Chapter 4

RESULTS
4.1: **Ectopic expression of LMP2A and EBERs in EBV non-associated epithelial cell lines.**

The EBV negative gastric cancer cells, AGS, and breast cancer cells, MCF7, were transfected with either pcDNA (vector control) or LMP2A/pDNA and stable clones constitutively expressing LMP2A were generated. LMP2A cDNA (kindly provided by Prof. R. Longnecker, Northwestern University, Chicago, USA) was cloned into the EcoR1 site of the vector pcDNA to obtain the plasmid LMP2A/pDNA (figure 4.1A). The expression of LMP2A was confirmed at the transcriptional level in clones 2, 5 and 8 of AGS-LMP2A and clones 1 and 3 of MCF-LMP2A (figure 4.1A.). The EBERs–DNA fragment including the promoter region was cloned into the Sal-I site of pREP7 plasmid and transfected into AGS produce constitutively EBERs-expressing stable cell clones (clones 3 and 8) (figure 4.1B). The plasmid pREP7 stably transfected cells were used as vector controls. AGS-LMP2AEBERs clones were generated by doubly transfecting AGS with pREP7 vector containing EBERs and pcDNA containing the LMP2A cDNA. Stable clones of transfectants expressing only vector pcDNA and LMP2A were selected in medium containing 800 µg/mL of G418 (Gibco, Carlsbad, CA). AGS clones stably expressing EBERs were selected in a media containing 400 µg/mL of Hygromycin (Invitrogen, Carlsbad, CA) whereas stable clones expressing both LMP2A and EBERs were selected with 400 µg/mL of Hygromycin and 800 µg/mL of G418. Since AGS-LMP2A (clone 5) and AGS-EBERs (clone 3) express the highest levels of LMP2A and EBERs respectively these were used in all further studies.

4.2: **Effect EBV latency 1 genes on cellular proliferation in gastric cancer cells.**

Flow cytometry assays were carried out to determine the effect of LMP2A/EBERs (Latency I genes expressed in gastric cancer cells) on cell proliferation. Cell proliferation is a process
whereby cells reproduce themselves by growing and dividing into two equal copies. Cells with high rate of proliferation will have increased cell population in the proliferative phase (S+G2/M) compared to non proliferative cells. The DNA content profile of AGS Control, AGS-EBERs and AGS-LMP2A was analyzed. Our data (figure 4.2) indicate that while AGS-EBERs show no significant increase in the percentage of cell population in the proliferative phase (S+G2/M), AGS-LMP2A shows an increased proliferative population compared to AGS Control cells.

Fig 4.1: Ectopic expression of LMP2A and EBERs in EBV non-associated epithelial cell lines. (A) The EBV negative gastric cancer cells, AGS, and breast cancer cells, MCF7, were stably transfected with either pcDNA (vector control) or LMP2A/pcDNA and stable clones constitutively expressing LMP2A were generated. The expression of LMP2A was confirmed at the transcriptional level in clones 2, 5 and 8 of AGS-LMP2A and clones 1 and 3 of MCF-LMP2A. (B) The EBERs–DNA fragment including the promoter region was cloned into the Sal1 site of pREP7 plasmid and transfected into AGS produce constitutively EBERs-expressing stable cell clones. The expression of EBERs was confirmed by quantitative RT-PCR in AGS-EBERs (clones 3 and 8).
EBRs lead to chemoresistance in gastric cancer cells.

EBRs although not contributing significantly to cell proliferation, display the property of chemoresistance and migration. To understand whether the expression of EBRs has any role in chemoresistance, MTT assay was performed to check cell viability after treating the EBRs-expressing clones of AGS and EBV positive SNU719 cells with chemotherapeutic agent Cisplatin at different concentrations (6, 12, 24 and 48 mM) for 24 h (figure 4.3A). Moreover since EBRs and LMP2A are the limited number of genes expressed in gastric cancer; we also checked the viability and chemoresistance in AGS cell expressing both LMP2A and EBRs simultaneously (figure 4.3A and B). The results revealed that EBRs protect the cells from Cisplatin induced cell death. In order to further investigate this apoptosis-resistance behavior of AGS-EBRs and AGS-EBRsLMP2A clones, Annexin V-FITC/PI assay (figure

Fig 4.2: Effect EBV latency 1 genes on cellular proliferation in gastric cancer cells. The cell cycle distribution data of DNA of AGS Control, AGS-EBRs and AGS-LMP2A. AGS-LMP2A reveal increased cell population in the proliferative G2/M phase. The results represent mean±S.E.M of three independent experiments.
4.3B) and cell cycle analysis (figure 4.3C) were done on cells treated with 12 μM Cisplatin for 24 h. The significantly higher (P< 0.001) percentages of early (Annexin V-FITC positive) and late (Annexin V-FITC and PI double positive) apoptotic cell populations of Cisplatin treated control cells with respect to that of EBERs-expressing cells as well as SNU719 clearly demonstrate that EBERs protect the AGS from chemotherapeutic drug induced apoptosis (figure 4.3B). Cisplatin treatment resulted in 4.270.9%sub-G0/G1 population in control cells, whereas AGS-EBERs (clone 3) and AGS-EBERsLMP2A (clone 1) cells showed only 0.9970.1% and 0.6970.1% population in the sub-G0/G1 phase. (figure 4.3C).

4.4: **LMP2A expression induces Epithelial Mesenchymal Transition and cell invasion.**

Since LMP2A has already been reported to induce cell migration in NPC cells and because we found LMP2A to be associated with enhanced cell proliferation, we therefore examined whether LMP2A could induce similar migration in gastric and breast cancer cells. In vitro wound healing scratch assay was performed on AGS as well as MCF7 cells stably expressing LMP2A and images were acquired at 0, 10, 24 and 48 hrs post introduction of scratch in a phase contrast microscope. Results indicate that LMP2A significantly increases the migratory ability of gastric cancer and breast cancer cells. Figure 4.4A shows that while AGS control cells show 16.17±1.18% migration at 48 hrs, AGS-LMP2A cells showed a 70.32±1.9% closure of wound at the same time point. Moreover, transwell migration assay further revealed that introduction of LMP2A leads to enhanced migration potential since control AGS cell shows 77±8 migrated cells/field while AGS-LMP2A shows 315±10 migrated cells/field on the lower surface of the membrane after 24 hrs (figure 4.4B). **Furthermore, while AGSEBERs show only a slight increase in cell migration, AGS cells expressing both LMP2A and EBERs also manifest a remarkably increased cell migration relative to AGS Control cells (Annexure II; Figure 1).**
Similarly while MCF7 control cells show 13.85±1.07% closure, MCF7-LMP2A reveals a 31.10±0.19% closure of wound at 48 hrs (figure 4.4C). Furthermore, quantitative real time PCR was performed to determine the transcript level expression of some important players of EMT viz. N-Cadherin, E-Cadherin, MMP-2 and MMP-14 in AGS and MCF7 cells. Both AGS-LMP2A and MCF7-LMP2A show upregulated mesenchymal markers (N-Cadherin, MMP-2 and MMP-14) and an almost unchanged E-Cadherin (epithelial marker) (figure 4.4D and E). In order to further confirm increased EMT in LMP2A expressing cells, the protein expression levels of some other prime EMT markers viz; Snail, Slug, Vimentin and Twist were also determined using immunoblotting in AGS/MCF7-LMP2A (figure 4.4F&4.4G) as well as in SNU719 (figure 4.4F) compared to their respective controls. The levels of all these markers were shown to be increased in LMP2A expressing epithelial cells relative to their respective controls.

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**Fig 4.3:** EBERs lead to chemoresistance and migration in gastric cancer cells: (A) MTT assay to determine cell viability upon treating AGS Control, AGS-EBERs, AGS-EBERsLMP2A and SNU719 with Cisplatin (6, 12, 24 and 48 mM) for 24 h. (B) Annexin V/PI assay to determine percentage apoptotic population when AGS Control and AGS-EBERs are treated with 12 mM Cisplatin for 24 hrs. (C) Cell cycle Analysis of was performed on cells treated with 12 mM Cisplatin for 24 h. Wound healing Scratch assay to determine cell migratory potential in AGS, AGS-EBERs and AGS-EBERsLMP2A at 0, 24 and 48hrs post introduction of scratch. Quantitation was performed by measuring percentage closure using Image J software. All the above results are represented as means±s.e.m of n≥3 experiments.
Fig 4.4: LMP2A expression induces epithelial mesenchymal transition and cell invasion. (A) LMP2A induces migration in gastric cancer cells. In vitro wound healing scratch assay was performed on AGS cells stably expressing LMP2A and images were acquired at 0, 10, 24 and 48 hrs post introduction of scratch under 10X magnification in a phase contrast microscope. Quantitation of closure of the wound at 10, 24 and 48 hrs calculated using Image J software (NIH). denotes the width of the wound that is free of any migrated cells. A scale bar of 0.5 inches has been included. The results shown are a representation of N ≥ 6. *, ** and *** denote P<0.05, P<0.005 and P<0.001 respectively as obtained by t-test. (B) AGS-control and AGS LMP2A were added into BioCoat matrigel invasion chamber and incubated for 24h. The invading cells migrated on the lower surface by penetrating through the matrigel. The cells on the undersurface of the membrane were stained with Haematoxylin and then visualized and photographed at 10x magnification using a phase-contrast light microscope. The photographs are representative of different microscopic fields from three independent experiments. (C) Cell invasion assay was carried out in MCF7 cells stably expressing LMP2A and results represented as percentage closure values. (D) Transcript level changes in few EMT markers in AGS stably expressing LMP2A by quantitative RT-PCR. (E) mRNA levels of N cadherin, E cadherin, MMP-2 and MMP-14 detected in MCF7 LMP2A compared to vector control cells. (F) The protein expression levels Snail, Slug, Vimentin and Twist in AGS-LMP2A and SNU719 with respect to control cells. (G) Immunoblotting to detect the expression of Vimentin, Snail, Slug and Twist in MCF7-LMP2A compared to MCF7 control cells. The mean values ± standard error obtained from three independent experiments are shown. *, ** and *** denote P value <.05, <0.005 and < 0.001 respectively.

4.5: Effect of LMP2A and EBERs on self renewal pathways.

EBV Latency I genes especially LMP2A have been shown to modulate some very important self renewal pathways in other cancers such as nasopharyngeal carcinoma and Burkitt lymphoma [40, 43, 44]. In order to understand the role of LMP2A/EBERs in modulating different self-renewal pathways in gastric cancer and breast cancer cells, LMP2A/EBERs-expressing AGS and MCF7 cells was subjected to quantitative RT-PCR analysis to detect the expression of various associated genes of self renewal pathways viz; Notch, Sonic Hedgehog and the Wnt pathway. It was observed that LMP2A significantly upregulated the mRNA expression of Notch1 and Notch target genes (Hes1, Hes5 and Hey1) in AGS-LMP2A, MCF7-LMP2A and SNU719 compared to their respective vector control cells (figure 4.5A). LMP2A also showed Sonic Hedgehog pathway and Wnt activation as indicated by increased expression of Sonic Hedgehog (Figure 4.5B) and Wnt pathway genes (Figure 4.5C) in AGS-LMP2A, MCF7-LMP2A and SNU719 cells. To study the effects of EBERs on self-renewal pathways, real-time RT-PCR was performed for the various associated genes in EBERs-expressing and control AGS and MCF7 cells. A very slight decrease or no significant change was noticed in the expression of Notch1
target genes (figure 4.5D), Sonic Hedgehog genes (figure 4.5E) or Wnt pathway genes (figure 4.5F).

**Fig 4.5:** Effect of LMP2A and EBERs on self renewal pathways. (A) Quantitative RT-PCR determination of Notch downstream genes Hes1, Hes5 and Hey1 in AGS-LMP2A and SNU719 cells. (B-C) Real Time PCR determination of Sonic Hedgehog pathway (B) and Wnt pathway (C) genes in LMP2A expressing gastric cancer and breast cancer cells relative to control cells. (D-F) EBERs do not significantly activate the self renewal pathways. Quantitative RT-PCR estimation of Notch (D), Sonic Hedgehog (E) and Wnt pathway (F) genes in AGS/MCF7-EBERs. The mean values ± standard error obtained from three independent experiments are shown. *, ** and *** denote $P$ value <.05, <0.005 and < 0.001 respectively.

4.6: **LMP2A expression is associated with increased mitochondrial fission.**

Since mitochondrial dynamics alteration has been correlated with epithelial cancers we proceeded to determine if LMP2A/EBERs may have any effect on the dynamic balance of mitochondria. Clones of AGS and MCF7 cells stably expressing LMP2A were subjected to quantitative RT-PCR to estimate the relative transcriptional levels of the key genes associated with mitochondrial dynamics. Results reveal that AGS-LMP2A show a marked upregulation of...
Drp1 (key player of mitochondrial fission) compared to any of the other players upon expression of LMP2A. As shown in figure 4.6A, AGS-LMP2A (clone 2, clone 5 and clone 8) and shows a 6.21±0.4, 9.21±0.48 and 8.21±0.5 fold upregulation respectively of fission gene Drp1. Since LMP2A and EBERs are among the limited genes expressed in latency I, the relative gene expression levels of mitochondrial dynamics were also determined in AGS-EBERs and AGS-LMP2A-EBERs. While AGS-EBERs showed no significant enhancement of mitochondrial fission genes over the fusion genes (figure 4.6B), AGS-LMP2A-EBERs (figure 4.6C) did reveal an upregulated Drp1 relative to vector control cells thereby establishing the fact that only LMP2A and not EBERs bring about this dynamic shift. Similarly MCF7-LMP2A (clone 1 and 3) indicates a 9.19±0.2 and 11.1±0.26 fold upregulation of Drp1 (figure 4.6A). As AGS-LMP2A (clone 5) and MCF7-LMP2A (clone 3) show similar level of expression of Drp1 compared to SNU719, these clones were subsequently used in all further experiments. Alterations of mitochondrial protein levels were also estimated using immunoblotting where AGS-LMP2A (clone 5) and MCF7-LMP2A (clone 3) indicate an elevated Drp1 (2.93±0.15 and 1.8±0.13 fold), and a 0.64±0.03 and 0.56±0.08 fold downregulation in Mfn1 respectively (figure 4.6D and 4.6E).

In order to further establish the role of LMP2A in elevated fission, expression of Drp1 was evaluated in EBV positive gastric cancer cells SNU719. It was observed that SNU719 manifests an elevated fission relative to EBV negative gastric cancer cells AGS (figure 4.6A and 4.6D).

Further confirmation of imbalance in mitochondrial dynamics was done by evaluating mitochondrial morphology in a confocal microscope. pAcGFP1-Mito (Clontech, CA) was transfected into AGS/MCF7 stably expressing either pcDNA or LMP2A as well as in SNU719 cells. 48 hrs after transfection cells were mounted on a slide and observed in a confocal microscope. It was evident that LMP2A expressing clones of AGS and MCF7 showed a more
punctuate and fragmented mitochondrial morphology compared to their respective vector controls (figure 4.6F and 4.6G). SNU719 cells also showed a punctate distribution of mitochondria (figure 4.6F). Length of mitochondria was calculated using Image J software (National Institute of Health, USA) and results represented as relative change in mitochondrial length compared to vector control cells. The percentage of mitochondria that show punctate morphology in these cells was also analyzed as discussed in Materials and Methods and represented (figure 4.6F and 4.6G). Our results clearly indicate that LMP2A expression leads to alteration of the fission-fusion equilibrium, shifting the balance towards fission. To assess whether the observed elevation of Drp1 could be indeed attributable to LMP2A, we knocked down LMP2A mRNA transcripts using LMP2A siRNA in a time dependent manner in SNU719 cells. Transfection of LMP2A siRNA caused over 80% of reduction in LMP2A expression compared to scrambled siRNA (figure 4.6H), while a concomitant reduction in Drp1 expression in a time dependent manner was also observed in the SNU719 cells (figure 4.6I). The decreased mitochondrial fission is also evident on observing a reduction in punctate mitochondrial morphology in SNU719 cells with 20nM LMP2A siRNA for 72hrs compared to untransfected control cells (Annexure II; figure 2). Results are representative of n≥3 and only p values <0.05 was considered statistically significant.
Increase of Drp1 upon LMP2A overexpression is responsible for cell migration. Since Drp1 has previously been associated with migration and invasion of breast cancer cells, we therefore determined if overexpressed Drp1 is responsible for the increased cell invasion and EMT observed in our cell lines. Our results clearly show that indeed upregulation of Drp1 due to LMP2A expression is one of the factors leading to enhanced cell migration because introduction of the dominant negative construct, dnDrp1 (Drp1K38A) (plasmid kindly provided by Prof. Alexander M. van der Bliek, Department of Biological Chemistry, UCLA School of Medicine, Los Angeles, California) hinders cell migration in AGS-LMP2A cells (figure 4.7A and B) as evidenced by scratch assay (at 0, 10, 24 and 48hrs) and transwell migration assay. Transfection of dnDrp1 also decreases cell invasion in MCF7-LMP2A (figure 4.7C) as well as in EBV positive gastric cancer cell SNU719 (figure 4.7D). Real time PCR analysis of EMT markers revealed a downregulation of mesenchymal markers and a marked upregulation in E-Cadherin expression upon introduction of dnDrp1 in AGS-LMP2A, MCF7-LMP2A and SNU719 cells (figure 4.7E, F and G respectively). Furthermore, overexpression of wtDrp1 in AGS as well as MCF7 resulted in enhanced wound closure when compared to untransfected control cells (figure 4.7H and I). In addition AGS-Drp1 and MCF7-Drp1 displayed increased EMT gene expression relative to their respective control cells (figure 4.7J and K).
4.8: **LMP2A increases cell migration via the Notch pathway.**

In order to understand the mechanism by which LMP2A may be causing increase in mitochondrial fission and invasion, the Notch pathway was investigated. We determined whether inhibition of the Notch pathway activated in LMP2A expressing cells regulates cell migration by using the γ-secretase inhibitor, GSI. AGS-LMP2A, MCF7-LMP2A and SNU719 were subjected to various doses of γ-secretase inhibitor (0.5, 1.0, 1.5 and 2.0µM) and cell invasion was measured at 0, 10, 24 and 48 hours. The scratch assay results along with quantitation of closure of wound with 1.0 µM GSI is represented in figure 4.8 A, B and C. It was observed that GSI markedly reduces the migratory potential of these cells. To further confirm the involvement of Notch in cell migration, dominant negative dnCBF1 was transfected in the above cells and migration assay carried out 48 hours later. Figure 4.8 D, E and F demonstrate that the rate of migration is hampered upon introduction of dnCBF1 in LMP2A expressing gastric cancer and breast cancer cell lines. Transcript level changes of key EMT genes were also estimated in AGS-LMP2A/MCF7-LMP2A/SNU719 treated with GSI/dnCBF1. Studies revealed a decrease in mesenchymal markers and increase in epithelial marker expression with GSI/dnCBF1 treatment (figure 4.8 G, H and I). Moreover, introduction of Notch downstream gene Hey1 in AGS as well as MCF7 cells bring about an increase in cell invasion (figure 4.8J and 4.8K respectively). Data shown is a representation of mean±s.e.m n≥3 experiments.
Fig 4.8: LMP2A increases cell migration via the Notch pathway. (A) Reduced cell migration of AGS LMP2A with increasing dose of GSI (0.5, 1.0, 1.5 and 2.0µM). Scratch assay images shows the closure of wound of AGS LMP2A treated with 1.0 µM in a 48 hrs assay compared to AGS LMP2A and the percentage closure values are plotted in a bar graph. denotes the width of the wound that is free of any migrated cells. A scale bar of 0.5 inches has been included. (B) Dose response plot of MCF7 LMP2A when treated with various doses of GSI for 48 hrs. In vitro wound healing assay represents the closure of wound at various time points in a 48 hrs duration with 1.0 µM GSI. Percentage closure values were calculated using Image J software (NIH). (C) Response of migratory potential of SNU719 cells upon treatment with 0.5, 1.0, 1.5 and 2.0µM of GSI at 0, 10, 24 and 48 hrs. Closure of wound of SNU719+1.0µM is pictorially represented. The percentage closure values represent means±s.e.m of three independent experiments. (D-F) Rate of cell migration is hampered upon introduction of dnCBF1 in LMP2A expressing gastric cancer (D, F) and breast cancer cell lines (E). Images are acquired in a phase contrast microscope under 10X magnification and data represented as percentage closure of wound at various time points (0, 10, 24 and 24 hrs) post introduction of scratch. denotes the width of the wound that is free of any migrated cells. A scale bar of 0.2inches has been included. (G-I) Transcript level changes in key EMT genes when AGS LMP2A (G)/MCF7 LMP2A (H)/SNU719 (I) are subject to GSI and dnCBF1 treatment. A marked increase in epithelial marker E cadherin is observed. The percentage closure values were calculated using image J software (NIH) and data represents average of n≥3. (J-K) Introduction of Notch downstream gene Hey1 in AGS (J) as well as MCF7 (K) cells bring about an increase in cell invasion.
4.9: **LMP2A induced Notch pathway mediated migration is associated with Drp1 elevation.**

Since our previous results did clearly indicate that Drp1 is upregulated upon LMP2A expression, we proceeded further to determine the relative expression levels of Drp1 when LMP2A expressing cells are treated with either GSI or dnCBF1 as shown in figure 6. Drp1 levels were estimated both at the RNA and protein levels with the help of quantitative RT PCR and Western blotting. Our experiments showed that treatment of AGS/MCF7-LMP2A/SNU719 with 1µM GSI lead to downregulation of Drp1 RNA (0.5±0.05, 0.6±0.02 and 0.5±0.08 folds respectively) (figure 4.9A, B and C) and protein levels (0.3±0.05, 0.12±0.09 and 0.36±0.05 folds respectively) (figure 4.9D, E and F) while an upregulation was observed in the markers of mitochondrial fusion. Treatment with dnCBF1 also showed similar results (figure 4.9A-F). In order to confirm that treatment with GSI/dnCBF1 does indeed inhibit the Notch pathway the expression of Notch downstream targets were analyzed upon treatment of AGS/MCF7-LMP2A and SNU719 with GSI and dnCBF1(figure 4.9G-I). Results demonstrate a decrease in Notch targets with GSI/dnCBF1 treatment. Moreover treatment of LMP2A expressing gastric cancer and breast cancer cells with Sonic hedgehog pathway inhibitor Forskolin and Wnt/β catenin pathway inhibitor PNU74654 does not significantly alter expression of mitochondrial fission genes (figure 4.9J-L) thereby indicating that the above phenomenon is Notch pathway specific. Therefore, our data clearly indicate that LMP2A mediated Notch pathway disturbs the fission-fusion equilibrium by elevating Drp1 which among other factors brings about enhanced cell migration that may lead to increase in metastatic potential in EBV infected gastric cancer and breast cancer cells.
4.10: **LMP2A expression is associated with activation of PI3K/Akt pathway in gastric cancer and breast cancer cells.**

Since the PI3K/Akt pathway occurs in crosstalk with the Notch pathway and because Akt is a downstream molecule of the Notch pathway, we extended our studies of mitochondrial dynamics to the above pathway in LMP2A expressing cells. LMP2A has been reported to activate the PI3K/Akt pathway in Nasopharyngeal carcinoma cells [25], we however checked the activation in our cellular models viz: gastric cancer and breast cancer cells. In order to study the activation of PI3K/Akt pathway in LMP2A expressing gastric cancer and breast cancer cells, clones of AGS and MCF7 stably expressing LMP2A were subjected to immunoblotting assays. Results displayed increased p-Akt levels with an almost unchanged Akt levels in gastric cancer as well as breast cancer cells stably expressing LMP2A compared to their respective control cells (figure 4.10A and 4.10B). Similarly protein blotting assay performed in EBV positive gastric cancer cells SNU719 also displayed elevated p-Akt when compared to AGS Control cells. In order to confirm the specificity of PI3K/Akt pathway activation in LMP2A expressing cells, LMP2A was knocked down using a siRNA mediated approach in EBV positive SNU719 cells. Results showed that a decrease in LMP2A expression was also accompanied with a decrease in p-Akt protein expression in a time dependent manner upon siRNA treatment (figure 4.10C) thereby highlighting the specificity of LMP2A in activating the PI3K/Akt pathway.
LMP2A activated PI3K/Akt expression increases mitochondrial fission associated with Drp1 elevation.

To study the association between the PI3K/Akt pathway and mitochondrial dynamics if any, in LMP2A expressing cells, LMP2A expressing gastric cancer and breast cancer cells were treated with PI3K inhibitor LY294002 (20μM, 24hrs) and quantitative Real Time PCR experiments were carried out to determine the effect of this inhibition on the mRNA expression levels of key mitochondrial fission (Drp1 and Fis1) and fusion (Mfn1, Mfn2 and Opa1) genes. Results showed a decreased expression of fission genes especially Drp1 with an increased expression of fusion genes when AGS-LMP2A (figure 4.11A), MCF7-LMP2A (figure 4.11B) and SNU719 cells (figure 4.11C) was treated with inhibitor LY294002 relative to untreated LMP2A expressing cells. Furthermore western blotting assays also displayed a decreased Drp1 protein levels in

Fig 4.10: LMP2A expression is associated with activation of PI3K/Akt pathway in gastric cancer and breast cancer cells. (A) Western Blotting experiments to determine PI3K/Akt pathway activation in AGS-LMP2A and SNU719 cells relative to control AGS cells. Bar graph depicts the relative densitometric quantification of Akt, p-Akt and LMP2A levels. Results represent mean±S.E.M. of three individual experiments. (B) Immunoblotting experiments to determine the protein levels of Akt, p-Akt and LMP2A in MCF7-LMP2A relative to MCF7 Control cells. (C) Time dependent effect of LMP2A siRNA on the activation of the PI3K/Akt pathway in SNU719 cells. All results shown represent mean±S.E.M. of n≥3 experiments.

4.11: LMP2A activated PI3K/Akt expression increases mitochondrial fission associated with Drp1 elevation.
LY294002 treated gastric cancer and breast cancer cells (figure 4.11D-F). The inhibition of the PI3K/Akt pathway by LY294002 was confirmed by determining the protein levels of Akt and p-Akt in LY294002 treated cells relative to untreated cells. A decreased p-Akt level and an almost unchanged Akt protein expression confirmed the inhibition of the PI3K/ Akt pathway in our model systems (figure 4.11D-F). Moreover myristylated Akt was also transfected in control AGS and MCF7 cells. It was observed that Akt overexpression increased the expression of fission genes especially Drp1 and decreased fusion gene expression both at the mRNA (figure 4.11G-H) and protein levels (figure 4.11I-J) as obtained by quantitative Real Time PCR assays and western blotting experiments respectively. The effect of LMP2A mediated Drp1 activation on mitochondrial morphology was estimated using confocal microscopy imaging. Cells were transiently transfected with pAcGFP1-Mito (Clontech, CA) for 24hrs and imaged in a confocal microscope to study mitochondrial morphology. Treatment with LY294002 caused a shift in mitochondrial morphology from a predominantly fragmented morphology to a more filamentous distribution in PI3K/Akt inhibited AGS-LMP2A, MCF7-LMP2A and SNU719 cells (figure 4.11K). Mitochondrial length was calculated using Image J software (National Institute of Health, USA) and depicted in figure 4.11K. An increased mitochondrial length indicated a decreased fission in the above cells. Additionally transfection of Akt in AGS and MCF7 cells lead to a more punctuate mitochondrial morphology accompanied by a decreased mitochondrial length compared to untreated control cells (figure 4.11L) emphasizing on the fact that LMP2A mediated PI3K/Akt pathway activation also disturbs the fission-fusion balance towards elevated fission accompanied with Drp1 elevation.
Fig 4.11: LMP2A activated PI3K/Akt expression increases mitochondrial fission associated with Drp1 elevation. (A-C) Effect of inhibition of the PI3K/Akt pathway using LY294002 upon the transcript level expression of key mitochondrial fission and fusion genes in AGS-LMP2A (A), MCF7-LMP2A (B) and SNU719 cells (C). (D-F) Protein expression levels of fission player Drp1 and fusion player Mfn1 upon treating AGS-LMP2A (D), MCF7-LMP2A (E) and SNU719 (F) with LY294002 for 24 hrs. Bar graph represents the relative densitometric quantification of Drp1 and Mfn1 protein levels. (G) Quantitative RT-PCR performed to determine the relative mRNA levels of key mitochondrial dynamics genes in AGS-Akt, AGS- LMP2A and SNU719 cells. (H) Real Time PCR carried out to estimate the relative transcript expression of mitochondrial fission and fusion genes in MCF7-Akt and MCF7-LMP2A cells. (I) Levels of Drp1 and Mfn1 protein in AGS-Akt, AGS-LMP2A and SNU719 relative to control cells. (J) Immunoblot to determine the protein expression on Drp1 and Mfn1 in MCF7-Akt and MCF7-LMP2A relative to MCF7 control cells. (K) Effect of inhibition of the PI3K/Akt pathway on mitochondrial morphology. Mitochondrial length was estimated using Image J software (NIH, USA) and represented in the bar diagram. (L) Estimation of mitochondrial morphology upon overexpression of Akt in Control AGS and MCF7 cells. Mitochondrial length was determined and depicted in the bar graph. All the above results represent mean±S.E.M. of at least 3 independent experiments.
4.12: **Inhibition of the PI3K/Akt pathway decreases Drp1 mediated cell invasion.**

Since Drp1 elevation has been associated with increased cell invasion and because results in figure 4.10 and 4.11 showed LMP2A mediated PI3K/Akt activation leads to Drp1 elevation in gastric cancer and breast cancer cells, we further tried to determine the effect of PI3K/Akt pathway activation and inhibition upon cell migration and invasion in LMP2A expressing gastric cancer and breast cancer cells. Inhibition of the PI3K/Akt pathway using LY294002 decreased cell migration and invasion in AGS-LMP2A (figure 4.12A and 4.12D), MCF7-LMP2A (figure 4.12B and 4.12E) and SNU719 cells (figure 4.12C and 4.12F) as observed by *in vitro* wound healing scratch assay and Matrigel invasion assay. Furthermore overexpression of myristylated Akt in control AGS and MCF7 cells increased the rate of cell migration and invasion (figure 4.12G-J). Since elevated cell invasion is often associated with elevated Epithelial Mesenchymal Transition (EMT) and *vice versa*, Quantitative Real Time PCR was performed to determine the transcript level expression of epithelial marker E Cadherin and Mesenchymal markers N Cadherin, MMP-2 and MMP-14. Inhibition of PI3K/Akt pathway decreased mesenchymal markers and increased epithelial markers indicating reduced EMT in LMP2A expressing cells (figure 4.12K) whereas overexpression of Akt increased EMT in AGS and MCF7 cells (figure 4.12L). Moreover, earlier reports from our lab have shown LMP2A activated Notch pathway to enhance cell migration via Drp1 elevation. Therefore it can now be concluded that the LMP2A activated PI3K/Akt pathway along with the previously reported LMP2A activated Notch pathway together bring about Drp1 elevation which enhances cell migration and invasion in LMP2A positive gastric cancer and breast cancer cells.
Fig 4.12: Inhibition of the PI3K/Akt pathway decreases Drp1 mediated cell invasion. (A–C) Transwell migration assay to determine the effect on PI3K/Akt inhibition on the invasive potential of (A) AGS-LMP2A, (B) MCF7-LMP2A and (C) SNU719 cells. The photographs are representative of different microscopic fields from three independent experiments. (D–F) Wound healing scratch assay to assay the effect of LY294002 mediated PI3K/Akt pathway inhibition on the rate of cell migration of (D) AGS-LMP2A, (E) MCF7-LMP2A and (F) SNU719 cells. Images were acquired at 0, 10, 24 and 48 hrs post introduction of scratch under 10X magnification in a phase contrast microscope. Quantitation of closure of the wound at 10, 24 and 48 hrs calculated using Image J software (NIH). | denotes the width of the wound that is free of any migrated cells. A scale bar of 0.5 inches has been included. (G–H) Effect of Akt overexpression upon the rate of cell invasion in AGS (G) and MCF7 (H) control cells. (I–J) scratch assay performed to determine the effect of Akt overexpression on the rate of closure of wound in AGS Control (I) and MCF7 Control (J) cells. (K) Transcript level changes in few important EMT markers upon treating AGS-LMP2A, MCF7-LMP2A and SNU719 cells with LY294002. (L) Quantitative Real Time PCR performed to determine the relative mRNA expression levels of EMT regulators N-Cadherin, E-Cadherin, MMP-2 and MMP-14 in AGS-Akt and MCF7-Akt cells relative to control cells. *, ** and *** denote P<0.05, P<0.005 and P<0.001 respectively as obtained by t-test.