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# Molecular Aberrations in Bone Marrow Stromal Cells in Multiple Myeloma

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#### Abstract

Multiple myeloma (MM) is a B-cell malignancy characterized by an accumulation of malignant plasma cells within the bone marrow. Bone marrow mesenchymal stromal cells (BMMSCs) represent a crucial component of MM microenvironment supporting its progression and proliferation. Alterations in BMMSC of MM (MM-BMMSC) have become an important research focus. In this study, we analyzed MM-BMMSC and their modification through interaction with plasma cells in 128 MM patients. MM-BMMSC displayed a senescence-like state that was accompanied by an increase in senescence-associated  $\beta$ -galactosidase activity, a reduced number of colony-forming units, an accumulation of cells in S phase of the cell cycle, and the overexpression of microRNAs (miR-16, miR-223, miR-485-5p, and miR-519d) and p21. MM-BMMSC showed a reduced expression of mitochondrial stress response protein SIRT3 and an increased mitochondrial DNA mass that led to a higher amount of reactive oxygen species compared to healthy donor BMMSC. The interaction between MM cells and MM-BMMSC is a complex mechanism that relies on multiple interacting signaling pathways. Observed aberrations in MM-BMMSC should be confirmed in an in vivo model in order to clarify the importance for the pathogenesis of MM. Eventually, the result of MM therapy could be improved by understanding the interaction between MM cells and MM-BMSCs.

Keywords: multiple myeloma, bone marrow stromal cells, molecular aberrations



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## 1. Introduction

Multiple myeloma (MM) is a B-cell malignancy characterized by an accumulation of malignant plasma cells within the bone marrow (BM) [1]. Contact between MM cells and their microenvironment plays a crucial role in MM survival and proliferation and is able to promote tumor progression and drug resistance. Bone marrow mesenchymal stromal cells (BMMSCs) represent a central component of MM microenvironment supporting its progression and proliferation [2–4]. Alterations in BMSC from MM patients (MM-BMMSC) have become an important research focus. Several studies and our previous data have suggested the genesis of constitutive abnormalities within the BMMSC population through direct and indirect interactions with MM cells [5-8]. The development of a senescence-like state in BMMSC and thereby a modulated secretory profile, worsened osteogenic differentiation potential and inhibition of the T-cell proliferation, was reported [6, 8, 9]. Senescent BMMSCs display an increased senescence-associated β-galactosidase activity (SAβGalA) and irregular cell morphology. Usually, the cell cycle of senescent cells is arrested at the G<sub>1</sub>/ S-transition point in combination with the overexpression of different cell cycle inhibitors as p21 and p16. In spite of the aberrant growth characteristics, senescent cells remain metabolically active, and therefore, the secretion of pro-inflammatory mediators could promote tumorigenesis in neighboring premalignant cells [10–12]. The secretion of pro-inflammatory mediators by senescent BMMSC could therefore promote tumorigenesis in neighboring premalignant cells [13].

Two imprinted clusters in the human genome might contribute to the generation of senescence and the induction of cellular changes in MM-BMMSC [14–17]. The DLK1-DIO3 imprinted domain is located on chromosome 14q32.2, and cluster C19MC is located on chromosome 19q13. The DLK1-DIO3 expresses the non-coding transcripts MEG3, anti-RTL1, 53 microR-NAs (miRNA), and 2 snoRNA clusters on the maternal chromosome. The paternal chromosome is responsible for the transcription of the protein-coding genes DLK1, RTL1, and DIO3 [18, 19]. Allelic expression of these genes is controlled through methylation of a regulatory region (IG-DMR) located upstream of the cluster [20]. The C19MC codes for 59 miRNAs are processed into one primary transcript from the paternal chromosome. Its expression strongly correlates with the epigenetic modulation of a CpG site located upstream [21].

In addition, there are evidence that the presence of cancer-associated fibroblasts (CAFs), characterized by high  $\alpha$ -SMA, FAP, and FSP-1 expression, in the BM samples of MM patients, contributes to altered, tumor favorable, cell-cell interactions and cytokine secretions [22–24].

BMMSC represents an essential part for assistance of MM partly by the secretion of tumor supportive cytokines as interleukin 6 (IL-6) and vascular endothelial growth factor (VEGF) [2]. Both of them play a major role in the aberration of multiple signaling pathways such as PI3K, JAK/STAT3, Raf, and NF- $\kappa$ B [25]. In addition, MM cells produce inflammatory molecules, such as TGF $\beta$  and TNF $\alpha$ , which lead to NF- $\kappa$ B activation tumor-promoting effects. The activation of the NF- $\kappa$ B pathway in both MM cells and BMMSC results in the downstream upregulation of adhesion molecules and a consequent increase in cell-cell interactions [26, 27].

It is known that the increased activation of NF-κB together with the overexpression of adhesion molecules can induce a therapy resistance [4, 26, 28, 29]. It can increase the secretion of tumor supportive soluble factors by BMMSC and may possibly lead to the generation of cell adhesion-mediated drug resistance [30–32].

Furthermore, additional interaction pathway, such as Notch signaling, is a factor between BMMSC and myeloma cells [33]. For example, it has been shown that malignant plasma cells overexpress the Notch ligand Jagged-2. An inhibition of Notch interaction induces myeloma cell apoptosis [34]. Notch signaling is important not only for the interaction of the myeloma cell with its surrounding cells but also for intercellular signaling between the malignant plasma cells. Downstream leads the Notch receptor-ligand interaction to an increased release of angiogenic and tumor-stimulating factors, such as VEGF, IL-6, and IGF-1 [35]. In addition, direct interaction between adhesion molecules (e.g., VCAM-1-VLA-4 interactions) and cell surface receptors such as Notch mediates therapy resistance and disease progression [36]. Furthermore, the formation of exosomes by BMSC, which actively transports modulatory substances, such as chemokines and miRNAs to the malignant MM cell, promotes survival and growth of MM cells. However, further investigations are needed to identify the exact mechanisms of exosome-mediated tumor promotion [37, 38].

It is known that cancer cells undergo dramatic alteration of metabolic pathways. Cancer cell survival and proliferation depend on metabolic processes, like glucose-uptake via altered glycolysis, also known as the Warburg effect. Sirtuins (SIRTs) are a family of deacylases and ADP-ribosyltransferases with clear links to regulation of cancer metabolism. Through their unique ability to integrate cellular stress and nutrient status in coordination with metabolic outputs, SIRTs are well poised to play pivotal roles in tumor progression and survival [39]. SIRT3 is the main mitochondrial deacetylase, which controls the activity of many metabolic enzymes in the mitochondria. SIRT3 deacetylates mitochondrial proteins that act in mitochondrial metabolism, including the oxidation of fatty acids, glutamine metabolism, and the production of mitochondrial reactive oxygen species (ROS) [40]. It was found that the increased level of cellular ROS observed with the loss of SIRT3 leads to a cancer resolution, coordinating the metabolic shift in the Warburg phenotype [41].

Despite some knowledge of the constitutive changes in the BMMSC of MM patients, the molecular mechanisms and pathways that induce abnormalities are largely unknown.

## 2. Patients and methods

#### 2.1. Patients and donor characteristics

BM samples from 116 MM patients were studied: 69 patients with MM at the time of diagnosis and 47 at relapse. All patients had indications for treatment. The main clinical characteristics

of patients are shown in **Table 1**. Twelve bone marrow aspirates were received from healthy donors (HDs) as control. Written informed consent was obtained from all patients and donors in accordance with the Declaration of Helsinki and the ethical guidelines of the Charité University School of Medicine, which approved this study (Votum No.: EA4/131/13).

#### 2.2. Isolation of BMSC and CD138+ plasma cells

BMMSCs from patients and donors (HD-BMMSC) were isolated using adhesion method and cultivated as previously described [42–44]. The colony-forming unit fibroblast (CFU-F) assay was used to study the self-renewal capacity of BMMSC. The staining was carried out with the Hemacolor Rapid Staining Kit from Merck according to the manufacturer's instructions. The evaluation was done by counting blue colonies.

Non-hematopoietic cell characteristics were identified by flow cytometry by the absence of CD105-FITC, CD90-FITC, CD45-PE, and CD34-PE (Miltenyi). Data were acquired and analyzed with a FACS Calibur Flow Cytometer (BD Biosciences). Control CD138<sup>+</sup> plasma cells were isolated from HD mononuclear cells using magnetic-activated cell sorting (MACS) with a CD138 antibody (Miltenyi) as recommended by the manufacturer's protocol and seeded in culture flask with RPMI media with 20% of fetal calf serum and antibiotic/antimycotic.

Characteristics	All patients ( <i>n</i> = 116)	Patients at diagnosis	Patients at relapse	Donors
				(n = 16)
		( <i>n</i> = 69)	(n = 47)	
Age, median (range)	63 (33–87)	64 (33–87)	62 (57–84)	69 (38–81)
Gender (M/F, %)	66/34	67/33	66/34	62/38
Ig expression (%)				
IgG	58	60	53	
IgA	13	14	13	
IgD	1	0	2	
Light chain	27	25	32	
Non-secretary	1	1	0	
Stage on Durie-Salmon (%)				
IA	10	13	7	
I B	6	8	2	
II A	8	6	11	
II B	5	3	7	
III A	55	54	57	
III B	16	16	16	
Bone marrow infiltration % median (range):	50 (10–100)	60 (10–100)	40 (5–90)	

Table 1. Patients and donor characteristics.

#### 2.3. Co-culture and transwell culture experiments

KMS12-PE cells received from DSMZ (ACC606) were cultured in enriched RPMI media. For cocultures, MM-BMMSCs were seeded in a six-well plate and incubated for 4 h. Then, KMS12-PE myeloma cells were added followed by incubation for 72 h. After incubation, KMS12-PE cells were removed. The absence of CD138+ cells was confirmed using microscopy and checked with FACS analysis. MM-BMMSCs were washed twice with PBS and applied for future analysis. Co-cultured KMS12-PE myeloma cells were suspended in TRIzol for future analysis.

For transwell cultures (0.4  $\mu$ M pore size, Corning), 2 × 10<sup>4</sup> MM-BMMSCs were seeded in the lower chamber of a 12-well plate and incubated for 4 h. Then, 2 × 10<sup>4</sup> KMS12-PE myeloma cells were added to the upper chamber. Incubation was performed for 72 h. Cultures without KMS12-PE cells served as negative control for transwell cultures and co-cultures.

#### 2.4. Detection of SAβGalA and cell cycle analysis

SA $\beta$ GalA was measured using the median fluorescence intensity (MFI) as previously reported [45]. Co-cultures of HD-BMMSC and HS-5 stromal cells (CRL-11882) were used as controls. In addition,  $\beta$ -galactosidase activity was analyzed using the "Senescence Cells Histochemical Staining Kit" (Sigma-Aldrich) as recommended by the manufacturer. Cell cycle analysis was performed using the "Cell Cycle Assay Kit" (Abcam) as recommended in the prescription. Data were studied using a logarithmic scale.

#### 2.5. Quantitative real-time PCR (qPCR)

Total RNA was extracted using TRIzol as described previously [46]. RNA was treated with DNase (Ambion) and poly(A)-polymerase (NEB) according to the manufacturer's instructions. About 800 ng of RNA was used for cDNA synthesis with a Transcriptor First Strand cDNA Synthesis Kit (Roche) and 2.5  $\mu$ l of poly(T)VN adaptor primer (10 pmol) in a 20  $\mu$ l reaction.

qPCR was performed with the FastStart Universal SYBR Green Master Mix (Roche). Primers were designed for each mRNA target using Primer3, OligoCalc, and OligoIDT. MiRNA detection was conducted using a specific miRNA primer and a universal reverse primer complementary to the adaptor sequence [47]. GAP-DH (for mRNA) and 5.8S rRNA (for miRNA) were chosen as housekeeping genes. QPCR was carried out with the Rotor Gene 6000 Real-Time PCR cycler. Cycling condition comprised 10 min at 95°C, 45 cycles of 15 s at 95°C and 60 s at 59°C, followed by a melting curve analysis from 60 to 98°C, rising by 1°/s. Efficiencies of qPCR were determined using linear regression analysis [48, 49] using LinRegPCR software, and relative quantifications were estimated with the Pfaffl method [50]. Received data were analyzed with the Rotor Gene 6000 software.

#### 2.6. Quantitative methylation-specific PCR (qMSP)

DNA isolation was performed using Puregene reagents (Qiagen) according to the manufacturer's instructions. Genomic DNA was subjected to bisulfite treatment with the EpiTect Fast Bisulfite Conversion Kit (Qiagen) as recommended in the manual. Primers were used as

described by Murphy et al. [51] for DLK1-DIO3 and Fornari et al. [52] for C19MC. Reactions were performed with 30 ng treated DNA using SYBR Green Master Mix (Roche). Quantification was carried out using a standard curve generated using a dilution series of fully methylated with unmethylated DNA (Applied Biosystems). Each sample was analyzed in duplicates, and Ct values above 32 were excluded.

#### 2.7. Copy number (CN) variation analysis

Three genomic regions located along each of the clusters were chosen for CN estimations of DLK1-DIO3 and C19MC. Assay qBiomarker Copy Number (Qiagen) was used. Genomic DNA from the stromal cell line HS-5 (CRL-11882) was applied as a calibrator. Analysis was performed with 5  $\mu$ l of SYBR Green Master Mix, 0.5  $\mu$ l of respective copy number assay, and 2 ng of genomic DNA in a total volume of 10  $\mu$ l. Relative quantification was achieved by the  $\Delta\Delta C_t$  method.

#### 2.8. Transfection of SIRT3 siRNA

The transient knockdown of SIRT3 was performed in HD-BMMSC using siRNA (Qiagen). The transfections were carried out in 6-well and 24-well plates. For a 24-well plate, 33 nM siRNA was mixed with 6  $\mu$ l HiPerFect Transfection Reagent in 100  $\mu$ l serum-free medium and incubated for 10 min at room temperature. The cells were incubated for 48 h and then used for future analyses. For a six-well plate, the cell number was constant, and the reagent volumes were scaled up accordingly.

# 2.9. Determination of mitochondrial membrane potential and reactive oxygen species

Investigation of ROS amount was carried out using the DCFDA—cellular Reactive Oxygen Species Detection Assay Kit (Abcam) as recommended in the instruction. Analysis was conducted using the median fluorescence intensity.

Analysis of mitochondrial membrane potential ( $\Delta \Psi m$ ) was performed with the Mitochondria Staining Kit (Sigma) using JC-1 dye. Results were analyzed using the ratio of JC-1 aggregates (median value of FL2 channel) to JC-1 monomers (median value of FL21 channel).

#### 2.10. Indirect enzyme-linked immunosorbent assay (ELISA)

Proteins from complete cell lysates of BMMSC were detected with a Coomassie (Bradford) Protein Assay Kit (Pierce) and were adjusted with BupH Coating Buffer (Pierce). Analyses were performed according to the commercially available indirect ELISA protocol from Abcam. Detection was performed with 1-Step pNpp-Substrate (Pierce). Absorption was measured at 405 nm. All measurements were performed with three technical replicates. A dilution series of complete cell lysates of the HS-5 cell line was used for standard curve generation.

#### 2.11. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 software (La Jolla, CA, USA). The data shown represent the mean ± standard error of the mean (SEM). Comparisons of

HD-BMMSC with MM-BMMSC were performed using the Mann-Whitney *U* test. The Wilcoxon signed-rank test was used for the analysis of co-cultures. Results were considered statistically significant when  $p \le 0.05$ .

### 3. Results

#### 3.1. MM-BMMSCs are characterized by high senescence state and cell cycle abnormalities

Analysis of  $\beta$ -galactosidase activity revealed a significantly higher SA $\beta$ GalA in MM-BMSC when compared with HD-BMDSC (**Figure 1A**). Since no significant differences in senescent cells between passages 1 and 4 were observed in both MM-BMMSC and HD-BMMSC, we can exclude the effect of cultivation on SA $\beta$ GalA. These results were confirmed by a histological  $\beta$ -galactosidase staining of HD-BMMSC and MM-BMMSC in passage 4.



**Figure 1.** MM-BMMSC exhibits a higher senescence state and a lower self-renewal capacity than HD-BMMSC. *P* values: \*<0.05; \*\*<0.01; \*\*\*<0.001; and \*\*\*\*<0.0001. All data were analyzed using the Mann-Whitney *U* test and unpaired *t*-test (ELISA analysis). (A) Flow cytometric analysis of SA $\beta$ GalA. ND-MM-BMMSCs and R-MM-BMMSCs displayed higher activity of SA $\beta$ GalA in passages 1 and 4 of cell cultures compared to HD-BMMSCs. (B) The colony-forming unit fibroblast (CFU-F) assay was used to study the self-renewal capacity of BMMSC. MM-BMMSC showed a lower self-renewal capacity compared to HD-BMMSC. (C) Cell cycle analysis showed a higher amount of cell in S phase and amount in G<sub>1</sub>/G<sub>0</sub> phase in MM-BMMSC compared to HD-BMMSC. (D) QPCR analysis displayed decreased cyclin E1, increased cyclin D1 and p21 expression in MM-BMMSC compared to HD-BMMSC. (E) Measurement of the protein level in HD-BMMSC and MM-BMMSC. Cyclin E1 was significantly decreased in MM-BMMSC compared to HD-BMMSC, whereas cyclin D1 and p21 were increased. The protein amount of p16 was slightly reduced in MM-BMMSC compared to HD-BMMSCs, healthy donor control.

The colony-forming unit fibroblast (CFU-F) assay was used to study the self-renewal capacity of BMMSC. MM-BMMSC showed a lower self-renewal capacity compared to HD-BMMSC (**Figure 1B**). Similar to the senescence study, MM-BMMSC obtained from relapsed patients showed a significantly lower self-renewal capacity than MM-BMMSC, which forms newly diagnosed patients.

MM-BMMSCs are characterized by a lower expression of cyclin E1 and an overexpression of cyclin D1 when compared with HD-BMMSC (**Figure 1D**). In addition, the cell cycle inhibitor p21 was upregulated in MM-BMMSC compared to HD-BMMSC (p < 0.05). No changes were observed in the mRNA level of p16. Changes in the mRNA levels were also confirmed using protein analysis (p < 0.03; **Figure 1E**). Cyclin E1 was decreased in MM-BMMSC compared to HD-BMMSC (p = 0.0416). Cyclin D1 and p21 protein levels were 1.5- to 1.8-fold increased. Protein measurement also showed a slightly reduced level of p16 in MM-BMMSCs, but this change was below 1.5-fold. These results correlated with a higher number of cells in S phase and a reduced number of cells in G<sub>1</sub>/G<sub>0</sub> phase compared to HD-BMMSCs (p < 0.008; **Figure 1C**).

# 3.2. Co-culturing of KMS12-PE cell line represses the senescence entry of MM-BMMSCs

Co-cultures of the KMS12-PE cell line with MM-BMMSC and HD-BMMSC were carried out to analyze whether MM cells can exert an influence on the senescence characteristics of BMMSC. Experiments were performed with MM-BMMSC (n = 20) and HD-BMMSC (n = 3). After co-culturing BMSC with MM cells, an inhibition of senescence entry in MM-BMMSC was observed. SA $\beta$ GalA activity was significantly reduced (**Figure 2A**). A similar effect was detected using transwell cultures to prevent cell-cell contact between MM-BMMSC and KMS12-PE cells (p < 0.0313; **Figure 2A**). No effect on the activity of SA- $\beta$ Gal was observed in HD-BMMSC and the HS-5 cell line co-cultured with KMS12-PE myeloma cells. Interestingly, CD138<sup>+</sup> plasma cells from healthy donors induced a downregulation of SA $\beta$ GalA activity in MM-BMMSC. However, this influence was three- to sixfold lower than that of observed in co-cultures with KMS12-PE cells. These results indicate that MM cells have a higher and more specificity proliferation stimulation effect on BMMSC compared to CD138+ plasma cells.

Also, mRNA expression of co-cultured and transwell cultured MM-BMMSCs was measured (**Figure 2B**). No changes were found for cyclin D1 and p16, whereas cyclin E1 was upregulated in both co-cultured and transwell cultured MM-BMMSC (p < 0.05). BMSC interaction with MM cells has induced an upregulation of p21. This effect was lower in transwell cultured MM-BMMSC compared to co-cultured MM-BMMSC (p < 0.008).

However, some contrary results were detected at the protein level. We have found a reduction in p21 in co-cultured MM-BMMSC (**Figure 2C**). In addition, cyclin D1 protein expression was 1.8-fold reduced upon co-cultivation with KMS12-PE myeloma cells, whereas no change was seen on mRNA level (p = 0.0033). The mRNA and protein analysis of cyclin E1 and p16 were concordant.

Next, we analyzed cell cycle distribution of co-cultured and transwell cultured MM-BMMSC (**Figure 2D**). Both cell culture systems led to a slight reduction in cells in S phase compared to MM-BMMSC cultured alone (p = 0.008) and an increase in the percentage of cells in G1/G0 phase

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**Figure 2.** KMS12-PE myeloma cells reduce SA $\beta$ GalA and modify cell cycle characteristics of MM-BMMSC. *P* values: \* <0.05; \*\* <0.01; \*\*\* <0.001; and \*\*\*\* <0.0001. All data were analyzed using the Wilcoxon signed-rank test and paired *t*-test (ELISA analysis). HS-5 (CRL-11882)—BMSC line. (A) KMS12-PE myeloma cells reduce SA $\beta$ GalA in MM-BMMSC upon co-cultivation and cultivation in transwell. The MFI in MM-BMMSC was significantly reduced in both culture systems. No changes were observed for co-cultured HD-BMMSC and HS-5 cells indicating specificity of the measured effect for MM-BMMSC. (B) Cell interaction with KMS12-PE myeloma cells induced increased cyclin E1 and p21 expression in MM-BMMSC compared to MM-BMMSC cultured alone. (C) Protein expression analysis of co-cultured MM-BMMSC (*n* = 3) compared to mono-cultured MM-BMMSC. Cyclin E1 was increased, whereas cyclin D1 and p21 were reduced in co-cultured cells compared to mono-cultures. No change was seen for p16. (D) Cell interaction with KMS12-PE myeloma cells induced an increase in cells in G<sub>1</sub>/G<sub>0</sub> phase and reduced the amount of cells in S phase in co-cultured and transwell cultured MM-BMMSCs (*n* = 8) compared to the same MM-BMMSC cultured alone.

when compared with mono-cultured MM-BMMSC (p = 0.008). Transwell cultured MM-BMMSCs showed the same tendency, but significant changes were not detectable.

#### 3.3. Deregulation of microRNA expression in MM-BMSC

We chose six microRNAs, which were previously reported to be deregulated in MM cells and to play a possible role in the generation of senescence or cell cycle arrest (miR-16, miR-485-5p, miR-519d, miR-221, miR-126, and miR-223). Analysis revealed an overexpression of miR-16, miR-223, miR-485-5p, and miR-519d (all with p < 0.025) in MM-BMMSCs compared to HD-BMMSCs. No expression differences were detected for miR-221 and miR-126 (**Figure 3A**).

We revealed the overexpression of miR-485-5p and miR-519d in MM-BMMSCs. These microRNAs are located on two imprinted clusters on chromosomes 14 (DLK1-DIO3) and 19 (C19MC), respectively, and are reported to play a role in senescence generation [21, 31, 32].



**Figure 3.** Overexpressed microRNAs in MM-BMMSC are associated with hypomethylation and CN accumulation of DLK1-DIO3 and C19MC. *P* values: \* <0.05; \*\* <0.01; \*\*\* <0.001; and \*\*\*\* <0.0001. All data were analyzed using the Mann-Whitney *U* test. (A) ND-MM-BMMSC and R-MM-BMMSC showed high overexpression of miR-16, miR-485-5p, miR-519d, and miR-223 compared to HD-BMMSCs. (B) The regulatory regions of DLK1-DIO3 and C19MC were hypomethylated in ND-MM-BMMSC and R-MM-BMMSC compared to HD-BMMSC. (C) CN analysis of C19MC displayed CN accumulation in all three regions in MM-BMMSC compared to HD-BMMSC. (D) CN analysis of DLK1-DIO3 displayed CN accumulation in all three measured positions in MM-BMMSCs compared to HD-BMMSC.

Given that the expression of both clusters is controlled by methylation of their regulatory regions, we analyzed their methylation status using qMSP (**Figure 3B**). Hypomethylation of both clusters in MM-BMMSCs compared to HD-BMMSCs was observed. For DLK1-DIO3, MM-BMMSC exhibited an approximate fivefold lower methylation level of the IG-DMR. The C19MC exhibited a 2.5-fold lower methylation level in MM-BMSC compared to HD-BMMSC (p = 0.0062). CN analysis of both clusters displayed CN accumulation in all three regions in MM-BMMSC (n = 38) compared to HD-BMMSC (n = 8; **Figure 3C** and **D**).

# 3.4. Co-culturing of MM-BMSC with the KMS12-PE cell line induces the changes of microRNA expression in both cell types

The expression of four miRNA (miR-16, miR-223, miR-485-5p, and miR-519d) after co-culturing and transwell cultured MM-BMMSC was measured using qPCR (**Figure 4A**). MiR-223 was down-regulated in co-cultured MM-BMMSC (p < 0.007), whereas no effect was detected in transwell



**Figure 4.** KMS12-PE myeloma cells downregulate miR-223 and miR-485-5p in MM-BMMSC. *P* values: \* < 0.05; \*\* < 0.01; \*\*\* < 0.001; and \*\*\*\* < 0.0001. All data were analyzed using the Wilcoxon signed-rank test. (A) Co-cultured MM-BMMSC (*n* = 25) displayed reduced expression of miR-223 and miR-485-5p. Transwell-cultured (*n* = 10) MM-BMMSC showed no changes in miR-223 expression but also decreased miR-485-5p levels. Intensity of changes in miR-485-5p decreased when cell-cell contact was prevented by transwell cultivation. (B) Cell interaction with MM-BMMSC induced changes in the microRNA expression of KMS12-PE myeloma cells (*n* = 10). MiR-221 was upregulated, whereas miR-223 and miR-519d decreased in co-cultured KMS12-PE myeloma cells.

cultured MM-BMMSC. In contrast, downregulation of miR-485-5p was detected in both cell culture systems (p < 0.03). Interestingly, cell-cell interaction also altered miRNA expression of KMS12-PE myeloma cells. We found upregulation of miR-221 and significantly downregulation of miR-223 and miR-519d (p < 0.02; **Figure 4B**). Expression of miR-485-5p was not detectable in KMS12-PE myeloma cells.

#### 3.5. KMS12-PE cells modulate the gene expression of MM-BMMSC

To explore the influence of KMS12-PE cells on gene expression of adhesion molecules, qPCR analysis of MM-BMMSC, co-cultured for 72 h with KMS12-PE cells in passage 4, was performed (n = 25). In mono-cultured BMSC, an upregulation of VCAM-1 (p = 0.33), ICAM-1 (p = 0.33), and IKK- $\alpha$  (p = 0.05) was demonstrated. Furthermore, the expression profile of miRNAs, targeting the analyzed genes or correlating with senescence, was studied (miR-16, miR-221, miR-126, miR-223, miR-485-5p, and miR-519d). MiR-16, miR-223, miR-485-5p, and miR-519d were significantly upregulated (p = 0.02; p = 0.004; p = 0.02; and p = 0.002, respectively), whereas miR-221 and miR-126 showed no considerable differences to BMSC obtained from healthy donors. After co-culturing of MM-BMSC with KMS12-PE cells, an enhanced expression of adhesion molecules was apparent. This includes the upregulation of VCAM-1 (p = 0.0078), ICAM-1 (p = 0.2425), and NF- $\kappa$ B activator IKK- $\alpha$  (p = 0.0573), though the values for ICAM-1 and IKK- $\alpha$  were not significant. Hence, MM cells seem to further boost the aberrant expression of adhesion molecules in MM-BMMSCs. Regarding microRNAs, a significant downregulation of miR-223 and miR-485-5p (p < 0.009) was detected. In addition, miR-16 and miR-519d showed a trend toward downregulation, though the changes were not significant. No expression alterations to miR-221 or miR-126 were detected (data not shown).

#### 3.6. Expression of metabolic regulators in MM-BMSC

We investigated whether metabolic changes in MM-BMMSC could be responsible for the early aging status of the cells. For this purpose, we analyzed the expression of the gene and protein of the metabolic molecules SIRT3 and UCP2 and the lactate transporter MCT1 and MCT4.

There were no significant differences in the gene expression of MCT1, MCT4, and UCP2 in MM-BMMSC compared to HD-BMMSC (data not shown). In contrast, a significant lower expression of SIRT3 was detected in MM-BMMSC (p < 0.001; **Figure 5A**). All data were reproduced at the protein level. In addition, it was investigated whether MM-BMMSCs have an increased mitochondrial mass in comparison with HD-BMMSC. For this purpose, mtDNA was quantified and was normalized to the content of nuDNA. It was shown that MM-BMMSCs show a significant increase in mitochondrial mass compared to HD-BMMSC (p = 0.0149; **Figure 5B**). These changes were not detected in MGUS-BMMSC (n = 4), suggesting an association with disease progression.



**Figure 5.** SIRT3 expression and mtDNA mass in MM-BMMSC. *P* values: \* <0.05; \*\* <0.01; \*\*\* <0.001; and \*\*\*\* <0.0001. All data were analyzed using the Wilcoxon signed-rank test. (A) MM-BMSC displayed a twofold decrease in the expression of SIRT3 compared to HD-BMSC. MGUS-BMSC showed no changes. (B) MM-BMSC showed a twofold increase in mtDNA mass compared to HD-BMSC. (C) Co-cultured MM-BMSC displayed a fourfold increase in SRT3 mRNA level. No changes were seen in transwell cultures. (D) Co-cultivation KMS12-PE and MM-BMSC induced depolarization of  $\Delta\Psi$ m. (E) Co-cultivation KMS12-PE and MM-BMSC reduced the amount of ROS in both cell systems.

To explore the influence of MM cells on SIRT3 expression in BMSC, co-culturing for 72 h with KMS12-PE cells (n = 20) and transwell experiments (n = 10) was performed. Interestingly, we found a fourfold upregulation of SIRT3 expression in MM-BMMSC when co-cultured with KMS12-PE myeloma cells (**Figure 5C**). No changes were seen in transwell cultures.

Moreover, co-cultivation induced depolarization of  $\Delta \Psi m$  leading to an approximately twofold JC1 monomers increasing in MM-BMSC and MM cells (**Figure 5D**). Co-cultivation of KMS12-PE and MM-BMSC reduced the amount of ROS in both cell systems (**Figure 5E**).

To further elucidate the involvement of SIRT3 in metabolic and senescence-like alterations of MM-BMMSCs, siRNA was used to transiently "knockdown" this gene in HD-BMMSC.



**Figure 6.** Influence of SIRT3 on ROS in HD-BMMSC. *P* values: \* <0.05; \*\* <0.01; \*\*\* <0.001; and \*\*\*\* <0.0001. (A) The knockdown of SIRT3 in HD-BMMSC caused an increase in the ROS content of all four siRNAs tested compared to the negative and transfection control. (B) Influence of SIRT3 on  $\Delta\Psi$ m in HD-BMMSC. The "knockdown" of SIRT3 in HD-BMMSC caused a reduction in the FL-2/FL-1 ratio. For siRNAs 2 and 3, only the proportion of FL-1 negative cells was reduced (R-4), whereas siRNAs 4 and 5 also caused an increase in FL-2 negative cells (R-3). (C) Influence of SIRT3 on cell cycle in HD-BMMSC. The "knockdown" of SIRT3 in HD-BMMSCs led to an accumulation of cells in S phase of the cell cycle (siRNAs 4 and 5). siRNAs 2 and 3 produced effects of the same tendency, but these were very low (<5%). (D) Influence of SIRT3 on senescence-associated  $\beta$ -galactosidase activity HD-BMMSC. Transfection of HD-BMMSCs with siRNAs 4 and 5 produced an increase in SA $\beta$ GalA. In contrast, no significant effects were observed for siRNA 2 and siRNA.

Subsequently, the ROS amount, mitochondrial membrane potential, cell cycle, and SA $\beta$ GalA of the cells were investigated. Two different HD-BMMSCs were used for these analyses, and from each study, 2–3 replicates were performed. The donors were 73 and 74 years old. Furthermore, four different siRNAs against SIRT3 were used. SIRT3 knockdown in HD-BMMSC induced 1.4-to 1.9-fold increase in ROS levels (p < 0.05; **Figure 6A**). This was associated with dissipation of  $\Delta\Psi$ M between 1.4- and 1.8-fold depending on the siRNA that was used for transient knockdown of SIRT3 (p < 0.04; **Figure 6B**). Furthermore, the inhibition of SIRT3 mimicked cell cycle arrest in S phase previously reported in BMMSC of myeloma patients. The percentage of BMMSC in S phase increased upon SIRT3 knockdown between 6.7 and 9.6% (p < 0.039; **Figure 6C**). In addition, it was investigated whether the depletion of SIRT3 increases senescence-associated  $\beta$ -galactosidase activity. It was found that transfection of HD-BMMSC with SIRT3 siRNAs 4 and 5 resulted in an approximately 1.5-fold increase in SA $\beta$ GalA (p < 0.03). In contrast, HD-BMMSCs transfected with siRNA 2 did not show any changes. Similarly, transfections with siRNA 3 caused only minimal changes in HD-BMMSCs (**Figure 6D**).

## 4. Discussion

MM-BMMSCs play a critical role in MM tumor growth and survival. Several studies suggest the existence of constitutive abnormalities in MM-BMMSC, and these lead to abnormal cell characteristics and increased tumor support [5, 6, 9, 23, 53, 54]. In this study, we explored the cellular and genetic aberrations of MM-BMMSCs in order to further identify the molecular mechanisms for these changes.

The enhanced and early senescence of BMMSC has been previously reported for different hematological disorders, including MM [9, 55]. Here, a significant higher senescence level of MM-BMMSC compared to HD-BMMSC was identified. When combined with our qPCR results that revealed an enrichment of cyclin D1 mRNA and the reduced expression of cyclin E1, an arrest of the cell cycle in G<sub>1</sub> phase can be assumed. In contrast, André et al. related senescence to an accumulation of MM-BMMSCs in S phase [9]. These contrasting results could be due to diverse patient samples as well as different cell isolation and culture treatment methods. However, early senescence indicates the impairment of MM-BMMSCs. With regard to the relapsed analysis group, therapy might lead to increased cellular stress for MM-BMMSCs resulting in higher senescence levels.

Distinct changes to gene expression profiles were also reported [24, 56–59]. In addition to the abovementioned changes in cyclin expression, an upregulation of the cell adhesion molecules VCAM-1 and ICAM-1, as well as the NF- $\kappa$ B member IKK- $\alpha$ , was found, consistent with previous studies [6, 9, 53]. Overexpression of the cell adhesion molecules and the NF- $\kappa$ B pathway without MM-BMMSC in contact with MM cells suggests the generation of a constitutive myeloma favorable microenvironment.

In contrast to the above studies, data relating to microRNA expression in MM-BMMSCs are limited. Here, overexpression of miR-16, miR-223, miR-485-5p, and miR-519d was identified. These microRNAs possibly influence cell cycle regulation, cell differentiation, and cell migration. Alterations to MM-BMMSCs could therefore result from the specific deregulation of microRNA expression and their corresponding downstream targets [15, 52, 60–66]. The relapsed analysis group displayed a higher senescence level and a strongly increased microRNA expression (mean fold change > 100), supporting their possible function as cell cycle modifiers. Therapy seems to enforce senescence in MM-BMMSCs due to higher cellular stress and could lead to an even more altered cellular phenotype at relapse.

Overexpressed miR-485-5p and miR-519d are associated with two imprinted clusters on chromosomes 14 (DLK1-DIO3) and 19 (C19MC), respectively. Since both clusters exhibit a complex composition, including tumor-suppressive as well as tumor-promoting microR-NAs, changes to their epigenetic regulation could account for important changes to the cellular characteristics of MM-BMMSCs [21, 66]. Here, analysis revealed hypomethylation and amplification of both clusters, possibly resulting in a higher transcriptional rate of cluster-associated genes. Several studies have reported the accumulation of genomic and global methylation changes due to in vitro cultivation of BMMSCs [67–72]. Indeed, minimal changes in the HD-BMMSC population, for example, hypo- and hypermethylation, as well as CN values between 2.2 and 2.8, were found. However, these alterations were less than those found in MM-BMMSCs, with distinct clustering of MM-BMMSC values below 20% methylation level and a mean value of more than 3.5 copies of the DLK1-DIO3 and C19MC genomic regions. The detected aberrations could be due to the existence of a CAF population in the MM-BMMSCs because some data highlight the presence of DNA hypomethylation and genetic instability in CAFs [24, 56, 73]. However, genetic instability in CAFs is controversial [74]. Hence, it cannot be excluded that CN variations of DLK1-DIO3 and C19MC result from hypomethylation or vice versa.

Moreover, the effect of MM cells on previously identified gene expression variations was investigated. In this context, a proliferation stimulating influence of KMS12-PE myeloma cells on MM-BMMSCs was apparent. Thus, KMS12-PE cells appear to repress MM-BMMSC senescence entry and increase the cell vitality. This modification could be associated with an increase in cyclin E1 mRNA levels.

Lastly, we investigated whether metabolic changes in MM-BMMSC could be responsible for the early aging status of the cells. For this purpose, we analyzed the expression of the gene and protein of the metabolic molecules SIRT3 and UCP2 and the lactate transporter MCT1 and MCT4. There were no significant differences in the gene expression of MCT1, MCT4, and UCP2 in MM-BMMSC compared to HD-BMMSC. In contrast, a significant lower expression of SIRT3 and a significant increase in mitochondrial mass compared were detected in MM-BMMSC. Interesting, no changes were detected in MGUS-BMMSC, suggesting an association with disease progression.

Our results suggested that MM cells influence the mitochondrial function of MM-BMMSC. This interaction leads to decrease the ROS levels in both cell types and could support their survival and growth. Moreover, the sustained induction of mitochondrial stress response could be the reason for premature senescence in MM-BMMSC. Therefore, the result of MM therapy could be improved through the disabling of metabolic interactions between MM cells and MM-BMMSC.

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