Author’s response to reviews

Title: Indirubin inhibits Wnt/β-catenin signal pathway via promoter demethylation of WIF-1

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Author’s response to reviews:

Dear editor,

Thank you very much for your patience and help, you provide me ample time to consult the materials and read a lot of literature. The comments of the two reviewers provide me a lot of help, and we thank the two reviewers for their contributions.

The following reply to the comments of reviewer 3(Every modification in our article is indicated in red font):

1. First of all, the English in the present manuscript is not of publication quality and require major improvement. Please carefully proof-read spell check to eliminate grammatical errors.

   Reply: We carefully proof-read spell check to eliminate grammatical errors.

2. The description in the introduction section about psoriasis pathogenesis is poor. The authors need to expand it (e.g. Rendon A. et al., Psoriasis Pathogenesis and Treatment, Int J Mol Sci, 2019 - Balato A. et al., Effects of adalimumab therapy in adult subjects with moderate-to-severe psoriasis on Th17 pathway, J Eur Acad Dermatol Venereol, 2014).

   Reply: We enhance the description in the introduction section about psoriasis pathogenesis. (Pag.3, line 1-13)

3. Pag.10, line 6-9: "We had previously detected the less expression of wif-1 after the treatment with different concentrations (0.04 μM, 0.2 μM and 1 μM) of indirubin…". Previously? Have the authors conducted these experiments in a previous manuscript?

   Reply: “Previously” (Pag.10, line 6-9:) express our study initiate stage in this study. We have modified this ambiguous sentence “we detected the expression of wif-1 was recovered after the treatment with different concentrations (0.04μM, 0.2μM and 1 μM) of indirubin in a concentration-dependent manner.”. (Pag.10, line 1-3)
4. Moreover, the authors should explain why they choose the selected indirubin concentrations in their experimental model.

Reply: Thank you for your reminder; we would like to add interpretation to explain the selected indirubin concentrations. When we first used indirubin, we checked the information about it. Indirubin is a potent cyclin-dependent kinase and GSK-3β inhibitor with IC50 of about 75nM and 0.19µM. The principle of selected concentrations is to incorporate established IC50 as much as possible and keep multiple appropriate gaps among different concentrations. Therefore, we chose these four concentrations. Concentration of 1µM showed the most active effects. So, the final indirubin concentration used in the experiment was 1µM.

5. Pag.10, line 12-18: "Similarly, we observed the mRNA expression of wif-1 was recovered analogously to Western blotting by qRT-PCR and ELISA (Fig. 1 b and c)." qRT-PCR result is reported in Fig. 1b? It is unclear. (Pag.10, line 3-5)

Reply: Figure 1b of the previous article is the semi-quantitative expression of Western blotting. Due to our negligence, the chart of qRT-PCR was not provided, and we have uploaded the chart of qRT-PCR in Figure 1, which is Figure b in Figure 1. We modified this sentence with ambiguous meaning to “Similarly, we observed the mRNA expression of wif-1 was recovered by qRT-PCR (Fig.1b), and protein expression of wif-1 was recovered by ELISA (Fig.1f).” (Pag.10, line 5-7)

6. - Pag.10, 31-39: "The research showed that wif-1 promoter hypermethylation in the HaCaT cells, the treatment with different concentrations (0.04 μM, 0.2 μM and 1 μM) of indirubin, the wif-1 promoter methylation level was recovered with concentration-dependent demethylation in HaCaT cells (Fig. 1d)." The result is interesting but the sentence is confusing.

Reply: We modified this sentence with ambiguous meaning to “The research showed that wif-1 promoter hypermethylation in HaCaT cells after the treatment with low(0.04μM), medial(0.2μM), and high(1μM) concentrations of indirubin, the wif-1 promoter methylation level was recovered with concentration-dependent demethylation in HaCaT cells.” (Pag.10, line 10-13)

7. - Pag.11, line 3-6: "To further investigate the effect of DNMT1 on the wif-1 promoter methylation, we performed the following experiments". What experiments? Please clarify.

Reply: We modified this sentence with ambiguous meaning to “To further investigate the effect of DNMT1 on the wif-1 promoter methylation, we performed the MSP experiments.” (Pag.11, line 3-5)

8. - "Indirubin promotes the transcriptional activity of wif-1 in HaCat cells and inhibits the transcriptional activity of β-cateninn" and "The ability of indirubin inhibits the proliferation and arrests cell cycle and induces apoptosis of HaCat cells" paragraphs: some data are redundant as well as some sentences confusing. I suggest shortening and reviewing the full paragraphs.

- Results are not arranged and numbered in the figures in order of appearance which is sometimes unclear.
Reply: We have made major adjustments to these two paragraphs. (Pag.12, line 2-23)

9. Figure legends should better support the article figures. Generally, I would suggest to improve them.
Reply: We have modified in detail Figure legends to support the article figures.

10. Abbreviations should be identified at the first time that they have been introduced in the paper.
Reply: We have changed the spelling of abbreviations in the article, include transcriptional activation of transglutaminase 1 (TGase1) (Pag.2, line 1) and Methylation-Specific PCR (MSP) (Pag.6, line 17)

The following reply to the comments of reviewer 4 (Every modification in our article is indicated in red font):

1. Although the title is neutral already the background of the abstract refers to psoriasis. However the effect was only tested on HaCaT cells that are not good models for psoriasis (Soboleva et al. Genetically predetermined limitation in HaCaT cells that affects their ability to serve as an experimental model of psoriasis. Russian J of Genetics, 2014.10:1081-1089) and the effect was not tested on psoriasis-like keratinocytes, so that a statement to psoriasis is not possible. Especially the sentence in the conclusion on page 17 that "these results suggest the factors by indirubin to ameliorate psoriasis, and they provide experimental evidence for indirubin treatment for psoriasis" cannot be drawn with these data, because no psoriasis model was used for the studies. Furthermore in the background as part of the introduction it is mentioned that the underlying cause of psoriasis is unclear and the treatment is largely still in exploratory stages. However nothing is mentioned on IL-17 and other cytokines as leading cytokine of psoriasis (e.g. Gaffen et al. The IL-23-IL-17 immune axis: from mechanisms to therapeutic testing. Nature Reviews Immunology.14:585-600) and there are many treatment optioned concerning systemic therapy of antiinflammatory targets as well as biologics and topic therapies. So many different treatment options are approved (Rendon et al Psoriasis pathogenesis and treatment. Int J Mol Sci. 2019.20(6):1475).

Reply:
Thank for your suggestions. For this question, I would like to make some explanations. Firstly, HaCaT cells may be not a perfect model for psoriasis researching. We admit that the conclusions on page 17 was not precise, and modifications would be re-generalize to include a proper consequence. However, HaCaT cells, a line of highly proliferating human keratinocytes that share some characteristics with psoriatic keratinocytes. Just only HaCaT cell line adopted for psoriatic research was available in many papers (1. Balato A et al. IL-33 is secreted by psoriatic keratinocytes and induces proinflammatory cytokines via keratinocyte and mast cell activation. Exp Dermatol 2012; 21(11):892-4. 2. Saelee C et al. Effects of Thai medicinal herb extracts with antipsoriatic activity on the expression on NF-kappaB signaling biomarkers in HaCaT keratinocytes. Molecules 2011; 16(5):3908-32). Furthermore, without psoriasis-like keratinocytes, HaCaT cells and My-La (CD8+ T cells) together called psoriatic relevant cell types were utilized to explore psoriasis GWAS loci, and the results were published in BMC...
Biology magazine in 2020 (Ray-Jones, Helen et al. Mapping DNA interaction landscapes in psoriasis susceptibility loci highlights KLF4 as a target gene in 9q31. BMC Biol.2020 May 04; 18(1):47). HaCaT cell line has been widely recognized and commonly used to investigate the mechanism for psoriasis in amounts of original article. Above all, we believe that HaCaT cells are a more mature model for psoriasis.

For the background of introduction part, it is valuable of the suggestions from reviewer, and we have added treatment progress in the manuscript. We deleted this sentence “these results suggest the factors by indirubin to ameliorate psoriasis, and they provide experimental evidence for indirubin treatment for psoriasis”, and modified it to “These results suggested that indirubin had inhibited cell proliferation and induced the apoptosis of HaCaT cells, reflecting its potential use in the treatment of proliferative diseases such as psoriasis.”. In addition, in the background as part of the introduction, we added an introduction to the background of psoriasis.

2. Some essential details in the M&M section are missing, e.g. the transfection method for the luciferase reporter gene assay, especially as HaCaT cells are not that easy to transfect. The test principle of the cell viability assay should be added on page 7. The information to the HaCaT cells that stand for human, adult, low calcium, high temperature, human adult skin keratinocytes are written with some capital letters. This cell line was characterized in the following publication: Boukamp et al. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line The Journal of Cell Biology.1988 106 (3): 761-771. These cells cannot be bought by ATCC but for example by CLS (cell line services). If they are bought by CLS this cell line has already passage 38, therefore it is not possible to use the first three passages for all experiments. There are some fundamental errors.

Reply:
We are very sorry for our carelessness of HaCaT incorrect writing, and mistakes have been rectified in the whole article. Other mentioned, such as transfection detail, cell viability assay principle, also could be found in the corresponding parts. HaCaT cell line we used was from lab of our hospital, which was brought from cell reseller with license, and the cells were guaranteed from ATCC. Some published articles in the method part show that HaCaT cells could be bought from ATCC (Lei Li et al. REGγ is critical for skin carcinogenesis by modulating the Wnt/β-catenin pathway. Nat Commun 2015 Apr 24; 6:6875. doi: 10.1038/ncomms7875). HaCat cells is a well-known immortalised human keratinocyte cell line. We wrote “first three passages for all experiments” in the method part. Actually, for this article, the cells thawed from liquid ammonia was supposed to be as the “primary cell” and passed beyond three generations were excluded from experiments. This sentence is likely to lead to ambiguity, and we are willing to delete this sentence.

3. The first sentence in the results section sounds confusing and should for example be changed to "..we previously detected a reduced expression of...

Reply: We change the first sentence of the results section to “we detected the expression of wif-1 was recovered after the treatment with different concentrations (0.04μM, 0.2μM and 1 μM) of indirubin in a concentration-dependent manner.”. (Pag.10, line 1-3)
4. Concerning figure 1b in the legend it is described that mRNA expression is shown however the description of the y-axe shows gray scale value for protein versus GAPDH. So it seems that the density of the bands of the Western blot in figure 1a are measured. 
Reply: Figure 1b of the previous article is the semi-quantitative expression of Western blotting. Due to our negligence, the chart of qRT-PCR was not provided, and we have uploaded the chart of qRT-PCR in Figure 1, which is Figure b in Figure 1. We modified this sentence with ambiguous meaning to “Similarly, we observed the mRNA expression of wif-1 was recovered by qRT-PCR (Fig.1b), and protein expression of wif-1 was recovered by ELISA (Fig.1f).” (Pag.10, line 5-7)

5. In figure 1c it is mentioned that the mRNA expression of wif-1 was promoted in HaCat cells by ELISA. If an ELISA was performed then not the mRNA was measured. It would be easier to change promote to increase. 
Reply: Thank you for your suggestion, however, we want to further verify our results by ELISA.

6. In figure 1d the description low, medial and high and in figure 1e and f the description of A; B;C;D should be explained. 
Reply: We re-explained the figure, including figure 1d (Pag.10, line11), figure 1e and f (Pag.25, line8-9).

7. In the result section DNMT1 as DNA methyltransferase should be explained again. 
Reply: In the results section “DNMT1 promotes wif-1 promoter hypermethylation”, we explained the function of DNA methyltransferase once again. (Pag.10, line20 and Pag.11, line1-5)

8. On page 12 the first sentence is misleading: "To analyze the relationship between wif-1 and indirubin, DNMT1, a luciferase reporter assay was carried out" leads to the impression that DNMT1 is an luciferase reporter assay. 
Reply: We deleted "To analyze the relationship between wif-1 and indirubin, DNMT1, a luciferase reporter assay was carried out" because of the ambiguity it caused, and made significant changes to this paragraph. (Pag.12, line4-14)

9. On page 13 the description that cells in the G0/G1 phase was large should be mentioned more precisely in addition of the percentage the cells that are in the G0/G1 phase. 
Reply: In the results section “The ability of indirubin inhibits the proliferation, arrests cell cycle, and induces apoptosis of HaCaT cells.”, We described in detail the percentage the cells that are in the G0/G1 phase, and modified it to “indirubin at 1μM increased the percentage of G0/G1 cells from 33.6% in control cells to 64.00% after 48h of treatment.” (Pag.12, line19-20)

10. The headline that indirubin treatment reduces the expression of key proteins on HaCat cells should be explained in more detail. Why are involucrin, keratin 17, TGase I chosen as key proteins in HaCaT cells? What do they detect? Why was keratin 17 tested? Involucrin are early and loricrin late differentiation markers. This should be outlined and explained.
Reply:
We elaborated on this issue in the discussion section.
As described below:
In psoriasis, Involucrin and TGase 1 are proteins expressed in the early stages of keratinocyte differentiation. Involucrin, as a protein precursor of the crosslinked envelope, is a marker of early differentiation of keratinocytes. TGase 1 expression may be regulated by β-catenin and glycogen synthase kinase. Keratin 17 has multiple biological functions and regulates cell proliferation, growth, and skin inflammation. In psoriasis, IL-17, IL-22 and INF-3 stimulate keratin 17 overexpression. Filaggrin and loricrin are proteins expressed in later stages of keratinocyte differentiation. (Pag.16, line19-22 and Pag.17, line1-4)

11. In the discussion line 6 it is stated "to explore the potential mechanism of psoriatic keratinocytes hyperproliferation, we explored the role of wif-1 in the pathology of psoriasis... This is not true as HaCaT cells are no psoriatic keratinocytes but an immortalized normal keratinocyte cell line (see arguments under point 2). Primary keratinocytes should ideally be treated with cytokines to create a psoriasis-like cell type to be able to make a statement to psoriasis.

Reply: In this part, the questions raised by the reviewers are similar to the first question, we have already explained and revised in the first part.

12. In the discussion on page 16 line 6 it is stated that one of the most important signaling pathway to induce psoriasis is the wnt signaling pathway. As reference the paper written by Lin et al (Gene 2017) is mentioned (reference 20), however this publication deals with gallbladder cancer and not psoriasis. Furthermore at least IL-17 as key cytokine of psoriasis should be discussed in this section.

Reply: We have deleted the reference 20, and discussed the role of IL-17 and wnt signaling pathway in psoriasis.
As described below:
Wnt signaling pathway plays a vital role in chronic inflammatory diseases as psoriasis, such as IL-17A, it inhibits the wnt signal pathway and rescues the expression of wnt target gene and bone formation. (Pag.15, line22 and Pag.16, line1-2)

13. on page 15 in the first sentence of the conclusion it is mentioned that the expression level of wif-1 was lower in HaCaT cells. "Lower" indicates a comparison but it is not mentioned with what HaCaT cells were compared.

Reply: The description in the original text is inaccurate. In order to avoid ambiguity, we deleted the description of the original text and changed it to “Our findings demonstrate that the expression level of wif-1 was recovered after treated with indircrubin in HaCaT cells.” (Pag.17, line13-14)
14. In general the central theme that the data provide evidence for psoriasis treatment and might be relevant for scientists or medicines are missing. Several experiments were described such as western blot, luciferase reporter assays, ger knock-down experiments but the explanation of the experiments and the conclusions from the experiments are insufficient.

Reply:
Thank you for your suggestion, we have explained the Figure legend of each part more clearly and rewritten the conclusion (Page 2, line 10-13 and Page 17, line 13-20).

Minor effects:
15. In the abstract there is an typing error in line 39 NDMT1 instead of DNMT1.

Reply: We have modified the writing of DNMT1. (Page 1, line 13)

16. In page 3 is stated that indirubin can be found in both healthy and disease-affected urine. If indirubin can be found in the uterine, the person is not healthy there is an infection with bacteria, isn't it.

Reply: As this sentence caused ambiguity, we have deleted it.

17. In the background section of the introduction the principal wnt-signaling pathway should be described a bit in detail.

Reply: We describe the wnt signal transfer pathway in detail in the background section. (Page 3, line 25-28 and Page 4, line 1-2)

18. JAK/STAT signal pathway is written in capital letters

Reply: We have changed JAK/STAT to a capital form. (Page 3, line 22)

19. Page 13 line 28 inolucrine should be changed in involucrin.

Reply: Through the word search function, we did not find the word “inolucrine”.

20. Page 12 line 26 or page 15 line 15 "when" should be written in small letters.

Reply: "when" should be written in small letters, we have made modified.