Myeloma cell growth suppression by osteoblast-derived extracellular vesicles: the creation of a non-permissive niche for myeloma cells by bone-forming osteoblasts

It is generally accepted that multiple myeloma (MM) cells preferentially grow in the bone marrow niche, where bone marrow stromal cells (BMSC) play a central role. BMSC are osteoblast (OB) precursor cells, and their differentiation into bone-forming, mature OB is inhibited by various factors in MM. In sharp contrast to BMSC, terminally differentiated OB actively producing bone matrices have been demonstrated to induce apoptosis in MM cells but not in normal hematopoietic cells.[1-3] However, the precise mechanisms for MM cell growth suppression by bone-forming, mature OB remain largely unknown. We demonstrate here that the expression of microRNA (miR)-125b, a tumor suppressor gene for MM cells, is repressed in MM cells but upregulated in mature OB actively producing bone matrix, and OB-derived miR-125b is transferred via extracellular vesicles (EV) into MM cells, causing MM cell death in parallel with reductions in the levels of expression of interferon regulatory factor 4 (IRF4) and MYC. These results suggest the importance of bone health with bone formation in MM treatment and therapeutic opportunities with synthetic miR-125b mimics.

MM has the unique propensity to develop and expand almost exclusively in bone marrow, resulting in bone destruction. MM cells enhance osteoclastogenesis while suppressing osteoblastogenesis, leading to devastating bone destruction with rapid loss of bone. MM cell-induced cell types in MM bone lesions, namely osteoclasts, vascular endothelial cells and BMSC with defective osteoblastic differentiation, play an important role in creating a cellular microenvironment suited for MM cell growth and survival as feeders for MM cells. Intriguingly, tumor regression has been demonstrated to occur within bone along with induction of bone formation in MM animal models treated with bone-anabolic agents, such as an anti-DKK1 antibody,[4] lithium chloride,[5] and transforming growth factor-β inhibitor[2] although these agents alone did not impair MM growth *in vitro*. These findings suggest a correlation between the pharmacological induction of active bone formation and tumor regression in bone. Consistent with these *in vivo* observations, we and others reported that terminally differentiated OB actively producing bone matrices induced apoptosis in MM cells but not in normal hematopoietic cells, whereas BMSC, OB precursor cells, enhanced MM cell growth and viability.[1-3] Importantly, the suppressive activity on MM cell growth was produced exclusively by terminally differentiated OB with mineralized nodule formation, while OB at earlier stages of differentiation with increased alkaline activity without mineralized nodule formation did not reduce the growth and viability of MM cells.[2] However, the precise underlying mechanisms for MM cell growth suppression by terminally differentiated, bone-forming OB remain largely unknown.

IRF4 has been demonstrated to be a master transcription factor vital for MM cell growth and survival, and it is regarded as a therapeutic target specific for MM.[6,7] IRF4 has been reported to be a target of miR-125b.[8,9] Morelli *et al*. reported that miR-125b expression was repressed in MM cells, especially in those residing in the bone marrow microenvironment, and that enforced expression of miR-125b downregulated IRF4 and MYC expression in patient-derived MM cells and MM cell lines, and thereby suppressed MM cell growth.[10] In regard to miR-125b, OB-derived extracellular matrix vesicles have been demonstrated to be highly enriched with miR-125b.[11] There is a similarity between extracellular matrix vesicles and exosomes as EV in terms of containing microRNA. In the present study, we explored the mechanisms of MM cell growth inhibition by terminally differentiated, bone-forming OB, with special reference to OB-derived EV.

Consistent with previous observations,[1-3] the viability of MM cell lines (Figure 1A, *Online Supplementary Figure S1A,* B) and primary MM cells (*Online Supplementary Figure S1C*) was reduced over time in co-cultures with mature OB with mineralized nodule formation differentiated from MC3T3-E1 pre-osteoblastic cells by BMP-2. However, MC3T3-E1 pre-osteoblastic cells without OB differentiation did not impair MM cell growth. Similar suppressive activity for MM cell lines was produced by mature OB with mineralized nodule formation differentiated from IDG-SW3 OB (Figure 1B) and primary BMSC (*Online Supplementary Figure S1D*). In addition, co-cultures with mature OB substantially reduced the protein levels of the vital survival factors IRF4 and MYC in MM cells (Figure 1C).

We next isolated EV from supernatants of MC3T3-E1 and IDG-SW3-derived OB with mineralized nodule formation and examined the effects of the isolated EV on MM cell growth. Addition of mature OB-derived EV induced cell death in MM cell lines and primary MM cells (Figure 2A). MM cells underwent apoptosis by treatment with OB-derived EV (*Online Supplementary Figure S2A, B*). To detect the incorporation of EV, we labeled RNA in EV isolated from mature OB, and incubated the RNA-labeled EV with MM cells. After 24 hours, the RNA-labeled EV were detected in MM cells (Figure 2B). However, treatment with

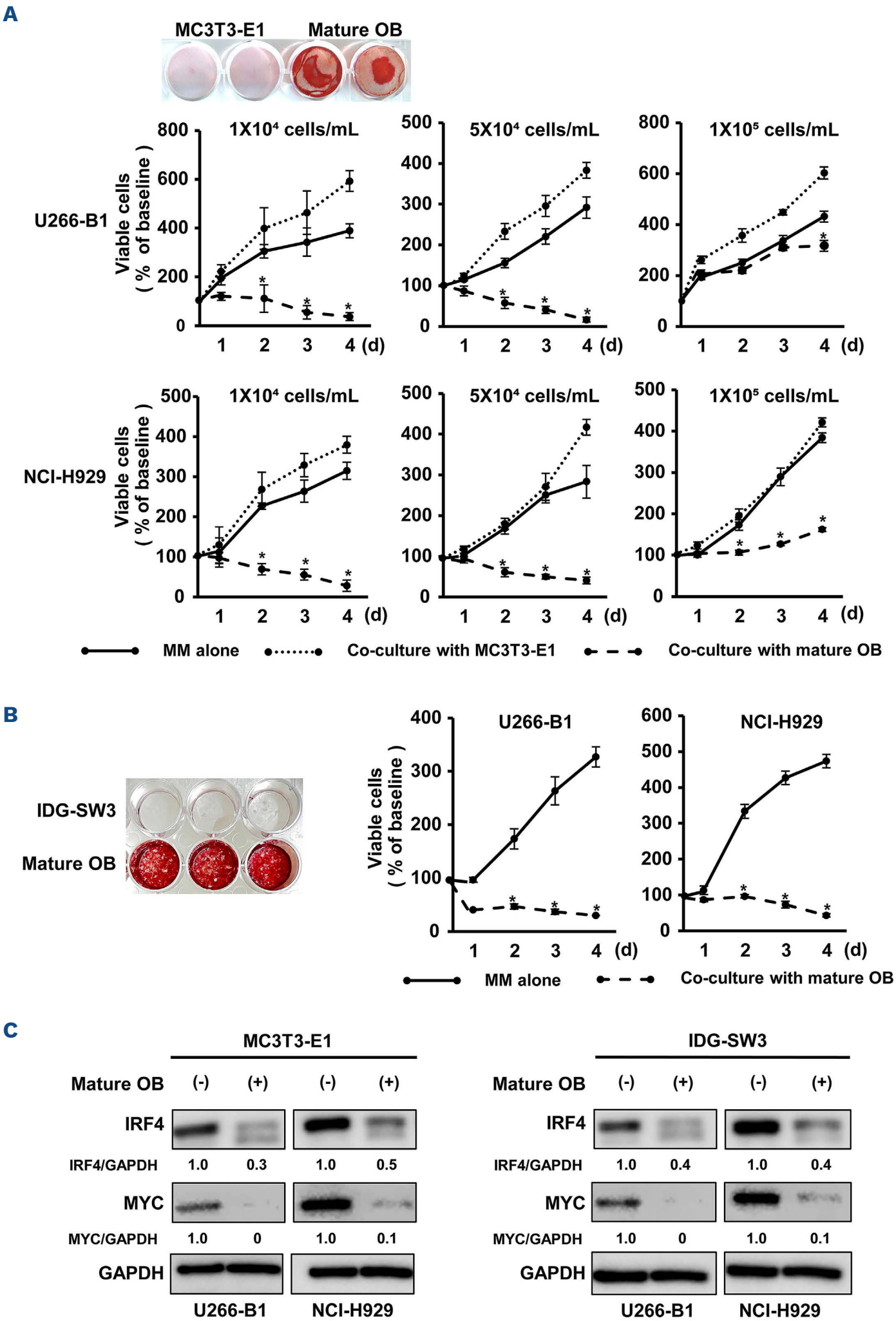

Figure 1. Mature osteoblasts suppressed the proliferation of myeloma cells. (A) MC3T3-E1 pre-osteoblastic cells were cultured in osteogenic media (25 ng/mL bone morphogenetic protein 2, 10 mM β-glycerophosphate, and 50 mg/mL ascorbic acid). After confirming the formation of mineralized nodules as indicated at the top, the cells were used as mature osteoblasts (OB). The multiple myeloma (MM) cell lines U266-B1 and NCI-H929 were cultured in triplicate at 1×10^4/mL, 5×10^4/mL, 1×10^5/mL alone (solid line), or co-cultured with either untreated MC3T3-E1 cells (dotted line) or mature OB derived from MC3T3-E1 cells (dashed line). (B) IDG-SW3 osteoblastic cells were untreated or cultured in osteogenic media to differentiate into mature OB as shown in the far left panel. U266-B1 and NCI-H929 cells were cultured in triplicate at 5×10^4/mL alone (solid line) or co-cultured with the mature OB (dashed line). Viable MM cell numbers were counted at the indicated time points. Percents of baseline were compared

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with those in MM cells cultured alone. The values are mean ± standard deviation (*P<0.05). (C) U266-B1 and NCI-H929 cells were cultured for 24 hours at 5×10^4/mL alone or co-cultured with mature OB differentiated from MC3T3-E1 cells (left) and IDG-SW3 (right). Protein levels of interferon regulatory factor 4 (IRF4) and MYC were examined by western blotting analysis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control.

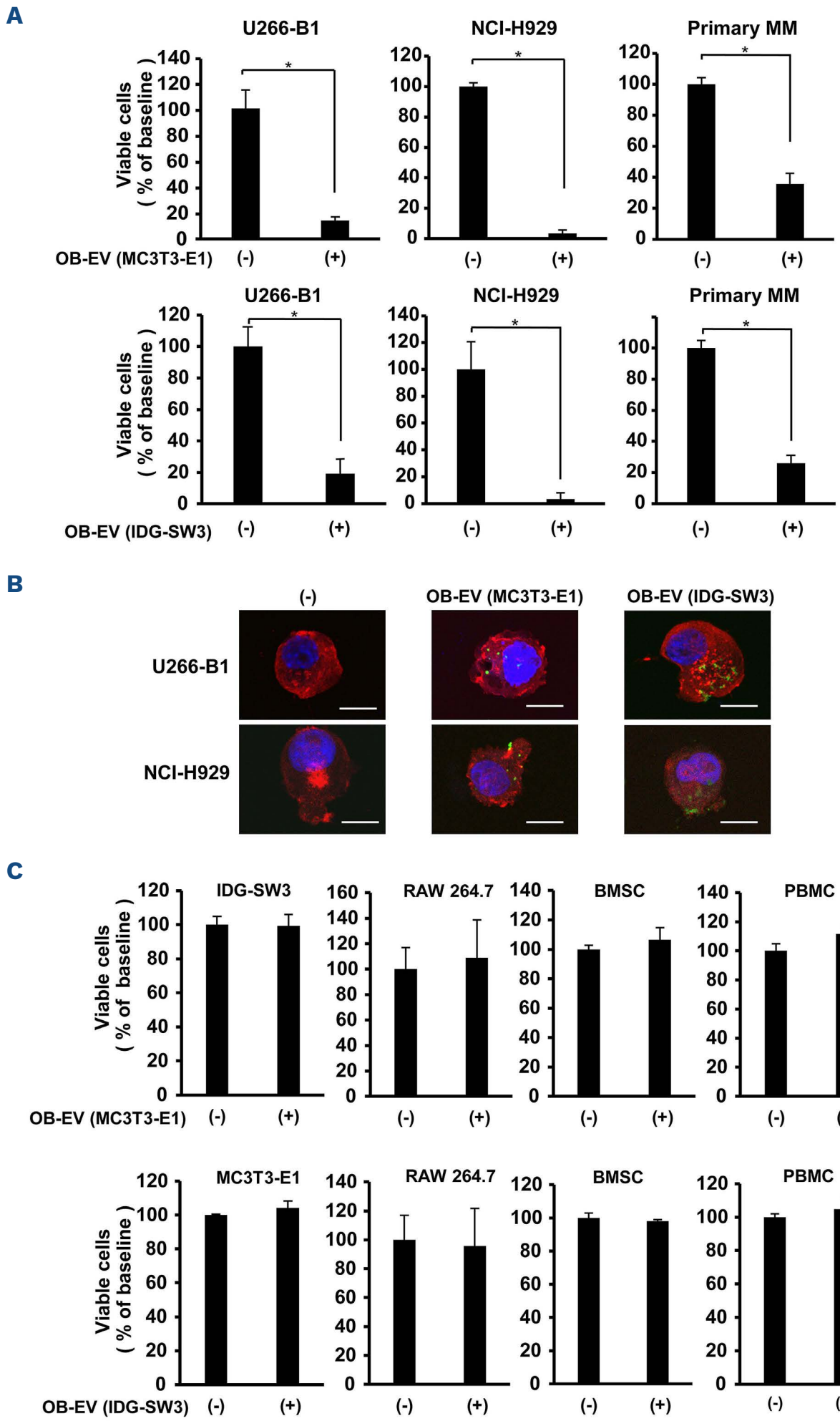

Figure 2. Mature osteoblast-derived extracellular vesicles induced death of multiple myeloma cells. (A) Extracellular vesicles (EV) were isolated from culture supernatants (10 mL) of mature osteoblasts (OB) derived from MC3T3-E1 or IDG-SW3 cells using

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EXORPTION® (Sanyo Chemical) and resuspended in 1 mL (10× concentration). MM cell lines, U266-B1 and NCI-H929, and primary MM cells were cultured at 5.0×10^4 cells/mL in triplicate for 4 days in culture media with or without EV. The EV solutions were added to the indicated wells to be diluted 1/10 (approximately 10^9 EV particles/mL). Viable cell numbers were counted, and percents of baseline were compared. The values are mean ± standard deviation (*P<0.05). (B) RNA in EV (EV-RNA) isolated from mature OB was labeled using an ExoGlow labeling kit (System Biosciences, LLC). MM cell lines U266-B1 and NCI-H929 were cultured with RNA-labeled EV from mature OB for 24 hours. The incorporation of EV into MM cells was detected by confocal microscopy. The actin filaments and nuclei were stained with phalloidin (red) and 4',6-diamidino-2-phenylindole (DAPI) (blue), respectively. Scale bars represent 10 μm. (C) The indicated types of cells were incubated in triplicate for 4 days with or without the EV from mature OB derived from MC3T3-E1 or IDG-SW3 cells. MC3T3-E1 or IDG-SW3 were seeded at 2×10^5 cells/mL; RAW264.7 cells at 2×10^5 cells/mL; bone marrow stromal cells at 4×10^4 cells/mL; and peripheral blood mononuclear cells at 1×10^6 cells/mL. Viable cell numbers were counted, and percents of baseline were compared. OB-EV: osteoblast-derived extracellular vesicles; BMSC: bone marrow stromal cells; PBMC: peripheral blood mononuclear cells.

the EV uptake inhibitor Dynasore blocked incorporation of the RNA-labeled EV, although not completely (*Online Supplementary Figure S2C*), and alleviated EV-induced MM death (*Online Supplementary Figure S2D*). Notably, the addition of mature OB-derived EV did not affect the viability of different types of normal cells other than MM cells, including peripheral blood mononuclear cells, BMSC, bone marrow macrophages, osteoclasts, and OB (Figure 2C). Therefore, OB-derived EV are suggested to selectively kill MM cells but not other types of cells in the MM tumor microenvironment in the bone marrow.

Among others, miR-125b has been reported to be produced in OB[11] and act as a tumor suppressor microRNA for MM cells.[10] The mouse and human miR-125b sequences share 100% homology. We next looked at the expression of miR-125b in terminally differentiated OB with mineralized nodule formation, which can suppress MM cell growth. The expression of miR-125b was substantially upregulated in terminally differentiated OB with mineralized nodule formation derived from MC3T3-E1 and IDG-SW3 cells (Figure 3A). Consistent with the previous report,[10] miR-125b was only marginally expressed in MM cell lines and primary MM cells compared with MC3T3-E1 cells (Figure 3B, *Online Supplementary Figure S3A*). Similar to its cellular expression, miR-125b levels were higher in EV isolated from culture supernatants of mature OB differentiated from MC3T3-E1 and IDG-SW3 cells than those from untreated MC3T3-E1 and IDG-SW3 cells, respectively (Figure 3C). miR-125b was marginally detected in EV isolated from culture supernatants of osteoclasts but not from MM cells. Notably, although MM cells cultured alone only marginally expressed miR-125b, miR-125b was detected in MM cells after co-culture with OB forming mineralized nodules (Figure 3D). Similar to the effects of mature OB (Figure 1C), the addition of OB-derived EV decreased the protein levels of IRF4 and MYC in MM cells (Figure 3E), suggesting a role of OB-derived EV in suppression of the IRF4-MYC axis in MM cells. However, transfection of a miR-125b inhibitor into MM cells suppressed MM cell death induced by OB-derived EV (Figure 3F). These results suggested that miR-125b plays an important role in MM cell death induced by OB-derived EV.

Together with the *in vivo* observations of MM tumor regression in a bone-forming milieu,[1-5] the present study suggests that OB with bone-forming activity might be a distinct cell type that creates a non-permissive niche for MM growth and survival in bone marrow. Proteasome inhibitors exert bone anabolic actions in bone lesions in responders to them; therefore, the expression of miR-125b in primary MM cells may be increased when bone formation is restored after treatment with proteasome inhibitors, although bortezomib treatment did not directly affect miR-125b expression in MM cells or OB *in vitro* (*Online Supplementary Figure S3B*). Alteration of miR-125b levels in primary MM cells during disease progression and their correlation with response to treatment should be clarified to elucidate MM biology in terms of MM cell-bone marrow interactions. Active OB distinguishably produce a large amount of extracellular matrices; therefore, the role of extracellular matrix produced by active OB has been studied in relation to the regression of MM growth. The small leucine-rich proteoglycan decorin is abundantly produced by mature OB and its role in mature OB-mediated MM regression has been reported.[12] However, decorin alone did not induce apoptosis in MM cells as potently as mature OB did in our experimental conditions.[2] Multiple factors produced by mature OB may act together to impair MM cell growth and survival. The present study suggests that EV-mediated transfer of miR-125b from mature OB into MM cells causes at least partial suppression of MM cell growth and survival, which contributes to the formation of an OB-mediated non-permissive niche for MM cells. OB-derived miR-125b has also been reported to suppress osteoclast differentiation,[11,13] further suggesting the therapeutic roles of OB-derived EV in the amelioration of bone disease together with MM tumor containment. Consistent with a previous study,[10] we demonstrated that miR-125b suppresses the expression of IRF4 and MYC to induce apoptosis in MM cells. miR-125b can inhibit translation through binding to the 3′ untranslated regions of various target mRNA, and has been demonstrated to repress the expression of various important mediators for growth and survival signaling pathways, including NF-κB, PI3K/Akt/mTOR, ErbB2 and Wnts.[14] Therefore, EV transfer of miR-125b into MM cells may repress the expression of various genes responsible

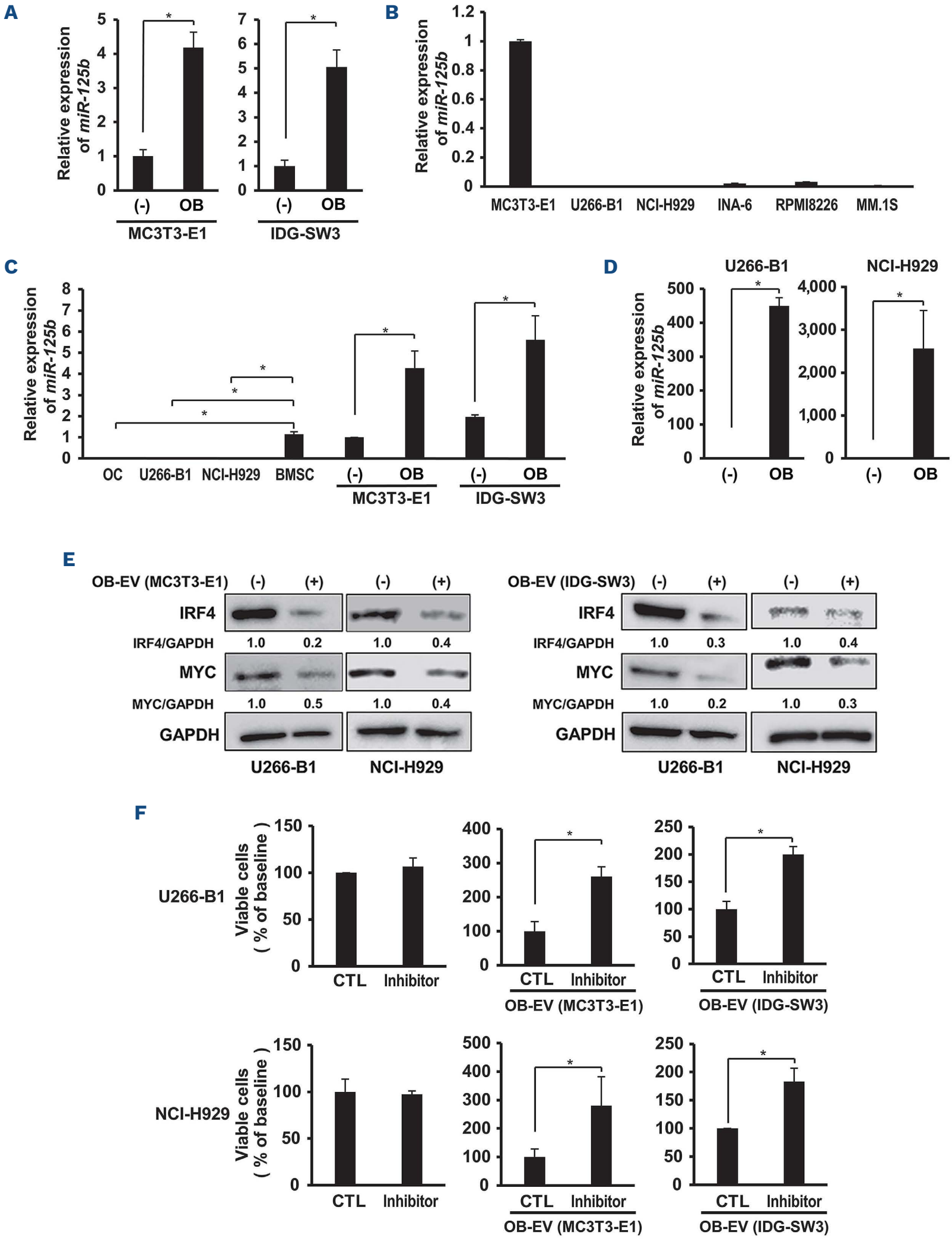

Figure 3. Extracellular vesicle-mediated transfer of miR-125b suppressed multiple myeloma cell growth and survival. (A) MC3T3-E1 cells and IDG-SW3 cells with or without osteoblast (OB) differentiation were harvested (N=3). OB differentiation was induced after culturing for 10 days in osteogenic media supplemented with 25 ng/mL bone morphogenetic protein-2, 10 mM β-glycerophosphate, and 50 mg/mL ascorbic acid. Total RNA was then collected, and miR-125b expression was analyzed by TaqMan MicroRNA Assays (Thermo Fisher Scientific). *U6* served as an endogenous control to normalize each sample. (B) miR-125b levels were analyzed by TaqMan MicroRNA Assays in the indicated cell lines. *U6* served as an endogenous control to normalize each sample. (C) The expression of miR-125b in extracellular vesicles (EV) isolated from human osteoclasts, multiple myeloma (MM)

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cell lines, U266-B1, and NCI-H929, mouse primary bone marrow stromal cells, OB precursors, MC3T3-E1 and IDG-SW3, and mature OB differentiated from MC3T3-E1 and IDG-SW3 cells. Synthetic *cel-miR-39–3p* was added to EV from an equal number of cells during RNA extraction, and the levels of *cel-miR-39–3p* were used as exogenous spike-in control for data normalization following TaqMan MicroRNA Assays. (D) The indicated MM cell lines were cultured with or without mature OB from IDG-SW3. After culturing for 4 days, MM cells were collected and miR-125b expression was analyzed in MM cells by TaqMan MicroRNA Assays. *U6* served as an endogenous control to normalize each sample. Each experiment was repeated three times and data are shown as mean ± standard deviation. **P*<0.05. (E) U266-B1 and NCI-H929 cells were cultured for 24 hours with or without of EV isolated from mature OB derived from MC3T3-E1 or IDG-SW3 cells. Protein levels of IRF4 and MYC were examined by western blotting analysis. GAPDH was used as a loading control. (F) U266-B1 and NCI-H929 cells were transfected with scrambled (CTL) or a synthetic miR-125b inhibitor using a Human Nucleofector Kit (Lonza). Then, MM cells were cultured in triplicate with or without the EV isolated from mature OB derived from MC3T3-E1 or IDG-SW3 cells for 2 days, and viable MM cell numbers were counted. Percents of baseline are shown. **P*<0.05. OC: osteoclasts; BMSC: bone marrow stromal cells; OB-EV: osteoblast-derived extracellular vesicles; IRF4: interferon regulatory factor 4; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

for MM cell growth, survival and function, which remains to be clarified. Delivery of isolated mature OB-derived EV or engineered nanoparticles containing synthetic miR-125b mimics may open an avenue to offer innovative therapeutic opportunities to treat MM.

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Contributions

SHK, JT and MA designed the research and conceived the project. SHK, JT, TH and TT isolated the extracellular vesicles and performed polymerase chain reactions. SHK, MH, AO and TH conducted the flow cytometry. SHK, JT, RA, AB-E, and HT performed immunoblotting. SHK, JT, AS and YS carried out the EV transfer assays. SHK, JT, MH, RA, AB-E, AO, HT, MT and TH performed the cell cultures. SHK, JT, MH, RA, AB-E, AO, HT, KIM, ET, TH, TT and MA analyzed the data. JT and MA wrote the manuscript.

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Data-sharing statement

The corresponding authors are available to share any requested original data and protocols with other investigators upon reasonable request.

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