Author’s response to reviews

Title: The use of Matrigel has no influence on tumor development or PET imaging in FaDu human head and neck cancer xenografts

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Author’s response to reviews:

Reviewers pointed out a number of points to improve manuscript, and we have evaluated these to improve the manuscript. Below are inserted comments, for each point given by reviewers, to address and evaluate the relevance for the manuscript.

Reviewer #1: The authors compare tumor growth, proliferation, and angiogenesis in a xenograft model. The FaDu cells that they used had a high take rate so the comparisons of +MG and -MG parameters were not affected by initial take rates. They showed very few differences between +MG and -MG measurements. Strengths include the quality of the data and data analysis.

Major weakness: Only one cell line was assessed, so the general applicability of these results cannot be assessed. The authors themselves state this. Analysis of a second, unrelated cell line would suggest whether or not their results are cell type dependent. The title, "The use of Matrigel has no influence on tumor development or PET imaging in human head and neck cancer xenografts" suggests that the results presented indicate the absence of an effect for all head and neck xenografts, not just the FaDu cells.

We accept the limitations of the study. However, we find the study relevant and the consideration and questions it addresses relevant. The aim of this study was to investigate the FaDu cell line in regards to the effects of matrigel on relevant growth and imaging characteristics.
Other title suggestions to clarify the aim of study:

"The use of Matrigel has no influence on tumor development or PET imaging in FaDu human head and neck cancer xenografts"

"The use of Matrigel has no influence on tumor development or PET imaging in a human head and neck cancer xenograft"

Minor issues:

1) This paper need to be edited extensively for English grammar and usage.

2) Figures were not discussed in the Results section adequately. Some were not even mentioned or only 1 panel was discussed. When results are dis-cussed in the Results and Conclusion section the figure number and panel letter should be indicated.

Only the unforeseen results and significant results are mentioned in the dis-cussion, but all results are shown in figures. Sections have been rewritten to make sure that reference to figure exists throughout section.

3) Did the +MG and -MG tumors in sub study II reach the sizes indicated at similar times? The results section indicates that the +MG group grew faster since these mice were sacrificed before Day 22. Did the -MG mice survive to Day 22? How many mice/group were sacrificed per time point in sub study II since there were only 10 mice/group to start with. Is each tumor or mouse considered a separate point?

Tumors in sub study II were collected over 14 days, where a tendency of the +MG group to be collected first due to slightly faster growth. The tumors were collected in order to create similar population number in each group, and no predetermined time points were set. Every tumor was considered a separate point, and tumors originating from the same mouse could be sepa-rated and allocated to two different groups according to size at time of sac-rifice. 10 mice in each group resulted in 20 data points for each group (a to-tal of 40 data points for Sub Study II). All mice were sacrificed on Day 22 in Sub Study I, but in Sub Study II the last mouse was euthanized on day 26 post implant. Allocation procedure does not allow for a valid statistical analysis of the collection time in Sub Study II, and this is therefore not in-cluded.
In Sub Study I tumors on mice in +MG group exceeded the permitted tumor size, in the predetermined humane endpoints, on day 19, and were sacrificed. For the –MG group mice were scanned and monitored on day 22 as the latest and sacrificed. Data from day 22 is not included in figures because no comparison could be made between groups. Since results from day 22 is not included in manuscript, description will only cover methods and data until day 19 in Sub study I.

4) Figure 5. The graphs should be consistent. The bars for each size group should have the same pattern throughout the figure. Labels on axes were too small to read easily.

Changes made in Figure:

1. +MG groups are now marked as blue bars, consistent with the form of the graph of growth and tumor uptake.

2. Every boxplot for each groups are now consistent in all figures.

3. Axes titles are enlarged.

5) Figure 6. The white balance (background) is different between the 4 IHC panels making it very difficult to see staining, especially in panels A and B.

Panel A and Panel B is the same staining image, but in panel B a filter is applied by the CAIMAN online tool in order to quantify vessel staining, the original background is hereby the same. On reproduced figure arrows now indicate DAB staining of vessels and the corresponding detection by the CAIMAN segmentation in Panel B. The figure legend is rewritten to clarify this.

In panel C and D these are also the same IHC image of Ki-67 staining. Comparison of Panel A and C is hereby not necessary in relation to staining, since these are not comparable.

6) Does tumor density inversely correlate with tumor size?
No correlation to be found in either of the groups (Pearson correlation test-ed).

-MG:

Number of XY Pairs  14
Pearson r       0,01958
95% confidence interval     -0.5165 to 0.5446
P value (two-tailed)       0,9470
P value summary           ns
Is the correlation significant? (alpha=0.05)  No
R squared                0,0003832

+MG:

Number of XY Pairs  19
Pearson r       0,3367
95% confidence interval     -0.1389 to 0.6860
P value (two-tailed)       0,1587
P value summary           ns
Is the correlation significant? (alpha=0.05)  No
R squared                0,1133

Does cell density correlate with tumor volume better in -MG than +MG?

The results above show a better correlation for the +MG group, but no accepted correlation found.

How does proliferative index compare with cell density?
-MG

Number of XY Pairs  14
Pearson r     -0.1391
95% confidence interval   -0.6237 to 0.4228
P value (two-tailed)   0.6354
P value summary       ns
Is the correlation significant? (alpha=0.05)   No
R squared     0.01934

+MG

Number of XY Pairs  19
Pearson r     0.01904
95% confidence interval   -0.4391 to 0.4693
P value (two-tailed)   0.9383
P value summary       ns
Is the correlation significant? (alpha=0.05)   No
R squared     0.0003626

No true tendency or correlation found here either.

Further statistical comparisons between these parameters could support the authors' speculations about the reasons for apparent discrepancies between tumor proliferation and cell density.

Conclusions. The questions raised in the manuscript about variability and apparent conflicts could have been addressed by having more 'n' in the sub-study 2 group. Further analyses of
correlations between parameters also might answer some of these questions. More cell lines would also determine the applicability of these results to other xenograft studies. The authors suggest that there is more variability in the +MG data. Can this be difference in variability be evaluated statistically?

The hypothesis of the study was that Matrixgel could be excluded from a cost-benefit perspective in the xenograft model using human FaDu cells for establishment, and the study is designed to evaluate this overall hypothesis. The number of animals included in study and the chosen characteristics to be detected was selected to reject the overall hypothesis and based on power calculations.

Minor hypotheses set before study included;

Growth delay for the –MG group is present at initially but is equalized in time.

No differences in tracer uptake (18F-FDG) between the two study groups in Sub study I.

No significant differences in tumor characteristics between groups including cell density, vessel formation, and proliferation at different size points.

The outcome of the study answers the questions raised beforehand, but, as many other studies, also caused further questions to be raised. We are under the impression that the written conclusion can be stated from the evaluated data, but if further questions should be answered in relation to correlations between tumor characteristics, a new study with new hypotheses should be designed. Differences in variability were found between groups in study and the design did not implement a further analysis of these group differences. Characteristics included in study were evaluated to be informative for the tumor development on level needed for present study, but cannot be used for further definite conclusions without restrictions.

Reviewer #2: Review:

The authors present an in-depth analysis of effect of Matrigel basement membrane matrix on the development, growth and metabolism of FaDu cancer xenografts in NMRI nude mice. Tumor size and uptake of 18FDG PET tracer were assessed at multiple times for 3 weeks after subcutaneous implantation with and without Matrigel. In addition, immunohistochemical staining was performed with similar timings to evaluate vascularization, hypoxia, proliferation rate and necrosis during tumor development. The authors conclude that Matrigel has no beneficial effect in this tumor model. Importantly, vascularization is reduced in matrigel implants relative to cells implanted without Matrigel. The results are interesting. The authors are careful to point out that these findings may not hold true for other xenograft models.
The report is well-organized, though will benefit from grammatical proof-reading. Other minor concerns should be addressed before publication.

Methods:

1) Clarify that the same number of cells were implanted for -MG and +MG groups. What was the total volume injected for each group?

Was described in article, but have been rewritten to be expressed even clearer. Is both described in the method section, but also discussed in the discussion in relation to the initial results. +MG group had a volume of 100 µL injected subcutaneously and the –MG group only 50 µL, but the number of cells was the same for the two groups (2.5 x 106 cells).

2) Clarify that tumor size and mouse weight was measured starting day 5 post-implant.

Is now corrected in method section.

3) In figure six, Panel B does not look significantly different from Panel A. Perhaps this image can be made more visually distinct as it is result of segmentation of A.

The filter is made by the CAIMAN tool. Description in the figure legend is corrected in order to more clearly state the difference/correlation between the different panels. Furthermore, arrows have been added in figure to point out the positive staining of vessels and the marking of these by the segmentation tool.

Both reviewers asked for proof-reading of manuscript, which has been made.

Furthermore a completed ARRIVE checklist has been uploaded in accordance to editorial policies.