A NOTCH3-CXCL12-driven myeloma-tumor niche signaling axis promotes chemoresistance in multiple myeloma

Hayley M. Sabol,[1] Cody Ashby,[2,3] Manish Adhikari,[1] Aric Anloague,[1] Japneet Kaur,[1] Sharmin Khan,[1] Samrat Roy Choudhury,[3,4] Carolina Schinke,[3,5] Michela Palmieri,[6] C. Lowry Barnes,[7] Elena Ambrogini,[6] Intawat Nookaew[2,3] and Jesus Delgado-Calle[1,3]

[1]Physiology and Cell Biology, University of Arkansas for Medical Sciences; [2]Department of Biomedical Informatics, University of Arkansas for Medical Sciences; [3]Winthrop P. Rockefeller Cancer Institute, University of Arkansas for Medical Sciences; [4]Pediatric Hematology-Oncology, Arkansas Children's Research Institute, University of Arkansas for Medical Sciences; [5]Myeloma Center, University of Arkansas for Medical Sciences; [6]Division of Endocrinology and Metabolism, Center for Osteoporosis and Metabolic Bone Diseases and Center for Musculoskeletal Disease Research, University of Arkansas for Medical Sciences and Central Arkansas Veterans Healthcare System and [7]Department of Orthopedic Surgery, University of Arkansas for Medical Sciences, Little Rock, AR, USA

Correspondence: J. Delgado-Calle
jdelgadocalle@uams.edu

Received: October 10, 2023.
Accepted: February 13, 2024.
Early view: February 22, 2024.

https://doi.org/10.3324/haematol.2023.284443

Abstract

Multiple myeloma (MM) remains incurable due to disease relapse and drug resistance. Notch signals from the tumor microenvironment (TME) confer chemoresistance, but the cellular and molecular mechanisms are not entirely understood. Using clinical and transcriptomic datasets, we found that *NOTCH3* is upregulated in CD138$^+$ cells from newly diagnosed MM (NDMM) patients compared to healthy individuals and increased in progression/relapsed MM (PRMM) patients. Further, ND-MM patients with high *NOTCH3* expression exhibited worse responses to bortezomib (BOR)-based therapies. Cells of the TME, including osteocytes, upregulated *NOTCH3* in MM cells and protected them from apoptosis induced by BOR. NOTCH3 activation (NOTCH3^{OE}) in MM cells decreased BOR anti-MM efficacy and its ability to improve survival in *in vivo* myeloma models. Molecular analyses revealed that NDMM and PRMM patients with high NOTCH3 exhibit CXCL12 upregulation. TME cells upregulated CXCL12 and activated the CXCR4 pathway in MM cells in a NOTCH3-dependent manner. Moreover, genetic or pharmacologic inhibition of CXCL12 in NOTCH3^{OE} MM cells restored sensitivity to BOR regimes *in vitro* and in human bones bearing NOTCH3^{OE} MM tumors cultured *ex vivo*. Our clinical and preclinical data unravel a novel NOTCH3-CXCL12 pro-survival signaling axis in the TME and suggest that osteocytes transmit chemoresistance signals to MM cells.

Introduction

Multiple myeloma (MM) is a hematological cancer characterized by the accumulation of malignant plasma cells in the bone marrow and the overproduction of monoclonal proteins (M-proteins). First-line therapy in MM includes proteasome inhibitors, such as bortezomib (BOR), administered alone or in combination regimens.[1] Despite high response rates to BOR-based therapies, MM remains incurable due to the development of chemoresistance and disease recurrence after transient remissions.

Although MM cells exhibit genetic and molecular heterogeneity, they depend highly on the bone marrow niche. MM cells localize in specialized niches in the marrow where tumor microenvironment (TME) cells promote their proliferation and allow them to escape anti-MM therapies by promoting *de novo* chemoresistance.[2-4] Notch signaling activation downstream of the four NOTCH (1-4) receptors in MM cells plays a critical role in transforming the bone marrow into a permissive niche for MM progression and chemoresistance.[5,6] Pharmacologic pan-inhibition of Notch in the TME induces apoptosis in MM cells and enhances sensitivity to chemotherapy.[6,7] Notch signals from stromal cells contribute to *de novo* drug resistance to proteasome inhibitors in MM cells.[8] Yet, the role of other TME cells and how MM cells integrate and execute TME Notch signals are not completely understood.

Prior studies have focused on NOTCH1 or 2, as they are expressed at relatively higher levels than *NOTCH3* or 4 in NDMM patients.[9] However, we recently reported that 30% of NDMM

patients exhibit *NOTCH3* expression levels comparable to *NOTCH1* or *NOTCH2*.[9] Moreover, we showed that osteocytes, the most abundant bone cells,[10] rapidly upregulate *NOTCH3* expression in MM cells,[9] emphasizing the need to understand the role of NOTCH3 in MM further. In this study, we describe a novel NOTCH3-CXCL12 signaling axis of TME-mediated chemoresistance and identify the osteocyte as a new TME cell capable of influencing MM therapeutic responses to BOR-based regimes.

Methods

Study population

The mRNA expression of *NOTCH* receptors was studied in CD138$^+$ plasma cells from a previously described institutional cohort of NDMM patients (N=52; t(4;14=8; t(11;14)=10; t(14;16)=9; t(14;20)=6; D1=12; D2=7) and age-matched healthy donors (N=4).[11] In order to study the impact of *NOTCH3* on the transcriptome and clinical outcomes of MM patients, we obtained clinical and gene expression data from NDMM patients from the Multiple Myeloma Research Foundation (MMRF) CoMMpass registry (*clinicaltrial gov. Identifier: NCT01454297*, version IA15).

Bioinformatic analyses

Gene expression and mutation analyses are described in the *Online Supplementary Appendix*.

Cell culture

MM osteocyte (5:1) or MM stroma (5:1) co-cultures were established as described before.[9,12] Co-cultures were treated with plerixafor (25 uM), BOR (3 nM), VRd (BOR: 2 nM, lenalidomide: 1 uM, dexamethasone: 10 nM) and refreshed every 24 hours (h). Cell characteristics, reagents, and methods for apoptosis/proliferation assays are described in the *Online Supplementary Appendix*.

Gene expression

Methods to quantify mRNA (quantitative polymerase chain reaction [qPCR]) and protein expression (western blot and enzyme-linked immunosorbant assay) are described in the *Online Supplementary Appendix*.

Genetic inhibition/activation in multiple myeloma cells

Methods used to manipulate *NOTCH3/CXCL12* expression are described in the *Online Supplementary Appendix*.

Ex vivo organ cultures

Ex vivo MM murine bone organ cultures were established as described before.[12] *Ex vivo* MM human bone organ cultures were established with human cancellous bone fragments similar in size obtained from femoral heads discarded after hip arthroplasty (see the *Online Supplementary Appendix* for details).

Animal studies

Seven-week-old immunodeficient littermate NSG female and male mice were injected intravenously with 5x10^5 OPM2-Scr MM cells, OPM2-Notch3^{OE} MM cells, or saline. Equal numbers of female and male mice were used per group. After 1 week, mice were randomized based on tumor burden and bone disease to two groups (1) vehicle (saline) or (2) 0.1 mg/kg BOR (intraperitoneally [i.p.]) 5x/week (wk) for 3 wks. In order to assess survival, the health of mice was monitored daily, and mice were euthanized at first sign of back leg paralysis. The sample size was calculated based on previous studies.[13,14] MicroCT analyses were performed as shown before.[12,14]

Statistics

Data were analyzed using GraphPad (GraphPad Software Inc, San Diego, CA, USA). Differences in means were analyzed using a combination of unpaired *t* test, one-way or two-way ANOVA tests, followed by pairwise multiple comparisons (Tukey *post hoc* test). Values were reported as means ± standard deviation (SD). *P* values ≤0.05 were considered statistically significant. Data analysis was performed in a blinded fashion.

Study approvals

All procedures involving animals were performed in accordance with guidelines issued by the University of Arkansas for Medical Sciences IACUC (protocol #2022200000489). Collection and de-identification of human bone samples was coordinated by the UAMS Winthrop P. Rockefeller Cancer Institute TBAPS and approved by the UAMS Institutional Review Board (IRB) (protocol # 262940). All participants provided written, informed consent before study procedures occurred, with continuous consent ensured throughout participation. NDMM patients and healthy donors were consented with IRB approval (protocol IRB #260284) for bone marrow aspirates for CD138$^+$ cell selection.

Results

NOTCH3 expression is increased in newly diagnosed patients by a non-mutational, tumor microenvironment cell-dependent mechanism

In order to investigate the integration of Notch signals by MM cells, we first compared the expression of the NOTCH receptors in CD138$^+$ plasma cells using an institutional cohort of NDMM patients of major molecular MM subgroups, including primary translocations t(4;14), t(11;14), t(14;16) and t(14:20), hyperdiploid subgroups D1 and D2, and age-matched healthy donors (Figure 1A). We found no differences in the expression of *NOTCH1* or *NOTCH2* in NDMM patients compared to healthy donors, except for a *NOTCH2* upregulation detected in the t(14;16) MM subgroup. *NOTCH4* was decreased in all the MM subgroups, except the t(4;14) MM subgroup. In contrast, *NOTCH3* ex-

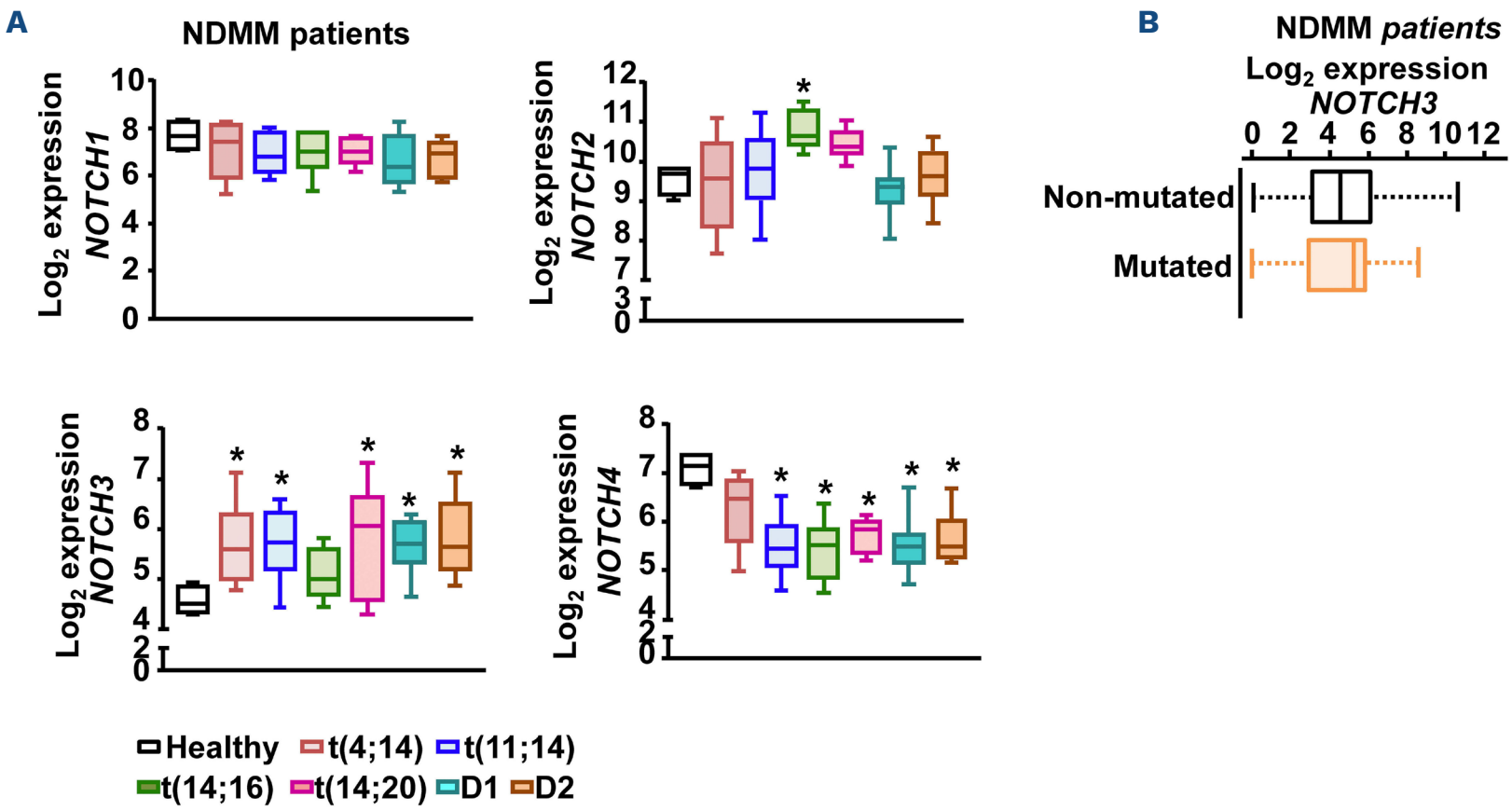


Figure 1. *NOTCH3* expression is increased in newly diagnosed multiple myeloma patients and regulated by the cells of the tumor niche. (A) Gene expression of *NOTCH* 1-4 receptors in CD138$^+$ cells from newly diagnosed multiple myeloma (NDMM) or healthy donors. N=52 patients. *P<0.05 *versus* healthy donors by one-way ANOVA, followed by a Tukey *post hoc* test. Boxes show the data interquartile range, the middle line in the box represents the median, and whiskers the 95% confidence interval of the mean. (B) *NOTCH3* gene expression in CD138$^+$ cells from NDMM patients with or without point mutations in the *NOTCH3* gene. N=725 (12 with mutations) NDMM patients.

pression was elevated in all NDMM subgroups, although it did not reach statistical significance in the t(14;16) subgroup. In order to understand the mechanisms behind *NOTCH3* upregulation in NDMM, we next mined the MMRF CoMMpass cohort dataset to investigate whether *NOTCH3* expression levels in CD138$^+$ plasma cells from NDMM patients correlated with gain- or loss-of-function mutations in the *NOTCH3* gene. We identified several mutations in the *NOTCH3* gene (*Online Supplementary Table S1*) but did not find an association with *NOTCH3* expression (Figure 1B). In addition, we investigated the presence of *NOTCH3* mutations in a panel of 69 human MM cell lines and identified only one cell line, FLAM76 (t11;14), with a frameshift mutation (AGGGG/AGGG). Next, we investigated if constituents of the TME upregulate *NOTCH3* in MM cells. As shown before,[9] co-culture with osteocytes upregulated *NOTCH3* and increased the expression of the Notch target gene *HES1* in several murine and human MM cell lines (*Online Supplementary Figure S1A-C*). In contrast, no changes were detected in *NOTCH1*, *2*, or *4*. Like osteocytes, co-culture with stromal cells, another important cellular component of the MM-TME,[2] selectively upregulated *NOTCH3* expression and activated Notch signaling in MM cells (*Online Supplementary Figure S1D-F*). Together, these data support that non-mutational, TME cell-dependent NOTCH3 activation occurs in specialized niches in the bone marrow of NDMM patients.

NOTCH3 expression correlates with worse responses to bortezomib-based therapies in newly diagnosed multiple myeloma patients

Further bioinformatic mining of the CoMMpass cohort revealed that NDMM patients with high *NOTCH3* expression exhibited upregulation and enrichment in genes associated with processes involved in chemoresistance, including responses to drugs (Figure 2A) and cell-adhesion pathways (Figure 2B). Moreover, gene set enrichment analysis (GSEA) revealed that the transcriptome of high *NOTCH3* NDMM patients is enriched in genes associated with poor responses to BOR therapy (Figure 2C).[15] Poised by these observations, we next investigated if high expression levels of *NOTCH3* are associated with poor prognosis in NDMM patients. No significant correlations were observed between the expression of *NOTCH3* and OS or PFS (*Online Supplementary Figure S2A, B*). However, after stratification by therapy (combined graph is shown in *Online Supplementary Figure S2C*), we observed that NDMM patients with high *NOTCH3* had significantly worse PFS when receiving BOR-based therapies *versus* other therapies not including BOR (Figure 2D). In contrast, NDMM patients with low *NOTCH3* levels exhibited similar PFS regardless of the therapy received. Next, we rationalized that if CD138$^+$ MM cells expressing high *NOTCH3* are chemoresistant, the expression of *NOTCH3* should increase in PRMM patients. Consistent with this notion, we found an increase in *NOTCH3* expression in PRMM patients using

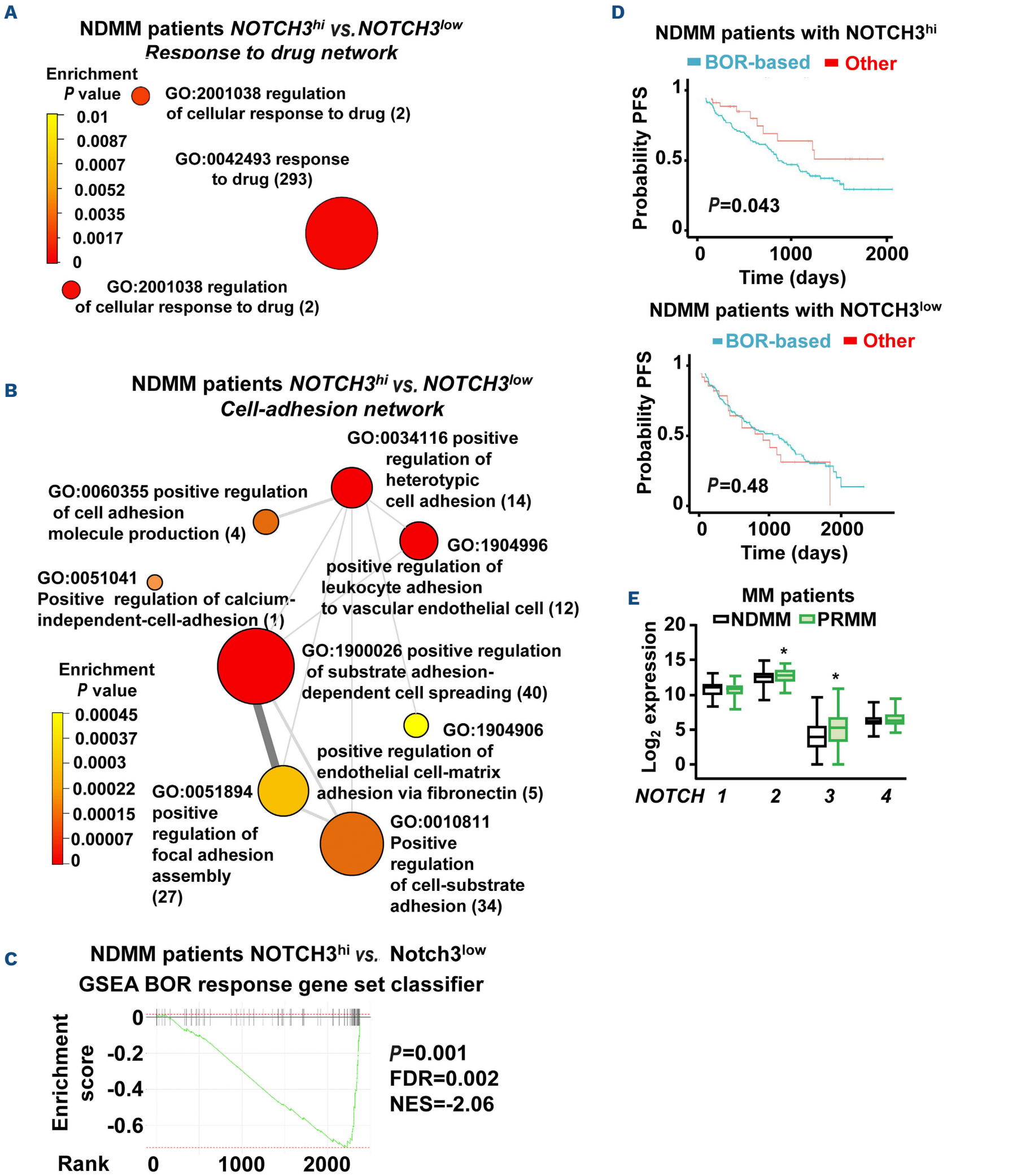


Figure 2. *NOTCH3* expression is increased in relapsed multiple myeloma patients and correlates with poor responses to bortezomib-based therapies. Network plot of selected upregulated functional enrichment analysis of gene ontology (GO) terms related to (A) responses to drugs or (B) cell-adhesion in CD138+ cells from newly diagnosed multiple myeloma (NDMM) patients with high *versus* low *NOTCH3* expression. The size of the circles represents the number of genes in the individual GO terms. The

Continued on following page.

thickness of the lines represents the number of overlapped genes between the individual GO terms. (C) Gene set enrichment analysis (GSEA) shows NDMM patients with high *NOTCH3* have enrichment in genes involved in poor responses to bortezomib (BOR) therapy in MM patients compared to NDMM patients with low NOTCH3 expression. Data were analyzed using a weighted Kolmogorov-Smirnov-like statistical test. (D) Kaplan-Meier plot of the progression-free survival (PFS) of NDMM patients with high (top) *versus* low (bottom) *NOTCH3* expression receiving BOR-based (blue line) *versus* other therapies (other) not including BOR (red line). N=708 patients. Data were analyzed using a log-rank (Mantel-Cox) test. (E) Gene expression of *NOTCH* 1-4 receptors in $CD138^+$ cells from paired diagnosis (NDMM) and progression/relapsed MM (PRMM) patients. N=70/group. **P*<0.05 *versus* diagnosis by Student's *t* test. Boxes show the data interquartile range, the middle line in the box represents the median, and whiskers the 95% confidence interval of the mean. FDR: false discovery rate; NES: normalized enrichment score.

paired diagnosis-relapse samples of MM patients included in the CoMMpass cohort (Figure 2E). These results suggest that NOTCH3-regulated transcriptional reprogramming of MM cells promotes drug resistance to BOR-based therapies and associates with poor clinical outcomes.

NOTCH3 signaling mediates tumor microenvironment-mediated bortezomib chemoresistance in multiple myeloma cells

In order to investigate the impact of NOTCH3 signaling on drug resistance to BOR-based therapies, we selected murine 5TGM1 and human U266 MM cells, which exhibit higher levels of NOTCH3 expression/activation,[9] and human OPM2 MM cells, with lower NOTCH3 levels (*Online Supplementary Figure S3A*), and determined that BOR therapy does not affect NOTCH expression in these cell lines (*Online Supplementary Figure S3B*). Then, we knocked down *NOTCH3* (*NOTCH3*^{KD}) in 5TGM1[9] and U266 MM cells (*Online Supplementary Figure 3C*) and established co-cultures with cells of the TME (Figure 3A). Control (Scr) and *NOTCH3*^{KD} MM cells cultured alone exhibited similar apoptotic responses to BOR and the triple regime VRd (BOR + dexamethasone + lenalidomide), frequently used in induction therapy for NDMM. Co-culture with osteocytes decreased by ~50% the apoptosis induced by BOR or VRd in control MM cells, while this protection was lost in Notch3^{KD} MM cells (Figure 3A, B). Second, we used CRISPR-mediated transcriptional activation from the endogenous *NOTCH3* loci to promote a more physiological activation of NOTCH3 in OPM2 MM cells (*NOTCH3*^{OE} cells) while permitting further *NOTCH3* regulation by the TME (*Online Supplementary Figure S3D*). NOTCH3 activation did not alter the anti-MM efficacy of these therapies in MM cells cultured alone but enhanced the pro-survival effects of osteocytes in *in vitro* co-cultures exposed to BOR or VRd (Figure 3C). Similar responses to *NOTCH3* inhibition/activation in MM cells exposed to BOR or VRd were also observed in co-cultures with stromal cells (*Online Supplementary Figure S4A-C*). Co-culture with osteocytes or genetic manipulation of *NOTCH3* did not affect the baseline levels of apoptosis in MM cells cultured in the absence of BOR (*Online Supplementary Figure S4D-E*).

Next, we injected Scr and *NOTCH3*^{OE} OPM2 MM cells in immunodeficient mice and treated them with BOR after the tumors engrafted. BOR decreased tumor progression, reduced tumor burden (~60%), and improved survival in mice injected with control MM cells (Figure 4A-C). In contrast, BOR only reduced tumor burden by 23% in mice bearing *NOTCH3*^{OE} MM cells and had no impact on survival. Lastly, to assess the effects of *NOTCH3* inhibition on responses to BOR-based therapies, we established *ex vivo* MM bone organ cultures (Figure 4D), a system that recapitulates the spatial dimension, cellular diversity, and molecular networks of the TME in a controlled setting.[16] Scr or *NOTCH3*^{KD} MM cells were allowed to colonize calvarial bones, treated with BOR, and MM-secreted paraprotein levels were quantified in the media to assess tumor growth (Figure 4E, F; *Online Supplementary Figures S5A, C*). BOR exhibited higher anti-MM efficacy in bones bearing 5TGM1 or U266 *NOTCH3*^{KD} MM cells compared to Scr MM cells (Figure 4E, F). Similarly, treatment of bones bearing 5TGM1 *NOTCH3*^{KD} MM cells with VRd resulted in better tumor reduction compared to bones bearing control 5TGM1 tumors (*Online Supplementary Figure S5B*). Together, this set of experiments supports that NOTCH3 integrates TME-mediated Notch signals in MM cells and confers chemoresistance/sensitivity to BOR-based therapies.

Because we previously reported that NDMM patients with high NOTCH3 have a gene signature consistent with increased osteoclastogenic potential,[9] we examined the impact of NOTCH3 activation on MM-induced bone disease. We found that *NOTCH3*^{OE} MM tumors led to greater reductions in cancellous bone mass and higher levels of the bone resorption biomarker CTX compared to control tumors (*Online Supplementary Figure S6A-C*), but no differences were detected in the levels of the bone formation marker P1NP (*Online Supplementary Figure S6D*). BOR therapy improved cancellous bone mass and P1NP and reduced CTX in mice bearing control MM cells, but had no effect on *NOTCH3*^{OE} MM-bearing mice. Consistent with increased MM osteoclastogenic potential, *NOTCH3*^{OE} MM cells expressed higher mRNA levels of *RANKL*, which were further enhanced by co-culture with osteocytes or stromal cells (*Online Supplementary Figure S6E*).

NOTCH3 transcriptional reprogramming increases the expression of CXC chemokines in multiple myeloma cells

In order to determine the molecular mechanism(s) by which TME-mediated NOTCH3 signaling dictates responses to BOR-based therapies in MM cells, we compared the transcriptome of NDMM patients with high *versus* low *NOTCH3*

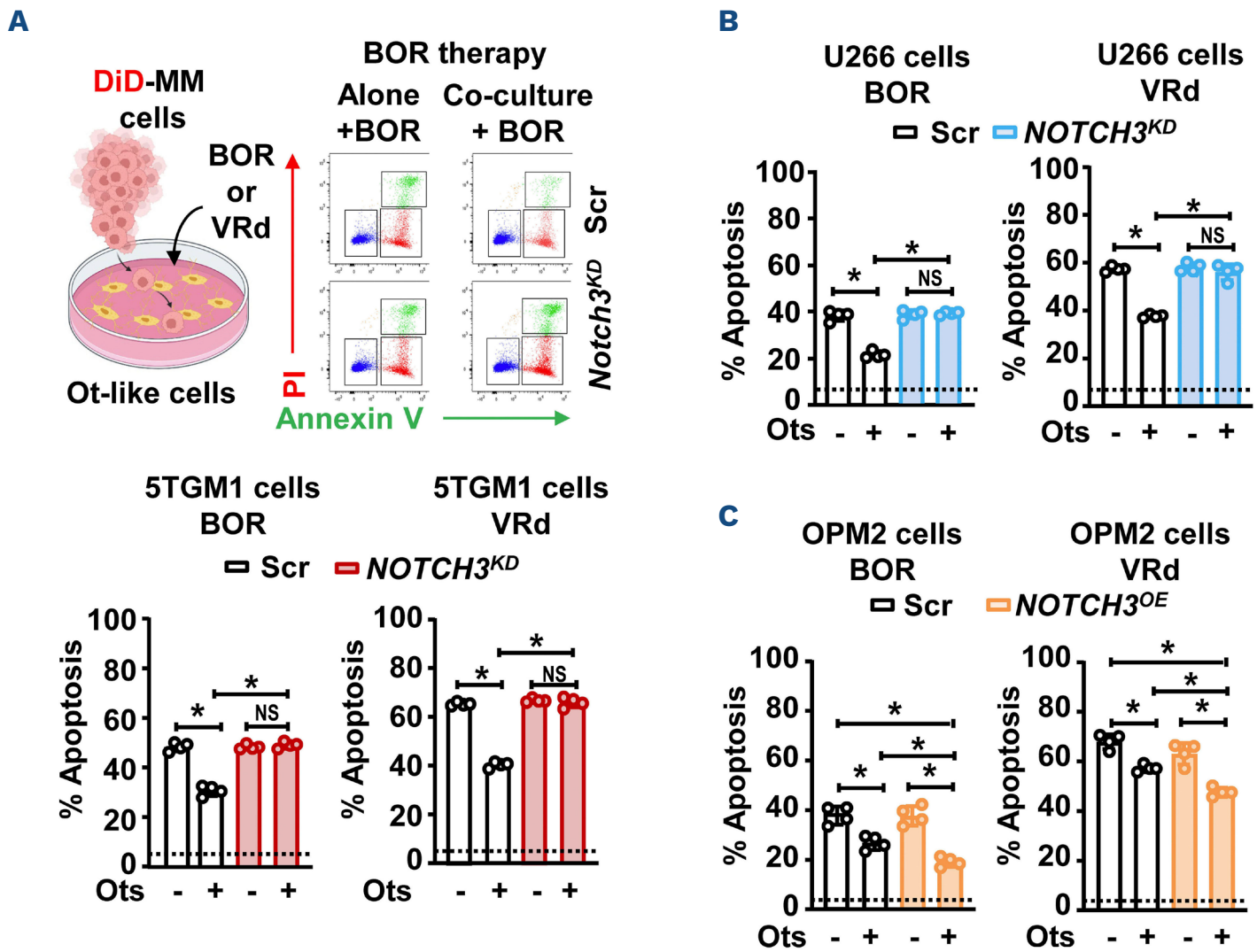


Figure 3. NOTCH3 integrates tumor micorenvironment-mediated signals dictating multiple myeloma cell responses to bortezomib-based therapies. (A) Multiple myeloma (MM) cells were co-cultured with osteocytes (Ots) and treated with bortezomib (BOR; 48 hours [h]) or dexamethasone + BOR + lenalidomide (VRd; 24 h). Percent apoptosis in scramble (Scr) or *NOTCH3* knockdown (*NOTCH3*^{KD}) 5TGM1 or U266 (B) MM cells, and in Scr or *NOTCH3*-activated (*NOTCH3*^{OE}) OPM2 (C) MM cells co-cultured in the absence/presence of Ots and treated with/without BOR or VRd. N=4/group. *P<0.05 by two-way ANOVA, followed by a Tukey *post hoc* test. The dotted line represents the percent apoptosis in vehicle-treated Scr MM cells cultured alone. NS: non-significant. Data are shown as mean ± standard deviation; each dot represents an independent sample. Representative experiments out of 2 are shown. DiD: cell label dye; PI: propidium iodide.

expression (*Online Supplementary Figure S7*). We found enrichment in GO terms related to chemokine signaling, CXCR chemokine signaling, chemotaxis, and cell adhesion (*Online Supplementary Figure S7A-C*), and upregulation of cytokine-cytokine receptor interaction, chemokine signaling pathways, and cell adhesion molecules pathways in NDMM patients with high *NOTCH3* expression (Figure 5A). Moreover, several members of the CXC chemokine family were upregulated in NDMM patients with high *NOTCH3* (*Online Supplementary Figure S7D*). We focused on *CXCL12* because it has been previously linked to cell adhesion-mediated drug resistance in MM.[17,18] We found a strong positive correlation between *CXCL12* and *NOTCH3* expression in NDMM and PRMM patients (Figure 5B). Similar to *NOTCH3*, the expression of *CXCL12* also increased in PRMM patients compared to levels at diagnosis (Figure 5C). Additionally, we mined a previously published single-cell RNA-sequencing data set of CD138^{+} plasma cells from PRMM patients[19] and found co-localization of *NOTCH3* and *CXCL12* expression in a subset of CD138^{+} plasma cells from patients with primary refractory MM (*Online Supplementary Figure S8*). Based on this clinical data, we hypothesized that TME-mediated NOTCH3 signaling increases *CXCL12* expression in MM cells. Osteocytes increased the expression of *CXCL12* in murine and human MM cells. This increase was prevented in *NOTCH3*^{KD} cells and further increased in *NOTCH3*^{OE} MM cells (Figure 5D-F). A similar regulation of *CXCL12* by NOTCH3 was seen in co-cultures with stromal cells (*Online Supplementary Figure S9*). Together, these clinical and cellular data demonstrate that NOTCH3 signaling regulates *CXCL12* expression in MM cells.

Autocrine CXCL12-CXCR4 signaling mediates NOTCH3-induced chemoresistance in multiple myeloma cells

Next, we investigated the contribution of CXCL12 to the NOTCH3-mediated acquired chemoresistance triggered by the TME. We found that TME osteocytes activated NOTCH3 signaling by cleaving NICD3, but not NICD1 or 2 (*Online Supplementary Figure S10*) and increased the phosphorylation of the CXCL12 receptor CXCR4 and the downstream targets ERK 1/2 and AKT in MM cells. These effects were fully prevented in *NOTCH3*^{KD} and enhanced in *NOTCH3*^{OE} MM cells

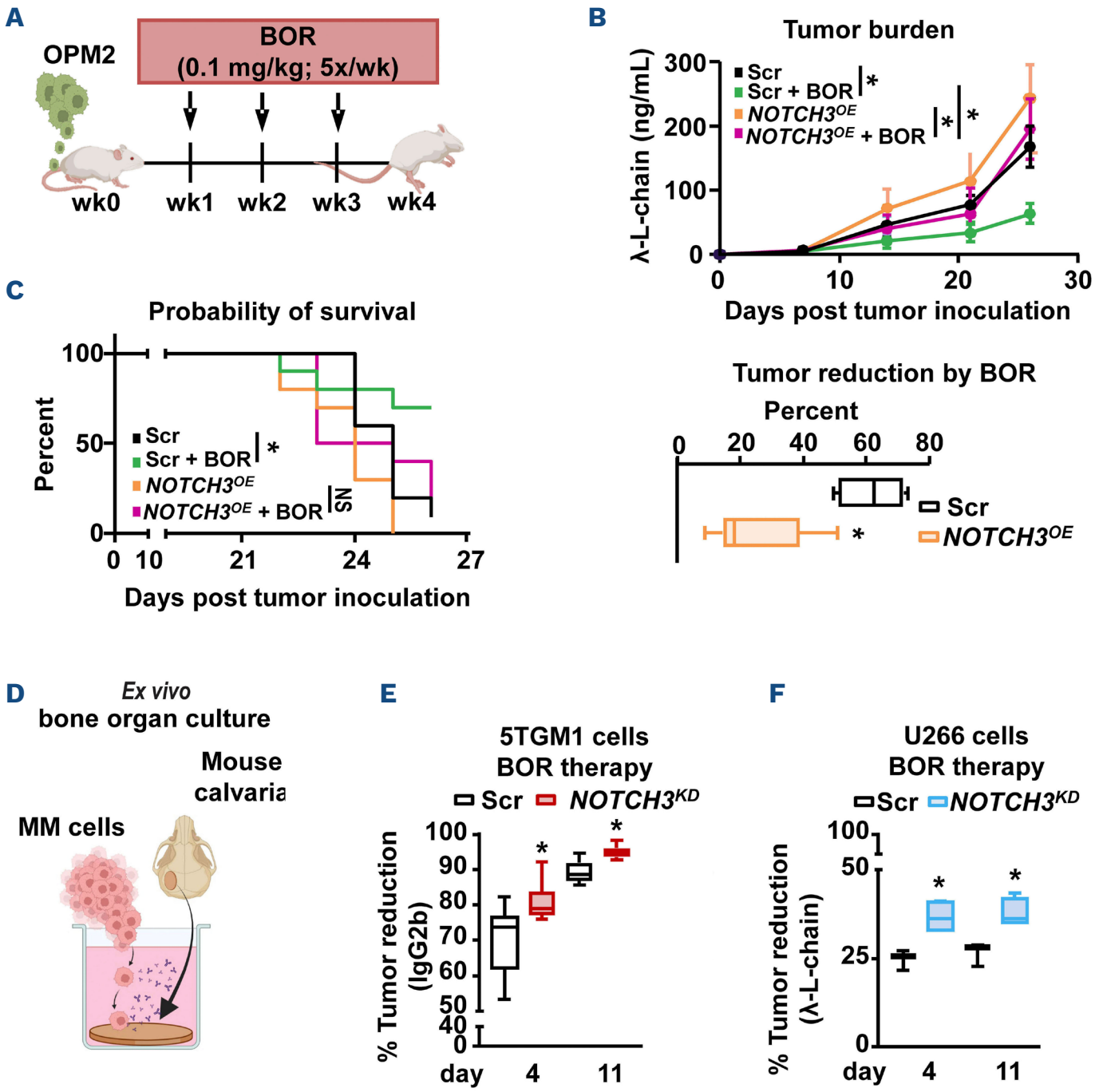


Figure 4. NOTCH3 activation in multiple myeloma cells promotes chemoresistance to bortezomib therapy. (A) Experimental design. Tumor progression and bortezomib (BOR)-induced tumor reduction (B) and probability of survival (C) in mice injected with scramble (Scr) or *NOTCH3*-activated (*NOTCH3*^{OE}) OPM2 MM cells treated with/without BOR. N=6/11 mice/group. A two-way ANOVA test was used for (B, endpoint), followed by a Tukey *post hoc* test. Tumor reduction by BOR therapy by Student's *t* test, **P*<0.05 *versus* mice bearing Scr tumors treated with BOR. For (C), a log-rank (Mantel-Cox) test was performed. (D) Tumor reduction by BOR in *ex vivo* organ cultures of calvarial disc bones from KaLwRijHsd bearing murine 5TGM1 (E) or NSG mice bearing human U266 (F) Scr/*NOTCH3* knockdown (*NOTCH3*^{KD}) MM cells. N=4-8/group. **P*<0.05 *versus* Scr MM cells treated with BOR by Student's *t* test for each time point. NS: non-significant. wk: week. Boxes show the data interquartile range, the middle line in the box represents the median, and whiskers the 95% confidence interval of the mean (D, E, F).

(Figure 6A, B; *Online Supplementary Figure S11*), indicating that CXCL12-CXCR4 signaling in MM cells depends on NOTCH3 signals. In order to further explore the role of CXCL12-CXCR4 signaling on responses to BOR-based therapies, we first employed plerixafor, a selective inhibitor of CXCR4. Plerixafor fully restored sensitivity to BOR and VRd in *NOTCH3*^{OE} MM cells co-cultured with osteocytes or stromal cells (Figure 6C, D; *Online Supplementary Figure S12A*). Because cells of the TME are thought to be an abundant source of CXCL12 in the MM-TME,[20,21] we examined the specific contribution of MM-derived CXCL12 by silencing *CXCL12* in *NOTCH3*^{OE} MM cells (*Online Supplementary Figure S12B*). As seen with plerixafor, genetic inhibition of *CXCL12* in MM cells restored the anti-MM efficacy of BOR and VRd to control levels (Figure 6E; *Online Supplementary Figure S12C*). These data identify the existence of a novel autocrine NOTCH3-CXCL12-CXCR4 signaling axis promoted by the TME in MM cells.

Pharmacological inhibition of CXCL12-CXCR4 or Notch signaling increases bortezomib sensitivity in high NOTCH3 multiple myeloma cells

Prompted by our *in vitro* studies with plerixafor, we explored further the use of this agent in combination with BOR-based therapies using MM *ex vivo* 3D organ cultures

established with murine and human bone. As seen *in vivo* with BOR, VRd's anti-MM efficacy was significantly reduced in murine bones bearing *NOTCH3*^{OE} *versus* control MM cells (Figure 7A, B). Co-administration of plerixafor increased the sensitivity of *NOTCH3*^{OE} MM cells to VRd. We also tested the effects of VRd + plerixafor in a novel human MM-human bone *ex vivo* system, which allowed us to study MM cell responses to chemotherapy in a TME closer to the one in patients (Figure 7C). MM cells engrafted human bones, and tumor growth was evident after 4 days (Figure 7C). *NOTCH3*^{OE} MM cells exhibited resistance to VRd therapy compared to control MM cells, and co-administration of

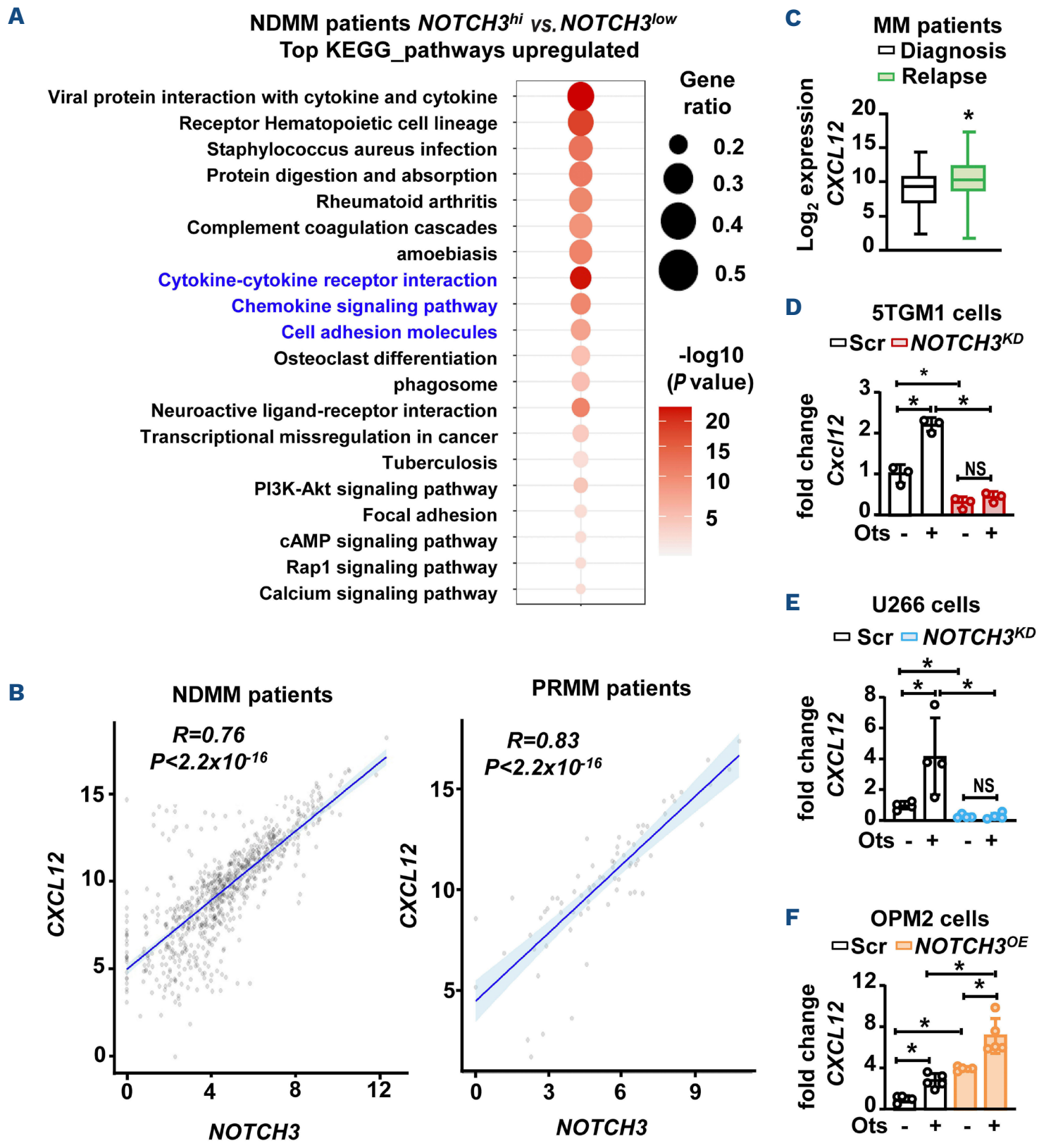
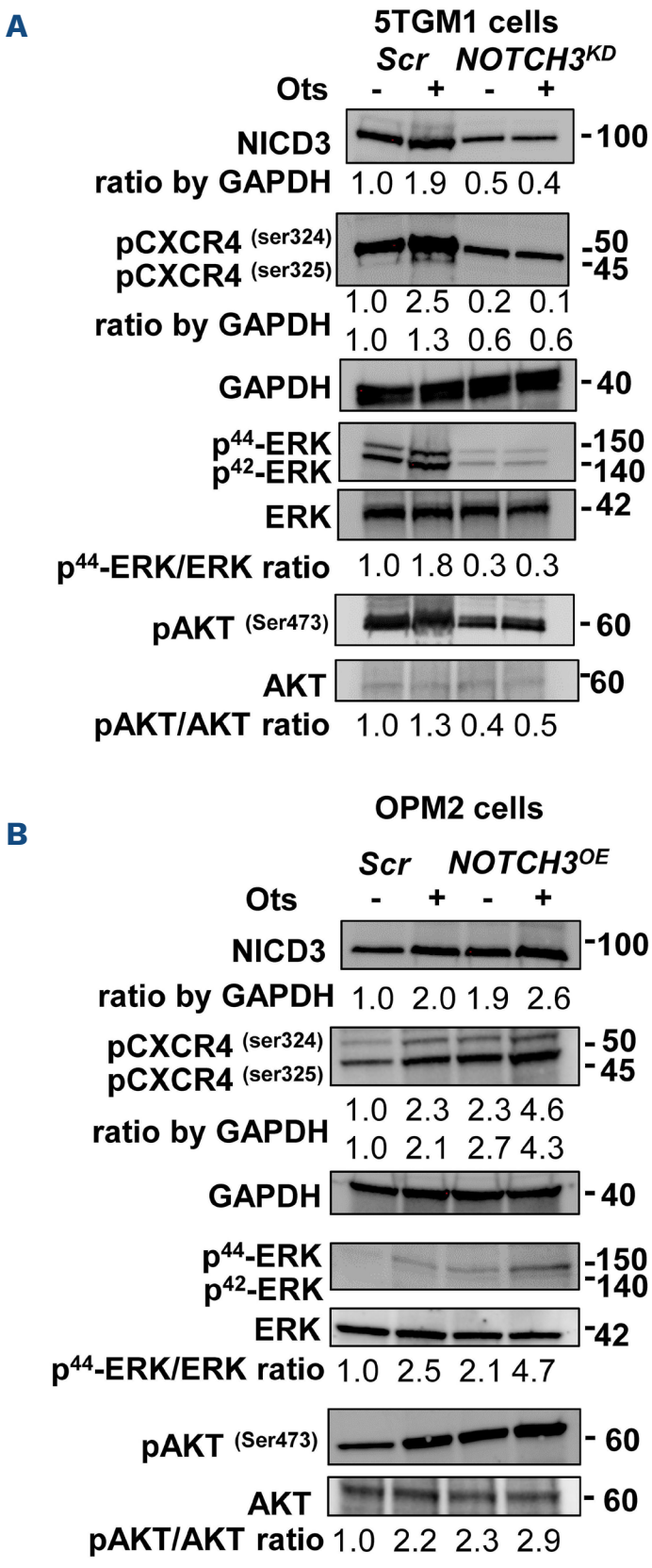


Figure 5. Activation of NOTCH3 transcriptional reprogramming by the tumor microenvironment increases *CXCL12* expression in multiple myeloma cells. Top 20 most significantly upregulated pathways (A) in newly diagnosed multiple myeloma (NDMM) patients with high *versus* low *NOTCH3* expression. N=768 patients. (B) *CXCL12* and *NOTCH3* expression correlation in CD138^{+} cells from NDMM and progression/relapsed (PRMM) patients. N=70/group. For (B), Pearson's correlation tests were performed. (C) Gene expression of *CXCL12* in CD138^{+} cells from paired diagnosis and relapsed MM patients. N=70/group. *P<0.05 *versus* diagnosis by Student's *t* test. Boxes show the data interquartile range, the middle line in the box represents the median, and whiskers the 95% confidence interval of the mean. *CXCL12* gene expression in scramble (Scr)/*NOTCH3* knockdown (*NOTCH3*^{KD}) 5TGM1 (D) or U266 (E) MM cells and Scr/*NOTCH3* activated (*NOTCH3*^{OE}) OPM2 (F) MM cells cultured in the absence/presence of osteocytes (Ots). N=4/group. *P<0.05 by two-way ANOVA, followed by a Tukey *post hoc* test. NS: non-significant. Data are shown as mean ± standard deviation; each dot represents an independent sample; representative experiments out of 2 are shown (F).

plerixafor restored VRd's anti-MM efficacy to control levels (Figure 7D). Lastly, we investigated if bone-targeted pan inhibition of Notch signals with a novel compound recently developed by our laboratory (BT-GSI)[13] overcomes the chemoresistance conferred by NOTCH3 activation in MM cells. Using *ex vivo* cultures established with murine bone and *Notch3*^{OE} MM cells, we found that co-administration of BT-GSI doubled the anti-MM efficacy of BOR (Figure 7E-F). Together, these studies highlight the potential of combining BOR-based therapies with CXCL12-CXCR4 or



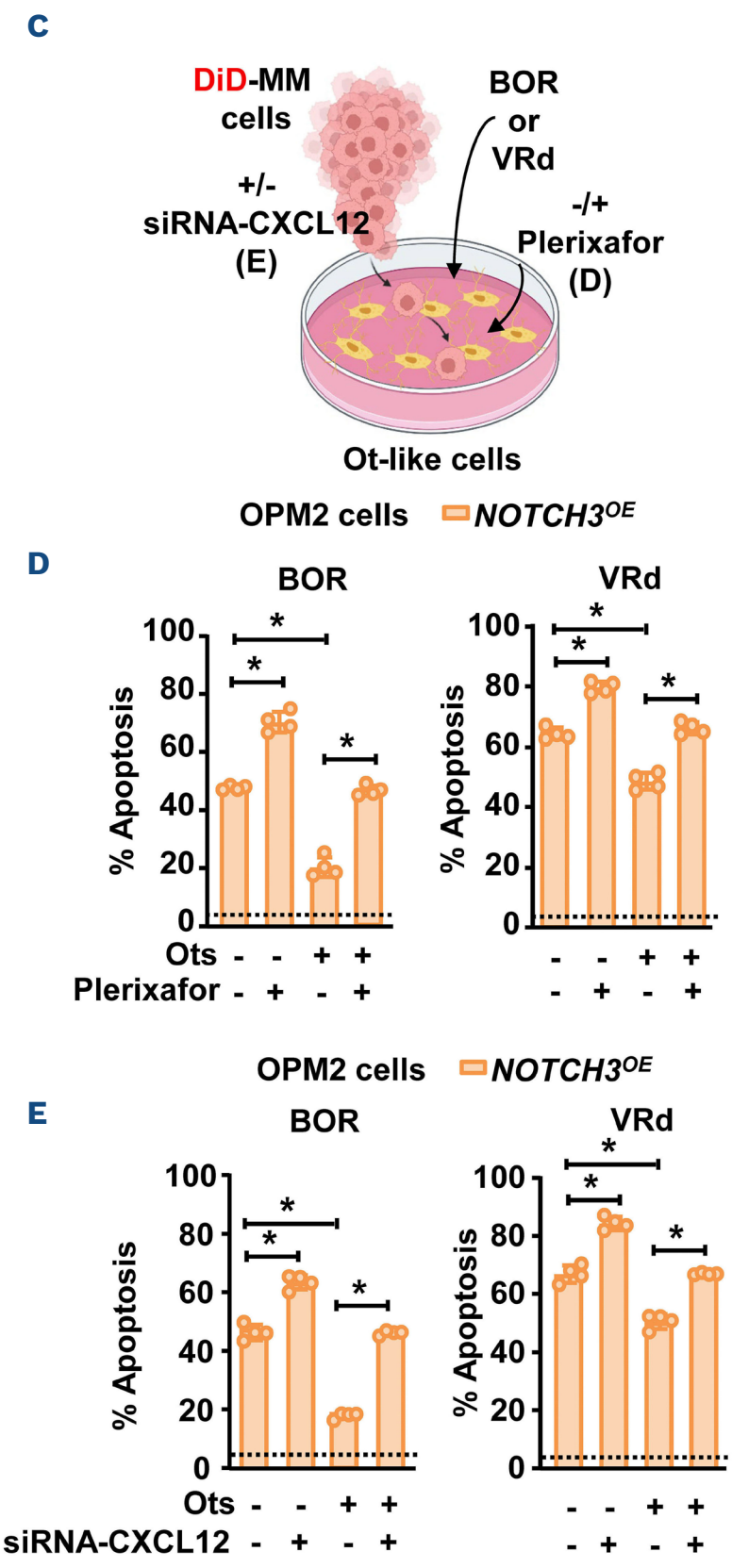


Figure 6. NOTCH3-CXCL12-CXCR4 signaling mediates tumor microenvironment-induced chemoresistance in multiple myeloma cells. Effects of osteocytes (Ots) and manipulation of NOTCH3 signaling in MM cells on protein levels of activated NOTCH3 receptor (NICD3), phosphorylated (p) CXCR4, pERK, pAKT in (A) scramble (Scr)/*NOTCH3* knockdown (*NOTCH3*^{KD}) 5TGM1 MM cells and (B) Scr/*NOTCH3*-activated (*NOTCH3*^{OE}) OPM2 MM cells. Representative images from 3 independent experiments are shown (see *Online Supplementary Figure S11*). (C) Experimental design. (D) Percent apoptosis of OPM2 *NOTCH3*^{OE} MM cells treated with/without plerixafor, bortezomib (BOR), or dexamethasone + BOR + lenalidomide (VRd) in the absence/presence of Ots. N=4/group; BOR=48 hours (h), VRd=24h. (E) Percent apoptosis of OPM2 *NOTCH3*^{OE} MM cells with/without *CXCL12* silencing and treated with BOR or VRd in the absence/presence of Ots. *P<0.05 by two-way ANOVA, followed by a Tukey *post hoc* test. The dotted line represents the percent apoptosis in vehicle-treated OPM2 *NOTCH3*^{OE} MM cells cultured alone. NS: non-significant. DiD: cell label dye. siRNA: small interference RNA. Data are shown as mean ± standard deviation; each dot represents an independent sample; representative experiments out of 2 are shown.

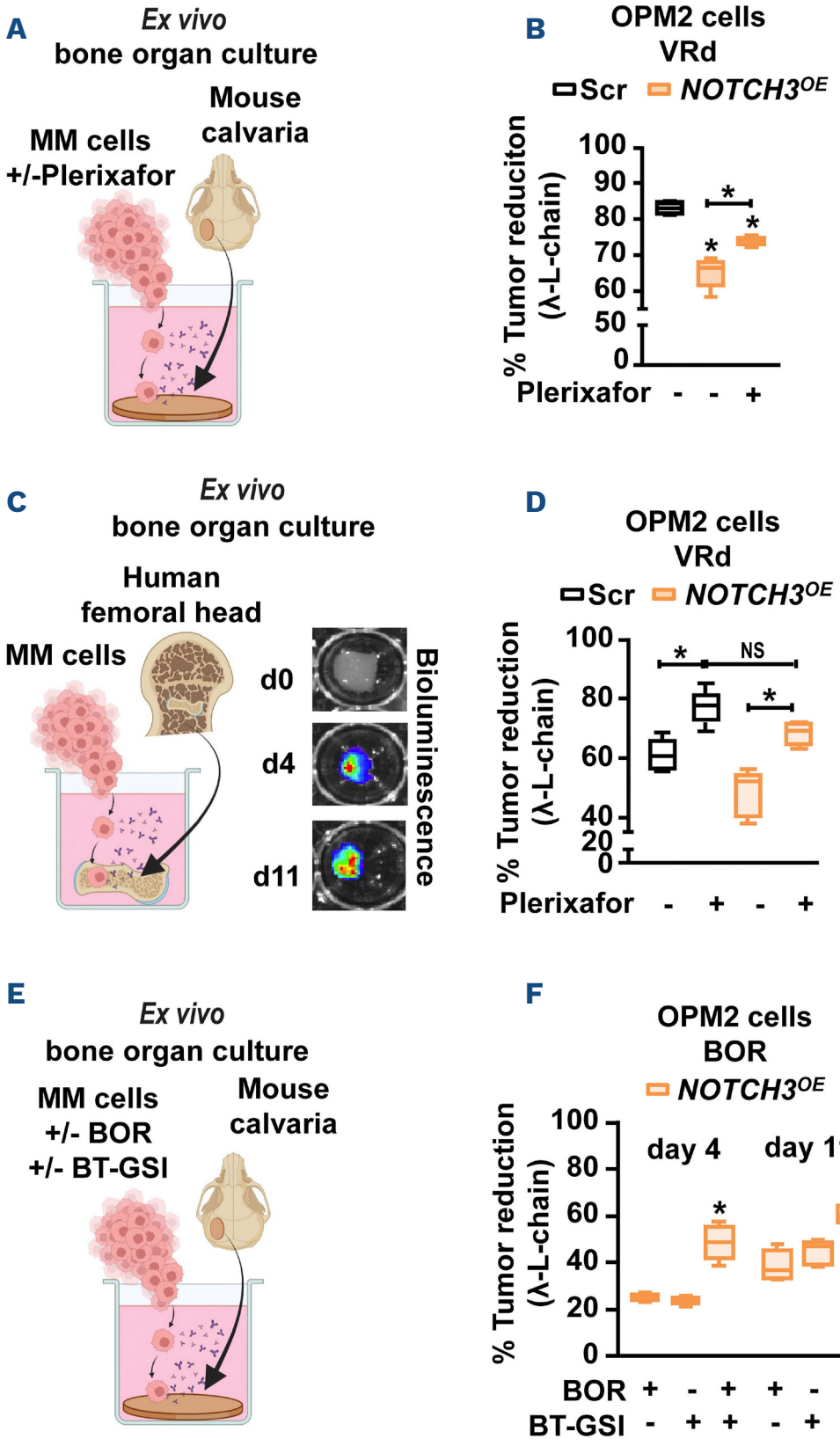


Figure 7. Pharmacological inhibition of CXCL12-CXCR4 or Notch signaling enhances therapeutic responses to bortezomib-based therapy in NOTCH3-activated multiple myeloma cells. (A) *Ex vivo* bone-multiple myeloma (MM) organ cultures established with scramble (Scr)/ *NOTCH3*-activated (*NOTCH3*^{OE}) OPM2 human MM cells and calvarial disc bones from NSG mice. (B) Percent tumor reduction by co-administration of dexamethasone + bortezomib (BOR) + lenalidomide (VRd) and plerixafor. N=6/group. *P<0.05 *versus* bones bearing Scr MM cells treated with VRd alone for 11 days by one-way ANOVA, followed by a Tukey *post hoc* test. (C) *Ex vivo* bone-MM organ cultures established with Scr/*NOTCH3*^{OE} OPM2 human MM cells and femoral head bone fragments from healthy human donors. Representative bioluminescence images of human bones showing engraftment and growth of human MM cells through the length of the experiment. (D) Percent tumor reduction by co-administration of VRd and plerixafor. N=6/group. *P<0.05 by two-way ANOVA, followed by a Tukey *post hoc* test. (E) *Ex vivo* bone-MM organ cultures established with *NOTCH3*^{OE} OPM2 human MM cells and calvarial disc bones from NSG mice. (F) Percent tumor reduction by co-administration of BOR and bone-targeted γ-secretase inhibitor (BT-GSI) after 4 and 11 days. N=6/group. *P<0.05 *versus* bones bearing *NOTCH3*^{OE} MM cells treated with BOR alone by one-way ANOVA, followed by a Tukey *post hoc* test. NS: non-significant. Boxes show the interquartile range, the middle line in the box represents the median, and whiskers the 95% confidence interval of the mean. d: day.

bone-targeted Notch inhibitors to overcome TME-mediated drug resistance.

Discussion

Chemotherapy resistance is the leading cause of relapsed/refractory disease, decreased survival, and a major obstacle to more successful clinical outcomes in MM. In this study, we demonstrate that a signaling pathway involving NOTCH3 activation by the extrinsic TME in MM cells promotes resistance to BOR therapeutic regimes. Our data highlight that this pathway is present in NDMM patients, upregulated in PRMM patients, and predicts worse clinical responses to BOR-based chemotherapy. Further, genetic activation of NOTCH3 in MM cells is sufficient to promote resistance to BOR therapies. Conversely, we show that genetic or pharmacologic interruption of NOTCH3 signals in MM cells increases sensitivity to BOR and decreases tumor burden. Our clinical and preclinical data position NOTCH3 inhibition as a rational target to improve clinical responses to first-line regimes based on BOR in MM patients.

Osteocytes are best known for their role in bone remodeling, where they function as paracrine and endocrine cells controlling the activity of bone cells in the bone marrow and distant organs.[10] Work from our group and others uncovered that osteocytes are also important components of the TME, capable of directly interacting with tumor cells, and have a pivotal role in tumor growth and cancer-induced bone disease.[22-24] Yet, the role of osteocytes in chemoresistance

has not been explored until now. Our studies extend beyond previous work on stromal cell-mediated mechanisms of resistance[3,4,8] and identify the osteocyte as a new cell type of the TME contributing to resistance to chemotherapy via Notch communication. Along the same lines, another recent study reported that osteocytes can confer MM resistance to chemotherapy via exosomes.[25] Because osteocytes are 95% of the cells in bone and, as stromal cells, can live for decades, these two cell types represent a major and long-lasting source of pro-survival signals for MM cells in MM. Future studies are needed to characterize further the contextual microenvironments and disease stages where these cell types preferentially operate.

It has been long appreciated that Notch signaling mediates communication between MM cells and other cells of the TME, supports tumor growth and bone destruction, and contributes to drug resistance and survival;[5,26] furthermore, functional studies have suggested that inhibiting Notch activation downstream all NOTCH receptors with γ-secretase inhibitors (GSI) decreases tumor burden, bone disease, and improves sensitivity to chemotherapeutic agents.[6,27] Although the evidence for the influence of NOTCH signals in MM progression is strong, the specific contribution of individual NOTCH components is less clear. Notably, our paper uncovers that the basal expression of *NOTCH3* is dynamic and selectively upregulated by TME cells and describes a previously unknown role for NOTCH3 in MM chemoresistance. Previous *in vitro* studies suggested that NOTCH1 and 2 are the main mediators of stroma-MM communication.[28,29] In contrast, our prior work showed that osteocytes preferentially employ NOTCH3 to communicate with MM cells.[9] Although we cannot exclude the contribution of other NOTCH receptors to TME-mediated chemoresistance, this study suggests that NOTCH3 activation is a common molecular mechanism that TME cells utilize to communicate with MM cells and plays a pivotal role in promoting drug resistance. We reported before that homotypic NOTCH3 signaling mediates MM cell proliferation but does not affect MM cell viability.[9] Consistent with this observation, homotypic NOTCH3 signaling (between MM cells) did not affect MM cell apoptotic responses to BOR or VRd, underlining their dependence on TME-derived Notch ligands for chemoprotection. Further studies beyond the scope of the current manuscript are granted to identify the TME Notch ligand(s) responsible for the activation of NOTCH3 and chemoresistance in MM cells.

We noted fascinating differences in the transcriptome of NDMM that are mechanistically dependent on NOTCH3 and lead to a gene signature predictive of poor clinical outcomes. Our data show that NOTCH3 integrates signals from cells of the TME to increase CXCL12 expression in MM cells and provide evidence of NOTCH3-CXCL12 co-expression in CD138^{+} plasma cells from patients. Previous *in vitro* observations showed that inhibition of all NOTCH receptors with GSI decreases CXCL12 production in MM cells.[30] Our findings are consistent with this study and support that NOTCH3 is a major molecular regulator of CXCL12. Although stroma-derived CXCL12/CXCR4 is a well-established symbiotic bridge linking MM cells and their stromal neighbors in oncogenic communication/drug resistance,[8,17,18,20,21,32] this report is one of the first indications suggesting the existence of an active autocrine CXCL12-CXCR4 signaling axis in MM cells promoted by the TME. We show that interruption of NOTCH3 signals by inhibiting NOTCH3 cleavage at the membrane level (BT-GSI) or suppressing CXCL12 expression (small interfering RNA) or signaling through CXCR4 (plerixafor) led to comparable prevention of TME-induced BOR resistance in MM cells. Remarkably, we validated these observations in human *ex vivo* models, which showed that bones infiltrated with NOTCH3-activated MM cells have worse responses to VRd and, importantly, a robust reduction in tumor burden after co-administration of VRd and plerixafor. Therefore, human (and murine) *ex vivo* organ cultures represent a powerful tool to model responses to chemotherapy in a physiologically relevant environment. Collectively, these findings support that NOTCH3 is activated in MM cells by the TME in specialized niches, resulting in a transcriptional response downstream of CXCL12 binding to CXCR4, which leads to chemoresistance.

In addition to its role in drug resistance, we confirmed that NOTCH3 signaling in MM cells exerts bone catabolic actions. We showed before that inhibition of NOTCH3 in MM cells reduces MM-induced bone disease.[9] Conversely, we found that mice-bearing MM cells with activated NOTCH3 exhibited worse bone destruction in this study. Two potential mechanisms, not mutually exclusive, may account for this observation. One, NOTCH3 activation by TME cells increases in MM cells the expression of RANKL, a pro-osteoclastogenic cytokine with a key role in the development of bone disease in MM patients.[33,34] Second, TME-derived signals integrated by NOTCH3 stimulate the proliferation of MM cells and lead to greater tumors and, therefore, more bone disease. Our studies did not address the contribution of each potential mechanism, as both occur simultaneously in our model.

These findings have important clinical implications. Our results suggest the potential added value of NOTCH3 expression in MM treatment decision-making for NDMM and PRMM patients. Further validation of the impact of NOTCH3 expression in other cohorts is needed to strengthen this argument, which we acknowledge as a limitation of our study. In addition, these findings raise the possibility that NOTCH3 might be a useful target for MM patients. Anti-*NOTCH3* antibodies have shown efficacy against solid tumors[35-37] but have not yet been evaluated in MM models. Further, our data suggest the potential of a therapy targeting NOTCH3 to simultaneously decrease tumor growth, improve responses to BOR regimes, and stop bone destruction, as pharmacological inhibition of *NOTCH3 in vivo* is sufficient to decrease bone resorption in naïve mice.[37]

Of note, *NOTCH3* antibodies do not exhibit dose-limiting side effects[37] seen with pan-inhibition of Notch signaling in humans and mice.[13,38] Similarly, our novel bone-targeted GSI inhibitor shows great potential to overcome drug resistance mediated by NOTCH3, and other NOTCH receptors, while circumventing toxicities. Lastly, our work provides a cellular and molecular rationale to combine BOR regimes with plerixafor as a chemosensitization strategy in MM patients, a strategy proven successful recently in a small clinical trial (*clinicaltrials gov. Identifier: NCT00903968*).[39] A better understanding of the cellular and molecular events leading to disease progression/relapse in MM is needed to bypass drug resistance. This study unravels a crucial role of NOTCH3 as a mediator of TME-mediated chemoresistance in MM. Complementary clinical and preclinical data and pharmacologic and genetic approaches in human and mouse systems support this conclusion. Further, we identified a previously unknown function of osteocytes as providers of Notch signals in the TME conducive to resistance to chemotherapy. Lastly, we demonstrate the beneficial effects of targeting NOTCH3 and its downstream signals to restore sensitivity to BOR-based therapies in MM cells. In summary, our findings support using existing and novel pharmacologic tools to interfere with NOTCH3 signals to overcome drug resistance and improve bone health in MM.

Disclosures

No conflicts of interest to disclose.

Contributions

JDC conceived and supervised the project. HMS, CA, CS, and JDC designed the experiments. HMS, CA, MA, AA, JK, SK, SRC, MP, CLB, IN, and EA performed the experiments and/or collected data. HMS, CA, IN, and JDC contributed to the data analysis and interpretation. JDC and HMS wrote the manuscript. All authors reviewed the manuscript.

Acknowledgments

The authors would like to acknowledge the services provided by the TBAPS of the UAMS Winthrop P. Rockefeller Cancer Institute and the MMRF for providing the CoMMpass IA15 dataset. These data were generated as part of the Multiple Myeloma Research Foundation Personalized Medicine Initiatives (https://research.themmrf.org and www.themmrf.org). The authors thank Dr. Onal for her assistance with CRISPR NOTCH3 activation and sgRNA design.

Funding

This work was supported by the National Institutes of Health (NIH) R37CA251763, R01CA209882, R01CA241677 (to JDC), P20GM125503 (to JDC and IN), and F31CA284655 (to HMS), the UAMS Musculoskeletal Hub Award (to JDC and IN), the UAMS Translational Research Institute (TRI), grant KL2 TR003108 through the National Center for Advancing Translational Sciences of the NIH (to CA), and the UAMS Winthrop P. Rockefeller Cancer Institute Seeds of Science Award and Voucher Program awarded (to JDC).

Data-sharing statement

The IA15 datasets used for the analyses described in this work were downloaded from the Multiple Myeloma Research Foundation CoMMpass (MMRF CoMMpass [SM] [Relating Clinical Outcomes in MM to Personal Assessment of Genetic Profile] study [www.themmrf.org]) researcher gateway. Other non-public datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

References

1. Rajkumar SV, Kumar S. Multiple myeloma current treatment algorithms. Blood Cancer J. 2020;10(9):94.
2. Maiso P, Mogollón P, Ocio EM, Garayoa M. Bone marrow mesenchymal stromal cells in multiple myeloma: their role as active contributors to myeloma progression. Cancers (Basel). 2021;13(11):2542.
3. Ria R, Vacca A. Bone marrow stromal cells-induced drug resistance in multiple myeloma. Int J Mol Sci. 2020;21(2):613.
4. Nefedova Y, Landowski TH, Dalton WS. Bone marrow stromal-derived soluble factors and direct cell contact contribute to de novo drug resistance of myeloma cells by distinct mechanisms. Leukemia. 2003;17(6):1175-1182.
5. Colombo M, Galletti S, Garavelli S, et al. Notch signaling deregulation in multiple myeloma: a rational molecular target. Oncotarget. 2015;6(29):26826-26840.
6. Nefedova Y, Sullivan DM, Bolick SC, Dalton WS, Gabrilovich DI. Inhibition of Notch signaling induces apoptosis of myeloma cells and enhances sensitivity to chemotherapy. Blood. 2008;111(4):2220-2229.
7. Wang Z, Li Y, Ahmad A, et al. Targeting Notch signaling pathway to overcome drug resistance for cancer therapy. Biochim Biophys Acta. 2010;1806(2):258-267.
8. Colombo M, Garavelli S, Mazzola M, et al. Multiple myeloma exploits Jagged1 and Jagged2 to promote intrinsic and bone marrow-dependent drug resistance. Haematologica. 2020;105(7):1925-1936.
9. Sabol HM, Amorim T, Ashby C, et al. Notch3 signaling between myeloma cells and osteocytes in the tumor niche promotes tumor growth and bone destruction. Neoplasia. 2022;28:100785.
10. Delgado-Calle J, Bellido T. The osteocyte as a signaling cell. Physiol Rev. 2022;102(1):379-410.
11. Choudhury SR, Ashby C, Tytarenko R, et al. The functional epigenetic landscape of aberrant gene expression in molecular subgroups of newly diagnosed multiple myeloma. J Hematol Oncol. 2020;13(1):108.
12. Delgado-Calle J, Anderson J, Cregor MD, et al. Bidirectional Notch signaling and osteocyte-derived factors in the bone

marrow microenvironment promote tumor cell proliferation and bone destruction in multiple myeloma. Cancer Res. 2016;76(5):1089-1100.
13. Sabol HM, Ferrari AJ, Adhikari M, et al. Targeting Notch inhibitors to the myeloma bone marrow niche decreases tumor growth and bone destruction without gut toxicity. Cancer Res. 2021;81(19):5102-5114.
14. Delgado-Calle J, Anderson J, Cregor MD, et al. Genetic deletion of Sost or pharmacological inhibition of sclerostin prevent multiple myeloma-induced bone disease without affecting tumor growth. Leukemia. 2017;31(12):2686-2694.
15. Mulligan G, Mitsiades C, Bryant B, et al. Gene expression profiling and correlation with outcome in clinical trials of the proteasome inhibitor bortezomib. Blood. 2007;109(8):3177-3188.
16. Bellido T, Delgado-Calle J. Ex vivo organ cultures as models to study bone biology. JBMR Plus. 2020;4(3):10.
17. Waldschmidt JM, Simon A, Wider D, et al. CXCL12 and CXCR7 are relevant targets to reverse cell adhesion-mediated drug resistance in multiple myeloma. Br J Haematol. 2017;179(1):36-49.
18. Bouyssou JM, Ghobrial IM, Roccaro AM. Targeting SDF-1 in multiple myeloma tumor microenvironment. Cancer Lett. 2016;380(1):315-318.
19. Cohen YC, Zada M, Wang SY, et al. Identification of resistance pathways and therapeutic targets in relapsed multiple myeloma patients through single-cell sequencing. Nat Med. 2021;27(3):491-503.
20. Ullah TR. The role of CXCR4 in multiple myeloma: cells' journey from bone marrow to beyond. J Bone Oncol. 2019;17:100253.
21. Ren Z, Lantermans H, Kuil A, et al. The CXCL12gamma chemokine immobilized by heparan sulfate on stromal niche cells controls adhesion and mediates drug resistance in multiple myeloma. J Hematol Oncol. 2021;14(1):11.
22. Anloague A, Delgado-Calle J. Osteocytes: new kids on the block for cancer in bone therapy. Cancers (Basel). 2023;15(9):2645.
23. Atkinson EG, Delgado-Calle J. The emerging role of osteocytes in cancer in bone. JBMR Plus. 2019;3(3):e10186.
24. Pin F, Prideaux M, Bonewald LF, Bonetto A. Osteocytes and cancer. Curr Osteoporos Rep. 2021;19(6):616-625.
25. Cheng F, Wang Z, You G, Liu Y, He J, Yang J. Osteocyte-derived exosomes confer multiple myeloma resistance to chemotherapy through acquisition of cancer stem cell-like features. Leukemia. 2023;37(6):1392-1396.
26. Fairfield H, Falank C, Avery L, Reagan MR. Multiple myeloma in the marrow: pathogenesis and treatments. Ann N Y Acad Sci. 2016;1364(1):32-51.
27. Colombo M, Platonova N, Giannandrea D, Palano MT, Basile A, Chiaramonte R. Re-establishing apoptosis competence in bone associated cancers via communicative reprogramming induced through Notch signaling inhibition. Front Pharmacol. 2019;10:145.
28. Nefedova Y, Cheng P, Alsina M, Dalton WS, Gabrilovich DI. Involvement of Notch-1 signaling in bone marrow stroma-mediated de novo drug resistance of myeloma and other malignant lymphoid cell lines. Blood. 2004;103(9):3503-3510.
29. Muguruma Y, Yahata T, Warita T, et al. Jagged1-induced Notch activation contributes to the acquisition of bortezomib resistance in myeloma cells. Blood Cancer J. 2017;7(12):650.
30. Mirandola L, Apicella L, Colombo M, et al. Anti-Notch treatment prevents multiple myeloma cells localization to the bone marrow via the chemokine system CXCR4/SDF-1. Leukemia. 2013;27(7):1558-1566.
31. Di Marzo L, Desantis V, Solimando AG, et al. Microenvironment drug resistance in multiple myeloma: emerging new players. Oncotarget. 2016;7(37):60698-60711.
32. Azab AK, Runnels JM, Pitsillides C, et al. CXCR4 inhibitor AMD3100 disrupts the interaction of multiple myeloma cells with the bone marrow microenvironment and enhances their sensitivity to therapy. Blood. 2009;113(18):4341-4351.
33. Raje N, Terpos E, Willenbacher W, et al. Denosumab versus zoledronic acid in bone disease treatment of newly diagnosed multiple myeloma: an international, double-blind, double-dummy, randomised, controlled, phase 3 study. Lancet Oncol. 2018;19(3):370-381.
34. Silbermann R, Roodman GD. Myeloma bone disease: pathophysiology and management. J Bone Oncol. 2013;2(2):59-69.
35. Varga J, Nicolas A, Petrocelli V, et al. AKT-dependent NOTCH3 activation drives tumor progression in a model of mesenchymal colorectal cancer. J Exp Med. 2020;217(10):e201921515.
36. Choy L, Hagenbeek TJ, Solon M, et al. Constitutive NOTCH3 signaling promotes the growth of basal breast cancers. Cancer Res. 2017;77(6):1439-1452.
37. Yu J, Siebel CW, Schilling L, Canalis E. An antibody to Notch3 reverses the skeletal phenotype of lateral meningocele syndrome in male mice. J Cell Physiol. 2020;235(1):210-220.
38. Golde TE, Koo EH, Felsenstein KM, Osborne BA, Miele L. γ-Secretase inhibitors and modulators. Biochim Biophys Acta. 2013;1828(12):2898-2907.
39. Ghobrial IM, Liu CJ, Zavidij O, et al. Phase I/II trial of the CXCR4 inhibitor plerixafor in combination with bortezomib as a chemosensitization strategy in relapsed/refractory multiple myeloma. Am J Hematol. 2019;94(11):1244-1253.