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ORIGINAL ARTICLE

# Anti-angiogenic pathway associations of the 3p21.3 mapped *BLU* gene in nasopharyngeal carcinoma

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*Zinc-finger, MYND-type containing 10* (*ZMYND10*), or more commonly called *BLU,* expression is frequently downregulated in nasopharyngeal carcinoma (NPC) and many other tumors due to promoter hypermethylation. Functional evidence shows that the *BLU* gene inhibits tumor growth in animal assays, but the detailed molecular mechanism responsible for this is still not well understood. In current studies, we find that 93.5% of early-stage primary NPC tumors show downregulated *BLU* expression. Using a PCR array, overexpression of the *BLU* gene was correlated to the angiogenesis network in NPC cells. Moreover, expression changes of the *MMP* family, *VEGF* and *TSP1*, were often detected in different stages of NPC, suggesting the possibility that *BLU* may be directly involved in the microenvironment and anti-angiogenic activity in NPC development. Compared with vector-alone control cells, *BLU* stable transfectants, derived from poorly-differentiated NPC HONE1 cells, suppress VEGF165, VEGF189 and TSP1 expression at both the RNA and protein levels, and significantly reduce the secreted VEGF protein in these cells, reflecting an unknown regulatory mechanism mediated by the *BLU* gene in NPC. Cells expressing *BLU* inhibited cellular invasion, migration and tube formation. These *in vitro* results were further confirmed by *in vivo* tumor suppression and a matrigel plug angiogenesis assay in nude mice. Tube-forming ability was clearly inhibited, when the *BLU* gene is expressed in these cells. Up to 70–90% of injected tumor cells expressing increased exogenous *BLU* underwent cell death in animal assays. Overexpressed BLU only inhibited VEGF165 expression in differentiated squamous NPC HK1 cells, but also showed an anti-angiogenic effect in the animal assay, revealing a complicated mechanism regulating angiogenesis and the microenvironment in different NPC cell lines. Results of these studies indicate that alteration of *BLU* gene expression influences anti-angiogenesis pathways and is important for the development of NPC.

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## INTRODUCTION

Human chromosome 3p21.3 was the first tumor suppressive region functionally identified in nasopharyngeal carcinoma (NPC).[1] This region was confirmed to be a tumor suppressor gene (TSG) cluster that contains several TSGs associated with the development of many human tumors.[2–5] *BLU* was one of TSGs mapped in this region and has been repeatedly reported to be involved in the development of NPC[6–9] and many other tumors, including lung cancers,[5,10,11] glioma,[12] neuroblastoma,[11] cervical neoplasia,[13] esophageal squamous cell carcinoma,[14] liver tumor[15] and myelodysplastic syndrome.[16]

The *BLU/ZMYND10* gene spans 4.5 kb on human chromosome 3p21.3; alterative splicing is observed in different tissues. It is not clear if or how *BLU* contributes to the early or late stages of tumor progression.[3,8,11] The hypermethylated *BLU* was detected in 66–74% of primary NPC samples.[6,7] Compared with the immortalized nasopharyngeal epithelial line, *BLU* was downregulated in NPC cell lines such as HONE1 and HK1.[6–8] The exogenously expressed *BLU* has an *in vitro* growth-inhibitory effect in lung, kidney and ovarian carcinomas, neuroblastoma and NPC cell lines.[5,9,11,17] However, the mechanisms responsible for inducing the functional tumor suppression in these cells have yet to be elucidated.

We provided the first *in vivo* functional evidence that stable *BLU* transfectants derived from NPC HONE1 cells suppressed tumor growth in nude mice.[8] However, the detailed molecular mechanism responsible for tumor suppression is still not understood. NPC studies, compared with other common cancers, have been greatly hampered by the lack of sufficient tissues for detailed investigation, as primary tumor specimens are often only available in small quantities and both tumor and control nasopharynx tissues exhibit extensive inflammation. Subsequent treatment of NPC basically relies on radiotherapy; this further restricts the availability of NPC tissues for studies. In addition, current knowledge on the molecular mechanisms of *BLU*, compared with other TSGs mapping to the human chromosome 3p21.3, is very limited, which further impedes the exploration of tumor suppressive networks mapped to this region in human tumors.

We previously showed that *ADAMTS9* suppressed tumor growth *via* its anti-angiogenesis activity through interactions with *MMP* and *VEGF* families.[18,19] *ADAMTS9* and its other family members interact with *Thrombospondin-1* (*TSP1* or *THBS1*) to control tumor progression, angiogenesis and metastasis.[18,20–23] *BLU* is regulated by *E2F*[7] and *E2F* directly controls *TSP1* expression.[24] The *MMP* family, a group of genes regulating extracellular matrix, was suggested to regulate NPC cell invasive properties in association

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with *VEGF*.[25,26] However, how *TSP1* and *VEGF* regulate angiogenesis, progression and metastasis processes in NPC is not known.

It is well established that members of the *MMP* family, *VEGF* and *TSP1* interact in the control of angiogenesis, invasion and metastasis pathways in tumor development.[22,26–30] These reports and our previous findings suggest a possibility that *BLU*-mediated signaling networks may include multiple gene families related to anti-angiogenesis activities in NPC. Therefore, we hypothesize that *BLU* is involved in the anti-angiogenesis processes through its interactions with *TSP1*-, *VEGF*- or other gene-mediated pathways to ultimately lead to the tumor suppression in NPC.[8,22,31–34] Genes mapping to the 3p21.3 TSG cluster are critical for tumor development as reported in a number of sporadic human cancers.[2–5] The *BLU* gene may be one of the key components of signaling networks controlling tumor microenvironment and angiogenesis. Here we report our novel findings on the tumor suppressive mechanism controlled by *BLU* in NPC cells.

## RESULTS

### Downregulated *BLU* expression occurs early in NPC development and is associated with angiogenesis

*BLU* was previously reported to show reduced mRNA levels and promoter hypermethylation in the majority of primary NPC samples. To assess the timing for *BLU* alterations in different stages of NPC, we screened 101 pairs of NPC samples at different clinical stages. Matched non-tumor tissues from the nasopharynx and NPC samples were collected from the same patients. A total of 84.2% of all NPC samples showed downregulation of the *BLU* gene. Among them, 93.5% (29/31) from Stages I and II were downregulated. In advanced stages of NPC, *BLU* expression was also decreased in 77.3% of stage III tumors and 82.6% in stage IV tumors (Figures 1a and b). To verify these PCR results, we utilized immunohistochemical (IHC) staining of NPC tissue microarray (TMA) slides to detect expression levels of BLU proteins in NPC. These examinations revealed that the BLU protein was expressed in 47 of 49 (95.9%) of normal nasopharyngeal tissue controls, but was significantly downregulated in 73 of 100 (73%) primary NPC samples ($P < 0.001$) and in 12 of 16 (75%) metastatic NPC samples ($P < 0.001$) (Table 1). In TMA samples, downregulation of BLU protein was found in stages I (66.7%), II (84.2%), III (64%), and IV (66.7%) in all informative samples, but no statistically significant association with clinical data was observed (Supplementary Table 1). These results strongly suggest that *BLU* alterations occur as an early event in NPC development and are present in both early and metastatic stages of NPC.

We recently reported that the anti-angiogenic activity of *MMP19* was essential for the NPC development.[26] BLU was found to be downregulated in NPC samples that had downregulated expression of MMP19 in informative cases for both genes. IHC staining showed the coordinately decreased expression of BLU and MMP19 in primary tumors (52 of 95, 54.7%) as compared with normal epithelium tissues (2 of 42, 4.8%) ($P < 0.001$) (Figure 1c and Table 2). To investigate the possibility that BLU may be involved in the angiogenesis regulatory network in NPC, we used an angiogenesis PCR array to compare established stable NPC *BLU* transfectant cells and vector-alone (VA) control cells.[8] These results indicated that MMP family members such as *MMP9*, *MMP14* and *MMP19* were clearly upregulated in *BLU*-overexpressing cells, but *MMP2* was downregulated in these cells. These results, especially the role of *MMP2,* were consistent with previous findings in other TSG studies such as *Fibulin-2*-mediated anti-angiogenic activities and confirmed a relationship between *BLU* and the *MMP* family in NPC development[8,35] (Figure 1d).

It is well-known now that both NPC and nasopharynx control tissues often contain extensive inflammation areas in the tumor microenvironment that may affect the angiogenesis process. To reflect real-expression changes of critical genes related to downregulation of *BLU*, total RNAs from paired tissues were analyzed as shown in Figure 1a. As *MMPs* have opposing functions related to the disease stage as previously reported,[36,37] we examined *MMP2* expression in *BLU*-downregulated NPC samples. In stages I and II, 82% (9/11) of tumors have an upregulated *MMP2* expression. Tumor samples with downregulated *MMP2* are 25% (1/4), 14.3% (1/7), 33.3% (4/12) and 75% (6/8) at stages I, II, III and IV, respectively (Figure 1e).

Besides the MMP family, the PCR array results also indicate that *BLU* influences NPC angiogenesis networks that include multiple genes, such as those encoding angiogenic factors, cytokines, growth factors and receptors, adhesion molecules and other matrix proteins (Supplementary Table 2). These data suggest that overexpression of *BLU* gene may trigger multiple signaling pathways or induce negative feedback responses in the regulation of NPC cell growth. Interestingly, one of the well-characterized angiogenesis regulators, *TSP1*, was downregulated in *BLU*-overexpressing NPC cells (−2.7-fold).

### *BLU* expression is associated with *TSP1* and *VEGF* expression and influences angiogenesis networks

To further determine whether *BLU* may control anti-angiogenic activities in NPC, we examined two well-studied genes, *TSP1* and *VEGF,* that have critical roles in regulating angiogenesis, invasion and metastasis in other tumors, but whose roles in NPC development are not well-known. Therefore, NPC RNA samples showing downregulation of *BLU* at each clinical stage were randomly investigated for *VEGF* and *TSP1* expressions. There were no differences in levels of upregulation of *VEGF* (66.7%, 20/30) by tumor stages (I/II vs III/IV). However, this is in contrast to upregulation of *TSP1* from 38.5% (5/13) of tumors at stages I/II, which increased up to 68.2% (15/22) for stages III/IV tumors (Figure 2a). Average upregulation of *TSP1* increased from 1.62-fold at stages I/II to 5.78-fold at stages III/IV (Figure 2b). Furthermore, the expression of TSP1 proteins can be detected in 63% (19/30) of examined TMA samples. Interestingly, the expression of TSP1 in the periphery of cancer nests is stronger than that in other regions, as compared with control tissues (Figure 2c).

To verify whether *BLU* may directly regulate expression of *VEGF* and *TSP1* in NPC cells, *BLU*-overexpressing NPC cells were examined. The presence of secreted VEGF proteins in the conditioned medium of stable *BLU* transfectants[8] was strongly inhibited to 0.46 pg/μl in c14 and 0.54 pg/μl in c16, compared with 1.8 pg/μl in VA control cells ($P < 0.01$), as detected by enyzme-linked immunosorbent Assay (ELISA). Transient transfection of *BLU* (>80% cells were *BLU* overexpressed) also caused inhibition of VEGF proteins in lentivirus-infected HONE1 (middle) and c666-1 (right) cells, as shown in Figure 3a.

qPCR analyses indicated RNA expression levels of both *TSP1* and *VEGF* were obviously inhibited in *BLU* transfectants (Figure 3b). The expression of *VEGF-T* (total), *VEGF165* and *VEGF189* was downregulated, compared with control VA cells in all three different clones.

To further verify whether downregulated *BLU* in NPC samples correlates with the upregulations of *VEGF* and *TSP1*, protein expression in stable *BLU* transfectants c14, c16 and c19 was assayed by western blotting. VEGF165, VEGF189 and TSP1 proteins were clearly downregulated in these BLU-overexpressing cell lines compared with control VA c4 cells (Figure 3c), which is fully consistent with findings detected by qPCR analyses (Figure 3b). Downregulated *BLU* RNA expression is observed in most NPC cell lines, including poorly differentiated HONE1 and differentiated squamous HK1 cells.[7,8] The lentivirus infection was used to induce overexpression of *BLU* in HK1 cells. The expression of VEGF165 protein was clearly inhibited in BLU-overexpressing HK1 cells. No obvious change of VEGF189 and TSP1 expression was detected in

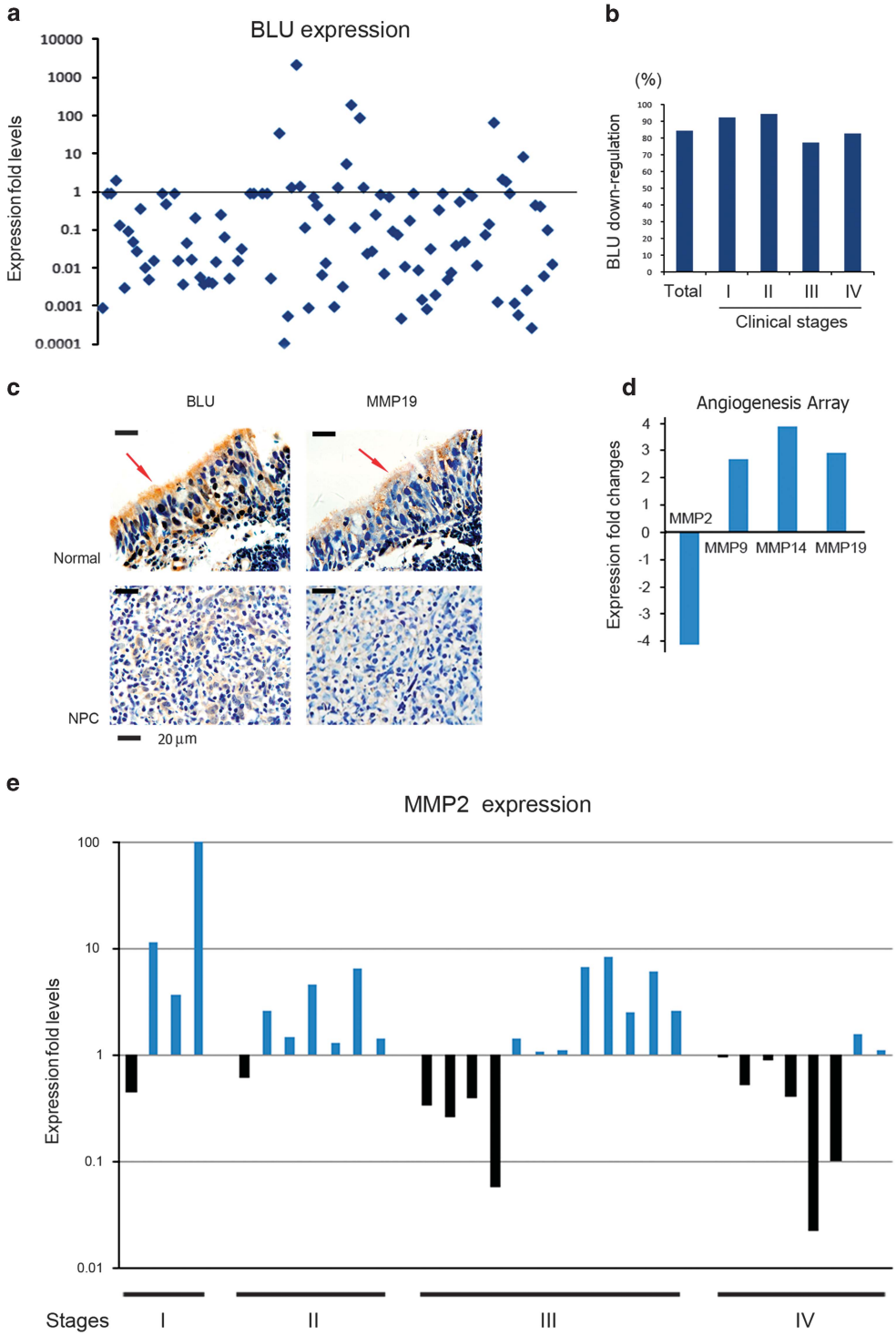


**Figure 1.** Downregulation of *BLU* is observed from both the early and late stages of NPC and is associated with the expression of MMP family. (**a**) qPCR analyses show up- or downregulated *BLU* expression in NPC samples compared with those of non-tumor nasopharyngeal tissues from the same patients. $< 1$, downregulation of *BLU* expression. (**b**) Percentages of NPC samples with downregulated *BLU* expression at each clinical stage. (**c**) IHC staining in tissue biopsies. BLU and MMP19 protein expression in normal nasopharyngeal mucosa (top), and downregulation in undifferentiated NPC cells (bottom). The arrowheads indicate BLU and MMP19 expression in epithelium (Table 2). (**d**) *MMP9*, *MMP14* and *MMP19* are upregulated, and *MMP2* is downregulated in *BLU* transfectants, compared with VA cells (Supplementary Tables 2 and 4). (**e**) qPCR analysis of *MMP2* expression. Thirty-one pairs of RNA samples, tumors and corresponding non-tumor tissues from the same patients showing downregulation of *BLU* at each clinical stage were investigated. Compared with stages I and II, *MMP2* expression is often inhibited in advanced stages (stage III/IV) of NPC ($P < 0.05$).

this cell line (Figure 3c). To confirm the signaling network controlled by both *BLU* and *TSP1* in NPC cells, *TSP1* cDNA was transfected into HONE1 cells by lentivirus infection. Western blotting showed that overexpressed TSP1 clearly downregulated the expression of endogenous BLU, but there was no obvious alterations of VEGF proteins levels in these cells (Figure 3d).

As expected, human umbilical vein endothelial cells (HUVEC) tube formation was strongly inhibited in both HONE1 (Figure 3e) and HK1 (Figure 3f) *BLU* transfectants compared with their VA cells. Relative tube-forming abilities were determined by comparison between *BLU* and VA transfectants, which further confirms that *BLU* is involved in the tube formation process in NPC cells.

*BLU* expression affects invasion and migration abilities of NPC cells in *in vitro* assay

As both *VEGF* and *TSP1* are involved in the migration and angiogenesis processes in tumor cells,[23,32,38] and *BLU* affects the expression of these genes, we performed two functional assays to determine the abilities of *BLU* transfectants in the control of cellular invasion and migration processes. Compared with control VA c4 cells, *BLU* stable transfectants showed delayed invasion ability and exhibited reduced migration ability immediately after seeding of cells (Figure 4a). Although cell growth dynamics are different, HK1-derived *BLU* transfectants also showed marked inhibition of both invasion and migration abilities, compared with their control VA cells (Figure 4b).

Another functional assay, wound healing, is well-known to be used in the determination of VEGF expression, as reported previously.[39] *BLU* expression was correlated with delayed wound healing in both HONE1 (Figure 4c) and HK1 *BLU* transfectants (Figure 4d), suggesting that *BLU* may be an important gene involved in regulation of both angiogenesis and migration in NPC cells.

As the microenvironment and pericytes in tumor angiogenesis are now recognized as critical factors affecting the regulation of tumor invasion and progression,[40–42] we examined changes of macrophages, cancer-associated fibroblasts and mesenchymal cells in NPC and control tissues by IHC staining. Our preliminary results, stained by CD68, F4/80, Vimentin and α-smooth muscle actin (α – SMA) antibodies, indicated that these pericytes are markedly increased in some of the NPC cases, especially in the later-staged tumors (Supplementary Figure 1).

**Table 1.** BLU expression in non-tumor and NPC samples by IHC staining

| *Tissue type*[a] | *Expression* | *Downregulated expression* | P-*value*[b] |
|---|---|---|---|
| Normal[c] | 47/49 (95.9%) | 2/49 (4.1%) | – |
| Primary NPC | 27/100 (27%) | 73/100 (73%) | $< 0.001$ |
| Metastatic NPC | 4/16 (25%) | 12/16 (75%) | $< 0.001$ |

Abbreviation: NPC, nasopharyngeal carcinoma. [a]Information for patients and informative cases are listed in Supplementary Table 1. [b]Compared with downregulated normal group. [c]Normal includes non-tumor nasopharyngeal mucosa and adjacent non-neoplastic mucosal tissues from same patient.

*BLU* has anti-angiogenesis and tumor suppressive functions in animal assays

Tumor suppression and matrigel plug angiogenesis assays were performed to examine survival and growth of tumor cells after injection into nude mice. As expected, injected tumor cells went through necrosis with *BLU* transfectants c14 and c16, as seen in all examined tissue sections after 1 week. Compared with control VA c4, ~70–90% of tumor nests disappeared and many necrotic cells were observed in the central region of tumor nodules of *BLU* transfectants c14 and c16 (Figure 5a). These results confirm our previous observations and provide novel findings, namely, *BLU* expression was correlated with the tumor suppression *in vivo*, and *BLU* caused a prolonged latency period or complete suppression of tumor growth in animals.[8] To determine whether tumor suppression was induced due to the anti-angiogenic effect induced by *BLU*, the newly formed blood vessels in the transplanted gel plugs were examined using antibody CD34 staining for both control and *BLU* transfectants. Numerous endothelial cell tubes were formed in the gel plugs with growing tumor cells in control VA c4 cells, but this was not observed in central regions of tumor nodules in *BLU* transfectant cells. Vascular capillaries were rarely detected in the remaining cancer nests that were undergoing necrosis in *BLU* transfectants. Representative images of these results are shown in Figures 5a and b. Compared with *BLU*-expressing HK1 transfectants, more vascular capillaries were observed by both hematoxylin & eosin and CD34 staining around cancer nests in HK1 VA control cells. The representative images and quantitative comparisons are shown in Figures 5c and d. Growing vascular capillaries were inhibited in HK1 *BLU* transfectants, but no significant difference of tumor suppression was detected between both *BLU* transfectants and VA cells. The TSP1 protein is easily detected in the extracellular matrix around cancer nests in VA control cells, but it is not detected in necrosis areas of *BLU* transfectants (Figure 5e)

## DISCUSSION

NPC is a unique cancer that is particularly prevalent among the southern Chinese, but is rare in most areas around the world. Genetic factors are regarded as early events that contribute to the development of NPC. Although extensive and random DNA losses in chromosome 3p and other chromosome regions were often observed in NPC tissues and many other tumors, DNA deletion mapping had little chance to precisely define TSG locations in sporadic NPC tissues.[3,5,43,44] Using cell fusion and chromosome transfer approaches, we previously identified functionally critical regions mapping to chromosome 3p21.3 and chromosome 9p21 region containing the known TSG *p16* for NPC development.[1,43] Currently, the 3p21.3 region has been regarded as one of the most important tumor suppressive regions for sporadic tumors in the human genome.[2,3] It is widely accepted now that TSGs in this

**Table 2.** Concomitant downregulated expression of MMP19 and BLU proteins in NPC by IHC staining[a]

| *Tissue type* | *Informative cases for MMP19 and BLU* | *Concomitant expression* | *Downregulated expression* | P-*value*[b] |
|---|---|---|---|---|
| Normal | 42 | 35/42 (83.3%) | 2/42 (4.8%) | – |
| Primary NPC | 95 | 13/95 (13.7%) | 52/95 (54.7%) | $< 0.001$ |
| Metastatic NPC | 14 | 1/14 (7.1%) | 7/14 (50%) | $< 0.001$ |

Abbreviation: NPC, nasopharyngeal carcinoma. [a]Contiguous sections from the same case were stained with MMP19 and BLU antibodies. [b]Compared with downregulated normal group.

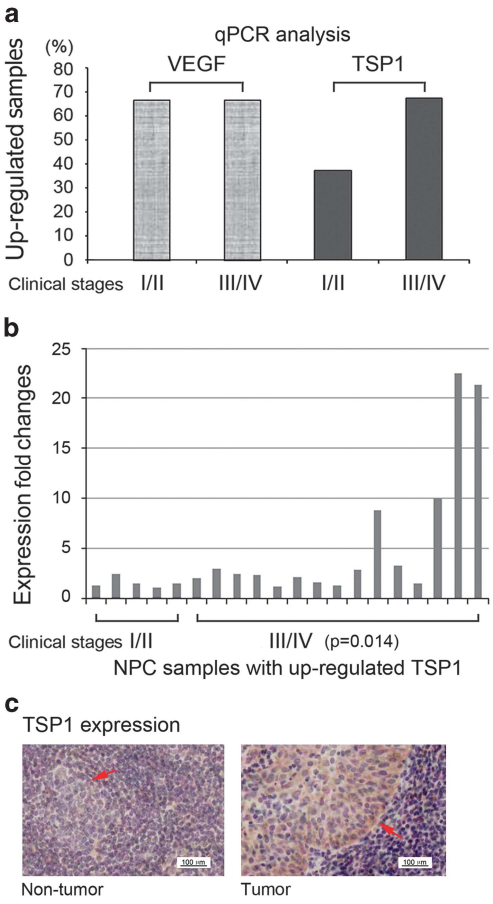


**Figure 2.** Upregulated *VEGF* and *TSP1* expression in NPC with a downregulated *BLU* expression. (**a**) qPCR analysis of *VEGF* and *TSP1* expression. Percentages of upregulated *VEGF* samples (66.7%) are not changed in the early (10/15) and later (10/15) stages, but samples with an upregulation of *TSP1* are increased from 38.5% at stages I/II (5/13) to 68.2% at stages III/IV (15/22). (**b**) Thirty-five pairs of RNA samples, tumors and corresponding non-tumor tissues from the same patients showing downregulation of *BLU* at each clinical stage were investigated. The upregulated expression of TSP1 samples in different stage is listed. Average *TSP1* RNA expression at stages III/IV (15 samples) is higher than that of samples at stages I/II (5 samples). (**c**) IHC staining shows that TSP1 protein is expressed in the cancer nests (red arrow) surrounded by lymphocytes, compared with non-tumor tissues that contain large numbers of inflammation cells around epithelium (red arrow).

region, such as *BLU,* are usually inactivated by hypermethylation of gene promoters rather than being lost or deleted in NPC.[6–8] As a result, the epigenetically silenced genes would not have been recognized by the mapping of deleted regions.

*BLU* is expressed in various fetal tissues and the normal upper respiratory tract, including the nasopharynx, but is rarely expressed in most other normal tissues.[7] In this current study, we examined *BLU* expression by using matched non-tumor tissues obtained from adjacent mucosa in the nasal cavity and tumor specimens from the same patients, which is a more reliable method to evaluate changes of BLU expression and other microenvironment-related factors in NPC patients. As down-regulated BLU expression is closely associated with expression changes of the MMP family in NPC, it is reasonable to speculate that *BLU* may have a certain role in the NPC angiogenesis network. As expected, PCR array analyses indicate that introduction of overexpressed *BLU* into NPC cells triggers many signaling activities associated with both pro- or anti-angiogenic and growth regulatory genes, reflecting unknown regulatory activities to achieve a new balance in the treated cells following the stimulation of exogenous *BLU*. Negative feedback regulatory mechanisms are extensively observed in human cells. Therefore, it is necessary to determine which critical genes are directly regulated by the alteration of *BLU* expression in NPC. As a result, two important angiogenic regulators, *VEGF* and *TSP1*, were found to be influenced by *BLU* in the NPC cells.

To reveal the consequence of expression of exogenous *BLU* in HONE1 cells, we performed the *in vivo* assays that now provide direct evidence for *BLU*-associated tumor cell death in animals and which provides a model to study TSG anti-angiogenic function in the *in vivo* assay. The anti-angiogenic activity of *BLU* was also detected in another NPC cell line HK1 that also showed a downregulated endogenous expression of *BLU*. Interestingly, BLU only inhibited VEGF165, but not TSP1 in HK1 cells. Compared with results derived from HONE1 cells, no obvious tumor suppression in HK1 cells was detected during the first week after injection, but vascular tube formation was inhibited in HK1 *BLU* transfectants. As both TSP1 and VEGF proteins were inhibited in HONE1 *BLU* transfectants, this may explain why exogenous *BLU* in stable HONE1 transfectants had a stronger anti-angiogenic effect than that in differentiated HK1 cells. These results also confirm the findings detected in NPC samples, namely, *TSP1* alterations are often involved in some, especially at advanced stages of NPC, such as poorly differentiated HONE1 cells. Differences in cell growth kinetics may also contribute to tumor survival seen at week one between *BLU* overexpression in HONE1 and HK1 *in vivo*. As $>90\%$ of NPC tumors are poorly- or undifferentiated, like HONE1, it is possible that *BLU*-mediated alteration of the *VEGF*/*TSP1* signaling may affect the majority of NPC cases.

Our current findings resolve a long-term unanswered question, namely, how *BLU*-induced tumor suppressive function in NPC cells. The roles of *TSP1* in angiogenesis and cancer growth control are complex and controversial, and its function varied in different tissues and cells,[22,27,28,45] as seen in HONE1 and HK1 cell lines. Although many studies indicated that *TSP1* inhibited angiogenesis, ample evidence also showed that *TSP1* had pro-angiogenic effects and had variable functional roles in the control of tumor invasion and metastasis.[22,27,38,46–48] Recent studies showed that *TSP1* was a major factor in the microvascular niche and was concentrated within the basement membrane to regulate tumor dissemination.[49] We observed increased expression of TSP1 in the tumor periphery in NPC, which was also consistent with findings in our animal studies. These results suggest BLU may have critical roles in both the tumor microenvironment and angiogenesis process through regulation of VEGF and TSP1 expression. The dual roles *TSP1* plays in cancer development are not fully understood now, but they can be influenced by TSP1 expression level, *p53* status, cell or tissue type and microenvironment.[30,38] To our knowledge, investigations on *TSP1* expression in NPC specimens have not yet been reported. We now provide novel results showing that *TSP1* expression is progressively upregulated when *BLU* is downregulated, during the development of NPC. In contrast, *VEGF* was reported to be widely expressed in NPC specimens (60–67%)[50,51] and was downregulated by candidate TSGs *Fibulin-2*, *MMP19* and *ADAMTS9* in NPC.[18,26,35] In addition, overexpressed TSP1 in NPC HONE1 cells suppressed the endogenous BLU. These results suggest that *TSP1*-mediated signaling acts in a tissue-specific and time-specific manner in NPC, as seen in other tumors. These studies also indicate that NPC cells are genetically heterogeneous in the regulation of angiogenesis network, and accumulation of multiple alterations is necessary for the NPC development as reported previously.[44,52]

The *BLU* gene can be controlled by environmental stresses, heat-shock factor, E2F family proteins and *p53* signaling.[2,7,17] Interestingly, *TSP1* is regulated by the *p53* gene,[53] and NPC, unlike other common tumors, has an infrequent mutation rate of the *p53* gene.[43,54,55] It is possible that *BLU* plays roles through the

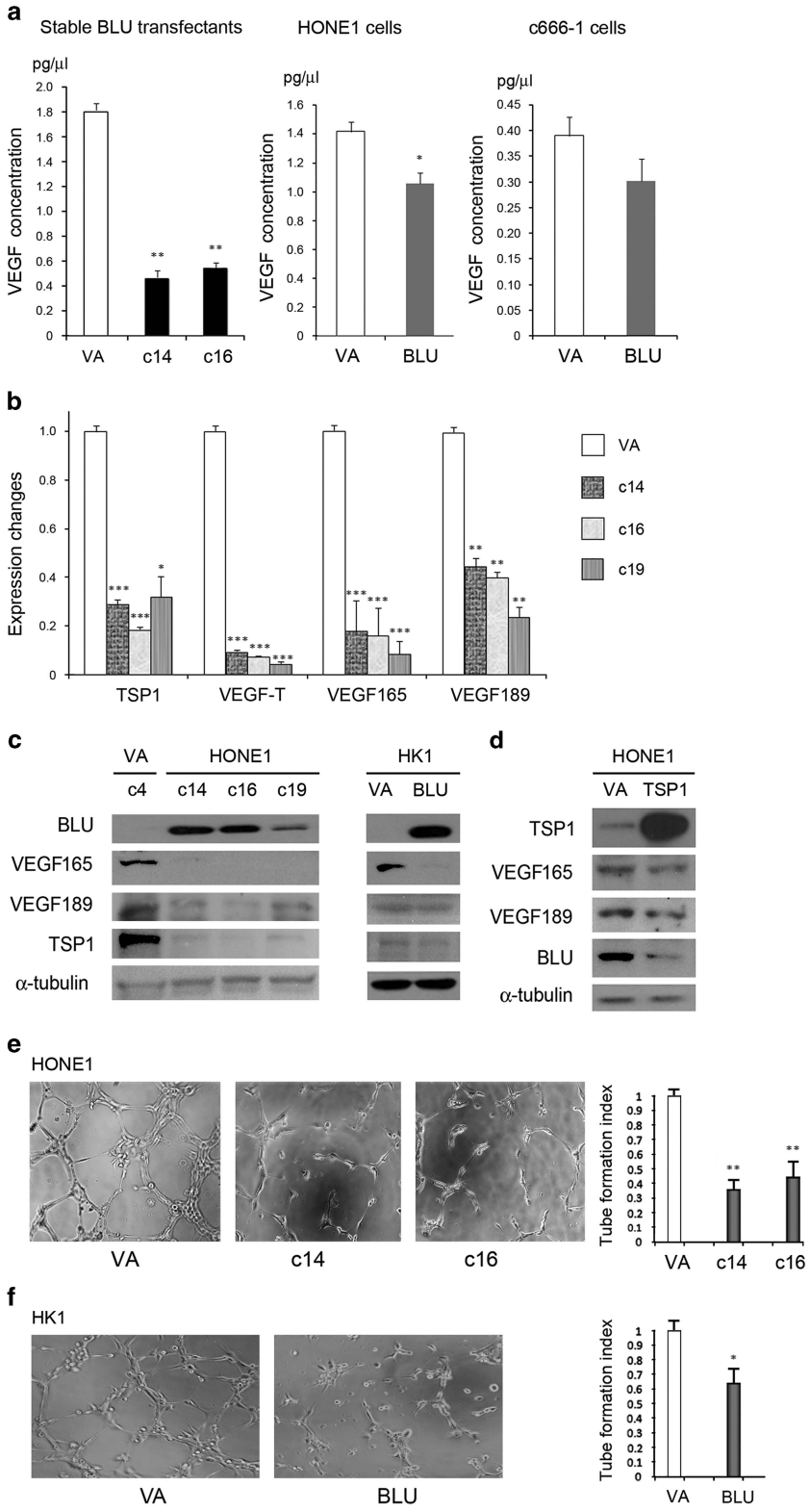


**Figure 3.** Inhibition of VEGF and TSP1 expression is associated with decreased tube formation abilities in NPC cells. (**a**) ELISA assay shows that VEGF secretion is inhibited in both stable (c14 and c16) and transient (HONE1 and c666-1) *BLU* transfectants, compared with VA control cells. $^{*}P < 0.05$, $^{**}P < 0.01$. (**b**) qPCR analyses reveal strong inhibitions of *TSP1*, *VEGF-T*, *VEGF165*, and *VEGF189*, in three different *BLU* transfectants, c14, c16 and c19, compared with VA control. Three independent assays were performed for each sample. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. (**c**) Western blotting shows that VEGF165, VEGF189 and TSP1 proteins are downregulated in three stable HONE1 *BLU* transfectants, c14, c16 and c19. Only VEGF165 is downregulated in *BLU*-overexpressing HK1 cells. (**d**) Overexpressed TSP1 in infected HONE1 cells clearly reduces the expression of endogenous BLU protein. (**e**, **f**) The HUVEC tube formation is inhibited by adding conditioned media from trasfectants in HONE1 stable *BLU* transfectants, c14 and c16, and HK1 *BLU* transfectants. The small and complete tubes are seen in VA cells, but incomplete tubes are observed in all *BLU* transfectants. The relative length of tubes in *BLU* transfectants was compared with that of VA cells (equal to 1). $^{*}P < 0.05$, $^{**}P < 0.01$.

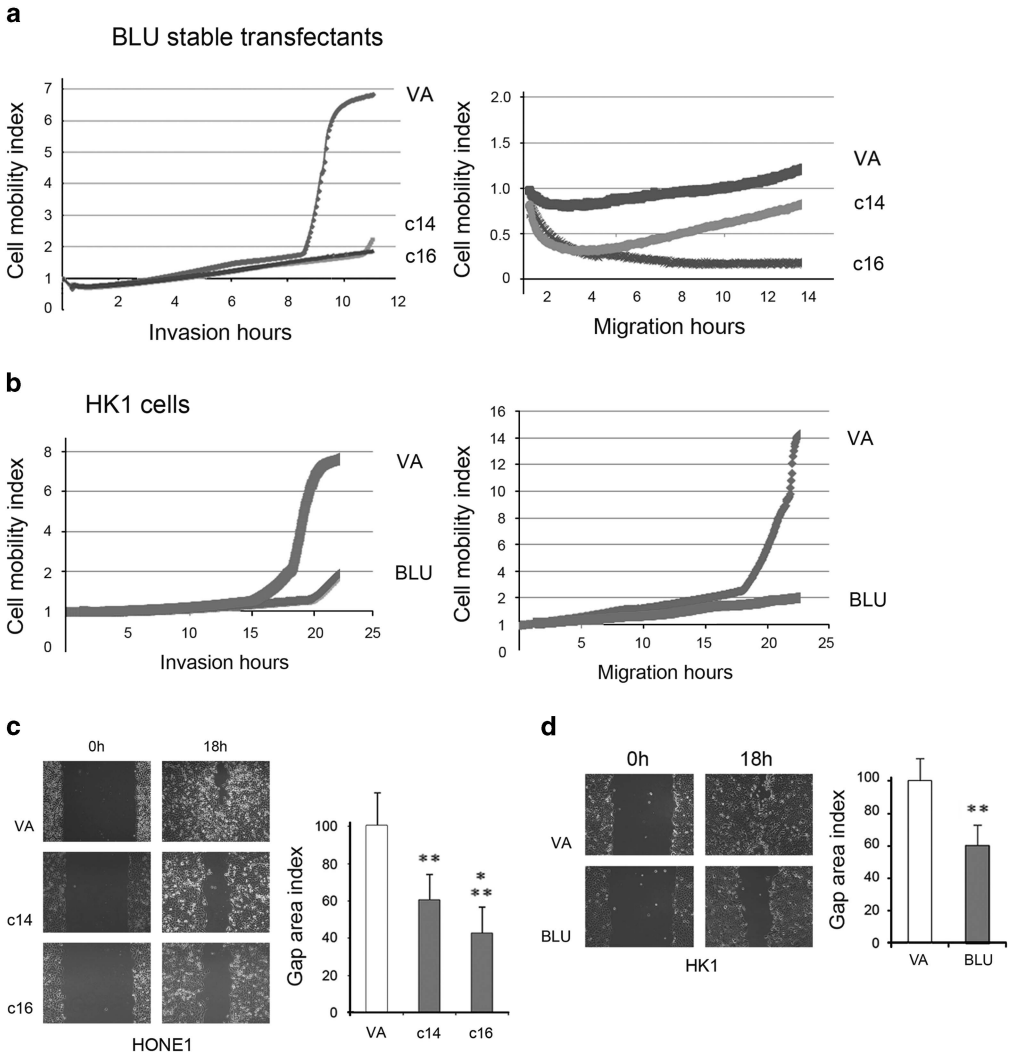


**Figure 4.** BLU suppresses invasion and migration abilities of NPC cells. (**a**, **b**) Cells were assayed by real-time *in vitro* invasion (left) and migration (right) assays. The cell mobility index reflected ability of tested cells passing through membrane in certain time interval compared with control (VA) cells. Both *BLU* stable transfectants (top) and HK1 *BLU* transfectants inhibit cell invasion and migration compared with VA control cells. (**c**, **d**) The wound healing assay was performed in both HONE1 and HK1 *BLU* transfectants showing reduced abilities for cell migration and proliferation, compared with their control VA cells. $^{**}P < 0.01$ and $^{***}P < 0.001$.

*VEGF/TSP1* signaling interactions with *p53* and other cell cycle regulators such as *E2F* and *Cyclin D1*.[7,9,17,56] However, direct evidence showing the relationship between functional tumor suppression in animals and *BLU* molecular regulation in cell growth is not yet available. More detailed studies in *BLU*-mediated angiogenesis networks and their regulatory mechanisms are currently in progress.

In conclusion, we provide novel evidence that *BLU* suppresses human tumor growth through its anti-angiogenesis activities involved in NPC development. These findings are further strengthened by *in vivo* studies and verified in different NPC cell lines and primary samples. Furthermore, current results provide future directions for the exploration of *BLU*-mediated therapeutic targets in NPC and signaling regulatory mechanisms involved in the 3p21.3 region and angiogenesis networks in human tumors.

## MATERIALS AND METHODS

### Cell lines and culture conditions

Stable pETE-Bsd *BLU* transfectants, c14, c16 and c19, were identified with overexpression of the *BLU* gene in NPC HONE1 cells. All HONE1 *BLU* transfectants, VA transfectant c4, HK1, c666-1 and HUVEC cells were maintained as previously described.[8,18,35]

### Lentiviral infection

A 1.3 kb *BLU* cDNA from pETE-Bsd vector[8] was excised and recloned into the pWPI vector (Addgene Plasmid 12254). The 12 540 bp *BLU* construct was confirmed by PCR and sequencing analyses of the vector pWP1-BLU. The *TSP1* cDNA (Plasmid 12993, pGEM2 hTSP-1) was obtained from Addgene. Empty vectors, BLU and TSP1 vectors were prepared for lentiviral infection. HEK-293 T cells were incubated at 37 °C overnight and transfected with psPAX2 packaging plasmid, pMD2.G envelope plasmid and FuGENE HD in DMEM medium, following the Addgene protocol.

### TMA and IHC staining

Matched normal nasopharyngeal and NPC biopsies from 101 NPC patients were used for qPCR analysis. Construction of the NPC TMA and IHC staining was previously described.[26] Antibodies are described in Supplementary Table 3. Non-tumor NP and NPC biopsies from NPC patients were collected from Queen Mary Hospital between 2005 and 2008 in Hong Kong. The study protocol was approved by the Hospital Institutional Review Board of Hong Kong and written consents were obtained from all patients. The immunostaining of TMA was reviewed by two independent pathologists.

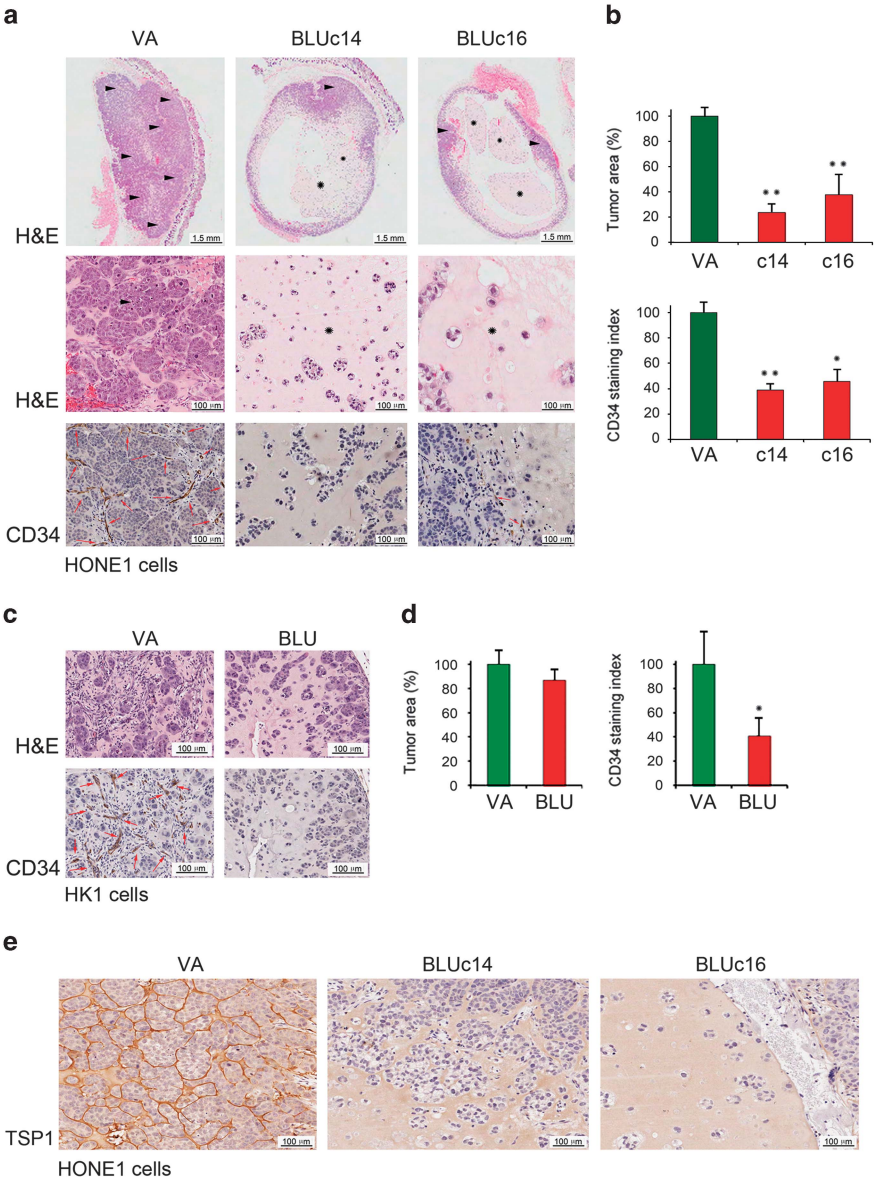


**Figure 5.** The matrigel plug *in vivo* assays show that *BLU* suppresses tumor development through inhibition of blood vessel formation. (**a**) The top panel shows whole image of excised tumor nodules from mice. The VA c4 nodule is actually full of growing cancer cells, indicated by black arrows. In contrast, large portions in *BLU* c14 and c16 nodules were necrotic (indicated by *), and dying cancer cells are observed in the center and periphery of nodules. The middle panel shows the high-power images of cancer nests in the central area of c4 nodule and necrosis areas in both c14 and c16 tumors. The bottom panel shows that CD34-stained endothelial cells indicated by red arrows. (**b**) Comparison of growing tumor areas and CD34 endothelial cell staining between *BLU* transfectants and VA cells. Scoring of vessel formation was confined to the areas containing viable tumor growth. (**c**, **d**) Comparison of growing tumor areas and CD34 endothelial cell staining between HK1 *BLU* transfectants and their VA control cells. *$P < 0.05$. (**e**) TSP1 expression could be detected in basement membrane and interstitial tissues of tumor nests in VA control cells that showed progressive growth in mice, but it was not detected in the necrosis areas in HONE1 *BLU* transfectants.

For evaluation of the staining, the nonmalignant and malignant tissues were scored for genes by assessing the site of positive staining in the cytoplasm. A random four views were counted under ×400 microscopy. When >70% of cells were positively stained, then the expression of genes was considered normal. Downregulated expression of genes was noted when <70% of cells were positively stained. Cases with <50 cells were excluded from statistical analysis.

## qPCR analysis and PCR array analysis

Total RNA from cell lines and tissues was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Primers and RT–PCR and qPCR conditions are summarized in Supplementary Table 4. Human *GAPDH* was used as an internal control for all PCR reactions. For qPCR analysis, duplicate or triplicate PCR reactions were performed using the LightCycler 480 Real-Time PCR Instrument (Roche Diagnositics GmbH, Mannheim, Germany). Human Angiogenesis Pathway PCR Arrays (PAHS-024 ZF-2) were obtained from SABIOSCIENCES, a Qiagen Company (Frederick, MD, USA). RNAs from *BLU* transfectant c16 and VA c4 cells[8] were used for analyses. All reaction procedures and data analyses were performed following the manufacturer's manual ($RT^2$ Profile PCR Array User Manual version 5.01) and provided analysis software ($RT^2$ Profiler RCR Array Data Analysis version 3.5).

## Western blotting

Western blot analysis of BLU, TSP1 and VEGF was performed as previously described.[26] The antibodies used in this assay are described in

Supplementary Table 3. The a-tubulin was used as a loading control for all experiments.

### ELISA

Human VEGF Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA) was used. The conditioned media from *BLU* transfectants, and vector-alone cells were collected, when cell confluence reached ~80–90%.[26] Three independent experiments in duplicate were performed for each sample.

### Invasion and migration assays

The CIM-plate 16 (Roche Applied Sciences, Mannheim, Germany) used for the invasion study is a thin layer of matrigel basement matrix, while the chamber used for migration study consists of an 8.0-μm pore size PET membrane at the bottom of the inserts. The real-time invasion and migration abilities of cells were determined by the number of cells passing through the membrane and the matrigel basement matrix, respectively. All procedures were performed following the manufacturer's manual.

### HUVEC tube formation assay

A total of $4 \times 10^4$ HUVEC resuspended in 100 μl conditioned media from transfectants supplemented with 1% serum were seeded in each well. The 96-well plates were pre-coated with 50 μl growth factor reduced-matrigel (BD Bioscience, San Jose, CA, USA). The HUVEC cells were then incubated at 37 °C for 6 h to allow the formation of tube-like structures. Images of the tubes formed were captured under inverted microscopy at ×10 magnification, and the total tube length formed by each sample was measured by SPOT imaging software (Diagnostic Instruments, Sterling Heights, MI, USA), as previously described.[35] Three independent experiments were performed for each sample.

### Wound healing assay

Each group had 12 culture wells for cell growth. The treated cells were seeded into culture wells and reached ~70–80% confluence as a monolayer 24 h later. The gaps were produced by scratching across the surface of the well. Cells were grown for an additional 18 h, then fixed and stained for examination. Gap distance was quantitatively evaluated using ImageJ software.

### *In vivo* tumor suppression and matrigel plug angiogenesis assay

The animal *in vivo* assays were performed as previously reported.[35] A total of $1 \times 10^7$ cells mixed with 50 μl DMEM and 250 μl ice-cold matrigel was injected into nude mice subcutaneously to allow gel plug formation. Cells from both control and experimental groups were injected to animals and each group had five injection sites. All nodules were formed subcutaneously and maintained for 7 days in animals. Excised tumors were fixed and embedded for staining. The slides were scanned by ImageScope v10 software (Aperio, Vista, CA, USA). All animal experiments were approved by the Government of Hong Kong Special Administrative Region and the University of Hong Kong.

### Statistical analysis

Differences between two groups were scored for statistical significance ($^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, mean ± s.d.) using Student's *t*-test or $\chi^2$-test.

## ABBREVIATIONS

MMP, matrix metalloproteinase; NPC, nasopharyngeal carcinoma; TSG, tumor suppressor gene; TSP1, thrombospondin-1; VEGF, vascular endothelial growth factor; ZMYND10/BLU, Zinc finger, MYND-type containing 10.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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