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Original Article

Tumor suppressor BLU exerts growth inhibition by blocking ERK signaling and disrupting cell cycle progression through RAS pathway interference

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Abstract: We have previously reported that the 3p21 tumor suppressor *BLU* regulates cell cycle by blocking JNK/MAPK signaling. Another member of the MAPK family, extracellular signal response kinase (ERK), is induced by the RAS-RAF-MEK-ERK pathway and is targeted in anticancer therapy. The effects of *BLU* on tumor growth were evaluated by measuring the size of nasopharyngeal carcinoma (NPC) xenografted tumors intra-tumorally injected with *BLU* adenovirus 5 (*BLU* Ad5) and the viability of NPC cells transferred with *BLU*. Tumor size was correlated with downregulation of the ERK pathway by *BLU*. Phosphorylation of ERK and Elk reporter activities were assayed. The regulated cyclins D1 and B1 were measured by *CCND1* and *CCNB1* gene promoter activity by co-transfection of *BLU, RAS* V12G, together with *BLU+RAS V12G, pCD316+RAS V12G*. The cell cycle phase distribution was determined by FACS-based DNA content assay. The data showed that growth of the xenografted tumor was inhibited and viability of HONE-1 cells was reduced by recombinant *BLU*. *BLU* down-regulated ERK signaling by reducing protein substrate phosphorylation, inhibiting Elk reporter activity, and blocking promoter activities of the *CCND1* gene and reduced cyclins D1 expression to arrest the cell cycle at the G1 phase. The population of G2/M cells was also remarkably decreased. *HRAS V12G* activated ERK and cyclin D1 and B1 promoters, and the effects were antagonized by *BLU*. Taken together, our results suggested that *BLU* inhibited ERK signaling, downregulated cyclins D1 and B1, and prevented cell cycle progression through interfering with *HRAS V12G* signaling to exert tumor suppression.

Keywords: *BLU*/ZMYND10, H-*RAS*, MAPK/ERK, cyclin D1, cyclin B1, cell cycle

Introduction

Carcinogenesis is a multistep, multifactorial process involving anomalies at the cytogenetic and molecular levels, including a loss of homozygosity (LOH) on chromosomal regions, gene amplification, and hypermethylation at the promoters of tumor suppressor genes (TSGs) [1].

BLU is a candidate TSG mapped to a frequently lost chromosomal region, 3p21. Its inactivation has been seen in a variety of tumors of epithelial origin, including breast, lung, cervical, and nasopharyngeal cancers [2, 3]. In nasopharyngeal carcinoma (NPC), *BLU* expression is down-regulated due to hypermethylation on the promoter of its coding gene [4, 5]. The mechanisms underlying tumor suppression by *BLU* are poorly understood. Proteins with similar structures recognize specific sites on histones, and catalyze post-translational modifications like methylation on lysine residues, and regulate gene transcription [6, 7]. We have shown that BLU inhibits NF-κB signaling and hence NF-κB dependent anti-apoptotic factors cFLIP and cIAP2, to promote apoptosis induced by TRAIL [8].

Similar with the tumor suppressor RASSF1A, which mapped to the same chromosomal region, 3p21 [9, 10], *BLU* down-regulates c-Jun

N-terminal kinase (JNK), which is a member of the mitogen-activated protein kinase (MAPK) family that integrates signals of growth, proliferation, and apoptosis, and inactivates the promoter of the cyclin D1 coding gene CCND1, and dampens expression of cyclin D1 to arrest the cell cycle at the G1 phase [11].

MAPK family proteins are activated by epidermal growth factor receptor (EGFR), the most frequently employed target of anticancer therapy, through activation of RAS proteins which transmit signals of receptor occupied growth factors, cytokines, and other stimuli [12]. Mutation-activated RAS proteins are implicated in malignant transformation [13, 14] and are modulated by interacting with a group of proteins that display a conserved domain to bind the small molecule guanidine nucleotide associating protein. Members of this family comprise RASSF1 and RASSF5/NORE1, which negatively regulate the activity of RAS proteins [15, 16]. Activated RAS proteins induce a kinase cascade involving kinases RAF and an MAPK family protein, extracellular signal-regulated kinase (ERK) [17, 18].

In the present study, we tested whether tumor suppression by *BLU* is exerted by targeting the RAF-MEK-ERK pathway, through interfering with the RAS oncogene with a high frequency of mutational activation in human cancers.

Materials and methods

Cells, plasmids and packaging of viral vector of BLU

HONE1 cells derived from a Chinese patient with undifferentiated NPC [19, 20] were maintained in RPMI-1640 that was supplemented with 10% fetal calf serum (FCS) (Gibco Biotech nology, Guangzhou, China) and 1% penicillin+ streptomycin (PEST) antibiotics. SKOV3 cells were derived from a serious ovarian cancer patient, and purchased from Cell Bank, Institute of Life Science, Chinese Academy of Science (CAS) Shanghai Branch. *BLU* pCD316 was constructed by inserting the cDNA of BLU/ZMYND10 into the cytomegalovirus-derived shuttle plasmid pCD316 as described previously [8, 11]. The recombinant shuttle plasmid was co-transfected to packaging 293 cells with at least two viral structural protein coding genes to generate recombinant *BLU* adenovirus type 5 (BLU Ad5) which express *BLU* on infection of target cells. The purified cell lysate was used in infection to transfer *BLU*. The expression vector/plasmid of H-*RAS V12G* carrying a point mutation on codon 12 of H-*RAS* and the empty vector pBABE were a kind gift from Professor Bob Weinberg (Massachusetts Institute of Technology, Boston, MA, USA) [21] and Dr. Elena Kashuba (Karolinska Institutet, Stockholm, Sweden) [22]. The ERK reporter plasmids pFA+pFR with Elk were purchased from Strategene (Guangzhou, China) [23]. Reporter plasmids for the promoters of the CCND1 and CCNB1 genes were kind gifts from Professors Richard Pestell (Temple University, Philadelphia, PA, USA) [24] and Kurt England (University of Leipzig, Leipzig, Germany) [25, 26].

Antibodies

The murine anti-human cyclin B1 mAb, GNS3 (8A5D12; Millipore, Guangzhou, China); rabbit anti-human cyclin D1 polyclonal antibody (Cell Signaling Technology, Guangzhou, China); goat anti-human BLU/ZYND10 polyclonal (Abcam, Cambridge, U.K.); Anti-p44/42 MAPK/ERK1/2 Rabbit mAb (#4695), and anti-Phospho-p44/42 ERK1/2 (Thr202/Tyr204) Rabbit mAb (#4370) purchased from Cell Signaling Technology were used in probing the proteins transferred to nitrocellulose filter. The reaction of primary antibodies binding was developed with ultrared labelled Odyssey antibodies against the immunoglobulin (Ig) of the primary antibodies (Li-COR Biosciences).

Cell viability assay

Up to 1,000 cells were seeded into each well of a 96 well tissue culture plate. After overnight (ON) incubation cells were challenged with *BLU* Ad5 at doses of 0, 5, 10, 20, and 40 PFU/cell in triplicate for 24 h. The cells were then washed twice with sterile PBS and incubated with the Cell Counting Kit-8 (CCK-8) reagent (Dojindo, Guangzhou, China). Absorbance values were read on a BioTek ELISA plate reader.

Western blotting

Monolayer cells transfected with BLU were harvested, washed with sterile PBS, and lysed with RIPA+lysis buffer. DNA was sheared by sonication, and the lysate was boiled at 95°C for 5

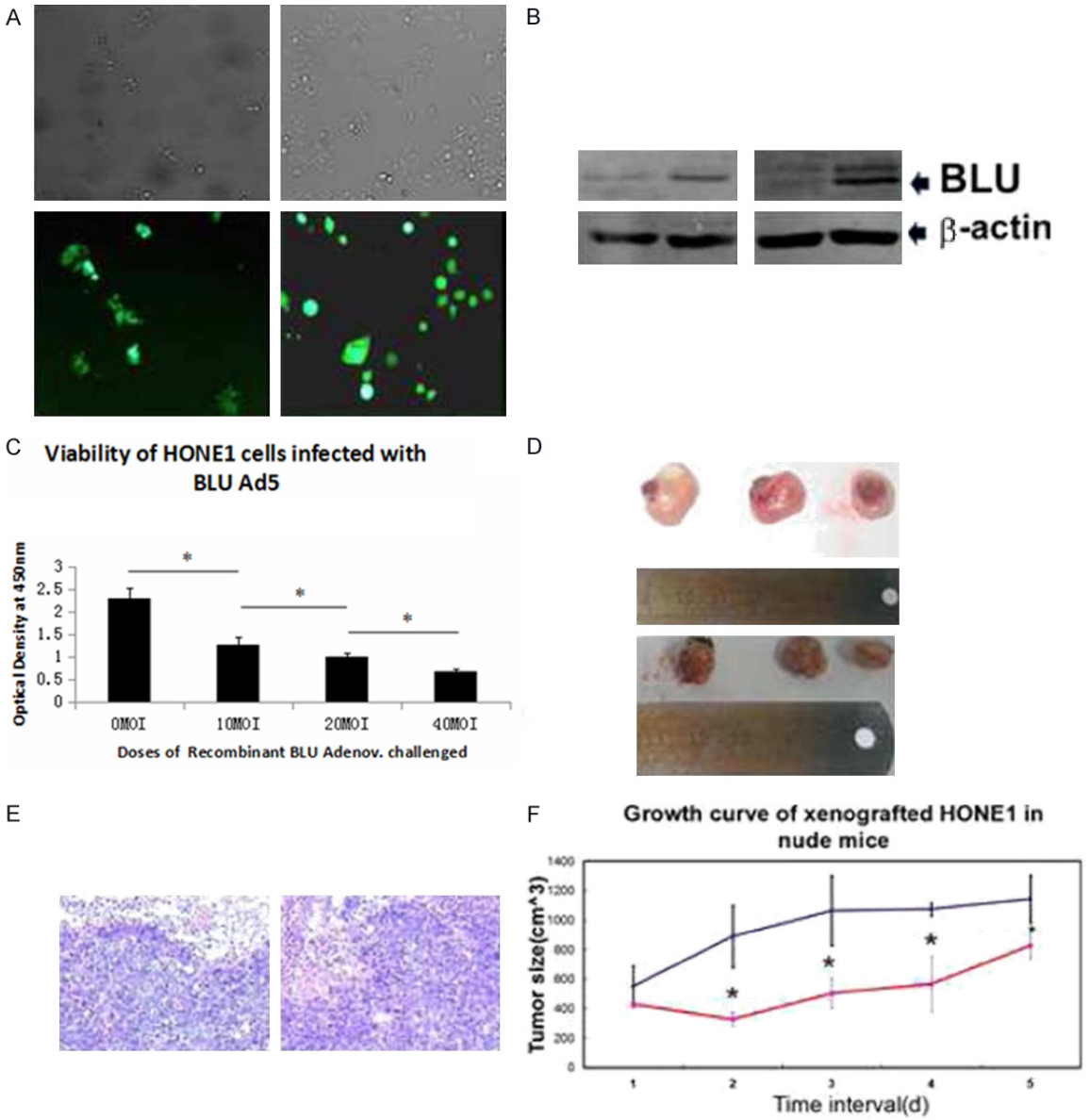
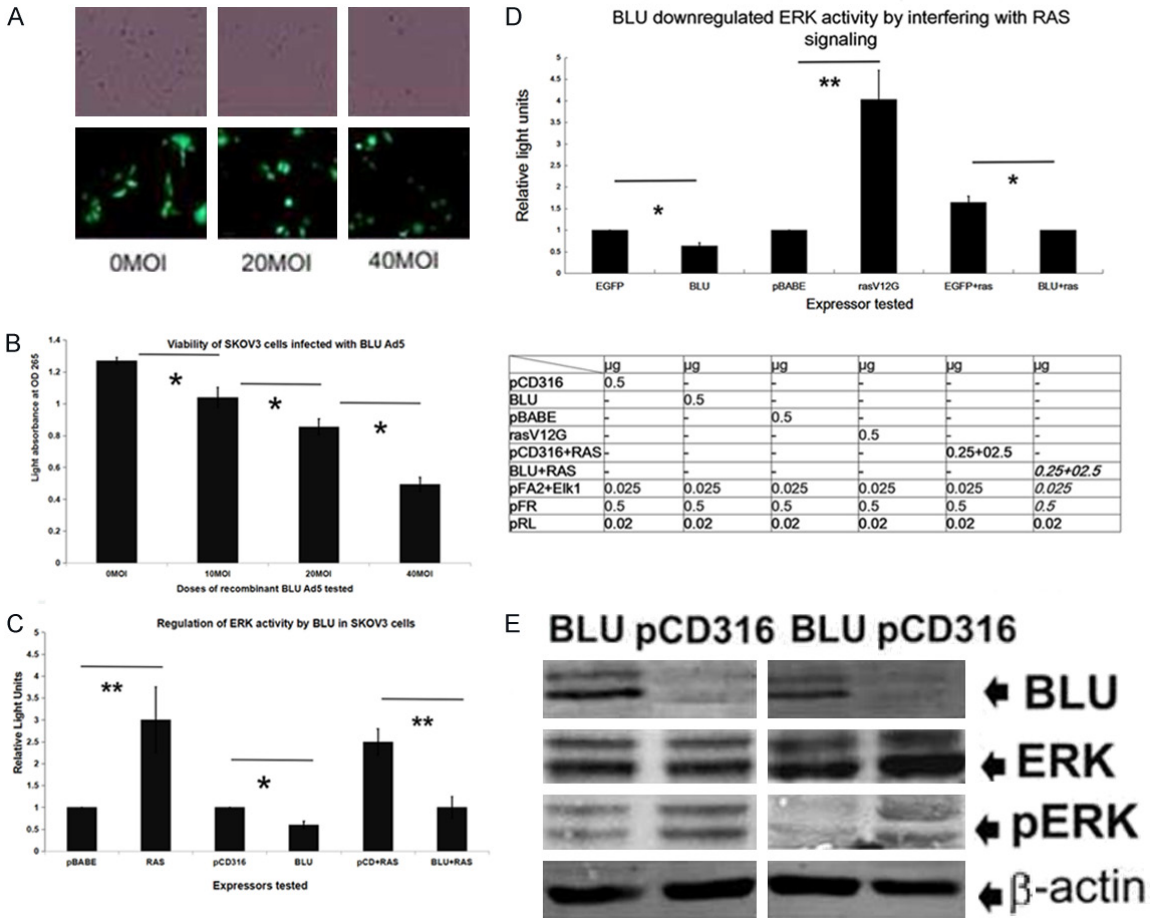


Figure 1. Re-expression of BLU inhibited *in vitro* and *in vivo* growth of HONE1 cells. HONE-1 cells were infected with BLU Ad5 and viewed under a fluorescence microscope to screen for tagged EGFP (A); Total proteins of the infected cells (left panel) and from the xenografted tumors (right panel) were tested with immunoblotting, probing with specific antibodies for the presence of BLU (B). The cells were challenged with BLU Ad5 at the indicated doses, andviability was evaluated. Data are presented as the mean ± SD derived from at least three independent analyses; and the asterisks (*) denoted $P<0.05$ (C). After injected intra-tumorally with BLU Ad5, the xenografted tumor grew for two weeks before harvest, and BLU Ad5 injected (Upper) and control tumors (Lower) were compared (D). The tumors of control (E, left panel) and injected with BLU Ad5 (E, right panel) were incised and sectioned, stained with hematoxylin and eosin (HE) and viewed microscopically. Original magnification, ×200. During the period, sizes of the tumor xenograted to BALB/C nude mice were calculated. The data are presented as mean ± SD on the growth curve. The asterisk (*) indicates $P<0.05$ of each compared pairs of grouped data (F).

min, and stored at -20°C until use. Total proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE), and electroblotted. After blocking with 5% skim milk in PBS for 1 h at RT or overnight at 4°C, the filters were probed with specific primary antibodies. Membranes were washed with 0.1% Tween 20 in PBS. The reaction was developed with ultra-red labelled Odyssey antibodies against the Ig of primary antibodies.



	μg	μg	μg	μg	μg	μg
pCD316	0.5	-	-	-	-	-
BLU	-	0.5	-	-	-	-
pBABE	-	-	0.5	-	-	-
rasV12G	-	-	-	0.5	-	-
pCD316+RAS	-	-	-	-	0.25+02.5	-
BLU+RAS	-	-	-	-	-	*0.25+02.5*
pFA2+Elk1	0.025	0.025	0.025	0.025	0.025	*0.025*
pFR	0.5	0.5	0.5	0.5	0.5	*0.5*
pRL	0.02	0.02	0.02	0.02	0.02	0.02

Figure 2. Expression of BLU inhibited ERK signaling in HONE-1, and the ovarian cell line SKOV3 cells. The presence of *BLU* in the infected SKOV3 cells was confirmed by tagged EGFP signals (A). The viability of SKOV3 cells infected with recombinant BLU Ad5 virus at indicated doses was assayed with CCK8 kit. Data are represented as the mean ± SD derived from at least three independent tests, and the asterisks(*) denoted $P<0.05$ (B). The *BLU*, mock transfected HONE-1 and SKOV3 cells were co-transfected with the reporter plasmids pFA2+pFR. Also with pCD316+*RAS V12G, BLU+RAS V12G,* and pFA2+pFR to test the activity of ERK reporter (C). Data are presented as the mean ± SD that were derived from at least three independent tests, and the asterisks(*) denoted $P<0.05$. Total ERK (middle, upper panel) and phosphorylated ERK (middle, lower panel) of BLU Ad5 and mock infected HONE-1 and SKOV3 cells were tested with specific antibodies (D). Presence of BLU was confirmed simultaneously by probing with specific anti-BLU antibody (Upper panel). The expression of BLU by HONE1 (left panel) and SKOV3 (right panel) cells on adenoviral infection was confirmed by immunoblotting, probing with specific anti-BLU antibody the samples of were indicated; the proteins obtained from BLU Ad5 infected and mock cells as indicated were also tested for phosphorylation of ERK by probing with antibodies against total and phosphorylated ERK. The loading was calibrated by probing with anti-β-actin (E).

Transfection

Nearly confluent HONE-1 and SKOV3 cells were seeded in 6- or 12-well plates. After ON incubation, plasmids *BLU* or *HRAS V12G* and their corresponding empty vectors (pCD316 and pBABE) were co-transfected with the plasmids pFR+pFA2, the Elk1 reporter, or the reporters of the *CCNB1, CCNB2* and *CCND1* promoters with the internal control by mixing with FuGene HD (Roche, Shanghai, China).

Flow cytometry

After synchronizing, HONE-1 cells were transfected with *BLU* pCD316, the pCD316 vector, *RAS* V12G pBABE, and pBABE for 24 or 48 h, as described above. Harvested cells were fixed in 80% ethanol overnight at -20°C. Then resuspended in 100 μg/ml propidium iodide (PI; Sigma Aldrich). Flow cytometry was carried out on a BD FACScan (San Jose, CA, USA), with data analyzed using Cycle 32 software (Phoenix

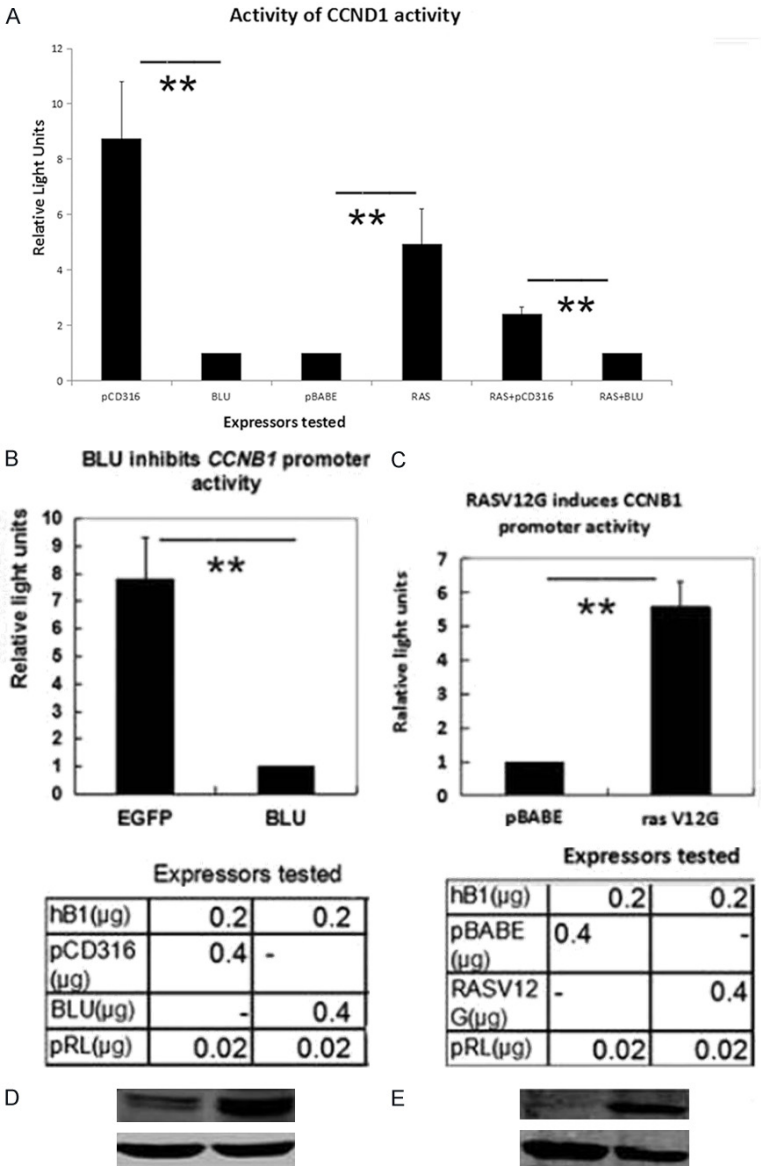


Figure 3. The activities of CCND1 and CCNB1 genes were inhibited and induced by *BLU* and *RAS V12G*. HONE-1 cells were co-transfected with *BLU,* pCD316*, RAS V12G,* pBABE*,* together with CCND1 (A) and CCNB1 (hB1) (B and C) promoter reporter plasmids. pCD316+*RAS* and *BLU+RAS V12G* were also included. The data in relative light units is presented as the mean ± SD that is derived from at least three independent experiments. The symbols *indicated P<0.05, and **indicated P<0.01 on when comparing values from two adjacent columns. Levels of cyclins D1 and B1 were measured with Western blotting (D and E). The blots were probed with specific anti-cyclins D1 and B1 antibodies (upper panel; left: BLU, right: pCD316) and anti-b-actin mAb (lower panel).

Flow Systems, Inc., San Diego, CA, USA). Cell cycle distribution was then assessed.

Xenografted tumors in nude mice

The protocols of animal experiments were approved by the Ethics Committee of Guangdong Medical University Affiliated Hospital (Permit no. 2015-051KT). BALB/c nude mice (4-6 weeks old) of both genders were used. Up to 2×10^6 HONE-1 cells were re-suspended in 0.2 ml serum-free RPMI, and injected subcutaneously into mice. Palpable tumors were identified two weeks thereafter. For treatment, up to 3.5×10^6 PFU *BLU* Ad5 was intra-tumorally injected. Tumors were allowed to grow for an additional two weeks before harvesting. Before the injection and during this period, the longest and shortest tumor diameters were measured with a Vernier caliper. Tumor volume was derived as $v = ab^2/2$ (where *a* and *b* are the longest and shortest diameters, respectively). At the time of harvest, the tumors were weighed. The means ± standard deviation (SD) of tumor sizes was calculated to establish growth curves in the treatment and control groups.

Statistical analysis

Statistical analysis was performed using the SSSP software package. Quantitative data were presented as mean ± SD from at least three independent experiments. The Student's T-test was used for group comparisons and a value of $P<0.05$ was considered statistically significant.

Results

Re-expression of the tumor suppressor BLU inhibits in vitro and in vivo growth of HONE1 cells and viability of SKOV3 cells

BLU was re-expressed by transfer with vector BLU Ad5 in two lines negative for BLU: NPC-derived HONE-1 cells, and ovarian cancer-

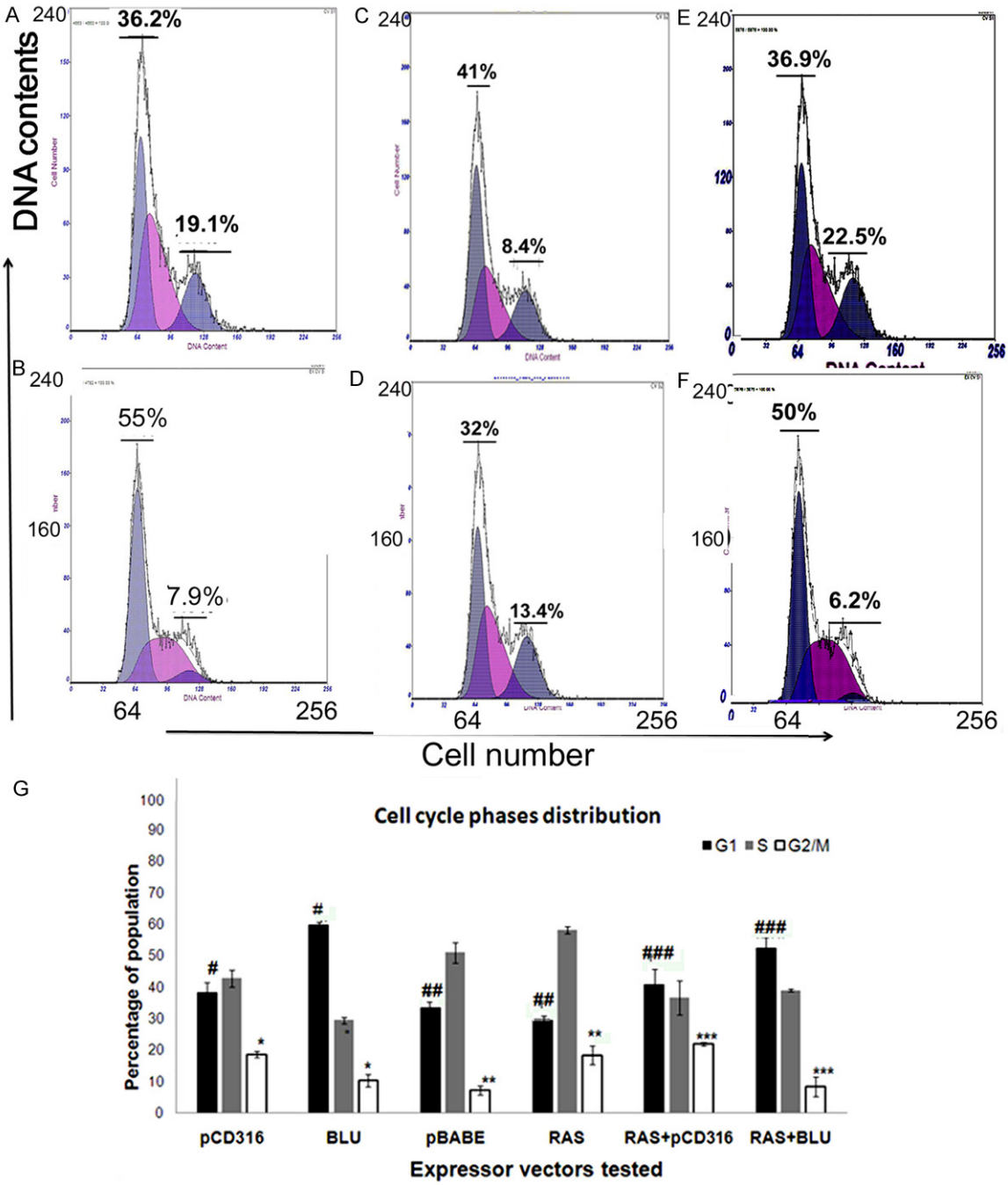


Figure 4. Alteration of cell cycle phase distribution by *BLU*. Cells were transfected with 1 μg of the plasmids pCD316 (A), *BLU* (B), pBABE (C), *RAS V12G* (D), PCD316+*RAS V12G* (E) and *BLU*+*RAS V12G* (F) and DNA content was flow cytometrically determined. Mean values of the percentage of cell populations in each phase of the cell cycle were calculated and the data are presented as mean ± SD with results derived from at least three independent experiments (G). The symbols #, ##, ###, and *, **, ***indicated P<0.05 when comparing each pair of values.

derived SKOV3 cells. The expression was evidenced by the fluorescence of the tagged EGFP signal viewed microscopically (**Figures 1A** and **2A**) and immunoblotting, probed with specific antibody (**Figures 1D** and **2B**). Transferred BLU Ad5 by viral infection reduced viability of HONE-1 cells in a dose-dependent manner (**Figure 1B**); the finding was also noted in ovarian cancer-derived SKOV3 cells (**Figure 2C**). When HONE-1 cells were xenografted in nude mice by

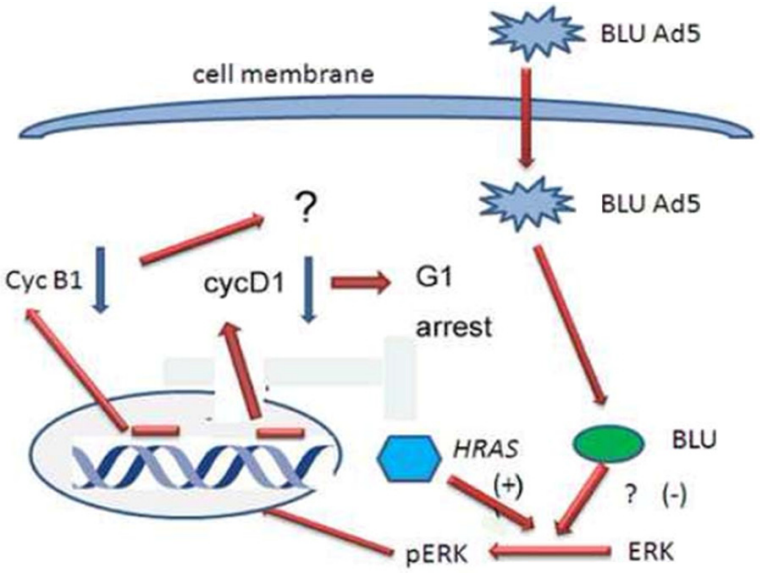


Figure 5. Scheme of engagement of ERK pathway by *BLU*. The tumor suppressor *BLU* was re-expressed by viral transduction or gene transfection. The expression of *BLU* inhibited the phosphorylation of ERK that was catalyzed by an upstream kinase(s), which opposed the effect of RAS proteins. Next, the activity of the *CCND1* and *CCNB1* gene promoters was down-regulated, leading to reduced expression of cyclins D1 and B1, and induction of G1 cell cycle arrest. It remains to be elucidated whether reduced proportions of cells in the G2/M phase of the cell cycle was related to the change of cyclin B1 level.

subcutaneously injection, palpable lesions of tumor were identified, and the radius of the tumors were recorded and size was calculated according to an established formula. The BLU Ad5 suspension and medium control were then intra-tumorally injected to the tumors. During the period of observation, the size of the xenografted tumors was calculated as above. After two weeks of growth, the tumors were harvested by excision, The incorporation of the human NPC cells and expression of BLU were confirmed hisopathologcally and immunoblotting respectively (**Figure 1C** and **1D**); the size of the BLU transferred and control tumors was compared (**Figure 1E**) and the growth curve was plotted according to the recorded data (**Figure 1F**). The inhibition of growth by BLU was suggested.

BLU down-regulated ERK signaling when compared to RAS V12G, and phosphorylation of ERK in HONE1 and SKOV3 cells

The RAS-RAF-MEK-ERK pathway is activated in a cascade manner when integrating extracellular stimuli, including ligation of growth factors with cognate receptors, to stimulate cell growth and proliferation. The signaling of ERK is regulated by oncoproteins and coding products of TSGs. In SKOV3 and HONE-1 cells, ERK signaling was downregulated by *BLU*, but induced by mutant *RAS* as demonstrated by co-transfection with *BLU* pCD316 and *RAS V12G* plasmids, their corresponding empty vectors, together with reporter plasmids pFA2 and pFR (**Figure 2B** and **2C**). ERK phosphorylation in SKOV3 and HONE-1 cells was markedly reduced by *BLU*. Co-transfection of *BLU* and *RAS V12G*resulted in lower levels of reporter activity when compared with pCD316+*RAS V12G*, suggesting an antagonistic effect of *BLU* on the induction of ERK activity by *RAS* (**Figure 2D**). ERK with low level of phosphorylation has weak potential to exert downstream effects; the data suggest that *BLU* inhibited ERK signaling through interfering with *RAS* activity.

BLU inhibited the promoter activity of CCND1 and CCNB1 and reduced expression levels of cyclins D and B1 in HONE1 cells

Cyclins are activated in a chain of phosphorylation and dephosphorylation and the phosphorylation of several amino residues is catalyzed by ERK and other MAPK family kinases. Activated cyclins serve as regulatory subunits of the cyclin-cyclin dependent kinase (CDK) complex [27, for a review see 28]; groups D and B cyclins function to promote transition of G1/S and G2/M phases in cell cycle respectively. The reporter plasmid for the promoters of cyclin D1 and cyclin B1 coding genes *CCND1*, and *CCNB1* were co-transfected with *BLU, HRAS* V12G vectors, and their corresponding empty vectors. Since cyclin B1 is also induced by activation of the RAF-MEK-ERK pathway, *CCNB1* promoter activity and expression of cyclin B1 were measured by the luciferase assay and Western blotting. The data showed that activity of *CCNB1* and CCND1 promoters was inhibited by *BLU*, and elevated by *HRAS* V12G (**Figure 3A-C**). The

level of cyclins D1 and B1 was reduced in *BLU* expressing cells, correlating with ERK inhibition by *BLU* (**Figure 3D** and **3E**). These findings suggested that BLU exerts proliferation inhibition and tumor suppression by inhibition of cyclin D1 and B1 gene promoter, i.e. transcriptional blocking of cyclin B1 and cyclin D1 expression and thus prevented cell cycle progression. The assay of reporter activity of *CCND1* suggests that *BLU* was antagonistic with the induction of *CCND1* promoter by *HRAS* through RAS-RAF-MEK-ERK.

The expression arrested cell cycle of HONE1 at G1 phase and correlated with its effect on cyclin D1 expression

The progression of cell cycle is regulated by complexes of cyclin and CDK. BLU has been shown to downregulate cyclin D1 and cyclin B1. The arrest of cell cycle at the G1 phase was well-correlated with alterations of cyclin D1 caused by *BLU* (**Figure 4A** and **4B**). In line with the published finding that *HRAS V12G* induced G2/M arrest, we found that the G2/M population of HONE-1 cells that had been transfected with *H-RAS* was remarkably increased in comparison with the mock (**Figure 4C** and **4D**) control. In BLU expressing cells, the G2/M population was decreased as revealed by DNA content. It might be reasoned that the change in DNA content might result from an accumulation of cells in the G1 phase of the cell cycle due in part to cell cycle arrest, as previously reported [29]. There is, however, the possibility that entry of cells from the S to the G2/M phase of the cell cycle was prevented because cyclin B1 expression was blocked by *BLU* (**Figure 3B**). In a co-transfection system with *BLU*+*HRAS V12G*, G1 arrest was registered, and the G2/M cell population was reduced by *BLU* when compared with cells expressing pCD316+*RAS V12G* (**Figure 4E** and **4F**). The data further suggested that BLU could interfere with the signaling of the mutant HRAS (**Figure 4G**).

Our results suggest that the tumor suppressor *BLU* inhibits tumor growth by blocking ERK signaling and downstream expression of effectors like cyclin D1. The changes then led to arrest of the cell cycle in the G1 phase. Moreover, downregulation of the ERK pathway also reduced cyclin B1 expression. *BLU* also reduced the population of cells in the G2/M phase of the cycle as compared with induction of G2/M arrest by *HRAS V12G*. ERK targeting growth and tumor suppression by *BLU* is summarized in **Figure 5**.

Discussion

BLU was mapped to the minimal region of differentially deleted fragments of varying sizes on chromosome 3p21. It is frequently lost in a variety of human tumors, notably of epithelial origin [2, 3, 30]. Its coding product is a protein with a zinc finger myeloid Nervy DEAF-1 (ZMYND) motif on its carboxylterminus, shared by members of a protein family. The activities of modified histone reader, transcription repression have been described in other members [9, 10].

BLU has been reported to inhibit cell cycle progression by engaging the RAF-MEK-JNK signaling pathway, similar to the tumor suppressor and the RAS effector RASSF1A [15] and inhibits angiogenesis by reducing VEGF levels and other cytokines [31], and enhances chemotherapeutic agent-induced apoptosis [32, 33]. Our observation suggested that BLU inhibited NF-κB signaling, down-regulated NF-κB dependent anti-apoptotic factors like cFLIP and cIAP2 and potentiated death receptor-induced apoptosis [8]. Its effect on NF-κB signaling could be explained by BLU-mediated post-translational modifications (PTMs) of IKKα, a kinase that phosphorylates IkappaB whose dissociation from NF-κB leading to its activation.

Currently EGFR serves as a frequent target of anti-cancer therapy. The signaling pathway is activated by guanine nucleotide binding proteins H-, Ki- and N-RAS. Alterations of the coding gene *RAS* lead to the persistent activation of several intracellular signaling pathways to stimulate growth and proliferation, and is implicated in the genesis of human cancers [34, 35]. A MAPK family member, ERK is involved in this process [17, 18]. The signal in turn induces the MAPK pathway, activates the MAPK family, and catalyzes phosphorylation on the downstream signaling molecules. Cyclins, which are key regulators of the cell cycle, are activated by phosphorylation to promote cell cycle entry.

Our previous observations that re-expression of *BLU* inhibited cyclin D1, a downstream effector of JNK that induces G1 arrest, suggested that growth suppression exerted by *BLU* was

targeted to the RAF-MEK1/2-JNK pathway [11]. We reasoned whether *BLU* also targeted pathways that involved other members of the MAPK family to exert tumor suppression. We thus compared regulation of ERK activated by, proto-oncogenes *RAS* and its downstream effectors cyclins D1 and B1 by *HRAS V12G* and *BLU*, and tested their interaction in a co-transfection system.

In the present study, it was shown that in HONE1 and SKOV3 cells, BLU inhibited signaling of ERK. In the context, BLU behaved similarly to a family of proteins that include RASSF. *RASSF1A*, the founding member of the family and *RASSF5/NORE* which inhibit tumor growth and proliferation by targeting signaling molecules of the MAPK family [9, 10]. MAPK proteins promote G1/S cell cycle phase transition by inducing cyclin D1, whose over-expression contributes to neoplastic transformation [36, 37]. *RASSF5/NORE* also targets cyclin B1 which promotes G2/M cell cycle progression by complexing with the CDK1 complex [38, 39]. Cyclin B1 plays an indispensable role in regulating cell entry into mitosis [40]. Growth inhibition by suppressing the cyclin B1/CDK1 complex and upstream signaling pathway(s) involves networks include the EGFR/ERK pathway [41].

Similar with that BLU arrested cell cycle at the G1 phase in CNE-2 cells [11]; it also induced G1 arrest in HONE-1 cells (**Figure 4A** and **4B**). The data supported that G1 arrest is the frequent target of tumor suppressors. However, we found that the G2/M phase in BLU expressing cells decreased. In line with previous reports, *HRAS V12G* induced G2/M arrest by activating the RAF-MEK-ERK pathway [42, 43]. While a decrease in the G2/M population might be explained by an increase in the G1 population, we observed that cyclin B1, which regulates entry into the G/M phase,was also reduced by BLU, and that G2/M arrest that is induced by *RAS* V12G was reversed by BLU (**Figure 4E** and **4F**). We reasoned that BLU could prevent entry into G2/M by affecting cyclin B1 activity, a finding that warrants future in depth investigations.

Effects of BLU on the ERK-cyclin D1 axis may account for its *in vitro* and *in vivo* growth and proliferation inhibition. In the present study, BLU inhibited the human NPC xenograft in nude mice upon intra-tumoral injection, and prevented tumor cell growth *in vitro* by inducing G1 cell cycle arrest. These effects correlated with down-regulation of *ERK* signaling, which were activated by *HRAS* V12G. Recently, it was reported that silencing of cyclin B by microRNA 15a/16-1, inhibits tumor growth [44, 43]. Together with the finding of inhibition of cell cycle progression by cyclin B1 by BLU, it is proposed that cyclin B1 might serve as an anti-cancer therapeutic target.

Our data suggested that xenografted tumor inhibition by *BLU* was incomplete, prompting further efforts to test whether concurrent administration of an additional tumor suppressive factor would completely eliminatethe tumors. Indeed it was reported that adenoviral transfer of IL-24 and TRAIL, leads to complete eradication of xenografted human hepatoma in mice [46]. Our recent publication suggested that *BLU* enhanced TRAIL-induced apoptosis [8]. The efficacy of concurrent administration of *BLU* and a pro-apoptotic factor in anti-tumor biotherapy could be evaluated in the *in vivo* system described above.

In conclusion, a tumor suppressor *BLU,* down-regulated ERK signaling and its downstream effector cyclins D1 and B1, which disrupted cell cycle progression and subsequent suppression of tumor growth. The process might be achieved by interfering with RAS-mediated signaling.

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Disclosure of conflict of interest

None.

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