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Lgr5 intestinal stem cells have high telomerase activity and randomly segregate their chromosomes

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(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 06 December 2010

Thank you very much indeed for your submission that has been seen by two expert scientists. I have further communicated with a third referee that essentially supports the views expressed by the two written statements. As such, I am in a position to reach a decision on your work to not unnecessarily delay the proceedings of your study. Both of the reports clearly emphasize the importance of the reported intestinal stem cell features. However, ref#1 also very strongly illustrates certain limits. Thus, a couple of further experiments seem necessary to make the results on cell cycle length and chromosome segregation more convincing as well as to definitely exclude the possibility of asymmetric segregation. In contrast to the referee however, I do prefer the individual presentation of the two telomere-length experiments and at this stage would not insist on more statistical (and very time-consuming) expansion. I therefore very kindly ask you to particularly attend to points 3 and 4 of ref#1's comments in corroborating your findings.
Conditioned on such expansions as well as some requested minor clarifications, we would be delighted to assess a thoroughly revised paper for eventual publication here.

Please be reminded that it is EMBO_J policy to allow a single round of revisions only and that the final decision still entirely depends on the content within the last version of your manuscript.

Yours truly,

Editor
The EMBO Journal

REFEREE REVIEWS

Referee #1 (Remarks to the Author):

Adult stem cells (SC) reside in adult tissues for extended periods of time and are responsible for tissue homeostasis and repair. The maintenance of genomic integrity is essential to sustain SC functions. The long-term persistence of SC in the adult tissues renders them at high risk of accumulating deleterious mutations that may result in cancer formation and ageing. In addition, considering the extensive self renewal capacities of intestinal stem cells, this characteristic may lead to cellular senescence in the absence of specialized mechanism protecting against telomere attrition during DNA replication. Therefore, understanding the mechanisms used by adult SC to protect their genome against replication induced mutations and telomere attrition is of great interest.

In this manuscript, Arnout and colleagues investigated whether Lgr5-positive intestinal SC exhibit higher telomerase activity and whether lgr5 positive intestinal SC are protected against mutations arising from DNA replication through asymmetrical DNA strand segregation. They showed Lgr5-positive intestinal SC presented a higher telomerase activity. Despite the higher telomerase activity, telomere length diminishes with age. Furthermore, using EdU-based staining they attempted to accurately estimate the length of the cell cycle of Lgr5+ cells (in combination with pH3), and to test the "immortal strand" hypothesis in the intestine (in combination with E-Cadherin), concluding that intestinal SC segregate their chromosomes randomly, in contrast to the recently published data from Quyn et al (Cell Stem Cell 2010).

The data presented in this work are generally of high quality. However, the data dealing with the length of the cell cycle and the chromosome segregation are relatively limited, and need to be strength before the paper will be suitable for publication in EMBOJ.

Specific comments
1. The authors claim in the abstract that "maintenance of genome integrity of Lgr5+ve adult stem cells involves telomerase expression". However, they don't show at any point in their manuscript that telomerase expression and/or activity is involved in the maintenance of genome integrity. This phrase should be removed from the abstract or experimentally tested.
2. The data of the telomere length are presented in term of relative telomere length. It would be interesting to determine to which extend the telomerase activity limit telomere attrition that would normally occurred based on the number of cell division that intestinal SC did undergo during the 5, 30, and 70 weeks of the study. Also, the errors and standard deviation between the two mice studied (which is very limited) should be shown.
3. In the experiments estimating the length of the cell cycle in Lgr5+ cells, the authors do not use a marker for SC. They should perform the same experiment using Lgr5+GFP staining. Moreover, they should confirm their results with double labelling pulse-chase experiments, using two different uridine analogs (such as CldU and IdU). Estimation of the double-labelled cells upon injection with the second analog at different time points will correspond to the cells undergoing cell cycle re-entry at this time-point.
4. The data regarding the random chromosome segregation are nicely performed but very limited, since the authors took into account only 36 cell divisions, and furthermore, they did not use a SC marker. Based on the low number of cell counted, they cannot exclude that asymmetric segregation takes place within rare subpopulation of Lgr5 positive cells. To be able to provide quantitative data, as well as to rule out the possibility of asymmetric chromosome segregation in rare intestinal SC (always to the detection limit of the assay used), the authors should first count more mitotic divisions using also a co-staining for Lgr5-GFP, and second perform FACS analysis in double IdU/CldU pulse-chase experiments, gating in the Lgr5+ population.
5. A recent paper show that immortal strand segregation may occur during DNA replication of colonic epithelial cells (Falconer E, Nature 2010). The authors should also determine Lgr5 stem cells in the colon present or not asymmetrical strand segregation.
6. The number of replicates and the statistical significance should be mentioned for each experiments displayed.

Referee #2 (Remarks to the Author):

In this short paper Schepers et al. describe results of telomerase and telomere length measurements using various cell types of the mouse intestine. They also studied the distribution of EdU in crypt

cells after a second cell division following injection with EdU.

The results show that cells at the bottom of the crypt have the highest telomerase activity and the longest telomere length, that telomeres in the crypt seem to shorten with age and that EdU seems to be randomly distributed between daughter cells.

The results are convincing and add to the extensive work on stem cells in the gut from the Clevers lab.

Minor comments

The decline in telomere length with age is remarkable and of interest. Is this only observed in cells of the gut or also in nucleated blood cells and cells of e.g. the brain? Error bars in Figure 3 should be shown and examples of flow dot plots to illustrate the differences between cell types with age should be shown.

The role of telomerase is mysterious and should be discussed more extensively in view of the fact that telomerase KO mice (without any telomerase activity) do not show a clear gut phenotype (at least not in the first generation). If telomerase is required to sustain the proposed hundreds of cell divisions in Lgr5 stem cells would the authors not have expected a gut phenotype?

The Edu experimenst do not exclude the possibility that a minority of cells show immortal strand segregation. The limitation imposed by the number of observations should be mentioned.

1st Revision - authors' response 04 January 2011

Referee #1

Adult stem cells (SC) reside in adult tissues for extended periods of time and are responsible for tissue homeostasis and repair. The maintenance of genomic integrity is essential to sustain SC functions. The long-term persistence of SC in the adult tissues renders them at high risk of accumulating deleterious mutations that may result in cancer formation and ageing. In addition, considering the extensive self renewal capacities of intestinal stem cells, this characteristic may lead to cellular senescence in the absence of specialized mechanism protecting against telomere attrition during DNA replication. Therefore, understanding the mechanisms used by adult SC to protect their genome against replication induced mutations and telomere attrition is of great interest.

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Specific comments

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A: Agreed. As this is not a central point of the study, this phrase is now removed from the abstract.

2. The data of the telomere length are presented in term of relative telomere length. It would be interesting to determine to which extend the telomerase activity limit telomere attrition that would normally occurred based on the number of cell division that intestinal SC did undergo during the 5, 30, and 70 weeks of the study. Also, the errors and standard deviation between the two mice studied (which is very limited) should be shown.

A: The initial experiments were actually not done with individual mice. For each data point, we pooled the cells from two mice before sorting. This was not clearly described in the methods-section. For each time point, we have now added two additional samples, such that we are now able to perform a statistical analysis. This is now explicitly described in the materials and methods. SEM values are now given in the new figure 3.

3. In the experiments estimating the length of the cell cycle in Lgr5+ cells, the authors do not use a marker for SC. They should perform the same experiment using Lgr5+GFP staining. Moreover, they should confirm their results with double labelling pulse-chase experiments, using two different uridine analogs (such as CldU and IdU). Estimation of the double-labelled cells upon injection with the second analog at different time points will correspond to the cells undergoing cell cycle re-entry at this time-point.

A: In two previous papers (Sato et al, 2010; Snippert et al, 2010), we have described that every cell at a crypt bottom is either an Lgr5 stem cell or a Paneth cell. In other words: every cell that touches a Paneth cell is an Lgr5 stem cell. Location adjacent to a Paneth cell can thus be used as a Stem Cell marker. Nevertheless, we have now added co-stainings for Lgr5-GFP, together with PH3 and EdU (figure 4).
To ensure that labelled cells re-enter the cell cycle, sequential double labelling has been performed with EdU and BrdU (injections separated by 21.5 hours). This reveals double-positive cells at 26.5 hrs (the second peak in figure 4), implying that –indeed- cells re-enter the cell cycle. This data is included in supplementary figure 1.

4. The data regarding the random chromosome segregation are nicely performed but very limited, since the authors took into account only 36 cell divisions, and furthermore, they did not use a SC marker. Based on the low number of cell counted, they cannot exclude that asymmetric segregation takes place within rare subpopulation of Lgr5 positive cells. To be able to provide quantitative data, as well as to rule out the possibility of asymmetric chromosome segregation in rare intestinal SC (always to the detection limit of the assay used), the authors should first count more mitotic divisions using also a co-staining for Lgr5-GFP, and second perform FACS analysis in double IdU/CldU pulse-chase experiments, gating in the Lgr5+ population.

A: More divisions have now been analysed to get a p-value of less than 0.025, which is described in the text. In 51 cells, we fail to see a single asymmetric DNA-segregation event. As argued above, location adjacent to Paneth cells is equivalent to expression of the Lgr5 stem cell marker. We have added an example in which we have performed co-staining for Lgr5-GFP (remark #3). Obviously, independent of the number of cell scored, it can never formally be excluded that asymmetric DNA-segregation occurs in a (very) small subpopulation. This is now discussed.
Of note, the original Potten paper (Potten et al, 2002) gives only an image of a single cell undergoing asymmetric segregation. Quin et al (2010) analyse a total of 46 cells, identified as label-retaining in a damage-response model.

5. A recent paper show that immortal strand segregation may occur during DNA replication of colonic epithelial cells (Falconer E, Nature 2010). The authors should also determine Lgr5 stem cells in the colon present or not asymmetrical strand segregation.

A: The Falconer-paper is very interesting because it makes elegant use of the fixed orientation of major satellite DNA on all chromosomes. It describes partial asymmetric strand segregation (we cite from the paper: "Of note, we did not observe 100% asymmetric segregation of sister chromatids in any pair of mitotic colon cells".) More importantly, their observations were not done in stem cells but randomly in any proliferative crypt cell, the overwhelming majority of which are TA cells. Both phenomena (partial segregation, in TA cells) are not the outcomes of the Immortal Strand Hypothesis of John Cairns.

We have elected to confine our study to small intestinal crypts, where Potten and Nathke studied asymmetric strand segregation previously, and where we have defined Lgr5 stem cells most extensively.

6. The number of replicates and the statistical significance should be mentioned for each experiments displayed.

A: Error bars/standard deviation are given for each experiment and statistical significance of the number of symmetrical divisions is described.

Referee #2 (Remarks to the Author):

In this short paper Schepers et al. describe results of telomerase and telomere length measurements using various cell types of the mouse intestine. They also studied the distribution of EdU in crypt cells after a second cell division following injection with EdU.
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Minor comments

The decline in telomere length with age is remarkable and of interest. Is this only observed in cells of the gut or also in nucleated blood cells and cells of e.g. the brain?

A: This is interesting but we feel that this goes beyond our study. We refer to two excellent papers that have reported similar phenomena in other tissues. The Blasco-lab gives explicit data on stem cell telomeres of different tissues and different ages (in Flores et al, Genes & Dev 2008)

Error bars in Figure 3 should be shown and examples of flow dot plots to illustrate the differences between cell types with age should be shown.

A: This has now been done. See comment to point 2 of referee 1.

The role of telomerase is mysterious and should be discussed more extensively in view of the fact that telomerase KO mice (without any telomerase activity) do not show a clear gut phenotype (at least not in the first generation). If telomerase is required to sustain the proposed hundreds of cell divisions in Lgr5 stem cells would the authors not have expected a gut phenotype?

Indeed, it is mysterious that it takes several generations before telomerase KO mice go into telomere crisis, given that Lgr5 cells go through hundreds of divisions in each generation. However, essentially the first organ to be affected is the intestinal epithelium (Lee et al 1998). We have expanded the discussion on this point.

The Edu experimenst do not exclude the possibility that a minority of cells show immortal strand segregation. The limitation imposed by the number of observations should be mentioned.

More divisions have been analysed, we now explicitly state that the possibility of asymmetric divisions in a small subpopulation can NOT formally be excluded. A statistical analysis is given.