

ORIGINAL ARTICLE

Lactotransferrin acts as a tumor suppressor in nasopharyngeal carcinoma by repressing AKT through multiple mechanisms

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LTF (lactotransferrin, also known as lactoferrin) is a key component of innate immune defense. It has recently been found to have anti-tumor and anti-metastatic activity in different cancers. We previously reported *LTF* to be the most significantly downregulated gene in nasopharyngeal carcinoma (NPC) specimens relative to normal nasopharyngeal epithelial tissues, and it was also negatively associated with the progression and metastasis of NPC. However, the mechanism underlying this remains unclear. In the current study, we revealed that LTF can suppress 3-phosphoinositide-dependent protein kinase 1 expression via the mitogen-activated protein kinase/c-Jun pathway and thus repress AKT signaling. We also showed that LTF interacts with keratin 18 (K18) and so blocks the formation of the K18–14-3-3 complex, leading to downregulation of K18-mediated AKT activation. Thus, LTF suppresses AKT signaling by two separate mechanisms, leading to inhibition of NPC tumorigenesis. This is the first report on the tumor suppressive effects of LTF through repression of AKT signaling in NPC. It suggests that both LTF and AKT signaling merit further study in the field of NPC research.

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INTRODUCTION

Human lactotransferrin (LTF), also known as lactoferrin, is an iron-binding glycoprotein produced by mucosal epithelial cells.¹ LTF has been referred to as a ‘moonlighting’ protein owing to its wide range of functions, including iron homeostasis, anti-microbial and immunomodulatory effects and anti-tumor activity.^{2,3} Several studies have demonstrated the protective effect of LTF against chemically induced carcinogenesis.^{2,4–6} In addition, LTF has been shown to reduce tumor growth and metastasis both *in vitro* and *in vivo*.^{7–9} LTF may have a direct effect on tumor cell growth, as suggested by the fact that LTF and a splice variant thereof are downregulated or absent in some cancers.^{10–13} This hypothesis is supported by the fact that LTF can arrest tumor cell growth at the G1 to S transition of the cell cycle,^{14,15} and has been shown to affect the expression and activity of critical cell cycle regulatory proteins, including cyclin-dependent kinases, cyclin-dependent kinase inhibitors p21 and p27, and retinoblastoma protein.^{14–16}

In previous reports, we demonstrated that LTF was strongly downregulated in nasopharyngeal carcinoma (NPC) and negatively associated with the progression and metastasis of NPC.^{17,18} We also showed that LTF inhibits NPC cell proliferation, induces cell cycle G₁/S arrest, and modulates the mitogen-activated protein kinase (MAPK) signaling pathway and cyclin D1-related proteins, such as cyclin D1, phospho-Rb, p21 and p27.¹⁸ Here, by using gain-of-function and loss-of-function approaches, we have confirmed the negative effects of LTF on growth and metastasis of NPC cells both *in vitro* and *in vivo*. We have shown that LTF downregulates the level of 3-phosphoinositide-dependent protein kinase 1 (PDK1) transcription and subsequently inhibits

the activation of AKT signaling. In addition, we also have demonstrated that LTF interacts with keratin 18 (K18) in NPC cells, and the interaction blocks the formation of the K18–14-3-3 complex, leading to downregulation of K18-mediated AKT activation. Our results show LTF to be a negative regulator of the AKT signaling pathway in NPC and reveal an important mechanism by which LTF suppresses the growth and metastasis of NPC.

RESULTS

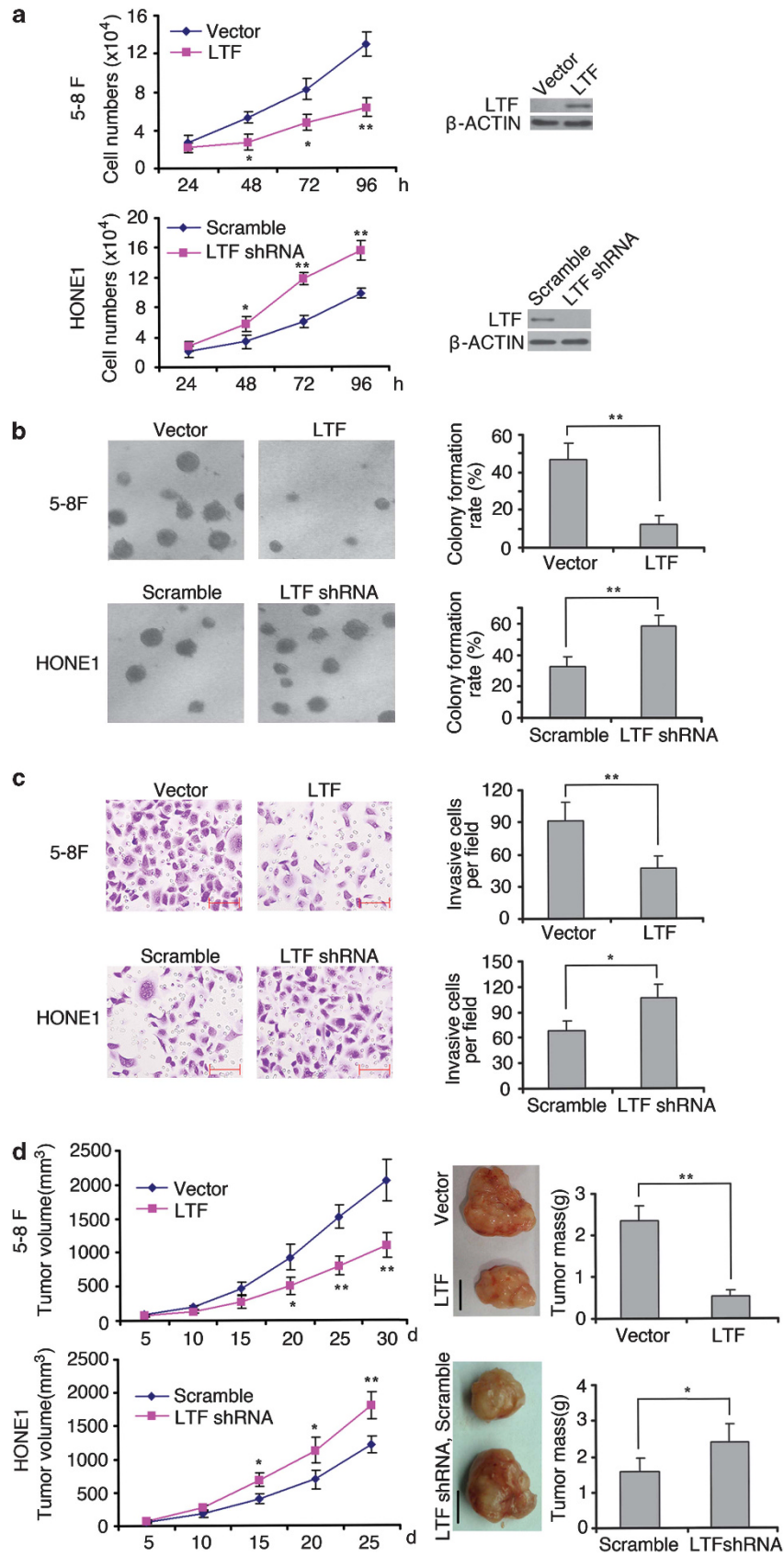
LTF inhibits tumor cell growth and invasion in NPC

Our previous investigation showed that both overexpression of LTF and treatment with recombinant LTF protein could inhibit the proliferation of NPC cells.¹⁸ LTF protein expression levels in normal nasopharyngeal epithelial cell line (NP69) and multiple NPC cell lines were investigated. NP69 and NPC cell lines HONE1, 6–10B had relative normal LTF levels, whereas most of the NPC cell lines had no LTF proteins (Supplementary Figure 1). Figure 1a shows that stably expressing LTF in NPC cell line 5–8F, which has no LTF expression, results in reduced monolayer growth and in reduced soft-agar colony formation relative to control cells (Figure 1b, upper panel). The same phenomena were observed in one additional NPC cell line, CNE2 (Supplementary Figure 2a). Conversely, in HONE-1 cells, which have a relatively high level of LTF expression, depletion of endogenous *LTF* by introduction of lentivirus-mediated small hairpin RNA (shRNA) caused a significant increase in monolayer growth (Figure 1a, right panel) and in anchorage-independent growth (Figure 1b, lower panel). In

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addition, overexpression of *LTF* inhibited the invasiveness of 5–8F and CNE2 cells, whereas knockdown of *LTF* stimulated this ability in HONE1 cells (Figure 1c and Supplementary Figure 2b).

Next, we determined whether LTF could have a role in tumorigenesis by using a nude mice xenograft model. We found that overexpression of *LTF* in 5–8F cells significantly suppressed tumor growth in nude mice (Figure 1d, upper panel). In contrast, knockdown of *LTF* in HONE1 cells was found to promote tumor growth in mice (Figure 1d, lower panel). These results indicate that LTF may repress NPC tumorigenesis and metastasis.

LTF suppresses PDK1 expression in NPC cells

To determine the mechanisms by which LTF inhibits NPC tumorigenesis, we used the Affymetrix gene expression microarray system to study differentially expressed genes between 5–8F/*LTF* and 5–8F/vector. We consistently identified 754 genes, the expression of which changed by more than two-fold in two independent experiments (*P*-value cutoff values <0.01). Their functions were categorized based on DAVID Functional Annotation Database (<http://david.abcc.ncifcrf.gov>). We found significant enrichment in the expression of genes involved in the regulation of cell proliferation, cell cycle and mitosis, apoptosis, adhesion and cell migration. The expression levels of 21 key genes among these differentially expressed genes involved in above cell processes were validated by quantitative reverse-transcriptase PCR in 5–8F/*LTF* and 5–8F/vector cells (Supplementary Table 1). PDK1 attracted our attention immediately because it was strongly reduced by LTF, by more than five-fold, and as a Ser/Thr kinase required for activation of protein kinases in the AGC kinase superfamily, it has important roles in cancer progression, such as activation protein kinase B (also known as AKT), protein kinase C and S6K.^{19,20} To determine whether LTF actually suppresses PDK1 expression, we examined the levels of PDK1 expression in multiple NPC cell lines transfected with *LTF* vector. As shown in Figure 2a, PDK1 expression at the mRNA and protein levels was markedly reduced in 5–8F and CNE2 cells overexpressing LTF. Conversely, in HONE1 cells expressing shRNA targeting *LTF*, PDK1 expression levels were found to be substantially increased.

LTF represses PDK1 via downregulation of MAPK pathway

We next assessed whether LTF downregulates PDK1 expression by decreasing its transcription. Luciferase reporter assays indicated that PDK1 downregulation was achieved through suppression of *PDK1* promoter activity (Figure 2b). Delta-LTF is a well-known intracellular LTF isoform that acts as a transcription factor.²¹ But here, we speculated that LTF-dependent PDK1 downregulation was due to repression of a transcriptional regulator of PDK1 expression by LTF. As LTF reduces c-Jun expression in NPC cells¹⁸ while the transcript factor c-Jun serves to activate transcription of *PDK1*,²² we speculated that LTF-induced PDK1 downregulation depends on the repression of the MAPK pathway and c-Jun. The results show that knockdown of *LTF* activated extracellular signal-regulated kinase and JNK (c-Jun N-terminal kinase) MAPKs, and

induced c-Jun and PDK1 expression, whereas treatment of the *LTF* shRNA/HONE1 cells with either PD98059 (MEK inhibitor) or SP600125 (JNK inhibitor) resulted in reduction of c-Jun levels, leading to reduced PDK1 levels (Figure 2c, upper panel). However, no obvious alterations in c-Jun or PDK1 were found in cells treated with phosphoinositide-3 kinase (PI3K) inhibitor LY294002 (Figure 2c, upper panel). Upon treatment with *c-Jun* siRNA, LTF-depleted cells showed a decrease in the expression of c-Jun associated with a reduction in the expression of PDK1 (Figure 2c, lower panel). These results suggest that the MAPK/c-Jun pathway is involved in LTF-induced PDK1 downregulation in NPC cells.

Effects of LTF on the activation of AKT signaling in NPC

We next analyzed the effect of LTF on AKT signaling, as AKT is the crucial target of PDK1. As shown in Figure 3a, the phosphorylation of AKT was markedly decreased in LTF-overexpressing cells (Figure 3a, left panel). Conversely, LTF-silenced cells showed a significant increase in the activated AKT (Figure 3a, right panel). For full activation, AKT requires phosphorylation at Thr308 and Ser473 by PDK1 and mTORC2, respectively.^{23,24} Surprisingly, AKT Ser473 phosphorylation was reduced to an extent greater than that of T308, the site directly phosphorylated by PDK1 on AKT (Figure 3a). We also measured phosphorylation levels of GSK-3 β and FOXO3 α , the downstream target proteins of AKT, after LTF overexpression or knockdown. We observed that the alteration in AKT phosphorylation was accompanied by corresponding changes in phosphorylation of GSK-3 β and FOXO3 α in LTF-overexpressed or -ablated cells (Figure 3a).

Importantly, overexpression of either wild-type *PDK1* or *PDK1*-A280V (a constitutively active form of PDK1)²⁵ in LTF-overexpressing 5–8F cells efficiently restored AKT phosphorylation at Thr308 but not at Ser473 (Figure 3b, left panel). On the other hand, knockdown of *PDK1* in LTF shRNA/HONE1 cells blocked upregulation of AKT phosphorylation only at Thr308. LY294002 (PI3K inhibitor) treatment reduced the rate of phosphorylation of AKT at both residues (Figure 3b, right panel). The aforementioned results suggest that PDK1 downregulation is one mechanism by which LTF can inhibit AKT activation, but another mechanism might also contribute to the negative regulation of AKT activity by LTF.

Interaction of LTF with K18

To investigate the mechanisms underlying LTF-mediated inhibition of AKT activity other than PDK1 downregulation, we decided to identify LTF-interacting proteins. 5–8F cells overexpressing Flag-tagged LTF plasmid or vector alone were lysed, and LTF was immunoprecipitated using an anti-Flag antibody. Next, the resulting immunocomplexes were resolved by SDS–polyacrylamide gel electrophoresis and stained with Coomassie blue to detect proteins co-immunoprecipitated with the Flag-tagged LTF constructs. A protein of about 45 kDa was found to be specifically associated with Flag-tagged LTF (Figure 4a). The identity of the protein was initially determined by mass

Figure 1. LTF inhibits tumor cell growth, colony formation and invasion *in vitro*, and reduces tumor growth *in vivo*. (a) 5–8F cells stably transfected with the vector pRES or pRES/*LTF* (upper panel) and HONE1 cells stably expressing LTF shRNA or scrambled shRNA (lower panel) were seeded in 12-well plates at the desired cell concentrations and maintained in medium containing 10% fetal bovine serum. The cells were counted at the indicated time points in triplicate and their growth rates were recorded. Western blots of LTF protein levels in 5–8F/*LTF* and HONE1/*LTF* shRNA transfectants are shown in the right panel. (b) Anchorage-independent growth assays were performed on the stable cell lines in soft agar. The results from three separate assays were averaged together and graphed (right panel). Representative images of the assays are shown (left panel). Original magnification $\times 40$. (c) The invasive properties of the cells were analyzed by an invasion assay using a Matrigel-coated chamber. Migrated cells were plotted as the average number of cells per field of view from three different experiments, as described in Materials and methods. Scale bar: 200 μ m. (d) Tumor growth in mouse xenograft models. Upper panel: 5–8F cells stably transfected with the LTF or control vector were injected subcutaneously into nude mice. Lower panel: HONE1 cells stably expressing LTF shRNA or scrambled shRNA were transplanted into nude mice. Tumor size was measured every 5 days. After 25 or 30 days, the mice were killed, necropsies were performed, and tumors were weighed. Scale bar: 1 cm. Error bars represent s.d. **P*<0.05; ***P*<0.01.

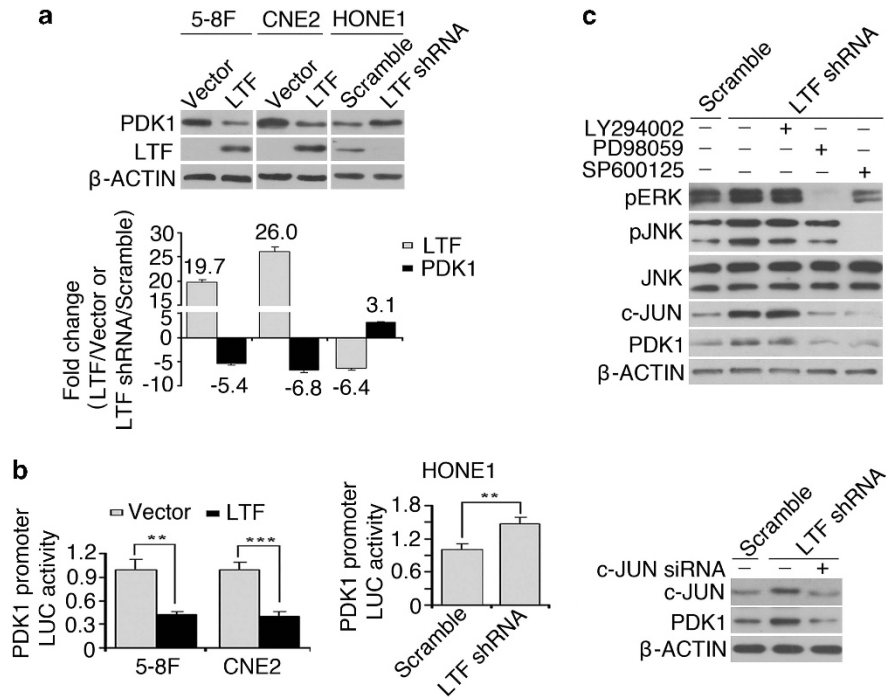


Figure 2. LTF represses PDK1 by downregulation of MAPK activity and c-Jun expression. **(a)** Upper panel: western blot showing expression of LTF and PDK1 in the indicated cells. Lower panel: fold changes of LTF and PDK1 mRNA levels were analyzed by real-time reverse-transcriptase PCR. A representative result of three different experiments is shown. Error bars indicate s.d. ($n=3$). **(b)** Luciferase activity assay under the control of human PDK1 promoter construct was measured in the indicated cells in triplicate. PDK1 promoter activity in the control group was normalized as 1. Error bars represent s.d. ($n=3$). $^{**}P<0.01$; $^{***}P<0.001$. **(c)** Upper panel: HONE1/LTF shRNA cells treated with LY294002 (50 μ M), PD98059 (20 μ M) or SP600125 (10 μ M) for 48 h were lysed to determine the expression levels of pERK, pJNK, JNK, c-Jun and PDK1 by western blot. Lower panel: western blot analysis on the expression levels of c-Jun and PDK1 in HONE1/LTF shRNA cells transfected with c-Jun siRNA.

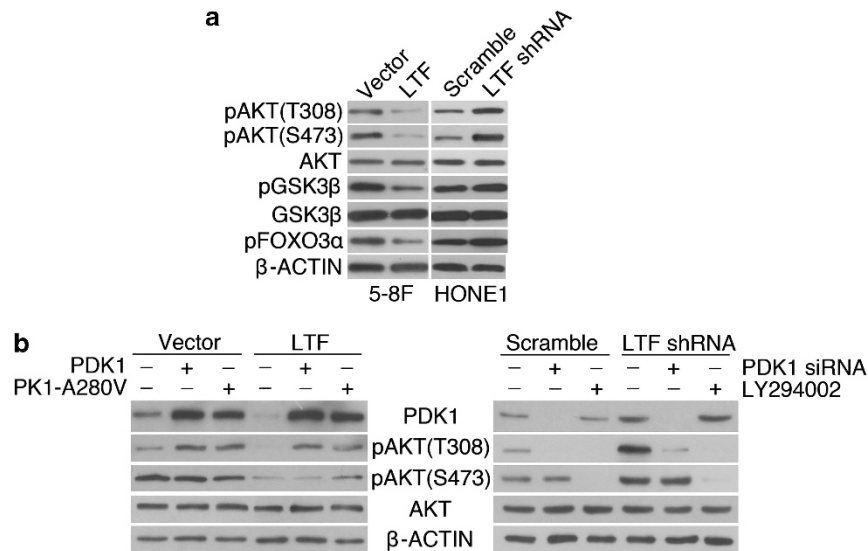


Figure 3. LTF inhibits the activation of AKT signaling in NPC. **(a)** Effect of LTF levels on AKT pathway determined by western blot. 5-8F cells stably transfected with the vector or LTF (left panel) and HONE1 cells stably expressing LTF shRNA or scrambled shRNA (right panel) were lysed. Cell lysates were immunoblotted with antibodies against pAKT (at Thr 308 or Ser 473), AKT, pGSK3 β , GSK3 β and pFOXO3 α . **(b)** Left panel: overexpression of either wild-type PDK1 or PDK1-A280V (a constitutively active form of PDK1) in 5-8F/LTF cells efficiently restored Thr308 AKT phosphorylation but not that of Ser473 AKT in 5-8F/LTF cells. Right panel: knockdown of PDK1 in LTF shRNA/HONE1 cells, blocked upregulation of AKT phosphorylation at only T308, whereas LY294002 treatment (50 μ M for 48 h) opposed the phosphorylation of AKT on both residues (Thr308 and Ser473).

spectrometric sequencing, and it was subsequently confirmed by co-immunoprecipitation and western blot analysis to be the intermediate filament protein K18 (Figures 4b and c). In a reverse

co-immunoprecipitation western blot, LTF in 5-8F cells stably expressing LTF was also co-precipitated with the K18 antibody (Figure 4d). Immunofluorescence staining also showed that LTF

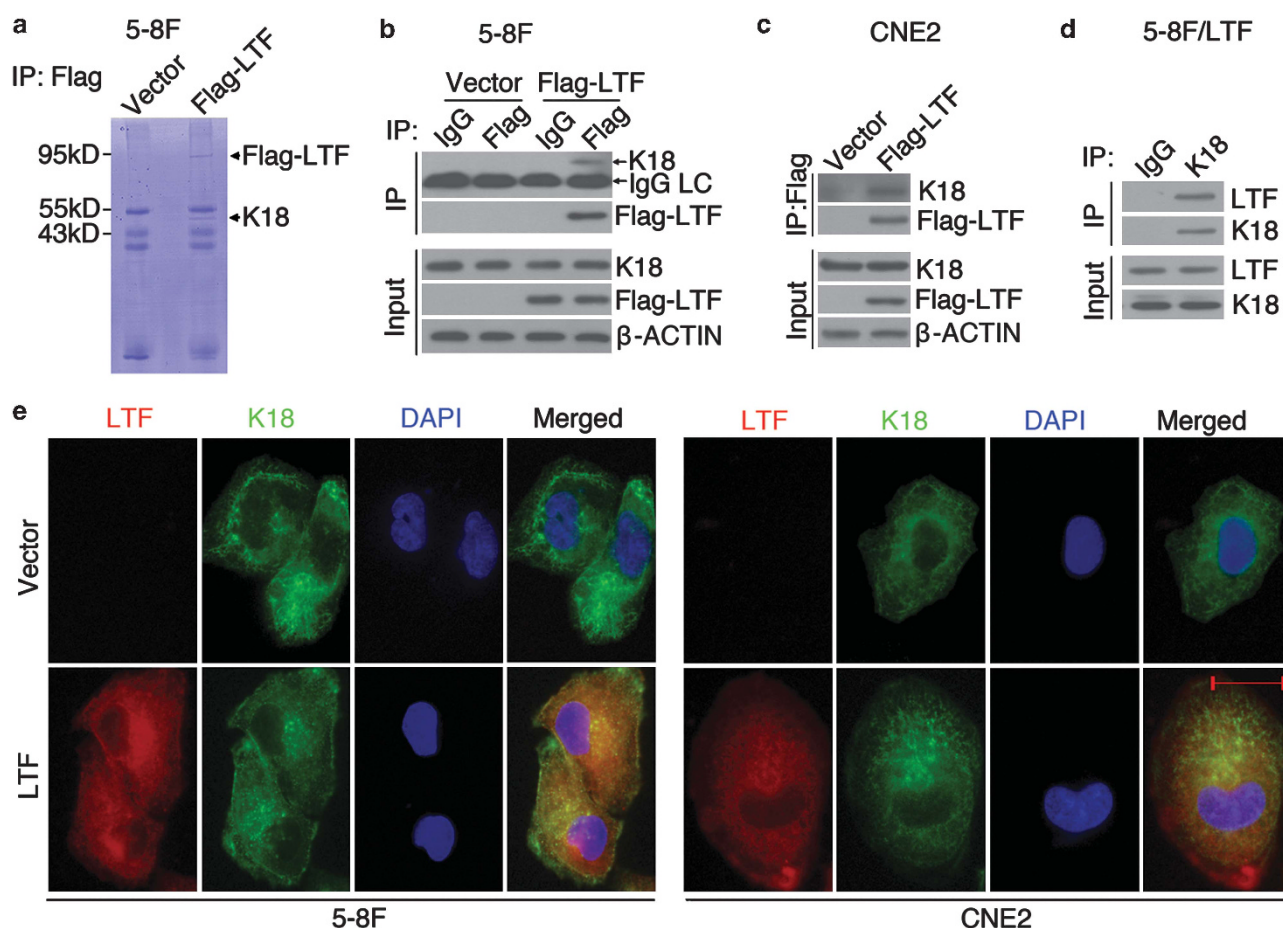


Figure 4. LTF interacts with K18 in NPC cells. (a) Immunoprecipitations with Flag antibody (IP:Flag) were performed on extracts of 5-8F cells overexpressing an empty vector or Flag-tagged LTF. The resulting immunocomplexes were detected by Coomassie blue staining and identified by mass spectrometry. (b) 5-8F cells overexpressing the empty vector or Flag-tagged LTF were immunoprecipitated using a Flag antibody (IP:Flag) or IgG (as control). Co-precipitation of endogenous K18 was detected by western blot. To confirm that equal amounts of each cell extract were used for immunoprecipitation, whole-cell extracts were immunoblotted with K18, Flag antibody (input). IgG LC: IgG light chain. (c) Flag-LTF in CNE2 cells transfected with Flag-LTF was precipitated using a Flag antibody, and co-precipitation of endogenous K18 was detected by western blot. Cell extracts were also immunoblotted with the indicated antibodies (input). (d) Endogenous K18 was immunoprecipitated from 5-8F cells stably expressing LTF and co-precipitation of LTF was detected by western blot. Cell extracts were also immunoblotted with the indicated antibodies (input). (e) Co-localization of LTF (red) and K18 (green) proteins in LTF-overexpressing cells by immunofluorescence. Scale bar: 10 μ m.

colocalizes with K18 in the cytoplasm of 5-8F and CNE2 cells overexpressing LTF (Figure 4e). These results demonstrate that LTF can interact with K18 in NPC cells.

Interestingly K18, a member of the keratin family, has been extensively used as a marker of epithelial tumors and metastasis because it is persistently expressed in many carcinomas.^{26,27} A recent study showed that the keratin family member K17 acts as a scaffold protein to bring together the necessary signaling proteins to trigger PI3K/AKT by binding the adapter protein 14-3-3 σ in a phosphorylation-dependent manner and retaining 14-3-3 σ in the cytoplasm.²⁸ K18 also can bind to 14-3-3 σ proteins.²⁹⁻³¹ Here we confirmed that K18 bound 14-3-3 σ in cells, and the degree of binding was markedly increased upon treatment with okadaic acid (a serine/threonine phosphatase inhibitor). That is, K18 binds 14-3-3 σ in a phosphorylation-dependent manner (Supplementary Figure 3a). Moreover, immunofluorescence staining showed that 14-3-3 σ was present mainly in the cytoplasm of control 5-8F cells, but it was present predominantly in the nucleus of K18 knock-down cells (Supplementary Figures 3b and c), suggesting that K18 is required for the retention of 14-3-3 in the cytoplasm. These findings led us to consider the possibility that K18, like K17,

contributes to trigger the PI3K/AKT pathway. This, in fact, turned out to be the case, as shown by the data in Figure 5a. K18 siRNA significantly decreased the level of AKT phosphorylation at Thr308 and Ser473. Moreover, treatment with K18 siRNA in 5-8F cells was found to decrease the level of phosphorylation of AKT downstream targets, such as GSK3 β and FOXO3 α (Figure 5a). K18 knockdown in 5-8F cells exhibited significantly decreased growth and soft-agar colony formation relative to control cells (Figure 5b). Taken together, these results suggest that K18 can trigger the PI3K/AKT pathway through interaction with 14-3-3 σ and induce growth in NPC cells. This implies that the association between LTF and K18 may be involved in the LTF-inhibition of the AKT pathway.

In addition, we estimated a possible role of K18 in LTF-mediated downregulation of the MAPK pathway and PDK1 expression because of LTF's role as a negative regulator of this pathway. K18 knockdown was found to have little effect on the activity of the MAPK pathway and c-Jun expression (Supplementary Figure 4). We also found that the PDK1 level remained unchanged after K18 knockdown (Supplementary Figure 4). These data imply that inhibition of MAPK signaling and PDK1 downregulation by LTF is K18-independent.

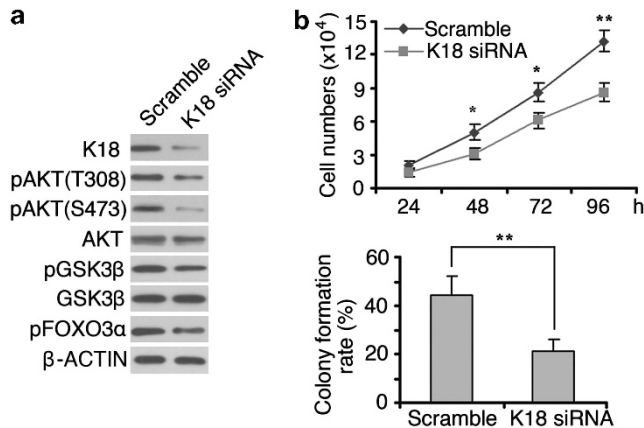


Figure 5. K18 facilitates AKT activation and tumor cell growth. (a) Effects of K18 knockdown on AKT signaling. 5–8F cells were transfected with K18 siRNA or scrambled siRNA for 48 h. Cell extracts were immunoblotted with the indicated antibodies. (b) Cells were transfected as indicated for 24 h. Cells were then split and subjected to the cell growth analysis (upper panel) or colony formation assay in soft agar (lower panel). Error bars represent s.d. * $P < 0.05$; ** $P < 0.01$.

LTF–K18 interaction blocks the ability of K18 to bind to 14-3-3 σ and to stimulate AKT activity

We next examined whether the association between LTF and K18 could influence the ability of K18 to bind 14-3-3 σ . As shown in Figure 6a, overexpression of LTF, which induces LTF–K18 binding, blocked the interaction of K18 and 14-3-3 σ , whereas K18 phosphorylation remained unaffected. In this way, it influenced the subcellular localization of 14-3-3 σ in NPC cells. As shown in Figure 6b, 14-3-3 σ localized to both the nucleus and cytoplasm in control cells, but it was mainly detected in the nuclei of LTF-overexpressing cells. Given that K18 can activate the PI3K/AKT pathway through its interaction with 14-3-3 σ , the fact that LTF binding to K18 results in disassembly of K18–14-3-3 σ complexes implies that the association of LTF with K18 may have significance in the regulation of this pathway. AKT phosphorylation was found to be dramatically increased in 5–8F cells transfected with Myc-K18 relative to control cells. However, the introduction of the LTF expression plasmid in K18-overexpressing cells abolished this effect (Figure 6c). In addition, overexpression of LTF also inhibited the ability of K18 to enhance the growth and soft-agar colony formation of 5–8F cells (Figure 6d). These results demonstrate that LTF suppresses AKT signaling and thereby reduces tumorigenesis partially through its interaction with K18.

LTF attenuates tumor cell growth and invasion via AKT pathway To determine whether LTF suppresses NPC cell proliferation and invasiveness by inhibiting AKT signaling, we used the PI3K/AKT-specific inhibitors Wortmannin and LY294002 to treat HONE1, in which endogenous LTF had been silenced with shRNA. We observed that knockdown of LTF significantly increased cell proliferation rates, promoted anchorage-independent growth and promoted invasiveness, whereas both Wortmannin and LY294002 treatment in HONE1/LTF shRNA cells counteracted these effects (Figures 7a–c). This indicated that LTF attenuates NPC cell growth and invasiveness in NPC at least partially by inhibiting PI3K/AKT signaling.

We further examined the expression levels of LTF, pAKT (Thr308 or Ser473) and total AKT by immunohistochemically analyzing tissue microarrays^{18,32} containing normal nasopharyngeal epithelia ($n = 33$), normal epithelia adjacent to NPC ($n = 23$) and

NPC in varying progressive stages (see Table 1 for characteristics of the 138 NPC cases). The levels of LTF expression were significantly decreased in NPCs relative to normal tissues (Figures 7d and e, and Supplementary Figure 5) and inversely correlated with tumor metastasis and clinical stage ($P = 0.009$ and 0.025 , respectively; Table 1). In contrast, the phosphorylation levels of AKT at Thr308 or Ser473 were upregulated when the expression levels of total AKT were unchanged in NPCs compared with normal tissues (Figures 7d and e, and Supplementary Figure 5). Statistical analysis revealed that LTF expression negatively correlated with pAKT Thr308 ($r = -0.225$, $P = 0.008$) and pAKT Ser473 ($r = -0.345$, $P = 0.001$) levels in NPC tissues, which revealed an inverse relationship between low LTF expression and hyperactivation of AKT in human NPC, further supporting a model of LTF downregulating AKT signaling.

Taken together, our results identify LTF as a negative regulator of AKT signaling through two different pathways and support the idea that LTF acts as tumor suppressor by repressing AKT signaling in NPC tumorigenesis (Figure 7f).

DISCUSSION

LTF has been shown to have anti-tumor activities in numerous studies.^{2,8,9,33} In the current study, we demonstrated that modulation of LTF levels in NPC cells may affect the their proliferation and invasiveness phenotypes (Figure 1). We also demonstrated that LTF suppresses the level of PDK1 transcription (Figures 2a and b). We also showed that LTF downregulates PDK1 expression via extracellular signal-regulated kinase/c-Jun pathway (Figure 2c), although the exact mechanism by which LTF regulates the MAPK pathway and PDK1 expression remains to be established.

PDK1 was originally identified as an upstream kinase for protein kinase B/AKT.²³ It is recognized as a master protein kinase for the regulation of multiple cell-signaling pathways.^{19,20} AKT signaling regulates many biological functions, including cell proliferation, survival, metabolism, cell migration and metastasis. The inhibition of AKT signaling by LTF may contribute to LTF's role in modulation of several important cell cycle regulators (such as cyclin D1, pRb, p21 and p27).¹⁸ The role of activated PI3K/AKT pathway is well documented in various human malignancies and it is sometimes correlated with an aggressive phenotype.³⁴ AKT activity is regulated through its phosphorylation at Thr308 and Ser473 by PDK1 and mTORC2, respectively.³⁵ Here, we demonstrated that LTF-dependent PDK1 downregulation has a direct and major impact on AKT activation in NPC. Elevated LTF levels and reduced PDK1 expression were found to result in hypoactivation of AKT, which was correlated with hypophosphorylation of GSK-3 β and FOXO3 α (Figure 3a), both acting downstream of AKT.

Further studies showed the interaction of LTF with K18 in multiple NPC cells overexpressing LTF (Figure 4). Recently, it has been suggested that keratins may be involved in tumorigenesis. For example, K19 is overexpressed in several cancers.^{36,37} Keratins have been found to be involved in the AKT pathway. Activation of protein kinase B/AKT requires the keratin filament for signal transmission.³⁸ In addition, K17 has been shown to trigger the PI3K/AKT pathway by binding the adapter protein 14-3-3 σ , retaining this protein in the cytoplasm to promote epithelial cell growth.²⁸ Recent reports have shown that K18 interacts with 14-3-3 family proteins, such as 14-3-3 σ , γ and ζ .^{29–31} These findings, combined with our own findings that K18 retains 14-3-3 σ in the cytoplasm of NPC cells by interacting with this protein (Supplementary Figure 4), leading to activation of the AKT pathway (Figure 5), imply that the association between LTF and K18 may be involved in LTF inhibition of the AKT pathway. This was confirmed by our observation that the association of LTF with K18 results in the dissociation of K18 from 14-3-3, which in turn

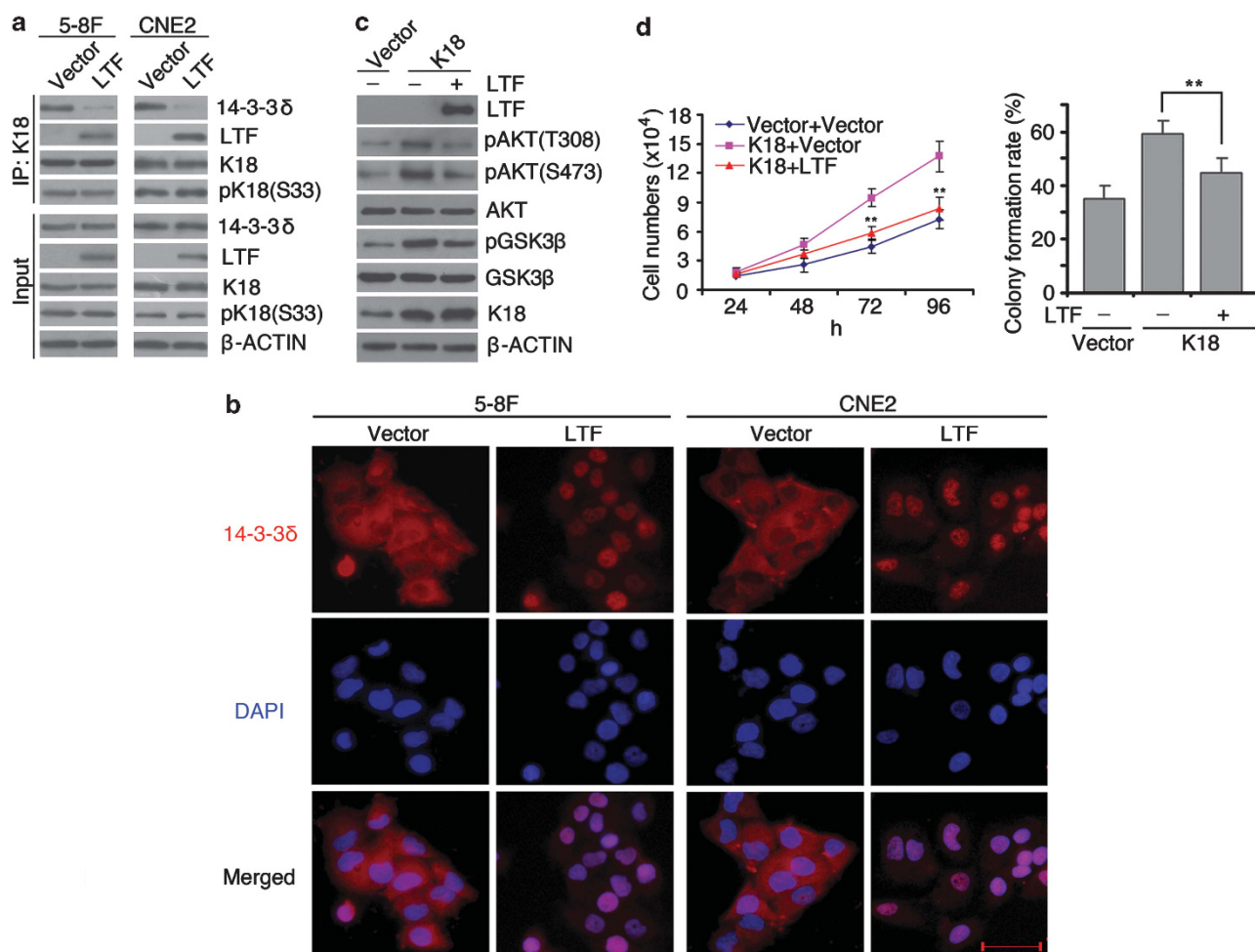


Figure 6. LTF–K18 interaction blocks the ability of K18 to bind to 14-3-3 σ and stimulate AKT activity. **(a)** K18 immunoprecipitates from extracts of 5-8F cells stably expressing either control vector or LTF were immunoblotted with 14-3-3 σ , LTF, K18 and pK18 antibodies. Cell extracts were also immunoblotted with the indicated antibodies (input). **(b)** The effect of LTF overexpression on the subcellular localization of 14-3-3 σ proteins (red) was examined by fluorescent microscopy. Scale bar: 20 μ m. **(c)** LTF (or control vector) transfection following K18 transfection 24 h later opposes AKT activation by K18. AKT pathway activity was measured by pAKT and pGSK3 β immunoblotting. **(d)** The growth-inhibiting properties of LTF overexpression or a control vector in K18 overexpression cell lines (5-8F) were assessed by counting the cells and by anchorage-independent growth assays as described in Figures 1a and b. Error bars indicate s.d. ** $P < 0.01$ (K18 + vector versus K18 + LTF).

sequesters 14-3-3 into the nucleus, blocking AKT activation and tumorigenesis (Figure 6).

In addition to 14-3-3, K18 binds several different cytoplasmic proteins involved in signaling pathways regulating cell growth, death and motility.³⁹ For this reason, we cannot exclude the possibility that LTF, through association with K18, affects K18-interacting protein signaling processes other than the 14-3-3-mediated AKT pathway. Further study is required to confirm this.

14-3-3 proteins are involved in a wide variety of signal-regulated cellular processes, including receptor-mediated signal transduction, apoptosis, cell cycle progression and checkpoint activation, through interaction with phosphorylated consensus sequences in protein targets, such as RAF-1, BAX, BAD, CDC25, K18, TSG1-2 and PI3K.^{40–42} Despite its binding to 14-3-3 proteins, K18 knockdown shows very little effects on MAPK signaling, and thus on the expression of c-Jun and PDK1, implying that the interaction between LTF and K18 are not involved in the LTF-induced inhibition of either MAPK activation or PDK1 expression.

In this present study, the observation that LTF binding to K18 results in the disassembly of the K18–14-3-3 complex suggests

that the association of LTF with K18 may have significance in the regulation of 14-3-3 availability. This implies that K18 may affect various intracellular signaling and cell cycle control pathways by modifying 14-3-3 availability, as observed with AKT pathway in this study.

In the current study, we further confirmed that PI3K/AKT pathway was involved in the inhibition of LTF in NPC tumorigenesis by PI3K/AKT inhibitor treatment (Figures 7a–c). At last, we found that LTF expression levels were significantly downregulated in NPC specimens and negatively correlated with phosphorylation levels of AKT (Figures 7d and e, and Supplementary Figure 6), which further support the idea that LTF may serve as a negative regulator of AKT activity in NPC development. As LTF levels correlated with tumor metastasis and clinical stage (Table 1), this data implied LTF could be used as a biomarker of NPC, and continuing evaluation is warranted. Shaheduzzaman *et al.*⁴³ also reported LTF was significantly downregulated in prostate cancer tissue and has the potential to become a prognostic marker of prostate cancer.

In conclusion, LTF has an important role in suppressing tumorigenesis of NPC through inhibition of the AKT pathway.

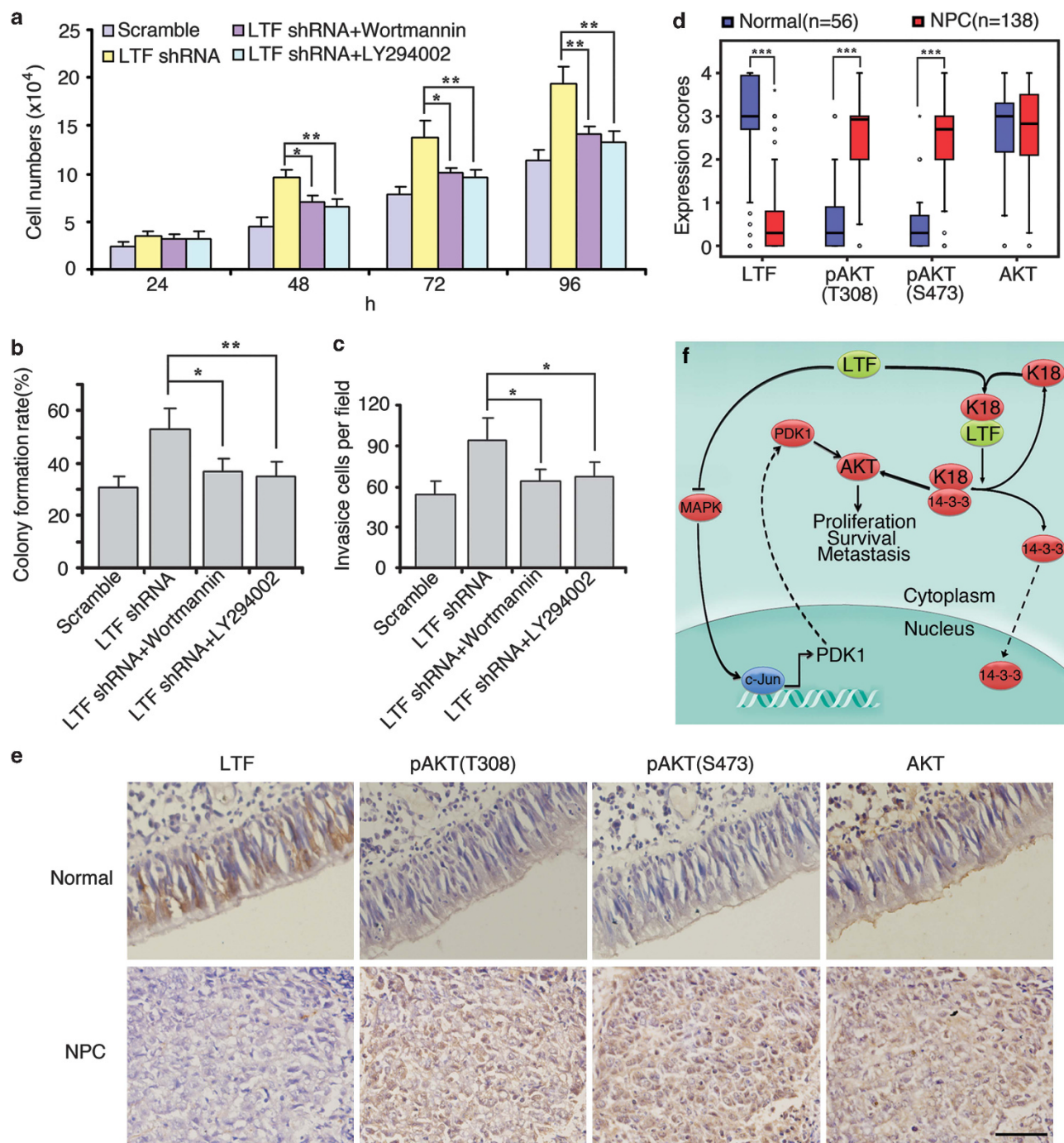


Figure 7. LTF inhibits tumor cells proliferation and invasion via AKT signaling. **(a)** Growth analysis of four groups of HONE1 cells (LTF shRNA, scramble shRNA, LTF shRNA with 100 μ M Wortmannin treatment and LTF shRNA with 50 μ M LY294002 treatment) by counting cells every 24 h. **(b)** Anchorage-independent growth assays were performed on the four groups of HONE1 cells as in soft agar. The results of three separate assays were averaged together and graphed. **(c)** The invasive properties of the four groups of HONE1 were analyzed by an invasion assay using a Matrigel-coated chamber. Migrated cells were plotted as the average number of cells per field of view from three different experiments, as described in Materials and Methods. **(d)** Immunohistochemistry was performed on the tissue array containing normal nasopharyngeal epithelia and NPC samples with anti-LTF, anti-pAKT (T308), anti-pAKT (S473) and anti-AKT antibodies. Expression scores are shown as box plots, with the horizontal lines representing the median, the bottom and top of the boxes representing the 25th and 75th percentiles, respectively, and the vertical bars representing the range of data. **(e)** Representative expression levels of LTF, pAKT (T308), pAKT (S473) and total AKT by immunohistochemistry. Bars, 50 μ m. **(f)** Schematic representation of a model for the major molecular mechanisms of LTF-inhibited AKT signaling in NPC. Error bars indicate s.d. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

LTF can repress AKT signaling through two different mechanisms (Figure 7f). First, LTF reduces PDK1 level by suppressing MAPK pathway and downregulating c-Jun, leading to the inhibition of AKT

activity. Second, LTF, through interaction with K18, blocks the formation of the K18-14-3-3 complex. This causes the sequestering of 14-3-3 in the nucleus and decreases AKT activity. Our findings

Table 1. Analysis of the correlation between expression of LTF in primary NPC and its clinicopathological parameters

Viable	Number of cases	Median expression of LTF (range)	P-values
Gender			
Male	109	0.56 ± 0.08	0.767
Female	29	0.61 ± 0.12	
Age (years)			
≥60	30	0.77 ± 0.18	0.117
<60	108	0.52 ± 0.07	
TNM stage			
Stage I or II	66	0.80 ± 0.10	0.025
Stage ≥III	72	0.53 ± 0.09	
Lymph node status			
Metastasis	94	0.46 ± 0.08	0.009
No metastasis	44	0.83 ± 0.12	

Abbreviations: LTF, lactotransferrin; NPC, nasopharyngeal carcinoma.

uncover important and previously unrevealed mechanisms by which LTF suppresses growth and metastasis of NPC.

MATERIALS AND METHODS

Expression vector

The full-length *LTF* CDS was subcloned into the expression vector pIRES (Clontech, Mountain View, CA, USA). *PDK1* expression plasmids and *myc-K18* plasmids were from Origene (Rockville, MD, USA). The *PDK1* A280V mutant was mutated by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA).

RNA interference

Recombinant lentiviruses, containing the shRNA human *LTF* sequences 5'-CCGGCCTGATCCTAACTGTGTGGATCTCGAGATCCACACAGTTAGGATCAGGTTTGTG-3', were purchased from Sigma-Aldrich (St Louis, MO, USA). *C-Jun*-specific, *PDK1*-specific and *K18*-specific siRNAs were obtained from Invitrogen (Carlsbad, CA, USA). The siRNA sequences are as follows: *c-Jun*, 5'-GAGAGCGGACCTTATGGCTACAGTA-3'; *PDK1*, 5'-GGAAGGATACGACCTCTT-3'; and *K18*, 5'-GCCTCATCTGCGATATAA-3'.

Cell culture

NPC cell lines 5-8F, CNE2 and HONE1 were grown in RPMI1640 medium supplemented with 10% fetal bovine serum. Immortalized nasopharyngeal epithelial cell line NP69 cells were cultured in keratinocyte-SFM medium (Invitrogen) supplemented with murine pituitary extract and recombinant EGF (Sigma-Aldrich). Expression constructs and siRNAs were transfected into cells using Lipofectamine 2000 (Invitrogen). Cells stably expressing LTF vector or control vector were generated by selection with 400 µg/ml G418 (Invitrogen). To establish stable LTF knockdown cell lines, HONE1 cells were transduced with the lentivirus containing LTF shRNA and selected with 5 mg/l puromycin (Invitrogen, San Diego, CA, USA).

Cell proliferation assay

Cells were plated in 12-well plates at the desired cell concentrations. Cell counts were estimated by trypsinizing the cells and performing analysis using a Coulter Counter (Beckman Coulter, Fullerton, CA, USA) at the indicated points in time in triplicate.

Anchorage-independent growth assay

Six-well plates were covered with a layer of 0.6% agar in medium supplemented with 20% fetal bovine serum. Cells (1×10^4) were prepared in 0.3% agar and seeded in triplicate. After the plates were incubated at 37 °C for 2–3 weeks, the colonies were counted. Each experiment was repeated a minimum of three times.

In vitro invasion assay

Cell invasion assay was performed as described previously.⁴⁴ The cells that had invaded were counted and imaged using an inverted microscope (Olympus, Tokyo, Japan).

cDNA microarray analysis

RNAs of 5-8F/vector and 5-8F/*LTF* stable-expression cell lines were detected for gene expression profiling; each sample was repeated twice. Biotinylated cRNA were prepared and hybridized to Affymetrix Human Genome U133 Plus 2.0 (Affymetrix, Santa Clara, CA, USA) oligonucleotide microarrays as per the manufacturer's protocols. These data have been deposited in NCBI's Gene Expression Omnibus (GSE36972) (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=ndafpcsaygckcdk&acc=GSE36972>).

Quantitative reverse-transcriptase PCR analysis

Real-time PCR was carried out using an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Reactions were run in triplicate in three independent experiments. Human *GAPDH* was used as control. The primer sequences are provided in Supplementary Table 2.

Western blot

Western blot was carried out as described previously.¹⁸ The following primary antibodies were used: rabbit anti-AKT, phos-AKT(T308), phos-AKT(S473), phos-ERK, PDK1, GSK3β, phos-GSK3β, phos-FOXO3α, c-Jun, JNK, phos-JNK (Cell Signaling Technology, Beverly, MA, USA); mouse anti-14-3-3σ, LTF, phos-K18(S33) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); rabbit anti-K18, pP38MAPK, P38MAPK (Epitomics, Burlingame, CA, USA); rabbit anti-LTF (Upstate, Chicago, IL, USA); and anti-Myc, Flag (Sigma-Aldrich).

Immunoprecipitation

Cell extracts (500 µg) were pre-cleared with protein G beads before incubation with a particular antibody for 2 h, followed by the addition of protein G beads for 2 h. The beads were washed with cell lysis buffer and boiled in SDS sample buffer. The eluted proteins were subjected to SDS-polyacrylamide gel electrophoresis and western blotting.

Identification of K18 as a LTF-binding protein

Coomassie blue staining of proteins resolved by SDS-polyacrylamide gel electrophoresis was performed. A ≈45-kDa band present in Flag-LTF immunoprecipitates was excised from the gel, digested with trypsin and peptide-sequenced by mass spectrometry (Bruker Daltonics, Ferment, CA, USA).

Immunofluorescence analysis

Cells were fixed in medium containing 3.7% paraformaldehyde for 1 h. The cells were permeabilized using 0.2% Triton X-100 and blocked using normal goat serum. The primary antibodies (LTF, K18, Fibronectin, or 14-3-3σ) were added and incubated at room temperature for 2 h and washed with phosphate-buffered saline. Alexa Fluor-488- and -568-conjugated secondary antibodies (Invitrogen) were then added and incubated for 1 h. DAPI (4',6-diamidino-2-phenylindole) was used to stain nuclei. Control experiments were carried out without primary antibody. The staining was examined using a laser confocal microscope (FluoView FV1000 Confocal Microscope; Olympus).

Luciferase assays

The *PDK1* promoter was amplified from genomic DNA from human blood cells and cloned into pGL3 reporter vector using *KpnI* and *HindIII*. The primer sequences are as follows: 5'-GACTCGAGGCGACAGAGACTCTGTTCA-3' and 5'-CGAAGCTTTCCTCCTCCCGAAGCGGAG-3'.

5-8F and CNE2 cells were transfected with reporter plasmid along with *LTF* vector. HONE1 cells were co-transduced with reporter plasmid and *LTF* shRNA lentivirus. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) after 48 h.

In vivo tumorigenesis

Four- to six-week-old male nude athymic BALB/c nu/nu mice were used to examine tumorigenicity. Different NPC cells were propagated and

inoculated subcutaneously into the dorsal flanks of nude mice (1×10^6 cells in 0.2 ml volume). Tumor size was measured every 5 days. After 25 or 30 days, the mice were killed. Tumor volumes were determined according to the following formula: $A \times B^2/2$, where A is the largest diameter and B is the perpendicular diameter. The experiments were performed using five or six mice per group. All animal procedure were performed in accordance with institutional guidelines.

Immunohistochemistry

Tissue microarray for nasopharyngeal epithelia tissue sections were constructed as previously described.³² Tissue microarray slides containing normal nasopharyngeal epithelia ($n=33$), normal epithelia adjacent to NPC ($n=23$) and NPC ($n=138$; see Table 1 for characteristics of the 138 NPC cases). Immunohistochemistry was performed on tissue microarray sections using rabbit anti-LTF (Upstate), anti-phos-AKT (T308), anti-phos-AKT (S473), and anti-AKT (Cell Signaling Technology). Protein expression levels were semi-quantitatively assessed in tissue samples. The intensity of staining was scored from 0 to 4, and the extent of staining was scored from 0 to 100%. The final quantitation of each staining was obtained by multiplying the two scores. The slides were analyzed by two independent pathologists. All the nasopharyngeal tissue samples were obtained from the Xiangya Hospital (Hunan, China), with written informed consent from patients and with approval for experiments from the ethical review committees of the appropriate institution.

Statistics

Two-sample comparisons used the two-tailed Student's *t*-test with pooled variance if there was no evidence of inhomogeneity of variances between groups. Two-way analysis of variance is used to compare the difference among three or more experiment groups. Spearman's correlation test was used to evaluate the pairwise association of AKT or phos-AKT levels with LTF levels in tissue microarray immunohistochemistry experiment. A *P*-value less than 0.05 was considered statistically significant for all comparisons.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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