Peer Review File

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Reviewer A

General comments:

While the effect of PAK4 on tumor suppression has been well reported in the literature, its role in cancer cell autophagy remains largely unknown. In this paper, the author reported an autophagy-inhibiting effect of PAK4 in hepatocellular carcinoma (HCC) cell lines. This is an interesting finding and should open up opportunities to further characterize the role of PAK in tumor autophagy and also the downstream effects of this role on both tumor cell function as well as tumor microenvironment.

However, this study also can be improved in multiple ways.

1.The English of the manuscript should be improved. It is quite difficult to understand what the authors want to convey, and the logic behind the statements is not obvious. This is especially the case for the abstract and the introduction.

Reply: We improved the language of the manuscript especially for the abstract and the introduction.

Changes in the text: modified as advised in the full text.

2. Only a single cell line is used in this study, which fails to provide a generalized conclusion on HCC. The observed effect can be cell-line-dependent rather than disease dependent.

Reply: Appreciate the piece of advice. In our published study (reference 32), two cell line on HCC were used only for proliferation, apoptosis and so on but autophagy. We focus on HepG2 cell lines but HCC in the study for restricted time and expenditure for a student about to graduate. We will further study as advised.

Changes in the text: No changes.

3. While the authors try to prove that PAK4 inhibition increase p53, which inhibits mTOR and induces autophagy. This cannot be proved by the study design, as it only suggests that PAK4 inhibitions increase p53, inhibit mTOR and induce autophagy but fail to demonstrate any inter-connection. This is the same for the observed G2/M cell cycle arrest, which cannot be proved to be related to autophagy induction.

Reply: We agree with the comment. The study fails to demonstrate the inter –connection of p53, mTOR, and G2/M cell cycle arrest although the references indicate.

Changes in the text: Modified as advised (see Page7, line 282-283 and Page8, line

324-325)

4. In vivo evidence is not provided by the study, so it remains uncertain whether the observed effect in HCC cell lines can be translated into animal models.
Reply: Appreciate the piece of advice. We will study it using animal models.
Changes in the text: No changes.

Introduction:
1. "An overexpressed gene characterized as an inhibitor of PAK4 increases the the proportion of leukemia stem cells in G0 by interfering with nuclear localization of PAK4 in acute myeloid leukemia."
- The authors should specify the gene's name.
Reply: Added the gene's name *INKA1*.
Changes in the text: Modified as advised (see Page 2, line 72-73)

2. "p53, a molecule upstream to PAK4 by interacting with p21[10], protects cells via cell-cycle surveillance on G1/S phase [11]. So, the downregulated PAK4 is speculated to impair proliferation due to p53-mediated G1/S arrest in the cancer cells."
- If P53 is upstream to PAK4, PAK4 downregulation should not affect P53 function. It should be the other way around.
Reply: Yes, as you think, 'Pak4 downregulation should not affect P53 function' according to the reference 10, p53 is upstream to PAK4. But there was report PAK4 affected P53, which was indicated next in reference 14, so "It should be the other way around", which was indicated in last sentence this paragraph.
The sentence means, regarding references, that p53 should affect G1/S and p53 should not be impacted by Pak4, but subsequently adverse evidence in reference 11-13 and 14.
Changes in the text: we modified the sentence. "Therefore, p53-mediated G1/S arrest is speculated to impaire proliferation in PAK4-blockdown cancer cells." (see Page 2, line 78)

3.In paragraphs three and four, the authors summarized current evidence on the association between PAK and autophagy. To my knowledge, currently, there is no evidence to connect PAK4 with autophagy. This probably should be pointed out here.
Reply: Thanks for the advice.
Changes in the text: Modified as advised (see Page 2, line 93-94)

4. "The autophagy is performed by mTOR/AKT signaling in a few of studies. The activator of mTOR increase the expression of PAK1, p-PAK1 and autophagic

molecule of LC3B1 in prostate cancer cells."
- mTOR activation inhibits autophagy, which should decrease LC3B conversion.
Reply: the ratio of LC3B2/ LC3B1 was considered as an autophagy marker in the cite.
Changes in the text:We modified as advised. (see Page 2, line 96)

Results
1.In figure 1, figures 1a and 1f showed the same result and probably don't need to be repeated.
Reply: Figure 1a and 1f are MTT assay for transient and stable PAK4-knockdown HepG2 cell lines, respectively.
Changes in the text: We modified the second title with a few of changes on its text in METHODS. (see Page 3-4, line 146-162)
And added a photograph on western blot in figure1A for evidence of transient PAK4-knockdown HepG2 cell and statement in results 1. (see Page 5, line 235-239 and figure1A)

2. In figure 1d, fluorescence intensity was measured by average optical density. However, the authors did not specify how average optical density is determined in either method or result session.
Reply: average optical density was measured by the software ImageJ.
Changes in the text: We modified a few of changes on its text in METHODS. (see Page 5, line 142-144)
3. In figure 3, the author used LC3B immunoblot and MDC staining to determine the autophagy level in PAK4 knocked-down cell lines. However, as certain autophagy inhibitors (such as chloroquine or hydroxychloroquine) can also increase LC3B level and MDC staining by decreasing lysosomal acidity. It remains a possibility that the observed effect of PAK4 knockdown can be from decreased degradation of autophagosome rather than induced formation.
Reply: Thanks for the good question. Autophagy is detected by static (MDA staining) and dynamic (western blot for the ratio of LC3-II/ LC3-I) measures. The sencond assay suggests the induction.
One of the most frequently used methods for autophagy is staining with acidotropic dyes such as monodansylcadaverine (MDC) in the study. MDC and other acidotropic dyes are label for later autophagosomes. MDA may demonstrate an accumulation of autophagosomes by measuring steady state levels that are static in nature, and so MDA reflect the induction of autophagy and/or inhibition of autophagosome as reviewer mentions above.
Hence, the dynamic process of autophagy needs to be detected. LC3 is initially

synthesized in an unprocessed form, proLC3, which is converted into a proteolytically processed form lacking amino acids from the C terminus, LC3-I, and, during the process of autophagy, is finally modified into the PE-conjugated form, LC3-II (as an only protein marker that is reliably associated with completed autophagosomes). In mammalian cells, the total levels of LC3 do not necessarily change, as there may be increases in the conversion of LC3-I to LC3-II during the dynamic process of autophagy. the ratio between LC3-I and LC3-II appears to correlate with changes in autophagy and provides a more accurate measure of dynamic autophagy.
Changes in the text: No changes

4. In figure 4, the downregulation of p53 and mTOR by PAK4 inhibition was demonstrated. However, a direct conclusion cannot be made between p53 and mTOR, as it is possible that PAK4 acts on both directly rather than affecting mTOR through p53.
Reply: Yes, it is.
Changes in the text:We modified as advised.(see Page 7, line 282-283)

Discussion
1.In the first paragraph, the author again suggests that p53 is upstream of PAK4. However, if PAK4 inhibition can suppress p53 expression then p53 should be a downstream mediator of PAK4.
Reply: Yes, the statement is unnecessary here.
Changes in the text: Deleted it. (see Page 7, line 290)

2. In paragraph three, the authors concluded that PAK4 inhibition induces autophagy through increased p53. Again, similar to mTOR above, this study did not provide evidence on the role of p53 on this effect.
Reply: Yes, it is.
Changes in the text:We modified as advised.(see Page 8, line 313)

3. Furthermore, while this study provides evidence of the role of PAK4 in HepG2 cell autophagy, it did not connect this induced autophagy with G2M cell cycle arrest. In order to prove this, the author will need to demonstrate inhibition of autophagy itself can cause cell cycle arrest.
Reply: Yes.
Changes in the text:We modified as advised.(see Page 8, line 323-324)

Reviewer B

In this article the authors provide evidence that PAK4 inhibition promotes cells to undergo autophagy. Lentiviral shRNA particles were used to block PAK4 expression and several different assays were performed. PAK4 knockdown suppresses proliferation and induced cell cycle arrest. The cells showed induction of autophagy. Mechanistically, the authors demonstrate the role of p53 aktmTOR axis in the process.

Specific comments:

1.The entire premise is depedent on one cell line. A second cell line can be used for some validation.

Reply: Appreciate the piece of advice. In our published study (reference 32), two cell line on HCC were used only for proliferation, apoptosis and so on but autophagy. We focus on HepG2 cell lines but HCC in the study for restricted time and expenditure for a student about to graduate. We will further study as advised.

Changes in the text: No changes.

2.What in p53 mutant or knockout cell lines? will similar results be observed?

Reply: The question is intresting. p53 mutant or knockout might be expected to show different results with lots of evidences, while lack of Pak4 knockout special for cell autophagy. The study demonstrated the knockdown of Pak4 affected proliferation and autophagy, and p53, mTOR and AKT involved in thecell events as preliminary exploration.

Changes in the text: No changes.

3.Autophagy staining can be supported by high resolution images of autophagy morphology

Reply:the brightspots are label for later autophagosomes.

Changes in the text: changed image as advised. (see figure 3)

4.Typos were found that should be checked and corrected

Reply: We improved the language of the manuscript.

Changes in the text: modified as advised in the full text.

5.Some figures were poor quality

Reply: Improved.

Changes in the text:(see Page8 , Figure 1;Page11 , Figure 3;Page12,Figure4)