Reviewer's report

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

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Reviewer: Julia Reichelt

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Major compulsory revisions:

1) Most importantly, the manuscript lacks novelty. The authors point out that it has been difficult in the past to use murine keratinocytes for organotypic 3D cultures of wild-type or genetically modified mice due to the lack of methods available for long term cultivation of murine keratinocytes. This is not right, as Hager et al. and Caldelari et al., both cited in the manuscript, published methods describing long-term culture of murine keratinocytes. Furthermore, Segrelles et al. missed another previous publication which describes a method to establish permanent murine keratinocyte lines (Reichelt and Haase, 2010, Methods Mol Biol 585, 59-69).

2) The stated use of defined medium conditions requires reconsideration. Segrelles et al. used cell culture media from the company Cellntec and a method recommended by Cellntec to establish a murine keratinocyte cell line which they named COCA. The author’s claim to having used defined media (CnT-07 and Cnt-02-3D, Cellntec) is not fully vested. Although, Cellntec state that their media are defined and most of the ingredients are named on their website, the amounts of the individual components are not given and “up to five proprietary components” are not named. Cellntec also indicate that the list of medium components on their homepage is not valid for their 3D media (e.g. CnT-02-3D, used by Segrelles et al. to differentiate CACO keratinocytes). Furthermore, Segrelles et al. always switched temporarily from CnT medium to FCS-containing medium after splitting their cultures.

3) The first method used to test for tumorigenicity has not been cited (cell injections into the flanks of mice). Is it an established method? As the authors mention, the conditions met at the injection site may have been adverse for the grafted keratinocytes resulting in cell death. A possible way to test for viability of the injected cells would be labelling of the cells prior to injection in order to identify them at the end of the experiment.

4) Fig. 1B shows large differences in proliferation between P21 and P51 in low Ca2+-medium and also at 24 hours in high Ca2+-medium. It is not clearly described how the error bars were determined, statistical analysis has not been done and the reasons for any significant differences have not been discussed.

5) In the second paragraph of the discussion the authors say that they
established a non-transformed keratinocyte line. However, CACO keratinocytes could be passaged for more than 75 times (which is far beyond the proliferative capacity of normal senescent cell lines) and chromosome analysis revealed highly heterogeneous clonal growth (Table 1): chromosomal instability, aneuploidy including cells bearing a translocation. It is known that tumorigenic lines stochastically appear in murine keratinocyte cultures (Yuspa et al., 1980, Cancer Res 40, 4694-4703). Transformation of individual clones within the CACO cell line can therefore not be excluded. Figure 3D shows an in-vivo generated epidermal-like structure which displays areas of hyperproliferation and disturbed differentiation. It is possible that such hyperproliferative foci developed from specific subclones which might give rise to tumours if grown for longer periods of time. The tumorigenic assay used by Segrelles et al. does not convincingly exclude tumorigenetic lineages in the CACO cell line. More detailed information on the control experiments which were only briefly mentioned in the manuscript (last results chapter, first paragraph and last sentence) concerning the use of genetically modified keratinocytes might serve to support the statements made by Segrelles et al..

6) The 3D epidermal model grown at the air-liquid interphase (Fig. 2D) shows expression of K10 in isolated suprabasal cells and an abnormal expression profile of loricrin and filaggrin which were detected in few isolated cells and often before K10 expression was switched on but not in the granular layer where these two differentiation markers are normally found in skin.

7) The cell grafting assay used by Segrelles et al. was adapted from Strachan and Ghadially (2010). In contrast to the original protocol, however, which analysed the grafts 9 weeks after transplantation, Segrelles et al. analysed the grafts at 4 weeks. According to Strachan and Ghadially, it takes 7 weeks before transit amplifying cells are lost from the grafts and any further growth results from keratinocyte stem cells. It would be interesting to test whether the keratinocyte line, COCA, established by Segrelles et al. supports growth beyond 7 weeks after grafting, which would indicate that these keratinocytes have stem cell properties and show long-term survival in vivo. (Discretionary revision).

More importantly, in the in vivo grafts transit amplifying cells divide, according to Strachan and Ghadially (2010), on average only 3 times before they die. A dysplasia or malignant transformation is therefore probably not detectable in the chosen experimental setting of analysis at 4 weeks after grafting (last results chapter, last paragraph). (Major revision).

Minor essential revision:

8) The antibody directed against K6 was not specified. This is important, however, for the interpretation of the observed suprabasal staining shown in Fig. 2C.

Discretionary revision:

9) In the conclusions of the abstract, COCA keratinocytes are proposed to represent a suitable control for keratinocyte lines established from genetically
modified mouse lines. There might however be mouse strain differences between lines established from genetically modified mice and COCA cells which were derived from the mixed mouse strain background C57BL/DBA.

**Level of interest:** An article of limited interest

**Quality of written English:** Needs some language corrections before being published

**Statistical review:** Yes, and I have assessed the statistics in my report.

**Declaration of competing interests:**

I declare that I have no competing interests