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Triptonide inhibits human nasopharyngeal carcinoma cell growth via disrupting *Lnc-RNA THOR*-IGF2BP1 signaling

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ABSTRACT

Advanced stage nasopharyngeal carcinoma (NPC) has a poor prognosis. Triptonide ("TN") is a small molecule monomer extract from the ancient Chinese herb *Tripterygium wilfordii Hook*. We show that TN, at nanomolar concentrations, potently inhibited survival and proliferation of multiple established and primary human NPC cells. TN induced NPC cell cycle arrest and apoptosis activation. NPC cell migration and invasion were also inhibited by TN. Importantly, TN was non-cytotoxic to nasopharyngeal epithelial cells. TN treatment in NPC cells disrupted *LncRNA THOR* ("*Lnc-THOR*")-IGF2BP1 association, causing depletion of *Lnc-THOR* and down-regulation of IGF2BP1 mRNA targets (*Myc*, *IGF2* and *Gli1*). *Lnc-THOR* or IGF2BP1 knockout by CRISPR/Cas9 gene-editing methods mimicked and abolished TN's actions in NPC cells. Conversely, ectopic *Lnc-THOR* over-expression inhibited TN-induced cytotoxicity in NPC cells. Significantly, *Lnc-THOR*, IGF2BP1 and its mRNA targets are elevated in human NPC tissues and cells, but almost undetectable in nasopharyngeal epithelial tissues and cells. *In vivo*, intraperitoneal TN administration significantly inhibited subcutaneous NPC xenograft growth in mice. Similarly, *Lnc-THOR*-knockout HONE-1 xenografts grew significantly slower than control tumors. Thus, TN inhibits human NPC cell growth *in vitro* and *in vivo* via disrupting *Lnc-THOR*-IGF2BP1 signaling.

1. Introduction

Nasopharyngeal carcinoma (NPC) is a malignant nasopharynx epithelial tumor with diverse etiopathogy and histopathology [1–3]. Epstein-Barr virus (EBV) is key risk factor of NPC [1–3], and is an important contributor to cancer-related human mortalities worldwide [1–3]. A large proportion of NPC patients respond well to radiotherapy, alone or in combination with chemotherapy agents [1–3]. However, NPC cells with pre-existing and/or acquired resistance do not respond well to conventional therapies and molecularly-targeted agents [1–3]. It is therefore urgent to develop novel, more efficient, but less toxic anti-NPC agents [1–3].

A recent study by Pan et al., testing the anti-leukemia activity of over 120 novel traditional Chinese medicinal herbs, found that Triptonide ("TN", chemical name diterpene triepoxide), a small molecule monomer extract from the herb *Tripterygium wilfordii Hook*,

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potently inhibited leukemia cell growth *in vitro* and *in vivo* [4]. Subsequent studies have confirmed the superior anti-cancer activity of TN [4–8]. Unlike other Chinese herb medicines, TN efficiently kills human cancer cells, with an IC-50 in the nanomolar (nM) range [4–8]. The potential activity of TN against NPC cells and its underlying mechanisms of action, have not been studied thus far.

Long non-coding RNAs (*LncRNAs*) were previously considered as non-functional transcripts over 200-nt length [9]. Recent studies have confirmed that *LncRNAs* can function as molecular signals, scaffolds, or enhancers/inhibitors to regulate important cellular behaviors, including cell survival, growth, proliferation, and apoptosis-resistance [9–11]. Emerging evidence demonstrate that *LncRNAs* dysregulation plays a pivotal role in tumorigenesis and progression of many human cancers [12–14].

Hosono et al., have characterized a conserved but oncogenic *LncRNA*, *THOR* ("*Lnc-THOR*", ENSG00000226856) [15]. *Lnc-THOR* expression is detected in normal testis and in human cancers, functioning by a conserved interaction with insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1) to stabilize its mRNA targets [15–18]. *Lnc-THOR* silencing or knockout downregulates IGF2BP1's mRNA targets, including *IGF2 (insulin-like growth factor 2)*, *Gli1(glioma-associated oncogene homolog 1)*, c-Myc and *CD44*, to potently inhibit cancer cell growth *in vitro* and *in vivo* [15–18].

It has been recently proposed that the *Lnc-THOR*-IGF2BP1 pathway is a novel therapeutic target for cancer treatment [15–17,19]. *Lnc-THOR* expression is significantly increased in NPC tissues and in non-adherent NPC spheres, which is correlated with a larger tumor size, and a higher rate of lymph node metastasis [16]. In the current study, we show that TN disrupts *Lnc-THOR*-IGF2BP1 signaling to inhibit human NPC cell growth *in vitro* and *in vivo*.

2. Materials and methods

Ethics. The study was approved by the Ethics Review Board of all authors' institutions.

Chemicals and reagents. Triptonide (TN, Purity ≥98%) was purchased from Jiuzhi Chemicals (Shanghai China). TN was dissolved in dimethyl sulfoxide (DMSO) at concentration of 5 mM as a stock solution, and diluted with cell culture medium before each experiment. The pre-experiment results show that DMSO vehicle control (0.1/0.5%), as expected, had no effect on NPC cell functions. Hoechst-33342, puromycin, mitomycin and other chemicals of this study were provided by Sigma-Aldrich (St. Louis, Mo). All antibodies of this study were obtained from Cell Signaling Tech (Shanghai, China).

Cell culture. The established human NPC cell lines, HONE-1, CNE-1, CNE-2, 5–8F, 6–10B, were acquired from the Cell Bank of Shanghai Institute for Biological Science (Shanghai, China). Cells were cultured in RPMI 1640 medium (Life Technologies, Shanghai, China) supplemented with 10% fetal bovine serum (FBS, Life Technologies), 1% penicillin-streptomycin in a 37 °C incubator containing 5% CO_2. Throughout the *in vitro* study, the final DMSO concentration was less than 0.05%.

Primary culture of human NPC cells. Two written-informed primary NPC patients (Stage-III, 55/57 year old, both male), administrated at the Affiliated Hospitals of Soochow University, were enrolled. The patients received no therapy before surgery. As described [20], the surgery-dissected NPC tissues and matched surrounding epithelial tissues were separated carefully under microscopy. Hematoxylin-eosin (HE) staining was employed to verify pathology. Tissues were mechanically dissociated, digested and filtered as described [21]. The individual cells were pelleted, rinsed, and primary cultured [20]. Primary human NPC cells from the two patients were named as "Pri_Can-1/-2", and the matched primary nasopharyngeal epithelial cells were named as "Pri_Epi-1/-2". The primary human cells at passage 3–10 were utilized for *in vitro* experiments. The protocol of using human tissues was approved by the Internal Review Board (IRB) and Ethic Review Board (ERB) of Soochow University, in according to Declaration of Helsinki.

Cell viability assay. Cells were seeded onto 96-well plates (5×10^3 cells per well), treated with different concentrations of TN for applied time. Cell Counting Kit-8 (CCK-8, Dojindo, Japan) was employed to evaluate cell viability according to the manufacturer's instructions. CCK-8 optical density (OD) at 450 nm was measured under a microplate reader (Bio-Rad Laboratories).

Colony forming assay. HONE-1 cells were incubated with indicated concentration of TN for 24 h, washed in RPMI1640 medium, and seeded onto the plastic dish (3×10^3 living cells per dish). After incubation for 8 days, colonies were counted.

EdU assay. HONE-1 cell proliferation was evaluated by EdU Apollo-488 *In Vitro* Imaging Kit (Ribo Bio, Guangzhou, China) according to the manufacturer's instruction. After the applied TN treatment the fluorescence dye 5-ethynyl-20-deoxyuridine (EdU) with a final concentration of 10 μM was added to cultured cells for additional 2 h. Cell nuclei were stained with Hoechst-33342 for 30 min, visualized under a fluorescent microscope (Leica, DM 4000, Germany). For each condition at least 500 cells in five random views were analyzed for EdU ratio (EdU/Hoechst-33342 × 100%).

Cell cycle distribution assay. Following treatment cells were harvested, fixed in ice-cold ethanol at 4 °C overnight, and stained with propidium iodide (PI)-RNase staining buffer (BD Biosciences, Shanghai, China) for 30 min at room temperature. DNA content was analyzed by using a Navios Flow Cytometer (Beckman Coulter, Brea, CA). Cell cycle distribution was recorded.

Annexin V assay. Annexin V assay was applied to identify cells undergoing apoptosis. In brief, after the applied TN treatment cells were harvested, washed twice with a cold PBS, and re-suspended in 1X Binding Buffer. Then, cells were stained with FITC-conjugated Annexin V (10 μg/mL, BD Pharmingen) and PI (10 μg/mL) for 15 min. The percentage of Annexin V positive cells was analyzed using a Navios Flow Cytometer (Beckman Coulter, Brea, CA).

Other cell proliferation, apoptosis and death assays, including the BrdU incorporation ELISA assay, Histone DNA ELISA assay, TUNEL intensity assay, Trypan Blue cell death staining assay were performed as previously described [21–25].

Wound healing assay. HONE-1 cells with TN treatment were carefully scratched with a 10 μL plastic tip to produce a straight line and washed three times with PBS. Cells were imaged under a light microscope to monitor the wound healing process. Mitomycin (2.0 μg/mL Sigma) was always added to exclude the influence of cell proliferation.

***In vitro* cell migration and invasion assays.** HONE-1 cells with TN treatment were digested, and seeded to the Corning chambers with 8 μm pore filters (Corning, New York, NY), pre-coated with or without 1 mg/mL Matrigel (BD Biosciences, Shanghai, China). Cells (5×10^4 cells of each chamber) starved overnight were added to the upper chamber in serum-free medium, and the lower chamber was filled with completed medium (with 10% FBS). After 24 h, cells that invaded to the lower surface of the chamber were fixed, stained and counted. Mitomycin (2.0 μg/mL Sigma) was always added to exclude the influence of cell proliferation.

Quantitative real-time PCR (qRT-PCR). The protocol of qRT-PCR, by the ABI Prism 7600H equipment using the SYBR Green PCR kit, was performed using the previously described method [24,26–28]. *mRNA* expression was quantified by $^{\Delta\Delta}$Ct method [29] with *GAPDH* as the internal control [26]. The melt curve analysis was always performed to calculate product melting temperature. The primers for detecting *Lnc-THOR* (F: 5′-CAAGGTGCTTCTCTCTGGATTT-3′ and R: 5′-GCCAAAGTC ATTTGTTGGGTAT-3′) were described early [15]. The mRNA primers for c-Myc, *IGF2*, *CD44* and *Gli1* were previously described [15]. The primers for *U6 mRNA* were also reported early [30]. All the primers were provided by Genechem (Shanghai, China).

Western blotting assay. Western blotting assay was performed by well-established protocols as previously described [26,31,32]. To examine expression of different proteins on the same blot, the blot was

stripped and re-probed. Alternatively, the lysates were run on separate (sister gels). The intensity of each protein band was quantified using the densitometric analysis via NIH ImageJ software.

Stable expression of *Lnc-THOR*. The pLenti6-GFP *Lnc-THOR* expression construct ("LV-Lnc-THOR") was provided by Dr Liang [17]. LV-Lnc-THOR was transfected to HONE-1 cells. Stable cells were selected by puromycin (2.0 μg/mL). Overexpression of *Lnc-THOR* was verified by the qRT-PCR assay. Control cells were infected with the pLenti6-puro-GFP vector control ("LV-C").

***Lnc-THOR* knockout.** The BbsI-linearized PX458 (pSpCas9(BB)-2A-GFP) plasmid encoding *Lnc-THOR* sgRNA (F: 5′-CACCgAGGGTGTAGC GCGGGCTAGA-3' [17]) was provided again by Dr. Liang [17], which was transfected to cells by Lipofectamine 2000. To obtain stable clones, GFP-positive cells were FACS-sorted as a single cell into 96-well plate. Cells were further cultured for additional three weeks, and genotyped by PCR assay. One stable HONE-1 cell line with complete depletion of *Lnc-THOR* ("THOR-KO") was established. Cells with control sgRNA vector were utilized [17] as the control cells.

IGF2BP1 knockout. The lentiCRISPR/Cas9-GFP IGF2BP1 knockout construct, encoding IGF2BP1 sgRNA (5′-GAGCACAAGATCTCCTA CAG-3′), was provided by Dr. Zhao [19]. The lentiviral construct was transfected to HONE-1 cells, and the GFP-positive cells were FACS sorted as a single cell into 96-well plate. After culturing for another three weeks, cells were distributed into two 24-well plates, followed by genotyping of depleted region of *IGF2BP1*. IGF2BP1 KO was further confirmed by Western blotting/qRT-PCR assay.

RNA Immunoprecipitation (RIP). RIP was performed as described [33]. In brief, cells were collected by trypsinization, washed, and incubated with 0.3% formaldehyde andglycine (0.125 M). Thereafter, cells were washed, and the pellets were resuspended in the described RIP buffer [33]. The cleared cell lysates were incubated with the anti-IGF2BP1 antibody. Pellets were washed three times, and re-suspended and incubated with the buffer containing proteinase K. IGF2BP1-bound RNA was then isolated. Expression of *Lnc-THOR* was tested by qRT-PCR assay.

RNA Pull-Down Assay. Biotin-labeled full-length *Lnc-THOR*, synthesized by Genechem (Shanghai, China), was transcribed using a Biotin RNA Labeling Mix Kit and T7 RNA polymerase (Invitrogen), which was then isolated with the RNeasy Mini kit (Invitrogen). Biotinylated *Lnc-THOR* was folded in RNA structure buffer, immediately put on ice, and then transferred to room temperature. For each assay, 1.0 mg cleared nuclei lysates of HONE-1 cells were mixed with folded *Lnc-THOR* and Dynabeads MyOne Streptavidin C1 magnetic beads ("Beads", Thermo-Fisher). Beads were washed five times, and the retrieved proteins were tested by the Western blotting assay.

Tumor xenograft assay. Briefly, control or "THOR-KO" HONE-1 cells (1×10^7 cells per mouse, in 100 μL DMEM and 100 μL Matrigel, no serum) were inoculated (via *s.c.* injection) to the female severe combined immuno-deficient (SCID) mice (4–5 week old, 17–18 g weight). Within 3–4 weeks, the tumors reached the average volume of 100 mm^3, the mice were intraperitoneally injected daily with either Triptonide (TN, 10 mg/kg daily) or saline ("Vehicle") for 21 consecutive days. Tumor volumes, recorded weekly, were calculated by: $\pi/6 \times$ larger diameter $\times$ (smaller diameter)^2. Estimated daily tumor growth (in mm^3 per day) was calculated as described [34]. The establishment of *s.c.* xenograft tumors using the primary human NPC cells ("Pri_Can-1") was performed similarly. All studies were performed with approval from the Institutional Animal Care and Use Committee (IACUC) of all authors' institutions. Human endpoints were always applied for decreasing suffering. All injections were performed via anesthesia method [35].

Human NPC tissues. A total of twelve (12) NPC patients were enrolled from the Kunshan First People's Hospital, the Affiliated Hospitals of Soochow University, Shanghai Xin-chao (Shanghai, China). All participants provided written consent. The NPC cancer tissues and matched nasopharyngeal epithelial tissues were separated carefully. Hematoxylin-eosin (HE) staining was again performed to confirm the pathology. Western blotting and real-time PCR assays testing fresh human tissues were described previously [26]. The protocols were conducted according to the Declaration of Helsinki, with approval from the Ethics Review Board of Soochow University.

Statistical analysis. Data were presented as mean ± standard deviation (SD). Statistics were analyzed by one-way ANOVA followed by the Scheffe' and Tukey Test using SPSS software (SPSS 21.0, Chicago, CA). Significance was $\boldsymbol{p} < 0.05$. To determine significance between two treatment groups, a 2-tailed unpaired *t*-test was applied (Windows Excel 2007).

3. Results

3.1. Triptonide is extremely cytotoxic to human NPC cells

First, we tested the potential effect of TN on established NPC cell lines. A total of five cell lines were utilized, including HONE-1, CNE-1, CNE-2, 5–8F and 6–10B. Employing a CCK-8 assay, the percentage of CCK-8 OD reduction was utilized as the indicator of cytotoxicity. As demonstrated, TN treatment dose-dependently inhibited viability of all tested NPC cells (Fig. 1A–E). A significant viability reduction was detected following 5 nM of TN treatment (72 h) in all NPC cells (Fig. 1A–E). The IC-50 of TN, the concentration that induced 50% reduction of cell viability, was between 20 and 200 nM (Fig. 1A–E). Among the tested cell lines, TN exhibited the highest potency in HONE-1 cells (Fig. 1A–E), with an IC-50 of 23.56 nM (Fig. 1A). Significantly, TN treatment (5–500 nM) was non-cytotoxic to normal nasopharyngeal epithelial NP-69 cells [36], as the viability OD was unchanged following TN treatment (Fig. 1F). HONE-1 NPC cell morphology images in Fig. 1G further confirmed the cytotoxic effect of TN. Performing a HONE-1 cell clonogenicity assay, we found that following the TN (5–25 nM) treatment the number of viable HONE-1 cell colonies was significantly decreased (Fig. 1G), further confirming the cytotoxic effect of TN.

The potential effect of TN in the primary human cells was also tested. As described, primary human NPC cells derived from two different NPC patients ("Pri_Can-1/-2") as well as the matched primary human nasopharyngeal epithelial cells ("Pri_Epi-1/-2"), were treated with TN. CCK-8 assay results show that TN treatment (25 nM, 72 h) led to over 60–70% reduction of cell viability in the primary NPC cells (Fig. 1I). In contrast, the viability of the epithelial cells was not significantly affected by TN treatment (Fig. 1I). These results further confirm the selective cytotoxicity of TN against cancerous cells.

3.2. Triptonide inhibits NPC cell proliferation and cell cycle progression

The potential effect of TN on NPC cell proliferation was tested on HONE-1 cells treated with 5–50 nM of TN. Using the EdU staining assay, we observed that TN (36 h) dose-dependently decreased EdU ratio (EdU/Hoechst-33342 × 100%) in HONE-1 cells (Fig. 2A), significantly ($\boldsymbol{p} < 0.05$ vs. "Ctrl" cells, Fig. 2B). Further, TN dose-dependently inhibited BrdU incorporation in HONE-1 cells (Fig. 2C), again confirming its anti-proliferative activity. Performing the BrdU ELISA assay, we also found that TN (25 nM) significantly inhibited proliferation of the primary human NPC cells ("Pri_Can-1/-2") (Fig. 2D). Conversely, in the primary human nasopharyngeal epithelial cells ("Pri_Epi-1/-2"), BrdU incorporation was not significantly affected by TN (Fig. 2D), again showing a selective activity by TN against cancer cells.

Cell cycle progression is vital for cancer cell proliferation. A PI-FACS assay was performed with HONE-1 cells to test cell cycle distribution in TN-treated cells. TN decreased the percentages of G0/1-phase and G2/M-phase cells (Fig. 2D), while increasing S-phase cell percentage (Fig. 2E). Quantitative cell cycle analysis integrating five replicated PI-FACS assays show that TN-induced cell cycle arrest was significant and dose-dependent (Fig. 2F). Notably, expression of cell cycle-associated

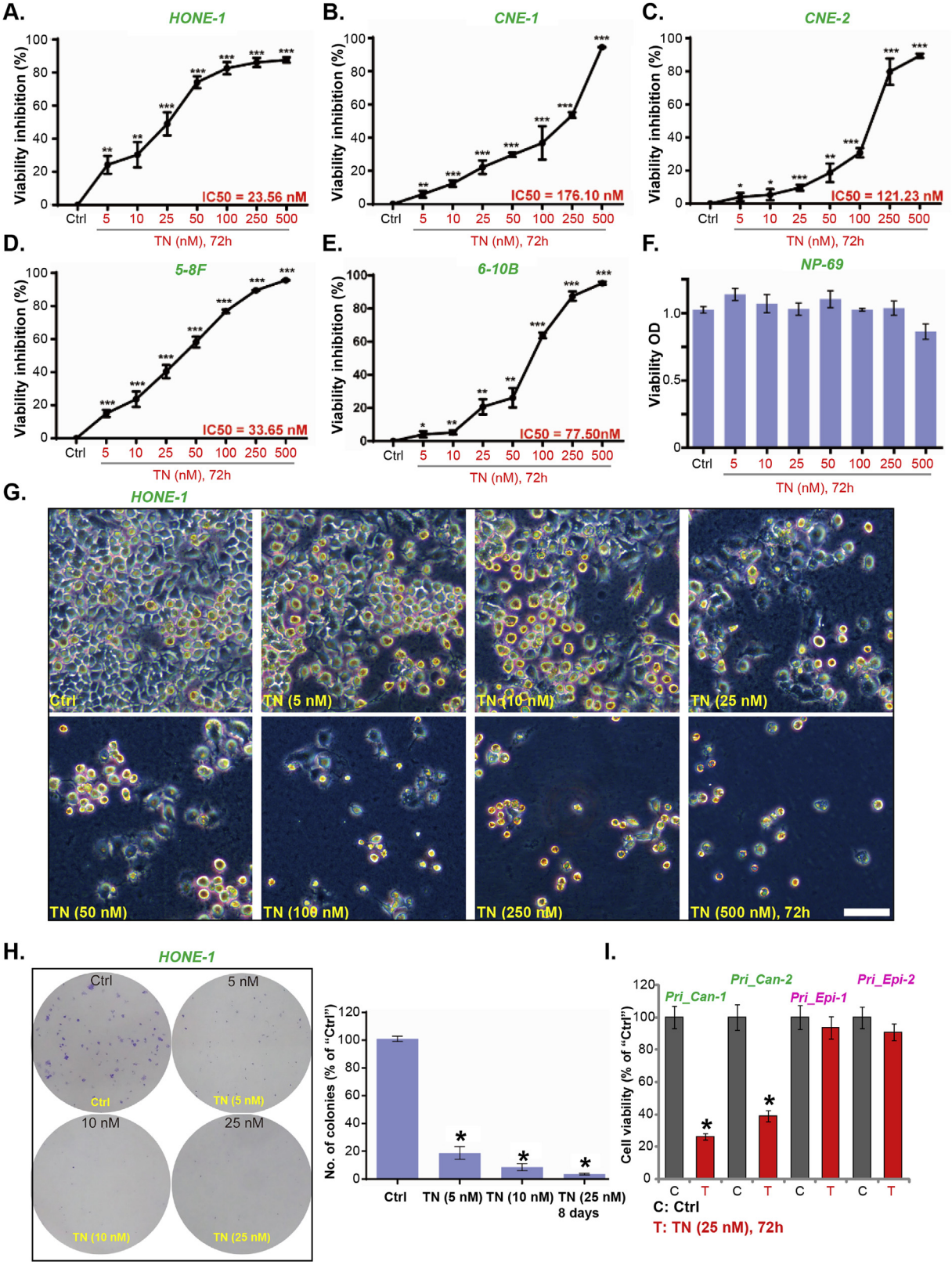

Fig. 1. Triptonide is extremely cytotoxic to human NPC cells. Established human NPC cells (HONE-1, CNE-1, CNE-2, 5–8F and 6–10B lines) (**A-E**, **G** and **H**), the nasopharyngeal epithelial NP-69 cells (**F**), the primary human NPC cells ("Pri_Can-1/-2") (**I**) or the matched human nasopharyngeal epithelial cells ("Pri_Epi-1/-2") (**I**), were either left untreated ("Ctrl") or treated with applied concentration (5–500 nM) of Triptonide ("TN"), cells were then cultured in TN-containing medium for indicated time, cell viability was tested by the CCK-8 assay (**A-F**, and **I**) and clonogenicity assay (**H,** TN-containing medium was renewed every two days); The representative HONE-1 cell morphology images were presented (**G**, Bar = 100 μm). Data were expressed as the mean ± standard deviation (S.D.). For each assay, n = 5. *$p < 0.05$ vs. "Ctrl" cells.**$p < 0.01$ vs. "Ctrl" cells.***$p < 0.001$ vs. "Ctrl" cells. In this figure, experiments were repeated three times, and similar results were obtained.

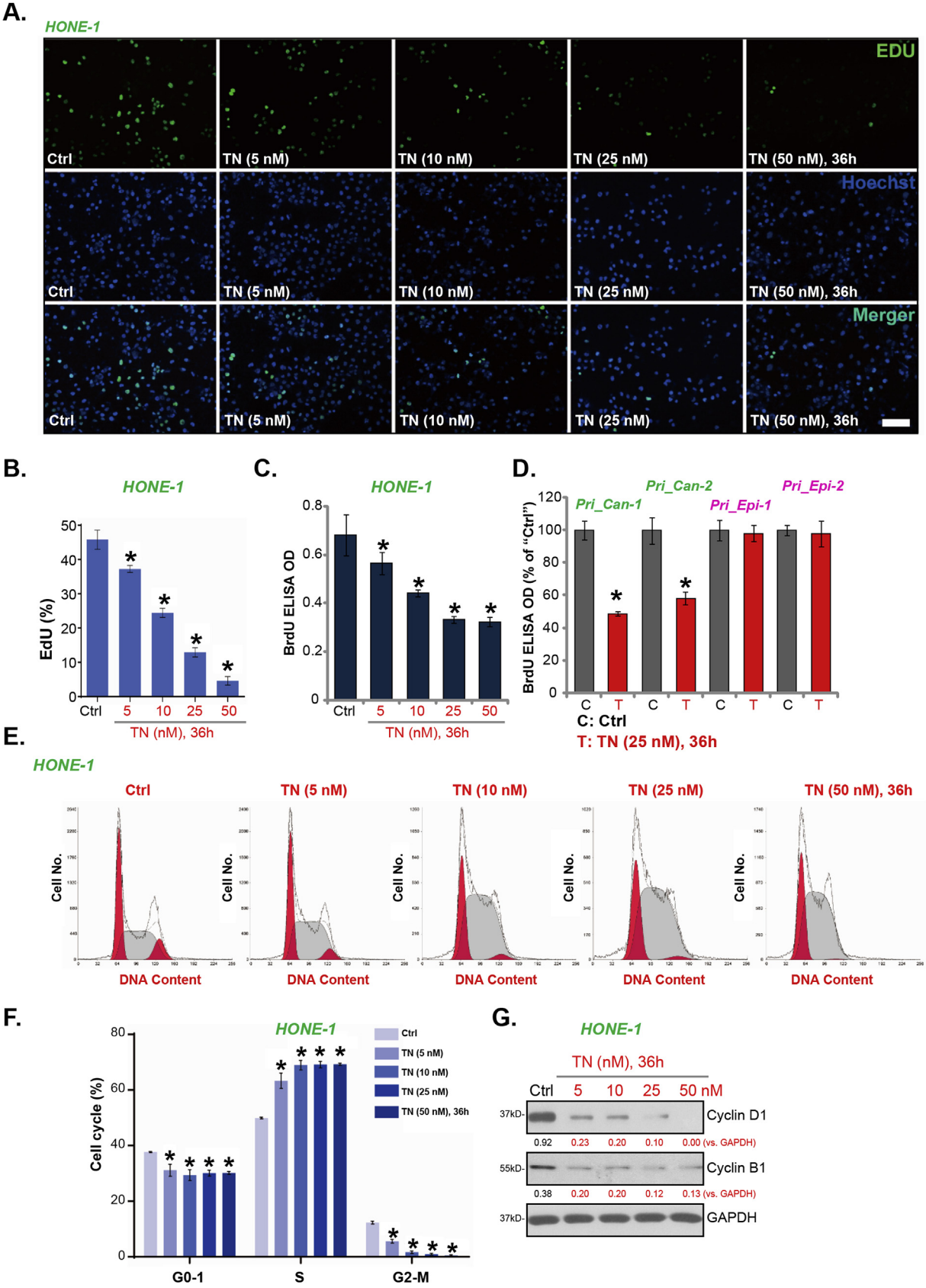

Fig. 2. Triptonide inhibits NPC cell proliferation and cell cycle progression. HONE-1 cells (**A-C**, **E-G**), the primary human NPC cells ("Pri_Can-1/-2") (**D**) or the matched human nasopharyngeal epithelial cells ("Pri_Epi-1/-2") (**D**), were either left untreated ("Ctrl") or treated with applied concentration (5–50 nM) of Triptonide ("TN"), cells were then cultured in TN-containing medium for indicated time, cell proliferation (**A–D**), cell cycle progression (**E** and **F**) and expression of listed cell cycle proteins in the total cell lysates (**G**) were shown. For Western blotting data in (**G**), expression of cyclin D1 and cyclin B1 were quantified, the their values were normalized to the loading control GAPDH. Data were expressed as the mean ± standard deviation (S.D.). For each assay, n = 5. *$p < 0.05$ vs. "Ctrl" cells. In this figure, experiments were repeated three times, and similar results were obtained each time.

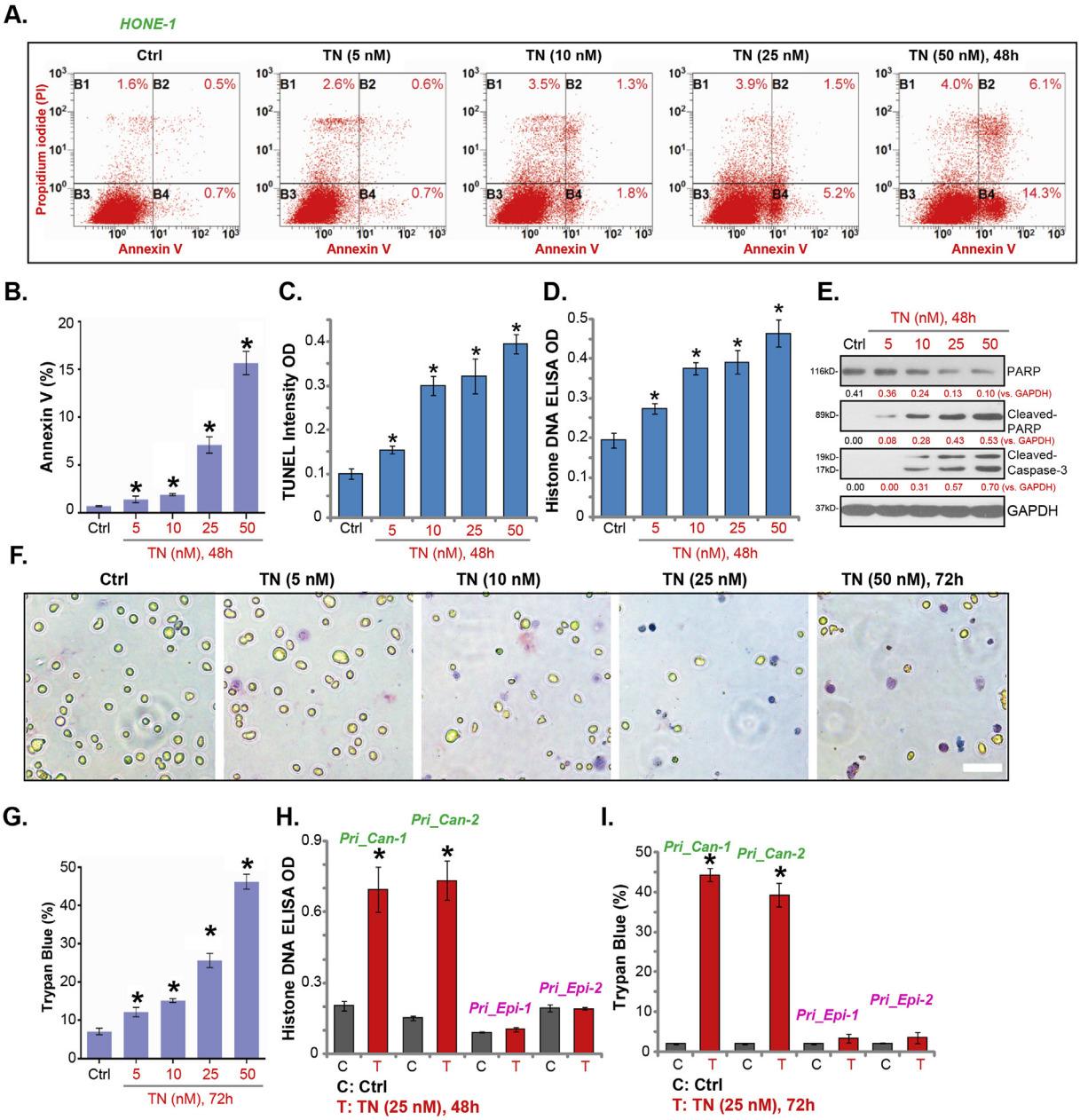

Fig. 3. Triptonide potently induces NPC cell death and apoptosis. HONE-1 cells (**A–G**), the primary human NPC cells ("Pri_Can-1/-2") (**H** and **I**) or the matched human nasopharyngeal epithelial cells ("Pri_Epi-1/-2") (**H** and **I**), were either left untreated ("Ctrl") or treated with applied concentration (5–50 nM) of Triptonide ("TN"), cells were further cultured in TN-containing medium for indicated time, cell apoptosis (**A-D**, **H**), cell death (**F**, **G** and **I**) and expression of listed proteins in the total cell lysates (**E**) were tested by mentioned assays. For Western blotting data in (**E**), expression of indicated apoptosis-associated proteins were quantified, and their values were normalized to the loading control GAPDH. Data were expressed as the mean ± standard deviation (S.D.). For each assay, n = 5. *$p < 0.05$ vs. "Ctrl" cells. In this figure, experiments were repeated five times, and similar results were obtained each time.

proteins, including cyclin D1 and cyclin B1, were significantly decreased in TN-treated HONE-1 cells (Fig. 2G), which might explain TN-induced cell cycle arrest and proliferation inhibition [37]. Collectively, these results show that TN inhibits human NPC cell proliferation and cell cycle progression.

3.3. Triptonide potently induces NPC cell death and apoptosis

The activity of TN on cell apoptosis was examined. By performing the Annexin V FACS assay, we show that TN treatment (5–50 nM) dose-dependently increased the percentage of Annexin V-positive HONE-1 cells (Fig. 3A). The quantitative analysis of five replicated FACS assays confirmed the increased Annexin V ratio by TN was significant (Fig. 3B). Further studies show that TN dose-dependently increased TUNEL intensity (Fig. 3C) and Histone-bound single-strand DNA levels (Fig. 3D) in HONE-1 cells. Additionally, Western blot assay results show a prominent cleavage of poly-ADP-ribose polymerase (PARP) and caspase-3 following TN treatment (Fig. 3E). The Trypan Blue staining assay was employed to quantify cell death. Results show that TN treatment, in a dose-dependent manner, increased the percentage of Trypan Blue-positive HONE-1 cells (Fig. 3F and G). These results clearly show that TN efficiently inducesHONE-1 cell death and apoptosis. Similarly, in the primary human NPC cells ("Pri_Can-1/-2"), TN treatment increased Histone-bound single-strand DNA levels (Fig. 3H) and Trypan Blue ratio (Fig. 3I), indicating apoptosis and cell death. However, TN treatment in the primary nasopharyngeal epithelial cells again failed to cause

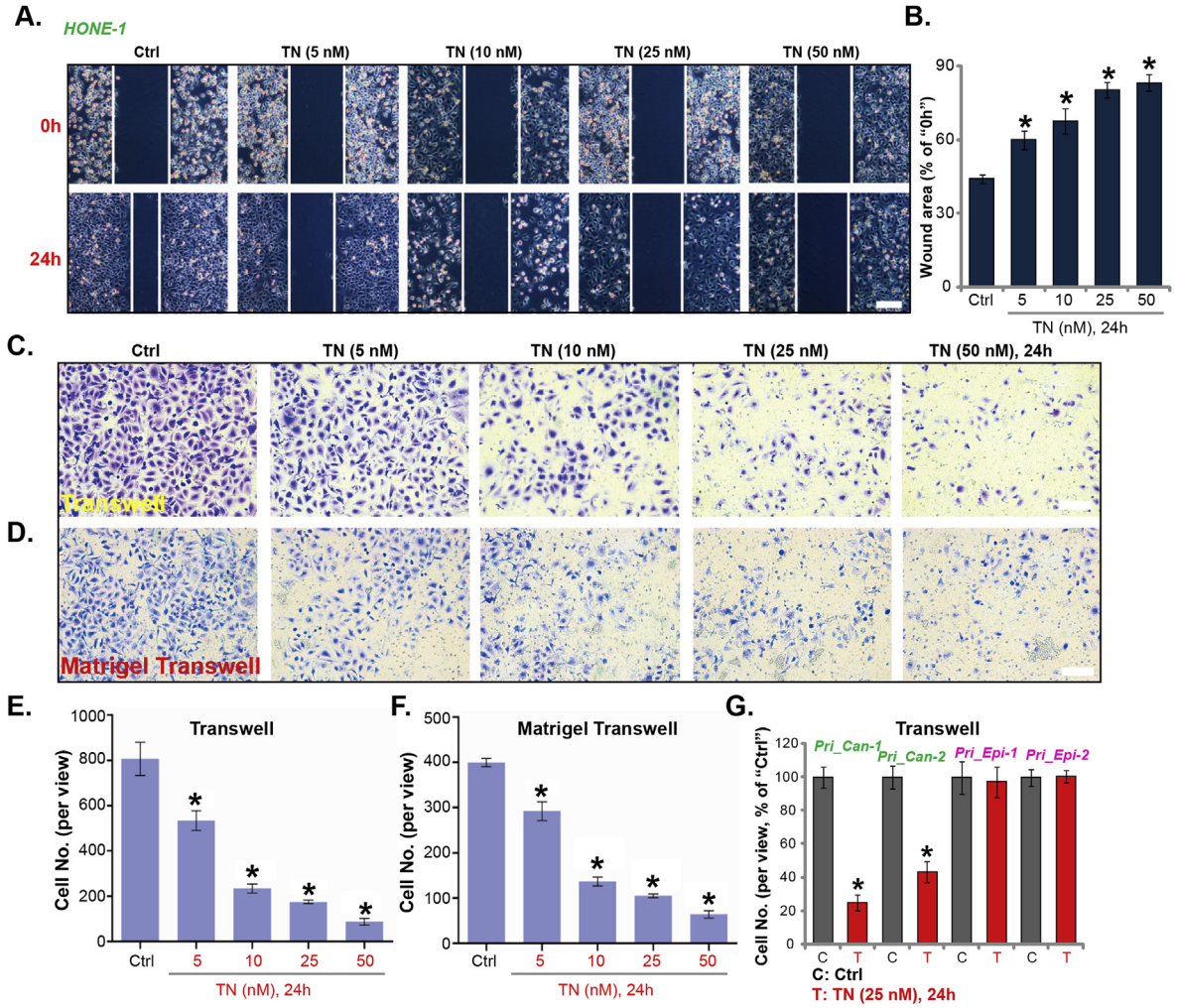

Fig. 4. Triptonide inhibits NPC cell *in vitro* migration and invasion. HONE-1 cells (**A–F**), the primary human NPC cells ("Pri_Can-1/-2") (**G**) or the matched human nasopharyngeal epithelial cells ("Pri_Epi-1/-2") (**G**), were either left untreated ("Ctrl") or treated with applied concentration (5–50 nM) of Triptonide ("TN"), cell migration and invasion were tested by "starch wound-healing assay" (**A** and **B**), Transwell assay (**C**, **E** and **G**) and "Matrigel Transwell" assay (**D** and **F**). Data were expressed as the mean ± standard deviation (S.D.). For each assay, n = 5. *$p < 0.05$ vs. "Ctrl" cells. In this figure, experiments were repeated five times, and similar results were obtained each time.

significant apoptosis (Fig. 3H) and death (Fig. 3I).

3.4. Triptonide inhibits NPC cell in vitro migration and invasion

NPC cell migration and invasion are essential for cancer progression [1,3]. The potential effect of TN on NPC cell migration and invasion was tested. Results from a scratch wound-healing experiment show that treatment with TN (5–50 nM) dose-dependently inhibited wound closure in HONE-1 cells (Fig. 4A). Quantitative analysis of the wound area demonstrated that HONE-1 *in vitro* cell migration (at 24 h) was significantly inhibited by TN (Fig. 4B). Performing a Transwell assay (Fig. 4C) and the Matrigel Transwell assay (Fig. 4D), we further demonstrated that TN (5–50 nM) can potently suppress HONE-1 cell invasion. Counting migrated cells (per field), we confirmed that TN significantly and dose-dependently inhibited HONE-1 cell invasion *in vitro* (Fig. 4E and F). Significantly, Transwell assay results demonstrate that TN (25 nM) inhibited *in vitro* migration of primary human NPC cells ("Pri_Can-1/-2") (Fig. 4G). Conversely, no significant inhibition of cell migration was detected following TN treatment in nasopharyngeal epithelial cells (Fig. 4G). Together, these results show that TN inhibits *in vitro* NPC cell migration and invasion.

3.5. Triptonide disrupts Lnc-THOR-IGF2BP1 signaling in NPC cells

Long non-coding RNAs ("*LncRNAs*") are a large family of transcribed non-coding RNAs, over 200 nucleotides in length [9–11]. *LncRNAs* do not directly encode proteins, but can alter protein expression and/or function via gene silencing, splicing regulation and chromatin remodeling [9–11]. Emerging studies have shown that *LncRNA* dysregulation is critically involved in the NPC tumorigenesis and progression [38,39]. A *LncRNA* microarray analysis (Genechem, Shanghai, China) was performed to identify the differentially expressed *LncRNAs* after TN (10/25 nM, 12 h) treatment in HONE-1 cells. Quantitative real-time PCR (qRT-PCR) was performed to quantify and validate the identified *LncRNAs*. Our results show that expression of one particular *LncRNA*, *THOR* ("*Lnc-THOR*"), was significantly decreased in TN-treated cells (Fig. 5A). *Lnc-THOR* has been reported be expressed in human cancers [15,17], and *Lnc-THOR*-IGF2BP1 association is essential for IGF2BP1's function [15,17]. *Lnc-THOR* silencing or knockout could efficiently inhibit human cancer cells via disabling IGF2BP1 [15,17].

As TN decreases *Lnc-THOR* in NPC cells, it would be anticipated to deplete IGF2BP1 mRNA targets. Performing a qRT-PCR assay in HONE-1 cells, we show that *IGF2*, *Gli1* and *c-Myc* mRNA levels were significantly decreased by TN (10/25 nM, 12 h) (Fig. 5B), resulting in decreased levels of IGF2, Gli1 and c-Myc proteins (Fig. 5C), although

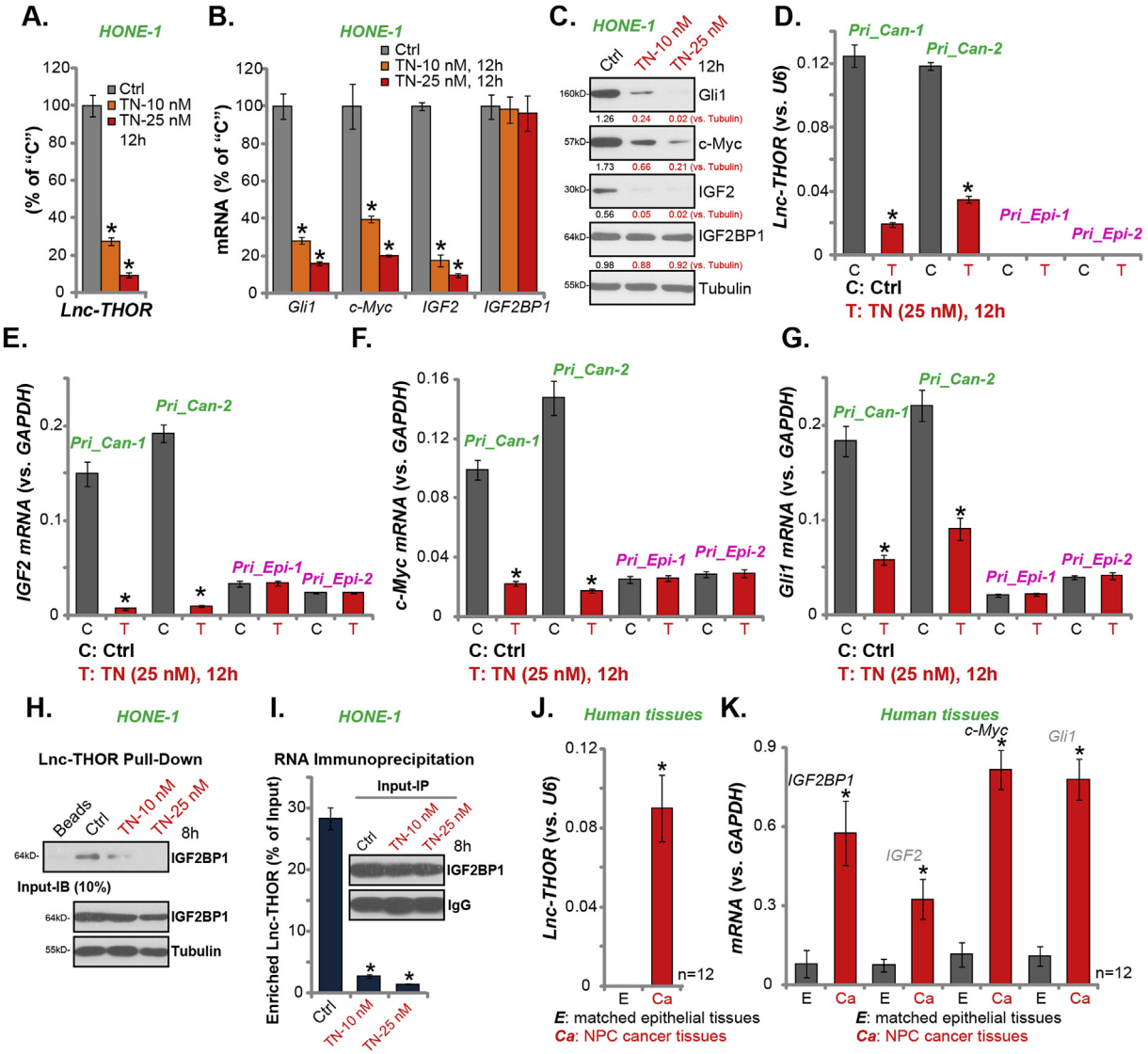

Fig. 5. Triptonide disrupts *Lnc-THOR*-IGF2BP1 signaling in NPC cells. HONE-1 cells (**A–C**), the primary human NPC cells ("Pri_Can-1/-2") (**D–G**) or the matched human nasopharyngeal epithelial cells ("Pri_Epi-1/-2") (**D–G**), were either left untreated ("Ctrl") or treated with applied concentration (10/25 nM) of Triptonide ("TN"), cells were further cultured in TN-containing medium for indicated time, *Lnc-THOR* expression was tested by qRT-PCR assay (**A** and **D**); mRNA and protein expression of listed genes were also shown (**B** and **C, E-G**). Western blotting assay of IGF2BP1 protein retrieved by *in-vitro*-transcribed *Lnc-THOR* in HONE-1 cells with/without TN treatment (**H**). qRT-PCR analysis of *Lnc-THOR* enriched by IGF2BP1 protein in HONE-1 cells with/without TN treatment (**I**). Western blotting of IGF2BP1-IP is shown ("Input-IP", upper) (**I**). Expression *Lnc-THOR* (**J**) and listed mRNAs (**K**) in twelve (12) pairs of fresh NPC cancer tissues ("Ca") and matched surrounding nasopharyngeal epithelial tissues ("E") were shown. Expression of indicated IGF2BP1 pathway proteins were quantified, and their values were normalized to the loading control GAPDH (**C**). Data were expressed as the mean ± standard deviation (S.D.). For each assay, n = 5 (**A–G**). n = 12 stands for twelve pairs of human tissues (**J** and **K**). *$p < 0.05$ vs. "Ctrl" cells (**A–G**).*$p < 0.05$ vs."E" tissues (**J** and **K**). In this figure, experiments were repeated five times, and similar results were obtained each time.

IGF2BP1 mRNA and protein expression were not significantly affected by TN (Fig. 5B and C). These results show that TN depletes *Lnc-THOR* to inhibit IGF2BP1 function in HONE-1 cells. In the primary human NPC cells ("Pri_Can-1/-2"), TN (25 nM, 12 h) potently downregulated *Lnc-THOR* (Fig. 5D) and IGF2BP1 mRNA targets (*IGF2*, c-Myc and *Gli1*, Fig. 5E–G). Significantly, *Lnc-THOR* expression was not detected in the primary nasopharyngeal epithelial cells (Fig. 5D). Expression of IGF2BP1 mRNA targets (*IGF2*, c-Myc and *Gli1*) were also substantially lower in the epithelial cells (Fig. 5E–G).

To verify the direct association between *Lnc-THOR* and IGF2BP1 protein, *a Lnc-THOR* pull-down assay was performed. We show that IGF2BP1 protein is co-precipitated with the *in-vitro*-transcribed biotinylated *Lnc-THOR* in HONE-1 cell nuclei (Fig. 5H). Such association was inhibited by TN (10/25 nM, 8 h). Additionally, employing a RNA Immunoprecipitation (RIP) assay, we again demonstrated the direct binding between endogenous *Lnc-THOR* and IGF2BP1 protein in HONE-1 cells (Fig. 5I). The *Lnc-THOR*-IGF2BP1 binding was also inhibited by TN (Fig. 5I). These results show that TN blocks *Lnc-THOR*-IGF2BP1 association, which could explain *Lnc-THOR* depletion in NPC cells.

The expression of *Lnc-THOR*-IGF2BP1 pathway components in human NPC tissues was analyzed. A total of twelve (12) pairs of fresh NPC cancer tissues ("Ca") and matched surrounding nasopharyngeal epithelial tissues ("E") were analyzed. qRT-PCR results show that *Lnc-THOR* was detected only in the cancer tissues, but not in the epithelial tissues (Fig. 5J). Significantly, the expression level of *IGF2BP1* and its mRNA targets (*IGF2*, *Gli1* and *c-Myc*) were significantly higher in cancer tissues, as compared to the epithelial tissues (Fig. 5K). Thus, *Lnc-THOR*, IGF2BP1 and its mRNA targets (*Myc*, *IGF2*, and *Gli1*) are elevated in human NPC tissues.

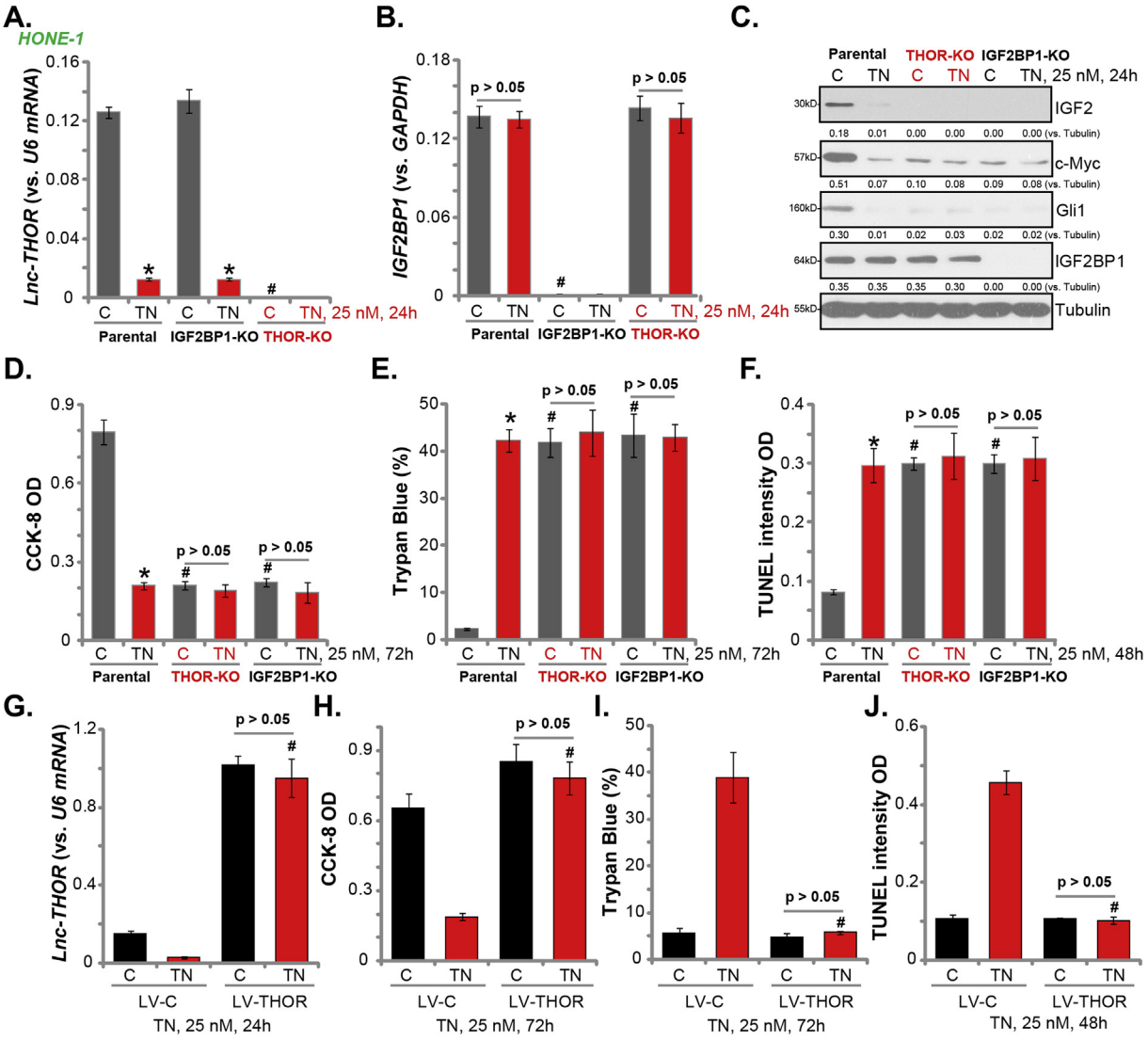

Fig. 6. *Lnc-THOR*-IGF2BP1 inhibition mediates Triptonide-induced cytotoxicity in HONE-1 cells. Stable HONE-1 cells with lentiviral CRISPR/Cas9 *Lnc-THOR* knockout construct ("THOR-KO" cells) or the CRISPR/Cas9 IGF2BP1 knockout construct ("IGF2BP1-KO" cells), as well as the parental control cells ("Parental") were treated with TN (25 nM) for indicated time, *Lnc-THOR*(**A**) and *IGF2BP1 mRNA*(**B**) were tested by qRT-PCR assay; Expression of listed proteins in total cell lysates were shown (**C**); Cell viability, death and apoptosis were tested by MTT assay (**D**), Trypan Blue staining (**E**) assay and TUNEL assay (**F**), respectively. Stable HONE-1 cells with the lentiviral *Lnc-THOR* expression construct ("LV-THOR") or the vector control ("LV-C") were treated with TN (25 nM) for indicated time, *Lnc-THOR* expression was tested (**G**); Cell viability (**H**), death (**I**) and apoptosis (**J**) were tested similarly. Expression of indicated IGF2BP1 pathway proteins were quantified, and their values were normalized to the loading control GAPDH (**C**). For the *in vitro* functional assays, the same number of viable cells of different genetic modifications were initially plated into each well/dish. Data were expressed as the mean ± standard deviation (S.D.). "C" stands for no TN treatment. $^{*}p < 0.05$ vs. "C" (**A–F**); $^{\#}p < 0.05$ vs. "C" of "Parental" cells (**A–F**). $^{\#}p < 0.05$ vs. TN treatment of "LV-C" cells (**G–J**). For each assay, n = 5. In this figure, experiments were repeated five times, and similar results were obtained each time. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.6. Lnc-THOR-IGF2BP1 inhibition mediates Triptonide-induced cytotoxicity in HONE-1 cells

To test whether *Lnc-THOR*-IGF2BP1 inhibition mediates TN-induced cytotoxicity in NPC cells, CRISPR/Cas9 gene-editing method was applied to knockout *Lnc-THOR*. As described, the lentiviral CRISPR/Cas9-*Lnc-THOR* KO construct was transfected into HONE-1 cells, stable cells selected by FACS sorting, and *Lnc-THOR* knockout confirmed by qRT-PCR ("THOR-KO" cells) (Fig. 6A). Additionally, the CRISPR/Cas9 method was applied to knockout IGF2BP1 in HONE-1 cells. Results show that *IGF2BP1* mRNA (Fig. 6B) and protein (Fig. 6C) expression were depleted in "IGF2BP1-KO"cells. Expression of IGF2BP1-dependent genes, including IGF2, Gli1 and c-Myc, were significantly decreased in the *Lnc-THOR*- and IGF2BP1-KO cells (Fig. 6C). Significantly, *Lnc-THOR*-or IGF2BP1-KO resulted in a reduction of cell viability (Fig. 6D), increasing cell death (Fig. 6E) and apoptosis (Fig. 6F) in HONE-1 cells, similar to TN activity. Significantly, treating *Lnc-THOR*- or IGF2BP1-KO cells with TN did not further inhibit expression of IGF2BP1-dependent genes (Fig. 6C). TN was also unable to further affect viability, death and apoptosis of *Lnc-THOR*- or IGF2BP1-KO cells (Fig. 6D and F). These results show that *Lnc-THOR*-/IGF2BP1-KO can mimic and abolish the action of TN on NPC cells.

If *Lnc-THOR*-IGF2BP1inhibition is the main mechanism for TN-induced cytotoxicity in NPC cells, ectopic overexpression of *Lnc-THOR* should rescue cells. To test this hypothesis, a lentiviral *Lnc-THOR* expression construct ("LV-THOR", from Dr. Liang [17]) was transfected into HONE-1 cells. Following puromycin selection, stable HONE-1 cells were established. *Lnc-THOR* RNA levels were significantly elevated in

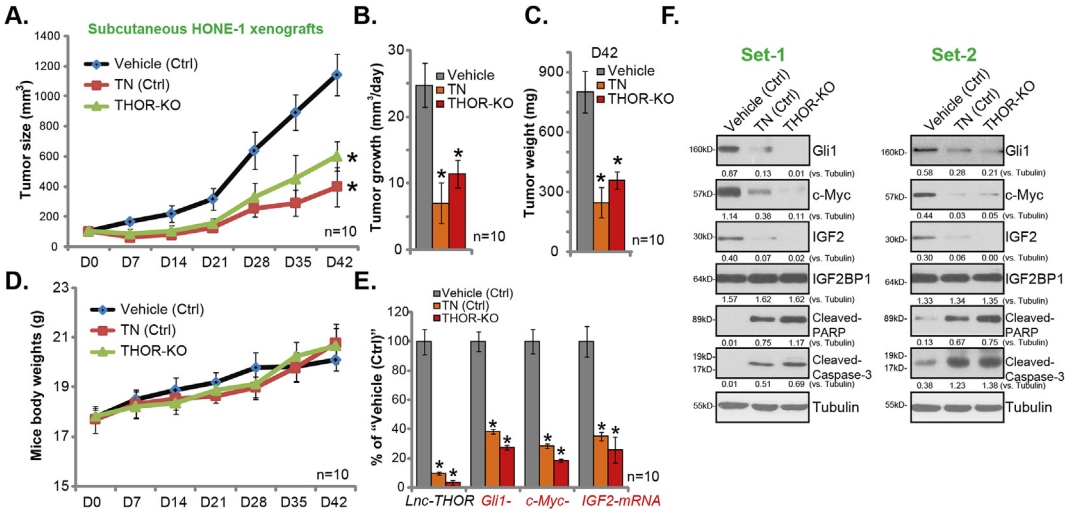

Fig. 7. Triptonide or *Lnc-THOR* knockout inhibits subcutaneous NPC xenograft tumor growth in SCID mice. Control, "THOR-KO" HONE-1 cells (**A–F**) were *s.c.* inoculated to the flanks of the SCID mice (1×10^7 cells per mouse) to form xenograft tumors, TN (*i.p.*, daily, 10 mg/kg body weight, for 21 consecutive days) or saline control ("Vehicle") treatment was started when tumor volume was around 100 mm^3 ("Day-0"), thereafter tumor volumes (**A**) and mice body weights (**D**) were recorded every seven days for a total of 42 days. Estimated daily tumor growth (mm^3 per day) was calculated as described (**B**); Mice were euthanized at Day-42, tumors were weighted individually (**C**). Expression of *Lnc-THOR* and listed genes (mRNA and protein) in fresh tumor tissue lysates (at Day-14) were tested (**E** and **F**). Expression of indicated proteins were quantified, and their values were normalized to the loading control Tubulin (**F**). The number of mice per group, n = 10. $^*p < 0.05$ vs. Vehicle-treated control mice.

"LV-THOR" cells, even after the TN treatment (Fig. 6G). Remarkably, TN-induced cell viability reduction (Fig. 6H), cell death (Fig. 6I) and apoptosis (Fig. 6J) were almost completely abolished in "LV-THOR" HONE-1 cells. Thus, ectopic overexpression of *Lnc-THOR* prevents TN-induced cytotoxicity, supporting the mechanism that *Lnc-THOR*-IGF2BP1inhibition mediates TN-induced actions in NPC cells.

3.7. Triptonide or Lnc-THOR knockout inhibits subcutaneous NPC xenograft tumor growth in SCID mice

The potential anti-NPC activity of TN *in vivo* was tested by using a xenograft SCID mice model. "THOR-KO" HONE-1 cells or the control cells were injected *s.c.* into the SCID mice. After HONE-1 xenograft tumors were established, mice were treated with TN (10 mg/kg, *i.p.* daily, for 20 days, for control tumors). Recording tumor volume every 7 days for a total of 42 days, we found that TN administration significantly inhibited HONE-1 xenograft tumor growth, causing significant reduction of tumor volume (Fig. 7A). Similarly, as compared to the control tumors, "THOR-KO" HONE-1 tumors grew significantly slower (Fig. 7A). The estimated daily tumor growth was calculated (tumor volume at day-42 subtracting tumor volume at day-0/42), and results further confirmed that TN administration or "THOR-KO" significantly inhibited HONE-1 tumor growth *in vivo* (Fig. 7B). At day-42, tumors of all groups were isolated and weighted, and control HONE-1 tumors with TN treatment or "THOR-KO" tumors weighted less than the control tumors with vehicle treatment (Fig. 7C). Mouse body weights were not significantly different between the groups (Fig. 7D). No apparent toxicities, including fever, fatigue, skin damage and vomiting, were noticed in the experimental mice.

At day-14, one tumor xenograft from each group was separated. Assaying the fresh tumor by qRT-PCR showed that *Lnc-THOR* expression levels were significantly decreased in TN-treated and "THOR-KO" HONE-1 tumors (Fig. 7E), and the expression of IGF2BP1 mRNA targets, *IGF2*, *Gli1* and *c-Myc*, were decreased as well (Fig. 7E). Protein levels of IGF2, Gli1 and c-Myc were downregulated in TN-treated and "THOR-KO" HONE-1 tumors (Fig. 7F), where cleaved-caspase-3 and cleaved-PARP levels were elevated (indicating apoptosis activation, Fig. 7F). Notably, IGF2BP1 protein expression was equivalent between the three groups (Fig. 7F). These results show that TN treatment inhibited the *Lnc-THOR*-IGF2BP1 pathway *in vivo*.

4. Discussion

Recent studies have investigated the potential anti-cancer activity of TN. Pan *et at.,* show that TN can induce leukemia cell senescence and apoptosis by inhibiting TERT and c-Myc transcription, while simultaneously upregulating p16 and p21 [4], and by inhibiting Lyn pathways [6]. Furthermore, TN treatment has been reported to strongly inhibit colony formation-, migration-, and invasion-promoting capacities of gastric cancer-associated fibroblasts (GCAF) [7]. TN abolished GCAFs potential of epithelial-mesenchymal transition into gastric cancer cells [7]. TN has also been shown to block the canonical Wnt/β-catenin signaling pathway by targeting β-catenin, to inhibit Wnt-dependent cancer [8].

In the present study, we show that TN potently inhibited survival and proliferation of established and primary human NPC cells, with an IC-50 lower than 50 nM. TN induced cell cycle arrest and apoptosis activation in NPC cells, and significantly inhibited human NPC *in vitro* cell migration and invasion. Importantly, we show that TN was non-cytotoxic to the primary human nasopharyngeal epithelial cells. *In vivo*, daily intraperitoneal TN injection significantly inhibited subcutaneous NPC xenografts growth in SCID mice, without any apparent toxicity. These results highlight the promising anti-NPC cell potential of TN.

Hosono et al., recently identified a conserved IGF2BP1-bound *LncRNA*, namely *Lnc-THOR* [15]. *Lnc-THOR* expression is detected in testis and different cancers [15,16,19]. *Lnc-THOR* directly binds to IGF2BP1, which is essential for IGF2BP-mediated stability of key oncogenic mRNAs [15–17,19]. *Lnc-THOR* silencing or depletion can inhibit cancer cell growth *via* disabling IGF2BP1 [15–17,19]. Conversely, ectopic overexpression of *Lnc-THOR* can enhance IGF2BP1-mediated pro-cancer functions [15].

In the current study, we show that TN downregulates Lnc-THOR and disrupts Lnc-THOR-IGF2BP1 association in NPC cells, causing significant decreases of IGF2BP1's mRNA targets (*Myc*, *IGF2* and *Gli1*). In TN-treated NPC xenograft tumor tissues, *Lnc-THOR* as well as IGF2BP1 pathway components were significantly downregulated. Importantly, *Lnc-THOR* or IGF2BP1 knockout by CRISPR/Cas9 gene-editing method mimicked and abolished TN-induced actions in NPC cells. Conversely,

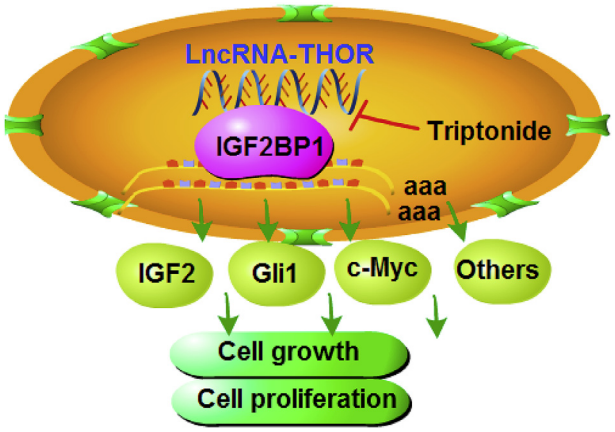

Fig. 8. The proposed signaling pathway of the current study. Triptonide (TN) downregulates *Lnc-THOR* and disrupts *Lnc-THOR*-IGF2BP1 association, leading to downregulation of IGF2BP1's targets (*Myc*, *IGF2*, *Gli1* and possible others), eventually inhibiting nasopharyngeal carcinoma (NPC) cell growth and proliferation. IGF2: insulin-like growth factor 2; IGF2BP1: insulin-like growth factor 2 mRNA-binding protein 1; Gli1: glioma-associated oncogene homolog 1.

ectopic overexpression of *Lnc-THOR* almost reversed TN-induced cytotoxicity in HONE-1 NPC cells. These results suggest that disruption of the *Lnc-THOR*-IGF2BP pathway could be the primary mechanism of TN-induced cytotoxicity in NPC cells (See the proposed signaling pathway in Fig. 8).

Significantly, expression of *Lnc-THOR*, IGF2BP1 and its mRNA targets (*Myc*, *IGF2* and *Gli1*) are significantly elevated in human NPC tissues and cells, but barely detectable in the nasopharyngeal epithelial tissues and cells. These results further indicate that the *Lnc-THOR*-IGF2BP pathway could be a valuable and novel therapeutic target for NPC treatment. This is supported by our finding that *Lnc-THOR* or IGF2BP1 knockout significantly inhibited NPC cell survival and proliferation *in vitro*. *In vivo*, *Lnc-THOR*-knockout HONE-1 xenografts grew significantly slower than control tumors. Further studies will be needed to develop novel strategies targeting this pathway to better NPC treatment.

5. Conclusion

In summary, our results show that TN potently inhibits human NPC cell growth *in vitro* and *in vivo* via disruption of *Lnc-THOR*-IGF2BP1 signaling. TN is a valuable therapeutic candidate that should be further tested for NPC.

Conflicts of interest

All the listed authors in this study assure that the data in the manuscript is original and the manuscript is not under consideration elsewhere. None of the manuscript contents have been previously published. All authors have made significant contributions of this work, and have read and approved all versions of the manuscript, its all content, and its submission to the ***Cancer Letters.***

The authors have no conflict of interests.

Competing interests

The authors declare that they have no competing interests.

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