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BIOTECHNOLOGICAL MANAGEMENT OF SKIN BURN INJURIES: CHALLENGES AND PERSPECTIVES IN WOUND HEALING AND SENSORY RECOVERY

GIRARD Dorothée*, PhD[1], LAVERDET Betty*, PharmD[1], BUHÉ Virginie*, PhD[2], TROUILLAS Marina, PhD[3], GHAZI Kamélia, PhD[4], ALEXALINE Maia M., PhD[3], EGLES Christophe, PhD[4], MISERY Laurent, MD, PhD[2], COULOMB Bernard, PhD[3], LATAILLADE Jean-Jacques, MD, PhD[3], BERTHOD François, PhD[5], DESMOULIÈRE Alexis, PharmD, PhD[1]

* Contributed equally to this work.

[1]University of Limoges, Myelin maintenance and peripheral neuropathies (EA 6309), Faculties of Medicine and Pharmacy, Limoges, France;

[2]University of Western Brittany, Laboratory of Neurosciences of Brest (EA 4685), Brest, France;

[3]Paris Sud University, INSERM U 1197 and Hôpital Percy, Clamart, France;

[4]Université de Technologie de Compiègne, CNRS UMR 7338 "BioMécanique et BioIngénierie", Compiègne, France;

[5]Centre LOEX de l'Université Laval, Centre de recherche du CHU de Québec and Département de Chirurgie, Faculté de Médecine, Université Laval, Québec, Canada.

Corresponding author:

Alexis Desmoulière

Department of Physiology, Faculty of Pharmacy, University of Limoges

2 rue du Dr. Marcland, 87025 Limoges cedex, France

Phone: +33 (0)5 55 43 58 73

E-mail address: alexis.desmouliere@unilim.fr

GIRARD Dorothée

University of Limoges, Myelin maintenance and peripheral neuropathies (EA 6309), Faculties of Medicine and Pharmacy, 2 rue du Dr. Marcland, 87025 Limoges cedex, France

Tel: +33 (0)5 55 43 58 25, fax: +33 (0)5 55 43 59 50, e-mail: dorothee.girard@unilim.fr

LAVERDET Betty

University of Limoges, Myelin maintenance and peripheral neuropathies (EA 6309), Faculties of Medicine and Pharmacy, 2 rue du Dr. Marcland, 87025 Limoges cedex, France

Tel: +33 (0)5 55 43 59 98, fax: +33 (0)5 55 43 59 50, e-mail: betty.laverdet@unilim.fr

BUHÉ Virginie

University of Western Brittany, Laboratory of Neurosciences of Brest (EA 4685), avenue Camille Desmoulins, 29238 Brest Cedex, France

Tel: +33 (0)2 98 01 81 21, fax: +33 (0)2 98 01 81 23, e-mail: virginie.buhe@univ-brest.fr

TROUILLAS Marina

Inserm U1197 - IRBA/CTSA, 1 rue du lieutenant Raoul Batany, 92141 Clamart, France

Tel: +33 (0)1 41 46 72 09, fax: +33 (0)1 41 46 72 81, e-mail: trouillasmarina@gmail.com

GHAZI Kamélia

Université de Technologie de Compiègne, CNRS UMR 7338 "BioMécanique et BioIngénierie", rue Roger Couttolenc, 60203 Compiègne, France

Tel: +33 (0)3 44 23 42 53, fax: +33 (0)3 44 23 79 42, e-mail: k.ameliaghazi@hotmail.fr

ALEXALINE Maia M.

Inserm U1197 - IRBA/CTSA, 1 rue du lieutenant Raoul Batany, 92141 Clamart, France

Tel: +33 (0)1 41 46 72 09, fax: +33 (0)1 41 46 72 81, e-mail: maia.alexaline@gmail.com

EGLES Christophe

Université de Technologie de Compiègne, CNRS UMR 7338 "BioMécanique et BioIngénierie", rue Roger Couttolenc, 60203 Compiègne, France

Tel: +33 (0)3 44 23 44 22, fax: +33 (0)3 44 23 79 42, e-mail: christophe.egles@utc.fr

MISERY Laurent,

University of Western Brittany, Laboratory of Neurosciences of Brest (EA 4685), avenue Camille Desmoulins, 29238 Brest Cedex, France

Tel: +33 (0)2 98 22 33 15, fax: +33 (0)2 98 22 33 82, e-mail: laurent.misery@chu-brest.fr

COULOMB Bernard,

Inserm U1197 - IRBA/CTSA, 1 rue du Lieutenant Raoul Batany, 92141 Clamart, France

Tel: + 33 (0)1 41 46 72 72, fax: + 33(0)1 41 46 72 81, e-mail: bernard.coulomb@inserm.fr

LATAILLADE Jean-Jacques

Inserm U1197 - IRBA/CTSA, 1 rue du lieutenant Raoul Batany, 92141 Clamart, France

Tel: + 33 (0)1 41 46 72 62, fax: + 33(0)1 41 46 72 81, e-mail: jjlataillade@gmail.com

BERTHOD François

Centre LOEX de l'Université Laval, Centre de recherche du CHU de Québec, 1401, 18e Rue, Québec, QC G1J 1Z4, Canada

Tel: +1 418 990 8255, fax: +1 418 990 8248, e-mail: Francois.Berthod@fmed.ulaval.ca

DESMOULIÈRE Alexis

University of Limoges, Myelin maintenance and peripheral neuropathies (EA 6309), Faculties of Medicine and Pharmacy, 2 rue du Dr. Marcland, 87025 Limoges cedex, France

Tel: +33 (0)5 55 43 58 73, fax: +33 (0)5 55 43 59 50, e-mail: alexis.desmouliere@unilim.fr

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Abstract

Many wound management protocols have been developed to improve wound healing after burn with the primordial aim to restore the barrier function of the skin and also provide a better aesthetic outcome. Autologous skin grafts remain the gold standard in the treatment of skin burn but this treatment has its limitation especially for patients presenting limited donor sites due to extensive burn areas. Deep burn injuries also alter the integrity of skin sensitive innervation and have an impact on patient's quality of life by compromising perceptions of touch, temperature and pain. Thus, patients can suffer from long-term disabilities ranging from cutaneous sensibility loss to chronic pain. The cellular mechanisms involved in skin reinnervation following injury are not elucidated yet. Depending on the depth of the burn, nerve sprouting can occur from the wound bed or the surrounding healthy tissue but somehow this process fails to provide correct reinnervation of the wound during scarring. In addition, several clinical observations indicate that damage to the peripheral nervous system influences wound healing, resulting in delayed wound healing or chronic wounds, underlining the role of innervation and neuromediators for normal cutaneous tissue repair development. Promising tissue engineering strategies including the use of biomaterials, skin substitutes and stem cells could provide novel alternative treatments in wound healing and help improving patient's sensory recovery.

Keywords: burn injury, cutaneous wound healing, skin engineering, nerve fiber regrowth

Running title: Burn wound healing and sensory recovery

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1. Introduction

A burn injury may be induced by thermal agents but also by radiations, radioactivity, chemicals or friction. The most common causes of burns are fire for adults and scald for children (1,2). In France, burns requiring medical attention affect approximately 500,000 people per year. Ten thousands need hospitalization and among those patients, 10% die (3). In Europe, the rate of death in hospitalized patients ranges from 1.4 to 18% across countries (4). In 2004, the World Health Organization revealed that fire burn affects 11 million people and account for more than 300,000 deaths per year (5). However, it unequally affects populations, since low- and middle-income countries have the highest mortality rates.

Whatever the cause of the burn, the severity of an injury mainly depends on its depth and extent. Besides, the assessment of these two features is crucial to providing a proper treatment without delay, especially for extensive burns (6). Then, when an injury extended over more than 10-15% of the total body surface area (TBSA), admission to critical care units is required. In that case, the first hours following the injury are dedicated to the prompt fluid resuscitation to prevent the hypovolemic shock which occurs secondary to a persistent œdema and outflow of osmotically active molecules such as proteins (7). After these potential vital treatments, burn wound management is performed using occlusive dressing or, if necessary, wound excision and skin grafting.

The post-burn mortality rate which is highly correlated to the age, the TBSA and the inhalation injury, has been decreasing over the past decades (4,8,9). This is likely due to the improvement of resuscitation procedures, treatment of infection and the wound healing management. Currently, the challenge is to improve the patients' rehabilitation and, hence, their quality of life. Indeed, in addition to any post-traumatic stress disorder, burned patients may suffer from their scars. At best, they are unaesthetic because of depigmentation,

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hyperpigmentation or skin thickening (hypertrophic scar). For some, scars become very disabling when scar contractures occur (10). Overall, itching and pain are frequent *sequelae* that may disturb daily life (11,12). The occurrence of these symptoms suggests that injured sensory nerve fibers regenerate improperly or insufficiently. This demonstrates that efforts in wound healing management should still be made to improve nerve fiber regeneration. In addition to the improvement of sensory perceptions, it would allow a significant progress in the wound healing process, since nerve fibers are known to be involved in skin repair and cutaneous homeostasis (13,14).

In this review, we will first overview the skin repair process and the regeneration of nerve fibers. After describing the current deep wound management and the possible post-burn *sequelae*, we will address the question of innovative strategies to improve wound healing and nerve fiber regeneration.

2. Skin repair process and peripheral innervation

2.1. Overview of wound healing

Wound healing consists in the restauration of the integrity of the damaged tissue. In a similar manner, the cutaneous healing process relies on complex cellular dialogues and can be divided into three sequential and intercorrelated phases. The inflammatory and vascular phase starts as soon as the damage occurs. The skin is richly vascularized and the disruption of blood vessels in the dermis, and in the hypodermis if the injury is more severe, leads to the formation of a blot clot and of a provisional matrix mainly composed of fibrin and fibronectin. Platelets involved in the blood clot have also a major role in the recruitment of inflammatory cells such as neutrophils, macrophages and mast cells to the wound due the

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local release of cytokines and chemokines. Fibroblasts and endothelial cells are also drawn to the wound by chemotaxis and will be major actors in the second stage of wound healing, the proliferation phase. The hallmark of the proliferation phase is the formation of the granulation tissue in which fibroblasts are stimulated to proliferate and undergo major cellular changes characterized by the expression of α-smooth muscle actin. They are consequently called myofibroblasts and display contractile properties that are essential in the maturation of the granulation tissue overtime (15). They also secrete and deposit extracellular matrix, mainly collagen type III that will progressively replace the provisional matrix. Most myofibroblasts derive from resident fibroblasts but it is important to note that different subpopulations of fibroblasts presenting their own proper capacities of differentiation are present in the dermis (16). Other sources of myofibroblasts have been highlighted such as local stromal stem cells, blood circulating progenitors and bone marrow-derived stem cells (17). To support the strong cellular activities occurring during the proliferation phase, endothelial cells recruited to the wounded area also proliferate and contribute to the angiogenesis process. A dense network of capillaries can then deliver all the necessary nutrients to the healing area (15). The third and last phase of skin wound healing, the remodeling phase, leads to the progressive formation of the scar. The scarring process involves two major phenomena: re-epithelialization and final maturation of the granulation tissue. At the edges of the wound, keratinocytes display a migratory phenotype. They express specific integrins allowing re-epithelialization and wound closure (18). Upon wound closure, the maturation of the granulation tissue is marked by the synthesis of collagen type I and the disappearance of the myofibroblast population by apoptosis (19). The persistence of myofibroblasts in the granulation tissue is a major cause of well documented pathological conditions involving hypertrophic scarring and tissue deformation (20). Both myofibroblast differentiation and apoptosis are driven by specific signals such as the release of the cytokine transforming growth factor (TGF)-β1 which is the

major inductor of the myofibroblast differentiation, intercellular and/or matrix interactions and finally mechanical stress (21,22). It is known that a stiffer environment leading to a lower rate of myofibrobalst apoptosis is a cause of hypertrophic scar (23). In addition, keratinocytes and the epithelium certainly play a role in the normal evolution of the granulation tissue and in myofibroblast apoptosis. Indeed, it has been shown that perturbation of dermal-epidermal interactions can lead to excessive scarring. Interestingly, in such pathological situations, a neurogenic inflammation seems to be involved (24).

2.2. Cutaneous innervation and role of sensory receptors in skin perceptions

In the skin, different nerve endings are implicated in the detection and transmission of sensitive information to the central nervous system.

Nerve fibers express neuromediators. Without stimulation, there is a basal expression of these neuromediators whereas after chemical injury, physical damage, or inflammation, the quantity of neuromediators dramatically increases. These mediators have been described to be involved in different physiological and pathological situations, including wound healing (for review, see (25)). Autonomic nerve fibers present in the skin play a major role in body thermoregulation by acting on smooth muscles in arterioles, on erector pili muscles and on sweat glands (Figure 1). Sensory information are detected by specific receptors present on sensory nerve fibers (Figure 1). These information are transmitted to the cell body located in dorsal root ganglia (close to the spinal cord) and finally to the central nervous system for integration.

Cutaneous nerve fibers can detect stimuli such as thermal and tactile sensation or pain (26,27). After skin lesion, these nerve fibers and their receptor are damaged or sometimes destroyed but neuronal cell bodies are still present in the dorsal root ganglia (Figure 2).

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Mechanoreceptor present on Aβ and Aδ fibers can detect mechanical stimuli while temperature and pain are detected respectively by thermoreceptors and nociceptors present on Aδ and C free nerve endings also called small fibers (for review, see (25)).

2.3. Role of innervation in skin healing and therapeutic options

It has been recently shown that cutaneous innervation play important roles in normal and pathological repair processes (14,28). However, the precise roles of sensory and autonomic innervation during wound healing remain to be clearly established. Not only keratinocytes and melanocytes but also fibroblasts and myofibroblasts express different neurotrophins such as nerve growth factor (NGF), neurotophin-3 (NT-3), brain-derived neurotrophic factor (BDNF) and their receptors which promote their proliferation and differentiation (29,30). Neuropeptides such as calcitonin gene related peptide (CGRP), substance P, and vasoactive intestinal peptide can modulate the activity of matrix metalloproteinase (MMP)-2 and MMP-9 which are major actors involved in granulation tissue remodeling and scar formation. In addition, these neuropeptides also act on collagen type I and type III production during skin wound healing and promote the adhesion of dermal fibroblasts and their differentiation into myofibroblasts (31). The effects of these neuropeptides on the extracellular matrix composition and arrangement are certainly essential as it is well established that the mechanical microenvironment organized by the extracellular matrix could interfere with fibroblast to myofibroblast differentiation (14). In addition, the modulation of MMPs acts on the subsequent MMP activation of latent TGF-β1 (32).

Skin damages induce the release by the immune cells and the sensory nerve endings of inflammatory mediators including interleukin (IL)-1β, tumor necrosis factor-α (TNF-α), bradykinin, substance P, CGRP, NGF, and prostaglandins, contributing to the "inflammatory soup" (33). It has been shown that altered substance P levels could be involved in impaired

cutaneous healing responses observed in diabetes mellitus (34) or during hypertrophic scar formation (35). It has also been shown *in vitro* that direct contact of fibroblasts with neurites is able to induce myofibroblastic differentiation increasing then collagen gel retraction which is an important process during wound healing (36).

In keloid, the density of nerve fibers is significantly higher than in the normal skin samples (37) and symptoms such as itch and pain, abnormal thermosensory thresholds to warmth as well as cold and heat pain are present suggesting that small nerve fibers are involved in the pathogenesis of this disease (38). In hypertrophic scar, data in the literature are not coherent with either a decrease (39) or an increase (40) of the number of observed nerve fibers. Nevertheless, in burn patients with chronic pain, abnormal cutaneous innervation is reported (41). Recently, in a mouse model of hypertrophic scarring induced by mechanical loading, Li et al. suggest that both inflammation and the cutaneous nervous system contribute to hypertrophic scar formation (42).

Animal models of skin denervation have helped investigating a possible role of sensory innervation in skin wound healing. Skin denervation models have been designed using surgery, chemicals or genetically engineered murine strains. Thus, studies have shown that surgical denervation induces delayed wound healing with reduced inflammatory cell infiltration, altered wound contraction and delayed re-epithelialisation (43,44). Another skin denervation model using chemical sympathectomy induced by intraperitoneal administration of 6-hydroxydopamine (6-OHDA) also interferes with wound healing. (6-OHDA)-induced sympathectomy modifies wound healing with an increase in wound contraction, a reduction of mast cell migration and a delayed re-epithelialization. These modifications are associated with a decrease in neurogenic inflammation (45,46). Capsaicin, a potent agonist of TRPV1, has also been used in order to induce the depletion of neuropeptides (substance P and CGRP) from Aδ and C-fibers. When administered to neonatal rats, capsaicin can provoke total

sensory denervation while in adults it can be used to promote transient sensory neuropathy. Studies have shown that capsaicin induces delayed wound healing and further highlight that neuropeptides released by sensory fibers play a major role in this process (47–49). Moreover, Toda et al. have also shown that angiogenesis and wound closure were significantly suppressed in a CGRP knockout mouse model (48).

Topical application of sensory neuropeptides following cutaneous wounding has also been investigated. Several studies have highlighted a beneficial therapeutic effect of substance P on wound closure and angiogenesis (34,50,51). The intraperitoneal or intradermal injection of CGRP has also been investigated with positive outcomes on wound contraction (52). Altogether these studies indicate that sensory innervation and neuropeptides such as substance P and CGRP can modulate the overall cutaneous wound healing process (for review see (28)) and could offer promising therapeutic options.

2.4. Mechanisms of nerve regeneration during cutaneous healing

Following skin damage, the mechanisms involved in nerve regeneration are not fully elucidated. Nevertheless, during wound healing, the remodeling of regenerating nerve fibers is observed and nerve fiber density is modified. During healing of a burn injury in guinea pigs, it has been shown that the number of substance P-containing nerve fibers acutely decreases after the burn and then gradually increases with a maximum on day 14 post burn. Following that peak, the fiber density gradually decreases to end up lower than controls (53).

Interactions during wound healing between myofibroblastic differentiation necessary for granulation tissue formation and innervation certainly play a major role. Indeed, myofibroblasts possess neurotrophic properties and are able to regulate innervation during healing. They synthesize and secrete all neurotrophins and express neurotrophin receptors (30) being certainly involved in the high levels of neurotrophins such as NGF observed in the

wound site (54). Myofibroblasts also produce extracellular matrix components (55) such as laminin, which are known to promote neurite outgrowth (56).

Relationships between nerves and myofibroblasts during cutaneous wound healing in the developing rat have been studied by Liu et al (57). Indeed, it is well known that changes in wound healing capacities occur with age with a delay in wound healing observed in elderly. Liu et al (57) show that, in neonatal animals, rapid wound closure is associated with an important myofibroblast proliferation and a marked increase in innervation density; in contrast, in adult rats where a delayed wound closure occurs compared with neonatal animals, the appearance of both myofibroblasts and nerves are reduced compared with younger rats. The early regeneration of nerves associated with the proliferation of myofibroblasts could at least in part be responsible for the rapid and efficient healing process observed in neonate animals. In mature rats, altered nerve-myofibroblast relationships may contribute to reduce healing.

In the skin, it seems clear that nerve fibers are located close to the vascular tree and relationships could exist between these structures (58). Interesting studies have been performed using MRL/MpJ mice, which present an accelerated ability to heal ear punch wounds without scar formation whereas wounds on the dorsal surface of the trunk heal with scar formation. Indeed, during dorsal skin healing (leading to scar formation), the wounded area becomes rapidly hyper-vascularized by as early as day 7 post-wounding while at that time, peripheral nerve regeneration is only found in the outer regions of the wound where nerve fibers have begun to sprout into the wound area from surrounding healthy tissue. In contrast, in the ear wound (which heals without scar formation), nerve regeneration precedes vascularization, recapitulating early mammalian development (59). In addition, denervation of the ear obliterates the regenerative capacity of the MRL/MpJ mice, and also has a severe negative effect on the ear wound repair mechanisms of the C57BL/6 strain (a control strain

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known to have a poorer regenerative capacity) (44). It suggests that innervation may be important not only for regeneration but also for normal wound repair processes.

Interestingly, it has been shown that the human intervertebral disc aggrecan inhibits both endothelial cell adhesion and neurite extension, repelling sensory neurite growth (60,61). These studies underline once more the role of extracellular matrix components in angiogenesis and nerve fiber regeneration.

In utero, fetal wounds heal in a regenerative manner without scar (62). Antony et al. suggest that during development, neurotrophins regulate peripheral innervation formation and that, after injury, these factors promote the survival and the regeneration of peripheral neurons (63). Identification of this pattern of neurotrophin and neurotrophin receptor expression in fetal skin which could be different in adult skin could provide new insights into understanding the fetal scarless repair mechanisms in response to injury.

In damaged skin, at the level of the nerve fiber, the classical Wallerian degeneration process cannot be involved as far as the distal part of the nerve ending is destroyed. However, we can imagine that at the edges of the lesion, similar process can develop. It is well admitted that macrophages and Schwann cells are actors in the clearance of debris. Surprisingly, it has been shown in Zebrafish skin that epidermal cells also phagocytose debris generated after injury to peripheral axons (64). Schwann cells that surround the axon of the fiber ending certainly play a major role to promote and to guide axon sprout. The growth of these sprouts is supported by growth factors produced by Schwann cells, particularly neurotrophic factors including neurotrophins (65). In addition, mesenchymal stem/stromal cells (MSCs) such as skin derived precursors (SKPs) present in the dermis (*e.g.* SKPs located within the dermal papillae at the base of the hair follicle) (see below) can certainly release factors able to act on nerve regeneration (66).

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3. Deep burn wound management

Skin damages may have multiple causes including genetic disorders, acute trauma, chronic wounds or surgical interventions. Among them, burn trauma represents a type of injury that can be caused by heat, freezing, electricity, chemicals, radiation or friction. In 2004, fire burn injuries affected 11 million people around the world, including superficial and severe cases (67). Despite significant improvements in terms of mortality, severe burns cause considerable functional, cosmetic and psychological *sequelae* and represent a major public health concern (4).

3.1. Classification of burn depths and gravity

Severity of burn wound and prognosis depend on injury depth and extent of the affected surface area (68,69). The depth of burn wound varies over time and patient needs to be evaluated for depth of the wound regularly (Table 1). A first degree burn involves only the superficial layer of the epidermis without affecting the basal layer. A second degree burn affects all the epidermis and part of the dermis from a superficial to a deep degree. A third degree burn or full thickness-burn involves the destruction of all the epidermis and dermis and may extend to deeper tissues (forth degree burn affects fat layer, muscle or bones). The severity of the damage is also evaluated by the extent of the surface affected and is expressed as a percentage of the whole body. For a rapid estimation of the extent of burn wounds, the "rule of nine" is used (70). However, the Lund-Browder chart provides accurate estimation of the extent of burn wounds in pediatric patients (68). A calculation program can also be used for a better estimation (71). Other criteria are also important during the assessment of the burn

severity, including patient's age, smoke inhalation, location of burns and medical state of the patient.

3.2. Burn pathophysiology

Skin burns produce a significant imbalance in tissue homeostasis, and result in both local and systemic responses. The local tissue damage may be divided into three zones (72). At the center of the injury, proteins coagulation results in irreversible tissue loss called "coagulation zone". This area of necrosis can extend to the adjacent zone of stasis characterized by decreased tissue perfusion. Indeed, the central zone may damage the adjacent tissue by the release of inflammatory factors and reactive oxygen or nitrogen species (73). The external zone of burn wound is called "zone of hyperaemia", which is characterized by vasodilation and inflammatory changes without structural damage. If the tissue in the "zone of hyperaemia" could almost always recover, the evolution of the zone of stasis depends on the resuscitation technique necessary to rapidly revascularize the tissue (74).

Beyond 10% of burned area, the local damage may become systemic and induce hypovolemia due to the destruction of the skin barrier function, increased vaso-permeability and plasma exudation (75). The burned tissue is highly toxic. Indeed, between 100 °C and 500 °C, melting lipids and membrane proteins create toxic lipid-protein complexes responsible for serious systemic problems (73,76). These lipid-protein complexes may in part be responsible in low survival rate of severely burned patients given that administration of anti-lipid-protein complex serum in burned mice greatly increase their survival (76).

Necrosis also triggers the release of inflammatory mediators that generate local inflammation. The inflammatory response accompanied by eventual infections may contribute to systemic effects inducing a systemic inflammatory response syndrome (SIRS) and organ dysfunction, with a threshold around 20–30% of TBSA burned (77). Systemic disease may cause

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pulmonary edema, severe organ failure, requiring specific care in burn treatment centers. The inflammatory response is complex and characterized by an early secretion of pro-inflammatory factors such as TNF-α and IL-6 followed by prolonged anti-inflammatory response linked to IL-4, IL-10 and TGF-β production (78), leading to a temporary immune-suppression. Therefore, patients become more susceptible to pathogenic microorganism contaminations (76).

3.3. Current management of burn injuries

Management of deep burn injuries depends on both depth and surface area of burn wounds (Table 1). In the particular case of burns, re-epithelialization of injuries of first or superficial second degree remains possible by the migration of keratinocytes from the edges of the wound, from hair follicles and sweat glands followed by their proliferation, stratification, and re-differentiation to form an intact epithelium (79). Antimicrobial creams and occlusive dressings are applied on the wound to avoid infection, to limit wound progression and to improve epithelialization progression (80).

In contrast, in more severe skin burns such as deep partial thickness or full-thickness burn, epithelial regenerative elements residing in the basal layer of the epidermis and in the dermis (*i.e.* epidermal appendages such as hair follicles) are fully destroyed. In these cases, only a re-epithelialization from the edges of the wound is possible (81). Full-thickness wounds larger than 1 cm diameter need special treatment to prevent delayed re-epithelialization and extensive scar formation that reduces mobility and induces cosmetic deformities (79). To date, standard medical treatment for severe skin burns consists in rapid eschar excision and split-thickness skin autograft taken from healthy skin of the same patient. The grafts are usually taken several times from the same area, once the donor site has had sufficient time to

regenerate (79,82). Skin grafts are meshed to stretch the graft and so that they can cover a larger area.

Besides being slow to heal and painful, skin autograft is very difficult to perform in patients with burns affecting over the 50-60% of the TBSA because of the poor availability of healthy tissue. Different techniques are currently available over the different burn treatment units around the world. The main objective is to reconstitute permanently the dermis and epidermis in the injured area. The first, and faster, alternative is the grafting of allogeneic skin, coming from cadaveric skin that can be obtained from skin banks. However, allografts cannot cover the patient wounds permanently because the epidermis is rapidly rejected even if burned patients are immune-suppressed (83). Sandwich techniques can be applied for more permanent covering alternatives where widely meshed split-thickness skin autograft are covered with narrowly meshed allografts (84) or where widely expanded postage stamp autografts regularly distributed over the wound bed (Meek technique) (85) are combined with an overlay glycerol preserved allograft (modified Meek technique) (86).

In 1975, Rheinwald and Green described for the first time the culture of epidermal sheets (Cultured Epidermal Autograft, CEA) produced with human autologous keratinocytes derived from a small sample of uninjured skin (87). Several burn treatment units used the technique of Cuono which consists on the early debridement of all burned tissue in the wound and the coverage of it with meshed expanded cryopreserved allografts coming from cadaveric skin. Later, allografts are abraded to remove mechanically allogeneic epidermis and CEA are applied directly to the allogeneic dermal bed, taking benefit of a pre-revascularized matrix (88). Since then, several combined procedures have been developed to overcome the lack of donor sites. Among these methods, the combination between the Meek technique and sprayed autologous cultured keratinocytes (89) has given interesting outcomes.

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Other alternative methods such as the "combined technique" can also be used (90). The first steps of Cuono technique can be applied until epidermis abrasion. Then, widely meshed autografts are grafted followed by the application of CEA. Keratinocytes from CEA will colonize mesh autograft and play a trophic role for epidermal regeneration. If this technique seems interesting in terms of percentage of engraftment, it needs enough available healthy skin to collect autografts. That's why the Cuono technology remains widely used despite a varying degree of graft take.

Grafting efficiency of CEA is highly variable and depends mainly of the metabolic status of the patient. However, nowadays, there is no other option to enhance patient survival and to provide enough surface for the epidermal barrier (91,92). However, several drawbacks with the use of CEA have been noticed such as poor dermo-epidermal junction maturation, their high cost, their fragility, the use of animal proteins and/or cells in the culture process, and the variable grafting efficiency (93). Several kind of acellular biomaterial can be used in combination or not with CEA grafting to improve grafting efficiency (Table 2).

For example, to overcome these weaknesses, researchers have cultured CEA on fibrin matrices firstly obtained from purified fibrinogen (127,128) and more recently on fibrin matrices obtained from clotted human plasma (human plasma-based epidermal substitute) (129,130) (Figure 3).

4. Chronic sensory disabilities following deep burn injuries

The local destruction of the cutaneous nerve fiber network during a burn injury leads to an immediate neuropathy, which is obviously more serious in the context of a full-thickness burn (see above). Although nerve fibers may regenerate after a skin grafting and subsequent wound

healing, their density often remains lower than before the injury (39,131–133). The persistence of the neuropathy is shown to be associated with some risk factors. For instance, the electrical cause is the more deleterious (134,135). Especially, a low-voltage electrical burn induces more frequent *sequelae* than a high-voltage injury and correlates with the occurrence of a mononeuropathy (136,137). Other additional factors promote the neuropathy. For example, the prevalence is higher in adults and in people displaying a large TBSA (over 20%), a full-thickness burn or a hypertrophic scar (39,134,138).

As the cutaneous innervation is crucial for wound healing, the persistence of a neuropathy delays it (14). Furthermore, neurological symptoms such as sensibility losses, itch, paresthesia and pain may occur. These complications are common in the first months following the burn injury but often gradually decrease with time. However, depending on the anatomic site of the scar or on the injury severity, they can impact the patient's quality of life and even delay their overall rehabilitation (139–141).

4.1. Sensibility losses

A lot of burned patients complain of a transient or permanent loss of sensibility, which affects their perception of temperature, pressure or touch and is very often associated with painful sensations and paresthesia (142). Hermanson et al. were among the first to assess the sensibility in burned patients using quantitative sensory measurements (143). They demonstrated that in years following burn, the touch threshold is increased at the scar site compared to the uninjured contralateral side skin. Such an abnormality is noticed in spontaneously healed scars and in early and late excised grafted scars. These findings suggest that treatments fail to improve the touch sensibility and that the severity of the burn injury doesn't influence the occurrence of a sensibility loss. A more recent study that enrolled a larger number of patients confirmed that the touch threshold is increased in scars compared to

uninjured skin from healthy volunteers. However, deep burns requiring skin graft displayed significantly higher touch threshold than superficial burns (142). It was the same for the heat pain threshold and the two-point discrimination, which measures the spatial tactile acuity. The assessment of the cold sensibility revealed that this threshold is significantly lower in deep burns than in superficial burns, still confirming that the scar sensibility loss does depend on the severity of the injury. Other studies focusing on grafted patients also reported impaired sensory thresholds in their scars and demonstrated that the sensibility loss was correlated with the amount of the neuronal structures within the burned area (132,133,144). However, a local deficiency of these structures is insufficient to explain a sensibility loss, since a lot of patients also exhibit slightly impaired sensory thresholds at their uninjured contralateral side (142,143,145). These data highlight that a sensibility loss also results from an altered processing of the afferent or the efferent information by the central nervous system.

4.2. Itching and paresthesia

A paresthesia is an abnormal perception that may be a long-term *sequelae* after burn. The severity, the frequency of this symptom and the impact on the quality of life is assessed using questionnaires. They revealed that more than two thirds of patients suffer from paresthesia, which the most frequent are tingling, stiffness, numbness or pinpricks (146,147). Itching is also a common post-burn paresthesia and affects at least 70% of burned patients at 1 or 2 years post-burn, and still around 40% in the following decade (12,148,149). The itching prevalence also depends on the injured anatomic sites. Contrary to the face and the neck, legs are typically affected, especially in the first months (150,151). Furthermore, itching is generally more intense during the first 3 months post-burn and its severity significantly decreases between the 3rd and 12th month post-burn, a timeframe consistent with the improvement of the scar quality (148,151,152). Sometimes, however, it delays the healing

because of frequent scratching and alters the quality of life (153). Moreover, numerous risk factors promote the itching persistence and severity. Willebrand et al. demonstrated that it is positively associated with the total burned skin area while Kuipers et al. rather showed that it is stronger as the number of itchy body surface areas is high (151,154). This apparent discrepancy between the two studies likely stems from the fact that the post-burn time was around few years in the first one and only around few months in the second one. Furthermore, participants were slightly older and had average TBSA and percentage of full-thickness burn twice higher in the Willebrand's study. Overall, itching appears to be related to the severity of the burn injury since other data highlighted its positive association to the time required for wound healing and to the number of surgical interventions (150,155). Moreover, grafted burn scars are itchier than non-grafted scars, especially in the first months (151). This findings support previous outcomes showing more substance P-immunoreactive fibers in grafted skin in the first years post-burn, although the number of the total nerve fibers was decreased (132). Interestingly, substance P was shown to trigger a release of histamine, promoting itching (156,157). This corroborates outcomes showing that this neuropeptide is especially elevated in case of hypertrophic scars, a complication tightly correlated with thermal injury and highly associated with itching (158–160). All of these data support many neurophysiological studies demonstrating that itching results from neuropathic mechanisms (161). Finally, the post burn itching mechanism is close to that observed in numerous peripheral neuropathic diseases (162).

4.3. Pain

Post-burn pain characteristics differ depending on the stage after the injury. Post-burn pain is first acute pain but becomes chronic pain in the rehabilitation phase. Three main subtypes of acute pain are distinguished (163). The procedural pain occurs during treatments, whereas the

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background pain continuously affects patients even immobile. Afterwards, with the decrease in analgesic medications and the increasing ability to move, patients may complain of upsurge in pain called “breakthrough pain”. The mechanism of acute pain is directly related to the tissue lesions, which lead to inflammation and damaged nerve structures. Inflammation is mediated by cytokines such as IL-6, which promotes hyperalgesia (164). For their part, injured nerve fibers exacerbate this inflammation by releasing neuropeptides such as substance P and CGRP, well known to mediate the neuropathic pain. The chronic pain arises later during the recovery phase and may persist for a long time. Indeed, it affects at least one third of patients in the first years post-burn (146,147). A survey among 336 burned patients reported that 52% of them complained of pain although their injury happened ten years before (11). For those with ongoing pain, at least half declared that it impeded their daily life and even delayed their rehabilitation. Chronic pain especially affects older patients and those displaying higher grafted burned skin areas (165). In addition, pain is exacerbated by factors such as temperature changes, light touch and also positions, especially when injuries affect extremities (165,166). It is worth noting that psychological aspects should also be addressed. For instance, anxiety and depression are associated with greater pain (167). Conversely, pain raises the level of anxiety and depression (167). This highlights that emotional distress needs to be considered to minimize pain, even if pain often significantly decreases between the 3rd and the 12th month post-burn (152,165). As well as for itching, this positive trend corresponds to the improvement of the scar quality. The understanding of the mechanism of chronic pain mainly focuses on substance P and CGRP. Although pain has been related to the release of these two neuropeptides, chronic neuropathic pain was rather found to be related to the release of CGRP (41,168).

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5. Strategies to improve wound healing and nerve regrowth

5.1. Biomaterials

The quality of wound healing relies also to the capacity to recover the sensitivity of the repaired areas, contributing also to promote tissue repair. In order to support nerve regrowth and therefore improve the recolonization of wounded regions by neuronal extensions, various biomaterials have been studied. These biomaterials can be separated into two families: materials of biological origin and synthetic materials. In both cases, the addition of specific molecules was tested in order to improve the adhesion of the nerve fiber endings onto the material and to enhance their growth.

The biomaterials aimed at the fabrication of a nerve support must have specific properties such as biocompatibility, biodegradability, and mechanical strength. Several bioengineered conduits have already been commercialized for clinical applications in order to replace a sectioned or crushed nerve (169), yet none of these products present a full functional recovery. Moreover, these kind of tridimensional materials do not address directly the problem of skin reinnervation and some modifications in the structure or the shape of the biomaterials are needed to be used to this specific aim.

5.1.a. Material properties

Biomaterials aimed at guiding axonal regrowth need to present various properties. Their biocompatibility is linked to the interactions between the material and its biological environment. Tissue-material interactions should not provoke irritation or create significant inflammatory response.

Moreover, the material needs to be flexible in order to react to the movements of the skin without breaking or creating a rigidity of the wounding (170). Ideally, electrical conductivity

could help nerve regeneration by stimulating axon regrowth and orientation due to the charged membrane surface. To date, most of the materials described in the literature are non-degradable (171). The disadvantages of the non-biodegradable materials or non-fully absorbable materials resides in the risk to provoke a reaction of the immune system which can lead to scarring or prolonged inflammatory responses. Additionally, a second surgical intervention is often required to remove the material.

In the specific case of wound healing, a biomaterial must tolerate modifications in physico-chemistry during the various phases of cell proliferation, re-epithelialization and extracellular matrix reorganization. Mechanical properties (traction force, elongation at rupture, tenacity) of the materials need to be tested together with the other cell types from the area (mainly fibroblasts and keratinocytes) in order to verify the efficacy of the biomaterial and its potential interactions with other cells from the healing area.

5.1.b. Natural materials

Because of their enhanced biocompatibility and specific structural motifs, natural polymers have been commonly used (172). Chitosan, derived from chitin, is an amino polysaccharide significantly studied in the literature. This material is considered non-toxic and biocompatible with many applications in tissue engineering and particularly for wound healing (173). It is used to create matrices presenting adjusted degrees of porosity. In addition, chitosan has been described to interact with laminin, fibronectin, and collagen type IV, molecules from the extracellular matrix able to promote adhesion, migration and differentiation of cells from the nervous system (174). Another promising candidate, collagen, the main structural protein in the body, is often employed as a scaffold supporting cells (175). The use of collagen to make nerve conduits restores partially the nerve functionality (176,177). Nevertheless, mechanical

properties and biodegradation rates of chitosan and collagen are not optimal (173). So, the studies are directed to other natural materials such as hyaluronic acid, keratin or silk fibroin.

The ability of hyaluronic acid to augment keratinocyte proliferation, fibroblast migration, and endothelial cell angiogenic responses in the wound makes it a useful biopolymer for wound healing (178). Hyaluronic acid can limit scar tissue and can facilitate a functional recovery of the neo-formed tissues (179). It is interesting to underline that fetal skin which is rich in hyaluronic acid heals without scar (180). Moreover, this molecule can accelerate nerve regeneration (178,181).

As for hyaluronic acid, mouse fibroblasts proliferate well on keratin covered surfaces, demonstrating the biocompatibility of this molecule (182). Furthermore, tridimensional materials made of a scaffold of keratin have been used for specific bioapplications such as wound dressings or hydrogels or scaffold guiding the growth of neural tissues (183–185). *In vivo* study showed that keratin hydrogel stimulated Schwann cells' migration and dedifferentiation from the proximal nerve ending. Moreover, these materials could block the infiltration of macrophages described during the Wallerian degeneration of the distal nerve part (186).

Silk fibroin, another natural polymer, has been used for various applications such as cosmetics or food additives. In recent literature, silk proteins have also been described as having vast promise in biomedical and engineering fields because of its specific biological properties, such as biocompatibility, biodegradability, and induced limited inflammatory responses *in vivo* (187–190). These promising properties have encouraged development of silk fibroin-based nerve conduits. Indeed, the use of silk fibroin allows high structural integrity and nervous tissue colonization (191) (Figure 4). Moreover, silk has robust mechanical properties, no toxicity towards neurons, and can be biofunctionalized permitting the acquisition of new physico-chemical properties (192–194).

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5.1.c. Synthetic materials

Synthetic materials also can be used in tissue engineering: they are structurally stable for implantation, are biomimetic and able to support repair and regeneration. Moreover, these materials are not toxic for cells of the original tissues or organs.

Poly(ε-caprolactone) (PCL) is a synthetic polymer presenting good mechanical properties while being biocompatible and biodegradable (195). The nanofibrous PCL is a dependable substrate supporting the growth and differentiation of a variety of cell types (196). PCL is also used in the development of tubular nerve guidance systems (197). Poly lactic acid (PLA) is another example. Gautier et al. have demonstrated the qualities of resorption and biocompatibility of this material specifically using Schwann cells and neurons from the spinal cord (198). Despite some concerns about the structural stability of the material, PLA scaffolds loaded with Schwann cells and surgically inserted in transected rat spinal cord allowed the regrowth of neural tissues and their revascularization, proving the high interest of this material (199).

Poly (d, l-lactic-co-glycolic acid) (PLGA), a copolymer from lactic acid and glycolic acid, has also been used as therapy vectors for the release of active molecules or cells. This copolymer typically offers a higher primary stability and is more amenable to macro/micro-structure formation than natural biomaterials. Among the various use of this material, nanospheres or microspheres made of PLGA have gained popularity, mainly because of their tissue compatibility and biodegradability (200). Chang et al. showed that animals implanted with conduits made of PLGA and supporting cultured Schwann cells, presented a higher number of myelinated axons (201).

Others polymers are also used such as polyvinyl chloride (PVC), polyethylene glycol (PEG), polyamidoamine (PAA) with some success. Indeed, an improvement in the density and size of

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the axons as well as greater myelin thickness were observed following the use of PAA nerve conduits (202). Other teams have shown that the use of PVC improves myelination and high structure integrity (203). Koob et al. have shown greater improvement in exploratory behavior of injured PEG-treated rats (204).

5.1.d. Biofunctionalization of the biomaterials

Various strategies have been tested in order to give specific functions to the biomaterials either based on structural modifications of the material in order to enhance cell adhesion (205) or to stimulate cell growth at its contact (169). Specifically for neuronal-related application, the option of grafting or adding a neurotrophic factor to the biomaterial has been widely studied. The most common factor inserted is NGF, followed by glial cell-line derived neurotrophic factor (GDNF), BDNF, NT-3, and neurotrophin-4/5 as these molecules have been demonstrated to improve peripheral nerve regeneration. These proteins have been added either in microspheres or in microgels (206–208) in order to diffuse in the microenvironment or in regenerative conduits (209,210) aimed at guiding the peripheral nerve regeneration.

Nevertheless, if neurotrophic factors seem in fact the most accepted candidates to biofunctionalize materials aimed at helping reinnervation, other molecules have been shown to have interesting potentials. For example, bone morphogenetic protein-2 (BMP-2) was demonstrated as able to increase the number of axons and their diameter (211).

No study has been found in the literature where growth factors specific to the epidermal layer were added to biomaterials to help skin reinnervation although fibroblast growth factors were described as allowing a faster rehabilitation after peripheral nerve injury (212).

5.2. Skin substitutes

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As mentioned above, to allow the coverage of deep and extensive burns over a TBSA of more than 50 to 95%, tissue-engineered epithelial sheets made of patient's own keratinocytes were developed in the 70s by Rheinwald and Green (87,213–215). These CEA were successfully grafted on wounds promoting efficient epidermal healing, with esthetical and functional results not as good as split-thickness skin grafts, but efficient to cover burns (216). The technique was improved over the years, allowing to prepare the sheets in about two weeks in sufficient amounts (217). The production of CEA was manufactured as Epicel® in USA under the Humanitarian Device Exemption regulations by Genzyme (which sold this division to Aastrom Biosciences in 2014). The main advantage of using CEA is the reduction of the delay to achieve a complete coverage of patient's extensive burns, leading to a better survival and a shorter stay in the burn unit (218). Its main drawback is the high cost of the treatment (that may exceed 100,000US$ per patient) that could be compensated by the reduced cost of the shorter hospitalization, and the lower need for subsequent reconstructive surgeries.

For a better healing quality of the wound, the combination of the epidermal autograft with a dermal compartment would be desirable (219). However, since dermis is a three-dimensional tissue, its *in vitro* reconstruction proved to be much more complex than the epidermis. Beside the development of acellular dermal substitutes (112), the first attempt to produce a living dermal substitute was performed by Bell in 1979 by culturing fibroblasts embedded in a collagen gel (220) (Table 2). This dermal tissue was then seeded with keratinocytes to produce a tissue-engineered skin (221). This living skin equivalent permitted to demonstrate the importance of the presence of dermal fibroblasts in skin substitutes to rapidly promote the formation of a functional neo-dermis in humans (222) (Table 3).

This skin substitute was then manufactured by Organogenesis as Apligraf®, made of human fibroblasts and keratinocytes. Since it is a heterologous tissue, it is only intended to treat venous leg and diabetic foot ulcers as temporary biological dressing, but not burns, which

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require autologous epidermal graft for permanent coverage (238). Some attempts were made to apply Apligraf over meshed split-thickness autografts transplanted on burn wounds and showed cosmetic and functional advantages, but the cost/benefit ratio of this approach is questionable (239). Moreover, whereas Apligraf was shown to efficiently improve ulcer healing, it is rarely used in the clinic because of its high cost, and the availability of much cheaper dressings with nearly similar efficacy and much easier handling (240). Several other dermal substitutes were developed to produce tissue-engineered skin, based on the culture of fibroblasts in a deepidermized dermis (241,242), a collagen sponge (231,243), a biodegradable mesh (244) or a self-assembled fibroblast sheet (245), to name a few. Most of these models were transplanted in mice and showed good results in terms of take or dermal and epidermal remodeling (245,246). One aspect has recently been given more attention, the delay of complete vascularization of the graft. Indeed, it was shown that even if skin substitutes were rather thin, a compromised survival of the epidermis could be feared in dermal compartments thicker than 100 micrometers, exceeding the maximal distance for diffusion of oxygen and nutrients from the wound bed (247). These skin substitutes would then require specific strategies to enhance vascularization of the dermis, through the incorporation of endothelial cells to promote capillary formation in the tissue prior to graft (248–250). A complete vascularization of the graft was observed only four days after transplantation, instead of two weeks in the control without capillaries, through the inosculation of the network of capillaries from the endothelialized skin substitute with the vascular network of the wound bed (250).

Another important aspect of the application of an autologous skin substitute to cover deep and extensive burns is to what extent it may improve nerve regeneration and sense of touch recovery. It was shown that transplantation of skin substitutes on mice promoted nerve migration into the graft after 3 to 4 months (251,252). However, the major advantage of

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reconstructing skin *in vitro* is that it is possible to incorporate into it molecules or cells that could specifically enhance nerve regeneration (253). Moreover, it is possible to investigate the potential benefit of these approaches *in vitro* through the design of an innervated reconstructed skin. This model was developed by the incorporation of sensory neurons extracted from mouse embryo dorsal root ganglions. They were seeded on the fibroblast-populated sponge one week before keratinocytes to form a nerve network (Figure 5). The *in vitro* impact on nerve migration of any molecule or cells incorporated into the model can be analyzed by quantification of the number of sensory neurites (254). These neurons, whereas they were of mouse origin, were shown to release neuropeptides (substance P) efficiently modulating the human keratinocyte behavior (255). Thanks to the high versatility of these tissue-engineered skin models, it was possible to perform a wound in the epidermis to analyze the effect of innervation on re-epithelialization *in vitro*, compared with a control without nerves. Wound closure was shown to be twice faster in presence of nerves, because of their release of substance P. Indeed, this effect was completely abolished after blocking the NK1 receptor for substance P with an antagonist (255). This experiment showed that nerves promote a direct enhancement of re-epithelialization, independently of their induction of neurogenic inflammation *in vivo*, which is well-known to improve wound healing (31).

To enhance *in vivo* nerve regeneration of skin substitutes after graft, different approaches were investigated. Laminin, a natural component secreted by Schwann cells and known to facilitate axon migration was added into a tissue-engineered skin, and induced a major increase in nerve migration after graft. It allowed complete functional recovery of all the three types of cutaneous nerve fibers (*i.e.* Aβ, Aδ and C fibers) (256). Since laminin is a stable and large molecule, it could be easily incorporated in skin substitutes. The addition of Schwann cells in the tissue-engineered skin also demonstrated an enhancement of nerve regeneration and pain and temperature perception recoveries, but should be more complex and expensive

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to use for a clinical application (254). Target cells for sensory nerves, such as Merkel touch domes (251) or immature hair follicles (257) could increase the speed of nerve regeneration and promote a guided nerve migration and a potential sense of touch recovery through the connection of nerves with a sensory unit, but are not yet feasible in a human context for a clinical application. Even if some of these techniques have been proved to be efficient to increase nerve regeneration, the question of the quality and functionality of this neoinnervation remains to be clearly demonstrated in clinical studies.

These encouraging results point out the potential of skin substitutes to markedly improve sensory recovery. However, split-thickness skin also contains Schwann cells and Merkel touch domes, but its graft does not always promote good sense of touch recovery. The main reason for that might be the anarchic structure of the wound bed, which may compromise efficient nerve regeneration (142,145). Thus, the time required to prepare these skin substitutes could become an important limitation in their use, since a delay to cover burns could induce an unfavorable remodeling of the wound bed preventing further nerve migration. In addition, all these exciting improvements of skin substitutes with more sophisticated characteristics and enhanced potential for tissue function recovery face the challenge of their manufacturing, which emerged as a bottleneck to translate these skin substitutes to the clinic. As observed with the CEA technology, whereas it was beneficial to patients, its high cost has always limited its application. Moreover, this complex manufacturing process has even probably never been profitable for the company itself. The reason is the need to use patient's own cells for each treatment, and one can easily see how the extraction of fibroblasts in addition to keratinocytes, and the reconstruction of the dermal compartment may dramatically increase the cost and the time of the tissue production, that may not be affordable to most burn units. An alternative could be to develop a local non-profit unit of production of skin

substitutes linked with regional burn units, but that would require highly qualified personal and regulatory approval, such as those established in Europe and Canada.

Finally, these autologous skin substitutes are clearly highly beneficial to the burn patients, and may be not that expensive on a long-term perspective. This is why it is still so important to continue developing an ideal tissue-engineered skin, easy to manufacture and as efficient as possible to achieve complete cutaneous recovery of function, including tactile and pain perception.

5.3. Mesenchymal and induced pluripotent stem cells

Biomaterials and skin substitutes can be associated with stem cells as another strategy to promote nerve sprouting from the surrounding healthy tissue and guide axonal regrowth within the forming scar. MSCs have the capacity to generate different cell lineages and offer a wide range of future therapeutic approaches in skin healing and sensory recovery (258). Because of their multipotency, large *ex vivo* expansive potential and immunotolerance properties, autologous MSCs represent an attractive source of stem cells that could be included in a wound management protocol (259). Another major drawback in the study of skin reinnervation is the limited sources of human mature sensory neurons that can be used in *in vitro* and *in vivo* experimental models. Using MSC-derived neurons or induced pluripotent stem cells (iPS) could help overcoming this issue in futur experimental investigations.

5.3.a. Therapeutic potential of adult mesenchymal stem/stromal cells

The skin and more precisely the dermal compartment is a source of adult MSCs named SKPs. These SKPs possess capacities of self-renewal and multipotency and they can differentiate into both mesodermal and neural progeny (260). Neural crest stem cells have a similar broad

potential and contribute to development of the dermis and, in this regard, SKPs form a neural crest-related stem cell niche that arises in the skin during embryogenesis and persists in lower numbers into adulthood (261). SKPs are present in several locations in the dermis hence translating cellular heterogeneity. The largest and most studied source is located within the dermal papillae at the base of the hair follicle (262,263). Other sources of dermal SKPs include the hair bulge, sebaceous gland, sweat gland as well as a perivascular niche recently described (262–264). After isolation, SKPs are maintained in culture as spheroids and express specific markers such as nestin, vimentin and fibronectin (265,266). Neuronal differentiation is achieved using AMPc and a cocktail of neurotrophins such as BDNF, NT-3 and NGF while glial differentiation into Schwann cell is promoted by the addition of forskolin and heregulin 1β to the culture medium (267–270) (Figure 6). Little is known regarding the role of SKPs in skin wound healing or a potential involvement in sensory nerve regrowth but several studies have shown that SKP-derived Schwann cells help promoting sciatic nerve regeneration in rodents (271–273). It suggests that SKP-derived Schwann cells are fully functional in supporting axonal regrowth following injury. Recently, Ke et al. have shown that collagen sponges seeded with SKPs facilitate skin wound healing in diabetic mice by promoting local vascular regeneration (274). Another study has also shown that intradermal injections of SKPs around full-thickness excisional cutaneous wounds in diabetic mice mediate faster wound closure and re-epithelization, earlier angiogenesis and might promote wound reinnervation (275). Interestingly, another *in vivo* study has highlighted that SKPs transplantation in denervated cutaneous wounds on nude mice promotes wound closure and local secretion of neuromediators such as substance P, CGRP as well as NGF (276). More studies have to be performed in order to determine if local or transplanted SKPs can either differentiate into Schwann cell following skin injury or if they somehow help mediating the migration of local Schwann cells and/or axonal regrowth of nerve fibers during scarring. The

study of SKPs "secretome" could shed new light into factors contributing to this phenomenon. Thus, the isolation of SKP-derived autologous precursors from adult human skin represents an accessible and very promising source of neurons and Schwann cells to help restore normal innervation after skin damage.

The adipose tissue represents another valuable and abundant source of adult MSCs. It has the advantage of being accessible using liposuction procedures. Adipose-derived stem cells (ASCs) can be easily expanded *ex vivo* by isolating the stromal vascular fraction from the adipocytes using enzymatic digestion. Like SKPs, autologous ASCs can be driven towards neurogenic or glial differentiation (277,278). Many studies have shown the ability of ASCs-derived Schwann cells in promoting peripheral nerve regeneration and wound healing but again, little is known about their potential in mediating cutaneous sensory recovery following skin damage. The subcutaneous adipose tissue could then be of interest as a close by reservoir of ASCs following skin injury. Recently, Tomita et al. have shown that in rats, Schwann cell-like cells differentiated from ASCs could improve the cutaneous nerve regeneration in skin flaps by producing NGF and BDNF (279).

Bone marrow (BM)-derived MSCs have also been used in the treatment of skin wounds (280). BM-derived MSCs are isolated using bone marrow aspirate and selected *in vitro*. The bone marrow aspirate is an invasive method and the number of MSCs present in the BM swab is limited (0.001 to 0.01% of total BM nucleated cells). The selection of BM-derived MSCs relies on their ability to adhere to plastic before expansion. Interestingly, BM-derived MSCs have been suggested to participate in tissue repair. They are able to migrate to the damage tissue and differentiate into wound healing (myo)fibroblasts (281). BM-derived MSCs have also been shown to differentiate into neurons and Schwann cells (282,283).

In addition, extra-fetal tissues are a source of great interest. In extra-fetal tissues, MSCs have been described in the amniotic fluid and in different layers of placenta, principally the amnios

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and chorion. They have also been described in Wharton's jelly around cord vessels. These cells have particularly interesting immunological features and hepatocyte-like differentiative capacities (284). It has also been shown that progenitor cells are present in gingival connective tissue (285). Based on their ability to differentiate into several lineages, to proliferate from single cells, to induce calcium deposits, and to secrete collagen *in vivo* after transfer on hydroxyapatite carriers, these cells correspond to gingival multipotent progenitor cells. The exceptional healing capacity of the gum can be correlated with the presence of these progenitor cells which also represent new safe therapeutic strategy for wound healing.

It gradually became apparent that MSCs ability to change a pathological environment and enhance wound healing is not only related to their capacity of differentiation but also to their ability to modulate the behavior of other cell types. Their activities mainly go through the secretion of different kinds of bioactive molecules (*e.g.* growth factors, cytokines, chemokines) (286). They are also able to realize mitochondrial transfer and to produce microvesicles and exosomes containing protein, mRNA, miRNA or mitochondrial fragments (287,288). Thereby, Zhang et al. have shown that exosomes derived from perinatal MSCs are able to accelerate the healing of skin burns by increasing the re-epithelialization and angiogenesis process via Wnt and PI3K/AKT signaling pathways (289). Finally, it is possible to optimize MSCs efficiency by modulating their culture environment with various kind of stimulation, called priming or licensing (288). This optimization has two objectives: (i) to prepare them to the environment in which they will be injected to and (ii) to modulate their behavior to counterbalance or promote a physiological reaction. For example, a pre-treatment with hypoxia (290) or with cytokines such as TGF-β1 (291) or TNF-α (292) can enhance wound healing. Studying the paracrine communication of MSCs in both their differentiated or naive state could also be of foremost interest.

5.3.b. Induced pluripotent stem cells

Induced pluripotent stem cells or iPS were first generated in 2006 using both embryonic and adult mouse fibroblasts (293,294). The experimental protocol consists in the genetic reprogramming of somatic cells into pluripotent stem cells by targeting four specific genes: Oct4, Sox2, Klf4 and c-Myc. Thus, human iPS display characteristics of embryonic stem cells and can generate a wide range of cell types including neurons (294–296). The generation of iPS-derived Schwann cells has not been reported so far. The major advantage of iPS is the availability of the source material, a simple skin biopsy being necessary to collect dermal fibroblasts. However, the genetic reprogramming of fibroblasts, maintenance and differentiation of iPS is technically challenging and time-consuming. Moreover, in order to reduce safety concerns associated with viral vectors, protocols using plasmids or recombinant proteins channeled into the cells have been developed (297,298).

As an alternative to iPS, the direct conversion of fibroblasts into neurons using small molecules has recently been described. Using a cocktail of chemicals and neurotrophic factors such as forskolin and CHIR99021, a selective inhibitor of glycogen synthase kinase 3, researchers were able to generate functional neurons in 21 days (299,300). This method represents a new advantageous tool to generate mature human neurons that could be used in future experimental approaches.

5.4. Bioprinting

Since several years, printing technology has rapidly progressed from two dimensional (2-D) to three dimensional (3-D) where different kinds of material can be used. Therefore, the field of tissue engineering has benefited from this technology to improve the seeding of a wide range of cells, onto solid and biodegradable scaffolds. It allows reproducing the complex 3-D structure of extracellular matrix components and designing tissues by adding bio molecules.

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Several 3-D bioprinting techniques exist such as inkjet bioprinting, microextrusion bioprinting, and laser-assisted bioprinting (301–303). Laser-assisted printing is the most favorable technique to maintain cell viability and print good quality vertical structures with high resolution. Microextrusion is the best technique to apply ink with high viscosity and inkjet bioprinters are used when low cell density is needed.

Materials or bio-inks must be easily printable to facilitate handling and deposition. They must be biocompatible for long-term transplantation, must degrade at rates that matche the ability of cells to produce their own extracellular matrix while displaying short-term stability.

Several tissues and organs can be printed efficiently. For example, a proof of concept for skin bioprinting has been demonstrated by several teams with a good cell viability and architecture of the tissue (304) and also with a bio-printed vascularization (305). Moreover, Skardal et al. show that it was possible to bioprint dermal substitutes combined with MSCs directly *in situ* inducing faster wound closure (306). However, functional vascularization, that need to be fully addressed in order to allow engineered tissue to survive, could be improved with the use of Pluronic F127 as a sacrificial bio-ink that can form open lumens concurrently with the printing of encapsulated cells around the vessels (307). Innervated bio-printed skin has not yet been produced but fabrication of a synthetic nerve graft by printing cell-dense tubes of Schwann cells and MSCs have been shown to be a promising approach for nerve regeneration (308). While bioprinting technology is promising in wound healing, several improvements have to be made in terms of rapidity of printing and of bio-engineering complex hollow structures.

6. Conclusions and perspectives

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The skin is a protective barrier but also serves as interface between our body and the external environment. It is indeed a highly sensitive organ. In addition to different cell types expressing many sensory receptors (309), skin comprises several sensory nerve fiber subtypes that perceive and convey various external stimuli, such as temperature variations, pain or tactile stimuli.

In addition to their sensory role, cutaneous nerve fibers are known to be tightly involved in a variety of physiological and pathological processes (25). It has been shown in several clinical observations that injury to the peripheral nervous system impairs wound healing, sometimes leading to the development within the affected area, of chronic wounds. Wound healing may be delayed, as demonstrated by studies using *in vivo* models of peripheral neuropathies by denervation or chemical impairment of nerve fibers (47). Likewise, patients with peripheral neuropathies due to lepromatous leprosy, spinal cord injury or diabetes mellitus develop ulcers that fail to heal (14). In elderly, cutaneous repair processes are also less efficient (310), partly due to a degeneration of the nerve fibers within the skin (311). Moreover, a defective innervation and/or inadequate levels of neuropeptides can negatively influence healing processes underlining that innervation and neuropeptides are major players for normal cutaneous repair. Promoting normal reinnervation and adequate levels of neuropeptides during the healing process are certainly crucial to improve skin healing and to avoid the appearance of pathological situations.

When a major skin injury occurs such as a deep burn, sensory nerve endings are destroyed while cell bodies in the dorsal root ganglia along the spinal cord are maintained. Cutaneous nerve regeneration and progressive reinnervation of the scar is possible and may result either from the regeneration of injured nerve fibers present in the wound bed or from the sprouting of nerve fibers located in the adjacent uninjured area. However, the nerve regeneration process is imperfect, as suggested by frequent impairment of skin perceptions or the

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occurrence of chronic pain and disabilities. After wound healing, itching and pain tend to decrease (152). However, cutaneous nerve fiber populations have been shown to be modified in scars compared to matched uninjured skin. Interestingly, the density of the C fibers, which are involved in pain perception, is higher in scars (132). Not surprisingly, this density is also increased in scars from patients with chronic pain compared to scars from patients without pain (41). These outcomes suggest that unmyelinated small C fibers involved in the pain detection regenerate faster than Aδ and Aβ myelinated fibers. Overall, it becomes clear that the regeneration of the destroyed nerve fibers needs to be improved during skin healing management and medical treatment.

Until now, various techniques have been used in wound care. It includes occlusive dressings, autograft application, dermal allograft and skin substitute, or highly expanded autograft, depending on the size of the lesion (see Table 1). Currently, the development of more sophisticated skin substitutes is in progress and aims to improve a patient's rehabilitation. New designs of skin substitutes, innovative biomaterials and stem cells represent promising therapeutic strategies that could both promote correct wound healing and sensory recovery. In these innovative products, the presence of neuronal cells, Schwann cells and/or the addition of neurotrophins could favor the development of a more physiological innervation in the repaired skin and minimize *sequelae* often associated with burn scar. The 3D bio-printing technology could especially offer new opportunities. This recent approach in which cells and materials are directly deposited on or in a patient (312) could be particularly interesting after extensive burns. However, these new biotechnological approaches are still challenging to apply in burn wound management. Limitations such as cost, ethical issues for stem cells and complex designs of skin substitutes still need to be addressed. Moreover, technical limitations related to the incorporation and/or the "selection" of appropriate innervation structures, especially tactile corpuscles, have to be overcome.

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Reprint author

Alexis Desmoulière

Department of Physiology, Faculty of Pharmacy, University of Limoges

2 rue du Dr. Marcland, 87025 Limoges cedex, France

E-mail address: alexis.desmouliere@unilim.fr

Figure legends

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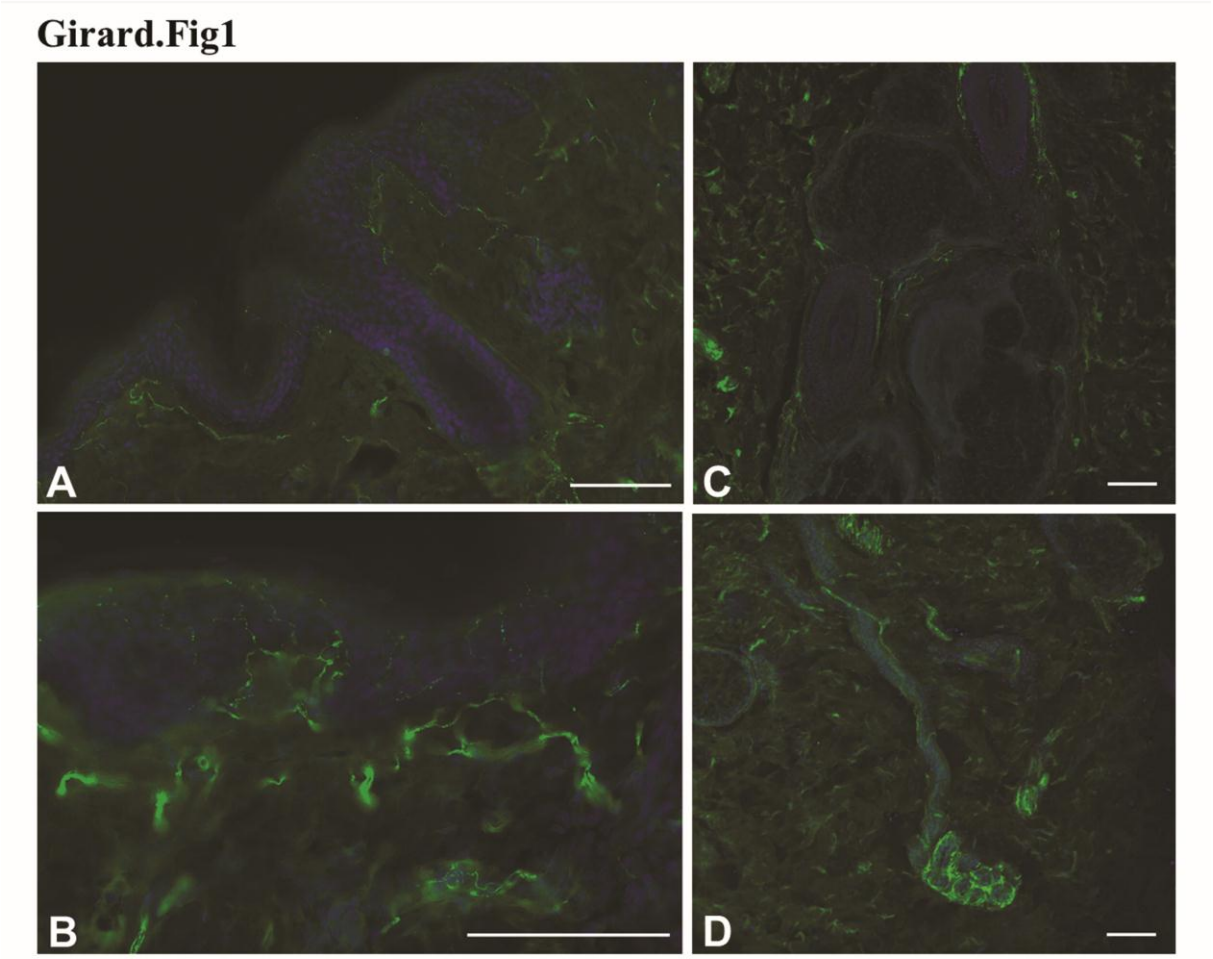

Figure 1: Skin innervation. In these pictures, cutaneous nerve fibers are labeled using an anti-PGP 9.5 antibody revealed by a secondary FITC-conjugated antibody (the cell nuclei are colored with DAPI). The superficial nerve plexus follows the dermal-epidermal junction in the dermis and the small sensory nerve fibers Aδ and C sprout into the epidermis reaching its upper layers (A, B). Autonomic nerve fibers are the main fibers innervating the skin appendages, *i.e.* hair follicles (C) and sweat glands (D). Scale bars: 100 µm.

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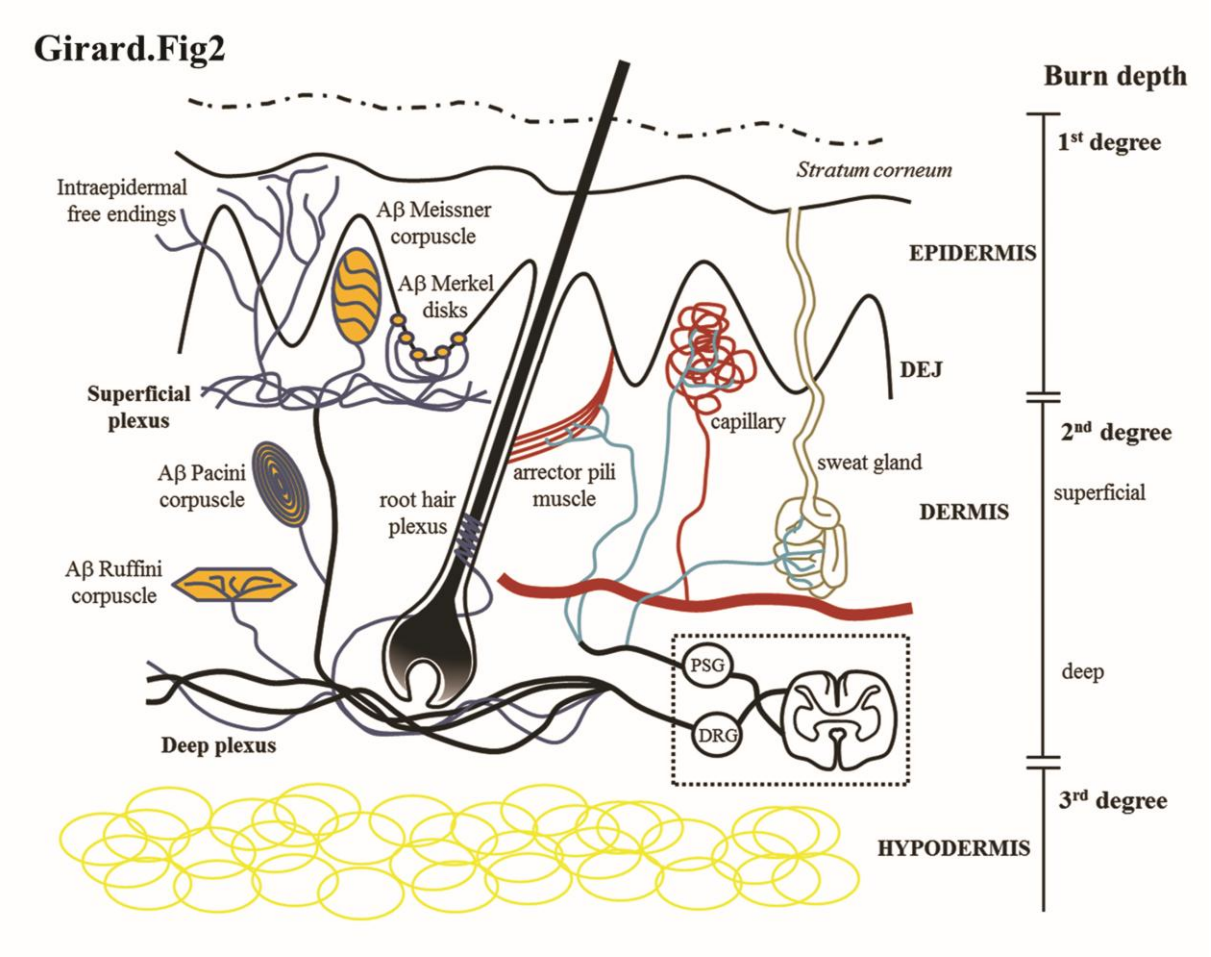

Figure 2: Schematic representation of the skin innervation. Skin nervous structures are endings of sensory and sympathetic neurons which have their cell bodies located respectively in dorsal root ganglia (DRG) and paravertebral sympathetic ganglia (PSG) along the spinal cord. Sensory and sympathetic skin structures are located respectively on the left and on the right part of the schema. Concerning sensory intraepidermal free endings, they are constituted of Aδ fibers and of peptidergic and non-peptidergic C fibers. In addition, axonal free endings are also present in the dermis. DEJ: dermal-epidermal junction.

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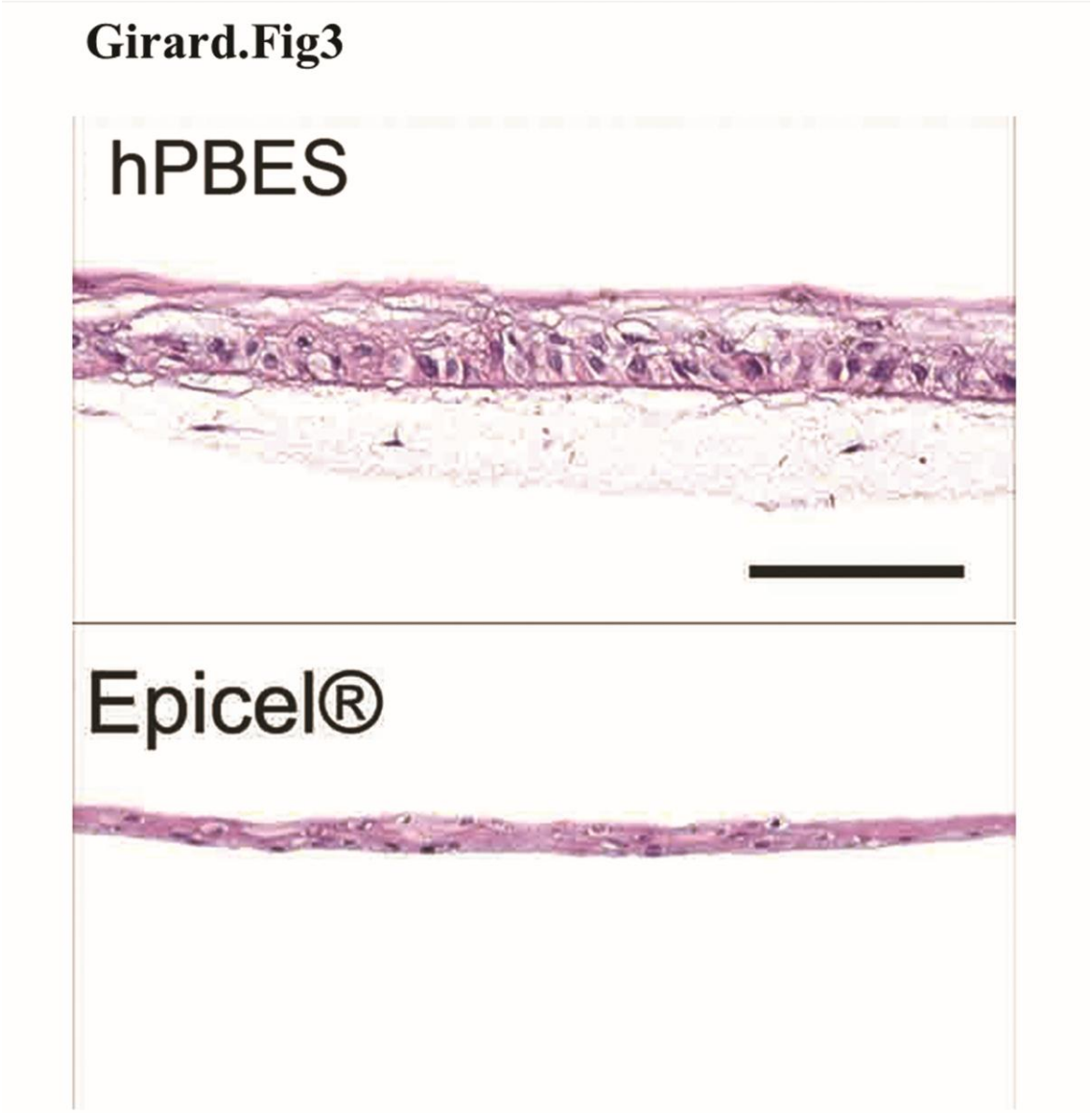

Figure 3: Hematoxylin phloxine saffron staining of a human plasma-based epidermal substitute (hPBES) and of a cultured epithelial autograft Epicel®. For the hPBES substitute, a well-organized basal layer of cuboidal or columnar keratinocytes is observed, similar to healthy skin. Scale bar: 100 µm.

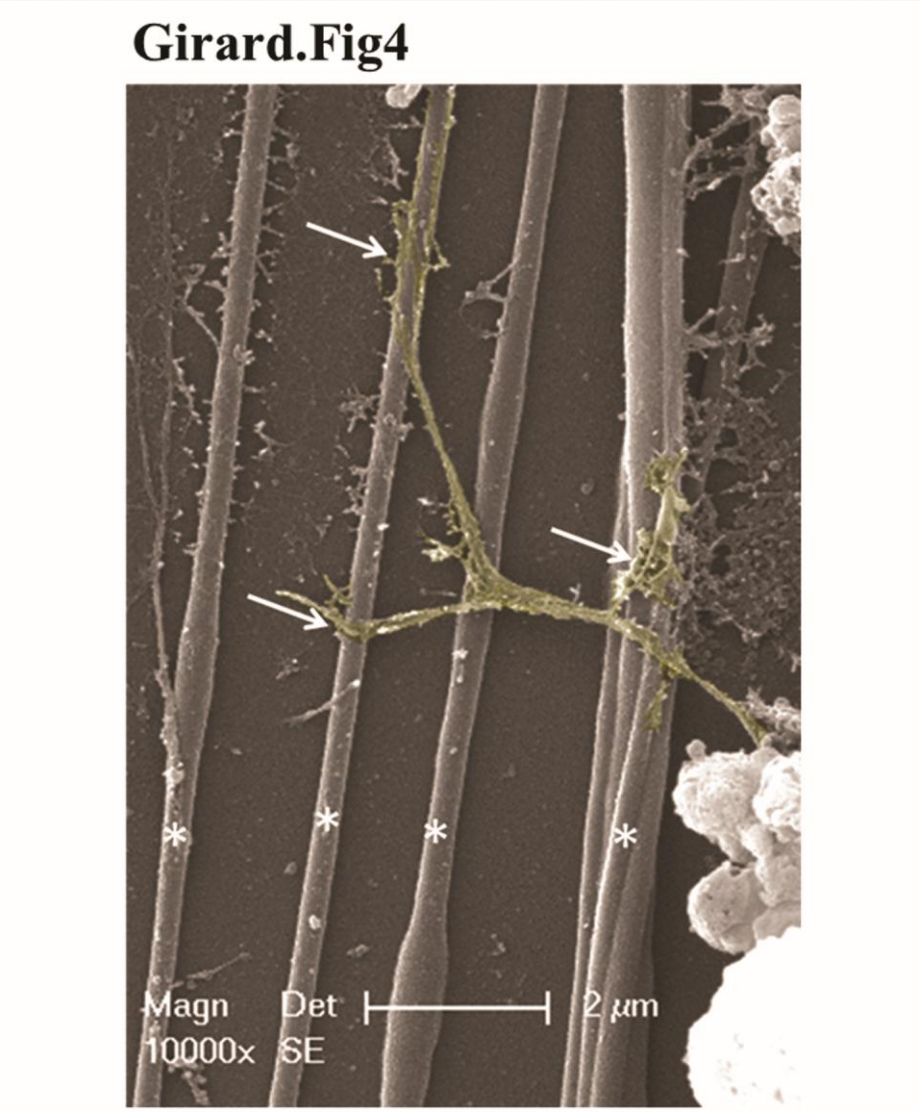

Figure 4: Scanning electron microscopy observation of neuron cells on electrospun fibroin nanofibers. Primary cell culture of dorsal root ganglia cells obtained from young male Sprague Dawley rats (1–3 months old) are seeded on electrospun fibroin nanofibers (*). Close interactions between axonal growth cones and fibroin nanofibers are visualized (arrows).

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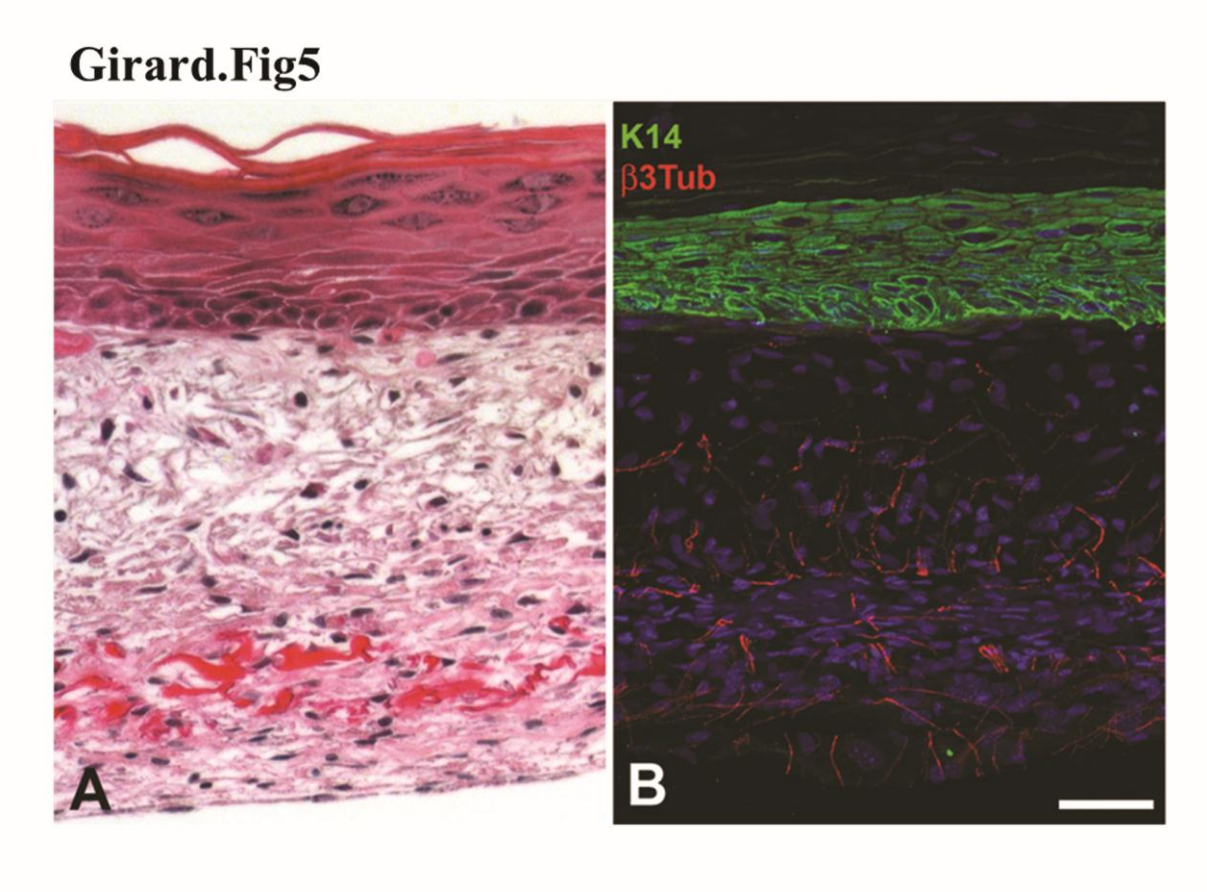

Figure 5: *In vitro* characterization of an innervated reconstructed skin. (A): The tissue-engineered skin is made of human keratinocytes and fibroblasts cultured in a collagen-chitosan sponge biomaterial for 42 days, in which mouse sensory neurons are incorporated at day 14 (one week before keratinocytes) on the opposite side compared to epidermis. Keratinocytes form a well-differentiated epidermis over the fibroblast-populated dermis as seen on hematoxylin-eosin histological cross-section. (B): Keratinocytes express keratin 14 (stained in green) and sensory axons, anti-β III tubulin, a neuronal marker (stained in red), and generate a homogeneous network of neurites migrating from the bottom of the dermis (where neurons are located) up to the epidermis. Cell nuclei are stained with DAPI. Scale bar: 50 µm.

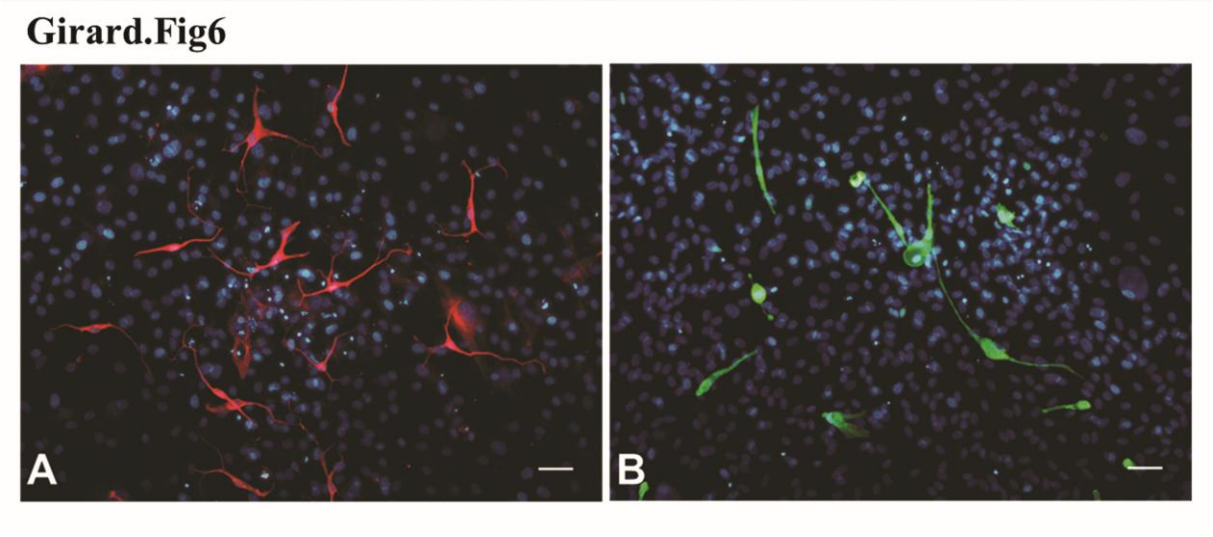

Figure 6: Neuronal and glial differentiation of human skin-derived precursors (SKPs). (A): Neuron-like SKPs express β III tubulin (red). (B): Schwann cell-like SKPs express S100β (green). DAPI is used for nuclear staining (blue). Scale bars: 100 µm.

Girard.Table1

Table 1: Description of clinical characteristics of burn wounds of various depths and treatments (Adapted from (58)). TBSA: total body surface area.

Degree	**Injured skin layer**	**Wound aspect**	**Healing time**	**Treatment**	**Prognosis**
Superficial or 1st degree	Suprabasal epidermis	Red, no blister, dry	3-7 days	Topical treatment	Good
Superficial partial thickness or 2nd degree	Epidermis and superficial or papillary dermis	Red, blister, moist, blanches with pressure	1-3 weeks	Topical antimicrobial agents, and occlusive dressings	Good
Deep partial thickness or 2nd degree	Epidermis and dermis (papillary and reticular)	White, non-blanching, dry	3-6 weeks, with scars	Topical antimicrobial agents, and occlusive dressings for small surface or eschar excision and autograft application	Scar
Full thickness (3rd degree)	Full thickness of skin including subcutaneous fat or deeper	hard texture, white, dry	Does not heal by primary intention	Eschar excision and autograft application if burn TBSA < 50%, or dermal allograft and skin substitute or autograft highly expanded if burn TBSA> 50%	Scar, weak skin

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Girard.Table2

Table 2: Acellular biomaterials commercially available and/or used in clinics for burn treatment (dermal replacement and /or skin repair).

Source of biomaterial		**Product/Company**	**References**
Human skin or dermis	Cadaveric skin (cryopreserved, glycerolized, lyophilized or acellularized)	Tissue Bank, Alloderm® Life cell Corporation (NJ, USA), Gammagraft® Promethean Life Science (USA), Glyaderm® Euroskinbank (The Netherland)	(77,94–102)
Animal dermis	Porcine acellularized dermis	Strattice Tissue Matrix® Life cell Corporation (NJ, USA), Epiflex® DIZG, EZ Derm® Mönlycke healthcare (USA)	(103–106)
	Lyophilized porcine intestinal mucosa with growth factor	Oasis Wound Matrix® Johnson and Johnson	(105)
	porcine tendon derived atelocollagen type I +bFGF	Pelnac + bFGF	(106)
	Lyophilized bovine dermis	Matriderm® MedSkin Solutions Dr.Suwelack,	(107–111)

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		Terudermis® Olympus Terumo Biomaterials (Japon)	
	Bovine collagen and chondroitin 6-sulfate		(112)
	Bovine collagen and glycosaminoglycans	Integra® Integra Lifescience	(113–115)
Synthetic polymer	Polylactide, trimethylene carbonate and e-caprolactone copolymer	Suprathel® Polymedics Innovations GmbH (Germany)	(116–118)
	Nylon coated with porcine peptides	Biobrane® Smith and Nephew (USA), AWBAT® Aubrey Inc. (USA)	(119–121)
Biopolymer	Derivatives from hyaluronic acid	Hyalomatrix PA® Fidia advanced biopolymers (Italy)	(122–125)
	Allogenic fibrin	Engineered skin substitute	(126,127)

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Girard.Table3

Table 3: Cellularized biomaterials commercially available and/or used in clinics for burn treatment (skin repair).

Source of biomaterial		**Cells**	**Product/Company**	**References**
Synthetic polymer	PGA/PLA	Neonatal foreskin fibroblasts	Dermagraft® Organogenesis, USA	(223,224)
Animal	Bovine collagen	Neonatal fibroblasts	Apligraf® Organogenesis, USA	(225–228)
	Bovine collagen	Autologous fibroblast		
	Bovine collagen	Neonatal fibroblasts and keratinocytes	Orcel® Forticell Bioscience, USA	(229)
	Bovine collagen	Autologous cultured keratinocytes and fibroblasts	Tissue-cultured skin autografts	(230)
	Bovine collagen + GAG	Autologous cultured keratinocytes and fibroblasts	Engineered skin substitute Amarantus USA	(231–233)

Human	Autologous fibrin	Autologous cultured keratinocytes and fibroblasts	MyDerm® Cell Tissue Technology, Malaysie	(234,235)
	Autologous Plasma	Autologous cultured keratinocytes and allogenic fibroblasts	Engineered skin substitute	(236,237)

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