



Published in final edited form as:

Mol Carcinog. 2019 March ; 58(3): 411–425. doi:10.1002/mc.22938.

Lymphotoxin- β receptor-NIK signaling induces alternative RELB/NF- κ B2 activation to promote metastatic gene expression and cell migration in head and neck cancer

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Abstract

Head and neck squamous cell carcinomas (HNSCC) preferentially spread to regional cervical tissues and lymph nodes. Here, we hypothesized that lymphotoxin- β (LT β), receptor LT β R, and NF- κ B-inducing kinase (NIK), promote the aberrant activation of alternative NF- κ B2/RELB pathway and genes, that enhance migration and invasion of HNSCC. Genomic and expression alterations of the alternative NF- κ B pathway were examined in 279 HNSCC tumors from The Cancer Genome Atlas (TCGA) and a panel of HNSCC lines. LT β R is amplified or overexpressed in HNSCC of the larynx or oral cavity, while LT β , NIK, and RELB are overexpressed in cancers arising within lymphoid oropharyngeal and tonsillar sites. Similarly, subsets of HNSCC lines displayed overexpression of LT β R, NIK, and RELB proteins. Recombinant LT β , and siRNA depletion of endogenous LT β R and NIK, modulated expression of LT β R, NIK and nuclear translocation of NF- κ B2(p52)/RELB as well as functional NF- κ B promoter reporter activity. Treatment with a NIK inhibitor (1,3[2H,4H]-Iso-Quinoline Dione) reduced the protein expression of NIK and NF- κ B2(p52)/RELB, and blocked LT β induced nuclear translocation of RELB. NIK and RELB siRNA knockdown or NIK inhibitor slowed HNSCC migration or invasion *in vitro*. LT β -induces expression of migration and metastasis related genes, including hepatocyte growth/scatter factor receptor MET. Knockdown of NIK or MET similarly inhibited the migration of HNSCC cell lines. This may help explain why HNSCC preferentially migrate to local lymph nodes, where LT β is expressed. Our findings show that LT β /LT β R promotes activation of the alternative NIK-NF- κ B2/RELB pathway to enhance MET-mediated cell migration in HNSCC, which could be potential therapeutic targets in HNSCC.

Keywords

LT β /LT β receptor; NIK; RELB/NF- κ B2; migration; HNSCC

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Conflict of Interest Statement: The authors have declared that no conflict of interest exists.

1 Introduction

Head and neck squamous cell carcinoma (HNSCC) arises from epithelia of the upper aerodigestive tract, with an annual incidence of ~40,000 new cases, and affects over 300,000 survivors in the U.S. (1-3). Regional cervical spread and lymph node metastasis is a hallmark of HNSCC, and increased cell migration is an early and crucial event. Accordingly, many HNSCC tumors are first identified from lymph node metastasis, which is an important predictor of worse prognosis in HNSCC, decreasing survival by ~50% (4). Few investigations have addressed the molecular and biological mechanisms underlying cell migration of HNSCC. It is not very clear which factors and pathways promote migration of the primary tumor cells to lymph nodes, where the tumor cells gain growth and survival advantages.

Lymph nodes are secondary lymphoid organs, containing T and B lymphocytes and other immune cells. Many immune regulatory cytokines are produced in the lymph nodes, which nurture and modulate immune cell proliferation, maturation, migration, and defense functions. Among these, Lymphotoxin- β (LT β) is one of the crucial cytokines produced by lymphocytes and lymphoid tissues (5-7). LT β is an anchored transmembrane protein of 244 aa (18.5KDa), that can form soluble heterotrimers (LT α 1 β 2, or LT α 2 β 1), which are shed from the cell surface by proteolytic cleavage. LT β heterotrimer LT α 1 β 2 is capable of binding to receptor LT β R, which mediates signaling when internalized, and is then resynthesized (8). LT β and LT β R play a critical role in controlling lymphoid organ development and facilitating efficient immune and inflammatory responses (6, 9-11). Interestingly, LT β R is implicated in the migration of T cells and neutrophils (12, 13), a feature deregulated in both hematopoietic and solid malignancies. Further, LT β R is not only expressed in lymphoid cells, but also in a wider range of normal tissues and tumor types, including many solid tumors (14, 15). However, the role of LT β /LT β R mediated signaling and its involvement in HNSCC migration and other features of the malignant phenotype have not been characterized.

It has been well established that LT β /LT β R signaling mediates activation of NF- κ B inducing kinase (NIK), Inhibitor- κ B Kinase- α (IKK α), and transcription factors NF- κ B2/RELB in cells of the hematopoietic lineage and lymphoid system (16) (17). The NF- κ B family contains five members, NF- κ B1 (p105/p50) and NF- κ B2 (p100/p52), RELA (p65), RELB, cREL. NF- κ B1, RELA, are mainly involved in the canonical NF- κ B pathways, while NF- κ B2 and RELB participate in the non-canonical alternative NF- κ B pathway (18, 19). Upon signaling from LT β /LT β R, the kinase function of NIK is essential for IKK α induced NF- κ B2/p100 processing to p52, which forms a complex with RELB that translocate to the nucleus to activate transcription of target genes (20, 21). Our laboratory previously identified the aberrant nuclear activation of canonical and alternative NF- κ B/REL family members in HNSCC cells and tissues (22, 23). We and others previously documented that activation of NF- κ B can modulate cancer-related inflammation and promote phenotypic alterations, such as cell proliferation, apoptosis evasion, therapeutic resistance, migration and metastasis (18, 22, 24, 25). However, the mechanism and role of LT β and activation of the alternative NF- κ B pathway in the malignant phenotype of HNSCC remain unknown.

Here, we hypothesized that $LT\beta/LT\beta R$ mediated signaling can activate alternative pathway and $NF-\kappa B2/RELB$ nuclear activation and promote migration of HNSCC. We discovered that $LT\beta$ and $LT\beta R$, NIK and RELB were overexpressed in subsets of HNSCC patient tissues and cell lines. In the HNSCC cell lines, $LT\beta$ induced activation of the alternative $NF-\kappa B$ pathway through NIK. NIK inhibitor or siRNA reduced protein expression of NIK, IKK α , RELB, and $NF-\kappa B2$, and blocked RELB and $NF-\kappa B2$ nuclear localization. Knockdown of NIK or RELB inhibited cell migration, and $LT\beta$ induced expression of genes implicated in metastasis and migration, including MET, which we previously showed promotes migration and metastasis of SCC (26). Our data revealed a novel role of $LT\beta$ mediated NIK activation of the alternative $NF-\kappa B$ pathway in expression of MET, which contributes to migration of HNSCC. Inhibition of migration by a NIK inhibitor provided evidence for the feasibility of therapeutic targeting of the alternative pathway. These signaling molecules involved in the alternative $NF-\kappa B$ pathway warrant further investigation as prognostic markers and therapeutic targets for HNSCC.

2 Materials and Methods

2.1 Cell lines and culture condition

A panel of 10 HNSCC cell lines was obtained from the University of Michigan squamous cell carcinoma (UM-SCC) series from Dr. T.E. Carey and cultured under standard growth conditions in MEM media with 10% FBS, penicillin and streptomycin (100 μ g/mL) and 1% L-glutamine. Authentication of UM-SCC and other lines was done at the University of Michigan by DNA genotyping of alleles for 9 loci, D3S1358, D5S818, D7S820, D8S1179, D13S317, D18S51, D21S11, FGA, vWA, and the amelogenin locus in 2015, as described (27). These cell line stocks were used within 3 months of thawing and passage. UM-SCC 1 $NF-\kappa B$ Blazer reporter stable cell line, which contain stably expressed $NF-\kappa B$ transcription factor response elements upstream of the β -lactamase reporter gene, was established in our laboratory (28). This cell line is maintained in standard growth conditions supplemented with 10% FBS, penicillin and streptomycin (100 μ g/mL), 1% NEAA, and Blasticidin (5 μ g/ml). All culture media, amino acids solutions, and antibiotics were purchased from Life Technology/Thermo Fisher Scientific. Cell DNA was sent for genotyping in 2008 and fall 2010 to compare and verify their unique origin from original stocks, as recently described (27). Human primary oral keratinocytes (HOK) from oral gingival mucosa were purchased from Science Cell Research laboratories and used as a control cell line. The cells were cultured in serum free Oral Keratinocyte Medium with supplements (Science Cell) for less than 4 passages.

2.2 Protein isolation and Western blot

The UM-SCC 46 cells were plated in 6 well plates at 3×10^5 cells per well or in 10 cm dishes at 1×10^6 cells per plate. Cells were treated with NIK inhibitor 1, 3[2H, 4H]-Isoquinolinedione (AK Scientific, Inc) (29, 30) for 24 hours starting from 500nM, 10 μ M and 25 μ M or with $LT\beta$ ($LT\alpha_1\beta_2$, 100ng/ml, R&D Systems, CAT# 678-LY-010) stimulation for 4 hours and 24 hours. After 48 to 72 hours whole cells were harvested using NP40 lysis buffer (Life Science Technology) supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). Nuclear and cytoplasmic proteins were isolated using

the Active Motif Kit (Active Motif). Proteins were quantitated by the Pierce BCA Protein Assay Kit (Life Technology). The equal amounts of lysates mixed with 5µl loading dye were run on NuPage® pre-cast 4-12% gradient Bis-Tris gel in MOPS SDS running buffer after heat denaturation. Samples were transferred for 6 minutes to nitrocellulose membranes using the Invitrogen iBlot system, according to the manufacturer's protocol. Membranes were blocked for one hour in milk blocking buffer and incubated with primary antibodies overnight at 4°C. The antibodies were visualized with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (Santa Cruz Biotechnology) using enhanced chemiluminescence (Amersham and Thermo-scientific). Protein quantification was done by protein densitometry normalized to β -tubulin or actin with ImageJ 1.45k software. The following primary antibodies were used for Western blots: LT β R polyclonal antibody (Novus), NIK polyclonal antibody (Cell Signaling, Abcam), RELB monoclonal antibody (Cell Signaling), and NF- κ B2/p52 monoclonal antibody (Millipore) and phospho-IKK α (Ser176) monoclonal antibody (Cell Signaling).

For testing NIK antibody's specificity using the blocking peptide, UM-SCC 1 cell cytoplasmic lysates were prepared using a Nuclear Extraction Kit from Active Motif (Carlsbad, CA), and the proteins were quantitated using BCA assay. Duplicate samples of 15µg cytoplasmic proteins were loaded on Nu Page® pre-cast 4-12% gradient Bis-Tris gel after heat denaturation. After running, samples were transferred to Nitrocellulose membrane using the Invitrogen iBlot system according to the manufacturer's protocol. Membrane was split in the middle to produce two identical blots. The NIK antibody (Abcam, ab19144, 0.5µg/ml) was pre-incubated with the blocking peptide (Abcam, ab8385 1µg/ml) or control buffer solution with agitation overnight at 4°C. Then the NIK antibody with or without blocking peptide were incubated with the membrane containing protein lysates overnight at 4°C, followed the standard Western blot protocol.

2.3 RNA isolation and qRT-PCR

For RNA isolation, cells were harvested using Trizol reagent (Life Technologies). The RNeasy Mini Kit from Qiagen (Santa Clarita, CA) was used for RNA isolation based on manufacturer's instructions. cDNA synthesis was performed on a GeneAmp® PCR system 9700 machine using the High-capacity cDNA Reverse Transcription Kit from Applied Biosciences (Foster City, CA) according to manufacturer's instructions. Quantitative Real-time PCR was performed using TaqMan assay kit (Applied Biosystems). TaqMan Gene Expression RT-PCR primers includes *LT β R*, *NIK*, *MET*, *BIRC3*, and *SERPINE 1*, were synthesized by Applied Biosystems. 30 ng of cDNA combined with primers and TaqMan Universal Master Mix (Applied Biosystems). The reactions were run in ViiA7 (AB Applied Biosystems). Relative gene expression was normalized to 18S endogenous control. Samples were assayed in triplicate and data are presented as the mean \pm standard deviation. Statistical Significance was determined using the student's t-test and *p* values < 0.05 were considered statistically significant.

2.4 Gene knockdown by siRNA

siGENOME™ SMART pool siRNAs for LT β R, NIK and MET or non-targeting siRNA controls were purchased from IDT (Coralville IA, USA) and Dharmacon (Lafayette, CO,

USA), and Ambion respectively. Individual siRNAs were tested and three duplexes of siRNAs with best knockdown efficiency and specificity for each target gene were selected. Cells were plated one day before transfection, and then transfected with transfection reagent alone as a control for nonspecific siRNA effects, or with 50 nM (Dharmacon) or 5 nM (IDT) of each siRNA, individually or in combination. Transfections were performed with Lipofectamine® 2000 or Lipofectamine® RNAiMAX transfection reagent, and Opti-MEM® reduced serum medium according to manufacturer's instructions (Life Technologies). Cells were harvested at 48, 72, and 96 hours after transfection or with treatment of LTβ (100ng/ml) in respective wells 24hours before harvesting (31).

2.5 Reporter gene assay

UM-SCC 1 NF-κB Blazer reporter stable cell line was established by stable transfection of NF-κB reporter construct (gene blazer) and sorted in responding to TNF-α (28). Cells were plated in 96-well plates one day before transfection. β-lactamase reporter system (Life Technologies) was used to measure the LTβR and NIK knockdown effect on the NF-κB function by measuring the β-lactamase activity, which was recorded at 96 hours after siRNA transfection, with 24hours LTβ (100ng/ml) treatment before adding substrate (32). All measurements represent the mean of 6 replicates in each experimental condition.

2.6 Immunofluorescent microscopy

UM-SCC 46 cells were plated in Lab-Tek^R II chamber slide (Life Technologies) at 15,000 cells per well in 500μl complete media. Upon achieving 70 to 80% cell confluent, NIK inhibitor (1, 3[2H, 4H]-Isoquinolinedione) was added to individual wells followed by LTβ (100ng/ml) stimulation for another 4 or 12hours. Cells were then fixed using ice cold methanol for 15minutes, and permeabilized on ice (0.5% Triton X-100 and 0.05% SDS). Then cells were blocked on ice for 1 hour using blocking solution (0.1% Tween-20 and 3% BSA). Anti-RELB antibody (Santa Cruz) or Anti- NIK antibody (abcam) was added to each well at 1:100 dilution and incubated for 1 hour at room temperature. Cells were incubated with AF-594-linked IgG (1:1000 dilution) for 45 minutes in dark. The slides were mounted with DAPI VECTASHIELD mounting media, and were visualized on LSM 780 confocal microscope. Confocal images were analyzed using Zen 2012 SP1 software (black and blue editions).

2.7 Migration assay

UM-SCC 1 NF-κB Blazer reporter stable cells were seeded at 4×10^5 cells/well in 6-well plates and transfected with 100 nM siRNAs (Dharmacon) against NIK, RELB and MET alone, or in combination of NIK plus MET. Forty-eight hours later, scratches were made on the cell monolayers. For NIK inhibitor cells were treated for 24hours by adding NIK inhibitor at different concentrations after the cells reached 70–80% confluency. Wound closure was monitored at 0, 12, and 24 hours on an EVOS microscope (Life Technologies). Wound healing was quantitated by ImageJ 1.45k software (33) and plotted as a function of time.

2.8 Invasion Assay

QCM TM 24- Well kit (Fluorometric) ECM 554 used. UMSCC 1 cells that has been passaged 2-3 times and that are 80% confluent starved with serum free media for 24 h and then used for the invasion assay in presence and absence of FBS, LTB and inhibitor according to the protocol as directed in the Kit. Fluorescence measured in Synergy 2 fluorescence plate reader using 480/520 nm filter set at gain setting 65.

2.9 Statistical Analysis

Data were presented as mean \pm standard deviation and significance was determined using the Student's t-test p values of less than 0.05 were considered statistically significant.

3 Results

3.1 Overexpression and genetic alterations of LT α / β , LT β R, NIK (MAP3K14), and RELB in HNSCC tissues and cell lines

We previously showed that canonical and alternative NF- κ B/REL subunits display aberrant nuclear activation in HNSCC tumors and cell lines (22, 23). However, the genomic status and expression of upstream LT β /LT β R, NIK (MAP3K14) signal and RELB transactivating components of the alternative NF- κ B pathway in HNSCC tumors has not been established. We explored if there are significant genomic and expression alterations of LT β /LT β R, NIK, and RELB, using datasets from the HNSCC TCGA project comprising 279 human HNSCC cases (34). In Figure 1A, we present the oncoprint for individual HNSCC tumors (individual bars) with primary tumor sites labeled on the top, and those tumors displaying homozygous genetic and expression alterations of LT β R, LT α , LT β , NIK, and RELB genes, below. Among 279 samples surveyed, 65 cases (23%) exhibited alterations in these molecules, mainly showing gene amplification and/or increased mRNA expression. The highest percentage of cases displayed alterations of LT β R (13%), with amplification and overexpression mainly seen in HNSCC from larynx and oral cavity. Alternately, increased mRNA expression of LT α (6%) and LT β (5%) lymphotoxin subunits, as well as NIK (5%) and RELB (6%) were often co-occurrent ($p < 0.05$, Fisher's exact test), and observed mainly in oropharyngeal /tonsillar cancers enriched for lymphoid tissues. Interestingly, patients carrying amplification and overexpression of LT β R are mutually exclusive from those bearing the alterations of other molecules involved in the alternative NF- κ B pathway ($p < 0.0001$, Fisher's exact test, Figure 1A). Furthermore, the association between RNA expression and DNA copy number variation (CNV) for all of the above mentioned molecules was examined, and a highly significant correlation was found for CNV and expression of LT β R and RELB (Figure 1B). Thus, our data provide evidence for genomic alterations and/or increased expression affecting LT β R, LT α , LT β , NIK, and/or RELB in HNSCC tumor tissues. We next surveyed the relative protein expression of alternative pathway signaling components LT β R, NIK and RELB in a panel of HNSCC (UM-SCC) cell lines and primary human oral keratinocytes (HOKs). Several UM-SCC cell lines showed increased LT β R, NIK, and/or RELB protein expression as compared to HOKs (Figure 1C, Supplemental Figure 1). Among these, two cell lines, UM-SCC 1 and 46, were selected for further studies.

3.2 LT β and NIK promoted RELB and NF- κ B2 protein nuclear translocation and induced NF- κ B reporter activity

Next, we examined the effects of classical and alternative pathway ligands TNF- α and LT β to stimulate NF- κ B subunits in UM-SCC 46 cells, which displayed increased protein expression of LT β R, NIK and RELB (Figure 1C). TNF- α induced rapid and strong activation of canonical pathway subunits RELA or cREL within one hour, as well as slower induction of alternative pathway subunit RELB over 3-4 hours (Figure 2A, Supplemental Figure 2), consistent with prior reports that RELA can secondarily induce RELB expression and nuclear translocation respectively (22). In contrast, LT β , which is a major upstream signal for the alternative NF- κ B pathway in lymphoid tissues, did not strongly modulate canonical RELA/c-REL subunits, but selectively promoted a gradual increase in nuclear translocation of RELB and NF- κ B2(p52) (Figure 2B, Supplemental Figure 3).

To study the functional effects of endogenous LT β R on NF- κ B activation, we performed gene knockdown of LT β R by siRNA, and verified decreased LT β R mRNA between 48h-96h in the UM-SCC 46 cell line (Supplemental Figure 4A). We observed decreased LTBR protein expression by 96h after transfection (Figure 2C). Knockdown of LT β R decreased downstream alternative pathway NIK, RELB, and processed p52 proteins (Figure 2C, Supplemental Figure 5A). We next confirmed the effects of NIK siRNA knockdown, which significantly inhibited NIK mRNA between 48h-96h in UM-SCC 46 cells (Supplemental Figure 4B). To confirm the siRNA knockdown and LT β stimulation effects in a second HNSCC cell line, we selected UM-SCC 1 cells, which displayed increased expression of the alternative pathway components (Figure 1C), and for which we have established a stable NF- κ B reporter cell line. We examined the effects of LTBR or NIK siRNA on their protein levels without or with LT β stimulation in UM-SCC 1 cells. Knockdown LTBR by siRNA decreased LTBR, and NIK protein levels, when normalized for protein loading (Figure 2D, Supplemental Figure 5B). Knockdown of NIK protein was also associated with decreased LTBR protein expression (Figure 2D, Supplemental Figure 5B). In addition, NIK gene knockdown significantly decreased LT β induced NIK mRNA expression (Supplemental Figure 4C), and RELB gene knockdown significantly decreased basal and LT β induced RELB gene expression in UM-SCC 46 cells (Supplemental Figure 4D).

We next examined the effects of LT β stimulation on functional NF- κ B reporter activity after LT β R or NIK knockdown in the UM-SCC 1 NF- κ B reporter line. LT β treatment significantly increased the reporter activity in cells, and knockdown of LT β R or NIK reduced NF- κ B reporter activity induced by LT β stimulation (Figure 2E, Supplemental Figure 5C). Thus, we observed that LT β modulates signals through alternative pathway proteins LT β R, NIK, RELB and NF- κ B2 p52, and NF- κ B reporter activity, in independent HNSCC cell lines.

3.3 NIK inhibitor (1, 3[2H, 4H]-Isoquinolinedione) decreased NIK protein and components of the alternative NF- κ B pathway

Next, we examined the effects of the NIK inhibitor (1,3[2H,4H]-Isoquinolinedione) over a range of concentrations on the downstream components of the alternative NF- κ B pathway in UM-SCC 1 cells. We tested the NIK inhibitor in this cell line, because we have been

successfully established cell migration assay and invasion assay using this adherent cell line. NIK inhibitor significantly inhibited NIK, p-IKK α , RELB, and NF- κ B2/p100, and p52 protein expression in the cytoplasm in a dose dependent fashion (Figure 3A). After treatment, the NIK inhibitor partially reduced expression of all of the proteins examined at the dose of 0.5 μ M, and completely blocked p-IKK α and RELB protein expression at the doses of 10 μ M and 25 μ M (Figure 3A). In addition, significant reductions in nuclear protein expression of both RELB and NF- κ B2/p52 were observed by the treatment of NIK inhibitor at different dosages (Figure 3B, Supplemental Figure 6A). As phosphorylation of NIK by IKK α has been reported as a doublet as observed in Figure 3A, we confirmed the NIK antibody specificity for both bands using a NIK-specific blocking peptide. A loss of the double bands of NIK protein was detected by the antibody after incubation with the blocking peptide (Supplemental Figure 6B). We next examined the effect of NIK inhibitor under LT β stimulation for 4h and 24h (Figure 3C). LT β stimulation increased both RELB and NF- κ B2/p52 in nuclear fractions, while more abundant nuclear RELB and NF- κ B2/p52 proteins remained when treated with both the NIK inhibitor and LT β stimulation (Figure 3C). Our data shows that NIK inhibitor decreased the levels of NIK and downstream proteins involved in the alternative NF- κ B pathway, while LT β stimulation partially diminished the effects of the NIK inhibitor on RELB and NF- κ B2/p52 nuclear protein. In these experiments, we used Lamin A as the protein loading control for the nuclear fraction, and did not observe significant effects of NIK inhibitor on Lamin A protein expression.

3.4 NIK inhibitor decreased NIK and RELB expression and cytoplasmic/nuclear localization

To further confirm the effects of the NIK inhibitor and LT β on NIK and RELB expression and cytoplasmic/nuclear localization in another model, we treated UM-SCC 46 cells with the NIK inhibitor for 24 hours, or plus LT β stimulation for 4 hours, and performed immunofluorescent staining using antibodies against NIK and RELB (Figure 4). The concentration range of the NIK inhibitor at 0.5 and 1 μ M in this assay was based on effects observed in the previous titration (Supplemental Figure 6A). 1 μ M NIK inhibitor decreased the diffuse staining pattern of NIK in the perinuclear cytoplasm by immunofluorescence (Figure 4A, left panels; 4B, upper panel), LT β stimulation for 4 hours attenuated this inhibitory effect on NIK staining (Figure 4A, right panels, and 4B; lower panel), consistent with stabilization of NIK. RELB staining exhibited diffuse punctate nuclear staining pattern, while decreased RELB protein expression were observed when treated by NIK inhibitor at 0.5 μ M (Figure 4C, D). A strong inhibition of co-localized RELB staining with nuclear DAPI staining was observed, supporting an effective blockage of RELB nuclear localization and activation (Figure 4E, F). Together, these data show that the NIK inhibitor blocked NIK in the perinuclear cytoplasm and inhibited RELB nuclear localization, while LT β stimulation enhanced NIK and partially reversed these effects.

3.5 Knockdown of NIK and RELB inhibited cell migration in the HNSCC cells.

To explore the functional roles of alternative NIK signaling and RELB, we knocked down NIK or RELB individually, and examined their effects on cell migration as measured by wound closure in UM-SCC 1 cells. The assay examined cell migration as the wound closure for 12 and 24 hours after scratching a gap in the confluent monolayers. Knockdown of NIK

(Figure 5A, B) or RELB (Figure 5 C, D) individually suppressed migration and closure. This effect was not attributable to anti-proliferative activity, as we did not observe significant effects on cell proliferation 24 hours after siRNA knockdown of LTBR and RELB, when measured by WST1 assay (Supplemental Figure 7A). We further analyzed cell migration affected by NIK inhibitor, and observed a dose dependent inhibitory effect on UM-SCC 1 cell migration (Figure 5E). The inhibitory effect on the cell migration was not due to the drug suppression of cell proliferation, as we have titrated the NIK inhibitor and showed there is no inhibitory effects on cell proliferation from day 1 through day 3 using the doses up to 20 μ M (Supplemental Figure 7B). Furthermore, we performed an invasion assay using UM-SCC 1 cells. While the cell migration/wound closure assay is to examine the collective cell migration in two dimensions, the invasion assay is using the transwell to analyze the ability of single cells to directionally respond to various chemo-attractants and migrate through a physical barrier and invade into a 3D matrix (matrigel). In this invasion assay, we observed that 10% FBS as the chemo-attractant, significantly increased cell invasion, while NIK inhibitor suppressed basal and FBS induced cell invasion (Figure 5F). Compared to FBS, LT β alone only slightly enhanced invasion, indicating that serum may contain additional factor(s) that promote invasion. Thus, our data from NIK siRNA and inhibitor, as well as RELB siRNA, support the hypothesis that NIK and RELB activate the NF- κ B alternative pathway and promote cell migration and invasion.

3.6 LT β and NIK modulated expression of survival and metastasis genes, and knockdown of NIK and MET by siRNA inhibited cell migration

We previously identified NF- κ B modulated genes that promote cell survival and migration in HNSCC (35-37). Of these, pilot oligonucleotide expression array screening for genes modulated by LT β , identified BIRC3 (inhibitor of apoptosis 2, IAP2), PLAUI and SERPINE1 proteinases, and MET receptor (38). However, how LT β and NIK are involved in the regulation of expression of these genes has not been studied. Using quantitative RT-PCR, we confirmed the time course observed by expression profiling for these 4 genes in UM-SCC 46 cells (Figure 6A). Accordingly, LT β treatment induced more than a 20-fold increase of *BIRC3* expression by 6 hours. Induction of *PLAU* is relatively more rapid, peaking at one hour after treatment, while *SERPINE1* and *MET* expression steadily increased between 6 hours and 24 hours.

Since knockdown of NIK and RELB suppressed cell migration, we asked whether the activation of alternative NF- κ B signaling molecules modulated gene expression involved in cell survival and migration. We showed that LT β stimulation induced gene expression of *NIK*, *BIRC3*, *SERPINE1*, and *MET* in an independent experiment using UM-SCC 46 cells (Figure 6B), consistent with the observations in Figure 6A. Knockdown of NIK by siRNA completely blocked LT β induced *NIK* and *MET* expression, while partially inhibiting LT β induced *SERPINE1* and *BIRC3* gene expression (Figure 6B). Interestingly, MET is an hepatocyte growth factor receptor implicated in promoting cell migration, and we previously showed that MET overexpression promotes migration and scattering of murine SCC cells (26). In this experiment, we knocked down either NIK or MET alone, or in the combination. We found that knockdown of either gene individually significantly decreased cell migration in UM-SCC 1 cells, while the knockdown both genes together did not exhibit additive

effects (Figure 6 C and D), consistent with MET being a target of the LT β -NIK pathway. Together, our data shows that LT β and NIK promote *MET* gene expression, and *NIK* and *MET* knockdown have similar inhibitory effects on cell migration.

4 Discussion

Most previous studies related to LT β /LT β R signaling are in lymphocytes and lymphoid tissues, where LT β /LT β R play critical roles in the communication between lymphocytes and stromal cells during lymphoid organogenesis (6, 7, 10), and in host defense for infectious, inflammatory, and autoimmune diseases (6, 11, 15, 18). Aberrant activation of LT β /LT β R signaling and the effects on the malignant phenotype have not been well studied in cancers, especially in solid tumors. In this study, we provide evidence that genetic and expression alterations of signaling molecules involved in the alternative NF- κ B pathways, including LT β /LT β R, NIK and RELB, occur in HNSCC tissues and cell lines (Figure 1, 2). In HNSCC, LT β /LT β R is an upstream modulatory signal for activation of NIK and alternative NF- κ B subunits RELB and NF- κ B2/p52 (Figure 2). Knockdown of LTBR and NIK by siRNA or a chemical NIK inhibitor diminished activation of the alternative NF- κ B pathway (Figure 2-4, Supplemental 4-6), inhibited cell migration and invasion, and suppressed expression of the MET gene involved in cell migration (Figure 5, 6, Supplemental Figure 8). Our results revealed a novel mechanism whereby LT β /LT β R signaling mediated activation of the alternative NF- κ B pathway promotes migration and other mediators implicated in pathogenesis of HNSCC. The finding that LT β activation of the alternative pathway promotes expression of MET and migration, could help explain why and how HNSCC preferentially spread to local lymph nodes, where LT β is abundantly expressed by lymphoid cells (26, 39, 40).

In HNSCC tumor samples from the TCGA datasets, we observed tissue site-specific alterations of the signaling molecules involved in the alternative NF- κ B pathway. LT β R genomic amplification at the chromosome 12p13.3 locus and/or its overexpression occurs in ~13% HNSCC cases, particularly those arising from larynx and oral cavity, which are mostly HPV (-). LT β R and RELB exhibited significant correlations between CNV and mRNA expression, which support a genetic basis as a contributing factor for the activation of alternative NF- κ B signaling pathway in these HNSCC (Figure 1B). In contrast, other molecules, such as LT ligands, NIK and RELB are amplified and/or overexpressed mainly in oropharyngeal tumors, which are enriched for HPV (+) HNSCC, and tonsil tissue containing lymphoid cells. Consistent with this, we observed aberrant nuclear immunostaining of NF- κ B2/RELB in a series of predominantly oropharyngeal tumors (23). These tissues provide a microenvironment rich in lymphokines and cytokines, which could induce the overexpression of LT ligands, NIK and RELB genes. Interestingly, frequent LT β R gene amplification was also previously found in nasopharyngeal carcinoma (NPC), a distinct type of head and neck cancer associated with infection with Epstein-Barr virus (EBV) that was not represented in TCGA (41). NPC also often first presents clinically as swollen lymph nodes in the neck, and the tumors arise in a microenvironment heavily infiltrated with lymphoid cells. Together, these observations suggest that either amplified or overexpressed LT β R, as well as LT ligands, NIK and RELB, could contribute to aberrant activation of the alternative NF- κ B signaling in different HNSCC subtypes.

Consistent with TCGA tumor tissue data, several HNSCC cell lines studied here exhibited increased protein expression of LT β R, NIK and RELB compared with HOK cells (Figure 1C). LT β R protein is profoundly increased in UM-SCC 1, 11B, 38, and 46 lines, which are derived from oral cavity or larynx, consistent with data in HNSCC tissues from TCGA datasets (Figure 1A, 1C). Furthermore, overexpression of NIK and RELB protein were observed in most of cell lines, indicating the broader downstream alterations affecting these HNSCC cell lines. We validated the significance of these results in two cell lines, using UM-SCC 46 and UM-SCC 1 cells in siRNA knockdown, NIK inhibitor, Western blot and/or reporter gene analyses. The results presented here are also consistent with our previous publications demonstrating the effects of LT β and expression and function of IKK α and IKK β functional mutants on alternative and classical pathway activation in UM-SCC 1 (22, 42), and LT β and TRAF3 in HPV(+) line UM-SCC 47 (43). In those studies, IKK α and TRAF3 were also shown to modulate alternative pathway subunits as well as migration.

These tumor cell lines reflect similar alterations in expression of alternative pathway components observed in human HNSCC tissues, and provide *in vitro* models for mechanistic studies. As expected, TNF- α induced rapid and strong induction of the canonical pathway as well as expression of alternative NF- κ B pathway components (Figure 2A, Supplemental Figure 2), as described in previous studies of other tumor cell lines by us and others (27, 36, 42, 44-46). This study provided direct evidence that LT β /LT β R-NIK signaling mediates alternative NF- κ B2/RELB activation. LT β preferentially induced NF- κ B2(p52)/RELB nuclear translocation in UM-SCC 46 cells (Figure 2B, Supplemental Figure 3). We previously treated UM-SCC 1 cells with LT β , and demonstrated modulation of the alternative NF- κ B pathway components IKK α and p100/p52 (22). In addition, we also studied the effects of LT β in an HPV(+) HNSCC cell line, UM-SCC 47, and observed results consistent with those presented for HPV(-) line in Figure 1C (43). Knockdown of LT β R and NIK decreased NIK, RELB, NF- κ B2/p100 and p52 protein expression, and suppressed the NF- κ B reporter function by β -lactamase assay (Figure 2C-E). LT β treatment stabilized NIK protein expression levels, and partially blocked the effects of NIK inhibitor on RELB and NF- κ B2(p52) nuclear localization as shown by immunofluorescent staining (Figure 4). These data support the importance of LT β signaling mediated activation of the alternative NF- κ B pathway in HNSCC, as well as potential to counter the effects of NIK siRNA knockdown or a pharmacologic NIK inhibitor.

NIK has been reported to be the downstream kinase of LT β R in the alternative NF- κ B pathway, and LT β /LT β R binding enhances NIK stability (11, 47, 48). We demonstrated here that targeting of NIK either by siRNA or small molecule inhibitor 1, 3[2H, 4H]-Isoquinolinedione attenuates protein expression of NIK, p-IKK α , NF- κ B2(p100/p52), and RELB as well as nuclear translocation of NF- κ B2 and RELB (Figure 2C, D, and 3A, 4B). From the NIK structure (49, 50), it is known that the C-terminal domain has phosphorylation sites for IKK α (17, 21), consistent with the doublet observed in HNSCC by western in our study. The association of NIK with active IKK α mediates NF- κ B2/p100 phosphorylation. Consistent with this, it appeared the NIK inhibitor beginning at a concentration of ~0.5 μ M diminished IKK α and the slower migrating NIK band, followed by blocking NF- κ B2/p52 and RELB heterodimer and nuclear translocation.

HNSCC often migrate to the lymph nodes, where high levels of LT β may be produced and expressed locally. LT β presence in the local lymph nodes and tumor microenvironment may attract and promote tumor cell migration and metastasis (6). The critical steps of tumor metastasis include cancer cell migration and invasion, which could be assayed *in vitro* by cell migration/wound closure assay and transwell invasion assays. The cell migration assay, also known as wound healing assay, is an *in vitro* technique to examine the collective cell migration in two dimensions. In our experiment, the monolayer of HNSCC cells was scratched with a pipette tip and created a gap in the monolayer. The cells were migrated into the gap, remaining in contact during their coordinated movement. The cell migration was imaged over 24 hours using a light microscope. This experiment is testing the rate of wound closure, a measure of the speed of the collective motion of the cells. The migration of cancer cells is one important step related to cancer metastatic progression, where cancer cells must migrate to a distant site and form foci. The cell invasion is another critical step in cancer metastatic progression. The cancer cells must invade through extracellular matrix, intravasate into blood circulation, and attach to a distant site to form new cancer colonies. The invasion assay is using the transwell to analyze the ability of single cells to directionally respond to various chemo-attractants, migrate through a physical barrier, and invade into a 3D matrix. In this study, we obtained direct evidence that knockdown of NIK or RELB inhibited cell migration (Figure 5A-D). The modulation of cell migration by the alternate NF- κ B and other pathways was supported by evidence that LT β stimulation regulates expression of several genes implicated in cell migration, metastasis, and other features of the malignant phenotype. LT β induced genes involved in cell survival (*BIRC3/cIAP2*), and cell migration and metastasis (*PLAU*, *SERPINE1*, *MET*) in HNSCC cells. Knockdown of NIK by siRNA completely inhibited LT β induced *NIK* and *MET* expression, and partially blocked LT β induced *BIRC3/cIAP2* and *SERPINE1* expression (Figure 6A, B). *BIRC3/cIAP2* is a member of the inhibitor-of-apoptosis protein family, providing the anti-apoptosis signaling downstream of TNFR through TRAF1 and TRAF2 (51). *PLAU/uPA* converts plasminogen to plasmin, and stimulates cell migration via a *PLAUR* signaling complex containing TYK2 and PI3K (39, 40). *SERPINE1* is involved in the extracellular matrix digestion, and could promote cell migration (52). We showed previously that *MET*, the receptor for hepatocyte growth factor/scatter factor, promotes cell migration and metastasis of SCC (26, 37). Knockdown of NIK or *MET* alone, and in combination, as well as treated with NIK inhibitor, directly inhibited cell migration (Figure 5 and 6). Supporting the contribution of other factors to migration, tumor cells cultured in 10% FBS, which contain EGF and HGF, more strongly induced invasion than LT β alone (Figure 5). Together, these findings provide a missing link connecting LT β R and NIK mediated alternative NF- κ B activation with *MET*/HGF expression and cell migration. Future studies in syngeneic murine models of metastatic SCC could enable dissection of the role of LT β , LTBR and the alternative pathway using KO mice and cell lines. Our data could help explain the underlying mechanisms of early migration of HNSCC to the regional lymph nodes. Blocking activation of the alternative NF- κ B pathway mediated through LT β and NIK signaling could be a potential strategy for developing targeted therapy for local-regional HNSCC spread and metastasis. The recent development of specific NIK inhibitors (47) may increase the feasibility of *in vivo* studies in syngeneic preclinical models and human clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This project is supported by NIDCD intramural projects Z01-DC-00016, 73, 74. We thank NIH Fellows Editorial Board and NIH library Editor Cindy Clark for their helpful suggestion in editing our manuscript. We thank Drs. Hong Yin (Feist-Weiller Cancer Center, LSUHSC-Shreveport) and Chiju Wei (Shantou University) for reading this manuscript and providing helpful suggestions. We like to express special thanks for technical supports from Sophie Carlson, and the caption service from Ms. Frances Freeman and Felicia Pickering, NIH Interpreters, Access Interpreting, Inc.

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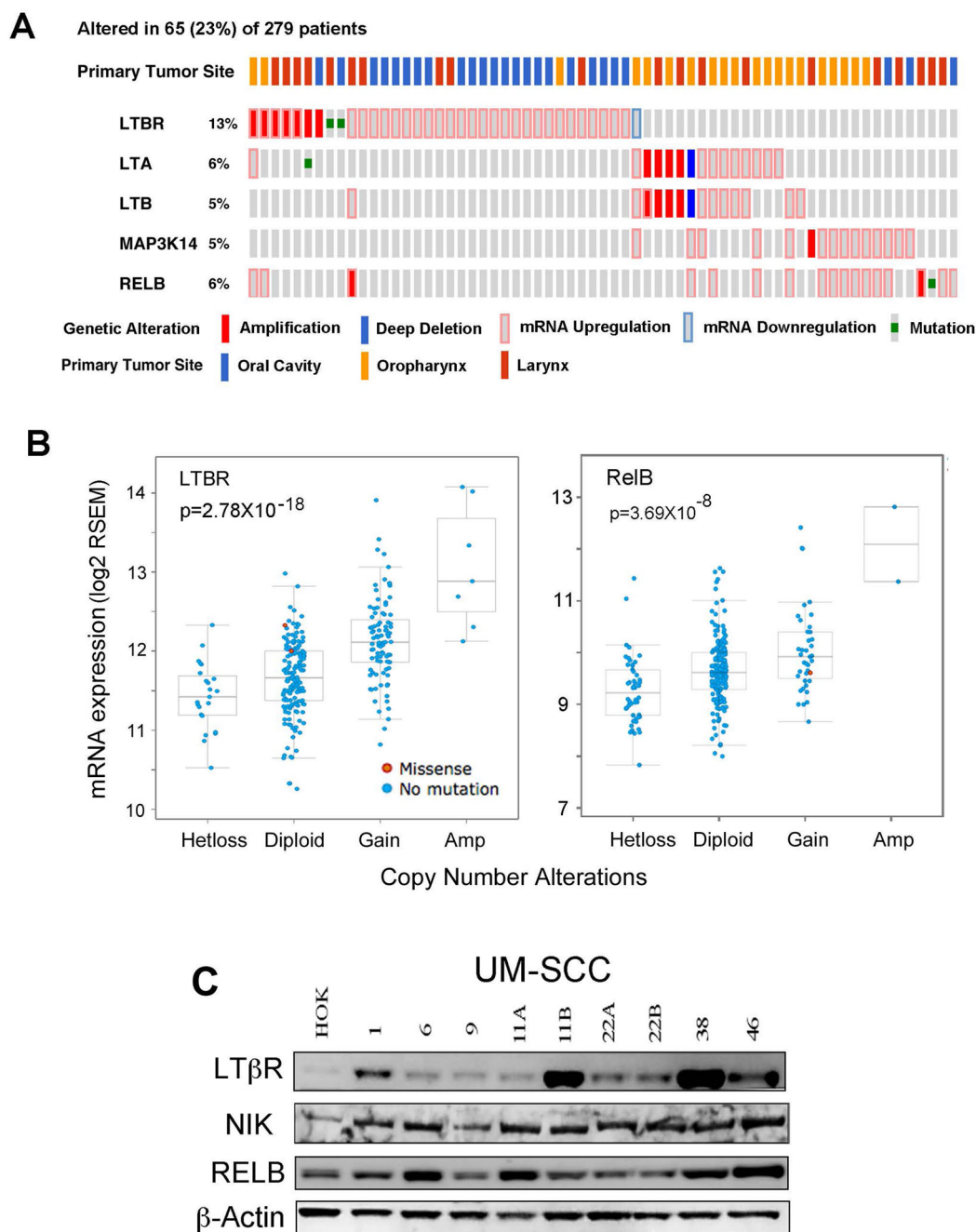


Figure 1. The genetic and expression alterations of LTBR, NIK (MAP3K14), and RELB in HNSCC tissues and cell lines.

Data of genetic and expression alterations were extracted from TCGA HNSCC project through cBioportal. (A) Oncoprint presents individual cases (each bar) with genetic and expression alterations of LTBR, LTA, LTB, MAP3K14, RELB. 65 (23%) cases with genetic and/or expression alterations were shown. Genetic and expression alterations are presented as solid red: homozygous amplification; solid blue, homozygous deletion; green: mutation; grey bar with blue frame: mRNA down-regulation compared with tumor mean; grey bar with pink frame: mRNA up-regulation compared with tumor mean. Primary tumor sites are

indicated at the top, blue: oral cavity; orange: oropharynx; red: Larynx. % on the left represents the percentage of cases with alterations. **(B)** RNA expression and DNA copy number variation (CNV) were correlated and presented. The statistical correlation of RNA expression with CNV was examined and presented as p value. The X-axis showed DNA CNV, as Hetloss (shallow deletion), Diploid (normal), Gain (one copy gain), and AMP (amplification of two copies or higher). Y-axis is mRNA expression and presented as log₂ RSEM (RNA-Seq by Expectation-Maximization). Mutation status, blue: no mutation; red: missense mutation. The relationship between CNV and RNA expression is tested by Pearson correlation. **(C)** Protein expression of LTβR, NIK and RELB in whole cell lysates from a panel of UM-SCC lines and HOK cells were detected by Western blot, using β-actin as a loading control.

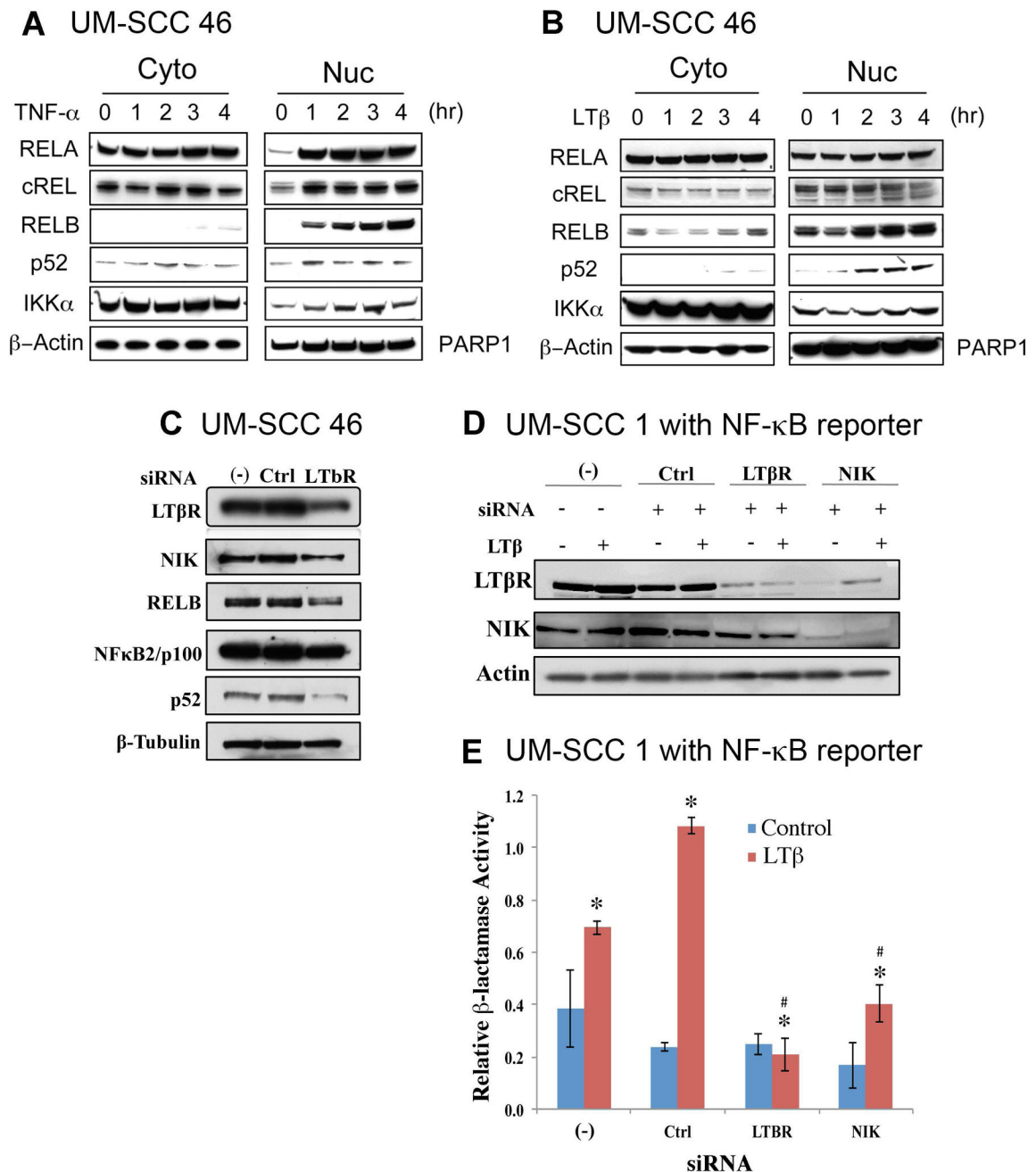


Figure 2. TNF- α and LT β stimulated and LT β R and NIK knocking down inhibited NF- κ B proteins and function activity in HNSCC cell line.

The HNSCC cell line UM-SCC 46 was treated with TNF α (25ng/ml, **A**) or LT β (100ng/ml, **B**), and the protein levels of NF- κ B subunits were measured at different time points via Western blot. β -actin was used as a loading control for the cytoplasmic fraction, and PARP1 for the nuclear fraction. (**C**) Knockdown of LT β R modulated its target kinase NIK, RELB, NF- κ B2/p100/p52 protein expression in UM-SCC-46 cells. Cells were transfected with LT β R siRNA, whole cell lysates were harvested 96h after transfection, and measured for LT β R, NIK, RELB and NF- κ B2/p100/p52 by Western blot. β -tubulin was used as loading

control. **(D)** Protein expression of LT β R and NIK after knockdown of LTBR and NIK by siRNAs in stable NF- κ B Blazer Reporter UM-SCC-1 stable cell line. Whole cell lysates were harvested by sonication of samples from each well of the β -Lactamase assay plate. Western blot was performed and β -Actin was used as loading control. **(E)** Knockdown of LTBR and NIK by siRNAs affected NF- κ B reporter function in NF- κ B Blazer Reporter UM-SCC-1 stable cell line. Relative β -Lactamase units were measured after stimulation with LT β (100ng/ml) for 24h before harvesting the cells at 96h. * indicates statistical significance induced by LTB treatment, and # indicates statistical significance after siRNA knockdown (p-value<0.05 by *t*-test). Data were calculated from triplicates of a representative experiment.

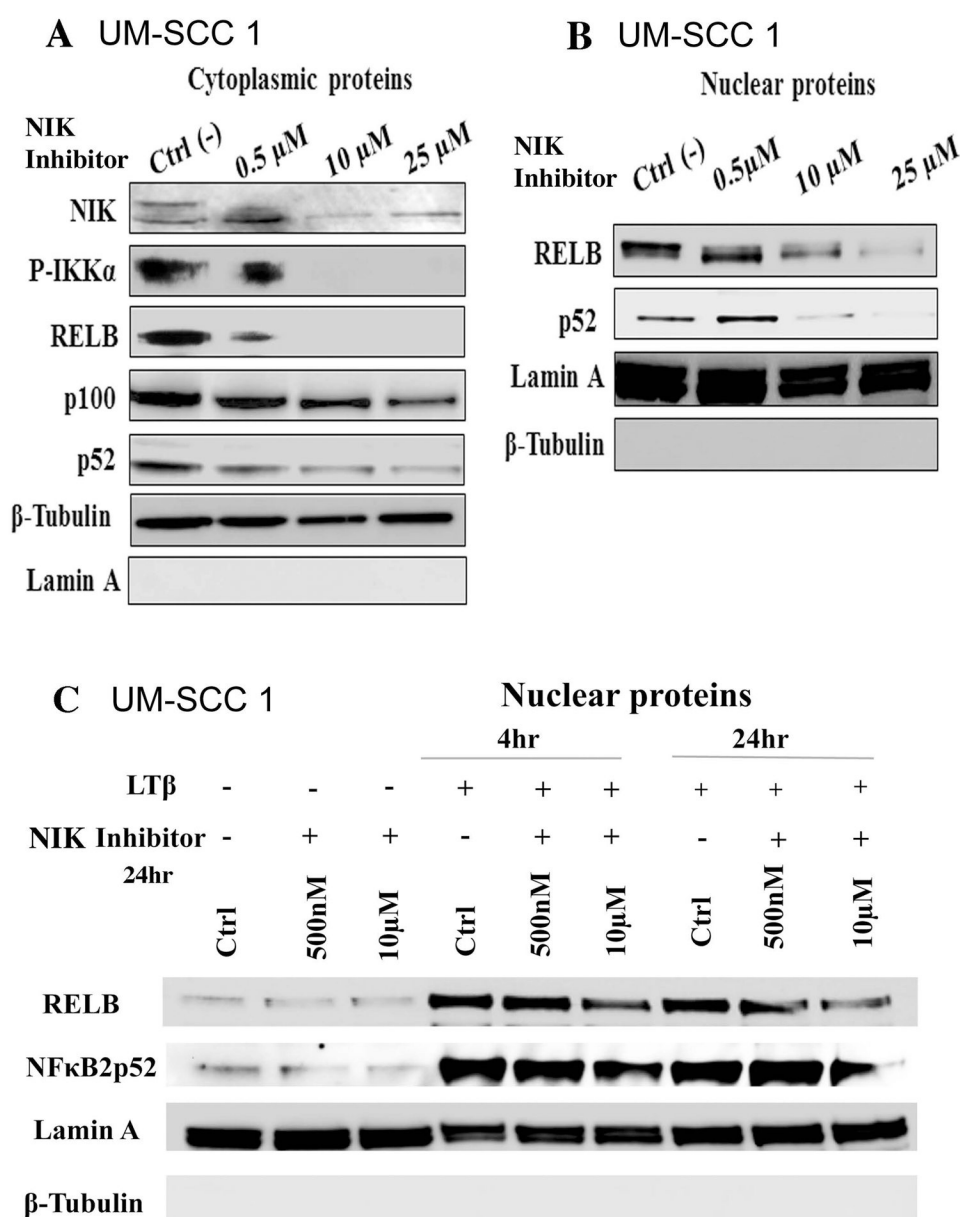


Figure 3. NIK Inhibitor (1, 3 [2H, 4H]-Isoquinolinedione) reduced expression and nuclear translocation of alternative NF- κ B proteins.

The UM-SCC1 cells were treated with NIK inhibitor (1, 3 [2H, 4H]-Isoquinolinedione) at the concentration of 500nM, 10 μ M, 25 μ M for 24h. Cells were fractionated into cytoplasmic (A) and nuclear fractions (B) and analyzed by Western blot. Lamin A (Nuclear) and β -tubulin (cytoplasm) were used as loading controls. (C) Cells were treated with NIK inhibitor at 500nM, 10 μ M, for 24h, followed with LT β (100ng/ml) treatment for 4h and 24h. RELB and NF- κ B2/p52 were examined in the nuclear protein fractions. Lamin A and β -tubulin were used as loading controls.

UM-SCC 46

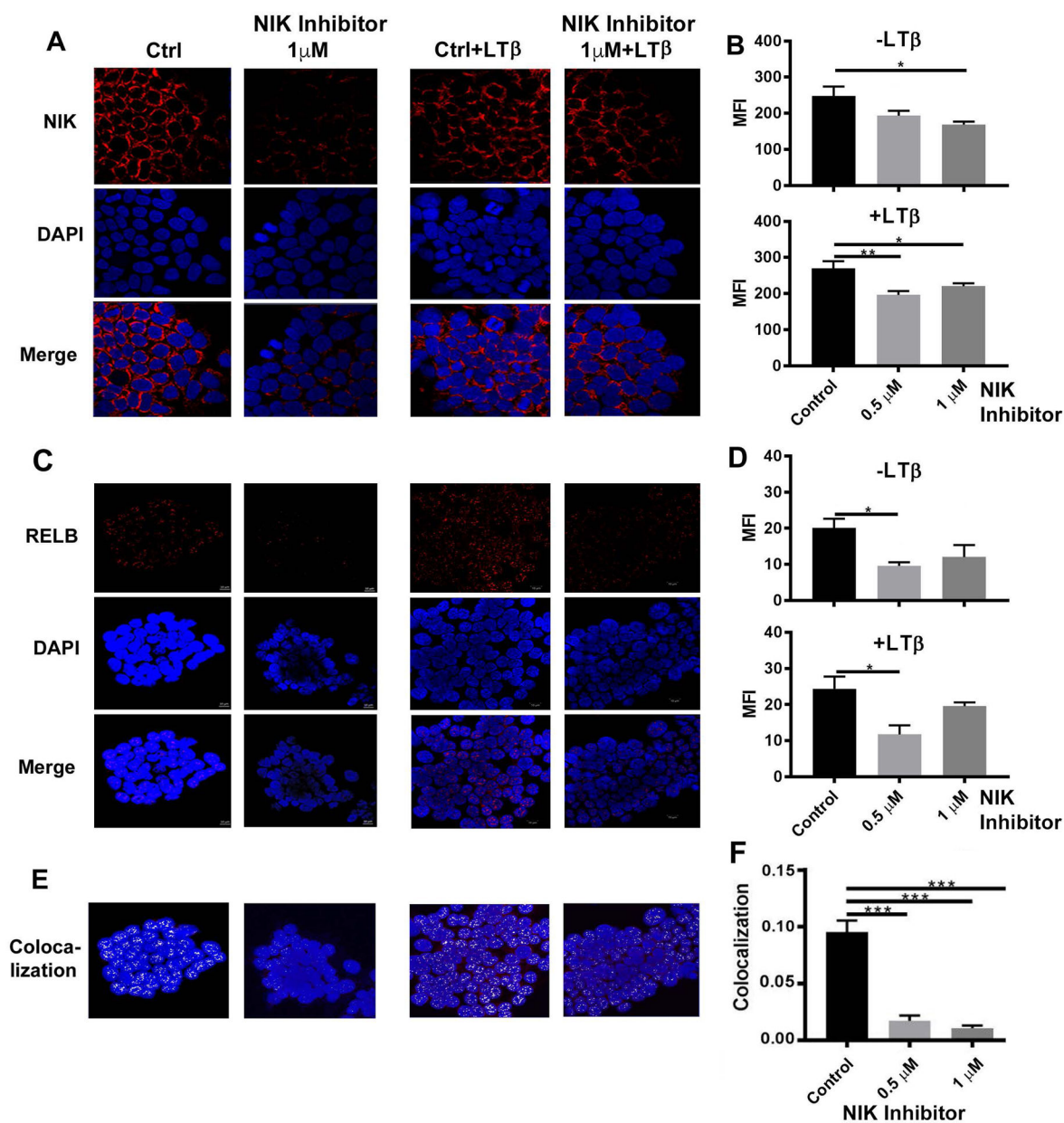


Figure 4. NIK stability in perinuclear cytoplasm and RELB nuclear localization are inhibited by NIK inhibitor 1, 3[2H, 4H]-Isoquinolinedione.

(A) UM-SCC-46 cells were pre-treated with NIK inhibitor at 1µM for 24h (left panels), and then stimulated with LTβ for 4h (100ng/ml, right panels). Cells were stained with anti-NIK antibody (red) and DAPI for nuclear staining (blue). The images were captured by immunofluorescence microscopy. (B) The quantifications of the images were presented as before (upper panel) or after treatment (lower panel) with different doses of NIK inhibitors (0.5 and 1µM). (C) The images of RELB immunostaining were presented, the left panels showed cells treated with NIK inhibitor but without LTβ stimulation. The right panels showed cells treated with NIK inhibitor plus LTβ stimulation. (D) Quantification of the

images presented in panel C. Upper panel is from the cells treated with NIK inhibitor but without LTB treatment (left panels in C). The low panel is the quantification of the images from the right panel of C. RELB co-localization with DAPI nuclear staining (E) and quantification (F). The photography was taken by confocal microscopy with 63X. Statistical analysis: Student T-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

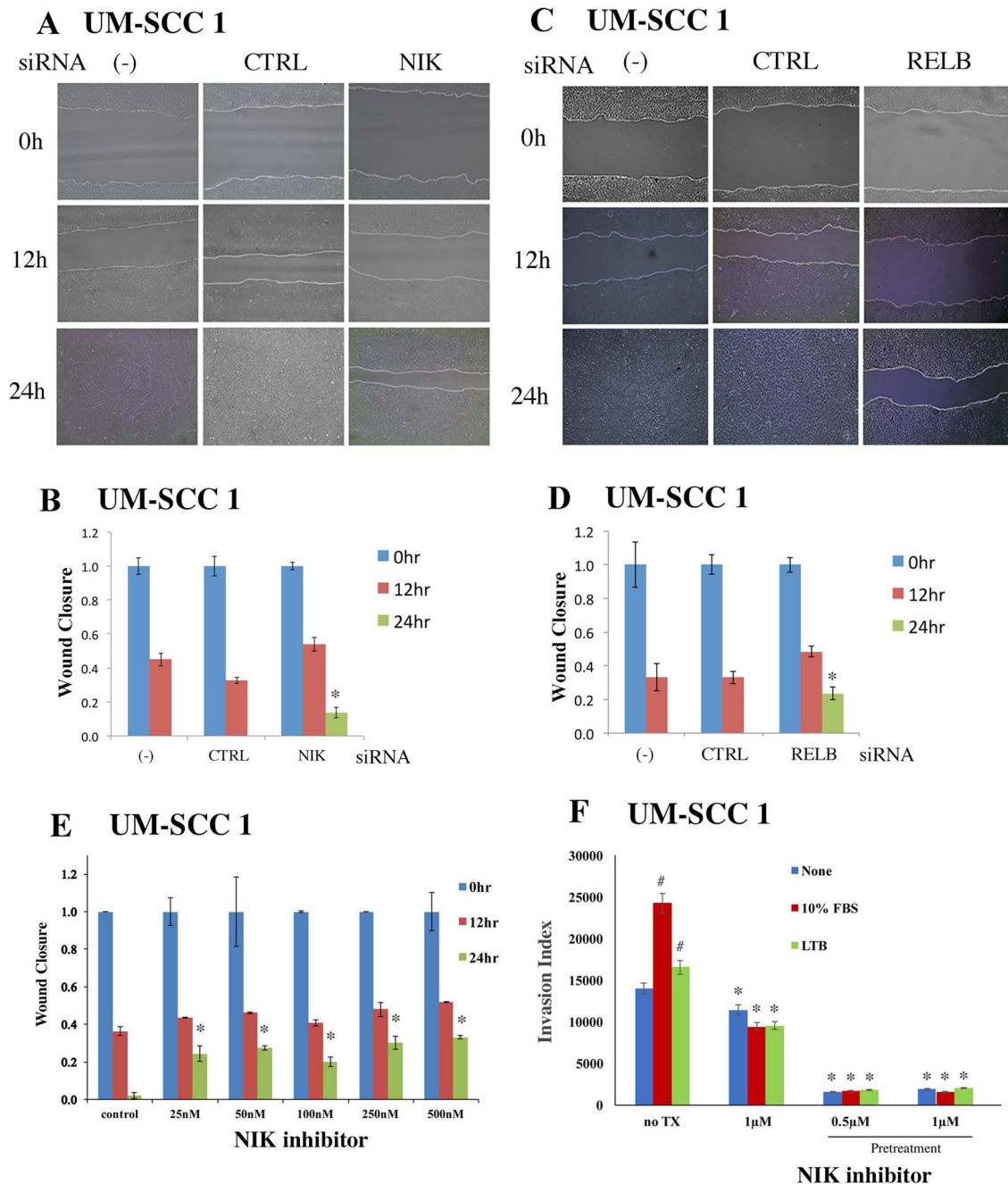


Figure 5. Knockdown of NIK or RELB by siRNAs or by NIK inhibitor inhibited migration of UM-SCC1 cells *in vitro*.

UM-SCC1 cells were transfected with siRNAs for NIK (A) or RELB (C) for 48h. Scratches were made on cell monolayers, and wound closure was monitored at 0, 12, and 24 h. (B, D) Quantification of wound healing from images was done using Image J program, and statistical significance was calculated. The distances between the wounds were measured, and significant differences by individual knockdowns were examined by t-test (*p < 0.05). (E) UM-SCC1 cells were pretreated with the NIK inhibitor, and after 24h the cell monolayers were scratched and treated with the inhibitor. Wound closure was measured at

0h, 12h, and 24h from images using Image J program. * indicates a statistical significance when compared with controls (p-value <0.05 by t-test). (F) UM-SCC1 cells were starved in serum free media for 24hr, and plated in 100mm plates at 1×10^6 cells per plate, with or without NIK inhibitor pretreatment for 4hr. The cells were subjected to invasion assay in EC matrix coated chamber in serum free media with chemoattractant in the outer wells, either adding 10% fetal bovine serum (FBS, red bar) or LTB (green bar). The invasion index was measured and calculated following the manufacturer's protocol. # indicated a statistical significance of FBS or LTB induced invasion, and * indicates the statistical significance of NIK inhibitor decreased cell invasion compared to the corresponding controls (p-value <0.05 by t-test).

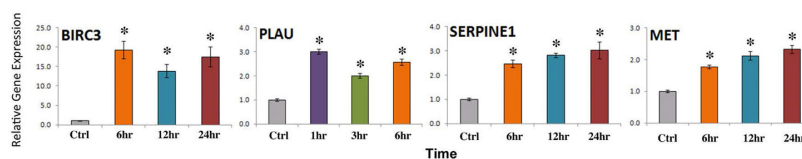
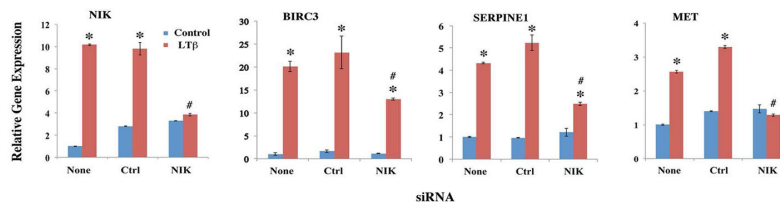
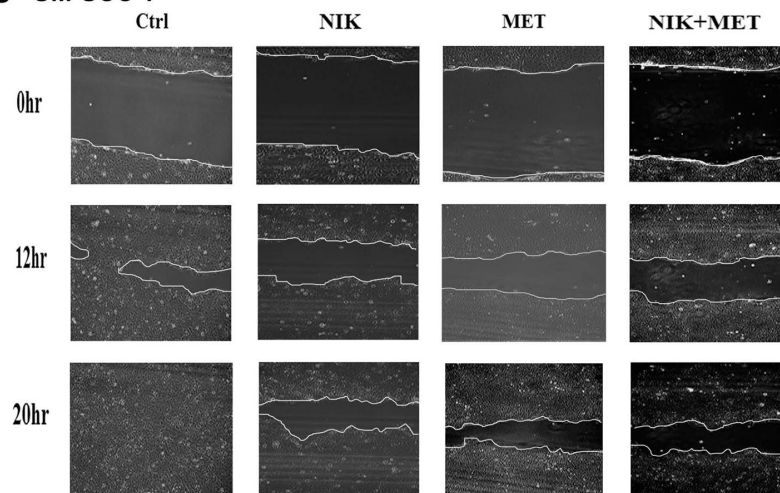
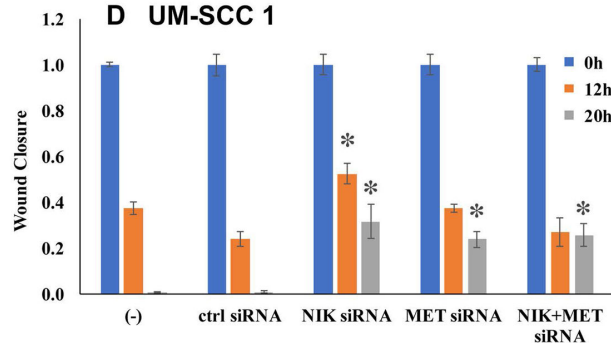
A UM-SCC 46**B UM-SCC 46****C UM-SCC 1****D UM-SCC 1**

Figure 6: LT β , NIK, and MET modulated expression of survival and metastasis genes, and promoted cell migration.

(A) UM-SCC 46 cells were treated with LT β (100ng/ml), and the relative mRNA expression of LT β upregulated genes were examined by qRT-PCR at different time points. The data were compared with time 0 (no treatment), and calculated with three replicates as mean + SD. An asterisk indicates p-value <0.05 using t-test. (B) UM-SCC46 cells were transfected with 50nM NIK siRNA for 48h, mRNA expression of *NIK*, *BIRC3*, and *SERPINE1*, and *MET* were measured by qRT-PCR. Statistical significance differences were calculated with

three replicates at *p 0.05 by t-test. **(C)** UM-SCC1 cells were transfected with NIK or MET siRNA alone, or in combination for 48h. Scratches were made on cell monolayers, wound closure was monitored at 0, 12 and 20h, and images were taken using EVOS microscope. **(D)** Quantification of wound healing with statistical significance. The distances between the wounds were measured, and significant differences were examined using image J. * indicates a statistical difference calculated with three replicates and shown with p-value <0.05 by *t*-test.