Gene silencing of the BDNF/TrkB axis in multiple myeloma blocks bone destruction and tumor burden in vitro and in vivo

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Osteolytic bone diseases are a prominent feature of multiple myeloma (MM), resulting from aberrant osteoclastic bone resorption that is uncoupled from osteoblastic bone formation. Myeloma stimulates osteoclastogenesis, which is largely dependent on an increase in receptor activator of NF-κB ligand (RANKL) and a decrease in osteoprotegerin (OPG) within the bone marrow milieu. Recently, brain-derived neurotrophic factor (BDNF) was identified as a MM-derived factor that correlates with increased RANKL levels and contributes to osteolytic bone destruction in myeloma patients. Because tyrosine receptor kinase B (TrkB), the receptor of BDNF, is abundantly expressed in osteoblasts, we sought to evaluate the role of BDNF/TrkB in myeloma–osteoblast interactions and the effect of this pathway on the RANKL/OPG ratio and osteoclastogenesis. Coculture systems constructed with noncontact transwells revealed that, in vitro, MM-derived BDNF increased RANKL and decreased OPG production in osteoblasts in a time- and dose-dependent manner. These effects were completely abolished by a specific small interfering RNA for TrkB. BDNF regulates RANKL/OPG expression in osteoblasts through the TrkB/ERK pathway. To investigate the biological effects of BDNF on myeloma in vivo, a SCID-RPMI8226 mice model was constructed using lentiviral short hairpin RNA-transfected RPMI8226 cells. In this system, stable knockdown of BDNF in MM cells significantly restored the RANKL/OPG homostasis, inhibited osteolytic bone destruction and reduced angiogenesis and tumor burden. Our studies provide further support for the potential osteoclastogenic effects of BDNF, which mediates stroma–myeloma interactions to disrupt the balance of RANKL/OPG expression, ultimately increasing osteoclastogenesis in MM.

Key words: multiple myeloma bone disease, brain-derived neurotrophic factor, osteoclastogenesis, receptor activator of nuclear factor κappa B ligand, osteoprotegerin

Abbreviations: BDNF: brain-derived neurotrophic factor; ELISA: enzyme-linked immunosorbent assay; MM: multiple myeloma; OPG: osteoprotegerin; RANKL: receptor activator of nuclear factor-κB ligand; RT-PCR: real-time polymerase chain reaction; TrkB: tyrosine receptor kinase B

Additional Supporting Information may be found in the online version of this article.

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Multiple myeloma (MM) is a B-cell neoplasm characterized by clonal expansion of plasma cells within the bone marrow (BM). Bone destruction is the most common feature of MM, which results from the deregulation of osteoclast and osteoblast activities adjacent to myeloma cells.1 Signs of skeleton involvement in MM include osteolytic bone lesions, spine compression and fractures, which significantly impact morbidity and mortality. A variety of MM-derived factors that stimulate osteoclast formation and activity, either directly or indirectly, has been identified. These osteoclast-activating factors (OAFs) include interleukin-1β (IL-1β), interleukin-11 (IL-11), tumor necrosis factor-1β (TNF-1β), hepatocyte growth factor (HGF), stromal-derived factor-1α (SDF-1α), RANKL and the more recently identified macrophage inflammatory protein-1α (MIP-1α).2-6 Among all these factors, RANKL plays a predominate role.7 Osteoclasts are activated by binding of RANKL to its cognate receptor, RANK, whereas osteoprotegerin (OPG), a soluble member of the tumor necrosis factor receptor family, acts as a nonfunctional decoy receptor to compete with RANK and inhibits the terminal stages of osteoclast differentiation and activation.8 Mice deficient in OPG showed severe osteoporosis,9,10 whereas mice overexpressing OPG resulted in osteopetrosis.8 Most recently, the RANKL/OPG ratio was identified as a novel prognostic index for myeloma bone destruction before
Brain-derived neurotrophic factor (BDNF) is an essential neurotrophic factor that participates in neuronal modulation, transmissions and plasticity. In recent years, numerous studies have revealed the diverse biological effects of BDNF in the development of various tumors, such as neuroblastoma, prostate cancer, ovarian cancers, breast cancer and MM. Tyrosine receptor kinase B (TrkB), the high-affinity receptor of BDNF, was even recently proposed as a drug target for anticancer therapy. The critical roles of the BDNF/TrkB axis in MM were manifested by their high expression in plasma cells and in most HMCLs, as well as its potential effects on MM migration and survival in vitro. BDNF has also been described as a potential angiogenic factor because it significantly increased VEGF production and microvessel density in a murine model of human myeloma disease. More importantly, recent studies have revealed the close association of BM BDNF levels, BM RANKL levels and bone destruction severity in MM patients. We also found that MM-derived BDNF increased RANKL secretion by BM stromal cells to promote osteoclast formation in vitro. However, there is no established mechanism linking the role of BDNF in osteoblast differentiation or the effects of BDNF on OPG expression and RANKL/OPG ratio in the BM microenvironment.

Elucidation of the interactions between MM cells and BM milieu would benefit the development of effective therapies to improve the outcome of MM patients. Osteoblasts are important sources of RANKL and OPG in BM milieu, and TrkB, the high-affinity receptor of BDNF, is abundantly expressed by osteoblasts. Therefore, in our study, we sought to investigate the involvement of BDNF in the cross-talk between MM cells and osteoblasts, as well as the molecular mechanism of BDNF-induced deregulation of RANKL/OPG ratio in vitro. In addition, endogenous expression of BDNF in RPMI8226 cells was knocked down by short hairpin RNA (shRNA) to investigate whether gene silencing of BDNF blocks osteoclastogenesis, angiogenesis and tumorigenesis in SCID-RPMI8226 mice model.

### Material and Methods

#### Cell lines and reagents

The human MM cell lines (HMCLs) RPMI8226, KM3 and U266 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) where they were characterized by DNA Fingerprinting, mycoplasma detection, isozyme detection and cell vitality detection. These cell lines were immediately expanded and frozen such that they could be restarted every 3 months from a frozen vial of the same batch of cells. Human recombinant BDNF (PeproTech, Princeton, NJ), the TrkB-specific inhibitor K252a (Calbiochem, San Diego, CA), a neutralizing antibody to human BDNF (Oncogene Research, San Diego, CA), a neutralizing antibody to human RANKL (Biolegend, San Diego, CA) and recombinant human macrophage colony-stimulating factor (M-CSF) (R&D Systems, Minneapolis, MN) were obtained and reconstituted according to the manufacturers’ specifications. Anti-human BDNF, RANKL and OPG, and anti-mouse CD34 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho ERK1/2, anti-ERK1/2, anti-phospho p38MAPK and anti-p38MAPK antibodies were purchased from Cell Signaling (Danvers, MA). Human BDNF, RANKL, OPG, VEGF, MIP-1α, IL-6 and human lambda immunoglobulin light chain (λ-IgLC) ELISA kits were obtained from R&D Systems (Minneapolis, MN). Osteogenic differentiation medium was purchased from Cyagen (Cyagen Biosciences, Guangzhou, China). A leukocyte acid phosphatase kit for tartrate-resistant acid phosphatase (TRAP) staining was purchased from SIGMA (St. Louis, MO). U0126 and SB203580 were purchased from Promega (Southampton, Hants, UK). Transwell inserts with 0.4-μm pores were obtained from Costar (Corning, NY). Dentine slices were prepared as previously described.

#### Primary cell culture and coculture systems

Human BM mesenchymal stromal cells (MSCs) were prepared as described in our previous studies. MSCs (2 × 10^3 cells per milliliter) in passages 3–5 were incubated in osteogenic differentiation medium in the absence or presence of BDNF to obtain osteoblasts. Identification of human osteoblasts by alizarin red S and alkaline phosphatase staining is
shown in Supporting Information Figure S1A. The effects of BDNF on osteoblast differentiation are shown in Supporting Information Figures S1B and S1C. Peripheral blood mononuclear clear cells from MM patients were cultured at 2.5 × 10⁶ cells per milliliter in α-MEM supplemented with 10% FBS, antibiotics and M-CSF (25 ng/ml). After 12–24 hr, the cultures were washed gently with fresh medium to detach and remove nonadherent cells. The remaining adherent cells were considered osteoclast precursors (pre-OCs). Osteoblast-pre-OC coculture systems and MM-osteoblast coculture systems were constructed as shown in Supporting Information Figures S2A–S2AC. RANKL expression in osteoblasts of these coculture systems was detected by PCR in Supporting Information Figure S2D. Additional details are shown in the Supporting Information Methods. All experiments involving human participants were approved by the ethics committee of Union Hospital, Huazhong University of Science and Technology, and written informed consent was obtained from all participants.

**RNA interference on expression of TrkB in osteoblasts**

Small interfering RNA (siRNA) was chemically synthesized by Genechem (Shanghai, China). The sequence of the sense strand of human TrkB siRNA was GAAUUGACGAUGGUGCAAATTT as previously described by Fujikawa et al.²⁷ Osteoblasts (80–90% confluence) were transfected with TrkB siRNA or the scrambled control-siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 36 hr of culture, knockdown efficiency was measured by Western blot analysis (Supporting Information Fig. S3A).

**Stable transfection of BDNF shRNA in RPMI8226 cells**

To directly determine the biological effects of BDNF on MM, an antisense construct to BDNF (shRNA) was designed as previously described.²⁷ The sequences of the two strands were 5′-CCGGCGGCGGATTGGAACCTCCCAGTGTTC AAGA CGCAGTGGGAAGTTCCAATGCCTTTTTT-3′ and 5′-AAT TCAAAAAAG GCATGGGAACCTCCCAGTGCGTCTTGAAC ACTGCGGATGTTCAAATGCGG-3′. RPMI8226 cells were transfected with BDNF antisense shRNA (AS-RPMI8226) or empty-vector shRNA (EV-RPMI8226) by replication-incompetent lentiviral vectors. Stable cell lines were selected by puromycin over a period of 4 weeks. Downregulation of BDNF protein expression was confirmed by Western blot analysis (Supporting Information Fig. S3B).

**Real-time polymerase chain reaction**

Total RNA was extracted from osteoblasts with Trizol reagent (Invitrogen) and subjected to reverse transcription-polymerase chain reaction with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Then, SYBR Green RT-PCR Kit (TAKARA) was used to the amplified cDNA under the following typical cycling conditions: denaturation at 95°C for 1 min, annealing at 60°C for 30 sec and extension at 72°C for 30 sec. The samples were amplified for 38 cycles. GAPDH was used as an internal control. The following primers were used: for RANKL, forward primer 5′-CAA GAG GAC AGA CTC ACT TTA T-3′, reverse primer 5′-TAT CGT TGG ATC ACA GCA C-3′; for OPG, forward primer 5′-GAT CTG CAT GAT CTG TAT CTA CTG A-3′, reverse primer 5′-GTA AGG CAC AAT GGA GTC TA-3′ and for GAPDH, forward primer 5′-GAC ATC AAG AAG GTG GTG AA-3′, reverse primer 5′-TGT CAT ACC AGG AAA TGA GC-3′. The PCR products were mixed with bromophenol blue-loaded buffer, separated by electrophoresis and photographed.

**Western blotting**

Total cell lysates were separated on a 10% SDS-PAGE gel and transferred to a PVDF membrane. After blocking for 1 hr with 5% BSA in TBST, membranes were incubated with 1:1,000 diluted BDNF, RANKL and OPG antibodies, 1:500 diluted phospho-ERK1/2, ERK1/2, phospho-p38MAPK and p38MAPK antibodies and 1:2,000 diluted β-actin antibodies at 4°C overnight. After washing with TBST, membranes were incubated with the corresponding secondary antibody for 1 hr at room temperature, and proteins were detected using the ECL Plus detection system.

**SCID-RPMI8226 mice model and in vivo treatments**

Six-week-old female NOD/SCID mice were obtained from Beijing Hua Fukang Bioscience Company (Beijing, China) and irradiated with 250 rad using a Co60 source. Twenty-four hours after irradiation, AS-RPMI8226, EV-RPMI8226 and wild-type RPMI8226 (WT-RPMI8226) cells (2.5 × 10⁶ cells per mouse) in 50 μl of phosphate-buffered saline (PBS) were injected intravenously into the mice. Murine blood samples were collected weekly, and the sera were analyzed for human lambda immunoglobulin light chain levels as an indicator of tumor burden in SCID mice. Radiographs were taken weekly with an AXR Minishot-100 beryllium source instrument (Associated X-Ray Imaging Corp, Haverhill, MA). Changes in total-body bone mineral density (BMD) of mice skeleton were measured by a PIXImus dual-energy X-ray absorptiometry (DEXA) device (GE Medical Systems LUNAR, Madison, WI). At the end of the experiment, mice were deeply anesthetized with pentobarbital and euthanized by cervical dislocation. To determine BDNF, RANKL, OPG, VEGF, MIP-1α and IL-6 levels, tibial BM was obtained by flushing the bones repeatedly with 1 ml of PBS and analyzed by ELISA. For microscopic observation, mouse tibiae and vertebrae were fixed, decalcified and embedded for sectioning. Bone sections were stained with hematoxylin and eosin staining or TRAP staining. For immunohistochemistry analysis, sections were incubated with 1:200 diluted CD34 monoclonal antibodies and corresponding secondary antibodies. All animal experiments were carried out according to protocols approved by the Animal Ethics Committee of the Tongji Medical College, Huazhong University of Science and Technology (Permit Number: S229).
Statistical analysis

In vitro experiments for all assays were performed in triplicate, and the results are reported as the mean ± SEM. Statistical analysis of differences observed between treatment and respective control groups was performed using Student’s t-test. Statistical analysis of the differences between animal groups was estimated by a one-way ANOVA. 

*p < 0.05 was considered statistically significant.

Results

BDNF promotes osteoclast formation in a transwell coculture system of osteoblasts with pre-OCs

Osteoblasts were cocultured with pre-OCs in a noncontacted transwell system and treated with various conditions. As shown in Figure 1a, BDNF (25 ng/ml) significantly increased TRAP(+) multinucleated osteoclast formation compared to the control. Importantly, osteoclast formation in this coculture system was almost completely inhibited by RANKL-neutralizing antibody (anti-RANKL) (Figure 1a). BDNF also increased the number of resorption pits on dentine slices; this effect was reversed by K252a and nearly completely abolished by anti-RANKL, as shown in Figure 1b (*p < 0.05). The time course of osteoclast formation is shown in Figure 1c. These data reveal that BDNF increased osteoclast formation and may function directly through the RANKL pathway. Based on these results, there should be a relationship between BDNF and the secretion of RANKL in osteoblasts.

MM-derived BDNF increases RANKL expression and decreases OPG expression in osteoblasts

Osteoblasts were treated with various drug concentrations at several time points to investigate the direct effects of BDNF on RANKL/OPG production in osteoblasts. Figure 2a shows that BDNF promoted RANKL mRNA and inhibited OPG mRNA expression in osteoblasts in a dose- and time-dependent manner. Treatment with 1–100 ng/ml BDNF for 48 hr caused a significant increase in RANKL mRNA and decrease in OPG mRNA expression compared to controls (*p < 0.05).

**Figure 1.** Osteoclast formation and function in an osteoblast-pre-OC coculture system. Coculture systems were treated with basic medium alone, 25 ng/ml BDNF, 25 ng/ml BDNF + 50 nM K252a, 20 μg/ml anti-RANKL or 25 ng/ml BDNF + 20 μg/ml anti-RANKL. (a) TRAP staining of multinucleated osteoclasts (indicated with black arrows). BDNF promotes osteoclast formation in cocultures; however, neutralizing anti-RANKL antibody markedly abolished osteoclast formation at concentrations of 20 μg/ml (magnification ×100). (b) BDNF at concentrations of 25 ng/ml significantly stimulated osteoclast formation and function in osteoblast-pre-OC cocultures (*p < 0.05 vs. controls with basic medium alone). K252a and anti-RANKL antibody abrogated osteoclast formation and function stimulated with BDNF (#p < 0.05 vs. BDNF treatment). The data represent the number of osteoclasts (black bars) and resorption pits on dentine slices (gray bars). Similar results were observed in three independent experiments. Bar represents the mean ± SEM. (c) Time course of osteoclast formation in coculture of osteoblasts with pre-OCs. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
After treatment of 25 ng/ml BDNF for 24 hr, the RANKL mRNA levels increased and OPG mRNA decreased in osteoblasts. This tendency became more significant at 48 hr ($p < 0.05$). Consistent with these results, BDNF also promoted RANKL and inhibited OPG protein levels in culture medium as detected by ELISA, as shown in Figure 2b ($p < 0.05$). Then osteoblasts were transfected with TrkB-siRNA or control-siRNA using Lipofectamine 2000. HMCLs (RPMI8226, KM3 and U266 cell lines) were cocultured with osteoblasts to confirm the effects of MM-secreted BDNF on RANKL/OPG expression in osteoblasts. As shown in Figure 2c, RANKL protein levels increased, whereas OPG levels decreased when control-siRNA-transfected osteoblasts were cocultured with RPMI8226, KM3 and U266 cells. Importantly, transfection of TrkB-siRNA in osteoblasts partially reversed these effects at the gene level (Fig. 2d), suggesting that BDNF is an important factor secreted by MM cells that disrupts the balance of RANKL/OPG expression in osteoblasts.

**BDNF disrupts the balance of RANKL/OPG expression in osteoblasts through the TrkB-ERK1/2 signaling pathway**

To identify the downstream signaling molecules activated by BDNF in osteoblasts, we analyzed the phosphorylation of its related signal proteins MEK/ERK and p38 MAPK by Western blot. Cells were stimulated with 25 ng/ml of BDNF for different time periods. As shown in Figure 3a, the phosphorylation of ERK1/2 in osteoblasts increased, with a peak at 5 min, and then diminished over 120 min. The total level of ERK protein remained unchanged. No obvious change in phosphorylation of p38 MAPK was detected up to 120 min. To examine whether TrkB, the high-affinity receptor of
BDNF, is involved in the BDNF-induced phosphorylation of ERK, TrkB-siRNA was transfected into osteoblasts. As shown in Figure 3b, knockdown of TrkB significantly abolished BDNF-induced phosphorylation of ERK in osteoblasts. Furthermore, as shown in Figure 3c, BDNF-induced enhancement of RANKL and decrease of OPG protein was nearly completely reversed when osteoblasts were transfected with TrkB-siRNA. Similar results were obtained for RANKL and OPG mRNA expression, as shown in Figures 3d and 3e. The enhancement of RANKL (or attenuation of OPG) induced by BDNF was distinctly reversed when osteoblasts were transfected with TrkB-siRNA or treated with U0126, an inhibitor of MEK/ERK (p < 0.05). No significant effect of SB203580 (an inhibitor of p38MAPK) on the RANKL/OPG ratio was detected in our study. These findings indicate that TrkB/ERK is the main downstream signaling target in BDNF-regulated RANKL/OPG expression in osteoblasts.

Stable knockdown of BDNF in MM inhibits osteolytic bone destruction and reduces tumor burden in SCID-RPMI8226 mice model

Equal numbers (2.5 × 10⁶ cells per mouse) of AS-, EV- and WT-RPMI8226 cells were injected intravenously into SCID mice to investigate the effects of BDNF on the pathophysiology of MM in vivo. As shown in Figures 4a–4d, mice in the AS group (b and d) suffered less from vertebral compression and punched-out lesions in the tibiae compared to controls in the EV group (a and c). Over 56 days, nine of 12 mice in the AS group were able to move their hind limbs, whereas only three of 12 mice in the EV group were unable to move their hind limbs (Fig. 4e, p < 0.01). Animals in the AS group, which were inoculated with RPMI8226 cells expressing lower levels of endogenous BDNF, had lower incidence of vertebral compression and a lower mean number of bone lesions in their hind limbs compared to either the WT
To better quantify the osteolytic burden, the groups were compared according to changes in total-body BMD. As shown in Figure 4h, the total-body BMD in the EV group decreased by 63.79% ± 7.92% compared to pretreatment BMD, whereas in the AS group, BMD decreased by only 23.46% ± 10.51% (n = 12 for each group), indicating that the osteolytic burden in the AS group was significantly lower than in the EV group (p < 0.05). In addition, bones harboring EV-RPMI8226 cells demonstrated representative puncture of the cortex and rupture of the trabecula (Fig. 5a), whereas no obvious osteolytic lesion was detected in most of the AS group mice (Fig. 5b). Osteoclast-like cell (OCL) number per square millimeter of bone in the AS-RPMI8226 group was also significantly reduced compared to the controls (Fig. 5c). To better investigate the molecular mechanism by which antisense inhibition of BDNF reduces osteoclast formation in SCID-RPMI8226 mice, RANKL and OPG levels in the BM plasma of mice tibiae were evaluated by ELISA. Figures 5d–5f show that AS-RPMI8226-infiltrated bones have significantly lower expression of RANKL as well as markedly higher expression of OPG compared to WT- and EV-RPMI8226-infiltrated bones (p < 0.05). Decreased levels of soluble MIP-1α and IL-6 were also found in tibial BM of AS group mice compared to WT and EV group, as shown in Table 1. Moreover, microvessel density (MVD) was immunohistochemically detected using an anti-CD34 monoclonal antibody on myeloma cell-infiltrated bone sections. Figures 6a–6d show significantly lower MVD in bones harboring AS-RPMI8226 cells compared to those harboring WT and EV-RPMI8226 cells (p < 0.05). With regard to tumor burden, we also observed that human lambda immunoglobulin light chain levels in the blood serum from the AS-RPMI8226 group were significantly lower than in the EV-RPMI8226 group (Fig. 6e). Furthermore, markedly prolonged overall survival was observed in AS-RPMI8226 mice compared to control mice, as indicated by the Kaplan–Meier curves in Figure 6f (p < 0.01).

**Discussion**

Our study demonstrates that human recombinant BDNF increased osteoclast formation and function in osteoblast-pre-OC coculture systems. This is consistent with our previous studies, which demonstrated the direct promotion of osteoclast formation by BDNF on *in vitro*.

28 In addition, anti-RANKL could nearly completely abolish BDNF-induced osteoclast formation in these cocultures, suggesting that BDNF may also induce osteoclastic differentiation indirectly through the RANKL signaling pathway. Consistent with this
possibility is the recent finding that BDNF stimulates human BM stromal cells to secrete RANKL and promotes osteoclast formation in vitro. However, little is known about the effects of BDNF on the differentiation of osteoblasts or OPG expression in osteoblasts. Therefore, MSCs were treated with osteogenic medium in the absence or presence of BDNF, but no significant effects of BDNF on osteoblast differentiation were found in our study (Supporting Information Figs. S1B and S1C). On the other hand, human recombinant BDNF promoted RANKL and inhibited OPG expression in human osteoblasts in a dose- and time-dependent manner. Moreover, consistent with previous findings reported by Pearse et al., RANKL expression increased and OPG expression decreased when osteoblasts were cocultured with three types of HMCLs. These effects were mostly reversed by TrkB-specific siRNA, identifying BDNF as an important factor secreted by MM cells that disrupts the RANKL/OPG balance in osteoblasts.

Although the results of our study identified BDNF as a soluble factor secreted by MM cells and contributes to osteoclastogenesis, direct contacts between MM cells and stromal/osteoblastic cells may also play important role in RANKL secretion and myeloma bone resorption. Therefore, we constructed a cell-to-cell contact transwell system and found that BDNF significantly increased RANKL mRNA levels in these cocultures (Supporting Information Figs. S2C and S2D). Combined with our previous data that BDNF promotes RANKL expression in BM stromal cells and osteoblasts (Figs. 2a and 2b), all these findings raise the possibility that BDNF may also play a role in a contact-dependent ability of MM cells to stimulate osteoclastogenesis. Details about the involvements of BDNF in this process remain to be elucidated in the future.

In our study, we found that the ERK signaling pathway was the main downstream mediator involved in BDNF-regulated RANKL/OPG expression in osteoblasts. RANKL and OPG are mainly secreted by stromal/osteoblastic cells in the BM milieu, accompanied by the activation of mitogen-activated protein kinase (MAPK). Moreover, the MAPK pathway has also been demonstrated as a downstream mediator of Trk signaling in response to a wide array of cytokines, including BDNF. These reports are consistent with our results from the aspect of the MEK/ERK pathway being involved in promoting RANKL expression and inhibiting OPG expression. In addition, it is well demonstrated that the biological functions of BDNF are mediated by two receptor systems: the high-affinity receptor TrkB and the low-affinity receptor p75NTR. Binding of BDNF to the high-affinity receptor TrkB leads to the phosphorylation of TrkB and the...
We used TrkB-siRNA to specifically downregulate the expression of TrkB in osteoblasts. Pretreatment of osteoblasts with a specific TrkB siRNA markedly reversed the imbalance in the RANKL/OPG ratio induced by BDNF. Therefore, it is possible that BDNF, a newly developed osteoclastogenic factor in myeloma, regulates RANKL/OPG secretion in osteoblasts through the TrkB/ERK pathway.

We also investigated the effects of BDNF on myeloma pathophysiology in vivo. RPMI8226 cells were transfected with BDNF shRNA lentiviral particles and intravenously injected into SCID mice. Gene silencing by shRNA, a powerful tool to analyze gene function in vivo, makes prolonged selective inhibition of target gene possible in various types of cells, including HMCLs.27,41 Here, we observed that stable knockdown of BDNF markedly inhibited osteolytic bone destruction in SCID-RPMI8226 mice. Several possibilities may explain these results: the first is that blocking BDNF activity suppresses RANKL and enhances OPG expression by osteoblasts in the BM milieu, as confirmed in Figures 2 and 5 of our study. Further support for the role of BDNF on RANKL pathway in MM comes from our recent studies, which demonstrate BDNF stimulates BM stromal cells to secrete RANKL both in vitro and in vivo.29 Moreover, decreased levels of soluble MIP-1α and IL-6 were also detected in BM of AS group mice (Table 1), reminding us that BDNF may contribute to other OAFs secretion in microenvironment. Consistent with this hypothesis are the results of Rezaee et al. who reported that neurotrophins stimulate BM stromal cell to secrete IL-6,42 a factor recently identified to be involved in myeloma-induced RANKL expression in T lymphocytes by Giuliani et al.43 Combined with our previous data that BDNFs are correlated with MIP-1α, and β2-microglobulin levels in patients with active myeloma,28 these data suggest that the complex interaction between BDNF and other OAFs in BM appears to be responsible in part for myeloma bone disease. Second, previous studies have demonstrated that antisense inhibition of BDNF blocked the secretion of VEGF in a subcutaneous myeloma model in immunodeficient mice.27 In our study, we also observed that stable knockdown of BDNF downregulated VEGF secretion and inhibited angiogenesis in SCID-RPMI8226 mice model.
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(Table 1, Figs. 6a–6d). Because VEGF is an important factor in the vicious cycle between angiogenesis and osteoclastogenesis in MM, it is possible that stable knockdown of BDNF in MM would break this cycle and ultimately restore osteoclastic bone resorption. Third, BDNF has been well defined as an important factor for MM cell growth, as confirmed by both us (Fig. 6e) and others. Because MM cells produce OAFs, such as IL-3 and MIP-1a, to promote osteoclastogenesis, inhibition of BDNF in MM may block both myeloma growth and myeloma-secreted OAFs, ultimately resulting in attenuated osteoclastogenesis in the BM milieu.

In conclusion, our data demonstrate that myeloma-derived BDNF disrupts the balance of RANKL/OPG expression in osteoblasts through the TrkB/ERK pathway and that anti-sense inhibition of endogenous BDNF in MM cells inhibits bone destruction, angiogenesis, and tumor burden in vivo. These findings suggest targeting of BDNF as a new therapeutic strategy to improve outcome in MM patients.

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