From skin to the treatment of diseases – the possibilities of iPS cell research in dermatology

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Abstract: Induced pluripotent stem cells (iPSCs) can be generated from different somatic cell types through ectopic expression of a set of transcription factors. iPSCs acquire all the features of embryonic stem cells (ESCs) including pluripotency and can thus give rise to any cell type of the body. iPSCs comparable with ESCs are amenable for the correction of gene mutations by homologous recombination. Patient-derived iPSCs may thus be an ideal source for studying diseases *in vitro* and for treating different disorders in the clinic. In this review, we summarize recent advances and possibilities of iPSC research with focus on the field of dermatology.

Sources of pluripotent cells

Pluripotent cells have the potential to differentiate into any of the three germ layers: endoderm (e.g. epithelium of the gastrointestinal tract or the lungs), mesoderm (e.g. muscle, bone and blood cells) or ectoderm (skin and nervous system).

Cells derived from the inner cell mass of blastocyst-stage embryos possess the property of pluripotency, the capacity to generate every cell type in the mammalian body including the germline. Embryonic stem cells (ESCs) can be derived from the inner cell mass of a blastocyst. They can undergo symmetrical self-renewal to produce identical stem cell daughters and have the ability to differentiate in various cells types. The first isolation of murine ESCs in 1981 was a major breakthrough (1,2). Thomson et al. succeeded to establish primate ESCs from Rhesus monkeys (3) and 3 years later human embryonic stem cells (hESCs) (4) (see also Table 1).

The quest for finding sources of pluripotent cells raises the question as to whether adult somatic cells are restricted to one's fate. Nuclear transfer studies proved that genes are not lost or permanently silenced during cell determination and differentiation. The pilot project was conducted by Briggs and King. They transferred nuclei from blastocysts into enucleated frog oocytes, which then gave rise to swimming tadpoles (5). Somatic cloning–derived ESCs – so called 'nuclear transfer embryonic stem cells' (ntESCs) – provide a new source of pluripotent cells. ntESCs allow the creation of pluripotent cells that are genetically identical to the patient, and differentiated cells derived from these cells will not be rejected after transplantation.

The establishment of human ESCs dramatically elevated the interest in this field with regard to therapeutical potential of ESCs, but ethical and practical considerations restrict the study of human embryos and raise the question of alternative sources of pluripotent cells in the mammalian body. It was shown that primordial germ cells, the precursors of the germline, continued to proliferate in the presence of certain cytokines and converted into cells resembling undifferentiated, pluripotent ESCs after several days (6,7). Germline-derived pluripotent stem cells derived from the testis of adult mice contributed efficiently to chimeras and showed germline transmission (8).

Recently, another alternative source of pluripotent cells was established by direct reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) with defined factors. This technique was pioneered by Takahashi and Yamanaka (9) and created a great deal of enthusiasm in the scientific community.

Reprogramming towards pluripotency by transcription factors

The overexpression of single transcription factors can lead to dramatic changes in the fate of somatic cells. First experiments in the 1980s showed that the ectopic expression of a homeotic gene in Drosophila melanogaster results in a change of the body plan (10). In mice, overexpression of the tissue-specific transcription factor MyoD resulted in the conversion of fibroblasts into myogenic cells (11). Together, these and many other studies (12) exemplify the potential of somatic cells to change their fate and provide the attempts to reprogram somatic cells directly into iPSCs.

Direct reprogramming into a pluripotent state has been achieved by Takahashi and Yamanaka (9) by reprogramming mouse fibroblasts into embryonic stem cell–like cells. A set of 24 genes expressed by ESCs were chosen as candidate factors for their ability to induce and maintain pluripotency. The generated clones were examined for the reactivation of stem cell–specific genes using a reporter gene construct containing a drug resistance gene under the control of the promoter of Fbx15, a target of Oct4 and Sox2. With this method, four key transcription factors were identified to be capable for generating cells resembling ESCs: Oct4, Sox2, Klf4 and c-Myc. To compare iPSCs and ESCs, further investigations into global gene expression pattern and DNA methylation status showed similarities, but iPSCs were not identical to ESCs. Even though the generated iPSCs did give rise to teratomas
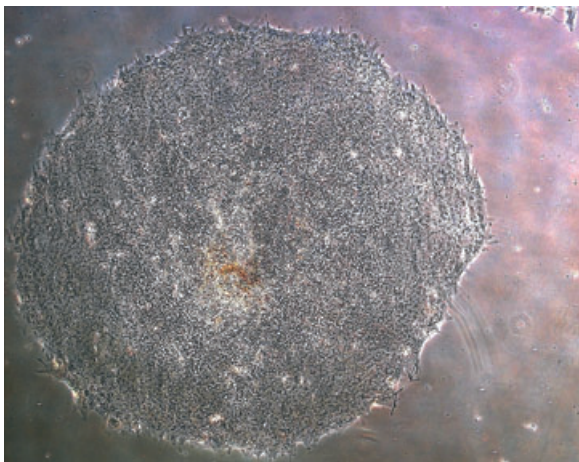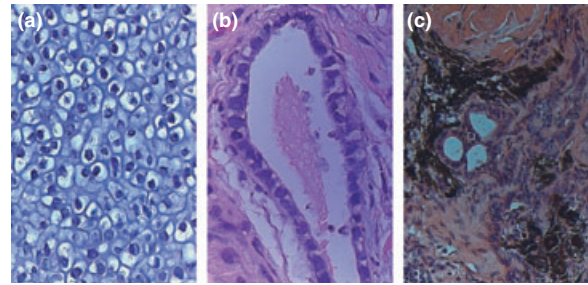
Experimental Dermatology

Table 1. History of cellular reprogramming

Method	References
Nuclear transfer in Amphibians	(5,84)
Reprogramming of somatic cells after fusion with teratocarcinoma cells in mice	(85)
Isolation of murine embryonic stem cells from the epiblast of a delayed-implantation embryo	(1)
Isolation of murine embryonic stem cells from the inner cell mass of a late blastocyst	(2)
Proof of pluripotency in primordial germ cells	(6,7)
Nuclear transfer of mammalian somatic cells into unfertilized oocytes, the cloned sheep 'Dolly'	(86)
Isolation of human embryonic stem cells	(4)
Somatic cloning using terminally differentiated cells	(87)
Cell fusion experiments with human cells	(88,89)
Reprogramming of somatic cells into induced pluripotent stem cells in mice	(9)

Figure 2. Potential of human induced pluripotent stem cells to differentiate into various cell types (a) cartilage, (b) epithelium, (c) pigmented cells in a teratoma forming assay.

after injection into immunodeficient mice, it was impossible to obtain viable chimeras.

Based on these findings, two groups in Boston and Shinya Yamanakas' group in Kyoto improved the generation of iPSCs from fibroblasts using Nanog or Oct4 as a selection marker or by just picking the developing iPSC clones on the basis of their morphology (13–15). Such generated iPSCs were indistinguishable from ESCs regarding histone modification and DNA methylation. They were also capable of forming adult chimeras and generate functional germ cells. This finding shows that fully reprogrammed iPSCs can be generated by the transduction of somatic cells with four transcription factors. In further investigations, a wide range of murine cells was tested for their reprogramming potential (Table S1).

Shortly after the successful reprogramming of mouse fibroblasts, human fibroblasts were reprogrammed, using either the same factor combination like in mouse (16) or the combination of Oct4 and Sox2 with Nanog and Lin28 (17). These human iPSCs are highly similar to human ESCs in terms of morphology, proliferation, gene expression and epigenetic status of pluripotency specific genes. Besides human fibroblasts (16–20), also other cutaneous cell types were successfully reprogrammed (Figs 1 and 2; Table S1).

Figure 1. Human induced pluripotent stem cell colony.

The replacement of reprogramming factors with factors among the same or other gene families lead also to fully reprogrammed cells, e.g. Sox1 or Sox3 can substitute for Sox2; Klf2, Klf5 or Esrrb can substitute for Klf4, and c-Myc can be substituted by N-Myc and L-Myc (21–23).

In addition, Wernig and colleagues (24) could show that c-Myc is not strictly required for the reprogramming of mouse fibroblasts, but the reprogramming time period increases, while the efficiency decreases. Moreover, Sox2 shows to be dispensable for the reprogramming of cells of neuroectodermal origin such as neural progenitor cells (25–29). The skin cell types of neuroectodermal origin such as melanocytes show an endogenous expression of Sox2 (30) and are thus amenable for reprogramming in the absence of exogenous Sox2 (31). Similarly, Tsai and colleagues (32) accomplish to reprogram dermal papilla cells from skin using only Oct4 and Klf4. As many cell types exhibit endogenous expression of some of the reprogramming factors, it has to be under examination whether the endogenous gene expression levels are sufficient for the reprogramming process.

During the reprogramming process, the expression of somatic cell–specific markers decreases, while the cells start to express stage-specific embryonic antigens (SSEA). Mouse ESCs and successfully reprogrammed mouse iPSCs express SSEA-1, whereas human ESCs and iPSCs express SSEA-3 and SSEA-4. Additionally, they reactivate endogenous genes required for pluripotency (e.g. Oct4 and Nanog), and silence exogenous factors which were necessary to initiate the reprogramming (Sox2, Oct4, Klf4 and c-Myc). Fully reprogrammed iPSCs show the same properties as ESCs. The most stringent test to determine whether generated iPSCs are fully pluripotent is the tetraploid blastocyst complementation assay. Different investigators succeeded in deriving adult fertile mice completely from iPSCs (33–36) and proved that differentiated somatic cells can be completely reverted into pluripotent iPSCs. This is especially the case when the Dlk1-Dio3 gene cluster on chromosome 12qF1 is normally expressed in iPSCs (36).

One major problem of the iPSC technology is the inefficiency of this technique. Two models have been constructed to explain this problem (37). The 'elite' model proposes that only a small amount of cells are amenable to reprogram into a pluripotent state. As somatic stem and progenitor cells are developmentally not terminally differentiated and present in most adult tissues, they are potential candidates for 'elite' cells. The 'stochastic' model describes reprogramming as a stochastic event, where cells have to pass different steps of epigenetic events to acquire pluripotency.

All cells represent the same reprogramming potential, but some cells may fail to pass all steps, but rather arrest at one stage or undergo senescence (38). The number of steps cells have to go through is unclear and could possibly vary between different cell types. However, the 'elite' model is controversial, because iPSC colonies were generated also from terminally differentiated cells. A 'stochastic' model with integrated 'elite' cells might be the most supposable explanation, as progenitor cells show a higher reprogramming efficiency, possibly due to fewer stochastic steps to pass.

The reprogramming process by itself comprises molecular changes in somatic cells, like downregulation of somatic markers, followed by the activation of pluripotency genes until the cells become independent from exogenous factor expression. The finding that early and late iPSCs differ with regard to telomere length (39) or DNA methylation pattern (40) shows reprogramming as an event with many roadblocks cells have to go through.

Fibroblast, keratinocyte or other cell types? – Which is the best source for generating iPSCs?

Induced pluripotent stem cells have been derived from multiple cell types, reprogrammed by several methods and with different efficiencies (41–43).

The efficiency varied in primary reprogramming systems in a range of 0.01% up to 1%, dependent on the used protocol and cell type. It was shown that mouse stomach and liver cells reprogram with a higher efficiency compared with fibroblasts (44) or that the efficiency to reprogram human keratinocytes is 100-fold higher than that of human fibroblasts (19,45), possibly due to a higher endogenous c-Myc expression level. Furthermore, it was demonstrated that a deficiency in the p53-p21 pathway resulted in a faster appearance and a significant increase in the efficiency of producing iPSC colonies. Moreover, cell types that normally fail to reprogram such as cells showing multiple types of DNA damage were rescued and gave rise to iPSCs (46–48).

The gene expression pattern of the donor cell plays an important role in the reprogramming process. It was shown that fibroblasts that are a product of epithelial-to-mesenchymal transition (EMT) pass through a mesenchymal-to-epithelial transition (MET) during the reprogramming process (49). MET can be achieved by the four transcription factors: Sox2 and Oct4 suppress Snail (EMT mediator), Klf4 induces epithelial genes and c-Myc downregulates transforming growth factor ß1 (TGFß1), resulting in successfully reprogrammed fibroblasts. In conclusion, the cell type of origin plays a pivotal role regarding the reprogramming process and the efficiency, because the gene expression pattern of each cell type has a different effect on the reprogramming factors.

To use iPSCs for studying developmental processes and diseases, the question has to be resolved whether there are fundamental differences between iPSCs derived from different donor cell types and which iPSC line exhibits the best differentiation potential. In recent studies, the direct comparison of different early passaged iPSC lines demonstrated a persistent cell-specific gene expression depending on the donor cell (50,51). However, it was shown recently by Jose Polo and colleagues that iPSCs retain a transient genetic memory of their cell of origin at early passages, but this transcriptional and epigenetic memory is lost upon continuative passaging. In differentiation studies, cells of early passages (p4) indicate aberrant differentiation potentials; through

further passaging (p16) these differences seemed to be eliminated and the matched cells become very similar to each other (40).

For therapeutic purposes of the iPSC technology, the chosen cell types have to be easily accessible in the patient. Skin cells like dermal fibroblasts, keratinocytes, dermal papilla cells or melanocytes can be easily isolated by punch biopsies from skin with minimal risk. Fibroblasts are the most commonly used cell type for reprogramming experiments. The plain culture conditions and their easy accessibility make them the source of choice for the majority of published studies. In contrast, the efficiency of reprogramming fibroblasts remains significantly low, typically under 0.01%, and the length of time until the first colonies appear is relatively long, compared with that of other cell types. In addition to fibroblasts, keratinocytes can be isolated from a skin biopsy. Although the expansion of the cells in vitro takes a long time, keratinocytes show a 100-fold increase in reprogramming efficiency compared with fibroblasts (19,45). Melanocytes or dermal papilla cells can also be isolated under defined conditions from skin biopsies, show a higher reprogramming efficiency and require no exogenous Sox2 (31,32). However, skin cells are exposed to UV light resulting in an increased DNA mutation rate. To avoid the reprogramming of cells with DNA damages, biopsies from more covert skin parts should be used like the inner upper arm or the armpit.

In addition to the skin as an easy accessible source of donor cells, iPSCs were shown to be generated from peripheral blood as from CD34^{+} cells (52). These cells were isolated from patients that were G-CSF-treated for several days. From 100 ml of peripheral blood, over 10[6] cells can be isolated, indicating an opulent source for cells. However, G-CSF treatment often results in uncomfortable side effects. Another easy accessible source is the cord blood. Giorgetti and colleagues (53) showed the successful reprogramming of CD133^{+} cells, using just Oct4 and Sox2. In another report, endothelial cells derived from the cord blood were reprogrammed using Oct4, Sox2, Nanog and Lin28 (54). But this source is limited to the patients that have banked their cord blood at birth. Recently, iPSCs were also generated from adult adipose stem cells by four transcription factors (Oct4, Sox2, Klf4 and c-Myc) (55). The cells of origin were derived from lipoaspiration, which is a minimally invasive procedure and represent a suitable source.

Generating iPSCs for therapeutic purposes

The use of patient-specific iPSCs possesses the possibility of customized therapy of diseases. Recent studies show that iPSCs generated from a patient with, e.g., amyotrophic lateral sclerosis give rise to motor neurons, after differentiation procedure in vitro (56). Other investigators isolated somatic cells from a patient with Parkinson's disease; after successful reprogramming, they were able to differentiate these cells into dopaminergic neurons (57). This and many other studies provide a major breakthrough in the field of ESC and iPSC technology and underscore the importance of this tool in regenerative medicine (Fig. 3).

The first attempts of reprogramming somatic cells into a pluripotent status used retroviral vectors, which were known to undergo silencing in the ESC state (58,59). This self-silencing property provided an advantage for the initial attempts as the temporal requirement of factor expression had not yet been determined. It was shown that retroviral silencing occurs gradually dur-

ing reprogramming (60). iPSCs generated with retroviral vectors show genomic integrations into the host genome and often maintain viral gene expression (56,61). Using an inducible lentiviral system facilitates the integration into the host genome and permits temporal control over factor expression (60,62).

The use of potentially harmful genome-integrating viral vectors reduces the applicability of the iPS cell technology in clinical therapy. Therefore, methods were applied to use small molecules that can replace certain reprogramming factors and thus reduce the number of viral integration sites. Such molecules are, e.g., TGFbeta inhibitors or BIX-01294 together with BayK8644 that can replace Sox2 and c-Myc (63–65). In addition, new strategies of non-viral reprogramming were established to avoid viral insertion events and adverse effects of reprogramming genes that become reactivated as a consequence of viral integration. The strategies of generating viral vector–free iPSCs are summarized in Fig. 4. It is possible to generate human iPSCs using a lentiviral system where the reprogramming factors are flanked by loxP sites and can be removed with exogenous expressed Cre-recombinase (57,66,67). However, the loxP site residuals still remain in the host genome.

Ongoing studies demonstrated also a successful reprogramming by transient transfection of cells with expression plasmids, carrying Oct4, Sox2, Klf4 and c-Myc (68). However, this method is very inefficient, and iPSCs need to be tested for frequent genomic integrations.

An improved method is the piggyBac transposition. PiggyBac transposons together with the polycistronic transgene containing the reprogramming factors were applied for creating iPSCs. The piggyBac transposon elements integrate into already existing sites in the genome and require only the inverted terminal repeats flanking the transgene and the transposase enzyme to catalyse the insertion. After successful reprogramming, the transgene can be removed by piggyBac transposase without leaving any residuals in the host genome (69). However, point mutations or chromosomal rearrangements are possible. iPSCs need to be analysed for these rarely occurring events.
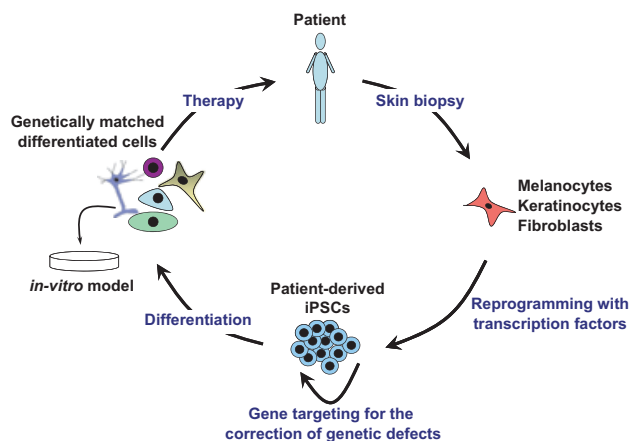
Another possibility for exogenous DNA-free reprogramming is the use of non-genomic DNA integrating adenoviral vectors. iPSCs reprogrammed with adenoviral vectors showed all properties of ESCs including generation of chimeric mice and provided the proof for the dispensability of viral integration for effective reprogramming (70).

Recent studies developed methods to create iPSCs without using genetic material. Therefore, stable HEK293 lines were generated to express the four reprogramming factors, which were fused to a HIV transactivator of transcription (HIV-TAT) protein, a
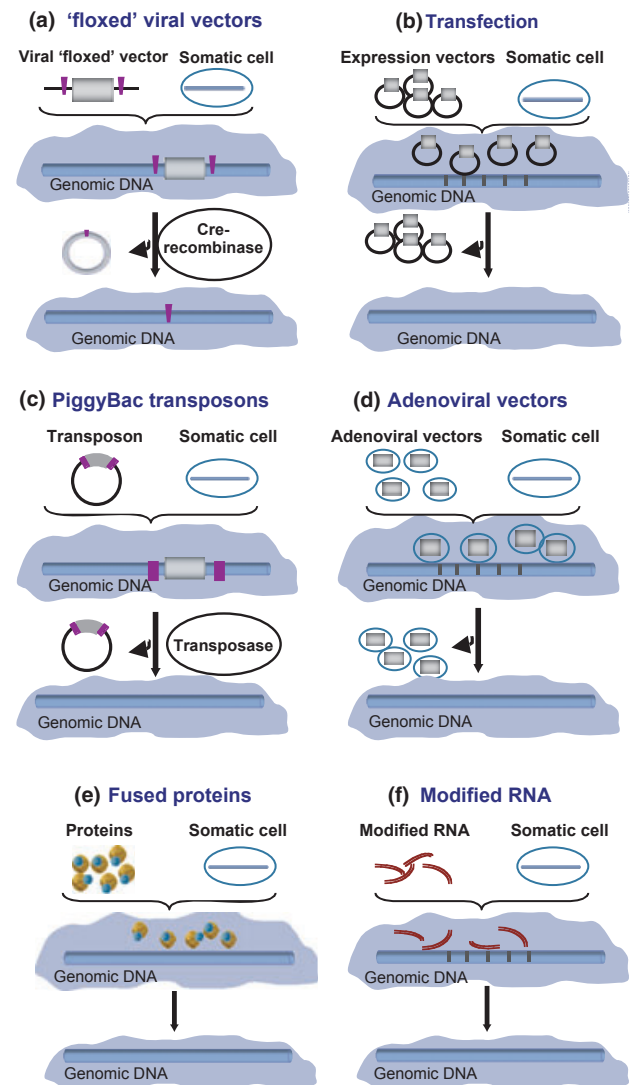
Figure 4. Methods for generating induced pluripotent stem cells free of viral DNA: (a) Lenti- or retroviral vectors carrying the reprogramming transcription factors are inserted into the host cell and can be floxed out after successful reprogramming. The loxP site residuals remain in the host genome. (b) Cells are transfected transiently by multiprotein expression vectors consisting of the reprogramming factors. (c) Reprogramming via piggyBac transposons that carry a polycistronic transgene. After reprogramming, the transgene can be removed with the enzyme transposase without any residuals. (d) Reprogramming somatic cells by adenoviral vectors, which do not integrate into the host genome. (e) Reprogramming without genetic material using purified recombinant reprogramming proteins fused to HIV-TAT or poly-arginine, to penetrate the cell membrane barrier. (f) Reprogramming via modified mRNA.

Figure 3. Therapeutic potential of the induced pluripotent stem cells (iPSC) technology: in future, it might be possible to generate patient-specific iPSCs from human skin cells (e.g. fibroblast, keratinocytes or melanocytes). Potential genomic mutations can be corrected in iPSCs, and these iPSCs can be differentiated into various other cell types (e.g. liver cells, pancreatic beta cells or keratinocytes). These cells can be used for *in vitro* models (e.g. drug screening) or for therapeutic purposes as for the treatment of genetic skin diseases such as xeroderma pigmentosum or dystrophic epidermolysis bullosa.

protein that possesses the ability to overcome the cell membrane barrier. Human fibroblasts were cultured in ESC medium supplemented with HEK293 extracts and could successfully be reprogrammed (71). To improve the technique of generating iPSCs viral- and nucleic acid-free with recombinant proteins, Zhou et al. (72) used purified recombinant reprogramming proteins. A polyarginine protein transduction domain was fused to the C-termini of the reprogramming factors to ensure their intrusion into the host cell. But, the protein-mediated generation of pluripotent cells is inefficient (0.001%), long-lasting and technically difficult. Recently, another non-integrating strategy for reprogramming cells based on the administration of synthetic modified mRNA was described. This approach can reprogram multiple human cell types to pluripotency with efficiencies that greatly surpass established protocols (73). Hence, reprogramming somatic cells viral free using recombinant proteins (PiPSCs) or modified mRNA (RiPSCs) eliminates the risk of viral integrations and allows the generation of safe iPSCs for therapeutic use.

Induced pluripotent stem cells are a great tool to create or correct specific mutations via gene targeting. Tailtip fibroblasts from sickle cell anaemia (SCA) mice were isolated and reprogrammed then transduced with a targeting construct and selected. The resulting iPSCs have undergone gene correction by homologous recombination and were differentiated into haematopoietic precursor cells before injection into SCA mice (74). Gene augmentation is the technique to rescue a single recessive mutation by providing an additional copy of the wild-type allele. In a recent study, cells from patients with Fanconi anaemia, corrected using vectors carrying FANCA or FANCD2 genes, could successfully be reprogrammed. The resulting iPSCs were defect free and could be differentiated into defect-free haematopoietic progenitors (75).

Nevertheless, patient-derived iPSCs are only safe if their tumorigenicity is eliminated. It is still a matter under discussion how to eliminate or even reduce the tumorigenicity, e.g. sorting of progenitor cells, addition of suicide genes or stemotoxic agents (76).

Recent studies demonstrated the direct conversion of somatic cells into other cell types, without the step of generating iPSCs. It was shown that the overexpression of Oct4, using either lentivirus for transduction (77) or virus free by transient transfection (78) generates 'half-way' reprogrammed multipotent cells. These cells exhibit the potential to differentiate into various cell types, when exposed to defined media conditions.

Possibilities of iPSCs in the therapy of skin diseases

Cellular therapies using genetically modified cells may offer new perspectives to our current therapeutic approaches and give hopes to patients with skin diseases (79). iPSC research is extremely important to the field of dermatology in that for the first time pluripotent stem cells were shown to be generated from adult human tissue and in that such iPSCs are first and facilely produced from the most accessible human tissue, skin. Successful reprogramming of differentiated human somatic cells into a pluripotent state would allow the creation of patient-specific and disease-specific stem cells to be used for the research and development of therapeutics, including transplantation medicine. Here, the differentiation of iPSCs in different cutaneous lineages (as keratinocytes or melanocytes) and the establishment of organotypic skin cultures are of great interest. It was shown that chimeric mice generated from iPS-

Cs show a normal skin and fur without any signs of differentiation abnormalities (e.g. 13,31). Recently, it was reported that iPSCs can be differentiated in vitro into keratinocytes by sequential applications of retinoic acid and bone morphogenetic protein-4 and growth on collagen IV-coated plates (80). Melanocytes can be differentiated in vitro from iPSCs by the supplementation of the differentiation medium with Wnt3a, SCF and ET-3 (81). However, it needs to be proven that these in vitro differentiated cells are completely identical to their skin equivalents in terms of functionality.

In the field of dermatology, iPSCs and their progenies may have potential applications in a broad spectrum of hereditary dermatologic diseases (see Table 2). In these genetic skin diseases caused by single-gene mutations such as xeroderma pigmentosum, congenital ichthyoses or epidermolysis bullosa, patient-specific iPSCs can be gene-targeted by homologous recombination to correct the gene defect.

Agarwal and colleagues recently showed that iPSCs can be derived from patients with dyskeratosis congenita, a disorder of telomere maintenance with degeneration of multiple tissues. A cardinal feature of iPSCs is the acquisition of indefinite selfrenewal capacity, which is accompanied by the induction of the telomerase reverse transcriptase gene. It was demonstrated that reprogramming restores telomere elongation in dyskeratosis congenita cells despite genetic lesions affecting telomerase and shown that strategies to increase TERC expression may be therapeutically beneficial in patients with dyskeratosis congenita (82).

Recently, iPSCs were derived from patients with recessive dystrophic epidermolysis bullosa (RDEB) harbouring a defect in the COL7A1 gene-encoding type VII collagen (Col7). It was reported that Col7 is not required for stem cell renewal and that RDEB iPSCs could be differentiated into both haematopoietic and nonhaematopoietic lineages. COL7A1 gene-corrected RDEB-iPSCs might be an ideal source of cells to generate autologous haematopoietic grafts and skin cells with the inherent capacity to treat skin and mucosal erosions in this disease (83).

Besides genetic skin disorders, other possible applications of the iPSC technology are wound healing, depigmenting disorders, and the use in aesthetics or restorative dermatology.

The era of stem cell biology employing adult human somatic cells as sources for unmodified or genetically modified iPSCs has been heralded through the use of skin-derived cells. Dermatologic application of these seminal research works will be at the forefront of the stem cell field for use in treating different skin diseases as well as in antiaging medicine.

Table 2. Examples of hereditary skin disorders that might be treated in the future by genetically corrected iPSCs

	Disorder	Gene defect
Keratin abnormalities	Ichthyosis hystrix	Keratin 1
	Ichthyosis bullosa of Siemens	Keratin 2e
Disorders of cholesterol metabolism	X-linked recessive ichthyosis	Steroid sulfatase
Connexin defects	HID syndrome	Connexin 26
Collagen defects	Dystrophic epidermolysis bullosa	COL7A1 (collagen VII)
DNA repair disorders	Xeroderma pigmentosum	XPA – XPG, XPV

iPSCs, induced pluripotent stem cells.

Acknowledgements

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Supporting Information

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

Table S1. Examples of somatic cell sources where iPSCs were derived from.

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