Eur J Dermatol 2015; 25(Suppl. 1): 12-7

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Pluripotent stem cells as a cellular model for skin: relevance for physiopathology, cell/gene therapy and drug screening

The skin represents the largest tissue in the human body. Its external part, the epidermis, accomplishes vital functions such as barrier protection, thermoregulation and immune function. The mammalian skin epidermis has been for decades the paradigm for studying the molecular events that occur in tissue homeostasis and repair. Many genes and signaling pathways have been identified by the use of manipulated transgenic and KO mice. However, despite numerous elegant transgenic mice experiments, absence of an appropriate *in vitro* model system has hampered the molecular study of the early events responsible for epidermal and dermal commitments, stages at which congenital genetic alterations are responsible for hundreds of rare skin diseases. For most of them, etiology and treatment are still missing. Here we review the last decade of studies aimed at designing cellular models from pluripotent stem cells (PSC) that recapitulate *in vitro* the main molecular steps of skin formation. As described below, PSC-based models are powerful tools to (i) clarify early molecular events that occur during epithelial/mesenchymal interactions, (ii) produce in large amount skin cells that could become an alternative for cell/gene therapies and (iii) screen for therapeutic compounds to treat genodermatoses.

Key words: pluripotent stem cells, drug screening, genodermatoses, skin diseases, epidermolysis bullosa, ectodermal dysplasia syndromes

Pluripotent stem cells and embryonic epidermal differentiation

Pluripotent stem cells

By definition, PSC are cells able to expand infinitely *in vitro* while maintaining their potential to spontaneously differentiate into any cell type of the three germ layers. In 1981, the first murine embryonic stem cell lines (mESC) have been established from pre-implantation mouse embryos, simultaneously by Martin Evans and Matt Kaufman [1] and by Gail Martin [2]. mESC were intensively used to study molecular events that occur during early mammalian development. In 1998, James Thomson and Joseph Itskovitz-Eldor reported the derivation of embryonic stem cell (ESC) lines directly from human blastocysts (hESC) [3]. This major breakthrough opened a new avenue to model human congenital pathologies but also to produce in large amount specific committed/differentiated cells with therapeutic potential. Few years later, Shinya Yamanaka found an incredibly simple way to reprogram *in vitr*o murine (in 2006) and human (2007) somatic cells into PSC. Exogenous expression of 4 genes (oct-4, sox-2, Klf4 and c-myc) coordinately expressed into somatic cells is sufficient to force the conversion of fibroblasts (or any somatic cell) into ESC-like cells, named by Yamanaka *induced pluripotent stem cells* (iPSC) [4]. Usually, hiPSC are produced from fibroblasts that can be easily isolated from skin biopsy. We have also designed a protocol to derivate hiPSC from few plucked hair follicles, a painless alternative to skin biopsy [5].
Because the somatic cells used to produce iPSC could be derived from patients, and thus carry the patient's genetic background, iPSC are useful tools for modeling of diseases and drug development. In addition, scientists hope to use them in regenerative medicine, theoretically avoiding immune rejection as autologous cells [6]. This revolution in cell biology and generative medicine justified S. Yamanaka's award of the Nobel Prize of Medicine in 2012.

Embryonic epidermal differentiation

PSC can recapitulate most of the embryonic steps *in vitro*, providing a good knowledge on the molecular events that occur *in vivo*. During vertebrate embryogenesis, epidermal and neuronal precursors derive from a common neuroectodermal precursor. Reciprocal instructive signals are secreted during ectodermal-mesodermal commitments for the formation of embryonic mammalian skin. Epidermal commitment is induced by mesenchymal BMP4 in the ventral part of the egg, while its absence within the dorsal part is due to BMP4 antagonists [7]. The developing neuroectoderm gives rise to single-layer ectodermal cells expressing the cytokeratins K8 and K18 until day 8.5 in mouse embryo. Soon after, ectodermal cells are committed to stratification, an event marked by the onset of expression of cytokeratins

doi:10.1684/ejd.2015.2537

To cite this article: Levy A, Petit I, Aberdam D. Pluripotent stem cells as a cellular model for skin: relevance for physiopathology, cell/gene therapy and drug screening. *Eur J Dermatol* 2015; 25(Suppl. 1): 12-7 doi:10.1684/ejd.2015.2537

K5 and K14 in the basal layer, then followed by stratification markers K1, K10 and Involucrin. The molecular events governing this multi-step process are far from being all identified. Most studies use powerful genetically engineered mice models obtained by crossing loxP-dependent KO mice with K14-cre mice, inducing the deletion of a specific gene within the K14+ epidermal compartment. This approach however, excludes the early embryonic steps before the onset of K14 expression. In this regard, PSC can bring complemental insights into early key events of skin biogenesis.

Murine pluripotent stem cells

In 1996, Fiona Watt's team first observed that mESC produce epidermal cells through spontaneous differentiation and that this commitment is dependent on integrin beta1 expression [8]. In 2003, we reported that mESC have the potential to recapitulate the reciprocal instructive ectodermal-mesodermal commitments, characteristic of embryonic skin formation [9]. Exposing mESC to mesenchymal extracellular matrix, ascorbate and transiently the morphogen BMP-4, known inducers of embryonic epidermal fate identified *in vivo*, leads to the production of epidermal cells but remarkably of fibroblasts as well from the same culture. As a matter of fact, when cultivated in organotypic reconstitution assay, this mixed cell population formed a two-compartment skin made of a multilayered epidermis positioned above an underlying dermis, similar to native skin [9].

Thus, PSC methodology provides a powerful tool for studying the molecular mechanisms controlling epidermal and dermal commitments. Accordingly, we demonstrated that BMP-4 controls neuroectodermal crossroads by selective apoptosis of sox-1^+-neural precursors to allow ectodermal commitment [10]. The resulting ectodermal precursors sequentially need activation of ΔNp63, a member of the p53 gene family, and its target genes to differentiate into proliferating keratinocytes [11]. This epidermal commitment is partially due to release of ΔNp63 from repression by miR-203, a regulator of epidermal stratification [12], elegantly confirmed *in vivo* [13]. A comparative study between laser-captured ectodermal cells from p63-deficient mouse embryos and genetically-manipulated mESC revealed that ΔNp63 acts as an embryonic gatekeeper of ectodermal progenitors, inducing epidermal commitment while repressing mesodermal fate [14].

Human pluripotent stem cells

The accessibility of human ESC (from 1998) and iPSC (from 2007) lines and their promise as cell/gene therapy tools prompted several laboratories to challenge these PSC lines for the production of epidermal cells and fibroblasts from normal and pathological sources. A major improvement to the original protocol on mESC [9] came in 2008 from Sean Palecek's team by the addition of retinoic acid (RA) along with BMP-4 [15]. The rationale is that RA is required after gastrulation to repress FGF, an inhibitor of BMP-4. Moreover, RA prevents epidermal terminal differentiation by activation of ΔNp63 gene expression [16]. Association of these two known neural repressors thus enhanced ectodermal cell production, ΔNp63 activation and, in synergy with BMP-4, epidermal commitment and stratification of hESC [15].

Efficient differentiation of pluripotent stem cells into skin cells

In 2009, a team led by Gilles Waksman and Marc Peschanski made a further step to the potential use of PSC in cell therapy. With a protocol similar to our original one (fibroblast feeder, ascorbate and BMP-4) [9] except that BMP-4 was added daily for 40 days, they generated a pure population of basal keratinocytes from hESC that formed a pluristratified epidermis *in vitro* but also *in vivo* onto immunodeficient mice [17].

Two years later, Angela Christiano's group produced similar epidermis from hiPSC with RA and BMP-4 in a defined medium [18]. With a similar protocol and strategy, Dusko Ilic and coauthors obtained a pure population of epidermal cells from hiPSC that could be selected and amplified by their preferential adherence to collagen IV [19]. The resulting cells display functional permeability barrier. In the later two studies, comparative transcriptome analyses strongly suggest that the iPSC-derived epidermal cells are similar to their somatic counterparts.

By the use of hESC, Jonathan Garlick's team nicely demonstrated plasticity of committed cells that could become either epithelial- or fibroblast-like cells according to the environmental cues. When these cells were incorporated into the stromal and epithelial tissue compartments of 3D tissues, they generated multilayer epithelia similar to those generated with foreskin-derived epithelium and fibroblasts [20]. When compared to their original somatic cells, PSC-derived fibroblasts displayed similar transcriptomic molecular profiling and ability to structure dermal equivalent, polymerize collagen fibres and support above epidermal stratification in 3D skin [21].

The epidermis is also populated with melanocytes and skin appendages – hair follicles (HFs), sebaceous and sweat glands – which participate in skin homeostasis, in close interactions with the dermis underneath. Skin equivalents derived from PSC should include those other types of cells. Several groups have reported the production of melanocytes, which originate from the neural crest, from PSC [22, 23]. Transplantation of hiPSC-derived epidermal cells in mice leads to the formation of a stratified epidermis lacking skin appendages, suggesting those cells are unable to form HFs [17]. However, Veraitch *et al.* showed that hPSC-derived ectodermal cells could upregulate HF markers when co-cultured with mesenchymal dermal papilla cells *in vitro* and participate to HF formation *in vivo* [24]. More convincingly, Xu *et al.* demonstrated that hiPSC could differentiate into HF cells by sequential addition of EGF and isolation of CD200+/ITGA6+ progenitors, containing K15+ HF stem cells and capable of HF formation *in vivo* [25]. This study also reported the formation of sebocytes in vitro. Altogether, those studies pave the way for the elaboration of a fully organized PSC-derived skin equivalent, engineered with all components of the epidermis and dermis from a single PSC source.

Standardization of PSC differentiation protocols will be key to successful applications. Additional studies are still needed to determine the optimal protocol to obtain a homogenous population of highly proliferating keratinocytes that could be routinely expanded and scalable-either for therapies or for high throughput screening studies. When considering the use of those cells in

regenerative therapies for skin burns or chronic ulcers, the advantages of PSC-derived epidermal cells over expanded autologous skin cells as currently successfully used in clinics might not appear obvious or worthwile. They could however provide temporary skin substitutes for patients awaiting autologous grafts as a possible alternative to cadaveric cells. Moreover, they could easily provide relevant 3D organotypic cell cultures for toxicology and cosmetology studies and become an alternative of choice to animal testing banned by the European Union for cosmetics.

Multipotent epithelial progenitors

Ectodermal cells are multipotent progenitors of epithelial lineages, able to further differentiate into epidermal cells, corneal cells, hair follicles and other skin appendages. Obtaining those cells from PSC is particularly interesting for therapies as they are proliferative and could respond to differentiation cues in vivo, leading to improved engraftment. We described an alternative robust method to differentiate hESC into a homogenous ectodermal precursor cell population that retained multipotency [26]. It is known that PSC display a truncated cell cycle with a short G1 phase and no checkpoint. Remarkably, we found that the hESC-derived ectodermal cell lines display somatic cell cycle kinetics, normal karyotype and do not produce teratoma; therefore, contrary to their undifferentiated counterparts, these cells could be safe for cell therapy. In 2011, Denis Roop's group demonstrated the multipotency of miPSC-derived keratinocytes, able to reconstitute normal skin and HFs after grafting them along with freshly isolated dermal fibroblasts onto mice back [27]. Such multipotent epithelial progenitors have been produced recently from hiPSC by Yang *et al.* [25] and also by Sean Palecek's group [28]. These precursors could be instructed to differentiate into keratinocytes, HFs or sebocytes.

Modelling skin diseases with pluripotent stem cells

hiPSC could be used to model human congenital diseases when derived from patient cells. Following cell reprogramming, the cells are induced to skin (keratinocytes or fibroblasts) cell differentiation, as described above. Despite the easy access to diseased skin cells through biopsy procedure, PSC methodology is particularly relevant to cutaneous pathologies as diseased epidermal cells have reduced proliferative capacities in vitro and as multiple biopsies are not desirable in particular for paediatric patients. Therefore, since they have unlimited proliferative capacity, and are permissive to homologous recombination to correct *in situ* the disease-causing mutation, differentiated hiPSC could be used as a constant cellular source for specific genodermatoses for which no treatment is yet available. Such *in vitro* models that could be scaled up are also highly relevant to screen for small compounds able to rescue the disease defects.

In the last two years, three skin congenital diseases have been modelled by the use of hiPSC derived from patients, p63-related ectodermal dysplasia (EEC) and two epidermolysis bullosa (EB) syndromes. As described below, these cellular models demonstrate the utility of iPSC technology to discover potential therapeutic drugs and to provide alternative tools for cell/gene therapy.

Ectodermal dysplasia syndromes

Ectodermal dysplasia is a group of congenital syndromes affecting a variety of ectodermal derivatives. Among them, ectrodactyly, ectodermal dysplasia and cleft lip/palate (EEC) syndrome is caused by single point mutations in the *p63* gene, which controls epidermal development and homeostasis [30]. Phenotypic defects of the EEC syndrome include skin defects and limbal stem cell deficiency (LSCD). LSCD alters corneal transparency with dense vascularized corneal pannus, eventually leading to visual impairment. Recently, we designed a novel cellular model that recapitulated major embryonic defects related to EEC. Fibroblasts from healthy donors and EEC patients carrying two different point mutations in the DNA binding domain (DBD) of p63, member of the p53 gene family, were reprogrammed into induced pluripotent stem cell (iPSC) lines [30]. EEC-iPSC from both patients showed early ectodermal commitment into $K18^+$ cells but failed to further differentiate into $K14^+$ cells (epidermis/limbus) or $K3/K12^+$ cells (corneal epithelium). The finding of a more severe phenotype *in vitro* than in the patient is a frequent feature in cellular models where no compensation occurs. In search of drugs that could revert the defects, since the DBD is highly conserved between the p53 gene family members, we tested a small compound that restores the pro-apoptotic function of mutated p53 and is already used with success in clinical trials in patients with haematological malignancies or prostate cancer [31]. We reported that PRIMA-1^{MET} could revert epithelial lineage commitment and reinstate normal p63-related signalling pathway [30]. This study illustrates the relevance of iPSC for p63 related disorders and paves the way for future therapy of EEC. It demonstrates that iPSC technology is powerful for drug screening of skin congenital diseases.

Epidermolysis bullosa

Epidermolysis bullosa (EB) is a group of severe inherited blistering disorders due to mutations in genes encoding for components of the epidermal-dermal junction of the skin. Dystrophic epidermolysis bullosa (DEB) is caused by mutations in the COL7A1 gene encoding type VII collagen, the major component of anchoring fibrils [32]. Patients with recessive DEB (RDEB) develop severe skin phenotypes, as well as mutilating scarring and increased risk of developing highly aggressive and metastatic squamous cell carcinomas in early adulthood. Junctional EB (JEB) is caused by mutations on genes encoding anchoring filaments (laminin 322 and type XVII collagen). Patient's skin is very fragile and blisters easily in response to minor injury or friction [33]. There is currently no cure for EB in general. Development of iPSC-based therapies for EB patients necessitates the correction of large amounts of cutaneous stem cells for long-term tissue repopulation of large affected surface area. Four major studies established the basis for EB therapy. In 2013, Osborn *et al.* corrected a mutation on the COL7A1 gene on RDEB patient fibroblasts with TALEN technology. The corrected cells were

reprogrammed into reverted hiPSC to produce normal keratinocytes able of secreting collagen VII [34].

More recently, three independent teams reported this month in back-to-back papers in *Science Transl Med* a major breakthrough to cell/gene therapy for EB. With different approaches, they provided strong support that EB patients could be treated in the future with iPSC.

Antony Oro's team used genetically defined repair approach with non-integrating highly recombinogenic AAV-DJ variant to restore *COL7A1* gene expression on RDEB patient iPSC and avoid safety risks associated with random integration [35]. This AAV variant has a high tropism to epithelial cells and displays high site-specific recombination frequency, as compared to conventional ZFNs, TALENs, and CRISPR/Cas9 enzymatic systems [36]. Moreover, their analysis suggests a lack of distinct mutational selection during both reprogramming and correction, although random occurrence of deleterious mutations cannot be ruled out. After gene correction, the iPSC were induced to epidermal commitment as described previously [17, 18] with an additional step of microscopic embryoid bodies before differentiation. Of note, no undifferentiated PSC remained in the differentiated cell population, ruling out the risk of potential teratoma formation after transplantation. The keratinocyte cultures stratify and deposit type VII collagen in vitro and in vivo, demonstrating the ability of corrected iPSCs to form epidermal sheets [35]. However, the iPSC-derived epidermis apparently did not last for long on mouse skin, probably due to the xenograft nature and increase of senescence. It is probable that autologous epidermis may function with improved survival when grafted onto patients rather than as a xenograft.

The second study described by Penninger's lab confirmed the relevance of iPSCs for the clinical treatment of EB in a murine model [37]. They used a *Col7a1* hypomorphic mouse model that recapitulates RDEB disease. RDEB-miPSC produced from these mice were corrected for the mutation by flippase recognition-mediated excision followed by their differentiation into fibroblasts. These mesenchymal cells injected intradermally onto the RDEB mice fully restored type VII collagen deposition and long-term increase in skin integrity and resistance to mechanical stress.

In a third study, Angela Christiano and Marcel Jonkman's teams elegantly used revertant mosaicism to generate iPSCs from spontaneous revertant keratinocytes of a JEB patient with compound heterozygous COL17A1 mutations [38]. Spontaneous correction of a pathogenic gene mutation occurs naturally in limited areas of patients affected by skin diseases, including in EB patients [39]. These patches,

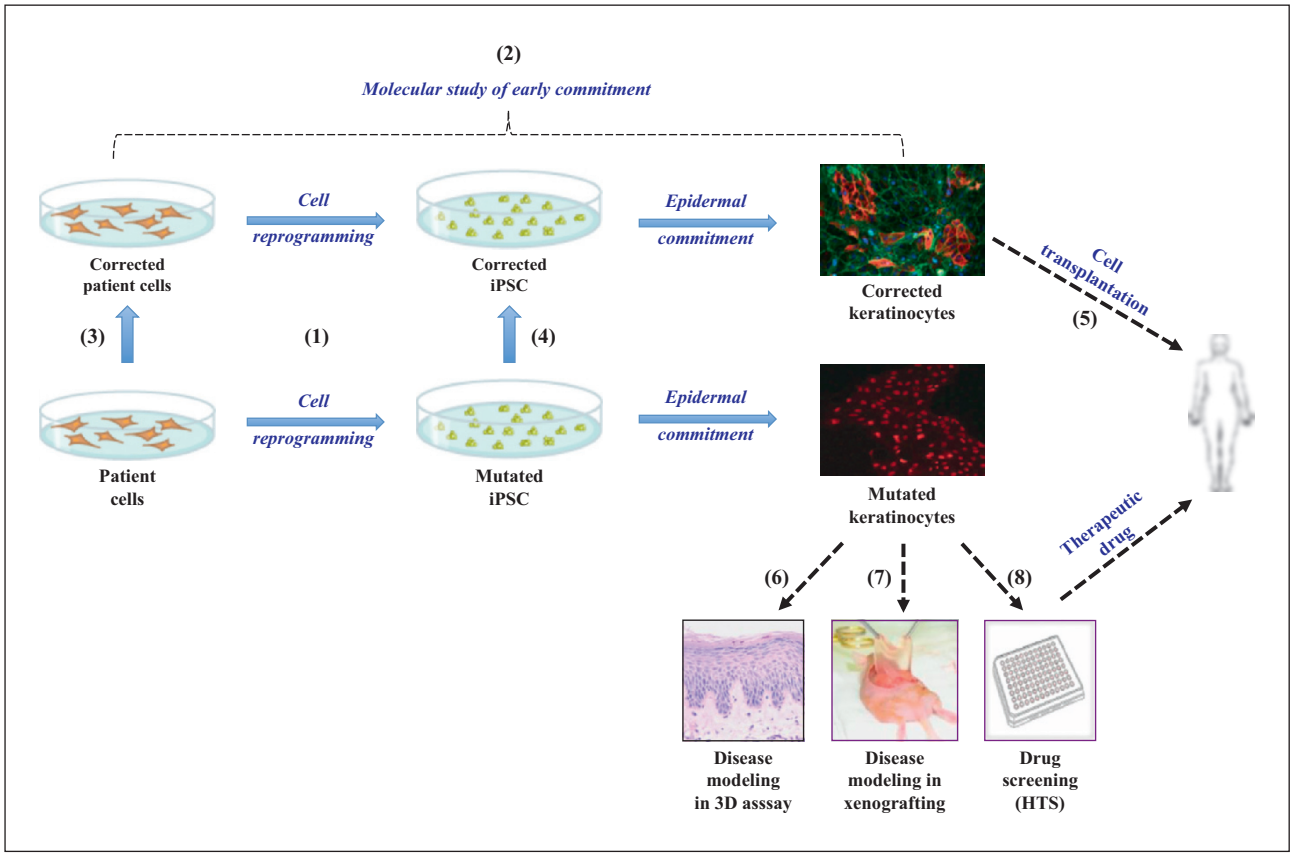

Figure 1. Schematic representation of the different steps underlying iPSC technology for skin physiology and disease modeling, drug screening and regenerative medicine. Somatic cells (fibroblasts, HFs, etc.) are reprogrammed into iPSC (1) and induced to epidermal differentiation (2) to study early molecular events. When isolated from patients with monogenic disease, somatic cells or derived iPSC are corrected by homologous recombination (3 or 4), the reverted cells are induced to differentiation for the production of graftable epidermis (5). Alternatively, the mutated iPSC-derived skin cells are used for modelling in 3D organotypic reconstitution (6) and transplantation into mice (7) or drug screening (8).

named revertant mosaicism, contain cells that acquired a secondary mutation that counteracts the effect of the primary EB mutation. These naturally genetically corrected iPSCs were then differentiated into keratinocytes that expressed type XVII collagen (Col17) and form in vitro skin equivalents that can be grafted in vivo in mice, both of which expressed Col17 in the basal layer. The study did not inquire if the grafted epidermis was maintained in the long term.

These four approaches represent thus an important step forward in generating renewable patient-specific cells for EB gene/cell therapy but also a proof of concept for the many other congenital skin diseases for which no treatment is available.

Perspectives

Although relatively recent, iPSC technology already demonstrated its considerable value to the characterization of skin congenital pathologies, development of alternative gene and cell therapy and search for targeted therapeutic tools (*figure 1*). Since the field is progressing rapidly, the actual limitations will be undoubtedly solved in the next years. These include methodologies to avoid aberrant mutagenesis during (i) cell reprogramming, (ii) cell amplification of the PSC and (iii) homologous recombination to correct patient mutations. Recent genomic and epigenetic analyses reported accumulation of mutations and aberrant DNA methylation in iPSC [40]. Moreover, the protocol of differentiation must be efficient, produce committed cells still able to proliferate and be cost effective. Indeed, translation of PSC-derived skin cells into clinical use is also largely dependent on economical constraints that should already be addressed. Time-effective and robust protocols for the culture of PSC and their derivatives should be designed in compliance with GMP restrictions. Those manufacturing limitations may prevent the development of autologous PSC therapies in favor of allogeneic universal PSC lines. In that case, the immunogenicity issue must also be resolved. Some studies suggest low immunogenicity of differentiated iPSC [41] while others claim the opposite [42]. Moreover, expression *de novo* of a protein that was absent in patient could induce an immune response and eventually rejection of the grafted tissue. Nevertheless, some clinical trials phase I/II have already started in Japan and UK to treat late age-related macula degeneration with differentiated hiPSC committed to retinal pigmented epithelial (RPE) cells. It will be critical to carefully follow the outcome from these pioneer treatments.

Besides genetic skin disorders for which iPSC technology will obviously become a new therapeutic and drug discovery tool (EEC, EB), production of cutaneous cells from iPSC was proposed for wound healing and chronic leg ulcers cell therapy. However, since the technique for growing somatic epidermal cells in large scale from a small skin biopsy has been utilized for decades, the advantage of iPSC over somatic cells for chronic wounds is not obvious. On the other hand, severe burn patients need large amount of epidermal cells that cannot be amplified from the healthy region and, in that case, iPSC could become a great advantage. In addition, efforts should be made to produce dermal fibroblasts from PSC, as dermis is mandatory for successful engraftment of autologous epidermal cultures. Production of skin appendages (sweat and sebaceous glands, hair follicles) and melanocytes, which are essential along with keratinocytes for a complete, good quality, epidermis should be taken in consideration.

We have demonstrated that iPSC derived from patients are a powerful tool to test drugs that could revert the developmental defects. Screening of drug libraries using iPSC-derived cells from other genodermatoses could identify small molecules able to bypass the mutation. To achieve this goal, efforts must be made to adapt the current protocols of PSC-derived epidermal cells to the constraints of high throughput technology and to develop adequate readout models. In conclusion, iPSC technology should accelerate our molecular knowledge about skin physiopathology and holds great promise for various skin disorders either directly in cell/gene therapies or in drug discovery. ■

Disclosure. *Acknowledgements: We would like to thank Xavier Nissan for critical reading of the manuscript. We have tried to be as exhaustive as possible and would like to apologize for any bibliographic omission. Financial support: AL is supported by a postdoctoral fellowship from the Fédération pour la recherche médicale (FRM). The work described here has been funded by ANR GENOPAT 2008, FP6 Integrated Project Epistem LSHB-CT-2005-019067 and ANR-Emergence 2011 (to DA). Conflict of interest: none.*

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