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Multiple myeloma exploits Jagged1 and Jagged2 to promote intrinsic and bone marrow-dependent drug resistance

Michela Colombo,[1] Silvia Garavelli,[1] Mara Mazzola,[2] Natalia Platonova,[1] Domenica Giannandrea,[1] Raffaella Colella,[1] Luana Apicella,[1] Marialuigia Lancellotti,[1] Elena Lesma,[1] Silvia Ancona,[1] Maria Teresa Palano,[1] Marzia Barbieri,[3,4] Elisa Taiana,[3,4] Elisa Lazzari,[1] Andrea Basile,[3] Mauro Turrini,[5] Anna Pistocchi,[2] Antonino Neri[3,4] and Raffaella Chiaramonte[1]

[1]Department of Health Sciences, Università degli Studi di Milano, Milano; [2]Department of Medical Biotechnology and Translational Medicine, Università degli Studi di Milano, Milano; [3]Department of Oncology and Hemato-Oncology, Università degli Studi di Milano, Milano; [4]Hematology, Fondazione IRCCS Cà Granda, Ospedale Maggiore Policlinico, Milano and [5]Department of Hematology, Division of Medicine, Valduce Hospital, Como, Italy

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ABSTRACT

Multiple myeloma is still incurable due to an intrinsic aggressiveness or, more frequently, to the interactions of malignant plasma cells with the bone marrow (BM) microenvironment. Myeloma cells educate BM cells to support neoplastic cell growth, survival, acquisition of drug resistance resulting in disease relapse. Myeloma microenvironment is characterized by Notch signaling hyperactivation due to the increased expression of Notch1 and 2 and the ligands Jagged1 and 2 in tumor cells. Notch activation influences myeloma cell biology and promotes the reprogramming of BM stromal cells. In this work we demonstrate, *in vitro, ex vivo* and by using a zebrafish multiple myeloma model, that Jagged inhibition causes a decrease in both myeloma-intrinsic and stromal cell-induced resistance to currently used drugs, i.e. bortezomib, lenalidomide and melphalan. The molecular mechanism of drug resistance involves the chemokine system CXCR4/SDF1α. Myeloma cell-derived Jagged ligands trigger Notch activity in BM stromal cells. These, in turn, secrete higher levels of SDF1α in the BM microenvironment increasing CXCR4 activation in myeloma cells, which is further potentiated by the concomitant increased expression of this receptor induced by Notch activation. Consistently with the augmented pharmacological resistance, SDF1α boosts the expression of BCL2, Survivin and ABCC1. These results indicate that a Jagged-tailored approach may contribute to disrupting the pharmacological resistance due to intrinsic myeloma cell features or to the pathological interplay with BM stromal cells and, conceivably, improve patients' response to standard-of-care therapies.

Introduction

Multiple myeloma (MM) is the second most common hematologic malignancy. It is still incurable, with a median overall survival that has not been substantially extended since the introduction of anti-myeloma agents such as melphalan, lenalidomide, and bortezomib.[1] The typical clinical course of MM displays a remission-relapse pattern due to the appearance of drug-resistant malignant cells, reducing the numbers of effective salvage regimens.[2] Therefore, a more stable response requires the development of a therapeutic approach that prevents drug resistance.

Multiple myeloma cells accumulate in the bone marrow (BM), where they establish anomalous signaling loops with BM-residing non-tumor cells, resulting in the exchange of anti-apoptotic factors which critically induce drug resistance.[3]

The Notch pathway includes four transmembrane receptors (Notch1-4) activat-

Correspondence:

RAFFAELLA CHIARAMONTE
raffaella.chiaramonte@unimi.it

MICHELA COLOMBO
michela.colombo@ndcls.ox.ac.uk

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ed by the interaction with five ligands (Jagged1-2 and Dll1-3-4) on adjacent cells.[4-6] Notch receptors and ligands have been found to be aberrantly expressed in MM cells.[7-10] We recently demonstrated that Jagged1 and the Notch transcriptional target HES5 are increasingly expressed in MM and in primary plasma cell leukemia.[11] Moreover, Jagged1 and Notch1 are over-expressed during progression from the benign monoclonal gammopathy of uncertain significance (MGUS) to MM,[12] while Jagged2 overexpression is already detected at the MGUS stage[13] and can be ascribed to aberrant acetylation of its promoter[14] or to altered post-translational processing due to aberrant expression of the ubiquitin ligase Skeletrophin.[15] Finally, Notch2 hyperexpression is associated with the high-risk translocations t(14;16)(q32;q23) and t(14;20)(q32;q11).[16]

Recently, we and other groups pointed out the importance of Jagged ligands in providing MM cells with the ability to shape the surrounding microenvironment, interacting with osteoclast progenitors,[17] and promoting a release of BM stromal cell (BMSC) key factors, including IL6, IGF1 and VEGF.[11,13]

Aberrant levels of Notch signaling are associated with pharmacological resistance in different tumor settings[6] and correlate with the expression of anti-apoptotic genes, such as *BCL2*[18] and *Survivin/BIRC5*,[19] or regulates the expression of *ABCC1*,[46] which contributes to multidrug resistance in MM.[20]

Given this, we hypothesized that the aberrant expression of Notch receptors and ligands in MM cells may foresee the development of drug resistance by inducing autonomous activation of Notch in MM cells, and by triggering Notch signaling in the surrounding BMSC and boosting their ability to support MM cell drug resistance.[21,22]

Previous studies investigated how BMSC support the development of drug resistance in MM cells by activating Notch signaling.[23-25] *Vice versa*, here we show that also the overexpression of MM cell-derived Jagged ligands triggers Notch signaling dysregulation in the BM niche and promotes MM cell intrinsic pharmacological resistance as well as BMSC-dependent drug resistance.

Methods

Cell lines and primary cells

The human MM cell lines (HMCL), OPM2 (ACC-50) and U266 (ATCC® TIB-196) were purchased from the DSMZ and ATCC, respectively. Primary cells were isolated from patient BM aspirates and MM cells were purified using the Human Whole Blood CD138$^+$ Selection Kit EasySep (StemCell Technologies). Detailed information is available in *Online Supplementary Table S1*. Primary BMSC were isolated as previously reported.[11] The Ethical Committee of the Università degli Studi di Milano, Italy, approved this study (approval n. 8/15).

Details of all cell treatments are available in the *Online Supplementary Appendix*.

Luciferase reporter assay

HS5 cells were transiently transfected with a Notch reporter plasmid pNL2.1 carrying a 6xCSL Notch responsive element[26] and with the vector constitutively expressing the firefly luciferase upon the thymidine kinase promoter (pGL4.54[luc2/TK]). After 24 hours (h), HS5 cells were cultured alone or placed in co-culture with scrambled (Scr) or Jagged1 and Jagged2 knockdown (J1/2KD) HMCL and incubated for 24 h. Luciferase activity was measured using Nano-Glo® Dual-Luciferase® Reporter assay kit (Promega) on the Glowmax instrument (Promega).

In vivo experiments on xenografted zebrafish embryos

Zebrafish AB strains obtained from the Wilson lab, University College London, UK, were maintained according to the national guidelines (Italian Ministerial Decree of 4/03/2014 2014, n. 26). All experiments were conducted within five days post fertilization.

Dechorionated zebrafish embryos were injected with Scr or J1/2KD U266 cells stained with the CM-Dil dye into the yolk (200 cells in 10 nl, 5-20 nl injection volume/embryo) with a manual microinjector (Eppendorf, Germany) using glass microinjection needles.

Xenograft-positive embryos divided randomly into the following groups: Scr-injected embryos treated with DMSO, Scr-injected embryos treated with 10 nM bortezomib, J1/2KD-injected embryos treated with DMSO, and J1/2KD-injected embryos treated with 10 nM bortezomib. Tumor growth was evaluated 48 h post injection (hpi) by fluorescence microscopy. Further details are available in the *Online Supplementary Appendix*.

Further details and information concerning cell cultures, RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR), RNAi assay, apoptosis assays, flow cytometry, ELISA, western blot and statistical analysis can be found in the *Online Supplementary Appendix*: experimental procedures.

Results

Jagged1/2 inhibition improves multiple myeloma cell response to standard-of-care drugs by increasing the anti-apoptotic background

To assess if Jagged1 and Jagged2 contribute to MM intrinsic drug resistance, we took advantage of an established knockdown (KD) approach using specific siRNAs for Jagged ligands[11,17] and analyzed MM cell response to three standard-of-care drugs: bortezomib (Bor), melphalan (Melph), and lenalidomide (Len). Two HMCL, OPM2 and U266 cells, were transfected with Jagged1 and Jagged2 (J1/2KD) or the scrambled control (Scr) siRNAs and then were treated with 6 nM Bor or 30 μM Melph or with 15 or 30 μM Len (respectively for U266 and OPM2 cells) (Figure 1A). The efficacy of J1/2KD was assessed by evaluating the expression of Jagged ligands and the active forms of the two Notch receptors expressed in MM cells, Notch intracellular domains 1 and 2 (NICD1 and NICD2), by western blot (Figure 1B).

The apoptosis rate of J1/2KD HMCL treated or not with Bor, Melph and Len was analyzed by flow cytometry. Figure 1C shows the effect of Bor, Melph and Len on HMCL normalized on DMSO-treated cells compared to J1/2KD HMCL treated with the drugs and normalized on untreated J1/2KD HMCL. J1/2KD induced an appreciable increase in HMCL sensitivity to standard-of-care drugs, with statistical significance reached in all cases, with the exception of U266 cells treated with Bor and Melph (P=0.06), that in any case confirmed the trend (Figure 1C). The basal apoptotic effect of J1/2KD is shown in *Online Supplementary Figure S1*. Concerning Len treatment, it is worth mentioning that, although Scr HMCL are resistant to this drug, J1/2KD cells acquire drug sensitivity. The selective inhibition of Jagged1 or Jagged2 is clearly less

effective in comparison with the simultaneous J1/2KD, that maximizes the biological outcome (*Online Supplementary Figure S2*).

These results indicate that the expression of Jagged1 and 2 stimulates autonomous Notch activity in MM cells that, consequently, may be inhibited by Jagged silencing. This evidence prompted us to verify whether the increased pharmacological sensitivity of MM cells induced by J1/2KD was associated to variations in the expression of recognized anti-apoptotic Notch targets, such as BCL2[18] and *Survivin/BIRC5*,[19] or with the levels of *ABCC1* reported to have a significant impact in MM.[19,20,27] J1/2KD, validated by the decrease in Jagged1, 2 and HES1 and 6 gene expression, significantly inhibited the expression of the studied anti-apoptotic genes analyzed by qRT-PCR (Figure 2A and B). The effect of J1/2KD on gene expression was seen not to be due to an increased apoptosis rate in HMCL (approx. 15%) (*Online Supplementary Figure S1*). J1/2KD effect on anti-apoptotic effectors was assessed at protein levels by flow cytometry (Figure 2C and D and *Online Supplementary Figure S3*) and western blot (*Online Supplementary Figure S4*). By contrast, the selective inhibition of Jagged1 or Jagged2 was not sufficient to significantly down-regulate the expression of these genes (*Online Supplementary Figure S5*).

Jagged1 and Jagged2 silencing contributes to multiple myeloma cell ability to promote bone marrow stromal cell-mediated drug resistance

Multiple myeloma cells localize within the BM and interact with several cell types, hijacking their functions to promote tumor progression. BMSC are a crucial target in this process that sustains malignant cell proliferation and survival.[22] Since Jagged-mediated activation of Notch pathway is involved in cell-cell communication,[6] we hypothesized that MM cell-derived Jagged ligands could activate Notch in BMSC, possibly determining BMSC-mediated drug resistance.

To explore this hypothesis, we first verified that HMCL-derived Jagged1 and Jagged2 were able to trigger the activation of Notch signaling in a BMSC line, HS5, using a Notch reporter assay. Scr HMCL are able to activate Notch signaling in co-cultured HS5 cells (Figure 3A), while this ability is lost by J1/2KD HMCL, indicating that MM-derived Jagged may activate Notch signaling in BMSC.

To verify if Jagged-mediated activation of Notch in BMSC affected the ability of these cells to promote drug resistance in MM cells, we used flow cytometry to analyze the apoptotic rate of Scr or J1/2KD HMCL cultured alone or co-cultured with HS5 cells after treatment with standard-of-care drugs. As expected, HS5 cells show a clear

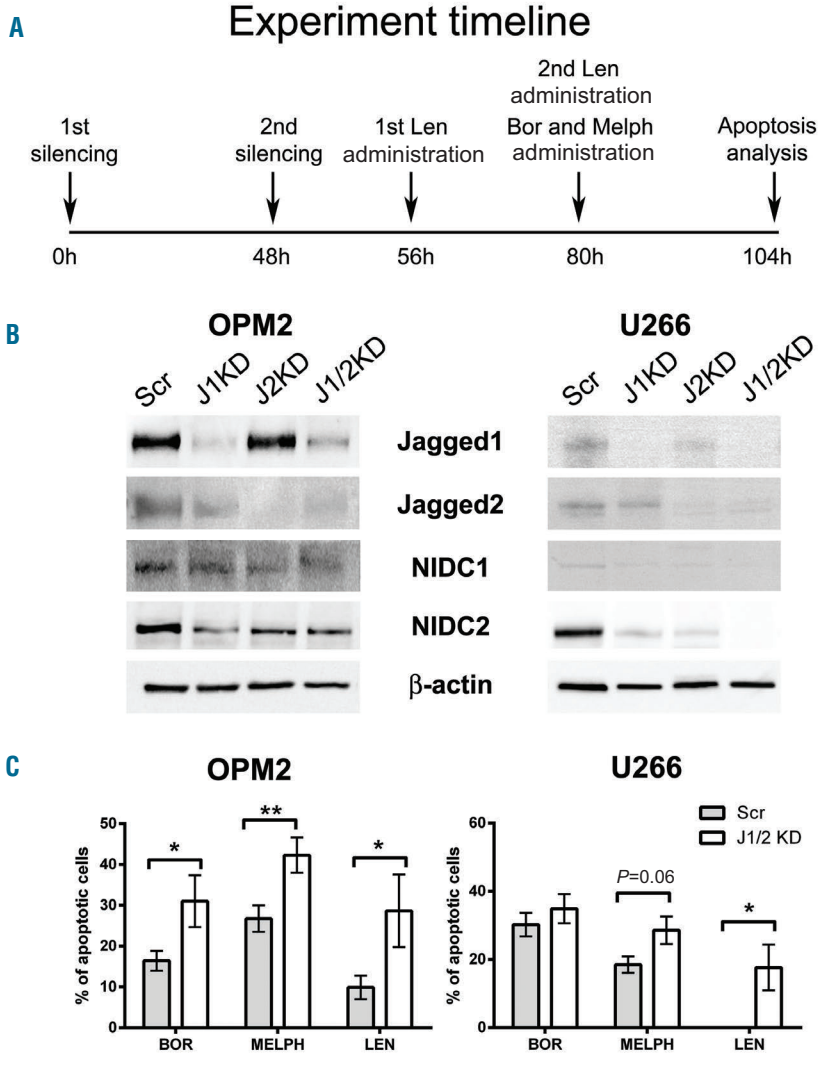

Figure 1. J1/2 silencing increases drug response in multiple myeloma (MM) cells. (A) Timeline of the experiment to study J1/2KD effect drug response in MM cell lines (HMCL). h: hour. (B) Representative western blots showing the expression of Jagged1, Jagged2, NICD1 and NICD2 in OPM2 and U266 cells following single and combined Jagged1 and/or Jagged2 silencing. β-actin was used as loading control. (C) The effect of J1/2KD on OPM2 (left) and U266 (right) cell response to bortezomib (Bor), melphalan (Melph) and lenalidomide (Len) was evaluated by Annexin V staining. MM cells were transfected with two specific siRNAs targeting Jagged1 and Jagged2 (J1/2KD) or the corresponding scrambled control (Scr) and treated with Bor, Melph or Len. Values of apoptosis of Scr HMCL were normalized to the corresponding DMSO-treated controls and values of J1/2KD HMCL treated with drugs were normalized to DMSO-treated J1/2KD HMCL. Results are shown as the mean±standard error of at least three independent experiments, and statistical analysis was performed using Mann-Whitney test (*P<0.05; **P<0.01).

trend of protection of HMCL from apoptosis induced by Bor (15% in OPM2 and 26% in U266), Melph (20% in OPM2 and 11% in U266), and Len (14% in OPM2) (Figure 3B and C), although the statistical significance was reached only in the case of OPM2 treated with Bor. Conversely and more importantly, J1/2KD induced a statistically significant increase in apoptosis, re-establishing HMCL drug sensitivity by hampering BMSC-mediated protection (HS5 cells do not display any significant increase in apoptosis; *data not shown*). Notably, although U266 cells were resistant to Len treatment in culture alone or in the presence of HS5 cells, apoptotic rate increased up to approximately 20% upon J1/2KD. The basal apoptotic effect of J1/2 KD on MM cells cultured with HS5 cells is reported in *Online Supplementary Figure S6*. As before, the selective Jagged1 or Jagged2 silencing was less effective than the simultaneous J1/2KD (*Online Supplementary Figure S7*).

Since HS5 cells could act as a source of paracrine/autocrine Jagged ligands, we wondered why they cannot rescue J1/2KD in MM cells. Western blot analysis indicates that the expression levels of Jagged1 and Jagged2 in HS5 cells are significantly lower than those expressed by OPM2 and U266 cells (*Online Supplementary Figure S8*). This can reasonably explain why, in our co-culture system, Notch signaling activated in HMCL by BMSC is not sufficient to rescue the loss of Jagged1 and Jagged2 in MM cells.

We further explored whether Jagged-mediated Notch activation in BMSC could promote the pharmacological resistance of MM cells by up-regulating the anti-apoptotic effectors previously analyzed, Survivin, BCL2, and ABCC1. To evaluate gene expression changes, we took advantage of a co-culture system including OPM2 or U266 cells with a non-human mimic model of BMSC, the murine cell line of NIH3T3 fibroblasts. This approach enabled us to precisely assess the expression levels of human (HMCL-derived) anti-apoptotic genes in co-culture by using species-specific primers. Results showed that BMSC were able to promote the expression of the anti-apoptotic effectors *Survivin, BCL2,* and *ABCC1* in Scr HMCL, while BMSC co-cultured with J1/2KD HMCL lost this ability (Figure 4A and B). Importantly, using an entire-

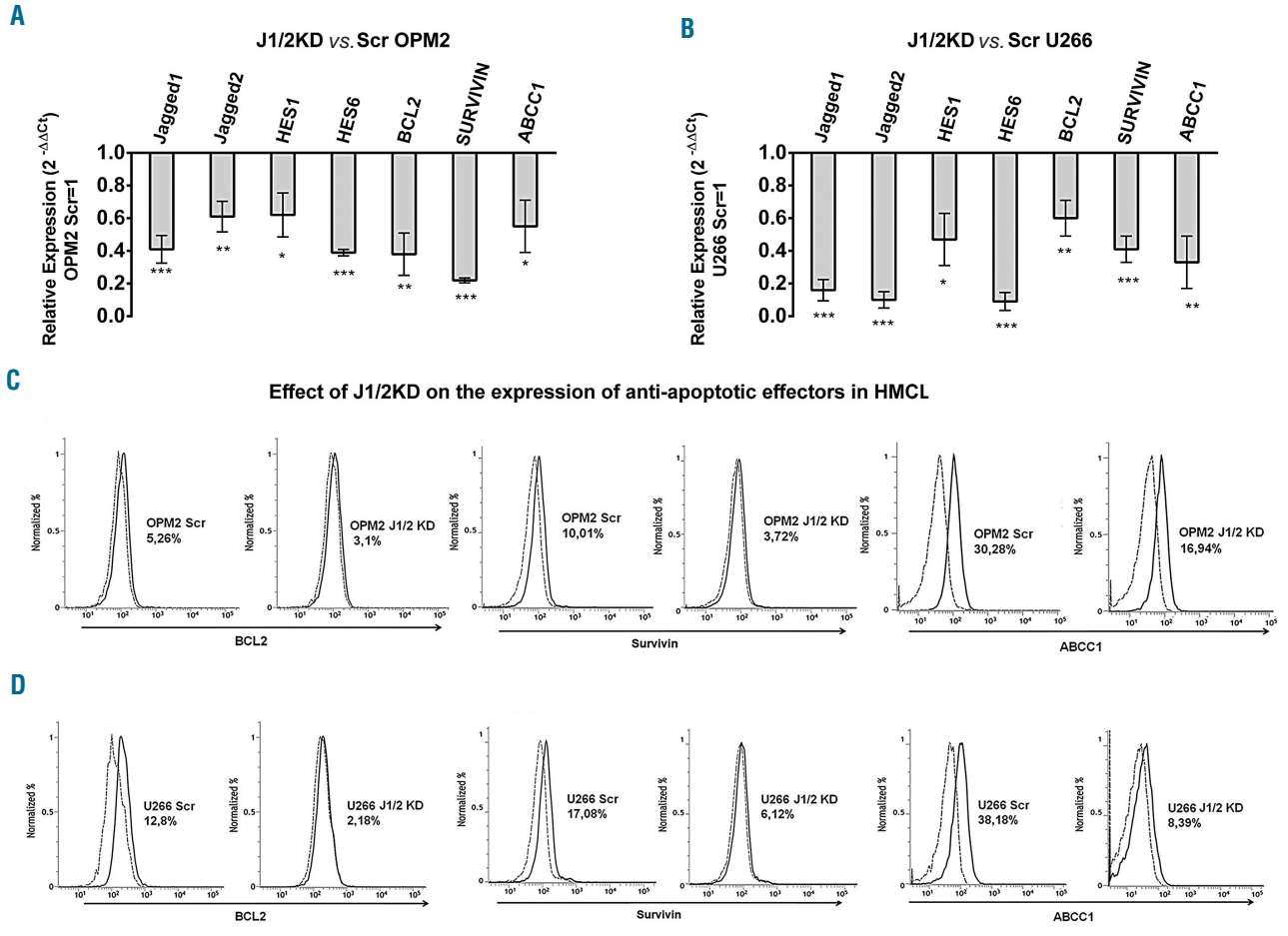

Figure 2. J1/2 withdrawal affects multiple myeloma (MM) cells anti-apoptotic background. We analyzed how J1/2KD affects Notch activation and the expression of anti-apoptotic genes in human multiple myeloma cell lines (HMCL). (A and B) Confirmation of J1/2KD efficacy in OPM2 (A) and U266 (B) cells was obtained by quantitative polymerase chain reaction (qPCR) assay assessing the relative gene expression variation of Jagged1 and Jagged2 and Notch target genes HES1 and HES6 (normalized to GAPDH) in cells transfected with J1/2 siRNA compared to cells transfected with Scr siRNA, calculated by the $2^{-\Delta\Delta Ct}$ formula. The expression levels of the anti-apoptotic effectors BCL2, Survivin, and ABCC1 were also analyzed. Data are expressed as mean± standard deviation of at least three independent experiments. Two-tailed *t*-test confirmed statistically significant downregulation of the tested genes; **P*<0.05; ***P*<0.01; ****P*<0.001. (C and D) Histograms display the levels of BCL2, Survivin and ABCC1 protein (black lines) analyzed by flow cytometry in J1/2KD OPM2 or Scr OPM2 (C) and J1/2KD U266 or Scr U266 (D) and an isotype-matched control (gray line). Histograms are representative of at least three independent experiments.

ly human co-culture system, we observed the same effects when we used flow cytometry to measure the protein expression of Survivin, BCL2, and ABCC1 in Scr or J1/2KD HMCL co-cultured with human GFP+ HS5 (Figure 4C-E and *Online Supplementary Figure S9*).

The CXCR4/SDF1α axis is a mediator of Notch pathway ability to determine drug resistance in multiple myeloma

To further study the molecular mechanisms underlying BMSC-induced drug resistance generated by Notch activation in the MM microenvironment, we explored the possible involvement of the chemokine system CXCR4/SDF1α, a key player in MM development and progression, and a downstream regulator of Notch signaling.[28,29] We hypothesized that Notch ability to promote pharmacological resistance in MM cells might be mediated by SDF1α. We reasoned that the main source of SDF1α in the BM was the stromal cell population. Therefore we explored if Jagged ligands, expressed by MM cells, could trigger the BMSC-mediated production of SDF1α and if J1/2KD might inhibit this effect.

The analysis was performed by taking advantage of co-culture systems of Scr or J1/2KD HMCL grown on a layer murine (NIH3T3) or human (HS5) stromal cells to measure the variations in SDF1α gene or protein expression. Results obtained by qRT-PCR with murine-specific primers (Figure 5A) indicate that HMCL promoted the activation of Notch signaling (HES5) and SDF1α gene expression in NIH3T3 cells, which could be reverted by J1/2KD.

Similar results were observed at protein level as assessed by flow cytometry analysis (Figure 5B and *Online Supplementary Figure S10*) on co-cultures composed of HMCL and the human GFP+ HS5 cells. Of note, the selective inhibition of Jagged1 or Jagged2 is clearly less effective if compared with the simultaneous J1/2KD, that maximizes the outcome on SDF1α inhibition (*Online Supplementary Figure S11*). Flow cytometric results were validated by ELISA on conditioned media (Figure 5C) indicating that MM cell-derived Jagged can increase SDF1α production by BMSC. We further confirmed that the variation in SDF1α expression was the consequence of Jagged-activated Notch signaling in BMSC by an assessment that showed that the stimulation with Jagged1 and/or Jagged2 peptides can increase HS5 cell-mediated secretion of SDF1α, measured by ELISA (Figure 5D). Additionally, we knocked down Notch1 expression in HS5 cells (N1KD HS5) by using a specific siRNA, as previously reported,[11] and observed that SDF1α expression significantly decreased in comparison to control HS5 cells (Figure 5E). Since Notch1 silencing does not significantly affect HS5 cell viability (On*line Supplementary Figure S12*), we could exclude the possibility that reduction of SDF1α expression might be due to HS5 cell apoptosis.

On the other hand, we verified that J1/2KD was associated to a reduced CXCR4 expression in HMCL used in co-culture experiments. J1/2KD HMCL significantly decreased CXCR4 expression in comparison to Scr HMCL (Figure 5F and *Online Supplementary Figure S13*).

We assessed the outcome of SDF1α stimulation on the anti-apoptotic background of HMCL by analyzing the levels of *Survivin, BCL2,* and *ABCC1* in U266 cells treated with 500 ng/mL SDF1α for 48 h. We observed an increase in *Survivin, BCL2*, and *ABCC1* gene expression by qRT-PCR analysis (Figure 5G) confirmed at protein level by western blot (Figure 5H). These results suggest that SDF1α can promote MM cell ability to survive to drug administration, at least in part, by stimulating tumor cell anti-

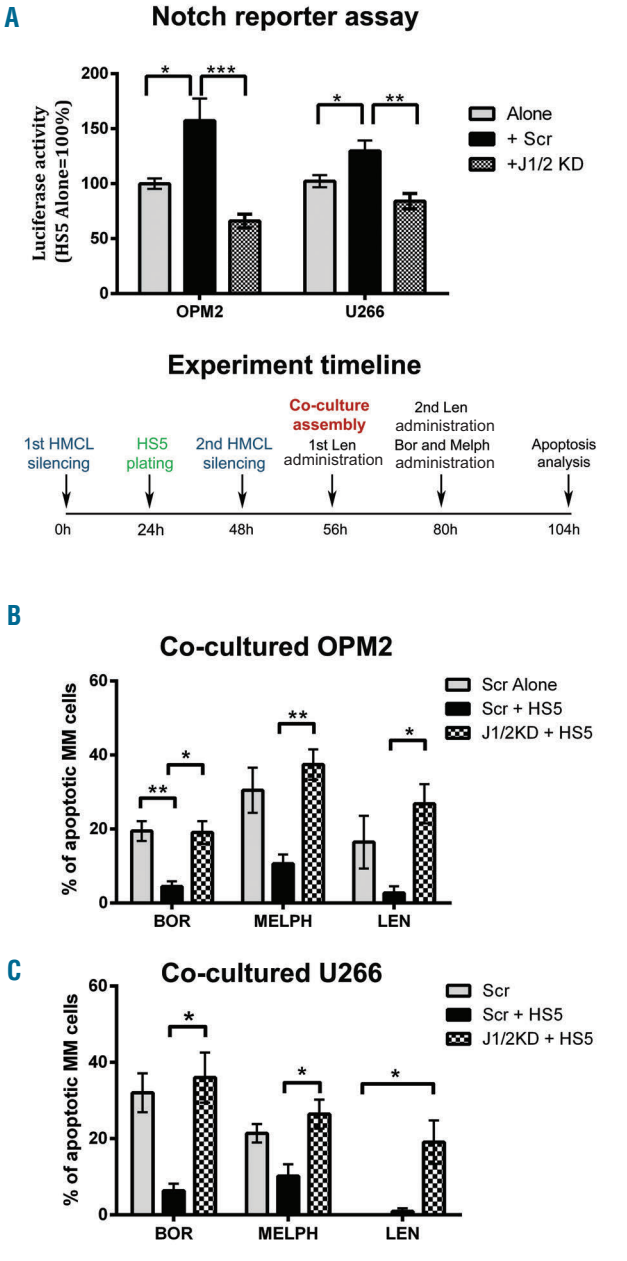

Figure 3. Effect of J1/2 inhibition on multiple myeloma (MM) cells ability to promote bone marrow (BM)-induced drug resistance. (A) A Notch-responsive dual luciferase assay was carried out in HS5 cells cultured alone or in the presence of Scr or J1/2KD human multiple myeloma cell lines (HMCL) for 24 hours (h). Data were normalized on luciferase activity in HS5 cells cultured alone (=100). Mean±standard deviation of three independent experiments are shown. Statistical analysis was performed using one-way ANOVA and Tukey post-test (*P<0.05; **P<0.01; ***P<0.001). (B and C) Co-cultures of J1/2KD or Scr HMCL with the BM stromal cell (BMSC) line HS5 were established to evaluate the effect of J1/2KD on BMSC-induced drug resistance. The experimental timeline is reported. Graphs show the percentage of apoptotic OPM2 (B) or U266 (C) cells (Annexin V+/GFP-). Values of apoptosis of each type of culture (Scr alone, Scr + HS5 and J1/2KD + HS5) treated with drugs are normalized to the corresponding controls treated with DMSO. Results are shown as mean±standard error of at least three independent experiments. Statistical analysis was performed using Kruskal-Wallis and Dunn post-test (*P<0.05; **P<0.01).

apoptotic defenses (Survivin, BCL2) and detoxification ability (ABCC1). Consistently, the treatment of U266-HS5 co-culture system with 50 μM AMD3100 (an antagonist of SDF1 binding to CXCR4), abrogated BMSC-induced resistance to the analyzed drugs (Figure 5I).

Translational potential of approaches inhibiting Jagged-mediated Notch activation in a multiple myeloma microenvironment

We further verified whether the ability of MM cells to promote BMSC-induced drug resistance was dependent on Jagged1 and Jagged2 expression by using primary co-culture systems of highly purified CD138$^+$ MM cells and BMSC isolated from BM aspirates of patients at MM onset (*Online Supplementary Table S1*).

Primary CD138$^+$ cells were transduced with the lentiviral vector pLL3.7 carrying Jagged1/2 shRNAs or Scr shRNAs and the efficiency was assessed by flow cytometry (*Online Supplementary Figure S14*). In order to maintain CD138$^+$ cell viability during *ex vivo* drug administration, after lentiviral transduction, they were co-cultured with primary BMSC stained with PKH26. Co-cultures were maintained for 72 h and treated for the last 24 h with 6 nM Bor (8 patients) or 30 μM Melph (10 patients), or for the last 48 h with 15 μM Len (9 patients) or the corresponding vehicle. The apoptotic rate of MM cells (expressing the GFP codified by the pLL3.7 vector) was detected by flow cytometry analyzing the GFP$^+$/Annexin-V-APC$^+$ subpopulation (Figure 6A). Results showed that J1/2KD significantly increased apoptosis of primary MM cells treated with

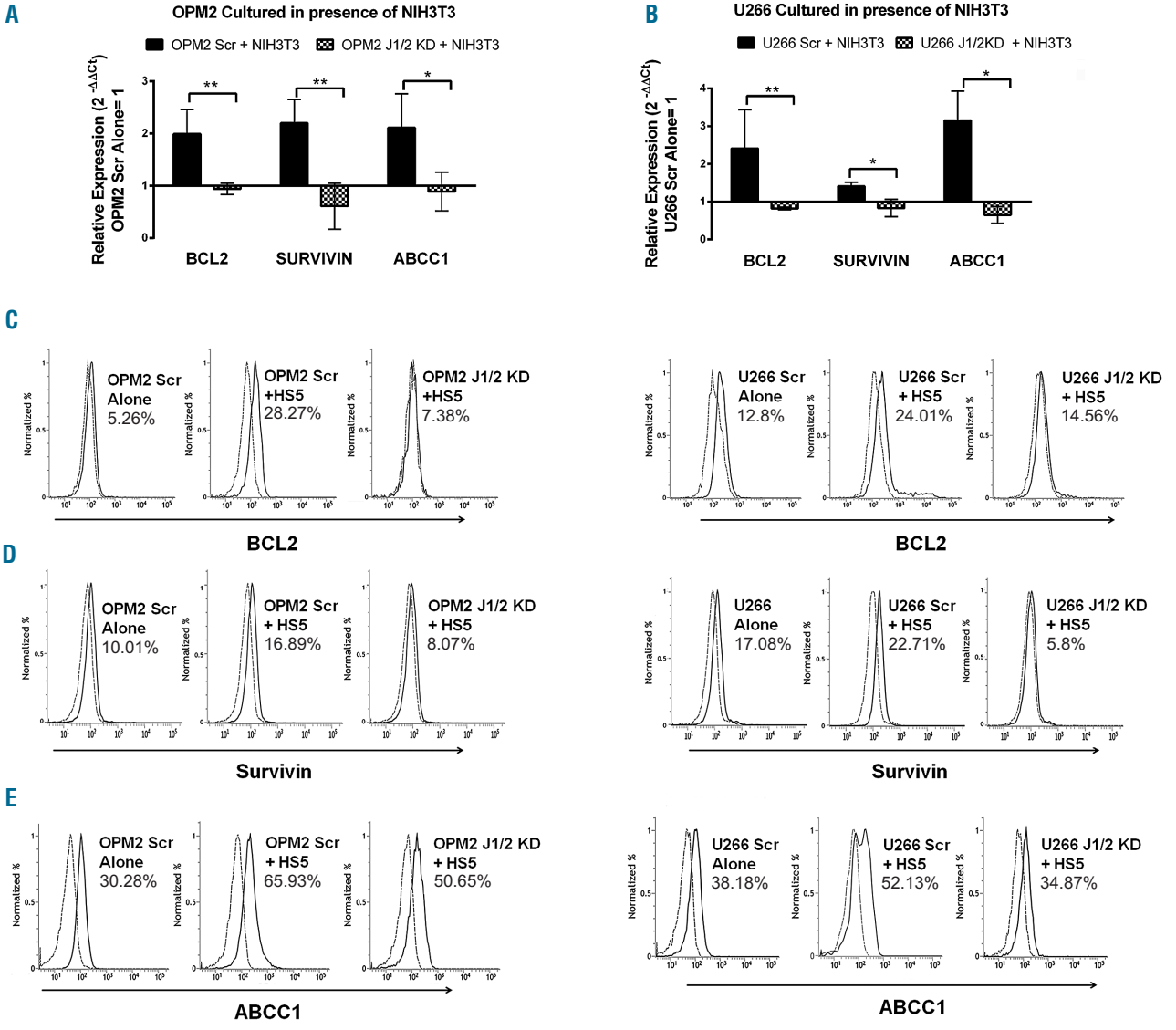

Figure 4. J1/2KD is crucial to determine bone marrow (BM)-induced drug resistance. We investigated how J1/2KD affects the molecular outcome of multiple myeloma (MM) cells crosstalk with BM stromal cells (BMSC). Quantitative real-time polymerase chain reaction (qRT-PCR) for BCL2, Survivin and ABCC1 gene expression in Scr or J1/2KD OPM2 cells (A) and Scr or J1/2KD U266 cells (B) cultured in the presence of the NIH3T3 cell line. Graphs show the relative expression levels normalized to GAPDH and compared with Scr cells cultured alone (=1), calculated by the $2^{-\Delta\Delta Ct}$ formula. Mean±standard deviation are shown. Statistical analysis was performed using two-tailed *t*-test (*P<0.05; **P<0.01). Histograms show the levels of intracellular BCL2 (C), Survivin (D), and ABCC1 (E) (black lines) analyzed by flow cytometry in Scr or J1/2KD OPM2 cells (left panels) and Scr or J1/2KD (right panels) in single culture or co-cultured with GFP$^+$ HS5 cells. The isotype-matched control is shown in gray. Histograms are representative of at least three independent experiments.

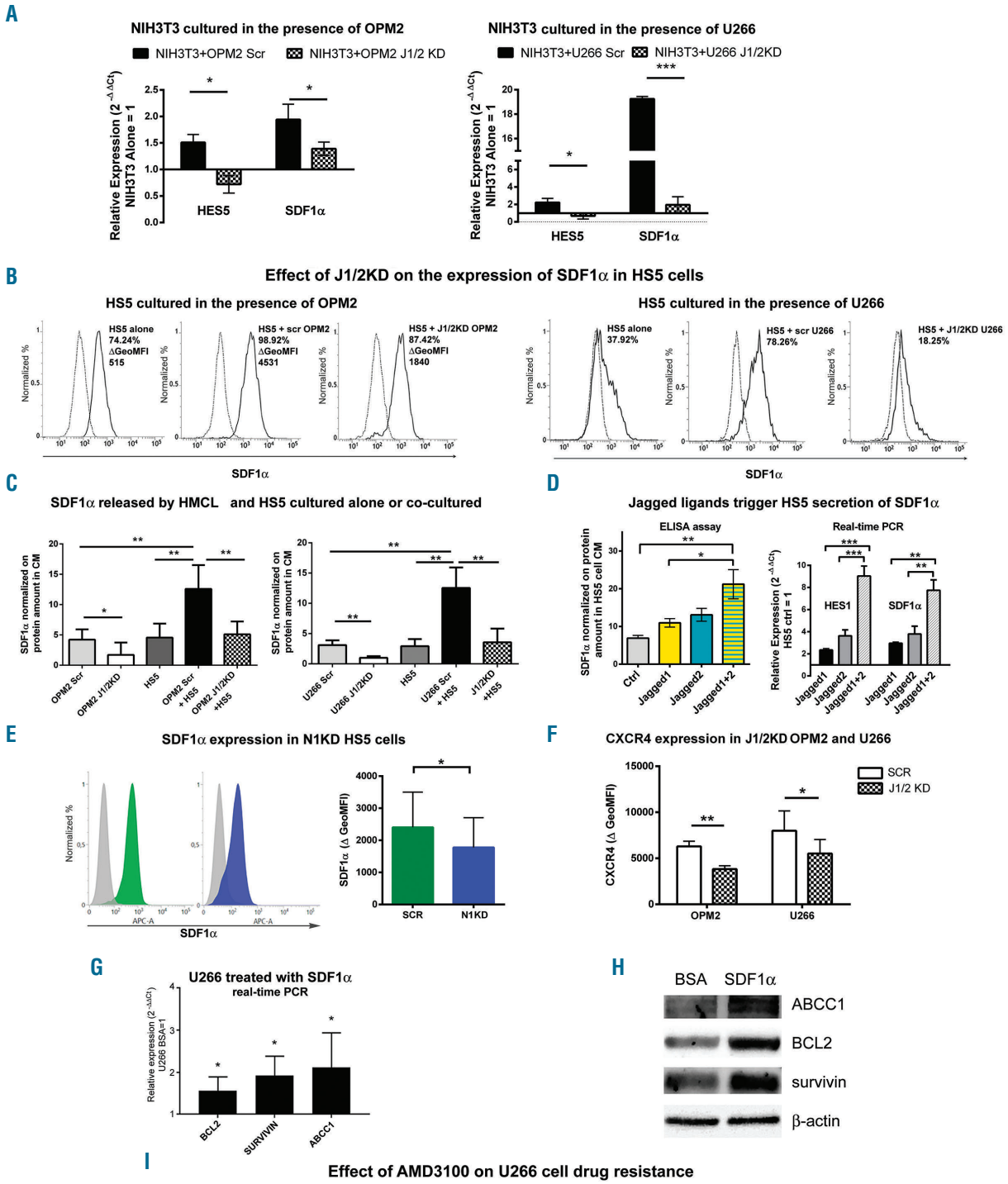

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Figure 5. Multiple myeloma (MM) cell-derived Jagged ligands promote resistance to apoptosis through the modulation of the CXCR4/SDF1α axis in the bone marrow (BM) niche. We evaluated the effect of J1/2KD in human multiple myeloma cell lines (HMCL) on the CXCR4/SDF1α axis in the BM and the consequence on the pharmacological resistance. (A) qRT-PCR for SDF1α and HES5 gene expression in NIH3T3 cells co-cultured with J1/2KD or Scr OPM2 cells (left panel) or J1/2KD or Scr U266 cells (right panel) compared to NIH3T3 cultured alone (=1), calculated by the $2^{-\Delta\Delta Ct}$ formula. HES5 was used as a control for Notch pathway activity. Mean±standard deviation of four experiments are shown. Statistical analysis was performed using two-tailed *t*-test (*P<0.05; ***P<0.001). (B) Intracellular SDF1α level in HS5 cells co-cultured with J1/2KD HMCL. Histograms show the levels of intracellular SDF1α (black lines) analyzed by flow cytometry in GFP$^+$ HS5 cells cultured alone or co-cultured with J1/2KD or Scr OPM2 cells (left panel) and J1/2KD or Scr U266 cells (right panel), and the isotype-matched control (dotted line). Histograms are representative of at least three independent experiments. Due to a high percentage of SDF1α expressing HS5 cells cultured with OPM2, we also show ΔGeoMFI. The apparent discrepancy between the two different basal levels of SDF1α produced by HS5 cells used as control in the co-culture systems with OPM2 or U266 cells is due to the effect of the different HS5 cell concentrations (see *Online Supplementary Methods*). (C) SDF1α levels in conditioned media of Scr or J1/2KD HMCL, HS5 cells or co-culture systems have been assessed by ELISA. Statistical analysis was performed using one-way ANOVA and Tukey post-test (*P<0.05; **P<0.01). (D) Effect of stimulation with Jagged1 and Jagged2 peptides on the secretion of SDF1α by HS5 cells. Statistical analysis was performed using one-way ANOVA and Tukey post-test (*P<0.05; **P<0.01; ***P<0.001). (E) Contribution of the Notch pathway to the ability of stromal cells to produce SDF1α. SDF1α levels were measured in Scr or N1KD HS5 cells. Flow cytometry histograms (left) and graphs (right) display the levels of intracellular SDF1α (ΔGeoMFI) analyzed in HS5 Scr (green) or HS5 N1KD cells (blue) and an isotype-matched control (gray); the graph shows mean±standard error of mean (SEM) of SDF1α expression levels. Statistical analysis was performed by *t*-test (*P<0.05). (F) Status of CXCR4 expression in Scr or J1/2KD HMCL used in co-culture experiments with HS5 cells. Values in the graph represent mean±SEM of CXCR4 expression levels (ΔGeoMFI) measured by flow cytometry. Statistical analysis was performed by *t*-test (*P<0.05; **P<0.01). (G) To evaluate if SDF1α contributes to BCL2, Survivin and ABCC1 expression, U266 cells were cultured in the presence of 500 ng/mL recombinant SDF1α for 48 h and analyzed by qRT-PCR. Graphs show the relative expression levels of the indicated genes compared with the corresponding values in BSA-treated cells (=1), calculated by the $2^{-\Delta\Delta Ct}$ formula. Mean values +/- standard deviations of three independent experiments are shown. Statistical analysis was performed using two-tailed *t*-test (*P<0.05). (H) Results were further confirmed by western blot analysis. Images were acquired using the UV-tech Alliance system and are representative of three independent experiments. (I) To assess if the SDF1α/CXCR4 axis affects MM cell drug resistance, U266 cells cultured alone or with GFP$^+$ HS5 cells were treated with 6 nM bortezomib (Bor), 30 μM melphalan (Melph), 15 μM lenalidomide (Len) or DMSO in the presence or absence of 50 μM AMD3100. Apoptotic MM cells were measured by flow cytometry as Annexin-V$^+$/GFP$^-$ cells. Graph shows mean±SEM of at least three independent experiments. Statistical analysis was performed using one-way ANOVA and Tukey's post-test: *P<0.05; **P<0.01; ****P<0.0001.

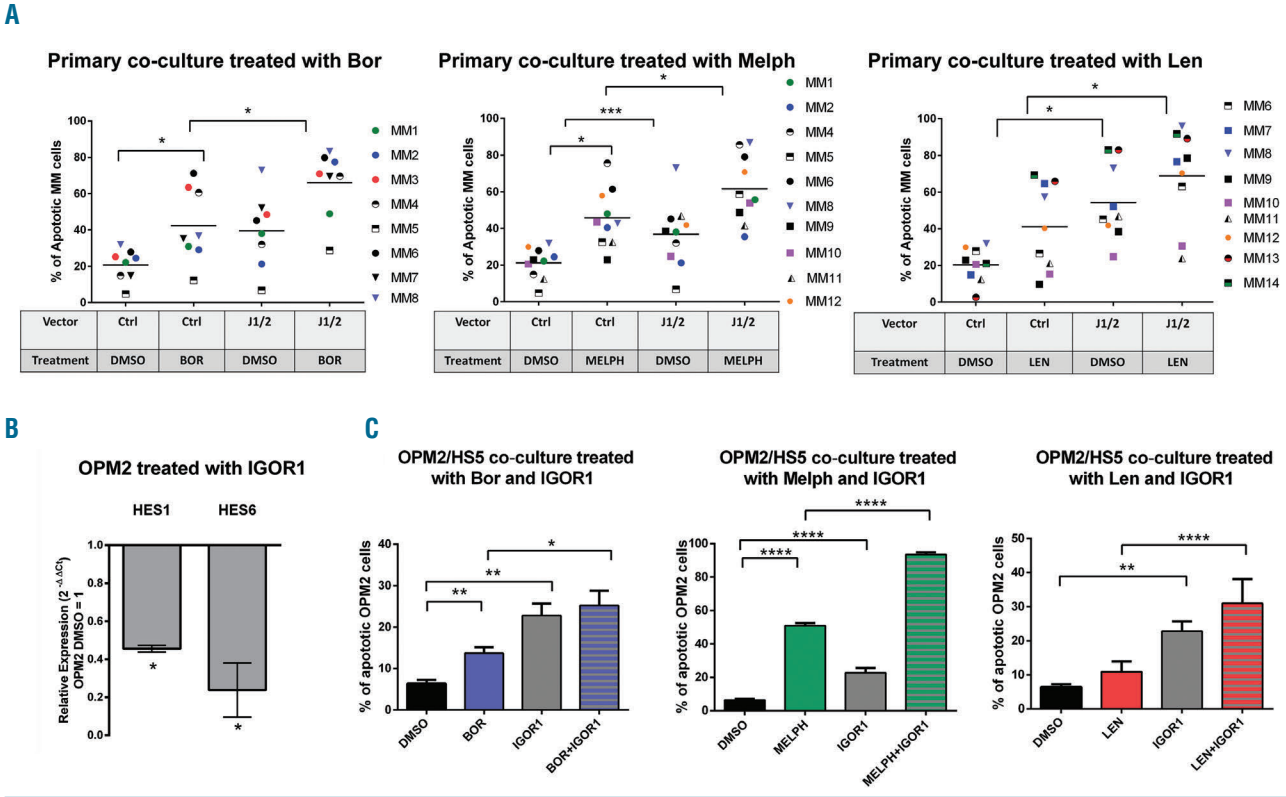

Figure 6. Translational potential of Jagged1/2 inhibition: outcome on *ex vivo* cultures of multiple myeloma (MM) patients' cells and treatment with small molecule affecting Notch-Jagged interaction. (A) Outcome of J1/2KD on primary CD138$^+$ MM cells response to standard-of-care drugs in a primary co-culture system with bone marrow stromal cells (BMSC). Levels of apoptosis were analyzed by flow cytometry on primary MM cells transduced with the lentiviral vector pLL3.7 codifying for the Jagged1 and 2 shRNAs (J1/2) or the corresponding control (Ctrl), and then co-cultured with BMSC from MM patients. Co-cultures were maintained for 72 hours (h) and treated for the last 24 h with 6 nM bortezomib (Bor) (left panel; 8 patients) or 30 μM melphalan (Melph) (central panel; 10 patients) and for 48 h with 15 μM lenalidomide (Len) (right panel; 9 patients) or DMSO. The percentage of infected MM cells that underwent apoptosis (GFP$^+$/AnnexinV$^+$) was detected by flow cytometry. Statistical analysis was performed using one-way ANOVA and Tukey post-test (*P<0.05; **P<0.01; ***P<0.001). (B and C) Effect of the inhibitory small molecule, IGOR1, on MM drug resistance. OPM2 cells treated with 30 μM IGOR1 were cultured on a monolayer of HS5 GFP$^+$ cells in the presence or the absence of different drugs as described in the Methods. (B) Quantitative polymerase chain reaction assay shows that IGOR1 inhibits Notch pathway in OPM2 cells, as demonstrated by the downregulation of Notch target genes, HES1 and HES6. Relative gene expression variation was normalized to GAPDH and calculated by the $2^{-\Delta\Delta Ct}$ formula. Mean values +/- standard deviations of three experiments are shown. Statistical analysis was performed using two-tailed *t*-test (*P<0.05). (C) The levels of apoptosis of OPM2 cells treated with IGOR1 and the indicated drugs were measured by staining with Annexin-V-APC (C). Graph shows mean values +/- standard deviations of at least three independent experiments. Statistical analysis was performed using one-way ANOVA and Tukey's post test (*P<0.05; **P<0.01; ****P<0.0001).

all the analyzed drugs, in agreement with the findings obtained *in vitro*.

To verify if the inhibitory approach based on J1/2KD had a translational potential, we recapitulated the experiments of MM-BMSC interplay by using IGOR1, a novel small molecule recently developed in our laboratory[30] to uncouple Notch-Jagged interaction. IGOR1 is able to inhibit Notch activation in OPM2 cells and significantly increases the efficacy of the administered drugs, with a higher efficiency for Mel and Len (Figure 6B and C).

Jagged1 and Jagged2 blockade promotes sensitivity to bortezomib in a zebrafish xenograft myeloma model

Bortezomib is one of the most commonly used drugs for the treatment of newly-diagnosed and refractory MM patients.[31] In recent years, several studies have supported the hypothesis that the development of resistance to such treatment is strongly dependent upon the BM microenvironment, with a significant contribution of the CXCR4/SDF1α axis.[32-34] Due to the results obtained *in vitro* concerning the role of this chemokine axis in the development of pharmacological resistance to Bor, we validated the effect of J1/2KD on MM cell resistance to Bor by taking advantage of a novel zebrafish xenograft MM model.

Zebrafish embryos were recently validated as a complementary *in vivo* model for MM that allows the rapid screening of MM cells response to chemotherapeutic drugs.[35] Moreover, this model fully recapitulates the cytokine milieu present in the human BM, since zebrafish-secreted growth factors, such as IL6 and SDF1α, support MM cells growth *in vivo*.[33,35] To validate our *in vitro* and *ex vivo* findings, Scr or J1/2KD U266 cells vitally labeled with the fluorescent dye CM-Dil were injected in the yolk area of 48 hpf zebrafish embryos. Xenotransplanted embryos were visualized by fluorescent microscopy to verify the presence of MM cells at the injection site at 2 hpi (Figure 7A-D), treated or not with 10 nM Bor and, visualized at 48 hpi for tumor cell growth (Figure 7A'-D'). Representative images of whole embryos are shown in *Online Supplementary Figure S15*.

As shown, the addition of 10 nM Bor to the embryo medium inhibited tumor growth of approximately 57% compared to controls (Figure 7A' and B'), without affecting embryo viability. A similar effect was induced by J1/2KD (Figure 7A'-C'), while the combination of J1/2KD and Bor significantly reduced tumor growth in comparison to all other experimental groups (-82% in comparison to the control) (Figure 7A'-D').

Discussion

Multiple myeloma progression is characterized by development of drug resistance causing patient relapse

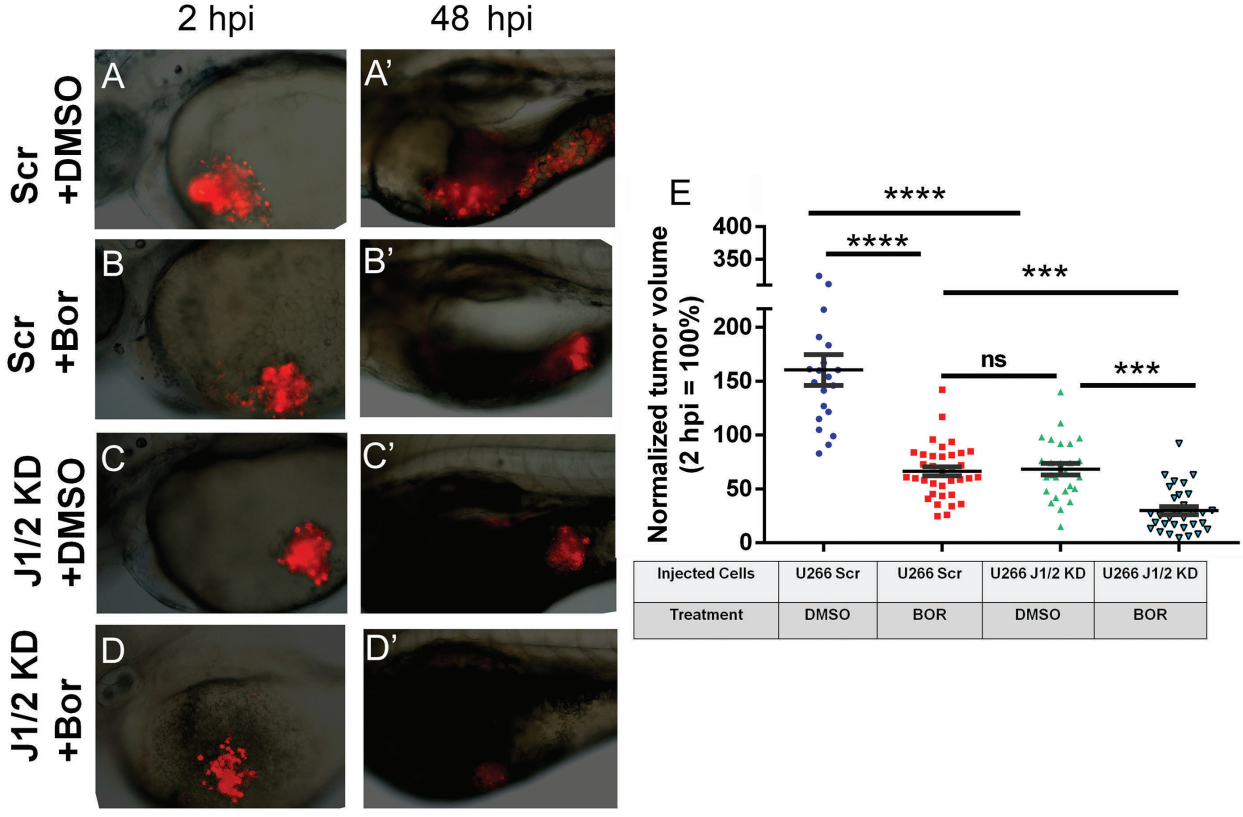

Figure 7. Evaluation of tumor growth inhibition of myeloma cells xenotransplanted in zebrafish embryos. Fluorescent microscopy images of CM-DiI stained multiple myeloma (MM) xenografts at 2 hours (h) post injection (hpi) (A-D) and 48 h post injection (hpi) (A'-D') into the yolk of zebrafish embryos. (A'-D') Tumor growth analyses indicates that MM xenografts are responsive to treatment with bortezomib (Bor) (compare A' and B'). Xenotransplanted J1/2KD cells also show reduced tumor growth (compare A' and C'). These effects are increased combining the injection of J1/2KD cells with Bor treatment (compare A', B', C' and D'). (E) Dot-plot shows the increase in tumor burden at 48 hpi, normalized to tumor area at 2 hpi (Scr+DMSO= 20 embryos; Scr+ Bor= 26 embryos; J1/2KD+ DMSO= 35 embryos; J1/2KD+ Bor=31 embryos). Statistical analysis was performed using one-way ANOVA and Tukey post-test (***P<0.001; ****P<0.0001).

and contributing to the fatal outcome of this disease. The close interaction of MM cells with healthy BM cells represents an important source of factors able to promote malignant cell growth and survival.

The Notch pathway is capable of mediating the cell-cell communication. Current evidence provided by different groups, including ours, highlighted the importance of Jagged ligands in the pathological communication between tumor and healthy cells within the myeloma BM. MM-derived Jagged ligands activate Notch receptors in the nearby BM cells inducing osteoclastogenesis, osteolysis,[17] angiogenesis,[36] and BMSC-mediated release of key cytokines including IL6, IGF1 and VEGF.[11,13] Moreover, the activation of Notch signaling in MM cells, induced by tumor cell-derived[37,38] or BMSC-derived Jagged[1,25] stimulates MM cell proliferation,[38] resistance to apoptosis,[37] and a decrease in drug sensitivity.[25]

This work is specifically focused on the pathological communication of MM cells and BMSC mediated by Notch signaling and on its outcome on MM drug resistance. Notably, the Notch pathway is known to be a key player in BM-induced drug resistance in other hematologic malignancies. Indeed, Krampera's group provided evidence of how the BM-driven activation of Notch3 and Notch4 in B-ALL[39-41] and Notch1, Notch2 and Notch4 in chronic lymphocytic leukemia,[42] results in chemoresistance, while Notch1-Jagged1 crosstalk supports BM-induced drug resistance in AML.[43]

As far as MM is concerned, in spite of the recent advances in the field, we still do not have a complete picture of the bidirectional crosstalk between BMSC and MM cells, which is indicated by the expression of Notch receptors and ligands on both cell types.[11,12,16,23,25,44] This work aims to fill some of those gaps by providing novel information about the effects of the aberrant expression of MM-derived Jagged ligands on the intrinsic tumor cell drug resistance and by investigating a key aspect that has never been previously explored, i.e. the outcome of MM-derived Jagged ligands on BMSC-induced drug resistance.

To address these issues, we interfered with the mRNA expression of MM-derived Jagged ligands and investigated J1/2KD outcomes in tumor cells and in surrounding BMSC. We observed *in vitro* that MM cell-derived Jagged ligands could trigger Notch signaling in the nearby MM cells by homotypic interaction. Notch activation resulted in the increased expression of anti-apoptotic effectors including BCL2, Survivin, and the multidrug resistance transporter ABCC1, along with the increase in MM cell survival to standard-of-care drugs, such as Bor, Len, and Melph. Notably, besides observing homotypic activation of Notch signaling among MM cells, we found that HMCL can trigger Notch signaling in the neighboring BMSC and, in turn, Notch activation boosts the ability of BMSC to increase the pharmacological resistance of MM cells. This effect was clearly dependent on MM-derived Jagged ligands, since J1/2KD completely abrogated BMSC support. At least in part, the pro-tumor effect of Notch-"educated" BMSC was due to their ability to increase SDF1α levels in the BM microenvironment. Indeed, soluble or MM cell-derived Jagged ligands may induce a Notch-dependent increase in SDF1α secretion by BMSC; on the contrary, J1/2KD HMCL lose this ability and N1KD interferes with BMSC to release SDF1α.

The Notch-dependent activation of SDF1α secretion by BMSC is potentially more important than the previously observed secretion induced by Notch activation in MM cells,[29] since BMSC are the most effective producers of this cytokine in the BM.

To complete the picture of a Notch signaling effect on the SDF1α/CXCR4 axis in myeloma BM, we also demonstrated that MM cell-derived Jagged ligands may further enhance the anti-apoptotic signaling of SDF1α by stimulating the expression of its receptor CXCR4 on the MM cell surface.

The contribution of the SDF1α/CXCR4 axis to MM pharmacological resistance was confirmed by the ability of the antagonist molecule AMD3100 to abrogate U266 cell resistance to (Bor), (Melph) and (Len) induced by BMSC, consistent with the findings of Azab *et al*.[32]

Although the downstream molecular mechanisms of Notch-associated drug resistance in MM still need to be fully elucidated, we showed that the secreted SDF1α can stimulate general mechanisms, including tumor cell anti-apoptotic background, by up-regulating BCL2 and Survivin, or drug extrusion mediated by ABCC1. These antiapoptotic proteins are particularly relevant to MM. Indeed, BCL2 and Survivin are over-expressed in MM cells, where they play an important role in cell survival, and significantly correlate with disease stage;[20,27,45] on the other hand, xenobiotic transporters, such as ABCC1, are well known mediators of MM multidrug resistance,[20] modulated by Notch in different cancer settings.[46]

The general validity of these novel findings stems from the observed improvement in drug-response promoted by J1/2KD in *ex vivo* co-culture systems of CD138$^+$ MM cells and BMSC from BM aspirates of newly-diagnosed MM patients.

Additionally, *in vivo* validation of these findings in a zebrafish xenograft MM model engrafted with U266 cells confirmed that J1/2KD promoted an increased sensitivity to Bor *in vivo*, showing a wider decrease in tumor burden compared to the control.

The present results provide novel and important information to help improve the current picture on the effect of Notch-mediated communication in myeloma BM. Indeed, since both BMSC and MM cells carry Notch receptors and ligands, their bidirectional crosstalk needs to be taken into consideration. We sought to fill the gap in the available information on the role of MM cells, such as Notch signaling sending cells in the BM. Here we discuss our findings according to the previous literature data in order to summarize the overall picture (Figure 8). Previous work reported the consequences of Notch activity in MM cells (mainly using γ-secretase inhibitors), identifying the following molecular mechanisms: i) upregulation of p21 induced MM cell growth inhibition and increased survival;[23] ii) Notch/HES1 mediated downregulation of the pro-apoptotic protein Noxa;[24] iii) Notch up-regulated expression of integrin αvβ5 resulting in increased adhesion to vitronectin and consequent protection from pro-apoptotic drugs;[47] iv) upregulation of the enzyme cytochrome P450,[44] implicated in drug metabolism and in the onset of several malignancies.[48] Concerning the contribution of Notch in BMSC-dependent drug resistance, previous investigations were focused on the autonomous contribution of BMSC-derived Notch ligands in MM cell behavior (Figure 8).[23,25,44]

In this work, we found that the alteration induced in the BM by the presence of MM cells aberrantly expressing Jagged ligands is a key step in "educating" the tumor microenvironment to a pro-tumor type of behavior.

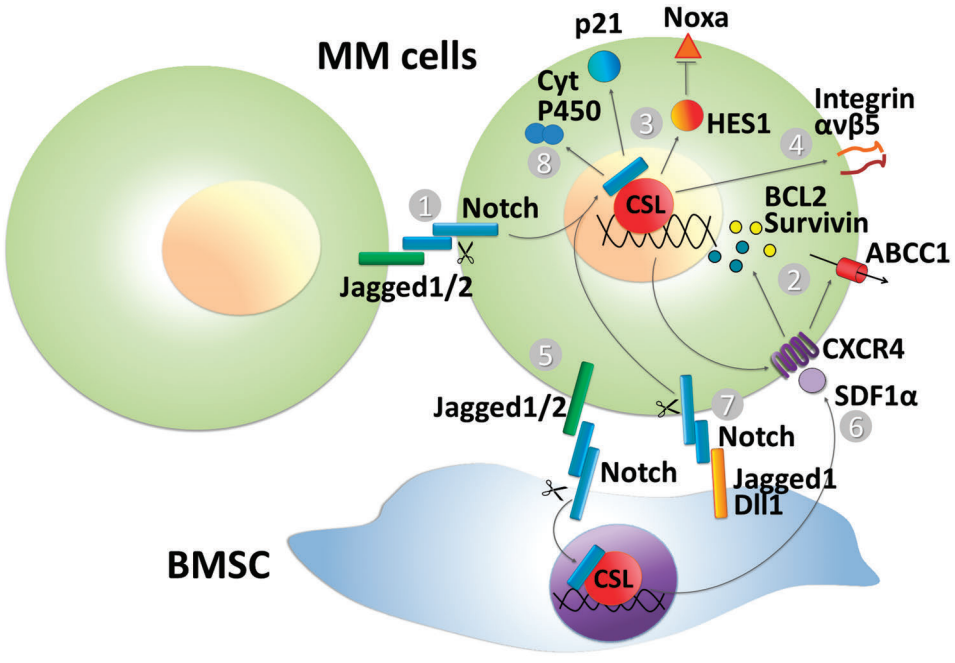

Figure 8. Mechanism underlying Notch ability to promote drug resistance in multiple myeloma (MM) microenvironment. Jagged1/2 overexpression in MM cells causes hyperactivation of Notch signaling in the bone marrow (BM) milieu, which, in turn, promotes drug resistance by modifying both MM cell and BM stromal cell (BMSC) behavior. Indeed, (1) Notch activation in MM cell triggered by Jagged1/2 through homotypic interactions sustains resistance to drug-induced apoptosis in different ways. Notch can (2) promote the expression of the pro-survival factors BCL2, Survivin, and ABCC1 and the chemokine receptor CXCR4; (3) up-regulates HES1, which in turn inhibits the expression of the pro-apoptotic protein Noxa; (4) promotes the expression of integrin αvβ5, thus enhancing MM cell adhesion to vitronectin. (5) MM-derived Jagged1/2 may also activate Notch in BMSC, (6) boosting its ability to produce SDF1α, which in turn, by activating CXCR4 signaling in MM cell, promotes the expression of the anti-apoptotic factors BCL2, Survivin, and ABCC1, improving MM cell pharmacological resistance. On the other hand, (7) BMSC activate the Notch pathway in MM cells through their basal expression of Jagged1 and Dll4, (8) promoting the expression of cytochrome P450 and p21, thereby supporting MM cell resistance to therapy.

Indeed, MM cell-derived Jagged1 and 2 may switch on Notch signaling in tumor and non-tumor BMSC by triggering Notch signaling, activating MM cell anti-apoptotic background, increasing SDF1α level in the BM, and, finally, resulting in supporting MM cell resistance to standard-of-care drugs (Figure 8).

Overall, our findings provide the proof-of-principle that selective targeting of Jagged ligands in MM cells can restore tumor cell sensitivity to therapy, laying the foundation for the development of combined low-toxic therapeutic options to restore drug sensitivity and overcome fatal drug resistance of relapsing MM patients. Recently, inhibitory small molecules[30] or neutralizing antibodies[49] directed to inhibit the activation of Notch signaling mediated by Jagged ligands have been developed. This prompted us to confirm the translational potential of our results by testing the anti-tumor effect of an inhibitory small molecule developed in our laboratory, IGOR1, which was directed to uncouple Notch-Jagged interaction.[30] *In vitro* results showed that IGOR1 had the ability to increase MM cell pharmacological response, with higher efficacy if combined with Melph and Len.

The importance of our results stems from the evidence that a Jagged-tailored therapy might represent a more suitable clinical approach to achieve the inhibition of Notch signaling in the BM of MM patients. Indeed, it lacks the potential adverse effects of pan-Notch blockade obtained with γ-secretase inhibitors (GSI), that provided promising results in an *in vivo* MM model by increasing the chemotherapeutic effect of doxorubicin and melphalan,[24] but that were associated with severe gastrointestinal toxicity due to intestine metaplasia.[50,51]

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