Microvesicles derived from normal and multiple myeloma bone marrow mesenchymal stem cells differentially modulate myeloma cells’ phenotype and translation initiation

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Abstract

Multiple myeloma (MM) cells’ interaction with the bone marrow (BM) microenvironment critically hinders disease therapy. Previously, we showed that MM co-culture with BM-mesenchymal stem cells (MSCs) caused co-modulation of translation initiation (TI) and cell phenotype and implicated secreted components, specifically microvesicles (MVs). Here, we studied the role of the BM-MSCs [normal donors (ND) and MM] secreted MVs in design of MM cells’ phenotype, TI and signaling. BM-MSCs’ MVs collected from BM-MSCs (MM/ND) cultures were applied to MM cell lines. After MVs uptake confirmation, the MM cells were assayed for viability, cell count and death, proliferation, migration, invasion, autophagy, TI status (factors, regulators, targets) and MAPKs activation. The interdependence of MAPKs, TI and autophagy was determined (inhibitors). ND-MSCs MVs’ treated MM cells demonstrated a rapid (5 min) activation of MAPKs followed by a persistent decrease (1–24 h), while MM-MSCs MVs’ treated cells demonstrated a rapid and continued (5 min–24 h) activation of MAPKs and TI (↑25–200%, \textit{P} < 0.05). Within 24 h, BM-MSCs MVs were internalized by MM cells evoking opposite responses according to MVs origin. ND-MSCs’ MVs decreased viability, proliferation, migration and TI (↓15–80%; \textit{P} < 0.05), whereas MM-MSCs’ MVs increased them (↑10–250%, \textit{P} < 0.05). Inhibition of MAPKs in MM-MSCs MVs treated MM cells decreased TI and inhibition of autophagy elevated cell death. These data demonstrate that BM-MSCs MVs have a fundamental effect on MM cells phenotype in accordance with normal or pathological source implemented via TI modulation. Future studies will aim to elucidate the involvement of MVs–MM receptor ligand interactions and cargo transfer in our model.

Background

The role of the bone marrow (BM) microenvironment in multiple myeloma (MM) progression is by now undeniably established, particularly its major contribution to disease relapse and mortality (1). Continuous efforts to understand the interactions of the malignant cells with the cellular, soluble and extracellular matrix (ECM) constituents of the BM niche have led to some major findings but the acquired knowledge is still incomplete and MM remains fatal (1–4). In the past several years, we have studied the interaction of the MM cells with BM-resident mesenchymal stem cells (BM-MSCs) (5–7). MSCs are stroma precursors responsible for organ structure, repair and (re)generation (8). Interestingly,
they show tropism to inflammatory and cancerous sites and are reported to display both anti- and pro-cancer activity (8,9). In previous research, we have shown that the cross-talk between the MM and BM-MSCs populations reciprocally affects cells’ phenotype and proteome via translation initiation (TI) (5). Importantly, we observed that the effect of the BM-MSCs is contingent on their origin with a tumor promoting effect ascribed to MM BM-MSCs and a non-supportive effect attributed to normal donors (ND) BM-MSCs (5). We have established that the activation of the TI factors eukaryotic translation initiation factor 4E (eIF4E) and eukaryotic translation initiation factor 4GI (eIF4GI) generates the MM promoting effect of the MM-MSCs and involves the up-regulation of key signals such as NFκB, cyclin D1, HIF1α, chymase, ERKs and SMAD5 (5,10) all implicated in MM progression (11–15). Additionally, we have determined that soluble factors participate in the interaction between the MM cells and co-cultured BM-MSCs. Moreover, we identified an involvement of microvesicles (MVs) in the interaction that piqued our interest and led to the current study (5).

MVs are extracellular vesicles (EVs) created by outward budding and fission of the cell membrane sized 100–1000 nm (13,14). As all EVs, MVs deliver signals between cells yet because of their genesis, they uniquely carry many constituents of their origin cells’ plasma membrane (16,17). Most research of EVs in MM focused on the vesicles derived from the malignant cells and not their surroundings (18–23). The few studies that did address the role of EVs secreted from cells in the MM microenvironment, particularly BM-MSCs, focused on exosomes, which originate from the endocytic system and are significantly smaller than MVs (40–100 nm) (20,24–26). Paucity of data regarding the role of MVs in MM progression is in striking contrast to the emerging understanding that EVs play a significant role in both (16). Importantly, EVs reflect the cells from which they originate and the changes the cells undergo. BM-MSCs, being stem cells are by definition plastic and highly responsive to signals from their surroundings a fact that is reflected in the dynamic change of their shed MVs. In turn, the shed BM-MSCs MVs modify the BM microenvironment and dictate continuous evolution. In these surroundings, the MM clones constantly adapt and eventually develop drug resistance (3,4).

Based on these accumulating data, we hypothesized that the MSCs’ secreted MVs afford a versatile platform for adaptable communication with MM cells and that this flexibility may be reflected in the different effects of MVs derived from ND-MSCs compared to those originating from MM-MSCs.

Indeed, our observations support this conjecture. We witnessed a profound effect of the BM-MSCs derived MVs on the malignant cells’ major characteristics with significant differences between MVs from ND-MSCs and MM-MSCs.

### Materials and methods

#### Cell lines

Multiple myeloma cell lines U266, ARP1, MM1S, OPM2 and RPMI 8266 were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), antibiotics and glutamine (Biological Industries, Kibbutz Beit Haemek, Israel) as previously (5,7,27). U266 and RPMI 8266 (ATCC) were authenticated (STR of 15 loci, 2014) by Genomics Core Facility of Biochip Technologies and the Rappaport Research Institute, Technion, Israel. Authenticated MM1S and OPM2 were obtained from Karin Joehrer lab, Austria (authenticated by STR of 15 loci, 2015). ARP1 (a gift from Prof. Joshua Epstein, Little Rock Arkansas) cannot be authenticated due to absence of initial genomic profile. All cell lines were propagated upon receipt/authentication and frozen (−80°C) in aliquots. Each experiment was conducted with freshly defrosted aliquots of these authenticated cell lines’ aliquots.

#### BM-MSCs sample isolation and propagation

BM samples were obtained from femur head BM samples of ND, generally healthy, undergoing elective full hip replacement surgery (n = 13) and MM patients. BM aspirates mature MM aspirations taken for medical purposes (n = 9) at Meir Medical Center, Israel. All participants signed informed consent forms approved by Meir Medical Center Helsinki Committee. MSCs were isolated, propagated and their identity verified as described by us before (5,7,27). Briefly, MSCs were isolated from BM samples on a Ficoll (Sigma-Aldrich) gradient and seeded in flask at 40 000 cells/cm2 with RPMI 1640 supplemented with 10% FBS (Biological Industries). Non-adherent cells were removed with the medium within the first 10 days of culture, leaving the adhered MSCs in the culture dish. Media were replaced twice weekly until the culture was nearly confluent (2–3 weeks); at which time, the cells were harvested for identity validation [(vimentin+, keratin−, CD271+, CD34−, CD45−) (immunocytochemistry, flow cytometry (FACS)) (5,7,27)].

#### Microvesicles isolation and application to MM cell lines

Microvesicles were isolated from conditioned media collected from 80% confluent BM-MSCs cultures (2–6 weeks) (27). Briefly, media was obtained after cell removal by centrifugation at 800× g for 5 min and then centrifuged at 4500× g for 5 min to discard large debris. After additional centrifugation at 20 000× g (Beckman Ti70 rotor; Beckman Coulter) for 60 min at 4°C, the MVs were washed and re-suspended in phosphate-buffered saline. Microvesicles total protein concentration was measured at 280 nm using NanoDrop spectrophotometer (Thermo Scientific Nano-Drop 1000; Thermo Fisher Scientific). Dose response (peIF4E, peIF4GI; cell count) of the MM cell lines MM1S, OPM2 and RPMI 8226 was conducted with freshly defrosted aliquots of these authenticated cell lines (5,7,27).

#### Flow cytometry (FACS)

Microvesicles derived from BM-MSCs were identified by size and validated by Annexin attachment to exposed membrane phosphatidylserine. For analysis of MVs uptake the MVs were stained with the PKH67 dye, according to the manufacturer’s instructions (Sigma-Aldrich). The PKH67-labeled MVs were incubated with U266 cells at 37°C for 5 min (1,4 and 24 h). Thereafter, the cells were washed with phosphate-buffered saline and the fluorescence was analyzed by flow cytometry (FACS) (Navios Flow Cytometers, Beckman Coulter). For inhibition of MVs uptake, U266 cells were pre incubated for 30 min with cytochalasin D (20 μM), Chloroquine (40 μM), sucrose (0.45 M) and Dynasore (50 μM) (Sigma-Aldrich); then incubated with PKH67 labeled MVs at 37°C for 24 h. After rinsing excess MVs, the MM cells were assayed by FACS for PKH67 fluorescence. U266 cells incubated only with PKH67-MVs were used as positive control.

#### Confocal microscopy

Microvesicles derived from BM-MSCs were stained with the PKH67 dye, according to the manufacturer’s instructions (Sigma-Aldrich). The
PKH67-labeled MVs were incubated with U266 cells at 37°C for varying time periods (5 min, 1, 4 and 24 h) then washed with phosphate-buffered saline, attached to poly-L-lysine–coated slides for 1 h at 37°C and fixed with 2% paraformaldehyde. The slides were covered with fluoromount (Sigma) and analyzed with a Zeiss Laser confocal microscope (Zeiss, Oberkochen, Germany).

**Trypan blue**

Total cell counts as well as the respective proportion of viable and dead cells were assayed by Trypan blue dye. Cells were automatically counted by Countess (Invitrogen). Live cells remained unstained while dead cells assimilated the dye (5,7,27).

**Cell viability assay**

Assessment of viability was performed on MM cell lines using cell proliferation reagent WST-1 (Roche, Basel, Switzerland) as described before (5,7,27).

**Western blotting**

Cells were lysed, proteins were extracted and western blot was performed as described elsewhere (5,7,26). The following proteins were detected using rabbit/mouse anti-human: peIF4E (Ser209)/total eIF4E, peIF4GI(Ser1108)/total eIF4GI, pe4EBP(Ser65)/total 4EBP, pmTOR(Ser2448)/total mTOR, pMNK(Thr197/Thr202)/total MNK/pERK1/2 and pJNK (Cell Signaling Technology, Danvers, MA, USA); SMAD5 (Epitomics, Burlingame, CA); c-Myc, HIF1α, NFkB, PCNA (Santa-Cruz, CA); tubulin, LC3-II (Sigma). Bound secondary antibodies and products were visualized as done by us previously (5,7,27).

**Transwell assay**

100 000 MM cells were cultured in the upper chamber of transwell plate 8.0 µm (corning) with RPMI 3% FBS. The lower chamber contained fibronectin (human plasma, Sigma, 20 µM) dissolved in RPMI 10% FBS. BM-MSCs’ MVs were added to the cells in the upper chamber, migrated cells present in the lower chamber were enumerated after 24 h using the automatic Countess (Invitrogen).

**Zimogram assay**

MM cell lines treated with BM-MSCs’ MVs for 3 days. Media (40 µl) were collected and electrophoresed at non-reducing conditions, in 10% polyacrylamide gel containing 1 mg/ml gelatin type A (Sigma). Gels were washed in 2.5% Triton X-100 and incubated overnight in 50 mM Tris–HCl (pH 7.5) and 5mM CaCl2. Coomassie blue staining followed by destaining (20% methanol, 7% acetic acid in DDW) allowed visualization of areas where the gelatin was digested and thus evident as clear zones against the blue background. Optical densities of the clear zones were measured by using LAS3000 Image reader. Results were normalized to background values using the Multi-gauge V3.0 program (Fujifilm).

**Inhibitors**

MAPKs inhibitors

U266 cells were treated with SP600125 (20 µM, JNK inhibitor, Biomol Int.), U0126 (10 µM, MEK1/2 inhibitor, CST, dissolved in DMSO) 1.5 h before...
incubation with BM-MSCs' MVs. The cells harvested and analyzed. eIF4E/eIF4G Interaction Inhibitor: cells were incubated with 4EGI-1 (35 μM, EMD Millipore, dissolved in DMSO) 3 days.

**Autophagy inhibitor**

Autophagy was inhibited using the 3 methyladenine (3MA) (7.5 mM) (Sigma) dissolved in ddH2O for 3 days.

**Statistical analysis**

Student's paired t tests were applied in the analyses of differences between cohorts. An effect was considered significant when P value was equal to or less than 0.05. All experiments were conducted at least three separate times. An additive effect was verified by drugs' interaction formula \[ q = \frac{P(A+B)}{P(A)+P(B)} - P(A)*P(B) \] \[ q < 0.85 – \text{antagonist}; \]
\[ 1.15 > q > 0.85 – \text{additive} \] (22). All experiments were conducted 3–7 separate times.

**Results**

**BM-MSCs MVs isolation and characterization**

As previously reported, we isolated and propagated BM-MSCs from MM patients bone marrow aspirates (MM-MSCs) and femur heads removed in total hip replacement surgeries of otherwise healthy subjects (ND-MSCs) (5–7). Once we had bona fide 80% confluent BM-MSCs cultures, we began collecting 3–4 days secretomes (conditioned culture media) from which we isolated the MVs (as described in the Materials and methods) (5–7). Validation of the MVs identity was based on several procedures. Firstly, we assayed the MVs size and morphology by transmission electron microscope and staining with uranyl acetate that demonstrated double membrane vesicles at the appropriate size (100–1000 nm) (6). In this study, we added corroboration of the MVs size by comparing them to commercial fluorescently tagged beads by flow cytometry (FACS) (and positive staining with Annexin (Figure 1A). Next, using confocal microscopy and FACS, we determined a time dependent (5 min, 1, 4 and 24 h) uptake of PKH-67 stained MVs into MM cell line (U266) that reached 94% uptake at 24 h (Figure 1B and C). We also determined that the uptake of the MVs into the MM cells was an active process that required energy by showing that incubation of MM cells with MVs at 4°C drastically abrogated the process (↓80%, P < 0.05) (Figure 1D). Concordantly, cytochalasin D (phagocytosis inhibitor), chloroquine and sucrose 0.45 μM (endocytosis inhibitors) but not dynasore (dynamin inhibitor) down-regulated the MVs uptake (↓30–50%, P < 0.05) indicating that the active incorporation of the MVs involved both phagocytosis and endocytosis (Figure 1D). When we combined endocytosis and phagocytosis inhibitors (cytochalasin D and chloroquine) we achieved an additive and most significant inhibition of the MVs uptake into the MM cells (additive q = 0.95, ↓75%) (Figure 1D), and when we combined two

![Figure 2. BM-MSCs MVs effect on MM cells MAPKs signaling and TI: first 24 h. U266 MM cell line was cultured for 5', 1, 4 and 24 h with MVs (50 μg/ml). Cells were harvested, lysed and immunoblotted for MAPKs pERK1/2, pJNK and for TI factors peIF4E/peIF4GI. Graphical presentation of analysis (A) and representative immunoblots (B) are presented. (C) U266 MM cells were pre-treated with MAPKs inhibitors for 1.5 h and incubated with MM-MSCs MVs for 1 h. Cells were harvested, lysed and immunoblotted for MAPKs and TI factors phosphorylation. Tubulin served as loading control. Results are presented in graphs (left) and representative immunoblots (right) and expressed as percent (mean ± SE, n ≥ 4) of respective protein expression in control cells not treated with MVs (dotted line). Asterisks depict statistical significance ($, &, * P < 0.05, ** P < 0.01).
endocytosis inhibitors (chloroquine and sucrose) we achieved an additive and substantial inhibition of the MVs uptake into the MM cells as well (additive $q = 0.85$, ↓65%) (Figure 1D). Finally, we assayed the dose response of the MM cell lines to BM-MSCs’ MVs (ND, MM) and determined 50 µg/ml per 100 000 cells as the optimal dose (detailed in Materials and methods).

MM BM-MSCs MVs have an immediate effect on MM cell lines’ MAPKs and TI’s activation

The transfer of information between MVs and recipient cells may be mediated rapidly by contact signaling or at a slower rate by delivery of their contents into the cells. We wanted to assay the time course of the MM cell lines’ response to administration of BM-MSCs MVs. Therefore, we assayed MM cell line (U266) for the activation of MAPKs (pERK1/2 and pJNK) and TI factors (peIF4E and peIF4GI) at several time points post treatment with MVs (5 min, 1, 4 and 24 h). We observed an immediate response of the MM cell lines to the MVs of MM-MSCs (within 5 min) manifested in increased phosphorylated MAPKs ($\uparrow70–85\%$, $P < 0.05$) (Figure 2A line graphs, B representative immunoblot). Correspondingly, within an hour we also registered elevated phosphorylated TI factors in MM cell lines treated with MM-MSCs MVs ($\uparrow75–80\%$, $P < 0.05$) (Figure 2A and B). An effect that was generally maintained for the next 24 hours ($\uparrow50–150\%$, $P < 0.05$) (Figure 2A and B). On the contrary, we registered phosphorylation of ERK1/2 and JNK in MM cell lines after only 5 min with ND-MSCs MVs ($\uparrow30–80\%$, $P < 0.05$) (Figure 2A and B), yet this response was weaker ($\downarrow25–35\%$, $P < 0.05$) and did not translate into phosphorylation of the TI factors nor was it maintained after 1h post treatment (Figure 2A and B).

Finally, in order to validate that ERK1/2 and JNK activation is instrumental to phosphorylation of eIF4E and eIF4GI in MM cell lines treated with MM-MSCs’ MVs we inhibited their activity (U0126 and SP600125, respectively) then re-assayed expressions of phosphorylated eIF4E and eIF4GI. Indeed, we witnessed decreased levels of phosphorylated TI factors in the U266 (Figure 2C, representative immunobLOTS and analysis) and RPMI 8226 (Supplementary Figure 2, available at Carcinogenesis Online, representative immunobLOTS and analysis) that received both MAPKs inhibitors and MM-MSCs’ MVs in comparison to MVs treated cells ($\downarrow75–90\%$, $P < 0.01$) or inhibitors only treated cells ($\downarrow50–75\%$, $P < 0.05$).

Figure 3. ND/MM MSCs MVs effect on MM cell lines’ phenotype and TI: prolonged effect. U266, OPM2, MM1S and ARP-1 MM cell line was incubated with ND/MM MVs (50 µg/ml). (A) All four cell lines were assayed for viability (WST1), cell count (trypan blue), and proliferation (PCNA immunoblot). Representative floating U266 and slightly more adherent MM1S were also assayed for migration (transwell), invasion (zymogram) and autophagy (LC3II, Beclin immunoblot). (B) MM cells were incubated with ND/MM MSCs MVs for 72 h, harvested, lysed and immunoblotted for TI factors, regulators and targets. Tubulin served as loading control. Results are expressed as percent (mean ± SE, n ≥ 4) of respective protein expression in control cells not treated with MVs (dotted line). Asterisks depict statistical significance (*$P < 0.05$, **$P < 0.01$).
BM-MSCs MVs cause a prolonged and differential effect on MM cell lines’ phenotype and TI in accordance with source

Once we established that BM-MSCs MVs affect MM cell lines signaling, we wanted to expand our assessment of this influence. Therefore, we harvested MM cell lines (U266, RPMI 8226, ARP-1, OPM2 and MM1S) treated with BM-MSCs MVs (ND, MM) (24 and 72 h) and tested several aspects of their phenotype. Protein was also extracted for analyses of TI status (regulators, factors, targets).

Our analyses demonstrated that MM cell lines (U266, ARP-1, OPM2 and MM1S) treated with MM-MSCs MVs (72 h) had augmented viability (↑30–100%, P < 0.05) (Figure 3A), live cell counts (↑30–60%, P < 0.05) (Figure 3A), and PCNA proliferation marker expression (↑25–130%, P < 0.05) (Figure 3A). Furthermore, the representative floating MM cell line U266 and partially adherent MM1S exposed to MVs from MM-MSCs (24 h) demonstrated increased cell migration (↑85–110%, P < 0.01) (Figure 3A) and metalloproteinase 9 activity (MMP9) (↑25–60%, P < 0.05), suggesting elevated invasion capabilities (Figure 3A). On the contrary, there were decreased viability, live cell counts (72 h) and migration (24 h) in MM cell lines treated with ND-MSCs’ MVs (↓20–50%, P < 0.05) (Figure 3A). No consistent effect of BM-MSCs MVs (ND, MM) on MM cell lines dead cell counts was registered (Figure 4C).

In our analyses of the TI profile in MM cell lines treated with MM-MSCs’ MVs (72 h), we witnessed a comprehensive activation of the treated cells’ TI regulators (mTOR, MNK1/2, 4EBP) and factors (eIF4E, eIF4GI) (↑20–270%, P < 0.05) (Figure 3B). Importantly and in proof of increased translational activity, we also observed significantly increased levels of central proteins with established roles in MM pathogenesis (NFκB, SMAD5, cyclin D, HIF1α, cMyc) (↑25–180%, P < 0.05) (Figure 3B). In contrast, MM cell lines treated with ND-MSCs MVs displayed decreased levels of TI regulators, factors and targets (↓up to 90%, P < 0.05) (Figure 3B).

We have previously substantiated that TI was critical for the MM cell lines phenotype, particularly when modulated by extracellular factors such as BM-MSCs in co-culture or BM-MSCs secretomes (5,7,26,27). Thus, we speculated that MM-MSCs MVs’ activation of the TI machinery is upstream of MM cell lines proliferation and migration. In order to test this conjecture, we inhibited eIF4E/eIF4G complex inhibitor (4EGI-1) and tested the MM cell lines (U266 and RPMI 8226) treated with MM-MSCs MVs for cell count, migration and PCNA and cyclinD1 expressions. In corroboration of our hypothesis, TI inhibition indeed prevented increases in live cell counts, PCNA and cyclinD1 expressions and migration (P < 0.05) (Figure 4A analysis, B-representative immunoblots).

The involvement of autophagy in MM and its frequent supportive function in the cells’ survival and proliferation was...
also established by us in past studies (7,27,28). Therefore, we surveyed the activation of LC3II and Beclin, both essential for autophagosomes’ formation, in MM cell lines (U266 and MM1S) treated with MM-MSCs’ MVs. An increase in autophagy markers was registered in MM cell lines treated with MM-MSCs MVs (↑60–195%, P < 0.05), yet there was no change in their expressions in MM cell lines treated with ND-MSCs’ MVs (Figure 3A). Inhibition of autophagy (3 methyladenine-3MA) in MM cell lines (U266 and MM1S) that received MM-MSCs MVs resulted in decreased LC3-II (representative immunoblot Figure 4D) and elevated dead cell counts (↑40–100%, P < 0.05) (Figure 4C top graph). Further analysis of death mode by FACS demonstrated increased apoptosis (↑270–320%, P < 0.05) (Figure 4C bottom graph and exemplary FACS dot plots, right).

Discussion
This study illuminates a novel communication route between the BM-MSCs and the malignant MM cells via secreted MVs. Moreover, while MM-MSCs’ MVs activate cancer promoting signals (MAPKs/TI/oncogenes) ND-MSCs’ MVs do not elevate these signals and at times even down regulate them. The consequences of the MVs function are clearly evident in the treated MM cell lines’ phenotypes, respectively. Thus, the MM-MSCs MVs caused increases in MM cell lines’ viability, proliferation, migration, invasion and autophagy whereas the ND-MSCs MVs did not affect these cell functions or down regulated them (Figure 5).

In a series of studies, we have repeatedly shown that TI regulates the selective expression of proteins with critical roles in MM (5,27,29–31). Here again, we show that the cancer microenvironment, specifically BM-MSCs MVs from MM patients stimulate eIF4E and eIF4GI functions and elevate the expressions of HIF1α, SMAD5, NfkB, cMyc and cyclin D1. With the exception of cyclin D1 that critically regulates cell cycle, proliferation, and migration (32,33), these proteins are major transcription factors that promote the cancerous traits of the MM cells. In such a manner, HIF1α is already recognized as an important regulator of MM cells’ survival and angiogenesis (34). Moreover, there are testaments that HIF1α expression in the MM cells is instrumental to cancer promoting cytokine signaling, adhesion to ECM in the BM stroma and maintenance of the Warburg effect. These signals are all integral to the hallmarks of cancer: sustaining proliferative signaling, evading growth suppression, angiogenesis, and deregulating cellular energetics (35,36). Similarly, TGFβ (upstream of SMAD5 (37)) supports MM progression by stimulating growth promoting cytokines, inducing protective autophagy on top of its crucial influence on osteoclastogenesis (38). Thus, TGFβ/

SMAD signals promote evasion of cell death and sustained proliferation cancer hallmarks. NfkB is a well-recognized promoter of MM cell survival, proliferation and drug resistance and critically important to MM extramedullary expansion (33,39). Indeed, numerous microenvironmental cues activate the NfkB cascade thereby actively contributing to the malignant plasma cells’ fundamental traits. The elevated expression of cMyc is a characteristic of hyperdiploid MM, yet the mechanism of
overexpression is only explained by translocations in some of the cases (40,41). Its expression is associated with MM survival, extramedullary expansion, hypercalcemia and shorter overall survival (40,41). Indeed, cMyc is an established promoter of survival, proliferation and glycosylation all integral to the malignant profile (42). In conclusion, the MM-MSCs’ MVs mediated activation of eIF4E/elF4GI instigates the expression of key signals integral to the MM cancerous hallmarks and its progression. These observations are in concordance with our findings in the co-culture model of MM cell lines and BM-MSCs (5).

The kinetics and mechanism of the BM-MSCs’ MVs uptake into the MM cells correspond to previous publications (43-45). We witnessed a time dependent internalization of the stained MVs that peaked at 24 h post-application. Moreover, the uptake mechanism was a combination of phagocytosis and endocytosis as evidenced by the additive effect of chloroquine and cytochalasin D or chloroquine and sucrose. Interestingly, a 5-min exposure of MM cells to the BM-MSCs’ MVs (MM, ND) was sufficient to induce a significant phosphorylation of ERK and JNK. Yet, we first detected MVs in the MM cells after an hour of co-incubation, by which time the MAPKs phosphorylation in MM cell treated with ND-MSCs MVs already decreased and returned to the base line levels observed in un-treated control MM cells. This suggests that the initial phosphorylation of the MAPKs in the MM cell lines may be attributed to a membranal interaction and not the MVs internalization. Further studies are needed to clarify this possibility and identify the MVs membranal signaling entity. In contrast, MM cell lines treated with MM-MSCs’ MVs displayed a phosphorylation of the MAPKs and TI factors that persisted for 24 h and corresponded to the kinetics of the MVs uptake into the MM cells. Lastly, MM-MSCs MVs caused a significantly higher phosphorylation of MAPKs in the MM cells compared to ND-MSCs MVs suggesting different membrane initiated signaling pathways may be involved.

With the use of MAPKs and eIF4E/elF4GI inhibitors, we demonstrated the hierarchy of the signaling cascade: MM-MSCs MVs activate MAPKs and the MAPKs instigate TI and elevate onco-genic targets and alter MM cells’ phenotype. These findings correspond to our previous observations with the MM cell lines and MM-MSCs co-culture model (5) and suggest that MVs are at least partially responsible for the dialogue between the BM-MSCs and MM cells.

The role of autophagy in cancer generally and in MM specifically has been extensively discussed previously by others and us. Indeed, the protective function of autophagy in MM cells treated with MM-MSCs MVs is in concordance with previous reports. This underscores the central setting of autophagy at an intersection between proliferation/metabolism and death, setting the stage for its targeting in anti-myeloma/cancer treatment.

Our findings once again show that the MM microenvironment differs from the normal one. This is critical to the understanding of the cancerous systemic state. For effective cessation of the BM supportive role we will need to manage the dynamic crosstalk between cells such as the BM-MSCs, with the malignant cells and prevent their transformation into cancer collaborators. Specifically, we need to block the transfer of essential signals embodied in the MM-MSCs MVs. For this purpose, we are currently characterizing the MM-MSCs MVs’ unique cargo (compared to ND-MSCs MVs) and the ensuing responses in the recipient MM cell lines. Once we identify communication that significantly alters the MM cells’ phenotype and promotes their malignant traits we will try to block it with the hope of developing a new anti-myeloma strategy.

### Supplementary material

Supplementary data are available at Carcinogenesis online.

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### Conflict of Interest Statement:

None declared.

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