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LITAF and TNFSF15, two downstream targets of AMPK, exert inhibitory effects on tumor growth

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Abstract

LPS-induced TNFα factor (LITAF) is a multiple functional molecule whose sequence is identical to small integral membrane protein of the lysosome/late endosome (SIMPLE). LITAF was initially identified as a transcription factor that activates transcription of proinflammatory cytokine in macrophages in response to LPS. Mutations of the *LITAF* gene are associated with a genetic disease, called Charcot-Marie-Tooth syndrome. Recently we have reported that mRNA levels of LITAF and tumor necrosis factor superfamily member 15 (TNFSF15) are upregulated by AMPK. The present study further assesses their biological functions. Thus, we show that AICAR, a pharmacological activator of AMPK, increases the abundance of LITAF and TNFSF15 in the LNCaP and C4-2 prostate cancer cells, which is abrogated by shRNA or dominant negative mutant of AMPK α1 subunit. Our data further demonstrate that AMPK activation upregulates the transcription of LITAF. Intriguingly, silencing LITAF by shRNA enhances proliferation, anchorage-independent growth of these cancer cells, and tumor growth in xenograft model. In addition, our study reveals that LITAF mediates the effect of AMPK by binding to a specific sequence in the promoter region. Furthermore, we show that TNFSF15 remarkably inhibits the growth of prostate cancer cells and bovine aortic endothelial cells *in vitro* with a more potent effect toward the latter. In conjuncture, intratumor injection of TNFSF15 significantly reduces the size of tumors and number of blood vessels and induces changes characteristic of tumor cell differentiation. Therefore, our studies for the first time establish the regulatory axis of AMPK-LITAF-TNFSF15. They also suggest that LITAF may function as a tumor suppressor.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

AMPK; LITAF; TNFSF15; p53; tumor suppressor; tumorigenesis

Introduction

Lipopolysaccharide-induced tumor necrosis factor alpha factor (LITAF) was initially characterized as a transcription factor that activates transcription of cytokines, such as TNFα, IL6, sTNF-RII, and CXCL in macrophages in response to lipopolysaccharide (LPS) (Myokai *et al.*, 1999). It was also described as a p53-inducible gene (PIG7) (Polyak *et al.*, 1997). Upon stimulation, LITAF translocates to the nucleus and binds to a specific sequence of the TNFα promoter to cooperate with other transcription factors such as STAT6(B) to upregulate the transcription (Tang *et al.*, 2005). Therefore, LITAF plays an important role in inflammatory response. Interestingly, the sequence of LITAF is identical to the Small Integral Membrane Protein of the Lysosome/late Endosome (SIMPLE), whose mutations are associated with a genetic disease called Charcot-Marie-Tooth disease (CMT), characterized by demyelinating disorders of peripheral nervous system (Niemann *et al.*, 2006). It is not clear whether the mutations disable the transcriptional activity of LITAF or other functions.

Tumor necrosis factor superfamily member 15 (TNFSF15) was first identified as an inhibitor of vascular endothelial cell growth factor, thus also named VEGI (Sethi *et al.*, 2009). This TNF subfamily contains three isoforms resulting from different splicing; the first one consists of 174 amino acids, which shares 20 to 30% homology to other TNF family members and, two additional isoforms comprise 192 and 251 amino acids, respectively (Sethi *et al.*, 2009). All the three isoforms are identical in the carboxyterminal 151 amino acids. TNFSF15 has been shown to potently inhibit the growth of vascular endothelial cells (Yue *et al.*, 1999; Zhai *et al.*, 1999a) as well as tumor cells (Haridas *et al.*, 1999). Several tumor xenograft studies have revealed that TNFSF15 suppresses tumor growth (Hou *et al.*, 2005; Pan *et al.*, 2004; Zhai *et al.*, 1999b). In addition, the longer form, VEGI-251, also called TNF-like ligand 1 (TL1A), has been implicated in autoimmunity and inflammation-induced immunity (Bayry, 2010; Sethi *et al.*, 2009).

AMP-activated protein kinase (AMPK) is a highly conserved serine/threonine protein kinase, consisting of three subunits, a catalytic subunit (α) and two regulatory subunits (β and γ) (Hardie, 2007). In mammals, each subunit of AMPK contains two to three isoforms (α1, α2; β1, β2; γ1, γ2, γ3). AMPK serves as a fuel gauge in maintaining energy homeostasis. Thus, it is activated under metabolic stress that increases intracellular levels of AMP, which servers as an allosteric activator by binding to the γ regulatory subunit. In addition, AMPK is phosphorylated and activated by upstream kinases, such as the tumor suppressor LKB1/Stk11 (Hardie, 2007; Luo *et al.*,2010). Under physiological conditions, AMPK is activated by hormones and cytokines, including leptin (e.g. in skeletal muscle) and adiponectin secreted from adipocytes, interleukin 6 (IL6), and ciliary neurotrophic factor (CNTF) (Zhang *et al.*, 2009). In addition, AMPK can be activated by a variety of pharmacological agents. The prototypical activator is 5-aminoimidazole-4-carboxamide 1-D-ribonucleoside (AICAR), a cell permeable agent that is phosphorylated after entering the

cell and converted to ZMP, an AMP analogue. Importantly, two clinically used anti-diabetic drugs, metformin and thiazolidinediones, have been known to activate AMPK (Zhang *et al.*, 2009).

Recently, we have demonstrated that inhibition of AMPK exacerbates the malignant behavior of prostate cancer cells (Zhou *et al.*, 2009). In exploring the mechanism underlying the inhibitory effects of AMPK, we have identified several interesting targets, among which are LITAF and TNFSF15. In the present study, we aim to explore the relationship of these molecules with AMPK and assess their biological effects on tumor cell growth and tumor development. Our results show that AMPK activation by AICAR upregulates LITAF, which in turn increases TNFSF15 expression. All these regulations occur at the level of transcription. We further show that knockdown of LITAF expression by shRNA enhances cell proliferation, anchorage-independent growth of prostate cancer cells and tumor development in xenograft model. Finally, we show that TNFSF15 inhibits the growth of prostate cancer cells in vitro and in vivo, with a more potent effect on endothelial cell growth and tumor angiogenesis.

Results

AMPK upregulates protein levels of LITAF

Previously we have shown that the expression of LITAF mRNA is reduced in prostate cancer cells containing the dominant negative mutant of AMPKα1 subunit or shRNA, whereas it is upregulated by AICAR treatment (Zhou *et al.*, 2009). In the present study, we assessed if AMPK plays a role in regulating the promoter activity of LITAF. Toward this end, we cloned 500 bp 5′ upstream of the transcriptional initiation site of the LITAF gene and inserted to a luciferase reporter plasmid. We then transfected LNCaP cells with this plasmid together with a plasmid encoding Renilla luciferase in the presence or absence of the plasmid for wild type α1 subunit of AMPK. After 48 hours, the cells were treated with AICAR for 8 hours. Fire fly luciferase activity was assayed and normalized with Renilla luciferase activity. As shown in Figure 1A, AICAR enhanced the luciferase activity by about two folds, while transfection of AMPK α1 subunit caused a 1.5-fold increase, which was further increased by the treatment with AICAR. To ascertain if the change holds true at protein levels, we treated a prostate cancer cell line, LNCaP cells, with AICAR for different periods of time and examined the expression of LITAF. As shown in Fig 1B, the treatment progressively increased the abundance of LITAF, whereas the induction was diminished in the cells expressing shRNA. A similar result was obtained with the dominant negative mutant of α1 subunit (Data not shown).

LITAF inhibits malignancy of tumor cells

To assess the biological effects of LITAF, we decided to knock down LITAF expression by shRNA. Out of 4 shRNA retroviral plasmids, one was found to yield more than 80% of silencing effect. Thus, the retrovirus for this clone was packaged in HEK293T cells and used to infect into LNCaP and C4-2 cells (Figure 2A). We also included p53 shRNA as a positive control, as previous studies identified LITAF as one of p53-induced genes. However, our current data revealed that knocking down p53 did not affect LITAF expression, or its

response to AICAR (data not shown). We then compared behaviors of prostate cancer cells with or without silencing LITAF using MTT assay and found that silencing LITAF significantly accelerated cell proliferation (Figure 2B). Furthermore, it enhanced anchorage-independent growth of prostate cancer cells on soft agars (Figure 2C). All these effects were as great as those by knocking down p53. We then inoculated these cells into athymic nude mice and assessed tumor development. As shown in Figure 3, our results revealed that prostate cancer cells containing LITAF shRNA form tumors at a significantly higher rate than the cells without knockdown.

LITAF mediates AMPK activation to upregulate TNFSF15

Previous studies have shown that LITAF responds to LPS to induce expression of proinflammatory cyokines such as TNFα. We have reported that AMPK activation increases expression of TNFSF15 in prostate cancer cells where TNFα is barely detectable (Zhou *et al.*, 2009). In this study, we were curious to ask if AICAR-induced expression of TNFSF15 was mediated by LITAF. Toward this end, we treated LNCaP and C4-2 cells containing LITAF shRNA or scrambled shRNA with AICAR and analyzed mRNA abundance by quantitative PCR. The data revealed that AICAR increased levels of TNFSF15 mRNA in control cells, while the induction was markedly reduced in cells where LITAF was silenced (Figure 4A & 4B). To examine if the change of mRNA is attributed to the regulation of transcription, we isolated 1000 bp 5′ upstream of the transcriptional initiation site of TNFSF15 and placed it to upstream sequence of luciferase reporter. We then transfected the plasmid to LNcaP cells carrying scrambled or LITAF shRNA and analyzed the luciferase activity. As shown in Figure 4C, AICAR significantly stimulated the luciferase activity in control cells, whereas the stimulation was diminished by LITAF shRNA. As a putative LITAF-binding element (CTCCC) is present in the promoter region of TNFSF15 (Tang *et al.*, 2003), we asked if LITAF directly binds to this sequence. In the ChIP assay, we found that the sequence surrounding the binding element was specifically pulled down by LITAF antibody, which was increased by the treatment of LNCaP cells with AICAR.

TNFSF15 inhibits angiogenesis and tumorigenesis

To assess if TNFSF15 plays a role in tumor development, we cloned cDNA encoding the longest version of TNFSF15, VEGI-251, from LNCaP cells by reverse transcription coupled PCR and then expressed the carboxyterminal 151 amino acids, which are identical among all three isoforms (Figure 5). We then examined if the carboxyterminal piece of TNFSF15 exerted an inhibitory effect on the growth of LNCaP cells and bovine aortic endothelial cells (BAEC). In this experiment, the cells were treated with different doses of TNFSF15 for 24 and 48 hours and assayed by MTT. At 24 h, OD570 of LNCaP cells exhibited a nearly linear increase with increasing doses of TNFSF15, reaching a maximal 20% at 1 nM ($P<0.01$), while at 48 h, the OD progressively decreased with increasing doses ($P<0.01$. The reason for the increase of MTT readings at 24 hours is not clear. It possibly reflects increased biogenesis of mitochondria within this specific time frame. In contrast, BAEC cells were markedly suppressed and appeared to be more sensitive to TNSF15 treatment at both 24 and 48 hour points ($P<0.01$). Two-way ANOVA analysis indicates that differences between different groups (cell types and times) are significant ($P<0.05$).

To test if TNFSF15 inhibits tumor growth *in vivo*, we inoculated LNCaP cells into nude mice and allowed tumor grow for 10 days. When tumors were palpable, intratumor injection of TNFSF15 was performed every two days for two weeks. In the end, mice were sacrificed and tumors removed and weighed. Our data revealed that the average weight of tumors from TNFSF15-admininstered mice was half of that injected with PBS vehicle (Figure 6A). In general, tumors contained fewer visible blood vessels. To evaluate tumor vascularization, the tumor tissues were sectioned for examining histopathological morphology and immunohistochemical reactivity to antibody for Von Willebrand Factor. As shown in Figure 6B, the tumor tissues of the control animals displayed uniform and anaplastic structure without evidence of differentiation. In contrast, experimental animals exhibited multiple lobular structure surrounded and separated by connective tissue. This structure apparently manifests a likelihood of differentiated tumor. In addition, the number of blood vessels formed in the tumor tissue of the control animals was significantly more than that of the experimental animals.

Discussion

Recently, we have shown that inhibition of endogenous AMPK by expression of the dominant negative mutant of AMPK α1 subunit or its shRNA augments malignant behaviors of prostate cancer cells, whereas activation of AMPK by AICAR or introducing LKB1 to cancer cells containing loss-of-functional mutations causes changes in an opposite direction. The effects of LKB1/AMPK might be mediated by concerted actions of various downstream targets (Zhou *et al.*, 2009). Among them, we have identified two novel targets, LITAF and TNFSF15. In the present study, we have for the first time demonstrated that activation of AMPK stimulates expression of LITAF, which in turns upregulates TNFSF15, and that knockdown of LITAF accelerates prostate cancer cell proliferation and tumor development. Our data also show that TNFSF15 remarkably inhibits the growth of prostate cancer cells and angiogenesis and induces morphological transition from malignant to benign tumors.

AMPK has been shown to play important roles in mediating the tumor suppressive function of LKB1. To understand the underlying mechanisms, our exciting but challenging task is to identify new targets downstream of AMPK in addition to those well characterized, such as mTOR, FASN, p53, p27 and FOX3a (Luo *et al.2010*). In this study, we have found that AMPK regulates the transcription of LITAF, which then binds to a specific sequence in the promoter region of TNFSF15 and regulates its transcription. Currently, we do not know how AMPK regulates the transcription of LITAF. It is possible that AMPK indirectly exerts such an effect through regulation of an unidentified transcription factor. However, our data do not support that this occurs through p53. Therefore, it will be our interest to identify the transcription factor that mediates this effect.

Existing data support that LITAF is a transcription factor involved in regulating production of proinflammatory cytokines in response to LPS (Tang *et al.*, 2005; Tang *et al.*, 2006). An additional function of LITAF relates to sorting and degradation of plasma membrane proteins by lysosome and late endosome (Niemann *et al.*, 2006). Mutations of LITAF/SIMPLE have been suggested to alter this second function, which may play a role in the

pathogenesis of CMT1C (Beauvais *et al.*, 2006; Bennett *et al.*, 2004; Latour *et al.*, 2006). Thus far, no direct evidence indicates a role of LITAF in tumorigenesis. However, several recent studies suggest that there is such a link. For instance, homozygous deletion the *LITAF* gene and promoter hypermethylation are reported in some types of lymphomas (Mestre-Escorihuela *et al.*, 2007). Secondly, LITAF was found to associate with WW domain oxidoreductase (WWOX) (Ludes-Meyers *et al.*, 2004). The latter has been found altered in multiple types of cancer and its ectopic expression inhibits xenograft tumor growth of breast cancer cells (Chang *et al.2010*). Therefore, it will be interesting to assess the direct effect of LITAF on tumorigenesis and elucidate the underlying mechanisms.

Although LITAF is known to upregulate transcription of TNFα, little information has been documented on its role in regulating other TNF superfamily members. A possible link between LITAF and TL1A has been suggested by the findings that their expressions are increased in macrophages of inflammatory bowel diseases such as Crohn's disease and ulcerative colitis (Picornell *et al.*, 2007; Stucchi *et al.*, 2006; Young and Tovey, 2006). Interestingly, one study has so far reported that bacterially expressed chicken LITAF or LITAF expressed, secreted and purified from COS7 cells, stimulates transcription of TNFSF15 in chicken macrophages (Hong *et al.*, 2006). This report is coincidently in line with our findings where we placed LITAF as an upstream modulator of TNFSF15 in response to AMPK activation.

A clinical investigation has indicated that high levels of TNFSF15 are associated with increased survival rate of breast cancer patients (Parr *et al.*, 2006). Numerous studies have reported that exogenous application of different isoforms of recombinant TNFSF15 to culture media or to animals inhibits tumor cell growth and tumor development and angiogenesis (Haridas *et al.*, 1999; Hou *et al.*, 2005; Pan *et al.*, 2004; Yue *et al.*, 1999; Zhai *et al.*, 1999a; Zhai *et al.*, 1999b). Our present study demonstrates that bovine aorta endothelial cells are more sensitive to TNFSF15 than LNCaP cells, which is in accord with previous reports that TNFSF15 exerts anti-tumor effects mainly through inhibition of angiogenesis (Sethi *et al.*, 2009). Consistently, our animal study showed that the treatment of xenograft tumors with TNFSF15 not only inhibits angiogenesis, but also induces morphological changes characteristic of benign tumors.

In the end, we would emphasize that our present study has for the first time demonstrated that AMPK upregulates LITAF, which in turn increases the expression of TNFSF15. The regulation occurs at the transcriptional level. Collectively, our data suggest that LITAF and TNFSF15 may function as tumor suppressors. Therefore, we propose a model illustrated in Figure 7. LITAF serves as one of downstream targets of AMPK to inhibit cancer cell growth, possibly by multiple mechanisms, one of which is TNFSF15 that potently inhibits angiogenesis. As for other functions of LITAF, such as those related to lysomal/late endosome trafficking and degradation of membrane proteins, it is worthwhile to explore if they participate in tumorigenesis. Overall, our work points to a new direction in investigating the correlation of tumor progression and prognosis with expression levels and activity of AMPK, LITAF and TNFSF15 and the mechanism by which they are involved in tumorigenesis. Likewise, our data also suggest that targeting AMPK pathway would

represent a promising approach for prevention and treatment of cancer, as it acts on both tumor cells and angiogenesis.

Materials and Methods

Materials

AICAR was purchased from Toronto Research Chemicals Inc (North York, ON, Canada). Antibodies for total and phospho-T172 of AMPK alpha subunit were from Cell Signaling Technology, Inc. (Danvers, MA). Antibody for p53 was from Millipore (Billerica, MA). Monoclonal antibodies for β-actin and flag epitope (M2) were purchased from Sigma (Saint Louis, MO). Monoclonal antibody for LITAF was purchased from BD Biosciences (San Jose, CA).

Stable cell lines

Retroviral plasmids for LITAF shRNA and scrambled shRNA were purchased from Open Biosystems (Huntsville, AL). A set of four plasmids (Catalog number: RHS4529-NM_001136472) were transfected into HEK293T cells to examine a clone that produces the most efficient silencing effect and one (V2HS_247509) was selected. ShRNA for p53 was in pSuperRetro vector, a gift from Dr. Zhixiong Jim Xiao. Retrovirus was packaged in HEK293T cells and the virus supernatant was infected into two prostate cancer cell lines, LNCaP and C4-2. Two days after infection, the cells were selected with puromcyine and maintained in RPMI1640 medium supplemented with 10% FBS. The stable cell lines for the dominant negative mutant of human AMPK α1 catalytic subunit (D139A) and α1 shRNA were described previously (Zhou *et al.*, 2009).

Immunoblot

Equal amounts of cell extracts (25 μg) were separated onto SDS-PAGE and electrophoretically transferred to PVDF membranes (Millipore). The membranes were sequentially blotted with the first and second antibodies, and developed by the enhanced chemiluminescence (ECL) method, according to the protocol provided by manufactures.

Soft Agar Assay for Colony Formation

Assays on anchorage independent growth were carried out on soft agar, as described previously (Zhou *et al.*, 2009).

Expression and purification of TNFSF15

Total RNA was prepared from LNCaP cells and cDNA synthesized. TNFSF15 was amplified by PCR using the primers based on the sequence of VEGI-251 (forward: 5′ ATGGCCGAGGATCTGGGACTGAGC 3′, reverse: 5′ CTCTCCTCCTATAGTAAGAAGGCTCC 3′) and subcloned into pCR-BluntII-TOPO (Invitrogen, Carlsbad, CA). After sequencing validation, the cDNA segment encoding the carboxyterminal 151 amino acids was amplified by PCR using the primers (forward: 5′ agctggatccGTTGTGAGACAAACTCCCACACAGC 3′, reverse: 5′ agctgtcgacCTCTCCTCCTATAGTAAGAAGGCTCC 3′). The PCR product was subcloned

into pMal-C2 plasmid at the BamHI and SalI sites. The plasmid was transformed into JM109 cells and the recombinant TNFSF15 was induced by IPTG, purified using Amylose beads, cleaved with Factor X, and purified further to remove Factor X, according to the manufacture's protocol (New England BioLabs, Ipswich, MA).

MTT assay

Cells (5×10^3) were seeded into 96-well plates and cell viability was measured at different days using MTT kit according to the manufacture's protocol (Invitrogen).

Quantitative Real-time PCR Analysis

the expression of mRNA were examined by real-time PCR with the ABI 7300 Real-time PCR System (Applied Biosystems, Foster City, CA), using SYBRGREEN PCR Master Mix 2× reagent in 20 μl reaction volume. The primers for RT-PCR were designed as described previously (Zhou *et al.*, 2009). Each sample was amplified in triplicate and normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. Results were evaluated by the comparative threshold cycle value method ($2^{-\Delta\Delta Ct}$) for relative quantification of gene expression.

Xenograft model

Stable LNCaP cells (2×10^6) bearing LITAF shRNA or scrambled shRNA were resuspended in 100 μl phenol red-free RPMI medium and mixed with 100 μl matrigel (BD Biosciences, Bedford, MA) for each injection. The cell suspensions were then injected subcutaneously into the flank of nude mice (Charles River Laboratories, Wilmington, MA). Each group consisted of 10 mice and two spots were inoculated for each mouse. Ten days after inoculation, tumors were measured with calipers and tumor volume was calculated using the formula $W^2xL/2$ (W, shorter diameter; L, longer diameter) (mm^3).

For TNFSF15 administration, when tumors grew palpable, TNFSF15 prepared in PBS (100μl, 10 μg/ml) or PBS as vehicle control was injected inside tumors every 2 days. After two weeks injection, mice were euthanized and tumors removed and weighed.

Immunohistochemical staining procedures

Tumor tissues were snap-frozen and sectioned immediately or stored at −80°C. The frozen sections were fixed with pre-cooled acetone/methanol (1:1) fixative for 10 min at RT, endogenous peroxidase activity was quenched with 3% H_2O_2 in methanol for 10 min. The slides were washed in Tris-buffered saline (TBS) three times 10 min for each, blocked with 5% calf serum in TBS at RT for 1 h. They were then briefly rinsed with TBS and incubated with rabbit polyclonal antibodies against human Von Willebrand Factor (Catalog number, ab6994, Abcam, Cambridge, MA) in 1:200 dilution with 5% calf serum-TBS at 4°C overnight. Non-immunized rabbit IgG (Catalog number, I-1000, Vector Laboratories, Burlingame, CA) was used as a negative control. The specimens were washed in TBS three times with gentle agitation and incubated with HRP-conjugated anti-rabbit IgG antibodies (Catalog number, NEFB 12001EA, PerkinElmer, Covina, CA) at RT for 1 h. After washing, the DAB substrate (Catalog number, SK-4100, Vector Laboratories) was applied and signals

were developed following the manufacture's instruction. The specimens were counterstained with hematoxylin and dehydrated and mounted.

Luciferase Assay

The promoter regions for human LITAF (500 bp upstream of the transcription initiation site and human TNFSF15 (1000 bp upstream of the transcription initiation site) were subcloned into pGL3 vector that expresses fire fly luciferase as a reporter (Promega, Madison, WI), respectively. The plasmid was cotransfected with pNull-Rluc plasmid encoding Renilla luciferase into LNCaP cells using Lipofectamine 2000 (Invitrogen). Two days later, the cells were treated with or without AICAR for 8 hours and reporter gene assays conducted using a Dual luciferase assay kit purchased from Promega, as previously described (Xiang *et al.*, 2002).

Chromatin immuoprecipitation (ChIP)

ChIP assay was performed as described by Nelson et al (Nelson *et al.*, 2006). Briefly, LNCaP cells at an 80% confluent density were treated with or without AICAR for indicated times (see Figure legend). The cells were then cross-linked with formaldehyde, quenched and lysed in lysis buffer. Cell lysates were centrifuged. The supernatant was saved for Western blot. Chromatin pellets were resuspended in the lysis buffer, sonicated and centrifuged again. The supernatant was immunoprecipitated with LITAF antibody (BD Biosciences). The immunoprecipitate was assayed by Western blot and PCR to amplify the sequence (258 bp) containing a potential LITAF binding motif (CTCCC) using the primers (up: 5′ GACAGAGGGCTAGGCAGCAC 3′ and down: 5′ GCTCATGTGCTCCTTCCTTCG 3′. The PCR product was analyzed on agarose gel electrophoresis.

Statistic analysis

Difference between paired groups was tested by student's t test. Trend difference within groups and between groups at different time points or doses was assessed by one-way and two-way ANOVA methods, respectively. Significance was set at $P<0.05$

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Abbreviation

AMPK	AMP-activated protein kinase
AICAR	5-aminoimidazole-4-carboxamide 1-D-ribonucleoside
BAEC	bovine aortic endothelial cells
CMT1C	Charcot-Marie-Tooth type 1C disease, an autosomal genetic disease with demyelinating periphery nervous system

GAPDH	glyceraldehyde 3-phosphate dehydrogenase
LKB1	liver kinase B, also named serine/threonine kinase 11 (STK11)
LITAF	lipopolysaccharide-induced tumor necrosis factor alpha factor
LPS	lipopolysaccharide
SIMPLE	small integral membrane Protein of the lysosome/late endosome
TNFSF15	tumor necrosis factor superfamily member 15

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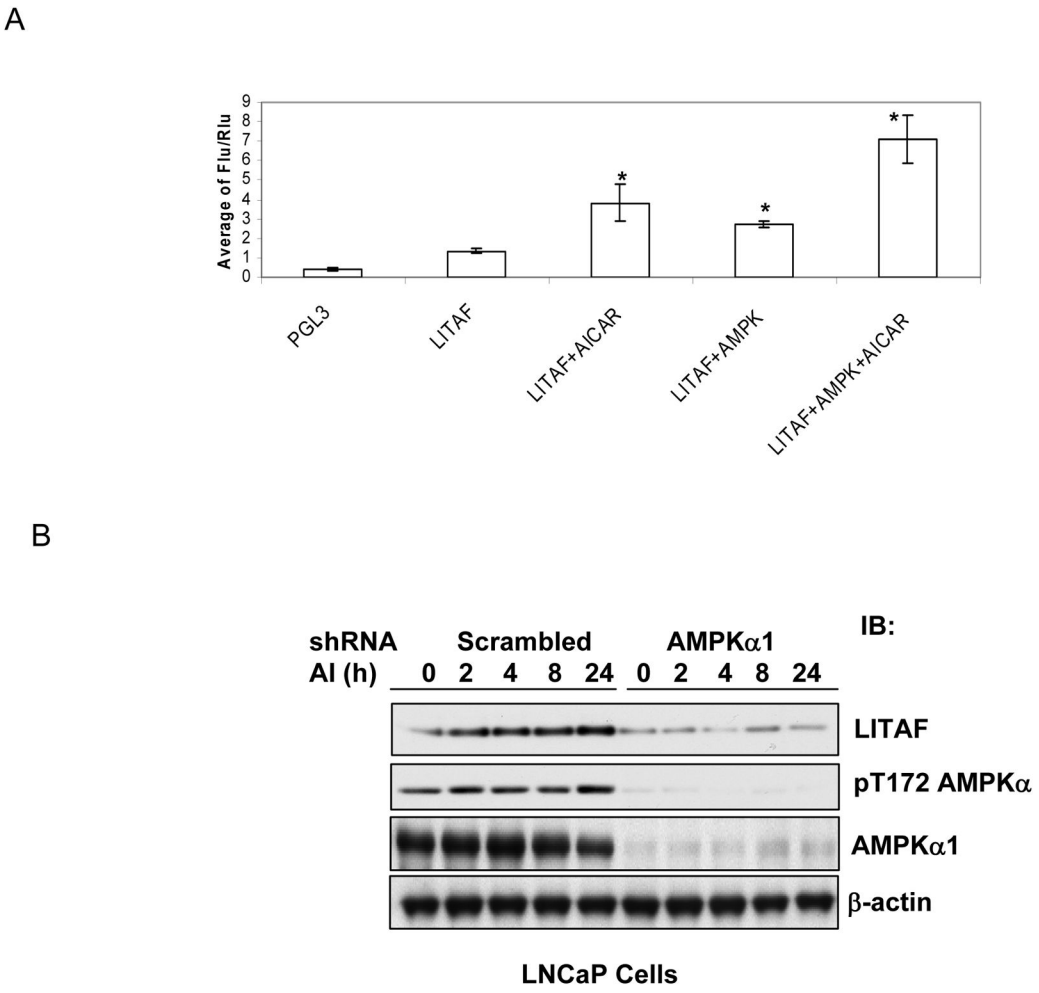

Figure 1. AMPK-dependent induction of LITAF by AICAR

A. Promoter activity assay. LNCaP cells were transfected with a promoter-less fire fly luciferase reporter plasmid (PGL3) or the plasmid with insertion of LITAF promoter region and Renilla luciferase plasmid in the presence or absence of a plasmid encoding wild type AMPK α1 subunit. Two days later, the cells were treated with AICAR for 8 hours and luciferase assay was conducted as described in Materials and Methods. The luciferase activity was normalized with Renilla lucicerase activity and expressed as ratios (Means ± SD, n=3). "*" denotes $P<0.01$ as compared to basal LITAF promoter activity. *B.* LNCaP cells stably infected with α1 shRNA or scrambled shRNA retrovirus were treated with AICAR (AI, 1 mM) for indicated times. Cell lysates were blotted with antibodies, as indicated.

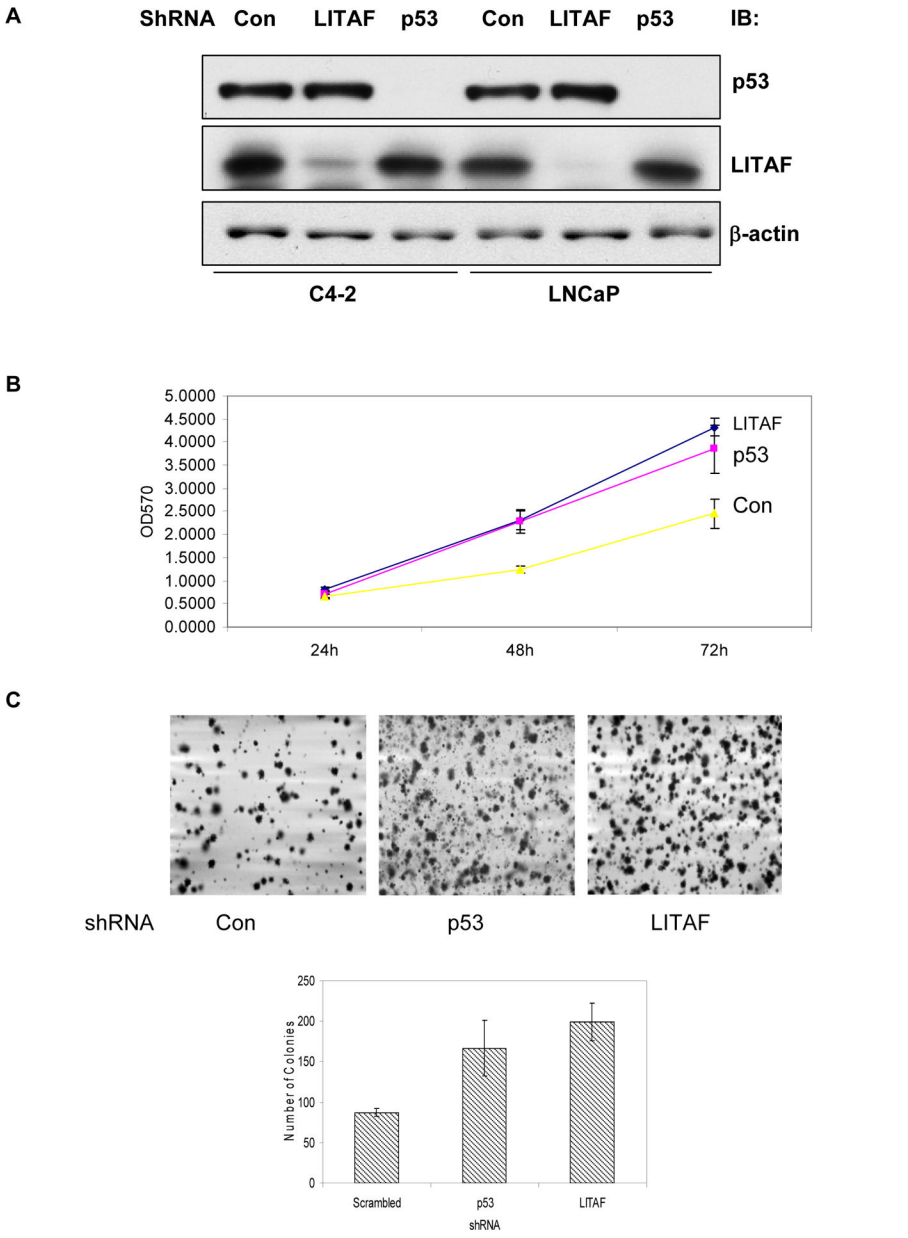

Figure 2. LITAF inhibits malignant phenotype of prostate cancer cells

A. C4-2 and LNCaP cells were stably infected with shRNA for LITAF, p53 and scrambled control (Con) and cell extracts were blotted with antibodies, as indicated. *B.* Proliferation of LNCaP cells carrying LITAF, p53 and scrambled (Con) shRNA was assayed by MTT method. *C.* Anchorage-independent growth of LNCaP cells with different shRNA on soft-agar. One representative of triplicates was shown. The graph represents means ± STDV (Student's t test, $P<0.05$).

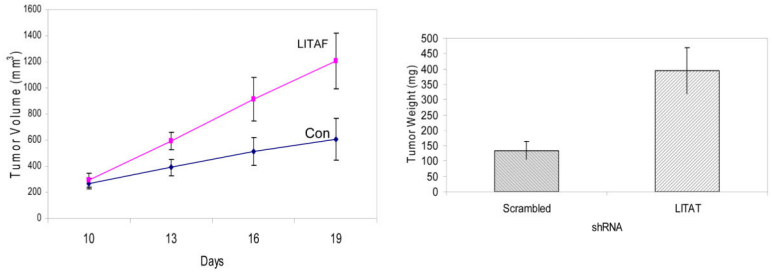

Figure 3. LITAF inhibits tumor growth in xenograft model

LNCaP cells bearing LITAF or scrambled (Con) shRNA were inoculated subcutaneously on the flank of athymic nude mice. Ten days after inoculation, tumors were measured every three days and volumes calculated (Left). At the end of experiment, tumor tissues were removed and weighed. The graph represents average volumes (means ± SE, n=10). Statistic test was conducted using two-way ANOVA method ($P<0.05$). The bars show differences in average weight of tumors in two groups (mean ± SE, Student's t test, $P<0.01$)

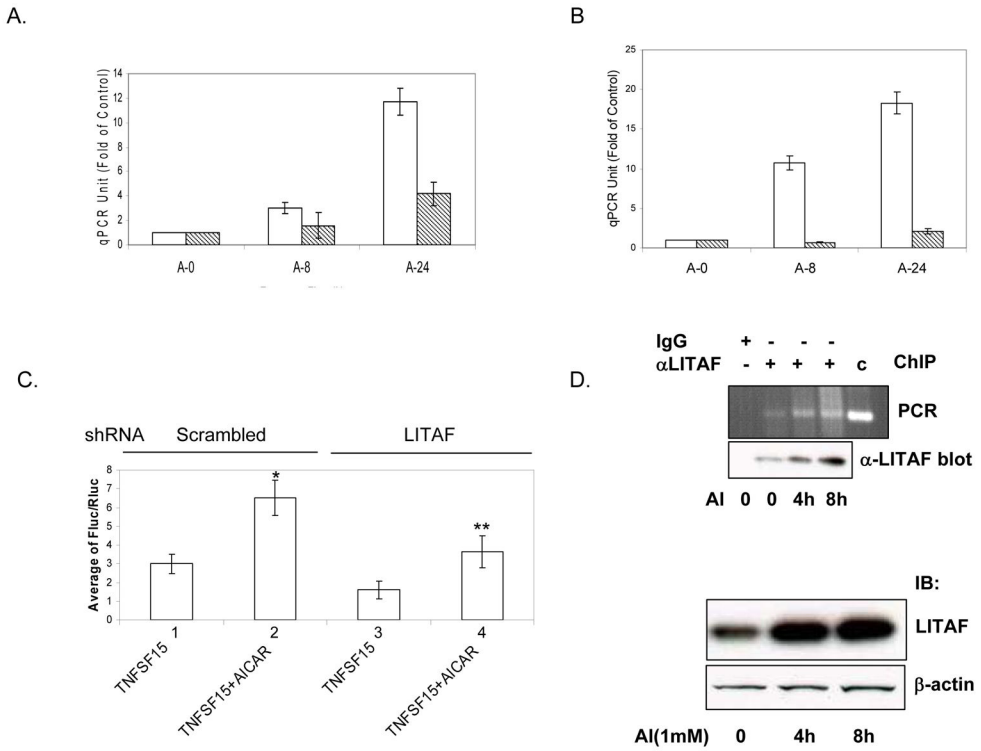

Figure 4. LITAF mediates the effect of AMPK on TNFSF15 expression

A. & B. C4-2 and LNCaP cells bearing LITAF shRNA or scrambled shRNA were treated with or without AICAR (1 mM) for 8 and 24 hours (A-0, A-8, and A-24), respectively. Total RNA was isolated and reverse transcription performed, followed by quantitative PCR. Each sample was assayed in triplicate and normalized as opposed to GAPDH. Units are expressed as fold of control without AICAR treatment. Bars represent mean ± SD (n=6, two-way ANOVA analysis shows $P<0.01$). *C.* LNCaP cells carrying scrambled and LITAF shRNA were transfected with the plasmids containing TNFSF15 promoter-driven fire fly luciferase reporter and Renilla luciferase, and treated with or without AICAR for 8 hours. Luciferase activity was assayed as for Figure 1A. Bars represents means ± SD (n = 4). "*" denotes $P<0.01$ (2 vs 1); "**", $P<0.05$ (4 vs 2). *D.* ChIP assay. LNCaP cells were treated with or without AICAR (1 mM) for indicated times and chromatin immunoprecipitation was performed using control IgG or anti-LITAF antibody as described in Materials and Methods. The immunoprecipitates were analyzed by PCR and resolved on agarose gel electrphoresis. Geneomic DNA was amplified using specific primers as a positive control (C). Aliquots of ChIP and cell extracts were examined by Western blot with antibodies against LITAF and actin.

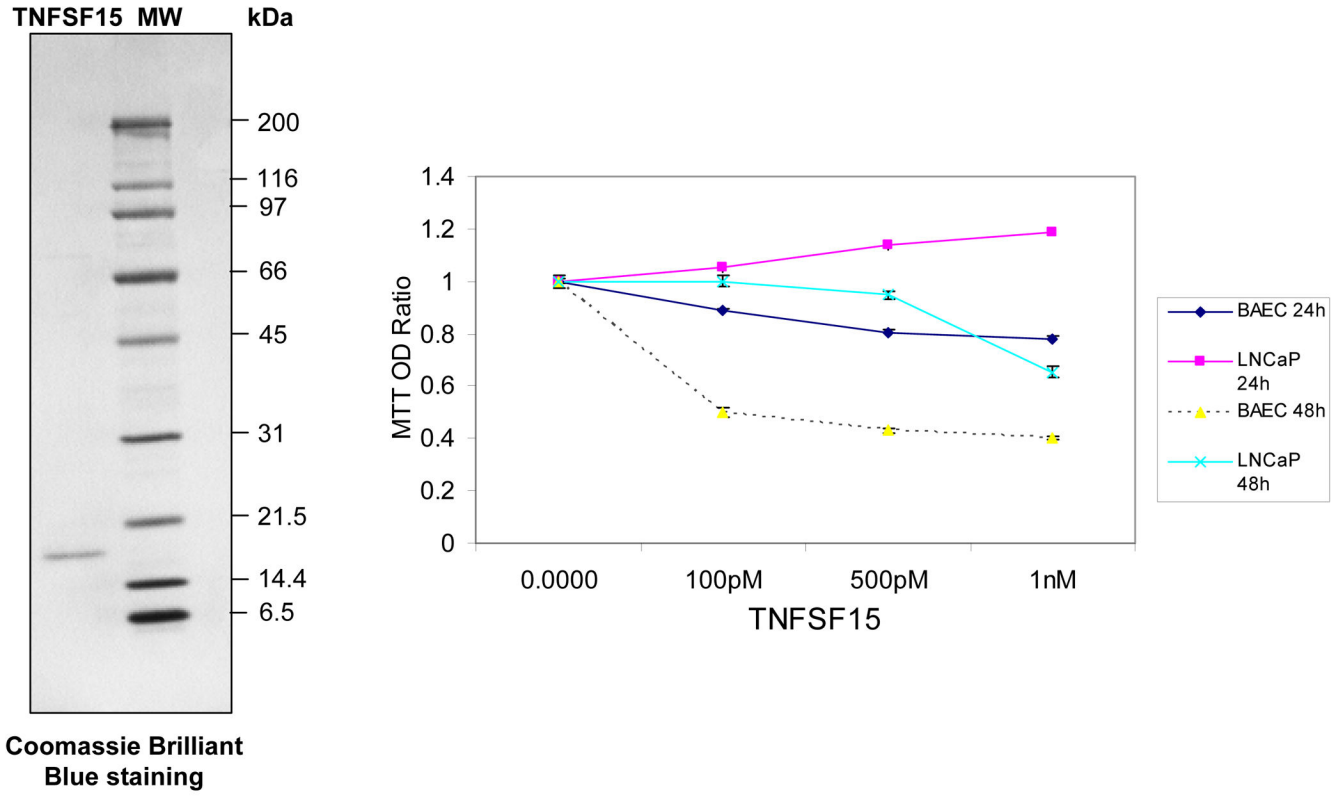

Figure 5. TNFSF15 inhibits cell proliferation in vitro

TNFSF15 was expressed as a fusion protein aminoterminally tagged by maltose-binding protein, purified with amylose agarose and cleaved by Factor X. The purified protein (300 ng) was separated onto SDS-PAGE and stained by Coomassie Brilliant Blue (Left). Recombinant TNFSF15 was added to LNCaP and BAEC cells at different concentrations and cell culture continued for 24 and 48 hours, respectively. The effects were evaluated by MTT assays and expressed as ratio of untreated cells cultured in parallel. The curves represent means ± SD (n=6). Statistic analysis was performed using One-way and Two-way ANOVA methods, respectively ($P<0.05$).

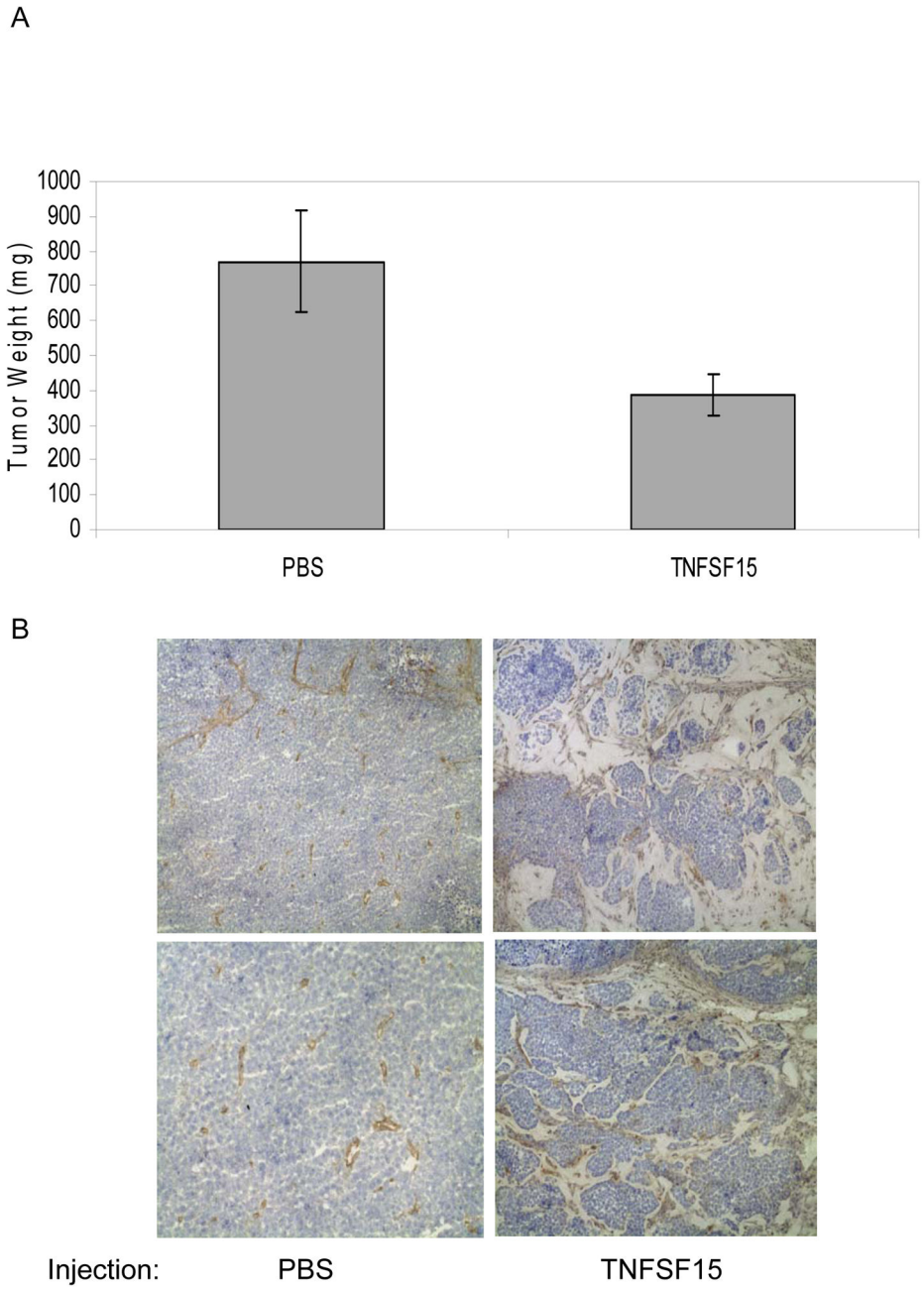

Figure 6. TNFSF15 inhibits tumor growth in xenograft model

A. LNCaP cells were injected into nude mice as described for Figure 3. Ten days after inoculation, intratumor injection of TNFSF15 was performed (10μg/ml, 100 μl for each injection) every two days for two weeks. In the end, mice were sacrificed and tumors removed and weighed. The bars represent average weights ± SE (n=10, Student test, P=0.012953). *B.* Tumor tissues isolated from *A* were snap-frozen and sectioned. Histopathological morphology of tumors and immunohistochemical staining with antibodies against Von Willebrand Factor for visualizing blood vessels were examined. Two representative views from tumors injected with TNFSF15 and PBS are presented. Tumor

tissues of the control animals exhibited uniform and anaplastic structure without evidence of differentiation, while experimental animals exhibited multiple lobular structure surrounded and separated by connective tissue. This structure apparently indicates the likelihood of differentiated tumor. In addition, the number of blood vessels formed in the tumor tissues of the control animals was significantly more than that of the experimental animals.

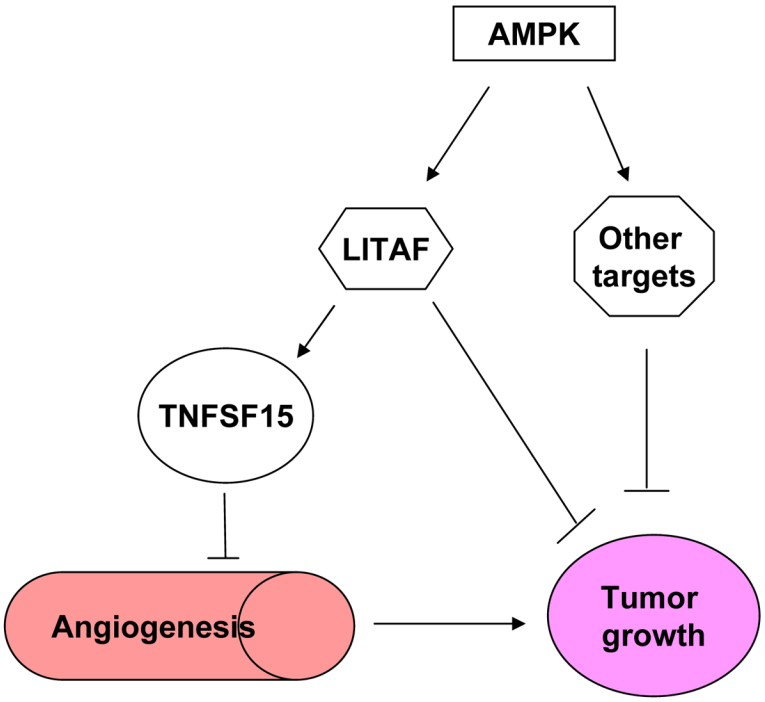

Figure 7. Model of LITAF regulation of tumorigenesis

LITAF mediates the inhibitory effects of AMPK on tumorigenesis by directly regulating TNFSF15, which in turn inhibits angiogenesis, and other unidentified targets.