Author's response to reviews

Title: Establishment of a murine epidermal cell line suitable for in vitro and in vivo skin modelling

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Version: 5 Date: 16 February 2011

Author's response to reviews: see over
We thank the reviewers for their valuable comments. We have tried to address all the reviewers’ observations and we think this has improved the quality of the manuscript, which we hope is now ready for its publication in BMC Dermatology. We provide here a list of the changes that have been made (in red in the manuscript) in response to the revisions requested.

REFEREE 1:

The observations made by the referee regarding the pattern of expression of differentiation markers (K10, Loricrin and Filaggrin) in the experiments described in figure 2 are now discussed in the text (Results, second section, last paragraph and Discussion, fifth paragraph).

Regarding Figure 3 illustrating COCA in vivo grafting after 4 weeks, we believe that there is an area of the tissue shown that appears misfolded (at the top left), such that some cell layers were lost from the section and this has possibly generated confusion. We have now added a new “Figure 3_revised”, with a tissue section we believe is better oriented. As already discussed in the text, there is an expansion of K5 staining to suprabasal layers, as well as K6 expression; however, K10, loricrin and filaggrin form a rather continuous layer.

REFEREE 2:

Major compulsory revisions:

1) It is not the aim of this manuscript to describe a new method for long-term culture of murine keratinocytes, but to establish 3D models using murine epidermal keratinocyte cell lines suitable for in vitro and in vivo skin modelling. This, to our knowledge, has not been previously achieved. It is true, however, that we have develop a new epidermal cell line that we too characterise here as it presents remarkable differences (and advantages) with cell lines previously developed, and its use can pose a benefit to the scientific community working in the field. The publication mentioned by the reviewer (Reichelt & Haase, Methods Mol Biol, 2010) has been included in the manuscript (Background, third paragraph).

During subculture, switching the cell cultures to FBS-containing medium for 8-14 hours enhances cell attachment in the initial passages. FBS-seeding media is not further needed once the cell line has been established. At this point, 8-14 hours incubation in CnT-07 medium containing 0.2mM CaCl2 is enough to ensure good cell attachment after trypsinisation. This has now been better clarified in Methods: Cell isolation and culture.
3) The referee questions how well-established is the tumorigenicity assay that we have used to test COCA tumour-formation ability. Subcutaneous injection of cells into NMRI nude or other strains of immunodeficient mice has been used for decades to test tumor growth. This is a widely used method in the field of cancer research (i.e. more than 14000 cites for “xenograft nude mice” and more than 1000 cites in PubMed for “nude mice tumorigenicity assay” - searched February 2011). We have added three references to the manuscript: the first one is by our group and uses nude mice to test tumorigenicity of non-transformed and Akt oncogene-transformed PB keratinocytes (Segrelles et al., Oncogene 2001); the other two are more general and date to the origin of this kind of approaches (Schmidt and Good, Lancet 1976; Sharkey and Fogh, Cancer metastasis reviews 1984).

As for the suggestion made by the referee to label the cells as a way to test their viability when injected into immunodeficient mice, it is not the aim of the experiment to test whether COCA cells can survive in this setting, but to test whether they can give rise to tumoral growth.

4) We have performed a more detailed statistical analysis of data in Figure 1B. Significant p values have been included (“Figure 1_revised”). Also, relevant statistical differences are discussed in the text (Results, second section, second paragraph and Figure 1, legend).

5) We agree with the reviewer it would be difficult to absolutely rule out the possibility of individual clone transformation within a cell line. We have tried to address this possibility using two different in vivo experimental approaches: i) subcutaneous injections, and ii) grafting in silicone chambers in immunodeficient mice. i) The first assay is a well-established, commonly-used method in the field of cancer to test for tumorigenic ability (see above answer to question number 3). We use it frequently in our lab, and other cells have shown to be tumorigenic in these settings, including ras-infected primary keratinocytes isolated from p16/p21 deficient mice (Paramio et al., J Biol Chem 2001), Akt oncogene-transformed PB keratinocytes (Segrelles et al., Oncogene 2001) and other cell lines derived from genetically modified mouse models such as K5myrAkt transgenic or K5myrAkt;p53 deficient mice (unpublished). ii) The use of silicone chambers to study tumorigenic potential is somehow more frequent in the field of skin biology. There is previous work using this assay to test tumorigenicity of keratinocyte cell lines (i.e. Fusenig et al., J Invest Dermatol 1983; Conti, Cancer Res 1988). In our lab, we have assayed the tumorigenic potential of the L84 cell line derived from K5myrAkt transgenic mice (Segrelles et al: Mol Biol Cell 2008, Cancer Res 2007). These cells have been isolated and cultured in the same conditions as COCA and were able to display features of malignant transformation in grafting assays. As requested by the reviewer, more detail on the control experiments concerning the use of genetically modified keratinocytes in these assays is provided in “Information for the Editor & Reviewers”.

COCA cells were unable to show any signs of tumoral transformation in these two experimental settings.

Regarding Figure 3 illustrating COCA in vivo grafting after 4 weeks, we believe that there is an area of the tissue shown that appears missfolded (at the top left), such that some cell layers were lost from the section and this has possibly generated confusion. We have now added a new “Figure 3_revised”, with a tissue section we believe is better
oriented. As already discussed in the text, there is an expansion of K5 staining to suprabasal layers, as well as K6 expression; however, K10, loricrin and filaggrin form a rather continuous layer.

6) Similar issues about Figure 2 have been raised by Reviewer 1. Observations made by both reviewers regarding the pattern of expression of differentiation markers (K10, Loricrin and Filaggrin) in the experiments described in Figure 2 are now discussed in the text (Results, second section, last paragraph and Discussion, fifth paragraph).

7) The reviewer suggests we adequate the timing of graft analysis after that set by Strachan & Ghadially.

While we have adapted the cell grafting assay used by Strachan & Ghadially (Methods Mol Biol (585) 2010) the method was not originally developed by these authors. In fact, they have modified it from others to use it for their aims (identification of long-term repopulating cells). Original studies (Fusening el al., Bull Cancer 1978 and J Invest Dermatol 1983; Conti et al., Environ Health Perspect 1986 and Cancer Res 1988) using silicone chamber were developed to assay abnormal growth and malignancy, and the grafting times analysed were 1-4 weeks.

On the other hand, the cells grafted are very different in our setting. Cells grafted by Strachan & Ghadially are primary keratinocytes isolated from newborn mice. In this experimental situation you would expect to have, among the population of keratinocytes grafted, transient amplifying cells (with a high but restricted proliferative potential) and a minor population of more quiescent long-term survival stem cells, just as suggested by the reviewer. Thus, in this setting, grafting times of 7-9 weeks are needed to assay for long-term repopulating units. In our experiments the cells have been maintained in culture for 75 passages (almost a year) after their isolation and before being grafted. Thus, the “loss” of transient amplifying cells that divide an average of three times before they die (as mentioned by the reviewer) and the “survival” of long-term repopulating cells is a process that has already taken place during the prolonged culture of these cells.

Due to this essential difference, we believe that Strachan & Ghadially grafting times are not applicable to our settings; instead, we considered more appropriated to set the grafting duration for our experiments using a grafting material more similar to COCA. This is the case of the L84 cell line derived from K5myrAkt transgenic mice in our lab (Segrelles et al: Mol Biol Cell 2008, Cancer Res 2007). These cells have been isolated and cultured in the same conditions as COCA but were able to display features of malignant transformation in grafting assays. It took less than 3 weeks for L84 grafts to develop papillomas that progressed into in situ carcinomas showing major alterations in cell proliferation and keratin expression (see “Information for the Editor & Reviewers”).

8) The anti-cytokeratin 6 antibody used in our experiments is from Covance. This is now specified in the manuscript (see “Methods: Histological procedures”).

Discretionary revision:

9) We fully agree with the reviewer’s comment and we now mention this consideration in “Abstract, Conclusions”.