Author’s response to reviews

Title: High-throughput RNA sequencing from paired lesional- and non-lesional skin reveals major alterations in the psoriasis circRNAome

Authors:

Liviu Moldovan (liviu.mldvn@inano.au.dk)

Lasse Kristensen (lasse@mbg.au.dk)

Thomas Birkballe Hansen (tbh@mbg.au.dk)

Morten Trillingsgaard Venø (mtv@inano.au.dk)

Trine Hauge Okholm (trine.okholm@clin.au.dk)

Thomas Levin Andersen (thomas.levin.andersen@rsyd.dk)

Henrik Hager (henrikhager@mac.com)

Lars Iversen (lars.iversen@clin.au.dk)

Claus Johansen (claus.johansen@clin.au.dk)

Jørgen Kjems (jk@mbg.au.dk)

Version: 2 Date: 31 Oct 2019

Author’s response to reviews:

None of the two reviewers requested any additional changes and both stated that their concerns had been satisfactorily addressed. Please see below:

Reviewer reports:

Yuanjie Su (Reviewer 1): I am satisfied with their response to my prior concern so I have no further questions.

Johann Shane Tian (Reviewer 2): Dear Authors,
I have read the changes and it has improved significantly. Please find my responses, point-by-point, to the changes.
Introduction:

On page 4, the authors considered only two means of circRNA biogenesis - via RNA binding proteins as an active approach, and inverted Alu elements as a passive approach. However, there are other means of circRNA biogenesis listed in the literature including canonical pathways of spliceosomes. In fact, there have been a more recent publication where ciRS-7 is not generated via Alu repeats but mammalian-wide interspersed repeats (MiRs; Yoshimoto et al. (2018)). Unless the authors have quantified/cited the majority of circRNAs' biogenesis to be influenced by RNA binding proteins and Alu repeats, the work would only cover a subset of circRNAs understood/studied thus far.

Our response: We stated the following in introduction: "Backsplicing is facilitated by the presence of homologous inverted repeats (e.g. inverted Alu elements (IAE)) in the regions flanking the involved exons." The MiRs, in the case of ciRS-7, are also homologous inverted repeats so the statement is correct. However, we have now added a reference to the mentioned article in the introduction of the revised version of our manuscript. We do not understand reviewer's comment about canonical pathways of spliceosomes should be different. Both the protein mediated- and inverted repeat mediated mechanism are believed to be facilitated by the spliceosome.

Review's response: Understood and thank you for the additional information. As to the second comment on "canonical pathways of spliceosomes", some information was mis-interpreted on my end while inferring from other articles. You are right that our current understanding of circRNA biogenesis (by repeats) are facilitated by spliceosomes, and have not been proven otherwise. For that, I apologize and would like to retract this statement.

On page 5, it is understandable that ciRS-7 (aka CD1as) is highly cited from Jeck et al. (2013), and it is very tempting to use the same approach. However, it is also arguable that there are more examples of miRNA sponges (from circRNAs) published in the literature. Maybe it would be more enticing if those examples were supplemented or used instead.

Our response: Please note that we have cited several other articles presenting circRNAs that function as miR sponges2-5. However, we have now added a few more6, 7 to that list. The reason we put emphasis on ciRS-7 is that it is undisputedly the best example in the existing literature of a circRNA that functions as a miR sponge, both when considering number of miR binding sites present in the sequence8 and when considering wet-lab data2, 4, 9, 10. Moreover, we selected this circRNA for further analyses in the present study (e.g. in situ analyses and NanoString nCounter analyses) making it logic to introduce in a bit more detail.

In addition, we have now deleted to part explaining basic knowledge about microRNAs and their functions to make the introduction more concise and added two more references11, 12 in the revised version of our manuscript.

Review's response: Thank you for considering the suggestions.
Methods:

On page 6, there are two cohorts that when analysed, were observed to be non-parametric. Therefore, non-parametric statistical analysis were used throughout the project. However, if the condition of psoriasis vulgaris was to be fairly common presentation in most patients, it may not be experimentally wrong to combine the two cohorts and re-test them for normality. Unfortunately, the authors did not indicate whether they have tested with a combined cohort. And even if the data is still not subjected to normality, it is not wrong (experimentally) to combine the two cohorts to form a larger sample size.

Our response: Throughout the result section, we have indicated within which cohorts given tests were performed. However, we agree that this information was less clear in the method section. Therefore, this section has now been updated so that it includes this information for the relevant subsections. Since RNA-seq was only performed on the first cohort, we think it is better to perform the analyses separately and prefer not to combine the two cohorts.

In addition, to make the method section more concise, we have collapsed the subsections concerned with the NanoString nCounter custom CodeSet in the revised version of our manuscript.

Review's response: Thank you for explanation. I understand it better now and agree with the authors.

From my understanding, the authors are trying to show that even within the same patient, there is a dramatic change in circRNAome between lesion and non-lesion cells. However, the data represented as expressions (throughout the article) do not seem to be very much different between conditions despite being statistically significant. Yet, non-parametric statistics may not be as powerful as parametric tests, and may be present the data as such.

Our response: We strongly disagree that the circRNA data do not seem to be very much different between conditions despite being statistically significant. In fact, the vast majority of circRNAs are downregulated more than two fold in the lesional skin compared to the non-lesional skin. On the contrary, only a handful of circRNAs were upregulated (Figure 2C). We used a non-parametric test to assess differences in the overall circRNA expression levels between lesional and non-lesional skin (Figure 2B). In spite the fact that this test may not be as powerful as a parametric test, the p-value was still less than 0.0001. Because the data were not following a Gaussian distribution, we believe that a non-parametric test is more appropriate.

Review's response: Thank you for explanation. The authors did make it clear to me that a non-parametric test is more appropriate.

Another question in mind is that psoriasis skin lesions (of patients) may recur in similar locations, but have little evidence of it not influencing surround cells. The authors then considered extracting samples 5cm apart from each punch. However, it is also arguable that patients apply medication (for psoriasis vulgaris) within the proximity. There is more reason to doubt the condition of non-lesion cells, and using them as controls. Being said, would it be more justifiable to compare the lesion cells with non-lesion cells of healthy participants? Because on
an experimental and medical point of view, the cells from healthy participants would be completely exclusive to the diseased patient, and a benchmark for treatment to be developed. If the authors want to justify that the non-lesion cells 5cm away from lesion cells are considered appropriate controls, then they should consider comparing with cells from healthy participants to rule out the doubt at least.

Our response: The rationale behind this study was to characterize the psoriatic plaque and compare it to the patient's non-lesional skin. We agree that psoriasis can be considered a systemic inflammatory condition, with a well-characterized cytokine profile expression. However, psoriasis is characterized by well-demarcated lesions in the skin that clearly separate lesional psoriatic skin from non-lesional skin. Therefore, it is also generally accepted that lesional and non-lesional psoriasis skin can be separated and compared (PLoS One 2012, 7(9):e44274, exp Dermatol 2017, 26(11) 1075-1082, plus many more). Furthermore, by comparing lesional and non-lesional skin from the same patient also eliminates inter-individual differences. However, in fact we did also perform analyses on skin samples from healthy participants (Supplementary Figure 3B).

None of the participants had used any systemically immunosuppressive medications for four weeks prior to the time of the biopsies and none had received local treatment at the site of biopsies for two weeks before the biopsies were taken. This is common practice in clinical studies involving diseased psoriatic skin.

Review's response: Thank you for explanation. I understand it better now and agree with the authors.

On page 7, it is fairly common for researchers to apply rRNA depletion (only) prior to RNA sequencing. With sufficient depth (and not stated in the article), the reads are able to pick up a large number of circRNAs after analysis. Moreover, researchers can still use the same data to analyse mRNA expression. But at the same time, it has been reported in the literature that one of the best circRNA identifiers - MapSplice - was only able to identify roughly 60% - 70% of circRNAs as true positives. To then improve the technique, RNase R treatment was added into the equation. Considering that the authors are intending to profile the circRNAome of the diseased cells (to control), then it may be suggestive to analyse the data more thoroughly, with and without RNase R treatment (on top of rRNA depletion); mRNA expression can be analysed with rRNA depleted only samples.

Our response: We agree with the reviewer that RNA sequencing is able to pick up a large number of circRNAs with sufficient depth. Indeed, we detected 2,066 and 2,626 unique circRNAs supported by at least two backsplicing junction-spanning reads in a single sample in the lesional- and non-lesional skin biopsies, respectively (see the result section, page 12). In the revised version of our manuscript, we have now indicated the sequencing depth for the samples in the methods section. Taking into account that we analyzed only the most abundant circRNAs and normalized using the total number of reads (RPM values); we believe that differences in sequencing depth among the samples have not affected the results. We decided not to perform RNase R treatment of the samples before RNA sequencing, as this treatment precludes analyses of circular-to-linear ratios and the analyses of other linear RNA species. In addition, not all circRNAs are resistant to RNase R treatment while some linear
RNAs are resistant due to internal double stranded structures. Moreover, we used two independent bioinformatics algorithms to detect circRNAs and manually inspected circRNA candidates not detected by CIRCexplorer to exclude mapping artefacts as previously described.

Review's response: The authors stated that the RNA-seq data were between 15 million and 66 million, with a median of about 30 million. I lack the experience to tell whether this is sufficient for circRNA analysis (from total RNA), it may be better to have considered much higher number of reads (~ 80 - 100 million) to increase the number of circRNAs captured. This may then explain why there were a smaller (on personal perspective) number of circRNAs detected by the authors. Nonetheless, there has yet to be a gold standard in establishing RNA-seq analysis (for circRNA), and they have established a list of validated candidates. Therefore, I agree with the authors in their response.

On page 7 and 8, RNA-seq data were analysed by applying find_circ and subsequently CIRCexplorer. I also recognise that this similar methodology was used in the last author's previous publication(s). In which, its methodology must have been accepted in academics. This is acceptable because there is no restriction in using any identification tool. On the contrary, it was then mentioned in the discussion that CIRI have low accuracy. Likewise, in the same article (Hansen et al. (2016)), it was also stated that find_circ, too, have low accuracy. In comparison, there was at least one recent article (Gao & Zhao, 2018) that indicated otherwise for CIRI; the number of true positives and percentage precision are comparable to both find_circ and CIRCexplorer. It is suggestive that find_circ (and subsequently CIRCexplorer) would have an under-estimated presentation of circRNAs identified.

Our response: We agree that find_circ has a low accuracy according to our previous publications. This is why we modified the algorithm to be much more stringent. Indeed, we find a much better correlation between our stringent version of find_circ and CIRCexplorer compared to the original version of find_circ (data not shown). We also agree that this added stringency may lead to an under-estimation of the number of circRNAs in our samples. However, as we observe a large overlap between the circRNAs detected by the two independent algorithms (see the result section, page 12), we do not believe that we severely under-estimate the number (please keep in mind that CIRCexplorer only detects circRNAs from previously annotated splice-sites and, therefore, for instance does not detect ciRS-7).

Review's response: Thank you for explanation. I agree with the authors.

To add on, in previous editions of circRNA identifiers, developers presented circRNA-linear mRNA ratios in their output as mentioned by the authors. However, this is questionable as they only considered counts as an ordinal measurement when even partial encompassment of read was observed. In more recent literature, researchers move pass this option and either validate them via qRT-PCR or ddPCR (to observe absolute counts). In bioinformatics, tools such as Sailfish-cir aid users to study expression of circRNA more accurately and precisely (Li, et al., 2107) by taking the coverage of the read (on the annotation/gene) into account. In fact, the use of reads per million (RPM) are not normalized (or was not mentioned in the manuscript if it was normalized), and may not be as accurate as measurements such as fragments per kilobase million (FPKM) or reads per kilobase million (RPKM). When comparing between samples, transcripts per million (TPM) would be the most appropriate. Aside
from that, circRNA-to-linear ratios were calculated and compared on a scatter graph with circRNA (RPM) or lesion/non-lesion. In such a manner, some information are unclear. And as mentioned before, comparison between samples with RPM may not be best means of measurement. Because the authors presented the data as such, it may explain why there are a lot more circRNAs showing RPM levels < 1 (supplementary fig 1) for example, and that their analysis mainly show downregulated circRNAs. The information presented in the article is still valid, but the authors may want to consider converting to TPM for better comparison or at least justify the use of RPMs in their data.

Our response: We believe our analysis of circRNA is both timely and performed with great care. While most circRNA experiments use only a single prediction algorithm, we used two to make sure false positives are removed. The suggested Sailfish-cir does indeed look like an interesting algorithm. This algorithm transforms a circular transcript to a pseudo-linear transcript. This must be why the reviewer suggest a benefit of using transcripts per million (TPM); when doing this transformation the circRNA is no longer defined purely by its back-splice junction (bsj), but now has a length, which will vary between circRNAs. Therefore, had we used Sailfish-cir, TPM or RPKM would have been the better choices. However, Sailfish-cir is by no means a gold standard approach in circRNA quantification. Its use seems quite limited in the scientific literature. The currently accepted way of quantifying circRNA is to count the number of bsj spanning reads and use this isolated measurement. Since quantifying by number of bsj reads does not have any length, reads per million (rpm) is an obvious choice for normalization to read depth. Since only using reads that cover the bsj, the RPM values will be relatively small.

Review's response: The authors mentioned that the analysis was based on a count method, and in my opinion, none of the methods stated by us are considered gold standards, yet. However, by providing more information to the analysis, may have provided more avenues to analyse the data differently. Coming back to the article at hand, their main point was to assess the data on whether there is or not a BSJ was detected. Therefore, I agree with the authors.

On page 8, the annotation used (release 71) for mRNA quantification does not seem to be identical to the one for circRNA quantification, or it was not indicated clearly. The authors may want to comment on why this is so.

Our response: The refSeq annotation was used for circRNAs. This was done because the less complex refSeq gene annotation might suit circRNA detection best, ensuring lower false positives due to exotic genes. For mRNA and lncRNA quantification, the more comprehensive Ensembl annotation was chosen to allow a wider field of RNAs to be discovered. This approach is in accordance with Zhao S et al. BMC Genomics (2015)17, which states that "Wu et al. [27] suggested that when conducting research that emphasizes reproducible and robust gene expression estimates, a less complex genome annotation, such as RefGene, might be preferred. When conducting more exploratory research, a more complex genome annotation, such as Ensembl, should be chosen."
Review's response: Thank you for explanation. Although it is known that earlier/different releases may have errors incurred between them, I agree with the authors on the notion of reproducibility.

On page 8, the authors took the top 50 circRNAs filtered from their bioinformatics analysis and designed probes to use in the NanoString nCounter technology. Yet, the authors have not validated whether these circRNAs are true positive since the analysis is not 100%. Moreover, it is also in assumption that each set of coordinates (or BSJ) presented in the output presents only one circRNA isoform. In figure 1c and 1d, the authors are trying to state that a gene (in their list) can form at least 1 circRNA. There are those that can produce 2 or more, and will be represented as two or more sets of coordinates in the output that fall under the same gene. This is also consistent with other circRNA literature. However, this does not tell us much of whether the same BSJ is able to present multiple isoforms. For example, a gene is predicted to form a circRNA with exons 1-5. But the output does not tell us much of whether the circRNA only forms with exons 1 to 5 or with a subset of the exons indicated. Therefore, there may be multiple isoforms of circRNAs presented per set of coordinates. To prove this, researchers should try to flank the BSJ with divergent primers and present it on the gel electrophoresis at least. Consequently, it also related to the part on page 10, where the authors stated that they "extracted the expected sequence of the mature circRNAs assuming splicing pattern similar to the host genes by which their share splice sites".

Our response: The main purpose of the NanoString nCounter experiments was actually to validate the findings of the RNA sequencing experiment to rule out whether the observed downregulation was caused by experimental bias. This technology is enzyme-free and therefore not subject to artefacts related to template switching and rolling circle amplification during reverse-transcription, as well as PCR bias, which are major concerns in the circRNA research field18-21. Indeed, since we were able to detect all these circRNAs using an enzyme-free method, these data strongly indicate that the false-positive rate of our bioinformatics strategy for detection of circRNAs in RNA sequencing data is very low. We agree, that our analyses do not assess the possibility that multi-exonic circRNAs are subject to alternative internal splicing or intron retention events. Analysing a high enough number of circRNAs to add significant information to the study using divergent primers and presenting it using gel electrophoresis would require way too much time given the deadline for this revision. Moreover, we do not have enough RNA left from the samples to perform such analyses. Nevertheless, we agree that this issue presents a potential caveat in our analyses and we have therefore added the following statement to the discussion section: "These analyses are limited by the possibility that a subset of the multi-exonic circRNAs analyzed could be subject to alternative splicing or intron retention events".

Review's response: Thank you for the explanation. I agree with the authors.

On page 11, the authors seem to only analyse inverted Alu elements between circRNA downregulated &gt; two folds. But in their data, there are also some circRNAs that are upregulated. The authors may want to comment on why they would omit those.

Our response: Assessing the distance range to inverted Alu elements has been considered solely in relation to downregulated circRNAs given the well known effect RNA binding proteins such
as ADAR1 and DHX9 have on circRNA downregulation. Other RBPs, such as FUS and QKI are, as mentioned, thought to enhance circularization not through ALU elements, but by binding to specific RNA motifs. In addition, and consistent with the analyses mentioned by the reviewer, we also found that the median distance to the nearest IAE did not differ significantly between circRNAs downregulated more than two-fold and the remaining circRNAs. Together, we consider our analysis of ALU mediated circRNA downregulation to be valid.

Review's response: Thank you for explanation. I agree with the authors.

The analysis for circRNA and mRNA expression was done with unpaired t tests with Welch's corrections. In which, it would be in assumption that the samples' variance are not the same. It was unclear whether the authors have done an F test for that analysis.

Our response: We would like to thank the reviewer for this comment, as we did not do the F test in the original manuscript. Therefore, we reanalyzed all this data (Figure 3A, 3B, 4C, 4D, 4E and 4F) to make sure the correct test was employed for each analyses. We have now specified in the manuscript that the Welch's corrections were only done when the samples' variance were not the same according to an F test. This did not lead to any circRNAs or mRNAs being significant, which were not significant in the original analyses, or vice versa, with the exception of QKI in Figure 3B.

Review's response: Thank you for considering the suggestions.

Results:
On page 12, the authors detected potential novel circRNAs that were not present in circBase. However, circBase's last update was in 2015, which the webmaster has not seem to continuously keep track of it. More updated ones such as NIH circular RNA interactome (https://circinteractome.nia.nih.gov/index.html). It may be better if the authors also make comparison with other circRNA databases (one other at least) in case others were captured.

Our response: None of the circular RNAs we investigated as being potentially novel can be found in the NIH circular RNA interactome. We have now also cited CIRCpedia, which has been recently updated22 and checked that the potentially novel circRNAs could not be found in this database either.

Review's response: Thank you for considering the suggestions.

For figure 2, it was stated that the analysis filtered 298 unique high-abundance circRNAs. It was unclear of why the authors did not want to consider the 170 (non-lesional) and 13 (lesional) mutually exclusive circRNAs. Have the authors considered looking into these?

Our response: We did actually consider those as the 170 (non-lesional) + 13 (lesional) + 115 (common) = 298 (unique high-abundance circRNAs).

Review's response: Thank you for explanation. I understand it better now and agree with the authors.
On page 14, the authors discussed about the influence of circRNA biogenesis from other factors. In this case, they focused on some RNA binding proteins and Alu repeats. Though it was stated that the factors are unlikely to be influencing the biogenesis, it is still questionable as there were factors significantly different in figure 4 and supplementary figure 4. It may be acceptable to know that the circRNA pool is a consequence of the disease, but knowing that the factors listed can influence biogenesis, a significant change in the factors' expression could alter the circRNA landscape then. Therefore, this may need more work to justify the claim.

Our response: In the RNA sequencing data, only very minor changes in these factors were observed; ADAR being the one showing the largest difference in expression between lesional- and non-lesional skin was only about 1.5 fold higher in lesional skin (Figure 3A). This was confirmed in the NanoString nCounter analysis (Figure 3B). Therefore, ADAR was the only factor that warranted further investigation. Since ADAR is known to decrease backsplicing through A to I editing of homologous inverted repeats (mainly inverted Alu elements) in the regions flanking the involved exons, we investigated whether the downregulated circRNAs in lesional skin were more prone to have Alu mediated biogenesis. However, it was very clear from the data that this was not the case (Figure 3C). Though QKI was significantly higher expressed in non-lesional skin it was only about 1.25 fold (Figure 3A). In light of the findings related to ADAR, which is known to influence a much larger number of circRNAs, we decided not to follow up on the findings related to QKI. Surprisingly, we also observed that HNRNPL was differentially expressed in the microdissected samples, but because this was not observed in the RNA-seq data it does not make sense to investigate this factor further using the RNA-seq data (similar to the analyses we did on ADAR). Also, HNRNPL was up-regulated in the lesional skin it is less likely to be responsible for the massive downregulation observed as it mainly promotes circRNA biogenesis.

Review's response: Thank you for explanation. I agree with the authors.

On page 15, the authors presented more concrete data of significantly downregulated circRNAs in lesional skin. This is interesting. Yet, they were not able to extract enough epidermis samples for analysis. Though this is understandable and relatable as an experimentalist, leaves a bigger question of whether the remaining circRNAs analysed portray similar trends. If the authors have a means of getting more samples or at least use cell models to illustrate this, it may improve the power of the claim.

Our response: We agree that the data from the microdissected FFPE tissues are not as good as for other samples. However, because of the microdissections we got much less material for these analyses. Yet the data are clearly showing that the downregulation of circRNAs is mainly occurring in the epidermis, which was also confirmed for ciRS-7 using in situ hybridization.

Review's response: Thank you for explanation. Based on the data presented, I agree with the authors.

On page 17, it was not fully explained on how the authors predicted miRNA binding sites in each of the individual circRNAs. Maybe it could be briefly explained in the methods. It was also observed that the analysis was between lesional and non-lesional skin samples. Yet earlier in the
manuscript, it seems to suggest that epidermis should be the source for analysis. Though it is more logical to use the current RNA-seq data for analysis, it may be more appropriate to extract epidermis for the RNA-seq analysis instead.

Our response: For the miRNAs analyzed, we determined the number of 8mer target sites present in the circRNA sequences. This was stated in the method section. This was done by searching for the reverse complementary sequences of the miRNAs within the circRNAs and adding an A (according to the definition of 8mer sites: An exact match to positions 2-8 of the mature miRNA (the seed + position 8) followed by an 'A')23, 24. We have added this information in the revised version of the manuscript.

Review's response: Thank you for explanation. I understand it better now and agree with the authors.

Figure 7a and 7b showed R2 values very close to zero. This would either mean there is truly no correlation between circRNA and miRNA in psoriasis vulgaris, or that method to compare is not appropriate. To add on, it was also not validated of the number of true miRNA binding sites that the circRNAs possess. Whether there is a means of doing so, it may not be the best means to draw the regression curve as such. Within the article, the amount of RNA used in the NanoString nCounter were dissimilar between circRNA and miRNA analysis. But maybe a better comparison could be done when the counts were normalized (with an appropriate set of controls), and then make comparison between them instead?

Our response: Regarding the different amounts of RNA used for the circRNA and miRNA analysis, as circRNAs are generally expressed at lower levels in comparison to miRNAs, the NanoString nCounter recommends using more RNA for a valid quantification. Nevertheless, the data were normalized and not compared directly. Considering the R2 values in Figure 7A and 7B, it is very clear from those analyses that the loss of circRNAs in lesional skin cannot explain the majority of the observed differences in miRNA expression. To apply maximum stringency to or analyses we considered 8mer sites in this analysis and still did not observe any correlation between the loss of 8mer sites and expression of the corresponding miRNAs (both when considering fold changes (Figure 7A) and absolute differences (Figure 7B). Therefore, it would be very unlikely that we would have observed any correlations if 6mer or 7mer sites had been analyzed. Validating the true miRNA binding sites that the circRNAs possess is not possible from the RNA sequencing data as discussed above.

Review's response: Thank you for explanation. I agree with the authors.

Discussion:
On page 17, the authors stated that the "circRNAome is extensively downregulated in lesional skin". The claim is rather bold, yet, only 7 circRNAs were validated from the epidermis extraction. The authors may want to consider this point.

Our response: Regarding the downregulation of circRNAs in the skin, considered as a whole, 148 circRNAs were more than two fold downregulated, all significant, while not a single one being significantly upregulated. Since we do not talk about the epidermis here, we do not feel that the statement is bold.
It was also observed that the authors have not discussed anything about Fus and Qki significant differences in figure 3B. The authors may want to consider discussing about them.

Our response: As already discussed above, the actual differences were only very minor; ADAR being the one showing the largest difference in expression between lesional- and non-lesional skin was only about 1.5 fold higher in lesional skin (Figure 3A). In addition, ADAR was the only factor that was significantly upregulated in lesional skin both when considering the RNA-seq data (Figure 3A) and the NanoString nCounter data in both psoriasis cohorts (Figure 3B and Supplementary Figure 4). Please also note that QKI is no longer significant in figure 3B in the revised version of our manuscript (see comment above).

Review's response: Thank you for explanation. I agree with the authors.

On page 18, the authors inferred that circRNA expression was previously observed to be increased in differentiated cells. And that they proposed the "highly proliferative keratinocytes" are preventing circRNA numbers from building. However, the authors have not carried out much bench work to illustrate a stronger connection in their proposal other than the RNA-seq data.

Our response: We agree that this is still somewhat speculative, but we think this is actually appropriate to mention in the discussion section. Although, we are currently trying to address this further, we think it is outside the scope of this profiling paper.

Review's response: Based on the current set of information, I agree with the authors.

On page 19, the authors seem to imply that psoriasis vulgaris is caused by viral infection. However, it is commonly known to be infected via Streptococcal (bacteria) or HIV (virus), though psoriasis vulgaris is likely to be an acquired disease of the latter. It is also known that increased lymphocyte counts could be a consequence of auto-immune disease, which psoriasis coincidentally is. Maybe the authors could try looking up for representative factors to search in their data before drawing a better conclusion.

Our response: While we did draw a comparison between circRNA downregulation in psoriasis and the reported case of viral infection, we did not imply that psoriasis is caused by viral infection. In fact, every report so far has only presented associations with viral infections, but not causality. In turn, because psoriasis is a chronic autoimmune disease, as pointed out by the reviewer, and infiltrating lymphocytes are, of course, a consequence of that immune process, it should also be noted that virus infections are a primary factor implicated in the initiation of some autoimmune diseases. However, psoriasis is considered a multifactorial disease.

Review's response: Thank you for the clarification. I agree with the authors.

On page 20, the authors stated that they cannot verify has_circ_0061012 in their analysis. It seems to imply that Qiao et al. (2018) may have non-specifically amplify their circRNAs' levels during analysis, and consequently featured it. Therefore, the former's analysis may not be as "powerful" or reliable as the current authors'. But this is highly probably that the two analyses are different. The current authors have two cohorts of patients with the second cohort's PASI ranging from 17.2 - 51.8, while Qiao et al. (2018) recruited patients with PASI ranging 16.4 - 29.
The latter also recruited healthy participants as compared to the current authors. Thus, has_circ_0061012 could be confined to less severe psoriasis or simply that the analysis was more mutually exclusive (and thus sensitive) in Qiao et al. (2018).

Our response: The absence of has_circ_0061012 was noted in our first cohort (for which we did RNA sequencing) with a PASI score ranging from 12 to 20.8. Hence, if this particular circRNA would be characteristic for a less severe disease, which stands as an interesting observation, even though the underlying mechanisms responsible for the plaque development are the same regardless of the severity, our analysis should have been able to identify it.

Review's response: Thank you for explanation. I agree with the authors.

Any other matter:
On page 27, reference 46 has an update in their manuscript details. The authors may want to update their manuscript’s reference as well.

Our response: We thank the reviewer for this comment and have now carefully checked all references in the revised manuscript.

Review's response: Thank you for considering the suggestions.

Figure 1 legends, it is stated of 10 black arrows to indicate top 10 alpha circRNAs, but there were only 8. Unless the authors are trying to say the arrows are representative of top 10 alpha circRNAs, and the red ones are not present in circBase.

Our response: What we indicated were circRNAs previously described as alpha circRNAs25. Among those ten alpha circRNAs, we found 8 to be in top 50 most abundant circRNAs in either sample type within our study.
Review's response: Thank you for your clarification.

Figure 3a stated normalized reads, but little was explained in the manuscript. The authors may want to consider briefly describing the method.

Our response: We thank the reviewer for pointing this out. We have now included a sentence in the method section describing how the normalization was done: "in which the normalization was done by use of DESeq2’s median of ratios method." In this method read counts are divided by sample specific size factors determined by the median ratio of gene counts relative to geometric mean per gene.

Review's response: Thank you for considering the suggestions.

Figure 5, it was stated that the scale bars are on the lower right corner, but it actually on the lower left corner. The authors should also present identical magnifications for both lesion and non-lesion samples. It is also observed that the ciRS-7 staining was found within the epidermis of non-lesional skin but in the dermis of lesional skin. The authors have not commented on this.
It is unclear whether there is any sort of invasive characteristics of the disease. But this is consistent to the data showing lesser ciRS-7 counts in lesional epidermis as compared to non-lesional epidermis.

Our response: We would like to thank the reviewer for noticing this. We have corrected the figure legend in the revised version of the manuscript. Regarding the use of identical magnifications for our CISH figure (Figure 5), we do agree with the reviewer that identical magnifications would have been the ideal way to present the data. However, by using a lower magnification for panel D in the figure, we were able to fully display all the epidermal and dermal layers of the psoriatic skin while not compromising on the definition of both the tissue architecture and single-molecule staining. Therefore, we prefer not to change the magnifications.

Our response: Finally, we have carefully gone through the entire manuscript to check for spelling mistakes and grammatical errors and made a few changes for better flow or accuracy. In addition, we have also now discussed the findings by Liu, C.X. et al.26, which were published after the original submission of our manuscript. These minor changes are highlighted in the revised version of the manuscript.

Review's response: Thank you for considering the suggestions.

Review's response: Finally, I would like to thank the authors, again, for the effort put into the project. The authors have also answered my concerns promptly. Congratulations on a well-written manuscript.