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Regulation of Human Lung Alveolar Multipotent Cells by a novel p38 MAPK/miR-17-92 axis

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 11 May 2012

Thank you very much for submitting your research paper on the identification of uniquely marked human lung stem cells for consideration to The EMBO Journal editorial office.

Please find enclosed two sets of complementary reports that outline the potential novelty and thus general interest in these findings, but at the same time raise crucial concerns on the current level of their characterization, at least compared to recent publication on this topic.

Given their explicit comments and based on our earlier communication, we are convinced that both the experimental as well as some presentation issues could be addressed within the timeframe available for one round of major revisions.

I am thus happy to offer you the chance to amend and develop the presented dataset according to the constructive suggestions from our referees. I urge you particularly to present both the experimental part carefully outlining the used methods and expand the comparative analysis with regard to earlier proposed lung stem cell markers to avoid misunderstandings during final scientific assessment from our valued referees.

Finally, I do have to formally remind you that The EMBO Journal considers only one round of major revisions with the ultimate decision solely depend on content and strength of your revised manuscript.

I am very much looking forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

General Comments:
The authors found that E-cadherin/Lgr6 double positive cells in human lungs had a potential to act as a lung endogenous stem cell. They also reported that the p38-alpha pathway through p53 activation and miR-17-92 regulation played a role in homeostasis of the lung stem cells. The most important advantage of this work is the newly identified cell surface markers of human lung stem cells. Because previous reported lung stem cells do not have a specific surface marker, this authors' work would open up a new direct isolation approach to evaluate the human lung stem cells.

Major Concerns:
1. Since human lung tissues were utilized in this study, the authors must state the ethical issues including informed contents provided from the patients. In addition, patients' characteristics should be presented.

2. The authors called the E-cad+/Lgr6+ cells as "lung alveolar multipotent cells (in Title)", "human lung stem cells (HLSC) (page 5)", and "human lung progenitor cells (page 13)". The name should be unified. Because differentiation into bronchial and alveolar epithelial phenotypes are only demonstrated in this manuscript, the E-cad+/Lgr6+ cells are not "alveolar multipotent" nor "lung stem", and "lung progenitor" would be the most applicable name.

3. Distribution of the E-cad+/Lgr6+ cells in normal lung tissue is not clear (Figure 1 and Suppl. Figure S1). In the left panel of the Figure 1A (the low magnification image), several yellow colored cells are present in alveolar walls. In addition, patchy distribution of Lgr6-positive cells (red color) are observed in alveoli in Supplemental Figure 1F. These observation suggest that some alveolar epithelial cells also express Lgr6. Please provide more clear images of the immunofluorescent staining. HE staining of the sequential mirror section of the same tissue shall be informative to understand the distribution of the stem cells in the peripheral lungs.

4. In Figure 2B, mRNA expression in the clonal cells are compared with that of lung tissues. However, whole lung tissue is a mixture of epithelial, endothelial, and mesenchymal cells; therefore, the aim of this comparison is not clear. The authors should compare the mRNA expression with the freshly isolated E-cad+/Lgr6+ cells.

5. Human lung explants were cultured and treated with bleomycin (Figure 3A-C and supplemental Figure S3A-B). However, there is no description of this study in the Methods section, therefore, the reviewer cannot evaluate these results.

6. Images of Figure 3D and 3E are too poor to determine the co-expression of epithelial markers with GFP. More clear confocal images are necessary. In addition, the authors should isolate GFP-positive cells from the lungs and analyzed by FACS with epithelial markers. It will give more clear idea of differentiation from the E-cad+/Lgr6+ cells.

7. E-cad+/Lgr6+ cells express SP-C on laminin-coated dish, suggesting differentiation into alveolar type II cells. Alveolar type II cells are well known to differentiate into type I-like cells after 5-7 days' culture. If the E-cad+/Lgr6+ cells are true lung stem cells and provide completely differentiated type II cells, can further differentiation into type I be observed?

Minor Comments:
1. "HLSC" should be spelled out at the first use.

2. Methodology for the clonal assay (Figure 1F), such as a culture condition, is missing.

3. Figure 1A. Since immunofluorescent images were taken with a confocal microscope, images of x-y and y-z axes should be shown to confirm the co-localization of Lgr6 and E-cadherin.

4. Figure 1E. The demonstrated gate (a red rectangle) in the Lgr6/E-cad scattergram contained both E-cad-negative and E-cad-positive population; therefore, the arrow indicates not only E-cad+/Lgr6+ but also E-cad-negative cells.

5. In the figure legend of Figure 1 (Page 21, line 12), (E) should be (F).

6. In page 8, line 10. "Fig. 4D" should be " Fig. 4G".

7. Bleomycin-treated mice were sacrificed at 5 days after instillation in the text (page 6), but analyzed after 10 days in the Methods section (page 16). Which is correct?

Referee #2:

This article by Oeztuerk-Winder et al. describes the isolation of putative human lung stem cells and identifies miR-17-92 as a regulator of their proliferative capacity. One concern is that the in vitro and in vivo analyses have been performed with cultured cells. This in vitro step may have induced changes and therefore these cells may not represent lung stem cells. In addition, the in vivo experiments are not highly convincing. In the human lung explants treated with bleomycin model (not described in the methods), are the cells proliferating and differentiating? Cell fusion with the endogenous cells cannot be ruled out and may occur in this setting. Similarly in the mouse bleomycin injury model, fusion with mouse cells could occur. The animals were sacrificed 5 days after the injury. This is a very early time point when no repair has yet occurred. Are the cells participating in repair and are human cells migrating to the lung regions with bleomycin-induced fibrosis?

The first part of the paper describes the in vitro and in vivo capacity of E-Cadh/Lgr6+ human lung cells. The expression of these markers was analysed after 2 days of culture of human lung cells. This culture may have affected the expression of cell surface markers. What is the FACS profile for these markers in freshly isolated tissue? Only a small proportion of the Lgr6+ cells are actually E-cadherin+. Are there any differences in behavior between E-Cadh+Lgr6+ vs ECadh-/Lgr6+ cells? Previous work by Kajstura et al. (NEJM 364, 2011) described c-kit as a marker of putative human lung stem cells. Are E-Cadh/Lgr6+ positive or negative for c-kit?

In Figure 1 F, was the clonogenic capacity similar between the 3 different patients? What does the Table represent? Distinct values for the different lung samples would be important to confirm that this observation was not specific to one patient or a pool of the three lung samples used.

The experiments presented in Figures 2, 3 and 4 present data regarding the Lgr6+ cells. Have similar experiments been conducted with Lgr6-negative cells as a comparison of their clonogenic capacity, gene expression profile, and in vivo repair capacity?

There are discrepancies between the level of expression of CC10 and Sp-C described in Figure 2A and the RNA expression presented in Figure B. Do the cultured cells express CC10?

What are the experimental details of the results presented in Figure 4G? Were the cells transplanted from lung to kidney? The methods indicate digestion of mouse lungs and then kidney capsule transplants. Were human and mouse cells mixed together or were the GFP-positive cells sorted? The small bronchiole CC10+ cells presented in Figure 4G are mainly GFP-negative so one wonders about the role of the HLSC in these transplants. More details on the serial transplantation analysis should be presented.

What are the level of p38 and mir17-92 expression in Lgr6+ compared to Lgr6- cells? Are these specific to the putative HLSCs?

1st Revision - Authors' Response 20 June 2012

We would like to thank the reviewers for their comments on our manuscript. We think that those comments have helped us to improve the strength of the manuscript and the discoveries contained in it.

We have addressed all the concerns of the reviewers adding new material and improving others. We have now included more information about the ethical issues with the human samples and as much as we have about the patients that we have access.

We have now added new images, clearly showing the E-Cadherin/Lgr6 co-expression, and the differential cellular expression with other markers as c-Kit and Integrin-alpha6. New flow cytometry plots show the distinct expression of c-Kit and Lgr6. Images showing further differentiation and cellular morphology have also been included.

Results from comparative analyses, in mRNA expression and functional capacity have also been added, using freshly isolated E-Cad+/Lgr6+ and E-Cad+/Lgr6-, and clonal HLSCs.

Further information about the specific techniques and experimentation have been extended in the Experimental Procedures section trying to clarify the details about the assays used to test the stem cell potential of the cells.

Finally we have corrected the mismatches between the text and figure numbers, and any other typos contained in the previous version.

I hope these additions can satisfy the reviewers concerns to allow the manuscript for publication in EMBO Journal.

Referee #1:

General Comments:

The authors found that E-cadherin/Lgr6 double positive cells in human lungs had a potential to act as a lung endogenous stem cell. They also reported that the p38-alpha pathway through p53 activation and miR-17-92 regulation played a role in homeostasis of the lung stem cells. The most important advantage of this work is the newly identified cell surface markers of human lung stem cells. Because previous reported lung stem cells do not have a specific surface marker, this authors' work would open up a new direct isolation approach to evaluate the human lung stem cells.

Major Concerns:

1. Since human lung tissues were utilized in this study, the authors must state the ethical issues including informed contents provided from the patients. In addition, patients' characteristics should be presented.

We have addressed that issue now and provide all the ethical and the available patient information we have access to from the hospital. All the samples provided come from healthy tissue biopsies used as a control from cancer patients. Samples from COPD or other inflammatory lung diseases that may produce fibrosis were avoided.

2. The authors called the E-cad+/Lgr6+ cells as "lung alveolar multipotent cells (in Title)", "human lung stem cells (HLSC) (page 5)", and "human lung progenitor cells (page 13)". The name should be unified. Because differentiation into bronchial and alveolar epithelial phenotypes are only demonstrated in this manuscript, the E-cad+/Lgr6+ cells are not "alveolar multipotent" nor "lung stem", and "lung progenitor" would be the most applicable name.

We understand that the different terms may induce to confusion. The term multipotent refers to their potential to differentiate into many cellular types. As we have just observed and analysed three cells types we could used the term tri-potent instead, but as we think these cells can still harbour other potentials we rather designate the cells as multipotent. Considering the cells “lung stem” is based in

their self-renewal and differentiation potential that we have demonstrated with serial transplantations. Progenitor cells, as it is accepted in the current literature, refers to cells with limited self-renewal and differentiation potential. We agree that may be the best denomination of this population would be bronchio-alveolar stem cells but we took the name "lung stem" as the use of the short name is more convenient, and we did not want to confuse with the BASCs population found in mice. This generalization is not unusual in other tissues, e.g. mammary or epidermis, where a number of different cells are considered stem cells although they have only limited differentiation potentials. However, as we state in the discussion we leave open the possibility of a hierarchy in the bronchioalveolar epithelium and we do not discard the existence of a population of stem cells in the human lung with a higher differentiation potential.

3. Distribution of the E-cad+/Lgr6+ cells in normal lung tissue is not clear (Figure 1 and Suppl. Figure S1). In the left panel of the Figure 1A (the low magnification image), several yellow coloured cells are present in alveolar walls. In addition, patchy distribution of Lgr6-positive cells (red colour) are observed in alveoli in Supplemental Figure 1F. These observation suggest that some alveolar epithelial cells also express Lgr6. Please provide more clear images of the immunofluorescent staining. HE staining of the sequential mirror section of the same tissue shall be informative to understand the distribution of the stem cells in the peripheral lungs.

We have now showing better images of the LGR6/E-Cad population (figures 1 and Supplemental 1 and 2). A major problem with lung tissue is the high auto fluorescence. This may have contributed to may look like different patches of yellow cells were present in the tissue. However, as we have observed in higher magnification that was just non-specific fluorescence. What we have observed by flow cytometry, and maybe is less clear in the confocal images that may have overlapping fluorescence quenching the E-Cad signal, is that all Lgr6 are E-Cad medium/low positive. All alveolar cells are E-Cad positive, so the possibility of other alveolar cells being positive for Lgr6, although plausible, is unlikely.

Human lung has a different distribution than mouse. The ratio of small bronchiolar tissue is much reduced than in mouse distal lung. What we have observed is the localization of the E-Cad/Lgr6 population is on the alveolar epithelium but always closed to bronchiolar or endothelial tissue, and always close to non-epithelial tissue. The specific cell morphology of these cells seems to be rounded and never with polygonal (as AT2 or Clara) or elongated as AT1 cells. We found the cells in pools ranging from 3 to 10-12 cells.

4. In Figure 2B, mRNA expression in the clonal cells are compared with that of lung tissues. However, whole lung tissue is a mixture of epithelial, endothelial, and mesenchymal cells; therefore, the aim of this comparison is not clear. The authors should compare the mRNA expression with the freshly isolated E-cad+/Lgr6+ cells.

We appreciate this concern and now we are addressing this point as requested. We show now the relative levels of lung specific, stem and epithelial mRNAs in freshly isolated and clonally derived cells.

5. Human lung explants were cultured and treated with bleomycin (Figure 3A-C and supplemental Figure S3A-B). However, there is no description of this study in the Methods section, therefore, the reviewer cannot evaluate these results.

We apologize for not having been more thorough in this respect and now we have included a more detailed protocol.

6. Images of Figure 3D and 3E are too poor to determine the co-expression of epithelial markers with GFP. More clear confocal images are necessary. In addition, the authors should isolate GFP-positive cells from the lungs and analysed by FACS with epithelial markers. It will give more clear idea of differentiation from the E-cad+/Lgr6+ cells.

We have now included better confocal images of the requested figures that show the co-expression of GFP and lung markers (Figures 3 and Supplemental S4 and S5). Supplemental Figure S8 (previous S6) includes Flow-cytometry analysis of lung markers from GFP cells isolated from lung

and kidney capsule engraftments.

7. E-cad+/Lgr6+ cells express SP-C on laminin-coated dish, suggesting differentiation into alveolar type II cells. Alveolar type II cells are well known to differentiate into type I-like cells after 5-7 days' culture. If the E-cad+/Lgr6+ cells are true lung stem cells and provide completely differentiated type II cells, can further differentiation into type I be observed?

This is an interesting point that we are again sorry of not have been sufficiently clear about, although Figure S9 had mRNA expression of lung markers (including AQ5), showing AT1 differentiation on laminin but not on fibronectin. As stated, part of the cells in laminin become ATI cells expressing AQ5. Now we have also added an image of AQ5 cells growing on laminin (Figure S2).

Minor Comments:

1. "HLSC" should be spelled out at the first use.

We have addressed this point.

2. Methodology for the clonal assay (Figure 1F), such as a culture condition, is missing.

Included now in Mat and Methods.

3. Figure 1A. Since immunofluorescent images were taken with a confocal microscope, images of x-y and y-z axes should be shown to confirm the co-localization of Lgr6 and E-cadherin.

We think that the improved images now clearly show the co-staining.

4. Figure 1E. The demonstrated gate (a red rectangle) in the Lgr6/E-cad scattergram contained both E-cad-negative and E-cad-positive population; therefore, the arrow indicates not only E-cad+/Lgr6+ but also E-cad-negative cells.

This confusion comes from the fact that most Lgr6+ cells are E-Cad medium/low positive. We hope that the new plot can better show this point.

5. In the figure legend of Figure 1 (Page 21, line 12), (E) should be (F).

We have addressed all the erroneous matches between text and figures.

6. In page 8, line 10. "Fig. 4D" should be " Fig. 4G".

Same as above. We thank the reviewer for this observation that skipped our attention.

7. Bleomycin-treated mice were sacrificed at 5 days after instillation in the text (page 6), but analysed after 10 days in the Methods section (page 16). Which is correct?

Apologies for the confusion. The correct time is 10 days after TVI, as stated in the Methods section.

Referee #2:

This article by Oeztuerk-Winder et al. describes the isolation of putative human lung stem cells and identifies miR-17-92 as a regulator of their proliferative capacity. One concern is that the in vitro and in vivo analyses have been performed with cultured cells. This in vitro step may have induced

changes and therefore these cells may not represent lung stem cells. In addition, the in vivo experiments are not highly convincing. In the human lung explants treated with bleomycin model (not described in the methods), are the cells proliferating and differentiating? Cell fusion with the endogenous cells cannot be ruled out and may occur in this setting. Similarly in the mouse bleomycin injury model, fusion with mouse cells could occur. The animals were sacrificed 5 days after the injury. This is a very early time point when no repair has yet occurred. Are the cells participating in repair and are human cells migrating to the lung regions with bleomycin-induced fibrosis?

The in vitro and vivo analyses have also been performed with freshly isolated and cultured cells. Only freshly sorted cells were used for clonogenic assays. All the in vivo assays (mouse bleomycin, kidney capsule, lung explants), have been performed with freshly sorted and cultured cells. The images show GFP labeled previously cultured cells, as they can be better spotted by the viewer, to prove their origin in the different host tissues and easily sorted for serial transplantations.

We have now introduced a more thorough explanation of the bleomycin model in the Material section.

Although cell fusion cannot be ruled out, it is highly unlikely this is happening, as we have used human and mouse lung tissues, besides an alien tissue as in the kidney capsule experiment. However, now we have also conducted a chromosomal analysis of the injected cells to avoid any doubt about this issue showing the normal human chromosomal composition of the GFP cells isolated from lung or kidney capsule.

As responded to the previous reviewer, I apologise for the confusion about the time in the bleomycin injury model. The correct time is 10 days, although we have analysed later time points, either in the mouse or the explant models.

The first part of the paper describes the in vitro and in vivo capacity of E-Cadh/Lgr6+ human lung cells. The expression of these markers was analysed after 2 days of culture of human lung cells. This culture may have affected the expression of cell surface markers. What is the FACS profile for these markers in freshly isolated tissue? Only a small proportion of the Lgr6+ cells are actually E-cadherin+. Are there any differences in behaviour between E-Cadh+Lgr6+ vs ECadh-/Lgr6+ cells? Previous work by Kajstura et al. (NEJM 364, 2011) described c-kit as a marker of putative human lung stem cells. Are E-Cadh/Lgr6+ positive or negative for c-kit?

As commented to reviewer one, the plot may have induced to confusion. Most Lgr6+ are E-Cad med/low and a small number E-Cad high, as it can be seen in the new-gated plot. We tested the potential of both (high or med/low) populations but not found differences in their stem cell potential. For that reason we use the whole pool.

We are also showing now flow cytometry (Supplemental Figure S2) and immnunofluorescent images (Figure 1 and Supplemental figure S2) showing that Lgr6 cells are negative for c-Kit.

In Figure 1 F, was the clonogenic capacity similar between the 3 different patients? What does the Table represent? Distinct values for the different lung samples would be important to confirm that this observation was not specific to one patient or a pool of the three lung samples used.

We are now including a table showing the clonogenic capacity of E-Cad/Lgr6+ cells isolated from four different patients (Supplemental Figure S2E).

The experiments presented in Figures 2, 3 and 4 present data regarding the Lgr6+ cells. Have similar experiments been conducted with Lgr6-negative cells as a comparison of their clonogenic capacity, gene expression profile, and in vivo repair capacity?

All assays had been also conducted with Lgr6- cells. We apologize for not having shown that data, and appreciate the reviewers concern. Now we are including those cells results in the clonogenic and kidney capsule experiments.

There are discrepancies between the level of expression of CC10 and Sp-C described in Figure 2A

and the RNA expression presented in Figure B. Do the cultured cells express CC10?

As mentioned in the text, Lgr6+ cells are not positive for SP-C or CC-10 in the tissue but they become positive in culture. We are now including new mRNA expression data to show the levels of lung markers in freshly isolated Lgr6+, Lgr6-, and clonally derived HLSCs.

What are the experimental details of the results presented in Figure 4G? Were the cells transplanted from lung to kidney? The methods indicate digestion of mouse lungs and then kidney capsule transplants. Were human and mouse cells mixed together or were the GFP-positive cells sorted? The small bronchiole CC10+ cells presented in Figure 4G are mainly GFP-negative so one wonders about the role of the HLSC in these transplants. More details on the serial transplantation analysis should be presented.

The serial transplantations were conducted with GFP sorted cells from lung or kidney capsule engraftments and then injected into the kidney capsule or tail vein.

What are the level of p38 and mir17-92 expression in Lgr6+ compared to Lgr6- cells? Are these specific to the putative HLSCs?

p38 levels (as that of other members of the MAPK superfamily, as ERKs or JNKs) are usually very constant in all cell types, and low protein levels have only been detected in tumours. Now we are showing the levels of miR-17-92 in freshly isolated Lgr6 (positive or negative), and clonal HLSCs cells. We believe this is not specific for HLSCs, although the outcome of this control may be more relevant in these cells as it influences their stem cell potential, what is not the case for differentiated cells that do not proliferate nor further differentiate. Most stem cell pathways as Wnt, Notch, or BMP, are not exclusive of stem cells, but their role is specific for cells with stem cell potential.

2nd Editorial Decision 22 June 2012

Thank you very much for the revised study that I now found the time to assess in quite some detail. Given the extensive revisions and concise responses to the points raised by the original referees, I am happy to inform you that the editorial office will soon be in touch with necessary paperwork related to official acceptance.

Please allow me to congratulate to such a fine dataset. I am very much looking forward to efficient publication.

Yours sincerely,

Editor
The EMBO Journal