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Molecular Therapeutics for Heritable Skin Diseases

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INTRODUCTION

Over the past two decades, there has been tremendous progress in molecular genetics of heritable skin diseases, and as many as 500 distinct human genes are now known to harbor mutations in these diseases (Feramisco *et al.*, 2009). The clinical implications of this progress are evident in the diagnosis and management of these diseases. For example, (a) identification of the specific mutations can be used for confirmation of diagnosis with prognostic implications; (b) identification of mutations has facilitated assessment of the precise mode of inheritance, particularly in cases with no previous family history of the disease; and (c) identification of candidate genes and mutations has formed the basis for DNA-based prenatal testing and pre-implantation genetic diagnosis in families at risk for recurrence (Uitto, 2009). However, in spite of significant progress in identification of the molecular basis of heritable skin diseases, there has been relatively little progress until very recently in developing effective and specific treatments. This synopsis will highlight some of the milestones in progress towards treatment and cure of heritable skin diseases, primarily focusing on epidermolysis bullosa (EB) as a paradigm of such conditions, with emphasis on development of gene-,protein-, and cell-based molecular strategies just entering the clinical arena (Uitto *et al.*, 2012).

RECLINICAL MODEL SYSTEMS

EB is a heterogeneous group of heritable blistering disorders due to fragility of the cutaneous basement membrane zone (link to *Milestone* article no. 1; Fine *et al.*, 2008; Intong and Murrell, 2012). The development of molecular therapies for skin diseases has been largely predicated upon development of animal model systems, particularly transgenic mice that recapitulate their clinical, histopathological, and ultra-structural features (Bruckner-Tuderman *et al.*, 2010; Natsuga *et al.*, 2010). In the case of EB, these include knockout mice with targeted ablation of the corresponding gene, such as those encoding type VII collagen, type XVII collagen, or the subunit polypeptides of laminin 332. Also, identification of naturally occurring spontaneous mutations in mice and other vertebrates has been helpful for development of preclinical approaches. In addition to transgenic animals, human xenograft

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CONFLICT OF INTEREST
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transplants onto immunocompromised mice have provided useful model systems (Siprashvili *et al.*, 2010). Finally, to our knowledge, previously unreported vertebrate model systems, such as zebrafish, have been explored as potential models for heritable skin diseases (Li *et al.*, 2011).

PROSPECTS OF GENE THERAPY

Information gleaned from the experiments utilizing preclinical animal models has been critical for the development of gene therapy approaches for EB. The first *Milestone* for applying gene therapy for EB took place in 2005, when cultured keratinocyte stem cells with holoclone potential were cultured from the skin of a patient with a junctional form of EB (Mavilio *et al.*, 2006). This patient was shown to have mutations in the *LAMB3* gene, which encodes the β3-subunit polypeptide of laminin 332. The patient, a 36-year-old man, had a form of non-Herlitz junctional EB caused by compound heterozygosity for a *LAMB3* null mutation and a missense mutation (E210K) that, in addition to the amino acid substitution, disrupts intron splicing. Holoclone-forming cells were not present in the blistering area of skin, most likely because the continuous proliferative stimulus associated with the chronic wound-healing process had depleted the stem cell pool. However, biopsy from the palm area, which blistered less, allowed a sufficient number of holoclones to be cultured. The cultured cells were transduced with a Maloney leukemia virus–derived retroviral vector expressing the *LAMB3* complementary DNA, and these cells were then used to prepare genetically corrected epidermal grafts in culture (Figure 1). The grafts were transplanted onto selected areas of the patient's legs, which were prepared for acceptance of the graft by laser ablation of the deficient epidermis. Engraftment was shown to result in synthesis and proper assembly of normal levels of functional laminin 332, which clinically resulted in development of adherent epidermis that remained stable and did not demonstrate blistering (Mavilio *et al.*, 2006). A published 3.5 year follow-up noted that on clinical examination, no blisters were observed in the transplanted area of skin, and the regenerated skin was stable, normal looking, and functionally resilient to mechanical trauma (De Luca *et al.*, 2009). There was no evidence of inflammation, and specific tests carried out 3 and 6 months after the transplantation procedure indicated absence of both humoral and T cell–mediated cytotoxic immune responses against the transgene product. It should be noted that one of the mutant alleles contained a single point mutation (E210K), which allowed residual expression of some laminin 332. Thus, the introduction of the complementary DNA–derived laminin β3 polypeptide was not recognized as a new protein by the patient's immune system, and no evidence of rejection has been noted.

Although this study clearly provided proof-of-principle of *ex vivo* keratinocyte therapy for EB, there have been significant concerns regarding this approach. One of them revolves around the safety of the retroviral vectors used to integrate the transgene into the genome, and specifically, the concern highlights the possibility that the random insertion of the transgene results in activation of proto-oncogenes or inactivation of tumor suppressive genes (De Luca *et al.*, 2009). These concerns were raised in response to clinical trials in which introduction of the curative transgene into hematopoietic progenitor cells of patients with X-linked severe combined immuno-deficiency caused activation of a T cell proto-oncogene, resulting in leukemia (Hacein-Bey-Abina *et al.*, 2003). Although it has been suggested that,

on statistical grounds, the probability of such an event to occur is extremely low, and that the retroviral integration into the genome may not be random, these concerns have resulted in the conclusion that the Maloney leukemia virus LTR-based vectors are not acceptable for genetic modification of stem cells, and such clinical trials are now banned by the regulatory agencies in many countries in Europe (see De Luca *et al*., 2009). Nevertheless, there have been significant improvements in vector design, and many of them have potentially a more favorable safety profile due to targeted integration sites and self-inactivating properties (Titeux *et al.*, 2010). On the basis of these considerations, clinical gene therapy trials focusing on the recessive dystrophic forms of EB have been recently initiated (Dr. Alfred Lane, Stanford University, personal communication).

In the context of *ex vivo* keratinocyte gene therapy, the phenomenon known as revertant mosaicism is of considerable interest (Lai-Cheong *et al.*, 2011; Pasmooij and Jonkman, 2012). Revertant mosaicism in skin diseases was originally noted in a patient with a junctional form of EB, where areas of skin were shown to reacquire the wild-type phenotype through naturally occurring mitotic gene conversion (Jonkman *et al.*, 1997). Thus, revertant mosaicism reflects “natural gene therapy,” which could provide corrected, patient-specific cells for grafting purposes (Gostynski *et al.*, 2009).

RNA INTERFERENCE TECHNOLOGY

Another gene therapy approach for treatment of inherited skin diseases utilizes silencing of the mutant RNA by short-interfering RNA (siRNA; Figure 2). Specifically, the safety and efficacy of a siRNA targeting mutant keratin 6A (N171K) has been recently reported in a patient with pachyonychia congenita (PC). The *Milestone* paper by Leachman *et al.* (2010) describes a patient with PC, a disabling keratinization disorder affecting the skin, nails, oral mucosa, hair, and teeth. This disease is inherited in an autosomal dominant fashion and often leads to painful lesions in the feet characterized by blistering and callus formation on the soles. PC is caused by mutations in either keratin 6, 16, or 17 genes, and the mutant polypeptides cause the clinical manifestation by dominant-negative interference (McLean *et al.*, 2011). The approach of this study was to elicit a selective depletion of the mutant keratin by injection of siRNA using a siRNA molecule carefully selected to be mutant-specific and not interfere with the wild-type K6a mRNA.

This double-blind study consisted of injection of the siRNA to one foot while the other foot received a vehicle–control solution (Leachman *et al.*, 2010). The treatment consisted of 17 weeks of two times weekly injections and was followed by a 3-month washout. Careful assessment of the clinical signs and symptoms revealed definitive improvement, and the size of the callus in siRNA-injected sites was getting smaller. Thus, this first-in-human double-blinded phase 1b clinical trial suggested the proof-of-principle of RNA interference technology for patients with keratinization disorders.

This study also highlighted some of the drawbacks of this approach. First, the siRNA was delivered by direct injection to the lesional skin, resulting in intense pain (Leachman *et al.*, 2010). Consequently, significant efforts are now focusing on improved delivery methods, such as pharmaceutical formulations for topical, non-invasive delivery for PC, and other

keratin disorders (Kaspar *et al.*, 2009; McLean and Moore, 2011). Another concern is that although there was a dramatic and specific response in the treated area of skin, the improvement appeared to be temporary, and upon discontinuation of the treatment, the lesions returned to their original size. Thus, continuous treatment by improved topical delivery system might be required for this approach (Smith *et al*., 2008). Finally, it has been pointed out that one of the disadvantages of the allele-specific gene silencing approach is that the US Food and Drug Administration and corresponding international agencies may consider each mutation-specific siRNA as a separate entity requiring individual toxicity studies and regulatory approval (see Uitto *et al.*, 2012). Thus, an alternative approach to the mutant allele–specific ablation, for example, in case of PC, would be total silencing of all *KRT6A* alleles, both mutant and wild type (Figure 2). It should be noted that the blistering phenotype does not occur in the complete absence of K6a because of functional redundancy with K6b and K6c, allowing normal intermediate filament network integrity. Clinical trials are now being contemplated using these approaches for PC and other keratinization disorders (Dr Irwin McLean, University of Dundee, personal communication).

ALLOGENEIC FIBROBLAST THERAPY

The *Milestone* article by Wong *et al.* (2008) reported on a clinical trial in which the potential for intradermal injections of allogeneic fibroblasts were assessed in individuals with recessive dystrophic EB (RDEB). These studies were based on the premise that human skin fibroblasts, in addition to keratinocytes, express type VII collagen (Stanley *et al.*, 1985; Chen *et al.*, 1994). Furthermore, previous animal studies have suggested that *COL7A1* gene–corrected human RDEB fibroblasts overexpressing type VII collagen, when injected intradermally into immunodeficient mouse skin or into a transplanted human RDEB skin xenograft, allowed sustained human type VII collagen deposition at the dermal epidermal junction, accompanied by anchoring fibril formation (Woodley *et al.*, 2003). These observations lead the authors to postulate that injection of a sufficient number of normal fibroblasts could lead to amelioration of the skin phenotype in patients with RDEB.

Five subjects were injected with cultured autologous fibroblasts into the edge of unblistered skin, and the expression of type VII collagen was evaluated by immunofluorescence in biopsies at 2 weeks and 3 months after a single injection (Wong *et al.*, 2008). Significant, up to ~2-fold increase in type VII collagen immunostaining was noted at sites injected with parental fibroblasts or from those of unrelated donors. The increased type VII collagen at the dermal–epidermal junction was accompanied with an increase in anchoring fibrils, although these were not fully developed. Nevertheless, this and complementary studies demonstrated the potential of allogeneic fibroblast therapy for treatment of RDEB (Petrova *et al.*, 2010; Yan and Murrell, 2010).

One of the observations in this study was that the allogeneic cells, as determined by Y chromosome positivity in female patients injected with male fibroblasts, were not detectable 2 weeks after the initial injection, yet type VII collagen deposition continued up to 3 months (Wong *et al*., 2008). Although initial observations of the injection sites did not reveal evidence of significant inflammation, subsequent studies suggested a subclinical immunological mechanism by which the allogeneic fibroblasts elicit increased type VII

collagen expression in the basal keratinocytes of the recipient's skin (Nagy *et al.*, 2011) (Figure 3). This conclusion was supported by the observation that increased type VII collagen deposition at the dermal–epidermal junction was particularly pronounced in those patients who expressed some type VII collagen at the baseline, and who, therefore, had an enhanced capacity to increase synthesis of their own mutant type VII collagen. These studies identified heparin-binding epidermal growth factor–like growth factor as a novel putative mediator induced in the recipient cells by allogeneic fibroblast injection (Figure 3).

Although these initial studies utilized a single injection of allogeneic fibroblasts to the skin, the increase in type VII collagen persisted for several months. This can be explained, in part, by the observation that type VII collagen, once deposited to the cutaneous basement membrane zone, has a relatively long half-life as judged from mouse studies (Kern *et al.*, 2009). Clinical trials utilizing multiple injections at regular intervals are now being contemplated to counteract the development of chronic wounds in patients with RDEB (Dr John McGrath, St John's Institute of Dermatology, personal communication).

BONE MARROW STEM CELL THERAPY

Cell-based therapy for EB and potentially other heritable skin diseases has recently been extended to include bone marrow–derived adult stem cells. Although these cells are known to have a critical role in skin homeostasis, it has also become clear that the plasticity of these cells enables their differentiation into cell types responsible for skin regeneration after injury (Badiavas *et al.*, 2003; Tamai *et al.*, 2009). The *Milestone* article describing the first clinical trial of allogeneic bone marrow transplantation for EB was reported by Wagner *et al.* (2010). In this study seven children with severe RDEB were enrolled to be recipients of bone marrow transplantation, using standard myeloablative approach (Wagner *et al.*, 2010). This study was predicated on previous animal studies, which have been conducted to evaluate the potential of bone marrow–derived stem cells to treat EB. For example, bone marrow transfer into the fetal circulation of mice that are deficient in type VII collagen resulted in deposition of type VII collagen in the skin, associated with reduction in the severity of the blistering in neonatal animals (Chino *et al.*, 2008). Also, hematopoietic and nonhematopoetic cell populations were infused into type VII collagen knockout mice at birth, and this treatment was shown to extend the survival of the recipient mice by several weeks or months (Tolar *et al.*, 2009). The EB phenotype was also rescued in a type XVII collagen knockout model by bone marrow transplantation (Fujita *et al.*, 2010). Although type VII collagen is synthesized both by keratinocytes and fibroblasts, type XVII collagen, a component of hemidesmosomes, is synthesized exclusively by keratinocytes. Collectively, these studies demonstrated that different bone marrow–derived stem cells, including mesenchymal stem cells, can ameliorate the clinical symptoms and increase the survival rate of EB mice, thus paving a way to clinical trials in patients with different forms of EB.

Bone marrow transplantation in the children with RDEB was noted to result in synthesis of new type VII collagen and clinical improvement that was sustained for at least 1 year after the transplantation (Wagner *et al.*, 2010; Tolar *et al*, 2011). Although these preliminary studies were promising and generated cautious optimism, it should be noted that two of the seven children died as a result of complications of the bone marrow transplant procedure,

which utilized traditional chemoablative preconditioning of the recipients. A second clinical bone marrow transplantation trial has been initiated with the approach to use reduced-intensity chemotherapy before transplantation, perhaps having lesser side effects with reduced morbidity and mortality (Kiuru *et al.*, 2010).

In addition to bone marrow transplantation, a pilot study on two patients with severe RDEB has tested the efficacy of intradermal injection of allogeneic mesenchymal stem cells into chronic ulcerative sites in these patients (Conget *et al.*, 2010). Improved wound healing lasting up to 4 months was attributed to replenishment of type VII collagen, which was undetectable before the procedure. Similar studies are now being developed to examine whether intradermal or intravenous injection of bone marrow–derived mesenchymal stem cells can improve the clinical outcome. Collectively, these early observations support the usefulness of bone marrow stem cell populations in the treatment of heritable skin diseases, such as RDEB.

NOVEL THERAPEUTIC APPROACHES

A number of new technologies are currently being developed for potential treatment of EB, but these have not reached the clinical trial stage as yet. One of such approaches focuses on induced pluripotent stem cells (iPSCs), which allow patient-specific cells to be corrected for the gene defect, followed by introduction of differentiated fibroblast and/or keratinocytes to the skin (Uitto *et al.*, 2012) (Figure 4). This approach would circumvent the difficulties in obtaining sufficient numbers of patient-specific cells and avoid the problem of immune rejection. Recently, patient-specific iPSCs have been generated from several human diseases to investigate the disease mechanisms, test potential drugs, and develop cell-based therapies. In the case of heritable skin diseases, patient-specific iPSCs have been generated from patients with dyskeratosis congenita as well as RDEB (Agarwal *et al.*, 2010; Itoh *et al.*, 2011; Tolar *et al.*, 2011). Although a number of technological issues still need to be resolved before iPSC-based therapy can be moved to the clinic, there is rapid technological progress in this area, and the first report of gene correction utilizing patient-specific iPSCs has already been reported in a case with gyrate atrophy (Howden *et al.*, 2011), providing a proof-of-concept for this technology.

Another approach to counteract the clinical manifestations of EB, which has not reached the clinical trial stage yet, is potential protein-replacement therapy. This concept is again predicated on the use of *Col7a1* knockout mice as a platform, which demonstrated that infusion of a purified type VII collagen results in significantly reduced blistering and extends the lifespan of these mice (Remington *et al.*, 2009). Again, once the type VII collagen has been properly deposited on the cutaneous basement membrane zone, the protein remains stably assembled for several months. Clinical trials utilizing GMP-purified type VII collagen are currently contemplated for patients with severe RDEB (Dr David Woodley, University of Southern California, personal communication).

Collectively, regenerative medicine for heritable skin diseases is moving very rapidly, and with novel technological innovations the field is making progress towards treatment and cure of these, currently intractable disorders.

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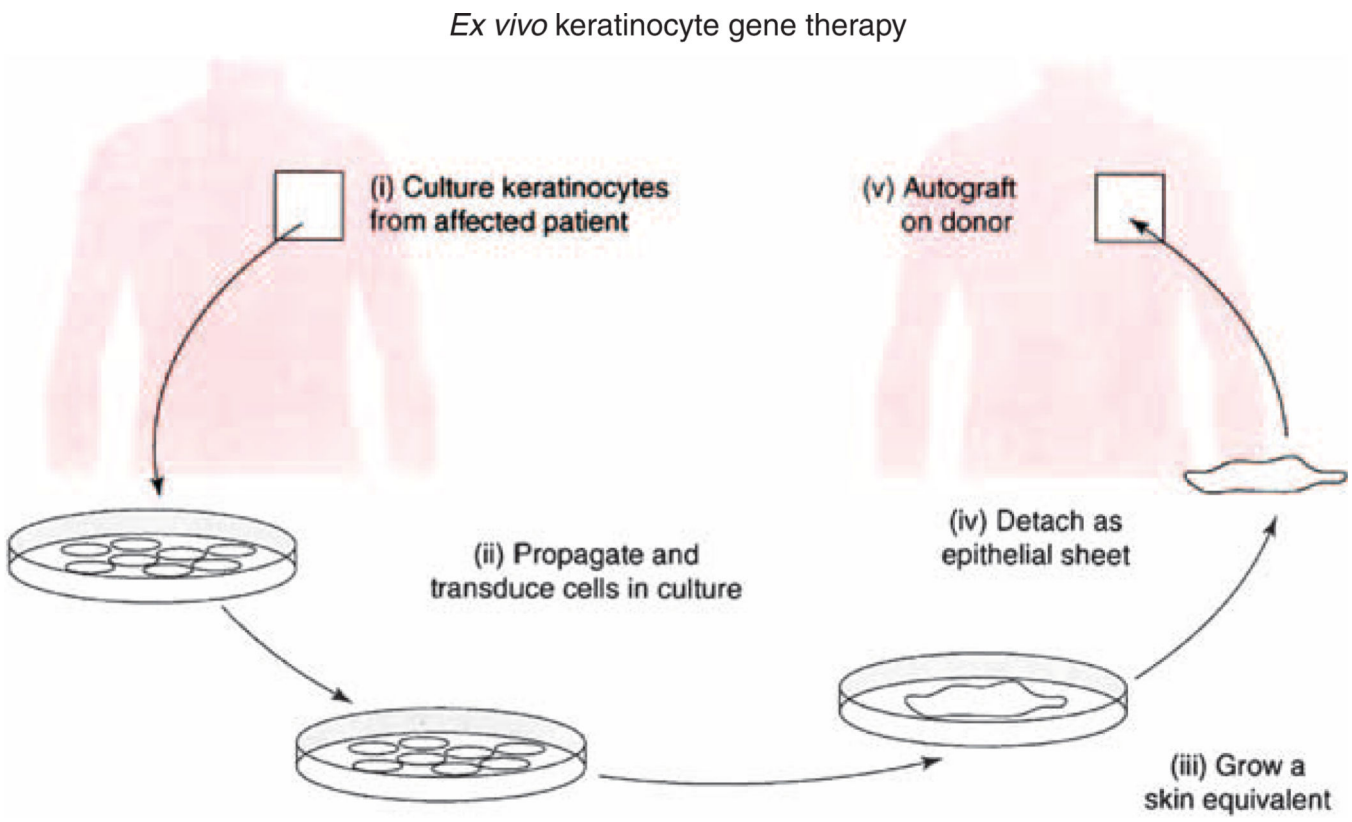

Figure 1. Strategy for an *ex vivo* patient-specific keratinocyte gene therapy

Keratinocytes cultured from the patient's skin biopsy are transduced with a vector expressing the transgene, and the transgenic cells are selected and grown into epithelial sheets that can be grafted back to the patient (adapted from Tamai *et al.*, 2009).

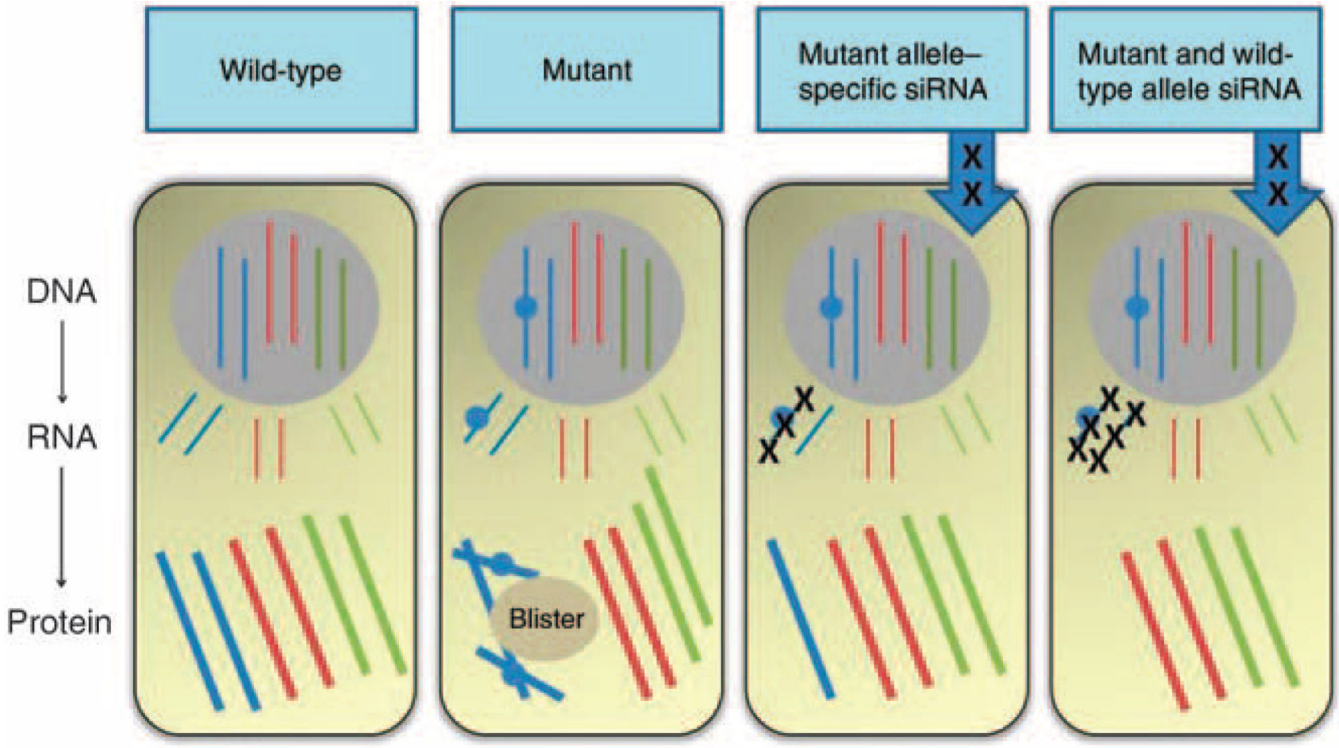

Figure 2. Small interfering RNA (siRNA) strategies for autosomal dominant keratin 6a disorders by targeting either mutant or both mutant and wild-type alleles

(a) In normal keratinocytes, synthesis of K6a (blue), K6b (red), and K6c (green) occurs; (b) in pachyonychia congenita keratinocytes with a heterozygous missense mutation in *KRT6A*, there is a dominant-negative interference between the wild-type and mutant K6a protein that perturbs the entire keratin network and compromises cell integrity, leading to skin blistering as a result of minor trauma; (c) one siRNA approach is to target the mutant *KRT6A* allele to leave only residual wild-type *KRT6A* allele expression; (d) an alternative siRNA strategy is to silence all *KRT6A*, both mutant and wild-type—blistering does not occur in the absence of K6a because of functional redundancy with K6b and K6c, allowing normal intermediate filament network integrity (reproduced from Uitto *et al.*, 2012).

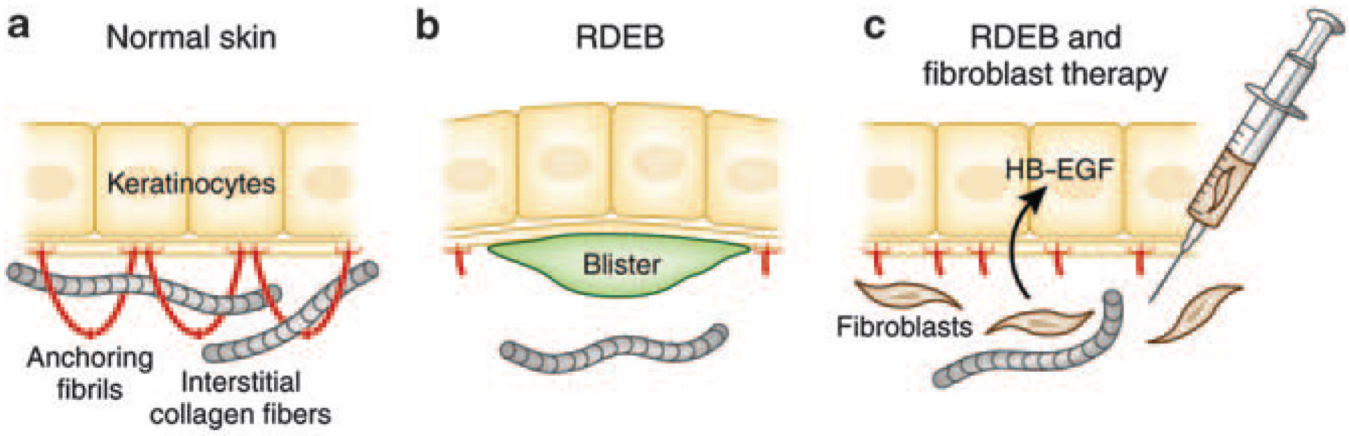

Figure 3. Postulated mechanism by which fibroblast therapy may ameliorate the blistering tendency in recessive dystrophic epidermolysis bullosa (RDEB)

(a) In normal skin, keratinocytes synthesize type VII collagen molecules (red), which assemble into anchoring fibrils. These fibrils entrap the interstitial collagen fibers in the dermis, securing the stable association at the dermal–epidermal junction. (b) In some patients with RDEB, there are only a few rudimentary anchoring fibrils, allowing formation of blisters below the lamina densa as a result of minor trauma. (c) Allogeneic fibroblasts injected directly into dermis elicit a subclinical immune reaction that leads to synthesis of heparin-binding epidermal growth factor–like growth factor (HB–EGF), which upregulates the synthesis and assembly of patient's own mutated type VII collagen. The increase in the rudimentary anchoring fibrils, which are partially functional, stabilizes the association of epidermis to the underlying dermis and ameliorates the blistering tendency (adapted from Uitto, 2011a).

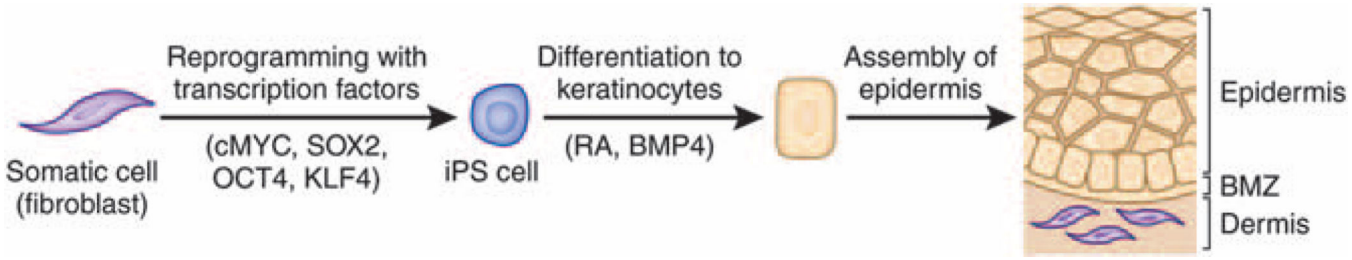

Figure 4. Schematic steps of reprogramming somatic cells, such as fibroblasts, to induced pluripotent stem (iPS) cells, and their differentiation into epidermal keratinocytes capable of forming skin-like structures

The reprogramming process is initiated by introduction of transcription factors (cMYC, SOX2, OCT4, and KLF4) into the somatic cells by transduction of expression vectors, synthetic mRNA, or recombinant protein. The iPS cells have characteristic features that allow their identification and enrichment. The iPS cells can then be differentiated into keratinocytes under specific culture conditions, e.g., medium supplemented with retinoic acid (RA) and bone morphogenic protein-4 (BMP4). BMZ, basement membrane zone (adapted from Uitto, 2011b).