

## cDNA Expression Array Analysis of Gene Expression in Human Hepatocarcinoma Hep3B Cells Induced By *BNIP-1*

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**Abstract** Bcl-2/adenovirus E1B 19 kDa interacting protein 2 like-1 (BNIP-1) is a novel human protein identified in our laboratory, which can interact with Bcl-2 and Cdc42GAP and induce apoptosis via the BNIP-2 and Cdc42GAP homology (BCH) domain. In the present study, we established the Hep3B-Tet-on stable cell line in which expression of BNIP-1 can be induced by doxycycline. The cell proliferation activity assay showed that the overexpression of BNIP-1 suppresses Hep3B cell growth *in vitro*. The differential expression profiles of 588 known genes from BNIP-1-transfected Hep3B-Tet-on and vector control cells were determined using the Atlas human cDNA expression array. Fifteen genes were differentially expressed between these two cell lines, among which seven genes were up-regulated and eight genes were down-regulated by BNIP-1. Furthermore, the differential expression result was confirmed by semiquantitative RT-PCR. Among these differentially expressed genes, *p16<sup>INK4</sup>*, *IL-12*, *TRAIL* and the lymphotoxin  $\beta$  gene involved in growth suppression or cell apoptosis were up-regulated, and *PTEN* involved in cell proliferation was down-regulated by BNIP-1. These results suggest that BNIP-1 might inhibit cell growth through cell cycle arrest and/or apoptotic cell death pathway(s).

**Key words** BNIP-1; Tet-on; Atlas human cDNA array; apoptosis

Bcl-2/adenovirus E1B 19 kDa interacting protein 2 like (BNIP) has been recently identified and characterized as an apoptosis-associated protein [1–3]. BNIP shares 72% homology (46% identity) with BNIP-2 [4] and has two variants, BNIP-1 (GenBank accession No. AF193056, also called BNIP- $\alpha$ ) and BNIP-2 (accession No. AY033000). The full-length amino acid sequence of BNIP-1 is 82 amino acids shorter at the N-terminus compared with that of BNIP-2. Previous studies have shown

that BNIP-1 interacts with Bcl-2 and Cdc42GAP, and induces apoptosis via the BNIP-2 and Cdc42GAP homology (BCH) domain [3]. However, the molecular mechanism by which BNIP-1 promotes apoptosis is not well defined so far.

Artificially regulated transgene expression systems are indispensable tools for studying the molecular mechanism of the differentiation process. Tetracycline (Tc)-regulated transgene expression systems, known as Tet-off and Tet-on systems, have been widely applied to a variety of biological materials, including mammalian cells [5,6]. The Tet-on system uses a reverse Tc-regulated transactivator (rtTA), which binds a Tc-responsive element (TRE) and induces transcription of the transgene in the presence of doxycycline (Dox) [7]. Microarray technology enables the monitoring of thousands of genes, and measuring the

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relative abundance of mRNA transcripts. There are a number of different microarray platforms available, which include cDNAs [8] or oligonucleotides [9] that are spotted on nylon membranes [10] or glass slides [11]. The cDNA expression array is a novel and robust method for comparing the expression of a large number of genes simultaneously and has been successfully used to study differential gene expression profiles [12].

In the present study, we established a Hep3B cell line with the doxycycline-regulated *BNIP-1* transgene system and identified differentially expressed genes regulated by *BNIP-1* in the Hep3B cell line using cDNA microarray technology.

## Materials and Methods

### Plasmid construction

The human *BNIP-1* full-length coding sequence was amplified by PCR using the primers containing *Bam*HI and *Cla*I restriction sites and the Myc-tag sequence (**Table 1**). The PCR product was subcloned into the Tet-on expression vector pTRE2hyg which contains the hygromycin-resistant gene. The recombinant plasmid construct, pTRE2hyg-*BNIP-1*, was confirmed by *Bam*HI/*Cla*I digestion (New England Biolabs Inc., Beverly, USA) and DNA sequencing.

### Cell culture

The human hepatocarcinoma cell line Hep3B was cultured in DMEM (Gibco BRL, Carlsbad, USA) containing 2 mM *L*-glutamine solution and 10% heat-inactivated Tet system-approved fetal bovine serum (Clontech, Palo Alto, USA). Cells were maintained at 37 °C in 5% CO<sub>2</sub>. They were plated in plates or dishes, and allowed to grow until the cultures were confluent.

### Cell transfection

Hep3B-Tet-on cells constructed by Wang *et al.* [13] were grown in 6-well plates to 70% confluence and transfected with pTRE2hyg-*BNIP-1* plasmid DNA using lipofectamine reagents (Invitrogen, Carlsbad, USA) under conditions recommended by the manufacturer. After 48 h, the medium was replaced with fresh DMEM containing 100 µg/L hygromycin to select the stable cell lines. Two weeks later, 43 hygromycin-resistant clones were obtained and subjected to further selection in the medium containing 25 mg/L hygromycin for more than three weeks, and then analyzed by Western blot using an anti-Myc monoclonal antibody (Invitrogen). *BNIP-1* expression in the cell line was induced by incubating cells with Dox, which is a Tetracycline analog (Clontech).

### Western blot

Whole-cell lysate was extracted by the T-PER™ tissue

**Table 1** Gene specific primers for PCR

Gene	GenBank accession No.	Sequence (5'→3')
<i>BNIP-1</i>	AF193056	Forward <u>CGGGATCCACC</u> <u>ATGGAACAAAACTCATCTC-</u> <u>AGAAGAGGATCTGCGCAAGCGTCTTTCT</u>
		Reverse <u>CCATCGATCTATGTCCCTCCTGAGCCATGGAG-</u> <u>ATCCCGGTCCAGCTGTCTGACAGC</u>
<i>β-actin</i>	NM_001101	Forward AAGTACTCCGTGTGGATCGG
		Reverse TCAAGTTGGGGGACAAAAG
<i>p16-INK4</i>	L27211	Forward CAGACATCCCCGATTGAAAG
		Reverse TTTACGGTAGTGGGGGAAGG
<i>Apo-2 ligand</i>	U57059	Forward GTTGTTGGTCTAAAGATGCAGA
		Reverse TGCTTTTCTTTCCAGGTCAG
<i>HBNF-1</i>	M57399	Forward AACACAGCCCTGAAGACCAG
		Reverse TCCTGTTTGCTGATGTCCTTT
Endothelin <i>ET2</i>	M65199	Forward ACACATTCCAGGTGGAGGAA
		Reverse ACAGAACTGCCTTGGACGAG

Restriction endonuclease recognition sites are underlined and the Myc-tag is double underlined.

protein extraction reagent containing 1% (V/V) proteinase inhibitor cocktail. Then, 10 µg of total protein from each sample was fractionated on 10% SDS-PAGE gels and electroblotted onto PROTRAN nitrocellulose membranes. After being blocked in TBST (20 mM Tris-HCl, pH 7.6, 130 mM NaCl, 0.1% Tween-20) containing 5% non-fat milk, the membranes were incubated with the primary anti-Myc antibody at 1:2000 dilution or monoclonal antibody actin pan Ab-5 (Neomarker, Westinghouse Drive, Fremont, USA) and then with horseradish peroxidase-coupled anti-mouse IgG (Santa Cruz, USA), and developed using SuperSignal west femto maximum sensitivity substrate (Promega, Madison, USA).

### Cell proliferation assay

Cell viability was determined by Cell-titer 96<sup>®</sup> aqueous one solution cell proliferation assay kit (Promega). Aliquots of  $2 \times 10^3$  cells/well were cultured in 96-well plates with or without 2000 µg/L Dox for 24, 48, 72, 96 and 120 h. The medium was removed at the end of the incubation. Then, 100 µl of DMEM and 20 µl of Cell-titer 96<sup>®</sup> aqueous one solution were added to each well and incubated for an additional 4 h. The absorbance at 490 nm was recorded with a 96-well plate reader. Each experiment was performed in triplicate and repeated at least three times.

### RNA extraction

Hep3B-Tet-on-BNIPL-1/20 cells were induced with or without 2000 µg/L Dox for 24 h. Cells were washed once with PBS, and total RNA samples were extracted from  $1 \times 10^7$  cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions, followed by treatment with RNase-free DNase at 37 °C for 20 min to avoid contamination of genomic DNA. The RNA quality and concentration were assessed using agarose gel electrophoresis and spectrophotometer readings.

### Probe preparation

Five micrograms of total RNA was reverse transcribed using 1 µl of CDS primer mix, 3.5 µl of [ $\alpha$ -<sup>32</sup>P]dATP (3000 Ci/mmol; Amersham Pharmacia Biotech, Piscataway, USA), 0.5 µl of 0.1 M DTT, 1 µl of dNTP mixture (containing dCTP, dGTP and dTTP), 1 µl of MMLV reverse transcriptase and 2 µl of 5×reaction buffer provided in the Atlas human cDNA expression array kit (Clontech). The <sup>32</sup>P-labeled cDNA probes ( $2$ – $10 \times 10^6$  cpm) were purified using a NucleoSpin extraction spin column to remove the unincorporated nucleotides. After purification, labeled cDNAs were denatured before use.

### Hybridization and imaging

Broad-scale expression profiling was performed using Atlas human cDNA expression array kit. Each array is spotted with duplicate cDNA fragments from 588 known genes and nine housekeeping genes or control sequences. The 588 known genes spotted on the Atlas membrane consist of cDNA fragments for cell-cycle control proteins, stress response proteins, apoptosis-associated proteins, DNA transcription factors, cell receptors, extracellular cell signaling and communication proteins, etc.. A complete list of the genes spotted on the array with their array positions and GenBank accession numbers is available at Clontech's website (<http://www.clontech.com>). Hybridization was carried out at 68 °C overnight in a hybridization oven. The membranes were washed thrice with 250 ml of 2×SSC containing 1% SDS and once with 250 ml of 1×SSC containing 0.1% SDS solution at 68 °C for 30 min. Then the membranes were wrapped and exposed to X-ray film for autoradiography at –70 °C for 48 h.

### Statistical analysis

The images for the hybridized Atlas arrays were scanned with the Cyclone<sup>™</sup> storage phosphor system (Packard Bioscience Company, Meriden, USA) and saved as \*.tiff files. The images were imported into the Quantarray<sup>®</sup> image system (Packard Bioscience Company) and analyzed. Housekeeping ubiquitin and  $\beta$ -actin genes were selected for normalization. The adjusted intensity was equal to the mean intensity of each duplicate gene minus the background value. The signal ratio of each given gene between two compared array membranes was then calculated. In the present study, the ratio threshold was set to be 2.0. Only those genes that showed a 2-fold or greater increase or decrease in number were considered to be differentially expressed.

### RT-PCR analysis

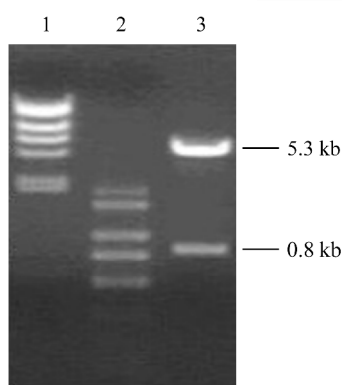
The expression of some prominently changed genes was further examined by RT-PCR to confirm the array results. First-strand cDNA was synthesized from total RNA using the SuperScript<sup>™</sup> first-strand synthesis system (Invitrogen). The PCRs were performed with first-strand cDNA for 26 cycles of 30 s each at 94 °C, 26 cycles of 30 s each at 60 °C and 26 cycles of 30 s each at 72 °C, followed by an extension for 3 min at 72 °C. The primers for the PCR are listed in **Table 1**. The RT-PCR products were subjected to electrophoresis in 2% agarose gels. To ensure that equal amounts of reverse-transcribed cDNA were applied to the PCR reaction, the  $\beta$ -actin gene was

also included in the PCR as a control.

## Results

### Construction and identification of the *BNIP1-1* expressing vector pTRE2hyg-*BNIP1-1*

The human *BNIP1-1* gene (0.8 kb) was cloned into the multicloning site of the pTRE2hyg vector (5.3 kb), designated as pTRE2hyg-*BNIP1-1* (6.1 kb), and verified by *Bam*HI and *Cla*I restriction endonuclease digestion (Fig. 1). The complete sequence of *BNIP1-1* was further verified by sequencing (data not shown).



**Fig. 1** 1% agarose gel electrophoresis analysis of recombinant pTRE2hyg-*BNIP1-1* digested with *Bam*HI and *Cla*I

1, Lambda DNA/*Hind*III marker; 2, SD 006 marker; 3, pTRE2hyg-*BNIP1-1* digested with *Bam*HI and *Cla*I.

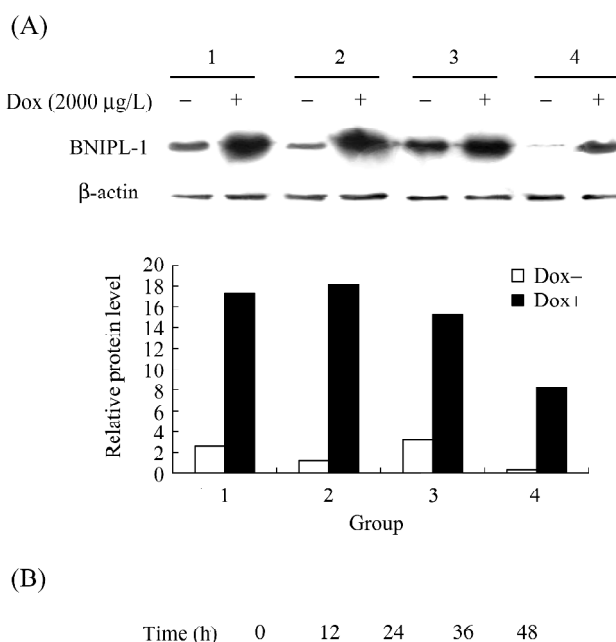
### Expression of *BNIP1-1* by doxycycline induction

After screening in the medium containing hygromycin for more than six weeks, a total of 43 independent hygromycin-resistant cell lines were obtained from the Hep3B-Tet-on cells transfected with pTRE2hyg-*BNIP1-1*. To verify the induction of *BNIP1-1* protein by doxycycline treatment, Western blot analysis was performed with equal amounts of total cell protein from different Hep3B-Tet-on-*BNIP1-1* cell lines. The clone Hep3B-Tet-on-*BNIP1-1*/20 showed a low background and high Dox-induced expression of *BNIP1-1*, and was selected as the cell line for further investigations. The amount of *BNIP1-1* induced in the clone Hep3B-Tet-on-*BNIP1-1*/20 cells treated with Dox (2000 µg/L) was 26-fold higher than

that in the cells without Dox treatment [Fig. 2(A)]. Moreover, the *BNIP1-1* expression increased in a time-dependent manner [Fig. 2(B)].

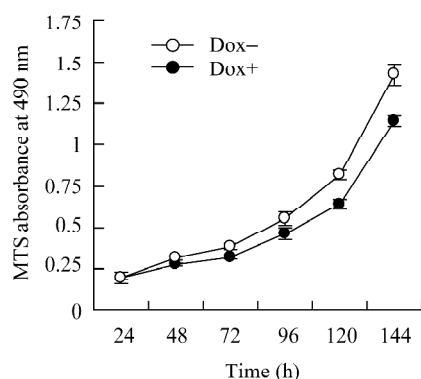
### Cell proliferation assay

To assess cell proliferation, we used the Cell-titer 96<sup>®</sup> aqueous one solution cell proliferation assay that is based on the metabolic conversion of a tetrazolium compound MTS to a colored product by living cells. The absorbance intensity of the MTS product is directly proportional to the number of viable cells in culture. As shown in Fig. 3, overexpression of *BNIP1-1* significantly inhibited Hep3B cell growth ( $P < 0.05$ ). At 120 h, *BNIP1-1* inhibited the proliferation rate by 21.7%. Each point in Fig. 3 represents the mean ± SD of triplicate experiments.



**Fig. 2** Western blot analysis of *BNIP1-1* expression in the presence or absence of Dox

(A) Different cell proteins in the absence and presence of Dox was obtained from 43 clones after hygromycin selection. 1, lysate of clone Hep3B-Tet-on-*BNIP1-1*/7; 2, lysate of clone Hep3B-Tet-on-*BNIP1-1*/10; 3, lysate of clone Hep3B-Tet-on-*BNIP1-1*/16; 4, lysate of clone Hep3B-Tet-on-*BNIP1-1*/20. The clone Hep3B-Tet-on-*BNIP1-1*/20 showed a low background and high Dox-induced expression of *BNIP1-1*. The intensity of each band was quantified by densitometry. (B) Clone Hep3B-Tet-on-*BNIP1-1*/20 cells were treated with Dox for different durations (0, 12, 24, 36 and 48 h). Equal amounts of total cell protein were analyzed using anti-Myc monoclonal antibody. The  $\beta$ -actin gene was used as an internal control.



**Fig. 3 Inhibition of cell growth of Hep3B cells by BNIPL-1**  
Clone Hep3B-Tet-on-BNIPL-1/20 cells were treated with or without Dox (2000  $\mu\text{g/L}$ ) for 24, 48, 72, 96, 120 and 144 h. Cell viability was determined by the Cell-titer 96<sup>®</sup> aqueous one solution cell proliferation assay. Each point represents the mean $\pm$ SD of triplicate experiments. Statistically significant differences were obtained ( $P < 0.05$ ;  $n = 6$ ).

### Identification of differentially expressed genes in clone Hep3B-Tet-on-BNIPL-1/20 cells

To identify differentially expressed genes regulated by *BNIPL-1*, we compared the gene expression profiles of 588 genes in clone Hep3B-Tet-on-BNIPL-1/20 cells treated with or without Dox (2000  $\mu\text{g/L}$ ) for 24 h. No signals were visible in the blank spots and negative control spots, indicating that the Atlas human cDNA array hybridization was highly specific. The density of housekeeping genes was very similar among the samples, indicating that the results were reliable. We used ubiquitin and  $\beta$ -actin genes to normalize the intensities among arrays. The comparison results analyzed by the Quantarray<sup>®</sup> image system showed that the expression of seven genes was up-regulated (Table 2) and that of eight genes was down-regulated (Table 3) following overexpression of BNIPL-1 in Hep3B cells. The genes involved in growth inhibition (*p16<sup>INK4</sup>* and *IL-12*) or cell apoptosis [*TRAIL* and lymphotoxin  $\beta$  (LTB) gene] were up-regulated, and the genes involved in proliferation (*PTN*) were down-regulated.

### Semiquantitative RT-PCR

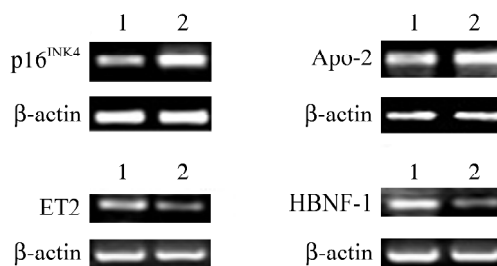
Although differences in gene expression regulated by *BNIPL-1* were observed with the cDNA array, it was essential to verify these results using other methods. We monitored the mRNA level of four selected genes shown to be differentially expressed in the Atlas arrays by RT-PCR. The results were consistent with the hybridization data for each of the genes monitored (Fig. 4); that is,

**Table 2 Up-regulated genes in BNIPL-1-transfected Hep3B-Tet-on cells**

Location	Gene description	Induction (fold)
E2n	interleukin-6 receptor	2.84
E3m	cytokine receptor (EBI3)	2.53
A6k	CDK4-inhibitor ( <i>p16<sup>INK4</sup></i> ); multiple tumor suppressor 1 (MTS1)	2.40
E3n	interleukin-12 receptor component	2.20
C4m	Apo-2 ligand; TNF-related apoptosis inducing ligand TRAIL	2.06
D2g	cAMP-responsive element-binding protein	2.04
C3n	lymphotoxin $\beta$	2.01

**Table 3 Down-regulated genes in BNIPL-1-transfected Hep3B-Tet-on cells**

Location	Gene description	Induction (fold)
F3h	nerve growth factor HBNF-1; PTN; HB-GAM; HBGF-8; OSF-1	0.22
D6d	R $\kappa$ B	0.38
F4b	endothelin ET2	0.40
D6l	ard-1	0.40
E5k	differentiation antigen CD19	0.43
B1l	tyrosine kinase receptor TKT	0.46
D7j	transcription factor TFIIIB 90 kDa subunit HTFIIIB90	0.46
B4m	serine kinase	0.50



**Fig. 4 Semiquantitative RT-PCR assay of *p16<sup>INK4</sup>*, *Apo-2* ligand, *HBNF-1* and *endothelin ET2* before and after BNIPL-1 overexpression**

The  $\beta$ -actin gene was also amplified as an internal control. The figure comprises the hybridization results. 1, before BNIPL-1 overexpression; 2, after BNIPL-1 overexpression.

*p16<sup>INK4</sup>* and *Apo-2 ligand* were up-regulated, and *HBNF-1* and *endothelin ET2* were down-regulated in *BNIP-1* overexpressed cells.

## Discussion

*BNIP-1* is homologous to *BNIP-2*, a previously known Bcl-2 and E1B-associated protein [4]. It contains a complete *BNIP-2* and *Cdc42GAP* homology (BCH) domain. The expression of *BNIP-1* or the complete BCH domain can cause extensive apoptosis in cells. *BNIP-1* forms a homophilic complex via a unique sequence motif within its BCH domain, and deletion of this interacting motif removes its pro-apoptotic effect [3]. However, the downstream signaling molecules that are involved in *BNIP-1*-induced apoptosis or cell growth suppression should be examined.

In the present study, we established a cell line, Hep3B-Tet-on-*BNIP-1/20*, in which the *BNIP-1* gene can be conditionally induced by Dox treatment. Dox activates the Tet-response element and significantly enhances the expression of the *BNIP-1* gene in Hep3B cells in a dose-dependent manner. A stable cell line with a low background and high Dox-induced expression of *BNIP-1* was obtained, which enabled us to investigate *BNIP-1*-induced gene expression efficiently and rapidly.

To define gene expression profiles and compare patterns of expression at different stages of inducement, we can use RT-PCR, RNase protection assays or Northern blot analysis, but these methods focus on only a few genes at a time. A more promising approach for analyzing multiple genes simultaneously is the hybridization of entire cDNA populations to nucleic acid arrays, a method that has been adopted for high-throughput analysis of gene expression [12]. It allows for the rapid detection of the gene expression profiles of hundreds or thousands of genes simultaneously. Here, we applied cDNA array technology to compare the expression profiles in the human hepatocarcinoma cell line Hep3B regulated by *BNIP-1*. Following the overexpression of *BNIP-1* in Hep3B cells, the expression levels of 15 genes were altered, of which seven were up-regulated and eight were down-regulated. Genes involved in growth inhibition (*p16<sup>INK4</sup>* and *IL-12*) or cell apoptosis (*TRAIL* and *LTB*) were up-regulated. P16 has been reported to induce apoptosis in K562 cells [14]. *IL-12* inhibits proliferation and increases the apoptotic rate of *IL-12Rβ2*-transfected B cell lines *in vitro* [15]. *TRAIL* is a member of the TNF gene superfamily that is able to induce apoptosis through the binding of two cell-surface

DRs (DR4 and DR5). *TRAIL* directly engages the cell-extrinsic apoptosis pathway through recruitment of the death-inducing signal complex and activation of initiator caspases, which activate effector caspases that ultimately execute programmed cell death. *TRAIL* can also engage the intrinsic apoptosis pathway through caspase-dependent activation of proapoptotic Bcl-2 family members [16]. The protein of *LTB* gene which is localized to the major histocompatibility complex region on chromosome 6p21.3 has an important role in the formation of germinal center reactions and regulation of immune response and apoptosis [17]. Genes involved in proliferation (*PTN*) and cell apoptosis (*ET-2*) were found to be down-regulated. In serum-starved NIH3T3 fibroblasts, *PTN* prevents apoptosis and induces cell growth. Anti-apoptotic signaling of *PTN* in NIH3T3 fibroblasts takes place via the MAP kinase pathway [18]. Endothelins (ET-1, ET-2 and ET-3) are a group of small vasoactive peptides (21 amino acids) with diverse paracrine/autocrine actions. ETs are expressed in a broad range of tumors; adding the ET-2 peptide to a culture medium moderately reduces the tumor cell death associated with hypoxia [19].

In conclusion, our results show that the combination of a Tet-on inducible system and cDNA microarray is an effective and powerful strategy to identify downstream signaling molecules induced by *BNIP-1*. Among these differentially expressed genes induced by *BNIP-1* in hepatoma Hep3B cells, *p16<sup>INK4</sup>*, *IL-12*, *TRAIL* and *LTB* were up-regulated, and *PTN* was down-regulated. These results suggest that *BNIP-1* might promote cell apoptosis and growth suppression through cell cycle arrest and/or apoptotic cell death pathway(s). However, the exact mechanisms need to be investigated further.

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