1. Introduction

Over the past decades, skin has become an increasingly interesting target for replacement therapies. Easy access plays a pivotal role in its widespread use in this context. Current cell culture techniques have optimized in vitro expansion of cells obtained from skin biopsies to be assembled in three-dimensional matrices and engineered skin equivalents that are amenable to clinical use. A wide range of natural scaffolds and synthetic materials are now available as matrices in organotypic skin cultures for skin regeneration (Shevchenko et al., 2010). Patients with severe skin loss require large-scale production of composite skin equivalents. We developed an improved whole autologous bioengineered skin based on the use of a fibrin three-dimensional dermal scaffold in which fibroblasts are embedded (World Patent WO/2002/072800) (Figure 1). We provided evidence that this plasma-based dermal equivalent adequately supports keratinocyte growth (Meana et al., 1998). Immunohistochemical studies over long follow-up periods showed that experimental grafting on immunodeficient mice yielded a healthy and mature skin with human architecture that persisted even after several epidermal turn-overs (Llames et al., 2004). Permanent skin regeneration requires preservation of epidermal cell stemness. The preclinical model fulfils this requirement (Larcher et al., 2007). Bioengineered human skin has been successfully employed in a clinical scenario (Figure 1) for permanent coverage in the case of extensive burns, necrotizing fascitis, removal of giant nevi, and graft-versus-host disease (Llames et al., 2004; 2006; Gómez et al., 2011). Currently, the use of bioengineered skin has spread to a wider range of applications such as the management of injuries of different aetiology including vascular and diabetic wounds and more recently the treatment of wounds associated with genetic rare diseases such as epidermolysis bullosa (EB). EB is characterized by skin blistering following minor friction or mechanical trauma. The condition varies from limited blisters in the skin to a form involving internal epithelial lining. The management of EB is mainly supportive with symptomatic
treatment, since currently no cure exists. Nevertheless, EB patients may benefit from the
treatment with new cell-based therapies. In this context, the EMEA awarded the Orphan
Drug Designation to a chimerical version of the substitute (orphan designation number
EU/306/369). Two additional strategies to treat EB, based on the use of bioengineered skin,
are being explored by our team.

Our approach to study the physiopathology of the skin evolved also toward disease modeling.
We have established a skin-humanized mouse model system based on bioengineered human
skin-engrafted immunodeficient mice (Del Rio et al., 2002b; Llames et al., 2004) (Figure 1).

Fig. 1. Human bioengineered skin. Human fibroblasts and keratinocytes isolated
from a skin biopsy are expanded in vitro. The tissue bioengineered skin equivalent is
based on a fibrin-based matrix containing live fibroblasts as a dermal component and
keratinocytes as the epidermal component. This bioengineered human skin has been
successfully transplanted to patients. The skin-humanized mouse model based on the
stable engraftment of this setting represents a useful pre-clinical platform to model
physiopathological process and to test innovative therapeutic protocols. Histopathological
features of the human bioengineered skin in vitro and after clinical and preclinical
This chimerical model involves the regeneration of human skin, vascularized and innervated by mouse vessels and nerves. This method allows for the generation of a large number of engrafted mice containing a significant area of homogeneous single donor-derived human skin in a relatively short period of time. We have deconstructed-reconstructed skin disorders using skin cells isolated from healthy donor or patient biopsies. Our work included different rare human monogenic skin diseases, such as the recessive form of dystrophic Epidermolysis Bullosa (RDEB), an inherited mechano-bullous disease (Gache et al., 2004; Spirito et al., 2006), the UV-sensitive cancer-prone disease Xeroderma Pigmentosum (XP) (Garcia et al., 2010), Pachyonychia Congenita (PC) (Garcia et al., 2011) and the Netherton Syndrome (NS) (Di et al., 2011), both debilitating skin disorders. With this model, we have succeeded in reverting the phenotype employing different gene therapy approaches for \textit{ex vivo} correction of cells. We were also able to generate a skin humanized mouse model of acquired conditions such as psoriasis, a common chronic inflammatory disease where the immune component plays a pivotal role (Guerrero-Aspizua et al., 2010). Finally, the model also serves to conduct studies in normal human skin, both in a physiological or pathological context, to gain insight into a process such as wound healing (Escámez et al., 2004, 2008; Martinez-Santamaría et al., 2009 and unpublished results). These wound healing models also allowed the validation of gene and cell therapy approaches to improve impaired wound healing conditions and to favour the efficacy of bioengineered skin substitutes in tissue regeneration.

2. The skin

Skin is the outermost tissue of the body and the largest organ in terms of both weight and surface area. It comprises an area of approximately 1.5-2 m$^2$ for an adult and represents about 8% of the body weight. The skin has a very complex structure that consists of many components and adnexa including hair follicles, sebaceous glands and sweat glands. The main function of skin is to act as a barrier to the surrounding environment dangers. It protects the body from friction and impact wounds with its flexibility and toughness. It also prevents water loss and regulates body temperature by blood flow and evaporation of sweat. Chemicals, bacteria, viruses and ultraviolet light are also prevented from entering the body by the skin. Furthermore, skin has a large amount of nerves and nerve endings that enable it to act as a sensory organ. When exposed to sunlight, skin can produce vitamin D, a critical molecule for calcium metabolism.

The skin is formed by anatomically, functionally and developmentally distinct tissues: the epidermis and the dermis. These layers are composed of different types of cellular elements. Hence, they are very different in terms of structure and function.

2.1 Epidermis

The epidermis is the outermost component of the skin formed mostly by a particular kind of epithelial cells known as keratinocytes. Other epidermis resident cells also include melanocytes, Merkel and Langerhans cells, responsible for important specialized functions. The epidermis is morphologically divided into different layers or strata. From the bottom (innermost), these layers are stratum basale (basal cell layer), stratum spinosum (prickle cell layer), stratum granulosum (granular cell layer), stratum lucidum (clear layer) and stratum corneum (horny cell layer). Keratinocytes produced in the basal layer, where cell proliferation is confined, move upward to the outer surface in a process named as epidermal
differentiation. During this turn-over, keratinocytes change their structures and physiological functions. One cycle of this turn-over process takes about 28 days. The differentiation process involves morphological and biochemical changes with temporal and spatial changes in gene expression. Specific proteins are characteristic of the cells at the different layers of the skin. Thus, the proliferating basal keratinocytes, which express keratin 5 (K5) and keratin 14 (K14), adhere to the basal membrane (BM). Basal keratinocytes mature into suprabasal keratinocytes. This transition is characterized by loss of contact with the BM, proliferation arrest, and downregulation of keratins K5 and K14 accompanied by upregulation of keratins K1 and K10. Finally, suprabasal keratinocytes undergo an apoptosis-related process called terminal differentiation which results in the formation of a layer of dead cornified cells, the stratum corneum. This layer is a main component of the protective skin barrier. The terminal differentiation process is accompanied by the expression of marker proteins such as transglutaminase, involucrin, filaggrin and loricrin among others. The hair follicles (HFs), which together with sweat and sebaceous glands form the epidermal appendages, are formed during embryogenesis as outgrowths of the epidermis.

2.2 Dermis
The dermis is the living layer that acts as a substrate and a support network for the epidermis. The essential dermal cell type is the fibroblast, which is responsible for the production and maintenance of the structural elements of skin. These elements, which include collagen and elastin, combine with non-fibrous substances such as glycosaminoglycans (GAGs) to form the extra-cellular matrix (ECM). The ECM also supports the basement membrane, ensuring the integrity of the dermo-epidermal junction (DEJ). Organized tissue renewal depends on the ECM. Normally, turnover of collagen is low, but occurs at a higher rate during damage repair. The vascular network, which is difficult to replace, is quite critical to skin regeneration. Without an adequate blood supply, repair is impaired, and if revascularization cannot be achieved, undesirable scar tissue formation is enhanced. Adequate regulation of the inflammatory and immunologic responses of the skin also plays a pivotal role in tissue regeneration. Imbalanced inflammation may prevent the progression of the regenerative (Eming et al., 2007; Pierce, 2001)
A complex BM composed by specialized proteins serves as an epidermal and dermal anchoring structure but also clearly establishes a boundary between epithelial and mesenchymal territories. Mutations in the genes coding for the BM proteins (i.e. collagen VII or laminin 5) are responsible for rare inherited mechano-bullous diseases.

2.3 Epidermal stem cells
Early tracing experiments performed in human and mouse epidermis demonstrated that within the basal layer of interfollicular epidermis there was a remarkable proliferative heterogeneity. However, these studies did not lead the way to the identification of bona fide functional markers for human epidermal stem cells (ESCs). Markers including, α6β1/CD71dim, and Lrig1+ were suggested to be useful to enrich for highly clonogenic cells (Li et al., 2004; Jensen et al., 2008). However, a criterion established more that 20 years ago, based on the in vitro proliferative capacity of keratinocytes remains the most reliable way to identify the putative stem cells of human interfollicular epidermis (Barrandon & Green, 1987). Based on these criterion three populations known as holoclones (clones with high clonogenic capacity and very high proliferative potential), meroclones (clones with less proliferative potential than holoclones, from committed progenitors or transitory amplifying
cells) and paraclones (clones with low proliferative capacity near terminal differentiation) were defined. This classification is still valid, and considers the holoclone as strictly derived from the human interfollicular epidermal stem cell. Thus, clonogenic assays appear to be the best predictors of “stemness”, at least in terms of extensive proliferative capacity of the putative interfollicular epidermal stem cells (Barrandon & Green, 1987; Mathor et al., 1996). These in vitro studies have demonstrated that either wild type or genetically modified cultured human epidermal cell clones named as holoclones, are endowed with an extraordinary replicative potential. One controversial point in the field of human epidermal stem cells in relation to regenerative medicine, is to establish whether there really is a cell subpopulation that has all the attributes of stem cells, or if, conversely, cells with limited proliferative capacity (defined as a population of transitory amplifying cells) can be programmed or reprogrammed to renew the epidermis in the long term (Li et al., 2004). However, neither both the actual proportion and performance, nor dynamics of the human epidermal repopulating clones in vivo have been studied in detail as done already with human hematopoietic stem cells. Although previous attempts to assess the putative stem cell behaviour of single genetically modified human clones (holoclones) in vivo were unsuccessful (Mathor et al., 1996), recent advances in organotypic cultures and surgical techniques have now made it possible to achieve this goal (Larcher et al., 2007).

Much of the current enthusiasm for the study of human embryonic stem cells (hSC) comes from the possible therapeutic use of somatic cells derived from them. While skin biopsies are the regular source of keratinocytes and ESCs, recent studies aim at generating keratinocytes from human embryonic stem cells. By assessing the sequential expression of specific transcription factors, Howard Green and co-workers followed the time- and migration-dependent development of the keratinocyte lineage from human embryonic stem cells in culture (Green et al., 2003). In a recent study these authors also established differences between post-natal keratinocytes and those derived from hSC showing that the latter have much lower proliferative potential in culture implying that hES-derived single keratinocytes cannot be expanded into mass cultures (Iuchi et al., 2006). They also showed that optimization of culture conditions improves the proliferation, but not sufficiently to permit their clonal isolation. However, our group, in collaboration with researchers at INSERM/UEVE U-861 (France) has recently succeeded in obtaining a homogenous population of keratinocytes derived from hSC. Following assembly in a proper scaffold and grafting to immunodeficient mice, these keratinocytes retained their ability to regenerate a fully differentiated self-renewing epidermis (Guenou et al., 2009). In relation with this issue, de-differentiation of adult cells into a pluripotent embryonic stage has been achieved. These cells are known as iPS (induced pluripotent stem cells) (Takahashi et al., 2007). While iPS cells have been generated from somatic cells, optimization of the process is still underway. The obtaining of differentiated cells from iPS or hES is still a major challenge. Recently, iPS cells have been obtained from EB patients (Tolar et al., 2011) and it is expected that iPSs from other skin diseases will soon be generated. So far, iPS cells differentiation to fully functional keratinocytes, as performed with hES, has not been reported. It is, however, a matter of further attempts and time to achieve this major goal.

3. Clinical applications of bioengineered skin

Skin is the most antigenic tissue in the body and it is refractory to currently known tolerance induction regimens. This fact has long precluded the use of allogenic skin grafts for
permanent tissue replacement. Allogenic cadaver skin grafts have been shown, however, to be of value as temporary skin replacement (i.e. temporary coverage of burn patients). When used in this context, a rejection of the epidermal layer of the grafted skin is clinically evident within 2-3 weeks post-grafting. Therefore, permanent skin regeneration has only been achieved with autologous ESCs transplantation (either as part of split-thickness grafts or of bioengineered skin equivalents).

The introduction of tissue-engineering in therapeutics opened the debate on the idea that allogenic skin equivalents are better tolerated by the host than the allogenic split-thickness grafts. In fact, allogenic bioengineered skin does not appear to evoke acute clinical rejection (Falanga et al., 1998). Instead, a gradual replacement of the allogenic cells by host cells occurs (silent rejection). This process is probably triggered by a response of the host immune system elicited by HLA-mismatch system (Hohlfeld et al., 2005). During this continuous replacement, cytokine release, structural support and provision of a moist wound environment supplied by the allogenic skin substitute would explain the improvement in the clinical course of wounds treated with allogenic bioengineered skin equivalents. Analysis of donor allogenic cell DNA in biopsies of healed wounds after application of a living skin equivalent (Apligraf®), for example, have demonstrated one-month persistence of allogenic cells in only a minority of venous ulcer patients, and the complete disappearance of these cells by two months post-application (Griffiths et al., 2004; Phillips et al., 2002). It is a widely demonstrated fact that allogenic keratinocytes do not persist and are progressively substituted by autologous keratinocytes of the patient in a process that lasts a few weeks. The fate of allogenic fibroblasts it is less clear. Some studies have reported persistence for up to 2.5 years (Otto et al., 1995). Within this context, it is now clear that allogenic bioengineered skin equivalents have a role only as temporary biological dressings with relevant healing promoting activity. Therefore, allogenic skin substitute transplantation is currently used to improve the healing of both acute and chronic wounds, including EB lesions (Eisenberg & Llewelyn, 1998; Falabella et al., 2000; Fivenson et al., 2003).

3.1 Permanent replacement of skin losses
In the mid seventies, Howard Green and co-workers set up the methods for serial culture and large expansions of human epidermal keratinocytes based on the use of a specific growth factor cocktail and the presence of lethally irradiated mouse fibroblasts acting as a feeder layer (Rheinwald & Green, 1975). Although grafting of pure epithelial sheets has helped to save the life of seriously burned patients around the world (Carsin et al., 2000; Compton, 1992; O’Connor et al., 1981), the approach showed various drawbacks including the fragility of the product, a limited engraftment efficacy, abnormal ultrastructure of the dermo-epidermal junction resulting in blistering and contracture leading to poor aesthetic clinical outcome (Mommaas et al., 1992; Woodley et al., 1988). Soon it became evident that a much more robust skin replacement system was needed. A race to develop and market such products started in the 80’s and still continues. As a result, bioengineered skin substitutes have emerged as the most carefully studied and proven of the advanced wound management technologies. While the initial impetus for their development was to replace autograft, allograft, and xenograft in acute skin loss applications, they have found even wider application in the treatment of chronic wounds. Bioengineered skin substitutes represent artificial alternatives to skin grafts that avoid the pain, potential complications and surface limitations of native skin harvesting. They should be easy to manipulate, resistant and always available in any quantity needed. In terms of function, the ideal skin substitute should mimic the physiology of normal skin, being highly
effective in achieving tissue regeneration and wound repair. It should be inexpensive, not subject to immune rejection by the host, and should have an extensive shelf-life. Tissue engineering of cultured skin substitutes is largely based on the strategy that the following three components are important in a bioengineered construct: 1) cell source, 2) tissue-regeneration-inducing factors, and 3) matrix or scaffold (Langer & Vacanti, 1993). A variety of cells, soluble mediators, and biopolymers have been tested in various combinations to engineer cultured skin substitutes. As already mentioned, epidermal sheets of cultured keratinocytes have been applied to wounds as allografts or autografts. Later, it was shown that replacing the connective tissue along with keratinocytes may increase mechanical strength of healed wounds and reduce ultimate scarring (Cuono et al., 1987; Desai et al., 1991; Gallico, 1990) so fibroblasts have been included in some artificial skin substitutes (Hansbrough et al., 1989; Llames et al., 2004; Meana et al., 1998). Others have used matrix-cultured dermal fibroblasts alone as a wound healing device (Marston et al., 2003). Due to difficulties in producing and marketing autologous skin equivalents, most current commercial bioengineered skin substitutes consist of sheets of a biomaterial matrix containing allogenic cells (keratinocytes, fibroblasts or both), which are typically derived from neonatal foreskin, a convenient tissue source with the added advantages of a higher content of putative keratinocyte stem cells, robust cell growth and metabolic activity, and reduced antigenicity.

The steps in creating and combining the components of bioengineered skin have been comprehensively discussed elsewhere (Boyce and Warden, 2002). Many recent reviews have summarized the history and current status of matrices and skin substitutes (Beele, 2002; Ehrenreich & Ruszczak, 2006; Hansen et al., 2001; Horch et al., 2005).

Although an ideal skin substitute has not yet been developed, we have contributed within this field with the development of a fibroblast-containing fibrin-based bioengineered skin product (WO/2002/072800) that does fulfil many of the clinical requirements. The fibroblast-containing fibrin-based bioengineered skin was devised by carefully looking at the wound healing process. Thus, fibrin was chosen as a matrix suitable to host dermal cells in a bioengineered skin equivalent. Fibrin is the primary and temporary wound healing matrix allowing blood clotting and migration of both, epithelial and mesenchymal cellular elements that, in turn, will repair the damaged tissue. The resistance and flexibility of the fibrin clot are ideally suited for grafting manipulations. In fact, acellular commercial fibrin gels have been used as carriers for human keratinocyte sheets grown, on top, using the standard Rheinwald & Green method. This system, replacing only the epidermal tissue, has been successfully used for grafting of burn patients (Pellegrini et al., 1999; Ronfard et al., 2000).

A major breakthrough was achieved with the demonstration that live human fibroblasts embedded in blood cryoprecipitate-derived fibrin gels were able to support human keratinocyte growth without the need of a feeder layer (Meana et al., 1998). Although soluble growth-stimulatory factors provided either by feeder cells or live human fibroblasts may be equivalent, the unique mechanical or nesting anti-differentiating signals attributed to feeder cells are somehow replaced by survival signals originated as a consequence of keratinocyte-fibroblast-fibrin interactions. However, fibrin (fibrinogen) may not be the only relevant factor since plasma cryoprecipitate also contains additional factors such as fibronectin or thrombospondin that may contribute to keratinocyte adherence and survival. More recently, a fibrin-based dermal matrix was obtained from pure plasma allowing the generation of fully autologous skin equivalents since keratinocytes, fibroblasts and fibrin may come from the same individual (Figure 1) (Llames et al., 2004). A major feature of fibroblast-containing fibrin-based scaffolds is that human keratinocytes can be seeded at low densities, decreasing
the amount of primary cells needed to generate a graftable skin equivalent. This is in greater contrast to collagen-based dermal equivalents such as Apligraf® in which keratinocytes are seeded at near confluence densities. Other features of the fibrin-based skin equivalent are its low cost and long shelf life. Moreover, the fibrin-based bioengineered skin developed by our team has been used successfully, in its autologous version, for permanent skin regeneration in different situations such as extensive burns, necrotizing fascitis, removal of giant nevi and graft-versus-host disease (Figure 2) (Llames et al., 2004; Llames et al., 2006; Gómez et al., 2011).

Fig. 2. Clinical applications of fibrin-based bioengineered skin. (A) Permanent skin regeneration on burn patients after autologous transplantation. (B) Skin regeneration on chronic ulcers by allogenic temporary coverage.

The treatment of more than 100 patients with extensive and severe burns, carried out in several Spanish hospitals, has achieved reasonable cosmetic results and encouraging graft take percentages. Two major causes were disclosed as responsible for cases of poor engraftment: 1) resistant infections of the graft recipient bed and 2) poor vascularised wound beds. These processes jeopardize the viability of bioengineered skin and are significantly affected by wound bed conditioning. Antimicrobial peptides (AMPs) are effective against a broad spectrum of pathogens, have low rates of bacterial resistance and in some cases favour the repair process. As such, they emerge as an alternative to conventional antibiotics. Concerning this issue, our studies support that cutaneous tissue engineering in combination with gene therapies may provide a strategy to promote neoangiogenesis (Lasso et al., 2007; Lugo et al., 2011) and combat infection at the same time (Carretero et al., 2004; Carretero et al., 2008).

3.2 Temporary dressing for chronic wounds
The primary difficulties associated with commercial autologous bioengineering skin substitutes related to high cost and logistics make them relatively unpopular products on the market. However, the use of allogenic skin equivalents for chronic wounds is a different story. The treatment of such hard-to-heal, chronic, open wounds has gained importance as
both, the aged segment of the population in the industrialized world and the incidence of comorbid states, such as diabetes mellitus and atherosclerosis have increased. Thus a potential market of 1-2% of the total population in the developed countries led to the development of several competing commercial products struggling to show their benefits in the clinics (Eisenbud et al., 2004; Herschthal & Kirsner, 2011; Límová M., 2010). Although great advances in terms of molecular mechanisms underlying the process of wound healing are being achieved, the science behind treatment of chronic wounds with skin substitutes is mostly empirical and not well understood. Several beneficial effects range from maintenance of a biochemically balanced moist wound environment to structural support for tissue regeneration and/or the provision of beneficial cytokines and growth factors to the wound bed. The latter is perhaps the best bet as skin substitutes made with younger donor cells appear to work better. In fact, Mansbridge et al. have reported that the viability and metabolic activity of the cellular component of a skin substitute is essential for therapeutic efficacy and have proposed that this is due to the need for ongoing cytokine expression in the wound bed following application (Mansbridge et al., 1998). In this regard, these authors also showed that metabolic activity, but probably not cell proliferative capacity, appears to be the critical event associated with healing efficacy.

As mentioned, allogenic skin substitutes provide cells that do not persist on the recipient site and thus, can be considered safe. In the case of chronic wounds, therefore, the goals of skin substitute therapy have evolved away from providing an immediate new skin (involving graft take) towards the more reasonable goal of providing a temporary biologic dressing that accelerates skin tissue regeneration and wound healing by stimulating the recipient’s own wound bed-derived skin cells. Defining the specific, discrete causes of the healing impairment may thus help to develop a combination of cell and gene therapy approaches aimed at providing a la carte solutions for the different subsets of chronic wound suffering patients. While the end point of wound closure is the most intensely studied, there is also increasing focus upon the quality of the healed wound and in pain control. Cell-based wound therapies have indeed the potential to reduce both wound contraction and pain. Of note, an allogenic version of the fibroblast-containing fibrin-based skin equivalent has been used as an efficient means for triggering/promoting healing at the patient’s own expense and releasing pain (Camblor-Santervas et al., 2003; Coto-Segura et al., 2007, 2008). A great efficacy/recurrence ratio has been achieved by using this allogenic bioengineered skin as a temporary cover. In particular, an 80% healing rate was attained by the weekly application of fresh allogenic bioengineered skin during an average period of 6.6 weeks. Relatively high percentage of ulcer recurrence (25%) is observed since these temporary substitutes do not cure the underlying ischemic or diabetic disease (Llames et al., 2008). As mentioned before, cutaneous tissue engineering in combination with gene therapy may provide strategies that extend the temporal effects at a local level, for example, by producing VEGF or other pro-angiogenic factors that enhance angiogenesis during the healing process (Lasso et al., 2007; Lugo et al., 2011).

3.3 Skin bioengineering for Epidermolysis Bullosa

The skin is the site of a wide variety of inherited diseases. In fact, genes involved in more than 80 skin disorders have been identified some of which are causative of rare diseases. Low prevalence, less than 1 affected in 2000 individuals, is the common feature shared by all rare diseases. Rare diseases are often chronically debilitating or even life-threatening and the impact on the quality of life of affected patients (of whom many are children) and their
family members is significant. The limited number of patients and scarcity of relevant knowledge and expertise conferred on them a remarkable research interest on rare diseases. The fact that this low prevalence conditions can serve as models for more common disorders and that their study/management frequently require multidisciplinary innovative approaches add value to the development of this field. To date, a very limited number of so-called orphan drugs are marketed, leaving the majority of rare diseases without any effective treatment. This fact has focused additional attention on new therapeutic approaches such as gene therapy. Cutaneous gene therapy has been one of most intensively explored fields (Del Rio et al., 2002a). In fact, the first successful gene therapy trial for junctional epidermolysis bullosa (JEB), a rare mechano-bullous genodermatosis, has been reported (Mavilio et al., 2006). Mavilio et al. transplanted genetically engineered epidermal stem cells from a JEB adult patient affected by laminin beta3-deficiency modified with a retroviral vector expressing LAMB3 cDNA. Moreover, long-term correction of RDEB using genetically modified human keratinocytes has been also achieved in pre-clinical assays (Del Rio et al., 2004; Spirito et al., 2006). Indeed, permanent correction involves vector-mediated transgene integration into the target-cell genome. Safety concerns related to insertional mutagenesis arose as a consequence of cancer development in two patients undergoing hematopoietic gene therapy for an inherited immunodeficiency (Hacein-Bey-Abina et al., 2003). Therefore, although gene therapy remains the golden standard for genetic disease correction, alternative therapeutic strategies such as cell therapy might be effective. On this regard, the previously mentioned orphan drug chimerical skin (autologous keratinocytes and allogenic fibroblasts) has been proved to be useful for RDEB treatment in the pre-clinical skin humanized mice as discussed further in this chapter. The attempt to improve the healing of EB lesions (including donor sites) by allogenic skin substitute transplantation (Eisenberg & Llewelyn, 1998; Falabella et al., 2000; Fivenson et al., 2003) has shown to report benefit and need to be explored in a systematic manner.

### 3.3.1 Allogenic bioengineered skin

Mitten deformities of the hands and feet occur in nearly every patient with the most severe form of RDEB (RDEB sev gen), and in at least 40–50% of all other RDEB patients. Hand deformities include adduction contractures of the first web space, pseudosyndactyly, and flexion contractures of the interphalangeal, metacarpophalangeal, and wrist joints. Surgical intervention is commonly performed to correct these deformities, but recurrence and the need for repeated surgery are common. Life-table analyses emphasize the need for early surveillance and intervention, since musculoskeletal complications may occur within the first year of life. The severity of the deformity worsens with age, and surgical correction becomes more difficult. Standard surgical procedures for the management of hand deformities in DEB includes incisional release of contracture and digits follow by autologous partial-thickness skin grafts transplantation to cover secondary wounds. Partial-thickness skin grafts are taken from patient’s own skin (e.g. the top segment of the leg), thus creating an additional open wound (a donor site).

Figure 3 shows a surgically created donor site during the standard programmed treatment for pseudosyndactyly and contracture. The skin obtained from the patient’s donor site (Figure 3F) is used to cover the wounds that result from incisional release of contracture and digits (Figure 3B-C). In the literature there is no consensus on the management of the split-thickness donor sites secondary to reconstructive surgery (Demirtas et al., 2010; Pan et al., 2011). On the other hand, allogenic bioengineered skin transplantation has proven to be of
clinical value when used as a healing device aiming at tissue repair/regeneration for chronic wounds (Camblor-Santervas et al., 2003; Coto-Segura et al., 2007, 2008; Eisenbud et al., 2004; Herschthal & Kirsner, 2011; Límová M., 2010) such as vascular ulcers and for full thickness excisional surgical wounds for skin cancer treatment (Donohue et al., 2005; Gohari et al., 2002). As previously discussed, this strategy provides a temporary biologic dressing that accelerates skin tissue regeneration promoting re-epithelialization from patient wound edges and release pain. On that basis, we are currently testing the clinical benefits of allogenic bioengineered skin transplantation on DEB patient donor sites to reduce pain and accelerate healing (Figure 3G-I).

![Fig. 3. Surgical management of hand contractures and pseudosyndactyly in RDEB.](A) Typical pseudosyndactyly and contracture of the thumb and fingers developed by RDEB patients. (B) Standard surgical procedure for the correction of these deformities by incisional release. (C) Autograft of secondary wounds with split-thickness skin from donor site. (D-E) Surgical generation of a donor site on the patient's upper-leg using a dermatome. (F) Split-thickness skin from donor site. (G-I) Donor site transplantation using allogenic bioengineered skin (panel H). Courtesy of Dr. Mir, Plato Clinic (Barcelona). The clinical images have been taken and reproduced with the signed consent of the patient.

3.3.2 Autologous “revertant” bioengineered skin
The term somatic revertant mosaicism refers to the occurrence of a natural phenomenon involving spontaneous genetic correction of a first pathogenic mutation in a somatic cell (Davis & Candotti, 2010). Different molecular mechanisms such as back mutation, intragenic crossover, mitotic gene conversion, and/or second-site mutation might underlie the in vivo reversion. Somatic mosaicism has also been reported in genodermatoses, including EB. In the skin of these patients, revertant mosaicism is manifested as small patches of clinically “better than expected” skin surrounded by easily blistering tissue (Jonkman & Pasmooij, 2009; Pasmooij et al., 2010). The incidence of this phenomenon of genetic reversion, thought
to be rare for years, appears to be more common than imagined (Lai-Cheong et al., 2011; May, 2011). As a matter of fact, to date the phenomenon of revertant mosaicism has already been found in three Spanish RDEB patients. These patients display patches of non-blistering unaffected skin (Figure 4A). The clinical reversion on these long-term persistent patches was further confirmed by the presence of type VII collagen that was almost absent in the non revertant skin (Figure 4B). COL7A1 pathogenic mutations leading to premature termination codons caused this blistering condition in all three patients. In one of these patients, a second-site mutation, present in revertant keratinocytes, resulted in reading frame correction and wild-type type VII collagen expression leading to restoration of skin function (Pasmooij et al., 2010).

Transplantation of autologous “revertant” bioengineered skin may be a tailored EB therapy for patients with somatic mosaicism and is currently being explored in our laboratory in collaboration with Marcel Jonkman’s team in the Netherlands. The ultimate goal of this strategy is the production of sufficient collagen VII from revertant epidermal stem cells to ensure adequate and long-term formation of the anchoring fibrils.

![Fig. 4. Revertant mosaicism in RDEB Spanish patients.](image)

(A) Clinical evidence of “revertant” skin patches (black dashed lines). (B) Type VII collagen expression detected by immunofluorescence shows almost complete absence of labelling at the dermal–epidermal junction in mutant skin but bright linear labelling in the revertant samples. White dashed line indicates dermal–epidermal junction. Asterisk depict sub-epidermal blistering.

4. The skin-humanized mouse

In vivo studies in the skin of human beings are obviously limited by ethical and practical constraints. Current knowledge mainly stems from the use of murine models, including
knockout and transgenic strategies. However, based on the significant differences existing between human and murine skin architecture and physiology, the question remains as to how far the results can be extrapolated to the human scenario. As an example, animal models such as the two-stage carcinogenesis model in the mouse are valuable tools to unravel critical mechanisms of disease, but do not faithfully recapitulate the human illness counterpart (Garlick, 2007). Studies in large animals such as pigs, whose skin architecture and dynamics resemble that of humans, are an alternative, but troublesome and expensive (Sullivan et al., 2001). Skin organotypic cultures represent a valid alternative to native skin \textit{in vivo} studies (Bernerd et al., 2001; Egles et al., 2010; Harrison et al., 2006). However, they are restricted, among other constraints, by their short culture life span and the absence or faulty pivotal mesenchymal responses such as angiogenesis. To circumvent these problems, researchers have often used xenogenic transplantation of donor/patient cutaneous biopsies to immunocompromised mice to perform relevant \textit{in vivo} experimentation in a human context. However, in addition to difficulties in sourcing, a major concern for this type of experiments is the marked heterogeneity of the graftable skin samples. In fact, differences in genetic background, body site or patient’s sun exposure history, among other factors, may severely hamper the outcome of the study. A possibility to overcome these drawbacks involves the stable regeneration of normal or diseased human skin in appropriate hosts by means of tissue engineering (Khavari, 2006). This approach, although realistic, represents a significant challenge that involves adequate human epidermal stem cell manipulation \textit{in vitro}, a technique that only a limited number of laboratories can handle. Hence, stable engraftment and regeneration of enough human skin for \textit{in vivo} studies upon grafting of skin substitutes to immunodeficient mice need to be standardized. Our group has developed a methodology enabling the generation of large numbers of mice engrafted with a significant area of single donor-derived human skin. The system, named as the skin-humanized mouse, is based on the optimized grafting of a fibrin-based bioengineered human skin (Del Rio et al., 2002b; Escámez et al., 2004; Llames et al., 2004). Using this setting, a mature, quiescent, homogeneous human skin is achieved avoiding the need for volunteers and overcoming major differences in tissue architecture and kinetics with mouse skin.

The technical procedure involves the deconstruction-reconstruction of the skin of healthy donors or patients suffering from the different diseases (Figure 1). That is, \textit{in vitro} isolation and amplification of cells (fibroblasts and keratinocytes, including the population of epidermal stem cells) from biopsies and their assembly as a bioengineered skin that is subsequently transplanted to immunodeficient mice (Del Rio et al., 2002b; Llames et al., 2004) (Figure 5).

The human regenerated skin showed the restoration of both epidermal and dermal skin compartments (Figure 6; Llames et al., 2004) indicating functional epidermal stem-cell preservation as further confirmed by the analysis of the regenerated skin after a secondary transplant protocol (Larcher et al., 2007). The secondary transplant protocol on immunodeficient mice is conducted by purifying epidermal and dermal cells from the regenerated skin after primary transplantation. These cells are secondary transplanted to immunodeficient mice as part of a human bioengineered skin. Stable regeneration of skin displaying a well-stratified and differentiated epithelium 40 weeks post-grafting is achieved, which is only possible with epidermal stem cells whose stemness has been preserved.
Fig. 5. **Bioengineered skin orthotopical transplantation procedure.** (A) Full thickness 12 mm circular wounds are created on the dorsum of a 6-week old nude mouse. (B-C) Mouse skin is de-vitalized by three frozen and thaw cycles. (D-F) The bioengineered skin is placed covering the wound. (G-H) De-vitalized mouse skin is used as a biological bandage and held in place by suture. (I) Human and mouse skin boundaries are outlined by a dashed line. (J) Immunostaining of human involucrin at the junction of regenerated human skin and murine host denoting the human origin of the regenerated epidermis. (K) Immunostaining of human vimentin at the junction of regenerated human skin and murine host denoting the human origin of the regenerated dermis.

Moreover, regenerated human skin retains the main physio-pathological characteristics of the donor/patient opening a range of possibilities for faithful recreation of different human skin pathologies *in vivo* (Gache et al., 2004; Spirito et al., 2006). The skin-humanized mouse model also offers the possibility of using genetically modified human keratinocytes and/or fibroblasts. These humanized models have been a unique platform on which to evaluate innovative therapeutic strategies in dermatology such as cell therapy using ESCs derived from both adult and embryonic stem cells (Escámez et al., 2009; Guenou et al., 2009; Larcher et al., 2008; Larcher et al., 2009) and gene therapy (Bergoglio et al., 2007; Del Río et al., 2002b; Di Nunzio et al., 2008; Escámez et al., 2008; Escámez et al., 2004; Larcher et al., 2001; Larcher et al., 2009).
et al., 2007; Lasso et al., 2007). In this chapter, we summarized our experience with the system in modeling various normal and pathologic skin processes.

**Fig. 6.** The skin humanized model recapitulates the main anatomo-pathological features of human skin. Presence of a stratified well-differentiated epithelium. (A) Keratin K5 immunostaining (green) and keratin K10 immunostaining (red) on the regenerated human skin. (B) Loricrin immunostaining on the regenerated human skin. (C) Masson’s trichrome staining showing a well-vascularized, mature, collagen-rich dermis on the regenerated human skin. (D) Laminin immunostaining of the dermo-epidermal junction denoting basal membrane restoration on the regenerated human skin.

### 4.1 Modeling rare monogenic skin diseases

The European Commission on Rare Diseases estimated that between 6000 and 8000 different rare diseases affect or will affect 29 million people in the European Union. In Spain, around 3 million people are affected by a rare disease. Poor availability of diagnostic and therapeutic options is a major consequence of the limited funding dedicated to research on rare diseases. Recently, a great effort is being made by the worldwide scientific community to optimize human and financial resources for the study of these diseases. In Spain, as an initiative of the Instituto Nacional de Salud Carlos III, the Centre for Biomedical Network Research on Rare Diseases (CIBERER), a network structure has been set up to pool and promote excellence in research devoted to the diagnosis and therapies of rare diseases. Within this picture, development of rare disease models, especially in a human context, would contribute to the basic and translational research toward individualized medicine by making patients and their families more immediately aware of potential medical interventions. Moreover, rare diseases can serve as models for more common diseases and the complexity of rare diseases often requires multidisciplinary innovative approaches. Based on our solid background in the field of dermatology and our consubstantiation with the objectives of CIBERER, our interest in translational research has grown over the years.
As a result, we have model a wide range of different rare genodermatoses including photosensitive conditions, some of which are described in the present chapter.

4.1.1 Mechano-Bullous genodermatosis: Recessive Dystrophic Epidermolysis Bullosa (RDEB)

As already mentioned, Epidermolysis Bullosa (EB) comprises a clinically and genetically heterogeneous group of rare skin disease characterized by skin blistering, either spontaneous or induced by minimal trauma. Based on the level of blister disruption, three types of EB are defined: Simplex (EBS), junctional (JEB) and dystrophic (DEB) (Fine et al., 2008). The prevalence of EB in Europe is estimated to be 0.60 per 10,000 individuals (Bruckner-Tuderman, 2008). In Spain the prevalence remains unknown because the genetic diagnosis has been recently settled by our team since 2006 with the scientific support of...
European Diagnosis Reference Centres\(^1\) and as part of the CIBERER network (Cuadrado-Corrales et al., 2010; Escámez et al., 2010; García et al., 2011). Studies carried out by our research team in collaboration with Dr. Meneguzzi team showed the possibility of achieving a lasting recapitulation of monogenic hereditary skin diseases of different subtypes of EB (Del Rio et al., 2004; Gache et al., 2004; Garcia et al., 2007). For instance, the recessive subtype of DEB (RDEB; OMIM: 120120), the most severe form of EB, is due to mutations in the gene encoding type VII collagen (\textit{COL7A1}). In particular, null mutations leading to complete absence of collagen VII entail physical deformities and increased risk of developing skin cancer which reduces their life expectancy dramatically. Extensive blistering of regenerated human skin obtained by orthotopic grafting of bioengineered cutaneous equivalents containing collagen VII-null RDEB keratinocytes was observed at a histological level, similarly to skin biopsies from RDEB patients (Figure 7C-D). Genetic modification of RDEB epidermal stem cells by retroviral vectors encoding human collagen type VII used in the generation of the skin equivalents (Figure 7A-B) resulted in a complete and permanent reversion of this phenotype. Phenotypic correction was also attained by a cell-based therapy based on the use of a chimerical bioengineered skin (Figure 7E-F).

\subsection*{4.1.2 Xeroderma Pigmentosum (XP): A photosensitive condition}

UV radiation is the main noxious and carcinogenic agent for human skin (Brash et al., 1996; Kraemer, 1997; Matsumura & Ananthaswamy, 2002; Mudgil et al., 2003; Setlow, 1974). There is compelling evidence that each of the three main types of skin cancer, basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and melanoma, is caused by sun exposure. As commented above, studies of UVB effects on the skin of volunteers are precluded by ethical and technical restraints and are inconceivable in cancer-prone patients. Molecular changes associated to UVB irradiation have been extensively characterized \textit{in vitro} in keratinocytes in culture (Li et al., 2001; Sesto et al., 2002). Although highly informative, these transcriptional profiling and other biochemical analyses are somewhat skewed by the fact that cultured keratinocytes represent only a mitotically activated basal cell compartment. The presence of differentiated cell layers of the epidermis achieved in 3D organotypic cultures allows for more accurate \textit{in vitro} models to study UV effects. However, the organotypic systems often maintain a (hyper) proliferative basal stratum as compared with quiescent native human epidermis and only allow for relatively short-term studies. Reliable \textit{in vivo} studies lack behind due to the ethical or practical constraints of using human volunteers or inaccurate animal models. We therefore challenged our system to assess whether it was capable of adequately responding to UV irradiation. To that end, we examined the effect of one biological efficient dose (BED) of UVB light in terms of sunburn cell formation and p53 induction, two well-described surrogate markers of UV action. As predicted, both effects were readily detected after irradiation (Figure 8).

Moreover, by using Caucasian or African-descent donor keratinocytes we were able to confirm the well known modulation of the UVB responses by the degree of skin pigmentation (Del Bino et al., 2006; Kobayashi et al., 1998). The model also proved satisfactory to test topical photoprotective agents as well as DNA damage repair kinetics after UVB irradiation in terms of epidermal hyperplasia and keratin K6 induction (Del Bino et al., 2004; Lee et al., 2002). Based on those results we have also established a photosensitive humanized skin models by grafting bioengineered skin containing Xeroderma

\(^1\) Dr. Zambruno (IDI, Italy), Dr. Meneguzzi (INSERM, France) and Dr. Batty (Ninewells Hospital, UK)
Pigmentosum (XP) patient cells (Figure 8). Xeroderma Pigmentosum (XP) is an autosomal and recessive disorder characterized by a severe deficiency in the most versatile DNA-repair mechanism in charge of the removal of bulky DNA adducts including UV-induced cyclobutane pyrimidine dimmers (CPDs) and pyrimidine pyrimidone photoproducts (6-4PPs). The first in vivo evidence of XP keratinocyte deficiency in nucleotide excision repair (NER) was obtained after acute UVB irradiation (Garcia et al., 2010). Our model recapitulated the findings of CPD persistence as previously described using XP-C organotypic skin cultures (Li et al., 2001) (Figure 8) and appears suitable to study chronic effects including mutagenesis and carcinogenesis.

Fig. 8. Skin humanized mice as a model to study UV responses and carcinogenesis-prone inherited cutaneous disorders. (A) Schematic diagram of the experimental design. (B) Macroscopic appearance of Caucasian (right) and African (left) descent-derived regenerated skins. (C) Histological appearance of a 1 BED irradiated Caucasian and (D) African descent derived skin 24 hours after irradiation. (F) CPD immunostaining of 1 BED-irradiated Caucasian and (G) African human regenerated skin 24 hours after irradiation. (E) Histological appearance of a 4 BED irradiated Caucasian- and (H) African descent-derived skin 24 hours after irradiation. (Arrows indicate sunburn cells). (I) Histological appearance of a representative section of a photoprotected (SPF 90), UVB-irradiated (4 BED) Caucasian-derived skin 24 hours after irradiation. Note the absence of acanthosis, epidermolysis, and sunburn cells. In vivo inability to repair DNA damage (CPD) in XP-C regenerated skin. CPD immunostaining of 4 BED-irradiated normal African-derived skin (control) section at (J) 2h and (L) 72 hours after irradiation. CPD immunostaining of 4 BED-irradiated XP-C regenerated skin section at (K) 2h and (M) 72 hours after irradiation. Note the persistence of CPD labeled cells in all epidermal strata indicating a DNA damage repair defect.
4.1.3 Pachyonychia Congenita (PC)
Pachyonychia congenita (PC) is a rare autosomal dominant keratin disorder characterized by thickened and dystrophic nails as well as painful palmoplantar keratoderma and blisters on or near the pressure points of the feet (Leachman et al., 2005; Smith et al., 2006).

Fig. 9. Clinical and histopathological features of human pachyonychia congenita (PC) skin. (A-C) Clinical appearance of engrafted mice. (A) Normal, (B) PC-regenerated skin from non-affected (buttocks) area and (C) PC-regenerated skin from affected foot sole-derived PC cells on immunodeficient mice. Development of a hyperplastic response in normal and pachyonychia congenita (PC)-regenerated human skin after tape stripping: Keratin K6 immunoperoxidase staining of (D,F) normal and (E,G) involved PC-regenerated skin sections at 72 and 360 hours after tape stripping (TS). (H) Constitutive expression of K6 in the foot sole-derived PC graft. (I) Expression of K9, which is characteristic and exclusively expressed in the palmoplantar suprabasal keratinocytes.
PC is caused by dominant-acting mutations in any one of the genes encoding the differentiation-specific and stress-inducible keratins, K6a, K6b, K16, or K17 (Smith et al., 2005; Wilson et al., 2011). The dominant-negative mutations on these keratins lead to defective intermediate filament formation responsible for all of the epithelial fragility symptoms associated with PC. Mouse models involving the PC-related keratin genes elicit only a subset of minor PC-specific epithelial lesions (Chen et al., 2008; Chen & Roop, 2005; Wong et al., 2005). Importantly, mouse models of PC are recessive, carrying loss-of-function alleles whereas, as mentioned, the human PC mutations are dominant negative (Smith et al., 2005; Wilson et al., 2011). Within this context, our group has contributed with the establishment of two skin-humanized models of PC (Figure 9). First model involves the use of bioengineered skin from an uninvolved area of PC patients carrying the same K6a mutation, displayed epidermal phenotypic changes consistent with a hyperproliferative response. Moreover, the use of keratinocytes from affected skin from another patient carrying a different mutation in the same codon of the keratin K6a gene resulted in the development of a constitutively expressed, bona fide PC phenotype. Currently we are evaluating the amenability of these humanized PC models to genetic intervention similar to that recently reported in PC patients (Leachman et al., 2010).

4.1.4 Netherton Syndrome (NS)
Netherton syndrome (NS) is a congenital skin disorder caused by mutations in the SPINK5 gene encoding the lymphoepithelial Kazal-type-related inhibitor (LEKTI) (Bitoun et al., 2003; Chavanas et al., 2000). It is characterized by defective keratinization, recurrent infections, and hypernatremic dehydration with a mortality rate of about 10% in the first year of life (Borgoño et al., 2007; Descargues et al., 2006; Ishida-Yamamoto et al., 2005). Grafting of human NS bioengineered skin onto immunodeficient mice made it possible to recapitulate the characteristic histological features of NS (Di et al., 2011), including psoriasiform changes and hypergranulosis with a parakeratotic stratum corneum and exfoliated corneocytes (Figure 10B). An ex vivo approach using a lentiviral vector to direct SPINK5 expression in keratinocytes resulted in reversal of skin abnormalities (Figure 10A) (Di et al., 2011). In this study we found that limited numbers of LEKTI-expressing cells mediate valuable beneficial effects likely through paracrine effects.

4.2 Modeling psoriasis, an inflammatory skin disease
Inflammatory and autoimmune cutaneous disorders are a major health and social concern worldwide. They can be disfiguring and disabling and take a toll in terms of the patient’s psychological distress. Skin infiltrating T lymphocytes play a pivotal role in triggering and maintaining common chronic inflammatory skin diseases such as psoriasis and atopic dermatitis, where an unequivocal deregulation in the Th1/Th2/Th17 balance accounts for the pathogenesis. In psoriasis this equilibrium is skewed towards Th1, whereas a Th2 phenotype is predominant in atopic dermatitis. Th17 cells are more abundant in both disorders (Di Cesare et al., 2008).

Reliable animal models for inflammatory cutaneous pathologies will contribute to the comprehensive knowledge of the basic mechanisms underlying the epidermal-immune cell interactions and the development of new therapeutic strategies. The adequacy of the animal model and its robustness to predict outcome will condition clinical success.
Fig. 10. *In vivo* assessment of Netherton syndrome (NS) keratinocyte correction in a humanized mouse model by gene therapy. Schematic diagram of the experimental design for the generation of skin-humanized mouse model: Histological appearance of a gene corrected (A) and non-corrected (B) NS engrafted mice.

Several transgenic and knockout animal models gave rise to psoriasis- or atopic dermatitis-like phenotypes (Danilenko, 2008; Nestle & Nickoloff, 2005; Schon, 1999; Shiohara et al., 2004; Zheng & Zhu, 2005). Although the differences in both architecture and function between mouse and human skin impose constraints on these models, nonetheless they contribute to elucidate the role of certain molecules in the underlying pathological processes. Xenotransplantation models of psoriasis closely mimic human disorders and have been used extensively (Gilhar et al., 1997; Nickoloff et al., 1995; Wrone-Smith & Nickoloff, 1996). However, the number of grafted mice that can be obtained from a single patient has ethical and practical limitations. Within this context, the bioengineered-skin humanized mouse model emerges as a powerful tool. One of the several potential advantages over other genetically modified or xenotrasplantation animal...
models is the feasibility of performing studies in a human context on homogeneous and large samples. This approach was recently used to generate a *bona fide* skin-humanized mouse model for psoriasis (Figure 11) (Guerrero-Aspizua et al., 2010). Activated specific lymphocyte subpopulations from the same patients (autologous approach) re-introduced by subcutaneous injection, coupled to tape stripping and the ensuing mild alteration of the epidermal barrier, triggered the psoriatic response. We demonstrated that a healthy normal human skin regenerated in immunodeficient mice using bioengineering technology, might give rise to a psoriasiform phenotype if the appropriate signals are present, i.e. a wounding stimulus and the appropriate cytokines produced by specific lymphocyte subpopulations (Th1/Th17) obtained from unrelated healthy donors (allogeneic approach). These signals play a pivotal role in the formation of the psoriatic plaque. This approach has contributed to elucidate the immunopathogenesis of psoriasis. Several genetic association studies revealed that a large range of susceptibility factors are paramount in the acquisition and/or severity of the disease (Roberson & Bowcock, 2010). However, a specific spatiotemporal combination of cytokines/factors can act directly on the normal lymphocyte-keratinocyte interacting pathways and produce the disease. Furthermore, accessible genetic manipulation of the individual cellular components of the bioengineered humanized skin will make it possible to assess the contribution of potential susceptibility factors to the pathogenesis of psoriasis using this model. Finally, the combined use of these technologies will allow for evaluation of the potential therapeutic effectiveness of novel compounds.

![Fig. 11. The skin humanized model develops a psoriasiform phenotype (A) after cytokine injection and tape stripping (TS). (B) Psoriatic phenotypic hallmarks included elongation (ERR) and fusion of rete ridges (FRR), parakeratosis (PK), and partial loss of the granular layer (LGL).](image)

### 4.3 Modeling a physiological process: Wound healing

Human cutaneous wound healing is a complex process not completely understood (Coulombe, 2003; Martin, 1997; Singer & Clark, 1999). Development of chronic ulcers
Bioengineered Skin

associated to a variety of diseases with a prevalence of 1% in the population are major problems of the health care system and carry a high social cost (Ramsey et al., 1999; Stockl et al., 2004). Even more importantly, those clinical situations may not only have severe effects on life quality but also condition the survival of patients, mainly due to the loss of the barrier function of the skin. Despite the absence of effective therapies, palliative treatments are available. Effective treatment of chronic ulcers is one of the greatest medical challenges (Langer & Rogowski, 2009). Currently around 100 clinical trials designed for this purpose are ongoing (Margolis et al., 2004; Senet et al., 2003). Within this context, the search for reliable human wound-healing models that allow us to address both mechanistic and therapeutic matters is warranted. To this end, our team developed an in vivo wound-healing model by creating excision wounds on the skin humanized mouse model that faithfully recapitulates all major features of cutaneous wound healing (Figure 12). A careful characterization of the healing process by monitoring the expression of various epidermal and mesenchymal markers showed that re-epithelialization, dermal matrix remodeling and basal membrane reorganization accurately mimic the process in humans (Escámez et al., 2004). This model also allows for the use of in vitro genetically manipulated human keratinocytes and/or fibroblasts during the amplification procedure, either to overexpress or silence specific genes, generating transgenic or KO humanized-skin respectively.

A central aim of regenerative medicine is to optimize healing and improve cosmetic outcome. In this sense, one of the main challenges is to create smart bioengineered products that can deliver growth factors and/or cytokines in a time-controlled fashion, promoting scar-free regeneration of embryonic or fetal skin. The combination of cell- and genetic-based therapy has made it possible to evaluate the promoting or detrimental wound-healing activities of specific factors using different model systems, such as transgenic mice, KO-mice or xenograft models (Davidson, 2001; Demarchez et al., 1986; Werner & Grose, 2003). As a proof of concept, we selected KGF, a well-characterized factor that is differentially regulated in normal and impaired healing, to compare the efficacy of different transient gene transfer strategies aimed at delivering smart factors to promote cutaneous repair in the wound healing skin-humanized model (Escámez et al., 2008). In the first approach, hKGF was delivered to wounds by intradermal injection of an adenoviral suspension. Although wound acceleration was achieved, the effect of hKGF was unreliable both in terms of the number of successfully targeted animals (versus the total number of treated animals) and re-epithelialization efficiency. In the second approach, KGF-encoding adenoviral vectors were immobilized in a fibrin gel carrier and applied immediately after wounding. In this case, the proportion of successfully targeted animals was higher than that achieved with the adenoviral injection method, and wound closure rose significantly. A third strategy was explored consisting in delivering hKGF protein from ex vivo adenoviral transduced fibroblasts that were, in turn, embedded in a fibrin matrix and used to treat the wound. In contrast to the two previous methods based on direct adenovirus delivery, this cell-mediated system did not depend on in vivo cell transduction. This method depends on the direct transfer of exogenous KGF therapeutic protein from gene targeted fibroblasts that was, in fact, achieved in all treated wounds, leading to a significant improvement in wound closure. Although all delivery systems achieved KGF protein overproduction at the wound site, with a concomitant re-epithelialization enhancement, only the use of genetically modified fibroblast-containing matrix as an in situ protein bioreactor was highly reproducible. This method appears the most reliable means to deliver growth factors to
wounds avoiding the potential danger of scoring cases of faulty administration as therapeutic failures and direct exposure to viral vectors. The bioengineered skin humanized mouse model of wound healing emerges as a unique platform for evaluating pharmacological, cell and gene therapy strategies for wound healing (Davidson, 2008).

Fig. 12. The skin humanized model truly recreates the human wound healing process. (A-C) Clinical and (D-F) histological features of the healing process of a wound until complete closure. (A) Non-wounded regenerated skin showing a multilayered human epithelium easily distinguish from the mouse epithelium. (D) Differences in the collagen deposition are also observed. (B) 1 day-wound plugged with a fibrin-rich clot (FC) (F) gradually populated by endothelial cells and fibroblasts (granulation tissue: GT) that by remodeling generates a mature dermis (MD) as observed on the older areas of a 7 day wound. The epithelial tongue observed in (E) migrates along the time until completely cover the damaged area by a neoeptithelium.

Diabetes is a systemic disorder with a high and continuously increasing incidence, affecting approximately 4% of the world population in developed countries. This index increases in relation to current lifestyle (Ramsey et al., 1999; Wild et al., 2004). Despite recent advances in the diagnosis and treatment of diabetes, its complications still represent a challenge for public health, since approximately 15% of diabetic patients develop a lower extremity ulcer.
during the course of their illness. In fact, over 25% of hospital admissions of diabetic patients are connected to problems with ulcers, particularly on the feet, which if not properly treated will result in the amputation of the affected limb (Moulik et al., 2003; Widatalla et al., 2009; Wu & Armstrong, 2005). Employing the widely used model of diabetes impaired-wound healing, i.e. leptin-deficient ob/ob mice, we examined the repair-promoting activities of the pleiotropic factor LL-37 antimicrobial peptide. This peptide has been shown to play a role in defense and favour repair as previously mentioned. We used adenoviral-mediated gene transfer to overexpress this factor around wound margins of full-thickness wounds generated in this animal model. We showed that LL-37 enhanced the re-epithelialization rate and granulation tissue formation in a healing-impaired context (Carretero et al., 2008). Although this and other animal models have been extensively useful in diabetes research (Frank et al., 2000; Michaels et al., 2007), the need to design appropriate models in a humanized context has become mandatory as previously discussed. To this end, our studies are mainly devoted to develop a humanized animal model of impaired wound healing (Martínez-Santamaría et al., 2009 and unpublished results).

5. Conclusion

Skin bioengineering has become a bright star in the field of regenerative medicine. The original feeble sheet of keratinocytes developed by Green and coworkers (Gallico et al., 1984) driven by the urgent need to cover a severe burn patient in the mid eighties has evolved into complex tridimensional products combining epithelial and mesenchymal cells together with a variety of matrices and scaffold materials. The spectrum of applications has also grown remarkably counting not only big skin losses (e.g. severe burns) but also various forms of chronic wounds including those of genetic origin. Recently, skin bioengineering has met gene therapy (Mavilio et al., 2006) and that couple is here to stay as new combined therapy protocols are foreseen not only with genetically manipulated keratinocytes but also with revertant, spontaneously corrected cells.

Major challenges remain. At the experimental level, human skin bioengineering has allowed the development of faithful skin disease models amenable to the screening of therapeutic approaches and mechanistic studies. Some but not all of the skin functions can be restored with existing autologous skin substitutes. In fact, bioengineered constructs offering the complete regeneration of functional skin, including all the skin appendages (hair follicles, sweat glands and sensory organs) are still awaiting development. Establishment of a functional vascular and nerve network and scar-free integration of current bioengineered products with the surrounding host tissue has neither fully achieved. New basic knowledge about epidermal stem cells and their interaction with neighboring mesenchyme will be a key to developing new enhanced tissue-engineered skin substitutes. We certainly cannot leave iPS cells out of any regenerative medicine equation. Attainable and safe production of genetically stable, truly iPS cells together with reliable procedures to obtain specific differentiated cell lineages, including epidermis, is conceivable and a door to an unlimited source of autologous cells. A smart combination of all these new advances to come hold the promise to fulfill the dream of perfect skin regeneration through off-the-shelf, next generation skin bioengineering.

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7. References


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Skin biopsy - perspectives is a comprehensive compilation of articles that relate to the technique and applications of skin biopsy in diagnosing skin diseases. While there have been numerous treatises to date on the interpretation or description of skin biopsy findings in various skin diseases, books dedicated entirely to perfecting the technique of skin biopsy have been few and far between. This book is an attempt to bridge this gap. Though the emphasis of this book is on use of this technique in skin diseases in humans, a few articles on skin biopsy in animals have been included to acquaint the reader to the interrelationship of various scientific disciplines. All aspects of the procedure of skin biopsy have been adequately dealt with so as to improve biopsy outcomes for patients, which is the ultimate goal of this work.

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