

1. Reviewer (03478635)

The manuscript describes about an important finding in which ALDH activity distinguishes differentiation capacity of mesenchymal stem cells. Please carefully check through the following and the whole manuscript again.

1. In Cell differentiation and immunofluorescence staining subsection of method section, **culture days** for the adipogenic and osteogenic differentiation of ADSCs may be added.

→Following phrases were added (indicated in red).

Briefly, for adipogenic differentiation, cells ( $3 \times 10^3$ /well) were cultured at 37 °C in a 5% CO<sub>2</sub> atmosphere in a 96-well plate in 100 µl adipogenic differentiation medium composed of  $\alpha$ -minimal essential medium ( $\alpha$ MEM) supplemented with 10% FBS, 1% PSM, l-glutamine, and 50 µl adipogenic supplement containing hydrocortisone, isobutylmethylxanthine, and indomethacin for **15 days** in 37 °C and a 5% CO<sub>2</sub> atmosphere. For osteogenic differentiation, cells were cultured in osteogenic differentiation medium composed of 5 ml  $\alpha$ -MEM basal medium and 250 µl osteogenic supplement containing ascorbate-phosphate,  $\beta$ -glycerolphosphate, and recombinant human bone morphogenetic protein-2 for **15 days** in 37 °C and a 5% CO<sub>2</sub> atmosphere.

2. In Cell differentiation to adipocytes and osteocytes subsection in result section, Fig.2B seems to indicate adipogenic differentiation, although it is described as osteocytes in the text. Please carefully check them.

→The figure numbers were confirmed and modified.

ADSCs that differentiated into adipocytes appeared as accumulated lipid droplets in the cytosol in each ALDH<sup>Hi</sup> and ALDH<sup>Lo</sup> subpopulation (Fig. 2A-B). Furthermore, immunofluorescence staining for osteopontin revealed that ADSCs that differentiated into osteocytes appeared as accumulated granules in the cytosol in each ALDH<sup>Hi</sup> and ALDH<sup>Lo</sup> subpopulation (Fig. 2C-D).

3. In conclusion section, the description about ribosome should be completed.

→The sentence was completed as follows.

'Ribosome biosynthesis is suggested to be a remarkable difference between ALDH<sup>Hi</sup> and ALDH<sup>Lo</sup> subpopulations.'

2. Reviewer (01047169)

This manuscript newly identified ALDH activity as a marker for adipose-derived stem cells subpopulation with higher potential of differentiation into adipocytes and osteocytes. Although ALDH activity was implicated in other stem cells and cancer cells, it was not reported in adipose-derived stem cells. Therefore, this information can provide better understanding and application.

However, there are some points to be revised.

1. Please check whether the indication of figures are right and enough at the present form. On page 14, in the middle. "ADSCs that differentiated into adipocytes appeared as accumulated lipid droplets in the cytosol in each ALDH<sup>Hi</sup> and ALDH<sup>Lo</sup> subpopulation (Fig. 2A-B?). Furthermore, antiosteopontin(?? It's not clear) immunofluorescence staining revealed that ADSCs that differentiated into osteocytes appeared as accumulated granules in the cytosol in each ALDH<sup>Hi</sup> and ALDH<sup>Lo</sup> subpopulation (Fig. 2C-D?)"

→It is rephrased as following;

After *in vitro* differentiation, immunofluorescence staining for FABP4 (marker of adipocytes) and *immunofluorescence staining for osteopontin (marker of osteocytes)* were performed (Fig. 2A–D). ADSCs that differentiated into adipocytes appeared as accumulated lipid droplets in the cytosol in each ALDH<sup>hi</sup> and ALDH<sup>lo</sup> subpopulation (Fig. 2A–B). Furthermore, *immunofluorescence staining for osteopontin* revealed that ADSCs that differentiated into osteocytes appeared as accumulated granules in the cytosol in each ALDH<sup>hi</sup> and ALDH<sup>lo</sup> subpopulation (Fig. 2C–D).

2. There seem some missing sentences in the last part of page 18. “Ribosome biosynthesis is” What?

→ *The sentence was completed as follows.*

*‘Ribosome biosynthesis is suggested to be a remarkable difference between ALDH<sup>hi</sup> and ALDH<sup>lo</sup> subpopulations.’*

3. Figure 2 and 3 can be combined. In that way, it is easier to understand the data.

→ *Figure 2 and 3 were combined.*

4. In Figure 2B, the cell numbers are too small, therefore it is difficult to compare the differentiation efficiency.

→ *An average of multiple visual fields were shown as a graph (Fig. 2E) with statistical significance.*

In addition, investigation on at least two markers per each cell-type differentiation is recommended to make sure.

3. Reviewer (00504800)

The authors use ALDH to sort an enriched stem cell population from murine ADSC. While by itself this is not terribly exciting, it does provide additional potential evidence between human and murine MSC, and perhaps between ADSC and other MSC sources. Of note, the finding of increased ribosome-related gene expression is potentially the most interesting finding of the manuscript, given recent interest in this topic, although this requires more discussion.

→ *Following statement was added to discussion.*

*We have few reports about relationship between ribosome biogenesis and MSCs.*

*One of these reports presented one of core proteins of 60S ribosome is necessary for differentiation of osteocyte from MSCs [18].*

Items to address:

The Abstract is brief and choppy. Gene set analysis is not mentioned in the Abstract methods, nor is the finding that ribosome-related genes have increased expression mentioned in the Abstract conclusion - I would think these are important to note in the Abstract.

→ *In conclusion section of abstract, we added following sentence.*

*Additionally, we suggested the importance of ribosome for differentiation of ADSCs by gene set enrichment analysis.*

The Methods can be shortened, particularly when referring to a product and stating that "the manufacturer's instructions were followed".

→ *We shortened them by deleting several statements. Such as following;*

*This was followed by incubation for 30 min at 37 °C, and cells were centrifuged and resuspended in 0.5 ml assay buffer.*

There are more papers now available on the topic of ribosome biogenesis and the regulation of stem cells. The authors spend only one short paragraph on this in the Discussion. Since this is potentially the most interesting finding of the manuscript (it already being known that ALDH can be used to sort/enrich various stem cell subsets), I would recommend that the authors enhance their discussion of stem cell regulation by ribosomes.

→ *Following statement was added to discussion.*

*We have few reports about relationship between ribosome biogenesis and MSCs.*

*One of these reports presented one of core proteins of 60S ribosome is necessary for differentiation of osteocyte from MSCs [18].*

In the final conclusion, the last sentence was cut off - please correct this.

→ *The sentence was completed as follows.*

*'Ribosome biosynthesis is suggested to be a remarkable difference between ALDH<sup>hi</sup> and ALDH<sup>lo</sup> subpopulations.'*

#### 4. Reviewer (01554116)

This is a fine manuscript describing the immunophenotypic characterization of a subpopulation of adipose-derived mesenchymal stem cells that present genomic and functional characteristics.

The work is well designed and performed, and the conclusions are supported by the results. The final phrase in the Conclusion appears to be cut in the middle of the sentence.

→ *The sentence was completed as follows.*

*'Ribosome biosynthesis is suggested to be a remarkable difference between ALDH<sup>hi</sup> and ALDH<sup>lo</sup> subpopulations.'*

#### 5. Reviewer (02445899)

Many thanks for the opportunity to review the article of Itoh et al. This is a very interesting and well executed piece of work, with suitable controls. An additional marker for a sub-population of ADSCs with preponderance to osteogenic and adipogenic differentiation is described and discussed in some detail, which adds to the current literature and knowledge about the heterogeneity of SC populations in general.

The abstract requires some attention to the English, but otherwise the article is clearly written.

I recommend that the article be published.

→ *Thank you.*

#### 6. Reviewer (01851506)

Comments to the authors General comments Itoh and colleagues have addressed the potential difference in differentiation between aldehyde dehydrogenase (ALDH)high subset and ALDHlow subset in the murine adipose-derived stem cells (ADSCs). They found that ALDHhigh subset has a strong propensity to differentiate into adipocytes and osteocytes as compared to ALDHlow subset accompanying upregulation of the genes relevant to the protein synthesis. Although the data are potentially interesting, there are several concerns.

(1) While they show the ALDHhigh subset in Figure 1A, ALDHlow subset is not indicated. This makes it impossible to review the results that they present here.

→ *ALDH<sup>lo</sup> is now indicated with tetragons (Fig. 1 A).*

(2) Though the authors use the mean, t-test, and one-way ANOVA for statistics, how they are sure that the data with which they deal are "parametric"?

→ *It is not possible to determine these data are parametric or non-parametric because of the limited sample size.*

*Following paper performed t-test for area ratio comparison (same experiment as we did). Tumour blood vessel normalisation by prolyl hydroxylase inhibitor repaired sensitivity to chemotherapy in a tumour mouse model (Koyama et al., Scientific Reports, 2017). So, we think it's not unusual to choose t-test.*

*However, even when we used Mann–Whitney U test (non-parametric) instead of t-test, statistical significance was confirmed. It's might be same when we choose Kruskal–Wallis H test instead of one-way ANOVA. So, conclusion is same regardless of parametric or non-parametric test we use.*

(3) While the authors report the difference in differentiation potential between ALDH<sup>high</sup> subset and ALDH<sup>low</sup> subset through visualization with the differentiation markers such as FABP4 and osteopontin (Figure 3), they do not observe any difference in transcripts pertinent to the adipocytes and/or to the osteocytes except for those implicated in the protein synthesis in the Gene Set Enrichment Analysis (Figure 4). This should be explained.

*→We agree with that it is an important question. We evaluated the expressions of differentiation markers after differentiation induction. However, GSEA was performed without differentiation induction. That is why we didn't find remarkable upregulation of osteogenic or adipogenic differentiation related genes.*

(4) Data are poor to support the conclusions of the authors.

Minor points

(1) English needs to be polished.

*→English was edited by a professional language editor.*

(2) Materials and Methods section should be more concise.

*→We shortened them by deleting several statements.*

(3) What stands for " cell proliferation rate" in Figure 1B?

*→It is data of WST-8 assay. Absorbance of wavelength 490nm correlates with cell numbers. If the cells proliferates a lot, absorbance will enhanced.*

7. Reviewer (02446280)

The paper is well written and provides comprehensive data on the ALDH activity usefulness for murine cells.

*→Thank you.*

8. Reviewer (00609371)

The concerns are:

1) how does the cell renewal ability correlate with ALDH activity?

*→We didn't assess the self-renewal ability. Knockout of ALDH1A1 is reported to elicit no detectable hematological abnormality, however, ALDH2 knockout is reported to develop aplastic anemia. It is unclear ALDH2 directly involves to self-renewal, however, at least indirectly involves the maintenance of hematopoietic stem cells. (Garaycoechea et al., Nature, 2012)*

2)does this has anything to do with constitutive vs inducible form of ALDH?

*→ALDEFLUOR reagent detects both forms of ALDH. So, we can't distinguish them from our current data.*

3) what's the specific clinical implication of this study?

*→One of the current issues of cell-based therapy using ADSCs is response rate to the therapy. Several explanations might be available, however, we are focused on heterogeneity of ADSCs. If there is (or are) subpopulation(s) of enhanced differentiation*

*ability, analysis of the subpopulation will be a key to provide more effective therapy. Or, a direct application of that subpopulation to therapy might be a candidate of more efficient therapy.*

4) Adherent ADSCs from passage 4 were used, why? is it possible to use the freshly isolated cells?

*→ We used ADSC of passage 4 because expanded cell number was needed for the experiments. We considered that freshly isolated ADSCs were not applicable because of limited cell number, contamination of erythrocyte and debris etc.*

5) the final conclusion is incomplete

*→ The sentence was completed as follows.*

*'Ribosome biosynthesis is suggested to be a remarkable difference between ALDH<sup>Hi</sup> and ALDH<sup>Lo</sup> subpopulations.'*