EBV- associated malignancies

The different viral gene expression programs have been seen in several human tumors, including Burkitt’s lymphoma, immunoblastic lymphoma in immunocompromised patients, nasopharyngeal carcinoma and Hodgkin disease (Münz & Moormann, 2008). These tumors are believed to have arisen originally from certain stages during EBV life cycle, and are somehow linked to disorders of the immune system (Fig. 1.2) (Thorley-Lawson, 2005). Burkitt’s lymphoma for example, expresses EBV
latency I program. Hodgkin disease, T-cell non-Hodgkin lymphoma, nasopharyngeal carcinoma and gastric carcinoma express type II latency. Type III latency has been seen mostly in immuno-compromised people and in HIV-associated lymphoproliferative disorders (Cesarman & Mesri, 1999; Kis, Takahara, Nagy, Klein, & Klein, 2006).

1.6 Epidemiology of EBV infection

1.6.1 Prevalence and geographic distribution

During the 1970s, IARC demonstrated the probability of more than 90% of adults worldwide infected with EBV, based on detecting antibodies against EBV, specifically antibodies to complement-fixing soluble (CF/S) antigens and viral capsid antigen (VCA) (de-The, Lavoue, & Muenz, 1978). It has been proven by many epidemiological studies the high prevalence of EBV worldwide (Black, Woodall, Evans, Liebhaber, & Henle, 1970; Tischendorf et al., 1970). The age at which the primary infection occurs differs globally, and the exposure to the virus is most likely because of socioeconomic factors (Evans, 1971) such as overcrowded places and poor living conditions where sanitation does not meet the required standards (de-Thé et al., 1975).

EBV is divided to two major types: EBV1 and EBV2, which vary geographically. The effect of EBV types in the development of different cancers is not known. Usually patient that are immuno-compromised, harbor both types of EBV (Borisch et al., 1992).
1.6.2 Transmission and outcome of primary infection

As mentioned before, young individuals are usually most susceptible to EBV, especially those from poor socioeconomic background, large families, and having poor hygienic lifestyle. Most of these individuals will become virus carriers by their early childhood. The virus infects primarily through oral transfer, but can also be transmitted by blood transfusion. In the developed countries, however, the infection usually occurs later in life, like in adolescence. Intimate act of exposure may be the primary cause in its transmission (Hjalgrim, Friborg, & Melbye, 2007). An estimated 50% of primary EBV infection during adolescence causes clinical infectious mononucleosis. The majority of individuals in Europe and the US also get infected with EBV at young age, but the one third, whose infection is delayed into adolescents are at increased risk for infectious mononucleosis (Balfour et al., 2013).

Following primary infection, EBV infected B cells circulates and stay undetected for life in a latent state (Thorley-Lawson & Gross, 2004; Young & Rickinson, 2004). Mucosal epithelial cells are also prone to EBV infection through intermittent replication of viral productivity (Frangou, Buettner, & Niedobitek, 2005). Resting memory B cells are considered as the true reservoir of the latent virus in healthy carriers (Table1.1). A restricted pattern of the viral gene expression occurs in these cells (Miyashita et al., 1994), which helps infected cells to persist in the face of an efficient cytotoxic T lymphocyte surveillance (CTL) (Masucci & Ernberg, 1994).
1.7 EBV associated diseases

EBV is one of the most successfully transforming virus that cause infections in 90-95% of people worldwide. However, the development of the diseases associated with EBV are considered rare (Khan & Hashim, 2014). There are several human malignancies that are linked to EBV, which are of epithelial and lymphoid origin (Griffin, 1998) including, Burkitt’s lymphoma (BL) (Schmitz et al., 2012), post-transplant lymphoproliferative disease (PTLD) (Gottschalk, Rooney, & Heslop, 2005), Hodgkin’s lymphoma (HL) (Vockerodt, Cader, Shannon-Lowe, & Murray, 2014) and X-linked lymphoproliferative disease (Marsh & Filipovich, 2011). Some of the epithelial malignancies that are associated with EBV include, nasopharyngeal carcinoma (NPC) (Lin et al., 2014) and gastric carcinoma (GC) (Iizasa, Nanbo, Nishikawa, Jinushi, & Yoshiyama, 2012). In addition to malignancies, EBV has been linked to several autoimmune diseases like multiple sclerosis (MS) (Hassani et al., 2018; Lünemann & Münz, 2007) and systemic lupus erythematosus (SLE) (Ascherio & Munger, 2015; Münz et al., 2008).

1.8 The lymphoblastoid cell line – an in vitro model of EBV infection and transformation

Lymphoblastoid cell lines (LCLs) are peripheral blood cells which when infected with EBV results in spontaneous outgrowth of EBV transformed cell lines (Rickinson et al., 1984). LCLs are produced when B cells are infected with EBV which produces immortalized cell line with individual genotype useful for genetic studies. Multiple copies of viral episomes in each cell of LCL, express a range of viral genes, including 3 LMPs 6 EBNAs, along with 2 non-coding RNAs (EBERs) (Fields et al.,
The transcription of these viral genes is regulated by several different promoters, including Cp, Wp and Qp, which appear to be activated in different latency programs (Brooks et al., 1992).

1.9 EBV encoded RNAs

1.9.1 Synthesis and expression of EBERs

EBV encoded RNA EBER1 and EBER2 are two small RNAs that are non-polyadenylated and noncoding. They are present abundantly in all latent stages and expressed at a very high numbers, up to \( \sim 10^7 \) copies per cell (Arrand & Rymo, 1982; Lerner et al., 1981). Their high expression in EBV infected cells makes them an ideal target for EBV detection through \textit{in situ} hybridization (Chang et al., 1992; Khan et al., 1992). Studies have shown that EBER1 is expressed \( \sim 10^6 \) and EBER2 is present approximately \( \sim 2.5 \times 10^5 \) per cell (Moss & Steitz, 2013). Both EBERs are transcribed approximately at equal rates but due to the longer half-life of EBER1, it is present at 10 fold higher levels compared to EBER2 (Clarke et al., 1992). Actinomycin D treatment has shown that the EBER2 half-life was 45 minutes while EBER1 was around 8-9 hours (Clarke et al., 1992). In newly infected cells, the expression of EBERs was observed after 36 hours while the higher levels were observed only after 70 hours post infections (Alfieri, Birkenbach, & Kieff, 1991). Is this difference in the half-life of EBERs of any importance? Till now it is not clear.

Recent studies have reported that EBERs are not seen in oral hairy leukoplakia, oral papilloma or lymphoma of salivary gland and Sjogren’s syndrome tissues by \textit{in situ}
hybridization, despite active EBV replication. Furthermore, within the switch from latent to lytic viral replication, the reduction in the expression of EBERs has been noted. It is reported that the expression level of EBERs diversity is heterogeneous among the tumor cells in the NPC individuals. These RNAs are expressed at high level in specific tumor cells whilst low levels are expressed by adjacent cells (Gilligan, Rajadurai, Resnick, & Raab-Traub, 1990; Knecht et al., 1990).

The transcription start site of EBERs are found on the EBV EcoRI J fragment of B95.8 genome (position number 6628-6796 and 6958-7129 bp) and is separated by 161 bp region. EBERs have both pol III and pol II promoter elements, but they are mostly transcribed by pol III (Rosa et al., 1981). Interestingly, downstream promoter elements including Box B (+50) and Box A (+10) are required by pol III (Rymo, 1979) for high EBERs expression level. It is reported that Box A mutation leads to reduction in 20% of EBERs transcription while Box B mutation reduces 97% of EBERs transcription in comparison to wild type (Howe & Shu, 1989). EBERs expression is further enhanced by three upstream elements along with the RNA pol II promoter element. These upstream elements including an ATF motif, Sp1-like and TGTA in the upstream EBER1 promoter or TATA box in the upstream EBER2 promoter stimulates the EBERs expression upto 50-fold in vivo. These findings suggested that EBERs are also transcribed by pol II (Ahmed et al., 2014; Yao et al., 1996). Two further enhancer elements CAGATG and CACGTG are required for EBER1 expression, but not EBER2 (Niller et al., 2003). Although the similarities of regulatory arrangement between EBER1 and EBER2 are at high level, any mutation or deletion in them leads to reduction of the EBERs expression (Howe & Shu, 1989).
The level of EBERs expression is influenced by a potent promoter and cellular and viral transcription factors. It has been reported that viral protein, EBNA1, plays a role as transcription factor for polymerase III C (TFIIC) and activation of ATF2 (transcription factor 2) (Felton-Edkins et al., 2006; Owen et al., 2010). In EBV infected cells, ATF2 is phosphorylated leading to interaction with EBERs promoter (Felton-Edkins et al., 2006). Furthermore, EBNA1 and 2 expression induces the binding of proto-oncogene c-MYC with two E box elements that promotes EBER1 expression (Niller et al., 2003; Spender, Cornish, Sullivan, & Farrell, 2002). Poly-thymidine region in the EBERs sequence acts as a termination signal for EBERs (Bredow, Sürig, Müller, Kleinert, & Benecke, 1990; Kickhoefer, Emre, Stephen, Poderycki, & Rome, 2003).

1.9.2 Structure of EBERs

EBER1 is 167, whereas EBER2 is 172 nucleotides in length and both have 54% homology in their sequences (Figure 1.3) (Lerner et al., 1981). EBERs are present close to the origin of replication (OriP) and transcribed from left to right by RNA polymerase III. Despite the relatively low sequence homology, they are similar in their secondary structures containing several short stem loops. The EBERs primary sequence is observed to be highly conserved among EBV strains, despite the few changes that had been observed. Similarly, polymorphism of EBERs has been shown in gastric cancer and NPC cases, in which EBER1 was observed to be highly conserved, except for three sequence variations in the intergenic region and six changes in the sequence of EBER2 were observed (Wang et al., 2010).
The conservation within EBERs sequences suggests that they are important in the EBV life cycle. Thus, their secondary structures and the conservation between the structures are probably critical for the EBER1 and EBER2 function. For example, in EBER1, the guanine residues replacement with inosine disables the binding of EBER1 secondary structure with PKR complex (Katze et al., 1991). Studies have shown that the secondary structure of adenovirus associated RNAs (VA1 and VA2) which are also non-polyadenylated are similar to that of EBERs (Rosa et al., 1981). VAs play an essential role in the replication and translation of the adenovirus. VA1 deletion was reported to have a significant effect on replication of the virus (Thimmappaya, Weinberger, Schneider, & Shenk, 1982). It was shown that EBERs were able to substitute the function of VA1 in VA1-deleted cell lines and rescue the adenovirus replication (Katze et al., 1991). The similarities between Herpesvirus papio (HVP) RNA type 1 and 2 and EBERs were also reported. HVP RNA type 1 is 83% sequence identical to EBER1 and HVP2 shares 65% similarity with EBER2, in addition to their secondary structures and regions of promoter (50-60%) (Iwakiri & Takada, 2010).
1.9.3 EBER localization

EBERs are mainly observed in the nucleus of EBV infected cells. This can be seen through *in situ* hybridization (ISH) staining of EBERs (Chang et al., 1992; Howe & Steitz, 1986; Khan et al., 1992). Previous studies indicated that using confocal laser scanning microscopy at high resolution ISH on Raji cells, EBERs may be associated with perinuclear region of the cells, corresponding to the locations of Golgi apparatus and endoplasmic reticulum (Fok et al., 2006; Schwemmle et al., 1992). Moreover, recent

Figure 1.3: EBERs secondary structure, EBER1 is 167 nucleotides, while EBER2 is 172 nucleotides long. EBER1 and EBER2 have 54% sequence homology. Image adapted from: (Iwakiri, 2014)
studies have shown that EBERs can also be secreted in exosomes along with lupus antigen protein (La) as a complex (Ahmed et al., 2014; Ahmed et al., 2018; Iwakiri, 2014).

1.9.4 EBERs interaction with cellular proteins

La protein

The La protein is one of the most abundantly expressed proteins in human cells (2x10⁷ per cell). Anti-La antibodies precipitated EBERs from nuclear RNP (ribonucleoprotein) complex in samples from systemic lupus erythematosus (SLE) patients (Lerner et al., 1981). Uridylate residues short sequence which presents the common motif in EBERs appears to be the interaction site with La antigen. In this complex, the interaction of La antigen with gene products of RNA polymerase III is lost at the 3'-end upon RNA maturation. For the most RNAs, this binding is not permanent, but is stable in the case of EBERs (Howe & Shu, 1988). Furthermore, the importance of this interaction is unknown. Although La is mainly localized in the nucleus, it is also observed in the cytoplasm under certain conditions. A previous study suggested that EBERs-La interaction is important in terms of helping EBERs to transport out from EBV infected cells to the neighboring cells (Ahmed, Attoub, Khan, & Philip, 2015; Iwakiri, 2014).

PKR

PKR, which is also known as human double-stranded RNA-regulated protein kinase, is a 551 MW enzyme. Its N-terminus contains two conserved 70-residue dsRNA-binding motifs (dsRBM I and II). However, its C terminus has a serine/threonine kinase catalytic
PKR is activated and auto phosphorylated by dsRNA, leading to the phosphorylation of the initiation factor eIF-2α thereby blocking initiation of protein synthesis and inducing apoptosis. EBER1 has been shown to bind to PKR at stem loop IV, and inhibits the phosphorylation of PKR (Lerner et al., 1981; Yoneyama & Fujita, 2007). This in turn blocks eIF-2α phosphorylation and inhibits apoptosis induced by IFN-α (Fok et al., 2006; Sharp et al., 1993).

Similar to EBER1, PKR is bound by VA RNAs of adenovirus, an IFN inducible threonine-serine kinase that acts as the antiviral key mediator (Vuyisich, 2002). Demonstrating that VAs/EBERs binding to the PKR de-phosphorylation form is as strong as other dsRNA activators, but VAs/EBER1 prevents trans-autophosphorylation of PKR by blocking the PKR dimerization.

L22

L22, a ribosomal protein, is part of the 60s eukaryotic ribosomal subunit and interacts with viral RNA and cellular proteins through histone H1 and human telomerase RNA (TERC) (Le, Sternglanz, & Greider, 2000; Ni, Liu, Hess, Rietdorf, & Sun, 2006). However, the importance of this interaction is not clear. Studies have shown that EBER1 and EBER2 bind to ribosomal protein L22 in EBV infected cells (Toczyski & Steitz, 1991). EBER1 binds with the L22 ribosomal protein by stem loop 1, 3 and 4 (Fok et al., 2006; Toczyski et al., 1994). Interaction between L22 and small RNAs are also noted in hepatitis C and herpes simplex virus (Leopardi et al., 1997; Wood et al., 2001).
Normally, L22 is found in cytoplasm and nucleoli of B lymphocytes. Interestingly, upon EBV infection, it is transferred to nucleoplasm by EBERs (Toczyski et al., 1994). In tumor cell types, re-localization of L22 from nucleoli to nucleoplasm results in decrease of L22 from ribosomes (Jennifer L. Houmani & Ruf, 2009; Toczyski et al., 1994). Studies suggested that the distribution of L22 may vary depending on the cell type. In the case of 293T cells, it is localized in nucleus whereas in the EBV-immortalized lymphoblastoid cells, it is in the cytoplasm (Gregorovic et al., 2011).

**Toll-like receptor 3**

Toll-like receptor 3 (TLR3) has a role in the innate immune response to evade pathogens by dsRNAs recognitions (Medzhitov et al., 1997; Rock et al., 1998). TLR3 receptor is expressed by variety of epithelial cells and conventional dendritic cells (Rock et al., 1998). Recently, it was demonstrated that EBERs-La complexes are released from infected cells through exosomes (Ahmed et al., 2014; Iwakiri et al., 2009) which are taken up by endocytosis. Therefore, intracellular TLR3 is activated leading to transcript IFN-α by the master key transcription factor NF-κB and pro-inflammatory cytokine production (Karikó, Ni, Capodici, Lamphier, & Weissman, 2004; Lee, Dunzendorfer, Soldau, & Tobias, 2006).

**AU-rich element binding factor1**

AU-rich element binding factor1 (AUF1) is able to bind to 3′-untranslated regions of precursor RNA by AU-rich elements (Lu et al., 2006; Sadri & Schneider, 2009). The role of the AUF1 protein in the nucleus associates with pre-mRNA to promote the
short-lived mRNA decay. Further to its binding with mRNA, involving cell cycle regulators, cytokines and proto oncogenes induce their decompositions (Loflin, Chen, & Shyu, 1999).

At the chromosome level, studies suggested that the AUF1 plays a role in the stabilization of particular transcripts (Lal et al., 2004). Also it is involved in telomerase maintenance, regulating telomerase expression and senescence suppression (Pant, Hilton, & Burczynski, 2012). A recent report showed that AUF1 is a novel partner of EBER1. Interestingly, electrophoretic shift assay showed that EBER1 competes with other mRNA for the AUF1 binding, and thus results in the inhibition of the short lived mRNA-AUF1 interactions (Lee, Pimienta, & Steitz, 2012). The EBER1-AUF1 interaction could lead to the increase in the specific ARE levels containing mRNAs resulting in over expression of AUF1 and therefore could contribute to EBV mediated oncogenesis (Gouble et al., 2002). AUF1 has also been shown to have the ability to control the anti-viral activity by inhibiting viral translation as is observed in human rhinovirus and enterovirus infected cells (Cathcart, Rozovics, & Semler, 2013). Binding of AUF1 with EBER1 could inhibit any potential antiviral effects during EBV infection. Also this interaction can lead to shuttling the AUF1 from nucleus to cytoplasm, as it is found in human polio virus and rhinovirus-infected cells (Rozovics et al., 2012).
1.9.5 Effect of EBERs on EBV infected cells

Resistance to Apoptosis by EBERs

The ability of EBERs to interact and inactivate a number of cellular factors that plays a role in the stimulation of apoptosis and innate viral sensing, suggests that EBERs may be involved in inhibiting apoptosis. Studies have shown that EBERs provide resistance in BL cell line (Akata, EBV-positive) to the glucocorticoid, hypoxic, cycloheximide stress and UV light apoptotic inducers (Komano et al., 1999). Similarly, in EBERs-transfected NPC cell line, which was exposed to poly(I:C), resulted in lower cell death compared to controls (Wong et al., 2005). Some studies reported that EBERs induce resistance to cell death in the serum depleted conditions in BJAB transfected cell lines. EBERs have also been shown to provide resistance to extrinsic apoptosis mediators such as FAS ligand in BJAB and epithelial cell lines (Nanbo et al., 2005; Yamamoto et al., 2000).

At the molecular level, the activation of PKR leads to IFN-α induction resulting in induction of antiviral cascade (Fuertes, Woo, Burnett, Fu, & Gajewski, 2013). However, EBERs cellular interaction with dsRNA-PKR, was shown to prevent the auto-phosphorylation and DAI activation (Mathews & Shenk, 1991). This DAI when activated, phosphorylates polypeptide chain initiation factor eIF-2α subunit (Clarke et al., 1992; Levin et al., 1980; Siekierka et al., 1985). This interaction results in inhibition of protein synthesis and subsequently induce apoptosis (Ahmed & Khan, 2014; Yoneyama et al., 1998). EBERs provide resistance to PKR induced apoptosis in Burkitt’s lymphoma cells (Nanbo, Inoue, Adachi-Takasawa, & Takada, 2002).
Role of EBERs in cell proliferation

In vitro studies have reported that EBERs enhance the growth rate in the epithelial cells derived from nasopharyngeal carcinoma and gastric cancer (Wong et al., 2005; Yoshizaki et al., 2007). Similarly, in vivo studies supported the significant role of EBERs in tumorigenesis by inducing lymphoma and hyperplasia in transgenic mice expressing EBERs (Repellin, Tsimbouri, Philbey, & Wilson, 2010). The increased growth rate in the EBERs-positive cells appears to be by inducing autocrine growth (IGF1) factor which has been shown to be expressed at a higher amount in biopsies of EBV-positive NPC samples compared to EBV-negative NPC samples. This suggests that EBERs are associated with developing epithelial cancerous cells by inducing autocrine growth (Iwakiri, Sheen, Chen, Huang, & Takada, 2005). Using various EBV infected cell lines, the role of EBERs in EBV mediated oncogenesis has been reported to be inducing various cytokines such as IL-9 and IL-10 (Kitagawa et al., 2000). IL-10 was observed to act as an autocrine growth factor for B cell activation and proliferation (Mege et al., 2006). Interestingly, in B cells, EBERs-positive EBV was 100 fold higher in B cell transformation than EBERs-deleted EBV, proposing that EBERs may contribute to efficient growth of B lymphocytes transformation (Yajima, Kanda, & Takada, 2005). However, in previous study showed that no difference was observed in B cell transformation between wild-type and EBER deficient EBV (Gregorovic et al., 2015). The different observations because of different EBV strains were used in the two studies. Some studies showed that the tumorigenicity and cell proliferation in B lymphocytes is induced by EBER1 (Houmani, Davis, & Ruf, 2009). In the T cells, IL-9 an autocrine growth factor has been reported to induce proliferation. Interestingly, 3 out of 4 biopsies of the EBV positive T/NK nasal
lymphomas have shown overexpression of IL-9, suggesting EBERs play a role in inducing IL-9 expression in these lymphomas (Yang et al., 2004). Still there is controversy in studies regarding which of the two EBERs are of importance for tumorigenicity and cell proliferation?

### 1.10 Hypothesis and aims of the study

A number of studies have shown that EBER1 can induce cell proliferation in EBV infected cells or EBER1 transfected cells (Clemens, 2006). Furthermore, EBER1 probably achieves this functional feature by interacting with the cellular proteins. The presence of EBERs could enhance the IL-10 production for cell proliferation (Yang, Aozasa, Oshimi, & Takada, 2004). Based on these observations, it is possible that EBER1 may promote potential mechanisms involved in the IL-10 pathway to induce proliferation in the immortalized cells. So, the specific aims for this study were:

1. To study the role of EBER1 in cell proliferation.
2. To investigate the potential molecular mechanism involved in EBER1 induced cell proliferation.
Chapter 2: Material and Methods

2.1 Cell lines and materials

2.1.1 Culture media and reagents

<table>
<thead>
<tr>
<th>Media (^*1)</th>
<th>Media (^*2)</th>
<th>Working Concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM (Dulbecco’s Modified Eagle’s Medium) containing L-glutamine (Gibco)</td>
<td>RPMI (Roswell Park Memorial Institute) 1640 (Gibco)</td>
<td>500 ml</td>
</tr>
<tr>
<td>Fetal Bovine serum (FBS)</td>
<td>Fetal Bovine serum (FBS)</td>
<td>50 ml (10%)</td>
</tr>
<tr>
<td>Antibiotic antimycotic (Santacruz)</td>
<td>Antibiotic antimycotic (Santacruz)</td>
<td>5 ml (10 µl/ml)</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>Gentamycin</td>
<td>0.5 ml (50 µg/ml)</td>
</tr>
<tr>
<td></td>
<td>L-glutamine (Gibco)</td>
<td>5 ml</td>
</tr>
<tr>
<td>Hygromycin B</td>
<td>Hygromycin B</td>
<td>150 µg/ml</td>
</tr>
</tbody>
</table>

Additional Reagents

- Phosphate buffered saline (PBS) (Gibco)
- Trypsin-EDTA solution (Gibco)
- Tissue culture flask T75 (Nunc)
- Tissue culture flask T25 (Nunc)

Culture Conditions

All cell lines were incubated in the incubator humidified at 37°C and supplemented with 5% CO\(_2\).
2.1.2 Type of cell lines

The cell lines used in this study are listed in Table 2.1 along with their appropriate culture conditions.

Table 2.1: The table shows the list of cell lines used in this study. The characteristic for each cell line with the appropriate culture conditions are given.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Features</th>
<th>Culture Requirements</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>293T-Hebo</td>
<td>Human embryonic kidney epithelial cell (stably transfected with pHEBo plasmid). Adherent cell line.</td>
<td>Media *1</td>
<td>(Ahmed et al., 2014)</td>
</tr>
<tr>
<td>293T-ER1</td>
<td>Human embryonic kidney epithelial cell (stably transfected with pHEBoEBER1 plasmid). Adherent cell line</td>
<td>Media *1</td>
<td>(Ahmed et al., 2014)</td>
</tr>
<tr>
<td>BJAB- Hebo</td>
<td>Burkitt lymphoma-derived B-cell (stably transfected with pHEBo plasmid) Suspension cell line</td>
<td>Media *2</td>
<td>Khan Lab</td>
</tr>
<tr>
<td>BJAB-ER1</td>
<td>Burkitt lymphoma-derived B-cell (stably transfected with pHEBoEBER1 plasmid). Suspension cell line</td>
<td>Media *2</td>
<td>Khan Lab</td>
</tr>
<tr>
<td>Jurkat-Hebo</td>
<td>Human T cell (stably transfected with pHEBo plasmid) Suspension cell line</td>
<td>Media *2</td>
<td>Khan Lab</td>
</tr>
<tr>
<td>Jurkat-ER1</td>
<td>Human T cell (stably transfected with pHEBoEBER1 plasmid) Suspension cell line</td>
<td>Media *2</td>
<td>Khan Lab</td>
</tr>
</tbody>
</table>
2.1.3 Cell thawing

The growth media (20% FBS) was warmed up at 37°C water bath. The frozen cell vials were taken out from liquid nitrogen tank, kept directly on ice and thawed quickly by keeping at 37°C water bath (until the sides were seen thawed but the insides still in a frozen state). After that, the cells were re-suspended in 3 ml of warm media using 5 ml pipette and transferred to a 15 ml conical tube. The cells were spun down at 1000 rpm for 5 minutes and the media was aspirated. The cell pellet was then re-suspended in 5 ml of 20% FBS media and moved into a labeled T25 flask and kept in the CO2 incubator.

2.1.4 Cell freezing

Cells at passage 3 or 4 were split and grown to 1 x 10^6 cells/ml in incubator for freezing in appropriate freezing media. For adherent cell lines, 1XPBS was used for washing the cells gently. Cells were then trypsinized with 1xtrypsin for about 2 minutes at 37°C, centrifuged at 1000 rpm for 5 minutes and counted using trypan blue (Sigma Cat# T8154) exclusion method. Non-adherent cell lines were collected and centrifuged in the same way, except that the initial trypsinization was not required. Supernatant was discarded and the cells were resuspened in 0.5 ml/vial of FBS. Freezing media (for both adherent and non-adherent cell lines) consisted of 10% dimethyl sulfoxide (DMSO) in RPMI or DMEM. Cells were gently mixed in freezing media 1:1 volume and aliquoted into 1 ml cryovials which were put in Mr. Frosty (Nalgene, Cat#5100) that contained isopropanol for slow freezing at -80°C. The following day, vials were transferred to liquid nitrogen for long-term storage.
2.2 Fixation and hybridization of cells

For embedding, 10 x 10⁶ cells/ml were collected and spun at 1500 rpm for 5 minutes. Cell pellet was washed once using cold 1XPBS and centrifuged again as mentioned above. After PBS was discarded, the pellet was re-suspended in 3 ml of 4% paraformaldehyde and kept for 2 hours at room temperature for fixation. After 2 hours, cells were spun down at 1200 rpm for 10 minutes and washed once with 1XPBS to remove the paraformaldehyde. The cell pellet was resuspended in 10 ml of 1XPBS and moved to a fresh 15 ml conical tube. As mentioned previously, resuspended cells were centrifuged and PBS was decanted gently and ~100 µl of left-over PBS was used to resuspend the cell clumps by tapping the bottom of the tube gently. 1 ml of 2% agarose in 1XPBS, cooled to 50°C, was added to the cells and pulse centrifuged at 1000 rpm so that the agarose would settle and solidify. Agarose cone was taken out of the 15 ml tube by gently tapping the bottom of the conical tube. The cone was divided into two halves by using sharp scalpel and transferred to labeled embedding cassettes. The cones were then processed to make paraffin blocks.

2.2.1 Embedding and sectioning of cells

The cells were dehydrated by incubating the cassettes in ascending concentration of ethanol, 30%, 50%, 70% and 90% for 30 minutes each. Further incubation was done in absolute ethanol (100%) for 60 minutes to make sure the remaining water was removed. To clear out agarose cones, they were incubated in ethanol-xylene mixture (1:1) for half an hour and in absolute xylene for 30 minutes.
After that, molten paraffin wax (Histoplast PE, Thermo Scientific, Cat# 8330) was used to impregnate the cones with two wax changes at 55°C for 30 minutes each. The flat surfaces of agarose cones were put in the mold's bottom, the cassettes were put on top and wax was added. The extra paraffin wax was removed from the sides after it cooled down to make sure it can fit properly in the microtome block holder.

**Microtomy**

For better sectioning, the paraffin blocks were incubated in the fridge overnight to cool down. The blocks were aligned and 10-15 µm thick sections were trimmed down to ensure that there was no excessive paraffin. Cells were sectioned at 5 µm thickness and floated on water bath at 40°C. Sections were collected using labelled slides coated with 3-aminopropyltriethoxysilane (APTES, Sigma, Cat# A3648) and dried on a hot plate.

**2.2.2 EBER in situ hybridization**

To detect EBERs in the transfected cell lines and EBV infected cell lines (B95.8) as positive control, EBERs in situ hybridization (EBERs-ISH) was done as previously described (Khan et al., 1992). For detection of EBER1 (30-mer oligonucleotides) sense and antisense probes were designed specifically. Each probe was labeled using digoxigenin (DIG) Oligonucleotide Tailing Kit which is commercially available and used according to manufacturer’s instructions (Roche, Cat#03353583910). EBER1 was used at 0.2 µg/ml final concentration for hybridization follows:

1. The slides were pre-warmed at 42°C for one hour, so that the paraffin wax would melt. Then, were washed twice in Xylene for 10 minutes each to remove paraffin.
Then, dehydrated in graded concentration of ethanol (70%, 90%, 100%) each for 5 minutes to remove Xylene from the sections.

2. The activity of endogenous peroxidase was inhibited by incubating the sections in 0.5% H₂O₂ (Panreac, Cat# 121076) in methanol (Panreac, Cat# 131091) for 20 minutes so that non-specific staining could be avoided. After that the slides were dehydrated by incubating them for 1 minute in graded concentration of ethanol (70%, 90% and 100%) and air dried.

3. The slides were pre-warmed in a humid chamber at 37°C for 10 minutes. To each section 200 µl of proteinase k (final concentration 100µg/ml) diluted in TE (Tris EDTA) were added and incubated for 10 minutes. The slides were washed in distilled water for 3 times and rinsed in graded ethanol (70%, 90%, and 100%). Slides were then air dried and sections were marked with a diamond pen before proceeding for hybridization.

**Hybridization**

Dig labeled EBER1 probe were diluted in hybridization buffer (see Appendix I), the probe's final concentration was 0.2 µg/ml (1:50 of 10 µg/ml working stock) in the hybridization mix.

1. To each section, 16 µl of the hybridization mix containing the probe was added and 22x32 mm coverslip was overlaid on the section (Sigma, Cat# 06522) to prevent evaporation.

2. The slides were hybridized in a humidified chamber that contains filter papers soaked in 2XSSC. The slides were heated in a domestic microwave (National,
model#5V52220389) at minimum power for 7 minutes and incubated at 42°C overnight in heated oven.

3. Next day, the slides were transferred to a coupling jar containing 2XSSC and the coverslips were removed by tapping gently. The slides were washed twice in 2XSSC for 5 minutes on an orbital shaker.

4. Stringent washes were performed to remove any excess probe, thus preventing non-specific binding. It was done by incubation of the slides in a coupling jar containing pre-warmed 0.1XSSC at 50°C in a shaking water bath. The incubation lasted for 10 minutes and the slides were washed twice.

5. The slides were washed twice for 10 minutes in 2XSSC followed by 1XPBS for 10 minutes.

**Signal detection**

6. Mouse anti-DIG monoclonal antibody, which is the primary antibody (clone D1-22, Sigma, Cat# D8156), was diluted to 1:2500 ratio in 1XPBS containing horse serum at 1:100 ratio. To each section, 150-200 µl was added very quickly without drying the sections. Incubation was done overnight in a humidified chamber at 4°C.

7. Next day, the sections were transferred to a coupling jar which contained 1XPBS and washed for three times, 10 minutes each by putting them on an orbital shaker.

8. Biotinylated anti-mouse secondary antibody (Thermo scientific, Cat # 35052) was diluted to a concentration of 1:200 in 1XPBS that contained horse serum at 1:100. As previously mentioned, the slides were incubated for an hour in a
humidified chamber at room temperature with 150-200 µl of secondary antibody for each section, followed by 3 washes in 1XPBS.

9. To prepare the ABC reagent (avidin biotinylated peroxidase complex, Thermo scientific, Cat# 32052), each reagent was diluted at 1:50 concentration in 1X PBS. For stabilization purposes, the reagent was prepared fresh 45 minutes before use. Around 150-200 µl of the reagent was added on each section and slides were kept for 30 minutes in humidified chamber at room temperature. Then the slides were washed 3 times in 1XPBS.

10. Signal detection was done using DAB substrate (3, 3’ diaminobenzidine, Sigma, Cat#D5637) which was fixed by adding 0.02% hydrogen peroxide and 1 ml of 25 mg DAB into 100 ml of 1XPBS. Slides were incubated in DAB solution for 30 minutes. Then the slides were washed three times in water.

11. The sections were counter stained with Harris hematoxylin (Thermo Scientific, Cat# 6765015) for about 2 minutes. Slides were instantly washed with tap water and in acid alcohol (1% HCl in 70% ethanol) for 1 minute to eliminate any non-specific staining and to better define the nuclei. Then, the slides were put under running tap water for 10 minutes to blue the sections.

12. In absolute ethanol, slides were dehydrated for 1 minute each, air dried and washed in Xylene. Using Xylene based DPX mounting medium, sections were mounted and coversliped. A light microscope was used to observe the slides closely.
2.3. Proliferation assays

2.3.1 Counting experiment

To compare cell proliferation of cells transfected with EBER1 and Hebo plasmids, experiments were carried out as follows. For adherent cells, $2.5 \times 10^4$ cells/well were plated onto a Nunclon™ DELTA Surface 24 well plate. The cells were grown in complete growth media at 37°C incubated with 5% CO$_2$ for six days. For the first three days, the cells were trypsinized each day with 50 µl of 1xtrypsin in 500 µl of complete media and counted. However, the last three days the cells were trypsinized with 100 µl and counted using trypan blue assay.

For non-adherent cells, $5 \times 10^4$ cells/well were plated in 24 well plates and cells were counted each day using the trypan blue exclusion assay. Each time point (1-6 days) was done in quadruplicates and three independent experiments were done. The mean growth curve was plotted along with the standard errors of the means (SEMs).

2.3.2 Cell viability assay

To differentiate the cell viability between the cells which were expressing EBER1 compared to the control, cells were grown in appropriate media and counted by trypan blue exclusion assay. Around $2.5 \times 10^3$ of 293T cells and $8 \times 10^3$ of BJAB and Jurkat cells per well were plated in 100 µl of complete media in 96 well white flat bottom plates (F96 MicroWell™ White Polystyrene plate, Cat# 136101, Thermo, USA). Negative control wells had equal volume of media without cells, and were grown in incubator supplemented with 5% CO$_2$ at 37°C.
The viability of the cells was measured by the CellTiter-Glo® Luminescent Cell Viability Assay Kit (Cat#G7570, Promega, USA) by following the manufacturer’s instructions. At different time points (24, 48, 72 and 96 hours) 50 μl of media was cautiously aspirated from the wells and replaced with 50 μl of the viability reagent and plate was covered by aluminum foil to avoid light sensitivity. The plate was incubated at room temperature for 10 minutes on a rocking platform. Readings were recorded using multilabel plate reader (Victor X3, PerkinElmer, USA) with luminescence settings. The viable cells were calculated using the following formula:

\[ Viability = \frac{OD \text{ of test cells (ER1)}}{OD \text{ of control cells (pHebo)}} \]

### 2.3.3 Serum deprivation

To investigate the proliferation activity in EBER 1 expressing cells compared to the control under serum deprivation condition. For 293T cells, 2.5×10⁴ cells per well, transfected with EBER1 or Hebo plasmids were plated in a Nunclon™ DELTA Surface 24 well plate. After 24 hours, the complete media was exchanged with serum depleted media. For next 6 days, the cells were trypsinized each day after 24 hours and collected in 500 μl of media and counted using trypan blue. However, for non-adherent cells, 5 × 10⁴ of EBER1 expressing cells and control were plated (1 ml/well) with serum free media in 24 well plates. The cells were collected in 1ml media and counted with trypan blue assay for every 24 hours. Each time point was counted in quadruplicates and three independent experiments were done.
2.3.4 Soft agar colony formation assay

To determine the effect of EBER1 on anchored independent growth, and its ability to form colonies in soft agar, the soft agar assay was performed in 6 well plates (Arafat et al., 2013). Briefly, 2.4% of agar (Bacto agar, Sigma) was dissolved in ddH$_2$O and was autoclaved. Agar was cooled down to 40-50°C and 1ml was added to each well. Gently, the plate was swirled so the agar would cover the well surface. The plate was kept at 4°C for 2 hours to solidify the agar. The plates were then transferred to 37°C incubator. The 293T, BJAB and Jurkat cells were counted using trypan blue exclusion assay. A total of 0.04 x 10$^6$ cells/well were resuspended in 2.5 ml of DMEM or RPMI media (according to the cell type) with 0.4 ml of warm agar. The cells were then gently layered onto the soft agar base and kept at 37°C incubator. PBS was used as negative control (without cells). Every two days, the media was gently aspirated and fresh 2 ml media was added in each well. The size of the colonies was monitored throughout for a period of 5-14 days using a light microscope.

2.4 Gene expression studies

2.4.1 RNA isolation from cells

For RNA isolation from cells, 2x10$^6$ cells were collected in a 15 ml conical tube, centrifuged at 1500 rpm at 4°C for 10 minutes and media was aspirated cautiously. Ice cold 1XPBS was used for washing the cell pellet, and cells were spun down under the same conditions as described above. RNA was extracted from cells using 1ml of TRIzol (Invitrogen, USA). The cells were incubated at room temperature for 10 minutes and the
transferred to a 1.5 ml eppendorf tube and 200 µl of chloroform was added. The eppendorf tubes were shaken vigorously and incubated for 2-3 minutes at room temperature. Samples were centrifuged at 13,000 rpm for 15 minutes at 4°C. After that, 3 layers were noticed with an aqueous phase that contained RNA. The upper layer was transferred to a fresh eppendorf tube and 600 µl of isopropanol was gently mixed with it. RNA was precipitated for 10 minutes at room temperature and was pelleted after centrifugation at 13,000 rpm for 10 minutes at 4°C. The white RNA pellet observed was rinsed with 1 ml of 75% ethanol and was spun at 8,000 rpm for 10 minutes to eliminate any possible contaminating proteins. The pellet was air dried for 15 to 30 minutes to remove any residual ethanol. The pellet was re-suspended in RNase-free H₂O. To ensure that the RNA pellet was dissolved properly, it was incubated for 10 minutes at 55°C. Nanodrop (Nanodrop 2000c, Thermoscientific) was used to quantify the RNA concentration. The samples were frozen at -80°C for downstream applications.

2.4.2 cDNA preparation

Of the isolated RNA, 4 µg was used for the cDNA synthesis. Dnase1 enzyme was used to eliminate any DNA contamination from the isolated samples (Promega). Dnase treatment was done as follows:

- Reconstituted RNA (4 µg) 30 µl
- 10X DNase buffer (1X) 3 µl
- RNasin (1 U/µg RNA) 1 µl
DNase1 (1 U/µg RNA) 2 µl

The RNA samples were incubated for 60 minutes at 37°C on a thermomixer, followed by addition of 3 µl of stop buffer (20 mM EGTA, Promega, Cat# M199A). The samples were incubated for 10 minutes at 65°C to stop the enzymatic activity. One micro liter of DNase treated RNA was tested by PCR to make sure the RNA was without any contaminating DNA. GAPDH primers were used for this process. After confirming the absence of amplification, DNased-RNA was converted into cDNA through the reverse transcription reaction using a commercially available Promega kit, following the manufacturer’s instructions (Reverse Transcription System, A3500 Promega).

Briefly, the DNase treated RNA was incubated in thermomixer for 10 minutes at 70°C and then immediately kept on ice. The reaction mixture was prepared as follow:

25 mM MgCl₂ (5 mM) 12 µl
10X Reverse Transcriptase Buffer (1X) 6 µl
10 mM dNTP’s (1 mM) 6 µl
Random Hexamer (0.5 µg/µl RNA) 2 µl
AMV Reverse Transcriptase (15 U/µg RNA) 3 µl
DNased-RNA ~30 µl

The samples were incubated for 10 minutes at room temperature, then for an hour at 42°C. Then to stop the reaction the samples were incubated for 5 minutes at 95°C. They were put on ice instantly, aliquoted and stored at -80°C.
2.4.3 Reverse transcriptase polymerase chain reaction (RT-PCR)

The quality of cDNA was examined by RT-PCR using housekeeping gene GAPDH and/or EBER1 specific primers. The final volume for each PCR reaction was set at 30 μl. The list of primer sets used are mentioned in table 2.2. The reaction mixture was prepared as follows:

10 XPCR mix (1X) 3 μl
25 mM MgCl₂ (2 mM) 0.5 μl
dNTPs (0.5 mM) 0.15 μl
Primer Forward (10 pM) 1 μl
Primer Reverse (10 pM) 1 μl
Taq Polymerase (1 U) 0.2 μl
Nuclease free H₂O 22.15 μl
Template 2 μl
The amplification profile was set as follow:

![Amplification Profile Diagram]

Each PCR batch included B95.8 cDNA as positive control and appropriate negative control. Applied Biosystems thermal cycler PCR System 2700 was used to set all PCR reactions. The amplified products were run by electrophoresis at 120 volts on 2.5% agarose gel and stained with ethidium bromide and visualized by gel documentation system.

2.4.4 Quantitative Real Time PCR (qRT-PCR)

Real time PCR was performed to determine the expression of proliferation markers (Ki67, PCNA and MCM2) at the RNA level in the stably transfected cell lines. Cellular RNA was isolated using TRIzol reagent as described in section 2.3.1. For this study, 4 μg of cellular RNA was converted into cDNA as explained above in section 2.3.2. Each experiment was repeated three times. The qPCR reaction was prepared as follows:

2X Sybr PCR mix (1X)  10 μl

Primer Forward (10 pM)  1 μl
Primer Reverse (10 pM) 1 µl

Nuclease free H2O 6 µl

Template 2 µl

The primer sequences of Ki67, PCNA and MCM2, together with the primers for the house-keeping gene β-actin are listed in table 2.2. Using MicroAmp® Optical 96-well plates (Applied Biosystems) in dark laminar flow hood, 20 µl of reaction was set as final volume and each sample was done in duplicates. Using MicroAmpTM optical adhesive film, the plate was covered, spun down at 2000 rpm for 1 minute and placed in QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems, Germany). The annealing temperature was set up at 55°C. The following formula was used to calculate the relative quantification (RQ).

TE = Tested Experimental.

TC= Tested Control

HE= Housekeeping Gene Experimental

HC= Housekeeping Gene Control

ΔCt Experimental (ΔCtE)= TE - HE

ΔCt Control (ΔCtC)= TC-HC

ΔΔCt = ΔCtE-ΔCTC

RQ= $2^{\Delta\Delta Ct}$
Table 2.2: List of primers, 10pM as working concentrations was used for amplification of viral and cellular genes.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Orientation</th>
<th>Sequences (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBER1</td>
<td>Forward</td>
<td>CCC AGA TCT AGG ACC TAC GCT GCC C</td>
</tr>
<tr>
<td>EBER1</td>
<td>Reverse</td>
<td>CCC AAG CTT AAA ACA TGC GGA CCA CCA GC</td>
</tr>
<tr>
<td>Ki67</td>
<td>Forward</td>
<td>TCC TTT GGT GGG CAC CTA AGA CCT G</td>
</tr>
<tr>
<td>Ki67</td>
<td>Reverse</td>
<td>TGA TGG TTG AGG TCG TTC CTT GAT G</td>
</tr>
<tr>
<td>PCNA</td>
<td>Forward</td>
<td>CTT TTG CAC TGA GGT ACC TG</td>
</tr>
<tr>
<td>PCNA</td>
<td>Reverse</td>
<td>GTG TCC CAT ATC CGC AAT TTT</td>
</tr>
<tr>
<td>MCM2</td>
<td>Forward</td>
<td>TGC CAG CAT TGC TCC TTC C</td>
</tr>
<tr>
<td>MCM2</td>
<td>Reverse</td>
<td>AAA CTG CGA CTT CGC TGT GC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>GCC TCC TGC ACC ACC AAC TG</td>
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<tr>
<td>GAPDH</td>
<td>Reverse</td>
<td>CGA CGC CTG CTT CAC CAC CTT CT</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward</td>
<td>TGT TAC CAA CTG GGA CGA CA</td>
</tr>
<tr>
<td>β-actin</td>
<td>Reverse</td>
<td>CTG GGT CAT CTT TTC ACG GT</td>
</tr>
</tbody>
</table>

2.4.5 Western blotting

To verify and identify the cellular proteins of proliferation markers between EBER1 expressing cells and control, western blot was employed. Cells were collected, washed with 1XPBS and saved in -8°C. The cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (see Appendix I). Every 1 ml of RIPA buffer had 50 μl of β-
mercaptoethanol (Sigma), 5 µl of proteinase inhibitor and 10 µl of phenylmethylsulfonyl fluoride (100 mM PMSF) (Sigma). The cells were kept on ice and 200 µl of RIPA buffer /1×10⁶ cells were added and incubated on ice for 10 minutes. 5 µl of each sample was taken to quantify by Bradford assay (Marion, 1976) using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Hercules, USA). In each assay, about 5-15 µg of the isolated protein was subsequently used. Adequate volumes of every protein was mixed with 6x loading buffer (see Appendix) and boiled at 100°C for 5 minutes for protein denaturation. SDS polyacrylamide gels (10%) of 1.5 mm thickness were prepared (see Appendix I). After the samples were loaded, and electrophoresis was done by running in 1X Running Buffer made from 10X stock (see Appendix I) at 78 V for 2.5 hours. Wet tank blotting system (Biorad) was used to electrotransfer the resolved proteins from the gel to the PVDF membrane. They were transferred in 1X transfer buffer made from 10X transfer buffer (see Appendix) and transferred overnight in a cold room at 30 V at 4°C.

Next day, after the transfer, the blot was washed in 1XPBST. To avoid non-specific binding of antibodies, the blots were blocked in 5% Bovine Serum Albumin (BSA) in 1XPBST for 2 hours on a rocker at room temperature. After blocking, the blots were transferred to hybridization bags and incubated with primary antibody diluted in 1% BSA overnight at 4°C. Next day, to remove the unbound antibody, the blots were washed three times in 1X PBST for 10 minutes and incubated in 20 ml of horseradish peroxidase (HRP) conjugated to anti-mouse or anti-rabbit secondary antibody (1:20,000 in 1% BSA) for 1 hour at room temperature. After the incubation, the blots were washed for three times with 1XPBST. To recognize the immune signals, ECL plus western blotting substrate was
used (Thermo Scientific) as recommended by the manufacturer. Typhoon FLA 9500 machine was used to visualize and capture the chemiluminescent signal.

Table 2.3: Details of antibody used for western blotting:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Cat. Number</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-MCM2</td>
<td>EPR4120</td>
<td>Abcam, ab108935</td>
<td>1:5000</td>
</tr>
<tr>
<td>Anti-PCNA</td>
<td>EPR3821</td>
<td>Abcam, ab92552</td>
<td>1:5000</td>
</tr>
<tr>
<td>Anti-B-actin</td>
<td>mAbcam 8226</td>
<td>Abcam, ab8226</td>
<td>1:5000</td>
</tr>
</tbody>
</table>

2.4.6 Immunocytochemistry

The level and intensity of the expression of proliferation markers like MCM2, PCNA and Ki67 were determined by immunocytochemistry which was performed as described below. Sections of human tonsil were included in each batch as controls:

1. The slide sections were incubated at 60°C in dry oven for 24 hours, dewaxed for 10 minutes each in xylene I and II and dehydrated in ascending concentration of ethanol (70, 90 and 100%) respectively.

2. The slides were incubated in 0.5% H₂O₂ in methanol to prevent endogenous tissue peroxidase activity and nonspecific background staining.

3. Slides were washed in water. For the antigen retrieval, tonsil sections were boiled in the citrate buffer for 10 minutes. However, the sections of cultured cells were more delicate and therefore were incubated in hot buffer (40-50°C) till buffer cooled down.
4. The slides were blocked in 200 μl of 5% BSA in tris buffer saline (1XTBST) in humidified chamber for 2 hours in order to eliminate nonspecific binding.

5. The slides were washed in 1XTBST (3 times for 10 minutes each) and incubated in 200 μl of the diluted primary antibodies for MCM2 or PCNA or Ki67 in 1% BSA and incubated at 4°C in humidified chamber (Table 2.4).

6. Sections were washed 3 times for 10 minutes each in wash buffer. The slides were incubated in 200 μl of 2° antibody, either anti-mouse or anti-rabbit diluted in 1XTBST at 1:200 in humidified chamber at room temperature.

7. Sections were washed and then ABC, DAB and H&E counter staining were performed as described in section 2.2.2, step 9-12.

### Table 2.4: Primary antibodies used in immunocytochemistry as follows:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Cat. Number</th>
<th>Incubation time</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-MCM2</td>
<td>EPR4120</td>
<td>Abcam, ab108935</td>
<td>2 hours</td>
<td>1:100</td>
</tr>
<tr>
<td>Anti-PCNA</td>
<td>EPR3821</td>
<td>Abcam, ab92552</td>
<td>2 hours</td>
<td>1:250</td>
</tr>
<tr>
<td>Anti-Ki67</td>
<td>SP6</td>
<td>Abcam, ab16667</td>
<td>overnight</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

### 2.4.7 Microarray analysis

In an attempt to understand the mechanisms involved in EBER1 induced proliferation, the Commercially available TaqMan® Array 96–Well Plate Human IL-10 Pathway array was used on the BJAB cell line. For each well of the microarray, 60 ng of cDNA was used. The cDNA and Master Mix were combined in microcentrifuge tube as follows:
cDNA sample + nuclease free water 10 µL

Master Mix 10 µL

Total volume 20 µL

The tube was vortexed to mix the contents thoroughly and centrifuged briefly to collect the contents at the bottom of the tube. For preparing the TaqMan® Array Plate, the plate package was removed and centrifuged briefly. 20 µL of cDNA Master Mix per well was added to the appropriate wells of the plate. The plate was sealed with MicroAmp™ Optical Adhesive Film, centrifuged briefly to collect the contents to the bottom of the wells and placed in QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems, Germany). The annealing temperature was set up at 60°C. The relative quantification was calculated by QuantStudio™ 7 Flex Real-Time PCR program.
Chapter 3: Results

3.1 Checking for EBER1 expression in stably transfected cells

EBER1 expression in the immortalized 293T, Jurkat and BJAB cells was achieved by transfecting the cells with the Hebo plasmid containing EBER1 sequence. The expression and the localization of EBER1 in the transfected cell line for 293T, BJAB and Jurkat were confirmed by RT-PCR and gold standard EBER in situ hybridization (EBER-ISH).

To confirm the efficacy of plasmid expressing EBER1, RNA was isolated from the stably transfected cell lines using TRIzol reagent and treated with DNasel to eliminate any DNA contamination, followed by reverse transcription using hexamer and A-MLV reverse transcriptase. To confirm the genomic DNA absence, prior to reverse transcription, PCR was performed on the DNaseI treated samples using the house keeping gene GAPDH primers (Figure 3.1 A). After confirmation of the absence of DNA contamination, RT-PCR was performed on the cDNA samples. The EBER1 transfected cells and the B95-8 were positive for EBER1 expression and the controls were clearly negative (Figure 3.1 B). GAPDH was used as internal control for reverse transcription (Figure 3.1 C).

The results of EBER-ISH using DAB staining showed the presence of EBER1 in the nucleus of EBER1 transfected cell lines (Figures 3.2, 3.3 and 3.4). The signal however was weaker than B95-8 cells used as positive control (Figure 3.2). As shown in the figure 3.2, no staining was seen in pHebo transfected 293T cells or when using sense probe,
confirming that the signals seen in EBER1 transfected cells were specific. Similar results were seen with EBER1 transfected BJAB cells (Figure 3.3) and Jurkat cells (Figure 3.4).
Figure 3.1: Checking for EBER1 expression in EBER1 and pHebo transfected cells. (A) PCR for GAPDH on DNase treated RNA (prior to reverse transcription) confirmed that there was no contaminating DNA. (B) PCR for EBER1 in cDNA indicated that EBER1 transfected Jurkat (J-ER1), BJAB (B-ER1) and 293T (293T-ER1) all expressed EBER1. Control (pHebo plasmid only) of these cell lines were clearly EBER1 negative. (C) GAPDH was however expressed in all cell lines. B95-8 was used as positive control.
Figure 3.2: EBER1 is expressed in stably transfected cell line 293T and B95-8. EBER1 expression was detected by EBER-ISH in EBER1 transfected cell lines and EBV positive B95-8. However, no signal was seen in control cells (293T-Hebo). Nucleus was stained with EBER1 giving positive brown signal with the anti-sense probe (left panel). No staining was observed with the sense probe (right panel).
Figure 3.3: EBER1 is expressed in stably transfected BJAB cell line. EBER1 expression detected by EBER-ISH in the stably EBER1 transfected cell line (left panel), but not in pHebo control or when using sense probe.
3.2 Proliferation activity of EBER1 expressing cells

We have hypothesized that EBER1 expressing cells have the ability to proliferate more compared to the control cells. To study the proliferation function of EBER1, stably transfected EBER1 cell lines and the control plasmid (Hebo) cells were plated onto a 24-well plate and cell count was quantified each day for 6 days using trypan blue exclusion assay.
Figure 3.5 shows that after 24 hours of plating, the cell numbers for EBER1 expressing cells and control remained similar. However, by days 2-4, EBER1 expressing cells started to show a higher proliferation rate compared to the control cells and this was statistically significant (Figure 3.5). The pattern of proliferation was not uniform for all cells. For example, epithelial cells (293T) reached 100% confluency by day 4-5 and were in the stationary phase. Interestingly, this condition did not limit the 293T-ER1 cells from proliferating more than the pHebo transfected control cells (Figure 3.5 A). As in the case of the suspension cells (BJAB and Jurkat), we noticed that even though there was gradual depletion of the growth factors and higher saturation densities in the culture, EBER1 expressing cells were growing at higher proliferation rate (Figure 3.5 B and C). By day 6 of culture, the difference in the proliferation rate between EBER1 transfected cells (B-ER1 and J-ER1) and their respective pHebo control cells was very pronounced and obvious.
Figure 3.5: Comparison of proliferation between pHebo control and EBER1 expressing cells. The EBER1 expressing cells exhibited an increase in growth compared to control cells transfected with pHebo plasmid. Each time point was counted four times and three independent experiments were carried out. The data presents the mean and standard error of the mean (SEMs).
3.3 Analysis of metabolic activity of EBER1 expressing cells

Proliferation activity can be determined by trypan blue exclusion assay or by measuring the metabolic activity of cellular enzymes. Metabolic activity is regulated by the pathways of signaling that control cell proliferation. To determine the metabolic activity in the EBER1 expressing cells, quantitation of ATP activity was used. Commercially available Cell Titre-Glo viability kit was used to estimate the ATP activity which was based on the viability of the cultured cells. Following the manufacturer’s protocol, 100 μl of the EBER1 transfected cell lines and control (suspension cells~8000/well, adherent cells ~2500/well) were plated in 96-well plates and incubated at 37°C in a cell-culture incubator for time points ranging from 24 to 96 hours. The time course experiments were performed for each cell line to determine the optimal period of culture that should be used for the subsequent assays on ATP activity measurements. This was important to avoid overgrowth and cell death which could influence ATP activity. For suspension cells, our data from these experiments showed that after 72 and 96 hours of plating, BJAB-ER1 and Jurkat-ER1 proliferated more than the control cell lines. However, culturing cells beyond these times triggered the cells to start dying. According to our results, the levels of ATP activity in cells transfected with EBER1 was significantly higher than the control cell lines (Figure 3.6). For epithelial cells (293T-ER1) which had optimal culture time of 48 hours, the ATP activity and hence, cell proliferation was three times higher than the corresponding control (293T-pHebo) cells significantly (Figure 3.6).
We investigated the proliferation of EBER1 expressing cells along with the corresponding controls under serum deprivation. For BJAB and Jurkat cells, the cells were washed and seeded onto 24 well plates with serum free medium. The growth of cells was monitored daily for 6 days. However, for adherent cells, the cells were seeded with serum containing growth media and incubated for 24 hours to allow the cells to adhere. The media was then replaced with serum free media. The cell viability was counted for 6 days using the trypan blue exclusion assay.

### 3.4 Growth rate of EBER1 expressing cells in serum deprived condition

![Proliferation assay](image)

Figure 3.6: Comparisons of proliferation based on ATP activity between the cells transfected with EBER1 and control (Hebo). The data plotted as a mean of the three independent experiments.
The three different cell lines, 293T, BJAB and Jurkat, behaved differently in response to serum deprivation. Surprisingly, 293T cells continued to grow for several days even in the absence of serum. Even under these conditions, there was a clear difference between EBER1 transfected cells and pHebo controls. 293T-ER1 proliferated at a higher rate compared to 293T-Hebo cells (Figure 3.7 A). However, by day 5 both cell lines started dying due to the lack of nutrients in the media. One possible explanation why 293T cells did not start dying from day 1 of serum depletion is that these cells were initially incubated for 24 hours in growth media to allow them to adhere and settle. Although the growth media was replaced with the serum depleted media, it is very likely that some of the growth media was still present to allow survival and proliferation of these highly resistant cells.

By contrast, the suspension cells (BJAB and Jurkat) were much more susceptible and started dying within first day of serum deprivation. For both cell lines however, EBER1 transfected cells proved to be more resistance to cell death compared to pHebo transfected control cells (Figure 3.7 B and C). These observations suggest that EBER1 may have the capacity to resist apoptosis and proliferate even under stressed conditions, as in the case of serum deprived state.
Figure 3.7: The growth of cells transfected with EBER1 compared to controls in serum free media condition. (A) Growth curves of 293T cells (293T-ER1 and 293T-Hebo). (B) Growth curves of BJAB cells (B-ER1 and B-Hebo). (C) Growth curves of Jurkat cells (J-ER1 and J-Hebo). The data are expressed as mean ± SD of three independent experiments.
3.5 Colony formation of EBER1 expressing cells: soft agar assay

In support of our hypothesis that EBER1 expressing cells have a huge rate of proliferation in culture, we wanted to determine the effect of EBER1 on anchorage independent growth and its ability to induce cell proliferation and colony formation in soft agar.

For this aim, we plated 293T cells in soft agar as described in our previous report (Ahmed et al., 2015). For the negative control, PBS was added to the wells instead of cells. Within five days of incubation, under the light microscope, we were able to see that the size and the colony numbers were almost similar in both transfected cells (Figure 3.8 A). However, by day twelve, the size and number of colonies in EBER1 expressing cells had gone up more than the control (Figure 3.8 B).

After eighteen days of incubation, further increase in the size and number of colonies in 293T-ER1 cells was observed, which was now visible by the naked eye. By contrast, most of the cells in the pHebo control showed much smaller and fewer colonies (Figure 3.9). These observations suggest that EBER1 expression has a significant effect on anchorage independent growth in immortalized epithelial cells.
Figure 3.8: 293T-EBER1 and control cells were grown in soft agar and allowed to form colonies for up to 12 days. (A) The 293T Hebo and ER1 showed similar number and size of colonies on day five of culture. (B) The colony size and number of 293T-ER1 increased more than 293T-Hebo by day twelve.
The data from our analysis of cell proliferation, metabolic activity and colony formation studies clearly indicated that EBER1 provided a proliferative advantage to EBER1 transfected cells. To understand what could be driving proliferation of EBER1 transfected cells, we looked for the expression of three well known markers of cell proliferation namely, MCM2, PCNA and Ki67. We used SYBR green qRT-PCR to study the relative expression of these proliferation markers at mRNA level. Expression was

3.6 Expression of proliferation markers in EBER1 expressing cells: qRT-PCR

Figure 3.9: 293T-EBER1 and control cells were grown in soft agar and allowed to form colonies for up to 18 days. Significant difference in the size and number of colonies was observed in 293T-ER1 after bromophenol blue staining on 18th day of post culturing. No colonies were observed in the PBS control group (negative control) (not shown).
measured in two cell lines, EBER1 transfected BJAB and Jurkat, together with pHelo-
transfected controls.

Both EBER1 transfected cell lines (BJAB and Jurkat) had higher levels of
expression of the proliferation markers compared to their control counterparts
significantly (Figure 3.10). PCNA was overexpressed more than 2.5 fold in BJAB-ER1
cells compared to the controls. EBER1 also increased mRNA expression of PCNA in
Jurkat cells approximately 1.5 fold (Figure 3.10 A). Similarly, the mRNA expression
levels of MCM2 were significantly higher in both cell lines transfected with EBER1
compared to controls (Figure 3.10 B). We also examined Ki67 expression, which was
found to be approximately 1.5-2.0 fold higher in EBER1 transfected cells and this was
statistically significant (Figure 3.10 C).
Figure 3.10: Effects of EBER1 on PCNA, MCM2 and Ki-67 mRNA expression. qRT-PCR was performed on the cDNA generated from 4μg of the RNA isolated from BJAB and Jurkat cell lines. Proliferation markers values were normalized to levels of β-actin mRNA. The data are expressed as mean ± SD of three independent experiments.
3.7 Expression of proliferation markers in EBER1 expressing cells: western blot analysis

Our data has consistently shown a higher rate of proliferation of EBER1 transfected cells compared to controls. Moreover, based on our qRT-PCR analysis, proliferation markers PCNA, MCM2 and Ki67 were up regulated at RNA level. To further understand the mechanism of cell proliferation at the cell cycle level, we looked at the expression of these markers at protein level.

We determined the level of expression of these markers by western blot using antibodies specific to these markers. Housekeeping gene β-actin was used as an internal control to check loading consistency. The Western blot analysis revealed that both PCNA and MCM2 were expressed at higher levels in EBER1 expressing cells compared to controls. Image J was used to quantify the difference in the expression profile. Indeed, in all three cell lines, 293T, BJAB and Jurkat, EBER1 transfected cells had higher levels of PCNA and MCM2 than the pHebo transfected controls (Figure 3.11).

The level of expression of PCNA and MCM2 was not uniform in all the cells. For example, EBER1 transfected BJAB cells expressed the highest level of PCNA (2-fold higher than control) whilst 293T and Jurkat cells expressed PCNA at approximately 1.5 times than observed in their controls (Figure 3.11 A2). We also found that, in EBER1 expressing BJAB and Jurkat cells, the expression level of MCM2 were elevated to 1.6 and 2.5 fold compared to controls, respectively. In 293T cells however, MCM2 level was only moderately increased (10%) (Figure 3.11 B).
Figure 3.11: Western blots for proliferation markers (PCNA and MCM2) on EBER1 expressing cells and control. β-actin was used as loading control. (A1) The level of PCNA protein was higher in all EBER1 transfected cells compared to the control. (A2) Using Image J program, the level was estimated to be 1.5-2.0-fold. Similar observations were seen for MCM2 expression (B1 and B2).
3.8 Expression of proliferation markers in EBER1 expressing cells:

immunocytochemistry

Since proliferation markers PCNA and MCM2 showed differences in their expression between cells with and without EBER1 by western blot analysis. We performed immunocytochemistry, to see whether this difference in expression could also be observed by this approach. The paraffin sections of EBER1 transfected and pHebo transfected cells embedded in agarose were used for this purpose. Sections of human tonsils were used to optimize the staining for PCNA, Ki67 and MCM2. In our study, the sections of tonsil gave positive staining for all three markers. As expected, the highly proliferating cells in the germinal centers expressed the highest level of these proliferation markers (Figure 3.12 A1). The negative controls remained negative throughout the experiments as expected (Figure 3.12 A2).

Ki-67 is a key marker for cellular proliferation and is detected as nuclear staining. Our results showed that the staining intensity was higher in EBER1 expressing cells compared to that of the control (Figures 3.13, 3.14, and 3.15, left panel). The highest signal intensity was observed in the Jurkat-ER1 cells compared to all the EBER1 transfected cell lines (Figure 3.15, left panel).

The expression of PCNA was also recognizably high in all EBER1 expressing cells compared to pHebo transfected controls (Figures 3.13, 3.14 and 3.15, middle panel). Nuclear immune staining for MCM2 showed high expression in the EBER1 expressing cells compared to the controls, except in the epithelial 293T cells where this distinction was very less. Both EBER1 transfected and pHebo transfected 293T cells expressed high
levels of MCM2 (Figure 3.13, right panel). From the three different cell lines, the effect of EBER1 on MCM2 expression was most obvious in BJAB cells (Figure 3.14, right panel). This was both in terms of staining intensity and the number of cells which were positive. Overall, our findings indicated that these cellular proliferation markers were expressed at higher levels, particularly in EBER1 transfected BJAB and Jurkat cells compared with their controls.
Figure 3.12: Immunocytochemistry for proliferation marker (PCNA, MCM2 and Ki67) on tonsil. The higher expression of proliferation markers was seen in the germinal center where the cells were proliferating and less expression was noticed in the non-germinal center (A1). No staining was seen in negative control (A2) ruling out non-specific staining possibilities.
Figure 3.13: Immunocytochemistry for proliferation markers (PCNA, MCM2 and Ki67) on EBER1 expressing 293T cells. Higher expression of Ki67 and PCNA was observed in 293T-EBER1 compared to the control (left and central panel), however, this difference was less clear in MCM2 expression (right panel).
Figure 3.14: Immunocytochemistry for proliferation markers on EBER1 expressing BJAB cells. Higher expression of Ki67, PCNA and MCM2 was seen in B-EBER1 compared to the control.
Figure 3.15: Immunocytochemistry for proliferation markers on EBER1 expressing Jurkat cells. Higher expression of Ki67, PCNA and MCM2 was seen in J-EBER1 compared to the control.
3.9 Microarray analysis of EBER1 transfected cells

In an attempt to understand the molecular pathways involved in EBER1 induced cell proliferation, we used microarray technology to determine which genes were expressed. Specifically, we used IL-10 microarray plates to determine gene expression profile in EBER1 transfected BJAB cells. This analysis identified a common set of genes that were regulated by EBER1 in BJAB transfected cell lines. The association of modulated genes with biological function revealed several pathways that may be affected by EBER1 to induce cell cycle and proliferation (Figure 3.16).

Our results showed that more than one pathway, including IL-10 and Toll receptor-4 (TLR-4) pathway may be activated by EBER1. Moreover, many genes were activated in more than one pathway. The genes which were upregulated in the EBER1 transfected cells where sub-grouped according to their functions. Our analysis indicated that IL-10 was upregulated by EBER1 more than 25-folds. Other highly upregulated genes were TLR-4, CCND2 and IL1B. Furthermore, IL-10 A and IL-10 B receptors were also upregulated up to 2 and 3 folds compared to that of the control. Similarly, TYK2, JAK and STAT3 were also upregulated more than 2-folds. Other related genes that appeared to be upregulated by EBER1 included, PIK family of genes, NF-κB family and MAPK genes. These genes are thought to be involved in a wide variety of cellular processes, including proliferation, transcription and inhibition of apoptosis. Interestingly, we also observed the expression of IL-6 at higher levels in EBER1 transfected cells. IL-6 is well known to induce proliferation of B-cells (Figure 3.16).
Figure 3.16: Up-regulation of genes in IL-10 and TLR-4 pathways. (A) Genes which are expressed 5-40 folds in EBER1 transfected cells compared to controls (pHebo). (B) Genes upregulated in the IL-10 pathways. (C) Genes up-regulated in the Toll receptor 4 pathway. (D) The downstream genes of both TLR4 and IL-10 pathways.
Chapter 4: Discussion

Epstein Barr virus (EBV), which is one of the most common and prolific Gamma Herpesviruses, infects more than 90% of the human population. EBV infects epithelial cells and B-lymphocytes and persists for life in memory B cells (Longnecker et al., 2013). The virus plays a role in several different malignancies of epithelial and lymphoid origin, as well as in some autoimmune disorders (Okano and Gross, 2012; Vockerodt et al., 2015). It has been estimated that malignancies triggered by EBV are responsible for causing ~1.8% of cancer deaths (Khan and Hashim, 2014; Cao, 2017), which has increased by 14.6% over a period of last 20 years. Thus, understanding the molecular details of EBV-associated pathologies is crucial for the development of possible interventions (Cohen et al., 2013). It has been known that EBV undergoes different latency programs in which a number of viral transcripts are expressed. Epstein-Barr virus encoded RNAs (EBER1 and EBER2) are two highly abundant transcripts, that are expressed in all forms of viral latencies (Longnecker et al., 2013).

EBER1 has been shown to be present at 10-fold higher levels in infected cells compared to EBER2, probably due to the longer half-life of EBER1 (Clarke et al., 1992). However, it remains poorly understood why EBER1 is expressed in large amounts. It has been shown that EBER1 stem loop structures have higher affinity towards RNA-protein interactions (Chavez-Calvillo, Martin, Hamm, & Sztuba-Solinska, 2018). It has also been reported that EBERs, specifically EBER1, contributes to cell proliferation in EBV infected cells or EBER1 transfected cells by interacting with a number of cellular proteins (Clemens, 2006). Moreover, previous studies have suggested that EBER expression may
stimulate the production of IL-10, leading to cell proliferation (Yang et al., 2004). Thus, it is plausible that EBER1 could be promoting cell proliferation in the transfected cells via IL-10 pathway. To understand the role of EBER1 in cell proliferation, we transfected EBER1 into different cell types, resulting in the generation of EBER1 expressing stable cell lines. Several different cell lines (293T, BJAB and Jurkat) were transfected with EBER1 plasmid along with the control plasmid (Hebo). Although all of the EBER1 transfected cells expressed EBER1, the level of expression of EBER1 in these stable cell lines was lower than the EBV infected B95-8 cells. The lower expression of EBER1 in the transfected cells could possibly be due to the low plasmid copy number per cell. Moreover, the absence of viral factors such as EBNA1, that has been shown to enhance the level of EBER expression, could also result in low EBER1 expression (Owen et al., 2010; Frappier, 2015).

Our results showed that EBER1 induce proliferation in different cell lines. In our growth and survival analysis, different growth properties between the EBER1 transfected cells and controls were observed under growth limiting conditions. Under normal growth conditions (10% FBS media) the proliferation was more in EBER1 transfected cells than that of control vector-transfected cells at the log phase. Similarly, the saturation density of EBER1 transfected cells was higher than that of controls at the stationary phase. These findings are consistent with previous studies which showed that proliferation of EBER expressing epithelial and B cells are higher than control cells (Komano et al., 1999; Wong et al., 2005; Yamamoto et al., 2000). However, little is known regarding the effect of EBER1 on the proliferation of T cells. Our study confirmed that EBER1 can also induce cell proliferation in T cells.
Furthermore, our results showed that EBER1 transfected cells can withstand serum deprivation conditions in comparison with control cells. Supporting our findings, EBERs were shown, in an earlier study, to confer resistance to apoptosis induced by low serum concentration in B cells (Komano et al., 1999; Yamamoto et al., 2000). Nevertheless, this has not been shown before in T cell lines. These findings support the notion that EBER1 possesses the ability to promote proliferation and at the same time show resistance to apoptosis when induced by saturation or serum deprivation. Additionally, our study demonstrates that EBER1 can promote malignant transformation in transformed 293T cells by the anchorage independent growth. Our results consistently showed the ability of EBER1 to induce proliferation in the epithelial, B and T-cells and enhance the transformation of immortalized epithelial cells. However, the molecular details behind the increase in cell number and subsequent increased proliferation are unknown.

4.1 Metabolic activity of the EBER1 expressing cells compared to control

Interestingly, nutrients via pathways that generate ATP molecules produce all the components necessary to duplicate the cell mass and cell division. Nutrient uptake is controlled by cell signaling pathways that also regulate cell proliferation and growth (Deberardinis, Sayed, Ditsworth, & Thompson, 2008; Vander Heiden, Cantley, & Thompson, 2009). These pathways of cell signaling play a role in the development of cancer, and their inappropriate activation in cancer is accompanied by an increase in nutrient uptake that has been described as a characteristic feature of malignant cells (Vander Heiden et al., 2009). Furthermore, ATP production plays a role at the restriction checkpoint and enhanced activation of cyclin/CDK for cell cycle regulation (Levine &
Puzio-Kuter, 2010). As a result, there is a linear correlation between ATP activity and cell proliferation (Vander Heiden et al., 2011). Our findings suggested that EBER1 expression may support increased ATP activity of the immortalized cells to increase cell proliferation. Our findings showed that EBER1 expression affected the metabolic activity of epithelial cells. These cells had a threefold higher metabolic activity compared to the control. Additionally, EBER1 expressing B and T cells also showed significantly increased metabolic activity compared to the controls.

4.2 The role of EBER1 at the level of cell cycle progression

To further understand the proliferation abilities of EBER1 transfected cells, we looked into the expression profile of a number of cellular proliferation markers. We studied this by examining the protein and RNA levels of selected proliferation markers in EBER1 transfected cells. Several cell proliferation markers have been identified which are present in growing or dividing cells. These markers, for example, MCM2, Ki67 and PCNA, serve as an indicators of cell division during different stages of cell cycle and have been used as prognostic markers in cancer diagnosis (Bologna-Molina, Mosqueda-Taylor, Molina-Frechero, Mori-Estevez, & Sanchez-Acuna, 2013). However, there is no study till date showing the relation between EBER1 and the proliferation markers. Our study showed for the first time that EBER1 has some effect on the expression of proliferation markers, MCM2, Ki67, and PCNA. Our results with these three potential proliferation markers, MCM2, Ki67 and PCNA, indicated that these markers are upregulated in EBER1 transfected cells compared to controls. We quantified the MCM2 gene expression by qRT-PCR which showed that MCM2 expression is upregulated in EBER1 expressing BJAB
and Jurkat cells, almost 2-fold compared to controls. The level of MCM2 protein expression observed by immunocytochemistry and western blot was also higher in BJAB and Jurkat cells transfected with EBER1 compared to that of the controls. Studies have indicated that the importance of MCM2 (Minichromosome Maintenance Protein 2) is for initiating the DNA replication of eukaryotic genome once per cycle. Higher expression of MCM2 is associated with several clinical pathological parameters, such as advanced tumor grade, poor prognosis, in breast cancer and colon cancer (Juríková, Danihel, Polák, & Varga, 2016). Similarly, our results showed elevated expression at mRNA and protein levels for PCNA and Ki67 in the B, T and epithelial cells transfected with EBER1. Both of these proliferation markers are expressed in G1, S, G2 and M phase and excluded in Go (Juríková et al., 2016). Although the exact molecular pathway by which Ki67 acts is not well understood, it is frequently used as tumor marker in the drug discovery field, pathology research and diagnostic studies (Bologna-Molina et al., 2013; Juríková et al., 2016). However, PCNA, (proliferating cell nuclear antigen) a non-histone protein is employed to repair the damaged and replicated DNA and act as an accessory for RNA polymerase alpha. PCNA as a marker of cell proliferation is heavily expressed for a long time in G1 and S phase where the cell goes to proliferation phase (Juríková et al., 2016; Kelman, 1997).

Overall, our result showed that the expression of cell proliferation markers such as MCM2, Ki67 and PCNA was increased in the EBER1 transfected cells. This upregulation of proliferation markers could be due to EBER1 expressing cells regulating certain genes to modulate the downstream pathways and activating cell cycle by these proliferation markers. However, these observations need further investigation.
4.3 Mechanisms of cell proliferation could be triggered by EBER1

A number of previous studies have shown that the expression of interleukin-10 (IL-10) improves growth of Burkitt’s lymphoma cells in response to EBER presence (Gregorovic et al., 2011). To further understand the mechanism of IL-10 induction, we used a commercially available microarray kit to look at the genes in the proliferation pathway which induce IL-10. Our results showed that, in EBER1 expressing BJAB cells, IL-10 was up-regulated along with other genes, as compared to the control. This result supports the previous findings showed that EBER expression can enhance the production of autocrine growth factors like IL-10 (Kitagawa et al., 2000). However, we also observed the induction of JAK/STAT and PI3K pathways in the EBER1 transfected BJAB cells. There has been studies which reported that activation of JAK/STAT and PI3K pathways promote the expression of genes involved in cell cycle regulation (Vaysberg, Lambert, Krams, & Martinez, 2009). Our results suggested that EBER1 may play a role to activate JAK/STAT and PI3K pathway in EBER1 transfected cells and regulate the cell cycle progression. Moreover, in the EBER1 transfected cells, we observed that the downstream genes such as BCL2L1, CCND1, 2 and 3 and IL-6 were triggered, and therefore may contribute to anti-apoptotic, cell-cycle progression and proliferation of B cells, respectively (Murray, 2007). A recent in vivo study showed that EBERs interact with TLR-3 to produce cytokines such as IL-6 and IL-10 (Li et al., 2015). Studies also have reported that depending on the cell type, EBERs stimulate production of cytokines, such as IL-10 and anti-apoptotic signals such as BCL-2 (Komano et al., 1999; Tycowski et al., 2015).
Chapter 5: Conclusion

In this study, we focused on understanding the role of EBER1 in EBV pathogenesis. Since EBV is known to infect different types of cells, we transfected EBER1 into three different cell types, namely 293T (epithelial cells), BJAB (B cells) and Jurkat (T cells), to understand the role of EBER1 in cell proliferation. Our results can be summarized as follows:

1) EBER1 has the ability to induce proliferation in epithelial, B and T cells, which was evident through high ATP activity in the transfected cells compared to the controls. Similarly, the cell counting assays also showed that EBER1 transfected cells had higher cell counts compared to the controls, despite been grown under similar conditions. Furthermore, EBER1 expression alone has significant effect on anchorage independent growth in immortalized epithelial cells by increasing in the size and number of colonies being formed.

2) EBER1 expressing cells showed higher production of proliferation markers such as Ki67, PCNA and MCM2, which are known to play a role in the cell cycle progression and subsequent increase in proliferation.

3) The microarray analysis showed that EBER1 transfected BJAB cells had increased levels of pro-inflammatory cytokines, anti-apoptotic and cell proliferation signals via IL10 pathway. Our future studies will be focused on analyzing the genes upregulated in our microarray analysis and further dissect out the possible mechanism of induction of various cytokines by EBER1 transfected cells.
References


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Ahmed, W., Tariq, S., & Khan, G. (2018). Tracking EBV-encoded RNAs (EBERs) from the nucleus to the excreted exosomes of B-lymphocytes. *Scientific Reports, 8*(1), 15438.


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Appendix

Freezing Media

- Adherent or non-adherent cell line: 10% Dimethyl sulfoxide (DMSO) in DMEM or RPMI

Hybridization Buffer

- Formamide deionized (Sigma, Cat# F9037) 2.5 ml
- Dextran sulphate (Sigma, Cat# D8906) 1 ml
- 20X SSC (saline Na$_3$C$_6$H$_5$O$_7$ (1L, pH 7.0), NaCl (175.3 g, 3 M), Na$_3$C$_6$H$_5$O$_7$ (800 ml), ddH$_2$O (800 ml)) 500 µl

- Tris-HCl (2 M, pH 7.5) 125 µl
- ddH$_2$O 875 µl

6x loading Buffer

- 6 g SDS, 5.91 g Trizma base, 9 ml β-mercaptoethanol, 48 ml glycerol, 30 mg bromophenol blue, volume was made up to 100 ml in dH$_2$O

RIPA Buffer

- 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% Sodium deoxycholate, 0.1% SDS, volume made up in dH$_2$O and filter sterilized

10X Running Buffer

- 10 g SDS, 30.2 g Trizma base, 144 g glycine, volume was made up to 1000 ml in dH$_2$O).
## SDS Gel Recipe

<table>
<thead>
<tr>
<th></th>
<th>10% Resolving Gel</th>
<th>4% Stacking Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH₂O</td>
<td>8 ml</td>
<td>ddH₂O</td>
</tr>
<tr>
<td>Tris pH 8.8</td>
<td>5 ml</td>
<td>Tris pH 6.8</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>6.7 ml</td>
<td>30% Acrylamide</td>
</tr>
<tr>
<td>10% SDS</td>
<td>200 μl</td>
<td>10% SDS</td>
</tr>
<tr>
<td>10% APS</td>
<td>200 μl</td>
<td>10% APS</td>
</tr>
<tr>
<td>TEMED</td>
<td>20 μl</td>
<td>TEMED</td>
</tr>
</tbody>
</table>

## 10X transfer Buffer

- 45.4 g Trizma base, 216 g glycine, volume was made up to 1000 ml in dH₂O.