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Novel Molecular Therapies for Heritable Skin Disorders

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Abstract

Tremendous progress has been made in the past two decades in molecular genetics of heritable skin diseases, and pathogenic mutations have been identified in as many as 500 distinct human genes. This progress has resulted in improved diagnosis with prognostic implications, refined genetic counseling, and has formed the basis for prenatal and presymptomatic testing as well as preimplantation genetic diagnosis. However, there has been relatively little progress in developing effective and specific treatments for these often devastating diseases. Very recently, however, a number of novel molecular strategies, including gene therapy, cell-based approaches, and protein replacement therapy have been explored for treatment of these conditions. This overview will focus on the prototypic heritable blistering disorders, epidermolysis bullosa and related keratinopathies, in which significant progress has been recently made towards treatment, and illustrate how some of the translational research therapies have already entered the clinical arena.

THE CLINICAL SPECTRUM OF GENODERMATOSES

Heritable skin disorders represent a broad group of conditions in which at one end of the spectrum the cutaneous findings can be relatively minor, primarily of cosmetic concern and limited to the skin, hair and/or nails. In contrast, at the other end of the spectrum, the cutaneous manifestations, often as part of multi-system pathology, can cause significant morbidity and untimely demise of the affected individuals. Heritable skin diseases can present a diagnostic challenge to practicing dermatologist for several reasons (Pulkkinen *et al.*, 2002). First, many of these conditions are relatively rare, and the physician may not be aware of the nuances and salient diagnostic features of the clinical presentations. This difficulty has been compounded by historically complex classification schemes, often riddled with eponyms. Secondly, in many of these conditions, there is considerable intra- and interfamilial heterogeneity, which, when combined with incomplete penetrance and/or late-onset manifestations, can obscure the timely diagnosis. Thirdly, in many of these diseases, the diagnosis is primarily made on the basis of clinical presentation, and histopathologic and ultrastructural findings are often non-specific or not diagnostic. However, over the past two decades, with the advent of molecular genetics in general and

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completion of the human genome project in particular, molecular diagnostics have become a standard for confirmation of the diagnosis, with potential for earlier detection. In fact, as many as 500 different genes are now known to harbor mutations in a manner that the genetic lesions explain the cutaneous manifestations in these conditions (Feramisco *et al.*, 2009).

Examination of the mutation databases in different heritable skin diseases reveals both obvious candidate genes as well as a number of surprises. An example of clearly identifiable candidate genes is epidermolysis bullosa (EB), a group of heritable blistering disorders manifesting with fragility of skin and mucous membranes (Fine *et al.,* 2008). In fact, just about two decades ago, when very little was known of the molecular pathology of this disorder, we made a prediction that mutations in the genes encoding the protein components of the structural attachment complexes at the epidermal-dermal basement membrane zone could explain the fragility of skin in different forms of EB (Uitto and Christiano, 1992). This prediction has since been proven correct by demonstration of a number of mutations in as many as 15 distinct genes encoding proteins necessary for the physiologic integrity of the skin (Uitto *et al.*, 2010).

In contrast the obvious candidate genes, many of the recently disclosed genes with mutations in heritable skin disorders have turned out to be surprisingly unpredictable, and their role in skin biology in many cases was not recognized before the identification of specific mutations in these genes. An example of such a condition is pseudoxanthoma elasticum (PXE), an ectopic mineralization disorder, which has been shown to result from mutations in the *ABCC6* gene that is expressed primarily in the liver and kidneys, and at very low levels, if at all, in affected tissues, including the skin. The *ABCC6* gene encodes a transmembrane efflux transporter protein, and it has been postulated that as a result of loss-of-function mutations there is an absence of physiologically circulating anti-mineralization factors, a situation which allows ectopic calcification of the peripheral connective tissues to ensue (Uitto *et al.*, 2011).

Apart from the tremendous progress in understanding the molecular basis of different genodermatoses, until very recently there has been relatively little progress in developing effective and specific treatment strategies for these, mostly intractable, disorders. However, identification of specific mutations in the candidate genes and elucidation of the consequences of such mutations at the mRNA and protein levels, have provided a basis for the development of novel therapeutic approaches, taking advantage of progress in molecular and cell biology in general. This overview will highlight a select number of genodermatoses, with a focus on EB and related blistering disorders, in which significant progress has been made towards treatment, and illustrate how some of these interventional modalities have recently entered the realm of clinical trials (Uitto *et al.*, 2010).

PRECLINICAL MODEL SYSTEMS

A number of model systems have been developed to examine the pathomechanistic consequences of mutations in heritable skin diseases, and many of these systems are also being utilized for development of molecular therapies. Particularly valuable towards understanding of diseasemechanisms has been the development of transgenic animal models which recapitulate the clinical features noted in patients; these genetically modified animals have played a major role in advancing our understanding of the disease mechanisms in different forms of EB (Bruckner-Tuderman *et al.*, 2010; Natsuga *et al,* 2010). Besides providing direct evidence for the structural role of many of the basement membrane zone adhesion molecules, the development of transgenic mice with EB phenotypes has provided novel information on the complex secondary effects mediated by signaling pathways and other systems that modify the EB phenotypes. In addition to transgenic animals, EB

phenotypes have been observed in a number of animal species, both domestic and wild, as a result of naturally occurring mutations (Jiang and Uitto, 2005; Bruckner-Tuderman *et al.,* 2010). In many cases, the suitability of these animal models of human disease for preclinical testing of gene-, protein-, and cell-based molecular therapies has been documented.

While traditionally, mice have provided the preferred platform to serve as a model for human diseases, often with remarkable similarity to the human phenotype both at the genetic, gross morphological, histopathological, and ultrastructural levels, mouse systems can also have considerable limitations. These include the relatively long developmental lifespan, and the time it takes to develop a knockout mouse. Moreover, in some cases, development of the knockout mouse as a model of the corresponding human disease has turned out not to be feasible due to the absence of the corresponding gene in the mouse genome, or because the mutations in the mouse gene result in embryonic lethality (Li *et al.,* 2007; Sercu *et al.,* 2007). Also, quite frequently, ablation of the mouse gene fails to result in a detectable phenotype. Such limitations, together with cost containment issues, have prompted the search for alternative model systems to study heritable skin diseases.

One of the alternate model systems utilizes zebrafish (*Danio rerio*), a small freshwater fish, which can be easily maintained in the laboratory setting (Lieschke and Currie, 2007). Zebrafish embryos develop and mature very rapidly, so that the development of various organs, including skin, is largely completed by days 5–6 post fertilization (dpf) (Li *et al.,* 2011). Specifically, at 6 dpf, the epidermis is composed of two cell layers clearly separated from the underlying connective tissue stroma by a basement membrane that depicts the presence of hemidesmosomal structures (Sonawane *et al.,* 2005). Examination of the developing zebrafish skin by scanning electron microscopy reveals well demarcated keratinocytes with the surface contour containing microridges which are well organized by 6 dpf. Also, the zebrafish epidermis and the dermal-epidermal basement membrane have characteristic landmark features, with demonstration of the corresponding gene expression. The zebrafish has, therefore, been suggested to serve as a suitable model system to study heritable skin diseases (Li *et al.,* 2011).

In addition to *in vivo* systems, cell cultures *in vitro*, including epidermal keratinocytes and dermal fibroblasts, provide experimental systems to study the consequences of gene mutations in heritable disorders. These cells can be examined for phenotypic consequences, such as migration and proliferation anomalies, as well as for changes in their gene expression profiles. The mutant cells can also be incorporated into artificial skin equivalent systems *ex vivo* with perturbations in the morphology and functionality of skin layers, such as the stratum corneum and its barrier function. Finally, treatment of cultured cells by corrective pharmacologic means, gene replacement approaches, or manipulation of the degree of differentiation has advanced our understanding of the diseases and provided a platform to develop novel treatments. Particularly intriguing are recent observations that cells, such as dermal fibroblasts, can be converted to induced pluripotent stem cells (iPSC) with subsequent differentiation to essentially any cell type in the body. This technology has the potential to develop unprecedented approaches for patient-specific regenerative medicine for heritable skin disorders.

MOLECULAR THERAPIES FOR EPIDERMOLYSIS BULLOSA

All forms of dystrophic epidermolysis bullosa (DEB) result from mutations in the *COL7A1* gene that encodes the anchoring fibril protein, type VII collagen, which is normally synthesized and secreted by both keratinocytes and fibroblasts (Stanley *et al.*, 1985; Chen *et al.*, 1994). In recessive forms of DEB (RDEB) the pathogenic mutations typically lead to reduced expression of type VII collagen (Christiano and Uitto, 1996; Aumailley *et al.*, 2006;

Chung and Uitto, 2011). The therapeutic goal in RDEB, therefore, is to increase type VII collagen expression at the dermal-epidermal junction (DEJ) to enable the skin to withstand trauma-induced blistering. To achieve this goal, various strategies are being pursued using gene, cell, protein and drug based approaches (Uitto *et al.*, 2010). Reported clinical trials in patients with RDEB, however, have thus far been limited to cell therapy, either involving intradermal injections of allogeneic fibroblasts, derived from unrelated adult or neonatal donors, or bone marrow derived stem cell therapy.

Allogeneic Fibroblast Cell Therapy for RDEB

In 2008, we reported a first-in-man clinical trial, in which single intradermal injections of allogeneic fibroblasts were assessed in five subjects with RDEB (Wong *et al.*, 2008). In skin biopsies from some individuals, fibroblast injection led to an increase in type VII collagen expression that was sustained for several months. Injection of male fibroblasts into a female subject, which allowed tracking of the cells with a Y-chromosome probe, indicated that the donor fibroblasts were undetectable 2 weeks after injection, yet there was no clinical evidence of inflammation, and the clinicopathologic benefits were sustained for several months (Wong *et al.*, 2008). Given the prolonged increase in type VII collagen following allogeneic fibroblast injection, therefore, a key question has been whether the new collagen at the DEJ is derived directly from the donor cells (“direct release”) or indirectly through enhanced synthesis of mutant type VII collagen by the recipient’s own keratinocytes and/or fibroblasts (“indirect release”).

In support of the “direct release” theory, injection of *COL7A1* gene corrected human RDEB fibroblasts or normal control human fibroblasts (at sufficient cell density) into immune deficient mice was shown previously to lead to deposition of new human type VII collagen at the dermal-epidermal junction (Woodley *et al.*, 2003). Moreover, injection of normal human fibroblasts into *Col7a1* hypomorphic mice generated new human type VII collagen at the mouse DEJ (Fritsch *et al.*, 2008; Kern *et al.*, 2009). Favoring the direct release of type VII collagen from fibroblasts were also the observations that (1) mutant fibroblasts did not exert any paracrine effect (in murine studies), (2) following fibroblast injection there was no upregulation of cytokines or growth factors known to increase type VII collagen expression, and (3) species-specific antibodies to type VII collagen showed that human type VII collagen was present at the mouse DEJ following injections of human fibroblasts (Kern *et al.*, 2009). In contrast, data consistent with “indirect release” stemmed from observations that allogeneic fibroblast injection was associated with (1) increased type VII collagen expression within basal keratinocytes (in humans), (2) an absence of mature anchoring fibrils following treatment, (3) increased expression of recipient patient-specific mutant *COL7A1* alleles at mRNA level, (4) a greater increase in type VII collagen at the DEJ in patients who expressed more type VII collagen at baseline and who therefore had an enhanced capacity to increase synthesis of their own mutant type VII collagen, and (5) some increased expression of type VII collagen following the irritant stimulus of saline injection (Wong *et al.*, 2008; Nagy *et al.*, 2011). Moreover, recent investigations in one patient with recessive DEB identified heparin-binding epidermal growth factor-like growth factor (HB-EGF) as a novel putative growth factor induced by fibroblast injections with the capacity to increase type VII collagen expression (predominately from the patient’s keratinocytes and therefore from mutant alleles) (Nagy *et al.*, 2011; Uitto, 2011a) (Figure 1). HB-EGF has not previously been known to influence type VII collagen expression, but exposure of patient and control keratinocytes and fibroblasts led to increased *COL7A1* gene expression with evidence of increased AP1 transcription factor probably accounting for the enhanced type VII collagen expression (Nagy *et al.*, 2011).

With regards to wound healing in RDEB, however, one randomized, double-blind study has shown no statistical differences in the extent or rate of re-epithelialization of chronic

erosions following injections of fibroblasts or saline, although other studies assessing the impact of allogeneic fibroblasts in wound healing are ongoing (Venugopal *et al.*, 2010; Yan and Murrell, 2010; John A. McGrath, unpublished data). One interesting observation has been that in the pre-injection monitoring of wounds in recessive DEB, many of the wounds, perhaps somewhat surprisingly, often show spontaneous healing as well as breakdown with change in the shape and size of eroded areas. Notably, within a particular area, the healing of some older wounds is often compounded by the appearance of adjacent newer wounds (John A. McGrath and Gabriela Petrof, unpublished observations). It is evident that careful selection and monitoring of wounds during clinical trials of fibroblast cell therapy in RDEB is paramount in evaluating whether the approach has clinical utility or whether alternative cell therapy or other therapeutic modalities might represent a better therapeutic intervention. For now, the current data and cumulative clinical experience indicate that for a subgroup of individuals with RDEB, notably those with mild-moderate severity of the disease and some baseline expression of type VII collagen at the DEJ, allogeneic fibroblast cell therapy might be clinically useful. From a practical perspective, injection of cell volumes of >0.25 ml per cm^2 to any one site can be painful and adequate analgesia (topical or systemic) may be necessary if multiple injections are planned. For patients with more generalized disease, associated with extensive scarring and contractures as well as with absence of type VII collagen protein at the DEJ, alternative cell therapy or other strategic approaches may be more effective.

Bone Marrow Stem Cell Therapy for RDEB

Cell-based therapy for heritable skin diseases has recently been extended to include bone marrow derived adult stem cells (BMDCs). These cells are known to play a crucial role in skin homeostasis, however, it has become clear that the plasticity of BMDCs also enables their differentiation into cell types responsible for skin regeneration after injury. Chronic wounding, such as in EB, has been shown to stimulate the engraftment of BMDCs to the skin and their incorporation and differentiation into non-hematopoietic skin structures (Badiavas and Falanga, 2003; Badiavas *et al.*, 2003; Tamai *et al.*, 2011).

A number of mouse studies have been conducted to evaluate the potential of BMDCs for the treatment of RDEB. For example, bone marrow transfer into the fetal circulation of *Col7a1*$^{-/-}$ mice resulted in deposition of type VII collagen around developing hair follicles, as well as reduction in severity of blistering in neonatal animals and extension of the overall lifespan of mutant mice (Chino *et al.*, 2008). In another study, hematopoietic and non-hematopoietic populations were infused into unconditioned *Col7a1*$^{-/-}$ mice at birth or soon thereafter (Tolar *et al.*, 2009). Strikingly, three of 13 (23%) recipient mice of adult bone marrow survived for several months, with evidence of skin engraftment of donor cells, production of type VII collagen in the skin, and formation of Col7a1-positive anchoring fibrils (Tolar *et al.*, 2009). Additionally, a model of milder junctional EB, the *Col17a1*$^{-/-}$ mouse, was used to test the skin differentiation capacity of various bone marrow-derived subpopulations of cells (Fujita *et al.*, 2010). These studies demonstrated that both purified hematopoietic stem cells (HSCs) and cultured mesenchymal stem cells (MSC) provided amelioration of clinical symptoms and an improved survival rate (Fujita *et al.*, 2010; Tamai *et al.*, 2011; Tolar *et al.*, 2009). These findings established that adoptive transfer of type VII collagen producing bone marrow cell populations is sufficient for the partial correction of the basement membrane zone defect in *Col7a1*$^{-/-}$ murine recipients. Collectively, these preclinical studies provide evidence for the potential of BMDCs as a source for regeneration of damaged skin in genetic skin diseases.

The first clinical trial of allogeneic whole bone marrow was recently reported in seven children with RDEB (Wagner *et al.*, 2010). New type VII collagen was noted at the dermal-epidermal junction and clinical improvement was sustained for at least 1 year after bone

marrow transplantation (BMT). Although the results are promising, two of the seven children died of complications of the procedure which utilized traditional chemoablative pre-conditioning of the recipient. A second clinical BMT trial has been initiated, using reduced intensity chemotherapy prior to transplantation (Kiuru *et al.*, 2010). In addition to bone marrow transplantation, a pilot study on two patients with severe RDEB suggested that intradermal injection of allogeneic MSCs into chronic ulcerated sites can accelerate re-epithelization of these wounds (Conget *et al.,* 2010). The improved wound healing, which lasted for four months, was attributed to replenishment of type VII collagen which was undetectable in these patients with RDEB prior to the procedure. Other studies are now examining whether intradermal or intravenous injection of BM-derived MSCs from the same donor as the transplanted cells in BMT improves clinical outcome. These early observations support the usefulness of BM stem cell populations in correction of the basement membrane defect in heritable skin diseases, such as RDEB (Petrova *et al.*, 2010).

INDUCED PLURIPOTENT STEM CELL THERAPY

The generation of induced pluripotent stem cells (iPSCs) from human fibroblasts was first reported in 2007 (Takahashi *et al.*, 2007; Yu *et al.*, 2007), in studies demonstrating that exogenous expression of a limited number of transcription factors was sufficient to reprogram somatic cells into an embryonic stem cell (ESC)-like state. iPSCs express ESC markers, have unlimited proliferative capacity, and can differentiate into all three germ layers, thus harnessing the full therapeutic spectrum attributed to ESC. Previous stem cell-based therapies were of limited success due to the difficulty in obtaining sufficient numbers of undifferentiated cells (adult stem cells, including BMDCs) and the problem of immune rejection (ESC). With the derivation of iPSCs, regenerative medicine was provided with a new method to circumvent these obstacles and obtain a renewable source of immunocompatible, patient-specific cells. In recent years, patient-specific iPSCs (PS-iPSCs) have been generated from several human diseases to investigate disease mechanisms, test potential drugs, and especially develop cell-based therapies. A proof of concept for iPSC-based therapy was previously provided in a study in which a sickle cell anemia mouse model was successfully treated with hematopoietic stem cells differentiated from gene-corrected iPSCs, derived from autologous mouse skin (Hanna *et al.*, 2007).

In the field of heritable skin disorders, PS-iPSCs have recently been generated from patients with dyskeratosis congenita (Agarwal *et al.*, 2010) and RDEB (Tolar *et al.*, 2010; Itoh *et al.*, 2011; Uitto, 2011b) (Figure 2). However, in order to move iPSCs-based therapy to the clinic, several technical challenges must be overcome. To begin with, integration factor-free iPSCs must be generated, because integration of viral transgenes carrying oncogenes, such as *MYC* and *KLF4*, into the genomic DNA of iPSCs may lead to their reactivation *in vivo* and subsequently to tumor formation (Okita *et al.*, 2007). Rapid development in this field, however, has recently produced several alternative methods, including plasmid-based derivation (Okita *et al.*, 2008), recombinant proteins (Kim *et al.*, 2009), integration-free viral vectors (Yu *et al.*, 2009; Zhou and Freed, 2009), and mRNA (Warren *et al.*, 2010), to generate integration-free iPSCs. Secondly, an efficient way to correct gene defects in PS-iPSCs must be employed. Several methods of correcting gene mutations have been developed, with the most promising approach using a type of homologous recombination (HR) based gene targeting. iPSCs, in contrast to other somatic cells, are suitable for HR-based gene correction due their unlimited, karyotypically stable proliferation. The first report of gene correction in PS-iPSCs from a patient with gyrate atrophy was recently published (Howden *et al.*, 2011), providing a proof-of-concept for this technique. However, the low frequency of HR in mammalian cells still prevents gene targeting from being applied in a broader therapeutic context. Recent advances in generating customized zinc finger nucleases (ZFNs), however, have paved the way for HR-based therapeutic strategies

(Cathomen and Joung, 2008). These artificial nucleases can increase the likelihood of HR at the mutation site up to 10,000 fold. This technology has been successfully applied to iPSCs, providing a more efficient strategy for gene-targeting as compared to conventional techniques (Zou *et al.*, 2009; Hockemeyer *et al.*, 2009).

Another option for mutation-free iPSC derivation from patients with skin disorders is taking advantage of natural gene therapy in the form of revertant mosaicism. This is a phenomenon wherein a subpopulation of cells re-acquires the wild-type phenotype through a naturally occurring second-site mutation or gene crossover/conversion (Lai-Cheong *et al.*, 2011). Revertant mosaicism has been observed in several human genetic disorders (Klein *et al.*, 1992; Wada *et al.*, 2004), including several types of EB (Jonkman *et al.*, 1997; Lai-Cheong *et al.*, 2011). Since revertant skin in the case of EB is both visible and easy to obtain via skin biopsy, it may serve as a natural, patient-specific source of gene-corrected cells. Taken together, these observations suggest that generation of gene-corrected PS-iPSCs can be achieved, enabling development of gene-targeted stem cell-based therapies for inherited human diseases.

A recent study demonstrated another possible challenge with iPSCs related to their immunogenicity (Zhao *et al.*, 2011a). It was shown that autologous mouse iPSC-derived teratomas were immune-rejected in syngeneic recipient mice with T cell infiltration. Although this study only suggested that tumor immunity, and not transplantation immunity, may be induced by undifferentiated iPSCs, elimination of the other populations from differentiated cell pools should be achieved for future clinical use of iPSCs. We recently demonstrated that both normal iPSCs and RDEB PS-iPSCs can be directly differentiated into functional keratinocytes, one of the relevant cell types for EB treatment (Itoh *et al.*, 2011). Moreover, the utility of keratinocyte-specific surface markers has been also shown to purify iPSC-derived keratinocytes (Itoh *et al.*, 2011), allowing for enrichment of keratinocyte lineage cells. In sum, iPSCs hold great promise to provide an unlimited and autologous source of cells for regenerative therapies for heritable skin diseases.

NOVEL THERAPEUTICS FOR KERATIN DISORDERS

The last two decades have seen tremendous progress in unravelling the molecular basis of keratinizing skin disorders – a large heterogeneous group of genodermatoses characterized by fragility and/or over-proliferation of epithelial tissues, typically affecting various regions of the epidermis and its appendages (Irvine and McLean, 1999; McLean and Irvine, 2007). The keratinopathies – disorders caused by mutations in genes encoding keratin intermediate filament proteins – are a good example of such conditions affecting epidermis, and mutations in 23 of the 54 human keratin genes have now been linked to human disorders of keratinization (Omary *et al.*, 2004). The archetypal keratin disease is epidermolysis bullosa simplex (EBS), which is caused by mutations in either of the genes encoding the basal keratinocyte-specific keratins K5 or K14 (Bonifas *et al.*, 1991; Coulombe *et al.*, 1991; Lane *et al.*, 1992). Heterozygous missense or in-frame insertion/deletion mutations in K5 or K14 act via dominant-negative interference to weaken the structural integrity of the intermediate filament cytoskeleton within the basal cell compartment of the epidermis. Functionally, this cytoplasmic filament network is responsible for maintaining the structural integrity of the basal cells when exposed to the high levels of mechanical trauma experienced by the skin in everyday life. Thus, failure of this system leads to skin blistering confined to the basal layer of the epidermis resulting in the clinical and histopathologic hallmarks of EBS (Irvine and McLean, 1999).

Most keratin mutations act through a dominant-negative pathomechanism because of the highly polymeric nature of the keratin cytoskeleton whereby the mutant keratin monomers

interact with the normal keratin to destabilize the entire keratin network within the cell. The great challenge in developing therapy for the common, dominantly inherited forms of EBS, and most other autosomal dominant keratinopathies, is to inhibit expression of the mutant allele without silencing the wild-type allele. Proof of concept for allele-specific gene silencing therapy comes from the study of unusual EBS families where homozygous loss-of-expression (typically nonsense or frameshift) mutations in K14 lead to recessively inherited EBS. In these kindreds, the affected individuals have complete absence of K14 in their basal keratinocytes, leading to cell fragility via a recessive, loss-of-function pathomechanism (Rugg *et al.*, 1994). Interestingly, the heterozygous carriers of these loss-of-function mutations, who only express one K14 allele, have clinically normal skin. Thus, if a means of silencing a dominant-negative mutant keratin allele could be found, these data strongly suggest that such a therapeutic approach would be likely to succeed (Kaspar, 2005; Lewin *et al.*, 2005).

RNA-interference Technology

Great strides towards therapy for keratin disorders have been made in recent years by application of RNA-interference (RNAi) technology, in the form of short interfering RNA (siRNA), to address the problem of allele-specific inhibition (Bumcrot *et al.*, 2006). siRNAs are 19-mer double-stranded RNA molecules with 2-nucleotide overhangs at either end, that are a perfect match for the mRNA that is to be silenced. Within the cell, these molecules are incorporated into the RNA-induced silencing complex (RISC) where the antisense strand is used to scan mRNA species. When an exact match is found, endonuclease components of RISC degrade the target message. For a given point mutation, there are 19 possible positions where 19-mer siRNA can be positioned relative to the single mutant base. Reporter gene systems, combined with a sequence walk methodology, facilitate the systemic determination of both potency and specificity of each of the 19 possible siRNAs to identify positions where the mutant reporter gene is strongly inhibited with minimal or negligible effect on the wild-type reporter. This methodology has been applied successfully to specific point mutations in keratins K6a causing pachyonychia congenita (PC) (Hickerson *et al.*, 2008), as well as EBS-causing mutations in K5 (Atkinson *et al.*, 2011).

PC is a rare genetic skin disorder that can be caused by dominant mutations in any one of the genes encoding keratins K6a, K6b, K6c, K16 or K17 (McLean *et al.*, 2011). These keratins are strongly expressed in the palmoplantar epidermis (Swensson *et al.*, 1998), and therefore, a major clinical feature of PC is painful and highly debilitating focal keratoderma, particularly affecting the soles of the feet (McLean *et al.*, 2011). The PC patient advocacy organization PC Project (www.pcproject.org), has been a key driving force in the development and implementation of siRNA therapy for this condition, in close collaboration with an international network of researchers and a small biotech start-up company, Transderm Inc. This work has recently led to a first-in-human double-blinded phase 1b clinical trial where an siRNA specific for the missense mutation N171K in the K6a protein was targeted (Leachman *et al.*, 2009). The therapeutic siRNA was delivered into hyperkeratotic plantar lesions by intradermal injection, which was both painful and highly inefficient. Nevertheless, significant clinical improvement was seen in the lesion that received the siRNA whereas no difference was observed in the lesion that received vehicle only. This study provides proof-of-concept for allele-specific siRNA therapy for dominant-negative skin diseases, however, an efficacious and non-invasive delivery method still needs to be developed (Figure 3). Cutaneous siRNA delivery is currently the focus of much attention in the keratin and RNAi fields where a number of potential delivery systems are being explored including chemical modification of the siRNA, topical formulation chemistry, nanoparticle technology and physical methods such as microneedle arrays.

One disadvantage of the allele-specific gene silencing approach is that the FDA and related agencies internationally may consider each mutation-specific siRNA as a separate entity requiring individual toxicological studies and regulatory approval. With this in mind, an alternative approach to treatment of certain keratin disorders, notably in the keratodermas such as PC, is a gene-specific approach where both wild-type and mutant alleles of the target gene is silenced. In palmoplantar epidermis in particular, there is significant keratin gene redundancy. For example, humans possess three versions of the K6 gene – encoding K6a, K6b and K6c, all of which are expressed in thick skin. The mouse genome has orthologs for only K6a and K6b. Single knock out of either of these muring genes produces a negligible phenotype (Wojcik *et al.*, 2001; Wong *et al.*, 2000). Only when both K6a and K6b genes are ablated is an epithelial fragility phenotype produced. This shows that there is considerable gene redundancy in certain epithelial tissues, such as palmoplantar epidermis. To this end, highly potent gene-specific siRNAs for human K6 genes have been developed and may be the weapon of choice in future clinical trials in PC (Smith *et al.*, 2008). In particular, most PC patients carry mutations in the K6a gene (Wilson *et al.*, 2011), and therefore, most could be treated with a single K6a-specific inhibitor (Smith *et al.*, 2008) (Figure 3).

Small Molecule Library Screen

With the increasing availability of chemical compound libraries and falling costs of robotic liquid handling technologies, recent years have seen considerable developments in academic drug discovery, which previously was solely within the domain of the pharmaceutical industry (Kozikowski *et al.*, 2006). Accordingly, increasing numbers of drugs entering clinical use have their origins in academic laboratories. Again, this technology has been applied to PC, where small molecules that reduce the expression of the PC-related keratins, such as K6a, have been sought through high-throughput screening using cell-based reporter gene assay systems. This has led to the discovery that the statins, drugs in common use for cholesterol control, have a modest inhibitory effect on K6a and certain other keratins (Zhao *et al.*, 2011b). This work has led to on-going clinical trials in a well-studied and genetically confirmed PC case series, coordinated by the International PC Research Consortium. Although this type of therapy may not be curative, it may help alleviate symptoms until more potent and specific gene silencing therapies enter the clinic.

Overall, the development of therapies from basic discoveries in genetics has been slow and gene therapy has been disappointingly slow to enter common clinical application. However, with the advent of RNAi, academic drug discovery and greater industry-academia partnerships (Vallance *et al.*, 2010), the landscape is changing rapidly, giving hope for patients with devastating inherited skin disorders.

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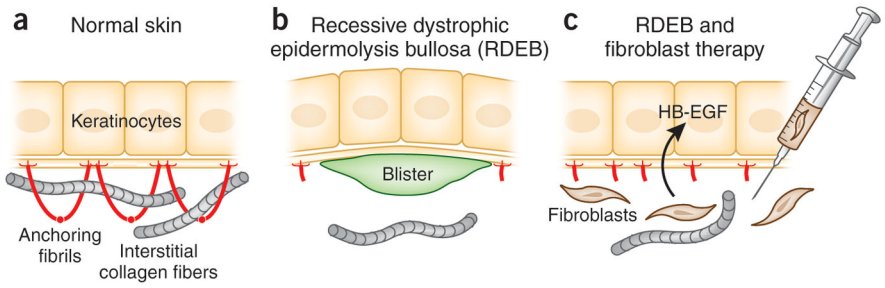

Figure 1.

Postulated mechanism by which fibroblast therapy may ameliorate the blistering tendency in 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-EGF-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).

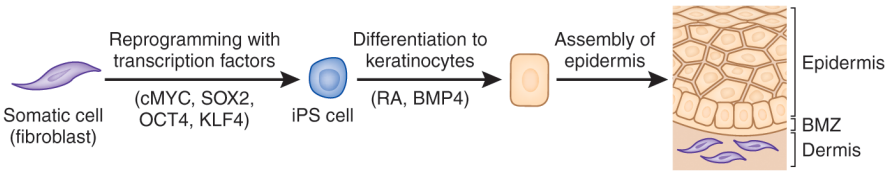

Figure 2.

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 (BMP-4). BMZ, basement membrane zone. (Adapted from Uitto, 2011b).

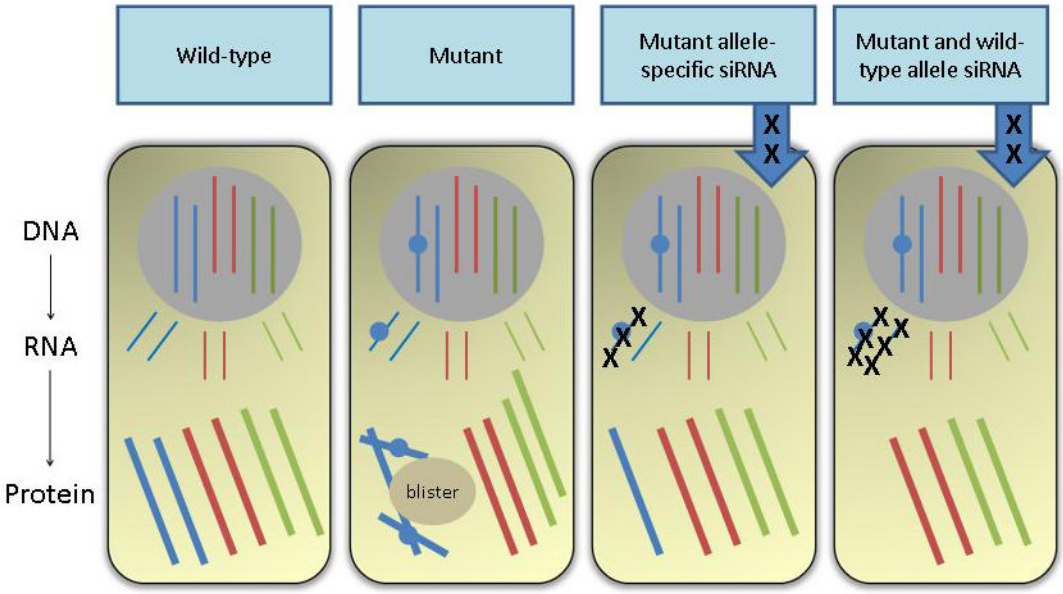

Figure 3.

siRNA strategies for autosomal dominant keratin 6a disorders by targeting either mutant or both mutant/wild-type alleles. (a) In normal keratinocytes, synthesis of K6a (blue), K6b (red) and K6c (green) occurs; (b) in PC keratinocytes with a heterozygous missense mutation in *KRT6A* there is dominant-negative interference between the wild-type and mutant K6a protein that perturbs the 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.