Title:
Brain-Derived Neurotrophic Factor Promotes Tumorigenesis via Induction of Neovascularization: Implication in Hepatocellular Carcinoma

Authors and Affiliations:
Chi-Tat Lam¹, Zhen-Fan Yang¹,², Chi-Keung Lau¹, Ka-Ho Tam¹, Sheung-Tat Fan¹,², and Ronnie T. P. Poon¹,²
¹Department of Surgery, ²State Key Laboratory for Liver Research, The University of Hong Kong, Pokfulam, Hong Kong, China

e-mail addresses:
Lam CT: ctlam88@hku.hk
Yang ZF: yangkitty@yahoo.com
Lau CK: lauck@hku.hk
Tam KH: ichristam@gmail.com
Fan ST: stfan@hku.hk
Poon RT: poontp@hkucc.hku.hk

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Address reprint requests to: Ronnie T. Poon, Department of Surgery, The University of Hong Kong, Queen Mary Hospital, 102 Pokfulam Road, Hong Kong, China. Phone: 852-2255-3641; Fax: 852-2817-5475; E-mail: poontp@hkucc.hku.hk.

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Translational Relevance

Brain-derived neurotrophic factor (BDNF) is a potential angiogenic factor. In this study, we showed that overexpression of BDNF confers angiogenic properties on endothelial cells (ECs). By establishing an in vivo co-transplantation model in nude mice, we demonstrated that high BDNF-expressing ECs could promote tumor growth, while its knock-down by shRNAs impaired the tumor-promoting effect. These data suggested a critical role of BDNF/tropomyosin receptor kinase B (TrkB) system in tumorigenesis. Interestingly, expression study by Western blot using 50 pairs of human hepatocellular carcinoma (HCC) tissues revealed overexpression of BDNF and TrkB in tumor tissues. By immunohistochemistry, aside from positive staining in tumor cells, ECs also showed strong BDNF reactivity in HCC tissues, implicating a role of tumor microenvironment in hepatocarcinogenesis. Patients with TrkB overexpression in tumors had significantly shorter overall survival, highlighting the clinical significance of BDNF/TrkB pathway in HCC.
Abstract

Purpose: Brain-derived neurotrophic factor (BDNF) has emerged as a novel angiogenic factor, and yet, its impact on tumorigenesis is unclear. This study aimed at investigating the roles of BDNF in angiogenesis and tumor development.

Experimental Design: BDNF was overexpressed in a mouse endothelial cell (EC) line by stable transfection, and angiogenic properties of the transfectants were assessed. Microarray analysis was employed to explore the molecular pathways. The impact of modulating BDNF levels in two mouse EC lines on tumorigenic potential of a transformed mouse liver cell line was evaluated by an in vivo co-transplantation model. BDNF and tropomyosin receptor kinase B (TrkB) protein levels were determined in 50 pairs of human hepatocellular carcinoma (HCC) tissues by Western blot and immunohistochemistry. Survival analysis was performed to determine their clinical significance.

Results: Overexpression of BDNF could promote EC proliferation, migration, invasion and survival. Microarray and molecular studies showed that RhoA, caspase-9, caspase-3, growth arrest specific 6 and vascular endothelial growth factor could mediate BDNF/TrkB-induced angiogenesis. The co-transplantation experiment demonstrated that high BDNF-expressing ECs could facilitate tumor angiogenesis and growth, while knock-down of BDNF by shRNAs impaired such effects. Furthermore,
examination on human HCC tissues revealed up-regulation of BDNF and TrkB protein levels in 46.0% and 33.3% of the cases studied, respectively. Immunohistochemistry disclosed strong BDNF reactivity in both tumor and endothelial cells. High TrkB expression was associated with shorter overall survival.

**Conclusions:** BDNF/TrkB system was crucial for tumor angiogenesis and growth, which may represent a potential target for antiangiogenic therapy in HCC.
Introduction

Antiangiogenic therapy is emerging as a therapeutic option for cancer patients because of relatively low toxicity, low risk of drug resistance (1), and high target selectivity (2). Recent insights into the molecular mechanisms of angiogenesis have led to identification of novel therapeutic targets, and brain-derived neurotrophic factor (BDNF) appears to be one of the candidates (3-5).

BDNF belongs to a class of growth factors called neurotrophins, which have well-documented functions in neural development and are linked to neoplasia (4, 6-12). The expression of BDNF and its receptor, tropomyosin receptor kinase B (TrkB), has been reported in tumor cells of several human cancers (9, 13, 14). Intriguingly, overexpression of TrkB and BDNF are often associated with aggressive phenotype and poor prognosis of the disease (13), implying the oncogenic characteristics of BDNF/TrkB signaling cascades. Indeed, it has been shown that BDNF/TrkB could promote cell proliferation and survival, and induces metastasis by suppressing anoikis in various cell types (8, 14, 15).

Several lines of evidence indicate that BDNF may play a role in angiogenesis. BDNF deficiency leads to an abnormal cardiac vessel system in knockout mice, while overexpression of BDNF in mouse gestational hearts increases capillary density (3).
BDNF also promotes angiogenic behaviors of rat brain endothelial cells (ECs) *in vitro* (16). Furthermore, BDNF is capable of recruiting TrkB⁺ ECs and bone marrow-derived hematopoietic progenitor cells in an ischemic mouse model (17). These findings support BDNF as a potential player in angiogenesis. However, its detailed roles in angiogenesis and tumor development remain unclear and warrant further studies.

It is known that microenvironment plays critical roles in tumorigenesis (18). Tumor microenvironment composing extracellular matrix, ECs and stromal cells, interacts with tumor cells by secreting soluble factors and by providing cell-cell and cell-matrix interactions (19, 20). Here, we hypothesize that overexpressing BDNF in ECs may promote angiogenesis and provide a favorable microenvironment for tumor growth. To validate this hypothesis, we performed *in vitro* and *in vivo* assays after modulating BDNF levels in ECs. In addition, human hepatocellular carcinoma (HCC) tissues were examined for BDNF/TrkB expression and their implication in hepatocarcinogenesis was evaluated.
Materials and Methods

Cell culture and reagents

Two mouse EC lines — MILE SVEN 1 (MS1) and SVEC4-10EE2 (SVEC4), and a transformed mouse liver cell line — BNL 1ME A.7R.1 (BNL) were purchased from American Type Culture Collection. Human umbilical vein endothelial cells (HUVECs) were obtained from Cascade Biologics. MS1 and BNL cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 100 units/ml penicillin and 100 μg/ml streptomycin. SVEC4 cells were cultured in DMEM with 10% horse serum. HUVECs were grown in Medium 200 supplemented with Low Serum Growth Supplement (Cascade Biologics). All cells were grown in a humidified 5% CO₂ atmosphere at 37°C.

Recombinant human BDNF and Trk receptor inhibitor, K252a, were obtained from Calbiochem. Warfarin was purchased from Sigma-Aldrich. Recombinant mouse Gas6 was obtained from R&D Systems.

Patients and sample collection

Tumor and corresponding non-tumorous tissues were collected from HCC patients undergone hepatectomy at Queen Mary Hospital, Hong Kong between 2003 and 2008. Fifty cases were randomly recruited in the current study. All patients had been
diagnosed with primary HCC and none of them had received treatment before surgery.

Pathological diagnosis was based on the histological examination of tumor specimens by experienced pathologists. For the total of 50 patients, 34 had cirrhotic livers and 16 had non-cirrhotic livers (13 chronic hepatitis and 3 non-cirrhotic). Six normal livers (from liver donors; 2 males and 4 females; aged 50-62) were included as controls. All tissues were obtained from consenting patients and approved by the Institutional Review Board of the University of Hong Kong. Tissues were immediately snap-frozen in liquid nitrogen after surgical resection and stored at -80°C prior to analysis. Parallel sections were formalin-fixed and paraffin-embedded for histological and immunohistochemical studies. The clinical data for the patients are summarized in Supplementary Table S1.

**Reverse transcription-PCR and quantitative PCR**

Total RNA was extracted from cells using RNeasy Mini Kit (QIAGEN) according to the user manual. Trizol Reagent was used to extract total RNA from mouse tissues following the manufacturer’s instructions (Invitrogen). cDNA was synthesized from total RNA using ImProm-II Reverse Transcription System (Promega). The primer sequences and PCR conditions are summarized in Supplementary Table S2.

Quantitative PCR (qPCR) was performed on a 7900HT Fast Real-Time PCR System
(Applied Biosystems) using SYBR Green PCR reagents. Relative quantification was performed using ΔΔCt method, normalizing to eukaryotic 18S rRNA. Dissociation curves were generated to evaluate PCR product specificity and purity.

**Construct preparation and stable transfection**

Total RNA was purified from adult BALB/c mouse brain, and cDNA was synthesized as described above. After reverse transcription using oligo(dT) primers, cDNA template was amplified by PCR using primers (Supplementary Table S2) designed to flank BDNF coding sequence (CDS; GenBank no.: NM_001048139). PCR products were cloned into pGEM®-T Easy Vector (Promega), and then sub-cloned into pcDNA3.1/ Hygro(+) vector (Invitrogen) at Not I site. The constructs were verified by restriction enzyme digestion and sequencing using T7 primer (5’ GTAATACGACTCACTATAGGGC 3’). BDNF construct (pCDNA3.1(+-)BDNF) was transfected into MS1 cells using FuGENE 6 Transfection Reagent (Roche) following the manufacturer’s protocol. Clones were selected in medium further supplemented with 400 μg/ml Hygromycin B (Invitrogen) and verified by measuring BDNF at mRNA and protein levels, using RT-PCR, Western blot and ELISA, respectively.

Preparation of short hairpin RNA (shRNA) constructs targeting against BDNF was described in Supplementary Materials and Methods. Transfections of empty vector
(pGE-1), negative control vector (pGE1-N; containing a scrambled shRNA sequence), and 2 shRNA constructs (pGE-1-shBDNF-1 and pGE-1-shBDNF-2) into SVEC4 were performed using the protocol described above. Clones were selected and maintained in culture medium with 400 μg/ml G418 (Invitrogen). The blocking efficacy of the shRNA clones was measured by Western blot.

**Western blot analysis**

Western blot analysis was performed as previously reported (21). The following primary antibodies were used: BDNF (1:250, N-20, Santa Cruz Biotechnology), TrkB (1:200, 794, Santa Cruz Biotechnology), mouse TrkB (1:200, 47/TrkB, BD Biosciences), VEGF (1:250, catalog #AF564, R&D Systems), Caspase-3 (1:1,000, 8G10, Cell Signaling Technology), Caspase-9 (1:1,000, C9, Cell Signaling Technology), and β-actin (1:100,000, AC-15, Sigma-Aldrich). Respective secondary antibodies conjugated with peroxidase were from DAKO (1:5,000).

**Enzyme-Linked Immunosorbent Assay (ELISA)**

Supernatant was collected from subconfluent culture of each cell line after growing in serum containing medium for 2-3 days. Media were centrifuged to remove any detached cells. BDNF concentration was quantified by using BDNF Emax®
ImmunoAssay System ELISA kit (Promega) following the manufacturer’s instructions. The samples were loaded in triplicate. The absorbance at 450 nm was recorded with a BioRad microplate reader (Model 680).

**Migration and invasion assays**

Cells (1x10^5) were serum-starved for 6 hr before seeding in 24-well migration Transwells (8.0-μm pore size polycarbonate filters, Corning) in 100 μl DMEM. DMEM/10% FBS was added to the bottom chamber of each well to serve as chemoattractants. After 12 hr incubation, the nonmotile cells at the top of the filter were removed and the motile cells at the bottom of the filter were fixed with ice-cold absolute methanol. The migrating cells were visualized by standard H&E staining and quantified by counting at least 6 fields randomly under an inverted microscope (Olympus CK40, Olympus Optical) at 200x magnification. Images were acquired with a Nikon COOLPIX E950 digital camera (Nikon) connecting to the microscope. Invasion assay was carried out by employing similar procedures as described above, except using BD BioCoat™ Matrigel™ Invasion Chambers (BD Biosciences). The chambers were transferred to the wells containing 0.75 ml DMEM/10% FBS, and cells were seeded at 1x10^5 cells/24-well chamber in 0.5 ml DMEM/1% FBS. Invasion was scored after 24 hr. Each experiment was repeated for at least three times.
**Rho activation assay**

The relative RhoA activity in MS1 and its BDNF transfectants was measured using Rho activation assay Biochem Kit (Cytoskeleton Inc) according to the manufacturer's recommendations. Cells were cultured in DMEM/10% FBS for 48 hr before growing in medium with low serum content (1% FBS) for 24 hr. Cell lysates were prepared and equal amounts (600 μg) of cell lysate were incubated with rhotekin-RBD beads. Bound RhoA proteins were detected by Western blot using a RhoA-specific antibody.

**Tumorigenicity assay: co-transplantation of transformed liver cells and ECs**

Male BALB/c nude mice of 4-8-week-old were obtained from Laboratory Animal Unit of the University of Hong Kong. One million BNL (transformed mouse liver) cells were injected with 1x10^5 ECs (MS1, MS1/EV 1, MS1/BDNF C3 or C4 clone) subcutaneously into the right flank of nude mice. One million BNL cells were injected into the left flank of the same animal for comparison. A minimum of eight mice were injected for each group. Meanwhile, at least eight sites were injected with ECs (1x10^5) alone to serve as an additional control. The dimensions of developing tumors were measured with sliding calipers weekly. Tumor volumes were calculated in cubic millimeters using the formula: L x W x H. The kinetics of tumor formation was
compared among these co-injection combinations. Mice were sacrificed 11 weeks after injection and tumors were excised. Half of each tumor was frozen and the other half was fixed in formalin for subsequent histological and immunohistochemical analyses. For the co-transplantation experiment using SVEC4/shRNA clones, the described protocol was applied. An empty vector clone (SVEC4/EV 1) and a negative control vector clone (SVEC4/NEG 1) were included as controls.

**Immunohistochemistry**

Immunohistochemistry was performed as previously described (21) using either anti-human BDNF (1:15, catalog #ab80124, Abcam) or anti-TrkB antibody (1:50, H-181, Santa Cruz Biotechnology).

Quantification of microvessel density (MVD) in mouse tumor xenografts was performed by using Blood Vessel Staining Kit (ECM590, Millipore) following the manufacturer’s protocol. The entire tumor section was scanned at 100x magnification to search for vascular hot spots (22, 23). MVD of tumors was quantified by viewing at least six hot spots of each section under the microscope at 200x magnification. Values were calculated from sections of at least three different tumors.

**Statistics**
SPSS version 14.0 and GraphPad Prism 4.0 were used for statistical analyses. One-way ANOVA, unpaired two-tailed Student’s $t$-test, Mann Whitney test and chi-squared test were used whenever applicable. Kaplan-Meier method was applied to calculate overall survival rates and log-rank test was used to assess the significance of the differences. $P$ value smaller than 0.05 was considered to be significant and is denoted in the figures with an asterisk(s).
Results

Overexpression of BDNF in ECs increased cell proliferation and protected them from apoptosis under serum starvation condition

To investigate the roles of BDNF in tumor angiogenesis, BDNF was overexpressed in MS1, a mouse EC line with undetectable BDNF level (Supplementary Fig. S1A). Three BDNF-overexpressing stable clones were obtained for further functional characterization (Fig. 1). The MTT assay showed that all MS1/BDNF clones exhibited significantly higher proliferation rates than the parental and empty vector controls (Supplementary Fig. S2A). Annexin V-binding assay demonstrated that up-regulation of BDNF could rescue the cells from serum-starvation induced apoptosis (Supplementary Fig. S2B). The most prominent effect was observed in MS1/BDNF C4 clone displaying down-regulation of active caspase-9 and caspase-3 (Supplementary Fig. S2C).

Up-regulation of BDNF enhanced migration and invasion abilities of ECs through inhibition of RhoA activation

To assess the effect of overexpressing BDNF on MS1 cells, cell motility of the three BDNF clones were compared with the parental and empty vector controls by migration assay (Fig. 2A). The number of migrated cells of all clones was increased
10.6- to 66.7-fold ($P<0.001$). Similarly, their invasive capacity was strikingly enhanced 14.9- to 52.7-fold ($P<0.05$ or 0.001) when compared with controls.

Rho has been indicated to be a pivotal protein for the regulation of actin cytoskeleton during cell movement (24). To explore the underlying molecular mechanisms by which BDNF regulates migration and invasion processes, the activity of Rho in MS1/BDNF clones was measured by Rho activation assay. Under low serum condition, levels of GTP-bound Rho in BDNF-overexpressing clones were suppressed when compared with the parental and empty vector controls (Fig. 2B). The results suggested that BDNF could promote EC migration and invasion through inhibition of RhoA activation.

We further extended the migration study to TrkB-expressing human ECs, HUVECs. Conditioned media from two clones expressing the highest level of secretory BDNF (MS1/BDNF clone C3 and C4; Fig. 1B) were used as chemoattractants for HUVECs (Fig. 2C). A significant enhancement (over 5-fold) of HUVEC migration was noted when compared with controls ($P<0.001$; Fig. 2D), demonstrating cross-species reactivity of BDNF. Notably, this enhancement was completely abrogated by the Trk receptor blocker, K252a, indicating that BDNF increased EC migration by acting
through TrkB receptor.

**Enhanced BDNF expression facilitated tumor growth, whereas knock-down of BDNF abrogated tumor-promoting activity**

To investigate the effect of elevated BDNF expression in MS1 cells on tumorigenesis, two clones showing the highest secretory BDNF levels and migration capacities (MS1/BDNF C3 and C4) were co-injected with a chemically transformed mouse liver cell line, BNL. Parental MS1 cells and empty vector clone were also co-injected with BNL cells to serve as controls. We found that BDNF transfectants could significantly promote tumor growth when compared with the controls ($P<0.05$ or $P<0.01$; Fig. 3A,i). At the end point, MVD in each tumor was quantified. Tumor xenografts derived from co-injection of BNL with MS1/BDNF clone C3 or C4 showed a significant increase (6.8- to 9.8-fold) in microvessels compared with tumors from BNL or those from co-injection with MS1 or empty vector clone ($P<0.001$; Fig. 3A,ii).

On the other hand, SVEC4, a mouse tumor-derived EC line (25, 26) which expresses a high level of BDNF protein (Supplementary Fig. S1A) was employed for the co-transplantation experiment. Three SVEC4/shRNA clones showing the most
effective suppression of BDNF (Supplementary Fig. S4) were co-injected with BNL cells. SVEC4 control vector clones (SVEC4/EV 1 and SVEC4/NEG 1) demonstrated a strong tumor-promoting ability upon co-injection with BNL cells (Fig. 3B,i). However, knock-down of BDNF in the shRNA clones could significantly abrogate such tumor-promoting effect ($P<0.05$), accompanied by a remarkable reduction in the number of microvessels in tumor xenografts (decreased 103- to 149-fold; $P<0.01$ or 0.001; Fig. 3B,ii).

**High BDNF-expressing ECs induced TrkB and vascular endothelial growth factor (VEGF) expression in BNL cells after in vivo co-transplantation and in vitro co-culture**

To understand the molecular mechanism underlying the interaction between BDNF-expressing ECs (MS1/BDNF C3 and C4) and BNL, TrkB and VEGF protein levels in tumor xenografts from the co-transplantation experiments were measured by Western blot. Both levels were enhanced in tumors derived from co-injection of BNL with MS1/BDNF clones (Fig. 4A). An *in vitro* co-culture study supported the finding by showing up-regulation of TrkB (increased by over 150%, $P<0.05$) and VEGF (elevated by over 110%, $P<0.05$) in BNL cells after co-culture with these ECs (Fig. 4B,ii). The results indicated that high BDNF-expressing ECs could stimulate BNL
cells by up-regulation of TrkB and elicit an angiogenic response via inducing VEGF expression.

Implication of growth arrest specific 6 (Gas6) gene in BDNF-elicited angiogenic responses

To explore the molecular pathways associated with BDNF-induced angiogenesis, microarray analysis (described in Supplementary Materials and Methods) was carried out to compare the gene expression profiles between MS1/BDNF clone C4 and the empty vector clone (MS1/EV 1). Around 1% (345 genes) of 34,000 genes covered by the Affymetrix Genechip exhibited >2-fold change upon overexpression of BDNF in MS1 cells. The genes were partially listed in Supplementary Table S3 and S4.

Based on the fold change magnitude and their known biological functions, twelve potential angiogenic genes were selected. qPCR was performed to validate microarray data (Fig. 5A). A high consistency between the data sets from microarray and qPCR was observed (Supplementary Table S5). Remarkably, we detected universal expression patterns of these genes in a panel of five BDNF transfectants by qPCR (data not shown).
The microarray data implied that Gas6 might have a role in modulating BDNF-elicited angiogenic responses. We investigated the effect of warfarin, a Gas6 inhibitor, on cell motility of the high BDNF-expressing ECs. Our result showed that warfarin could effectively suppress their motility by 30.3-fold ($P<0.001$; Fig. 5B), demonstrating the pivotal role of Gas6 in regulating BDNF-induced cell migration. Then, we examined the potential linkage between Gas6 and apoptosis. Treating the parental MS1 cells and empty vector transfectant with recombinant Gas6 protein did not protect cells from serum-starvation induced apoptosis, and blocking Gas6 by warfarin in the MS1/BDNF clone did not induce apoptosis (Fig. 5C). The results indicated that Gas6 did not account for the anti-apoptotic effect of BDNF on MS1 cells.

Clinical significance of TrkB expression in human HCC

To investigate the potential implication of BDNF/TrkB in hepatocarcinogenesis, their protein expression levels in human HCC tissues were detected by Western blot (Fig. 6A). BDNF was elevated in 46.0% (23/50) and TrkB was up-regulated in 33.3% (15/45) of HCC tissues when compared with the adjacent non-tumorous liver tissues and normal liver controls. Immunohistochemical staining showed strong BDNF reactivity in both tumor and ECs of HCC tissues, while TrkB expression was only
detected in tumor cells (Fig. 6B). Association analysis of BDNF level with TrkB expression showed that concurrent up-regulation of both proteins in HCC is infrequent [6.7% (3/45); $P<0.05$]. Kaplan-Meier survival analysis revealed that patients with tumors exhibiting high TrkB expression had significantly shorter overall survival ($P=0.0268$; Fig. 6C).
Discussion

Accumulating evidence suggests that neurotrophins including BDNF may serve as angiogenic factors (5, 27) and may play a pathological role in tumorigenesis (8). The implication of BDNF in tumor angiogenesis, however, remains obscure. This study attempts to identify the roles of BDNF in angiogenesis and tumor development. To examine the effects of BDNF/TrkB system on EC functions, we generated stable BDNF-overexpressing clones in the mouse EC line, MS1. The clones displayed enhanced cell proliferation under low serum condition, indicating that the ECs became less growth factor-dependent upon up-regulation of BDNF. Regarding cell survival, we reported that BDNF up-regulation was able to protect the ECs from serum-starvation induced apoptosis, which correlated with the decrease in caspase-9 and caspase-3 activation. The results suggested that BDNF exerted its anti-apoptotic effect on MS1 cells by regulating the levels of the active caspases.

Next, we showed significant increases in cell motility and invasiveness of MS1 cells upon overexpression of BDNF. Provided that Rho regulates signaling pathways linked to cytoskeletal remodeling (24) and is a critical player in cell migration and invasion (28, 29), its activity in the high BDNF-expressing clones was measured. Consistent with previous reports, we found that activated RhoA levels were
consistently reduced in all clones showing enhanced cell motility. In fact, inhibition of Rho activation has been reported to induce migration in several cell types, such as mesenchymal stromal cells (30), murine hematopoietic progenitor cells (31) and mouse embryonic fibroblasts (32). Though the detailed signal transduction pathway is not fully understood, it has been suggested that by regulating cytoskeletal rearrangement, Rho inhibition can promote migration (30). Additionally, we showed that conditioned media of high BDNF-expressing clones could substantially increase migration of HUVECs, and the effect was abrogated by the TrkB blocker. Taken together, we suggested that BDNF, by acting through TrkB receptor, could promote cell migration/invasion of MS1 ECs by inhibition of Rho activation.

We then provided functional evidence for the role of BDNF in tumorigenesis by using a co-transplantation model in athymic nude mice. MS1/BDNF transfectants were co-injected with BNL, the transformed mouse liver cell line which demonstrated low tumorigenic potential over a 3-month period. We found that BDNF-overexpressing ECs could significantly enhance tumor growth of BNL cells upon co-injection, accompanied by a prominent increase in the number of microvessels in the corresponding tumors. On the other hand, when BDNF expression in SVEC4, the tumor-derived EC line, was stably knocked-down by shRNAs, its tumor-promoting
effect was abrogated. Interestingly, we observed a significant reduction in microvessel density in the tumor xenografts obtained. The source of the ECs that form microvessels in tumors, however, remains uncertain. The injected ECs were likely to participate in vessel formation, though we cannot preclude the possibility that vascular ECs were recruited from the host animals. Yet, these findings provide compelling evidence that high expression of BDNF in ECs could promote tumor growth in vivo through enhancing neovascularization in tumors.

To further understand the molecular mechanisms through which BDNF regulates tumorigenesis, the expression levels of TrkB and VEGF in BNL cells were determined in both in vivo and in vitro settings. Their levels in BNL cells were elevated after co-injecting with the high BDNF-expressing ECs in vivo or after co-culture with these ECs in vitro. Collectively, these findings suggested that high BDNF-expressing ECs could stimulate BNL cells by up-regulation of TrkB and induced neovascularization via VEGF pathways, which eventually facilitated tumor growth.

Verification of the microarray data by qPCR disclosed similar expression patterns of twelve angiogenic genes in a panel of five MS1/BDNF clones. The finding reflected a
universal regulation of these genes by BDNF in the studied clones, and thus suggested their linkage with BDNF-induced angiogenesis. Among these genes, Gas6 exhibited the most dramatic up-regulation in MS1/BDNF clones. It is a vitamin K-dependent growth factor (33) primarily acting through Axl receptor (34) and is involved in angiogenesis and tumorigenesis (35). We linked Gas6 to BDNF-induced angiogenesis by showing that the migration-promoting effect of BDNF on the MS1/BDNF clone could be abolished by the Gas6 inhibitor. On the other hand, Gas6 showed no role in regulating the anti-apoptotic effect of BDNF.

As a follow-up to previous studies on examining expression of BDNF and TrkB in human malignancies (9, 13, 14), we performed a detailed BDNF and TrkB expression study in human HCC by Western blot and immunohistochemistry. We showed up-regulation of BDNF and TrkB in HCC tissues. Association analysis revealed that concurrent up-regulation of both BDNF and TrkB is infrequent, implying that elevation of either one of these proteins may be sufficient to promote hepatocarcinogenesis. Interestingly, immunohistochemical analysis disclosed the presence of high BDNF-expressing ECs in HCC, suggesting that tumor microenvironment comprising ECs may represent an important source of BDNF and contribute to tumorigenesis. From survival analysis, we noted that patients with high
TrkB levels had poor overall survival, unveiling the clinical significance of BDNF/TrkB pathway in HCC.

Based on the current findings, we suggested two mechanisms by which BDNF-expressing ECs could promote angiogenesis and hence facilitate tumor growth. First, by acting via TrkB receptors on ECs in an autocrine manner, BDNF induced angiogenesis through regulation of the molecules including RhoA, caspase-3, caspase-9 and Gas6. Second, BDNF from ECs could stimulate tumor cells by up-regulation of TrkB and may elicit angiogenic responses through VEGF pathways.

In conclusion, we demonstrated that BDNF was a potent angiogenic factor which facilitated tumor growth via promoting angiogenesis. To our knowledge, we are the first group to report the association of TrkB up-regulation in HCC with overall survival, suggesting a critical role of BDNF/TrkB pathway in HCC. We also showed overexpression of BDNF by ECs in HCC, implicating a role for the tumor microenvironment in hepatocarcinogenesis. Future studies are warranted to evaluate the BDNF/TrkB pathway as a potential target for antiangiogenic therapy in HCC.
Figure legends

Fig. 1. Detection of BDNF expression in MS1 cell line and its BDNF transfectants. A, increased cytosolic BDNF protein levels in indicated clones by Western blot. *, clones showed elevated BDNF levels. EV, empty vector. B, increased secretory BDNF levels in MS1/BDNF clones as detected by ELISA. Columns, mean values of two experiments, each performed in duplicate; bars, SEM.

Fig. 2. BDNF enhanced migration/invasion ability of ECs, which was blocked by TrkB inhibitor K252a. A, MS1/BDNF clones showed significant enhancement of cell migration and invasion. (Left) Images of indicated clones after 12-hr migration/24-hr invasion (magnification ×200). As a positive control, 10 ng/ml VEGF164 (from Sigma-Aldrich) was added to the bottom chamber of MS1 cells. (Right) Quantification of the assays (n=4). B, inhibition of RhoA activation in MS1/BDNF clones. Cells were cultured under low serum condition (1% FBS) for 24 hr prior to the assay. Equal amounts (600 μg) of cell lysate were incubated with rhotekin-RBD beads and bound RhoA proteins were detected by Western blot using a RhoA-specific antibody (top panel). The amount of total RhoA and actin in cell lysates are shown in the lower panels. Blots are representative of four experiments. C, experimental outline of HUVEC migration assay. TrkB-expressing...
HUVECs were starved overnight and seeded on the top chambers of 24-well migration Transwells in low serum medium (0.5% FBS). Conditioned media collected from 2-day culture of indicated cells were added to the bottom chambers in the presence or absence of 200 nM K252a (TrkB blocker). Cells were allowed to migrate for 6 hr. D, migration of HUVECs was induced by the conditioned media from the high BDNF-expressing clones (MS1/BDNF C3 and C4), but the increase was completely abrogated by K252a (left). Result summary of 3 independent experiments (right). Values are means ± SEM. *P<0.05; **P<0.01; ***P<0.001 versus parental (MS1) cells and empty vector control (MS1/EV 1); #P<0.001 compared between the groups with or without K252a treatment.

Fig. 3. BDNF expression conferred ECs tumor-promoting ability. A, tumorigenicity study for MS1 and its derivative clones. A,i, high BDNF-expressing ECs (MS1/BDNF C3 and C4) promoted tumor growth of BNL cells in nude mice. Tumor volumes are reported as means ± SEM (n=8). *P<0.05; **P<0.01 versus parental cells and empty vector control. A,ii, analysis of microvessel density (MVD) in tumor xenografts derived from indicated co-injection of BNL and MS1/BDNF clones. Representative images were shown (top; magnification ×200). –ve control, PBS substitution for the primary antibody; arrows, microvessels; bar, 50 μm. (Bottom) Quantitative analysis of vWF staining. ***P<0.001 versus controls. B, tumorigenicity study for SVEC4 and its shRNA clones. B,i, SVEC4
transfecting with negative control vector (SVEC4/NEG 1) or empty vector (SVEC4/EV 1) promoted tumor growth after co-injecting with BNL cells, but knock-down of BDNF in SVEC4 cells by shRNAs could abrogate such tumor-promoting effect. * \( P < 0.05 \) for BNL co-injecting with SVEC4/NEG 1 versus those co-injecting with shRNA clones. B, ii, MVD analysis for tumor xenografts derived from combined injection of BNL and indicated SVEC4 clones. Representative images (top; magnification \( \times 200 \)) and quantitative analysis are shown (bottom). ** \( P < 0.01 \); *** \( P < 0.001 \) versus negative and empty vector controls. Counts from six fields per section and a minimum of three tumors from each group were included for statistical analysis.

Fig. 4. Induction of TrkB and VEGF expression by high BDNF-expressing ECs in both in vivo and in vitro settings. A, protein levels of TrkB and VEGF were elevated in six representative tumor xenografts derived from co-injection of BNL with indicated MS1/BDNF clones when compared with that from co-injection with empty vector control. B, i, schematic illustration of the in vitro co-culture experiment. BNL \( (3 \times 10^5) \) cells were seeded onto a 6-well plate and serum-starved overnight. MS1/EV 1, MS1/BDNF C3 or C4 \( (1.5 \times 10^5) \) cells were seeded on the top chambers of Transwells \( (0.4 \mu m \text{ pore size}) \). Cells were co-cultured in low serum medium \( (0.5\% \text{ FBS}) \) for 8-24 hr. Expression levels of TrkB and VEGF in BNL cells were measured by Western blot.
B, ii, TrkB and VEGF protein levels in BNL cells were elevated after co-culture with the high BDNF-expressing clones (MS1/BDNF C3 and C4). The blots are representative of three independent experiments. (Right) Quantification of blots; *P<0.05 versus empty vector control.

Fig. 5. Gas6 modulated migration process initiated by BDNF. A, validation of microarray data by qPCR. Fold changes of twelve potential angiogenic genes in MS1/BDNF C4 clone using MS1/EV 1 as reference were shown. Detailed calculation formula is provided in Supplementary Materials and Methods. B, warfarin inhibited migration of the MS1/BDNF clone. ECs were seeded on the top chambers of Transwells in serum free medium, DMEM/10% FBS was added to the bottom chambers in the presence (+) or absence (-) of 10 μM of warfarin. (Top) Representative images after 24-hr migration. (Bottom) Quantification of the assay. Values are means ± SEM; n=3. ***P<0.001. C, Gas6 treatment of MS1 ECs did not reduce apoptosis, and blocking of Gas6 by warfarin in MS1/BDNF C4 clone did not induce apoptosis.

Fig. 6. Overexpression of BDNF and TrkB in human HCC tissues. A, by Western blot, BDNF protein level was up-regulated in 46.0% (23/50) of HCC tissues (T) when
compared with adjacent non-tumorous liver tissues (NT) and normal liver tissues. TrkB level was elevated in 33.3% (15/45) of HCC tissues. B, immunohistochemical staining of BDNF and TrkB in human HCC tissues. Representative images from selected cases are shown. (Upper panel) Both tumor (short arrows) and endothelial cells (long arrow) showed BDNF immunoreactivity. (Lower panel) TrkB was stained positively on both plasma membrane (solid arrows) and cytoplasm (open arrows) of tumor cells. Magnification ×200; inset, ×400; bar, 50 μm. C, Kaplan–Meier overall survival plot according to TrkB expression levels. Patients with more than 2-fold increase in TrkB expression in tumor compared to non-tumorous tissue were classified into the high expression group. High TrkB expression was significantly associated with poor survival of HCC patients (log-rank test, $P=0.0268$).
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Chi-Tat Lam, Zhen Fan Yang, Chi-Keung Lau, et al.

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