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Accessibility
Activin A promotes multiple myeloma-induced osteolysis and is a promising target for myeloma bone disease

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Understanding the pathogenesis of cancer-related bone disease is crucial to the discovery of new therapies. Here we identify activin A, a TGF-β family member, as a therapeutically amenable target exploited by multiple myeloma (MM) to alter its microenvironmental niche favoring osteolysis. Increased bone marrow plasma activin A levels were found in MM patients with osteolytic disease. MM cell engagement of marrow stromal cells enhanced activin A secretion via adhesion-mediated JNK activation. Activin A, in turn, inhibited osteoblast differentiation via SMAD2-dependent distal-less homeobox 5 down-regulation. Targeting activin A by a soluble decoy receptor reversed osteoblast inhibition, ameliorated MM disease, and inhibited tumor growth in an in vivo humanized MM model, setting the stage for testing in human clinical trials.

Tumor-related bone disease, specifically osteolytic disease, represents a major clinical burden in many cancers, including multiple myeloma (MM) (1, 2). Osteolysis is the consequence of a pathological imbalance between osteoblast (OB) and osteoclast (OC) activity in the bone marrow (BM) niche. Tumor cells activate OC through several well characterized cytokines and signaling pathways (3). However, relatively little is known about the effects of tumor cells on OB differentiation. Consequently, although the majority of clinical interventions have targeted OCs in osteolytic disease, therapeutic interventions that target OBs have been far less successful (4).

To identify potential pathways with immediate relevance to human cancer-induced bone disease, we performed broad cytokine profiling of BM plasma derived from MM patients with and without osteolytic bone disease. Forty-three cytokines associated with tumor development or involved in bone remodeling were profiled. We could not detect any expression for 25 cytokines independent of the presence of osteolysis. Of the 18 cytokines with detectable levels, only activin A demonstrated a significantly higher expression in patients with more than one OL versus patients with one or no OLs (9.7- and threefold increase, respectively; P = 0.03; Fig. 1A).

Interestingly, we observed that IL-16 and CD40 ligand were preferentially expressed in patients with osteolysis (five of six patients with osteolysis vs. three of six patients without osteolysis, P = 0.05; two of six vs. none of six expressed CD40 ligand). Whereas VEGF expression was associated with absence of osteolysis (one of six patients with osteolysis vs. three of six patients without osteolysis), but the differences did not reach statistical significance.

We further studied the association of activin A with osteolysis by comparing a larger group of MM patients at diagnosis with variable degree of bone disease versus non-MM patients. The average expression level of activin A was 112.07 pg/mL (SEM, 30.4) in MM patients with osteolytic disease (n = 15), versus 28.62 pg/mL (SEM, 6.2) in MM patients with one or fewer OLs (n = 13) and 30.6 pg/mL (SEM, 7.9) in the non-MM group (n = 10), respectively (P < 0.05; Fig. 1B). Importantly, activin A levels did not correlate with plasma IL-16 or CD40 ligand levels. This suggests that activin A is a critical pathway in tumor-induced osteolysis and establishes it as a therapeutic target with direct relevance to human cancer-related bone disease.

Results

Activin A Correlates with Osteolytic Disease in MM Patients. To identify pathways that might be involved in the pathogenesis of bone disease in MM, we performed broad cytokine profiling of BM plasma derived from MM patients with and without osteolytic bone disease. Forty-three cytokines associated with tumor development or involved in bone remodeling were profiled. We could not detect any expression for 25 cytokines independent of the presence of osteolysis. Of the 18 cytokines with detectable levels, only activin A demonstrated a significantly higher expression in patients with more than one OL versus patients with one or no OLs (9.7- and threefold increase, respectively; P = 0.03; Fig. 1A).

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not correlate with parameters reflecting tumor burden (Table S1), suggesting that activin A has a specific role in bone disease.

**JNK Activation Is Associated with MM-Induced Stromal Cell Secretion of Activin A.** To identify the main sources of activin A in the tumor niche, we analyzed activin A secretion by BMSCs, OBs, and OCs from MM patients, as well as MM cell lines and MM primary cells (Fig. 2A). BMSCs secreted high levels of activin A (average 1.8 ng/mL), whereas activin A secretion by OC was variable over a wide range. Differentiation of BMSCs to OBs markedly decreased activin A expression (6). MM primary cells and cell lines secreted very low or undetectable levels of activin A (P < 0.01). Of note, we observed that increased expression of activin A was a specific feature of tumor-conditioned BMSCs derived from MM patients compared with tumor-naïve BMSCs derived from healthy donors (P < 0.01; Fig. S1), suggesting that MM-conditioned stromal cells retain activin A secretion after ex vivo culture.

The enhanced expression of activin A in patients with MM bone disease and in tumor-conditioned BMSC (compared with tumor-naïve BMSCs or MM cells alone) led us to investigate whether activin A expression was being affected by the engagement of MM with BMSCs. We cultured several MM cell lines with tumor-naïve BMSCs expressing low basal levels of activin A. The engagement of MM cells with healthy donor-derived BMSCs significantly increased activin A levels in the coculture supernatant by 2.5- to 6-fold (P < 0.05; Fig. 2B). By analyzing the expression levels of inhibin-βA subunits, of which activin A is a dimer, and inhibin-α subunits, which form a heterodimer inhibin A, we confirmed that activin A is up-regulated in the stromal compartment by coculturing with MM cells (2.5-fold; P < 0.05; Fig. S2A).

To determine whether the induction of activin A expression was adhesion or cytokine-mediated, we used a transwell system that significantly inhibited activin A secretion (Fig. 2C), suggesting that direct MM-BMSC contact is necessary to induce activin A secretion. To further elucidate the ligand/receptor interactions leading to activin A secretion in coculture, we tested several molecules previously studied in the context of MM cell–BMSC adhesion, including CD40 ligand, osteopontin, intercellular adhesion molecule (ICAM)–1, and very late antigen (VLA)–4. Only neutralizing antibody against VLA-4 inhibited activin A secretion (20%; P < 0.05, Fig. 2C), albeit modestly. Of note, recombinant VLA-4 induced BMSC secretion of activin A, which was completely inhibited in the presence of neutralizing vascular cell adhesion molecule (VCAM)–1 antibody (Fig. S2B). These data suggest that the VLA4/VCAM-1 axis mediates activin A secretion, although the activation of other adhesion-mediated pathways likely contribute to activin A up-regulation in the presence of MM cells.

Activin A secretion by other cell types is known to occur via p38-dependent and JNK-dependent pathways (8). Because previous reports have particularly implicated activation of the JNK signaling pathway by cell-to-cell contact (9), and a highly conserved c-Jun–binding sequence is present in the JNHi64 promoter (10), we next investigated whether the JNK pathway was associated with activin A induction by coculture. Cell contact between BMSCs and MM cells was sufficient to activate the JNK pathway, evidenced by JNK phosphorylation in BMSCs by MM cells (Fig. 2D). Additionally, treatment with specific JNK inhibitor (SP600125, 20 μM) reduced activin A secretion by BMSCs alone and completely inhibited MM-induced secretion of activin A (Fig. 2E). Inhibition of the p38 pathway (SP202190) served as a positive control.
Activin A Inhibits Osteoblastic Differentiation via SMAD2-Mediated DLX5 Down-Regulation. Activin A is a TGF-β family member whose role in maintaining the balance of osteoclastogenesis and osteoblastogenesis remains controversial (5, 11, 12). Consistent with recent results, we found that activin A activated OC differentiation (Fig. S3) (13) while inhibiting OB differentiation from tumour-derived BMSCs, evidenced by decreased alkaline phosphatase (ALP) enzyme activity and by decreased mineralization (P < 0.05; Fig. 3A) (6).

We next investigated the signaling pathways responsible for activin A–mediated OB inhibition. Activin A induced SMAD2 phosphorylation in OB after 30 min of stimulation without affecting SMAD1 or β-catenin phosphorylation (Fig. 3B). Although SMAD2 downstream targets include RUNX2 and DLX5 genes (14), only DLX5 expression was markedly down-regulated in the presence of activin A by both mRNA and protein expression levels (Fig. 3C).

To determine the role of SMAD2 in OB inhibition, we transduced BMSCs with a lentivirus construct carrying a validated shRNA against human SMAD2. SMAD2 knockdown induced ALP gene expression (4.7-fold; P < 0.05) in pre-OB and addition of activin A partially reduced ALP expression but was unable to return expression to the baseline (2.26-fold increase; P < 0.05; Fig. 3D Right). Furthermore, SMAD2 knockdown derepressed DLX5 expression (1.5-fold; P < 0.05) whereas addition of exogenous activin A was unable to fully inhibit DLX5 expression (1.22-fold increase; P < 0.05; Fig. 3D Left).

We then evaluated whether DLX5 might be a crucial component of activin A–mediated impairment of OB differentiation. Osteogenic differentiation of cells transduced with control shRNA stimulated the ALP activity index (API), whereas addition of exogenous activin A decreased API by 58%. In contrast, DLX5 knockdown inhibited OB differentiation (32.3% inhibition of API) and the inhibitory effect of exogenous activin A on DLX5...
knocked-down OBs was relatively attenuated (41% inhibition; Fig. 3E) and no synergy was observed. Taken together, these data suggest that activin A affects OB differentiation mainly via SMAD2-mediated DLX5 inhibition, although other pathways may also be involved.

Finally, to determine whether activin A–induced DLX5 repression observed in our in vitro studies was directly relevant to the pathogenesis of human MM osteolysis, we performed immunohistochemistry (IHC) for DLX5 on BM biopsies from MM patients and correlated with activin A levels. Patients with high activin A levels (>50 ng/mL, n = 5) had an average of 27.8% (range, 8.9–41%) OB staining for DLX5, whereas patients with low activin A levels (<50 ng/mL, n = 5) showed 62.7% OB staining (Fig. 3F). Thus, the repression of DLX5 by activin A observed in vitro was corroborated in vivo by the reduction of DLX5-positive OBs in human MM BM samples with high activin levels and osteolytic disease.

**Activin A Inhibition by the Soluble Decoy Receptor RAP-011 Rescues MM-Induced Impairment of OB Differentiation.** As shown in Fig. S1, MM-conditioned BMSCs secrete high levels of activin A compared with naive BMSCs. To verify whether activin A might represent a potential therapeutic target against MM-induced osteolysis, we treated MM-derived BMSCs with an anti–activin A neutralizing antibody. A significant increase in OB differentiation was observed, confirmed by up-regulated ALP mRNA levels and enhanced ALP staining. This was also associated with a significant increase in bone mineralization assayed by alizarin red staining (three- and fivefold increase by 0.1 and 1 μg/mL, respectively; P < 0.05; Fig. S4).

Although activin A expression is down-regulated by OB differentiation (Fig. 2A) (6), we observed that MM cells stimulated activin A release in OB culture (Fig. S5), confirming our previous results (Fig. 2B).

We therefore hypothesized that if activin A was involved in coupling MM–OB interactions, its inhibition should reverse the OB impairment induced by MM cells, resulting in restoration of the pospho-SMAD2 and DLX5 axis. RAP-011 is a soluble activin A receptor that binds to activin A with high affinity (3.39 pM). Previous work in nonmalignant systems has shown that RAP-011 enhances OB mineralization and increases bone density in an osteoporotic mouse model (7). The humanized counterpart (ACE-011) is currently in clinical trials in patients with osteoporosis (15). Other than activin A, soluble activin A receptors bind four other ligands—bone morphogenic proteins (BMPs) 2, 3, 7 and inhibin A—at micromolar concentrations (Fig. S6A) (7, 16, 17).

As shown in Fig. S6B, activin A could be efficiently immunodepleted from MM-derived stromal supernatant, but we were unable to show any binding to BMP3, inhibin, BMP2, and BMP7. We therefore chose to use RAP-011 to achieve activin A inhibition in our models.

Exposure of BMSCs to both MM cell lines (MOLP5, INA6) and MM primary cells decreased OB differentiation, evident by decreased ALP activity; conversely, RAP-011 restored osteoblastic differentiation, suggesting implication of activin A in this inhibition (Fig. 4A). Several reports have also suggested that OBs reciprocally inhibit the proliferation and survival of MM cells (18, 19). We also observed that, compared with untreated BMSCs and OBs, OBs differentiated in the presence of RAP-011 did not support MM cell proliferation (Fig. S7). Therefore, RAP-011 stimulatory effects on OBs translated in inhibition of MM cell growth.

Consistent with our studies, MM engagement of BMSCs was associated with increased SMAD2 phosphorylation in OB that was inhibited by RAP-011 (Fig. 4B). Additionally, whereas MM cell lines and primary cells down-regulated DLX5 expression in cocultured OBs to levels found in undifferentiated BMSCs, treatment with RAP-011 rescued DLX5 expression to comparable levels as noted in OBs (Fig. 4C).

Taken together, these results suggest that RAP-011, an inhibitor of activin A, enhances OB differentiation in the presence of MM cells via rescuing DLX5 expression, and this results in an indirect inhibition of MM cell proliferation.

**RAP-011 Improves Osteolytic Disease and Exerts Antitumor Activity in a Humanized Myeloma Model.** To investigate whether activin A inhibition could overcome MM osteolytic disease and impact tumor growth in vivo, we used a well established mouse model of MM bone disease (SCID-hu model) (20).

In the presence of MM cells, bone volume per tissue volume (BV/TV%) was significantly decreased compared with tumor-naive bone chips [mean, 17.6% ± 9 (SD) vs. 52.5% ± 3.7, respectively; P < 0.01]. Treatment with RAP-011 markedly restored BV/TV% nearly back to tumor-naive levels, although bone fraction remained somewhat decreased even upon RAP-011 treatment (50.1% ± 25.6; P < 0.05; Fig. S4). Similarly, OB number per mm² was markedly decreased in the bone chips injected with MM cells compared tumor-naive bones (645.4 ± 476.1 vs. 2,490 ± 377, respectively; P < 0.01); conversely, RAP-011 treatment reversed this negative trend, although we did not observe a complete rescue (1,356.2 ± 270.48; P < 0.05; Fig. 5B). Of note, we observed a decreased OC number/bone area, suggesting that RAP-011 has a negative effect on OC (Fig. 5C).

High-resolution CT scan performed on the human bones demonstrated increased bone mass (a representative image is shown in Fig. 5D Left). A 75% decrease in the number of OLS was observed in the RAP-011–treated group (P < 0.02; Fig. 5D Right). Importantly, DLX5 expression analyzed by IHC was increased along the bone surface in the RAP-011 treated bones, whereas almost no DLX5 staining was evident on the trabecular surface of the untreated bones (Fig. S8A).

As increasing OB number may result in inhibition of MM proliferation, we next evaluated the effects of modifying the OC/OB niche on the tumor cell compartment. We quantified MM cell growth in our in vivo model using soluble human IL-6 receptor (sHuIL6R) secreted by INA-6 MM cells. Treatment with RAP-011 showed significant anti-MM effects as seen by a decrease in sHuIL6R levels observed as early as week 2 that persisted over the 4-week treatment period (n = 11; P < 0.01; Fig. 5E). Additionally, direct quantification of MM burden in the human bones by counting GFP + cells in midsection bones through the entire bone area also revealed a reduction of MM cells in the RAP-011–treated animals (P < 0.01; Fig. 5F and Fig. S8B).

These data support the conclusion that activin A is a promising target for the treatment of MM-induced OLS.

**Discussion**

Bone disease represents a significant clinical problem in the management of MM patients, yet current knowledge of the MM–microenvironment interactions leading to osteolysis is limited, as are the treatment options available to our patients. In this study we have identified activin A among a broad panel of cytokines as a critical player in MM-induced bone disease. We show that activin A is a stromally derived OB inhibitor induced by MM cells via the JNK pathway. MM-induced activin A down-regulates DLX5 gene expression via SMAD2 activation that can be effectively blocked by activin A inhibition with a soluble receptor, RAP-011. Our in vivo animal studies confirmed the anabolic effects of activin A inhibition. Furthermore, the antitumor activity observed with RAP-011 supports the notion that targeting the tumor niche cross-talk via activin A inhibition is a promising therapeutic strategy in tumor-related bone disease.

Activin A is a TGF-β superfamily member most commonly associated with embryogenesis and gonadal hormone signaling (21). In addition, activin A is involved in bone remodeling with growth stimulatory effects on OCS (13). In contrast, the effects on OB differentiation are still unclear; both inhibition and promotion...
of OB differentiation by exogenous activin have been reported (5, 6, 11). However, in vivo evidence supports the hypothesis of an inhibitory role on OBs for activin A. Both transgenic expression of inhibin or treatment with a soluble receptor for activin, RAP-011, result in enhanced bone formation rate and bone mass in vivo (7, 22). Our data confirmed these findings and demonstrate DLX5 down-regulation as the main mechanism of action for activin A. Indeed, DLX5 is a critical transcription factor in OB differentiation, regulating the expression of osterix (23). DLX5 is also a common gene target for other TGF-β family members as well as...
activin A secretion mediates OB inhibition both in vitro and in vivo and is regulated in part by SMAD2–DLX5 signaling. Targeting this unique pathway by using RAP-011, a soluble activin A receptor, we have demonstrated reversal of these effects. RAP-011 treatment prevents the development of Ols and inhibits tumor growth in a humanized model of MM bone disease. These results suggest that targeting the MM–microenvironment interac-
tions with the purpose of restoring bone homeostasis and creating a hostile niche for tumor cell growth may provide an alternative approach for the development of anticancer thera-
pies. Indeed, interventions that alter the malignant cell niche may be a promising dimension of anticancer therapeutics.

Experimental Procedures

Patients. West studied BM plasma from 28 patients with MM at diagnosis and 10 non-
MM plasma samples as control subjects including 3 acute leukemia (n = 5), non-
Hodgkin lymphoma (n = 1), thyroid cancer (n = 1), primary amyloidosis (n = 1), osteoporosis (n = 1), and anemia of chronic disease (n = 1). Additionally, 10 BM biopsy samples were obtained in MM patients to perform IHC analysis for DLX5. All patients provided written informed consent per the Declaration of Helsinki, and approval was obtained by the institutional review board of the Massachusetts General Hospital Cancer Center (Boston, MA).

Mouse Model. All animal studies were conducted according to protocols approved by the institutional Animal Care and Use Committee. The SCID-hu model was generated as previously described (20). Four weeks after iNAl injection, we started s.c. injections of RAP-011 (10 mg/kg twice per week) for 28d. Two weeks after the end of the treatment schedule, the mice were killed and eight matching bone chips harvested. Each bone was sectioned in half and processed for either cryosectioning or paraffin-embedding. Six non-
tumor-injected bones were obtained from fetal bones of similar age and processed like the tumor-injected bones, except that animals were not implanted with MM. These bones were used as controls. See SI Experimental Procedures for more information.

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