Peer Review File

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Reviewer A

Comment 1: Endothelial layer was thin, one layer in normal vascular wall. The number of the endothelial cells are not larger in the endothelial layer than that of the smooth muscle cells. Endothelial cells after vascular injury are considered to drop out from vascular wall and cause apoptosis because of strong inflammation. You would like to show the injured endothelial cells proceed to EndMT process without cell death. Could you explain the mechanism of escaping from cell death and proceed EndMT? Could you investigate the acute fate of endothelial cells?

Reply 1: Thanks for your constructive suggestion. EndMT and apoptosis are fundamental physiological processes [1]. A large number of studies showed that some risk factors, including TGF-β1 and oxidative stress, not only promote EndMT but also simultaneously induce apoptosis [2]. Furthermore, the study indicated that TGF-β1 induced EndMT or apoptosis were closely related with the cell cycle stage. Apoptosis was induced mostly in cells at G2/M phase, whereas EndMT was only induced in cells at G1/S phase [2]. The ultimate goal of the stimulus is to cause the apoptosis of endothelial cells, while EndMT is the early step of this death process [3]. These results suggest that there is a deep correlation between the regulation of EndMT and apoptosis.

In the face of acute injury, vascular endothelial cell death can be classified into pyroptosis, apoptosis and necrosis, etc [4]. Recent evidence indicated that pyroptosis is a form of inflammatory programmed cell death and is critical for cardiovascular disease [5,6]. Another group in our laboratory has also been investigating the regulatory effect of HDAC3 on endothelial cell pyroptosis. The results will be published in another journal in the future.

Comment 2: Do you discuss the origin of smooth muscle cells in the neointima after vascular injury? The smooth muscle cells might proliferate and migrate after injury. Please evaluate the relationship smooth muscle cells proliferation / migration and EndMT? What do you think

EndMT process play a role after the vascular injury?

Reply 2: Thanks for your comments and suggestion. Cooley BC *et al.* found that endothelial-derived cells contribute to neointimal formation through EndMT using *in vivo* murine cell lineage–tracing models. At day 0 to 14 after vein graft remodeling, endothelial-derived cells were gradually expressed VSMC markers, indicating that endothelial cells acquired a smooth muscle cell–like phenotype. By day 14 to 35, it almost did not detect expression of endothelial-specific markers in these neointimal endothelial-derived cells [7,8]. These endothelial-derived cells failed to develop a fully "mature" smooth muscle cell phenotype although they lost endothelial phenotype and acquired smooth muscle cell–like properties [7]. Therefore, we think that EndMT and smooth muscle cell proliferation/migration play different roles in different stages of the neointima after vascular injury. In the early stages of vascular remodeling, endothelial cells were transformed into smooth muscle cells. However, the proliferation and migration of smooth muscle cells play the major role in the advanced stages of neointimal hyperplasia.

Comment 3: Inflammatory response was considered important. Could you investigate expressions of inflammatory cytokines in the injured arteries treated with/without RGFP966 by qPCR?

Reply 3: Thanks for your good advice. We added new experiment about the expression of inflammatory cytokines IL-1β and IL-18 in the injured carotid arteries of mice treated with/without RGFP966 by quantitative real-time PCR.

Changes in the text: The detailed information was added in the Abstract and Results section (page 3 lines 50-51, page 3 lines 58-59, page 13 lines 262-265). Moreover, the results were shown as Figure 5B and 5C, and the figure legends were added in the revised manuscript (page 27 lines 580-582). In addition, the primer sequences of mouse IL-1β and IL-18 were also added in Table1.

Comment 4: Please perform immunostaining with CD31 and SMC actin on the in vivo specimens. Endothelial cells and smooth muscle cells should be identified and discussed in the

treatment with RGFP966.

Reply 4: Thanks for your good suggestion. Additional experiments were performed to detect the fluorescence intensity of endothelial cell marker CD31 and smooth muscle cell marker α-SMA in the hyperplastic intima of mice.

Changes in the text: The detailed information was added in the Abstract and Results section (page 3 lines 48-50, page 13 lines 269-273). Moreover, the results were shown as Figure 5F-H and the figure legends were added in the revised manuscript (page 27 lines 582-584).

Reviewer B

Comment 1: The title is very much overstated. It reads as a conclusion about neointimal hyperplasia. However, there is no any posttranslational modification data from the artery samples. In fact, even in the literature there is no evidence HDAC3 is an eraser of crotonylation.

Reply 1: Thanks for your insightful comments. We added new experiment about the changes of acetylation and crotonylation levels of neointimal hyperplasia in mice by immunofluorescence staining. Crotonylation is a recently described post-translational modification that occurs at multiple identified histone lysine crotonylation sites [9]. Accumulating studies suggest that class I histone deacetylases (HDACs), including HDAC1, HDAC2, and HDAC3, are major executors of histone decrotonylation [9-14].

Changes in the text: The detailed information was added in the Results and Discussion section (page 14 lines 294-299, page 17 lines 357-359, page 18 lines 372-373). Moreover, the results were shown as Figure 6F-I and the figure legends were added in the revised manuscript (page 28 lines 599-603).

Comment 2: There is no data either on EndMT from artery samples, therefore, at best the conclusion about neointima is that RGFP966 reduced neointima, which has been published by other. Therefore, using cell culture data to make conclusion about neointima can be misleading.

Reply 2: Thanks for your good suggestion. Additional experiments were performed to elucidate the effects of RGFP966 on EndMT in the carotid arteries of mice. First, the immunostaining of

EndMT makers CD31 and α-SMA was performed in the hyperplastic intima of mice. Then, the expression levels of endothelial markers (VE-cadherin, vWF) and mesenchymal markers (SM22α, FSP-1) in the carotid arteries were determined by quantitative real-time PCR.

Zhang M *et al.* performed the balloon injury model of left carotid artery in Sprague-Dawley rats (300 to 350 g), and they found that RGFP966 (2 mg/rat) treatment for 14 days slightly decreased lumen size of neointimal hyperplasia, although the changes were not statistically significant [15]. In our present study, C57BL/6J mice underwent the ligation of the left carotid arteries. Meanwhile, the mice were subjected to RGFP966 (10 mg/kg) for one day before surgery and then for 14 days after surgery. We found that RGFP966 treatment significantly alleviated neointimal hyperplasia compared to the vehicle-treated group. We speculate that the different effects of RGFP966 on neointimal hyperplasia in rats (5.7-6.7 mg/kg) and mice (10 mg/kg) may be related with the dose of RGFP966 treatment.

Changes in the text: The detailed information about CD31 and α-SMA immunostaining was added in the Abstract and Results section (page 3 lines 48-50, page 13 lines 269-273). Moreover, the results were shown as Figure 5F-H and the figure legends were added in the revised manuscript (page 27 lines 582-584). The detailed information about the expression levels of endothelial and mesenchymal markers was added in the Results section (page 13 lines 265-269). Moreover, the results were shown as Figure 5D-E and the figure legends were added in the revised manuscript (page 27 lines 580-582). In addition, the primer sequences of endothelial and mesenchymal markers were also added in Table1.

Comment 3: mRNA evidence from artery samples at best is from the mixed cells of the artery, conclusion cannot be made for endMT. For examples, aSMA can be expressed by many cell types in the artery, smooth muscle cell, fibroblasts, and even immune cells. To gain in vivo evidence, please perform immunohistochemistry for endMT markers.

Reply 3: Thanks for your good advice. Additional experiments were performed to detect the fluorescence intensity of endothelial cell marker CD31 and smooth muscle cell marker α-SMA in the hyperplastic intima of mice.

Changes in the text: The detailed information was added in the Abstract and Results section

(page 3 lines 48-50, page 13 lines 269-273). Moreover, the results were shown as Figure 5F-H and the figure legends were added in the revised manuscript (page 27 lines 582-584).

Comment 4: Making a conclusion by using only two markers (CD31 and SMA) is premature. Please determine at least E-cadherin, vimentin, sm22, Zeb2, and fibronectin. These markers together define EndMT.

Reply 4: Thanks for your advice. To further clarity the effect of HDAC3 on EndMT in the carotid arteries of mice, the expression levels of endothelial markers (VE-cadherin, vWF) and mesenchymal markers (SM22α, FSP-1) was added by quantitative real-time PCR.

Changes in the text: We have added the relevant detailed description about the expression levels of endothelial and mesenchymal markers in the Results section (see Fig 5D-E, page 13 lines 265-269). Furthermore, the figure legends of Fig 5D and 5E were added in the revised manuscript (see page 27 lines 580-582). In addition, the primer sequences of endothelial and mesenchymal markers were also added in Table1.

Comment 5: If available, can you quantify the immunofluorescence density for figure 2E and 4A by Image J to support your data due to the unequal background. In addition, the scale bar was not showed in these images.

Reply 5: Thank you for this suggestion. We performed the quantification of mean fluorescence intensity in Figure 2E and Figure 4A by Image J software. We are so sorry not to clearly show the scale bar in Figure 2E and Figure 4A. The scale bar was located in the right lower side of images by fluorescence microscope. However, the scale bar was not very obvious, it was enhanced in the revised manuscript and the scale bar was also described in the figure legends of Figure 2E and Figure 4A.

Changes in the text: The quantification results of mean fluorescence intensity were added in Figure 2F and Figure 4B, respectively. Meanwhile, the result description and figure legends were also added in the revised manuscript (page 11 lines 222-224, page 12 lines 247-249, page 26 lines 543-544, page 27 lines 565-567).

Comment 6: It is interesting finding that HADC3 inhibitor rescues the expression of histone 3 acetylation and crotonylation in vitro. The researchers also performed neointimal hyperplasia using ligation of the left carotid arteries to demonstrated the HDAC3 selective inhibitor RGFP966 suppress EndMT in vivo. However, the authors did not provide in vivo evidence of immunostaining data to support their conclusion on either histone modification or EndMT.

Reply 6: Thanks for your constructive suggestion. To further clarify the effects of HDAC3 selective inhibitor RGFP966 on EndMT by histone modification in mice, new experiments were added. First, additional experiments were performed to detect the fluorescence intensity of endothelial cell marker CD31 and smooth muscle cell marker α-SMA in the hyperplastic intima of mice. Then, we added new experiment about the changes of acetylation and crotonylation levels of neointimal hyperplasia in mice by immunofluorescence staining.

Changes in the text: The detailed information about the mean fluorescence intensities of CD31 and α-SMA were added in the Abstract and Results section (page 3 lines 48-50, page 13 lines 269-273). Moreover, the results were shown as Figure 5F-H and the figure legends were added in the revised manuscript (page 27 lines 582-584).

The detailed information about the changes of acetylation and crotonylation levels of neointimal hyperplasia was added in the Results and Discussion section (page 14 lines 294-299, page 17 lines 357-359, page 18 lines 372-373). Moreover, the results were shown as Figure 6F-I and the figure legends were added in the revised manuscript (page 28 lines 599-603).

Comment 7: Histone crotonylation is a novel identified epigenetic modification that regulates gene expression. In the study, did the researchers have other evidence to discover what is the role of histone crotonylation in the mechanism of HADC3 regulating EndMT.

Reply 7: Thanks for your good question. It is well established that EndMT is modulated by multiple integrative signaling pathways including TGF-β/Smad, BMP, Wnt/β-catenin and Notch. All these pathways converge and induce the expression of transcription factors, such as Snail, Slug, Twist, and promote or inhibit EndMT [16]. In the present study, we found that HDAC3 regulated EndMT by histone crotonylation modification. Therefore, we speculate that

HDAC3 alters histone crotonylation in the promoter, and regulates the transcription of EndMT-related signaling pathways and genes. Further studies are ongoing to identify histone crotonylation levels of EndMT-related signaling pathways that HDAC3 participates in neointimal hyperplasia.

Comment 8: In the figure 6, the researchers should clearly describe the lysine sites of histone acetylation or crotonylation you detected in the experiment. The quantified data of figure 6c and 6d was not showed. Please add it to your manuscript.

Reply 8: Thanks for your good suggestion. In this study, pan-anti-acetylated lysine and pan-anti-crotonylated lysine antibodies were used for the detection of histone acetylation or crotonylation, and all the lysine sites of histone acetylation or crotonylation are included in the images. Since the pan-anti-acetylated and pan-anti-crotonylated lysine antibodies were used, the acetylation or crotonylation of proteins just appeared changes in some sites by Western blotting, so we don't quantify data of Figure 6C and 6D. In the future study, site-specific antibodies will be used and the protein expression levels will be quantified.

Minor concerns:

Comment 1: If available, please enlarge the size of symbols such as "*" or "#".

Reply 1: Thanks for your good advice. The size of symbols "*" or "#" has been enlarged in the Figures.

Comment 2: It was a little confused about the Y axis of figure1c, 1d, 2c, 2d, 3b, 3d, 4c and 4d between western blotting and real-time PCR experiments. My suggestion is that "mRNA level" or "protein level" should be more obvious than before if you revise.

Reply 2: Thanks for your constructive suggestion. The Y axis of Figure1C, 1D, 2C, 2D, 3B, 3D, 4C and 4D has been modified to mRNA level or protein level, respectively.