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Title

Current and upcoming therapies to modulate skin scarring and fibrosis

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1

Abstract

Skin is the largest organ of the human body. Being the interface between the body and the outer environment, makes it susceptible to physical injury. To maintain life, nature has endowed skin with a fast healing response that invariably ends in the formation of scar at the wounded dermal area. In many cases, skin remodelling may be impaired, leading to local hypertrophic scars or keloids. One should also consider that the scarring process is part of the wound healing response, which always starts with inflammation. Thus, scarring can also be induced in the dermis, in the absence of an actual wound, during chronic inflammatory processes. Considering the significant portion of the population that is subject to abnormal scarring, this review critically discusses the state-of-the-art and upcoming therapies in skin scarring and fibrosis.

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[1] **Abbreviations: 5-FU:** 5-Flururoruracil; *α*-**SMA**: *α*- Smooth Muscle Actin; **AKT:** Protein Kinase B; **BLM:** Bleomycin; **BMP-1:** Bone Morphogenetic Protein 1; **CTGF/CCN2:** Connective Tissue Growth Factor; **ECM:** Extracellular Matrix; **EGFR:** Epidermal Growth Factor Receptor; **GF:** Growth Factor; **HA:** Hyaluronic Acid; **HGF:** Hepatocyte Growth Factor; **IGF-1:** Insulin-like Growth Factor 1; **IFN:** Interferon; **IL:** Interleukin; **KF:** Keloid-derived Fibroblasts; **LOX:** Lysyl Oxidase; **M6P:** Mannose-6-Phosphate; **MMP**: Matrix Metalloproteinase; **miRNA:** Micro RNA; **mRNA**: Messenger RNA; **mTOR:** Mammalian Target of Rapamycin; **NOX-4:** NADPH-Oxidase 4; **PAI1:** Plasminogen Activator Inhibitor 1; **PDGF:** Platelet-Derived Growth Factor; **PHI:** Prolyl Hydroxylase Inhibitor; **PI3K:** Phosphatidylinositol-4,5-Bisphosphate 3-Kinase; **PPAR:** Peroxisome Proliferator-Activated Receptors; **ROS:** Reactive Oxygen Species; **SiRNA:** Small Interfering RNA/Silencing RNA; **SMAD:** Small Mothers Against Decapentaplegic**; SSc**: Systemic Sclerosis; **TAC:** Triamcinolone Acetonide; **TGF-*β***: Transforming Growth Factor *β*; **Th:** T helper; **T-killer:** Cytotoxic T cell; **TLR:** Toll Like Receptor; **TNF-*α*:** Tumour Necrosis Factor α; **Treg:** Regulatory T cells; **Trm**: Resident Memory T cells

2

Contents

1   Introduction

Scar formation is the end result of the repair process after a tissue has been wounded. There is one exception to this feature in humans: the human foetus heals without, or almost without, scar formation in the first three months of a pregnancy [1]. Depending on the wounding mechanism, the scarring wound healing response has a pathological spectrum, ranging from cosmetic annoyance to grave functional impairment (i.e. scar traction across joints, impeding facial muscular movement). The classical wound healing stages in the skin that lead to scarring are well characterised [2-4]. It is however important to understand the stages of wound healing to identify key points for therapeutic intervention and to derive and employ efficacious pharmacological compounds (**Figure 1**). Thus, herein we briefly recount these phases. With the initial traumatic event comes bleeding and the initiation of coagulation cascade resulting in a blood clot (later, scab). Platelets are trapped within the blood clot and, in contact with exposed collagen, they bind to it and get activated, thus releasing multiple factors. As defined here, this represents stage zero of wound healing. The next stage, the inflammatory phase, sees the recruitment of cells of the innate immune system to the wound area (monocytes, neutrophil granulocytes), fighting local infection and phagocytosing local debris and damaged connective tissue, and also subsequently removing fibrin. The cells involved here produce inflammatory cytokines, such as transforming growth factor β (TGF-β) [5], interleukin 4 and 13 (IL-4 and IL-13) [6] and tumour necrosis factor α (TNF-α) [7]. This ushers in the proliferative phase, whereby fibroblasts enter the scene and begin to build up fresh connective tissue. For this to occur, these fibroblasts display an activated phenotype, the myofibroblast. Myofibroblasts are specialised contractile cells characterised by expression of α-smooth muscle actin (α-SMA) [8], a splice variant of fibronectin [9], an amine oxidase copper containing 3 protein [10] and a fibroblast activation protein [11] and produce and deposit large amounts of collagen. Myofibroblast origin can be traced back to different cells and it is still not completely understood. The major contribution comes from the *in situ* activation of resident fibroblasts in response to different triggers, such as TGF-β1, Jagged/Notch, Connective Tissue Growth Factor/CCN Family Member 2 (CTGF/CCN2), endothelin-

1, lysophosphatidic acid, and other signalling molecules, as well as hypoxia and mechanical stress due to increased extracellular matrix (ECM) stiffness [12]. Mesenchymal stem cells [13] and circulating cells, called fibrocytes, [14] have also been proposed as actors in the tissue repair process. Alternatively, myofibroblasts may arise from other cell types, including epithelial cells in the skin or lungs vascular smooth muscle cells and pericytes [12]. The cells partially break down collagen fibres and splice *de novo* collagen fibres with those bordering the tissue interruption. The fibroblasts are specialised to make connective tissue, and in the skin, the major component of the dermis, namely fibrillary collagen types I, III and V, but also basement collagens, such as type IV, and non-collagenous matrix molecules, such as fibronectin, fibrillin, elastin, not to forget proteoglycans, which play major role in storing growth factors (GF) and binding water molecules [15]. Collagen I represents the lion's share of the fibril forming collagens, while collagens III and V are admixtures and therefore contribute to the formation of heterotypic collagen fibrils [16]. The *de novo* deposition of collagen I and adjunct collagens and proteoglycans leads to the formation of a densely packed, collagen-rich ersatz tissue in place of the original microarchitectures [17]. Of note, both composition and microarchitecture deviate from the original connective tissue. The formed scar aims to restore tissue cohesion and replace lost tissue; preserving aesthetics and functionality, at this stage, have lower priority. The last step of wound healing is the remodelling phase. Here, the fibroblasts that have been active at the deposition of collagen / scar formation, retreat or go into apoptosis and remove some of the scar material [18]. Clinically, this is manifested as a palpable softening of the scar and the changing of its hue from pink to pale [19]. This colour change points to the role of microvasculature in the scar, which also needs to be re-established after a tissue defect has occurred. The proliferative phase is characterised not only by invasion and local proliferation of mesenchymal cells, but also of endothelial cells forming capillaries. It is remarkable that hypertrophic scars and in particular keloids show a high degree of hypervascularity, the latter essentially representing fibro-vascular tumours [20]. Sprouting and the formation of microvascular networks involve the activity of collagenases [matrix metalloproteinases (MMPs)], which contribute to the remodelling of the

formed scar. As hypervascularity recedes, the hue of the scar changes accordingly. The remodelling phase can take up to one year and only after this time a scar can be considered fully mature.

It has been claimed that the resulting scar tissue possesses about 80 % tensile strength of the original tissue [21], however, considering scarring as a quick repair system, it has been proven quite efficient in humans in the course of evolution. Actual tissue injury could also occur via infarction leading to acute tissue necrosis and re-perfusion injury, as has been observed in myocardial infarction [22]. The same wound healing stages apply in myocardial infraction, leading to the formation of a scar. The resulting scar can represent, depending on its size, a weakness in the heart wall, an impediment to normal movement, or disturbing electric signal conductance. Remodelling of the scar also plays a major role in recovering cardiac function [23].

From the wound stage model, it becomes obvious that an actual traumatic / wounding event is not required for a scarring process, as long as an inflammatory process can be initiated that can be maintained long enough to drive a fibroblastic proliferative state with subsequent continued ECM deposition. This is where a scarring process can involve a larger area of an organ, or affect it as a whole, with devastating consequences. We shall define here such a non-traumatic scarring process as fibrosis. On the basis of current health statistics from the U.S.A. government, 45 % of non-accident related deaths in the U.S.A are attributable to chronic fibro-proliferative disease [24]. Chronic inflammatory stimuli can be caused by toxins and viruses, exemplified by liver cirrhosis. Inhaled foreign bodies in the form of dust can cause silicosis of the lung. Irradiation of tissue for cancer treatment can lead to fibrotic transformation of the irradiated tissue volume [25]. In many cases, the underlying cause of a chronic fibrotic process cannot be identified with certainty. This is the case in idiopathic lung fibrosis, and particularly, in many chronic autoimmune diseases, including scleroderma, rheumatoid arthritis, Crohn's disease and systemic lupus erythematosus [26]. Of note, a tissue area undergoing fibrotic change might be a cradle for epithelial-mesenchymal or mesenchymal-epithelial transformation, preparing the ground for cancer formation. [27, 28].

Despite its enormous impact on human health, there has been no real breakthrough in fibrosis therapy. This may be due to the logic of antifibrotic treatment in general. Scar formation and fibrosis can be predicted in the case of implanting (often drug delivering) biomaterials or acute events like myocardial infarction or elective surgery. Thus, local or systemic delivery of antifibrotic substances in a more acute setting would have a prophylactic function. However, fibrotic diseases often have a smouldering course and can take decades to develop. Typically, patients will receive medical attention and a subsequent diagnosis only when the fibrotic process causes clinical symptoms [29]. At this stage, fibrosis might be quite advanced and current therapies can merely halt the progression. For example, at the time point of diagnosis of idiopathic lung fibrosis, the life expectancy of a freshly diagnosed patient ranges from 2.9 to 5 years [30]. Therefore, the clinical reality would postulate strategies to reverse a pre-existing fibrosis, which would be akin to initiate a remodelling process. This very feature would appear at the moment to be the holy grail of fibrosis treatment.

As we focus here on pathological scarring and fibrosis of the skin, we need to acknowledge that systemic sclerosis (SSc), scleroderma, keloid and hypertrophic scars, have different aetiologies. Whilst hypertrophic scars and keloids are localised [31], scleroderma can be limited to the dermis in circumscribed areas or affect the whole integument [32]. SSc, however, affects the whole skin and internal organs [33]. SSc is a chronic multi system autoimmune disorder characterised by overproduction of collagen from activated fibroblasts in the skin and some internal organs, microangiopathy and defects of the humoral and cellular immunity [34]. Scleroderma hallmarks are similar to SSc's, but without the involvement of internal organs [32]. Hypertrophic scars are raised, but unlike keloids, they stay within the confines of the original wound site and can undergo spontaneous regression [35]. The term keloid was first introduced in 1817 [36] to describe the lesions as chancroid and later used to refer to their 'crab claw-like appearance', as the original meaning in Greek would be. Keloids and hypertrophic scars generate after a disruption of skin integrity as a consequence of superficial or deep injuries, such as incisions, scratches and insect bites, but they can also occur after piercing, needle sticks, surgical procedures, thermal and chemical burns, or even

spontaneously after allergic reactions [31]. It is estimated that approximately 100 million people acquire scars each year after surgery [37], whilst burn wounds that do not heal within 21 days have 70 % or a greater risk to degenerate in hypertrophic scars [38]. Individuals of all ethnic backgrounds can form both types of scars as a familial predisposition, however the incidence of keloid formation is higher in pigmented ethnic groups than in whites, with an occurrence of 6 to 16 % [39, 40]. Additionally, people born with certain genetic disorders, such as Turner's syndrome, Opitz-Kaveggia syndrome, progeria and Rubinstein-Taybi syndrome have shown a tendency to develop keloids [41]. Intralesional corticosteroids injections have been the golden standard since the middle 1960's, however the mechanism of action of this treatment is mainly symptomatic, not completely understood and often followed by several side effects, such as atrophy of the surrounding normal skin, fat and muscle and osteoporosis and pain at the injection side [42]. Combination therapies, particularly surgical removal of scars followed by cryotherapy, pressure therapy, radiotherapy and application of silicon sheets [43, 44] seem to yield the highest rate of success and lowest recurrence rates, but a standardized therapeutic management of scars is still missing. Emerging techniques based on the use of inhibitors of TGF-β activation, IFN-γ and recombinant growth factors have been tested in clinical trials giving promising results [45, 46]). Current limitations in developing therapies for normal and pathological tissue repair are partially due to the broad range of imbalanced and interconnected signalling pathways underlying such pathologies, and the inherent difficulty in pinpointing exactly the affected pathways in each case.

All scarring conditions are associated with dysfunctional connective tissue metabolism, activated fibroblasts and excessive production of several ECM components [47]. This review will consider therapeutic approaches that tackle wound healing stages from the inflammatory phase onwards.

2   Methods of diagnosis and biomarker assessment

Although the main hallmarks of fibrotic conditions are fairly well established, they can reflect different stages of fibrotic progression and might be difficult to quantify at the early stage of the

disease. Therefore, there is interest in the search for different non-invasive and easily detectable biomarkers and methods for diagnosis of skin fibrosis, which can better illustrate the inflammatory and fibrotic activity in each specific case, thus helping in choosing the best possible therapeutic approach [48]. Several diagnostic methods and soluble biomarkers (e.g. MRI; use of a plicometer, elastometer or cutometer; analysis of the serum levels of TNF-*α,* procollagen precursors, different MMPs or Il- 6, 10, 4, 12, 13) have been correlated to some extent with fibrotic conditions, however their practical application can fall short due to their high costs, being very time-consuming and irreproducible between different observers, or just due to contradictory studies regarding the levels of the different markers assessed in a pathological condition [48, 49]. Currently, the most established and reliable methods of diagnosis and biomarkers assessed for skin fibrosis include: clinical assessment by the modified Rodnan score of skin thickness (current golden standard) [50], supported by its measurement using a Durometer (a device that applies pressure to the skin to measure its thickness) [51] and ultrasound [52]; determination of the levels of circulating fragments from collagen and collagen precursors [53], TGF-*β* [54], N-terminal CTGF [55] and MMP-9 [56] levels in serum; and TGF-*β* receptor presence in skin biopsies [57] as well as quantification of deposited collagen in skin biopsies [58]. More recently, promising new approaches, including the assessment of cartilage oligomeric matrix protein (COMP) levels [59], optical coherence tomography [60] and mRNA and gene expression analysis [61] have been positively correlated with the assessment of fibrotic conditions, paving the way for more efficient and personalised diagnosis methods.

3   Modulation of the inflammation phase

A wound healing sequence resulting in a tissue scar has local inflammation in a tissue as starting point; an actual trauma is not obligatory and can be replaced by other strong stimuli that set the cascade in motion. [62]. The recruitment of inflammatory cells and subsequent deposition of ECM following an injury is a physiological response in the process of wound healing, as cells in the vicinity of the wound become activated and migrate to fill the breach [63, 64]. In contrast to acute

9

inflammatory reactions, a pathogenic fibrotic response typically results from chronic inflammatory reactions that persists for several weeks or months and in which inflammation, tissue destruction and repair processes occur simultaneously (**Figure 2**) [26]. This leads to an aberrant production of growth factors, proteolytic enzymes, angiogenic factors and fibrogenic cytokines, which together stimulate the deposition of connective tissue that progressively remodels and destroys normal tissue architecture [65]. The evidence that inflammation is involved in the scarring process of skin fibrosis is copious; therefore, one widely-used therapeutic strategy is to target directly or indirectly the recruitment and persistence of inflammatory cells themselves or to interfere with inflammation mediators.

3.1   Targeting inflammatory cells

After injury that leads to bleeding, circulating platelets, activated upon encountering exposed ECM components and von Willebrand factor, start the coagulation process [62]. Following the proteolytic cleavage of prothrombin into thrombin, thrombin converts soluble fibrinogen into insoluble fibrin, which forms a fibrin clot together with aggregated platelets [26].

Neutrophils and monocytes are recruited to the site of the skin injury through the process of diapedesis, or extravasation from blood to tissues, due to the response of the activated complement pathway, degranulated platelets and by-products of bacterial degradation [26]. Neutrophils are the most abundant inflammatory cells at the early stages of wound healing. These cells are essential to amplify the wound-healing response by recruiting other inflammatory cells, such as monocyte-derived macrophages, to phagocytose the fibrin clot and eliminate tissue debris and dead cells.

Macrophages at the site of wound consist of two populations. The first is the resident tissue macrophage that is present in tissues at all times at low density and is indicated as M1 phenotype. The second major population is recruited by monocytes and is indicated as M2 phenotype [66]. There are different mediators that can stimulate macrophages differentiation into M1-macrophages, the most important being bacterial products like lipopolysaccharide and inflammatory cytokines like

10

interferon (IFN)-γ, whilst M2 macrophages are activated mainly by IL- 13 and 14 [67]. The two populations exhibit different functions: whilst M1 has antimicrobial properties, M2 is involved in wound healing, angiogenesis and the defence against parasitic infections, but also in allergy, asthma and fibrosis [66]. In a well-orchestrated situation, a balance between M1 and M2 macrophage populations is maintained throughout the wound repair process. During the inflammation phase, more M1 are needed for defence against possible pathogens and clearance of senescent cells, after which they undergo apoptosis. The few macrophages remaining in the wound area exert other functions that influence the wound healing process, like stimulation of collagen production, angiogenesis and reepithelialisation [68]. A switch between M1 and M2 phenotype is also possible as a consequence of the change in cytokine expression [69]. M2 macrophages are the main players of the proliferative phase and are intended to create an anti-inflammatory environment and promote healing and regeneration of wounds. Beside this, they are also a great source of TGF-β, which is involved in different aspects of wound repair, included wound contraction, ECM deposition, angiogenesis. If the injury persists, the chronic activation of M2 macrophages leads to continuous production of TGF-β and other GFs that promote proliferation of myofibroblasts and excessive ECM deposition [70].

Selectins are the mediators of neutrophil adhesion to endothelial cell and represent an interesting target for anti-inflammation strategies [71], with various inhibitors successfully tested in different models of chronic inflammation, such as monoclonal antibodies against the β2 integrin CD11/18 present on the neutrophil surface in clinical trials in phase I in a model of asthma [72], antibodies against the endothelial cell receptor intercellular adhesion molecule 1 in clinical trials in phase II in a model of smoldering multiple myeloma [73], monoclonal antibodies against the L-selectin receptor in a rabbit model of thromboembolic stroke in phase I [74], small molecules that mimic the binding site of selectin ligands, reducing selectin-mediated leukocyte adhesion and chronic inflammation in a mouse model of psoriasis in phase I [75], small molecule pan-selectin antagonist in phase II of clinical trials in models of psoriasis [76] and chronic obstructive pulmonary disease in phase II [77].

Leukotriene B4 (LTB4) receptor type I (BLT1), is also involved in recruitment of neutrophils and inflammatory cells, thus representing a clinical target for inflammatory based diseases, like asthma, arthritis and psoriasis [78-80]. Recently, evidence of the efficacy of BLT1 antagonists emerged for the treatment of lung fibrosis through decreased inflammation and alteration of TGF-β, IL-6, IL-13 and IFN-γ [81], making promising the use of this class of antagonist in other types of fibrosis as well. Whilst the influx of neutrophils, and monocytes as early cellular responders to wounding is essential in terms of fighting infections [82], their role in wound closure and subsequent scarring still remains controversial [63, 83].

Following infection, the localisation of neutrophils to the site of inflammation is crucial for clearance of the infection. Indeed, a reduction in neutrophil numbers in the blood leads to severe immunodeficiency in humans [84, 85]. However, when it comes to skin wound healing, neutrophils do not seem to play a major role. In the 1970's, it was carried out one of the first experiments to assess the neutrophil role in the wound healing process by depleting them with anti-neutrophil serum in a guinea pig model. These experiments showed that there was no difference between the control and the neutropenic wounds in terms of rate of wound debridement, cellularity or extent of the repair [86]. More recently, this has been confirmed using specific anti-mouse neutrophil antibodies and, furthermore, it has been proved that tissue repair is even more rapid in mice model, as long as sterile conditions are guaranteed [87]. This might be explained considering that neutrophils release oxidants, proteases and antimicrobial proteins that could be perpetual or non-resolving of the healing process. On the other side, the depletion of macrophages *vis-à-vis* wound healing outcome has given controversial results. Transgenic mice specifically depleted of macrophages that showed delayed re-epithelialization and reduced collagen deposition and angiogenesis [88-90]. In contrast, multiple studies in mice revealed that the depletion of the same cells resulted in a faster healing [91, 92]. A recent study developed a mouse model that allowed conditional depletion of macrophages at different stages of the repair, revealing that these cells exert a basic role in the early stage of repair, whereas a late stage depletion did not impact wound maturation [93]. Further studies have also highlighted a

12

different role for recruited and resident macrophages: whilst the former have often been reported to contribute to tissue injury and scar formation, the latter seem to have a more beneficial role in the healing process [94]. Thus, limiting the pro-inflammatory activity of recruited macrophages in late stages of the healing process might prove to be beneficial in chronic inflammatory and fibrotic diseases. Specific macrophage-targeted therapies have recently proved to be efficient in a variety of clinical indications, such as breast cancer [95] and diabetes [96] but, their application in skin fibrotic conditions has yet to be explored.

Activated macrophages and neutrophils release harmful products, such as reactive oxygen species (ROS) and nitrogen species that can further aggravate the inflammatory response, since they are interlinked with the activation of pro-inflammatory GFs and cytokines and the further progression of fibrosis [97]. Recently, some microRNAs (miRNAs) have been shown to be regulators of pro- and anti- fibrotic processes [98]. The expression of NADPH-oxidase 4, the major catalytic subunit of TGF-β-activated NOX involved in the production of ROS, can be down-regulated by specific miRNA called redoximiRS [99], which was able to modulate the TGF-β-induced transformation of human dermal fibroblasts in liver [100] and skin fibrosis [101].

Another class of cells involved in tissue repair are the T lymphocytes that become activated by the presentation of an antigen by the antigen presenting cells, such as dendritic cells or macrophages and are involved in direct microbial killing [102]. The category of effector T cell is broad and includes various cell types, such as T helper (Th), regulatory T cells, cytotoxic T cells (T-killer) and resident memory T cells (Trm). Th cells produce various cytokines, such as IL-4 and IL-13, that are considered pro-fibrotic and, since these cells can be found in proximity to fibroblasts, it has been hypothesised that their secretion products may trigger fibroblast shift to myofibroblasts [103]. T regulatory (Treg) cells are in charge of counterbalance abnormal activity of Th cells, with strong evidence that any impairment associated to Treg may lead to autoimmune diseases [104, 105]. For example, in SSc patients, autoimmune suppressive functions of Treg have been found to be diminished as a consequence of reduced CD69 surface expression and TGF-β secretion / expression [106]. A possible

treatment should aim to reduce self-reactive T cells and re-establish Treg role. On this line, Trichostatin A, a histone deacetylase inhibitor, permits the acetylation of Foxp3, a marker of Treg, thereby enhancing their function and expansion [107] and resulting in a promising therapy to treat inflammatory diseases.

3.2   Targeting the mediators of the inflammation response

Another strategy to suppress fibrotic degeneration comprises targeting and modulating essential mediators of the inflammation response, such as the genes responsible for cell migration, since inflammatory cells rely on this mechanism to reach the site of injury [108]. Some valid strategies to reduce inflammation-associated fibrosis might include the modulation of the toll like receptor 2/1 (TLR 2/1) and sphingosine 1-phosphate receptor signalling pathways, which have an important role in the regulation of dermal immune responses and cell motility [109], inhibition of Wiskott-Aldrich syndrome protein, involved in the recruitment of neutrophils and macrophages [110] and inhibition of microtubule polymerization with nocodazole [111].

Another category of mediators upregulated in inflammation and implicated in fibrotic degeneration includes fatty acid metabolites [112, 113]. Arachidonic acid, the main precursor, is transformed by lipoxygenase and cycloxygenase enzymes into inflammatory molecules, such as leukotrienes and prostaglandins [114]. The inhibition of these metabolites has shown promise in different pathophysiologies, including skin [115], lung [116], vascular [117] and cystic [118] fibrosis.

Thalidomide, a drug widely used in the 1960's as sedative and antiemetic in pregnant women, and later withdrawn from the market because of teratogenic side effects in new born [119], is now effectively used to treat a range of adult conditions, including multiple myeloma and complications of leprosy [120]. It was also reported to have a variety of biological effects, including anti-angiogenic, anti-inflammatory and immunomodulating properties [121]. Beside this, antifibrotic effects were recently reported, attributed to the ability of Thalidomide in inhibiting TGF-β1 expression and, as a consequence, decreasing fibronectin levels in a keloid mouse model [122]. Furthermore, ECM

14

fragments, including hyaluronan (HA), have been shown to actively drive chemokine and pro-inflammatory cytokine production by activation of macrophages [123].

Considering that TNF-α has been described as a powerful inflammation mediator, it is conceivable that interfering with it, either through antibodies or processing inhibitors, would ameliorate fibrotic processes. In fact the application of antibodies against TNF-α, here infliximab, was reported to be successful in cases of retroperitoneal fibrosis [124], sub-retinal fibrosis [125] silica-induced lung fibrosis [126], SSc and associated lung fibrosis [127] and localised scleroderma (morphea) [128, 129]. One open-label pilot study using infliximab in diffuse cutaneous SSc showed apparent stalling of the disease progress, and a drop of elevated biomarkers for collagen biosynthesis such as N-telopeptide of collagen III [130]. However, frequency of suspected infusion reactions may warrant additional immunosuppression in any future studies in SSc.

The recent use of Treg modulators and redoximiRS to regulate the production of ROS has emerged as promising therapies to target inflammation, whilst inflammatory mediators like genes involved in cells motility, fatty acid metabolites or ECM fragments have been modulated by the use of drugs, such as nocodazole and thalidomide. Other recent advancements in therapies targeting modulation of inflammation are further described in **Table 1**. The possibility to prevent fibrotic degeneration at early stages by modulating the inflammation response is an appealing scenario within the antifibrotic strategies, but much has still to be learned about the molecular pathways that disrupt the physiological inflammatory phase during repair, the specific mediators involved in the fibrotic shift and the exact time of action to drive a successful therapy. Furthermore, it is paramount that all these research findings are now extrapolated to the next level of clinical trials to widen the spectrum of available antifibrotic therapies in a meaningful and concrete way.

4 Modulation of the proliferative phase

Collagen is abundant in human tissue with 20 and 30 % of dry mass content [131], but its stoichiometric composition and microarchitecture make a local difference. Scar tissue is not only

characterised by a high concentration of collagen, but also by its difference in architecture: densely packed and parallel collagen fibres in tendon and corneal tissues vs a looser distribution of collagen in normal dermis tissue [132, 133]. The ratios of the different types of collagen can also be altered, with keloids presenting higher ratios of collagen I/III (possible to quantify, as described elsewhere [134, 135]) and cross-linking, contributing to an alteration in the normal tissue architecture [136]. This establishes collagen content, composition and architecture as hallmarks of scar formation. Therefore, the first point of interference would be to hinder collagen synthesis, extracellularisation and deposition, individually or simultaneously, to reduce local collagen concentration. Collagen is synthesised as a trimer composed of three pro-α chains which are assembled in the endoplasmic reticulum and post-translationally modified (prolyl hydroxylase, lysyl hydroxylase, glycosylation) immediately before three pro-α chains form a procollagen triple helix. Hydroxylation of prolyl residues ensures thermostability and thus intracellular unfolding of procollagen triple helices and therefore ensures export to the extracellular space. Here, C- and N- propeptides of procollagen trimers are enzymatically removed by bone morphogenetic protein 1 (BMP-1), so that collagen trimers result. The propeptides prevent premature collagen assembly. Upon their removal, a rapid supramolecular assembly of collagen triple helices ensues, leading to fibrillogenesis. These assemblies can be covalently cross-linked by lysyl oxidase (LOX) or transglutaminase 2 activities [137-139]. The stabilisation of collagen assemblies is also a valid point of interference in scarring, as less cross-linked collagen may be more susceptible to turn-over [140, 141] and remodelling, which, in turn is the last possible step of interference.

In the framework of scarring and fibrosis, there are several factors that can induce abnormal collagen deposition and organisation (**Figure 3**), leading to increased synthesis of collagen [142] (**Figure 4**), higher ratios of collagen I/III [136] and the formation of less organised and abnormally cross-linked collagen fibre bundles in fibrotic tissues, when compared to normal tissue [143]. A prominent example for such a factor causing aberrant collagen synthesis and deposition is TGF-β dysregulation, which plays a pivotal role in skin wound healing [144], but has also been proven to be involved in

16

the formation of scars [145]. In particular, it plays a role in generating the myofibroblast phenotype that is responsible for the collagen deposition and contractile forces in a healing wound [146] (**Figure 4**). This offers further opportunities to interfere with scarring: scavenging or neutralising fibrogenic GFs, or blocking their receptors, downregulating fibrogenic signalling pathways, and epigenetic reprogramming of myofibroblasts.

Although the inherent complexity of the subject makes it a difficult hurdle to surpass, it also opens up the door for different paths of research and therapy (**Table 2**), such as the use of INFs [147], corticosteroids [148], prolyl-4-hydroxylase inhibitors (PHI) [149], BMP-1 inhibitors [150, 151] and decorin [152, 153] among others. This prophylactic type of approach, although not always possible to apply, might prove to be advantageous in some cases, as it addresses the main outcome in a fibrotic condition, the excess of haphazardly synthesised and deposited collagen.

4.1 Inhibitors of post-translational modifiers of collagen

4.1.1 Prolyl hydroxylase inhibitors

One class of compounds that target the synthesis of collagen, known as PHIs, seems to show great promise in the treatment and prevention of skin fibrosis [149]. PHIs are a class of drugs that inhibit collagen prolyl 4-hydroxylases, which are iron (II) and α-ketoglutarate dependent dioxygenases [154] that are involved in the conversion of (2S)-proline residues into (2S,4R)-4-hydroxyproline residues, essential for stabilising the conformational structure of mature collagen triple helices [155].

One of the first described PHIs was alpha, alpha-dipyridyl [156]. Later on, pyridine-2,4-dicarboxylate was shown to be a selective suppressor of hydroxyprolyl biosynthesis, inhibiting its synthesis (essential for collagen formation) in dermal fibroblasts *in* vitro, while possessing low levels of cytotoxicity [157]. 5-oxaproline was also shown to be an inhibitor of prolyl 4-hydroxylase, leading to a decrease in the synthesis of 4-hydroxyproline and secreted collagen in dermal fibroblasts *in vitro* [158]. More recently, hydralazine has also exhibited its potential as a PHI. Fibroblasts treated with the drug *in vitro* showed marked deficiency of both hydroxyproline and hydroxylysine, two molecules

17

that are essential for proper collagen synthesis, resulting in deficient collagen biosynthesis [159, 160].

The concentration of hydralazine used plays an important role in the effect on collagen turnover, as seen in several *in vitro* studies [161, 162]. Hydralazine is a good example for indication discovery, as it has been previously approved for the treatment of high blood pressure [163] and heart failure [164], now paving the way for its application in skin fibrosis. Another PHI known as 1,4-dihydrophenanthrolin-4-one-3-carboxylic acid, was also shown to prevent the accumulation of collagen build up, supressing scavenger receptor A expression and limiting tissue ingrowth *in vivo* [165]. It is important to recognise that any drug that can chelate iron may also be classified as a PHI due to its necessity as a co-factor in collagen synthesis [166]. Interestingly, depending on their specificity, PHI can cross-react with the prolyl hydroxylase that modifies the angiogenic transcription factor hypoxia induce factor-α, thus potentially inducing angiogenesis and preventing scar formation [167, 168].

4.1.2 Lysine hydroxylase inhibitors

Hydroxylation of lysine residues is another essential step for the proper cross-linking and glycosylation of α-chains leading to the formation of functional collagen proteins [169, 170]. Like prolyl hydroxylases, these affect collagen in its pro-peptide stage, requiring the same co-factors to function [137]. Lysyl hydroxylase inhibitors, such as minoxidil, have shown to have potential beneficial effects on wound healing and regeneration *in vitro* by inhibiting the proliferation and migration of fibroblasts [171]. However, their exact potential as antifibrotic agents has yet to be elucidated [170], as they might lack the inhibitory efficacy necessary to have a therapeutic effect.

4.1.3 Inhibitors of procollagen conversion

Other important class of enzymatic inhibitors is the one targeting BMP-1 or procollagen C-proteinase, as it is also known [172]. This enzyme catalyses the proteolytic cleavage of the C-terminal propeptide of types I, II and III procollagens at the Gly-Asp and Arg-Asp sites, thus constituting an important

step in the formation of an insoluble collagen matrix [150] and their inhibitors showing great promise as antifibrotic compounds. Companies, such as Roche Bioscience [173, 174], Pfizer [175-178], Bayer AG [179] and FibroGen [180, 181], have investigated their potential therapeutic effects and more recently new classes of these compounds, such as sulphonamides [150] and succinyl hydroxamates [151], have surfaced. Using the latter as an example, it was observed that these compounds have high selectivity, with one of the compounds being specific to procollagen C-proteinase over other MMPs involved in wound healing and showing high effectiveness in crossing a skin *in vitro* model, while resulting in a decrease of collagen deposition in dermal fibroblasts *in vitro* [151].

4.1.4 Inhibitors of collagen cross-linking

LOX plays a major role in cross-linking of collagen assemblies [182, 183]. Its activity has been implicated in several fibrotic conditions and it has been proposed that inhibiting its activity could ameliorate collagen synthesis in fibrotic pathophysiologies [183, 184]. Despite most results being reported using LOX-2 inhibitors for peritoneal [185], cardiovascular [186], hepatic [187] or pulmonary fibrosis [188], it is hypothesised that similar results might be obtained in the future regarding skin fibrosis [189]. However, it is unclear if some of these compounds would ever be able to meet safety standards and reach the clinic. This is due to the fact that they are derived from plants such as the sweet pea (*Lathyrus odoratus*), whose consumption has been associated with lathyrism, a collagen cross-linking deficiency [190], caused by the presence of a LOX inhibiting peptide, beta amino proprionitrile [191], thus raising concerns regarding its application.

4.2   Modulation of myofibroblast activation

Myofibroblasts are activated fibroblasts, usually present in granulation tissue, that acquire a smooth muscle cell-like phenotype and are responsible for synthesising and depositing ECM components that replace the provisional matrix, playing a key role in the wound-healing process [146]. These cells have contractile properties, provided by the presence of microfilament bundles of α-SMA [192]. On

the third phase of the healing process, scar formation, a gradual remodelling of the granulation tissue and subsequent reepithelialisation take place. This process involves a progressive replacement of collagen type III for type I and production of elastin, with a normalisation of cell density through apoptosis of vascular cells and myofibroblasts [193]. However, if this process is disrupted, impaired granulation tissue remodelling and the formation of fibrotic tissue, such as in hypertrophic and keloid scars, can occur [143, 194]. The major features of this imbalance are myofibroblast hyperactivity, resistance to apoptosis and excessive collagen production [65, 195]. The major player in all the aforementioned processes is TGF-β1 [196], whose overexpression results in the abnormal synthesis of ECM components, such as fibrillary collagens and fibronectin and reduction of MMP activity [197]. Considering all these, it is normal that myofibroblast modulation has garnered attention as a potential mechanism of treatment for fibrosis [65].

One approach has focused on trying to block myofibroblast differentiation through inhibition of TGF-β1 signalling. It has been shown that peroxisome proliferator-activated receptors can play a role in modulating fibrosis, as they were found to be transcriptionally repressed in SSc, as well as in normal fibroblasts after TGF-β treatment, indicating a reciprocal inhibitory effect between them and illustrating the potential of PPAR treatment against fibrosis [198]. A study using a synthetic PPAR-γ agonist (2-cyano-3,12-dioxo-olean-1,9-dien-28-oic acid) has also observed an amelioration of fibrotic response in both normal and scleroderma explanted human skin fibroblasts and also on two mouse models of scleroderma [199].

A substance extracted from the Chinese herb *Radix Arnebiae*, shikonin, has been described as having anti-bacterial, anti-inflammatory, anti-angiogenic and anti-tumorigenic properties [200]. Recently, it has also been shown to attenuate the expression of TGF-β1 signalling-induced collagen and α-SMA expression, as well as cell contraction in hypertrophic-derived human skin fibroblasts, as it interferes with Small mothers against decapentaplegic (SMAD)/ERK signalling [201]. Although further testing in preclinical models is required, this substance seems to be a promising treatment agent for hypertrophic scarring.

One possible cause for the pathological role of myofibroblasts in fibrosis is their acquired resistance to apoptosis, due to down-regulation of p53 expression [202], a cell cycle gatekeeper associated with the activation of DNA repair mechanisms, cell cycle arrest and promotion of apoptosis [203]. Mechanical compression has been advocated as a possible approach to induce myofibroblast apoptosis [204, 205], due to activation of mechanoreceptors [206], leading to a decreased recurrence rate in keloids [205].

IL-4 and IL-13 are considered profibrotic cytokines since they activate the differentiation of fibroblasts into myofibroblasts and stimulate ECM production and deposition [207]. Inhibitors of IL-4 were found to reduce dermal fibrosis in a mouse model of scleroderma [208] and the blockade of CCL2, a key IL-13 regulated gene [209], and IL-13 [210] have been achieved with small molecules antagonist in asthma and rheumatoid arthritis conditions. Inhibitors of platelet-derived growth factor (PDGF) have also been successfully explored [211, 212]: the dual inhibition of Abelson kinase and PDGF, two key players of SSc, was accomplished by administration of Dasatinib and Nilotinib, tyrosine kinase inhibitors, usually used as chemotherapeutics for leukaemia, which reduced dermal thickness, the number of myofibroblast and the collagen content [212] in cultured fibroblasts derived from SSc patients and in a mouse model of bleomycin (BLM)-induced dermal fibrosis.

Histone deacetylases are responsible for the removal of acetyl groups from a histone molecule normally used to keep DNA tightly bound together [213]. Inhibitors of histone deacetylase therefore keep certain genes sterically accessible for continued transcription, but also exert effects on various non-histone proteins in the body that require acetylation [214]. Through inhibition of histone deacetylase, it is possible to limit cellular proliferation, and to modulate fibrosis-related gene transcription, thus having potential as an antifibrotic drug [215]. Trichostatin A is one of the most potent molecules that fall into this class of drugs, having been suggested as a potential antifibrotic compound due to its effect in decreasing collagen expression in SSc fibroblasts [216] and in *in vitro* models of fibrosis [217], while also decreasing ECM deposition in a mouse model of BLM induced skin fibrosis [216]. Another similar compound is valproic acid, which has been shown to promote

normal wound healing after radiation-induced injuries, preventing the formation of skin fibrosis in BALB/c mice [218]. Valproate is another example of an indication discovery as it is an approved drug for the treatment of epilepsy and bipolar disorder and to prevent migraine headaches [219]. It is useful for the prevention of seizures in those with absence seizures, partial seizures, and generalized seizures, with a new-found potential for the treatment of skin fibrosis.

4.3 Interfering with fibrogenic growth factors

Although a growing body of literature corroborates that inflammation plays an important role in fibrosis, it remains controversial which secreted factors (cytokines, growth factors, chemokines) are capable of locally activating resident or immigrating fibroblasts towards a fibrotic phenotype [220]. However, the role of some fibrogenic signalling molecules, including TGF-β [42], IL-4 and IL-13 [43], PDGF [44], TNF-α [45], CTGF, IGF-1, FGF and plasminogen activator inhibitor 1 (PAI1) [65, 221], have been well documented. The aforementioned GFs can also lead to increased myofibroblast activation and proliferation [146] or even increased matrix stiffness [222]. Matrix stiffness can play an important part in fibrosis, with certain ECM molecules, such as Fibulin-5, being upregulated in fibrotic conditions. Fibulin-5 is thought to be involved in the formation of elastic fibres, consequently causing increased tissue stiffness, which might lead to chronic inflammation, and further fibroblast activation, thus perpetuating this positive feedback fibrotic loop. As such, these molecules might also constitute important targets of interest in the development of new antifibrotic therapies [223].

Although several GFs can be associated with fibrosis, the prominent role of TGF-β in the development of the various forms of fibrotic pathologies is widely recognised [65]. TGF-β possesses 3 isoforms, with forms 1 and 2 being involved in the downstream signalling activation of SMAD and Wnt complexes, leading to the activation of fibroblasts, whilst form 3 acts as a receptor antagonist, resulting in inactivation of the signalling pathway [224, 225]. It is thought that a different isoform expression profile present in early gestational phases is responsible for a scar-free healing process, opposed to what happens normally [226].

TGF-β signalling begins by the binding of its ligands to receptors in the cell membrane (with TGF-β1 being stored in the ECM in a latent form that can be posteriorly recruited [227], where they can be phosphorylated and then lead to the activation of SMAD proteins [5]. These proteins are intracellular cytoplasmic messengers that act as nuclear transcription factors after activation [228] and transducers of the TGF-β signalling pathway [229]. They can either act as downstream messengers of the TGF-β signalling pathway (SMAD 2 and 3) or inhibit it (SMAD 7) [5], thus becoming attractive targets for pharmacological intervention, even if its therapeutic use has been hampered with challenges associated with its multifunctional nature [65]. Some of the first approaches to TGF-β modulation involved the use of antagonists of TGF-β receptors or even TGF-β specific antibodies, which have failed to reach clinical studies due to potential safety liabilities [230, 231]. More refined approaches aim to decrease TGF-β downstream signalling or even prevent its activation, thus circumventing some of the potential adverse side effects resulting from TGF-β inactivation. Regarding decreased TGF-β signalling, some studies have attempted to block some of the signalling molecules in the TGF-β pathway: SMAD 3, with inhibitors such as proteins of the Tryptophan Regulated Attenuation Protein 1 family, halofuginone, quercetin, trichostatin A and paclitaxel [216, 232-239] or SMAD 4, through the transfection of a mutant SMAD 4 gene using an adenovirus vector [240]. Another method involves the upregulation of SMAD 7, an inhibitor of TGF-β family-induced signals [241], through the use of asiaticoside [242, 243], tetrandrine [244] or even IFN-γ [245, 246], with overall reduction of collagen synthesis *in vitro*. On the other hand, some studies have also tried to prevent TGF-β1 and TGF-β2 activation, using molecules, such as mannose-6-phosphate (M6P) analogues [247], through inhibition of dipeptidyl peptidase IV-like enzymatic activity [248], administration of hepatocyte growth factor (HGF) [249], Tamoxifen [250, 251] or Tacrolimus [252]. Other strategies involve the activation of TGF-β3 by using avotermin, (successful in phase I and II clinical studies, failed in phase III) [253-256] or even potentially recurring to genetic therapy using miRNA (miRNA-4269, miRNA-382, miRNA-203, miRNA-205 and miRNA-29) associated to collagen synthesis in keloid scars and systemic sclerosis [257, 258]. Several new

peptides have also been proposed as new potential targets for scar treatment strategies, including DS-SILY [259, 260], Cav-1 cell-permeable peptides [261], AZX100, a heat shock protein analogue [262, 263] and thymosin β (4) [264]. Within the TGF-β based anti-scarring drugs, local application of M6P has been used in clinical trials (phase I and II) to accelerate the closure of split thickness skin grafts [45]. M6P binds to the cation-independent M6P receptor in the cell membrane, modulating the activation of a latent precursor of TGF-β, thus inhibiting TGF-β1 and TGF-β2 [247]. P144 peptide, a TGF-β1 inhibitor, is another promising anti fibrotic candidate tested in clinical trials Phase II in skin fibrosis by topical administration [265].

SMAD 3, a mediator in the TGF-β pathway, has been efficiently inhibited by quercetin [266] and paclitaxel [267], between other molecules, resulting in decreased collagen production in hypertrophic scars and keloids. Decorin, a proteoglycan [152], and Tacrolimus, an immunomodulator drug [252], are also known to suppress TGF-β activity and collagen synthesis in fibrotic conditions.

Several signalling pathways can be involved in the genesis of a fibrotic response, while at the same time, different parallel pathways can also be involved during this process, resulting from an imbalance of pro- and anti- fibrotic mediators, from which GFs play a major role [65]. In order to effectively modulate these processes, two possible approaches emerge as the most promising: using a therapeutic agent that can bind to more than one target or modulating a single target that can control several signalling pathways.

One prominent example of a therapeutic agent that can bind more than one target is decorin, a small, leucine-rich proteoglycan [268] which is a ubiquitous component of the interstitial matrix of the dermis and preferentially associates with collagen fibrils [152], modulating their assembly [269, 270]. It may also interfere with ECM production through its ability to inactivate several growth factors, such as TGF-β [269], CTGF/CCN2 [271] or even a myriad of cell surface receptors, including IGF-1 [272], epidermal growth factor receptor (EGFR) [273] and HGF receptor [274].

Considering its wide spectrum of action, it is easy to understand why decorin would be chosen as a potential therapeutic against skin fibrosis, despite already being a ubiquitous component of the ECM,

as when administered systemically it naturally targets blood vessels through protein dependent interactions, then passing to the wound site [275]. Several studies have used purified recombinant forms of decorin [276], namely with a wound-targeting peptide CAR [275, 277, 278] or even using microRNA to regulate decorin production [279]. In a study that blocked decorin by downregulating microRNA it was possible to increase decorin expression and decrease myofibroblast differentiation in hypertrophic scar-derived fibroblasts, demonstrating its therapeutic potential [279]. Studies using recombinant decorin have shown decreased contraction of collagen gels by both normal and hypertrophic scar fibroblasts, while also reducing the levels of α-SMA and plasminogen activator inhibitor *in vitro* [276], while *in vivo* it led to selective accumulation of the recombinant protein in wounds, promoting wound healing and suppressing scar formation [275].

An example of an approach using a single target capable of modulating several signalling pathways involves the modulation of the Phosphatidylinositol-4,5-Bisphosphate 3-Kinase/ Protein Kinase B (PI3K/AKT) signalling pathway, which may contribute to myofibroblast resistance to apoptosis [280]. Another target of this pathway is the mammalian target of rapamycin (mTOR) [281], involved in the regulation of cell growth, proliferation, motility and survival [282]. It has also been identified as a regulator of collagen type I expression in dermal fibroblasts [283], with increased expression in keloid tissues [282]. Sirolimus (rapamycin) is a macrolide antibiotic that can act as an inhibitor of mTOR [282] and PDGF [284], leading to a decrease of collagen and α-SMA expression in normal and keloid-derived fibroblasts (KF), as well as inhibiting ECM deposition both *in vitro* and *in vivo* [284]. Other inhibitors of the mTOR complexes have also shown potential as therapeutics for fibrosis, after inhibiting KF cell attachment, spreading and proliferation both *in vitro* and *ex vivo* [285].

CTGF is another hallmark of fibrosis in multiple tissues, including skin, heart, lung and kidney [286]. It is a co-factor of TGF-β, which acts through integrins and heparan sulphate proteoglycans to directly promote fibroblast adhesion. It has also been proved that CTGF contribute to myofibroblast recruitment in a BLM-induced skin fibrosis mouse model [287]. Pamrevlumab, a human antibody against CTGF/CCN2 currently under clinical trials Phase 2 for the treatment of idiopathic pulmonary

fibrosis [288], pancreatic cancer [289] and Duchenne muscular dystrophy [290] was recently tested in a skin fibrosis mouse model. The use of Pamrevlumab was shown to mitigate the effects of angiotensin II-induced systemic sclerosis, being comparable to the effects of genetic depletion of CTGF, resulting in reduced inflammation, myofibroblast accumulation in the skin, dermal thickness and decreased levels of CTGF and collagen [291].

Despite considerable challenges in the development of successful fibrosis treatments targeting imbalances in the proliferative phase, some therapies have managed to reach clinical studies, such as corticosteroids (phase IV) [292], cancer chemotherapeutics (phase IV) [293], inhibitors of TGF-β activation (phase II) [45], IFN-γ (phase II) [294] and recombinant GFs (phase II) [46], illustrating the potential of this type of intervention in the prevention and treatment of fibrotic conditions.

Several signalling pathways and GFs are involved in the genesis of fibrotic pathologies and often they are intertwined. This leads to an increased difficulty in developing new therapies, as modulating a single upstream element of these signalling networks can have vast ramifications in other cellular mechanisms. This can give rise to several potential side effects jeopardizing the success of therapies that are focused on a single molecular target (e.g. increased risk for tumour growth when using growth factor therapy or higher risk of morbidity and mortality). Many of the current therapies also fail to achieve clinically relevant results, as the modulation of a single element seems to be insufficient in most cases, due to heterogeneity in the different fibrotic conditions [295]. Therefore, it is imperative that the focus of new therapeutic approaches shifts from single to multi-target modulation therapies, capable of maximizing synergistic effects and reducing harmful side effects [296].

5   Post-scarring therapies to emulate remodelling

After the proliferative phase, scar formation can undergo a third phase of remodelling, with reduction of deposited scar tissue by MMP activity, which can last up to one year after wounding. This can result in the formation of a more mature scar tissue, failing again to achieve normal architecture of collagen fibre deposition [297]. Treatments intervening in this phase aim to reduce scar tissue and

revert collagen architecture, even preventing scar recurrence. From a clinical point of view, this would be considered a late intervention [35]. The treatment of scars at this stage relies heavily on the use of drug injections (triamcinolone, TAC) given trans-dermally [298], which can be quite painful. Several materials can also be used as drug carriers to cross the basement membrane. Occlusive dressings, such as silicone dressings, are also one of the gold standards for the treatment of raised scars. They are considered as a type of mechanical therapy and due to the fact that their mechanism of action is still not fully understood, their therapeutic use is still questionable [35]. Overall, several current and upcoming treatments aim to reverse scar formation and restore normal ECM architecture, namely collagen, fibrillin and elastin composition, and MMP activity, thus reversing the pathological phenotype present in fibrosis (**Figure 5**, **Table 3**).

5.1   Transdermal injections

After the process of scarring has been completed, there is a significant build up in the amount of collagen found in the affected area, compromising the skin's function and mechanical properties, resulting in cosmetic or functional tissue/organ impairment for the patient [35]. Treatment after scar formation can consist of either partial correction by surgery or, in many cases, injection of steroids directly into the scar tissue.

Intralesional corticosteroid injections have been the traditional treatment for scarring diseases since the 1960 [299], whilst topical administration of creams containing corticosteroids did not show the same rate of success [300]. Most of the known effects of corticosteroids are considered to result primarily from the suppressive effects on the inflammatory response and secondarily from reduced collagen and glycosaminoglycan synthesis and inhibition of fibroblast proliferation [301], however, the mechanism of action, as well as the most appropriate dosage, are still not certain. Corticosteroids, including hydrocortisone acetate, dexamethasone, methylprednisolone and TAC, have been the most widely used [302]. Although they have been shown to be fairly effective, they are associated with side effects, such as atrophy of the surrounding normal skin, fat and muscle, osteoporosis, glucose

intolerance, glaucoma, and pain at the injection side. One of the most used drugs in this process is TAC [299, 303]. TAC has been known to reduce fibroblast proliferation as well as modulate the activity of key factors, such as vascular endothelial growth factor (VEGF), TGF-β1 and the collagen degrading MMP-2 [304]. Other drugs, such as 5-FU, a chemotherapeutic agent, have been shown to have beneficial effects when combined with TAC and applied to a model of dermal keloid scarring *in vitro* [305]. This effect was the rational for the combination of both drugs in a potential treatment to reduce scar elevation and the recurrence of keloids in a clinical setting. However, the progression of this multi-approach therapy has been somewhat hindered by its undesirable side effects, since some studies have reported hyperpigmentation, local pain sensation and the occurrence of superficial ulceration [306]. Shorter-acting corticosteroids (e.g. dexamethasone or dexamethasone acetate) are sometimes administered in conjunction with TAC, as their administration was shown to be more effective than single administration treatments [307].

TAC has also shown promise when it was used in combination with the antibiotic glycopeptide BLM. A long-term study (phase II) investigated the effect of repeated injections of both TAC and BLM in small doses, every 3 months, for a period of up to 2 years. The results showed almost all of the keloids (over 97 %) softening after the first dosage. The exact manner by which BLM exerted these effects has not been fully elucidated, but it was hypothesised that the drug inhibited further collagen synthesis by TGF-β-activated fibroblasts directly, either by reduction in lysyl oxidase levels, or by increasing fibroblast apoptosis [308]. It has been concluded from previous studies (phase II) that BLM also produces very little side-effects and is generally well-tolerated in the human body [309]. Further investigation will be required to pinpoint the exact pathway altered by this combination.

The role of histone deacetylase inhibitors has been previously described regarding their effect during the proliferative phase; however, they can also be efficient during the remodelling phase, when scar tissue is already established. In a rabbit ear model of dermal scarring, it was shown that trichostatin A reduced the scar elevation index after intradermal injection into a re-epithelialized wound [310]. This reduction in scar elevation was credited to the reduction of collagen type 1 and fibronectin

expression and opened up the possibility of scar reduction following its formation. The drug has also seen similar success for the treatment of keloid scars and tumour cells in both *in vitro* and *in vivo* studies via the alteration of TGF-β1 activated collagen synthesis [311], thus addressing the issue of fibrosis both before and after collagen synthesis and in both healthy and pathological fibroblasts.

Hepatocyte growth factor (HGF), which has been reported to have mitogenic, morphogenic and anti-apoptotic properties [312, 313], has also been proposed as a potential antifibrotic treatment [314, 315], considering its role in the metabolism of collagen fibrils [316] and regulation of TGF-β1 [317]. Some studies have also assessed the effects of subcutaneous injections of HGF on the treatment of already established skin scars [318] or injection of vectors containing the HGF gene in scleroderma [319]. In the first case it was observed that treatment resulted in thinner collagen fibres and a significant decrease in scar elevation and formation, when compared to the control [318]. On the other hand, it was also observed that transfection with the HGF gene resulted in a decrease of hypodermal thickness, accompanied by a decrease in the expression of IL-4 and TGF-β1 mRNA, thus illustrating its potential as a therapeutic for skin fibrosis.

Despite transdermal injections being widely used and reasonably effective in promoting scar reduction and regression, the occurrence of several undesirable side effects and high recurrence rates severely hinder its therapeutic use in skin fibrosis. Therefore, the use of new delivery vehicles or complementary therapies that can mitigate the occurrence of side effects and recurrence rates is paramount for developing a more effective treatment.

5.2   Biomaterial-based approaches

Following the appearance of scar tissue on skin, it can often happen that the only option for the replenishment of damaged tissue is skin transplantation via surgical methods. This procedure is far from perfect and often leaves the patient with severe skin abnormalities and unwanted cosmetic differences, not to mention the high recurrence rates after surgery [320]. Advancements in the fields of tissue engineering and scaffolding techniques are bringing more alternative treatments closer to

becoming a therapeutic reality. The functionalisation of these biomaterials with various drugs and growth factors provides a well-controlled method of scar treatments.

Functionalised wound dressing have proven to accelerate wound healing, angiogenesis, regulate newly formed forms of collagen and improve the structure of already formed collagen to reduce its bulk around the site of injury [321]. In combination with techniques such as electrospinning, which uses electrical forces to create fibrous scaffolds in a nano to micro scale with controlled porosity [322], a form of advanced healing bandage can be fabricated. These scaffolds can be effective due to their capacity to mimic the natural ECM architecture [323], providing mechanical strength [324], supporting cell adhesion and proliferation, preventing desiccation and providing coverage, leading to tissue repair *in vivo* [325]. Their porous nature also enables controlled drug loading and release at the site of injury, improving their therapeutic potential for the delivery of antifibrotic compounds. One such example is seen in a study investigating the benefits of a silk-fibroin/gelatin electrospun nano-fibrous dressing that was functionalised with astragaloside IV [326], which has been previously shown to promote healing and inhibit full-thickness scar formation, due to its promotion of re-epithelization, angiogenesis and reorganisation of the ECM, by decreasing TGF-β1 secretion and the ratio of collagen type I/III [327]. The poorly soluble drug was applied to a deep partial thickness burn wound through this scaffold. The dressing demonstrated the ability to significantly reduce scar formation and enhance wound closure *in vivo* and was highly effective in delivering the fragile drug without any unwanted complications.

Another example of wound dressing is represented by a study on decellularised scaffolds obtained from pig peritoneum that was loaded with HA and epidermal growth factor (EGF) [328]. HA is a major component of the ECM and is implicated in the process of wound healing [329, 330], while EGF has roles in the migration of fibroblasts and proliferation of vascular endothelial cells [331]. The animal study compared two groups of rabbit spinal cord injury and reported significant enhancement of wound healing in those who had been treated with the HA/EGF scaffold. The synergistic effects

of the two molecules improved re-epithelisation and granulation tissue formation after injury in comparison to the untreated group. Further studies must be carried before bringing this to clinic [328]. The use of IFN-γ-loaded collagen scaffolds has also been shown to reduce total myofibroblast numbers following surgical procedures in a rat model of cleft palate repair [332]. Although seemingly contradictory, the use of collagen-based scaffolds can be beneficial in wound healing and treatment of fibrosis, considering its low immunogenicity and capacity to be degraded *in vivo* in a controlled manner, thus reducing inflammatory response and promoting wound healing and scar resolution [333]. A plastically compressed collagen system has also been widely described for the construction of cellularised scaffolds that can better mimic the *in vivo* architecture, which can then be used in regenerative therapies [334]. The group received the scaffold treatment experienced a more rapid influx of host cells and a marked reduction in myofibroblast levels. These results indicate that this type of scaffold is appropriate for use in oral surgery techniques, as repeated injections of IFN-γ can delay the wound healing process. Several other studies have confirmed the effectiveness and bioavailability of collagen as a scaffold, particularly in combination with IFN-γ [335, 336].

Several commercially available bilayer-wound dressings, such as Integra™ Bilayer Matrix Wound Dressing (a porous matrix of cross-linked bovine tendon collagen I and glycosaminoglycan and a semi-permeable polysiloxane) and Apligraf® (a living skin substitute, composed of a bottom layer of bovine collagen I seeded with human fibroblasts and an upper layer of keratinocytes) have also been in large keloids, following surgical resection and adjuvant corticosteroid therapy [337]. Although initially designed for the treatment of traumatic (e.g. abrasions, burn wounds), chronic (e.g. ulcers) or surgical (e.g. donor site/grafts wounds) wounds, these products can also have a positive effect on the treatment of fibrotic conditions, such as keloids, due to their capacity to promote cellular invasion and capillary growth, resulting in wound healing with a low prevalence of fibrosis [338, 339]. As such, they are of particular interest in the treatment of these conditions after surgical excision and when combined with adjuvant corticosteroid treatment.

31

Although therapies that prevent abnormal scar formation altogether are preferable, with a focus on targeting earlier molecular pathways, this is not possible in most cases in clinic, as patients tend to only seek assistance at a later stage of their pathology, when scar formation has already occurred. Despite this, several treatments are already applied in clinic to target fibrotic tissues, such as intradermal injections of steroids or functionalised wound dressings. In the future, it is expected that such treatments can combine wound healing and revert abnormal scar formation, leading to a more integrated approach against dermal fibrosis [65].

5.3   Non-pharmacological approaches

A variety of non-pharmacological approaches against skin fibrosis have also been described, with different degrees of success. These include minimally invasive therapies, such as topical application of onion extracts (phase II) [340], cryosurgery [341] and laser therapy [342]; and surgical approaches, such as excisions, typically followed by reconstruction with a skin graft (phase II) [343], radiotherapy [44], administration of corticosteroids [344] or silicon sheeting [345]. Some of these methods are painful and can result in high scar recurrence rates when adopted as monotherapy, however interesting combinations of them have proved to considerably improve final outcomes. Non-pharmacological approaches have also proved to be advantageous when compared to pharmacological treatments by reducing the occurrence of side effects, associated for example to corticosteroid injections, and by limiting the extent of the treatment to the site of scarring.

Liquid silicone made its appearance as therapy in the 1970s, followed by topical silicone sheets soon after. It is now widely accepted as an efficacious treatment of scar tissues, despite its exact mechanism of action still being unknown. It is thought that silicone sheets work by acting as an occlusive barrier to the stratum corneum, and by limiting oedema infiltration, they can reduce fibroblast proliferation and collagen deposition [346].

Surgical excisions of scars consist in full or partial removal of scarring tissue and are usually performed with adjuvant therapies, since the recurrence rate of the excision alone is ranging from 45

% to 100 % [347]. On the other hand, surgical excision followed by radiotherapy or application of silicone gel / sheeting is the most successful treatment for keloid scars, according to the international advisory panel on scar management with recurrence rates ranging from 0 % to 8.6 % [348].

Radiotherapy is a common adjuvant post-operative method with low recurrence rate and a mechanism of action still unknown. One shared theory is that radiotherapy prevents fibroblast repopulation after the excision or modulates cellular factors involved in fibroblast recruitments [349]. Radiation was traditionally applied by external devices, but the high irradiation dose required and the unnecessary overexposure of the surrounding tissue prompted the investigation of an internal radiation therapy, called 'brachytherapy' [350]. In this set-up, a catheter is incorporated into the wounded area, allowing the radiation source to be directed from the inside and leading to a more targeted treatment [44].

Another option for the treatment of fibrosis is cryosurgery. First introduced as monotherapy in the 1980s' [351], cryosurgery is thought to act by provoking cellular injury and necrosis of the injured tissue through primary cellular dehydration, which causes high solute concentrations within the cell and subsequent damage. This leads to the dysfunction of cellular membranes and the penetration of ice crystals inside the cells with lethal damage [352]. For decades, liquid nitrogen has been applied externally with a contact probe, leading to major damage to the skin, blistering and infections. Intralesional cryosurgery is gaining pace as has no side effects, due to an internal application of liquid nitrogen by using injection needles or internal cryoprobes [341].

Various laser and light approaches have also been explored for the treatment of fibrotic tissue, including argon, carbon dioxide and pulsed dye laser [342]. Following laser / tissue interaction, a photo-thermal effect is produced by directing energy to specific chromophores of the skin and avoiding collateral damage to the surrounding tissue. The first lasers made their appearance in the early 1980's and were based on argon, carbon dioxide and neodymium:yttrium-aluminum-garnet, but the early results were not convincing since the recurrence rate was between 36 % and 45 % [353]. Newest laser-based methods are based on pulsed and fractional lasers [354] and already showed promising results (phase II). Laser devices emit light directed to the skin, which produce

microthermal zones and thermal injury. The healing process of new collagen deposition and fibroblast proliferation takes place in these zones quicker than the ablative resurfacing, ensuring amelioration of skin appearance and stimulation of re-pigmentation [355].

Recently, a drug-free-patch was shown to reduce scar growth, through physical contact alone, particularly in the case of keloid and hypertrophic scars [356]. The self-manageable device, comprised of microneedles of a liquid crystal polymer Vectra® MT1300, is capable of significantly reducing dermal fibroblast proliferation activity *in vitro,* while *in vivo* it prevented dermis thickening, decreased the number of infiltrated inflammatory cells, with less disrupted dermis tissue architecture and presenting a more flattened appearance.

Successful examples of scar removal have been achieved by combinatorial approaches of the aforementioned therapies (**Table 3**), some of them already in clinical trials. A recent work has gathered updated fibrosis treatment algorithms based on the analysis of the size of the lesions, the outcomes, the recurrence rates and the satisfaction of the patients [298]. Optimal treatments continue to be represented by surgical approaches with adjuvant therapies, however the standard or ideal approach has yet to be defined. Therapies that directly target molecular imbalances are emerging and they are expected to prevent scar formation, that way rendering post-scarring treatments unnecessary. However, the complete prevention of scar formation is still several years away, so, in the meantime, combinatory or adjuvant therapies play a prominent role in the treatment of already established fibrotic scars.

6   Conclusions and future perspectives

To-date, there has been no drug approved as a complete inhibitor of fibrosis [357, 358]. Current limitations in the treatment of chronic and recurring fibrotic wounds can be accredited to both an incomplete understanding of the exact molecular mechanisms hindering repair and to the broad range of imbalanced signalling pathways underlying such pathologies. Skin fibrosis can have its genesis in

imbalances arising during several stages of the wound healing process (e.g. coagulation, inflammation, remodelling).

The possibility to prevent fibrotic degeneration at early stages by modulating the inflammation response and the proliferative phase is an appealing scenario within the antifibrotic strategies, with several drugs being tested in clinical trials. However, this is still far from being the golden standard in the treatment of fibrosis as at this stage it is still an asymptomatic condition, with limited and costly prospects of early diagnosis. Therefore, the majority of current and upcoming treatments act merely after scar formation has already taken place, aiming to reverse the fibrotic phenotype and restore normal ECM composition and architecture.

Considering the inherent complexity of fibrotic pathologies , we hypothesise that for their successful treatment, several signalling pathways must be taken into account and novel co-delivery therapies should be employed for increased synergistic effects, with the development of advanced multi-delivery vehicles taking centre stage [296]. Considering that several factors can act as trigger for fibrotic response and that these can vary greatly amongst patients, an emphasis on the development of custom-made diagnostic methods (e.g. RNA microarray screening) should also play a more prominent role, paving the way for an era of personalised treatment for skin fibrosis [359].

As the understanding of fibrosis evolves and new diagnosis become available and more accessible, more and more therapies are predicted to make their way into clinic, greatly reducing the burden imposed by these debilitating pathologies.

7　Figure legends

Figure 1: Pathological scarring cycle and associated therapeutic targets. Scarring phenomena share three common phases after wounding: chronic inflammation, proliferative and remodelling phase. In this review, the different therapeutic approaches that can be pursued in each phase will be highlighted, by discussing their advantages and limitations, for a better understanding of the therapeutic potential for the treatment of skin fibrosis.

Figure 2: Modulators of chronic inflammation in skin fibrosis. Different mediators can directly (e.g. immune cells, cytokines, reactive oxygen species and growth factors) or indirectly (e.g. genes involved in cell motility, fatty acid metabolites and ECM fragments) intervene in the exacerbation of inflammation, promoting the shift from acute to chronic inflammation. This makes them attractive targets for therapeutic intervention.

Figure 3: Modulators of the proliferative phase in skin fibrosis. Several mediators can lead to myofibroblast activation; increased collagen synthesis, secretion and deposition, amplified fibroblast adhesion and proliferation whilst inhibiting apoptosis and MMP activity, resulting in a prolonged fibrotic phenotype. Therefore, several of these molecules constitute important therapeutic targets for the treatment of fibrosis.

Figure 4: Mechanism of collagen fibre formation and fibroblast activation into myofibroblast. Glycosylation and Hydroxylation of Proline and Lysine residues lead to the formation of collagen α-chains, which can then assemble in a triple helix formation, leading to the formation of procollagen, which will be further processed and cleaved, leading to the formation of collagen fibrils that can then assemble in bundles, finally resulting in collagen fibres. Myofibroblast activation resulting from the action of several growth factors and in some cases, prolonged inflammation, leading to increased matrix synthesis and cellular proliferation and resistance to apoptosis.

36

Figure 5: Post-scarring therapies in skin fibrosis to emulate normal remodelling. Several treatments try to reverse scar formation and emulate normal wound healing remodelling, including transdermal injections, biomaterial-based approaches and non-pharmacological approaches.

8   Tables

Table 1: Indicative examples of latest modulators of inflammation used in skin fibrosis in the last 10 years.

Target pathway	Type of study	Therapeutic used	Molecular pathway	Outcome	Ref.
Modulation of intracellular mediators	*In vitro* and *in vivo*	Administration of Epigallocatechin-3-gallate to KFs and murine keloid model (normal fibroblasts and KFs implanted into the backs of nude mice)	Epigallocatechin-3-gallate, a polyphenol present in green tea, inhibits the signalling pathways PI3K, MEK/ERK, and STAT3	Inhibition of KFs proliferation more than normal fibroblasts and suppression of collagen production	[360]
Modulation of intracellular mediators	*In vitro, ex vivo* and *in vivo*	Transdermal delivery of 10,11-methylenedioxycamptothecin by HA-based nanoemulsion to KFs, *ex vivo* keloid model and full-skin mice	10,11-methylenedioxycamptothecin, a chemotherapeutic and cell cycle blocker leads to down-regulation of PAI-1 and up-regulation of SMAD 7	Inhibition of keloid fibroblasts proliferation	[361]

		Neutralization of PAI-1 on skin biopsy specimens of patients with diffuse and limited SSc and BLM-induced chronic skin fibrosis mice	Neutralization of PAI-1, an inhibitor of the fibrinolytic pathway leads to normalization of the coagulation–fibrinolysis balance and resolution of vascular injuries and MMP-1 activation	Resolution of dermal fibrosis and inflammation	[33]
Coagulation	*Ex vivo* and *in vivo*				
PDGF / c-abl	*In vitro* and *in vivo*	Administration of Dasatinib and Nilotinib, to fibroblasts derived from skin biopsies of SSc patients and to BLM-induced dermal fibrosis mice	Dasatinib and Nilotinib, chemotherapeutic drugs cause inhibition of c-abl and PDGF receptor signalling	Reduction of the number of myofibroblast, collagen content and ECM synthesis	[212]
CTGF	*Ex vivo*	Administration of Iloprost to fibroblasts derived from skin biopsies of patients with scleroderma	Iloprost, a synthetic analogue of prostacyclin PGE2, elevates cAMP by the prostacyclin receptor 27, which in turns blocks the induction of CTGF by TGF-β	Blockage of CTGF and reduction of collagen synthesis	[362]
TGF-β1 and CTGF	*In vivo*	Administration of Pomalidomide to BLM-induced dermal fibrosis mice	Pomalidomide, a thalidomide analogue and immunomodulator, causes inhibition of NFκB signalling, activation of natural killer cells and	Reduction of the expression of TGF-β target genes PAI-1, CTGF and	[363]

			cytotoxic T cells, shifts from a T helper cell 2 to a T helper 1 response	col 1a1, of dermal thickness, myofibroblast number and skin fibrosis content	
TGF-β1	*In vivo*	Topical application of P144 by lipogel emulsion onto BLM-induced dermal fibrosis mice	P144 is a peptide that inhibits TGF-β1 signalling	Reduction of number of myofibroblasts, dermal thickness and soluble collagen content	[239]
	In vivo	Oral administration of Paquinimod to Tsk-1 fibrotic mice (which develop skin fibrosis as a result of a partial in-frame duplication in the fibrillin-1 gene)	Paquinimod is an immunomodulator, causing a shift of macrophages from a pro-fibrotic M2 to an antifibrotic M1 phenotype and inhibition of TGF-β1	Reduction of dermal thickness and number of myofibroblasts	[364]
TGF-β1 / SMAD	*In vitro*	Administration of Quercetin to KFs derived from earlobe keloid scar samples	Quercetin, an anti-inflammatory flavonoid compound causes reduction of the expression of TGF-β1 and TGF-β2 receptors and	Inhibition of KFs proliferation and production of collagen	[266]

			inhibition of the formation of the SMAD 2 / 3 / 4 complex		
	Ex vivo	Administration of Paclitaxel onto skin biopsies from patients with SSc transplanted into mice	Paclitaxel, a chemotherapeutic, suppresses SMAD 2 and SMAD 3 phosphorylation	Inhibition of collagen deposition	[267]
TGF-β1 / SMAD 3 / p38	*In vitro*	Administration of Thalidomide to KFs	Thalidomide, an anti-inflammatory drug causes inhibition of p38 phosphorylation, SMAD 3 and DNA binding activity of AP-1 and SMAD 3 and 4	Reduction of TGF-β1 induced expression of fibronectin and number of fibroblasts	[122]
COX-2 / TGF-β1	*In vivo*	Topical application of Celecoxib to wound excisions in mice	Celecoxib, an anti-inflammatory drug causes a decrease in PGE2 and TGF-β1 levels and inhibition of COX-2	Reduction of inflammation levels in the early stages and decrease of scar formation in the later stages of wound healing	[365]
IL-1 and MMP-9	*In vivo*	Administration of Prednisolone through PDLL microspheres to wound excisions in mice	Prednisolone, an anti-inflammatory and immunosuppressive drug leads to a reduction in IL-1 and upregulation of MMP-9 levels	Reduction of inflammatory level and decrease in fibrosis and scar thickness	[366]

41

| CD4+ lymphocytes | *In vivo* | Administration of Xiamenmycin to wound excisions in mechanical stretch-induced mouse model | Xiamenmycin, an anti-inflammatory natural product leads to reduction of CD4+ lymphocytes; downregulation of the phosphorylation of FAK and p38 | Reduction of inflammation levels and mechanical stress resulting into decreased fibrosis | [367] |

AP-1: Activator Protein 1; BLM: Bleomycin; cAMP: Cyclic Adenosine Monophosphate; COX-2: Cyclooxygenase-2; c-abl: Abelson kinase; CTGF: Connective Tissue Growth Factor; ECM: Extracellular Matrix; ERK: Extracellular Signal-Regulated Kinase; FAK: Focal Adhesion Kinase; HA: Hyaluronic Acid; IL: Interleukin; KF: Keloid-derived Fibroblasts; MEK:Mitogen-Activated Protein Kinase; MMP: Matrix Metalloproteinase; NFkB: Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells; P13K: Phosphatidylinositol-4,5-Bisphosphate 3-Kinase; PAI-1: Plasminogen Activator Inhibitor 1; PDGF: Platelet-Derived Growth Factor; PDLL: Poly(D,L-lactide); PGE2: Prostaglandin E2; SMAD: Small Mothers Against Decapentaplegic; SSc: Systemic Sclerosis; STAT 3: Signal Transducer and Activator of Transcription 3

Table 2: Indicative examples of latest modulators of collagen synthesis and deposition used in skin fibrosis in the last 10 years

Target pathway	Type of study	Therapeutic used	Molecular pathway	Outcome	Ref.
Fibroblast proliferation	*In vitro*	Administration of c-Ski in skin fibroblasts *in vitro*	c-Ski acts as a co-repressor of the TGF-β1 / SMAD 3 pathway, inhibiting cellular proliferation	Increased fibroblast proliferation, without increased fibroblast-myofibroblast differentiation and reduced collagen type I expression	[368]
	In vitro	Administration of Tacrolimus in KF	Tacrolimus, an immunomodulator inhibits TGF-β1/SMAD signalling, decreasing KF proliferation and collagen production	Decreased KF *in vitro* proliferation, migration and collagen production	[369]
	In vitro/in vivo	Administration of dexamethasone and green tea polyphenols, using	Dexamethasone is an immunosuppressant corticosteroid used to inhibit cellular proliferation, while green tea	Increased suppression of human KF *in vitro* and induced degradation of collagen fibres in keloids *in vivo* when compared to normal silicone gel	[370]

		electrospun fibres in KF and in a keloid mice model	polyphenols possess antibacterial properties, preventing infection	sheeting, while maintaining normal viability to healthy cells	
	In vivo	Combinatorial administration of TAC and VPL through intralesional injection in a keloid mouse model (HS xenografts from human patients implanted into nude mice)	TAC is a corticosteroid that can inhibit fibroblast proliferation, while VPL is a calcium channel blocker that reduces ECM synthesis and increases collagenase synthesis	Decreased fibroblast proliferation and increased decorin expression, while promoting a decrease in scar weight, for both single and co-delivery	[148]
	Clinical trial (phase II)	Combinatorial administration of TAC, β-MET, DD and DF through intralesional injection and ointment application post-surgery in keloids	Corticosteroids are used as immunosuppressants, decreasing KF proliferation and collagen synthesis	Decreased fibroblast infiltration in the scar excision site, resulting in a reduction of lesion recurrence after surgery	[292]

	Clinical trial (phase II)	Administration of TAC or 5-FU, through intralesional tattooing in keloids	TAC, a corticosteroid, acts as an immunosuppressant, decreasing cellular proliferation and collagen synthesis while 5-FU is a pyrimidine analogue with antimetabolite action	Improvement in all the histological and clinical parameters assessed for both compounds, although improvement was more significant with 5-FU	[293]
	Clinical trial (phase II)	Combinatorial administration of IFN-α2b and TAC, through intralesional injection in keloids	IFN-α2b interferes in the production of basic fibroblast growth factor, inhibiting cellular proliferation, with TAC doing the same as an immunosuppressant	Decreased patient lesion depth and volume, when compared to treatments with single formulations	[294]
Collagen synthesis modulation	*In vitro*	Transduction of human dermal fibroblasts and KF with decorin-expressing adenovirus	Decorin acts as a TGF-β1 inhibitor, while interfering in the collagen assembly and degradation process	Reduced secreted TGF-β and EGFR expression, lower collagen levels (I and III) observed and upregulation of MMP-1 and MMP-3 mRNA	[152]

	In vitro	Administration of diethyl pyimDC or diethyl pythiDC *in vitro* in human cell lines	Biheteroaryl compounds pyimDC or pythiDC inhibit collagen prolyl 4-hydroxylase, essential in collagen synthesis	Inhibition of collagen synthesis at concentrations that neither cause cytotoxicity or disrupt iron homeostasis	[160]
	In vitro / In vivo	Administration of Tropisetron in a mouse model of scleroderma	Tropisetron acts an antagonist for an *α*7 nicotinic acetylcholine receptor for serotonin, which has been linked to ECM synthesis	Reduction of collagen synthesis in human dermal fibroblasts derived from patients with systemic sclerosis and prevention of induced dermal fibrosis, with a reduction of collagen content *in vivo*	[371]
	In vivo	Administration of CAR-decorin fusion protein in a wound model in mice	Decorin inhibits TGF-*β*1, while regulating collagen assembly and degradation. CAR-peptide is a wound targeting peptide	Selective accumulation of fusion protein in wounds, promotion of wound healing and enhanced suppression of scar formation at lower doses than when compared to non-targeted decorin	[275]
	In vivo	Administration of LPA receptor antagonist in a	LPA is involved in g protein-coupled signalling, with its	Decreased mRNA expression of TGF-*β*1, CTGF, MIP-1*α*, IFN-*γ* and collagen	[372]

46

		BLM-scleroderma mouse model	involvement reported in the pathogenesis of SSc, possibly through the activation of TGF-β1 and CTGF	α1(I) with decreased dermal thickness and a lower number of mast cells and phospho-SMAD 2/3- positive spindle cells in the treated group	
	In vivo	Administration of A$_{2A}$ receptor antagonist in a high adenosine mice model of fibrosis	Adenosine g protein-coupled A$_{2A}$ receptors are involved in the synthesis of collagen in dermal fibroblasts	Adenosine induced fibrosis was prevented through the use of a A$_{2A}$ receptor antagonist, resulting in decreased dermal collagen content and expression of profibrotic cytokines and growth factors.	[373]
Myofibroblast activation	*In vitro*	Administration of IL-1β into dermal fibroblasts previously exposed to TGF-β1	IL-1β is capable of inhibiting TGF-β1-induced myofibroblast activation and collagen synthesis	Inhibition of TGF-β1-induced myofibroblast formation and collagen synthesis, reduction of GLI1, involved in myofibroblast activation, increased levels of MMP1, -2, -9 and -14, with decreased levels of lysyl oxidase	[374]

47

	In vitro	Administration of rhMG53 to dermal fibroblasts *in vitro*	MG53 is a component of the cell membrane repair machinery, being shown to be involved in the modulation of the inflammatory response and myofibroblast activation	Reduced expression of α-SMA, collagen type I and fibronectin, through interference in the TGF-β signalling pathway, reducing myofibroblast activation	[375]
	In vitro / In vivo	Administration of a synthetic PPAR-γ agonist in *in vitro* and *in vivo* models of scleroderma	PPAR-γ has been described as a regulator of TGF-β activity, while also being involved on the modulation of cell proliferation and the inflammatory response	Reduction of collagen type I expression, thinning of scar tissue, attenuated loss of subcutaneous adipose layer and attenuated upregulation of multiple fibrotic marker genes	[199]
	In vivo	Subcutaneous administration of paricalcitol into mice models of fibrotic wounds	Paricalcitol, a vitamin D receptor agonist acts as a negative regulator of the TGF-β / SMAD pathway	Decreased collagen release and myofibroblast differentiation	[376]

	In vivo	Administration of rhEGF to murine skin wounds	EGF modulates TGF-β1 activity	Faster wound healing, smaller and thinner scars and more controlled collagen formation and deposition	[377]
	In vivo	Administration of antisense oligonucleotides against CTGF/CCN2 in rabbit models of hypertrophic scarring	CTGF/CCN2 is hypothesised to be a co-factor or downstream mediator of TGF-β	Inhibition of CTGF resulted in a reduction in the number of myofibroblasts in scars and decreased transcription of collagen type I and III, while showing no significant adverse effect on early wound closure	[378]
	In vivo	Administration of WIN55,212-2 as a modulator of pathologic fibrosis in a model of BLM-induced scleroderma	WIN55,212-2 is a cannabinoid receptor agonist, which can modulate the endocannabinoid system that has been connected to the development of pathological fibrosis	Similar levels of subcutaneous inflammatory infiltration, dermal thickness and collagen content when compared to the control group. Reduced activation of myofibroblasts and inhibition of TGFβ, CTGF and PDGF-BB expression.	[379]

49

| | Clinical trial (phase II) | Administration of rhEGF to thyroidectomy postsurgical wounds | EGF modulates TGF-β1 activity | Lower scores on the Vancouver scar scale, decreased scar pliability and thickness after surgery | [46] |

5-FU: 5-Fluroruracil; **α-SMA:** α- Smooth Muscle Actin; **β-MET:-** Methasone; **CTGF/CCN2:** Connective Tissue Growth Factor; **DD:** Diflorasone Diacetate; **DF:** Difluprednate; **ECM:** Extracellular Matrix; **EGFR:** Epidermal Growth Factor Receptor; **GLI1:** Glioma-Associated Oncogene Homolog 1; **HS:** Hypertrophic Scar; **IFN:** Interferon**; IL:** Interleukin; **LPA:** Lysophosphatidic Acid; **MIP:** Macrophage Inflammatory Protein; **MMP:** Matrix Metalloproteinase; **PDGF:** Platelet-derived Growth Factor; **PPAR:** Peroxisome Proliferator-Activated Receptors; **rhEGF:** Recombinant Human Epithelial Growth Factor; **SMAD**: Small Mothers Against Decapentaplegic; **SSc:** Systemic Sclerosis; **TAC:** Triamcinolone Acetonide; **TGF-β:** Transforming Growth Factor; **VPL:** Verapamil

Table 3: Indicative examples of latest modulators of post-scarring therapies used in skin fibrosis in the last 10 years

Target pathway	Type of study	Therapeutic used	Molecular pathway	Outcome	Ref.
Matrix remodelling	*In vivo*	Administration of kynurenic acid encapsulated in a nanofibrous wound dressing to wounds in a mice model	Kynurenic acid, a metabolite of L-tryptophan, regulates collagen and MMP expression and reduces fibroblast proliferation and migration	Reduced cellularity, collagen type I and fibronectin expression, paired with increased levels of MMP-1	[380]
	Clinical trial (phase II)	Administration of TAC in combination with ablative fractional laser therapy in hypertrophic scars	TAC inhibits fibroblast proliferation and collagen synthesis, while laser therapy creates ablation zones that activate heat shock proteins leading to collagen remodelling	General improvement on histological and functional scores (dyschromia, hypertrophy texture), after combination therapy was applied	[381]

	Clinical trial (phase II)	Administration of a porcine gelatin-dextran hydrogel post excision for the treatment of keloids	The hydrogel acts as lattice for fibroblast adhesion, leading to a more organised ECM	Low recurrence rate (19.2%) with these cases showing a high reduction rate in scar volume (less than 15% of original volume) with an almost perfect patient satisfaction score	[382]
Destruction of scar tissue	Clinical trial (phase II)	Use of intralesional cryosurgery for the treatment of keloid scars in a prospective study	Cryosurgery induces cellular destruction of the scar tissue while preventing recurrence due to renormalisation of fibroblast phenotype and absence of wound contraction	Decrease of average scar volume, sensation of pain and itchiness and overall increase in the histological scar scores, despite a 25% recurrence rate	[43]
Destruction of scar tissue/fibroblast proliferation	Clinical trial (phase II)	Combinatory administration of radiofrequency, ultrasound and TAC for hypertrophic scars	Radiofrequency treatment inhibits fibroblast proliferation while ultrasounds create microchannels for the delivery of TAC, which inhibits fibroblast proliferation and collagen synthesis	Significant improvement on scar morphology, with a mean severity attenuation of 67%, with regenerative changes observed in both the dermis and epidermis of the treated scars	[383]

	Clinical trial (phase II)	Combinatory therapy of UVA radiation, PUVA and RA for the treatment of localized scleroderma	UVA radiation inhibits fibroblast proliferation, while RA can reverse the abnormal expression of MMP-13	Improvement of all erythematous patches, with 90-100% softening, although some of the indurated patches did not heal completely	[384]
Fibroblast proliferation	Clinical trial	Use of adjuvant radiotherapy after keloid-post excision surgery in a retrospective study	Radiotherapy inhibits excessive fibroblast proliferation	High local control rate (88.25%) paired with a low recurrence rate (9.59%) and a low incidence of side-effects, while no treatment-associated cancers were reported over 19 years	[385]
	Clinical trial (phase II)	Combinatory administration of onion extract and TAC for the treatment of keloids and hypertrophic scars	Onion extract contains quercetin and heparin, which in conjunction with TAC possess anti-inflammatory capabilities, decreasing fibroblast proliferation and collagen synthesis and modulating its organisation	Significant reduction of pain-sensitiveness, itching and elevation but not erythema and induration in the combinatory treatment when compared to the administration of TAC alone	[386]

TLR7 activation	Clinical trial (phase II)	Administration of an imiquimod cream for the treatment of keloid scars	Imiquimod, a TLR7 agonist, modulates the inflammatory response in scar tissue, while increasing IFN-γ concentration, MMP activity, apoptosis and decreasing angiogenesis	Well tolerated treatment with a reduction in the recurrence rate over 6 months, although not statistically significant due to low number of patients in the study	[387]
TGF-β3	Clinical trial (phase II)	Administration of avotermin following scar revision surgery	TGF-β3 modulates the deposition and organisation of new extracellular matrix, the inflammatory response and myofibroblast differentiation in scar tissue	Improved scar appearance and greater reduction in scar area paired with better collagen organisation when compared to the placebo treatment	[255]
EGFR	Clinical trial (phase II)	Administration of rhEGF topic serum for the treatment of atrophic acne scars	EGF modulates TGF-β1 activity	Improvement in scar appearance scores, both external and self-evaluated when compared to the baseline	[388]

Fibroblast apoptosis	Clinical trial (phase II)	Use of custom-moulded ear clips for the treatment of ear keloids	Potential decrease in perfusion leading to decreased oxygen supply to the scar tissue and increased fibroblast apoptosis	Improvement in scar histological scores after treatment with a low recurrence rate	[205]
Muscle tension reduction	Clinical trial (phase II)	Administration of botulinum toxin type A for the treatment of hypertrophic scars	Botulinum toxin type A blocks neuromuscular transmission, reducing muscle tension and inhibiting fibroblast proliferation	Reduction in erythema, itching sensation and pliability scores for all patients, paired with high levels of patient therapeutic satisfaction	[389]

ECM: Extracellular Matrix; **EGFR:** Epidermal Growth Factor Receptor; **MMP:** Matrix Metalloproteinase; **PUVA:** Photosensitizer Psoralen; **RA:** Retinoic Acid; **rhEGF:** Recombinant Human Epidermal Growth Factor; **TAC:** Triamcinolone Acetonide; **TGF-β3:** Transforming Growth Factor Beta 3; **TLR-7:** Toll Like Receptor 7; **UVA:** Ultraviolet A

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10 Competing financial interests

The authors have no competing financial interests.

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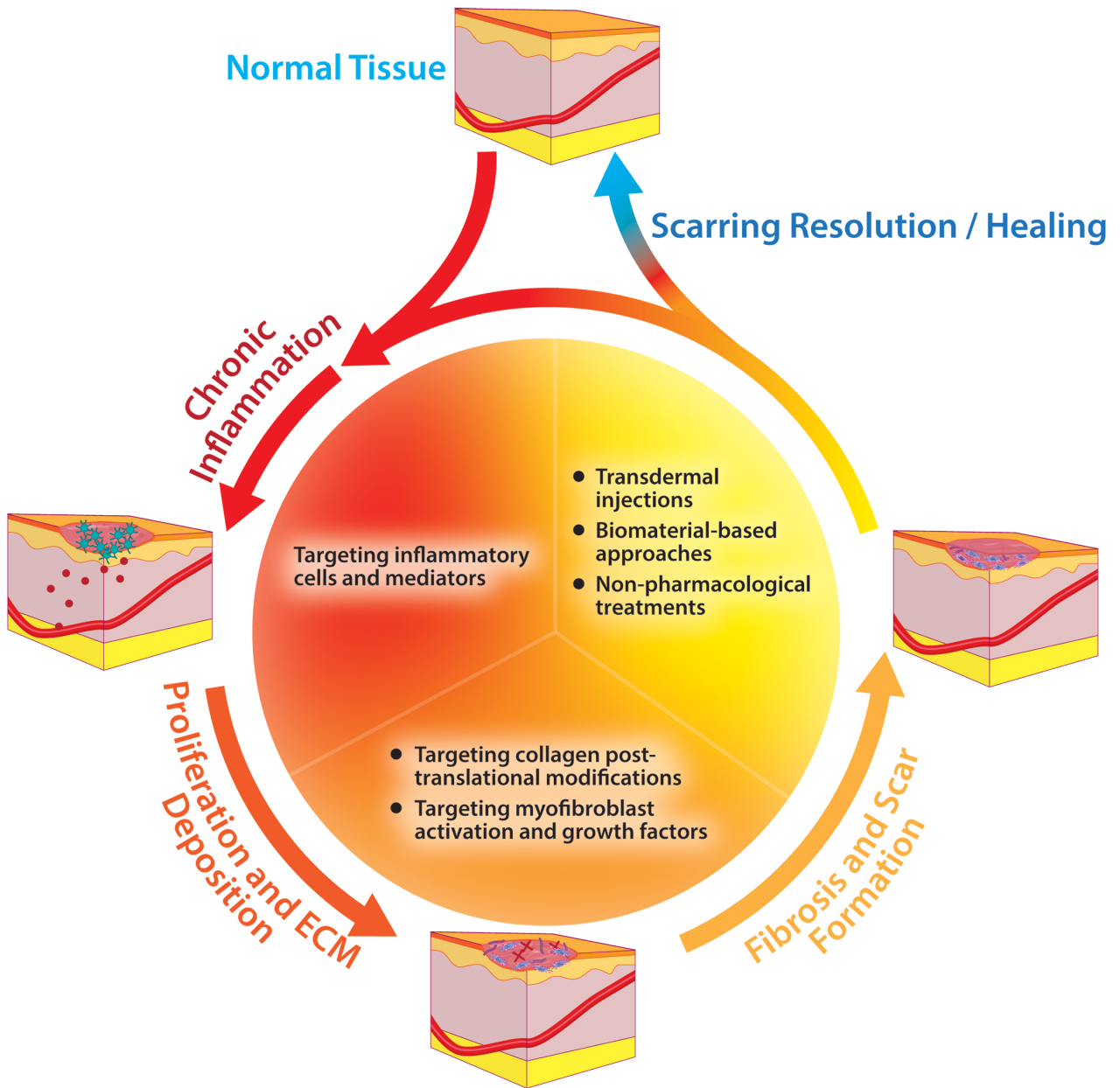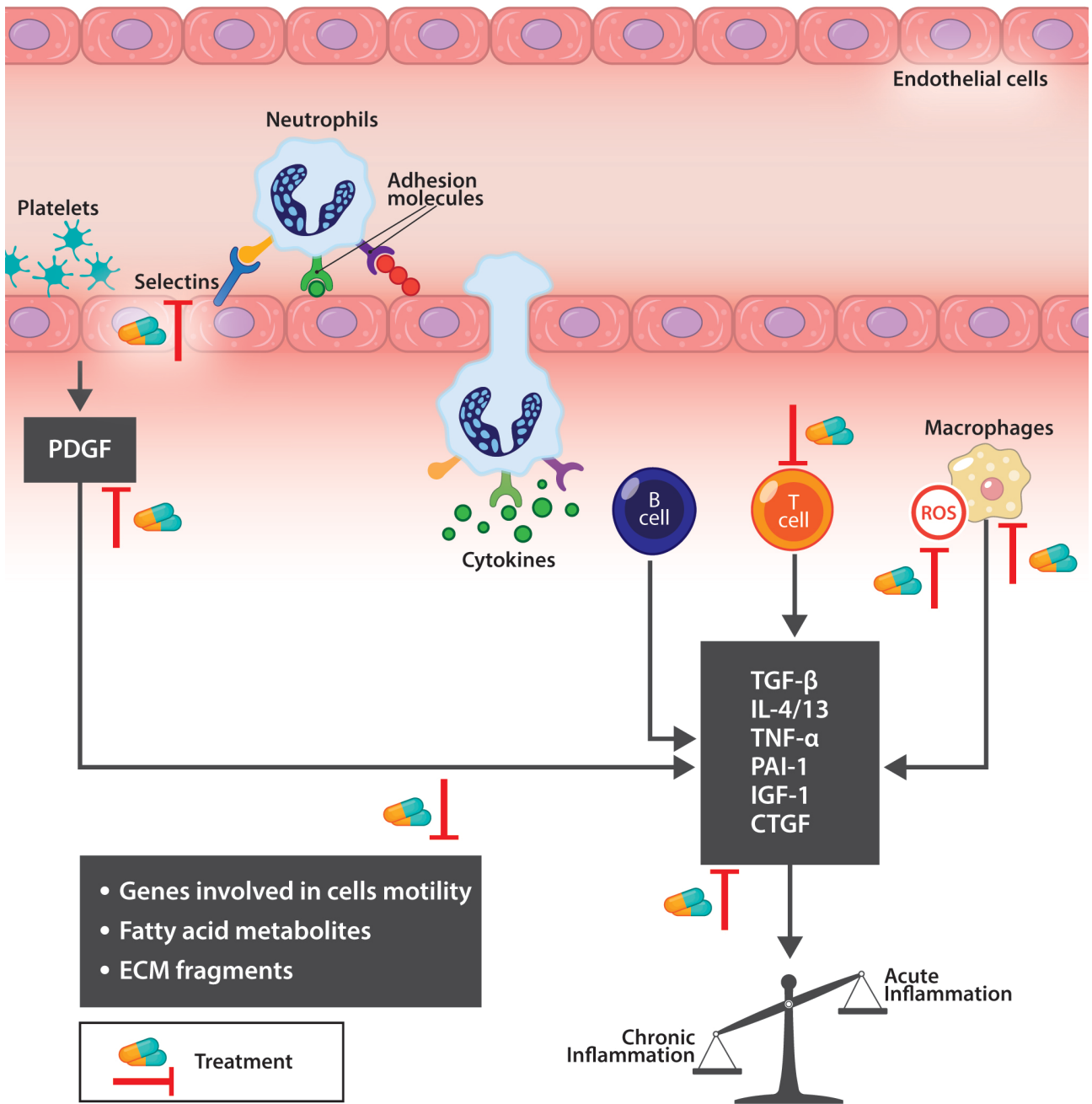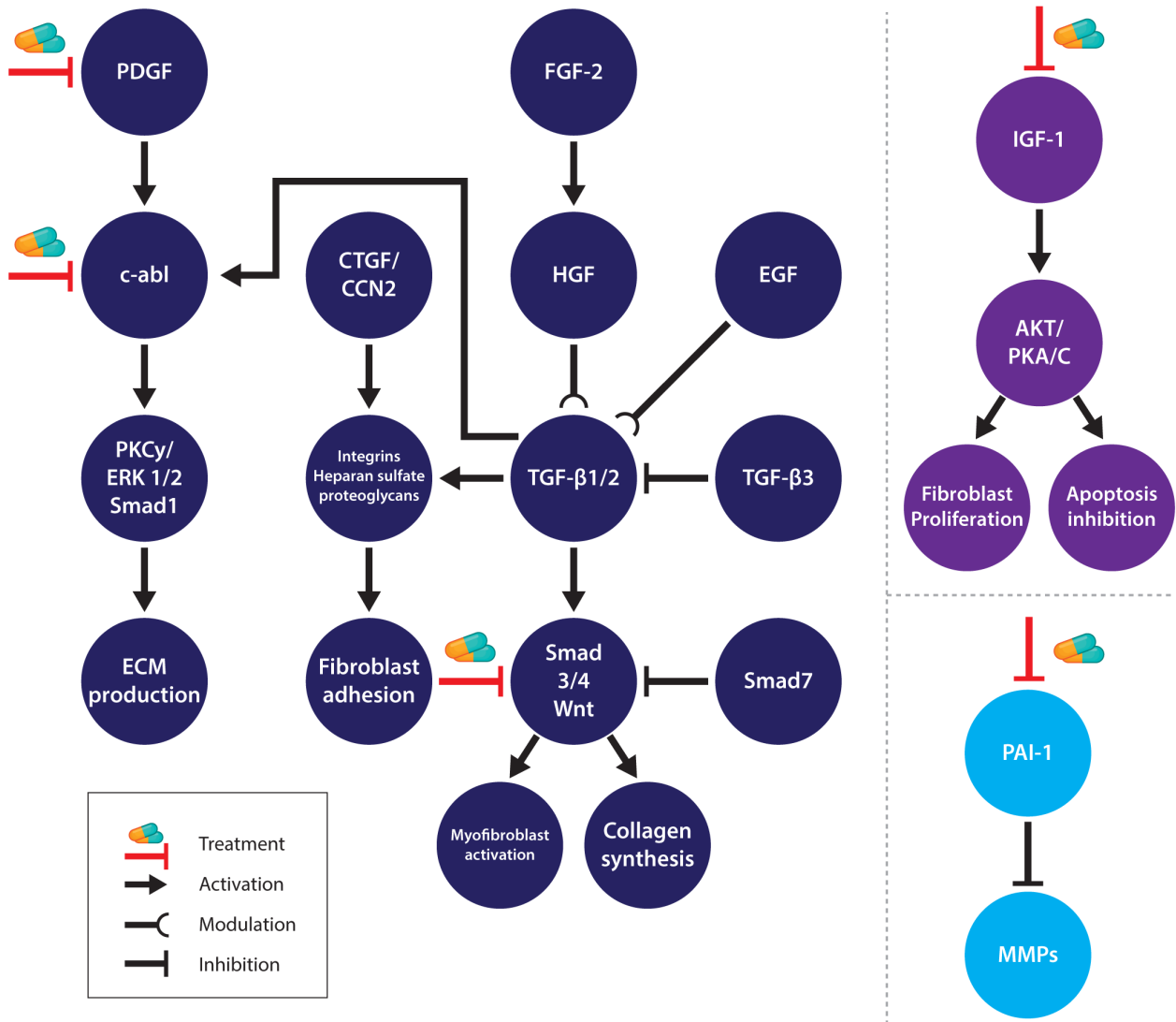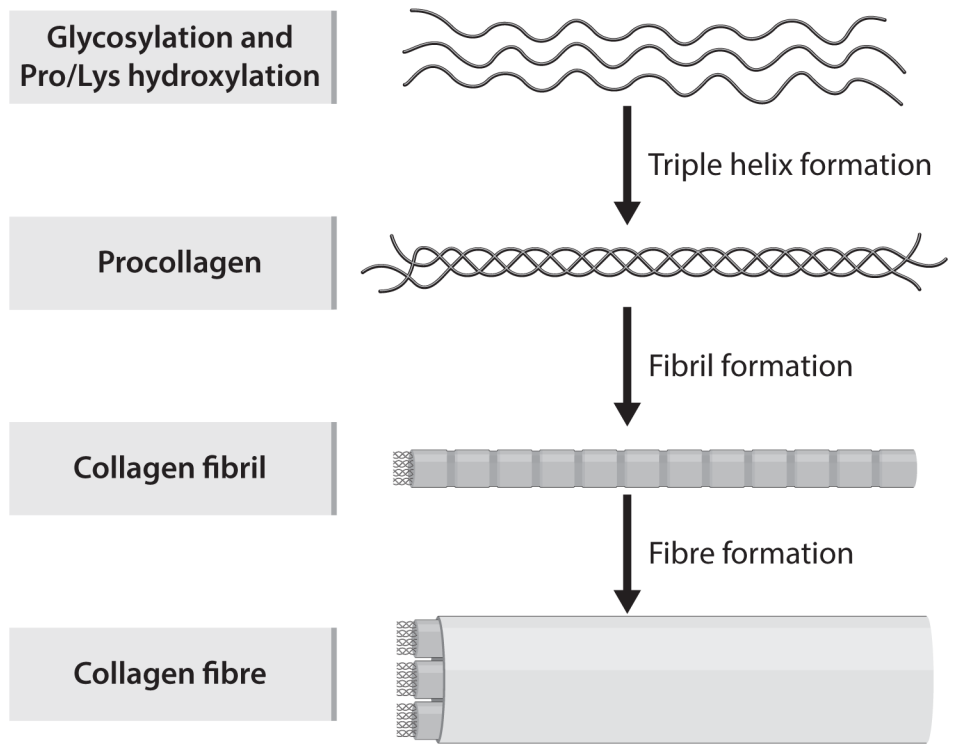
Figure 1

Figure 2

Figure 3

Glycosylation and Pro/Lys hydroxylation	
Procollagen	Triple helix formation
Collagen fibril	Fibril formation
Collagen fibre	Fibre formation

Myofibroblast activation

Fibroblast   Myofibroblast

Figure 4

Figure 5