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Teaser Activation of local endogenous skin stem cells inside the body offers a new strategy for scar-free skin regeneration and for preventing skin diseases or delaying aging skin cells in both clinical and cosmetic practice.

Therapeutic potential of endogenous stem cells and cellular factors for scar-free skin regeneration

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Injured human skin fails to regenerate, resulting in scar formation. Annually, 100 million new skin-scarring incidents, occurring as a result of surgery, disease, burns, or sports-related damage, remain untreated. Here, we review knowledge gained from scar-free experimental animal models that have natural regenerative mechanisms for scar-free skin recovery. We also focus on the unique role of endogenous stem cells and other cellular and molecular factors, including the balance of the transforming growth factor-beta (TGF-β) pathway in the context of human skin regeneration. This new strategy opens a new window in drug development for scar-free skin regeneration treatments in both the clinical and cosmetic practice settings.

Introduction

Scar formation is a therapeutic and cosmetic issue that can have devastating consequences for patients. Currently, there are no satisfactory or reliable treatments to treat or prevent scarring. Scar formation is a naturally occurring spontaneous healing procedure that occurs after trauma, injury, infection, and surgery to any tissue or organ in the body [1]. Scarring of the skin has significant psychological impacts, with a loss of self-esteem, seclusion, sentiments of defenselessness, and low social confidence [1,2]. Generally, a scar is caused upon injury as a result of the inability of the skin to naturally regenerate collagen production. The human skin has two major layers. The external layer, the epidermis, acts role as a barrier to the external environment, protecting the skin from ultraviolet (UV) radiation, for example. It contains layers of cells with regenerative capabilities [1,3], so that superficial injuries, such as scrapes, tend to have scar-free regeneration [1]. Underneath the epidermis is the dermis, which is one of the most important layers of the skin [1,3], containing a complex network of connective tissue that contributes to the strength and elasticity of human skin [1,3]. However, upon injury, these connective tissue networks are affected, resulting in the formation of injury-associated scars. Skin scarring includes

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Shibashish Giri is a stem cell scientist. He is currently deputy head of Applied Stem Cell Biology and Cell Technology, Center for Biotechnology and Biomedicine, Medical Faculty, University of Leipzig, Germany. He has 12 years of research experience in the location and isolation of endogenous stem cells in different human organs (heart, skin and liver) and animal models. He has more than 32 publications in international peer review journals. His main focus of research is the activation of endogenous stem cells for cell or organ regeneration. Currently, he is actively engaged in researching therapeutic interventions for scratch scar removal as well scar-free skin regeneration in human models. He is also developing a herbal extract-derived food supplement for the treatment of fatty liver disease.

Hans-Gfinther Machens After studying medicine in Hanover, G—ttingen, Heidelberg and Chicago, Hans-Gfinther Machens completed his PhD in 1988 in Heidelberg. He acquired his German medical board certification in surgery in 1995 and in plastic surgery in 1999. During this time, Professor Machens also earned his postdoctoral teaching qualification (habilitation) while conducting research into techniques for angiogenesis induction via cell-based gene transfer. After holding senior physician positions in Hanover and Lfibeck, in 2007 he was appointed Chair of Plastic Surgery and Hand Surgery at Technische Universitδt Mfinchen (TUM). His main research interests are in translational research in the fields of tissue engineering and tissue regeneration. His research focuses primarily on new techniques for matrix-based angiogenesis induction *in vitro* and *in vivo*. Currently he is working on a GMP process for the cell-based gene transfer from BFGF and VEGF by means of electroporation and on the development of systemically and locally applied rh-erythropoietin applications for local tissue regeneration. His main clinical interest is reconstructive surgery and innovative applications for

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hydrotropic, normotrophic, contracture, hypertrophic and keloid scars, which occur as a result of the overgrowth of collagen fibrous tissue that replaces normal skin during healing [1,3]. Hypertrophic scars are raised, oddly pigmented, and cause itching or unusual sensations [3]. Compared with keloid scars, hypertrophic scars stay within the limit of the area of initial damage and fade with time [3]. They comprise nodules containing myofibroblasts, which are differentiated fibroblasts that constitute alpha-smooth muscle actin, collagen fibres, and extracellular matrix (ECM) [3].

Estimates from the stem cell therapy market suggest that scar treatment will surpass US$202.77 billion by the year 2026 from US$12.25 billion in 2016 [4]. There are numerous endogenous stem cells in the human body that are usually dormant but actively participate in scar-free skin regeneration upon injury [5,6]. In addition, recent studies have also shown the scope of the topical application of recombinant growth factors or small molecules on scars, which has become an emerging interest in clinical and cosmetic settings [5,6]. Accumulating evidence suggests that stem cell-based scar-free skin regeneration could have broad application as drug therapies for preventing skin fibrosis and scarring, not only to improve healthy skin, but also the quality of life of patients undergoing scar-related treatments. Moreover, stem cell-based scar-free treatment could be an approach for the development of novel regenerative therapies for both scarred and aging skin.

Current scar treatments

Although diverse medications have been utilized for different kinds of scar, none have achieved satisfactory clinical acceptance. Steroid infusions, cryotherapy, weight treatment, and pharmaceuticals have been used, but with a low level of patient satisfaction; thus, scars remain a challenge in both the cosmetic and surgical setting [2,4,5]. The scar treatment industry is developing quickly because of the growing awareness of aesthetics [2,4]. Scars can negatively impact those with them, influencing their life style choices and social interactions [2,5], as seen with acne caused by inflammatory skin scars [2,4,5]. Stem cell-based therapies are receiving increasing interest as scar treatments that target the wound-healing processes [5], and skin cells in regenerative species that prevent scar formation have been well described [6]. Furthermore, recent studies have also highlighted the scope of the topical application of recombinant growth factors or small molecules on scars, which are an emerging interest in clinical and cosmetic practices [4–6].

To develop new medication for scar prevention and removal, researchers have focused on results from scar-free skin regeneration models, such as human fetuses, African spiny mouse, American black bear, bottlenose dolphin, reindeer, fish-scale gecko, salamander, zebrafish, and amphibians, which show significant regenerative and reparative capacities during wound healing by utilizing their endogenous stem cells to prevent scar formation (Fig. 1). Therefore, we focus here on the molecular and cellular basis of regeneration and tissue repair in these regenerative vertebrate models.

Animal models of scar-free skin recovery

African spiny mouse

Scar-free skin regeneration is found in two species of African spiny mouse (*Acomys kempi* and *Acomys percivali*) [7]. Unlike other mammals, include laboratory mice (*Mus musculus*), these mice recoup not only lost skin and new hair follicles, but also the sebaceous organs and tendons, resulting in no scars [7]. Seifert *et al*. [7] examined tissue recovery in *Acomys* spp. by punching a 4-mm diameter circle in the ears of the mice. Extensive regeneration was noted by the total recovery of the ligament, fat tissue, dermal, and epidermal layers of skin and new hair follicles in only 12 days. This observation was unique to *Acomys* and distinct from laboratory mice, the scars of which did not exhibit such efficient regeneration. In laboratory mice, keratin 17 expression occurs at day 14 following injury, but subsequently lost during the later phases of wound recuperation (day 26), without development of any hair follices. In *Acomys*, the Wnt pathway also induces the sequestration of fibroblasts underneath the hair germ and in dermal papilla upon utilization of lymphoid enhancer-binding factor (LEF1). This molecular signaling could be a target in the development of new hair follicles.

Furthermore, levels of a proinflammatory family of macrophages are low during skin regeneration in *Acomys* [8]. Gawriluk *et al*. demonstrated that *Acomys* regenerates ear tissue by forming a blastema [9]. According to the authors, three species of *Acomys* and New Zealand white rabbits were able to regenerate ear tissue in contrast to outbred and inbred strains laboratory mice, such

wound treatment. He is one of the founders of the Tissue Engineering Competence Centre in Lfibeck, director of various German Federal Ministry of Education and Research (BMBF) projects focusing on tissue regeneration, and is responsible for setting up a composite tissue allotransplantation team to perform extremity transplants at TUM's Klinikum rechts der Isar.

Augustinus Bader is a physician and biomedical scientist, and one of the leading experts in the field of stem cell research. His clinically most relevant inventions include a biological process that imitates bionic principles for stem cell activation and tissue regeneration. Most of his patents have a global coverage, with 27 currently active patent families with over 200 international filings. In 2010, Augustinus received the Cicatrix Prize, the largest European scientific prize organized by a patient organization, for the development of a therapeutic method to prevent scar formation following severe thermal injuries. Recently, he developed a skin cream that triggers existing endogenous skin stem cells, encouraging them to repair and renew the skin.

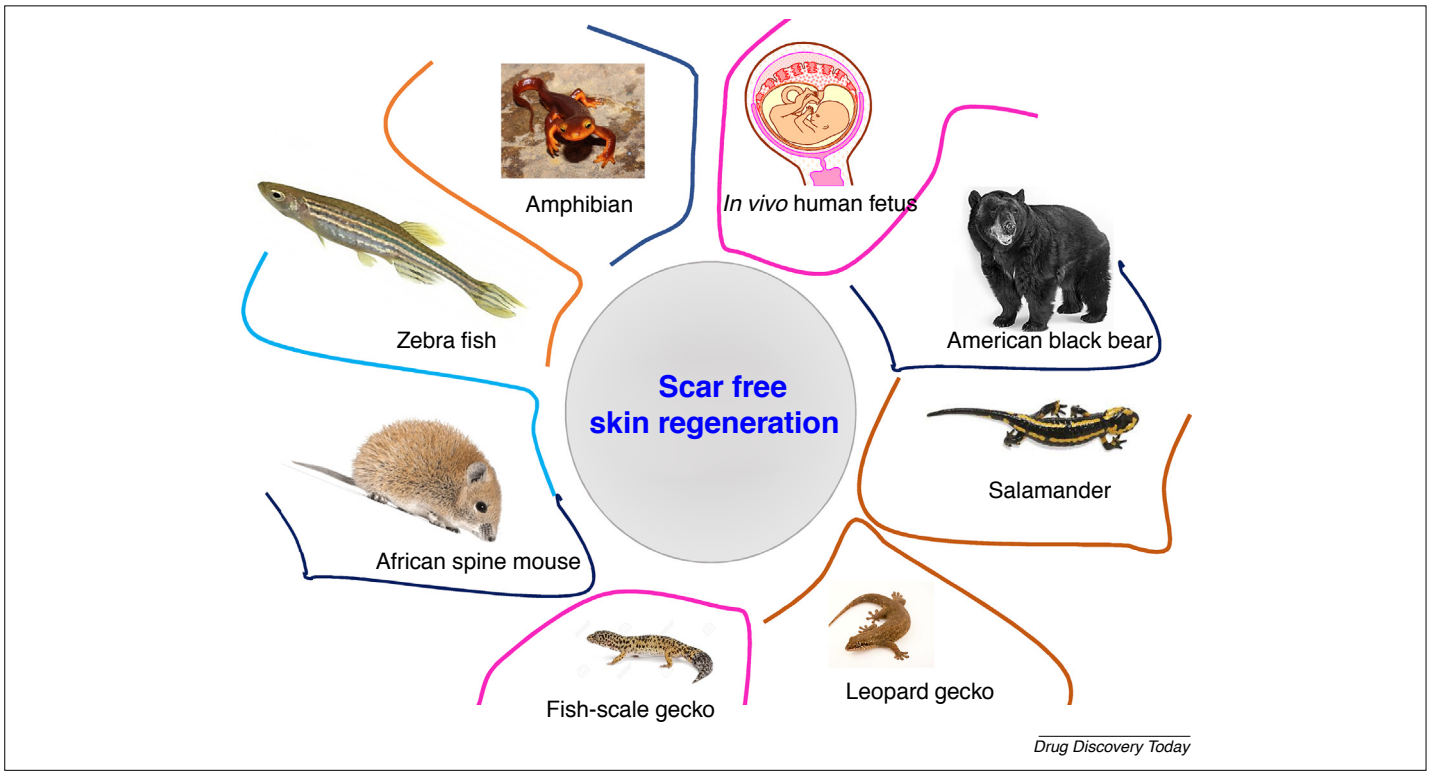

FIGURE 1

Scar-free skin regeneration in different animals and human fetuses.

as MRL healer mice, which failed to show signs of regeneration and instead healed the wounds by scarring. *Acomys* regenerated skin, sebaceous glands, hair follicles, adipose tissue, and cartilage even after full-thickness skin wounding [9]. In a study by Brant *et al.* [10], the effects of cytokines and immune cells on cell proliferation and scar architecture in scarring and nonscarring organisms were compared. The authors found that there were more mast cells at the site of wound formation in *Acomys* compared with laboratory mice. Most importantly, there were low levels of F4/80 macrophages, including proinflammatory cytokines, in the wound site in *Acomys*, which suggests that there are underlying factors responsible for the regenerative properties of the skin in this genus. However, cell proliferation in *Acomys* and *M. musculus*, either in the epidermis or mesenchymal tissues, did not account for any differences in the matrix composition of the wounds. Skin of *M. musculus* expresses eight types of collagen, which are upregulated at least fivefold in the scar-forming trichrome-positive matrix, whereas there are low levels of collagen in *Acomys*. Thus, the absence of many cytokines, resulting from the lack of macrophages, in *Acomys* skin is likely to be responsible for the failure to upregulate fibrotic collagens, a prerequisite for the regenerative response within the skin rather than the generation of a scar [10]. A histological study by Matias *et al.* revealed unique characteristics of the wound-healing patterns in *Acomys* versus *M. musculus* [11]. Therefore, a thorough understanding of the mechanistic details involved in wound healing in *Acomys* could help determine regeneration events at both the cellular and molecular levels that could be applied for the development of therapies for skin scarring in humans.

American black bear

In hibernating American black bears (*Ursus americanus*), their low blood flow, hypothermia, reduced body temperature, and limited capacity to burn calories are thought to contribute to the regeneration of scars during hibernation [12,13]. In a study conducted using 25 years of observation and with a sample size of more than 1000 American wild bears, it was found that wounds, such as those caused by bullets, bolts from hunters, or bites, healed without obvious scarring and independently of the use of any therapeutics during hibernation [14]. In addition, Laizzo *et al.* explored visual and histological skin regeneration in 14 wild mountain bears by initiating 2–5-mm full-thickness cutaneous injuries at the start of their hibernation [15]. Following 3 months of hibernation, skin regeneration was observed without any scarring. The authors reported the transformation of type III to type I collagen during hibernation, which could be exploited as a scar treatment [15]. Additionally, Chow *et al.* [16] conducted proteomic investigations of serum proteins in mountain bears during hibernation. In total, 70 serum proteins were differentially expressed, and the expression of 34 proteins was significantly induced during hibernation, including higher levels of hibernation induction trigger (HIT), a δ-opioid receptor agonist, and ursodeoxycholic acid (UDCA). Welinde *et al.* [17] investigated the differences in the levels of blood constituents between hibernating and nonhibernating brown bears. In hibernating bears, there was a 45-fold induction in globulin, a sex hormone, suggesting that this could be involved in skin regeneration. Significant induction of other proteins was also observed, including bile salt-activated lipase, which hydrolyzes both triglycerides and cholesterol esters and facilitates the

efficient use of energy during hibernation. Some key coagulation factors, such as fibrinogen, thrombin, and factor Xa, were enhanced during hibernation and are involved in wound healing by enabling the local formation of blood clots [17].

Therefore, the scar-free regeneration of skin in hibernating bears suggests that further studies are required to determine the molecular mechanisms involved, which could highlight compounds that could be developed as scar treatments in humans. Such treatments might be particularly relevant in patients with slow-healing skin injuries, such older patients and those with diabetes.

Zebrafish

Zebrafish (*Danio rerio*) are a model of quick scar-free skin regeneration. Zebrafish have the ability to regenerate a full-thickness injury at a rate of 250 μm/h [18], whereas re-epithelialization in a human wound injury is ~0.001 μm/h [19]. Zebrafish are useful for the *in vivo* tracking of skin cells, along with epithelial cellular responses, involved in scar-free skin regeneration. Furthermore, they have been used to identify the role of β-catenin/integrin signaling during skin re-epithelialization. The skin of zebrafish follows the three general phases of wound healing, similar to that seen in mammalian skin [20]. Fibroblast growth factor (FGF) is crucial for later epidermal remodeling and granulation tissue formation [18].

A recent study demonstrated the behavior of epidermal cells in the scar-free regeneration of smooth skin in zebrafish [21]. Two types of epithelial cell were found to be involved at the site of injury. The first type initially covered the wound but disappeared within a few days as a result of cellular apoptosis, whereas the second type of epithelial cells was recruited to the vicinity of the skin that contains the stem cells that actively participate in scar-free skin regeneration [21]. In addition, existing stem cells in the basal layer had a key role in scar-free skin regeneration. This study provides clues about the role of the autonomous proliferation of stem cells in the basal layer for scar-free skin regeneration [21]. However, further research is required to determine whether similar regenerative mechanisms involving resident skin stem cells occur in human skin and, if they do, whether they show potential for scar-free wound healing.

Amphibians

The multilayer epidermis in amphibians is similar to mammalian epidermis [21,22]. However, amphibians are able to regenerate scar-free multilayer epidermis, including the exocrine glands, an ability that is not lost as they age [23,24]. The activity of matrix metalloproteinases (MMPs), with keratinocyte relocation, is involved in the formation of an ECM during wound healing [24]. This activity was investigated using scar-free healing cellular responses of 8- and 15-month-old injured *Xenopus laevis*. Although the process was delayed in older frogs, the healing phases (i.e., inflammation, new tissue formation, and remodeling) remained the same, independent of age [25]. Otsuka-Yamaguchi *et al*. investigated the scar-free regeneration of skin in *Xenopus* after an excision injury, and suggested that cells other than skin cells facilitate skin regeneration. This research is now helping researchers understand how amphibians regenerate skin without any scar formation, even after deep injuries [26]. Such discoveries provide new avenues to further investigate the molecular properties of the subcutaneous tissues of *Xenopus* and to compare them with scar-forming cells in human skin.

Leopard gecko

The leopard gecko (*Eublepharis macularius*) is a nocturnal ground-dwelling gecko that lives in deserts and has caught the attention of herpetologists recently for several reasons [27]. *E. macularius* exhibits tremendous potential for epimorphic scar-free regeneration of skin wounds to its body and tail [27]. Skin loss, post-wound formation, is marked by replacement with small scales, without any sign of large cone-like scales [28,29]. Incomplete recovery of *de novo* pigmentation in newly regenerated tails has also been reported, with changes in the skin coloration pattern [28,29]. However, in response to experimental excisions, scar-free regeneration was observed accompanied by the recovery of skin pigmentation close to nonaffected areas, suggesting the migration of healthy pigmented cells to the newly repaired areas [27]. Upon investigation of this regeneration process, growth factors, including the VEGF family of proteins, SMAD, TGF-β1, activin βA and tyrosine kinase receptors (VEGFR1 and FGFR1), were observed to be regulated during the early phases of re-epithelialization [29,30]. Thus, preserving a portion of pigmented skin (close to the affected areas) could significantly aid regeneration, restoring pigment patterns. These recovery capabilities depend on where on the body of *E. macularius* injuries occur.

Crucially, not all reptiles demonstrate this scar-free regeneration ability [31]. Therefore, more research is required to fully understand the mechanisms involved in skin regeneration in this particular species, to determine whether it could contribute to the development of scar-free wound-healing therapies in humans.

Salamander

Salamanders also demonstrate scar-free skin regeneration. When immune cells, such as macrophages, are removed, lizards lose their capacity to recover an appendage and instead form scar tissue. Hence, macrophages appear to be essential factors for limb regeneration in salamander [32]. Macrophage depletion or the disruption of macrophages resulted in scarred skin in a murine model of a full thickness dermal damage [33]. Tylotoin, a wound-healing peptide derived from salamander skin, enhances the motility and expansion of keratinocytes, vascular endothelial cells, and fibroblasts, prompting quickened re-epithelialization and the arrangement of granular tissue at the site of wound development [34]. This peptide stimulates secretory factors, such as beta1 and interleukin (IL)-6, which are essential for scar-free skin regeneration [34]. The formation of new dermal ECM, along with the low levels of fibronectin and high levels of tenascin-C, also promote scar-free skin regeneration [35]. Taken together, these findings suggest that salamander is an excellent model to identify the molecular factors, signaling pathways, and cellular processes underlying scar-free regeneration.

Fish-scale geckos

The skin of fish-scale geckos is prone to tearing to enable the animals to escape from predatory attacks. New skin develops over several weeks without the formation of scars [36] and it is thought that larger scales tear more easily than do smaller scales [36]. The skin of fish-scale geckos has unusually large scales and attached only by a relatively narrow region which is adapted to tearing. The pre-formed splitting zone within skin is present beneath to large scales. Such *physiological* adaptation of make them especially active at escaping

from predators at the slightest touch and able to regenerate their scales back with no scar in weeks while other geckos able to lose their skin when grasped firmly and take long time regenerate the skin. These particular scales are thick and rigid [36], which could also explain the rapid formation of new scales without a scar. Although the mechanisms involved in the regeneration of fish-scale gecko are not well understood, it could have applications in human medicine in the future.

Reindeer

The skin on reindeer antlers, which is termed velvet, recovers from injury in a way that is not observable in any other large mammal. Experimentally inflicted wounds on reindeer velvet versus other parts of the animal resulted in permanent scarring in the latter wound sites, whereas, on the antlers, skin regenerated within a month accompanied by typical levels of pigmentation and hair follicles [37]. The annual regrowth of antlers represents epimorphic recovery, whereby a recovery blastema is formed that comprises mainly mesenchymal stem cells, resulting from the pedicle periosteum. These stem cells allow the growth of the new antlers, which comprise different cells, including those of skin, nerves, bone, and veins [37]. Mature males of all cervids recover their long antler nerve connections without a distal nerve piece [38,39]. The nerves develop at an indistinguishable rate from other antler tissues, peaking at 2 cm growth daily in the largest species [40]. This natural regenerative mechanism is a potential source of novel regenerative components that are engaged in fast axonal recovery, with scope for translation to the clinic.

Additionally, antler velvet provides insight into the variables related to neurite development, advancing our current understanding of the properties of murine embryonic dorsal root ganglion neurons [41]. Antler velvet secretes proteins that enhance and advance neurite development in trigeminal neurons. The skin advances neurite development, exclusively utilizing neuronal growth factor (NGF), while the velvet uses extra proteins. Antler velvet secretes more than 20 axon development promoters, including NGF and periostin. Periostin and NGF synergistically and effectively advance neurite development, imitating the impact of velvet that involves the expression of key embryonic stem cell markers, as observed during mammalian epimorphic regeneration [41,42].

These findings implicate reindeer as a prospective model for rapid scar-free regeneration. Interestingly, reindeer cells grow faster than do tumors but do not generate any tumors, resulting in scar-free regeneration. Thus, the regenerative properties of reindeer antlers could have clinical implications for quick scar-free healing of skin injuries in humans.

Bottlenose dolphins

Bottlenose dolphins are known of the exceptional healing of large soft-tissue wounds. These animal are able to heal large soft tissue wounds (30 cm long and 3 cm deep) in 4 weeks, without scar formation [43]. They form blood clots less readily than do terrestrial mammals [44] and the scar-free healing process utilizes blubber, which contains triglycerides, adipocytes, elastin fibers, and collagen. However, the mechanisms involved in such scar-free regeneration are currently unknown and further research is required before their potential for use in the clinic is realized.

In vivo *human fetal skin*

Fetal and adult skin heal via distinct mechanisms. In adults, the healing of deep wounds often leads to excessive scarring. By contrast, during early gestation, fetal skin has the ability to heal wounds without a scar. Several *in vivo* and *in vitro* models are described in the literature. Generally, fetal skin wounds at the beginning of pregnancy (<24 weeks) are repaired quickly without scarring [45]. The healing process relies upon both the gestational age and injury size. Human fetuses in early pregnancy (<24 weeks) recuperate from incisional wounds without scarring, whereas fetuses from 24 weeks onwards can form scars [45]. A few cells, cytokines, development factors, and parts of the extracellular framework are associated with fetal injury recuperation and have an essential role in scar-free treatment. To determine the factors involved in tissue regeneration and scarring, Coolen *et al*. developed a human fetal *ex vivo* wound model and compared it with an adult skin and scar tissue model [46]. Their findings indicated that the presence of fibroblasts in the wounded area was an important factor in scarless healing [46]. The fibroblast population has also been implicated in the rapid production of ECM components, such as fibronectin and tenascin [45,47,48]. Several studies demonstrated that both mechanical tension and profibrotic growth factors, such as TGF-β1 and platelet-derived growth factor (PDGF), are important for promoting myofibroblast activation at the site of wound healing [49]. Wulff *et al*. showed that the population of activated dermal mast cells in scarless wounds that were generated on embryonic day 15 (E15) were scarce and less mature, indicative of a lower inflammatory response [50].

ECM remodeling and wound healing comprises a family of proteinases known as matrix metalloproteinases (MMPs), including several collagenases, gelatinases, and stromelysins [51]. During scarless tissue repair, the proportion of MMPs is higher than the activity of inhibitors, such as the tissue inhibitor of metalloproteinases (TIMPs). This enzyme:inhibitor ratio favors ECM remodeling, with a lower accumulation of collagen. Additionally, keratins, such as K8 and K19, are present during fetal skin development but are undetected in adult epidermis [52]. Similar studies conducted using lamb fetuses also highlight the differences in collagen deposition patterns as a factor for scarless healing [53]. It is also known that the differences between the production of fetal and adult collagen results from interactions with specific cell surface receptors on fibroblasts. Recent studies highlight the TGF-β3:TGF-β1 expression ratio as a crucial determinant of scar formation in fetal wound healing [46,54]. In adults, TGF-β1 occurs at higher levels, and TGF-β3 at lower levels compared with fetal wounds [46]. Studies support a strong correlation between TGF-β3 expression and hypoxia. Hypoxia drives TGF-β3 expression proportionally, which supports the idea of the differential expression of TGF-β3 in early and postnatal wounds [54].

Akita *et al*. discussed the role of growth factors in amniotic fluid, such as basic fibroblast growth factor (bFGF) and PDGF, which were shown to stimulate the proliferation of dermal fibroblasts [55]. Preincubation of fibroblasts with a bFGF receptor blocker and an anti-bFGF antibody in the amniotic fluid demonstrated significant cellular proliferation [55]. At the molecular level, homeobox transcription factors were involved in patterning events and cell type specification during fetal development [56]. Furthermore, fetal repair without scarring was associated with the decreased expression of HOXB13 and the increased expression of homeobox factor PRX-2 [56]. PRX-2 activation is also important for stimulat-

ing dermal formation. In parallel, expression of HOXB13, a strongly expressed gene in normal skin from the second trimester of pregnancy, is significantly reduced upon response to an injury. This finding suggests that HOXB13 is an inhibitor of dermal proliferation and that its constant expression is an important factor for the maintenance of the dermal static architecture rather than for promoting dermal growth [57].

Cellular factors mediating scarless healing

Inflammatory response

Fetal scar-free healing often occurs without inflammation [58]. In postnatal wounds, it is important to reduce inflammation and to overcome the effects of scarring [59]. However, reports suggest that earlier inflammation in the skin induces a memory function that could stimulate the skin for faster healing [60] because the skin stem cells remember any previous injury, and respond more rapidly to skin regeneration [60]. Such skin regeneration is independent of local skin-resident macrophages or regulatory T cells. The different cellular sources of scar skin regeneration are described in Fig. 2, whereas Fig. 3 details the different types of secretory factor.

Platelets

The PDGFs, TGF-β1 and TGF-β2 are less secreted during the production of fetal platelets than in adults [61]. Early gestational fetal platelets do not show aggregation patterns, whereas late gestational fetal platelets exhibit aggregations similar to adult platelets. This suggests that we need to know more about the transition point between platelet scar-free repair and repairs that result in scars. The major platelet-associated growth factors and their activation are listed in Fig. 4.

Neutrophils

The immune response of the injured skin is marked by the infiltration of circulating neutrophils and natural killer T (NKT) cells to prevent infection. These events are followed by delayed healing and excessive scar formation. In a study by Dovi *et al.*, the depletion of neutrophils accelerated wound closure in a mouse model [62]. A histological analysis of stimulating wounds further supports the presence of a large number of infiltrated neutrophils within the scar granulation tissue, highlighting the infiltration of neutrophils as a driving factor in scar formation [63]. Neutrophils produce cytokines, growth factors, and other soluble mediators that activate the target cells, which is indicative of the inflammatory response [63]. This inflammatory response could be a mechanism for the delayed healing of wounds in the skin along with the formation of scars. Neutrophil-mediated inflammation also leads to the degradation of the ECM and tissue damage beyond the initial injury [63]. Similarly, NKT cells act as additional recruiters for other white blood cells to the site of injury [64]. Primarily, NKT cells produce secretory factors, such as cytokines and chemokines, which attract neutrophils and other white blood cells to the wound. The presence of activated NKT cells significantly slows the healing process, whereas the absence of these cells leads to faster wound closure. Therefore, a homeostatic balance is essential for achieving scar-free skin regeneration.

Fibroblasts to adipocytes

The skin epidermis contains many mature adipocytes, and supports the epithelial keratinocytes of the epidermis. Daily, human skin replaces approximately 500 million cells without any scarring [65]. A tiny cut is often filled by adipocytes, similar to that seen in newborn skin with a scar [65]. Scar tissue comprises cells known as myofibroblasts [65] and does not contain any fat cells or hair follicles [65]. The phenotype of a scarred skin is similar to old skin; as humans age, adipocytes are lost, thereby prompting discoloration and wrinkles. Research has shown that myofibroblasts can be changed into adipocytes, opening a new possibility to convert surrounding myofibroblasts to as fat instead of forming a scar [65]. However, there is a need to identify the possible molecular signaling that provokes hair follicles to develop in

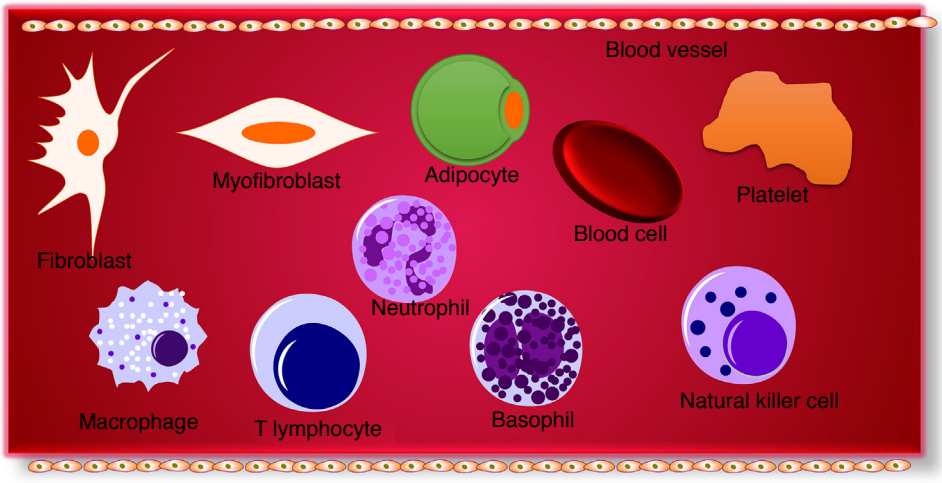

FIGURE 2

Role of different cellular sources for scar-free skin regeneration.

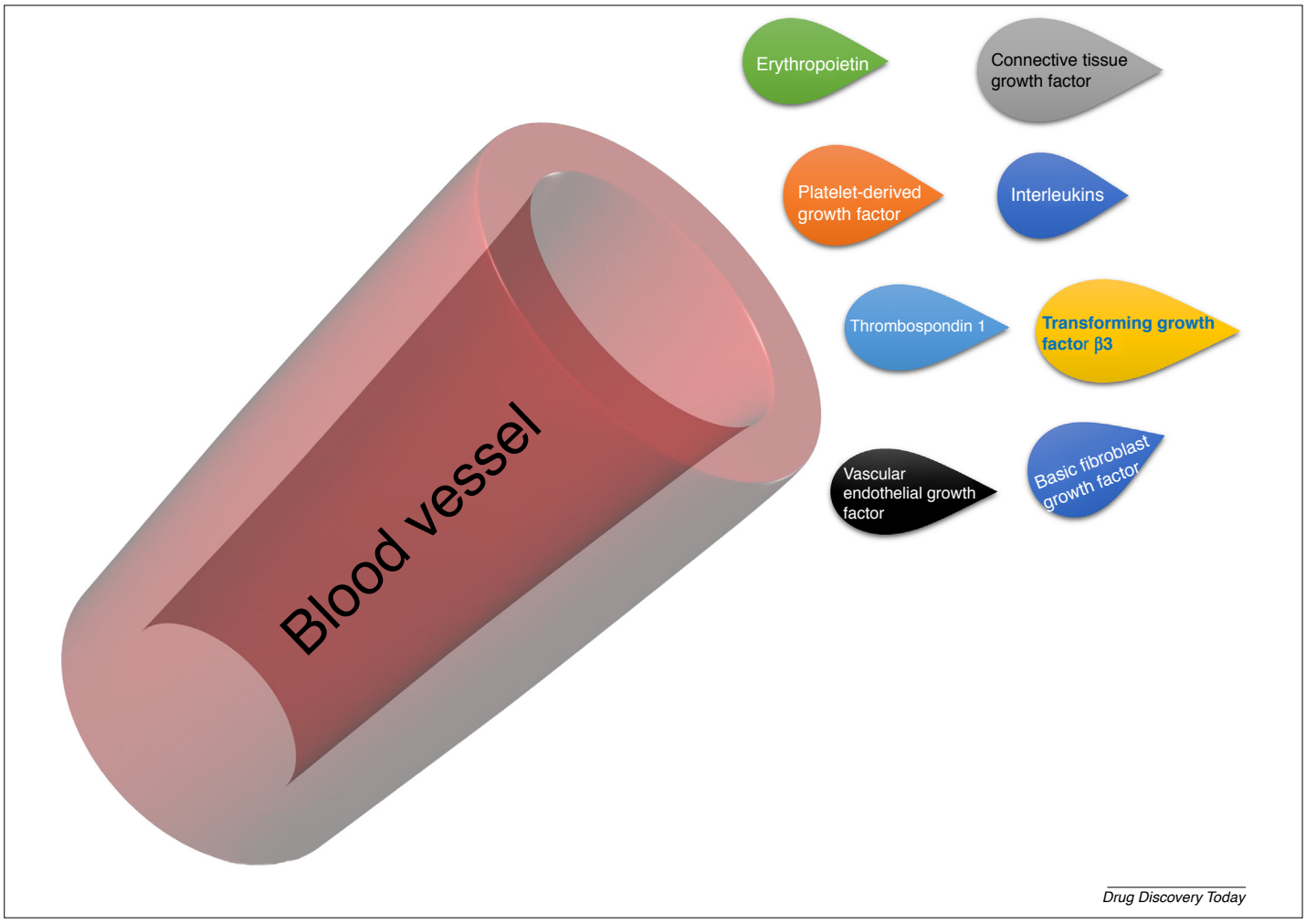

FIGURE 3

Role of different secretory factors for scar-free skin regeneration.

shaping scar tissue in mice and lab-developed human pores and skin tests.

Regulatory T cells

The communication between skin cells and immune cells is required for skin regeneration, a process that naturally declines with age [66]. A subset of immune cells called Foxp3$^+$ CD4$^+$ regulatory T cells (Tregs) have a key role in skin inflammation during the orchestration of stem cell-mediated hair follicle regeneration in the skin [67]. Thus, skin Tregs could provide novel therapeutic targets for the prevention and regeneration of scars. Roshan *et al.* demonstrated that dividing human skin cells switch their behavior between these two modes of maintenance and repair [68]. The authors reported differences in gene expression between wound-healing and balanced populations of cells. Tregs trigger stem cells in the skin to promote healthy hair growth. In the absence of Tregs, the stem cells show a lack of hair follicle regeneration, resulting in baldness. Epithelial stem cell differentiation is associated with Tregs in the microenvironment of the skin [69]. These findings help us understand the behavior of the skin under various conditions and the modes of repair and regeneration. Thus, insights gained from such studies will have broader implications in the area of tissue regeneration.

Myeloid lineage cells

Inflammation is a key event in the development of skin scarring because macrophages are the primary mediators associated with skin fibrosis during scarring [70]. Macrophages have a dual role. They are essential for early wound healing as well as for the formation of scars [70]. However, excessive macrophage activity and persistent inflammation are two major factors that cause skin fibrosis. Myeloid cells, particularly macrophages and neutrophils, are vital to the inflammatory phase of wound healing to protect the host against infection. Macrophages are associated with the development of skin fibrosis, where the number of macrophages correlates with the cellularity in the scar tissue of patients with hypertrophic scars [71]. Increased mast cells and macrophages (M2 subtype) are observed in the skin of patients with keloid scars [72]. Inflammatory macrophages are considered M1 macrophages, whereas anti-inflammatory macrophages are M2 macrophages. The balance between the inflammatory M1 phenotype and the anti-inflammatory M2 phenotype in wound healing and regener-

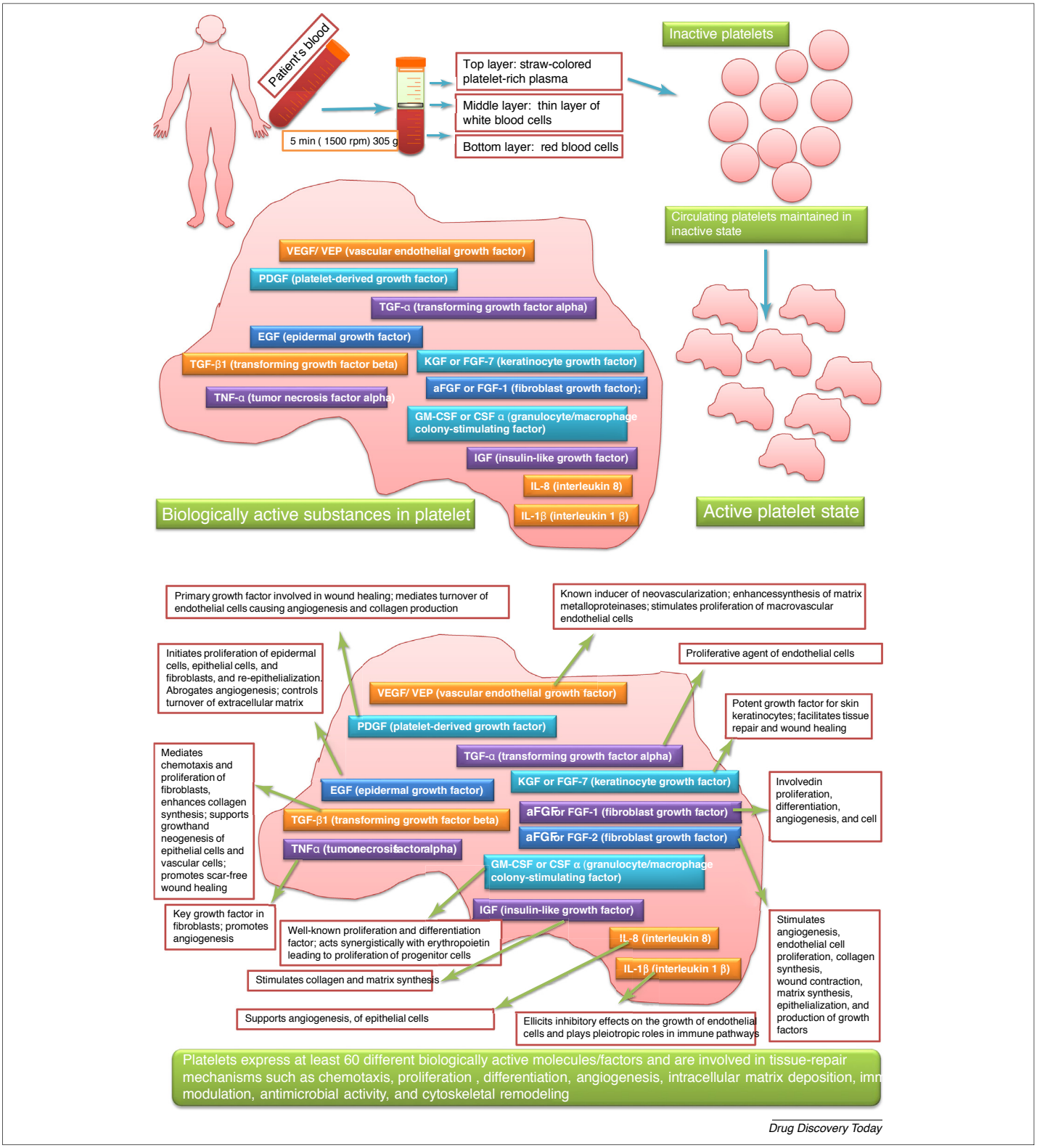

FIGURE 4

Platelet based regenerative factors and their regenerative potential for scar-free skin regeneration. (a) Biologically active regenerative factors for scar-free skin regeneration. (b) Functions of biologically active regenerative factors in scar-free skin regeneration.

ation events has been reviewed elsewhere [73]. Macrophages are essential in the regeneration of new hair follicles during skin regeneration. Chen *et al*. demonstrated that removing hair follicles at different densities caused the adjacent hair follicles to release CCL2, which induced macrophages to secrete tumor necrosis factor (TNF)-α [74]. The removal of hair follicles acts as an initiator for the recruitment of proinflammatory macrophages, providing unique molecular signals for hair regeneration. Therefore, macro-

phages have a significant role in the regenerative environment. Further research is required to gain a better understanding of the role of macrophage phenotypes in scar-free skin regeneration for future drug discovery.

Exosomes

Exosomes are a major paracrine factor primarily released by mesenchymal stem cells and have also been implicated in cellular communication [75–77]. Stem cell-based exosomes prevent the conversion of fibroblast to myofibroblasts and inhibit granular tissue formation [75–77]. Interestingly, exosomes increase the TGF-β3:TGF-β1 ratio *in vivo* [75,76]. Moreover, stem cell-based exosomes reduce the scarring process and switch to a low antiscarring ratio, similar to scar-free healing in human fetuses. Stem cell exosomes promote collagen modeling in a scarless pattern [75,76]. Enhanced angiogenesis reduces the scarring and re-epithelization that primarily occur upon the transplantation of stem cell-based exosomes [77]. Human induced pluripotent stem cell-based exosomes stimulate collagen synthesis and angiogenesis during skin healing [78]. Zhang *et al*. reported that adipose-derived stem cell *(*ADSC)-based exosomes contain a group of specific miRNAs (miR-21, miR-23a, miR-125b, and miR-145) that accelerate full-thickness skin wound healing and attenuate scar formation. The authors demonstrated that ADSC-derived exosomes stimulate fibroblast proliferation and migration, including collagen deposition, via activation of the PI3K/Akt signaling pathway [79]. Similarly, Fang *et al*. demonstrated increased collagen I and III production after the systemic administration of exosomes during the early stage of wound healing [80]. Interestingly, it was shown that exosomes inhibit collagen expression to reduce scar formation during the late stage of wound healing [80]. In a recent study, researchers found that umbilical cord-derived MSCs-Exos, enriched in specific miRNAs (miR-21, miR-23a, miR-125b, and miR-145), have an essential role in suppressing myofibroblast formation by inhibiting the TGF-β2/SMAD2 pathway [81]. Thus, the paracrine factors of stem cell-derived exosomes have significant potential as a cell-free therapy for scar treatment.

Major secretory regenerative factors for scarless repair

Role of TGF-β isoforms and scarring

Studies report that isoforms of the transforming growth factor beta (TGF-β) superfamily have an important role in scar-free skin regeneration. Although both TGF-β1 and TGF-β3 are important for wound healing, evidence also suggests that excessive production of TGF-β1 promotes scarring, whereas TGF-β3 promotes scar-free healing [82]. Thus, the development of a scar might be related to the relative expression levels of TGF-β3 to TGF-β1 and TGFβ2 [82]. However, there is some controversy associated with these conclusions, given that other evidence suggests that TGF-β1 and TGF-β3 do not exhibit different effects on scar hypertrophy. For example, a study using a rabbit ear wound model reported that topically applied TGF-β3 accelerated wound healing but did not reduce hypertrophic scarring compared with the vehicle control. Thus, further research is required to determine the molecular mechanisms underlying their differential effects on scar formation.

To date, no definitive treatment to either prevent or reduce any form of scarring exists. Clinical studies are often inadequate because of the small number of patients, the lack of well-designed controls, a lack of standardization in scar outcome measurements, and a lack of an in-depth understanding of the genomic and proteomic components of the molecules involved in such repair processes. Several mechanisms associated with the role of TGF-β and its isoforms have been proposed recently, of which some are implicated in manipulating the scarring potential. Considering these results, we propose a mechanistic model (Fig. 5) of the signaling mechanisms of TGF-β based on the molecular interactions known thus far.

TGF-β inhibitors

Enhanced TGF-β expression is reported in fibrotic skin scarring. Therefore, blocking TGF-β action by using inhibitors is expected to be beneficial in promoting antiscarring effects.

TGF-β3

As mentioned above, research on the expression and function of the TGF-β isoforms (β1, β2, and β3) in scar-free (embryonic) and scar-forming (adult) wound-healing models showed that the three TGF-β isoforms have different roles in scarring, with TGF-β1 and TGF-β2 showing proscarring effects and TGF-β3 displaying antiscarring effects [83]. The discovery of the antiscarring properties of TGF-β3 led to a clinical development program for evaluating recombinant human TGF-β3 (avotermin) as a therapeutic intervention (prophylactic) to reduce scarring in human surgical wounds. The prophylactic administration of avotermin was successful for the improvement of skin scarring in several double-blind, placebo-controlled, Phase I/II studies [84–86]. Avotermin was shown to significantly improve the visual appearance of scars [84–86], decrease the scar surface area, and promote collagen organization that more closely resembled that of normal skin in 14 of 19 cases [84]. Unfortunately, avotermin failed to show efficacy in Phase III trials, possibly because of the use of a different TGF-β3 standard, which led to a twofold overestimation of the TGF-β3 concentration and, therefore, a 50% lower dose of TGF-β3 was used in the Phase III clinical trial compared with the Phase I/II clinical trials [87].

Nonetheless, from a clinical perspective, the application of small molecules has significant advantages in terms of stability, sterility, shelf life, and regulatory hurdles [88]. In addition, the unique chemical composition of these compounds prevents their rapid degradation, as seen in both cell and growth factor-based therapies. The emerging small-molecule therapies for scar-free skin regeneration are based on the modulation of key signaling pathways involved in tissue repair, and are discussed below.

Connective tissue growth factor

Connective tissue growth factor (CTGF/CCN2) is a fibrogenic cytokine that regulates fibrosis and scarring in numerous tissues, including skin, induced by TGF-β. TGF-β-mediated CCN2 induction is specific to dermal fibroblasts via a complex network of transcriptional interactions requiring Smads, protein kinase C, and ras/MEK/extracellular signal-regulated kinase (ERK) and the Ets-1/transcription enhancer factor. In dermal fibroblasts, CCN2 activation appears to promote scar formation [89]. In adults, CCN2 is induced during wound healing [90] and is overexpressed in fibrosis [90]. The expression of CCN2 has been observed during both the early phases of human acute burn wound healing and murine excisional wounds. The presence of TGF-β1 induces CCN2

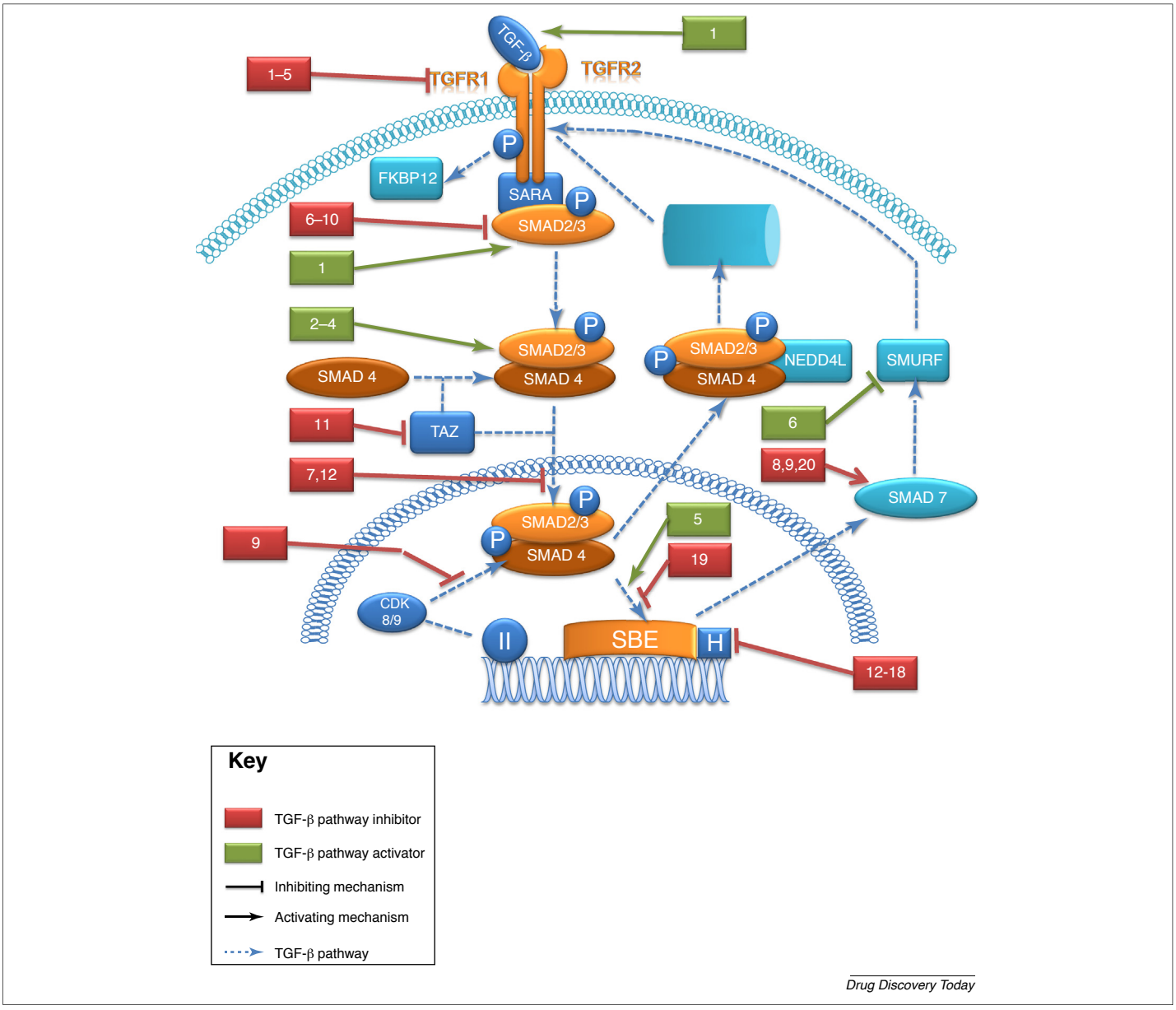

FIGURE 5

Transforming growth factor (TGF)-β pathway showing its activators and inhibitors. Activators of the TGF-β pathway include: (1) tuftsin; (2) aspirin; (3) chlorophyll-related compounds; (4) prostaglandin E2; (5) ΔNp73; and (6) withaferin A. Inhibitors of the TGF-β pathway for scar-free skin regeneration include: (1) 4-([1,2,4triazolo[1,5-apyridin-6-yl)-5(3)-(6-methylpyridin-2-yl)imidazole and -pyrazole derivatives; (2) SD-208; (3) TASP0382088; (4) C11; (5) 5-(1,3-benzothiazol-6-yl)-4-(4-methyl-1,3-thiazol-2-yl)-1H-imidazole derivatives; (6) caveolin-1; (7) silibinin; (8) asiatic acid; (9) compound *Astragalus* and *Salvia miltiorrhiza* extract; (10) PTTG1; (11) C19; (12) m-carboxycinnamic acid *bis*-hydroxamide; (13) C107; (14) C646; (15) melatonin; (16) curcumin; (17) cinnamoyl compounds 1a–c and 2a–d; (18) trichostatin A; (19) AFODN4; and (20) MG32.

expression with a concomitant downregulation in CCN5 expression [91]. Hu *et al.* reported the significant inhibition of CCN2-induced α-SMA and collagen I expression in human hypertrophic scar fibroblasts upon inhibition of ERK and c-Jun N-terminal kinase (JNK) [92]. In a rabbit ear model of a hypertrophic scar, JNK and ERK inhibitors significantly improved the architecture of the rabbit ear and reduced scar formation [92]. The results indicate that ERK and JNK mediate collagen I expression and scarring in the rabbit ear and could be considered as specific drug therapy targets for human hypertrophic scar fibroblasts [92]. Studies also indicate that phosphatase and tensin homolog (PTEN) is a potential regulator of fibrogenesis, where PTEN agonists might show antifibrotic characteristics [93]. Under normal conditions, PTEN knockout in fibroblasts resulted in the phosphorylation of Akt (via the Akt pathway) with an increased expression of CTGF/CCN2 [93]. Furthermore, inhibition of the phosphatidylinositol 3-kinase/Akt pathway led to reduced collagen production and CCN2 expression [93]. The inhibition of CCN2 was associated with a marked reduction in the number of myofibroblasts in scars and the decreased transcription of TIMP-1 and collagen (types I, III). Taken together,

these findings confirm the role of CCN2 as an important mediator of hypertrophic scarring [94]. Recently, it was also shown that the post-transcriptional regulation of CCN2 by cellular miR-143-3p regulates the Akt/mammalian target of rapamycin (mTOR) pathway, thereby inhibiting hyperplastic scar formation [95].

Unlike humans, regeneration potential can also be observed in zebrafish (e.g., spinal cord regeneration). Mokalled *et al.* [96] identified a growth factor in zebrafish that helps this process. The protein encoded by the *CTGFA* gene is a post-injury secretory factor that encourages the interaction between glial cells at the site of the lesion. Supplementation of this protein improves spinal cord repair in injured zebrafish [96]. These experiments provide clues to the regenerative features in zebrafish compared with mammals [96].

Wnt/β-catenin signaling

Wnt/β-catenin signaling is involved in many normal and disease biological phenomena, including cancer [97], and has been challenging to target therapeutically. Therapeutic candidates specifically targeting the Wnt/β-catenin signaling pathway were recently considered for clinical trials, but none have yet been approved [98]. The Wnt/β-catenin signaling pathway in pathology and regeneration is reviewed elsewhere [99]. However, what is important in this context is the fibroblast–myofibroblast transition, which has a key role in hypertrophic scar formation during wound healing [100]. β-catenin influences the fibroblast–myofibroblast transition in normal skin fibroblasts and negatively regulates the TGF-β1-induced myofibroblast transition. However, these two signaling pathways exhibit both synergy and antagonism during pathological scar formation [101].

Skin stem cell system

Another approach for improving regeneration therapies for the skin is achieved by using stem cells. The skin acts as an essential barrier, protecting organisms from their environment. Broadly, the skin comprises two parts: the epidermis, which is the cells that form the barrier, and the dermis, which provides support and nutrition to the epidermis. The microenvironment of the skin, with a special focus on the pool of local resident stem cells, is detailed in Fig. 6. Skin stem cells are localized to the base of the epidermis and the hair follicle bulge. Adult mammalian tissues are maintained by multipotent stem cells, many of which are highly responsive to soluble Wnt proteins. Two Tcf family members, Tcf3 and Tcf4, were shown to be involved in the development and maintenance of epithelial stem cells in the skin through Wnt-dependent and -independent processes [102]. Rinaldi *et al.* identified two proteins (Dnmt3a, Dnmt3b) to be crucial for the self-renewal of skin stem cells, wherein the proteins drive the first step of the genetic programming in these cells [103]. Sendoel *et al.* demonstrated new mechanisms behind a shift that occurs just before the development of skin cancer [104], whereas Mascré *et al.* demonstrated the existence of a new population of stem cells that give rise to progenitor cells that ensure the daily maintenance of the epidermis and demonstrated the major contribution of epidermal stem cells to wound healing [105]. Stem cells also have the capacity to develop into invasive tumours [106]. Rinkevich *et al.* identified multiple lineages of fibroblasts in the dorsal skin [107]. Engrailed-1, a fibrogenic lineage protein, has a central role in dermal development, wound healing, radiation-induced fibrosis, and cancer stroma formation. The inhibition of this lineage resulted in reduced melanoma growth and scar formation, with

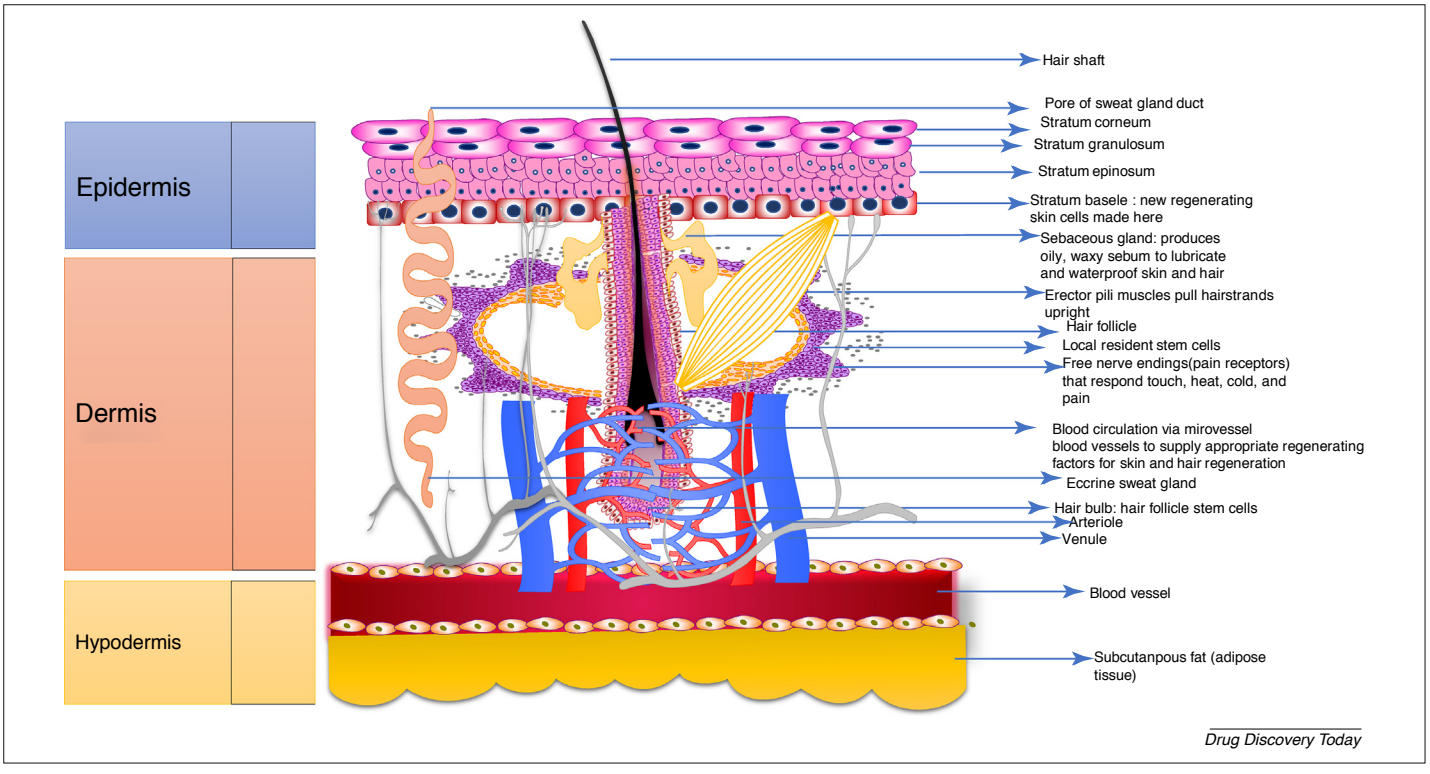

FIGURE 6

Microenvironment of the skin, with a special focus on the pool of local endogenous resident stem cells, for scar-free skin regeneration.

no effect on the structural integrity of the healed skin, thus indicating a therapeutic approach for treating fibrotic disease.

Hair follicle stem cells have the ability to regenerate all of the components of the epidermis. They are localized to the bulge region, which forms part of the outer root sheath [108,109]. Under normal conditions, these cells are naïve. However, trauma transforms them into epidermal stem cells [110]. With injury, the bulge cells rapidly migrate upward and repair the epidermis *in vivo* [111]. In addition, postnatal epidermal stem cells from aged mice show similar plasticity to neonatal epidermal stem cells [112]. Given the significant structural differences between fetal and postnatal skin, it is possible that the origin and location of stem cells in fetal skin are different from those in adult skin [113]. Nevertheless, recent work indicated that the specific role of fetal skin stem cells might be a determinant in scarless wound healing. The PDGFA–AKT signaling axis regulates the renewal of skin adipocyte stem cells [114]. Hair follicle regeneration requires immature adipocytes and the PDGF ligands of immature adipocytes stimulate hair regeneration. The self-organization of dividing stem cells in the skin is important for scar-free skin regeneration [115] as well as for the maintenance of skin tissue size and its appropriate architecture. Hair regeneration is more efficient in skin organoid models [116] Hair follicle stem cells are long-lived stem cells and spend much longer times in quiescence in the hair follicle. Flores *et al.* [117] demonstrated a new way to activate hair follicle stem cells for hair regrowth. When the researchers blocked the production of lactate in hair follicular microenvironment, it prevented activation of hair follicle stem cells. Upon an increase in the production of lactate, the activation of silent hair follicle stem cells occurred.

The bulge area is considered a pool of hair follicle stem cells. These local stem cells move down to the bulb and basal areas of the hair follicle, which produce the protein SCF, which has a key role in the generation of the hair shaft [118]. Hair shaft creation is associated with the production of the KROX20 protein. Thus, hair follicular stem cells move to the bulb areas because of KROX20 and SCF. After reaching the basal areas of bulb, the hair follicular stem cells interact with melanocytes to initiate the pigmentation of hair. The content of these two proteins and of melanocytes declines with age, as does the number of hair follicle stem cells. Without those two proteins, hair becomes gray in color; hence, this is a sign of aging. The differentiating fate of skin progenitors from sweat glands is controlled by competing signals, such as Bmp5. In a mouse model, dorsal-side skin cells generates hair follicles, whereas the ventral skin cells generate sweat glands. There is also high expression of mesenchymal-derived bone morphogenetic proteins in ventral skin cells compared with dorsal skin cells [119].

The endogenous stem cells in the hair follicles are in a quiescent state until they receive an external signal from the local microenvironment to regenerate. Hair follicle stem cells regulate melanocyte stem cells in the bulge area. The life cycle of hair is described in Fig. 7. Hair follicle stem cells have the ability to regenerate all the components of the epidermis, as described in Fig. 8. The activation of hair follicle stem cells is controlled by FOXC1. Without FOXC1, hair follicle stem cells retain one bulge in the hair follicle niche, whereas a normal hair follicle, with FOXC1, retains up to four bulge areas in the follicular region [120]. This research provides clues for activating hair follicle stem cells to generate new hair. The gene *COL17A1* also regulates hair follicle stem cells [121]. Periodically, hair follicle stem cells follow activation and quiescence to

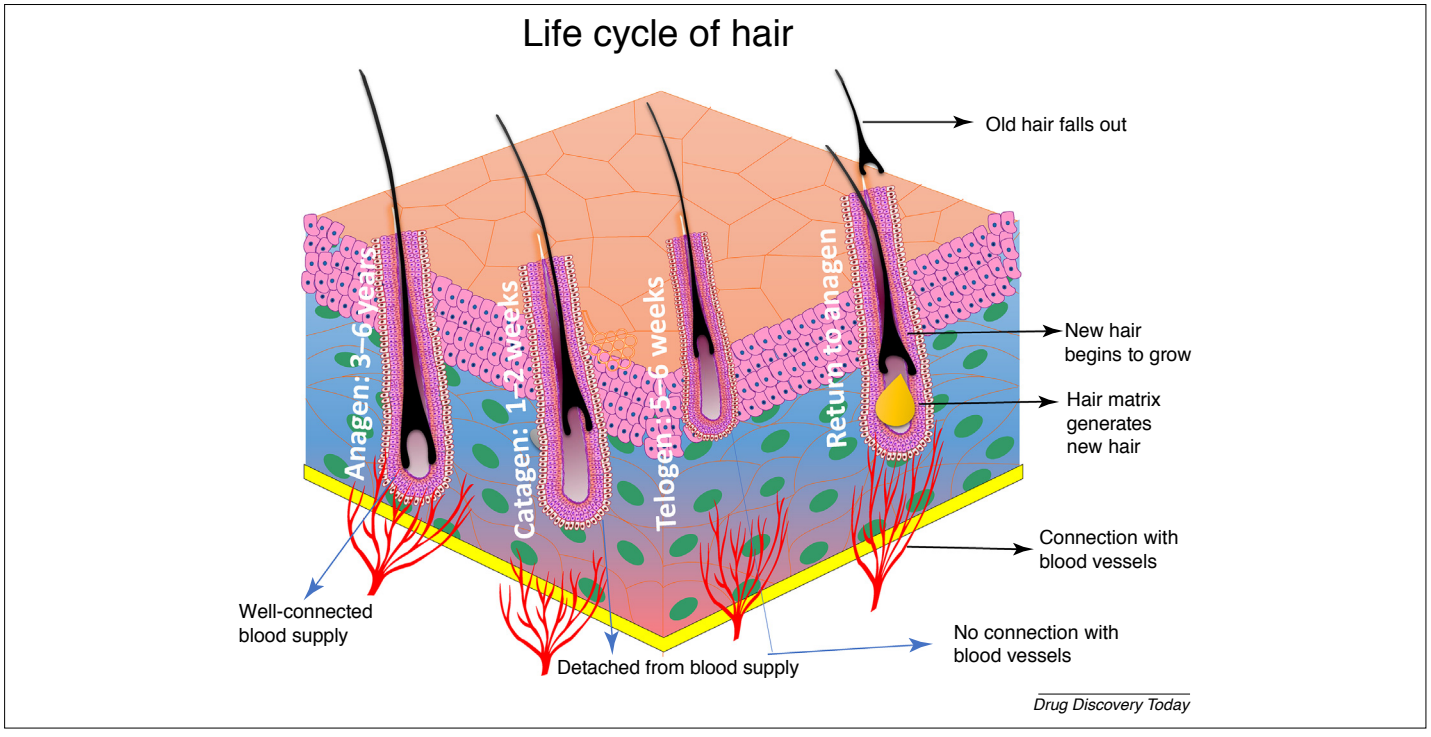

FIGURE 7

Life cycle of hair. Hair production is a cyclical rather than continuous process.

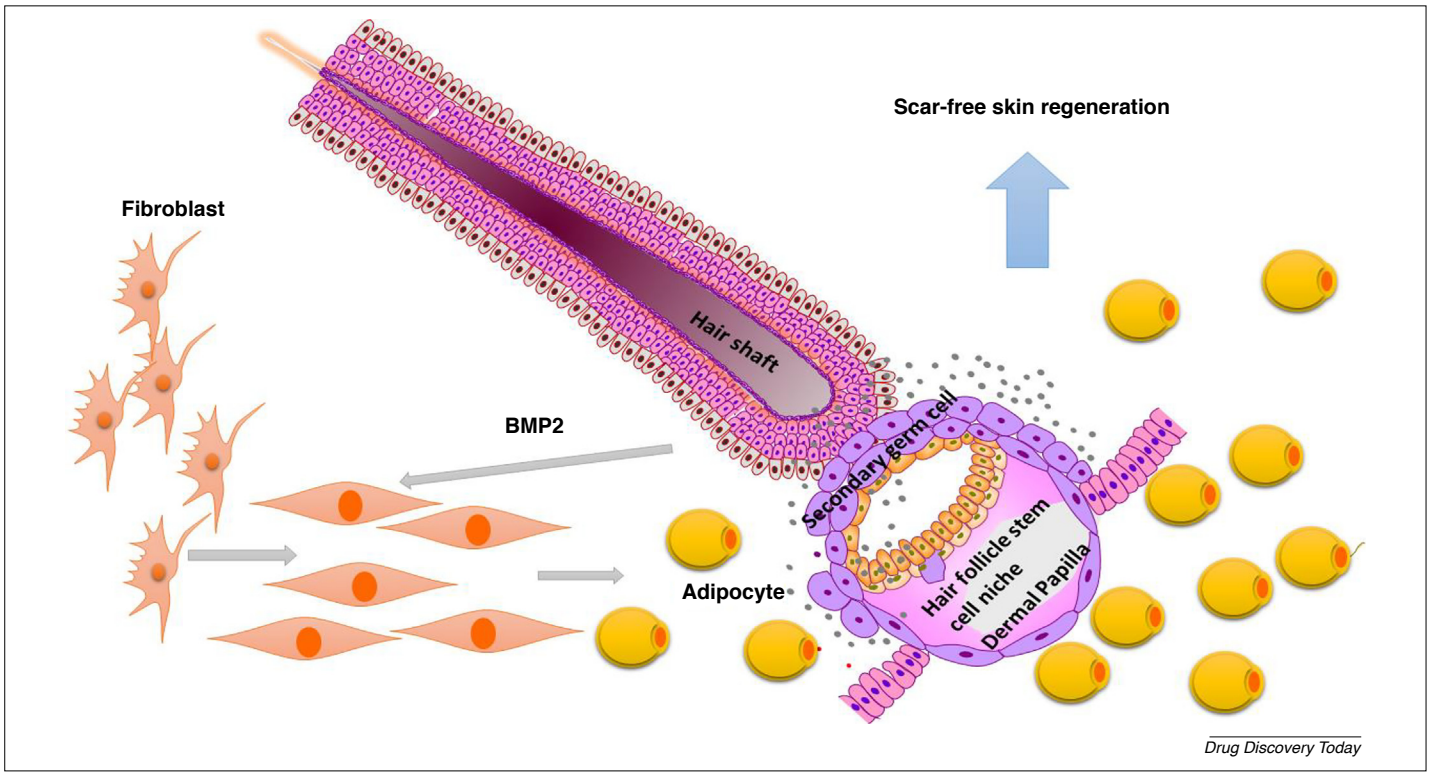

FIGURE 8

Potential targets in hair follicular areas for scar-free skin regeneration, including hair regrowth.

maintain the stem cell pool and generate new hair. The activation of FOXC1 is required in different phases of the hair growth cycle to produce new hair [122]. JAK inhibitors are also required for the activation of hair follicle stem cells to produce new hair. JAK inhibitors stimulated the generation of new hair in 10 days in a mouse model. Interestingly, no new hair generation was observed in control mice during the same time period. This suggests that it is important to find small molecules to inhibit the JAK/STAT signaling pathway to promote the generation of new hair [123]. The local microenvironment of the tiny hair follicle niche has a key role in the proliferation rate of skin stem cells [124]. However, under such conditions, immune cells are recruited at the site of injury and secrete regenerative signals that trigger the healing process and the regeneration of hair follicles [125].

The epidermis, dermis, and hypodermis are the main components of human skin [126]. The dermis is approximately ten times thicker than the overlying epidermis, and the vital cells of the dermis are fibroblasts [126]. The dermis contains a pool of stem cells, as reviewed elsewhere [127]. Interestingly, studies suggest that distinct fibroblast lineages have a crucial role in the regulation of the dermal architecture in skin development and repair [128]. Generally, two forms of skin fibroblasts arise from two distinct lineages (one forms the upper dermis and the second forms the lower dermis). The fibroblasts of the upper dermis regulate hair growth, hair follicle formation, and the arrector pili muscle (APM), which controls piloerection, while synthesizing the bulk of the fibrillar ECM, pre-adipocytes, and adipocytes of the hypodermis to contribute to skin regeneration [129]. Dermal fibroblasts are a heterogeneous population of cells with diverse features, and the identification and isolation of the dermal lineage, with intrinsic fibrogenic potential, is well established [128]. Paracrine cross-talk between dermal fibroblasts and epidermal keratinocytes also directs epithelial cells towards specific phenotypes [130].

Recently, researchers generated an entire human epidermis using transgenic stem cells. Long-lived stem cells ('holoclones') were able to regenerate a stable, self-renewing epidermis, without any visible scarring in the transplanted areas of skin of a young patient with epidermolysis bullosa [131]. Interestingly, upon transplantation of transgenic stem cells from another child, most of the cells from the graft disappeared after a few months, but a small population of holoclones were able to regenerate multiple colonies for the complete regeneration of the epidermis without any scars [131].

Concluding remarks

Although the TGF-β signaling pathway is considered a promising therapeutic target for the treatment and prevention of skin scarring as well as for the treatment for injured scars, currently, no satisfactory therapies based on this pathway are available. Based on knowledge accumulated over the past three decades, several strategies to modulate TGFβ signaling are under investigation to promote scar-free skin regeneration. Small molecules, which create the optimum balance of TGFβ1, TGFβ2 and TGFβ3, are potential antiscarring therapies. Regenerative vertebrate scar-free models provide clues to molecules and pathways that could be exploited for both the prevention of scar formation and scar-free skin regeneration. The activation of hair follicle stem cells, the

conversion of myofibroblasts to adipocytes, the use of long-lived stem cells (holoclones), and the stimulation of local skin progenitor cells are particularly relevant for scar-free skin regeneration. Further research in regeneration-competent vertebrates and using local skin stem cells is required, because this could lead to the identification of elements lacking in regeneration-incompetent skin, thus informing pharmacological strategies of broad applicability to both medical and cosmetic settings.

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