Critical Role of AKT in Myeloma-induced Osteoclast Formation and Osteolysis

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Running title: Myeloma activation of osteoclastogenesis

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Key words: Myeloma; AKT; ATF4; RANK; osteoclast; bone

Background: Myeloma cells cause abnormal osteoclast formation and osteolysis.

Results: Myeloma cells up-regulate AKT in osteoclast precursors and promote osteoclast formation. Systemic AKT inhibition blocks the myeloma-induced osteolysis and tumor growth in bone.

Conclusion: AKT is critical for the myeloma promotion of osteoclast formation and osteolysis.

Significance: AKT could be a useful target for treating patients with myeloma bone disease.

SUMMARY

Abnormal osteoclast formation and osteolysis are a hallmark of multiple myeloma (MM) bone disease, yet the underlying molecular mechanisms are incompletely understood. Here we show that the AKT pathway was up-regulated in primary bone marrow monocytes (BMM) from MM patients, which resulted in sustained high expression of receptor activator of NF-kappaB (RANK) in osteoclast precursors. The up-regulation of RANK expression and osteoclast formation in the MM BMM cultures was blocked by AKT inhibition. Conditioned media from MM cell cultures activated AKT and increased RANK expression and osteoclast formation in BMM cultures. Inhibiting AKT in cultured MM cells decreased their growth and ability to promote osteoclast formation. Of clinical significance, systemic administration of an AKT inhibitor LY294002 blocked the formation of tumor tissues in the bone marrow cavity and essentially abolished the MM-induced...
Osteoclast formation and osteolysis in SCID mice. The level of activating transcription factor 4 (ATF4) protein was up-regulated in the BMM cultures from MM patients. Adenoviral overexpression of ATF4 activated RANK expression in osteoclast precursors. These results demonstrate a new role of AKT in the MM promotion of osteoclast formation and bone osteolysis through, at least in part, the ATF4-dependent up-regulation of RANK expression in osteoclast precursors.

Multiple myeloma bone disease (MMBD) is characterized by progressive osteolysis associated with abnormally increased osteoclast formation and activation. Up to 90% of patients with multiple myeloma (MM) develop osteolytic lesions (1), which cause major clinical problems such as bone pain, pathological fractures, spinal cord compression, and hypercalcemia of malignancy (2-4). Studying the underlying molecular mechanisms will help to develop new therapeutic targets to treat osteolytic lesions and related complications in MM patients.

Osteoclasts originate from cells in the monocyte/macrophage lineage (5). Osteoclast formation and maturation are tightly regulated by osteoblast/stromal cell/osteocyte/hypertrophic chondrocyte-derived factors such as M-CSF, RANKL, and OPG. OPG is a soluble decoy receptor that blocks RANKL binding to RANK and thereby inhibits osteoclast differentiation (6,7). M-CSF binds to its receptor CSF1R on early macrophage lineage cells and activates RANK expression (8). This is a critical step for generating osteoclast precursors and early osteoclast differentiation. RANKL then binds to RANK on osteoclast precursors and recruits TRAF6, which results in the activation of multiple signaling pathways including IKK complexes (IKKα,β,γ and NIK-IKKα) and MAPKs (ERK1/2, p38, and JNK) (9,10), leading to activation of critical transcription factors such as NF-κB and c-FOS, and NFATc1 and eventually osteoclast formation (10-12).

Abnormal osteoclast formation and activation are mediated by factors produced and induced by tumor cells (13). For example, breast cancer cells-produced parathyroid hormone-related protein (PTHrP) induces osteoclast formation through up-regulation of RANKL by osteoblasts (14,15). Further, the NF-κB pathway in breast cancer cells promotes osteolytic bone metastasis by activating osteoclast formation via up-regulation of granulocyte-macrophage colony-stimulating factor (GM-CSF) (16). Monocyte chemotactic protein-1 (MCP-1) mediates prostate cancer- and lung cancer-induced osteoclast formation and bone resorption (17,18). Macrophage inflammatory protein (MIP)-1α promotes the development of osteolytic lesions in MM patients (19,20). Enhanced RANKL expression plays an important role in tumor-induced osteoclast formation and bone destruction (13,21). However, molecular mechanisms whereby the MM cells promote osteoclast formation and activation and osteolysis are not completely understood.

AKT, also known as protein kinase B (PKB), is a serine/threonine-specific protein kinase that plays a critical role in multiple cellular processes such as cell proliferation and survival and migration, glucose metabolism, and gene transcription (22,23). Accumulative evidence shows that the PI3K/AKT pathway plays a critical role in activating osteoclast differentiation (24,25). However, if and how
AKT mediates the MM-induced osteoclast formation and osteolysis has not been addressed.

EXPERIMENTAL PROCEDURES

Reagents—Tissue culture media, fetal bovine serum, and horse serum were obtained from Thermo Scientific HyClone (Logan, Utah). LY294002 was purchased from LC Laboratories (Worburn, MA), ascorbic acid and DMSO from Sigma Aldrich (St. Louis, MO), human recombinant M-CSF, RANKL, and TNF-α from R&D Systems Inc. (Minneapolis, MN). All other chemicals were of analytical grade.

Human bone marrow samples—Bone marrow aspirates were collected in heparin from normal donors (ND) and patients with MM. Protocols were approved by the respective IRB committees of the University of Pittsburgh and the Institute of Hematology & Blood Diseases, Chinese Academy of Medical Sciences.

DNA constructs, transfection, and adenoviral infection—pCMV/β-gal, pCMV/ATF4, pCMV/AKT-CA, and wild-type and mutant p4OSE1-luc plasmids (an ATF4 reporter plasmid) were previously described (26,27). Rank-luc reporter plasmid was constructed by PCR subcloning of a -1073/+79 mouse Rank gene promoter into the pGL3-luc vector (Promega, Madison, WI) in the project laboratory. For transfection experiments, the amounts of plasmid DNAs were balanced as necessary with β-galactosidase expression plasmid such that the total DNA was constant in each group. Adenoviruses expressing ATF4 or EGFP were described previously (28,29). The amount of adenovirus was balanced as necessary with a control adenovirus expressing EGFP such that the total amount was constant in each group.

Gene expression studies—RNA isolation and reverse transcription (RT) were previously described (30). Quantitative real-time RT-PCR analysis was performed to measure the relative mRNA levels using SYBR Green kit (Bio-Rad Laboratories Inc.). Melting curve analysis was used to confirm the specificity of the PCR products. Four to six samples were run for each primer set. The levels of mRNA were calculated by the ddCt method. Samples were normalized to Gapdh expression. The DNA sequences of human and mouse primers used for qPCR were summarized in Tables 1 and 2. Western blot analysis was performed as previously described (26,30). Antibodies used are from the following sources: antibodies against NFATc1, c-FOS, RANK, ATF4 and anti-rabbit or anti-mouse antibodies conjugated with horseradish peroxidase from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), antibodies recognizing phosphorylated and total AKT and PU.1 from Cell Signaling Technology Inc. (Beverly, MA), and mouse monoclonal antibody against β-actin from Sigma Aldrich.

In vitro osteoclast assays—For osteoclast studies using bone marrow cells from humans, nonadherent BMMs were isolated from MM patients and normal donors (NDs). For undifferentiated cultures, cells were cultured in the proliferation media (α-MEM media containing 20% horse serum and 10 ng/ml human recombinant M-CSF) for 10d. For differentiation studies, cells were cultured in the differentiation media (proliferation media containing 50 ng/ml human recombinant
RANKL) for 21d, followed by TRAP or 23C6 staining or gene expression studies. The anti-23C6 antibody, which recognizes the αvβ3 integrin, a receptor for vitronectin, on osteoclasts, was described previously (31-33). The TRAP- or 23C6-positive multinucleated cells (MNCs) (≥3 nuclei) were scored using an inverted microscope. For osteoclast studies using primary mouse bone marrow cells (BMMs), nonadherent BMMs were isolated from total bone marrow cells and cultured on tissue culture dishes for 48 h. For differentiation, cells were first cultured in the proliferation media (α-MEM containing 10% FBS and 10 ng/ml M-CSF) for 3d and switched to differentiation media (proliferation media plus 50 ng/ml RANKL) for 5-7d, followed by TRAP staining or gene expression studies. The TRAP-positive MNCs (≥3 nuclei) were scored using an inverted microscope.

Mouse model and histological analysis and bone histomorphometry-For intratibial injection, both left and right tibiae of 4-weeks-old male SCID mice (10 mice/group or 20 tibiae/group) were injected with 1x10^6 5TGM1 cells in 20 µl PBS or 20 µl PBS alone (control group without MM injection). One week later, mice were subcutaneously injected with LY294002 (40 mg/kg body weight) or vehicle (DMSO) twice a week for 3 weeks. Protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Nankai University. All specimens were fixed in 10% formalin at 4 °C for 24 h, decalcified in 10% EDTA (pH 7.4) for 10-14d, and embedded in paraffin. Then serial sections were prepared and stained with hematoxylin and eosin. Sections of tibiae from each group were used for TRAP staining as described previously (34). Bone histomorphometry such as osteoclast surface/bone surface (Oc.S/BS) and osteoclast number/bone perimeter (Oc.Nb/BPm) of tibiae was measured using an Image Pro Plus 7.0 software as previously described (25,35).

Statistical Analysis-Data was analyzed with a GraphPad Prism software (4.0) (San Diego, CA). A one-way ANOVA analysis was used followed by the Tukey test. Results were expressed as means ± standard deviation (SD). Differences with a P <0.05 was considered as statistically significant. All experiments were repeated at least 2 times and similar results were obtained.

RESULTS

MM activates osteoclast formation in primary bone marrow monocyte (BMM) cultures in the presence and absence of exogenously added RANKL. To study the mechanisms whereby MM cells activate osteoclast formation, we measured osteoclast differentiation in primary BMM cultures from MM patients in vitro compared to BMM cultures from normal donors (ND) with or without addition of exogenous RANKL. We measured the numbers of TRAP-positive multinucleated osteoclasts (MNCs) generated by each. Results showed that the number of TRAP+ MNCs (≥3 nuclei) in the RANKL-differentiated MM BMM cultures was dramatically increased compared to that in the ND BMM cultures (Figure 1, A and B). The MNCs formed in the RANKL-differentiated MM BMM cultures were much larger than those from the ND BMM cultures (Fig 1B). Surprisingly, we found a number of TRAP+ MNCs in the MM BMM cultures in the absence of exogenously added RANKL, which were
absent from the ND BMM control cultures (Fig 1, A and C). This could result from potential contamination of osteoblasts and stromal cells and bone marrow lymphocytes, which produce RANKL and OPG (36). For this reason, we measured the expression levels of both factors by qPCR analysis. Results showed that, although both mRNAs were detected in both cultures, the RANKL/OPG ratio was actually reduced in the MM versus ND cultures (Fig 1D). Therefore, the enhanced osteoclast formation in the MM cultures is not due to increased RANKL and/or reduced OPG expression produced by cells in the cultures. qPCR analysis showed that the mRNA levels of osteoclast differentiation marker genes including those encoding cathepsin K (Cat K), integrin β3 (β3), nuclear factor of activated T cells (NFAT) c1 (a master regulator of osteoclast differentiation), and matrix metalloproteinase-9 (MMP-9) were all significantly increased in the differentiated MM relative to ND BMM cultures (Fig 1E). The mRNA level of c-FMS, the gene that encodes the receptor for the macrophage colony-stimulating factor (CSF-1R), was slightly but significantly increased in the RANKL-differentiated MM versus ND BMM cultures (Fig 1E). In contrast, the mRNA level of PU.1 (an Ets family transcription factor that regulates osteoclast differentiation) was not different in the RANKL-differentiated MM and ND BMM cultures (Fig 1E). Western blot analysis revealed that the levels of NFATc1 protein were increased in the RANKL-differentiated BMM cultures from 5 MM patients compared to those in 5 NDs (Fig 1F). Collectively, these results suggest that osteoclast differentiation capacity of the MM BMMs is increased in both RANKL-dependent and RANKL-independent manners.

Sustained high expression of RANK is a major feature of the BMMs from MM patients.

To study the early molecular events in the MM BMM cultures, we measured the expression levels of genes that are known to regulate early osteoclast differentiation in undifferentiated BMM cultures from MM patients and NDs. Results showed that the level of RANK mRNA was increased by more than 10-fold in undifferentiated BMM cultures from MM patients compared to that from NDs (Fig 2A). In contrast, the levels of c-FMS, PU.1, c-FOS, TRAF6 and DAP-12 mRNAs were not significantly increased in the undifferentiated MM versus ND BMM cultures (Fig 2A). Western blot analysis confirmed that the level of RANK protein was increased in the undifferentiated MM relative to ND BMM cultures (Fig 2B). Importantly, the levels of RANK mRNA were significantly increased in the uncultured BMMs from 11 MM patients compared to those from the uncultured BMMs from 6 NDs (Fig 2C). Because RANK expression is a critical step for generating osteoclast precursors, these results suggest that MM cells greatly promote early osteoclast differentiation. Surprisingly, although RANK is a marker of osteoclast precursors, we found that its expression continued to increase even in the terminally differentiated osteoclast cultures from MM patients (i.e., in the presence of RANKL for 21d) (Fig 2D). Western blot analysis confirmed that the level of RANK protein was increased in the RANKL-differentiated MM relative to ND BMM cultures (Fig 2E).

Critical role of AKT in RANK expression and osteoclast formation in BMM cultures from MM patients. While the above results clearly
showed that RANK expression was up-regulated in osteoclast precursors from MM patients, the underlying mechanisms remain unknown. Recent studies showed that AKT plays a role in promotion of early osteoclast differentiation (24,25). We investigated whether AKT is up-regulated in the MM BMM cultures. As shown in Fig 3A, in the RANKL-differentiated MM BMM cultures, the levels of both phospho-AKT and total AKT proteins were up-regulated compared to those in the ND BMM cultures. Because RANK and AKT were similarly up-regulated in the MM BMM cultures, we determined whether AKT is required for the up-regulation of RANK expression in the MM BMM cultures. Results showed that LY294002, a specific AKT inhibitor, dose-dependently decreased RANK mRNA expression in the MM BMM cultures (Fig 3B). In contrast, the inhibitor did not reduce the expression of PU.1 mRNA in the MM BMMs (Fig 3B). LY294002 similarly decreased RANK protein expression in the MM BMM cultures in a dose-dependent manner (Fig 3C). Importantly, the AKT inhibitor blocked formation of the MNCs in the MM BMM cultures (Fig 3, D-F). Further, overexpression of a constitutively active form of AKT (AKT-CA) increased the mouse Rank promoter activity in COS-7 cells (Fig. 3F). Taken together, these results suggest that MM may activate RANK expression and osteoclast formation through, at least in part, up-regulation of AKT in osteoclast precursors.

ATF4 protein is up-regulated in MM osteoclast cultures and by 5TG1-CM and TNF-α. Our recent study demonstrates that loss of ATF4 impaired M-CSF induction of RANK expression in osteoclast precursors (25). Interestingly, we found that the level of ATF4 protein was increased in the RANKL-differentiated BMM cultures from 4 MM patients compared to that of 4 NDs (Fig 5A). The level of ATF4 mRNA was not increased in MM versus ND cultures (Fig 5B), suggesting a post-transcriptional regulation. The 5TG1-CM and TNF-α, a well-known osteoclastogenic factor produced by many cancer cells including MM cells, increased the ATF4 protein levels in the BMM cultures (Fig 5, C and E). In contrast, 5TG1-CM and TNF-α did not increase the levels of Atf4 mRNA (Fig 5, D and F). Interestingly, a slower migrating bands indicated by an arrow were observed in the MM BMM cultures (Fig 5A) and increased by treatments with 5TG1-CM (Fig 5C) or TNF-α (Fig 5E). These bands are
probably phosphorylated forms of ATF4 because similar bands in primary mouse BMMs disappeared with CIP phosphatase treatment (25).

ATF4 is phosphorylated and up-regulated by AKT. To determine whether ATF4 is a downstream of AKT, we performed several experiments. First, we found that AKT inhibitor LY294002 decreased the ATF4 protein levels in primary BMM cultures from MM patients in a dose-dependent manner (Fig 6A). Second, overexpression of a constitutively active form of AKT (AKT-CA) increased the level of ATF4 protein in COS-7 cells (Fig 6B). There was a slower migrating ATF4 band on Western blots that was observed in samples from the AKT-CA-transfected cells (Fig 6B). Third, transfection assays revealed that AKT-CA increased ATF4-dependent transcriptional activity (Fig 6C), which was abolished by introduction of a 3-bp point mutation in the ATF4 binding core sequence (Fig 6D). Fourth, we tested whether ATF4 can be directly phosphorylated by AKT enzyme by performing in vitro kinase assays using purified GST-ATF4 protein and AKT1 enzyme in the presence of \([\gamma^{32P}]\)ATP. As shown in Fig 6E, purified GST-ATF4 but not GST protein was directly and strongly phosphorylated by the AKT1 enzyme in vitro. Finally, adenoviral overexpression of ATF4 increased the level of Rank mRNA by 10-fold in osteoclast precursors (Fig 6F). In contrast, Dap-12 (DNAX-activating protein 12), a membrane protein expressed in both macrophages and osteoclasts, was not increased by ATF4 in BMMs (Fig 6E).

Blocking AKT reduces tumor burden in bone marrow cavity and inhibits the MM-induced osteoclast formation and osteolysis in SCID mice. To determine whether AKT plays a role in the MM-induced osteoclast formation and osteolysis in vivo, we injected 5TGM1 MM cells into the tibial marrow cavity of SCID mice. One week after the injection, animals were subcutaneously injected with LY294002 (40 mg/kg body weight) or vehicle (DMSO) twice per week for 3 weeks. This LY294002 dose is in the range used by other investigators to efficiently block the PI3K/AKT pathway in mice (38,39). We examined whether the MM cells stimulated osteoclast formation by staining histological sections for the osteoclast enzyme tartrate-resistant acid phosphatase (TRAP). Results showed that 5TGM1 cells increased osteoclast surface/bone surface (Oc.S/BS) and osteoclast number/bone perimeter (Oc.Nb/BPm) of tibiae by 2.3- and 1.8-fold, respectively (P <0.05 for both, PBS/DMSO versus 5TGM1/DMSO) (Fig 7, A-C). Strikingly, the MM-induced increases in Oc.S/BS and Oc.Nb/BPm were completely inhibited by LY294002 (Fig 7, A-C). We further determined whether blocking AKT affects the MM-induced osteolysis by examining histological sections. Results showed that, in DMSO-injected mice, the majority of the trabecular bone of the proximal tibial metaphysis was destroyed and that the marrow cavity was replaced by tumor tissues. In contrast, in those LY294002-injected mice, the bone destruction was largely prevented (Figure 7, D and E). The formation of tumor tissues in bone marrow cavity was suppressed by AKT inhibition (Figure 7D). It should be noted that, although AKT was reported to regulate osteoclast and osteoblast function in mice (24),
the LY294002 treatment under our experimental conditions only slightly decreased the basal level of the Oc.S/BS (P = 0.087, PBS/DMSO versus 5TGM1/LY). In addition, LY294002 did not cause any reductions in the OcNb/BPm and trabecular bone area (P >0.12 for both parameters, PBS/DMSO versus 5TGM1/LY).

**AKT is critical for the MM cell growth and promotion of osteoclast formation in vitro.**

We next performed in vitro experiments to examine whether AKT pathway in the MM cells is required for the tumor cell growth and promotion of osteoclast formation. Results showed that the LY294002 treatment significantly decreased the growth of MM1.S cells in vitro (Figure 8A). Western blot analysis confirmed that the level of phospho-AKT but not total AKT protein was decreased by the AKT inhibitor (Figure 8B). Finally, osteoclast formation induced by the CM from MM1.S cells was significantly reduced by treatment of the tumor cells with LY294002 (Figure 8, C and D).

**DISCUSSION**

Abnormal osteoclast differentiation is a major contributor to osteolysis caused by major cancers such as MM, breast, lung and prostate cancers. Bone-residing cancer cells promote osteoclast formation and activation (16,40-45). Increased osteoclast formation and activity and bone resorption increase releases of growth factors from the bone matrix that stimulate cancer cell growth (40-46). Therefore, there is a vicious cycle between osteoclast-mediated osteolysis and tumor growth and progression in bone marrow. Breaking the vicious cycle is of major clinical significance since osteolytic lesions and related complications such as severe bone pain, pathological fractures, spinal cord compression, and hypercalcemia of malignancy are common causes of morbidity and sometimes mortality (42,47).

In the present study, we found that AKT in osteoclasts and their precursors plays an important role in the MM promotion of osteoclast formation and activation and osteolytic lesions. Both AKT expression and phosphorylation are up-regulated in primary bone marrow osteoclast cultures from MM patients. Blocking AKT inhibits the MM-induced osteoclast formation in vitro and abolishes the MM-induced increase in osteoclast differentiation and osteolysis in bone. We demonstrate that AKT promotes osteoclast formation through, at least in part, up-regulation of RANK in osteoclast precursors. The expression of AKT and RANK are both increased in primary bone marrow osteoclast cultures from MM patients. AKT inhibition reduces the level of RANK mRNA and osteoclast formation in the MM BMM cultures. Overexpression of constitutively active form of AKT increases the RANK gene promoter activity. We further demonstrate that ATF4, which is up-regulated by AKT, TNF-α, and soluble factors produced by the MM cells, is a new upstream transcriptional activator of RANK gene expression in osteoclast precursors.

Increased RANK expression in osteoclast precursors in the MM bone marrow could greatly increase the sensitivity for RANKL to induce osteoclast formation and maturation, suggesting that, even under low concentrations of RANKL, osteoclast differentiation can be enhanced in the bone marrow of MM patients. This notion is supported by the following evidence: i)
osteoclast formation is greatly enhanced in the MM BMM cultures in the absence of exogenously added RANKL, in which the RANKL/OPG ratio is reduced (Fig 1D); and ii) the levels of RANKL from many tumor cells that can activate osteoclast formation are very low (17). Furthermore, sustained high expression of RANK even in highly differentiated osteoclast cultures (i.e., 21d in the presence of RANKL) from MM patients could also suggest that part of the BMM cells in the MM bone marrow are maintained at the osteoclast precursor state because RANK expression is a marker of osteoclast precursors. These precursor cells can actively proliferate and increase the osteoclast numbers in bone marrow, which could further contribute to the increased osteoclast formation and osteolysis in MM patients.

While results from the present study demonstrate that AKT is critical for the MM-induced osteoclast formation, how MM cells activate AKT remains unclear. Cancer cells can produce and induce factors that can promote osteoclast formation and activity, including granulocyte-macrophage colony-stimulating factor (GM-CSF) (16), monocyte chemotactic protein-1 (MCP-1) (17), macrophage inflammatory protein (MIP)-1α and MIP-1β (19,20), TNF-α (48), and PTHrP (14,15). Future studies will determine if any of those factors are responsible for MM cell activation of AKT in osteoclast precursors. It will be interesting to determine if AKT also mediates the osteoclast formation and osteolysis caused by other cancers such as breast, lung and prostate cancers in the future.

It should be noted that blocking AKT in MM cells decreases their growth in vitro and that systemic inhibition of AKT blocks the formation of tumor tissues in the bone marrow cavity in SCID mice. Interestingly, our in vitro studies show that AKT inhibition in the MM cells also reduces the ability of tumor cells to activate osteoclast formation, which should contribute to the reduced osteoclast formation induced by the tumor cells. Finally, although the AKT inhibitor essentially abolishes the MM-induced osteolysis and bone loss, it does not significantly affect the basal level osteoclast formation and bone mass under our experimental conditions. Collectively, these results suggest that AKT could be a potential target for inhibiting the MM-induced osteoclast osteolytic lesions as well as tumor growth and progression, thus breaking the vicious cycle in the MM patients.
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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Abnormal osteoclast differentiation in primary BMM cultures from MM patients in the presence and absence of exogenously added RANKL

(A-C) Non-adherent BMMs from MM patients (MM) and normal donors (ND) were seeded at the density of 1x10^5/well on 96-well plate in α-MEM containing 20% horse serum and M-CSF (10 ng/ml) with or without RANKL (50 ng/ml) for 21d, followed by TRAP staining (A). TRAP-positive MNCs from (A) with (B) or without (C) RANKL treatment were scored under a microscope. Experiments were repeated at least 6 times using samples from different MM patients and NDs. Similar results were obtained. *P <0.01 (versus ND). (D-E) Non-adherent MM and ND BMMs were cultured in α-MEM containing 20% horse serum and M-CSF (10 ng/ml) without (D) or with (E) RANKL (50 ng/ml) for 21d as in (A), followed by qPCR analysis. mRNA levels were normalized to GAPDH mRNA. *P <0.01 (versus ND). Experiments were repeated at least 3 times using samples from different MM patients and NDs. Similar results were obtained. (F) Non-adherent BMMs from 5 different MM patients and 5 different NDs were cultured in α-MEM containing 20% horse serum and M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 21d as in (A), followed Western blot analysis. β-ACTIN was used for a loading control for Western blot analysis.

Figure 2. RANK expression levels are dramatically up-regulated in uncultured, undifferentiated and RANKL-differentiated BMMs from MM patients

(A and B) MM and ND BMMs were seeded at the density of 1x10^6/dish into 35-mm dish in α-MEM containing 20% horse serum and proliferated by 10 ng/ml M-CSF for 10d, followed by qPCR (A) and Western blot analysis (B). Experiments were repeated at least 3 times using samples from different MM patients and NDs. Similar results were obtained. *P <0.01 (versus ND). (C) RNAs from uncultured BMMs from 11 MM patients and 6 NDs were used for qPCR analysis for RANK mRNA. *P <0.01 (versus ND). (D) MM and ND BMMs were seeded at the density of 1x10^6/dish on 35-mm dish in α-MEM containing 20% horse serum and 10ng/ml M-CSF with or without 50 ng/ml RANKL for 21d, followed by qPCR analysis for RANK mRNA. Experiments were repeated at least 3 times using samples from different MM patients and NDs. Similar results were obtained. *P <0.01 (versus ND). (E) BMMs from 3 MM patients and 3 NDs were differentiated for 21d as in Figure 1F, followed by Western blot analysis for RANK protein.

Figure 3. AKT is up-regulated and is critical for RANK expression and osteoclast formation in the MM BMM cultures

(A) BMMs from 3 MM patients and 3 NDs were differentiated for 21d as in Figure 1F, followed by Western blot analysis for total and phosphor-AKT using specific antibodies. (B and C) BMMs from MM
patients were differentiated for 10d. On day 11, cells were treated by the indicated concentrations of LY294002 for 24h, followed by qPCR (B) or Western blot analysis (C). (D) BMMs from MM patients were differentiated for 10d. On day 11, cells were treated by LY294002 for 24h and switched to differentiation media for another 10d, followed by staining for the 23C6+ (top) and TRAP+ (bottom) MNCs. (E and F) Statistical analysis of (D). *P < 0.01 (vs. 0 µM LY). (G) COS-7 cells were co-transfected with RANK-luc, in which a -1073/+79 mouse Rank gene promoter driving firefly luciferase gene expression, pRL-SV40 (for normalization) and indicated amounts of pCMV/AKT-CA or pCMV/β-gal, followed by dual luciferase assays. Experiments for B-F were repeated 2-4 times and similar results were obtained.

Figure 4. Conditioned media from MM cell line cultures increase AKT phosphorylation, RANK expression, and osteoclast formation
(A) Primary mouse BMMs were treated with equal volume of conditioned media (CM) from human MM1.S or mouse 5TGM1 MM cell lines or control medium (DMEM containing 2% FBS) for the indicated times, followed by Western blot analysis. (B) Primary mouse BMMs were treated with MM1.S-CM (0, 1/1000, 1/100, v/v) for 48h, followed by qPCR analysis for Rank mRNA. (C) Primary mouse BMMs were treated with MM1.S-CM (1/100, v/v) for the indicated times, followed by Western blot analysis for RANK protein. (D and E) Primary mouse BMMs were differentiated by M-CSF and RANKL in the presence or absence of indicated amounts of MM1.S-CM for 7d, followed by TRAP staining. TRAP-positive mononucleated (D) and multinucleated (3≥nuclei/cell) (E) osteoclasts (MNCs) were counted. *P<0.01 (vs. 0 MM1.S-CM). (F) CFU-GM assay. 2 x10^4 cells from mouse spleens were cultured in methylcellulose semi-solid medium in 35-mm dishes in the presence of 1.0 ng/ml of recombinant human GM-CSF in the presence or absence of indicated amounts of MM1.S-CM for 10d. The numbers of CFU-GM colonies were counted under an inverted microscope. (G) Statistical analysis of (F). Experiments for A-D were repeated 2-4 times and similar results were obtained.

Figure 5. ATF4 protein is up-regulated in MM BMM cultures and by 5TGM1-CM and TNF-α.
(A) BMMs from 4 MM patients and 4 NDs were differentiated by M-CSF and RANKL for 21d as in Figure 1F, followed by Western blot analysis. β-ACTIN was used for a loading control. (B) BMMs from 1 MM patients and 1 ND were differentiated by M-CSF and RANKL for 21d as in Figure 1F, followed by qPCR analysis. ATF4 mRNA levels were normalized to GAPDH mRNA. (C-F) Primary mouse BMMs were cultured in M-CSF-containing media for 3d and treated with 5TGM1-CM for the indicated times (C and D) or the indicated concentrations of TNF-α for 48 h (E and F), followed by Western blot (C and E) or qPCR analysis (D and F). The mRNAs were normalized to Gapdh mRNA. Experiments for B-F were repeated 2-3 times and similar results were obtained.

Figure 6. Effects of AKT activation or inhibition in the ATF4 expression and activity and phosphorylation
(A) Non-adherent BMMs from MM patients were differentiated for 10d. On day 11, cells were treated by the indicated concentrations of LY294002 for 24h, followed by Western blot analysis for ATF4
expression. (B) COS-7 cells were co-transfected with pCMV/ATF4 and increasing amounts of expression vectors for a constitutively active form of AKT (AKT-CA), followed by Western blot analysis for ATF4 and AKT. (C and D) COS-7 cells were co-transfected wild-type (wt) (C) and mutant (mt) (D) ATF4 reporter plasmid (pOSE1-luc) (49), pRL-SV40 (for normalization), pCMV/ATF4 with or without the indicated amounts of AKT-CA expression vector, followed by dual luciferase assays. *P<0.01 versus 0 µg AKT-CA. (E) Purified GST-ATF4 and GST proteins were incubated with AKT1 (Cell Signaling Technology, Inc., Beverly, MA) in the presence of [γ-32P]ATP. (F) Primary mouse BMMs were cultured in M-CSF-containing media for 3d and infected with equal amounts of ATF4 or EGFP adenovirus. Twenty hours later, cells were harvested for qPCR for Rank and Dap-12 mRNAs. The mRNAs were normalized to Gapdh mRNA. *P <0.01 versus Ad/EGFP. Experiments for A-F were repeated 2-3 times and similar results were obtained.

Figure 7. Blocking AKT inhibits the MM-induced osteoclast formation and bone loss and the tumor tissue formation in bone marrow cavity in SCID mice

(A-C) 1x10⁶ 5TGM1 cells in 20 µl PBS or equal volume PBS alone were injected into both left and right tibiae of 4-weeks-old male SCID mice (10 mice/group or 20 tibiae/group). One week later, mice were subcutaneously injected with LY294002 (40 mg/kg body weight) or equal volume of vehicle (DMSO) twice a week for 3 weeks. Osteoclast surface/bone surface (Oc.S/BS) and osteoclast number/bone perimeter (Oc.Nb/BPm) of tibiae were measured using an Image Pro Plus 7.0 software as previously described (25). (D and E) Tibiae from each group were sectioned from the midtibial metaphysis for at least 10 sections from each tibia (6 tibiae/group) and stained with H&E. Trabecular bone area versus total bone marrow area was measured using an Image Pro Plus 7.0 software. *P <0.01 (vs. PBS/DMSO), #P<0.01 (vs. 5TGM1/DMSO).

Figure 8. Blocking AKT inhibits the growth of MM cells and reduces their ability to activate osteoclast differentiation in vitro

(A) MM1.S cells were seeded at 5x10⁷/well on a 96-well plate and cultured for 2d in 10% FBS DMEM. Cells were then treated with LY294004 (10 µm) or equal volume of vehicle (DMSO) for the indicated times followed by the MTS assay as previously described(35). *P < 0.05, versus DMSO. (B) MM1.S cells were seeded at 5x10⁵/dish on 6-well plate and cultured for 2d in 10% FBS DMEM and then treated with LY294004 (10 µm) or DMSO for 2d, followed by Western blotting. (C and D) MM1.S cells were seeded in 10% FBS DMEM media and treated with or without LY294004 (10 µM) or equal volume vehicle (DMSO) for 24h. Cells were then switched to 2% FBS DMEM media for another 24h. Conditioned media (CM) from each group were harvested. 2% FBS DMEM was used as control. For in vitro osteoclast differentiation, primary mouse BMMs were differentiated by M-CSF (10 ng/ml) and RANKL (30 ng/ml) in the presence of equal volume of the indicated CMs or control media (1/200, v/v) for 7d, followed by TRAP staining (C). TRAP-positive MNCs (≥3 nuclei/cell) were counted (D). Experiments for A-D were repeated 2 times and similar results were obtained. *P <0.05 (vs. Ctrl Media), #P <0.05 (vs. MM1CM/DMSO).
### Table 1: human qPCR primers

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### Table 2: mouse qPCR primers

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<td>Rank</td>
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<td>AAGTTCTACACTCGCCGGCTAGA</td>
</tr>
</tbody>
</table>
Figure 1

A

M-CSF: + +
RANKL: - -

ND

MNCs/field (20x)

MM

B

C

D

E

RANKL

OPG

Relative mRNA

Cat K

β3

NFATc1

MMP-9

c-FMS

PU.1

ATF4

Relative mRNA

ND

MM

- NFATc1/C

- NFATc1/B

- NFATc1/A

- β-actin

F
Figure 3

A

B

C

D

LY(μM): 0 12.5 25 50

23C6 staining

TRAP staining

23C6(+) Cells/well

LY(μM): 0 12.5 25 50

TRAP(+) MNCs/well

LY(μM): 0 12.5 25 50

Rank-Luc activity

LY(μM): 0 12.5 25 50

β-gal(μg): 1.0 0.75 0.5 0

AKT-CA(μg): 0 0.25 0.5 1.0
Figure 5

A

B

C

D

E

F
Signal Transduction: Critical Role of AKT in Myeloma-induced Osteoclast Formation and Osteolysis

Huiling Cao, Ke Zhu, Lugui Qiu, Shuai Li, Hanjie Niu, Mu Hao, Shengyong Yang, Zhongfang Zhao, Yumei Lai, Judith L. Anderson, Jie Fan, Hee-Jeong Im, Di Chen, G. David Roodman and Guozhi Xiao

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