Elucidation of molecular basis of osteolytic bone lesions in advanced multiple myeloma

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Abstract

Osteolytic bone lesion is a major cause of lower quality of life and poor prognosis in patients with multiple myeloma (MM), but molecular pathogenesis of the osteolytic process in MM remains elusive. Fms-like tyrosine kinase 3 ligand (FLT3L) was reported to be elevated in bone marrow (BM) and blood of patients with advanced MM who often show osteolysis. Here, we investigated a functional link of FLT3L to osteolytic process in MM. We recruited 86, 306, and 52 patients with MM, acute myeloid leukemia (AML), and acute lymphoblastic leukemia (ALL), respectively. FLT3L levels of patients with hematologic malignancies were measured in BM-derived plasma and found to be significantly higher in MM than in AML or ALL, which rarely show osteolysis. FLT3L levels were further elevated in MM patients with bone lesion compared with patients without bone lesion. *In vitro* cell-based assays showed that the administration of FLT3L to HEK293T, HeLa, and U2OS cells led to an increase in the DKK1 transcript level through STAT3 phosphorylation at tyrosine 705. WNT reporter assay showed that FLT3L treatment reduced WNT signaling and nuclear translocation of β-catenin. These results collectively show that the FLT3L-STAT3-DKK1 pathway inhibits WNT signaling-mediated bone formation in MM, which can cause osteolytic bone lesion. Finally, transcriptomic profiles revealed that *FLT3L* and *DKK1* were predominantly elevated in the hyperdiploidy subtype of MM. Taken together, FLT3L can serve as a promising biomarker for predicting osteolytic bone lesion and also a potential therapeutic target to prohibit the progression of the osteolytic process in MM with hyperdiploidy.

Introduction

Multiple myeloma (MM) is the second most common hematologic malignancy involving malignant plasma cells. MM is characterized by the presence of clonal plasma cells producing fragmented monoclonal immunoglobulins of various heavy and light chain subtypes. In the US, the overall 5-year survival rate for patients with MM is 55%,[1,2] despite combination chemotherapy with potent target agents, and even high-dose chemotherapy and autologous hematopoietic stem cell transplantation. MM causes various complications, including osteolytic bone lesion, anemia, renal failure, hypercalcemia, and hyperviscosity syndrome.[3]

Osteolytic bone lesions at multiple sites are a unique feature of MM, which differs from other hematologic cancers such as acute and chronic leukemia or lymphoma. The chief complaint in about two-thirds of MM patients is bone pain due to the direct bone involvement of myeloma tumor cells or indirect effect of myeloma tumor cells or tumor microenvironment-secreting cytokines on bony skeleton. Skeletal-related events (SRE) in MM patients include intractable bone pain, fracture, and paralysis due to spinal cord compression,[4] which radically lower patients' quality of life (QoL) and place a substantial burden on healthcare resources.[5,6] Recent advances in treatment for MM have remarkably improved the median overall survival of patients with MM up to over 5 years.[7] Accordingly, QoL has become a more important issue for long-term MM survivors. Furthermore, bone osteolysis has a negative impact on the survival of MM.[8] Osteolytic bone lesions, a major cause of morbidity

and mortality in MM patients, are induced by an imbalance in bone remodeling characterized by the suppressed osteoblast formation and function, increased osteoclast formation and activity, as well as direct cell interactions between MM cells and osteoclasts, and the release of cytokines and growth factors.[9] One of the key mechanisms contributing to the development of osteolytic bone lesions is the activation of osteoclasts resulting in enhanced bone resorption and subsequent bone loss.[10] However, the molecular mechanisms underlying the pathogenesis of MM-induced osteolytic bone lesions are still not fully understood.

The putative mechanism of bone osteolysis in intercellular interactions between myeloma cells and osteoclast has been extensively studied. Cytokines of IL-6, IL-1β, TNF-α, MIP1α, and receptor activator of nuclear factor κ-B ligand (RANKL)[11] generated in the bone marrow (BM) microenvironment accentuate the interactions between myeloma cells and BM stromal cells, which enhance osteoclastic activity. In contrast, Dick Kopf-related protein 1 (DKK1), an extracellular antagonist of WNT signaling, is known to contribute to the formation of osteolytic bone lesion by depressing osteoblastic activity.[12] Specifically, the high expression levels of DKK1 in the BM microenvironment have been associated with osteolytic bone lesions in MM patients[13] and regulation of the WNT signaling pathway. Recent studies have investigated the molecular mechanisms underlying osteolytic bone lesions in MM, providing critical insights into the disease's pathogenesis. The cross-talk between MM cells and the BM microenvironment closely modulates the WNT/β-catenin signaling pathway, which promotes osteoblast activity and suppresses osteoclast differentiation.[14] Many studies have demonstrated the importance of the WNT/β-catenin pathway in healthy bone development through canonical WNT signaling participation in the regulation of osteoblast differentiation.[15] MM cells secrete WNT antagonists that disrupt BM regulation of osteoblastic differentiation, leading to osteolytic lesions.[16] Dysregulation of WNT pathway by DKK1, secreted from MM cells, affects the BM microenvironment, leading to altered bone homeostasis and osteolytic bone disease.[14] Several studies have investigated the expression pattern and functions of DKK1 in MM, revealing significantly higher DKK1 expression in MM patients compared to healthy controls, which is associated with poor prognosis.[17] Additionally, preclinical studies have demonstrated that targeting DKK1 using monoclonal antibodies or small molecule inhibitors can inhibit MM cell proliferation and induce apoptosis both *in vitro* and *in vivo*.[18]

Recently, Fms-like tyrosine kinase 3 ligand (FLT3L), a cytokine that plays a crucial role in hematopoiesis and immune regulation, has been reported to be elevated in BM and blood of patients with MM,[19] and correlated with vascular endothelial growth factor (VEGF) which may reflect the increased proliferative tumor activity based on the association between FLT3L level and Ki-67 positivity in MM.[20] FLT3L binds to the FLT3 receptor, which is expressed on hematopoietic stem cells and myeloid progenitor cells.[21] In MM, FLT3L has been shown to promote the proliferation and survival of malignant plasma cells,[20] as well as the recruitment of immunosuppressive cells to the tumor microenvironment.[22] Inhibiting FLT3L using a small molecule inhibitor has reduced proliferation and increased apoptosis in MM cells.[23] Similarly, targeting FLT3L with antibody-based therapy has demonstrated significant antitumor activity in MM.[24] While emerging evidence highlights the crucial role of FLT3L in MM pathogenesis and its potential as a therapeutic target, further studies are needed to fully understand the underlying mechanisms of FLT3L function in MM and develop more effective FLT3L-targeted therapies for the disease. Therefore, we investigated the link of FLT3L to bone osteolysis in MM and explored the molecular mechanism in view of the osteolytic phenomenon in MM.

Methods

Primary sample

The patients who had been diagnosed with hematologic malignancies at Seoul National University Hospital from March 2004 to December 2012 were enrolled in this study. BM-derived blood samples from patients with leukemia or MM were used to measure FLT3L. Primary samples were obtained with informed consent (Seoul National University Hospital [SNUH] Institutional Review Board [IRB] N. 1902-047-1008). The study was approved by the SNUH IRB (N. 1902-047-1008).

Cell lines and reagents

HEK293T, HeLa, U2OS, and MOLP8 cells were used to investigate the effect of FLT3L on intracellular signaling. HEK293T, HeLa, and U2OS cells were grown in high-glucose DMEM, and MOLP8 cells were cultured in RPMI. Culture media were supplemented with 10% FBS and 100 U/mL penicillin-streptomycin (all Gibco; Grand Island, NY, USA). Cells were maintained in a humidified 5% CO_2 at 37°C. Recombinant human FLT3L ligand (rhFLT3L) was purchased from R&D SYSTEMS (Minneapolis, MN, USA). Stattic (Sigma-Aldrich; St. Louis, MO, USA), a STAT3 inhibitor, was pre-treated for one hour before treatment with rhFLT3L in HEK293T cells.

Measurement of FLT3L

Plasma was extracted from patients' BM-derived blood by gradient centrifugation, and used to measure FLT3L. FLT3L level was measured using human FLT3L pre-coated enzyme-linked immunosorbent assay (ELISA) kit (Biogems, Westlake Village, CA, USA) according to the manufacturer's protocol. The standard curve was created by generating a 4-parameter logistic (4-PL) regression for the concentrations *versus* measured intensities. The concentration in the sample was taken as an estimate of the measured intensity using the standard curve.

Analyses for clinical factors

Clinical variables of patients with MM included age, sex, disease status, serum M-protein, and osteolytic lesion; these were reviewed in the electronic medical records and included in the description of patients' characteristics. MM-related variables of stage, subtype, the presence of anemia, hypercalcemia, azotemia, and osteolytic bone lesion were converted into categorical variables and used for survival analysis in view of prognostic factors.

RNA isolation and quantitative real-time polymerase chain reaction

Total RNA of the cells was extracted using RNeasy Mini Kit and QIAshredder (GIAGEN; Valencia, CA, USA) and synthesized into cDNA through the SuperScript III First-Strand Synthesis System (Invitrogen; Carlsbad, CA, USA). mRNA level was determined by 2XqPCRBIO SyGreen Blue Mix Lo-ROX (PCR Biosystems; Wayne, PA, USA) and normalized by that of an internal control GAPDH. All reactions were carried out in LightCycler® 96 (Roche; Indianapolis, IN, USA). Primer sequences used were as follows: hFLT3L sense: 5'-ACCTATCTCCTCCTGCTGCT-3', antisense: 5'-GGTAGTCAGACAGCTCACGG-3'; hDKK1 sense: 5'-GTCCAAGATCTGTAAACCTGTCCT-3', antisense: 5'-AGCCTAGAAGAATTACTGGCTTGA-3', hGAPDH sense: 5'-GCAAATTCCATGGCACCGTC-3', antisense: 5'-TCGCCCCACTTGATTTTGGA-3'; mDkk1 sense: 5'-TCCGTCTGCCTCCGATCATC-3', antisense: 5'-GCCTTTCCGTTTGTGCTTGG-3'; mGapdh sense: 5'- CATGTTCCAGTATGACTCCACTC-3', antisense: 5'-GGCCTCACCCCATTTGATGT-3'. SpectraMax i3x (NFEC-2017-12-241146) was used for quantitative real-time polymerase chain reaction (RT-qPCR) analysis at the Core Facility Center for Chronic and Metabolic Diseases at Sookmyung Women's University.

Statistical analysis

Statistical analyses for clinical parameters were performed using Stata version 13 (Stata Corp.; College Station, TX, USA) and GraphPad Prism v 5.00 (GraphPad Software Inc.; San Diego, CA, USA). Data were presented as mean ± standard deviation (SD). Statistical significance (*P*-values) was determined by one-way ANOVA followed by Dunnett's post hoc correction or two-way ANOVA with Tukey's post hoc correction. The Mann-Whitney test was used to compare the level of FLT3L between two groups. Interquartile range (IQR) was calculated to estimate the distribution of FLT3L level in each group. Linear regression analysis was used to investigate the association between FLT3L level and myeloma tumor cell burden in BM. Overall survival (OS) was defined as the interval between the initial diagnosis of MM and death due to any cause or the last clinical follow-up. Survival probabilities were estimated using the Kaplan-Meier method and analyzed using Cox proportional hazard model.

Analysis of transcriptomic data

We obtained mRNA expression data (GSE2658) from the Gene Expression Omnibus (GEO) database together with patient subtype (PAM cluster) and clinical information. As instructed in the original study,[22] we used the data from 414 samples in the dataset after removing the samples contaminated with myeloid or normal plasma cells. The probe intensities were first converted into $\log_2$-intensities and then normalized using quantile normalization.[23] To identify expressed genes, we fitted a Gaussian mixture model to the distribution of the normalized $\log_2$-intensities and selected the genes whose intensities were higher in half of the samples than the threshold intensity at which the 2 fitted Gaussian probability density functions meet. Next, we identified molecular signatures that define individual subtypes of patients with MM using the previously reported statistical method.[24] In brief, for the selected expressed genes, we calculated their $\log_2$-fold-changes with respect to their median values, and the $\log_2$-fold-changes were then normalized using quantile normalization. For each gene, we calculated an observed *t* statistic value in the comparison of one subtype *versus* the others. We then estimated an empirical null distribution of the *t* statistic value by performing random permutations of the samples 1,000 times, calculating *t* statistic values for

Table 1. Demographics of patients with multiple myeloma.

Variables	N (%) of patients
Age in years, median (range)	63 (33-87)
Diagnosis	
Multiple myeloma	86 (100.0)
Sex	
Female	37 (43.0)
Male	49 (57.0)
Subtype	
Heavy chain	58 (67.4)
IgG	36
IgA	19
IgM	1
IgD	1
Light chain	26 (30.2)
Kappa	13
Lambda	13
Non-secretory	2 (2.3)
Disease status at time of FLT3L measurement	
Initial diagnosis	43 (50.0)
Complete/partial remission	13 (15.1)
Relapsed/refractory	30 (34.9)
Osteolytic bone lesion	
No osteolytic bone lesion	33 (38.3)
Osteolytic bone lesion	51 (59.3)
Unknown	2 (2.3)
Serum M-protein in PB, g/dL, median (range)	1.41 (0-9.51)
Plasma FLT3L in BM, pg/mL, median (range)	161.75 (9.79-952.14)

BM: bone marrow; FLT3L: fms-like tyrosine kinase 3 ligand; N: number; PB: peripheral blood.

all expressed genes using each permuted dataset, and estimating a Gaussian kernel density function for the resulting *t* statistic values. Using the estimated empirical distribution, we computed the adjusted *P*-values for the observed *t* statistic value for each gene by performing two-sided testing. Finally, we identified molecular signatures as those that had *t* test $P<0.05$ and $\log_2$-fold-changes >1.5.

Enrichment analysis of cellular pathways and gene ontology biological processes

To identify cellular processes and pathways represented by the molecular signatures for each patient subtype, we performed the enrichment analysis of pathways and gene ontology biological processes (GOBP) for the selected molecular signatures using ConsensusPathDB.[25] The cellular pathways and GOBP represented by the molecular signatures for each subtype were selected as those with $P<0.05$, and the number of molecules involved in the pathway or GOBP ≥3.

Results

FLT3L reflects osteolytic bone lesion and prognosis in multiple myeloma

FLT3L level was reported to be elevated in BM and blood of patients with MM. To confirm this finding in Korean MM patients, and to further examine the association of FLT3L level with bone osteolytic bone lesion, we collected BM-derived plasma samples from 86, 306, and 52 patients with MM, acute myeloid leukemia (AML), and acute lymphoblastic leukemia (ALL), respectively. FLT3L level of monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma (SMM) was included (N=42), as MGUS/SMM involves no osteolytic bone lesion.[25] Patients' clinical characteristics are presented in Table 1. FLT3L levels were significantly ($P<0.05$) elevated in Korean patients with MM (median 161.8 pg/mL, interquartile range [IQR] 73.96-233.50) compared to those in AML (median 28.16, IQR 8.48-107.30) and ALL (median 46.11, IQR 12.52-172.80) (Figure 1A). Consid-

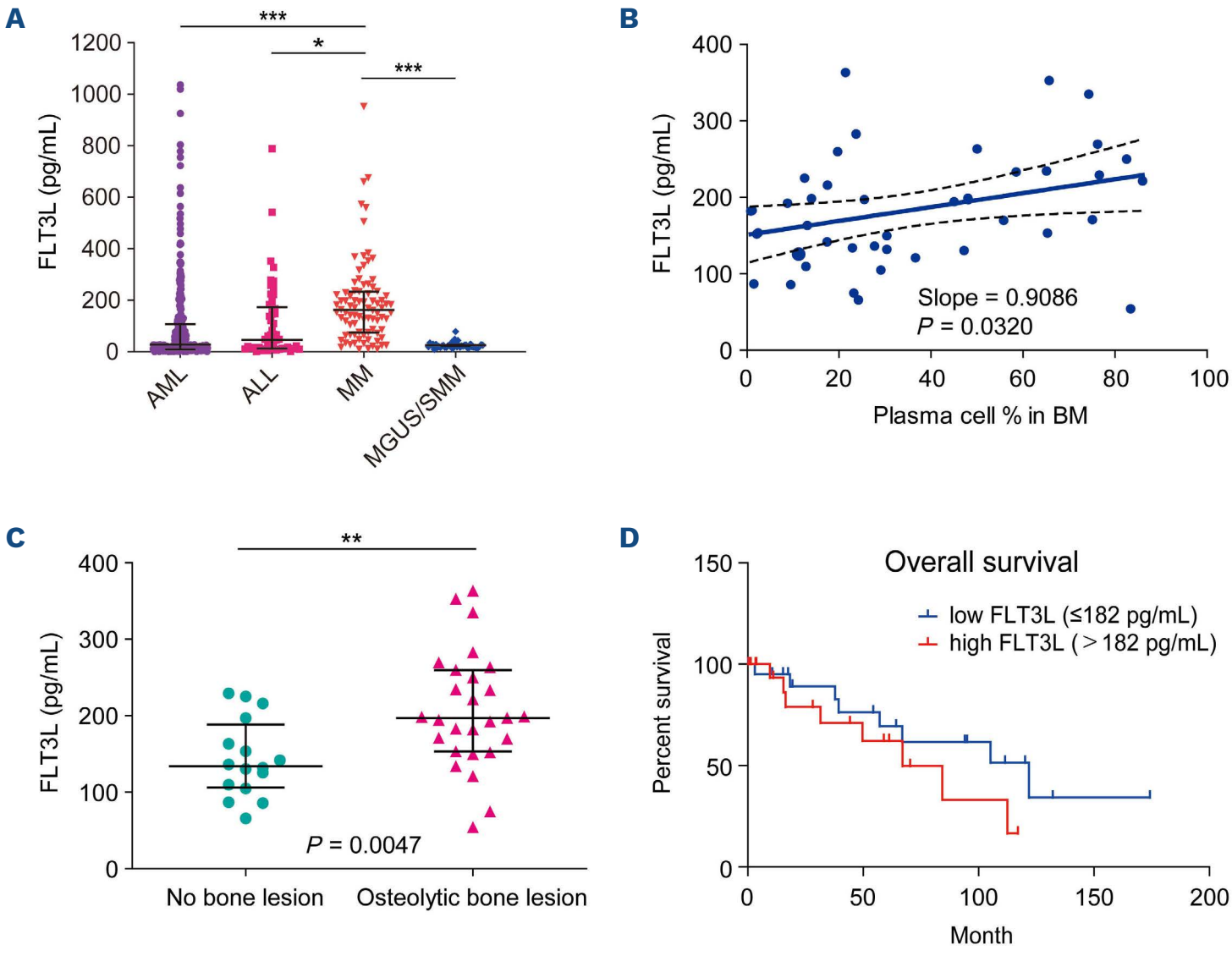

Figure 1. Plasma expression of Fms-like tyrosine kinase 3 ligand is elevated in multiple myeloma patients. (A) The plasma level of Fms-like tyrosine kinase 3 ligand (FLT3L) in bone marrow (BM) is significantly higher in multiple myeloma (MM) (N=86; median 161.8 pg/mL, interquartile range [IQR] 73.96-233.5) than in acute myeloid leukemia (AML) (N=306; median 28.16, IQR 8.48-107.3), acute lymphoblastic leukemia (ALL) (N=52; median 46.11, IQR 12.52-172.8), and monoclonal gammopathy of undetermined significance (MGUS) / smoldering multiple myeloma (SMM) (N=42). (B) The plasma level of FLT3L in BM of MM shows a linear correlation with the percentage of plasma cell / total nucleated cells in BM aspirates at the time of initial diagnosis (N=43). (C) The plasma level of FLT3L in BM is higher in MM with osteolytic bone lesion (N=27, median 194.70, IQR, 152.52-242.27) than in MM without bone lesion (N=16, median 141.62 pg/mL, IQR, 109.57-215.98) at the time of initial diagnosis ($P=0.0047$). (D) Kaplan-Meier survival curves for MM patients with low and high FLT3L expression levels. *$P<0.05$, **$P<0.01$, ***$P<0.001$, ns: not significant.

ering the infrequent occurrence of osteolytic bone lesions in ALL and AML, these findings indicate a potential association between FLT3L and bone osteolysis in MM.

FLT3L levels in BM aspirates and plasma were also reported to increase through MM progression and reach the maximal in patients with relapsed/refractory MM.[19] However, we were not able to determine a statistically meaningful increase in FLT3L in relapse/refractory MM or in disease status in our cohort (*Online Supplementary Figure S1A-C*). The observed heterogeneity in FLT3L levels could be due to a possible discrepancy in the sample collection (collection time and sample status). As an alternative measure to reflect tumor burden and aggressiveness of tumor microenvironment, we thus determined the percentage of plasma cells among total nucleated cells and then examined the association of the plasma cell percentage with FLT3L levels (N=43). The percentage of plasma cells showed a significant (P=0.032) positive correlation with the FLT3L level (Figure 1B), supporting the view that the FLT3L level increases through MM progression in Korean patients. To further investigate the association of the FLT3L level with bone osteolysis, we next grouped the 43 MM patients for whom the FLT3L levels were measured into 27 patients with osteolytic bone lesion and 16 patients without the lesion. The FLT3L level was significantly (P<0.01) elevated in patients with osteolytic bone lesion (median 194.70, IQR, 152.52-242.27) compared with those in patients without the bone lesion (median 141.62 pg/mL, IQR, 109.57-215.98) (Figure 1C). Moreover, we performed a multivariate

Table 2. Prognostic factors for overall survival of treatment-naïve multiple myeloma (N=43)

Parameter	Overall survival					
	Univariate			Multivariate		
	HR	95% CI	*P*	HR	95% CI	*P*
Age						
±1	1.02	0.97-1.07	0.44	1.06	0.91-1.23	0.45
Sex						
Female (referent)	-	-	-	-	-	-
Male	2.35	0.83-6.68	0.108	48.73	0.65-3663.87	0.078
ISS						
I	-	-	-	-	-	-
II	2.81	0.68-11.70	0.16	1.8	0.058-55.48	0.74
III	3.85	0.83-17.88	0.086	813.31	6.60-100259.2	0.006
Subtype						
Heavy chain, IgG	-	-	-	-	-	-
Heavy chain, non-IgG	0.94	0.20-4.48	0.94	0.84	0.014-50.29	0.94
Light chain/non-secretory	1.31	0.43-3.97	0.63	0.18	0.0053-5.84	0.33
Cytogenetics						
Euploidy	-	-	-	-	-	-
Hypodiploidy	3.73	1.047-13.29	0.042	9.2	0.051-1655.46	0.4
Hyperdiploidy	2.43	0.66-8.89	0.18	1.01	0.017-61.42	0.99
Anemia						
No (Hb≥10 g/dL)	-	-	-	-	-	-
Yes (Hb<10 g/dL)	1.04	0.36-3.01	0.94	0.34	0.030-3.82	0.38
Hypercalcemia						
No (Ca≥11.0 mg/dL)	-	-	-	-	-	-
Yes (Ca<11.0 mg/dL)	2.91	0.60-14.09	0.18	6.95	0.21-228.29	0.28
Azotemia						
No (Cr<1.4 mg/dL)	-	-	-	-	-	-
Yes (Cr≥1.4 mg/dL)	1.78	0.54- 5.88	0.34	0.018	0.000043-7.56	0.19
Hypoalbuminemia						
No (>3.5 g/dL)	-	-	-	-	-	-
Yes (≤3.5 g/dL)	1.78	0.58-5.45	0.32	0.17	0.0053-5.69	0.33
Osteolytic bone lesion						
Absent	-	-	-	-	-	-
Present	1.32	0.48-3.68	0.59	0.83	0.11-6.34	0.86
FLT3L						
≤182 pg/mL	-	-	-	-	-	-
>182 pg/mL	1.85	0.67-5.15	0.24	65.96	2.06-2109.60	0.018

N: number; HR: Hazard Ratio; CI: Confidence Interval; Hb: hemoglobin; Ca: calcium; Cr: FLT3L: fms-like tyrosine kinase 3 ligand.

logistic regression analysis using FLT3L as a bi-categorical dependent variable (≤182 pg/mL *vs*. >182 pg/mL) and clinical factors as independent variables in the treatment-naïve MM patients (N=43). This analysis showed that the percentages (%) of BM plasma cell and hyperdiploidy were significantly associated with FLT3L in MM (*Online Supplementary Table S1*), consistent with the finding in Figure 1B. This analysis suggests the associations of serum M-protein level and the presence of osteolytic lesions with FLT3L levels (*P*=0.071, 0.073, and 0.069, respectively). These data thus imply that these 4 factors may collectively contribute to FLT3L levels. We next examined the association of the FLT3L level with the OS of the 43 patients. The high level of FLT3L in BM (>182 pg/mL) was significantly (*P*=0.018) associated with poor survival in multivariate analysis (Hazard Ratio [HR]=65.96, 95% Confidence Interval [CI]: 2.06-2109.60), as indicated by the median survivals of 121.8 months in the low FLT3L group (N=22, ≤182 pg/mL) and 67.03 months in the high FLT3L group (N=21, >182 pg/mL) (Figure 1D). Interestingly, many clinical parameters, such as age, subtype of heavy and light chain, cytogenetics of hypo- and hyperdiploidy, anemia, hypercalcemia, azotemia, and hypoalbuminemia, were not significantly associated with OS (Table 2). On the other hand, sex tended to show a marginal (*P*=0.078) correlation with survival in multivariate analysis, and International Staging System (ISS) advanced stage (III) was a poor prognostic factor compared to stage I (HR=3.85 and 813.31, 95% CI: 0.83-17.88 and 6.60-100259.2; *P*=0.086 and 0.006 in univariate and multivariate analyses,

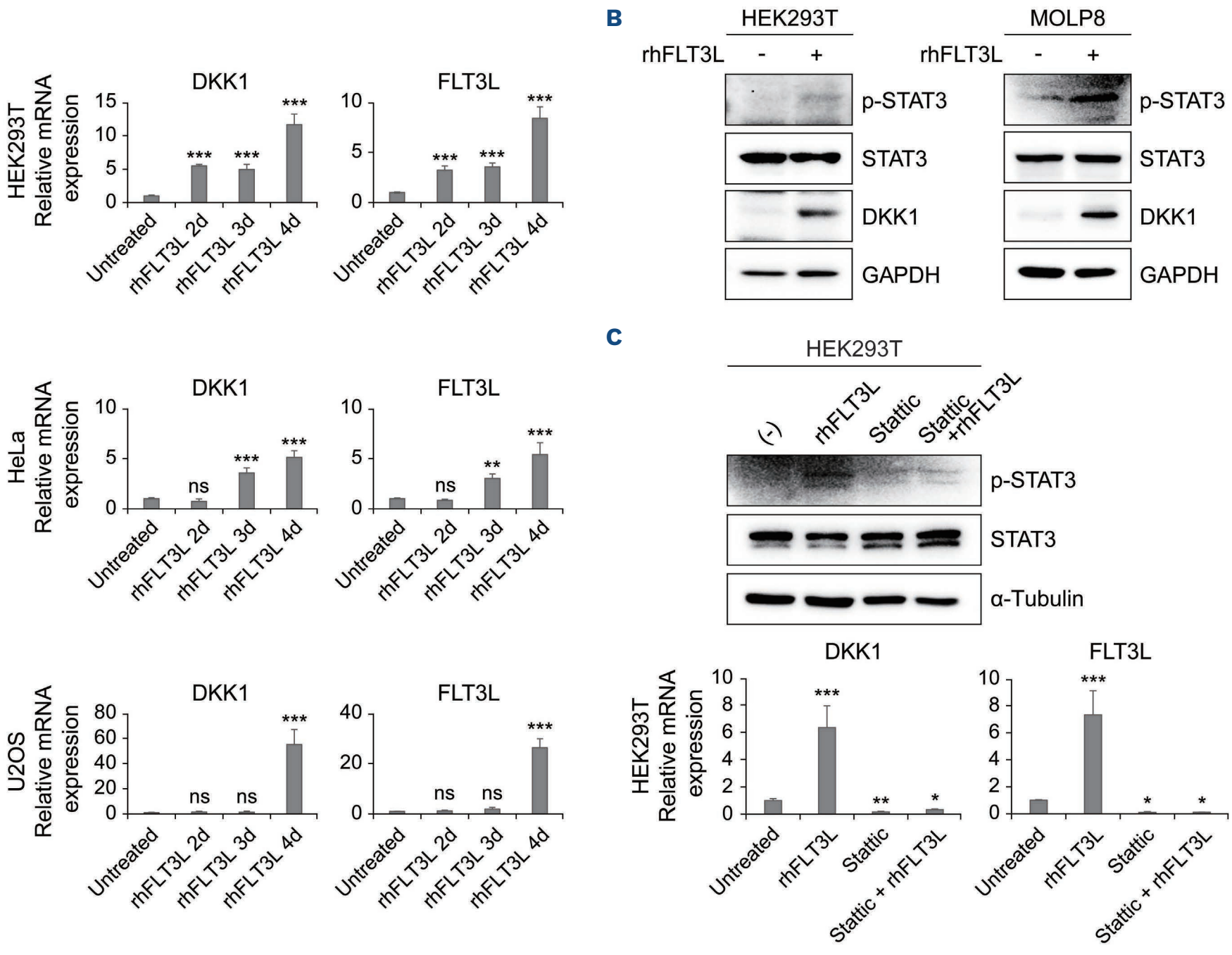

Figure 2. Fms-like tyrosine kinase 3 ligand enhances Dick Kopf-related protein 1 expression via STAT3 signaling. (A) The mRNA expression of Dick Kopf-related protein 1 (*DKK1*) and Fms-like tyrosine kinase 3 ligand (*FLT3L*) obtained from HEK293T, HeLa, and U2OS cells treated with 40 ng/mL rhFLT3L for from two to approximately four days, as analyzed by quantitative real-time polymerase chain reaction (RT-qPCR) (N=4). (B) Western blot analysis of phosphorylation of STAT3, total STAT3 and DKK1 under treatment with rhFLT3L (40 ng/mL) in HEK293T and MOLP8 cells for three days. (C) In HEK293T on treatment with rhFLT3L (40 ng/mL), Stattic (10 μM), or both for three days, protein level of phosphorylated STAT3 and total STAT3 and mRNA level of *DKK1* and *FLT3L* were analyzed by western blotting and RT-qPCR, respectively (N=8). Statistical analysis was performed by one-way ANOVA with Dunnett's multiple comparison. Error bars indicate the standard deviation of 3 independent replicates. **P*<0.05, ***P*<0.01, ****P*<0.001, ns: not significant (*P*>0.05).

respectively). Taken together, these data suggest that the FLT3L level can serve as a promising factor that reflects both osteolytic bone lesion and prognosis in MM.

FLT3L enhances DKK1 expression via STAT3 signaling

It has been reported that expression of the *DKK1* gene, a soluble inhibitor for WNT signaling, is significantly elevated in plasma cells from patients presenting with MM, and the enhanced DKK1 expression in turn leads to defects in osteoblast differentiation.[12] However, the molecular basis of how DKK1 expression is enhanced in MM has not been determined. As both FLT3L and DKK1 are increased in MM patients, we hypothesized that expression of DKK1 might be positively regulated by FLT3 signaling. To test this hypothesis, we treated FLT3L to HEK293T, HeLa, and U2OS cells and measured the *DKK1* transcription levels using RT-qPCR. Intriguingly, cells treated with FLT3L exhibited the increased *DKK1* transcript levels (Figure 2A). The *DKK1* transcript level increased over time during the culture of up to four days. Consistent with previous reports,[26,27] we confirmed that *FLT3L* transcript is also enhanced upon FLT3L treatment (Figure 2A, right).[28] Next, we attempted to elucidate the molecular basis of the increased *DKK1* expression through FLT3L signaling. FLT3L signaling was reported to be mediated by STAT3 phosphorylation.[29,30] We consistently found that STAT3 was activated by phosphorylation at tyrosine 705 in HEK293T and MOLP8, an MM cell line, upon treatment of FLT3L (Figure 2B). Again we were able to observe enhanced DKK1 expression in response to FLT3L treatment by western blot analysis (Figure 2B). In order to confirm that the enhanced DKK1 expression is mediated by STAT3 phosphorylation, we treated the cell with STAT3 signaling inhibitor, Stattic, and found that treatment of Stattic significantly reduced FLT3L-mediated STAT3 phosphorylation (Figure 2C, top). RT-qPCR showed that the inhibition of STAT3 results in a significant reduction in *DKK1* expression (Figure 2C, bottom). These results suggest that FLT3L signaling activates the *DKK1* transcription through STAT3 phosphorylation.

FLT3L-induced DKK1 attenuates WNT signaling to reduce osteogenesis

We next tested whether the enhanced DKK1 expression impairs WNT signaling, which can result in reduced osteogenic activity. To this end, we examined the β-catenin activity in HEK293T cells in response to DKK1 and/or FLT3L. It was seen that DKK1 actually reduced the β-catenin protein level, which became further exacerbated when FLT3L was added (Figure 3A). Correspondingly, FLT3L treatment led to reduced nuclear translocation of β-catenin (Figure 3B). To further confirm this FLT3L-mediated reduction of WNT signaling at the cellular level, we performed a WNT reporter assay using the construct in which the expression of luciferase was under the promoter containing β-catenin responsible elements. As expected, the reporter activity was reduced on FLT3L treatment, which was much further reduced when DKK1 was added (Figure 3C). In order to validate the impact of FLT3L on osteoblast differentiation at the cellular level, we performed alkaline phosphatase (ALP) staining analysis, which has been widely used for evaluating osteogenesis. We found a significant reduction in ALP staining and activity in the mouse pre-osteoblast cell line, MC3T3-E1, following treatment with FLT3L or DKK1, while the addition of BMP2 enhanced the osteoblast differentiation (Figure 3D, E). In addition, RT-qPCR with the cells in the same setting showed that treatment of FLT3L significantly enhanced *DKK1* expression at the transcriptional level (Figure 3F), demonstrating the detrimental effect of FLT3L on osteogenesis. To further explore the relationship of the FLT3L-DKK1 pathway with osteolytic bone lesions, we next measured DKK1 level by ELISA in a subgroup of MM patients whose BM plasma samples were available (N=35). FLT3L and DKK1 levels tended to show a positive correlation in MM patients with osteolytic bone lesion (Adjusted [Adj] R^2 0.0922, P=0.1809), but a negative correlation in MM patients without osteolytic bone lesion (Adj R^2 = -0.0183) (*Online Supplementary Figure S2A, B*). Next, we explored whether FLT3L would be also implicated in osteoclastogenesis, which could lead to bone loss. To this end, we established a TRAP assay using mouse BM and found that FLT3L treatment does not affect osteoclastogenesis (*Online Supplementary Figure S3A, B*). Taken together, these findings demonstrate that osteolytic bone lesions mediated by the FLT3L-DKK1 pathway is due to the reduced osteogenesis through the inhibited WNT signaling, and not to the enhanced osteoclastogenesis.

FLT3L and DKK1 are highly expressed in malignant plasma cells with hyperdiploidy

It has been reported that patients with hyperdiploidy (HY) showed the elevated level of *DKK1*, but only about 59% of these patients had osteolytic bone lesions.[12] This suggests that only a subset of patients show osteolytic bone lesion mediated by the FLT3L-DKK1 pathway. To explore this subset of patients, we obtained the previously reported mRNA expression profiles of plasma cells from 414 patients with MM (GSE2658[22] in GEO database). When the gene expression profiles from all patients were used, we found virtually no correlation (Spearman's correlation = -0.02) in mRNA expression levels of *FLT3L* and *DKK1* (*Online Supplementary Figure S4A*). We then examined mRNA expression patterns of *FLT3L* and *DKK1* across the previously defined 7 subtypes of MM patients and found that the HY subtype showed the highest median mRNA expression level of both *FLT3L* and *DKK1* (Figure 4A). Moreover, the HY subtype showed the largest percentage (43.10%) of patients showing high (≥ median expression level in all samples) expression levels of both *FLT3L* and *DKK1* compared with the other subtypes (Figure 4B, *Online Supplementary Figure S4B*). Correspondingly, the HY subtype had the smallest percentage (6.03%) of patients with low expression levels of *FLT3L* and *DKK1*. These results suggest that HY subtype may represent a subset of patients

with FLT3L-DKK1 pathway-mediated bone osteolysis.

To understand the characteristics of the HY subtype, we next identified 662 genes predominantly up-regulated in the HY subtype (*Online Supplementary Figure S5A*) and then examined cellular pathways represented by these genes using ConsensusPathDB[31] (Figure 4C). These genes defining the HY subtype were significantly (P<0.05) associated with FLT3 and JAK-STAT signaling pathways, consistent with our findings (see Figure 2). We further divided the HY subtype patients into those with high levels of both *FLT3L* and *DKK1* (*FLT3L*^{H}*DKK1*^{H} in Figure 4B) and the others (*FLT3L*^{H}*DKK1*^{L}, *FLT3L*^{L}*DKK1*^{H} and *FLT3L*^{L}*DKK1*^{L}), and identified 984 differentially expressed genes (DEG; 471 up-regulated and 513 down-regulated in *FLT3L*^{H}*DKK1*^{H}) between the 2 subgroups. The up-regulated genes most strongly represented cytokine response, and also, significantly, other pathways previously

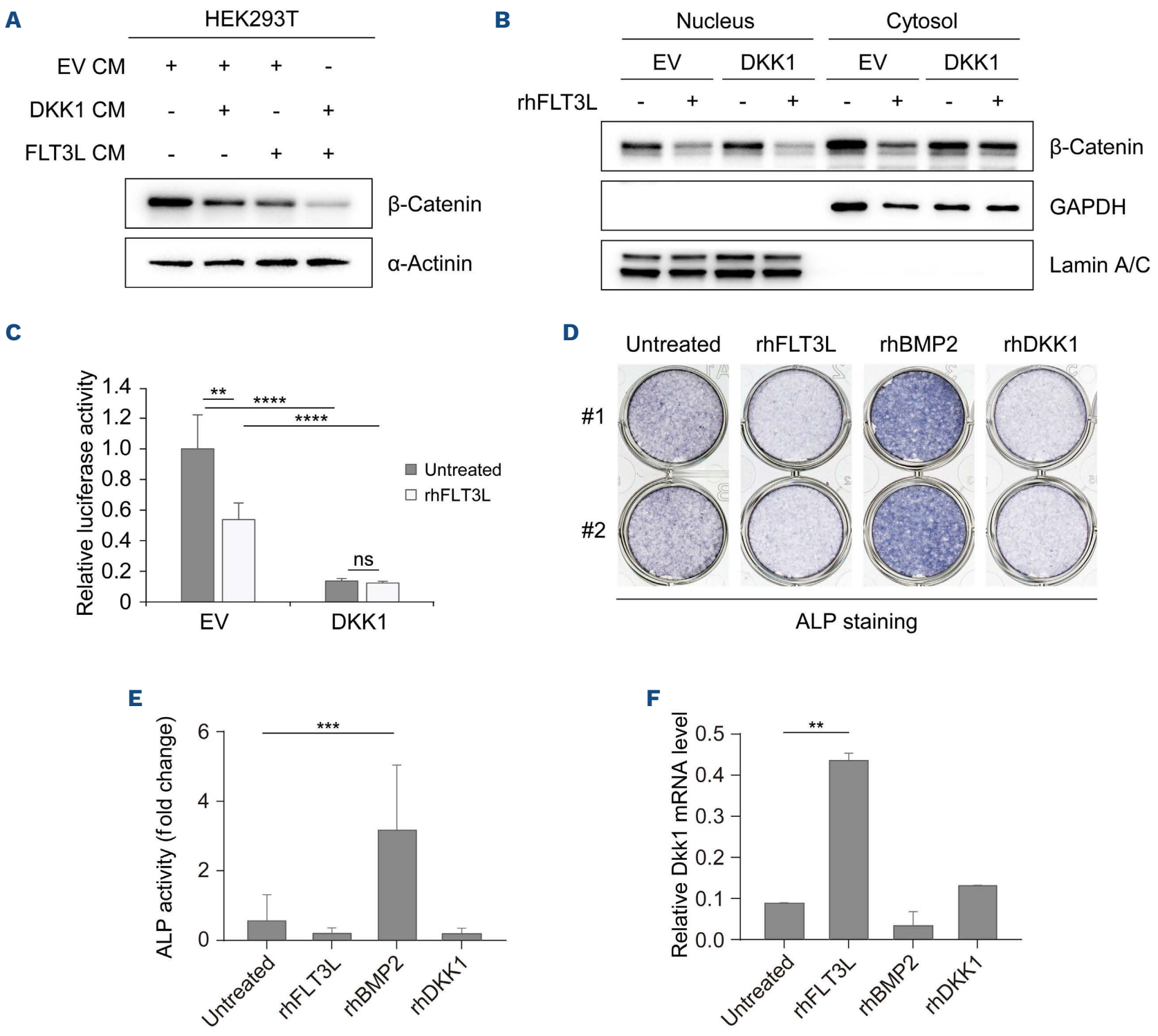

Figure 3. Fms-like tyrosine kinase 3 ligand-induced Dick Kopf-related protein 1 attenuates WNT/β-catenin signaling. (A) Conditioned media (CM) obtained from HEK293T transiently transfected with DNA construct, such as Dick Kopf-related protein 1 (DKK1), Fms-like tyrosine kinase 3 ligand (FLT3L), or both were treated into newly HEK293T cells for three days. Expression of β-catenin protein from cell lysates was analyzed by western blot assay. (B) EV (empty vector) and DKK1 transiently expressing HEK293T cells were treated with rhFLT3L (40 ng/mL) for three days. Nuclear and cytoplasmic fraction from the cell pellets was separated by Extraction kit. Protein levels of β-catenin and GAPDH (cytosol) and Lamin A/C (Nucleus) as loading controls were determined by western blotting. (C) Reporter gene assay for β-catenin-mediated transcriptional activation was conducted in HEK293T transiently transfected with plasmids carrying EV or DKK1 under treatment of rhFLT3L (40 ng/mL) for three days (N=4). (D) Alkaline phosphatase staining and (E) activity were evaluated with MC3T3-E1 cells treated with mock, rhFLT3L, rhBMP2 or rhDKK1 for seven days (N=6). (F) Relative mRNA expression level of *DKK1* was determined by qRT PCR. mRNA was prepared from the MC3T3-E1 cells treated with mock, rhFLT3L, rhBMB2 or rhDKK1 for seven days (n=3). Error bars indicate the standard deviation of at least 3 independent replicates. ALP: alkaline phosphatase **P<0.01, ***P<0.001, ****P<0.0001, ns: not significant (P>0.05).

reported to be associated with bone lesion or loss (Figure 4D, left: VEGF,[32] mTOR,[33] TNF[10] and MAPK[34] signaling pathways). In contrast, the down-regulated genes were associated with bone growth and its associated pathways (Figure 4D, right: cell development, response to EGF, ECM organization, calcium signaling, and bone resorption).

To focus on the cytokine response, we identified 9 secretory proteins among the DEG and found that *BMP5, NPY*, and *POSTN* were up-regulated in *FLT3L*^{H}*DKK1*^{H} while *CALCA, COMP, CTSK, CRYAB, NTF3*, and *TNC*[35] were down-regulated (*Online Supplementary Figure S5B*). Among them, 4 down-regulated genes (*CALCA*,[36] *CTSK*,[37] *CRYAB*,[38] and *TNC*[35]) are known to be associated with WNT signaling and osteogenesis, and showed the lowest expression levels in *FLT3L*^{H}*DKK1*^{H} across all subtypes of MM, suggesting that they may serve as potential effector cytokines leading to reduced bone osteogenesis by

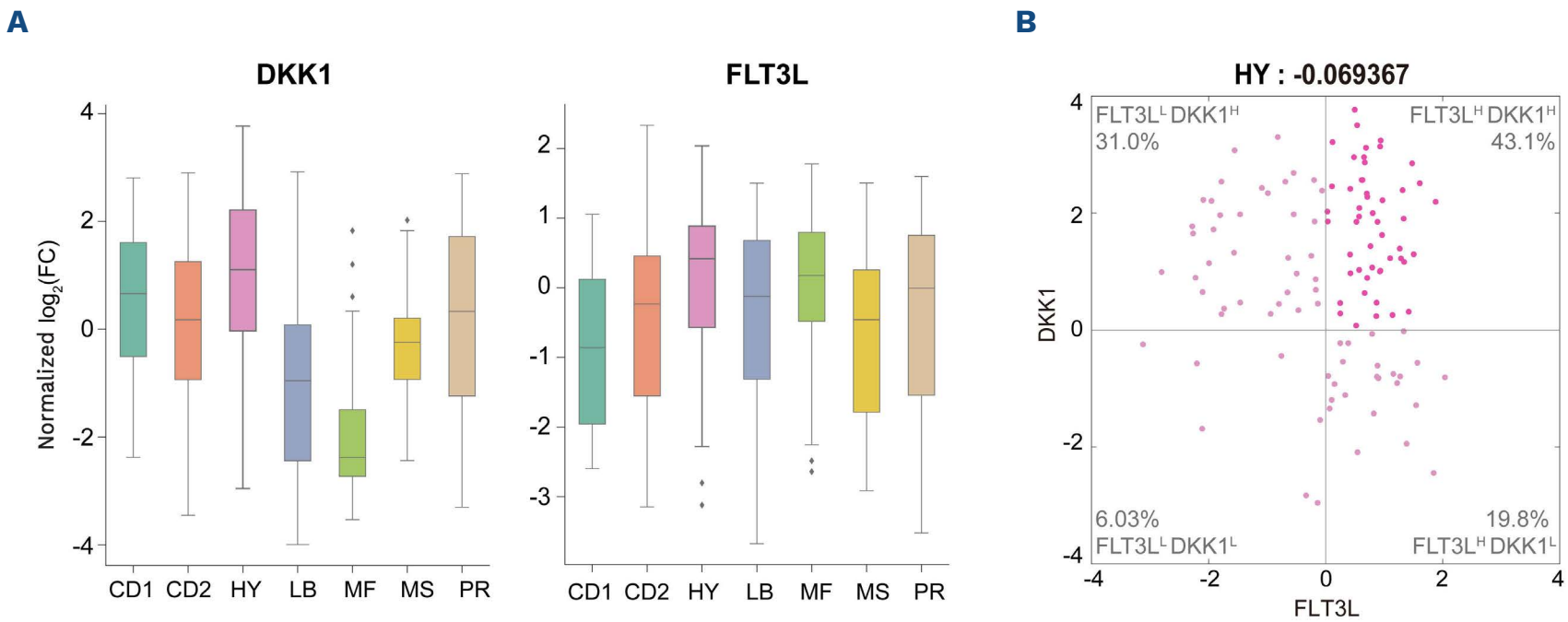

Figure 4. Fms-like tyrosine kinase 3 ligand and Dick Kopf-related protein 1 are highly expressed in plasma cells of multiple myeloma patients with hyperdiploidy subtype. (A) Boxplots showing distributions of log_2-fold-changes of mRNA expression levels of Fms-like tyrosine kinase 3 ligand (*FLT3L*) and Dick Kopf-related protein 1 (*DKK1*) across 7 subtypes (CD1, CD2, HY, LB, MF, MS and PR) reported by Zhan *et al.*[50] Log_2-fold changes were computed with respect to the median mRNA expression value in all samples. (B) Scatterplot showing log_2-fold changes of mRNA expression levels between *FLT3L* (x-axis) and *DKK1* (y-axis) in patients (N=116) of the hyperdiploidy (HY) subtype. Spearman's correlation between *FLT3L* and *DKK1* (top) and the percentage of patients in each quadrant are displayed in the HY subtype. Median expression value for each gene was used to define high expression of the gene. (C) Gene ontology biological processes (GOBP) and pathways represented by the signature genes for the HY subtype. (D) GOBP and pathways represented by the up-regulated (left) or down-regulated (right) genes in the HY subgroup with high expression of both *FLT3L* and *DKK1 (FLT3L*^{H}*DKK1*^{H}*)* compared to the other subgroups. Significance (*P*-value) of the GOBP and pathways being enriched by the genes is displayed as -log_{10}(*P*-value). Cut-off of enrichment *P*-value (0.05) is indicated by the line. Pathways used to build a network model are highlighted. GPCR: G protein coupled receptors.

FLT3L-DKK1 pathway. In summary, a network model (*Online Supplementary Figure S6*) shows that *FLT3L* binds to its receptor *FLT3*, and *FLT3* then activates STAT3 signaling to induce *DKK1*, which inhibits WNT signaling to decrease its target genes involved in bone formation.

Discussion

Osteolytic bone lesions greatly diminish the QoL for advanced MM patients. Unfortunately, the molecular mechanisms underlying the development of these bone lesions have not yet been determined, which poses a challenge for the development of effective therapeutic strategies. In this study, we aimed to address this knowledge gap by examining plasma samples derived from the BM of 86 MM patients, 306 AML patients, and 52 ALL patients. Our investigation revealed that the expression of FLT3L in MM patients was significantly higher compared to AML and ALL patients. Notably, elevated FLT3L levels were found to be positively correlated with a poorer prognosis in MM patients. *In vitro* analysis demonstrated that FLT3L-mediated STAT signaling pathways contribute to the upregulation of DKK1 expression, a soluble antagonist for the WNT signal cascade. Given the role of WNT signaling in osteoblastic differentiation, these findings suggest that the FLT3L-STAT3-DKK1 axis is involved in the development of osteolytic bone lesions in MM patients. Furthermore, our analysis of previously collected transcriptome data revealed that the activation of the FLT3L-STAT3-DKK1 pathway predominantly occurs in the HY subtype of MM. This finding holds significant promise for improving the accuracy of bone osteolysis prediction and treatment, as it provides a potential diagnostic marker to identify the involvement of the FLT3L-STAT3-DKK1 pathway. Taken together, these findings provide novel insights into the molecular mechanisms underlying osteolytic bone lesions in MM patients. By uncovering the FLT3L-STAT3-DKK1 axis as a key pathway, these studies shed light on the new opportunities for the diagnosis of MM and the development of potential therapeutic interventions for MM patients.

Hyperdiploidy has been seen to be a good prognostic factor in MM,[39,40] while, in our study, high FLT3L was associated with osteolytic bone lesion and poor survival. To explore the relationship between FLT3L, HY, and FLT3L-DKK1-mediated bone osteolysis, we performed multivariate logistic regression analysis. In this analysis, FLT3L served as a dependent variable, while clinical factors including HY and osteolytic bone lesions were treated as independent variables. We noticed that FLT3L was not only associated with hyperdiploidy, which is known as a good prognostic factor (P=0.044), but also associated with plasma cell fraction in BM, a surrogate marker of disease stage (P=0.029) (*Online Supplementary Table S1*). These findings may appear inconsistent with the claim in the current study that HY-MM subtype presumably represented FLT3L-DKK1 pathway-mediated pathological features, leading to subsequent deterioration in the QoL of the affected patients. However, recent studies have revealed that even among patients with the HY-MM subtype there is a considerable cytogenetic heterogeneity, resulting in distinct clinical outcomes and diverse prognoses.[39,41,42] Indeed, in our study, the enrichment of the $FLT3L^{H}DKK1^{H}$ group among HY-MM subtype patients led to discernible differences in transcriptomic profiles compared to the other HY-MM patients (Figure 4F). Therefore, detailed functional relationships of FLT3L-DKK1 pathway with the previously established MM subtypes, along with its potential use as biomarkers for MM prognosis prediction, still have to be clarified.

Phase III randomized clinical trials proved that pamidronate and zoledronate, the 2 most commonly used bisphosphonates in the clinic, reduce bone osteolysis in MM.[28,43] However, the prolonged use of these bisphosphonates can cause the serious complication of bisphosphonate-related osteonecrosis of jaw (BRONJ) or subtrochanteric femoral insufficiency.[44,45] Although denosumab, a RANKL inhibitor recently approved for bone-directed adjunctive therapy for MM, was proven to be more effective than the bisphosphonates,[46] the side effect of BRONJ has also been reported in patients treated with denosumab. The addition of bisphosphonate[46,47] or RANKL-targeted monoclonal antibody to the standard chemotherapy regimens can lessen bone resorption by suppressing osteoclastic activity, but these agents cannot fully inhibit the progression of the bone osteolytic process. Furthermore, the recovery of osteolytic lesions during treatment is rarely observed, which means that the current strategy to target osteoclastic activity in MM patients with bone involvement has still not been successfully achieved. Thus, it is important to develop alternative or complementary therapies that can target reduced osteoblastic activity that can, therefore, be used together with existing therapeutic options to improve the treatment of bone osteolysis in patients with MM.

New effective bone-directed therapeutic strategies need to be developed to overcome the adverse effect of the current standard therapies and improve MM patients' QoL. Targeting the FLT3L-STAT3-DKK1 pathway that primarily reduces the osteoblastic activity via suppression of WNT signaling might be a promising therapeutic approach because it can restore the reduced osteoblastic activity. Inhibition of FLT3L can thus provide a complementary therapeutic axis to the existing bisphosphonates and RANKL inhibitors that mainly target the osteoclastic activity. Targeting both osteoblastic and osteoclastic activities using a combined therapy of FLT3L/FLT3 inhibitors,[48] bisphosphonates, RANKL inhibitor and/or the standard chemotherapy are expected to effectively improve therapeutic outcomes for MM patients with osteolytic bone lesions. As a downstream regulator of FLT3 signaling, DKK1 induces inhibition of WNT signaling in osteoblastic differentiation in MM.[49] DKK1 inhibitors have been proposed as a promising therapeutic strategy to target the reduced osteoblastic activity in MM. The benefits and disadvantag-

es of FLT3L/FLT3 inhibitors over DKK1 inhibitors should be further investigated in a large cohort of patients.
We enrolled patients diagnosed with MM from 2004 to 2012, a period in which the ISS still had to gain popularity among clinicians, so we could not collect information from the ISS in our cohort. Nevertheless, we think that our information aligns well, not with the ISS, but with the old Durie-Salmon staging system, since the ISS does not include information about bone osteolysis, while the Durie-Salmon staging system does. Enrichment of patients having high expression of both *FLT3L* and *DKK1* in the HY subtype of MM suggested that FLT3L-mediated bone osteolysis might be prominent in the HY subtype. Accordingly, therapies to inhibit the FLT3L-mediated bone osteolysis might be most effective for patients in the HY subtype. These subtype-associated predictions should be verified in plasma cells derived from a large cohort of patients with MM.

Disclosures
No conflicts of interest to disclose.

Contributions
YK, SSY and DH designed and supervised the studies. DS recruited, treated, and followed the enrolled patients together with SSY. DS collected BM-derived plasma cells from patients. DS, DK, CL and KSA measured cytokines with DK, CL and KSA. MJK performed most of the in vitro experiments. EJ, BCL and DK performed the TRAP assay. SC analyzed transcriptome database. YK, DS, DH and SSY wrote the draft of the manuscript and all authors revised the final version for publication.

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Data-sharing statement
All the original data, reagents and protocols are available upon request.

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