Zinc-finger, MYND-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.

INTRODUCTION

Human chromosome 3p21.3 was the first tumor suppressive region functionally identified in nasopharyngeal carcinoma (NPC). Region of this region was confirmed to be a tumor suppressor gene (TSG) cluster that contains several TSGs associated with the development of many human tumors. BLU was one of TSGs mapped in this region and has been repeatedly reported to be involved in the development of NPC and many other tumors, including lung cancers, glioma, neuroblastoma, cervical neoplasia, esophageal squamous cell carcinoma, liver tumor and myelodysplastic syndrome. The BLU/ZMYND10 gene spans 4.5 kb on human chromosome 3p21.3; alternative splicing is observed in different tissues. It is not clear if or how BLU contributes to the early or late stages of tumor progression. The hypermethylated BLU was detected in 66–74% of primary NPC samples. Compared with the immortalized nasopharyngeal epithelial line, BLU was downregulated in NPC cell lines such as HONE1 and HK1. The exogenously expressed BLU has an in vitro growth-inhibitory effect in lung, kidney and ovarian carcinomas, neuroblastoma and NPC cell lines. 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. 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. ADAMTS9 and its other family members interact with Thrombospondin-1 (TSP1 or THBS1) to control tumor progression, angiogenesis and metastasis. BLU is regulated by E2F and E2F directly controls TSP1 expression. The MMP family, a group of genes regulating extracellular matrix, was suggested to regulate NPC cell invasive properties in association
with VEGF. However, how TSP1 and VEGF regulate angiogenesis, progression and metastasis processes in NPC is not well 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. 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. Genes mapping to the 3p21.3 TSG cluster are critical for tumor development as reported in a number of sporadic human cancers. 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. 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. 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 (Figure 1d).

It is well-known now that both NPC and nasopharynx control microenvironmental 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, 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 (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 IV) (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 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 enzyme-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.

cPCR 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. 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

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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 confirmed 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).

BLU has anti-angiogenic 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.3,35,43,44 Using cell fusion and chromosome transfer approaches, we previously identified functionally critical regions mapping to chromosome 3p21.3 and chromosome 9p21 regions containing the known TSG TSP1 and 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 1. BLU expression in non-tumor and NPC samples by IHC staining

| Tissue type | Expression | Downregulated expression | P-value<sup>a</sup> |
|-------------|------------|--------------------------|---------------------|
| Normal      | 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. *Information for patients and informative cases are listed in Supplementary Table 1.*<sup>a</sup>Compared with downregulated normal group. *Normal includes non-tumor nasopharyngeal mucosa and adjacent non-neoplastic mucosal tissues from same patient.

### Table 2. Concomitant downregulated expression of MMP19 and BLU proteins in NPC by IHC staining<sup>a</sup>

| Tissue type | Informative cases for MMP19 and BLU | Concomitant expression | Downregulated expression | P-value<sup>b</sup> |
|-------------|-------------------------------------|------------------------|--------------------------|---------------------|
| 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. *Contiguous sections from the same case were stained with MMP19 and BLU antibodies.*<sup>b</sup>Compared with downregulated normal group.
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 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.2,7,17 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.40,48 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%)30,31 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.40,52

The BLU gene can be controlled by environmental stresses, heat-shock factor, E2F family proteins and p53 signaling.2,4,17 Interestingly, TSP1 is regulated by the p53 gene,32 and NPC, unlike other common tumors, has an infrequent mutation rate of the p53 gene.33,34,55 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 transfectants 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.
VEGF/TSP1 signaling interactions with p53 and other cell cycle regulators such as E2F and Cyclin D1. 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.

**Lentiviral infection**

A 1.3 kb BLU cDNA from pETE-Bsd vector3 was excised and recloned into the pWPI vector (Addgene Plasmid 12254). The 12540 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. 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.

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**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.
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 Diagnostics 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 were used for analyses. All reaction procedures and data analyses were performed following the manufacturer’s manual (RT² Pro File PCR Array User Manual version 5.01) and provided analysis software (RT² Profiler RCR Array Data Analysis version 3.5).

Western blotting
Western blot analysis of BLU, TSP1 and VEGF was performed as previously described. The antibodies used in this assay are described in...
the tubes formed were captured under inverted microscopy at × 10 for 6 h to allow the formation of tube-like structures. Images of 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. Three independent experiments were performed for each sample.

**HUCt tube formation assay**
A total of 4 × 10^4 HUCt 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 HUCt 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. 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 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. A total of 1 × 10^5 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 χ²-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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**REFERENCES**
1. Cheng Y, Poulo NE, Lung ML, Hampton G, Ou B, Lerman MI et al. Functional evidence for a nasopharyngeal carcinoma tumor suppressor gene that maps at chromosome 3p21.3. Proc Natl Acad Sci USA 1998; 95: 3042–3047.
2. Hesson LB, Cooper WN, Latif F. Evaluation of the 3p21.3 tumor-suppressor gene cluster. Oncogene 2007; 26: 7283–7301.
3. Lerman MI, Minna JD. The 630-kb lung cancer homozygous deletion region on human chromosome 3p21.3: identification and evaluation of the resident candidate tumor suppressor genes. The International Lung Cancer Chromosome 3p21.3 Tumor Suppressor Gene Consortium. Cancer Res 2000; 60: 6116–6133.
4. Dammann R, Li C, Yoon JM, Chin PL, Bates S, Pfeifer GP. Epigenetic inactivation of a PAS association domain family protein from the lung tumour suppressor locus 3p21.3. Nat Genet 2006; 35: 315–319.
5. Zabarovskiy ER, Lerman MI, Minna JD. Tumor suppressor genes on chromosome 3p involved in the pathogenesis of lung and other cancers. Oncogene 2002; 21: 6915–6935.
6. Liu XQ, Chen HK, Zhang XS, Pan ZG, Li A, Feng QS et al. Alterations of BLU, a candidate tumor suppressor gene on chromosome 3p21.3, in human nasopharyngeal carcinoma. Int J Cancer 2003; 106: 60–65.
7. Qiu GH, Tan LK, Loh KS, Lim CY, Srivastava G, Tsai ST et al. The candidate tumor suppressor gene BLU, located at the commonly deleted region 3p21.3, is an E2F-regulated, stress-responsive gene and inactivated by both epigenetic and genetic mechanisms in nasopharyngeal carcinoma. Oncogene 2004; 23: 4793–4806.
8. Yau WL, Lung HL, Zabarovskiy ER, Lerman MI, Sham JS, Chu DT et al. Functional studies of the chromosome 3p21.3 candidate tumor suppressor gene BLU/ZMYND10 in nasopharyngeal carcinoma. Int J Cancer 2006; 119: 2821–2826.
9. Zhang X, Liu H, Li B, Huang P, Shao J, He Z. Tumor suppressor BLU inhibits proliferation of nasopharyngeal carcinoma cells by regulation of cell cycle, c-Jun N-terminal kinase and the cyclin D1 promoter. BMC Cancer 2012; 12: 267.
10. Marsit CJ, Kim DH, Liu M, Hinds PW, Wiencke JK, Nelson HH et al. Hypermethylation of RASSF1A and BLU tumor suppressor genes in non-small cell lung cancer: implications for tobacco smoking during adolescence. Int J Cancer 2005; 114: 219–223.
11. Agathangelou A, Dallol A, Zochbauer-Muller S, Morrissey C, Honorio S, Hesson L et al. Epigenetic inactivation of the candidate 3p21.3 suppressor gene BLU in human cancers. Oncogene 2003; 22: 1580–1588.
12. Hesson L, Bieche I, Koox D, Cimiere E, Hoang-Xuan K, Maher ER et al. Frequent epigenetic inactivation of RASSF1A and BLU genes located within the critical 3p21.3 region in gliomas. Oncogene 2004; 23: 2408–2419.
13. Lai HC, Lin YW, Chang CC, Wang HC, Chu TW, Yu MH et al. Hypermethylation of two consecutive tumor suppressor genes, BLU and RASSF1A, located at 3p21.3 in cervical neoplasias. Gynecol Oncol 2007; 104: 629–635.
14. Yi Lo PH, Chung Leung AC, Xiong W, Law S, Duh FM, Lerman MI et al. Expression of candidate chromosome 3p21.3 tumor suppressor genes and down-regulation of BLU in some esophageal squamous cell carcinomas. Cancer Lett 2006; 234: 184–192.
15. Tischler I, Markwarth A, Witzigmann H, Ulhmann D, Hauss J, Mirmohammadsadegh A et al. Allele loss and epigenetic inactivation of 3p21.3 in malignant liver tumors. Int J Cancer 2005; 115: 684–689.
16. Yang Y, Zhang Q, Xu F, Wu L, He Q, Li X. Tumor suppressor gene BLU is frequently downregulated by promoter hypermethylation in myelodysplastic syndrome. J Cancer Res Clin Oncol 2012; 138: 729–737.
17. Park ST, Byun HJ, Kim BR, Dong SM, Park SH, Jang PR et al. Tumor suppressor BLU promotes paclitaxel antitumor activity by inducing apoptosis through the down-regulation of Bcl-2 expression in tumorigenesis. Biochem Biophys Res Commun 2013; 435: 153–159.
18. Lo PH, Lung HL, Cheung AK, Apte SS, Chan KW, Kwong FM et al. Extracellular protease ADAMTS9 suppresses esophageal and nasopharyngeal carcinoma tumor formation by inhibiting angiogenesis. Cancer Res 2010; 70: 5567–5576.
19. Lung HL, Lo PH, Xie D, Apte SS, Cheung AK, Cheng Y et al. Characterization of a novel epigenetically-silenced, growth-suppressive gene, ADAMTS9, and its association with lymph node metastases in nasopharyngeal carcinoma. Int J Cancer 2008; 123: 401–408.
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20 Kaur S, Martin-Manoso G, Pendrak ML, Garfield SH, Izenberg JS, Roberts DD. Thrombospondin-1 inhibits VEGF receptor-2 signaling by disrupting its association with CD47. J Biol Chem 2010; 285: 38923–38932.

21 Koo BH, Coe DM, Dixon LJ, Somerville RP, Nelson CM, Wang LW et al. ADAMTS9 is a cell-autonomously acting, anti-angiogenic metallopeptidase expressed by microvascular endothelial cells. Am J Pathol 2010; 176: 1494–1504.

22 Lee YI, Koch M, Karl D, Torres-Collado AX, Fernando NT, Rothrock C et al. Variable inhibition of thrombospondin 1 against liver and lung metastases through differential activation of metalloproteinase ADAMTS1. Cancer Res 2010; 70: 948–956.

23 Tuszynski GP, Nicosis RF. The role of thrombospondin-1 in tumor progression and angiogenesis. Bioessays 1996; 18: 71–76.

24 Ji W, Zhang W, Xiao W. E2F-1 directly regulates thrombospondin 1 expression. PLoS ONE 2010; 5: e13442.

25 Yang EV, Sood AK, Chen M, Li Y, Eubank TD, Marsh CB et al. Norepinephrine up-regulates the expression of vascular endothelial growth factor, matrix metalloproteinase (MMP)-2, and MMP-9 in nasopharyngeal carcinoma tumor cells. Cancer Res 2006; 66: 10357–10364.

26 Chan KC, Ko JM, Lung HL, Selldreck R, Zhang ZF, Luo DZ et al. Cytolytic activity of Matrix metalloproteinase-19 is essential for tumor suppressor and anti-angiogenic activities in nasopharyngeal carcinoma. Int J Cancer 2011; 129: 1826–1837.

27 Zaslavska Y, Baek KH, Lynch RC, Short S, Grillo J, Folkman J et al. Platelet-derived thrombospondin-1 is a critical negative regulator and potential biomarker of angiogenesis. Blood 2011; 115: 4605–4613.

28 Sargianioudi I, Zhou J, Tuszynski GP. The role of thrombospondin-1 in tumor progression. Exp Biol Med (Maywood) 2001; 226: 726–733.

29 Nucera C, Porrello A, Antonello ZA, Mekal M, Neis MA, Giordano TJ et al. B-Raf (V600E) and thrombospondin-1 promote thyroid cancer progression. Proc Natl Acad Sci USA 2010; 107: 10649–10654.

30 Miyata Y, Sakai H. Thrombospondin-1 in urolological cancer: pathological role, clinical significance, and therapeutic prospects. Int J Mol Sci 2013; 14: 12249–12272.

31 Ancelin M, Buteau-Lozano H, Meduri G, Osborne-Pellegrin M, Sordello S, Plouet J et al. A dynamic shift of VEGF isoforms with a transient and selective progesterone-induced expression of VEGF189 regulates angiogenesis and vascular permeability in human uterus. Proc Natl Acad Sci USA 2002; 99: 6023–6028.

32 Herve MA, Buteau-Lozano H, Mourah S, Calvo F, Perrot-Plantanet M. VEGF189 stimulates endothelial cells proliferation and migration in vitro and up-regulates the expression of Flik-1/KDR mRNA. Exp Cell Res 2005; 309: 24–31.

33 Peng X, Li W, Tan G. Reversal of taxol resistance by cisplatin in nasopharyngeal carcinoma cells from nasopharyngeal carcinoma. Int J Cancer 2011; 129: 1826–1837.

34 Ancelin M, Cheillet-Martin S, Herve MA, Legrand C, El Benna J, Perrot-Applanat M. An recombinant NH(2)-terminal heparin-binding domain of the adhesive glycoprotein, thrombospondin-1, promotes endothelial tube formation and cell survival: a possible role for syndecan-4 proteoglycan. Matrix Biol 2002; 21: 311–324.

35 Law EW, Cheung AK, Kashuba VI, Pavlova TV, Zabarovsky ER, Lung HL et al. Progesterone-induced expression of VEGF189 regulates angiogenesis and vascular endothelial growth factor (VEGF) expression predicts outcome in 833 patients with primary breast carcinoma. Int J Cancer 2000; 89: 51–62.

36 Cheng Y, Stanbridge EJ, Kong H, Bengtsson U, Lerman MI, Lung ML. A functional investigation of tumor suppressor gene activities in a nasopharyngeal carcinoma cell line HONE1 using a monochromosome transfer approach. Genes Chromosomes Cancer 2000; 28: 82–91.

37 Liu HL, Cheung AK, Ko JM, Cheng Y, Stanbridge EJ, Lung ML. Deciphering the molecular genetic basis of NPC through functional approaches. Semin Cancer Biol 2012; 22: 87–95.

38 Linderholm B, Lindh B, Tavelin B, Granvist K, Henriksen R, p53 and vascular-endothelial growth-factor (VEGF) expression predicts outcome in 833 patients with primary breast carcinoma. Int J Cancer 2000; 89: 51–62.

39 Ferrari di Outeiro-Bernstein MA, Nunes SS, Andrade AC, Alves TR, Legrand C, Morandi V. A recombinant N(2)-terminal heparin-binding domain of the adhesive glycoprotein, thrombospondin-1, promotes endothelial tube formation and cell survival: a possible role for syndecan-4 proteoglycan. Matrix Biol 2002; 21: 311–324.

40 Lawler PR, Lawler J. Molecular basis for the regulation of angiogenesis by thrombospondin-1 and -2. Cold Spring Harb Perspect Med 2012; 2: a006627.

41 Abe M, Ohira M, Kaneda A, Yagi Y, Yamamoto S, Kitano Y et al. CpG island methylator phenotype is a strong determinant of poor prognosis in neuroblastomas. Cancer Res 2005; 65: 828–834.

42 Ghajar CM, Peinado H, Mori H, Matei IR, Evasion KJ, Brazier H et al. The perivascular niche regulates breast tumour dormancy. Nat Cell Biol 2013; 15: 807–817.

43 Oliver EP, Chan AT, Pezzella F, Turley H, To KF, Poon TC et al. Coexpression of hypoxia-inducible factors 1alpha and 2alpha, carbonic anhydrase IX, and vascular endothelial growth factor in nasopharyngeal carcinoma and relationship to survival. Clin Cancer Res 2002; 8: 2595–2604.

44 Krishna SM, James S, Balam P. Expression of VEGF as prognosticator in primary nasopharyngeal cancer and its relation to EBV status. Virus Res 2006; 115: 85–90.

45 Lung HL, Cheung AKL, Ko JM, Cheng Y, Lung ML. Identification of tumor suppressor genes via cell fusion and chromosomal transfer. In: Cheng Y (ed.) Tumor Suppressor Genes. InTech: Croatia 2012, pp 53–78.

46 Dameron KM, Volpert OV, Tainsky MA, Bouck N. Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. Science 1994; 265: 1582–1584.

47 Sun Y, Hegamy G, Cheng YJ, Hildesheim A, Chen YJ, Chen H et al. An infrequent point mutation of the p53 gene in human nasopharyngeal carcinoma. Proc Natl Acad Sci USA 1992; 89: 6516–6520.

48 Cheng Y, Cheung AK, Ko JM, Phoon YP, Chiu PM, Lo PH et al. Physiological β-catenin signaling controls self-renewal networks and generation of stem-like cells from nasopharyngeal carcinoma. BMC Cell Biol 2013; 14: 44.

49 Yoo HJ, Kim BR, Byun HJ, Park SY, Rho SB. BLU enhances the effects of antiangiogenic activity in combination with gemcitabine-based chemotherapeutic agents. Int J Biochem Cell Biol 2013; 45: 1236–1245.

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