Author's response to reviews

Title: Multiple myeloma cells alter the senescence phenotype of bone marrow mesenchymal stromal cells under participation of the DLK1-DIO3 genomic region

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Author's response to reviews: see over
Response to the concerns of the reviewers and editorial office

We are accepting the critique of the reviewers. We have revised the manuscript and modified specific points in accordance with the reviewer’s helpful criticism. All changes in the manuscript are marked yellow.

Replies to specific points of reviewer 1

1) As the authors manifest, “MM-BMMSCs play a critical role in MM tumor growth and survival”. Therefore, it would be interesting to test the effect of MSCs upon KMS12-PE multiple myeloma cell line. In my opinion, this is the real proof-of-concept to be tested, rather than the influence of MM cells on BMMSCs. For instance, the miRNAs analyzed were chosen because they are deregulated in MM cells. Then, why not to look at the expression of these miRNAs in the MM cell line co-cultured with MM-BMMSCs of different origin? This is an experiment not so difficult to perform, as RNA preps could be easily done from KMS12-PE cells. Likewise, expression of cyclins, p16, p21, etc...could also be tested.

It is well known that interaction between MM cells and BMMSCs is pivotal for the survival and proliferation of MM cells. Differently to other blood cancers as leukemia MM cells have an increased dependence on their BM tumor niche. A lot of studies focus on the effects that are induced in MM cells upon interaction with MM-BMMSCs. However, there were also reports that MM-BMMSCs are constitutively altered thereby providing a better platform for the progression and relapse of MM disease. Because different therapeutics target MM cells by the inhibition of their interaction with MM-BMMSCs it is important to clarify constitutive changes that are present or induced in stromal cells. Recently different studies have reported the premature senescence-like state of MM-BMMSCs. But it is unclear whether this characteristic is an attendant phenomenon for example through increased activation by MM cells during disease progression or could be also important for the interaction between MM cells and stromal cells. Our data indicates that this cell characteristic is more an additional effect and not a specific tumor supporting effect induced by MM cells, because the senescence-like phenotype is reduced by interaction with MM cells. It seems that MM cells benefit more from a proliferative and vital state of MM-BMMSCs and thus increase the viability of the stromal cell population.

For more clarity regarding the experimental design of the project we added following parts:

“Furthermore it is not clear whether alterations of MM-BMMSCs are important for the interaction between stromal cells and MM cells or are more an attendant phenomenon.”
(Line 104-106)

“In this context we wanted to analyze whether the premature senescence status of MM-BMMSCs could be a specific effect induced by MM cells for increased tumor support.”
(Line 120-22)

“Furthermore, the premature senescence phenotype of MM-BMMSCs seems to be an attendant phenomenon rather than a specific tumor supportive stromal cell characteristic.
The reduction of the senescence phenotype by MM cells indicates that interaction of MM cells and MM-BMMSCs does not rely on this cell characteristic. It is likely that MM cells benefit from a more vital cellular state of MM-BMMSCs. Therefore, the premature senescence phenotype of MM-BMMSCs could be a side effect possibly induced through increased activation of stromal cells by MM cells. However, further analysis is needed to determine whether the senescence-associated constitutive changes of MM-BMMSCs could support relapse of MM disease.” (Line 410-418)

Of course analysis of microRNA gene expression changes in KMS12-PE myeloma cells is also interesting. Therefore, we performed the suggested analysis for all the previously measured microRNAs. The results are shown in Figure 4.B. In addition, following parts were added to the manuscript:

“Co-cultured KMS12-PE myeloma cells were pelleted and resuspended in TRIzol for downstream analysis.” (Line 153-154)

“Interestingly, cell-cell-interaction also altered microRNA expression of KMS12-PE myeloma cells. MiR-221 increased 13.78-fold whereas miR-223 and miR-519d were 2.72-2.85-fold reduced in co-cultured KMS12-PE myeloma cells (p<0.02, Figure 4.B). Expression of miR-485-5p was not detectable in KMS12-PE myeloma cells.” (Line 311-315)

“Interaction of KMS12-PE myeloma cells with MM-BMMSCs also induced microRNA changes in the MM cell fraction. Cell-cell-interaction strongly increased the level of miR-221 in KMS12-PE myeloma cells. This microRNA acts as an oncogene by downregulation of different cell cycle inhibitors leading to an increased tumor cell proliferation [50]. In addition, miR-223 and miR-519d were downregulated in co-cultured KMS12-PE myeloma cells. As previously mentioned these microRNAs could have anti-proliferative effects on cells [21, 31, 45, 46]. Therefore, interaction with MM-BMMSCs seems to shift microRNA levels of KMS12-PE myeloma cells towards a more pro-proliferative gene expression pattern.” (Line 384-392)

“(B) Cell interaction with MM-BMMSCs 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.” (Line 653-656)

Analysis of p16, p21, cyclin D1 and cyclin E1 was performed in the context of senescence of MM-BMMSCs. Because this cellular state is not present in a myeloma cell line analysis of previously mentioned genes in KMS12-PE myeloma cells would not provide any further helpful information.

2) In addition, “the analysis of senescence in MM-BMMSCs displayed similar results as found by others (line 327)”’. This deprives of novelty to the initial characterization performed in this work.

This sentence was removed from the manuscript.
3) Co-culturing experiments: SA-betaGal essay must be done with another cell type (Hela?, fibroblasts? Or even better, B or plasma cells) as controls. Moreover, which is the effect upon HD-BMMSCs? These experiments are important in order to assess the specificity of the effect.

Additional measurements of SA-betaGal were performed with n=3 HD-BMMSCs co-cultured with KMS12-PE myeloma cells and n=3 cultures of HS-5 stromal cell line with KMS12-PE myeloma cells. The results were added to Figure 3A.i. Furthermore following parts were added to the manuscript:

“Co-cultures of HD-BMMSCs (n=3) and HS-5 stromal cells (n=3, CRL-11882) were used as controls.” (Line 164-165)

“No effect on the activity of SA-β Gal was observed in HD-BMMSCs and the HS-5 cell line co-cultured with KMS12-PE myeloma cells.” (Line 286-287)

“No changes were observed for co-cultured HD-BMMSCs (n=3) and HS-5 cells (n=3).” (Line 636-637)

4) As seen in Figure 1, senescence of Relapsed-MM-BMMSCs is higher than that of newly diagnosed. This point should be discussed.

Discussion of this point was added in following parts:

“R-MM-BMMSCs had a 1.4-fold increased SA-βGalA as compared to ND-MM-BMMSCs.” (Line 235-236)

“R-MM-BMMSCs showed a higher SA-βGalA compared to ND-MM-BMMSCs. Therefore, it seems that relapse of MM could increase the premature senescence phenotype of MM-BMMSCs.” (Line 345-347)

5) As gene and miRNA expression data are too disperse, at least Cyclins, p16 and p21 could be tested at protein level (western or IH). Histograms of cell cycle are not informative. The same is true for the co-cultures/transwell experiments.

It seems that because of the big cell size of BMMSCs the amount of total protein which can be extracted is very low. We were only able to receive a concentration between 300-200 µg/ml from a medium cell culture flask with 80% confluence. This would mean a concentration of only 4-6 µg protein for SDS-Page which is not nearly enough to perform western blots. Therefore, we would need a lot of primary material to perform western blotting. In addition, quantitative protein analysis is difficult with IH. Because the reviewers are right with the criticism regarding the post-transcriptional regulation we performed the cell cycle analysis to compensate the missing analysis of protein levels. We found a significant change of the cell cycle characteristics that was correlated with the changes in mRNA levels accounting for a possible functional downstream effect of these observed changes. We are planning to perform western blots in future investigations. We will therefore collect high amounts of primary material, but this will take us a longer period of time.
6) What is the purity of isolated BMMSCs? It would be useful to have this information.

The purity of isolated BMMSCs was specified in the methodology part under 'Isolation and cultivation of BMMSCs':

"Purity of isolated BMMSCs ranged from 94% to 99.5% (see Additional File 2, Figure S1: Purity of isolated BMMSCs)." (Line 144-145)

We have also provided some representative FACS measurements in Additional File 2, Figure S1.

7) BMMSCs can be removed from axis labels, as it is common to all groups.

Because we now also included gene expression analysis of KMS12-PE myeloma cells we think that it is more comprehensible to specify the cell compartment that was analyzed.

8) Some data are difficult to believe, for instance, the statistical significance in figure 4B (methylation status of co-cultured MSCs) should be revisited by a statistician.

You can find the values of the qMSP analysis in the table below. The statistic was analyzed with GraphPad Prism 6.0 using Wilcoxon-signed rank test. The received p-value was 0.0039.

<table>
<thead>
<tr>
<th>MM-BMMSCs</th>
<th>co-cultured MM-BMMSCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>11,705150</td>
<td>11,811970</td>
</tr>
<tr>
<td>0,183131</td>
<td>4,864091</td>
</tr>
<tr>
<td>7,145256</td>
<td>8,440101</td>
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<tr>
<td>24,654070</td>
<td>29,745690</td>
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<tr>
<td>6,030772</td>
<td>6,544557</td>
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<tr>
<td>9,242729</td>
<td>10,307260</td>
</tr>
<tr>
<td>7,387266</td>
<td>8,238097</td>
</tr>
<tr>
<td>12,212040</td>
<td>37,104400</td>
</tr>
<tr>
<td>10,818940</td>
<td>11,705150</td>
</tr>
</tbody>
</table>

9) Experiments with mimics and inhibitors of miR-485 are not informative.

Indeed the data received with miR-485-5p mimics and inhibitors is partly controversial. However, it shows that miR-485 influences the cell cycle characteristics and senescence of MM-BMMSCs and therefore provides a functional assay for the examination of the data measured in MM-BMMSCs before. In addition, the experiments showed that manipulation of miR-485-5p alone is not able to mimic the senescence-reducing effect that is induced by KMS12-PE myeloma cells and thus it provides further information regarding the role of DLK1-DIO3 for senescence.

Replies to specific points of reviewer 2

1) The levels of cyclin E1, cyclin D1, p16 and p21 (figs 1 and 3) have to be showed by western blot. Measuring just the mRNA levels does not account for post-transcriptional regulation that potentially could lead to different protein levels.

Please refer to point 5 of reviewer 1.