See related commentary on pg 1403

Keratin 14-Null Cells as a Model to Test the Efficacy of Gene Therapy Approaches in Epithelial Cells

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Skin fragility disorders caused by keratin mutations are incurable, and a better understanding of their etiology is needed to find new ways to improve and treat these conditions. The best-studied skin fragility disorder is epidermolysis bullosa simplex (EBS), an autosomal dominant condition caused by mutations in keratin 5 (K5) or K14. To analyze disease mechanisms and develop gene therapy strategies, we have used keratinocyte cell lines derived from EBS patients as model systems. Here, we describe two cell lines established from EBS patients with K14-null mutations. We analyze the responses of these cells to stress assays previously shown to discriminate between wild-type and keratin-mutant keratinocytes, to directly evaluate the efficacy of rescuing K14-null cells by supplementation with wild-type K14 complementary DNA (cDNA). The K14-null cells show elevated levels of stress correlating with reduced normal keratin function. By transfecting wild-type K14 into these cells, we demonstrate "proof of principle" that an add-back approach can significantly rescue the normal keratinocyte behavior profile. These K14-null cell lines provide a disease model for studying the effects of keratin ablation in EBS patients and to test the efficacy of gene add-back and other therapy approaches in keratinocytes.

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INTRODUCTION

Gene therapy strategies for dominant monogenic disorders, such as epidermolysis bullosa simplex (EBS), are often designed to silence the mutant gene first and then correct the pathological phenotype through addition of a wild-type gene. Different approaches from chimeric RNA/DNA oligonucleotides, small interfering RNAs (Richardson *et al.*, 2002; Hickerson *et al.*, 2006; Leachman *et al.*, 2008), antisense (Kurreck, 2003), and ribozyme technologies (Samarsky *et al.*, 2000; Kashani-Sabet, 2002) are being developed to switch off mutant genes. However, the consequences of silencing major structural genes such as keratins have not been well studied, in spite of published suggestions that total lack of keratin 14 (K14) may be less pathogenic than the presence of a defective dominant mutant K14 (Chan *et al.*, 1994; Jonkman *et al.*, 1996b; Batta *et al.*, 2000; Lanschuetzer *et al.*, 2003).

Keratinocyte cell lines with total absence of K14 provide an ideal model to study the effect of a defective keratin inactivation in EBS patients. EBS was the first disease in which intermediate filament mutations were identified. It is mainly an autosomal dominant skin disorder, caused by mutations in either K5 or K14 (Bonifas *et al.*, 1991; Coulombe *et al.*, 1991; Lane *et al.*, 1992). These keratin filament proteins are synthesized and assembled as co-polymers of K5/K14 in the basal cells of stratified epithelia. They form networks of bundled filaments that link into desmosomes and hemidesmosomes, and provide physical resilience across the whole epithelial sheet tissue. Mutations in either K5 or K14 compromise the whole filament network and lead to fragile epidermal basal cells that easily rupture on physical trauma to the epidermis, giving rise to intraepithelial blisters (reviewed in Porter and Lane, 2003). The different degrees of clinical severity of EBS are correlated with the position of K5 or K14 mutations (Letai *et al.*, 1993), with the most severe Dowling–Meara phenotype (DM-EBS) being caused by mutation hotspots at either ends of the central α-helical rod domain of the affected keratin (most frequently in the arginine codon at position 125 in K14) and the mildest EBS phenotype (Weber–Cockayne-EBS) being mainly caused by mutations in the linker regions of the keratin. Severely mutated DM patients present with clustered blisters at various sites of their bodies and have an elevated risk of developing basal cell carcinoma (Fine *et al.*, 2004), whereas blistering is generally limited to the hands and feet (highest stress sites) in the mild EBS patients. Cytoplasmic aggregates of misfolded keratin protein are classically seen in the basal keratinocytes of DM-EBS patients, and the presence of such aggregates activates a cascade of stress response processes that are likely to contribute to the disease phenotype (D'Alessandro *et al.*, 2002; Russell *et al.*, 2010).

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Abbreviations: cDNA, complementary DNA; DM-EBS, Dowling–Meara EBS; EBS, epidermolysis bullosa simplex; JNK, c-Jun N-terminal kinase; K, keratin; SAPK, stress-activated protein kinase

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Although most EBS mutations are genetically dominant, rare recessive cases exist. Some of these arise from junction protein mutations (Huber *et al.*, 2002; Jonkman *et al.*, 2002; Pasmooij *et al.*, 2004), but most are cases of homozygous or compound heterozygous ablation of K14 expression (Chan *et al.*, 1994; Rugg *et al.*, 1994; Jonkman *et al.*, 1996b; Batta *et al.*, 2000; Lanschuetzer *et al.*, 2003; Yiasemides *et al.*, 2008). These patients a show moderately severe phenotype and generalized blistering. Interestingly, total absence of K5, the co-polymerizing partner of K14, has never been observed in any individual, suggesting that ablation of K5 is lethal in humans. The existence of viable K14-null individuals opens another avenue for investigating the cellular mechanisms underlying EBS.

We previously developed a range of experimental stress assays, including heat shock, osmotic shock, and mechanical stretch (D'Alessandro *et al.*, 2002; Morley *et al.*, 2003; Russell *et al.*, 2004), and used them to show that EBS patient-derived cell lines expressing dominant-negative mutant keratins have an accelerated, augmented, and sustained response to stress, as seen by MAPK activation in response to osmotic shock (D'Alessandro *et al.*, 2002). K14-null cells, however, do not have to cope with the stress of misfolded protein, but neither do they have a reservoir of normal K14, so it was unclear how they might respond to stress. Here, we describe two cell lines established from patients with K14-null mutations. By comparing them with other EBS patient-derived keratinocytes and with isogenic lines re-expressing wild-type K14, we analyze the specific effect of K14 deficiency in the cells' response to experimental stress and the degree to which ''normal'' cell responses can be restored by adding back wild-type K14. The K14-null cell lines provide a useful disease model to study the effects of keratin inactivation. This will give important baseline information to design knockdown and replacement therapeutic strategies.

RESULTS

Addition of K14 into spontaneous K14-null EBS cells

The functional impact of K14 loss was assessed in the K14-null keratinocyte cell lines KEB-11 and KEB-13. KEB-11 was derived from an EBS patient with generalized blistering (Koebner EBS), caused by a homozygous *KRT14* mutation c.314delGC (p.Ala105TrpfsX3), leading to premature termination of both copies of the K14 mRNA (Rugg *et al.*, 1994). KEB-13 was derived from a patient with severe generalized skin blistering (III-2 in Jonkman *et al.*, 1996b), caused by the mutation c.526-2A>C in the 3′ acceptor splice site of *KRT14* intron 1 (Schuilenga-Hut *et al.*, 2002), which leads to misreading and premature terminations p.Ile176ValfsX2 or p.Ile176ProfsX30 in the helix 1B of the K14 protein (Jonkman *et al.*, 1996b). KEB-11 and KEB-13 were compared with the control keratinocyte cell line NEB-1 and the severely mutated KEB-7 (K14 c.374G>C and Arg125Pro) (Morley *et al.*, 2003).

Wild-type K14 complementary DNA (cDNA) was introduced into KEB-11 and KEB-13 using retroviral vectors, and following single-cell clonal selection, the levels of K14 expression in KEB-11 + K14 and KEB-13 + K14, and the ratio of K14 to endogenous co-polymerizing K5 were assessed (Figure 1a). Two clones showing a 1:1 ratio of K14 and K5 (clone J for KEB11 + K14 and clone C for KEB13 + K14; Figure 1a) were selected for further analysis.

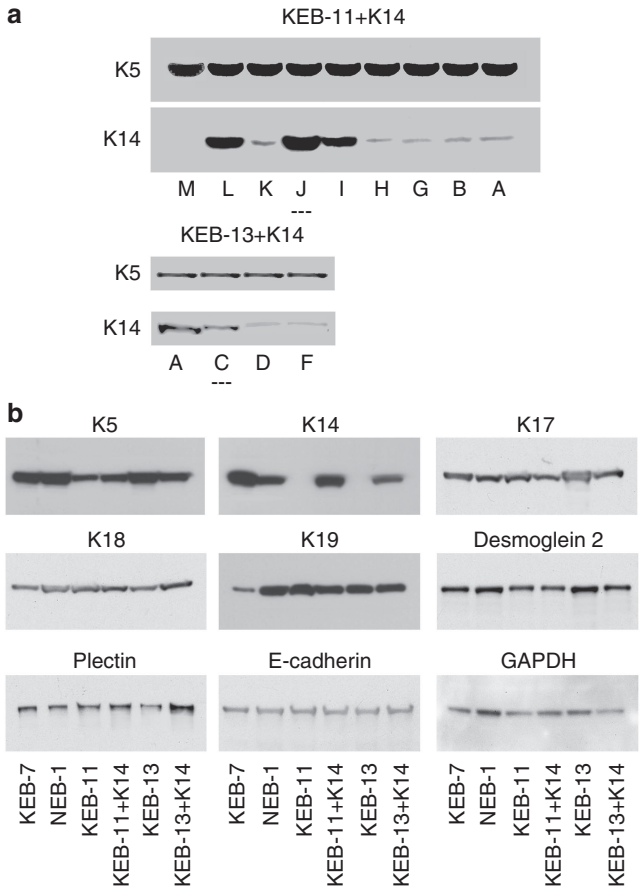

Figure 1. Immunoblotting analysis of cell extracts. (**a**) Immunoblotting analysis for clone selection. The levels of keratin 14 (K14) expression in KEB-11 + K14 and KEB-13 + K14 clones, and their ratio to the endogenous K5, were assessed. Clones J of KEB-11 + K14 and C of KEB-13 + K14, which have a 1:1 ratio of K14 to K5, were selected and further expanded. (**b**) Immunoblotting analysis of patient-derived cell lines. Total protein extracts from KEB-7, NEB-1, KEB-11, KEB-13, KEB-11 + K14, and KEB-13 + K14 were tested for keratins K5, K14, K17, K18, and K19, and for desmoglein 2, E-cadherin, and plectin. Apart from the lack of K14 protein in KEB-11 and KEB-13, and a significant increase of plectin expression in KEB-13 after K14 supplementation, all other keratins and epidermal proteins were present as expected. Protein loading was balanced for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels.

The introduction of K14 did not significantly affect the expression of other keratins or junctional and desmosomal proteins, apart from plectin. K5, K17, K18, and K19 levels did not significantly alter in KEB-11 and KEB-13 after K14 transfection (Figure 1b), nor was there any significant change in the levels of desmoglein 2 and E-cadherin (Figure 1b). However, plectin expression significantly increased in KEB-13 after K14 supplementation (Figure 1b).

After the addition of wild-type K14, the morphology of KEB-11 and KEB-13 remained unaltered (Figure 2a). K14 networks appeared well defined and fully extended to the cell junctions in both KEB11 + K14 and KEB13 + K14 cell lines

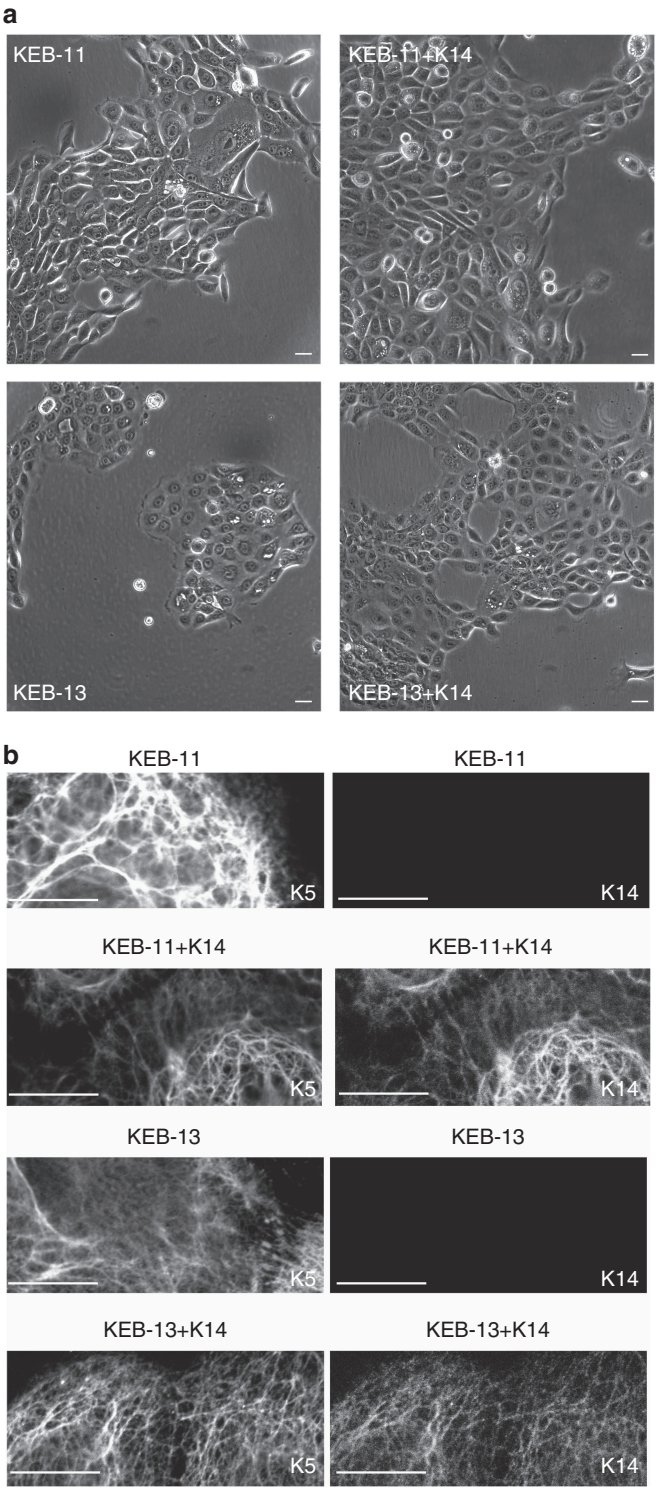

Figure 2. Morphology of keratin-null and keratin add-back cell lines. (**a**) Phase-contrast images of KEB-11, KEB-11 + K14, KEB-13, and KEB-13 + K14 show no significant morphological differences in the cells before and after transfection with keratin 14 (scale bar = 1 μm). (**b**) Immunofluorescence staining of keratin filaments in K14-null and add-back cell lines. KEB-11, KEB-13, KEB-11 + K14, and KEB-13 + K14 were fixed at 2 days after plating and double-stained with monoclonal antibody LL001 to K14 and polyclonal antiserum BLK18 to K5. K14 is absent in KEB-11 and KEB-13 but is strongly expressed, and colocalized with K5, in KEB-11 + K14 and KEB-13 + K14 (scale bar = 1 μm).

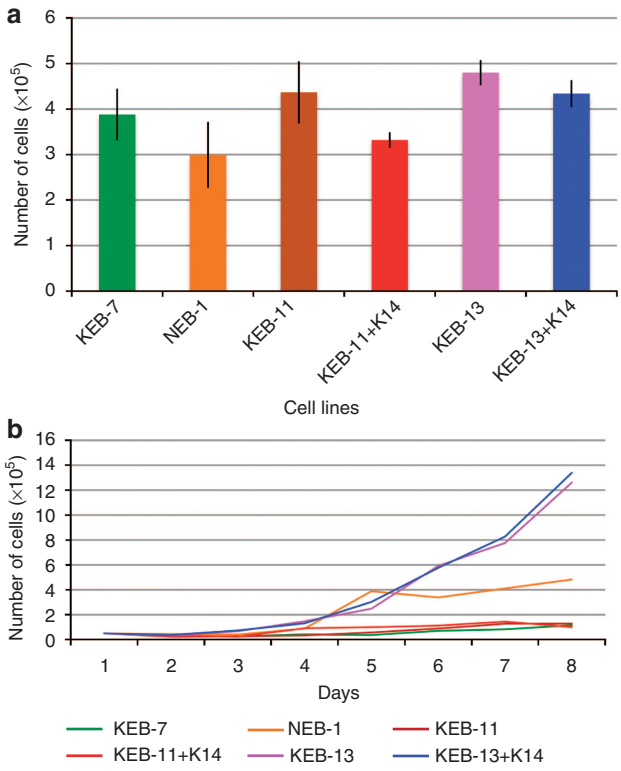

Figure 3. Comparison of cell proliferation rates between different cell lines. KEB-7, NEB-1, KEB-11, KEB-13, KEB-11 + K14, and KEB-13 + K14 were counted at: (**a**) 24 hours after plating and (**b**) over 8 consecutive days. (**a**) In all, 8×10^4 cells were plated onto Greiner ThinCert tissue culture filters, fixed after 24 hours, stained with DAPI, and counted. There was no significant difference in the proliferation of KEB-13 and KEB-13 + K14, and little difference between KEB-11 and KEB-11 + K14. (**b**) In all, 5×10^4 cells were plated into eight T25 flasks, and the cells in one flask were counted on each of the following 8 days. There was no significant growth rate difference between KEB-11 (brown curve) and KEB-11 + K14 (red curve), nor between KEB-13 (pink curve) and KEB-13 + K14 (blue curve). K14, keratin 14. KEB-11 and KEB11+K14 are the two steepest lines, NEB-1 is intermediate, and KEB-13, KEB-13+K14, and KEB-7 are the lower three lines.

(Figure 2b). Although no K14 was detectable before the add-back experiments, both KEB-11 and KEB-13 did contain filamentous keratin networks as seen with the antibody BL18 to K5 (Figure 2b). To sustain this filament network, one or more type I keratin must be co-polymerizing with K5 in the place of K14, as keratins are obligate heteropolymers. On double immunofluorescence staining, type I keratins K19 and K17 showed the most extensive colocalization with type II K5 (Supplementary Figure S1 online). K15 was excluded as a potential partner for K5 because of the low degree of colocalization (data not shown).

The growth and proliferation characteristics of KEB-11 and KEB-13 were also unaltered by the addition of K14 in either cell line (Figure 3), both in the short term (24 hours, Figure 3a) and in the long term (8 days, Figure 3b).

Effect of K14 expression on responses to stress assays

Migration assays. Using a battery of *in vitro* stress assays, we looked for evidence of functional rescue of KEB11 and KEB13

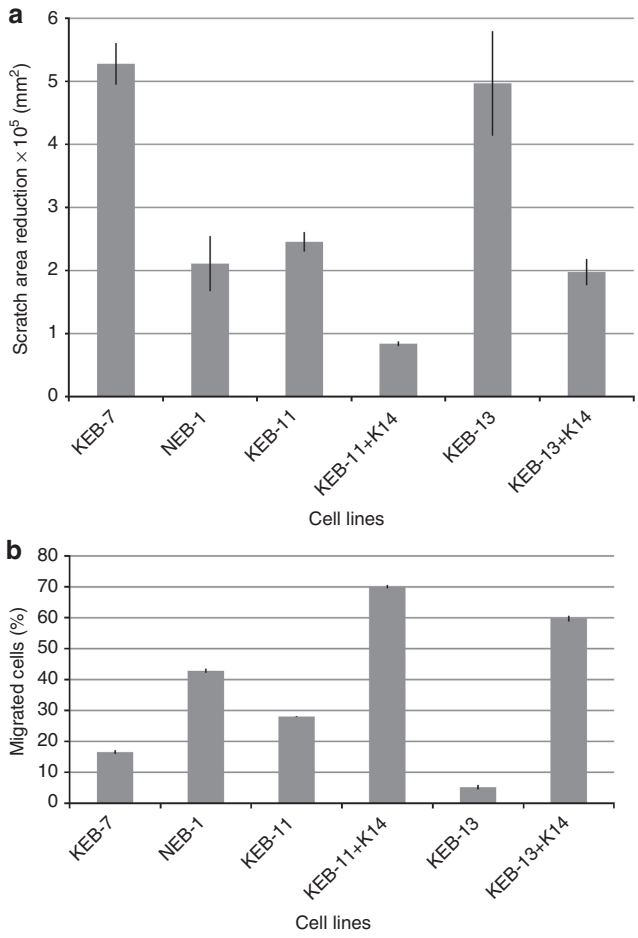

Figure 4. Migration assays on K14-null and K14-add-back cell lines. (**a**) Scratch wound closure assay. Scratch reduction after 8 hours is significantly slower in the wild-type NEB-1 than in KEB-7, as reported previously (Morley *et al.*, 2003). Addition of wild-type K14 reduces the closure migration rate in KEB-11 and KEB-13. (**b**) Transfilter migration assay. In this assay, KEB-7, KEB-11, and KEB-13 cells migrate less than the NEB-1: 18% KEB-7, 29% KEB-11, and 5% KEB-13 cross the filter compared with 42% NEB-1. After K14 add-back, both KEB-11 + K14 (70% now migrating) and KEB-13 + K14 (60% now migrating) show greatly improved transfilter migration. K14, keratin 14.

following K14 supplementation. To test cell migration, we used two models: (i) a scratch wound closure assay to assess the effects of K14 expression on epithelial sheet migration and (ii) a modified Boyden chamber assay, in which keratinocytes were challenged to migrate as single cells though 8 μm pores (Figure 4).

In the scratch wound closure assay, a single scrape was made across a confluent sheet of keratinocytes, and the extent of regrowth to close the scratch wound was measured after 8 hours (Figure 4a). As previously reported (Morley *et al.*, 2003), the reepithelialization process was significantly faster in the EBS dominant-negative mutant keratin cell line KEB-7 (K14 R125P) than in the control NEB-1 cell line (Figure 4a). This was interpreted as being linked to the observed hyperactivated stress responses of KEB-7 cells, when compared with the wild-type cells.

Reepithelialization was also significantly faster in the two K14-null cells, suggesting that they too might be in a physiological state of preactivation or stress. Reintroduction of K14 into KEB-11 and KEB-13 slowed down their migration, and the cells' behavior shifted much closer to the control speed of wound healing (Figure 4a).

The transfilter migration assay requires single cells to migrate through a filter. The ability of the mutant cells KEB-7 to migrate through the filter was significantly lower than the control NEB-1 cells, with only 18% of the plated KEB-7 cells detectable on the underside of the filter after 24 hours, against 42% of the wild-type NEB-1 (Figure 4b). The K14-null cells KEB-11 and KEB-13 were also inefficient, with only 29% and 5% of the cells, respectively, counted through the filter at 24 hours. Introduction of wild-type K14 into these cells significantly improved their ability to migrate, with 70% of KEB-11 + K14 and 60% of KEB-13 + K14 cells detectable on the underside of the filter by 24 hours (Figure 4b). The different rates of migration on the 24 hours time scale appear to be independent of cell proliferation, which is similar among all cell lines (Figure 3a).

Response to heat shock. In earlier studies, we showed that cells with severe dominant-negative mutations in K5 or K14 formed keratin aggregates on exposure to heat shock, which disappeared as the cells recovered (Morley *et al.*, 1995). When K14-null cell lines were exposed to heat shock, however, no alteration was observed in the keratin filaments neither in the KEB-11 or KEB-13 lines nor in their add-back transfectants (Supplementary Figure S2 online). Transient heat shock at 43° for 15 minutes caused keratin aggregates formation only in the mutant K14 cell line KEB-7. This result indicates that heat shock-induced aggregates are directly due to a mutant protein, rather than to the general instability of the rest of the keratin network caused by K14 deficiency.

Response to osmotic shock. Cells respond to the transient swelling caused by hypo-osmotic shock with the activation of the JNK (c-Jun N-terminal kinase)/SAPK (stress-activated protein kinase) pathway. We previously showed that cells expressing mutant keratins react to osmotic stress with faster JNK/SAPK activation of this pathway (D'Alessandro *et al.*, 2002, 2004). We tested the effect of K14 supplementation on JNK/SAPK activation in KEB-11 and KEB-13 cells before and after supplementation of K14. In KEB-11 and KEB-13, JNK/SAPK activation is visible at 5 minutes after stress, reaches a plateau at 20-minute recovery, and is still detectable at 2 hours after stress (Figure 5). In KEB-11 + K14 and KEB-13 + K14, JNK activation is significantly lower and 18 only detectable from 7.5 minutes until 1 hour after stress, with a pattern similar to the one shown by the wild-type NEB-1 (Figure 5).

DISCUSSION

Gene therapy approaches for dominant-negative disorders are usually designed to silence the mutant gene first and then correct the pathological phenotype through addition of the wild-type gene. But it is still not clear whether the

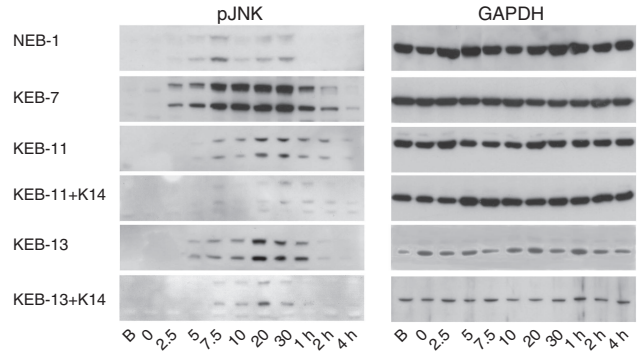

Figure 5. c-Jun N-terminal kinase (JNK) phosphorylation in response to hypo-osmotic shock. In KEB-11 and KEB-13, JNK1 (p46) and JNK2 (p54) activation is detectable at 5 minutes after cessation of the stress (weaker in KEB-11). The response continues to rise in all the epidermolysis bullosa simplex lines, peaking at 20 minutes and remaining detectable at 2 hours after stress. In KEB-11 + K14 and KEB-13 + K14, JNK1/2 activation is significantly attenuated, being only detectable at 7.5 minutes of recovery and never reaching high levels. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; K14, keratin 14; pJNK, phosphorylated c-Jun N-terminal kinase.

inactivation of a defective gene is less detrimental to the phenotype than the defective protein itself. EBS keratinocyte cell lines with total absence of K14 provide us with an ideal model to study the effects of inactivation of a defective protein in dominant-negative disorders.

The association of EBS with mutations in K5 or K14 is well documented, but the actual mechanisms by which these mostly missense mutations lead to cell fragility in the basal cells of the epidermis is still not fully understood. The most severe type of EBS, DM-EBS, is associated with aggregates of keratin in a non-filamentous state, and there is evidence that the misfolded K14 protein generates physiological stresses in the EBS cells. If these stresses were proven to contribute to the disease phenotype, then new therapeutic strategies for EBS, other than direct gene correction, could be explored (Russell *et al.*, 2010).

K14-null cells do not have to cope with the stress of misfolded proteins, but neither do they have a reservoir of normal K14. There is debate as to whether patients with the recessive form of EBS and total lack of K14 have a milder phenotype than patients with the DM-EBS dominant-negative form of EBS (Chan *et al.*, 1994). Although this has been hard to investigate systematically, a null phenotype should lend itself to add-back gene therapy approaches.

In this study, we used different stress assays to compare two cell lines generated from patients expressing no K14 protein before and after K14 addition. We also looked at the response to the same stresses in a cell line expressing a severe dominant-negative K14 mutation (KEB-7) and in a wild-type cell line (NEB-1).

A number of findings emerged from this study. First, we cast light on the compensatory co-assembly of K5 with an alternative partner to K14. *In vitro,* type I keratins can associate with many type II keratins (Hatzfeld *et al.*, 1987), but *in vivo* copolymerization is much more selective. In earlier EBS studies, both K15 and K17 have been suggested as alternative partners to K5 in the K14-null patients (Chan *et al.*, 1994; Rugg *et al.*, 1994; Jonkman *et al.*, 1996b). In the tissue culture environment of the present study, KEB-11 and KEB-13 cells both express many type I keratins in the absence of K14, but K19 is the most highly expressed. K19 is less differentiation restricted than most keratins, and is expressed in regions of flexible or borderline differentiation commitment (Stasiak *et al.*, 1989). It has been proposed to help maintaining cells in an indeterminate state before differentiation commitment and has been described as a potential stem cell marker in epidermis (Lane *et al.*, 1991; Michel *et al.*, 1996). However, K19 is a short, tail-less keratin that may be less able to generate a robust keratin filament network than the longer keratins (Lu *et al.*, 1993; Fradette *et al.*, 1998). This deficit could explain why the K14-null cells are nearly as severely affected by stress as the DM-EBS cell line KEB-7.

The addition of K14 to K14-null cells growing in monolayers did not affect the expression of E-cadherin or desmoglein-2 in adherens junctions and desmosomes, respectively. Plectin expression was, however, significantly increased in KEB-13. Plectin is a member of the plakin family of large cytoskeleton linker proteins, located in the desmosomes and hemidesmosomes, and binds keratin intermediate filaments (Bolling *et al.*, 2010). Plectin mutations are associated with clinical phenotypes highly related to EBS, such as the form of EBS associated with muscular dystrophy (McLean *et al.*, 1996; Smith *et al.*, 1996; Koss-Harnes *et al*, 2002). The observations of elevated plectin in a K14-null cell line indicate the existence of a feedback mechanism operating between keratins and plectin, whereby an abnormality in keratin filament composition triggers a possible compensatory upregulation of a keratin-binding protein.

The stress assays used in this study were specifically developed as tracking indicators of the EBS phenotype and of its amelioration in response to treatments. In all the assays conducted, when wild-type K14 was added back to the K14-null lines, the behavior of the transfected cells was shifted closer to that of wild-type cells, demonstrating a significant rescue of the EBS phenotype. Where they could be compared, relative reproducibility was high between these experiments and earlier ones (D'Alessandro *et al.*, 2002; Russell *et al.*, 2004; Liovic *et al.*, 2008). The lack of aggregate formation after heat shock in K14-null cells clarifies interpretation of the heat shock-induced aggregates as directly due to mutant protein rather than to the general instability of the rest of the keratin network, because of the deficiency of K14. KEB-11 and KEB-13 cells, in fact, do not make any abnormal protein that would contribute to misfolding and aggregate formation.

We included two different migration assays in this battery and, interestingly, they gave nearly opposite results. In the scratch wound assay, mutant cells migrated faster than wild-type cells, moving as a continuous epithelial sheet to close the scratch wound. In the transfilter migration assay, the mutant cells, moving individually, were less efficient at migrating through the filter pores than wild-type cells, as measured by net cell numbers across the filter after 24 hours. These two assays measure very different cell behaviors. Normal epithelial cells always migrate as a compact sheet, and the scratch wound

assay therefore reflects a more normal activity. The transfilter migration assay requires single cells to migrate through pores in a filter in a behavior that mimics metastatic epithelial cells rather than normal epithelial sheets. K14-null cells showed no obvious signs of metastatic behavior, although severe EBS has Indeed been associated with a greater incidence of skin cancer (Fine *et al.*, 2004). These results also emphasize the need for careful selection of appropriate functional assays.

We have previously obtained experimental evidence for a direct correlation between the severity of the mutation carried by an EBS cell line and the speed of JNK activation, with more severe mutations associated with faster and more sustained activation (D'Alessandro *et al.*, 2002). Here, we demonstrate that the addition of K14 to K14-null cell lines reduces their JNK activation levels, shifting their SAPK/JNK stress response pathway closer to that of cells with wild-type keratin.

The assays used in this study showed that the absence of K14 leads to a constitutive stress, as in the case of dominant missense mutations, even though in K14 null cells there are no visible protein aggregation consequences. However, the level of JNK/SAPK activation is clearly lower in the K14-null lines than in KEB-7, supporting the hypothesis that a K14-null phenotype is less severe than a DM-RBS phenotype (Chan *et al.*, 1993; Jonkman *et al.*, 1996a, b).

Curative therapy for genetic skin disorders will require some alteration of the genetic expression profile of the affected cells or some form of bypassing of the defect. But whatever methods are adopted for correcting the defects in cells and tissues (Wells, 2004), disease model culture systems will always be required to demonstrate the effectiveness at early stages of specific gene therapy development. Efficient models to better understand the disease mechanisms and discover more generic ways of therapeutically ameliorating tissue fragility disorders are needed (Gonzalez-Gonzalez *et al.*, 2009; Russell *et al.*, 2010; Wally *et al.*, 2010). The results presented here show that the two K14-null cell lines KEB-11 and KEB-13 can be used as such models for epithelial cells. By transfecting wild-type K14 into these cells and testing them with various stress assays, we were able to demonstrate that an add-back approach can significantly rescue the normal keratinocyte behavior profile.

MATERIALS AND METHODS

Cell culture

The keratinocyte-derived cell lines used in this study were NEB-1 (wild type), KEB-7 (K14, c. 374G>C) (Morley *et al.*, 2003), KEB-11 (K14, c.314delGC), and KEB-13 (K14, c.526-2A>C). Primary keratinocytes from the patients, obtained in accordance with the Declaration of Helsinki protocols and with informed consent, were immortalized using HPV16 E6^E7 and neomycin selection for NEB-1, KEB-7, and KEB-11 (Morley *et al.*, 2003). For KEB-13, LXSNE6E7 retrovirus was used (courtesy of Dr T Brown, Fred Hutchinson Cancer Research Center, Seattle; Roecklein and Torok-Storb, 1995). Experiments described in this study were carried out on mutant and control cell lines at passage 20 after immortalization. Cells were cultured in 75% DMEM and 25% Ham's F12 medium, with 10% fetal calf serum, plus additional growth factors hydrocortisone ($0.4\,mg\,ml^{-1}$), transferrin ($5\,mg\,ml^{-1}$), liothyronine (2×10^{-11} M), adenine (1.9×10^{-4} M), insulin ($5\,mg\,ml^{-1}$), and epidermal growth factor ($0.1\,ng\,ml^{-1}$). All cell lines were fibroblast feeder cell independent, and were cultured at 37°C in 5% CO_2.

Supplementation of wild-type K14 into K14-null cells

The human K14 wild-type cDNA (NM_000526) was cloned into the LZRS-LacZ-Ires-Blasto vector, derived from the previously described LZRS-LacZ vector (Kinsella and Nolan, 1996), using the *Bam*HI and *Xho*I restriction sites. This retroviral vector was transfected into Phoenix amphotropic-packaging cells (Pear *et al.*, 1993) by calcium–phosphate/chloroquine-induced transfection to produce viral particles (Kinsella and Nolan, 1996). KEB-11 and KEB-13 cells were infected with the viral particles, drug selected with blasticidin (D'Alessandro *et al.*, 2004), and single-cell clones selected. The levels of expression of transfected K14 in KEB11 and KEB13, and their ratio, compared with the endogenous wild-type K5 were assessed by immunoblotting with LL001 monoclonal antibody against K14 and BL18 rabbit polyclonal antibody against K5 (Purkis *et al.*, 1990). Clones with an approximately 1:1 ratio of K14 and K5 were further selected and expanded.

Keratin expression

Monoclonal antibodies used in this study were: LL001 against K14 (Purkis *et al.*, 1990); E3 against K17 (Guelstein *et al.*, 1988), LP34 reacting with keratins K1, K5, K6, and K18 (Lane and Alexander, 1990), and LP2K against K19 (Böttger *et al.*, 1995). A rabbit polyclonal antiserum, RbaK5 (BL18), was used against K5 (Purkis *et al.*, 1990). Cells were grown on coverslips for 2 days, fixed with ice-cold methanol: acetone (1:1 ratio) for 5 minutes, immunostained with different antibodies, and analyzed, as previously described (D'Alessandro *et al.*, 2002). Immunoblotting was carried out using the above antibodies plus LDK18 against K18 (EBL, unpublished), anti-Dsg2 against desmoglein 2 (a gift from late M Wheelock, University of Toledo, Ohio), HD121 against plectin (Hieda *et al.*, 1992), and 36/E-Cadherin against E-cadherin (BD Transduction Laboratories, East Rutherford, NJ).

Cell proliferation

The growth rates of all cell lines were measured by plating 5×10^4 cells into eight T25 flasks (Greiner Bio-One GmbH, Frickenhausen, Germany). On each of the 8 days after plating, cells were dispersed by trypsin treatment and counted with a Coulter counter (Beckman Coulter, Luton, UK). To determine the cells' growth rate on the Greiner ThinCert tissue culture filter (see below), over a 24 hours period, 8×10^4 cells were plated onto each membrane, fixed after 24 hours, stained with DAPI (Invitrogen, Carlsbad, CA) and counted.

Scratch wound assay

Simple scratch wounds were made with a yellow Gilson pipette tip in all wells of a 24-well plate of cells previously grown to confluence over 5 days. A total of 12 wells were fixed with methanol: acetone (1:1 ratio) at time zero and the remaining wells were fixed after 8 hours of continued growth. The areas of the wounds were measured using Axiovision 3.0 software (Carl Zeiss Vision GmbH, Oberkochen, Germany) and the reduction in wound size was calculated (Morley *et al.*, 2003).

Transfilter migration assay

ThinCert tissue culture inserts (8.0 μm pore size, translucent membrane) were used in a 6-well plate (Greiner Bio-One) to create an upper and a lower chamber. The lower chamber was filled with 2 ml of migration buffer (0.5% bovine serum albumin in culture medium, plus 10% fetal calf serum as chemoattractant). In all, 5×10^5 cells were resuspended in 1 ml of migration buffer and added to the upper chamber on the top of the insert. The plate was incubated at 37°C. After 24 hours, 1 ml of 4% paraformaldehyde solution was added to the lower chamber for 15 minutes to fix the cells that had migrated through the filter. Membranes were washed three times in phosphate-buffered saline, and cells remaining on top of the filter were removed with a cotton bud. One ml of a 1/5,000 solution of DAPI in phosphate-buffered saline was added to the lower chamber for 10 minutes to stain the nuclei of the migrated cells. The filter membranes were then cut away from the chamber and mounted on a slide. Migrated cells were counted using Axiovision 3.0 software. In all, 20 fields of view were counted per slide and the average was used, expressed as % of total cells plated at the start.

Heat stress assay

Heat stress assay was carried out as previously described (Morley *et al.*, 1995). Briefly, cells were plated onto coverslips and grown to near confluence in 48 hours. Medium at 37°C was exchanged for fresh culture medium at 43°C, and cells were maintained at 43°C for 15 minutes. Medium was then replaced and cells returned to normal temperature. After 15 minutes of recovery at 37°C, coverslips were removed, fixed with ice-cold methanol/acetone (1:1), and stained with monoclonal antibody LP34 against K1, K5, K6, and K18 (Lane and Alexander, 1990) and processed as above.

Osmotic stress assay

Cells were cultured to reach 80% confluence in 48 hours, and then subjected to hypo-osmotic shock by immersion in 150 mM urea for 5 minutes at 37°C. The hypo-osmotic solution of urea was replaced with normal tissue culture medium, and cells were allowed to recover for variable times (D'Alessandro *et al.*, 2002). Cells were lysed into extraction buffer (Liovic *et al.*, 2009) at 0, 2.5, 5, 7.5, 10, 20, and 30 minutes, and 1, 2, and 4 hours of recovery after osmotic shock. Protein concentration was determined by Bradford assay (Bio-Rad protein assay, Bio-Rad GmbH, Munich, Germany), and 5 μg of total protein per sample was loaded on denaturing protein gels (Invitrogen, Carlsbad, CA). Immunoblotting was carried out using overnight incubation at 4°C with antibodies to phospho-JNK/SAPK (New England Biolabs, Ipswich, MA, dilution 1:1,000 in 5 ml blocking buffer). Bound primary antibodies were detected using a secondary antibody of swine anti-rabbit Ig antiserum, conjugated to horseradish peroxidase (diluted 1:1,000 in 5 ml blocking buffer), and visualized by electrochemiluminescence. A GAPDH-HRP antibody (Abcam, Cambridge, MA) was used as loading control.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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