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DOI:
10.1002/path.5130

Activation of SREBP1-mediated lipogenesis by the Epstein-Barr Virus-encoded LMP1 promotes cell proliferation and progression of nasopharyngeal carcinoma

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Short Running Title: Activation of SREBP1-mediated lipogenesis by EBV-encoded LMP1

Conflict of Interest statement: The authors declare that there are no conflicts of interest.

Total Word No.: 3805 (Introduction to Discussion)

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/path.5130

Abstract

Nasopharyngeal Carcinoma (NPC) is closely associated with Epstein-Barr virus (EBV) infection. The EBV-encoded latent membrane protein 1 (LMP1), which is commonly expressed in NPC, engages multiple signaling pathways that promote cell growth, transformation and metabolic reprogramming. Here, we report a novel function of LMP1 in promoting *de novo* lipogenesis. LMP1 increases the expression, maturation and activation of sterol regulatory element-binding protein 1 (SREBP1), a master regulator of lipogenesis, and its downstream target: fatty acid synthase (FASN). LMP1 also induces *de novo* lipid synthesis and lipid droplet formation. In contrast, siRNA knockdown of LMP1 in EBV-infected epithelial cells diminished SREBP1 activation and lipid biosynthesis. Furthermore, inhibition of the mTOR pathway, either through the use of mTOR inhibitors or siRNAs significantly reduced LMP1-mediated SREBP1 activity and lipogenesis, indicating that LMP1 activation of the mTOR pathway is required for SREBP1-mediated lipogenesis. In primary NPC tumors, FASN overexpression is common, with high levels correlating significantly with LMP1 expression. Moreover, elevated FASN was associated with aggressive disease and poor survival in NPC patients. Luteolin and fatostatin, two inhibitors of lipogenesis, suppressed lipogenesis and proliferation of nasopharyngeal epithelial cells; effects which were more profound in cells expressing LMP1. Luteolin and fatostatin also dramatically inhibited NPC tumor growth *in vitro* and *in vivo*. Our findings demonstrate that LMP1 activation of SREBP1-mediated lipogenesis promotes tumor cell growth and is involved in EBV-driven NPC pathogenesis. Our results also reveal the therapeutic potential of utilizing lipogenesis inhibitors in the treatment of locally advanced or metastatic NPC.

Key words: Nasopharyngeal Carcinoma; Epstein-Barr virus; Lipogenesis; LMP1; SREBP1

Introduction

Non-keratinizing undifferentiated nasopharyngeal carcinoma (NPC) is a distinct type of cancer prevalent in southeast Asia and southern China. The unique feature of NPC is its strong association with Epstein-Barr virus (EBV) infection [1,2]. Among the EBV-encoded gene products expressed in NPC, latent membrane protein 1 (LMP1) is of particular interest as it displays oncogenic properties *in vitro* and *in vivo*. LMP1 is an integral membrane protein containing two signaling domains: CTAR1 and CTAR2. Through these two domains, LMP1 engages multiple signaling cascades that include the Ras-ERK-MAPK, PI3K-AKT, NF-κB, and p38-MAPK pathways, which modulate the expression of a variety of cellular targets that contribute to the transforming activities of LMP1 [1-3]. Previous studies have established a role for LMP1 in promoting cell proliferation, transformation, cell invasion and migration, aerobic glycolysis and metabolic reprogramming in nasopharyngeal epithelial cells [3-5]. These observations imply an essential role for LMP1 in the pathogenesis of NPC.

Deregulated lipid metabolism is an established hallmark of cancer. Cells obtain fatty acids either from the diet or through *de novo* lipid synthesis (lipogenesis). Normal cells rely primarily on dietary fatty acid for the synthesis of new structural lipids and lipogenesis is not universal. However, cancer cells extensively engage *de novo* lipogenesis to produce long-chain fatty acids that are essential for the synthesis of glycerophospholipid membrane and membrane signal molecules during rapid cell proliferation (supplementary material, Figure S5) Fatty acids are also necessary for energy storage as lipid droplets [6-8]. Lipogenesis is tightly regulated by sterol regulatory element-binding protein 1 (SREBP1), a transcription factor that regulates transcription of most genes involved in lipogenesis [9-11]. There are two SREBP1 isoforms (SREBP1a and SREBP1c) encoded from *SREBF1*. SREBF1a and SREBF1c are produced from different promoters, differing only in the length of their N-terminal transactivation domains. SREBPs are synthesized as precursor proteins bound to the ER membrane. After stimulation, the SREBP precursor undergoes proteolytic cleavage in the Golgi to release the transcriptionally active-N-terminal domain. Once mature, active SREBP1 translocates to the nucleus, where it binds to sterol regulatory element (SRE) within its promoter and those of its target genes [9-11]. mTOR complex 1 (mTORC1) and 2 (mTORC2) have been shown to regulate SREBP1 activity and lipogenesis. Activation of mTOR signaling by the Ras-ERK, and PI3K-AKT pathways increases expression of SREBPs and SREBP-mediated lipogenesis [12-14]. Numerous lipogenic genes, including fatty acid

synthase (FASN), are upregulated in a variety of cancers [7;8;10]. In this study, we demonstrate that FASN expression is common in primary NPC tumors, with higher levels correlating with LMP1 expression. Furthermore, LMP1 activates *de novo* lipogenesis and LMP1 activation of SREBP1-mediated lipogenesis contributes to cancer cell growth and tumor progression. These implicate the involvement of LMP1-mediated lipogenesis in the pathogenesis of EBV-infected NPC.

Materials and Methods

Cell lines, Chemicals, and Pharmacological inhibitors

C666-1 and HK-1 NPC cell lines were maintained in RPMI 1640 supplemented with 10% FBS. The SV40 large T-immortalized nasopharyngeal epithelial cell line NP69 was maintained in keratinocyte serum-free medium (Thermo Fisher Scientific, Waltham, MA USA). Torin 1, Torin 2, luteolin and fatostatin were obtained from Abcam, Cambridge, UK. Further details are presented in supplementary material, Supplementary materials and methods.

DNA Constructs and siRNA

Scrambled shRNA and LMP1 shRNA vectors were generated by inserting a fragment of synthesized oligo with a scrambled sequence or a sequence to LMP1 into pSUPER.retro.puro vector (Oligoengine). The pGL2-3xSRE luciferase vector was obtained from ATCC (Manassas, VA, USA). pGL3-FASN was kindly provided by Dr. Qiang Liu, University of Saskatchewan, Canada [15]. All siRNAs were purchased from Dharmacon Inc., Lafayette, CO, USA. Transient transfection of siRNA or DNA was performed using Lipofectamine 2000 (Thermo Fisher Scientific) or Fugene HD (Promega, Madison, WI, USA) respectively. See supplementary material, Supplementary materials and methods for addition detail.

Western blotting analysis

Total cell lysates (5–50 μg of protein) were separated by 10% or 4–12% SDS-PAGE and transferred to PVDF membranes prior to immunoblotting. Antibodies to LMP1 were purchased from Dako and α-tubulin from Santa Cruz. The anti-SREBP1 [2A4] was purchased from Abcam and Santa Cruz. All other antibodies were purchased from Cell Signaling Technology (Beverley, MA, USA). Additional details are provided in supplementary material, Supplementary materials and methods.

RT-qPCR

All RT-qPCR products were amplified using the Power SYBR green PCR Master Mix Kit (Thermo Fisher Scientific). Details including primer sequences are provided in supplementary material, Supplementary materials and methods.

Luciferase Reporter Assay

Ten thousand HeLa or 293 cells grown in 96-well plate were co-transfected with 20 ng of the luciferase reporter construct together with increasing amounts of an LMP1 expression vector (pCDNA3-LMP1) as indicated in the Figure. Renilla pRL-SV40 vector was transfected as an internal control to correct for transfection efficiency. Two days post-transfection, cells were lysed in reporter lysis buffer and then assayed for luciferase and Renilla activities with the Dual-Luciferase Reporter Assay System (Promega).

Immunofluorescence Staining

Immunofluorescence staining was performed as previously described [16] and as further outlined in in supplementary material, Supplementary materials and methods.

Lipid Droplet Fluorescence Staining

Nile Red fluorescence staining was assessed using the Lipid Droplets Fluorescence Assay Kit according to the manufacturer's protocol (Cayman Chemical, Ann Arbor, MI, USA). Details are provided in supplementary material, Supplementary materials and methods.

Immunohistochemical Staining

Immunohistochemical staining was performed as described previously [17]. Information regarding normal and tumor specimens, staining and intensity score methods are outlined in supplementary material, Supplementary materials and methods.

Cell Proliferation Assay

Cell proliferation assays were performed with cell proliferation reagent CCK-8 (Dojindo Molecular Technologies, Rockville, MD, USA and as described in supplementary material, Supplementary materials and methods.

De Novo *Lipogenesis Assay*

For the lipogenesis assay, cells were incubated in serum-free medium containing 2.5 μCi/ml [1-^{14}C] acetate for 8–12 h. After washing with PBS, cells were lysed in 0.5% Triton X-100/PBS. Lipids were extracted by successive addition of methanol and chloroform, following by centrifugation. The organic phase was air-dried and re-suspended in chloroform for scintillation counting. Results were normalized to total protein content. Details are provided in supplementary material, Supplementary materials and methods.

In Vivo *Tumorigenicity Experiments*

The University Animal Experimentation Ethics Committee, CUHK approved the study protocol. In brief, C666-1 cells (1×10^7) were inoculated subcutaneously into BALB/c nude mice. When the size of tumors reached about 50 mm^3, animals were injected intravenously with PBS (vehicle), luteolin, or fatostatin every 2–3 d for 3 wk. At the end point, animals were killed and tumors were harvested. For further detail see supplementary material, Supplementary materials and methods..

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 GraphPad Software Inc, La Jolla, CA, USA). *P*-value was calculated using either Fisher's exact test or unpaired two-tailed Student's t-test. The IC_{50} values of inhibitors were calculated by applying the four-parameter logistic equation to generate the Sigmoidal dose-response (variable slope) curves. Survival curves were analyzed using the Kaplan-Meier method, and were compared with a log-rank test. *P*-value <0.05 was considered to be statistically significant.

Results

LMP1 increases SREBP1 expression and activity

To examine the impact of LMP1 on SREBP1-mediated lipogenesis, increasing amounts of an LMP1 expression vector was transfected into the nasopharyngeal epithelial cell line, NP69. Under serum-deprived conditions, a dose-dependent induction of SREBF1a and SREBF1c mRNA was observed (Figure 1A). Similarly, LMP1 increased the levels of both precursor and mature forms of SREBP1 protein (Figure 1C). SREBP1 transactivates target genes by binding to SRE elements within the promoter region. Using a pGL2-3xSRE reporter construct containing 3 tandem copies of an SRE/SP1 element [18], we found that LMP1 increased SREBP1 transcriptional activity (Figure 1B**).** FASN is a transcriptional target of

SREBP1. FASN promoter activity was also strongly enhanced in response to LMP1 expression (Figure 1B). The expression of FASN mRNA and protein were also induced by LMP1 (Figure 1A & 1C). These findings suggest that FASN induction by LMP1 is mediated primarily through modulation of the expression and activity of SREBP1. Both the mTORC1 and mTORC2 pathways are involved in regulating SREBP1 activity [12-14]. LMP1 has been reported to activate the mTOR signaling pathway [18-20]. Here, we further confirmed that LMP1 induced phosphorylation of 4E-BP1 and p70S6K, two established downstream targets of mTOR signaling (Figure 1C).

In nasopharyngeal epithelial cells stably expressing LMP1 we also observed increased activities of the SREBP1 and mTOR pathways (Figure 1D). Immunofluorescence staining revealed overexpression of FASN in LMP1-expressing nasopharyngeal epithelial cells (Figure 1E). To determine whether LMP1 promotes *de novo* lipogenesis, LMP1-expressing cells were labelled with ^{14}C-acetate for 8–12 h under serum-deprived conditions. The ^{14}C labelled lipid fraction was then extracted for quantification. As shown in Figure 1F, LMP1-expressing cells produced more newly synthesized lipid than control cells. Furthermore, Nile Red staining revealed more intracellular lipid droplets in LMP1-expressing cells (Figure 1G, H). Overall, these data indicate a role for LMP1 in promoting lipid biosynthesis.

LMP1 Promotes SREBP1-Mediated Lipogenesis In EBV-Infected NPC Cells

In NPC xenografts (C17, X2117, and C15), western blotting analysis revealed higher levels of the precursor and mature forms of SREBP1 and FASN in LMP1-positive X2117 and C15 compared to the LMP1 negative C17 xenograft and EBV negative non-malignant NP69 nasopharyngeal epithelial cell line (Figure 2A). In NPC cell lines, the levels of mature SREBP1 and FASN proteins were higher in EBV-infected HK1-EBV and C666-1 cells compared to HK-1 cells (Figure 2B). Similarly, levels of the phosphorylated forms of 4E-BP1 and p70S6K were elevated in HK1-EBV and C666-1 cells (Figure 2B). A role for LMP1 in these effects was established, as siRNA silencing of LMP1 in EBV-infected cells resulted in reducing the expression of mature SREBP1 and FASN as well as the signaling activity of mTOR (Figure 2C). Furthermore, LMP1 silencing in C666-1 cells resulted in a decrease in FASN promoter activity (supplementary material, Figure S1) as well as lipogenesis, particularly under serum-deprived condition (Figure 2D). These data suggest that SREPB1-mediated lipogenesis in EBV-infected cells is induced by LMP1 through the mTOR signaling pathway.

LMP1 induces SREBP1-mediated lipogenesis through the mTOR signaling pathway

To investigate whether LMP1-induced lipogenesis was dependent on mTOR signaling, NP69-pLNSX and NP69-LMP1 cells were treated with the mTOR inhibitors, Torin 1 and Torin 2. Torin 1 is documented to inhibit both mTORC1 and mTORC2 complexes, while Torin 2 inhibits the mTORC1 complex [21,22]. ^{14}C-acetate incorporation assays revealed that these two mTOR inhibitors could suppress lipogenesis in both cell lines, although the effects were more profound in LMP1-expressing cells. As shown in Figure 3A, NP69-LMP1 cells exhibited 53-72% reduction in lipogenesis, while only a 15-39% reduction was observed in NP69-pLNSX cells. To further determine whether LMP1 induction of mTOR signaling promoted SREBP1-mediated lipogenesis, NP69 cells were transiently transfected with an LMP1 expression vector together with siRNAs targeting Raptor to inhibit mTORC1 signaling, Rictor to inhibit mTORC2 signaling, or mTOR to inhibit both mTORC1 and mTORC2 signaling pathways. As shown in Figure 3B, inhibition of either mTORC1, mTORC2 or both, reduced LMP1-induced FASN expression and SREBP1 maturation. Also, NP69-LMP1 cells exhibited 20-35% reduction in lipogenesis, while a 14–27% reduction was observed in control NP69-pLNSX cells (Figure 3C). These finding indicated that mTOR signaling contributes to LMP1-induced lipogenesis.

Expression of FASN correlates with LMP1 expression in NPCs and poor prognosis in NPC patients

To examine the expression of FASN in NPC, we performed immunohistochemical staining for FASN and LMP1 in 38 NPC primary tumors. The intensity of FASN staining was scored, and a graph of the statistical dot-plot of FASN staining intensity against LMP1 expression was generated (Figure 4B). Immunohistochemical staining revealed a negative or low expression of FASN (immunoreactivity score <3) in normal nasopharyngeal epithelium (Figure 4A, N1) and 16 of 38 (42%) NPC tumors in which LMP1 expression was barely detected (Figure 4A, representative NPC: T4). In contrast, moderate or high levels of FASN (immunoreactivity score ≥3) were observed in 22 of 38 NPC tumors (58%) (Figure 5A, representative NPC: T13, T22 & T34). In particular, LMP1-positive tumors displayed significantly higher levels of FASN (p=0.0003) (Figure 4B). In analysis of FASN expression with clinicopathological variables including gender, age, tumor size, lymph node and cancer stage, in NPC patients, we found that higher levels of FASN expression (score ≥3) significantly correlated to advanced primary tumor T3-T4 (p=0.012) and distant lymph node

metastasis N1-N3 (p=0.005) of NPC (supplementary material, Table S1). Also, Kaplan-Meier survival analysis revealed that elevated FASN expression significantly correlated to poor survival (p = 0.02) (Figure 4C). Overall, these findings indicate that FASN overexpression is common in NPC and correlated with LMP1 expression. Moreover, the elevated FASN is associated with aggressive disease and poor prognosis in NPC patients. These also imply a role of LMP1 in up-regulating FASN for NPC progression.

LMP1 induction of lipid synthesis contributes to cell growth

Next, we examine whether LMP1 induction of lipogenesis contributed to cell growth. NP69 and HK1 cells expressing pLNSX control or LMP1 were treated with luteolin, or fatostatin. Luteolin is an inhibitor which suppresses SREBP-mediated lipogenesis. Fatostatin is an SREBP specific inhibitor [23-25]. ^{14}C-acetate incorporation assays revealed that these inhibitors could dampen lipogenesis in all cell lines; however, the repression was more evident in LMP1 expressing cells (Figures 5A,B). Fatostatin and luteolin treatment reduced lipogenesis of NP69-pLNSX cells by 35-70%, while, a 54-87% reduction was observed in NP69-LMP1 cells (Figure 5A). Similarly, fatostatin and luteolin reduced lipogenesis in HK1-pLNSX cells by 28-46% and in HK1-LMP1 cells by 50-69% (Figure 5B). Western blot analysis confirmed that both luteolin and fatostatin effectively inhibited SREBP1 maturation and FASN expression in NP69-LMP1 and HK1-LMP1 cells (supplementary material, Figure S2).

To determine whether lipogenesis conferred a growth advantage to LMP1-expressing cells, pLNSX control and LMP1 expressing NP69 and HK-1 cells were grown in culture medium supplemented with 1% serum together with increasing doses of luteolin or fatostatin for 1–4 days, and the effects on cell growth examined. As shown in Figures 5D-5G, inhibition of fatty acid synthesis by luteolin or fatostatin reduced the proliferation of both control and LMP1 expressing cells, although NP69-LMP1 and HK1-LMP1 cells appeared more susceptible to the actions of both drugs. The IC_{50} values of luteolin at day 3 in NP69-pLNSX, NP69-LMP1, HK1-pLNSX and HK1-LMP1 cells were 2.6 μM, 1.14 μM, 12.4 μM and 5.6 μM, respectively. Similarly, the IC_{50} values of fatostatin at day 3 in NP69-pLNSX, NP69-LMP1, HK1-pLNSX and HK1-LMP1 cells were 6.5 μM, 2.9 μM, 5.6 μM and 3.3 μM, respectively. Also, these two inhibitors suppressed lipogenesis in C666-1 NPC cells by 50% (Figure 5C) and inhibited their proliferation; the IC_{50} value of luteolin and fatostatin at day 4

were 9.6 μM and 25.5 μM respectively (Figures 5H,I). These findings indicate that suppression of lipogenesis inhibits LMP1-induced proliferation in both malignant and non-malignant nasopharyngeal epithelial cells, implicating the induction of lipogenesis by LMP1 as an essential mechanism in facilitating cell proliferation.

Inhibitors of lipogenesis suppress NPC growth and induce apoptosis in vivo

Next, we examined whether blocking lipogenesis inhibited the growth of NPC tumor *in vivo*. C666-1 cells were subcutaneously injected into the flanks of nude mice. Once tumors reached $50mm^3$ in size, the mice were injected intraperitoneally with PBS, luteolin (20mg/kg), or fatostatin (15mg/kg) every 2-3 days for 19 days. Body weight and tumor size were measured every several days. At the endpoint, animals were killed and tumors were harvested. Both luteolin and fatostatin significantly inhibited tumor growth in mice ($p<0.0001$) (Figure 6A). In animals treated with luteolin or fatostatin, substantially lower tumor weights and smaller tumor size were observed ($p<0.0001$) (Figure 6B,C). Surprisingly, no noticeable weight loss was observed in treated animals with either drug (supplementary material, Figure S3). Immunohistochemical staining analysis of NPC tumors from these two groups of mice indicated that both luteolin and fatostatin effectively inhibit FASN expression (Figure 6D & supplementary material, Figure S4). In addition, histological investigations of tumor tissues revealed more pronounced necrosis in both luteolin (35%) and fatostatin (43%) treated groups compared to the control (5%) group (Figure 6D). An examination of cell proliferation by IHC staining for Ki67; and apoptosis by staining for cleaved capase-3 (CC3), revealed that both drugs significantly inhibited cell proliferation and increased apoptosis. As shown in Figure 6D, the amount of Ki67 positive cells was markedly lower in the tumor tissues of luteolin (56%) and fatostatin (45%) treated groups compared to the control group (70%). Also, the percentage of cleaved Caspase-3 (CC3) positive cells were significantly high in luteolin (36%) and fatostatin (31%) treated groups when compared to (12%) control group. Western blotting analysis further confirmed induction of CC3 and cleaved PARP in luteolin and fatostatin treated tumors (supplementary material, Figure S4). These findings indicate that inhibition of lipogenesis by luteolin and fatostatin causes cell apoptosis and necrosis, leading to tumor growth suppression.

Discussion

The oncogenic LMP1 protein is frequently found to be expressed in NPC tumors. While, LMP1 promotes cell growth, transformation, invasion and migration, it also plays a role in

modulating host cell metabolic pathways [1,3,26]. In previous studies, we have demonstrated an ability of LMP1 to promote aerobic glycolysis through constitutive activation of FGF2/FGFR1 signaling pathway and upregulation of c-Myc and HIF-1α, two primary regulators of aerobic glycolysis [5]. LMP1 also inactivates LKB1-AMPK to promote proliferation and anchorage-independent growth [4]. Here, we report a novel function of LMP1 in promoting SREBP1-mediated *de novo* lipogenesis, an effect that facilitates cell growth and tumor development (supplementary material, Figure S5).

In this study, we show that LMP1 increases SREBP1 expression and activity in nasopharyngeal epithelial cells. Luciferase promoter reporter assays and RT-qPCR analysis demonstrate that LMP1 upregulates SREBP1 at the transcriptional level (Figures 1A & 1B), while western blotting analysis revealed that LMP1 promotes SREBP1 maturation and the expression of its downstream target, FASN (Figures 1C,D). Using ^{14}C-acetate incorporation assay, we demonstrate an ability of LMP1 to promote lipogenesis (Figure 1F). Interestingly, silencing LMP1 in EBV-infected NPC cells resulted in a reduction in FASN expression and lipogenesis (Figures 2C,D). Furthermore, suppressing lipogenesis by inhibitors significantly reduced LMP1-induced proliferation (Figure 5). Collectively, these findings demonstrate that LMP1 induction of lipogenesis in NPC contributes to cell proliferation.

SREBPs tightly regulate lipogenesis. However, the mechanism underlying the expression and activity of SREBPs is still unclear. Hypoxia, glucose and insulin have been found to increase the expression of SREBPs [10]. Interestingly, we and others have reported a function of LMP1 in promoting HIF-1 expression and glucose uptake [5;26]. mTORC1 and mTORC2 have been shown to regulate SREBP activity and lipogenesis through multiple inputs [27-30]. Here, in line with other studies [18-20], we demonstrate that LMP1 increases the phosphorylation of 4E-BP1 and p70S6K, two established targets of mTOR, in nasopharyngeal epithelial cells (Figures 1-3). Using siRNAs targeting Raptor or Rictor, we show that both mTORC1 and mTORC2 signaling pathways are involved in LMP1-mediated lipogenesis (Figure 3). The mTOR signaling pathway is complicated as it contains both positive and negative feedback loops, and its downstream signaling interconnects with other cell growth and survival pathways [28-31]. mTORC2 is activated by ribosomes and PI3K signaling and is negatively regulated by a mTORC1-induced p70S6 kinase, which dampens PI3K signaling by inhibiting IRS-1 [28-31]. mTORC1 activity is induced by PI3K-AKT,

Ras-ERK-MAPK, IKKα-NFαB and IKKβ-NFαB pathways, all of which inhibit Tuberous Sclerosis Complex (TSC), a critical negative regulator of mTORC1. mTORC2-induced AKT signaling also activates mTORC1. In contrast, LKB1-AMPK inhibits mTORC1 through activating TSC2 and inactivating Raptor [28-31]. Interestingly, AMPK is an inhibitory target of LMP1 [4]. PI3K-AKT, Ras-ERK-MAPK, and NFκB are three major pathways engaged by LMP1 [2;3]. As such, LMP1 induction of mTOR-SREBP-induced lipogenesis appears to be mediated through multiple downstream targets and/or signaling pathways.

Overexpression of FASN has been documented in oral hairy leukoplakia (OHL), an EBV-associated benign lesion associated with robust EBV lytic replication [32]. FASN is induced by the EBV-encoded lytic protein, BRLF-1 through the p38-MAPK pathway and required for lytic viral gene expression [32]. In a similar vein, HBV and HCV lytic infection in liver cancer cells is associated with the induction of fatty acid synthesis for the formation of viral envelopes [33-35]. Another γ-herpesvirus, KSHV, has been shown to induce FASN expression and fatty acid synthesis for the survival of latently infected PEL cells [36]. Here, we found higher levels of SREBP1 activation and FASN expression in EBV latently infected NPC cells and xenografts, where LMP1 was expressed (Figure 2). While normal nasopharyngeal epithelium showed negative expression of FASN and LMP1 (Figure 4A), high level of FASN expression in NPC primary tumors was common and correlated significantly with LMP1 expression (Figure 4A,B). In line with previous studies, elevated FASN expression was significantly associated with T3-T4 staged primary tumors, N1-N3 lymph node metastasis and poor overall survival of NPC patients (Figure 4C & supplementary material, Table S1) [37]. These findings suggest that LMP1 induction of FASN and lipogenesis is involved in NPC progression. Of the LMP1-negative NPC tumors examined, 9/25 (36%) displayed a moderate or high level of FASN (Figure 4B). Our previous genetic analyses indicated that somatic mutations and/or aberrant expression of signaling proteins (LTBR, PIK3CA, p50, RelB, Bcl3, EGFR and RAS), or signaling regulators (INPP4B, TRAF3, TRAF2, A20, NFKBIA, TNFAIP3, and CYLD) are common in NPC, resulting in constitutive activation of the ERK1/2, NFκB and PI3K/AKT pathways [38-40]. Therefore, the genetic background of NPC tumors, in addition to LMP1 expression, is likely to contribute to FASN upregulation.

Given that FASN is commonly increased in virus-associated cancers, lipogenesis appears to be essential for viral infection and cancer progression [35]. As lipogenesis is not common in normal cells, targeting lipogenesis (or lipogenic pathways) might selectively inhibit the growth of virus-infected cells as well as highly proliferative cancer cells in the early stage of cancer development. In this study, we examined the effects of fatostatin and luteolin on cell growth and tumor development in relation to NPC. Fatostatin is a small molecule, which specifically blocks proteolytic activation of SREBPs [24]. Luteolin is a plant flavonoid, which possesses inhibitory effects on lipogenesis [23]. Both fatostatin and luteolin significantly inhibit SREBP1 activity, lipid synthesis and cell proliferation induced by LMP1 (Figure 5 & Supplementary Figure S2). Interestingly, luteolin has been reported to inhibit PI3K-AKT, MAPK-ERK and mTOR signaling activities [41], the major pathways required for SREBP-mediated lipogenesis by LMP1. Moreover, fatostatin and luteolin significantly inhibit the proliferation and tumorigenic growth of C666-1 cells. In nude mice, fatostatin and luteolin at a doses which effectively downregulates FASN expression, brought about a marked reduction in tumor growth (Figures 6 & supplementary material, Figure S4). Surprisingly, no significant weight loss was observed in animals treated with luteolin or fatostatin (supplementary material, Figure S3). The promising effects of these inhibitors in suppressing NPC tumor growth with low-level toxicity suggest a possible clinical utility in the treatment of NPC. Luteolin is a dietary flavonoid found in vegetables, fruits and herbs. In addition to its inhibitory effect on lipogenesis, luteolin also suppresses inflammation, angiogenesis, cell proliferation, and metastasis, all of which associated with cancer development [41]. Interestingly, luteolin has been shown to induce G1 arrest and inhibit EBV reactivation in NPC cells [42,43]. Given that luteolin is a natural polyphenolic compound and its toxicity is barely highlighted, it may prove to be a promising compound for the inhibition of EBV infection and the treatment of EBV-associated cancers. The potential of luteolin for the treatment of EBV-associated malignancies is worth to be further evaluated, particularly in clinical trials.

Acknowledgements

This work was supported by Research Grants Council of Hong Kong - GRF (14117316) to AKF Lo. KW Lo was supported by Core Utilities of Cancer Genome and Pathobiology, Research Grant Council, Hong Kong (Theme-based Research Scheme - T12-401/13-R; Collaborative Research Fund - C7027-16G; General Research Fund - 471413, 470312, 1404415, 14138016), Focused Innovations Scheme and Faculty Strategic Research

(4620513) of Faculty of Medicine, and VC's One-off Discretionary Fund (VCF2014017, VCF2014015), the Chinese University of Hong Kong.

Statement of author contributions

AKFL, RWML, CWD, LSY and KWL designed research, AKFL, RWML, CWD, CWK, WWY, WK, KFT, KWL performed research, AKFL, RWML, CWD, LSY and KWL analyzed data and drafted the manuscript. All authors discussed the findings, reviewed the data and commented on the manuscript.

Figure Legends

Figure 1. Induction of SREBP1 expression and activity by LMP1. (A) NP69 cells transfected with increasing amounts of an LMP1 expression vector as indicated were subjected to RT-qPCR analysis for *SREBF1a, SREBF1c, LMP1* and *FASN*. The mRNA expression of the target gene of interest was normalized to expression of the *TBP* gene. Relative mRNA levels were calculated using the sample without the LMP1 expression vector (set at 1). (B) HEK-293 cells were transfected with various amounts of an LMP1 expression vector together with luciferase promoter vector: pGL2-3xSRE or pGL3-FASN. After serum-deprivation for 12 h, cells were harvested for reporter activity analysis. The firefly luciferase activity was normalized to Renilla luciferase activity and plotted relative to the sample without the LMP1 expression vector (set at 1). (C) NP69 cells transfected with increasing amounts of an LMP1 expression vector as indicated or (D) NP69 and HK1 cells stably expressing LMP1 were incubated in serum-free medium for 12 h, prior to immunoblotting analysis; (E) immunofluorescence staining of FASN; (F) ^{14}C-acetate incorporation assay for measurement of *de novo* lipid synthesis; and (G) Nile Red fluorescence staining for lipid droplets and counterstained with Hoechst 33342 to stain cell nuclei. As a positive control for lipid droplet staining (Right panel), pLNSX control cells were incubated in medium supplemented with oleic acid for 12 h prior to staining. Nile Red fluorescence (ex/em 385/535 nm) and Hoechst 33342 fluorescence (ex/em 355/460 nm). (H) The relative amount of lipid droplet formation was calculated by normalising the Hoechst 33342 fluorescence to the Nile Red signal in each well and compared to pLNSX vector control sample (set at 1). Data represent the means of 8 determinations. Mean and SD. ***, p <0.001.

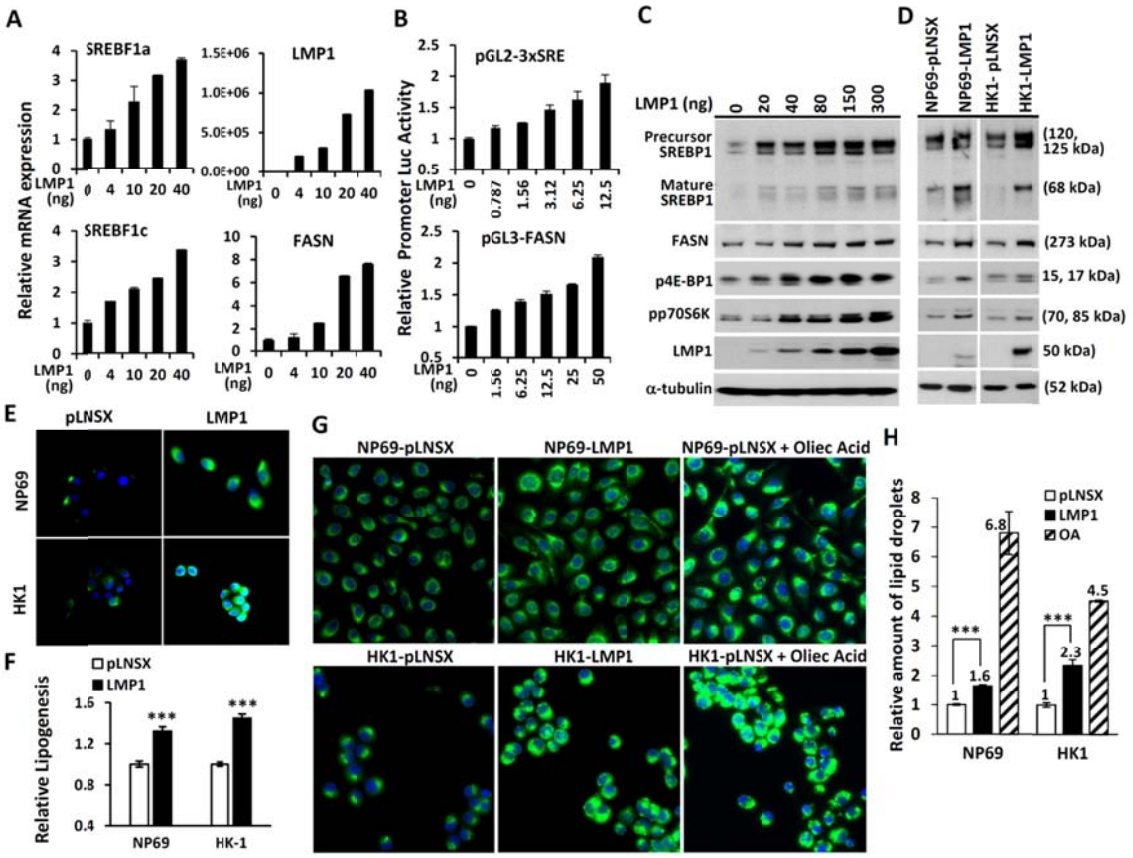


Figure 2. LMP1 Induction of SREBP1-mediated lipogenesis in EBV-infected NPC cells. (A) NPC xenografts (C17, 2117, and C15) together with the immortalized nasopharyngeal epithelial cell line, NP69, which was incubated in serum-free medium for 16 h prior to harvesting were subjected to western blotting analysis for the indicated proteins. (B) The EBV-infected NPC cell lines, HK1-EBV and C666-1 together with EBV-negative HK-1 cells were incubated in serum-free medium for 12 h prior to western blotting analysis. (C) HK1-EBV and C666-1 cells transfected with negative siRNAs control (si-Control) or siRNAs targeting LMP1 (si-LMP1) were subjected to western blotting analysis. (D) C666-1 cells expressing pSuper.retro-shRNA scrambled control (ShC) or shRNA LMP1 (ShLMP1) were incubated in serum rich (SR) or serum free (SF) medium containing 2.5 μCi/ml [1-^{14}C] acetate for 10 h and then subjected to measurement of lipid synthesis. NS*: Non-specific band. Mean and SD. ***, p <0.001.

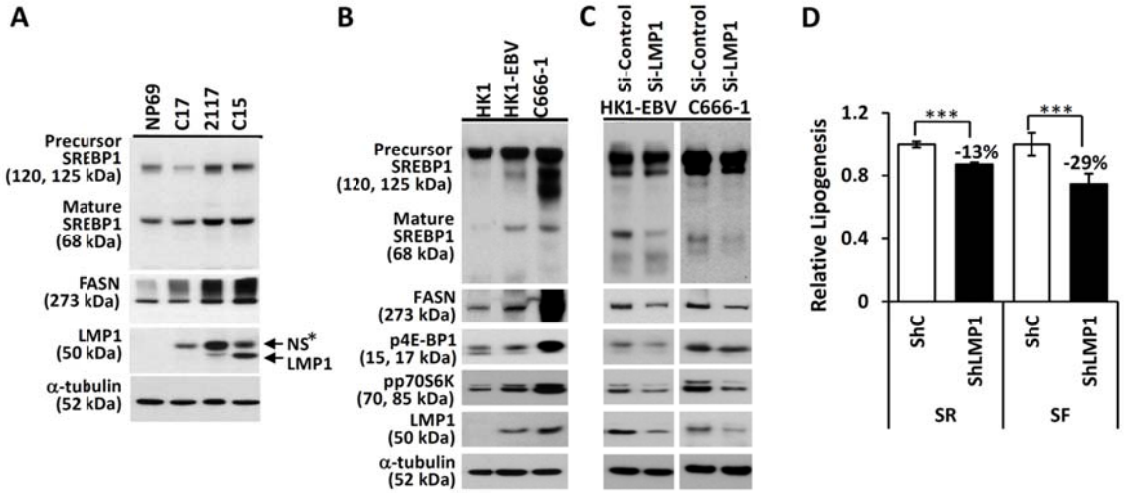


Figure 3. LMP1 activation of the mTOR signalling pathway is required for SREBP1-mediated lipogenesis. (A) NP69-pLNSX and NP69-LMP1 cells treated with vehicle alone (Blank), Torin 1 (0.3 μM) or Torin 2 (0.1 μM); and (B) NP69 cells transfected with either control pCDNA3 vector or pCDNA3-LMP1 expression vector, together with a control scrambled siRNA (siCtl), or siRNA specific for Raptor (siRaptor), Rictor (siRictor), or mTOR (simTOR) were subjected to western blotting analysis for the indicated proteins. (C) NP69-pLNSX and NP69-LMP1 cells transfected with indicated siRNAs for 24 h were subjected to lipid synthesis measurements. The asterisks indicate a significant difference (*p <0.05, **p <0.01, ***p <0.001).

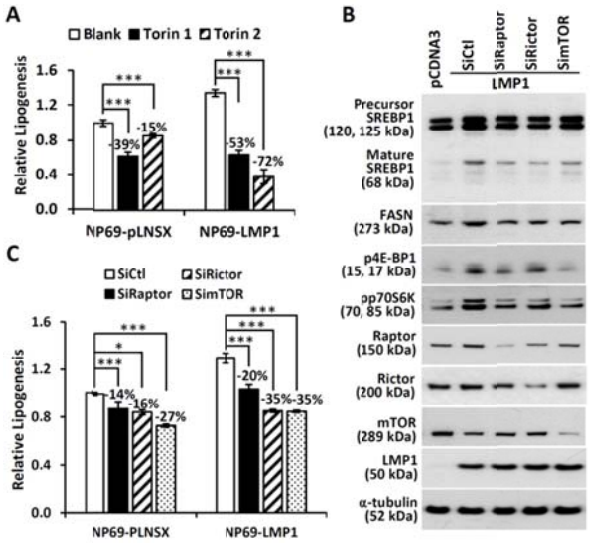


Figure 4. Elevation of FASN correlates with LMP1 expression in NPC tumours and is associated with poor prognosis in NPC patients. (A) Immunohistochemical staining of FASN and LMP1 in primary NPC and normal nasopharyngeal epithelium (NE) specimens. An absence of FASN and LMP1 expression was observed in NE (N1). In NPC tumor T4, which is LMP1-negative, low levels of FASN (score: 1) were observed. In NPC tumors T13, T22 & T34, which are LMP1 positive, moderate (immunoreactivity score: 4) and high (score: 6 and 8) levels of FASN were observed. Yellow arrow: normal nasopharyngeal epithelium; red arrow: NPC tumor cells. (B) Dot plot showing the immunoreactivity score of FASN staining within the group of tumors with and without LMP1 expression (N=38). All LMP1-positive NPC tumors (13/13) exhibited strong FASN expression (score ≥3). The median value of each group is shown in the horizontal line. The *p*-value between two groups is shown. (C) Kaplan-Meier survival curves for NPC patients with available follow-up information. High FASN expression (score ≥3, n=16) and low FASN expression (score <3, n=7).

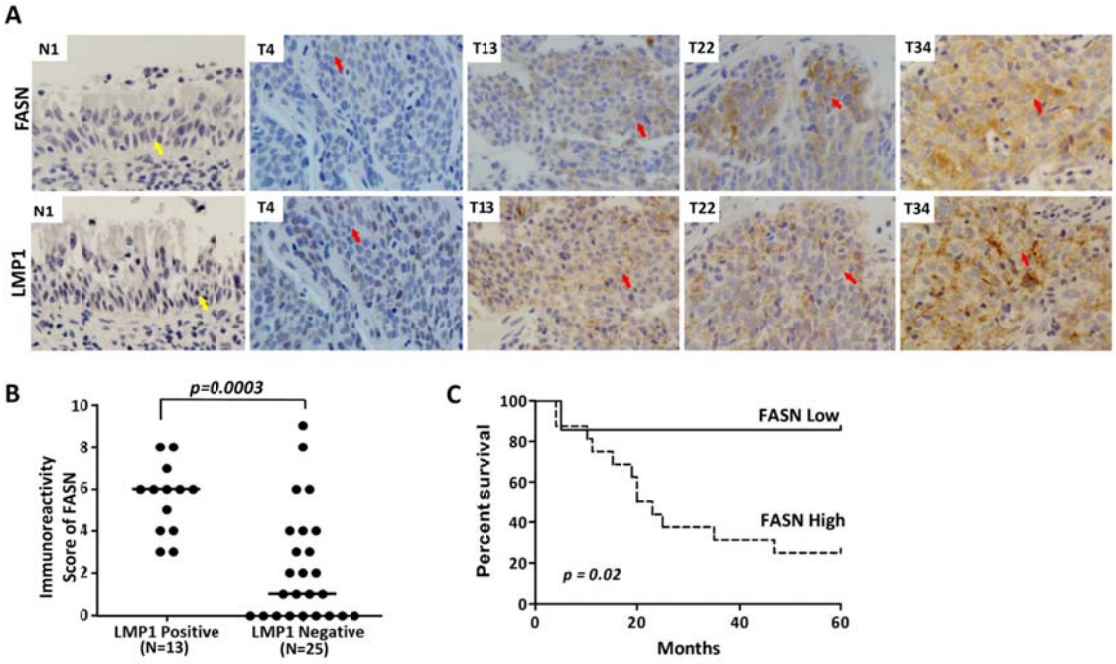


Figure 5. LMP1 induction of lipogenesis contributes to cell proliferation. (A) NP69-pLNSX and NP69-LMP1 cells, (B) HK1-pLNSX and HK1-LMP1 cells and (C) the EBV-positive NPC cells, C666-1 cultured with 1 % serum were treated with DMSO (vehicle), luteolin (20 μM), or fatostatin (15 μM) for 16 h prior to lipogenesis measurements. Relative lipogenesis was calculated using vehicle-treated cells as reference control (set at 1). (D,E) NP69-pLNSX and NP69-LMP1 cells, (F,G) HK1-pLNSX and HK1-LMP1 cells and (H,I) C666-1 cells were treated with increasing dose of inhibitors, as indicated, for 3 d prior to cell growth analysis. Relative cell growth was calculated using drug-free treated cells as reference control (set at 1). ***$p < 0.001$.

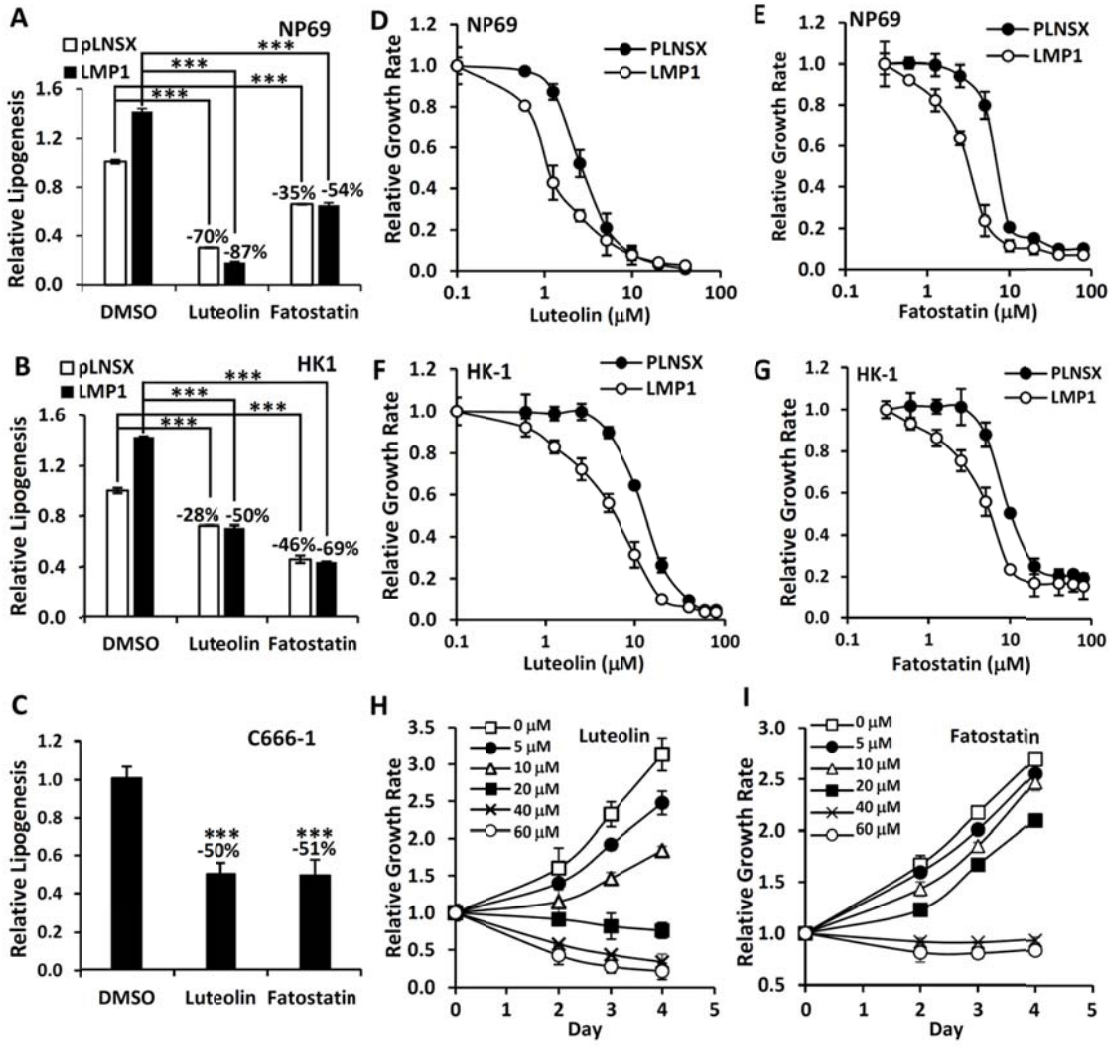
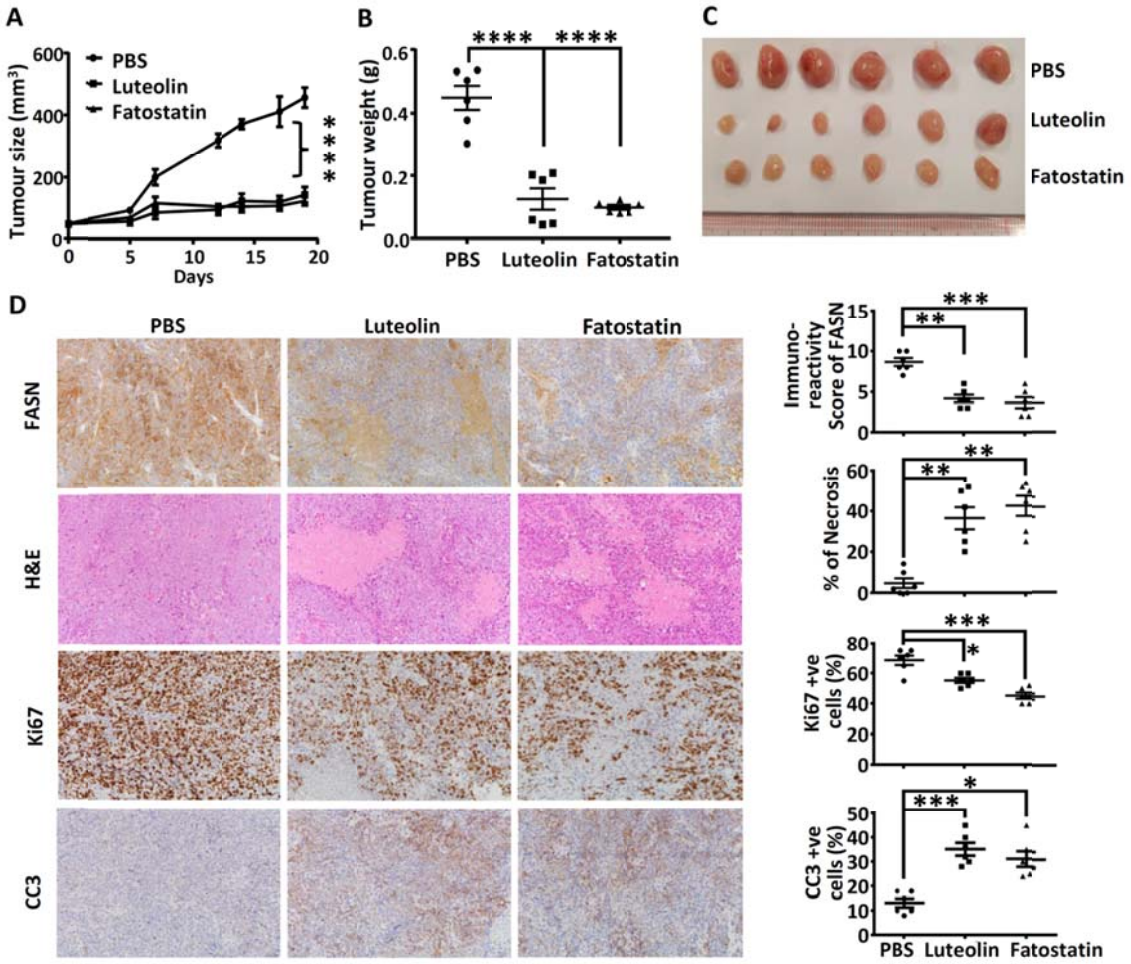


Figure 6. Suppression of NPC tumor growth by inhibitors of lipogenesis

Mice bearing C666-1 xenografts were treated with PBS (control), luteolin (20 mg/kg), or fatostatin (15 mg/kg) every 2-3 days for 3 weeks. (A) Tumor size was measured every 2–3 days for 19 days. At the end point, animals were killed, and tumors were (B) weighed and (C) photographed. (D, left panel) the harvested xenografts were embedded for H&E staining and IHC staining analysis. (D, right panel) The percentage of necrosis, positive staining of FASN, Ki-67 and cleaved caspase-3 were measured and are shown as dot plots. Lines and bars are mean and SEM of each experimental group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.



SUPPLEMENTARY MATERIAL ONLINE

Supplementary materials and methods YES

Supplementary figure legends No because legends are embedded in the Suppl Figures PDF

Figure S1. Silencing of LMP1 leads to a reduction of FASN promoter activity

Figure S2. The inhibitory effects of luteolin and fatostatin on SREBP1 maturation and FASN expression

Figure S3. Body weight of mice bearing C666-1 NPC xenografts during the course of treatment with either PBS (control), luteolin, or fatostatin

Figure S4. The inhibitory effects of Luteolin and Fatostatin on FASN expression, cell proliferation and apoptosis in NPC xenografts

Figure S5. The mechanism of LMP1 in upregulation of lipogenesis

Table S1. Clinicopathologic characteristics of patients with NPC

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