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STAT3 activation induced by Epstein-Barr virus latent membrane protein1 causes vascular endothelial growth factor expression and cellular invasiveness via JAK3 And ERK signaling

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ABSTRACT

The principal Epstein–Barr virus (EBV) oncoprotein, latent membrane protein 1 (LMP1), has been suggested to contribute to the highly invasive nature of nasopharyngeal carcinoma (NPC). Signal transducer and activator of transcription 3 (STAT3) is a master transcriptional regulator in proliferation and apoptosis and is newly implicated in angiogenesis and invasiveness, which, in turn, are likely to contribute to the highly invasive character of NPC. The fundamental molecular mechanisms of LMP1-regulated STAT3 activation in NPC cell invasion have not been completely explored. Here, we showed that LMP1 signals the Janus kinase 3 (JAK3) and extracellular signal-regulated kinase 1/2 (ERK1/2) pathways upon the activation of STAT3 as well as STAT transactivation activity. LMP1 induces vascular endothelial growth factor (VEGF) expression via the JAK/STAT and mitogen-activated protein kinase (MAPK)/ERK signalling pathways. Induction of STAT3 by the human viral oncoprotein LMP1 may contribute to the invasion of NPC.

1. Introduction

Nasopharyngeal carcinoma is the malignancy most strongly associated with Epstein–Barr virus (EBV).[1] In nasopharyngeal carcinoma (NPC), EBV expresses Epstein–Barr nuclear antigen 1 (EBNA1), latent membrane protein 1 (LMP1), LMP2A, Epstein–Barr virus-encoded RNAs (EBERs) and the BamHI-A rightward transcripts (BARTs).[2] Latent membrane protein 1

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is the EBV-encoded protein with oncogenic properties. As a transmembrane protein, LMP1 interacts with signalling molecules and results in activation of nuclear factor (NF)-κB,[3] activated protein 1 (AP-1),[4] phosphatidylinositol 3-kinase (PI3-kinase),[5,6] extracellular-regulated kinase (ERK)-mitogen-activated protein (MAP),[7–9] the c-Jun N-terminal kinase (JNK),[10,11] p38 mitogen-activated protein kinase (MAPK)[12] and Janus-activated kinase (JAK)/signal transducers and activators of transcription pathways.[13]

LMP1 is detected in at least 70% of NPCs at the protein level and in virtually all at the transcriptional level.[14] The frequent expression of LMP1 in undifferentiated NPC points to a role for this viral oncoprotein as a key effector molecule in NPC pathogenesis.[15] LMP1 is thought to play an essential role in the tumourigenesis of NPC. In addition to its transforming properties, LMP1 has been suggested to be associated with the promotion of invasiveness and metastasis.[16] Studies demonstrate that LMP1-positive NPCs show a more progressive attitude and an increased tendency towards lymph node metastasis than LMP1-negative NPCs.[17–19]

Signal transducer and activator of transcription (STAT)-family proteins are latent cytoplasmic transcription factors that convey signals from cytokine and growth-factor receptors to the nucleus. STAT proteins, particularly signal transducer and activator of transcription 3 (STAT3) and the STAT5 proteins, are constitutively activated and present in the nucleus in NPC cells.[20] Recently, it has been reported that STAT3 activation contributes directly to the invasiveness of nasopharyngeal cancer cells.[21,22] LMP1 has been found to associate with Janus kinase 3 (JAK3) or to upregulate the expression of IL-6 to activate STAT3 tyrosine 705 (Tyr^{705}) phosphorylation.[13,20] Recently we have also demonstrated that LMP1 CTAR2 and CTAR3 (CTAR, carboxyl terminal activator region) mediates JAK/STAT activation.[23,24] Additionally, STAT3 serine 727 (Ser^{727}) phosphorylation is required for the maximum activation of STAT3 signalling and Tyr^{705} phosphorylation alone is not sufficient for the obligatory role of STAT3 signalling in oncogenesis.[25–27] It has been demonstrated that MAPKs can phosphorylate Ser^{727} on STAT3 to modulate its transcriptional activity.[28,29] Whether (and if so, how) STAT3 activation regulated by LMP1 implicates migration ability and metastasis in NPC has not yet been completely examined. Understanding how LMP1-regulated STAT3 activation influences NPC cell invasion on a molecular level could open new ways towards additional therapeutic strategies in the treatment of NPC.

In the present study, we found that phosphorylation of STAT3 activated by LMP1 represents an important pathway for the cell invasion of NPC. STAT3 has been shown to be a direct transcriptional-activator of the vascular endothelial growth factor (VEGF) gene.[30] To explore the potential mechanisms underlying LMP1-induced VEGF expression and NPC cell invasion, we used mitogen-activated protein kinase kinase 1 (MEK1) specific inhibitor PD98059, JAK3 kinase specific inhibitor WHI-P131, antisense oligos and siRNA of STAT3 and LMP1-targeted DNAzyme to inhibit MAPK/ERK pathway, JAK/STAT pathway STAT3 expression or LMP1 expression respectively. Our results show that LMP1 activates STAT3 through JAK3/STAT3 and MEK1/ERK1/2 signalling pathways, which in turn results in induction of VEGF and finally enhances NPC cell migration.

2. Materials and methods

2.1. Materials

The MEK1 inhibitor PD98059 (#9900) was purchased from Cell Signaling Technology (Beverly, MA), it was dissolved in dimethyl sulphoxide (DMSO) and stored at −20 °C. The JAK3 kinase inhibitor, WHI-P131 (#420101), was purchased from CALBIOCHEM (Darmstadt, Germany). DNAzyme 1 (DZ1) is a LMP1-targeted DNAzyme that binds and cleaves LMP1 RNA in a highly sequence-specific manner.[19,31] The control oligonucleotide of DZ1 was designed by inverting the catalytic core sequence. The glycine-rich region (5)-luciferase (GRR[5]-Luc) reporter (kindly provided by Dr. Paul Brennan, section of infection and immunity, Department of Medicine, University of Wales College of Medicine) contains five tandem copies of the FcγR1 gamma interferon-activated sequence upstream of a minimal thymidine kinase promoter in the pBL-Luc vector.[11,32]

2.2. Cell culture

The human NPC cell line CNE1 is derived from a poorly differentiated NPC[33] and the CNE1-LMP1 cell line stably expressing LMP1 was stored in our laboratory.[34] The cells were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% v/v foetal bovine serum plus antibiotics at 37 °C in a 5% CO_2 atmosphere.

2.3. STAT3 oligonucleotide transfection

Oligonucleotides were obtained from Applied Biosystems (Foster City, CA) and also 4 specially modified oligomers each one used methylated 5 bases from both the 5′and 3′ ends at the 2-position of the ribose structure of DNA. The phosphate backbones are completely modified by phosphorothioate to be stabilized and antisense-activated with the strands. The STAT3 antisense oligonucleotides sequence was 5′-GCT CCA GCA TCT GCT GCT TC-3′ and control mismatch oligonucleotides sequence was 5′-GCT CCA ATA CCC GTT GCT TC-3′.[35–37] The human STAT3 antisense oligonucleotides (ASO) (base sequence 5′-GCT CCA GCA TCT GCT GCT TC-3′) hybridized to a sequence in the coding region (nucleotides 637–656). Control ASO that we used is a five-base mismatch oligonucleotide (5′-GCT CCA ATA CCC GTT GCT TC-3′). Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA) was used as the transfection reagent following the manufacturer's instructions. STAT3 oligonucleotides and control mismatch oligonucleotides were transfected into 60% confluent CNE1 and CNE1-LMP1 cells at a final concentration of 500 nmol/L. Twenty-four hours after transfection, the efficacy of knockdown was assessed by Western blotting.

2.4. STAT3 siRNA transfection

Short interfering RNAs (siRNAs) were designed according to the methods described by Elbashir et al.[38] RNAs of 21 nucleotides were chemically synthesised using Expedite RNA phosphoramidites and thymidine phosphoramidite (Invitrogen Life Technologies, Carlsbad, CA). The accession numbers are from GenBank. The sequence of siRNA STAT3 (Acc. No.

NM_003150) corresponded to the coding regions 987–1007 nucleotide of coding frame. STAT3 interfere RNA (5′-AAC AUC UGC CUA GAU CGG CUA dTdT-3′, 3′-dTdT GUA GAC GGA UCU AGC CGA-5′)[39,40] or control siRNA (5′-GGC UAC GUC CAG GAG CGC ACC dTdT-3′, 5′-UGC GCU CCU GGA CGU AGC CUU dTdT-3′), Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA) was used as the transfection reagent following the methods described by Elbashir *et al.*[38] Annealed siRNAs were transfected into 30–50% confluent CNE1 and CNE1-LMP1 cells at a final concentration of 100 nmol/L. Three days after transfection, the efficacy of knockdown was assessed by Western blotting.

2.5. Western blot analysis

The cells were treated or not treated with inhibitors and washed three times with ice-cold phosphate-buffered saline (PBS) and suspended in 100 μL of lysis buffer (50 mM Tris–HCl, pH 8.0; 1 mM EDTA, 2% w/v sodium dodecyl sulphate [SDS]; 5 mM dithiothreitol [DTT]; 10 mM phenylmethanesulphonyl fluoride [PMSF]; 1 mM NaF; 1 mM Na_3VO_4; and protease inhibitor cocktail). They were then denatured in boiling water for 5 min and sonicated for 30 s. The lysate was centrifuged at 12,000 rpm for 20 min. The supernatant fractions were saved for Western blot analysis; protein concentration was determined by bicinchoninic acid (BCA) assay. Equal amounts of protein were separated by 8% w/v (for LMP1, phospho-STAT3 and STAT3) or 12% w/v (for VEGF) sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and proteins were subsequently transferred to polyvinylidene fluoride (PVDF) membranes by electroblotting. The membranes were incubated in blocking buffer (Tris-buffered saline [TBS] containing 1% w/v milk, 5% w/v BSA and 0.1% v/v Tween 20) for 2 h. The level of phosphorylated STAT3 (Tyr^{705} and Ser^{727}), as well as total STAT3, LMP1, VEGF and β-actin protein, was selectively measured by Western blot using a specific antibody. The antibody-bound proteins were detected using the Supersignal-chemiluminescence system (ECL, Pierce, Rockford, IL), followed by exposure to autoradiographic film. The antibodies used were as follows: mouse LMP1-monoclonal antibody (M0897, DAKO, Carpinteria, CA), STAT3 (#9132, Cell Signal, Danvers, MA), phospho-STAT3 (Tyr^{705}) (#9136, Cell Signal, Danvers, MA), phospho-STAT3 (Ser^{727}) (#9134, Cell Signal, Danvers, MA), β-actin (A-5441, Sigma, St. Louis, MO) and VEGF (A-20) (Santa Cruz, CA).

2.6. Transient transfection and luciferase assay

CNE1 and CNE1-LMP1 cells were seeded in 24-well plates before transfection. Using SuperFect Transfection Reagent (Qiagen, Hilden, Germany) in a serum-free medium (according to the manufacturer's instructions), 0.9 μg of GRR5-Luc was co-transfected with 0.1 μg of pRSV-β-galactosidase (gal). Twenty-four hours after transfection, the cells were treated with inhibitors as designated for 24 h (DZ1) and 6 h (WHI-P131 or PD98059). Cells were then lysed and reporter gene-activity was determined with the Luciferase Assay System (Promega Corporation, Madison, WI) using a luminometer (Promega). β-Galactosidase activity was measured using the β-galactosidase enzyme assay system (Promega) and the system was used for the normalisation of transfection efficiency. All experiments were performed in triplicate and repeated at least three times.

2.7. Measurement of vascular endothelial growth factor

Cells (5×10^5) were plated in six-well plates and cultured with regulators for the desired time. Cell culture medium was collected, and the concentration of VEGF was measured using an ELISA kit (Quantikine, R&D Systems, Minneapolis, MN). Triplicates of each sample were analysed using SigmaPlot 8.0 software.

2.8. Matrigel invasion assay

Cell invasion was assayed using a Boyden chamber assay. In brief, polycarbonate membranes (8.0-μm pore size) were coated with diluted Matrigel in the upper compartment of Transwell culture chambers. Then 250-μL portions of cells (5×10^5) suspended in serum-free Dulbecco's modified eagle's medium (DMEM) (GIBCO, Grand Island, NY) were placed in the upper compartment and the lower compartment of the chamber was immediately filled with 500 μL of DMEM supplemented with 1% v/v foetal bovine serum. After 24 h of incubation, the membranes were fixed with methanol and stained with haematoxylin and eosin (H&E). Cells located on the upper surface of the filter were completely removed by wiping the filter with a moist cotton swab; cells that had invaded the Matrigel and had migrated through the membrane to the lower surface were counted using a light microscope. Each assay was repeated at least three times.

2.9. Statistical analysis

All statistical analyses were performed with Statistical Product and Service Solutions (SPSS) software, version 13.0. The data shown are mean values of at least three different experiments and are expressed as means ± SD (standard deviation). Student's *t*-test was used for comparison. $P < 0.05$ is considered statistically significant.

3. Results

3.1. The inhibition of JAK3 and ERK1/2 pathways blocks LMP1-regulated STAT3 phosphorylation, transactivation activity and VEGF production

Our previous studies have shown that both JAK3 and ERK1/2 could be activated by LMP1 in NPC cell lines.[7,19,23] LMP1 is capable of activating STAT3 involving MAPK/ERK and JAK/STAT pathways, which directly phosphorylate STAT3 at Ser^{727} and Tyr^{705}, respectively. To further confirm the involvement of the JAK3 and ERK1/2 pathways in LMP1-augmented STAT3 phosphorylation, WHI-P131, a specific JAK3 inhibitor and PD98059, an MEK1 inhibitor, were used to investigate STAT3 phosphorylation induced by LMP1. The levels of STAT3 Tyr^{705} and Ser^{727} phosphorylation were higher in CNE1-LMP1 cells than in CNE1 cells (Figs. 1A and 2A). Treatment of CNE1-LMP1 cells with both inhibitors resulted in a dose-dependent suppression of STAT3 phosphorylation upregu-

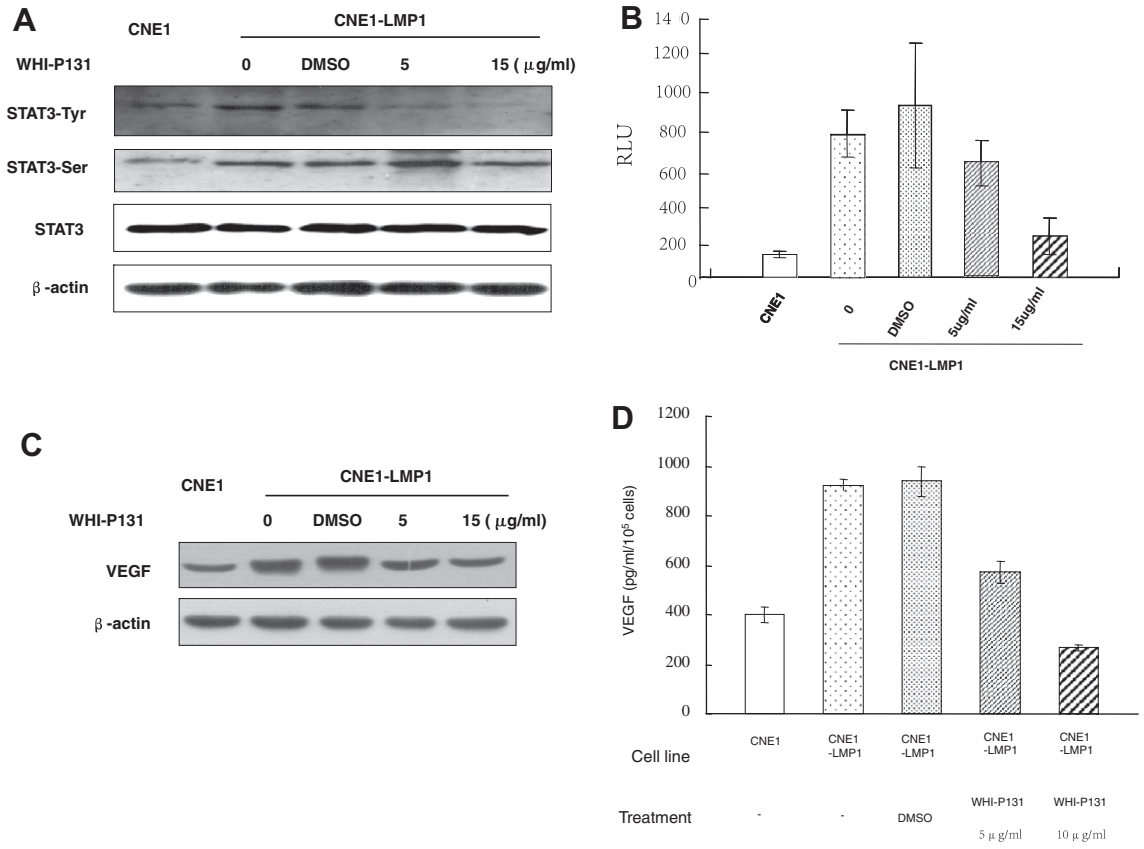

Fig. 1 – Effects of JAK3 inhibitor on LMP1-induced STAT3 phosphorylation, transactivation activity and VEGF production. (A) Analysis of STAT3 phosphorylation. NPC cells were incubated with 5 or 15 μg/mL WHI-P131 and DMSO control for 24 h at 37 °C. STAT3 activity was determined by immunoblotting with antibodies specific for phosphorylated STAT3 Tyr^{705} or STAT3 Ser^{727}. The quantity of protein added was verified by immunoblotting with antibodies specific for total STAT3 and β-actin. Results are representative of three separate experiments. (B) Analysis of STAT3 transactivation activity. NPC cells were seeded in 24-well plates. 0.9 μg pGRR5-Luc and 0.1 μg pRSV-β-gal constructs were co-transfected into cells. Twenty-four hours after transfection, cells were treated with WHI-P131 and DMSO control for 6 h. Values are means plus standard deviations of at least two independent experiments, each performed in triplicate and normalised to β-gal activity. The luminescence emitted was quantified as the relative luminescent unit (RLU). (C) Analysis of VEGF expression. NPC cells were incubated with 5 or 15 μg/mL WHI-P131 and DMSO control for 24 h at 37 °C. VEGF expression was determined by immunoblotting with antibodies specific for VEGF. The quantity of protein added was verified by immunoblotting with antibodies specific for β-actin. Results are representative of three separate experiments. (D) Analysis of VEGF secretion. Soluble VEGF were collected from conditioned medium and quantitatively determined by ELISA. Columns represent the mean of three different experiments; bars represent standard deviation.

lated by LMP1 as compared with using DMSO as a control (Figs. 1A and 2A).

To determine if transactivation activity of STAT3 is involved in the LMP1-induced JAK3 and ERK1/2 pathways, we transiently transfected CNE1 and CNE1-LMP1 cells with a reporter construct, GRR (5)-Luc luciferase reporter, which contains five copies of a gamma interferon-activating element. Luciferase-reporter assays show that WHI-P131 and PD98059 efficiently inhibited the transactivation activity of STAT3 induced by LMP1 (Figs. 1B and 2B). Overall, these results confirm the concept that upregulation of transactivation activity of STAT3 by LMP1 occurs via activation of JAK3 and MAPK pathways.

Then, we used JAK3- and MAPK-specific inhibitors to determine which pathway was responsible for LMP1-mediated VEGF regulation. When the JAK3 pathway was inhibited by WHI-P131, a concentration-dependent inhibition of VEGF expression was detected. The VEGF basal level of 922 ± 23 ng/ml in CNE1-LMP1 was reduced to 572 ± 44 ng/ml (by 38%, $p < 0.01$) and 268 ± 14 ng/ml (by 71%, $p < 0.01$) when cells were exposed to 5 and 15 mg/mL of WHI-P131, respectively, for 24 h (Fig. 1D). Similar studies were carried out to determine the inhibitory profile of the MAPK-specific inhibitor PD98059 in CNE1-LMP1. The VEGF basal level of 647 ± 14 ng/ml in CNE1-LMP1 was reduced to 470 ± 55 ng/ml (by 27%, $p < 0.05$) and 360 ± 29 ng/ml (by 53%, $p < 0.01$) when cells were exposed to 25 and 50 μmol/L PD98059, respectively, for 24 h (Fig. 2D). Next, Western blot analysis showed that protein expression level of VEGF is also downregulated by WHI-P131 and PD98059 (Fig. 1C and 2C). Taken together, our findings suggest that the inhibition of JAK3 and ERK1/2 pathways

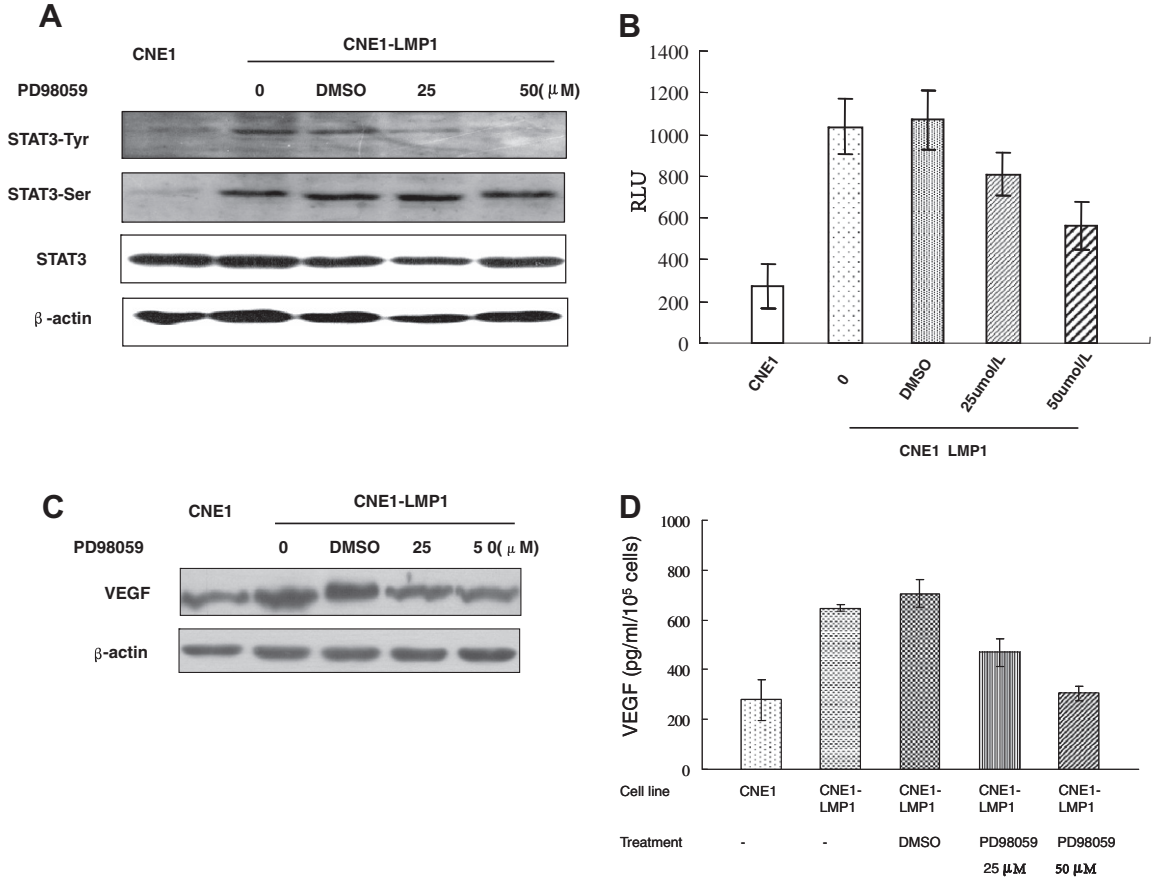

Fig. 2 – Effects of MEK1 inhibitor on LMP1-induced STAT3 phosphorylation, transactivation activity and VEGF production. (A) Analysis of STAT3 phosphorylation. NPC cells were incubated with 25–50 μmol/L PD98059 and DMSO control for 24 h at 37 °C. STAT3 activity was determined by immunoblotting with antibodies specific for phosphorylated STAT3 Tyr^{705} and STAT3 Ser^{727}. The quantity of protein added was verified by immunoblotting with antibodies specific for total STAT3 and β-actin. Results are representative of three separate experiments. (B) Analysis of STAT3 transactivation activity. NPC cells were seeded in 24-well plates. 0.9 μg pGRR5-Luc and 0.1 μg pRSV-β-gal constructs were co-transfected into cells. Twenty-four hours after transfection, cells were treated with PD98059 and DMSO control for 6 h. Values are means plus standard deviations of at least two independent experiments, each performed in triplicate and normalised to β-gal activity. (C) Analysis of VEGF expression. NPC cells were incubated with 25 or 50 μmol/L PD98059 and DMSO control for 24 h at 37 °C. VEGF expression was determined by immunoblotting with antibodies specific for VEGF. The quantity of protein added was verified by immunoblotting with antibodies specific for β-actin. Results are representative of three separate experiments. (D) Analysis of VEGF secretion. Soluble VEGF were collected from conditioned medium and quantitatively determined by ELISA.

blocks STAT3 phosphorylation and VEGF production upregulated by LMP1.

3.2. *The inhibition of LMP1 expression by LMP1-specific DNAzyme, decreasing STAT3 phosphorylation, transactivation activity and VEGF production*

We have designed and constructed specific DNAzymes that were shown to be effective in suppressing the expression of the target protein LMP1.[31] We used the LMP1-specific DNAzyme, DZ1, which is targeted to the transmembrane domains of LMP1, to investigate further the effect of LMP1 on the phosphorylation of STAT3. Western blotting results indicated that DZ1 obviously inhibited LMP1 protein expression in CNE1-LMP1 cells as compared with thiamine monophosphate (TMP) transfection reagent and non-specific DNAzyme controls. DZ1 could concomitantly downregulate STAT3 Tyr^{705} and Ser^{727} phosphorylation in the LMP1-positive cell line CNE1-LMP1 but had no effect on STAT3 expression (Fig. 3A). Then we transiently transfected CNE1 and CNE1-LMP1 cells with a reporter construct, GRR (5)-Luc reporter. Using TMP transfection reagent and non-specific DNAzyme as controls, luciferase reporter assays show that the DNAzyme DZ1 efficiently inhibited the transactivation activity of STAT3 induced by LMP1 (Fig. 3B). Furthermore, we explored whether targeting LMP1 by DZ1 could decrease the production of VEGF in CNE1-LMP1 cells, and Western blotting showed that DNAzyme DZ1 inhibits VEGF expression and ELISA analysis also showed DZ1 reduce VEGF concentrations in the conditioned medium (Fig. 3C and D). The

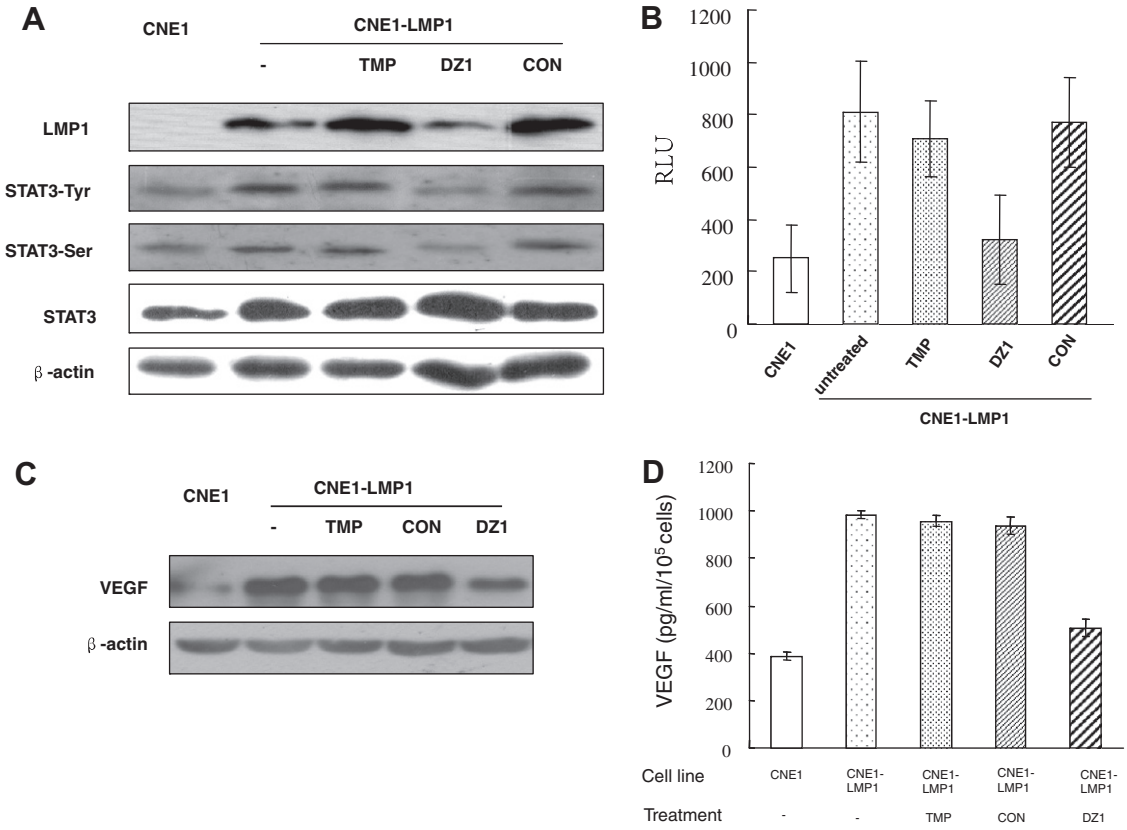

Fig. 3 – LMP1-targeted DNAzyme decreases STAT3 phosphorylation, transactivation activity and VEGF production. (A) Analysis of STAT3 phosphorylation. NPC cells grown in six-well plates were transfected with DNAzyme (DZ1 at 2 μM), a control oligonucleotide (CON) and TMP transfection reagent incubated in medium containing 10% FBS at 37 °C for 24 h. LMP1 expression and STAT3 activity was determined by immunoblotting with antibodies specific for LMP1, phosphorylated STAT3 Tyr^{705} and STAT3 Ser^{727}. The quantity of protein added was verified by immunoblotting with antibodies specific for total STAT3 and β-actin. (B) Analysis of STAT transactivation activity. NPC cells were seeded in 24-well plates. 0.9 μg pGRR5-Luc and 0.1 μg pRSV-β-gal constructs were co-transfected into cells. Twenty-four hours after transfection, cells were treated with the LMP1 DNAzyme (DZ1 at 2 μM), a control oligonucleotide(CON), TMP transfection reagent and untreated controls for 24 h. (C) Analysis of VEGF expression. NPC cells were incubated with DNAzyme (DZ1 at 2 μM), a control oligonucleotide (CON) and TMP transfection reagent at 37 °C for 24 h. VEGF expression was determined by immunoblotting with antibodies specific for VEGF. (D) Analysis of VEGF secretion. Soluble VEGF were collected from conditioned medium and quantitatively determined by ELISA.

result further suggested that LMP1-induced expression of VEGF is mediated by STAT3 activation.

3.3. *Interference RNA reduced VEGF production and intracellular levels of STAT3 protein*

To further confirm the involvement of STAT3 signalling pathways in LMP1-augmented VEGF expression, STAT3 siRNA was used to investigate STAT3 phosphorylation and its target gene VEGF expression as induced by LMP1. Using β-actin as control, treatment of CNE1 and CNE1-LMP1 with siRNA resulted in a significant decrease of STAT3 expression at the protein level, compared with STAT3 siRNA controls. Both the levels of STAT3 Tyr^{705} and Ser^{727} phosphorylation were significantly reduced, indicating that STAT3 siRNA could inhibit the activity of STAT3 (Fig. 4A). Moreover, as indicated by Western blot and ELISA assay analyses, the STAT3 siRNA inhibited VEGF expression in CNE1-LMP1 cells, compared with the STAT3 siRNA controls. We cannot observe significant change in the expression level of VEGF in CNE1 cells, but the secretion of VEGF in the cell culture supernatants was significantly reduced (compared with STAT3 siRNA controls, $p < 0.05$). These results confirm the concept that upregulation of VEGF by LMP1 occurs via STAT3 signalling (Fig. 4B and C).

3.4. *Antisense STAT3 reduced VEGF production and intracellular levels of STAT3 protein*

Using mismatch oligonucleotides as controls, CNE1 and CNE1-LMP1 cells were further transiently transfected with STAT3 antisense oligonucleotides. Western blot analysis of total STAT3 protein levels demonstrate that STAT3 antisense- (Fig. 5A), but not control-mismatch oligonucleotides, were able to diminish substantially STAT3 protein expression in CNE1 and CNE1-LMP1. This block in STAT3 expression specifically ablates the levels of STAT3 Tyr^{705} and Ser^{727} phosphory-

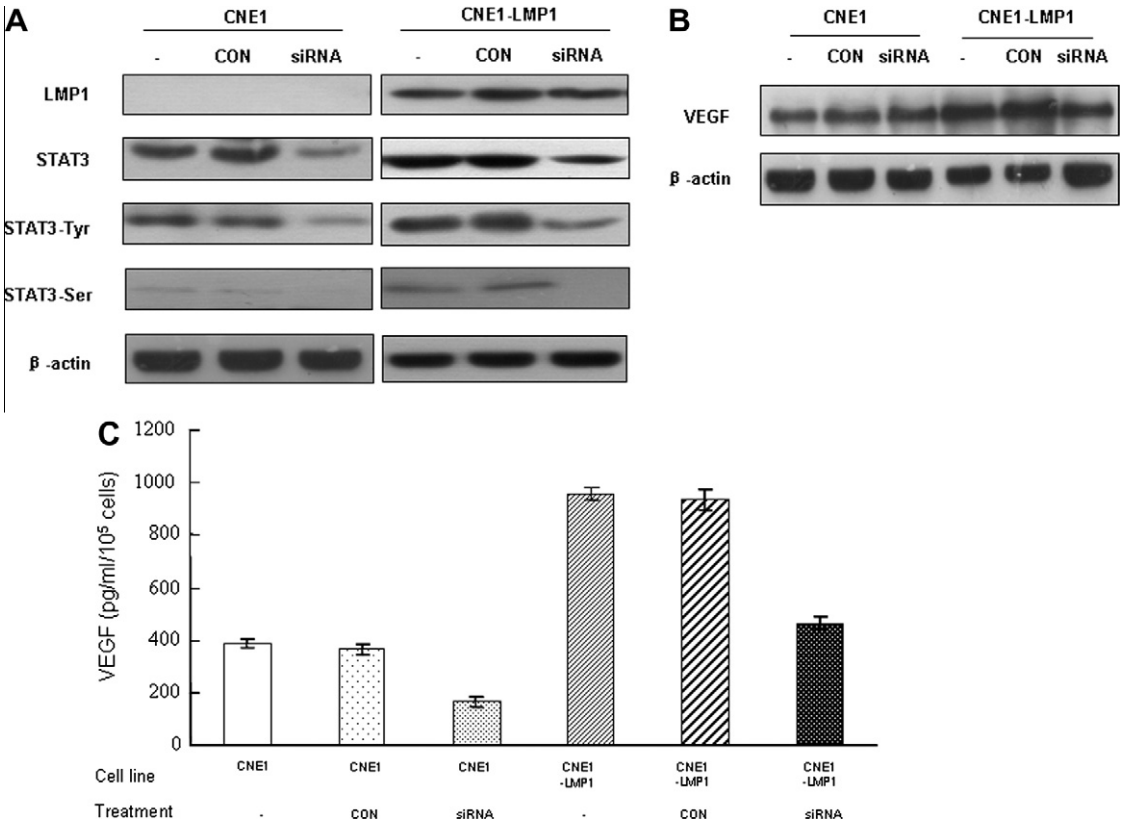

Fig. 4 – STAT3 siRNA inhibits LMP1-induced STAT3 phosphorylation and VEGF production. (A, B) Analysis of STAT3 activity. STAT3 activation, STAT3 and VEGF expression was determined by immunoblotting with antibodies specific for phosphorylated STAT3 Tyr^{705}, STAT3 Ser^{727}, STAT3 and VEGF. The quantity of protein added was verified by immunoblotting with antibodies specific for total β-actin. (C) Analysis of VEGF secretion. After NPC cells were cultured for 24 h after 100 nM STAT3 siRNA or the control siRNA (CON) treatment, the medium was changed and a further 48-h culture was performed, then the medium was collected for VEGF ELISA. Columns represent the mean of triplicate data points from three different experiments; bars represent standard deviation.

lation in both NPC cell lines. Then we estimated the effect of antisense-oligonucleotides for STAT3 on the production of VEGF regulated by LMP1 in CNE1-LMP1. Compared with control-mismatch oligonucleotides, the downregulation of STAT3 by antisense-oligonucleotides decreased the production of VEGF in CNE1-LMP1 and CNE1 (Fig. 5B and C). This result further demonstrates the pivotal role of STAT3 activation by LMP1 in VEGF expression in NPC.

3.5. *Suppression of LMP1-regulated STAT3 signalling decreased the ability for Matrigel transmembrane migration*

Increased cell motility and invasion are correlated with increased metastatic potential. To examine whether LMP1 is involved in NPC migration, we investigated the invasive ability of these human NPC cell lines in a Transwell chamber assay. Using the LMP1-specific DNAzyme, DZ1, we found that LMP1 can increase the migration ability of CNE1-LMP1 by 2.3 fold compared with CNE1. DZ1 significantly inhibited the migration ability by 65% in CNE1-LMP1 ($p < 0.01$). The control oligonucleotide DNAzymes did not ($p > 0.05$; Fig. 6A). To analyse further whether JAK3 and ERK1/2 activity was necessary for LMP1-induced cell migration, we performed Transwell migration assays in the presence of a JAK3 inhibitor (WHI-P131) and an MEK1 inhibitor (PD98059) for 24 h. WHI-P131 significantly inhibited the migration ability of CNE1-LMP1 by 53% at 5 μg/ml ($p < 0.01$) and by 69% at 15 μg/ml ($p < 0.01$, Fig. 6B). PD98059 significantly inhibited the migration ability of CNE1-LMP1 by 37% at 25 μmol/L ($p < 0.01$) and by 61% at 50 μmol/L ($p < 0.01$, Fig. 6C). Finally, we used the STAT3-specific ASO treatment and found that ASO significantly inhibited migration ability by 56% in CNE1 ($p < 0.01$). The control-mismatch oligonucleotides did not significantly inhibit migration ability ($p > 0.05$; Fig. 6D). In addition, ASO significantly inhibited migration ability by 65% in CNE1-LMP1 ($p < 0.05$), whereas the control-mismatch oligonucleotides did not ($p > 0.05$; Fig. 6D). Taken together, the results demonstrate that STAT3 activation regulated by JAK3 and ERK1/2 is involved in the LMP1-induced migration of NPC.

4. Discussion

In addition to its classic role in tumourigenesis, LMP1 has recently been implicated in the role of NPC metastasis.[41–50]

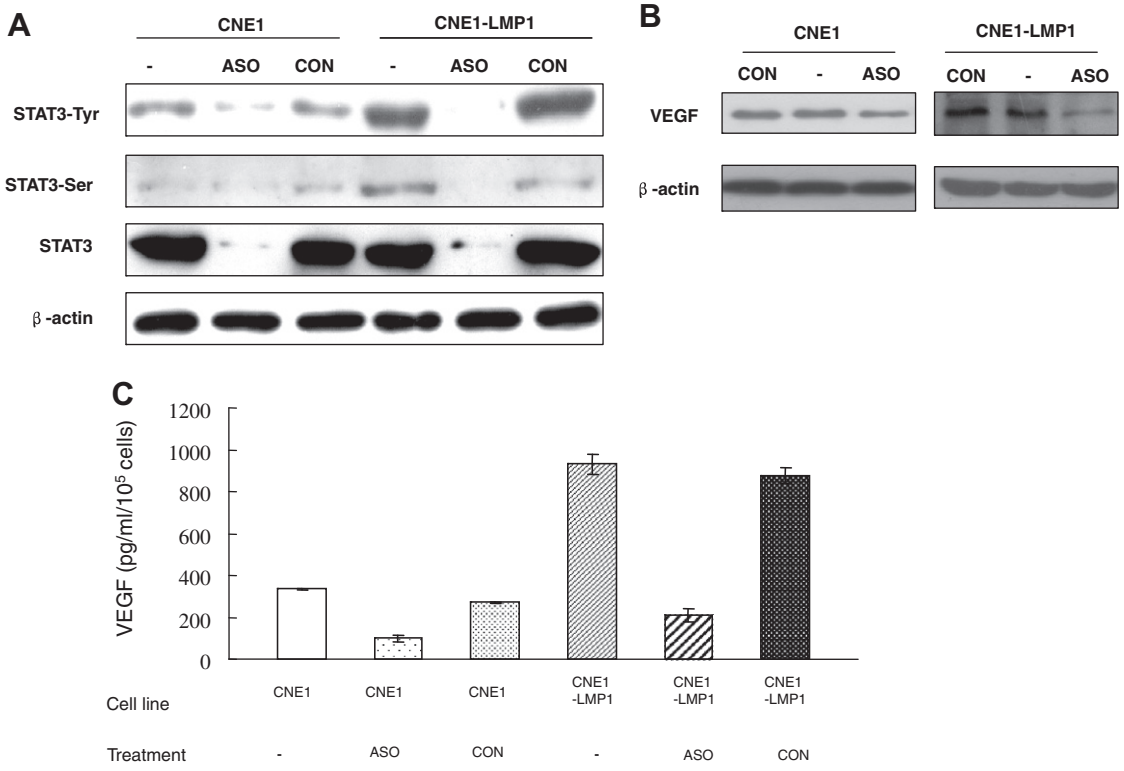

Fig. 5 – Antisense STAT3 oligonucleotides block LMP1-induced STAT3 phosphorylation and VEGF production. (A) Analysis of STAT3 phosphorylation and expression. NPC cells transfected with STAT3 antisense oligonucleotides (500 nM) or the control oligonucleotide(CON) for 24 h. STAT3 activation and STAT3 expression was determined by immunoblotting with antibodies specific for phosphorylated STAT3 Tyr^{705}, STAT3 Ser^{727} and STAT3. (B, C) Analysis of VEGF production. VEGF protein and soluble VEGF in the cell culture supernatant treated with antisense STAT3 oligonucleotides or the control oligonucleotide(CON) for 24 h, VEGF expression was determined by immunoblotting with antibodies specific for VEGF and the medium was collected for VEGF ELISA.

Studies have demonstrated that STATs contributes to malignant properties such as angiogenesis and immune-suppression.[51] However, the pattern of activation of STATs by LMP1 is unclear, perhaps because it is influenced by cell type.[20,52] Previous studies have confirmed the involvement of LMP1 in STAT3 activation in NPC.[19,20,52–54] Of the major findings reported here, the most important is the association of STAT3 activation with LMP1, the principal EBV oncogene, in one of the most invasive EBV-associated malignancies, NPC. Interestingly, the MAPK-ERK pathway is activated in CNE1-LMP1 cells and that the MEK1 inhibitor, PD98059, is able to inhibit this effect, which is consistent with our previous results.[7] Our initial investigations using LMP1 mutants demonstrated an essential role of CTAR2 and CTAR3 in JAK3/STAT3 activation.[23] The expression of LMP1 in NPC resulted in increased phosphorylation of STAT3 at Tyr^{705} and Ser^{727}, as well as an induction of STAT3-dependent luciferase activity. Phosphorylation of Tyr^{705} is required for cytokine-induced STAT3 dimerization, nuclear translocation and DNA binding, whereas full transcriptional activity of the homodimer is manifested only when Ser^{727} in the transactivation domain is also phosphorylated.[55]

As major upstream signalling molecule for STAT3, EGFR may contribute STAT3 activation in NPC. Recently, our data have demonstrated that interaction of EGFR and p-STAT3 increases cyclinD1 expression (data not shown). However, STAT3 localising upstream or/and downstream of the EGFR pathway in LMP1-positive NPC cell line still needs to be resolved in the future.

The expression of LMP1 and its role in regulating VEGF expression have been studied in NPC. Similarly, COX2 has been implicated in inducing VEGF production.[45] LMP1 has also been shown to be necessary for hypoxia-induced VEGF expression, further demonstrating the pivotal role of LMP1 in NPC angiogenesis.[48] Recently, STAT3 has been shown to participate in VEGF expression.[30,56] To assess whether STAT3 is necessary for LMP1-upregulated VEGF expression, we determined if blocking STAT3 signalling could inhibit VEGF expression induced by LMP1. Western blot and ELISA analyses of VEGF indicated that upregulation of expression of VEGF was inhibited by using LMP1-targeted DNAzyme and that WHI-P131 and PD98059, inhibitors of JAK3 and MEK1, respectively, inhibited LMP1-augmented VEGF expression in NPC cells. LMP1-positive NPC cells transfected with siRNA or ASO for STAT3 exhibited significantly decreased VEGF production compared with their parent cells. These results suggest that LMP1 elevated VEGF through the activation of the JAK/STAT and ERK1/2 signalling pathways.

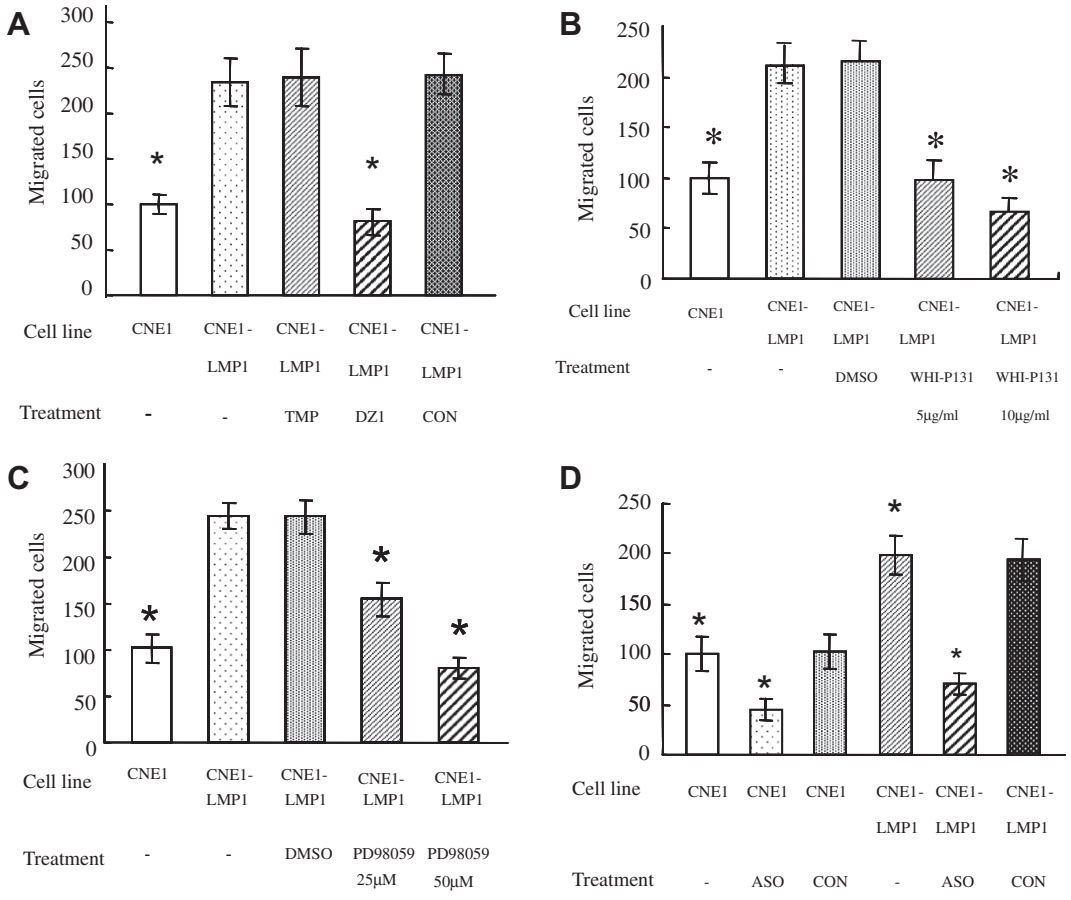

Fig. 6 – Effect of blocking JAK/STAT3 or MEK1/ERK signalling pathways on the migration ability of NPC cells. It was assayed migration ability of NPC cells with a Transwell apparatus. The number of cells that had migrated through the pores was quantified by counting five independent visual fields using a 20× microscope objective. Three independent assays were performed. Each point shows the means ± SD. Points represent the average number of cells that had migrated through the membrane to the lower surface; bars represent standard deviation. Differences were tested by paired t test. (A) After transfected with DZ1 for 24 h, NPC cells were seeded into the upper part of each chamber. Cellular migration ability was assayed. (B) NPC cells were seeded into the upper part of each chamber in the presence or absence of JAK kinase inhibitors WHI-P131. Cellular migration ability was assayed. (C) NPC cells were seeded into the upper part of each chamber in the presence or absence of MEK1 kinase inhibitors PD98059. Cellular migration ability was assayed. (D) After transfected with antisense STAT3 oligonucleotides treatment for 24 h, NPC cells were seeded into the upper part of each chamber. Cellular migration ability was assayed.

As a member of a family of latent cytoplasmic transcription factors, STAT3 has long been implicated in oncogenesis. More recently, evidence for the role of STAT3 in metastasis has become increasingly apparent. We found recently that STAT3 Tyr^{705} phosphorylation had more relevance with NPC clinical III and IV stage.[19] Constitutive activation of STAT3 in LMP1-overexpressing NPC and the imperative requirement of STAT3 in LMP1-induced VEGF production as well as cell migration have provided strong evidence to support the metastatic potential of STAT3. The present study provides insights into LMP1 correlating with migration activity in NPC cells. Data shows that LMP1 can enhance NPC cell migration and that LMP1-induced migration depends on the JAK/STAT and MAPK/ERK signalling pathways. Activation of the JAK/STAT and MAPK/ERK pathways induced by LMP1 is involved in the activation of STAT3, contributing to the attenuation of cell–cell adhesion, degradation of the extracellular-matrix and promoting the enhanced motility and migration of NPC cells. The findings also single out a potential target, STAT3, for molecular-directed therapy.

It is reported that LMP1 induces the expression of a series of cellular invasion and metastasis factors, such as matrix metalloproteinase 9 (MMP-9), which plays a critical role in the invasion of the basement membrane.[17,30,44,46,49] LMP1 expression also promotes cell migration and invasive growth via E-twenty-six-1 (Ets-1) expression which may contribute in part to the highly metastatic potential of NPC,[42] LMP1 induces interleukin-8 (IL-8) mainly through the activation of nuclear factor NF-κB and partly through AP-1 to take part in angiogenesis of NPC[57]; phosphorylation of ezrin and its recruitment to the cell membrane linked to F-actin and CD44 is a process required for LMP1-stimulated cell motility and invasion of NP

cells[58]; increasing Decoy receptor 3(DcR3) expression by LMP1 not only helps EBV-associated cancer cells gain survival advantage by preventing host immune detection but also increases the chance of cancer metastasis by enhancing cell migration and invasion[59]; LMP1 can directly induce Twist via nuclear factor-κB (NF-κB) in nasopharyngeal epithelial cells. Expression of Twist is correlated with LMP1 protein expression in human NPC tissues and NPC metastasis.[60] Our current study provides new pathways of LMP1 involving NPC cell invasion and clearly demonstrates that LMP1 activates STAT3 through JAK3/STAT3 and MEK1/ERK1/2 signalling pathways, which in turn results in induction of VEGF and finally enhances NPC cell migration.

Conflict of interest statement

None declared.

Acknowledgements

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