ANSWERING REVIEWERS

Oct 30, 2015

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 20957-Revised manuscript_text). The figures are in PDF format (file name: 20957-Revised manuscript_figures).

Title: Epigenetic alteration to activate Bmp2-Smad signaling in Raf-induced senescence

Author: Mai Fujimoto, Yasunobu Mano, Motonobu Anai, Shogo Yamamoto, Masaki Fukuyo, Hiroyuki Aburatani, Atsushi Kaneda

Name of Journal: *World Journal of Biological Chemistry*

ESPS Manuscript NO: 20957

The manuscript has been improved according to the suggestions of reviewers/editor:

1 Format has been updated. As for language, we asked one of the suggested professional English language editing companies, American Journal Experts, to check errors in our revised manuscript, and we corrected them accordingly (throughout the manuscript). The certificate by the company is attached. Regarding plagiarism, we faithfully understand the journal policy that all the revised manuscript is suggested to be checked for plagiarism before resubmission. Since we have already had an account for iThenticate, we check our manuscript for plagiarism using iThenticate as usual, instead of CrossCheck, which is powered by iThenticate and suggested to use for this purpose. If our manuscript is found to include any sentences similar to those written in previous reports, we make changes in the sentences throughout the manuscript. Screenshot image of the results of iThenticate for all the pages is attached.

2 Revision has been made according to the suggestions of the reviewers and editor. All the changes are highlighted.

(1) Editor:

"Biostatistics statement. This statement must be mentioned in the text, and a certificate of statistical review signed by a biostatistician must be provided in PDF format. Sample wording: The statistical methods of this study were reviewed by [name(s) of individual(s)] from [name(s) of organization(s)]…"

--WE agree to the editor. We moved this statement in the text, and PDF file of the certificate of statistical review is attached.

"Audio Core Tip. In order to attract readers to read your full-text article, we request that the first author make an audio file describing your final core tip. This audio file will be published online, along with your article. Please submit audio files according to the following specifications: Acceptable file formats: .mp3, .wav, or .aiff. Maximum file size: 10 MB. To achieve the best quality, when saving audio files as an mp3, use a setting of 256 kbps or higher for stereo or 128 kbps or higher for mono. Sampling rate should be either 44.1 kHz or 48 kHz. Bit rate should be either 16 or 24 bit. To avoid audible clipping noise, please make sure that audio levels do not exceed 0 dBFS."

--We attached Audio Core Tip in mp3 format.

(2) Reviewer 01299180:
"In this study, the authors examined the expression changes induced by oncogenic RAF. Previously, the authors have conducted similar study on oncogenic Ras. The main conclusion from this study is that oncogenic RAF and Ras cause similar patterns of gene expression, which is sort of expected, given that oncogenic Ras/RAF function in the same pathway that leads to cellular transformation. I suggest the authors to provide more explanations in the introduction section as why it is important to focus on oncogenic RAF here, given that the work on oncogenic Ras has already been done."
--WE thank the reviewer for careful reading of our manuscript and giving a helpful comment. We added the following sentences in the Introduction section: "These epigenetic alterations associated with Bmp2-Smad1 signal were found to occur specifically in Ras-activated cells, not in control cells with mock retrovirus infection[22]. In contrast, the epigenetic alteration at the Ink4a-Arf locus was commonly observed in Ras-activated cells and mock cells, i.e. both in cells under Ras-induced senescence and cells under replicative senescence[22]. It was suggested that there might be common epigenetic mechanisms in premature senescence and replicative senescence, and also different epigenetic regulation on specific signals between the two senescence programs. Within such molecular alterations specifically observed during premature senescence, there might be some common alterations and some different alterations in premature senescence induced by different stresses, but such analysis to compare different types of premature senescence has not been conducted in detail." (p.6, ll.19-30).

"A small typo in the first sentence of "Aim" (epigenomics)."
--WE are sorry for the typo. The word was corrected (p.3, l.2), and other grammatical errors were also checked by English editing service and corrected (throughout the manuscript).

(3) Reviewer 02608938:
"Fujimoto et al studied global changes of mRNA levels, H3K4me3, H3K27me3 in cells infected with retrovirus expressing oncogenic mutant of BRafV600E and compared these changes with their previously reported data in MEF cells infected by congenic Ras. Their data indicate that both oncogenic mutants of Ras and Raf induced cellular senescence and altered mRNA levels of a large number of genes. Among common changed mRNAs, they identified the Bmp2 and Smad6 genes. Their ChiP-Seq data also showed that both of these genes have similar enrichment or reduction of H3K4me3 and H3K27me3, respectively, except that H3K27me3 induction was not found from the Samd6 promoter in RafV600E infected cells. They further showed that knocking-down Bmp2 and overexpressing Samd6 in MEF cells prevented MEF cells from BRafV600E-induced senescence. Their data indicate potential common mechanism underlying cellular senescence induced by mutated Ras and Raf oncogenes. However, this study missed a critical control and a reasonable rationale of why commonly changed, but not differentially expressed, genes were selected for comparison of oncogenic Ras and Raf overexpression."
--WE thank the reviewer for carefully reading and appreciating our manuscript that our data indicate potential common mechanism underlying cellular senescence induced by mutated Ras and Raf oncogenes. And we also thank the reviewer for helpful comments. For a critical control, we added an analysis using retroviral vector with empty exogenous gene (Mock) as the reviewer suggested in the specific comment 2. The detailed explanation is written below in the answer to the comment 2. As for the reason why genes commonly changed in Ras- and Raf-induced senescence, i.e. Bmp2 and Smad6, were selected, we added the explanation in the Results section as follows. "We previously reported that the activation of Bmp2-Smad signaling by the harmonized epigenetic activation of Bmp2 and repression of Smad6 contributes to Ras-induced senescence[22]. In the present study, Bmp2 and Smad6 were found to be altered commonly in Ras- and Raf-induced senescence, but not in mock cells (Figure 5). In our recent targeted exon

sequencing analysis in colorectal tumors, mutation of genes in BMP signaling, e.g. BMPR2, BMP2, and SMAD4, were significantlydetected in BRAF-mutation(+) colorectal cancer[33]. It was thus suggested that activation of Bmp2-Smad signalling might be also critical in Raf-induced senescence and disruption of the signaling may play a role in tumorigenesis of BRAF-mutation(+) colorectal cancer." (p.14, ll.16-24)

"Specific comments. 1. Critical rationale of the current study is missing. Whether there is a difference between replicative cellular senescence and premature senescence is not described clearly in the introduction. It seems that oncogene(s) can induce both types of senescence. Whether different oncogenes will produce different senescence is unclear. Therefore, it is unclear why the comparison of gene expression in cells induced by different oncogenes should be performed."
--WE thank the reviewer for a helpful comment. We added the following sentences in the Introduction section: "These epigenetic alterations associated with Bmp2-Smad1 signal were found to occur specifically in Ras-activated cells, not in control cells with mock retrovirus infection[22]. In contrast, the epigenetic alteration at the Ink4a-Arf locus was commonly observed in Ras-activated cells and mock cells, i.e. both in cells under Ras-induced senescence and cells under replicative senescence[22]. It was suggested that there might be common epigenetic mechanisms in premature senescence and replicative senescence, and also different epigenetic regulation on specific signals between the two senescence programs. Within such molecular alterations specifically observed during premature senescence, there might be some common alterations and some different alterations in premature senescence induced by different stresses, but such analysis to compare different types of premature senescence has not been conducted in detail." (p.6, ll.19-30).

"2. A critical control is missed from this manuscript, i.e., retroviral vector with empty exogenous gene since viral infection itself may produce cellular reaction(s)."
--WE agree to the reviewer that data of the critical control was missing in the submitted version. In the revised version, we added expression array data of mock retrovirus infection as requested. In Figure 3, we showed genes altered commonly in Ras- and Raf-induced senescence. We added Figure 4 showing genes altered commonly in Ras- and Raf-induced senescence and in mock cells as well, and Figure 5 showing genes altered commonly in Ras- and Raf-induced senescence but not in mock cells. Bmp2 and Smad6 were included in the latter group, suggesting that their alterations were commonly observed in Ras- and Raf-induced senescence, but not occurred by the stress of viral infection itself. Text was also changed accordingly. (p.13, l.7 – p.14, l.19; Figures 4-6, and their legends)

"3. Why retroviral vector is used for the overexpression of BRaf and its mutant? What is the infection efficiency of cell population? Whether infected cells must express recombinant gene? Was this expression confirmed? Uninfected cell population may not enter into senescence and thus not undergo alteration of gene expression, therefore resulting in dilution of potential changes of mRNAs in infected cells due to analysis of total RNA from the whole cell population. Should we consider these histone binding as part of chromatin remodeling per se instead of epigenomic change?"
--WE thank the reviewer for helpful comments. We used retroviral vector for overexpression of activated *Ras* in the present study, and that is why we used the same method for the current analysis of *Raf*. Infection efficiency of retrovirus produced by plat-E packaging cells and pMX vector is known to be very high so that multiplicity of infection can be nearly 100%. When cells infected with mock retrovirus were cultured with and without G418, they did not show a difference in cellular growth, indicating that multiplicity of infection is nearly 100%. We added this in Methods section. (p.8, ll.2-5) For expression of the recombinant gene, we added the data in Figure 1A. (Figure 1A) We agree to the reviewer's comment that uninfected cells would not enter

into senescence and thus not undergo alteration of gene expression. But multiplicity of infection is suggested to be nearly 100%, and we added this explanation in Methods section. (p.8, ll.2-5) Also, the cells were exposed to 700μg/mL of G418 for selection, and it was confirmed that MEF cells without infection completely die under this condition by day 5. We added these in Methods section, and expression array analysis and ChIP-seq analysis were performed using cells on day5 or after. (p.8, ll.2-5) For epigenomic information, we analyzed H3K4me3 and H3K27me3. As the reviewer gave a comment, these changes can be considered as part of chromatin remodeling. We analyzed H3K4me3 for an active histone mark and H3K27me3 for a repressive histone mark, to gain insight into a role of epigenetic alteration in expression alteration as we previously did for *Ras*-induced senescence.

"4. Why was SA-b-gal analysis only done for cells infected for 7 days? Clearly, this analysis is cytochemical and thus differ from gene expression and ChIP analysis. How can authors explain the difference?"
--WE thank the reviewer for a helpful comment. We showed the data of SA-b-gal analysis for days 3, 5, 7, and 10 in the revised version, instead of day 7 only. We agree to the reviewer that this analysis is cytochemical, and does not give quantitative information of molecular alteration, but gives information of cellular state. This analysis was done in order to confirm that BrafV600E cells were senescent. We added these in the Methods and Results sections. (p.10, l.8; p.12, ll.2-9)

"5.Different from naive MEF cells, infected cells were screened by G418. Whether this screening will wipe out a special group of cells which are never infected by retroviron, thus producing bias? To the results that cells infected with RafWT showed the "similar" cell number to naive cells, considering that G418 removed non-infected cells, it should produce less cell number than naive cells. Why did these 2 groups showed similar number of cells? Is this caused by overexpression of Raf? In view of small error bars, statistical examination of cell numbers between RafWT and MEFs should be performed."
--WE thank the reviewer for a helpful comment. After infection, the cells were exposed to 700μg/mL of G418 for selection, and it was confirmed that MEF cells without infection completely die under this condition by day 5. Therefore, a bias due to contamination of non-infected cells could be observed in the data on day 3 for growth curve and SA-b-gal staining. But when mock-infected cells were cultured with and without G418, they did not show a difference in cellular growth. The multiplicity of infection is considered to be nearly 100%, and the possible bias due to contamination of non-infected cells on day3 must be tiny. We added these in Methods section. (p.8, ll.2-5) Expression array analysis and ChIP-seq analysis were done using cells on day 5 or after, so there should be no bias in these data due to non-infected cells. For growth curve of RafWT cells, while mock cells did not show a difference in cell number compared with naive MEF cells, RafWT cells showed slightly faster growth. We added statistical examination, and added sentences in the Results section. (p.11, l.24 – p.12, l.1; Figure 1B, and its legend)

"6.Why mRNA expression was extracted from cells infected with virus for 5, 7, 10 days, but mRNA expression are examined only for day 5 and 7 and ChIP was only done for day 7 after infection? What are biological repeats of array analysis?"
--WE thank the reviewer for a helpful comment. For expression array analysis, biological repeats are often performed, and we actually collected the data for day 5 and day 7 for this purpose. For ChIP experiments, ChIP-seq was not repeated, but ChIP assay itself was repeated twice for ChIP-PCR and the similar results were obtained. We added description about the repeats. The reason why we selected day 5 and day 7 is because growth curve data (Figure 1B) and SA-b-gal data (Figure 1C) indicate that RafV600E cells are senescent at least by day 5, and selection of cells by G418 basically finishes by day 5. We are sorry that critical information of these was missing in the submitted version, and we added the information in Figure 1, Methods and Results sections.

(p.10, l.8; p.11, l. 24 – p.12, l.9; p.31, ll.1-5; Figures 1 and 8)

"7. In figure 2, 3, 4, why were mRNAs in cells infected with virus expressing wild-type Raf not studied as a control?"
--WE thank the reviewer for a helpful comment. We agree that it would be better to add control data e.g. RafWT cells or mock cells. We added expression array data of mock cells as a control, and reanalyzed the data. (p.13, l.7 – p.14, l.19; Figures 4-6, and their legends)

"8.Writing needs improving. Careful editing should be conducted for the whole manuscript. Examples are listed below. 1) .Conceptual connection at the beginning of introduction is unclear regarding cellular senescence, replicative cellular senescence and premature senescence. 2) Consistence in uses of terms should be greatly taken care of. For examples, replicative cellular senescence and replicative senescence are used; BrafV600E, RafV600E an"
--WE thank the reviewer for a helpful comment. We carefully edit the revised manuscript. And we also asked one of the professional editing companies suggested by the editor, American Journal Experts, to check errors in our revised manuscript. We corrected all the errors accordingly throughout the manuscript. (Whole manuscript)

3 References and typesetting were corrected.

Thank you again for your careful handling of our manuscript and kind consideration on publication in the *World Journal of Biological Chemistry*

Sincerely yours,
Atsushi

Atsushi Kaneda, M.D., Ph.D.
Department of Molecular Oncology
Graduate School of Medicine, Chiba University
Inohana 1-8-1, Chuo-ku, Chiba-City
260-8670 Japan
Fax: +81-43-226-2039
E-mail: kaneda@chiba-u.jp