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Skin squamous cell carcinoma propagating cells increase with tumour progression and invasiveness

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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 03 April 2012

Thanks so much for submitting your research paper on tumor propagation frequency of cancer cells of different origins for consideration to The EMBO Journal editorial office.

You will easily recognize that all referees agree on the technical quality of the work but are rather divided when it comes to their assessment on overall novelty, biological significance and further reaching molecular underpinnings. The spectrum ranges from ref#2 that does not recognize any major conceptual advance, ref#3 seeing the merits of clarifying recent inconsistencies in the field as sufficient for publication and ref#1 though recognizing these merits but at least at this stage by no means convinced that resulting questions from the experimentation have sufficiently been addressed for presentation in a molecular title such as The EMBO Journal.

With these assessments, I am afraid I am unable to invite rather straightforward revisions and feel more comfortable returning the paper to you as to decide how to proceed with the study.

As referee #1 does provide the most balanced report and also offers very constructive suggestions, I believe you would be in a strong position to address these concerns and develop the current dataset to reach the necessary level of insight for eventual publication here.

I hope you understand the rationale of this decision solely based on the rather preliminary dataset and leaving the decision up to you to either thoroughly expand OR seek more rapid publication elsewhere.

I am really sorry that I cannot be more encouraging on this stage, but I hope that clear

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communication of EMBO_Js demands and expectations might facilitate efficient further proceedings on this project.

Please do not hesitate to get in touch for further clarifications (preferably via E-mail).

Sincerely yours,

Editor
The EMBO Journal

P.S. I include here some further recommendations from one of the referee that were transmitted to me via separate E-mail:

For publication the authors will need to address as a minimum:

The role of p53 in the progression of the tumor phenotype e.g. can papilloma cells from a chemical induction on a flox-p53 background suddenly convert to cancer propagating cells upon deletion of p53. This applies to the efficiency of chemically induced tumours. In the tumours the have generated it will be possible to analyse whether they serially select for cells with a mutated p53 genotype.

Add biological significance by further characterizing the highly enriched (3rd) versus the initial (1st) populations to determine which traits are selected e.g. by expression analysis.

Analyse both CD34 low and high populations from their serial grafts in order to determine whether the observed phenotype reflects the entire population or a subset.

The characterization of the dermal cells is optional.

On a technical note, the experimental set-up used for their serial grafting might compromise the quality of the data, as single mice have been used for hosts of multiple grafts. In addition, it is not clear whether the replicates are biological (multiple tumors) or technical (individual tumors).

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REFEREE COMMENTS

Referee #1:

The manuscript by Lapauge et al. analyses how tumor progression is associated with the appearance of cells with cancer propagating properties. The authors use a number of tumor models to initiate tumor formation in the skin. The approach taken enables them to acquire data from both genetically and chemically induced benign and malignant tumors. Interestingly, the authors find that despite very robust proliferative potential in vivo and in vitro, cells from benign tumors cannot initiate secondary tumor formation without the addition of tumor stroma. Malignant tumors have on the other hand been shown to contain a tumor propagating population (Schober and Fuchs, 2011; Malanchi et al., 2008); however, the cellular identity of these cells has remained controversial. Based on limiting-dilution experiments the authors demonstrate that both CD34high and CD34low epithelial cells are capable of initiating tumors, which can be serially propagated. This is the case from both chemically induced tumors and a validated genetic model, where p53 has been eliminated and K-Ras activated (White et al., 2011; Lapouge et al., 2011). Surprisingly, the authors find that serial grafting of CD34high cells enriches for cancer stem cell properties, whereas the CD34low cells losses their propagating potential over time. The authors conclude that tumor progression is associated with the acquisition of specific cellular properties such as stromal independent growth and tissue invasiveness.

The quality of the work is excellent, but there are major concerns with respect to the novelty and the actual biological significance of the data. The manuscript contains many interesting observations, which raises additional questions that will need to be addressed.

Major concerns:

1. What is the actual read-out from the CSC experiments? The data suggest that serial grafting assays simply select for p53 loss of function. The authors will need to address whether this is the essential basis for their cancer stem cell phenotype. Their data from papillomas, chemical induced SCCs and genetic experiments could indicate this and do provide a platform to address this experimentally. The authors could chemically induce papillomas on a p53 flox'ed background and assess whether loss of p53 in cells from a papilloma is enough to induce the cancer propagating phenotype. This could be combined with analysis of cells from both genetically and chemically induced tumor to determine the p53 status.

2. The actual propagating phenotype might on the other hand be driven by something else. This is an interesting observation, but there is currently no added biological understanding of the process. The authors here have a unique opportunity to try and address, what is enriched with respect to mutations and expression patterns in multiple samples.

3. Related to this, what is the significance of CD34 expression levels and tumor initiating potential? Is there a sudden change in the global expression patterns within these populations or in the pattern of CD34 expression in the grafted material - basal versus suprabasal expression? The data provided, although this is not entirely clear, relates to the continuous propagation of either CD34low or CD34high populations. How does the serial propagation of sub-populations of tumor cells relate to the entire population (EPCAM+ve, ItgA6+ve cells), CD34high or CD34low? In addition, will CD34high cells from a CD34low initiated tumor behave like CD34low cells or CD34high cells.

4. The authors state that papillomas have propagating potential, when provided with the appropriate microenvironment. The materials and methods mention the use of markers associated with the mesenchyme, but none of this data is included in the manuscript. In light of their own findings (Beck et al., 2011, Nature), the authors speculate that the presence of a perivascular niche is responsible for the effect. They will need to demonstrate whether these cells are indeed responsible for this effect. This will also shed light on the relevance of the grafting assay as a functional read-out for CD34 expressing cells, as the authors have shown that the perivascular niche has specific effects on these cells.

Minor comments:

The authors have used a K14Cre driven model for their SCC studies, but for the majority of papilloma models they have focused on the K19Cre model. Although the data is supported by their chemical models, it is not clear, why they have chosen this approach as they are potentially targeting different populations of cells in the epidermis. This can potentially explain the differences they observe between the chemical and genetic papilloma experiments.

The authors use 3 injections per mouse for their grafting assays this is a concern if they use these as triplicate samples for the same cell type. In addition, it is not clear what constitute the replicates for the experiments. Are these technical or biological replicates.

There are no clone numbers for the antibodies used.

How does the proportion of CD34low/high cells change during serial grafting

Referee #2:

This is a carefully performed but descriptive study to look at the tumor propagation frequency of different skin squamous cell carcinoma mouse models following transplantation into several different immunocompromised recipients. Although this study helps clear up some technical issues and discrepancies in the literature about the use of the single CD34 cell surface marker to distinguish tumor propagating cells(TPCs) in these different models, there is no new mechanistic information presented about the regulation of self-renewal of the TPCs. The overall conclusions about different frequencies of these cells in aggressive GEM vs. chemically induced SCC, and the

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effects of stromal and serial transplantation are not conceptually new. There are a number of minor concerns as well:

1. The clonal evolution and CSC models need not be mutually exclusive as nicely summarized in several recent reviews. The authors should present a more appropriate Introduction.
2. Likewise, it is a mistake to conclude that if the CSC model is correct, 'killing only CSC may result in long term to the eradication of the tumours", because of issues of plasticity of bulk tumor cells, and the need to target both populations. This again has been suggested in numerous reviews.
3. There is no reason to expect that CSC frequency will correspond to growth rate of primary tumors. Instead they might correspond to response to treatment. Has this been tested in these SCC models?
4. No difference in TPC frequency with CD34 hi or lo, no difference in TPC frequency in different immune compromised mice. Both of these are clean, but negative results. What about in syngeneic recipients? The innate immune system is known to play a critical role in tumor progression and metastasis.
5. Several grammatical errors: p. 5, tumours progress; p. 6 papillomas arising, p. 7, secondary tumours

Referee #3:

In this work, Lapouge and colleagues have set to study the frequency and tumor propagating potential of different subsets of tumor cells from squamous tumors (both benign and malignant). There have been recent papers that showed discrepancies in the nature, molecular characteristics, and functional relevance of tumor initiating and propagating cells in squamous tumors. In this work, the authors now provide clear data solving some of these discrepancies, and accurately measuring the frequency of different SCC models.

Of particular interest are the following conclusions: (A) that papilloma TECs can only form secondary tumors when co-transplanted with stroma cells (this must be highlighted since previous work by Malanchi et al, did not take this into account when setting up their methodology to purify putative cancer stem cells); (B) The CD34+ and CD34- SCC TECs are equally competent to form secondary tumors when transplanted directly (i.e. without prior growth in culture); (C) Most importantly, that the two previously identified populations of tumor TECs (CD34+ and CD34- populations), can be functionally and hierarchically classified based on their differential ability to propagate the tumor in the long term. This is an important conclusion in light of a recent paper by the group of Elaine Fuchs which suggested otherwise.

Altogether this work is of great quality, and the conclusions relevant to the field. I therefore think it will be suitable not only for the epidermal field, but also for the broader fields of cancer and stem cells. I have some minor points and suggestions for the authors that when addressed could, in my opinion, strengthen some of their conclusions:

1) Fig 1C: The immunofluorescent co-staining of K5/CD34 shows that some of the CD34+ cells are not K5 positive. Are these endothelial cells? Or are there CD34 bright cells that are dim for K5? I am asking this because if one looks at the FACS profiles of alpha6 and CD34 (Fig 1A), it seems that the brightest alpha6 cells are not the brightest CD34 (or similarly, that the brightest CD34+ cells fall within the alpha6 mid cells). This is something that all of us in the field see when performing alpha6/CD34 FACS of squamous tumors, but do not really pay much attention to (considering that we normally select large clouds in our FACS profiles when sorting). Do the authors think there is a CD34bright/alpha6dim population functionally distinct from the alpha6bright/CD34+ and alpha6bright/CD34- populations? It would be interesting if they showed some comparison of the clonogenic (culture) potential of these three populations.

2) Fig 2: It would be clearer if the authors state in the text again that they are comparing the CD34+/alpha6+ and CD34-/alpha6+ populations, rather than just CD34+ vs CD34-. Otherwise it could lead to confusion that they are not using alpha6 integrin as an additional selection marker.

3) Fig 2F: there seems to be a difference in the cell output of cells originating from chemically

versus genetically induced tumors. Is this difference statistically significant?

4) In the last paragraph describing Figure 2 the authors state ¨these cells are not clonogenic on their own, and rely...¨. Perhaps it would be more accurate if the authors said ¨tumorigenic¨ instead of ¨clonogenic¨, since papilloma TECs are capable of clonogenic growth in culture, but not tumorigenic potential in vivo.

5) CD34+ TECs are more tumorigenic in the long-term than CD34- TECs. The authors suggest that this might be due to intrinsic differences in their expression signature with regards to stemness genes (specially those related to EMT). This is an interesting hypothesis which ties very well with what has been observed by the group of Weinberg, as well as other laboratories, that EMT might promote stemness. Thus EMT might be a common feature correlating to stemness in many solid tumors. However, the results equally imply that CD34+ TECs are heterogeneous, with some % expressing markers of EMT and simple epithelia (K8), and others not. Is this the case? If so, can CD34+ TECs be subdivided on the basis of intensity of CD34? For instance, are the K8+ cells shown in Fig 4E positive for CD34 (co-staining)?

Resubmission 24 October 2012

We thank the reviewer for their interesting comments and suggestions. We have addressed most of their concerns and have performed a series of new experiments that helps us to strengthen the pre-existing data, to provide many novel and very interesting findings that substantiate our initial claims.

In summary,

- To define which stromal cells are required to propagate tumour epithelial cells into immunodeficient mice, we have performed new co-grafting cells experiments. We found that both tumour endothelial cells and tumour associated fibroblasts support the propagation of papilloma tumour cells into immunodeficient mice (Figure 2H)
- To define the enrichment of tumour propagating cells in $CD34^{HI}$ and $CD34^{LO}$ populations, we have performed transplantation of all Lin-/α6+/Epcam+ TECs from DMBA/TPA SCC in immunodeficient mice and found no increase in the frequency of tumour propagating cells in any of the three fractions, although tumours from CD34Hi cells were always growing more faster than the other two populations (Figure 3D, 3H).
- To define the similarity and difference between the $CD34^{LO}$ and $CD34^{HI}$, we have assessed their ability to give rise to reform the tumour heterogeneity found in primary SCC (Figure 4G), and assessed the rate of growth of their secondary tumours (Figure 3F, 4D).
- To determine the molecular difference between $CD34^{HI}$ and $CD34^{LO}$ from DMBA/TPA and genetic induced SCC, we have assessed by quantitative RT-PCR the expression of EMT related genes known to modulate the stemness of cancer such as Twist1, Snail, Slug in both populations (Figure 3H and 4J) and found a good correlation between the expression of these genes and the tumour propagating frequency.
- To determine the plasticity and interconversion of Lin-/α6+/Epcam+/$CD34^{LO}$ cells and Lin-/α6+/Epcam+/$CD34^{HI}$ cells, transplantation experiments, were assessed the frequency of CD34 expressing cells in tumour arising from $CD34^{HI}$ and $CD34^{LO}$ (Figure 5D, E).
- To determine the role of the immune cells in regulating tumour propagating cells, we have performed transplantation experiments in syngenic (FVB/N) mouse model from DMBA/TPA induced SCC, and found no difference in the frequency of tumour propagating cells in more immunodeficient mice (Figure 7A).
- We have now clearly presented our data by separating the biological from the technical replicates (Table1).
- We have also modified our text according to the reviewer comments.

This series of new data reinforces the notion that tumour progression, invasiveness and serial transplantation increase the frequency of tumour propagating cells.
We hope that reviewers will find that our revised manuscript has now incorporated their thoughtful comments and suggestions.

Referee #1:

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The manuscript by Lapouge et al. analyses how tumor progression is associated with the appearance of cells with cancer propagating properties. The authors use a number of tumor models to initiate tumor formation in the skin. The approach taken enables them to acquire data from both genetically and chemically induced benign and malignant tumors. Interestingly, the authors find that despite very robust proliferative potential in vivo and in vitro, cells from benign tumors cannot initiate secondary tumor formation without the addition of tumor stroma. Malignant tumors have on the other hand been shown to contain a tumor propagating population (Schober and Fuchs, 2011; Malanchi et al., 2008); however, the cellular identity of these cells has remained controversial. Based on limiting-dilution experiments the authors demonstrate that both CD34high and CD34low epithelial cells are capable of initiating tumors, which can be serially propagated. This is the case from both chemically induced tumors and a validated genetic model, where p53 has been eliminated and K-Ras activated (White et al., 2011; Lapouge et al., 2011). Surprisingly, the authors find that serial grafting of CD34high cells enriches for cancer stem cell properties, whereas the CD34low cells losses their propagating potential over time. The authors conclude that tumor progression is associated with the acquisition of specific cellular properties such as stromal independent growth and tissue invasiveness.

The quality of the work is excellent, but there are major concerns with respect to the novelty and the actual biological significance of the data. The manuscript contains many interesting observations, which raises additional questions that will need to be addressed.

We thank the reviewer1 for his/her positive assessment of our manuscript and his/her very constructive comments. We have addressed his/her additional questions, which we hope help to clarify the biological significance of our data.

Major concerns:

1. What is the actual read-out from the CSC experiments? The data suggest that serial grafting assays simply select for p53 loss of function. The authors will need to address whether this is the essential basis for their cancer stem cell phenotype. Their data from papillomas, chemical induced SCCs and genetic experiments could indicate this and do provide a platform to address this experimentally. The authors could chemically induce papillomas on a p53 flox'ed background and assess whether loss of p53 in cells from a papilloma is enough to induce the cancer propagating phenotype. This could be combined with analysis of cells from both genetically and chemically induced tumor to determine the p53 status.

We thank the reviewer for this interesting question.

We agree with the reviewer comment that in the $KRas^{G12D}$ induced tumors, the loss of p53 correlated with the progression of benign papiloma to invasive SCC (supplemental figure 7 from Lapouge et al., 2011 PNAS).
In this study, we found that indeed in genetically induced skin tumours, there is a very good correlation in the frequency of tumour propagating cells and p53 status upon transplantation of primary tumours (Figure2 – $KRas^{G12D}$; Figure4 – $KRas^{G12D}$:$p53^{fl/fl}$). However, the increased frequency of tumour propagating cells during serial transplantation (Fig 6B) is unlikely to be the consequence of the p53 status, since p53 is already deleted in the primary tumours. Similarly in chemical induced tumours, p53 status is also likely to influence the frequency of tumour propagating cells in primary tumours, as p53 is well known to be lost during the progression of papilloma to invasive SCC (Kemp et al., Cell 1993), suggesting that p53 indeed might play a critical role in the ability of squamous tumour cells to propagate in the absence of stromal cells. However, like for genetically induced tumours, the increase in tumour propagating frequency upon serial transplantation of DMBA/TPA induced tumours are unlikely to be related to p53 status.

2. The actual propagating phenotype might on the other hand be driven by something else. This is an interesting observation, but there is currently no added biological understanding of the process. The authors here have a unique opportunity to try and address, what is enriched with respect to mutations and expression patterns in multiple samples.

We fully agree with the reviewer that the propagating phenotype could be due to other mechanisms than simply p53 status. We have performed, as suggested by the reviewer, transcriptional profiling of $CD34^{HI}$ and $CD34^{LO}$ cells over serial transplantations. However, these analyses did provide (too) many putative candidates that might regulate the frequency of tumour propagating cells. Clearly, further functional experiments will be required to test the functional relevance of the genes that are differentially expressed in the $CD34^{HI}$ and $CD34^{LO}$ cells during serial transplantations. We believed that publishing these data at this stage without any functional experiments will add very little to our understanding and might just confuse the readers. The functional experiments might take years before completion and we feel that these experiments are beyond the scope of this current paper. We think however that these data, although descriptive, are really important for the field and will be very useful for those working with these tumour propagating assays as it established the standard for future more mechanistic experiments. Of note, we tried for more than 6 months and grafted dozen and dozen mice before realizing that tumour cells from papilloma need support from the stromal cells to propagate into immunodeficient mice.

In the revised manuscript, we have also added new experiments in which we show that tumour propagation is correlated with expression of genes related to EMT (Twist1, Snail1, Slug)(Figure 3H, 4J). Interestingly, in the most aggressive skin tumour model ($KRas^{G12D}$:$p53^{fl/fl)}$ these genes, which have been shown to regulate stemness in cancer cells (Mani et al., Cell 2008), are enriched in the $CD34^{HI}$ population that has the best propagating property in serial transplantation (Figure 6B). Of course, further functional experiments are required to properly study the role of these genes in tumour propagation but we think these experiments are beyond the scope of this current paper.

We believe that this present work already reports many novel and important findings that we feel will be important for the field of cancer and stem cells, clarify important controversies in these fields, which to our opinion will be worth publishing in EMBO Journal.

- We have compared in this study the ability of tumour cell to propagate into immunodeficient mice in 6 different tumour models (4 papilloma models: DMBA/TPA papilloma, genetic papilloma with 3 different promoters K14creER::$KRas^{G12D}$, K19creER::$KRas^{G12D}$ and Lgr5creER::$KRas^{G12D}$, and 2 SCC models: DMBA/TPA induced tumours and the genetic K14creER:: $KRas^{G12D}$::p53fl/fl carcinoma), which to our knowledge has never been done in any other tumour models so far (Figure 2E, 2H, 3D, 4C, 5A, 5B, 5C, 6A, 6B, 6C).
- We demonstrated that benign tumours (papilloma) from 2 different models depend on their microenvironment, notably endothelial cells and tumour associated fibroblasts, to propagate into immunodeficient mice (Figure 2E, 2H).
- We demonstrated that invasive SCC can propagate without stromal component (Figure 3D, 4C, 5A, 6A, table1).
- We demonstrated the relative frequency of tumour propagating cells correlates with tumour aggressiveness. More aggressive tumours have higher frequency of tumour propagating cells. (5B, 6B, table1)
- We found that loss of p53 induces formation of highly aggressive tumours with a high frequency of tumour propagating cells comparing to papilloma (K14creER::$KRas^{G12D}$ with vs K14creER::$KRas^{G12D}$::$p53^{fl/fl}$)(Figure 2 and Figure 5).
- We demonstrated that serial transplantation is required to assess the long term self-renewal properties of tumour propagating cells (Figure 5 and 6) and showed that $CD34^{HI}$ cells present greater self renewal capacity compared to $CD34^{LO}$ cells in serial transplantation (Figure 5B, 5E, 5F, 6B) and tumours arising from $CD34^{HI}$ from DMBA/TPA SCC grow faster than the ones from $CD34^{LO}$, clearly clarifying important controversy in the field (Malinchi et al. Nature 2008 and Schober PNAS 2011)
- We demonstrated that in mouse skin tumours, the immune system does not play a major role in the frequency of secondary tumour formation in sharp contrast to what has been found in human xenograft transplantation of melanoma cells (Quintana et al. Nature 2008) (Figure 7)
- Finally, the relative frequency of tumour propagating cells in transplantation assay (Figure 2H, Table 1) underestimates the frequency of tumour stem cell measured by lineage tracing in vivo (Driessens et al., Nature 2012).

3. Related to this, what is the significance of CD34 expression levels and tumor initiating potential? Is there a sudden change in the global expression patterns within these populations or in the pattern of CD34 expression in the grafted material - basal versus suprabasal expression? The data provided, although this is not entirely clear, relates to the continuous propagation of either CD34low or CD34high populations. How does the serial propagation of sub-populations of tumor cells relate to the entire population (EPCAM+ve, ItgA6+ve cells), CD34high or CD34low? In addition, will CD34high cells from a CD34low initiated tumor behave like CD34low cells or CD34high cells.

A. *what is the significance of CD34 expression levels and tumor initiating potential?*

CD34 marks around 20-30% of tumour epithelial cells in papillomas and up to 70% in SCC (Figure 1D), while the frequency of tumour propagation is much more rare. In addition, the proportion of tumour propagation is 50/100 times higher in genetic SCC compared to DMBA/TPA $CD34^{HI}$ cells. However, there is no difference in the proportion of CD34 expressing cells between these two models, suggesting that CD34 by itself is a poor predictor of tumour propagating cell frequency (Figure 1D). Supporting this notion, $CD34^{HI}$; $CD34^{LO}$ and total Epcam+ cells in DMBA/TPA SCC present the same frequency of tumour propagating cells in the first transplantation (FIG 3D).
However, the frequency of tumour propagating cells increases in the $CD34^{HI}$ cells over serial transplantation while it decreases in the $CD34^{LO}$ (Figure 5A, 5B, 6A, 6B). Moreover, tumour arising from $CD34^{HI}$ grew faster compared to the tumour arising from the transplantation of $CD34^{LO}$ or total Epcam+ in DMBA/TPA induced SCC (Figure 3F). Altogether, these data support the notion that $CD34^{HI}$ enriched for tumour propagating cells presenting higher long term renewing capacities compared to $CD34^{LO}$ cells. We have now included these data and clearly discussed them in the text.

B. *Is there a sudden change in the global expression patterns within these populations or in the pattern of CD34 expression in the grafted material - basal versus suprabasal expression?*

No difference of CD34 expression pattern was observed between the primary tumours, the first and the second transplantations (Figure 3G, 3D, 4D, 4C, 4E, 4G).

C. *How does the serial propagation of sub-populations of tumor cells relate to the entire population (EPCAM+ve, ItgA6+ve cells), CD34high or CD34low?*

We thank the reviewer for this excellent suggestion. To address this question, we compared the frequency of tumour propagation between total Epcam+ with $CD34^{HI}$ and $CD34^{LO}$ Epcam+ cells from the classical DMBA/TPA induced SCC. While we did not observe a significant difference in the frequency of tumour propagation between these different populations (Figure 3D), suggesting that CD34 is not predictive of the frequency of tumour propagating cells, we found however that $CD34^{HI}$ cells always grew much faster than $CD34^{LO}$ and Epcam total cells in serial transplantation assays (Fig 3F).

D. *will CD34high cells from a CD34low initiated tumor behave like CD34low cells or CD34high cells.*

Our results show that $CD34^{HI}$ from $CD34^{HI}$ Epcam+ cells and $CD34^{HI}$ from $CD34^{LO}$ Epcam+ cells give rise to the same proportion of secondary tumours (Figure5D, E). However, these tumours were growing faster as demonstrated by their larger volume 5 to 8 weeks following transplantation (Figure 5E) suggesting that the renewing potential of these populations are different and whereas $CD34^{LO}$ cells can give rise to $CD34^{HI}$ cells, suggesting a plasticity in CD34 expression in tumour cells and a certain degree of interconversion between $CD34^{HI}$ and $CD34^{LO}$, the long term tumour renewal capacities of these cells are not fully reversible.

4. The authors state that papillomas have propagating potential, when provided with the appropriate microenvironment. The materials and methods mention the use of markers associated with the mesenchyme, but none of this data is included in the manuscript. In light of their own findings (Beck et al., 2011, Nature), the authors speculate that the presence of a perivascular niche is responsible for the effect. They will need to demonstrate whether these cells are indeed responsible for this effect. This will also shed light on the relevance of the grafting assay as a

functional read-out for CD34 expressing cells, as the authors have shown that the perivascular niche has specific effects on these cells.

We thank the reviewer for this is excellent suggestion.
We have performed as suggested by the reviewer co-transplantation of TECs (Epcam+) from DMBA/TPA induced papilloma together with either endothelial cells (CD31+) or tumour associated fibroblasts (CD140a+), and assessed their ability to reform secondary tumour upon transplantation (Figure2E, 2H). These experiments showed that endothelial cells or tumour associated fibroblasts allow propagation of tumour epithelial cells from papilloma into immunodeficient mice.
We have now included and discussed these new data in our revised manuscript.

Minor comments:

The authors have used a K14Cre driven model for their SCC studies, but for the majority of papilloma models they have focused on the K19Cre model. Although the data is supported by their chemical models, it is not clear, why they have chosen this approach as they are potentially targeting different populations of cells in the epidermis. This can potentially explain the differences they observe between the chemical and genetic papilloma experiments.

We have now performed these experiments in several genetic models using three different types of CREEER (K14CreER, K19CreER and Lgr5CreER – $KRas^{G12D}$). We found that the genetic models irrespective of the CREER used led to the low frequency of tumour propagation when transplanted together with stromal cells with a frequency similar between genetic and DMBA-TPA induced papilloma.
Importantly, all our experiments indicate that genetically and chemically induced papillomas require stromal component such as endothelial cells to propagate tumours into immunodeficient mice (Figure 2E, 2H).
We have added the table of our grafting experiments in the figure 2 to make this point more clear.
We could not use the K19CreER:$KRas^{G12D}$:p53fl/fl mice to study the primary SCC since they die following TAM injection due to internal tumours (Lapouge et al., PNAS 2011).

The authors use 3 injections per mouse for their grafting assays this is a concern if they use these as triplicate samples for the same cell type. In addition, it is not clear what constitute the replicates for the experiments. Are these technical or biological replicates.

The methodology to assess the frequency of tumour propagating cells in a given tumour by limiting dilution required to assess the tumour formation with replicates of the same tumour at different dilutions (Hu, Y, and Smyth, GK 2009), 'technical replicates" as named by the reviewer. We have performed these experiments (technical replicates) usually with 5 biological replicates (different tumours from different animals).
We have now included the number of biological replicates as well as technical replicates in figures and table (Figure 2E, 2H, Table 1).

There are no clone numbers for the antibodies used.

We have added clone numbers for the antibodies used in the methods section.

How does the proportion of CD34low/high cells change during serial grafting

Interestingly, the proportion of $CD34^{LO/HI}$ cells did not change during serial grafting
We have now added these data in our revised manuscript (figure 3G, 4D).

Referee #2:

This is a carefully performed but descriptive study to look at the tumor propagation frequency of different skin squamous cell carcinoma mouse models following transplantation into several different immunocompromised recipients. Although this study helps clear up some technical issues and discrepancies in the literature about the use of the single CD34 cell surface marker to

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distinguish tumor propagating cells (TPCs) in these different models, there is no new mechanistic information presented about the regulation of self-renewal of the TPCs. The overall conclusions about different frequencies of these cells in aggressive GEM vs. chemically induced SCC, and the effects of stromal and serial transplantation are not conceptually new. There are a number of minor concerns as well:

We thank the reviewer for saying that our study is carefully performed.

We disagree with the reviewer that our study just clear up some technical issues.

We believe that our study does resolve a major controversy in the field of skin cancer and determine to which extend CD34 allow the enrichment of tumour propagating cells (The two recent studies addressing this question came up with completely opposite conclusions) (See comments of reviewer 3).

We also strongly disagree with the reviewer that different tumour propagating frequency between aggressive genetic SCC and chemically induced SCC, and the requirement of stromal cells to propagate papilloma were known. To our knowledge, only 2 studies up to now have looked at tumour propagating frequency in FACS isolated populations of chemically induced tumours and to our knowledge no studies has ever assess the frequency of tumour propagating cells in KRas:^{G12D}:p53 cKO mice and ever compared the tumour propagating frequency in different mouse models of SCC .
We also are not aware of any study that perform FACS isolation of tumour epithelial cells and perform combined transplantation of FACS isolated endothelial cells together with tumour epithelial cells from skin papilloma. We would be happy to cite any relevant literature that we would have overlooked.

We believe that this present work reports many novel and important findings that we feel will be important for the field of cancer and stem cells, and clarify important controversies in these fields, which to our opinion will be worth publishing in EMBO Journal.

- We have compared in this study the ability of tumour cell to propagate into immunodeficient mice in 6 different tumour models (4 papilloma models: DMBA/TPA papilloma, genetic papilloma with 3 different promoters K14creER:: KRas^{G12D}, K19creER::KRas^{G12D} and Lgr5creER::KRas^{G12D}, and 2 SCC models: DMBA/TPA induced tumours and the genetic K14creER::KRas^{G12D}::p53fl/fl carcinoma), which to our knowledge has never been done in any other tumour models so far (Figure 2E, 2H, 3D, 4C, 5A, 5B, 5C, 6A, 6B, 6C).
- We demonstrated that benign tumours (papilloma) from 2 different models depend on their microenvironment, notably endothelial cells and tumour associated fibroblasts, to propagate into immunodeficient mice (Figure 2E, 2H).
- We demonstrated that invasive SCC can propagate without stromal component (Figure 3D, 4C, 5A, 6A).
- We demonstrated the relative frequency of tumour propagating cells correlates with tumour aggressiveness. More aggressive tumours have higher frequency of tumour propagating cells. (5B, 6B, table1)
- We found that loss of p53 induces formation of highly aggressive tumours with a high frequency of tumour propagating cells comparing to papilloma (K14creER::KRas^{G12D} with vs K14creER::KRas^{G12D}::p53fl/fl)(Figure 2 and Figure 5).
- We demonstrated that serial transplantation is required to assess the long term self-renewal properties of tumour propagating cells (Figure 5 and 6) and showed that CD34^{HI} cells present greater self renewal capacity compared to CD34^{LO} cells in serial transplantation (Figure 5B, 5E, 5F, 6B) and tumours arising from CD34^{HI} from DMBA/TPA SCC grow faster than the ones from CD34^{LO}, clearly clarifying important controversy in the field (Malinchi et al. Nature 2008 and Schober PNAS 2011)
- We demonstrated that in mouse skin tumours, the immune system does not play a major role in the frequency of secondary tumour formation in sharp contrast to what has been found in human xenograft transplantation of melanoma cells (Quintana et al. Nature 2008) (Figure 7)

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- Finally, the relative frequency of tumour propagating cells in transplantation assay (Figure 2H, Table 1) underestimates the frequency of tumour stem cell measured by lineage tracing in vivo (Driessens et al., Nature 2012).

1. The clonal evolution and CSC models need not be mutually exclusive as nicely summarized in several recent reviews. The authors should present a more appropriate Introduction.

We agree with the reviewer comment that the clonal evolution and CSC models are not mutually exclusive. We have rewritten the introduction to make it clearer as requested by the reviewer.

2. Likewise, it is a mistake to conclude that if the CSC model is correct, 'killing only CSC may result in long term to the eradication of the tumours", because of issues of plasticity of bulk tumor cells, and the need to target both populations. This again has been suggested in numerous reviews.

We agree with the reviewer and remove this sentence in the revised manuscript.

3. There is no reason to expect that CSC frequency will correspond to growth rate of primary tumors. Instead they might correspond to response to treatment. Has this been tested in these SCC models?

This is an interesting question raised by the reviewer. If the CSC contribute to tumour growth, and tumour propagating cells would reflect the CSC contents, then indeed one would expect to find a good correlation between CSC frequency and the growth rate of primary tumours.
We agree with the reviewer comment that the response of CSC to therapy is a fascinating question that we feel is beyond the scope of this manuscript.

4. No difference in TPC frequency with CD34 hi or lo, no difference in TPC frequency in different immune compromised mice. Both of these are clean, but negative results. What about in syngeneic recipients? The innate immune system is known to play a critical role in tumor progression and metastasis.

We have performed as suggested by the reviewer, transplantation of Epcam+ tumour epithelial cells from DMBA/TPA SCC into syngenic mice (FVB/N) to investigate the impact of the immune system on the tumour propagating cell frequency. Interestingly and somehow unexpectedly, we did not find a significant difference in the frequency of tumour propagation in syngenic mice, suggesting that in our mouse model, immunity is not a strong barrier for tumour propagation at least in invasive SCC. We have now included and discussed these data in our revised manuscript. (Figure7A).

5. Several grammatical errors: p. 5, tumours progress; p. 6 papillomas arising, p. 7, secondary tumours

We thank the reviewer for pointing out these mistakes. We have corrected them in the revised manuscript.

Referee #3:

In this work, Lapouge and colleagues have set to study the frequency and tumor propagating potential of different subsets of tumor cells from squamous tumors (both benign and malignant). There have been recent papers that showed discrepancies in the nature, molecular characteristics, and functional relevance of tumor initiating and propagating cells in squamous tumors. In this work, the authors now provide clear data solving some of these discrepancies, and accurately measuring the frequency of different SCC models.

Of particular interest are the following conclusions: (A) that papilloma Epcam+ cells can only form secondary tumors when co-transplanted with stroma cells (this must be highlighted since previous

work by Malanchi et al, did not take this into account when setting up their methodology to purify putative cancer stem cells); (B) The CD34+ and CD34- SCC Epcam+ cells are equally competent to form secondary tumors when transplanted directly (i.e. without prior growth in culture); (C) Most importantly, that the two previously identified populations of tumor Epcam+ cells (CD34+ and CD34- populations), can be functionally and hierarchically classified based on their differential ability to propagate the tumor in the long term. This is an important conclusion in light of a recent paper by the group of Elaine Fuchs which suggested otherwise. Altogether this work is of great quality, and the conclusions relevant to the field. I therefore think it will be suitable not only for the epidermal field, but also for the broader fields of cancer and stem cells. I have some minor points and suggestions for the authors that when addressed could, in my opinion, strengthen some of their conclusions:

We would like to thank the reviewer for his/her positive assessment of our manuscript and I believe that the reviewer nicely summarize the novelty of our manuscript.

1) Fig 1C: The immunofluorescent co-staining of K5/CD34 shows that some of the CD34+ cells are not K5 positive. Are these endothelial cells? Or are there CD34 bright cells that are dim for K5? I am asking this because if one looks at the FACS profiles of alpha6 and CD34 (Fig 1A), it seems that the brightest alpha6 cells are not the brightest CD34 (or similarly, that the brightest CD34+ cells fall within the alpha6 mid cells). This is something that all of us in the field see when performing alpha6/CD34 FACS of squamous tumors, but do not really pay much attention to (considering that we normally select large clouds in our FACS profiles when sorting). Do the authors think there is a CD34bright/alpha6dim population functionally distinct from the alpha6bright/CD34+ and alpha6bright/CD34- populations? It would be interesting if they showed some comparison of the clonogenic (culture) potential of these three populations.

This is indeed an interesting observation that some CD34+ cells expressed low level of a6-integrin as determined by FACS. As shown in our recent publication (Beck et al 2011) as well as in the paper of Fuchs's group (Schober et al 2011), the vast majority of CD34+ Epcam+ cells are located at the epithelial stroma interface and these cells are positive for K5 and a6 integrin. We have not further investigated the clonogenic potential of a6 low tumour cells since *Schober* and colleagues have already demonstrated that these cells are not clonogenic in vitro and in vivo.

2) Fig 2: It would be clearer if the authors state in the text again that they are comparing the CD34+/alpha6+ and CD34-/alpha6+ populations, rather than just CD34+ vs CD34-. Otherwise it could lead to confusion that they are not using alpha6 integrin as an additional selection marker.

The reviewer is corrected and we have now used this nomenclature throughout the paper.

3) Fig 2F: there seems to be a difference in the cell output of cells originating from chemically versus genetically induced tumors. Is this difference statistically significant?

YES, this difference statistically significant- (FigureS1) p-value CD34-:0,00051 and p-value CD34+: 0,00269.

4) In the last paragraph describing Figure 2 the authors state "these cells are not clonogenic on their own, and rely..." Perhaps it would be more accurate if the authors said "tumorigenic" instead of "clonogenic", since papilloma TECs are capable of clonogenic growth in culture, but not tumorigenic potential in vivo.

The reviewer is corrected and we have now used the word tumourigenic rather than clonogenic.

5) CD34+ TECs are more tumorigenic in the long-term than CD34- TECs. The authors suggest that this might be due to intrinsic differences in their expression signature with regards to stemness genes (specially those related to EMT). This is an interesting hypothesis which ties very well with what has been observed by the group of Weinberg, as well as other laboratories, that EMT might promote stemness. Thus EMT might be a common feature correlating to stemness in many solid

tumors. However, the results equally imply that CD34+ TECs are heterogeneous, with some % expressing markers of EMT and simple epithelia (K8), and others not. Is this the case? If so, can CD34+ TECs be subdivided on the basis of intensity of CD34? For instance, are the K8+ cells shown in Fig 4E positive for CD34 (co-staining)?

A. Do CD34+ TECs are heterogeneous, with some % expressing markers of EMT and simple epithelia (K8), and others not?

The reviewer is corrected that CD34+ TECs are heterogeneous, with only some cells expressing markers of simple epithelia (K8). In DMBA/TPA SCC, K8 expression in CD34+ TECs is relatively rare, while it is more frequent, although still heterogenous in genetically induced SCC (Figure 3E, 4F, 5G, 6D).
We have investigated as suggested by the reviewer the expression of EMT markers by qPCR in 3 distinct stages of skin carcinogenesis (papilloma, SCC and spindle SCC) and found that expression of these genes (Twist1, Snail1, and Slug) increased in more invasive tumours. Interestingly, EMT marker expression has been shown to regulate stemness in cancer cells (Mani et al., Cell 2008), are enriched in the $CD34^{HI}$ population that showed the best propagating properties in serial transplantation (Figure 3H, 4J). Of course, further experiments are required to properly demonstrate the functional role of these genes in tumour propagation but we think these experiments are beyond the scope of this current study.

B. If so, can CD34+ Epcam+ cells be subdivided on the basis of intensity of CD34? For instance, are the K8+ cells shown in Fig 4E positive for CD34 (co-staining)?

We could not perform CD34 and K8 co-staining since both antibodies are from the same specie (Rat). Nonetheless, the vast majority of tumour epithelial cells are CD34 positive in SCC and quite stable between the different models (Figure1B, 1D) while the proportion of K8 is very rare in DMBA-TPA SCC (Figure 3E) and more abundant in more aggressive tumours (Figure 4C and 5D). Hence, it is likely that CD34 positive cells are heterogeneous regarding K8 expression. We have added a sentence in the revised manuscript to clarify this point.

2nd Editorial Decision 02 November 2012

One of the original referees has assessed the revisions provided. Based on this imput, I am pleased to accept your manuscript for publication in The EMBO Journal.

Yours sincerely,

Editor
The EMBO Journal

****
REFEREE COMMENTS

I think the authors have done a very good job in addressing most of the referee comments. There are some that are left unanswered, but they pertain to the molecular characterization of the populations of CSCs (CD34high versus CD34low), and I agree with the authors that this is simply beyond the scope of a single work (it will take years before we fully understand this). I am sure this paper will have considerable impact: it directly addresses a controversy in the field and nicely sets the standard for future work.