Scar Formation: Cellular Mechanisms

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L. Téot et al. (eds.), *Textbook on Scar Management*, https://doi.org/10.1007/978-3-030-44766-3_3

3.1 Background

Tissue repair after injury is a complex phenomenon involving intricate and coordinated mechanisms. Even though during the last decade, many studies have increased our knowledge on the different cellular players involved in this process [16], many gray areas remain, particularly concerning the dialogue between different cell populations acting during wound healing and scar formation. Interestingly, an abnormal course of the inflammatory phase at the beginning of the healing process appears to have effects long after scar formation. In addition, the relationships between cells and the extracellular matrix remain a key aspect in understanding the normal development and remodeling of scars.

3.2 Introduction

The skin provides the primary protection against external injuries for the body and is also essential in the maintenance of general homeostasis. Located beneath the epidermis, the dermis represents the thickest compartment of the skin and is composed mainly of a dense extracellular matrix network of collagen fibers supporting the specific dermal appendages, including hair follicles, sebaceous glands, and sweat glands. Dermal (myo)fibroblasts play a major role in the synthesis and maintenance of the extracellular matrix as well as in the wound-healing process [4]. During healing, fibroblasts proliferate and migrate into the wound space, though the origin of these cells remains to be clearly elucidated. Moreover, research on progenitor cells present in the skin, which can differentiate into many different cell types, represents an interesting source of cells that may be able to promote (when correctly stimulated) wound healing, improve the repair process in impaired or difficult-to-heal wounds or be able to modify the process of excessive scarring. In this chapter, these different aspects will be expanded on, including the biochemical, cellular, and physical factors that are involved in regulating healing and scarring in skin wounds (e.g., cytokines, mechanical tension, and innervation).

3.3 General Mechanisms of Scar Formation

Immediately after wounding, the healing process commences leading to (partial) restoration of the injured tissue. Wound healing passes through three dynamic and interrelated phases which temporally overlap [3]. Based on morphological changes seen in the wound tissue during the course of the healing process, these phases are defined as the inflammatory phase, the proliferative phase (with the development of the granulation tissue) and reepithelialization phase, and the remodeling phase which includes maturation and scar formation. The inflammatory phase begins with capillary damage, where blood loss results in a clot forming, which consists of fibrin and fibronectin and stops further blood loss. The wound space is thus filled with a provisional matrix that provides a scaffold into which various cells can migrate. The initial source of chemokines in the wound is platelets that are present in the clot, and these degranulate and provide multiple factors that stimulate the recruitment of inflammatory cells, neutrophils, and macrophages. At the same time, fibroblasts and endothelial cells are attracted by growth factors that are chemotactic for these cells. The proliferative phase of healing which follows includes angiogenesis, the growth of new vessels into the wound. Angiogenesis is vital for tissue repair since it provides vascular perfusion of the wound, delivering oxygen and nutrients and thus contributing to cell proliferation, including the proliferation of fibroblasts. The wound is initially hypoxic, as it lacks normal perfusion, and hypoxia itself is an important stimulus for the release of growth factors, including those that regulate angiogenesis. During the proliferative phase, angiogenesis proceeds and eventually reestablishes a more normal level of perfusion. The regulation of angiogenesis may represent a target for improving wound repair, particularly in those cases where delayed or abnormal angiogenesis has been suggested to play a role in healing impairment. Fibroblasts present in the granulation tissue become activated and are then termed myofibroblasts. These myofibroblasts are responsible for the synthesis and deposition of extracellular matrix components which progressively replace the provisional matrix [6]. Myofibroblasts also show contractile properties, playing a major role in the contraction and maturation of the granulation tissue. Scar formation, which is the third phase of healing, involves progressive remodeling of the granulation tissue (Fig. 3.1). A major role in this process is played by proteolytic enzymes, particularly the family of matrix metalloproteinases (MMPs) and their inhibitors (tissue inhibitor of metalloproteinases [TIMPs]). Synthesis of extracellular matrix is then reduced, though not completely stopped, and the components that make up the matrix are modified as the matrix is remodeled. Collagen type III, the major collagen present in granulation tissue, is progressively replaced by collagen type I, the main structural protein in normal unwounded dermis. Finally, elastin which is largely responsible for the elasticity of the skin and which is absent in granulation tissue also reappears. In deep human wounds, elastin fibers do not show complete regeneration after wound healing being both deficient and not showing normal structural

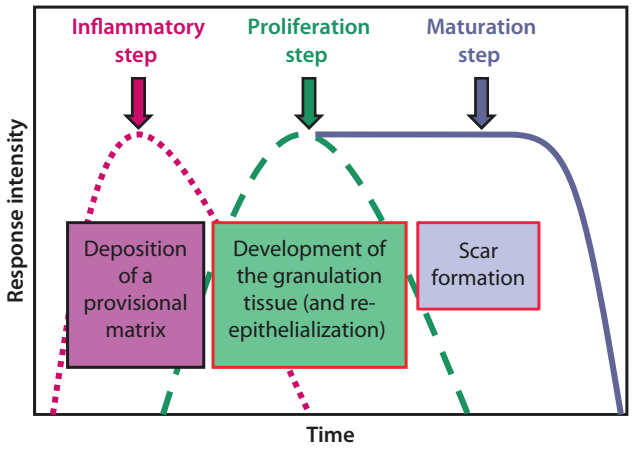

Fig. 3.1 The various phases of the healing process. After damage, inflammation leads to the formation of granulation tissue during which myofibroblasts appear. A significant neoangiogenesis is also observed. A new epidermis can then develop over this granulation tissue. Subsequently, remodeling of this granulation tissue occurs via apoptosis of the cells present in the granulation tissue (myofibroblasts and vascular cells), and the extracellular matrix is gradually reorganized

organization. During the resolution phase of wound healing, apoptosis of both vascular cells and myofibroblasts results in a significant reduction in cell number in the granulation tissue. Whether myofibroblasts can reacquire a quiescent phenotype thus returning to a "normal" dermal fibroblast phenotype is still open to question.

3.4 Morphological and Biochemical Characteristics of Myofibroblast Phenotype

The earliest descriptions of myofibroblasts, based on their electron microscopic morphology, identified ultrastructural specializations showing some similarity to those of smooth muscle cells, in particular prominent bundles of cytoplasmic microfilaments. Further ultrastructural and molecular markers that define myofibroblasts have since been identified, including cell-cell and cell-matrix adhesions, stress fibers, and expression of α-smooth muscle actin [9]. Both in vivo and in vitro, the presence of fibroblasts exhibiting prominent microfilament bundles in their cytoplasm (known as stress fibers) can be observed; however, these cells do not necessarily contain α-smooth muscle actin-positive microfilaments. In vitro, these fibroblasts can also be shown to secrete a splice-variant form of fibronectin, ED-A fibronectin. These cells have been termed proto-myofibroblasts, and their stress fibers contain only β- and γ-cytoplasmic actin. Proto-myofibroblasts can exert tractional forces in connective tissue, and they may be induced by mechanical stress; however, to undergo full differentiation into myofibroblasts, they require stimulation with transforming growth factor (TGF)-β1 (see below). Fully differentiated myofibroblasts are then capable of exerting increased force due to contraction. The expression pattern of myofibroblasts and smooth muscle cells shows several differences. Smooth muscle cells express smooth muscle myosin heavy chain, smoothelin, and h-caldesmon, while myofibroblasts are generally negative for these markers. Desmin, an intermediate filament protein which is normally expressed in muscle cells, has also been used as a negative marker of myofibroblasts, since during normal wound healing myofibroblasts have been found to be desmin-negative. In some conditions of pathological scarring, myofibroblasts have been observed to be desmin-positive. Overall though α-smooth muscle actin is also expressed in smooth muscle cells and pericytes, it still represents the most reliable phenotypic marker of myofibroblast phenotype.

Given the important roles of myofibroblasts in tissue repair and scarring, in particular their role in contraction, the exact mechanisms regulating contraction in such tissues need to be clearly identified. Examination of spontaneous intracellular Ca^{2+} oscillations has shown that intracellular Ca^{2+} oscillations are coordinated between contracting myofibroblasts via adherens junctions but occur randomly between fibroblasts and non-contacting cells. Therefore the following model of mechanical coupling for myofibroblasts can be proposed: individual cell contraction is transmitted via adherens junctions leading to the opening of mechanosensitive ion channels in adjacent cells. The resulting Ca^{2+} influx induces a contraction that can feed back on the first cell and/or stimulate other contacting cells allowing the cells to work like a syncytium. This mechanism of coordination of myofibroblast activity may improve the remodeling of cell-dense tissue [8].

3.5 Cellular Origins of Myofibroblasts

Recruitment of fibroblasts from the local connective tissue is generally accepted to be the major source of myofibroblasts in the wound. Dermal fibroblasts that are located at the wound margins can acquire a myofibroblastic phenotype and then play a role in tissue repair. However, there is considerable heterogeneity in fibroblastic cell subpopulations. These fibroblast subpopulations are present in different locations within the skin and have specific activation and deactivation properties. At least three subpopulations have been

identified in the dermis: superficial (or papillary) fibroblasts (the papillary dermis is approximately 300–400 μm in depth and is arranged as a ridgelike structure), reticular fibroblasts which are present in the deep dermis (which consists of thick collagen and elastin fibers that are arranged parallel to the surface of the skin), and fibroblasts which are associated with hair follicles. These cell subpopulations can be isolated for cell culture and then, depending on the age and nature of the skin sample, show distinct differences in phenotype in vitro.

It has been recently suggested that local mesenchymal stem cells may also be involved in the tissue repair process. These progenitor cells have been identified in the dermal sheath that surrounds the exterior of hair follicles, facing the epithelial stem cells. These cells are involved in regeneration of the dermal papilla and can also differentiate into wound-healing myofibroblasts after damage or injury.

Recent data has also suggested a role for circulating cells, termed fibrocytes, in the tissue repair process. Fibrocytes may enter damaged skin together at the same time as inflammatory cells and may then acquire a myofibroblastic phenotype. In burn wound, fibrocytes may infiltrate the wound where they both stimulate a local inflammatory response and additionally secrete extracellular matrix proteins, thus contributing to the pathological (hypertrophic) scarring that can be seen postburn injury.

Another bone marrow-derived circulating cell has also been suggested to play a role in tissue repair. Mesenchymal stem cells are bone marrow-derived nonhematopoietic precursor cells that may be present in both normal and damaged connective tissue, where they infiltrate the tissue and then contribute to the maintenance and repair of the tissue. Indeed, these cells have the capacity to seed into several organs and then differentiate into myofibroblasts, similar to those seen during wound healing. The degree to which damaged tissues or organs are infiltrated by these cells is dependent on the severity of tissue injury.

Lastly, epithelial- or endothelial-to-mesenchymal transition of either differentiated or malignant epithelium (or endothelium) can result in a phenotypic change to fibroblasts or myofibroblasts that are then responsible for extracellular matrix production. Although this mechanism is now accepted as playing an important role in fibrogenesis after tissue injury, it appears to play a less prominent role in normal tissue repair. Overall, myofibroblasts derived from circulating fibrocytes, mesenchymal stem cells, epithelial- or endothelial-to-mesenchymal transition, or bone marrow-derived cells may supplement local fibroblast recruitment and differentiation where their numbers are insufficient for the repair and remodeling process [14].

3.6 Regulation of Myofibroblast Phenotype

Wound healing and skin homeostasis are regulated by a number of cytokines and growth factors. Some growth factors act directly on granulation tissue formation and fibroblasts activity, while others have effects on vascular and epithelial cells. Of the factors with direct effects on fibroblasts, TGF-β1 is notable as it is a potent inducer of myofibroblast differentiation [18]. In addition to its role in inducing expression of α-smooth muscle actin, TGF-β1 also powerfully stimulates the synthesis of extracellular matrix proteins. TGF-β1 also favors the deposition of matrix by an effect on the balance between MMPs and their inhibitors, TIMPs, by reducing MMP activity while stimulating TIMP expression. The action of TGF-β1 on fibroblast to myofibroblast differentiation requires the presence of ED-A fibronectin, underlining the close relationship between growth factor activation, the extracellular matrix, and regulation of cellular function (◘ Fig. 3.2). It is interesting to note that granulocyte macrophage colony stimulating factor can also increase the number of myofibroblasts in vivo; however, this is most likely due to its activation and recruitment of macrophages which in turn increases the levels and availability of TGF-β1 [15]. Finally, microRNAs (miRNAs) have also been implicated in the induction of myofibroblasts in both fibrotic conditions and in cancer. Specifically, expression of miR-21 appears to be correlated with high levels of TGF-β1 stimulation of the myofibroblast phenotype. A recent study has suggested that the mechanism underlying this may be through effects on TGF-β1 inhibitory pathways. Improvements in our understanding of the effects of miRNAs in regulating fibrosis, potentially through actions on myofibroblast differentiation and activity, may allow miRNAs to be targeted in therapies aimed at inhibition of fibrosis and scarring.

3.7 Role of Myofibroblasts in Pathological Scarring and Fibrosis

In some cases, wound healing proceeds in a pathological course resulting in pathological scarring [17]. Such abnormal repair processes may be the result of impaired remodeling of the granulation tissue resulting in, for example, abnormal repair of the skin in the form of hypertrophic or keloid scars and to fibrosis in internal organs. In the case of excessive scarring in the skin such as hypertrophic scars, normal healing fails and the granulation tissue continues to expand, likely due to abnormal and excessive secretion of growth factors and/or to a lack of molecules that in normal healing are responsible for induction of apoptosis or remodeling of the

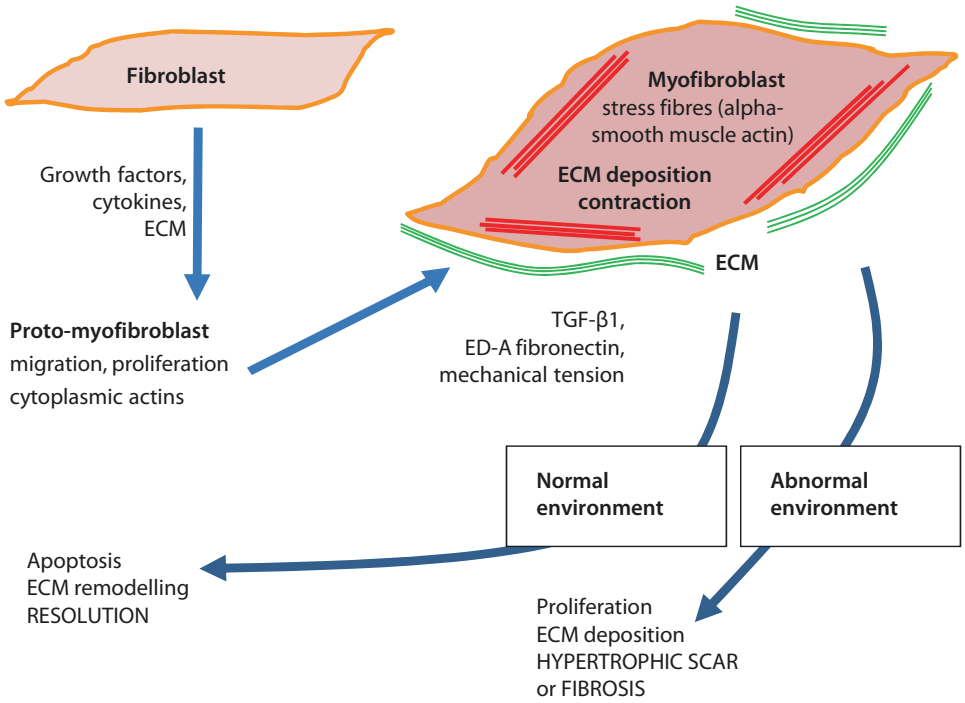

Fig. 3.2 Illustration showing the evolution of the fibroblast phenotype during normal and pathological conditions. Changes observed in fibroblast phenotype as cells differentiate towards a myofibroblast phenotype begin with the appearance of proto-myofibroblasts. These cells possess stress fibers composed of β- and γ-cytoplasmic actins. These then evolve, at least in some cases, into fully differentiated myofibroblasts. These cells possess stress fibers containing α-smooth muscle actin. Soluble factors such as transforming growth factor-β1 (TGF-β1), extracellular matrix (ECM) components such as ED-A fibronectin, and/or the mechanical microenvironment are all involved in myofibroblastic differentiation. The myofibroblast may then disappear by apoptosis, while deactivation, leading to a quiescent phenotype, has not been clearly demonstrated at least in vivo. If the remodeling phase of the granulation tissue does not occur (with no apoptosis of the myofibroblasts and vascular cells present in the granulation tissue nor reorganization of the extracellular matrix), myofibroblasts may then persist, leading to pathological conditions characterized by excessive scarring

extracellular matrix. When the stimulus driving the response to injury persists in internal organs, excessive deposition of extracellular matrix then leads to organ fibrosis. As is observed in pathological healing in the skin, the occurrence of fibrosis and its chronic nature may be a consequence of an imbalance between matrix deposition and matrix degradation. The mechanism underlying this is most likely an imbalance in the levels of MMPs and their inhibitors, TIMPs.

Hypertrophic scars and keloids are both characterized by an abnormal accumulation of extracellular matrix; however, they represent two very different cutaneous pathologies. In the case of hypertrophic scars, these do not extend beyond the periphery of the lesion, while keloids do extend beyond the margins of the original lesion. α-Smooth muscle-positive myofibroblasts are abundant in hypertrophic scars, and these cells contract, inducing retraction of the scar, particularly when the scar tissue is located in a region that is subjected to mechanical tension. This is particularly the case when the scar is adjacent to joints including the shoulders, elbows, wrists, knees, and ankles. Conversely, retraction does not occur in keloids. The development of hypertrophic scars is frequently observed after severe burns, and it appears that prolonged inflammation contributes to the increased risk of hypertrophic scarring. Conversely chronic wounds, such as venous leg ulcers, can also show prolonged inflammation with the mixture of pro-inflammatory cytokines and MMPs leading to a failure of matrix deposition and repair. It is therefore suggested that after cleansing of the wound, treatment should be aimed at producing the most rapid recovery possible with the aim of limiting the time spent in the inflammatory stage of healing.

3.8 The Role of Mechanical Tension

Due to both their contractile nature and their intimate relationship with the extracellular matrix, myofibroblasts are sensitive to their mechanical environment, and mechanical signaling has been shown to play important roles in regulating the activity of myofibroblasts [10]. Differentiation markers of myofibroblasts, including stress fibers, ED-A fibronectin, and α-smooth muscle actin expression, appear earlier in granulation tissue

that is subjected to increased mechanical tension. This has been shown in experiments using splinting of a full-thickness wound with a plastic frame and comparing this to unsplinted, normally healing granulation tissue. Additionally, mechanical forces can be altered by culturing fibroblasts on substrates of varying stiffness, and these experiments have shown alterations in fibroblast phenotype dependent on substrate compliance. In culture, fibroblasts grown on soft, compliant substrates do not show stress fiber expression, while increasing the stiffness of the substrate induces a rapid change in morphology and the appearance of stress fibers. In cultured fibroblasts, shear forces, from movement of fluid, are also able to increase TGF-β1 synthesis and thus stimulate fibroblast differentiation to a myofibroblast phenotype. Shear forces are able to affect fibroblast differentiation in the absence of other stimuli that are normally involved in their differentiation, for example, exposure to cytokines or pre-straining of the extracellular matrix which regulates TGF-β1 bioavailability. As mentioned above, the role of mechanical stress in stimulating myofibroblast activity has also been shown in experiments using mechanically stressed skin wounds in mice. These wounds are stretched or splinted, resulting in an increased mechanical load that in turn increases myofibroblast activity and results in increased scar formation. These models to some extent mimic hypertrophic scarring that is sometimes observed in humans. The mechanical environment of the wound and the tension present are thus essential factors that need to be taken into account and managed in order to reduce scarring. To this end controlled immobilization of the wound should be employed. Interestingly, devices that manage the mechanical environment and tension of the wound are now appearing on the market (e.g., Embrace® from Neodyne Biosciences, Inc. or Zip® from ZipLine Medical, Inc.).

3.9 Role of Innervation in Skin Healing

Recently it has been shown that innervation of the skin plays an important role in both normal and pathological wound healing. However, the precise roles of sensory and autonomic innervation during wound healing remain to be clearly established [12]. Keratinocytes, melanocytes, fibroblasts, and myofibroblasts have all been shown to express a variety of neurotrophins including nerve growth factor, neurotophin-3, brain-derived neurotrophic factor, as well as their receptors, and these promote cellular proliferation and differentiation. Neuropeptides including calcitonin gene-related peptide, substance P, and vasoactive intestinal peptide are able to modulate the activity of MMP-2 and MMP-9, both of which play important roles in granulation tissue remodeling and scar formation. Additionally, these neuropeptides also act on collagen type I and type III synthesis during skin wound healing, promoting dermal fibroblast adhesion and their differentiation into myofibroblasts. The effects of these neuropeptides on the composition of the extracellular matrix and its organization are certainly essential since it is well-established that the mechanical microenvironment, which is dependent on the organization of the extracellular matrix, can affect fibroblast to myofibroblast differentiation. Lastly, regulation of MMPs can also affect the subsequent activation of latent TGF-β1 which involves MMPs.

Injury to the skin induces the release of a number of inflammatory mediators, from both immune cells and sensory nerve endings. These include interleukin-1β, tumor necrosis factor-α, bradykinin, substance P, calcitonin gene-related peptide, nerve growth factor, and prostaglandins, and their release by these cells contributes to the "inflammatory soup" present in the wound. It has been suggested that changes in substance P levels may be involved in the aberrant wound-healing response seen in hypertrophic scarring. Furthermore, it has been observed that in cocultures of fibroblasts and neurites, the direct contact of these cells is able to induce myofibroblast differentiation leading to increased retraction of collagen lattices, mimicking the contraction seen in wound repair.

In keloids, nerve fiber density is significantly higher than in normal skin samples, and symptoms including itch, pain, abnormal thermal sensory thresholds to heat as well as cold, and pain associated with heat sensation are all reported suggesting the involvement of small nerve fibers in the pathogenesis of this disease. In hypertrophic scars, published data are inconsistent with either a decrease or an increase of the number nerve fibers having been reported. Nevertheless, in burn patients with chronic pain, abnormal cutaneous innervation has been reported. A recent pilot study has been published which compared healthy skin versus postburn scars from the same patient [2]. These authors examined the expression of genes involved in regulation of innervation and additionally looked at the intraepidermal density of nerve endings. Significant differences in the patterns of expression were observed when comparing healthy skin and postburn scars. Based on studies of a mouse model of hypertrophic scarring induced by mechanical loading, it has been suggested that innervation of the skin and the level of inflammation present may both play roles in the development of hypertrophic scars.

The role of the sensory nervous system in wound healing in the skin has been examined using several ani-

mal models of denervation. Surgical denervation has been employed, as has chemical denervation, and mice with genetic modifications resulting in denervation have also been used. Studies using surgical denervation have shown that wound healing is retarded in these animals, with a reduction observed in the number of inflammatory cells infiltrating the wound, delayed contraction of the wound, and a delay in reepithelialization. Another skin denervation model, which used chemical sympathectomy induced by intraperitoneal administration of 6-hydroxydopamine, also showed that denervation interfered with wound healing. 6-Hydroxydopamine-induced sympathectomy has also been shown to modify wound healing with an increase in wound contraction, a reduction in mast cell migration and delayed reepithelialization. These alterations in healing are associated with a decrease in neurogenic inflammation. Lastly, these studies have definitively shown that neuropeptides released by sensory fibers play an important role during wound healing, affecting the granulation tissue in particular.

3.10 Therapeutic Options

It has been recently shown, in human pulmonary fibrosis, that mechanical stretching of tissue can contribute to the development of fibrosis via mechanical activation of TGF-β1 [5]. Stretching of the extracellular matrix in the lungs, due either to breathing or to mechanical ventilation that is employed to support breathing in cases of lung injury or lung disease, may contribute to TGF-β1-driven disease progression. Since it is not possible to stop breathing to avoid mechanical TGF-β1 activation from established fibrotic tissue, therapeutic intervention might however be possible aimed at the cellular side of mechanical TGF-β1 activation. Thus, in organs that are subjected to mechanical stress, such as the lungs and skin, the role of TGF-β1 in excessive scarring and fibrosis is clearly crucial, and the mechanisms involved in activation of TGF-β1 represent an important therapeutic target.

The presence and activity of fibroblasts is vital for normal skin homeostasis, while the presence of myofibroblasts is crucial for tissue repair and has evolved to speed the process of normal tissue repair [1]. The importance of fibroblast activity in normal repair has been very well documented using in vitro models of dermal substitutes. For example, a living dermal equivalent (containing fibroblasts) that has been applied to skin graft beds was found to reduce pain, improve hemostasis, and also improve the mechanical and cosmetic properties of the graft, in particular, producing a normal undulating dermal-epidermal junction by 3–4 months after grafting and also leading to the presence of elastic fibers, which were detectable 6–9 months after grafting. Therefore, it seems apparent that tissue engineering approaches to normal repair require fibroblasts and myofibroblasts to be effective [7]. It is, however, very important to keep in mind that many different populations of fibroblasts exist and that these have different properties. These recent observations thus offer new perspectives for skin repair and for tissue engineering. In addition, promotion of normal reinnervation and adequate levels of neuropeptides during the healing process certainly appear to be crucial for improving skin healing and to avoid the occurrence of pathological repair processes and scarring [13]. Despite the existence of many areas that still require elucidation in myofibroblast biology, it seems clear that myofibroblasts are pivotal cells for the control of extracellular matrix deposition and remodeling during normal repair and are also important in pathological conditions such as excessive scarring. Myofibroblasts thus definitively represent an essential target to be considered when developing new therapeutic strategies.

3.11 Conclusion

Myofibroblasts are key cells during wound healing. It has become increasingly evident that a lack of myofibroblast apoptosis is a major mechanism leading to excessive scarring [11]. Blocking pro-survival mechanisms, particularly those linked to the mechanical environment, and modifying the myofibroblast phenotype to obtain cells able to remodel the excessive deposition of extracellular matrix certainly represent new ways to develop therapeutic options that could positively modulate scar formation.

Take-Home Messages

- Myofibroblasts play a major role during granulation tissue formation with transforming growth factor-β1 being the main soluble factor involved in myofibroblastic differentiation.
- Myofibroblasts, through mechanotransduction pathways, are very sensitive to their mechanical environment.
- Myofibroblast apoptosis and extracellular matrix remodeling are necessary for normal scar formation.
- Abnormal mechanical tension facilitates the development of hypertrophic scars.
- Normal innervation and neuropeptide secretion are necessary to maintain skin homeostasis and normal wound healing.

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