response for cathelicidin, and to a lesser (but not statistically significant) degree, TLR-9 in lesional skin. This finding supports the role that wounding may play in psoriasis induction and suggests that one factor affecting the efficacy of etanercept may be the blunting of this wounding response. Further studies with larger sample sizes, immunohistochemistry and qRT-PCR data are necessary to confirm this novel preliminary finding.

Author contributions

TH, MA, JM, MJ, DTA, PFK, FK, MG, RLG performed the research. MA, AMT, YL analysed the data. TH, RLG designed the study and wrote the manuscript.

Conflict of interests

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Quantitative real-time PCR.

Data S2. Statistical supplement for figure 1B, 1E and 2E.

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Letter to the Editor

Keratinocyte cell lines derived from severe generalized recessive Epidermolysis Bullosa patients carrying a highly recurrent *COL7A1* homozygous mutation: models to assess cell and gene therapies *in vitro* and *in vivo*

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Abstract: Recessive dystrophic epidermolysis bullosa (RDEB) is caused by deficiency of type VII collagen due to *COL7A1* mutations such as c.6527insC, recurrently found in the Spanish RDEB population. Assessment of clonal correction–based therapeutic approaches for RDEB requires large expansions of cells, exceeding the replication capacity of human primary keratinocytes. Thus, immortalized RDEB cells with enhanced proliferative abilities would be valuable. Using either the SV40 large T antigen or papillomavirus HPV16-derived E6-E7 proteins, we immortalized and cloned RDEB keratinocytes carrying the c.6527insC mutation. Clones exhibited high proliferative and colony-forming features. Cytogenetic analysis revealed important differences between T antigen-driven and E6-E7-driven immortalization. Immortalized cells responded to differentiation stimuli and were competent for epidermal regeneration and recapitulation of the blistering RDEB phenotype *in vivo*. These features make these cell lines useful to test novel therapeutic approaches including those aimed at editing mutant *COL7A1*.

Key words: cell lines – genodermatoses – keratinocytes – viral oncogenes

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Background

Recessive dystrophic epidermolysis bullosa (RDEB) is a devastating mechanobullous genodermatosis whose severity and classification depend on the extent of type VII collagen deficiency (1). Null *COL7A*1 mutations give rise to the severe generalized RDEB form (2). We have recently disclosed c.6527insC as a highly recurrent *COL7A1* mutation affecting around 50% of the Spanish RDEB population (3,4). Several therapeutic approaches for RDEB, including protein, cell and gene therapies, are currently being explored at preclinical and clinical stages (5,6). Although feasible through retroviral gene transfer (7), *ex vivo* gene therapy is evolving towards safer standards (8) requiring suitable test systems such as the immortalized cells herein described.

Questions addressed

Recurrent *COL7A*1 mutations such as c.6527insC are susceptible of tailored therapeutic strategies including homologous recombination, RNA trans-splicing or exon skipping, which nowadays remain inefficient. Evaluation of these approaches relies on the *in vitro* analysis of large number of cells and on stringent assays dealing with skin regenerative capacity of the modified cells *in vivo* (9–11). Although immortalized keratinocyte cell lines derived from several genodermatoses, including RDEB, have been reported (12–15), detailed characterization of the immortalization process, growth performance, cytogenetic status and skin regenerative capacity has not been thoroughly addressed.

Experimental design

Immortalization of keratinocytes derived from two RDEB patients carrying the c.6527insC *COL7A1* mutation was performed by retroviral transduction of either the SV40 virus large T antigen or papillomavirus-derived E6-E7 proteins (16,17). We designed experiments to comparatively characterize the immortalized cells with respect to proliferation, clonogenicity, differentiation and karyotype. We also tested their regenerative performance and capacity to recapitulate the RDEB disease phenotype *in vivo*. (For details see Appendix S1).

Results

RDEB Keratinocyte immortalization

Large T antigen- or E6E7-transduced cells (named RDEB-T and RDEB-E67, respectively) grew originally in the presence of 3T3 feeder layer. After approximately 25 population doublings, polyclonal cells became independent of feeder cells without apparent signs of proliferative crisis. Clones of both cells were isolated. Studies were continued with two clones, RDEB-TA4 and RDEB-E67A6, derived, respectively, from RDEB-T and RDEB-E67 parental cells. Both clones showed typical keratinocyte morphology (Figure S1a). Expression of the corresponding immortalization proteins and their concomitant activity on retinoblastoma protein degradation were confirmed (Figure S1b, c). Restriction fragment length polymorphism analysis demonstrated the persistence of the c.6527insC *COL7A1* pathogenic mutation (Figure S1d). Consistently, type VII collagen expression was absent (Figure S1e).

Proliferation and differentiation

Doubling times of 18 h 27 min and 19 h 30 min were determined for RDEB-E67A6 and RDEB-TA4, respectively (Fig. 1a). Colony-forming efficiency (CFE) assays showed that RDEB-TA4 cells formed large colonies (Fig. 1b). In contrast, RDEB-E67A6 exhibited low number of small and aberrant colonies (Fig. 1b). Seeding

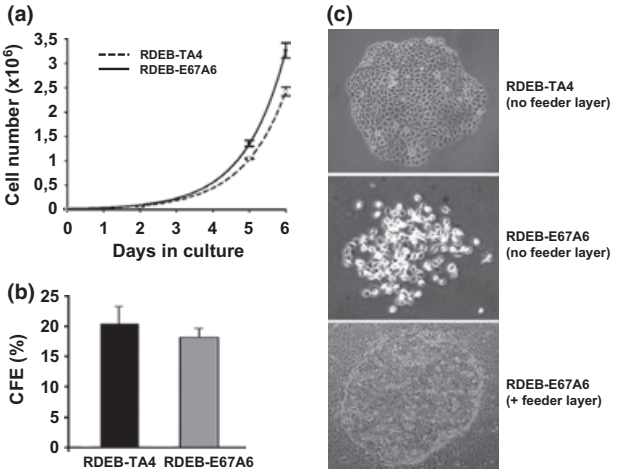

Figure 1. Growth properties of RDEB immortalized cell lines (a) Growth curves of RDEB-E67A6 and RDEB-TA4 cells. The number of cells was determined at 2, 5 and 6 days after seeding 1×10^4 cells. The graph shows curves within the interval of exact exponential cell growth. (b) Quantitative data of CFE. Black bar, RDEB-TA4 cells. Grey bar, RDEB-E67A6 cells. (c) Microscopic appearance of a representative colony from RDEB-TA4 cells (upper panel) in the absence of feeder layer and from RDEB-E67A6 cells with (lower panel) and without (middle panel) feeder layer.

E6E7-immortalized cells with lethally irradiated feeder cells overcame this drawback (Fig. 1b). CFE values were 18.3 ± 1.5% and 20.4 ± 2.9% for RDEB-E67 and RDEB-TA4, respectively (Fig. 1c), in the range of highly clonogenic human primary keratinocytes (18). Cell differentiation, assessed *in vitro* after a calcium concentration switch (19), showed that the overall number of keratins K1-/K10-positive cells in RDEB immortalized cells and normal keratinocytes was similar (Figure S2).

Cytogenetic changes

Karyotype analysis showed a marked aneuploidy in RDEB-TA4 cells with abundant structural clonal aberrations, including a translocation t(3,10) as one of the most remarkable changes. In contrast, the RDEB-E67A6 karyotype showed only a tetrasomy of chromosome 20 (Figure S3). Amplification of chromosome 20 sequences has been shown in other cells immortalized by E7 protein (20).

Skin regenerative potential and phenotypic features

Immortalized cells were not tumorigenic (data not shown). In short-term subcutaneous grafts (Figure S4a–d), both clones formed well-developed stratified epithelia but exhibited markedly different phenotypes. RDEB-TA4 cells showed aberrant differentiation, mild dysplasia in basal and parabasal layers with anisokaryosis. Dyskeratosis and immature parakeratotic stratum corneum was also observed (Figure S4e). In contrast, RDEB-E67A6 cells gave rise to a well-differentiated epithelium with gradual maturation and formation of well-developed stratum granulosum and stratum corneum with moderate parakeratosis (Figure S4e). Grafts from both cell lines showed dermo-epidermal blister formation, typical of RDEB. Immunofluorescence analysis showed absence of type VII collagen expression at the dermo-epidermal zone (Figure S4f). The quasi-normal behaviour of RDEB-E67A6 in subcutaneous grafts led us to test their performance in orthotopic grafts (21,22) after marking cells with GFP (Fig. 2). It is noteworthy that skin regenerated from RDEB-E67A6 resembled that attained with normal human keratino-

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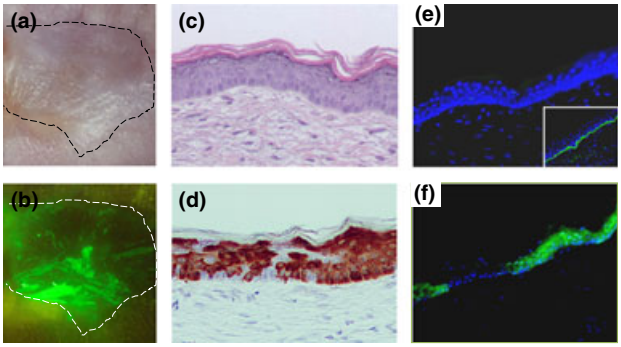

Figure 2. Skin regeneration after orthotopic grafting of bioengineered skins containing immortalized Recessive dystrophic epidermolysis bullosa (RDEB) cells. (a, b) Macroscopic appearance of regenerated skin 5 weeks after grafting of EGFP-transduced RDEB-E67A6 cells to immunodeficient mice. (a) White light epi-illumination of a graft (black dotted line). (b) GFP fluorescence of the same graft (white dotted line) shown in (a) (blue light illumination). (c) Histological appearance (H&E staining) of regenerated skin from RDEB-E67A6 graft. (d) GFP immunoperoxidase staining in a graft section consecutive of that shown in (c). (e) Immunofluorescence analysis of collagen VII expression. Note the absence of expression. The inset (lower right corner) shows a collagen VII immunofluorescence from skin regenerated after transplantation of bioengineered skin with normal human keratinocytes as positive control. (f) Immunofluorescence analysis of EGFP expression in a consecutive section of that showed in (e). Note that immunofluorescence analysis of EGFP expression was needed because EGFP fluorescence is lost after tissue fixation in methanol–acetone, the fixative of frozen sections for collagen VII immunofluorescence. (a, b) 6×. (c, d) 200×. (e, f) 100×.

cytes (Fig. 2a–d). Because tissue was dissected carefully to facilitate histological processing, mechanical stress was avoided and blister formation was not evident. However, immunofluorescence again demonstrated lack of type VII collagen expression (Fig. 2e).

Conclusions

Immortalized cells capable to reproduce disease phenotypes represent valuable tools to replace primary cells limited by senescence-imposed constraints. Despite variations in the immortalization procedures used, no significant differences were observed in terms of proliferation when cells were seeded at medium or high density. Under extreme isolation, however, RDEB-E67A6 cells had more complex growth factor and/or nichelike requirements. Immortalized RDEB cell lines resisted high calcium-induced growth arrest (23) without losing their differentiation abilities and consistently with their capacity to achieve epidermal stratification *in vivo*. However, although proficient for skin regeneration, T antigen-immortalized cells displayed a more aberrant epidermal differentiation than E6E7-immortalized ones. In view of the stringent growth requirements for clonal growth and nearly normal skin regenerative features, transduction of E6E7 proteins seems to drive RDEB cells to a less defiant immortalization state than that driven by the large T antigen.

Gross aneuploidy and chromosomal structural abnormalities were observed in T antigen-immortalized cells. Conversely, E6E7 immortalization rendered diploid or near diploid cells. Others have also shown that the T antigen produced a large number of chromosomal aberrations (24–27) perhaps unrelated to its ability to extend cellular lifespan but rather to interact with the spindle checkpoint machinery (28). Other less genotoxic forms to extend cells lifespan, including forced expression of telomerase, exist (29). However, keratinocytes require additional genetic changes to overcome senescence (30). While the most conceivable rationale for the differential cell behaviour relies on the degree of genetic instability produced by the different immortalization molecules, we cannot rule out intrinsic genetic or epigenetic background differences of the patients notwithstanding the presence of the same pathogenic *COL7A1* mutation.

The new immortalized cell lines herein described represent reliable cellular systems to test novel therapeutic approaches for RDEB.

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Author contribution

FL, MDR designed the study. CC, DA, BD, RM, ÁM, MG and MJE performed the experimental work. SGL, ÁM established primary keratinocyte cultures from patient skin biopsies. JCC and LE performed the cytogenetic analysis. CJC performed the histopathological analysis. RG and RM provided materials. FL, RM, MDR wrote and reviewed the manuscript.

Conflict of interests

All authors declare no conflict of interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Phenotypic and genotypic features of immortalized RDEB cell lines.

Figure S2. Calcium induced differentiation of immortalized RDEB cells.

Figure S3. Cytogenetic abnormalities of immortalized RDEB cells.

Figure S4. Subcutaneous bioengineered skin xenotransplantation procedure and characterization of skin regeneration from engrafted immortalized RDEB cells.

Appendix S1. Materials and methods.

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