Chapter 5

DISCUSSION
Viruses play an important role in generation of cancer [132]. As an intracellular parasite the virus can reprogram the host cellular machinery and signaling processes that monitor proliferation, differentiation and immune regulation and hence aid in cancer development. Epstein-Barr virus (EBV) association has been detected in various malignancies including gastric and breast carcinoma, but the contribution of EBV in epithelial cancer pathogenesis remains to be determined. EBV-infected gastric carcinoma cells express EBNA1, EBERs, LMP2A, and BARF0. EBERs have also been detected in malignant cells of EBV-associated breast tumors [22, 23]. Previously, our lab had reported that EBV is critically involved in immune evasion of gastric carcinomas [12], where LMP2A may have important contribution. Interestingly, EBV-association had been correlated with invasive breast cancer [22] and another clinical report has indicated the association of EBV in highly metastatic gastric carcinoma [82]. In spite of that, significantly little is known about the role of LMP2A and EBERs in EBV-associated malignancies, particularly gastric and breast cancer. This study addresses the contribution of EBV Latency I genes (LMP2A and EBERs) in alteration of tumorigenic parameters such as cell proliferation, activation of self renewal pathways and alteration of mitochondrial dynamics.

We tried to investigate the role of EBERs in epithelial cancer pathogenesis. EBERs are the only EBV latency gene products that are consistently expressed at high levels in all EBV-associated tumors [1]. Earlier reports indicated that EBERs induce the expression of several autocrine growth factors [9-11, 13, 14] and confer resistance to different apoptotic stimuli [8, 15, 19, 96]. In this study, we tried to understand the exact role of EBERs-expression in epithelial cancer particularly in EBV non-associated gastric cancer cell line AGS and EBV positive Korean gastric carcinoma cell SNU719 respectively. EBERs were also shown to protect AGS cells against the chemotherapeutic drug Cisplatin induced cell death. Moreover, EBV-associated
gastric cancer (SNU719) cells also showed apoptosis-resistance when treated with 12μM Cisplatin for 24h.

LMP2A on the other hand contributed to increase in cell proliferation in gastric cancer cells. In this study we further tried to addresses the role of Epstein Barr Virus latent protein LMP2A and its downstream activated notch pathway in mitochondrial dynamics alteration and cell migration in gastric and breast cancer cells. Previous reports indicate that LMP2A expression leads to activation of the Notch pathway in epithelial cells in order to auto regulate its own expression [40]; we therefore explored whether this activated Notch pathway has any role in LMP2A induced cellular migration. Furthermore, since alterations in mitochondrial dynamics have recently been linked to various patho-physiological disorders, we therefore proceeded to find out whether LMP2A mediated dynamic alteration has any role in cell migration. Our results revealed that LMP2A leads to increased fission and Drp1 elevation as well as increased rate of cell invasion.

The study of mitochondrial dynamics has undergone huge advances in the past few years. Adequate balance of mitochondrial dynamics is important not only for determining mitochondrial shape but also the functional state of mitochondria [46]. Moreover, potential role of mitochondria in tumorigenesis has recently been investigated [133-135]. Epithelial cell carcinogenesis usually shows metastatic behavior in advanced cases where cellular migration and invasion are among the hallmarks of metastasis [136]. A recent report [129] showed that mitochondrial fission player Drp1 is involved in metastasis of epithelial cells and that ATP was a prerequisite for the cytoskeleton reorganization during cell migration. Mitochondria are the cellular organelles responsible for ATP synthesis. Previous reports highlights the fact that mutations in mitochondrial DNA lead to enhanced metastatic potential along with increase of
Reactive Oxygen Species (ROS) in cell lines [137]. Furthermore, a well established relation already exists between EBV and mitochondria since EBV infected B cells has been shown to cause ROS elevation [138]. Moreover, our present study clearly highlights that EBV LMP2A leads to an alteration of mitochondrial dynamics with a significant elevation of mitochondrial fission. Increased Drp1 may then explain the observed increase metastatic behavior of EBV associated gastric carcinomas [82]. Tumor cells employ aerobic glycolysis to satisfy its ATP requirement. Importantly, previous studies indicate the involvement of mitochondrial fission in bringing about glycolytic reprogramming of cancer cells. [139]. It is therefore possible that Drp1 mediated modification of cellular energetics may be bringing about the observed migration alteration in LMP2A expressing cells. The increased fission mediated generation on greater number of mitochondria may be helping in production of larger ATP yields hence aiding in tumor progression. Furthermore, another member of the Herpesviridae family, pseudorabies virus was shown to alter mitochondrial morphology with reduction on mitochondrial length upon infection [140] shedding light on the possibility that mitochondrial dynamics may be one of the important feature of viral infection.

Our study demonstrates EBV LMP2A regulated Notch pathway to bring about increased cell migration along with an overexpression of EMT markers in both gastric cancer and breast cancer cells. Epithelial-mesenchymal transition (EMT), characterized by a gain of mesenchymal cell markers (N-cadherin, Twist), is a process whereby cells acquire molecular alterations that facilitate cell motility and invasion [141]. In an earlier study LMP2A was shown to induce ITAM/Syk and AKT dependent migration through αV-integrin membrane translocation [142]. It may be pointed out that a crosstalk between the Notch and the PI3K/Akt pathway is well established [143, 144] which thus establishes Akt as a critical effector of Notch signaling
Notch regulation of mitochondria has also been formerly reported, where activation of Notch pathway was shown to increase glucose consumption and lactate production with the activation of Akt pathway in glycolysis [145]. Moreover it has also been reported that mitochondrial dynamic players, MfnS are required for Notch mediated anti-apoptotic effects and cell survival in epithelial cells [146]. Interestingly several viruses including as Adenovirus, Human Papilloma Virus and Simian Virus 40 have been shown to activate the Notch pathway upon infection [147]. Moreover a very recent report emphasizes the role mitochondrial fusion mediated Notch pathway to promote cardiomyocyte differentiation [148]. Hence Notch may be a one of the critical pathway required for viral maintenance and function. Since the Notch and the PI3K/Akt pathway signaling cascades co-interact with each other, we also studied the relationship between the PI3K/Akt pathway and mitochondrial dynamics in gastric cancer and breast cancer cells. EBV LMP2A led to an increased activation of the PI3K/Akt pathway causing elevation of mitochondrial fission protein Drp1 in the above cells. This increased Drp1 further aided in increased metastatic parameters such as cell migration and invasion. The signaling crosstalk among the Notch and PI3K/Akt pathway may therefore together increase Drp1 mediated cell migration.

The role of LMP2A in activation and modulation of other important self renewal pathways such as Sonic Hedgehog pathway and Wnt/β catenin pathway have previously been investigated in nasopharyngeal carcinoma and keratinocytes. However activation of the above pathways in gastric and breast cancer cells has not been investigated so far. The Sonic Hedgehog pathway functions during normal embryogenesis to regulate tissue morphogenesis and patterning, sustained or aberrant activation in adult tissues can facilitate cancer progression and cancer stem cell maintenance [43]. Hedgehog signaling is initiated by binding of a Hedgehog ligand to
Patched (PTCH1), a transmembrane protein that represses the activity of Smoothened (SMO), a protein that displays similarities to G protein-coupled receptors. In response to HH ligand binding, PTCH1 repression of SMO is relieved, and the activity of three GLI transcription factors (GLI1, 2, 3) is induced through altered protein processing and/or transcriptional upregulation. The conserved Wnt/β-Catenin pathway regulates stem cell pluripotency and cell fate decisions during development. The Wnt ligand is a secreted glycoprotein that binds to Frizzled receptors, which triggers displacement of the multifunctional kinase GSK-3β from a regulatory APC/Axin/GSK-3β-complex. This is followed by translocation of β-catenin and the subsequent recruitment of LEF/TCF DNA-binding factors as co-activators for transcription. Our data show EBV LMP2A and not EBERs to activate the Hedgehog and the β-Catenin pathway in AGS, SNU719 and MCF7 cells. However the increase in Drp1 by LMP2A was specifically via the Notch pathway as inhibitors of the above pathways failed to alter Drp1 expression. We have however found the Sonic Hedgehog pathway to be responsible for immune evasion in gastric cancer cells, which is another important aspect of EBV tumor maintenance. The contribution of dysregulated cell signaling pathways to the etiology of this cancer especially immune evasion is not very well defined. Here we show the Sonic Hedgehog pathway principally Gli1 to have a role in LMP2A mediated HLA ABC downregulation. It is estimated that upwards of 25% of all cancers require autocrine or paracrine Hedgehog signaling plays an important role to sustain tumor cell growth and survival [149]. Moreover LMP2A was shown to activate stemmness genes; Bmi-1 and EZH2 in a Sonic Hedgehog dependent manner in Nasopharyngeal Carcinoma [43]. Stem cells in addition to its self renewal properties that aid in cancer progression have also been shown to confer immune suppression and bring about overall immune survival [150, 151]. We therefore explored the effect of LMP2A induced Sonic Hedgehog pathway on HLA class Ia
LMP2A has been found to downregulate the expression of Human Leukocyte Antigen Class 1a (HLA class I) via the Sonic Hedgehog pathway since treatment of the above cells with Sonic hedgehog pathway inhibitor Forskolin increased HLA ABC (HLA class Ia) expression (Annexure 1.A and 1.B). Furthermore this HLA class I downregulation was Gli 1 dependent as evidenced by increased HLA expressing upon siRNA mediated knockdown of Gli1in LMP2A expressing gastric cancer cells (Annexure 1.C). HLA class Ia is responsible for presentation of viral and tumor antigens to cytotoxic T Lymphocytes for immune mediated destruction. Decreased HLA expression hence hampers the above process and therefore leads to immune evasion.

Despite intense research, relatively few LMP2A and EBERs-regulated genes have been clearly characterized so far. This study was performed to improve our understanding on the role of EBV oncogenes, LMP2A and EBERs in oncogenesis. This work indeed revealed several novel functions of LMP2A and EBERs, particularly in gastric cancer cells. These oncogenes are found to differentially modulate cellular self-renewal and oncogenic pathways, although aiding in cancer progression. Importantly, the results of the EBV-associated gastric cancer (SNU719) cells support to ascribe the biological significance of our findings. The study proposes the first report suggesting that LMP2A exploits Notch pathway to induce cellular migration in gastric cancer cells. This is the first report showing that EBV Latency 1 gene LMP2A activated Notch pathway leads to imbalance of mitochondrial fission-fusion cycle that is manifested by increased fission. This alteration and imbalance leads to elevated cell migration and metastasis in epithelial cancer cells. Cell migration and metastasis are the markers of poor prognosis in patients with advanced stages of carcinogenesis especially epithelial cancers. To summarize it may be pointed out that in gastric cancer cells both EBERs and LMP2A are significantly contributing to tumor maintenance.
and progression. While EBERs is conferring the property of chemoresistance, LMP2A is providing a huge invasive potential and hence aiding in metastasis via the Notch pathway. Moreover LMP2A is utilizing yet another self renewal pathway, the Sonic Hedgehog pathway in bringing about HLA downregulation and hence tumor evasion. LMP2A and EBERs have been shown to manifest a differential effect on the self-renewal pathways. It may be possible that LMP2A being a membrane protein may have an increased ability to activate the common self-renewal pathways such as Notch, Wnt and Sonic Hedgehog pathways, all of which are initiated upon ligand binding on membrane receptor followed by downstream activation. Therefore, it is possible that targeting common self renewal pathways such as Notch and sonic Hedgehog as well as fission protein Drp1 may help in generation of new avenues of cancer therapeutics.