CELLULAR AND MOLECULAR MECHANISMS AT THE PROLIFERATION STAGE IN WOUND HEALING: FROM SCARRING TO TISSUE REGENERATION

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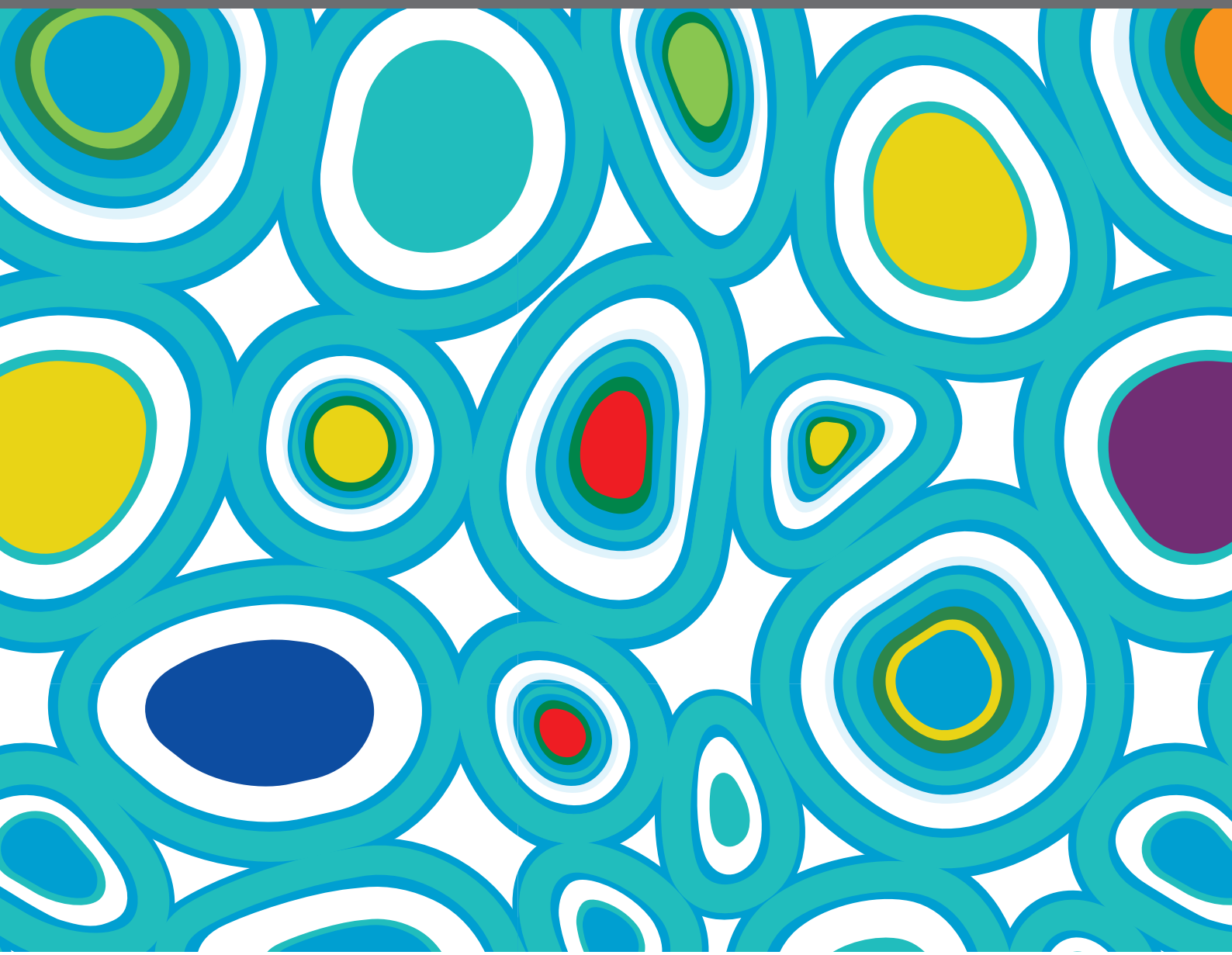

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CELLULAR AND MOLECULAR MECHANISMS AT THE PROLIFERATION STAGE IN WOUND HEALING: FROM SCARRING TO TISSUE REGENERATION

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EDITORIAL
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Editorial: Cellular and Molecular Mechanisms at the Proliferation Stage in Wound Healing: From Scarring to Tissue Regeneration

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Keywords: wound healing, regeneration, scar, proliferation stage, cell interaction

Editorial on the Research Topic

Cellular and Molecular Mechanisms at the Proliferation Stage in Wound Healing: From Scarring to Tissue Regeneration

Wound healing is a complex physiological reaction in our body to tissue injury that can lead to the impairment of the original organ functions depending on the area of tissue injury. Many cell types participate in the wound healing process, including but not limited to, tissue resident cells, cells of the immune system, vascular cells, fibroblasts, and tissue progenitor/stem cells. However, the cellular and molecular regulatory mechanisms of wound healing are not yet fully identified. In the proliferation stage, granulation tissues develop accompanied by matrix deposition and neovascularization, which lead to proper regenerative responses including epithelialization. If this reaction is impaired, then scar formation and non-regenerative healing may occur, in which case many of aggravating factors, such as growth factors, inflammation, and tensile forces, are involved.

This Research Topic consists of 11 published articles including 5 reviews, 2 mini-reviews, and 4 original research manuscripts. On behalf of the Topic Editors, we thank all the authors for their contribution. With their participation, we could draw a future perspective of wound healing research.

CELLS IN WOUND LESIONS

Faster wound healing is always better for patients, and it is desirable that defected wound lesions heal to regenerate original normal tissue. If fibrous/fibroproliferative tissue develops as hyperplastic scar and keloid, respectively, aggravated appearance and uncomfortableness will affect the patient's quality of life. Although delayed wound healing and fibrous/fibroproliferative healing are contradictory biological reactions, both provide serious issues in wound healing in clinical practice. Limandjaja et al. provide important knowledge regarding abnormal fibrosis, keloid. The review article details current research status and classification. The authors emphasized on several pathophysiological factors for keloids, such as inflammation-related environmental factors, keloid-prone topological factors and genetically predisposed individual factors. Defining the factors that contribute to the formation of wound lesion necessitates a demand for future basic and clinical research.

In the process of keloid development, abnormal cellular functions have been investigated, and fibroblasts are a major cell type that are responsible for fibrosis-prone lesions. Although endothelial

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cells are also known to be a key player not only in inflammation, but also in fibrosis, their functional analysis in keloids has not been done. Matsumoto et al. utilized a gene expression analysis of human endothelial cells isolated from keloid and normal skin in patients by using magnetic-activated cell sorting and identified that SERPINA3 and LAMC2 were overexpressed in keloid endothelial cells. Overexpression of the indicated genes may affect keloid pathogenesis by inhibiting matrix degradation and prolonging inflammation. However, additional experiments should be performed to confirm this.

Macrophages are multifunctional innate immune cells with scavenging/phagocytosis as their main function. In addition, they play an orchestrating role in the wound healing process. Barman and Koh summarized current knowledge of macrophage function in the pathogenesis of diabetes. Certain types of macrophages with pro-inflammatory and pro-healing phenotypes may play a dominant role during wound healing, whereas dysregulation of macrophage polarization could cause impaired wound healing. Thus, it is important to elucidate the relationship between macrophage polarization and the exacerbating factors in unhealed wounds.

WOUND HEALING THERAPY

New and effective biomaterials for wound therapy are desirable, which may lead to more effective treatment of unhealed wounds. In this Research Topic, Nurkesh et al. reviewed recent findings on using biomaterials, as drug delivery systems, to extend the activity of incorporated growth factors/cytokines for improving wound healing. Singampalli et al. described the roles of anti-inflammatory cytokine IL-10 and glycosaminoglycan hyaluronan in reducing scarring and improving tissue regeneration. A zwitterionic betaine-incorporated collagen sponge was newly designed for anti-oxidation and anti-inflammation by Chen et al.. Another approach in wound therapy is using a ubiquitin-specific peptidase 15 to enhance re-epithelization via an increase in keratinocytes that was reported by Zhao et al. in addition to the activation of TGF-β signaling pathway in fibroblasts. Development of new biomaterials/molecules may reveal a future strategy for wound healing therapy.

Collagen exists as an extracellular matrix protein and cellular scaffold in granulation tissue. However, its excessive deposition leads to the development of fibrous/fibroproliferative lesions in wound healing. Sato et al. described a novel population of mouse fibroblasts segregated by p75NTR expression and summarized their findings. The authors proposed that collagen degraded di-peptide, Pro-Hyp that arises in wounded tissue or from the blood via food intake, triggered fibroblast transition from a growth arrested subtype to a growth prone p75NTR-positive subtype. This new concept regarding collagen peptides in wound healing should be verified in the future.

The application of MSCs isolated from bone marrow or fat tissues has emerged as another approach for wound therapy. The review article by Jiang and Scharffetter-Kochanek suggested that supplemented MSCs may act as an environment sensing adaptive cells in granulation tissues during wound healing. It was recently reported that microtube-based organelle called primary cilia senses chemical and mechanical signals by Hosio et al. This paracrine effect, rather than their self-renewal and differentiation capacity into several lineages, meet the changing demands during wound healing progression.

ANIMAL MODEL

Many types of animal models for wound healing are currently available. However, a scar forming model in rodents is rare. Marchesini et al. created a wound scar model in rats using talc. This animal model is useful for studying the pathogenesis of hypertrophic scar formation and to assessing potential therapy for treatment.

CONCLUSION

The collection of papers published in this Research Topic further contributes to our understanding of the wound healing process and describes new approaches to reduce scarring and improve wound healing. We hope these studies will stimulate further research in this challenging area of wound healing.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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The Keloid Disorder: Heterogeneity, Histopathology, Mechanisms and Models

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Keloids constitute an abnormal fibroproliferative wound healing response in which raised scar tissue grows excessively and invasively beyond the original wound borders. This review provides a comprehensive overview of several important themes in keloid research: namely keloid histopathology, heterogeneity, pathogenesis, and model systems. Although keloidal collagen versus nodules and α-SMA-immunoreactivity have been considered pathognomonic for keloids versus hypertrophic scars, conflicting results have been reported which will be discussed together with other histopathological keloid characteristics. Importantly, histopathological keloid abnormalities are also present in the keloid epidermis. Heterogeneity between and within keloids exists which is often not considered when interpreting results and may explain discrepancies between studies. At least two distinct keloid phenotypes exist, the superficial-spreading/flat keloids and the bulging/raised keloids. Within keloids, the periphery is often seen as the actively growing margin compared to the more quiescent center, although the opposite has also been reported. Interestingly, the normal skin directly surrounding keloids also shows partial keloid characteristics. Keloids are most likely to occur after an inciting stimulus such as (minor and disproportionate) dermal injury or an inflammatory process (environmental factors) at a keloid-prone anatomical site (topological factors) in a genetically predisposed individual (patient-related factors). The specific cellular abnormalities these various patient, topological and environmental factors generate to ultimately result in keloid scar formation are discussed. Existing keloid models can largely be divided into *in vivo* and *in vitro* systems including a number of subdivisions: human/animal, explant/culture, homotypic/heterotypic culture, direct/indirect co-culture, and 3D/monolayer culture. As skin physiology, immunology and wound healing is markedly different in animals and since keloids are exclusive to humans, there is a need for relevant human *in vitro* models. Of these, the direct co-culture systems that generate full thickness keloid equivalents appear the most promising and will be key to further advance keloid research on its pathogenesis and thereby ultimately advance

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keloid treatment. Finally, the recent change in keloid nomenclature will be discussed, which has moved away from identifying keloids solely as abnormal scars with a purely cosmetic association toward understanding keloids for the fibroproliferative disorder that they are.

Keywords: keloid, cicatrix, hypertrophic, keloid anatomy and histology, keloid etiology, keloid pathology, keloid heterogeneity, keloid model, keloid phenotype

INTRODUCTION

As early as approximately 3000 B.C., the existence of keloid scars has been acknowledged in the description of a "*swelling on his breast, large, spreading, and hard,*" which felt like "*touching a ball of wrappings*" in the Edwin Smith Papyrus, the first known surgical treatise describing ancient Egyptian medical practice (Breasted, 1930; Berstein and Roenigk, 1996). Keloids are not mentioned in modern day literature until the early 19th century when Jean Louis Alibert, the father of French Dermatology, first described tumor-like scars which he initially referred to as '*les cancroïdes de la peau.*' When it became clear these cicatricial tumors were in fact non-cancerous, Alibert changed the name to '*cheloïde*' or '*keloïde*' in reference to the Greek word '*χηλί*' (khçlçé) for crab's claw and the suffix -oid meaning 'like.' Together this is meant to reflect not only the claw-like extension of the keloids but also refers to their horizontal invasive growth beyond the initial wound margins into the surrounding skin (Alibert, 1825; Delpech, 1881; Berstein and Roenigk, 1996).

The keloid incidence rate varies greatly and is known to be influenced by racial ethnicity. The risk of keloid development significantly increases with increasing pigmentation (Burd and Huang, 2005; Wolfram et al., 2009). In the Black and the Hispanic general population, the incidence varies from 4.5–6.2 to 16% (Cosman et al., 1961; Oluwasanmi, 1974; Rockwell et al., 1998); while the incidence in the Taiwanese Chinese and Caucasians is reported to be as low as <1% (Bloom, 1956; Seifert and Mrowietz, 2009; Sun et al., 2014). However, these numbers are largely based on studies from several decades ago with the oldest dating back to 1931. To our knowledge there are no new incidence numbers of keloid scarring in the general population. More recent data is available for very specific subpopulations: in head and neck surgical patients (Young et al., 2014) as well as women after caesarian section (Tulandi et al., 2011), the incidence of keloid scar formation was significantly increased in African Americans (0.8 and 7.1%, respectively) compared with the Caucasian (0.1 and 0.5%, respectively) and Asian or other (0.2 and 5.2%, respectively) population. Interestingly, in Africans with albinism the prevalence rate of 7.5% was not statistically different from the overall prevalence rate of 8.3% in the general population or the 8.5% observed in the normally pigmented African population (Kiprono et al., 2015). It would therefore seem that increased pigmentation in and of itself cannot solely explain the reported ethnic differences in incidence rates (Bran et al., 2009).

In addition to the obvious cosmetic disfigurement, keloids can also produce symptoms of itching and pain (Lee S. S. et al., 2004; Bijlard et al., 2017). A study comparing the quality of life in patients with keloids to that of psoriasis patients found that patients with abnormal scars demonstrated the same reduced quality of life levels as psoriasis patients when compared with healthy controls (Balci et al., 2009). Similarly, a cross-sectional survey on the burden of keloid disease (Bijlard et al., 2017) showed that having keloids was associated with considerable impairment of emotional wellbeing. In summary, keloids may affect a very specific demographic for reasons we do not yet know, but for those affected, these abnormal scars can have significant consequences beyond cosmetics.

The mechanisms behind keloid scarring in particular are still poorly understood (Slemp and Kirschner, 2006; Robles and Berg, 2007; Seifert and Mrowietz, 2009), and this is reflected in our inability to satisfactorily manage this abnormal scar (Niessen et al., 1999; Butler et al., 2008b). Known for its therapy-resistant nature, excision alone has recurrence rates of 55–100% and can even result in the development of a worse scar than before (Robles and Berg, 2007; Butler et al., 2008b; Balci et al., 2009; Shih et al., 2010). To further advance research on the pathogenesis underlying keloid scar formation, there is an urgent need for relevant, true-to-life keloid scar models that resemble the *in vivo* keloid phenotype accurately. This review on keloid scars will discuss histopathological characteristics, inter- and intralesional heterogeneity, the pathogenetic mechanisms, as well as existing scar model systems of keloids.

KELOID HISTOPATHOLOGY

Keloids are primarily a clinical diagnosis (Gulamhuseinwala et al., 2008), and as such are not usually sent in for further analysis by the pathologist. Although the histopathological definition of a keloid scar was not further detailed in the article, Gulamhuseinwala et al. (2008) found that retrospective analysis of H&E stainings of 568 clinically diagnosed keloids only proved accurate in 81% of the cases. Experienced plastic surgeons diagnosed keloids based on the following clinical criteria: the presence of a scar with a history of antecedent local trauma and growth extending beyond its boundary. The non-keloid diagnoses included acne keloidalis (11%), hypertrophic (6%), and even normotrophic (2%) scars and a single pilonidal abscess. Importantly though, no malignancies or dysplasias were reported. Based on these findings, the authors suggested that sending excised keloid tissue for histopathological examination is not necessary if the clinician is an expert and there is a strong clinical suspicion (Gulamhuseinwala et al., 2008). In response to this study, however, Wong and Ogawa pointed out that many clinicians would not be comfortable with the incorrect diagnosis rate of 19% and therefore advocate for

post-surgical histopathological confirmation (Wong and Lee, 2008; Ogawa et al., 2009).

The histopathological abnormalities of the full scarring spectrum and normal skin have been summarized in **Supplementary Table S1**, specific cellular abnormalities in keloid scars are summarized in **Supplementary Table S3** and will be elaborated upon in the section 'Keloid cellular abnormalities'. The histopathological findings on keloid scars will be briefly summarized in this section. The epidermal thickness in keloid scars has been described as anything from atrophic (Koonin, 1964; Bakry et al., 2014) and normal (Moshref and Mufti, 2009; Huang et al., 2014), to sometimes (Ehrlich et al., 1994; Materazzi et al., 2007) or always increased (Bertheim and Hellström, 1994; Chua et al., 2011; Syed et al., 2011; Sidgwick et al., 2013; Jumper et al., 2015; Suttho et al., 2017; Shang et al., 2018). However, the overwhelming majority supports the observation of increased epidermal thickness in keloid scars, and what is more, this was confirmed when thickness was measured in μm (Hellström et al., 2014) as well as number of viable cell layers (Limandjaja et al., 2017, 2019). Similarly, conflicting findings have been reported with regards to rete ridge formation. Reports range from normal rete ridge formation (Lee J. Y. Y.et al., 2004; Moshref and Mufti, 2009) to reduced (Koonin, 1964; Chong et al., 2015; Jumper et al., 2015; Suttho et al., 2017; Shang et al., 2018) or complete absence thereof (Ehrlich et al., 1994; Meenakshi et al., 2005; Huang et al., 2014), although none have attempted to objectively measure the extent of rete ridge formation. Overall, most studies, including our own histopathological studies (Limandjaja et al., 2017, 2019), appear to support the findings of a flattened epidermis with increased thickness. Epidermal differentiation appears mostly unaffected (Bloor et al., 2003; Ong et al., 2010; Limandjaja et al., 2017, 2019). Although increased epidermal activation/proliferation has been observed (Prathiba et al., 2001; Bloor et al., 2003; Ong et al., 2010), this is in contrast with the findings from our extensive immunohistochemical analysis of the keloid epidermis (Limandjaja et al., 2017). We showed normal levels of epidermal proliferation and differentiation in the thickened keloid epidermis, save for the precocious expression of terminal differentiation marker involucrin. We therefore proposed that increased epidermal thickness was not the result of epidermal hyperproliferation, but was associated with abnormal differentiation instead. Interestingly, keloid keratinocytes also showed increased expression of epithelial-mesenchymal transition (EMT) markers (Chua et al., 2011; Ma et al., 2015; Yan et al., 2015; Hahn et al., 2016; Kuwahara et al., 2016).

Histopathological studies of the keloid dermis showed that fibroblasts were present in higher numbers (Ueda et al., 1999; Tanaka et al., 2004; Meenakshi et al., 2009; Jiao et al., 2017). Other dermal cell types residing in keloids include myofibroblasts (Santucci et al., 2001; Kamath et al., 2002; Lee J. Y. Y.et al., 2004; Lee et al., 2012; Moshref and Mufti, 2009; Shin et al., 2016) and fibrocytes (Iqbal et al., 2012; Shin et al., 2016), both were present in increased numbers. In our whole biopsy image analysis of keloid tissue, CD34 expression was found to be absent from the keloid dermis, but constitutively and abundantly present in normal skin and normotrophic scars (Limandjaja et al., 2019). Interestingly, within these CD34− dermal regions, we found senescent (p16+) mesenchymal cells (vimentin+) as well as myofibroblasts (α-SMA+).

Overall trends in the dermal ECM composition include increased levels of collagen I and III (Naitoh et al., 2001; Syed et al., 2011) with increased collagen bundle thickness (Verhaegen et al., 2009); increased fibronectin (Kischer and Hendrix, 1983), glycosaminoglycans (Carrino et al., 2012), chondroitin sulfate (Ikeda et al., 2009), biglycan (Hunzelmann et al., 1996), versican (Carrino et al., 2012), tenascin (Dalkowski et al., 1999), and periostin (Zhou et al., 2010; Maeda et al., 2019); while levels of elastin (Szulgit et al., 2002; Ikeda et al., 2009; Theoret et al., 2013), and decorin (Carrino et al., 2012) were reduced. Dermal hyaluronic acid expression showed variable results (Alaish et al., 1995; Meyer et al., 2000; Ikeda et al., 2009; Yagi et al., 2013), but unlike normal skin, its expression was equal in both the keloid epidermis and dermis (Tan et al., 2011). Reports on vascularity are also highly variable, both increased (Ehrlich et al., 1994; Amadeu et al., 2003; Tanaka et al., 2004; Materazzi et al., 2007; Ong et al., 2007b; Syed and Bayat, 2012; Bakry et al., 2014) and decreased vascular density (Beer et al., 1998; Ueda et al., 2004; Kurokawa et al., 2010; Theoret et al., 2013) has been observed in keloids. Nerve fiber density appears to be increased in keloids compared with normal skin (Hochman et al., 2008; Drummond et al., 2017). Lastly, keloids also show increased immune cell infiltration (Amadeu et al., 2003; Sharquie and Al-Dhalimi, 2003; Tanaka et al., 2004; Shaker et al., 2011; Jiao et al., 2015; Luo et al., 2017), with higher quantities of macrophages (Boyce et al., 2001; Shaker et al., 2011; Jiao et al., 2015) and T-lymphocytes (Shaker et al., 2011; Murao et al., 2014; Jiao et al., 2015) in particular. An extensive review by Jumper et al. (2015) on the histopathology of keloid scars has reiterated most of the aforementioned findings, and further emphasizes the following as most frequently occurring and therefore most discerning features of keloid scars: a thickened, flattened epidermis; a tongue-like advancing edge in the dermis; haphazard, thick, hyalinized collagen bundles as the predominant dermal feature, with subsequent loss of the papillary-reticular boundary; increased dermal cellularity; signs of inflammation; and variable α-SMA expression.

Unlike their keloid counterparts, hypertrophic scars are raised scars whose growth remains within the borders of the original wound (Burd and Huang, 2005) and they can be difficult to distinguish histopathologically. Keloidal collagen (Cosman et al., 1961; Santucci et al., 2001; Ogawa et al., 2009) vs. α-SMA and dermal nodules (Ehrlich et al., 1994; Huang et al., 2014) have often been cited as pathognomonic features for keloids or hypertrophic scars, respectively, but conflicting reports abound (Muir, 1990; Ehrlich et al., 1994; Santucci et al., 2001; Lee J. Y. Y.et al., 2004; Ogawa et al., 2009; Ali et al., 2010; Bux and Madaree, 2010; Huang et al., 2014). In a histopathological study (Limandjaja et al., 2019) comparing both scar types, α-SMA and dermal nodules were present in both scars and while keloidal collagen remained a strong keloid marker, it was also observed in one of the hypertrophic scars. Additionally, a thickened epidermis with involucrin overexpression and a CD34−/α-SMA+/p16+ dermal cell population could be found in both scar types, although α-SMA and p16 immunoreactivity were present in higher degrees

in hypertrophic scars vs. keloids, respectively. In short, despite the clearly defined clinical distinction between the two abnormal scar types, the histological distinction between hypertrophic and keloid scars remains a source of contention, especially in the early stages (Burd and Huang, 2005).

INTER- AND INTRALESIONAL KELOID HETEROGENEITY

Interlesional Heterogeneity

Several reports suggest that distinct keloid phenotypes may exist. As early as 1960, Conway et al. (1960) discerned between nodular raised keloids and flat keloids often observed on the sternum. More recently, Bella et al. (2011) studied large multigenerational pedigrees of patients with familial keloids in three rural African tribes. The superficial spreading phenotype predominated in two of the tribes, while the raised vertical phenotype predominated in the remaining tribe (**Figure 1**). Superficial spreading keloids show irregular subepidermal spread with irregular areas of hyper- and hypopigmentation, they are mostly raised at the edges and are characterized by a central flattened and quiescent area. This central area is often regularly or hypopigmented, while the margins show hyperpigmentation. In contrast, raised keloid scars are prominently bulbous in shape with distinct borders and may have limited areas of central quiescence. Another form of interlesional keloid heterogeneity was proposed by Akaishi et al. (2010), who distinguished between regular keloids with a round shape and clear curving lines, and irregular keloids with irregular shapes and lines. The authors found that the irregularly shaped keloids showed a significant increase in infection and previous surgery rates, and proposed that in contrast, the shape of regular keloids was determined by skin tension alone. In predilection body sites exposed to constant stretching (e.g., scapula, chest, and shoulder), the butterfly, the crab's claw or the dumbbell have also been described as typical keloid shapes which are predominantly determined by local mechanical factors (Huang et al., 2017). In short, several distinct keloid phenotypes have been described, the division of which appears to be based predominantly on the keloid's growth pattern and/or the resulting shape.

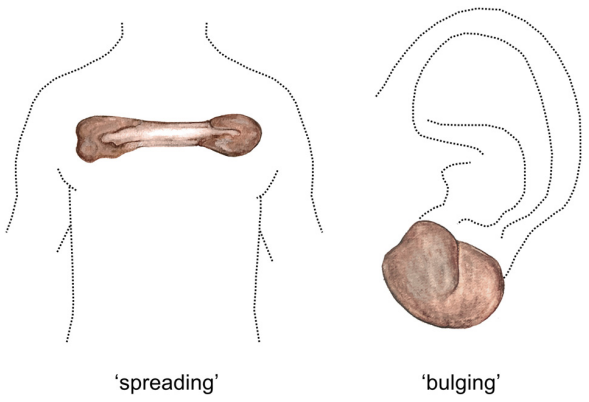

FIGURE 1 | Spreading vs. bulging keloid phenotypes. Watercolor illustration. Left figure shows a typical keloid of the 'spreading' phenotype located on the anterior chest, with quiescent center and an actively growing peripheral margin. Right figure shows a keloid of the 'bulging' phenotype, which are bulbous in shape and can often be observed on the earlobe. Figure first published in Limandjaja (2019), used with permission.

Intralesional Heterogeneity

It is also likely that heterogeneity exists within keloid scars (see **Supplementary Table S1**). Based on clinical observations, the most often described distinction is that of a red, raised peripheral margin which actively invades the surrounding skin and more depressed, lighter colored center showing clinical regression (Louw et al., 1997; Lu et al., 2007a; Seifert et al., 2008). This peripheral-central distinction matches the description of the superficial spreading keloid phenotype (Bella et al., 2011). Symptoms of strong itching (Lee S. S. et al., 2004) predominate in the more pigmented keloid margin (Louw et al., 1997; Le et al., 2004; Lu et al., 2007a; Bella et al., 2011), together with hypercellularity (Appleton et al., 1996; Ladin et al., 1998; Akasaka et al., 2001; Varmeh et al., 2011; Huang et al., 2014), increased vascularity (Appleton et al., 1996; Le et al., 2004; Touchi et al., 2016) and immune cell infiltration (Appleton et al., 1996; Le et al., 2004). Reduced apoptosis (Lu et al., 2007a; Seifert et al., 2008), increased proliferation (Varmeh et al., 2011; Suttho et al., 2017) and increased cellular activity (Louw et al., 1997), all contribute to enlarging the pool of ECM-producing fibroblasts in this region and support the hypothesized increased keloid activity (see **Supplementary Table S2A**). In contrast, the central keloid region shows either hypopigmentation or regular pigmentation (Louw et al., 1997; Le et al., 2004; Lu et al., 2007a; Bella et al., 2011) with pain as the main symptom (Lee S. S. et al., 2004), as well as hypocellularity (Appleton et al., 1996; Ladin et al., 1998; Akasaka et al., 2001; Varmeh et al., 2011; Huang et al., 2014) and reduced vascularity (Appleton et al., 1996; Le et al., 2004; Touchi et al., 2016) (see **Supplementary Table S2A**). Fibroblasts derived from this central region generally show signs of inactivity (Louw et al., 1997), as well as reduced proliferation (Varmeh et al., 2011; Suttho et al., 2017), increased apoptosis (Lu et al., 2007a; Seifert et al., 2008) and senescence (Varmeh et al., 2011). Taken together with the increased expression of ECM-degrading genes (Seifert et al., 2008), the central region appears to be the area of relative quiescence.

Overall, the majority of studies support the concept of an actively developing periphery and a more quiescent central region, but the reverse has also been postulated with an active role for the central region rather than the periphery. The keloid center has been reported to show increased proliferation (Giugliano et al., 2003; Tsujita-Kyutoku et al., 2005), the absence of apoptosis (Appleton et al., 1996; Sayah et al., 1999; Akasaka et al., 2001), increased expression of fibrosis-associated genes (e.g., TGFβRI, SMAD 2, and SMAD3) (Tsujita-Kyutoku et al., 2005) and certain wound healing mediators (IL-6 and VEGF) (Giugliano et al., 2003), all of which support a more pro-active role for this region. In line with these findings, our *in vitro* reconstructed (Limandjaja et al., 2018b) different keloid regions showed that differences existed between the regions in terms of scar parameter expression. The central deep keloid region

showed the more exaggerated keloid phenotype with respect to increased contraction, increased epidermal thickness, reduced HGF secretion and reduced collagen type IV α2 chain dermal gene expression.

Comparison of the keloid heterogeneity findings remains difficult due to the varying definitions of what constitutes the periphery and the center within a keloid, as this may differ significantly between studies. Additionally, we suspected that the apparent dichotomy between results supporting an active keloid periphery and those whose findings ascribe this active role to the keloid center, may be explained by the existence of different keloid phenotypes (Limandjaja et al., 2018b). Various keloid phenotypes have been described in the previous paragraph, but we would like to propose an even simpler classification based on the work of Bella et al. (2011) and Supp et al. (2012b): namely that of a more concave 'superficial spreading' and raised or 'bulging' keloids (see **Figure 1**) (Bella et al., 2011). Depending on the phenotype, the actively expanding region could be located in the periphery or the deeper central region (Supp et al., 2012b). As all the keloids included in the study were of the 'bulging' phenotype, it follows that the central deep region would show the most aggressive keloid phenotype.

Furthermore, heterogeneity has also been observed in regions other than the periphery and the center, these are summarized in **Supplementary Table S2B**. An often-used division is that between different dermal layers (Russell et al., 1989, 1995; Luo et al., 2001; Supp et al., 2012b; Chong et al., 2015, 2018; Jiao et al., 2017), in which case the middle or deepest dermal layers were usually found to act more aggressively compared with the more superficial layers. The conflicting reports on the peripheral and central keloid regions notwithstanding, it is clear that differences exist between different lesional sites within a keloid and these abnormal scars should therefore not simply be considered or treated as a homogenous growth. Because of this, we strongly advocate for the inclusion of a description of the keloid phenotype/shape and the use of schematic drawings to indicate from where within a keloid samples were taken for experimentation.

Normal Skin Surrounding Keloids

The normal skin directly adjacent to and surrounding keloid scars is also rarely included in keloid research. Although similarity to normal skin has been reported (Jumper et al., 2017), the majority of reported results suggest that the surrounding normal skin behaves more like keloid tissue than normal skin. For example, the surrounding normal skin often itches like the keloid periphery (Lee S. S. et al., 2004) and shows increased blood flow compared with unaffected normal skin (Liu et al., 2016). On a cellular level, increased staining of the hematopoietic stem cell marker c-KIT (Bakry et al., 2014) and heat shock protein 70 (Lee et al., 2013) has been observed in both keloids and their surrounding normal skin, while keratinocytes and fibroblasts from the surrounding normal skin shared the abnormal expression of many of the same genes in keloid-derived keratinocytes and fibroblasts (Hahn et al., 2013). In the dermal compartment, the epidermal appendages lost from keloid tissue reappear in the surrounding normal skin in reduced capacity; and the dense and excessive collagen deposition of the keloid can extend into the ECM of the surrounding normal skin, which is otherwise loosely organized, with thin, wavy or even fragmented collagen fibers (Lee et al., 2013; Lee W. J. et al., 2015; Bakry et al., 2014; Jiao et al., 2017). Similarly, portions of nodules from the adjacent keloid have also been found to extend into the surrounding normal skin (Kischer and Pindur, 1990). The dermis adjacent to keloids is more cellular but less crowded compared with healthy skin, and shows significant lymphocyte infiltration (Bakry et al., 2014; Lee W. J. et al., 2015; Jiao et al., 2017). The skin surrounding keloids also differed from unaffected healthy skin with respect to proliferation and apoptosis. Increased dermal proliferation and increased numbers of apoptotic keratinocytes (Appleton et al., 1996) were only observed in the surrounding normal skin, and absent from healthy skin. Lastly, Dohi et al. (2019) proposed an even more prominent role for surrounding normal skin as the driving force for keloid progression into the normal skin, via local preferential increased mechanical strain. Conversely, the surrounding normal skin has also been reported to differ from keloids. Compared with keloid scars, the surrounding normal skin has more blood vessels (Beer et al., 1998) and showed increased expression of the proliferative PCNA marker in the dermis, which was normally absent in both normal skin and keloids (Appleton et al., 1996). The surrounding skin also showed strong, increased levels of CD34 staining in contrast with the CD34 absence in abnormal scars (Erdag et al., 2008). It also lacks the abnormal thermosensory thresholds to warmth, cold, heat and pain sensations reported for keloids (Lee S. S. et al., 2004). All things considered, current literature suggests that the surrounding normal skin shares several features with the adjacent keloid and is therefore a relevant area to include for further investigation.

In our histopathological analysis of keloids, we found that the normal skin directly adjacent to the keloids mostly resembled normal skin or mature normotrophic scar (Limandjaja et al., 2019). An epidermis of normal thickness and rete ridge formation with normal differentiation and proliferation could be seen, together with a CD34+/α-SMA−/p16− phenotype instead of the CD34−/α-SMA+/p16+ phenotype associated with keloid scars. However, the most interesting findings emerged from our *in vitro* work (Limandjaja et al., 2018b), where changes were observed which in and of itself were not statistically significant but overall formed a clear pattern of intermediate abnormal expression. Across all the abnormal scar parameters present in the *in vitro* keloid scar model, the *in vitro* reconstructed surrounding normal skin showed a phenotype more extreme than true normal skin but less aggressive than the peripheral keloid models. With respect to contraction, α-SMA immunoreactivity, HGF secretion and collagen type IV α2 gene expression, the surrounding normal skin showed intermediate between truly unaffected normal skin and keloid scar.

Taken together, we and others have shown that heterogeneity exists within keloid scars. For future studies it would therefore be imperative to mention the shape and growth pattern of the keloid (superficial spreading or bulging) and additionally, it should always be mentioned where in the keloid any tissue for experimentation was obtained from, preferably with a schematic overview for unambiguous clarification. Additionally, we would

argue that it is worth including the surrounding normal skin in any keloid research study when possible.

KELOID SCAR PATHOGENESIS: WHERE DO WE STAND?

The development of a human *in vitro* keloid scar model resembling *in vivo* keloid tissue would not only benefit drug development and testing, it would also greatly aid research into the underlying mechanisms leading to keloid scarring. The fact that keloid pathogenesis remains so poorly understood (Slemp and Kirschner, 2006; Wolfram et al., 2009), has been the bane of affected patients and clinicians alike. Despite the many theories proposed by experts, no single unifying hypothesis has been put forward (Seifert and Mrowietz, 2009). In **Figure 2**, adapted from Wolfram et al. (2009), the various abnormalities reported in keloid tissue and keloid-derived cells are organized in several tiers: patient, topography or special skin sites, and environmental factors all contribute to the expression of abnormal cellular responses, which eventually lead to keloid scar formation. In the following sections, recent and relevant findings for each of these factors will be discussed.

Patient-Related Factors Influencing Keloid Scarring

Ethnicity, genetic predisposition, gender and age are patient characteristics which may influence keloid predilection (Wolfram et al., 2009). There is no conclusive evidence in favor of differences in occurrence based on gender. Some have reported that keloids are more likely to occur in women than men (Bayat et al., 2005; Burd and Huang, 2005; Seifert and Mrowietz, 2009; Middelkoop et al., 2011; Young et al., 2014), but this may also reflect at least in part, the overall greater awareness of unaesthetic scarring in women and a consequent higher tendency to seek medical assistance (Marneros et al., 2001; Burd, 2006). Young age is also associated with increased risk of abnormal scarring (Middelkoop et al., 2011). Keloids can develop at any age, but incidence is highest between the ages of 10–30 years (Bayat et al., 2005; Seifert and Mrowietz, 2009; Young et al., 2014; Lu et al., 2015). Because of the peak in incidence immediately post-puberty, exacerbations during pregnancy and resolution after menopause, a potential role for endocrinological hyperactivity in keloid pathogenesis has also been proposed (Rockwell et al., 1998; Seifert and Mrowietz, 2009; Huang and Ogawa, 2013a; Glass, 2017).

Perhaps the most relevant patient-related factor is the genetic predisposition for keloid formation in certain individuals. Ethnic differences in prevalence showed that darker pigmented individuals are at higher risk of developing keloid scars (Marneros et al., 2001; Al-Attar et al., 2006). Additionally, having a family member with keloids is associated with increased keloid prevalence (Bayat et al., 2005; Kiprono et al., 2015; Lu et al., 2015). This is predominantly the case for first degree relatives, as demonstrated by a heritability of 72, 41, and 17% for first, second, and third degree relatives in the Chinese population (Lu et al., 2015). Individuals with a family history of keloids are also at higher risk of developing multiple keloids and developing keloids of greater severity (Bayat et al., 2005; Lu et al., 2015). The familial heritability, the increased prevalence in certain ethnicities and common occurrence in twins, all strongly support the concept of genetic susceptibility in patients with keloid scars (Marneros et al., 2001; Shih and Bayat, 2010; Glass, 2017). Other lines of evidence pointing to a genetic influence on keloid predisposition include familial inheritance patterns, linkage studies, case-control association studies, and gene expression studies (Shih and Bayat, 2010; Glass, 2017). Different modes of inheritance have been reported, varying from autosomal recessive and X-linked to autosomal dominant (Shih and Bayat, 2010; Glass, 2017). Shih and Bayat (2010) have previously reviewed the available evidence and suggest that most evidence points to an autosomal dominant inheritance pattern with incomplete penetration and variable expression, this then also explains why carriers do not always express the keloid phenotype and why keloid patients do not always respond to trauma with keloid scarring. Despite their valuable contribution to our understanding of genetic predilection for keloids, familial inheritance studies have not led to the discovery of any particular predisposing genes (Glass, 2017). Multiple gene mapping methods as well as targeted gene pathway investigations have identified several gene polymorphisms (*NEDD4, FOXL2, MYO1E,* and *MYO7A* also *HLA*) associated with keloids (Nakashima et al., 2010; Zhu et al., 2013; Velez Edwards et al., 2014; Glass, 2017), but the underlying mechanism is still unclear. Similarly, various abnormalities in gene expression have shown highly variable results between studies (Shih and Bayat, 2010), but affected genes are known to be involved in the ECM, inflammation and apoptosis (Shih and Bayat, 2010). Aside from these inherited gene mutations, acquired altered gene expression in the form of epigenetic modification may also play a role in keloid pathogenesis and further complicates matters (Glass, 2017; He et al., 2017). Ultimately, however, the specific genetic variation responsible for keloid scarring has yet to be identified, but likely involves more than a single gene. Additionally, different keloid patients probably carry different gene polymorphisms which can all lead to keloid scar formation, this would explain the variations in keloid phenotype observed in different people (Shih and Bayat, 2010; Velez Edwards et al., 2014; Glass, 2017).

Topography-Related Factors Influencing Keloid Scarring

It is important to note that patients with a history of keloid scarring do not necessarily form keloids after every injury (Slemp and Kirschner, 2006), two identical incisions can generate one normal scar and one keloid in the same individual (Fong et al., 1999; Fong and Bay, 2002; Al-Attar et al., 2006). Certain body sites are more prone to keloid scarring, thus the location of the wound influences risk of keloid scar formation (Wolfram et al., 2009; Middelkoop et al., 2011). The earlobe, neck, sternum, upper back, shoulders and upper limbs all constitute keloid-prone anatomical sites (Murray et al., 1981; Bayat et al., 2005; Burd and Huang, 2005; Bella et al., 2011; Middelkoop et al., 2011).

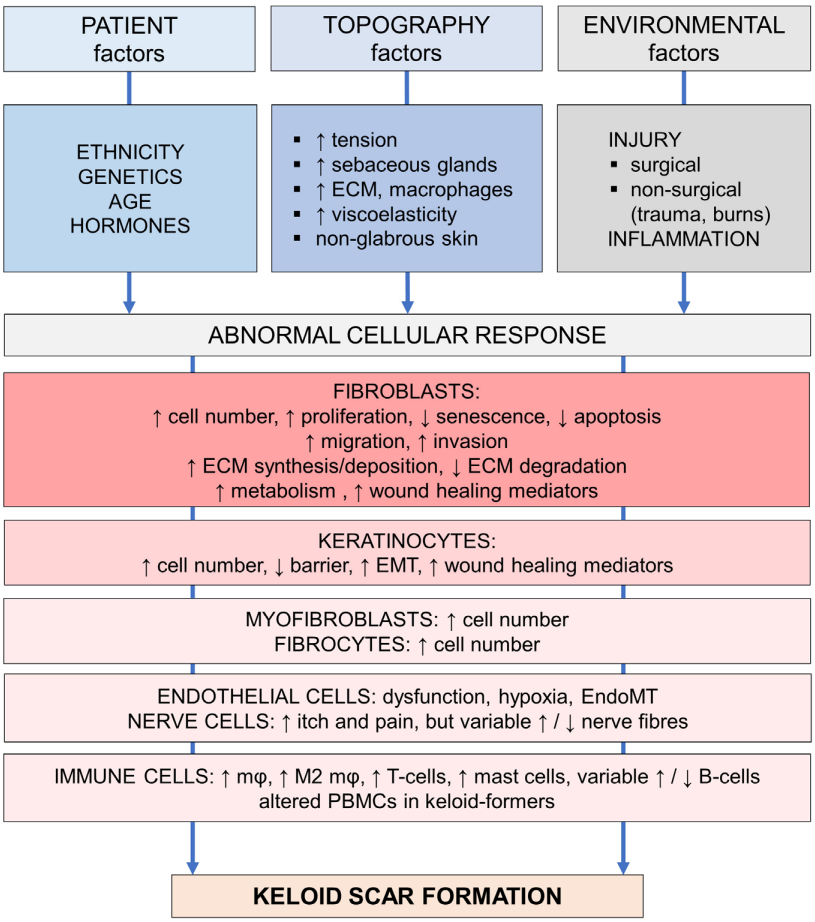

FIGURE 2 | Pathogenesis of keloid scarring. Overview of the various factors involved in keloid pathogenesis, adapted from Wolfram et al. (2009). This figure is meant to provide a provisionary framework to help organize the multitude of pathogenetic findings in a logical and systematic way. Abbreviations; ECM, extracellular matrix; EMT, epithelial-mesenchymal transition; EndoMT, endothelial-mesenchymal transition; mφ, macrophage; M2, alternatively activated, pro-fibrotic macrophage subtype; PBMCs, peripheral blood mononuclear cells. Figure first published in Limandjaja (2019), used with permission.

Although the keloid-prone earlobe and anterior chest have been described as tension-free areas (Brody, 1990; Ogawa et al., 2003; Al-Attar et al., 2006), the most popular explanation for why keloids occur more frequently at certain body sites remains the theory that these are regions of increased skin tension that are subject to constant stretching during normal movement (Peacock et al., 1970; Rockwell et al., 1998; Butler et al., 2008a; Bux and Madaree, 2012; Ogawa et al., 2012). There is no consensus on whether elasticity may explain the differences between keloid-prone and keloid-protected sites. Bux and Madaree (2012) reported that keloid-prone sites are characterized by high tension with low stretch and low elastic modulus. In contrast, Sano et al. (2018) observed that with exception of the earlobes, sites less prone to pathologic scarring (e.g., palmoplantar regions) were "*comparatively hard,*" characterized by low distensibility and reduced elasticity. In contrast, keloid susceptible sites showed high distensibility and increased elasticity. Additionally, high sebaceous gland density (Yagi et al., 1979; Fong et al., 1999; Fong and Bay, 2002; Al-Attar et al., 2006), increased collagen and decreased M1 macrophage numbers (Butzelaar et al., 2017), are all characteristics of keloid-prone skin which may promote keloid scar formation in genetically predisposed individuals.

Environmental Factors Influencing Keloid Scarring

Although spontaneous keloid scar formation has been reported (Jfri et al., 2015), it is a rare occurrence that has been reported in association with certain syndromes such as Rubenstein-Taybi and Goeminne syndrome (Jfri et al., 2015), or may simply reflect a laps in memory (Robles and Berg, 2007; Jfri et al., 2015). Environmental factors are therefore generally an essential prerequisite to keloid scar formation as some form of assault to the skin has to take place to incite keloidogenesis (Bran et al., 2009; Wolfram et al., 2009; Shih and Bayat, 2010). These keloid-inducing events vary from minor to major antecedent trauma, as well as any process resulting in skin inflammation. Insect bites or vaccinations are examples of minor insults to the skin which may be so minor as to not be remembered by the patient at all, while major trauma is usually observed in the setting

of surgical and non-surgical wound healing. Examples of the latter group include lacerations, abrasions, piercings, tattooing or blunt trauma. Additionally, inflammatory skin conditions such as acne, (peri)folliculitis, chicken pox, herpes zoster and hidradenitis suppurativa, may also lead to the development of keloids (Nemeth, 1993; Murray, 1994; English and Shenefelt, 1999; Bran et al., 2009). Isotretinoin is often used to treat acne and has been suggested to act as an additional predisposing factor, though this has not yet been proven conclusively (Guadanhim et al., 2016). While burns have often been mentioned as one of the many potential keloid-inducing events (Trusler and Bauer, 1948; Nemeth, 1993; Murray, 1994; English and Shenefelt, 1999; Bran et al., 2009), they are usually associated with the formation of widespread hypertrophic scars (Middelkoop et al., 2011; Gold et al., 2014) rather than keloids. Fortunately, venipuncture has not been reported to induce keloid scarring (Yadav et al., 1995; Robles and Berg, 2007). Regardless of the type of injury however, the resultant keloid scar response is characteristically disproportionate to the original inciting injury (Tuan and Nichter, 1998).

To summarize, keloid formation is most likely to occur after an inciting stimulus such as dermal injury or inflammatory process (environmental factor) at a keloid-prone anatomical site (topological factor) in a genetically predisposed individual (patient-related factor). The specific cellular abnormalities this generates to ultimately result in keloid scar formation, are discussed next (**Figure 2**).

Keloid Cellular Abnormalities

Although the keloid fibroblast is still considered the main culprit responsible for keloid scar formation, recent studies have shifted the focus to recognize the potential role of the epidermal compartment and the immune system in keloidogenesis. This section will address the trends in reported cellular abnormalities across the entire spectrum of cells present in skin and/or involved in wound healing (see **Supplementary Tables S3A–D**).

Abnormalities in Keloid Epidermal Cell Population

Often overlooked or even designated as 'normal appearing' (Butler et al., 2008b; Hollywood et al., 2010), the keloid epidermis has only recently started garnering attention in keloid research. A summary of the reported keloid epidermal abnormalities has been listed in **Supplementary Table S3A** and a summary of the histopathological epidermal abnormalities can be found in the paragraph 'Keloid Histopathology' and in **Supplementary Table S1**. The abnormalities of the keloid epidermis are not limited to those visible by histopathology alone, there is growing evidence that its barrier function is also affected as measurements of transepidermal water loss (TEWL) and high-frequency conductance suggest keloid scars may show altered stratum corneum function compared with healthy skin (Suetake et al., 1996; Sogabe et al., 2002). In line with these findings, we found that the specific abnormal overexpression of terminal differentiation marker involucrin was not only associated with increased epidermal thickness, but also with disorganization of the stratum corneum as visualized by transmission electron microscopy (Limandjaja et al., 2017). The following paragraphs will focus on abnormalities reported for keloid keratinocytes and melanocytes. Langerhans cells also reside in the epidermal compartment, but will be discussed in the paragraph on keloid immune cells.

Keloid keratinocytes

Keloid keratinocytes may have a more direct role in keloid scarring than previously assumed. Increased expression of growth factors and cytokines such as CTGF (Khoo et al., 2006), HGF and its receptor c-Met (Mukhopadhyay et al., 2010), VEGF and PLGF (Ong et al., 2007b) have been demonstrated in keloid-derived keratinocytes. Furthermore, cultured keloid keratinocytes were found to differentially express 538 genes in a study by Li and Wu (2016) and of these, further functional analysis identified homeobox A7 (*HOXA7*), minichromosome maintenance 8 (*MCM8*), proteasome subunit α type 4 (*PSMA4*) and proteasome subunit β type 2 (*PSMB2*) as key differentially expressed genes. In another gene expression study, Hahn et al. (2013) found abnormal expression of genes involved in differentiation, cell–cell adhesion and increased motility. Keloid keratinocytes also contribute to keloid scarring by paracrine regulation of ECM synthesis in fibroblasts, as evidenced by their ability to induce a more profibrotic phenotype *in vitro* even in fibroblasts of normal skin origin (Lim et al., 2002).

Lastly, several studies support a role for epithelial-mesenchymal transition (EMT) in keloid scarring, a phenomenon by which epithelial cells undergo phenotypic changes and acquire more mesenchymal characteristics (Stone et al., 2017). EMT has been found to occur in wound healing, and plays a role in fibrosis by serving as a source of myofibroblast generation (Stone et al., 2017). The changes associated with EMT have been reported in keloid scars and involve the loss of epithelial cell markers such as E-cadherin (Ma et al., 2015; Yan et al., 2015; Hahn et al., 2016) and gain of mesenchymal characteristics such as vimentin and FSP-1 (fibroblast specific protein 1) expression (Yan et al., 2010, 2015; Ma et al., 2015; Hahn et al., 2016; Kuwahara et al., 2016), combined with changes in cell shape toward a more motile and migratory phenotype (Hahn et al., 2013, 2016; Supp et al., 2014; Stone et al., 2017). In short, the fundamental abnormalities found in the keloid keratinocytes with respect to wound healing mediator secretion, differentially expressed genes, paracrine effects on co-cultured cells and epithelial-mesenchymal transition, all support a more active role for keratinocytes in keloidogenesis.

Keloid melanocytes

Little has been published on the role of melanocytes in keloid pathogenesis (see **Supplementary Tables S1, S3A**), despite the long observed increased keloid incidence in individuals with darker pigmentation (Burd and Huang, 2005; Wolfram et al., 2009). To our knowledge, only Gao et al. (2013) addressed the potential role of melanocytes specifically in both hypertrophic and keloid scar formation and proposed that during wound healing, a damaged basement membrane allows the melanocytes to interact with the dermal fibroblasts. The ensuing increase in

fibroblast proliferation and collagen production together with activation of the TGF-β pathway, promote abnormal scar formation. They performed indirect co-culture experiments in which melanocytes were able to induce increased levels of proliferation, collagen I, TGF-β1 and its downstream p-SMAD 2/3 expression in normal fibroblasts compared with monocultured fibroblasts.

The increased melanin in keloid-prone patients may also contribute to keloid scar formation by inhibiting the senescence-inducing and anti-inflammatory effects of UVB radiation (Wirohadidjojo et al., 2011) and vitamin D (Cooke et al., 2005), respectively. However, variations in melanin levels alone cannot fully explain the association of keloids with darker pigmented individuals, as it has been reported that the African albino population shows similar keloid prevalence rates to the normally pigmented Africans (Kiprono et al., 2015). Aberrations have also been reported in the steps involved in melanogenesis, the process by which melanin is generated (Koonin, 1964; Slominski et al., 1993; Song, 2014; Wadhwa et al., 2016). For example, we now know that polymorphisms in the MC1R gene are in fact responsible for ethnic variations in pigmentation (Videira et al., 2013), and it has been demonstrated that this receptor is not only expressed on melanocytes but can also be found on dermal fibroblasts (Stanisz et al., 2011). In fact, Luo et al. (2013) reported that keloid scars and particularly keloid fibroblasts, showed reduced expression of the melanocortin-1 receptor. They proposed that this may negate the α-MSH-mediated suppression of collagen synthesis and myofibroblast formation, thereby stimulating keloid development. Additionally keloid fibroblasts have also been found to be resistant to inhibitory effects of TGF-β1 on POMC expression (Teofoli et al., 1997; Lotti et al., 1999). Thus, keloids association with increased pigmentation may very well not reflect a primary abnormality in the melanocytes, but a concomitant altered function of a shared receptor in the fibroblasts.

Abnormalities in Keloid Dermal Cell Population

By their very nature, hypertrophic and keloid scars are defined by the presence of raised, protruding scar tissue. The focus of most studies has therefore understandably been on the dermal component and more specifically on the extracellular matrix (ECM) and the ECM-producing fibroblasts. Both keloid and hypertrophic scars showed increased cellularity and were in excess of all three primary ECM components of water, collagen and proteoglycans. Notably, in keloids these processes were significantly upregulated compared with normal skin and hypertrophic scars (Ueda et al., 1999; Miller et al., 2003; Meenakshi et al., 2005).

A summary of the reported keloid dermal abnormalities has been listed in **Supplementary Tables S3B–D**, and a summary of the dermal histopathological abnormalities can be found in the paragraph 'Keloid Histopathology' and in **Supplementary Table S1**. The following paragraphs will detail abnormalities reported for keloid fibroblasts and keratinocyte/fibroblast interactions, myofibroblasts, fibrocytes, endothelial cells, and nerve cells.

Keloid fibroblasts

An overwhelming number of studies have been devoted to the keloid-derived fibroblast. However, the sheer multitude of papers published on keloid fibroblasts have made it impossible to discuss them all in this review and is outside our scope. For this reason, the focus of this review was limited to fibroblast abnormalities as they pertain to the main themes listed by Marneros and Krieg (2004) and Robles and Berg (2007): proliferation, ECM synthesis and degradation, expression of wound healing mediators and apoptosis. A summary of these publications is listed in **Supplementary Table S3B**, overall trends in these *in vitro* monoculture findings will be summarized in the following paragraphs.

There is an overall increase in the number of fibroblasts in keloids, most likely mediated by increased proliferation rates. Although some have also reported normal or even decreased proliferation rates in keloid fibroblasts, the overwhelming majority of *in vitro* monolayer studies support keloid fibroblast hyperproliferation (Russell et al., 1988; Concannon et al., 1993; Blume-Peytavi et al., 1997; Carroll et al., 2002; Carroll and Koch, 2003; Giugliano et al., 2003; Hanasono et al., 2003, 2004; Meenakshi et al., 2005; Lim et al., 2006, 2009; Yeh et al., 2006; Ghazizadeh et al., 2007; Ong et al., 2007a; Akino et al., 2008; Witt et al., 2008; Zhang G. et al., 2009; Jing et al., 2010; Romero-Valdovinos et al., 2011; He et al., 2012; Syed and Bayat, 2012; Jurzak and Adamczyk, 2013; Wang et al., 2013; Xin et al., 2017). In conjunction with increased proliferation, reduced apoptosis by any means would also lead to a cumulative net increase in the keloid fibroblast population. Despite some papers reporting increased apoptosis (Akasaka et al., 2000, 2005), overall, the majority of studies report reduced apoptosis (Ladin et al., 1998; Messadi et al., 1999; Chipev et al., 2000; Luo et al., 2001; Funayama et al., 2003; Tucci-Viegas et al., 2010; Wang et al., 2013). Apoptosis is also reduced in keloid fibroblasts by upregulation of apoptosis-resistance (Ohtsuru et al., 2000; Messadi et al., 2004; Lu et al., 2007b; Seifert et al., 2008), as well as telomere dysfunction and defective senescence. Findings of telomerase upregulation (Yu et al., 2016) and consequent telomere lengthening (Granick et al., 2011; Yu et al., 2016) in keloid fibroblasts support lifespan-prolonging effects of telomere dysfunction, although telomere shortening as a result of oxidative stress has also been reported in 30% of the keloids studied by De Felice et al. (2009). In normal wound healing, fibroblasts eventually become senescent and can then act as inhibitors in the regulation of fibroblast proliferation and ECM synthesis. In this way, defective senescence may also result in a net increase in fibroblast density (Blažić and Brajac, 2006), but literature on senescence in keloid fibroblasts has been sparse and even counterintuitive (Varmeh et al., 2011).

In line with their invasive nature, keloid fibroblasts also show increased migration (Fujiwara et al., 2005a; Lim et al., 2006; Witt et al., 2008; Wen et al., 2011; Syed and Bayat, 2012; Wang et al., 2013; Fang et al., 2016; Jumper et al., 2017; Hsu et al., 2018) and capacity for invasion in 3D invasion assays (Dienus et al., 2010; He et al., 2012; Syed and Bayat, 2012; Wang et al., 2018). Furthermore, increased metabolic activity

(Meenakshi et al., 2005; Vincent et al., 2008), increased ECM synthesis (McCoy et al., 1982; Abergel et al., 1987; Ala-Kokko et al., 1987; Babu et al., 1989; Berman and Duncan, 1989; Suzawa et al., 1992; Fujiwara et al., 2005a; Ong et al., 2007a; He et al., 2012) and deposition (Abergel et al., 1985; Uzawa et al., 2003; Fang et al., 2016) combined with reduced ECM degradation (Abergel et al., 1985; Berman and Duncan, 1989; Uchida et al., 2003; Yeh et al., 2006, 2009; Seifert et al., 2008; Russell et al., 2010; McFarland et al., 2011; Suarez et al., 2013), all contribute to the ECM overexpression and the resulting dermal protuberance that defines these scars (see **Supplementary Table S3B**). Increased levels of collagen I (Uitto et al., 1985; Ala-Kokko et al., 1987; Lee et al., 1991; Friedman et al., 1993; Sato et al., 1998; Chipev et al., 2000; Daian et al., 2003; Hasegawa et al., 2003; Lim et al., 2003; Hsu et al., 2006, 2018; Xia et al., 2007; Zhang G. et al., 2009; Dienus et al., 2010; McFarland et al., 2011; Lin et al., 2015; Suarez et al., 2015; Fang et al., 2016; Luo et al., 2017), a major constituent of the dermis, is likely responsible for the bulk of this increased tissue mass. Additionally, there is an increased collagen I:III ratio (Uitto et al., 1985; Abergel et al., 1987; Lee et al., 1991; Friedman et al., 1993; Zhang G. et al., 2009), despite variable reports on the levels of collagen III (Clore et al., 1979; Uitto et al., 1985; Ala-Kokko et al., 1987; Lee et al., 1991; Sato et al., 1998; Lim et al., 2002, 2003, 2013; Zhang G. et al., 2009; Lin et al., 2015; Hsu et al., 2018). The most reported trend is that of increased collagen III levels (Ala-Kokko et al., 1987; Lee et al., 1991; Sato et al., 1998; Lim et al., 2003, 2013; Zhang G. et al., 2009; Hsu et al., 2018). Other ECM constituents also expressed at higher levels in keloid fibroblasts include fibronectin (Kischer and Hendrix, 1983; Babu et al., 1989; Kischer and Pindur, 1990; Sible et al., 1994; Blume-Peytavi et al., 1997; Chipev and Simon, 2002; Liang et al., 2013; Suarez et al., 2015; Fang et al., 2016; Luo et al., 2017; Hsu et al., 2018), elastin (Russell et al., 1989, 1995; Lee et al., 1991), glycosaminoglycans (Berman and Duncan, 1989; Suarez et al., 2013), and both small and large proteoglycans (Yagi et al., 2013).

The profibrotic characteristics of keloid fibroblasts are at least in part, mediated by increased levels of several key wound healing mediators and their associated receptors (see **Supplementary Table S3B**). Major pathways upregulated in keloid fibroblasts include TGF-β1 (Mccormack et al., 2001; Mikulec et al., 2001; Carroll et al., 2002; Carroll and Koch, 2003; Funayama et al., 2003; Hanasono et al., 2003; Xia et al., 2004; Bock et al., 2005; Fujiwara et al., 2005b; Bran et al., 2010; Lim et al., 2013; Wang et al., 2013; Jurzak et al., 2014; Yang et al., 2014; Lin et al., 2015; Suarez et al., 2015; Fang et al., 2016), TGF-β2 (Xia et al., 2005; Bran et al., 2010; Suarez et al., 2015; Fang et al., 2016), and their receptors (Chin et al., 2001; Xia et al., 2004; Tsujita-Kyutoku et al., 2005); CTGF (Xia et al., 2004, 2007; Khoo et al., 2006; Russell et al., 2010; Jurzak et al., 2014; Fang et al., 2016); VEGF (Wu et al., 2004; Fujiwara et al., 2005b; Ong et al., 2007b; Dienus et al., 2010); interleukins IL-6 (Xue et al., 2000; Ghazizadeh et al., 2007; Do et al., 2012) and IL-8 (Do et al., 2012); as well as IGF-1 receptor (Yoshimoto et al., 1999; Ohtsuru et al., 2000; Phan et al., 2003; Hu et al., 2014) and its binding-related proteins (Phan et al., 2003; Seifert et al., 2008; Smith et al., 2008; Russell et al., 2010). Moreover, keloid fibroblasts not only produce higher levels of wound healing factors, they are also inherently more sensitive to the effects of many of these factors (see **Supplementary Table S3B**). Keloid fibroblasts showed increased collagen secretion, PAI-1 and PDGFα receptor expression, as well as increased proliferation and migration in response to IL-18 (Do et al., 2012), VEGF (Wu et al., 2004), TGF-β1 (Messadi et al., 1998), HDGF (Ooi et al., 2010), and CTGF (Luo et al., 2017), respectively, which was absent in normal fibroblasts. Similarly, keloid fibroblasts exhibited a greater response in ECM synthesis, proliferation, migration, invasion and inflammatory mediator secretion to TGF-β (Bettinger et al., 1996; Daian et al., 2003), HGF (Jin, 2014), PDGF (Haisa et al., 1994), and IL-18 (Do et al., 2012) stimulation, respectively, compared with normal skin fibroblasts.

In a similar fashion to normal fibroblasts (Kroeze et al., 2009), keloid-derived fibroblasts display mesenchymal stem cell (MCS) markers and possess the multipotency to differentiate into adipocytes, osteocytes, chondrocytes, smooth muscle cells, endothelial cells, and neural lineage cells (Moon et al., 2008; Iqbal et al., 2012; Plikus et al., 2017); thereby earning the descriptor of multipotent precursor cells. Interestingly, multipotency capabilities may differ between different scar types as demonstrated by the ability of keloid fibroblasts, but not their hypertrophic counterparts, to differentiate into adipocytes either by stimulation with BMP4 or when co-cultured with human scalp hair follicle cells (Plikus et al., 2017). Iqbal et al. (2010) further differentiated between MSCs of hematopoietic and non-hematopoietic origin, with the majority comprising the non-hematopoietic subtype located in the top and middle areas of the keloids. Regardless of the MSC subtype, however, all MSC markers showed progressive downregulation in culture with increasing cell passage. Based on the similarly progressive loss of the keloid phenotype with *in vitro* serial culturing and the abnormally proliferative nature of keloid fibroblasts, Moon et al. (2008) hypothesized that keloid fibroblasts may be stimulated by the aberrant keloid cytokine milieu to remain in an undifferentiated multipotent and proliferative stem cell state. By extension, Qu et al. (2013) proposed that these keloid stem cells are able to sustain themselves by asymmetric cell division due to their drug resistant and high self-renewing abilities. The continued generation of new aberrant keloid cells then sets the typical tumor-like keloid growth in motion, and also helps explain the high post-therapy recurrence rates. In fact, the pathological keloid microenvironment may also be responsible for generating the keloid stem cells in the first place. Qu et al. (2013) also hypothesized that a pathological niche exists in keloids that is the result of the pre-existing abnormalities in keloid-prone patients, namely the enhanced and persistent inflammatory response and the overexpression of growth factors and their receptors. The multipotent keloid fibroblasts, or rather keloid stem cells, are then transformed from normal dermal stem cells after exposure to this pathological keloid niche. Akino et al. (2008) co-culture experiments of mesenchymal stem cells with keloid fibroblasts may support this niche hypothesis, as mesenchymal stem cells showed similar fibrotic and myofibroblast-like changes after exposure to keloid fibroblasts in co-culture. Regardless of their cell of origin however, the multipotent stem cell nature of the keloid fibroblast

appears to play an important role in the genesis and maintenance of keloid scars.

Although highly informative, findings from fibroblast monolayer cultures are not without their limitations. Serial culturing, methods of fibroblast cell isolation (enzymatic vs. explant*), presence or absence of serum in culture medium, and 3D vs. monolayer culturing, are all potential culturing artifacts which have differed across studies and may influence outcome parameters significantly. It is important to consider the potential confounding effects of these culturing differences while interpreting different results. As a final consideration, it should be noted that keloid fibroblasts are usually compared with fibroblasts derived from healthy non-lesional skin, while in fact normotrophic scars represent the true standard against which keloids should be compared. This holds true for all the tissue and cellular components studied in keloid research, and is not limited to the comparison of keloid fibroblasts to normotrophic scar-derived fibroblasts. In conclusion, keloid fibroblast monocultures have generated a multitude of interesting findings, but it is important to remember the inherent limitations associated with monoculture model systems and consider the influence of differences in cell isolation and culture methods.

Abnormal keloid keratinocyte-fibroblast interactions

We know that the interactions between keratinocyte and fibroblasts are an integral component of the normal wound healing process (Lorenz and Longaker, 2003; Broughton et al., 2006) and findings from *in vitro* double chamber co-culture experiments have been particularly informative in this regard, as they allow us to study indirect paracrine interactions between the two cell populations (see **Supplementary Table S4D**). Co-cultures of keratinocytes with fibroblasts show increased proliferation, levels of ECM and growth factor expression compared with monocultures (Phan et al., 2002, 2003; Funayama et al., 2003; Lim et al., 2003; Xia et al., 2004; Khoo et al., 2006; Ong et al., 2007b; Ooi et al., 2010; Do et al., 2012), but this effect is generally greatest with keloid-derived cells. Keloid keratinocytes are able to induce the profibrotic keloid phenotype in normal skin fibroblasts (Lim et al., 2001, 2002, 2003; Funayama et al., 2003; Xia et al., 2004; Khoo et al., 2006; Ong et al., 2007b; Chua et al., 2011), while keloid fibroblasts are able to the propagate fibrosis markers even when co-cultured with normal keratinocytes (Lim et al., 2001; Phan et al., 2003; Xia et al., 2004; Supp et al., 2012b; Lee Y.-S. et al., 2015).

These findings all strongly support a role for abnormal keloid keratinocyte/fibroblast interactions in the pathogenesis of keloids and thereby provide a new point of interception for therapeutic strategies. In this light, Burd and Chan (2002) described an interesting case of a pediatric patient with a giant keloid covering most of the upper right leg and buttocks. In a multistep procedure, the keloid tissue was removed and an artificial dermal matrix was placed on the wound bed. This was followed by the addition of an autologous keratinocyte cell suspension fixated by a fibrin glue spray. During the 18-month follow-up there was no recurrence of keloid formation. This case report serves as an excellent example of a bench to bedside approach to negate the adverse keloid epidermis-dermis interaction, by removing the diseased dermal matrix and introducing normal keratinocytes.

Keloid myofibroblasts

Although Ehrlich et al. (1994) put forward the absence of myofibroblasts as a feature that differentiates keloids from hypertrophic scars, the opposite has also been observed (Santucci et al., 2001). In fact, the overwhelming majority of studies report the presence of α-SMA+ myofibroblasts in 33–81% of the keloids analyzed (Santucci et al., 2001; Kamath et al., 2002; Amadeu et al., 2003; Lee J. Y. Y.et al., 2004; Moshref and Mufti, 2009). Particularly when cultured *in vitro*, keloid fibroblasts can be shown to contain a significant portion of myofibroblasts (Chipev et al., 2000; Chipev and Simon, 2002; van der Slot et al., 2004; Ong et al., 2007a; Lee et al., 2012; Jin, 2014; Suarez et al., 2015; Luo et al., 2017; Shang et al., 2018). In our histopathological study on abnormal scar types and immature scars (3–5 weeks old), we identified a CD34−/α-SMA+ specific dermal cell population, which were largely senescent in the abnormal scars (p16+) but actively proliferating in the young scars (Ki67+) (Limandjaja et al., 2019). See **Supplementary Tables S2, S3B** for a summary of the histopathological results and cellular abnormalities, respectively.

In wound healing by secondary intention, macrophages stimulate wound bed-derived fibroblasts with TGF-β1 and PDGF to transform them into myofibroblasts (Broughton et al., 2006). In a recently published review, Lim et al. (2019) suggested that the aberrant fibroblasts and myofibroblasts in keloids may originate from an altogether different cell type, namely the embryonal stem cell-like cell population located in the endothelium of microvessels and on the perivascular cells within keloid-associated lymphoid tissue. After injury, these cells are thought to differentiate into abnormal fibroblasts and myofibroblasts through the process of endothelial-mesenchymal transition. Additionally, circulating fibrocytes or mesenchymal stem cells from the bone marrow may also migrate to the target site to generate the abnormal (myo-)fibroblast population. In other words, myofibroblasts in the keloid environment may have several sources of origin beyond the wound bed fibroblasts. Alternatively, mesenchymal stem cells may also serve as a myofibroblast source. Monolayer co-culture experiments with mesenchymal stem cells and keloid fibroblasts have shown that the latter are able to induce a myofibroblast-like phenotypic switch of the mesenchymal stem cells (Akino et al., 2008). In short, myofibroblasts may very well originate from several different sources in addition to the wound-bed fibroblasts.

In normal wound healing processes, we know that myofibroblasts can produce significant wound surface reduction through wound contraction (Lorenz and Longaker, 2003), but Plikus et al. (2017) published an interesting new theory on how myofibroblasts may contribute to the development of keloid scars. They found that hair follicles are essential for inducing myofibroblast-to-adipocyte reprogramming that allows for regeneration rather than scar formation. As hair follicles are absent from the keloid microenvironment, the myofibroblasts are left unable to convert to adipocytes, thereby triggering the scar response leading to keloid formation. In this way, hair follicles and adipocytes may be involved in keloid scarring by effecting myofibroblast dissipation from the wound bed, and serve as interesting new potential therapeutic targets for further research.

Keloid fibrocytes

Bucala et al. (1994) were the first to suggest that the surrounding connective tissue may not be the sole source of new fibroblasts in wound repair and described a blood-borne cell with fibroblast-like properties that enter sites of tissue repair (see **Supplementary Tables S1, S3B**). These so-called fibrocytes were characterized by a collagen+/vimentin+/CD34+ phenotype and not only produced ECM proteins and wound healing mediators, but were also capable of acting as antigen-presenting cells and differentiating into myofibroblasts (Bucala et al., 1994; Quan et al., 2004). Based on the limited available literature, there are increased numbers of CD45RO+/35F9+/MRP8/9+ fibrocytes in keloids compared with normotrophic scars (Iqbal et al., 2012), and moreover, PBMCs derived from keloid patients produced more LSP-1+/collagen1+ fibrocytes than PBMCs from healthy controls (Naylor et al., 2012). In further support of this, Mathangi Ramakrishnan et al. (2012) showed that keloid fibroblasts expressed increased levels of fibrocyte markers (CD34+/CD86+), which were absent in normal fibroblasts. This suggests an at least partial fibrocyte origin of the keloid-derived fibroblasts. Reduced fibrocyte numbers have also been reported (Ueda et al., 1999), but this was based on the presence of histologically identified slender nuclei rather than immunohistochemical phenotyping, and may therefore not be as reliable. Given the aforementioned findings of increased fibrocyte presence in keloids (Iqbal et al., 2012; Mathangi Ramakrishnan et al., 2012) and their potential differentiation into abnormal keloid myofibroblasts (Lim et al., 2019), fibrocytes may be significantly involved in keloidogenesis and therefore deserve further investigation.

Keloid endothelial cells

Both increased (Ehrlich et al., 1994; Amadeu et al., 2003; Tanaka et al., 2004; Materazzi et al., 2007; Ammendola et al., 2013; Bakry et al., 2014) and decreased (Beer et al., 1998; Ueda et al., 2004; Bux and Madaree, 2010; Kurokawa et al., 2010) vasculature have been reported in keloid scars in approximately equal measure (see **Supplementary Tables S2, S3C**). However, based on reports of microvessel occlusion and increased expression of hypoxia-induced factor 1α (HIF-1α) in abnormal scars, it has been proposed that keloids are relatively hypoxic tissues (Zhao et al., 2017). The ischemia hypothesis builds on this to explain how hypoxia can contribute to keloid scar development. Kischer et al. (1982) demonstrated that unlike normal skin, the overwhelming majority of hypertrophic and keloid scars have microvessels with occluded lumens and that this was likely due to endothelial cell proliferation. The authors considered this reaffirmation of their hypothesis that hypoxia "*is an integral factor in the generation of hypertrophic scar and keloid*" (Kischer et al., 1982), but whether or not this relative hypoxia promotes fibroblast and endothelial cell proliferation has still not been determined (Song, 2014).

Kischer (1984) also suggested another mechanism by which the microvessel abnormalities could generate both hypertrophic scars and keloids. They proposed that injury leads to regeneration of the microvessels, and suggest that the pericytes of the newly regenerating microvessels form the source of the fibroblasts generating the excessive collagen which characterizes these abnormal scars.

It has also been suggested that endothelial cell dysfunction plays a role in keloidogenesis. Ogawa and Akaishi (2016) proposed that local factors such as stretching tension together with genetic factors both act to induce endothelial cell dysfunction in the form of vascular hyperpermeability during the inflammatory phase of wound healing. This prolongs the influx of inflammatory cells and factors, thereby also prolonging the inflammatory phase. Consequently, dysfunction of the fibroblast cell population leads to the development of either hypertrophic or keloid scars. Lastly, endothelial cells may also contribute to keloid scar development by undergoing endothelial-mesenchymal transition (EndoMT) to acquire a mesenchymal phenotype (Lee Y.-S. et al., 2015). In this way, endothelial cells may directly serve as a source of the abnormal keloid fibroblasts.

Keloid nerve cells

Based on the symptoms of itching and pain, both sensations carried by small nerve fibers, there does appear to be a role for nerve cells in the development of keloid scars. Yet, thus far little has been published on the presence of nerve cells in keloid tissue (see **Supplementary Tables S2, S3C**). Sensory nerve fibers have also been mentioned in the context of Ogawa's mechanobiology theory on keloid pathogenesis (Ogawa, 2011). As part of the group of skin receptors perceiving mechanical forces, information from the sensory fibers is then relayed to the central nervous system leading to the release of neuropeptides, which can then modulate scarring by altering skin and immune cell functions. However, studies staining for nerve fibers in keloid tissue have reported both increased (Hochman et al., 2008; Drummond et al., 2017) and decreased (Saffari et al., 2018) nerve fiber densities. As different markers (PGP9.5 and S100 protein, respectively) were used to identify the nerve fibers, this could in part explain the different outcomes.

Keloid Immune Cells

Although both increased and reduced levels of certain immune cell types have been reported (see **Supplementary Tables S2, S3D**), overall there appears to be an increase in macrophages (Boyce et al., 2001; Shaker et al., 2011; Bagabir et al., 2012a; Jiao et al., 2015), T-lymphocytes (Boyce et al., 2001; Shaker et al., 2011; Bagabir et al., 2012a; Jiao et al., 2015) and mast cells (Kamath et al., 2002; Moshref and Mufti, 2009; Shaker et al., 2011; Bagabir et al., 2012a; Ammendola et al., 2013) in keloids that have been found to interact with each other, other immune cells and dermal fibroblasts on a cellular level (Boyce et al., 2001; Santucci et al., 2001; Shaker et al., 2011). Moreover, macrophages and T-lymphocytes from keloids also showed intrinsic abnormalities compared with their normal counterparts. Keloid-derived macrophages showed a high activation status, increased M2 polarization and overall increased expression of both M1 and M2 activation factors compared with normal skin macrophages (Jin et al., 2018). They were also more potent at inducing the regulatory T-cell phenotype when co-cultured with CD4+ T-lymphocytes from

keloid patients' blood. Thus, there was not just a general increase in T-lymphocytes, but specifically the regulatory (Jin et al., 2018) and memory (Chen et al., 2018) T-cells, as well as an increased CD4+:CD8+ ratio (Boyce et al., 2001; Bagabir et al., 2012a) in keloids. Furthermore, the altered cytokine production in keloid-derived memory T-cells (Chen et al., 2018) and the reduced mitogenic response of circulating T-cells to known mitogenic stimuli (Bloch et al., 1984), suggests that an abnormal T-cell response may contribute to keloid scarring. In this way, the previously discussed sebum reaction hypothesis is also an extension of this concept as the intrinsically abnormal, sebum-sensitive T-lymphocytes take center stage in this theory (Song, 2014).

Mast cells have also been found in abundance in keloid scars. Arbi et al. (2015) found that mast cells were closely associated with fibroblasts in keloid scars and that the phagocytosis of collagen fibrils by mast cells was a common ultrastructural feature. They hypothesized that the abnormal collagen synthesis observed in keloids and the consequent accumulation of collagen fibers, are able to induce increased mast cell recruitment and subsequent collagen phagocytosis. The resulting release of mast cell-derived mediators (interleukins, mediators, and growth factors) is then able to stimulate further collagen production and thereby aids further keloid scar development. Lastly, there have been variable reports on the presence of Langerhans cells in the epidermal compartment of keloid scars, both normal (Bagabir et al., 2012a) and increased (Jiao et al., 2015) numbers have been observed in both hypertrophic and keloid scars.

Several hypotheses centering on inflammatory processes have also been put forward. In the chronic inflammation hypothesis, Dong et al. (2013) posed that the presence of chronic inflammation in keloids indicates that local inflammation promotes keloid formation. The traumatic and inflammatory stimuli that trigger keloid scar formation result in the continuous upregulation of already highly sensitive pro-inflammatory genes in the keloid microenvironment. This keloid microenvironment fosters the development of abnormalities in the resident keloid fibroblast, which in turn is considered to be the driving force behind keloid scar formation. Ogawa (2017) has further expanded on this notion and postulated that chronic inflammation is responsible for the invasive growth of keloids and even suggests that both hypertrophic scars and keloids are principally inflammatory disorders of the reticular dermis rather than being skin tumors. In the neurogenic inflammation hypothesis, the inflammation is thought to arise from mechanical stress, such as skin stretching, which stimulates mechanosensitive receptors on sensory fibers to release neuropeptides. These then bind to receptors of various skin cell types including keratinocytes and fibroblasts, mast cells and endothelial cells. Vasodilation and vessel permeabilization, increased mast cell release of histamines and cytokine production (including TGF-β) take place as a result of this. Fibroblasts then become activated as a result of the neurogenic inflammation and the upregulation TGF-β, leading to keloid and hypertrophic scar formation (Akaishi et al., 2008).

Other Proposed Hypotheses on Keloid Scar Formation

The myriad of available treatment modalities is only matched by the multitude of proposed hypotheses to explain keloid scar formation. These are not mutually exclusive, and further support the notion that keloid scarring has a multifactorial genesis (Slemp and Kirschner, 2006). When appropriate, these have already been discussed in the appropriate paragraphs of the section on keloid cellular abnormalities. A brief discussion of additional hypotheses that could not be categorized in the previous paragraph, will follow next.

Keloid Triad Hypothesis

Perhaps one of the only proposed hypotheses encompassing multiple risk factors in one, the keloid triad hypothesis (Agbenorku et al., 1995) is defined as a group of three etiologic factors: genetic links, infective agent (bacterial, viral, or other) and surgery (e.g., sutures, tension of suture lines, location of sutures in relation to the relaxed skin tension lines); which must be simultaneously present and interact to develop keloid scarring. These three factors are further subdivided into major factors and minor etiological factors. Major factors include: African ethnicity, age 10–30 years, familial susceptibility or keloid-prone upper part of body site. Minor factors include: orientation of incisions/sutures with respect to RTSLs, wound or sutures under tension, healing by secondary intention, type of infection; determines whether or not a keloid scar is likely to develop. At least one major and two minor factors must be present for keloid scars to develop. A keloid is unlikely to develop if all three factors are minor or if only two factors are present, but a hypertrophic scar may form instead.

Incomplete Malignancy Hypothesis

When Ladin et al. (1998) studied apoptosis in keloid scars, they found that the level of apoptotic cells was significantly reduced in keloid tissue and fibroblasts compared with normal foreskin tissue and fibroblasts. However, keloid fibroblasts did show increased apoptosis upregulation in response to treatment with hypoxia, hydrocortisone or γ interferon, while normal fibroblasts were only responsive to high doses of hydrocortisone. Because of this, Ladin et al. (1998) suggested that keloids may represent a type of incomplete malignancy that has undergone some, but not all tumourigenic changes.

Viral Hypothesis

In this infection-based hypothesis (Alonso et al., 2008), the authors proposed a role for a normally quiescent, unknown virus in the bone marrow or lymphatic system which is activated in a genetically susceptible person with a wound. This virus can then reach the wound via fibrocytes that are chemoattracted to the wound site or via infecting virions in the saliva arriving at the wound bed. There the many chemical stimuli from the wound healing processes allow the virus to become activated, resulting in transcription of viral proteins which derail wound healing and eventually lead to keloid scarring.

Stiffness Gap Hypothesis

Born out of recent findings on the role of mechanotransduction in keloid scarring, this theory (Huang et al., 2017) proposes that the enlarged gap between ECM stiffness and cellular stiffness enables the constant and continued keloid progression. The ECM is not only a cellular scaffold storing important wound healing mediators, but its rigidity also influences fibroblast function and can induce processes such as fibroblast migration, proliferation and differentiation. When dermal fibroblasts sense the stiffness gaps between the ECM and themselves via mechanotransduction, alterations in fibroblast phenotype ensue which promote proliferation, migration and ECM synthesis and therefore contribute to keloid progression. However, systematic mechanobiological experiments to verify this hypothesis have yet to be performed.

Immunonutritional Hypothesis

It has been proposed by several authors that nutritional imbalances can promote prolonged inflammation and cytokine-mediated reactions which contribute to keloid scarring (Huang and Ogawa, 2013a). Nutritional imbalances have been reported for essential fatty acids and micronutrients in keloid-prone patients. For example, the keloid-prone black South African population were deficient in the essential fatty acids a-linolenic acid, eicosapentaenoic acid and docosahexaenoic acid, but showed an increased intake of linoleic acid and arachidonic acid. The levels of micronutrients (calcium, copper, iron, vitamin B2, vitamin C, vitamin A, and zinc) was also insufficient in their diet (Louw and Dannhauser, 2000), and this was also reflected in the serum as well as keloid scar tissue (Bang et al., 2002). Compared with non-keloid forming patients, keloid-prone patients showed higher levels of zinc in their serum, higher copper levels in non-lesional normal skin and in the keloid scar tissue. The essential fatty acid imbalance has also been observed in the membrane of keloid fibroblasts with lower linoleic acid and higher neutral lipid levels in the central keloid area compared with its periphery (Louw et al., 1997). Lipids are not only essential cell membrane constituents, but also have important roles in local inflammation and intracellular signal transduction. As such they are also active participants in the chronic inflammatory processes that result in the development and continued growth of keloids (Huang and Ogawa, 2013b). And finally, dietary compounds have been shown to affect keloid-derived cells at the *in vitro* level as well. Green tea and its extracts have been shown to reduce keloid fibroblast proliferation, migration and collagen synthesis (Zhang et al., 2006; Park et al., 2008).

Metabolic Hypothesis

It has also been theorized that the net overexpression of ECM components in the keloid dermis is also the result of both the increased number of fibroblasts and their increased intrinsic metabolic activity, aside from the usual suspects in abnormal signaling pathways (Butler et al., 2008a). Both keloids and hypertrophic scars showed increased numbers of fibroblasts compared with normal scars or normal skin (Ueda et al., 1999), but only keloid fibroblasts showed increased metabolic activity. Metabolic activity has also been measured as levels of ATP (Ueda et al., 1999; Vincent et al., 2008) and as total protein content synthesis together with endoplasmic reticulum staining (Meenakshi et al., 2005), all of which have proven to be upregulated in keloids compared with normal skin, normotrophic and hypertrophic scars. Modulating keloid metabolism may therefore be a way to reduce keloid scar growth (Butler et al., 2008a; Huang and Ogawa, 2013a).

Nitric Oxide Activity

Using mathematical modeling, Cobbold (2001) proposed that nitric oxide could be involved in both hypertrophic and keloid scar formation. Nitric oxide is a known source of free radical molecules which stimulates collagen synthesis in normal wound healing, it therefore follows that excessive amounts of nitric oxide can result in the elevated scar tissue characteristic of these abnormal scar types. Cobbold further speculated that the source of this excessive nitric oxide may be the basal epidermis, in part because the generated free radicals also stimulate melanin synthesis and melanocytes are located within this epidermal layer.

Psychoneuroimmune-Endocrine Hypothesis

Hochman et al. (2015) posed that the "brain-skin connection" may play a role in keloid scarring. The psychogenic component is a part of the pathogenesis in most skin diseases such as psoriasis and atopic dermatitis, where it can trigger integrated responses from the nervous, immune and the endocrine systems. Normal wound healing also depends on neurogenic factors, which in turn are influenced by psychological, immune and endocrine factors. Any change in these factors may interfere with the scar formation process. Hochman speculated that stressed patients themselves exacerbate the neuro-endocrine system, leading to the release of hormones, neural transmitters, and immune cells which creates an inflammatory microenvironment that stimulates fibrotic processes (Hochman et al., 2015). This has not been extensively studied for abnormal scars but, stress as measured by the sweating response during a stress-inducing task was found to be associated with an increase in keloid recurrence rates after surgery with postoperative radiotherapy (Furtado et al., 2012).

Hypertension Hypothesis

Dustan (1995) first hypothesized that the differences in growth factor abnormalities that are conducive to keloid scarring in the black population, could also be responsible for the development of hypertension and therefore explain the differences in hypertension severity between the black and white population. A significant increase in hypertension rates has in fact been reported in keloid-prone patients compared with non-keloid formers in both the African–American (Snyder et al., 1996; Adotama et al., 2016; Rutherford and Glass, 2017) and Caucasian population (Snyder et al., 1996). Ogawa et al. (2013) also reported that severe keloid cases (multiple or large >10 cm^2) in Japan were significantly more likely to have hypertension than patients with mild keloids (<2 or <10 cm^2). Furthermore, in patients with keloids, those with more severe hypertension were significantly more likely to have multiple and/or larger keloids than patients with normotension or less severe hypertension (Arima et al., 2015). The mechanisms by which hypertension could lead to

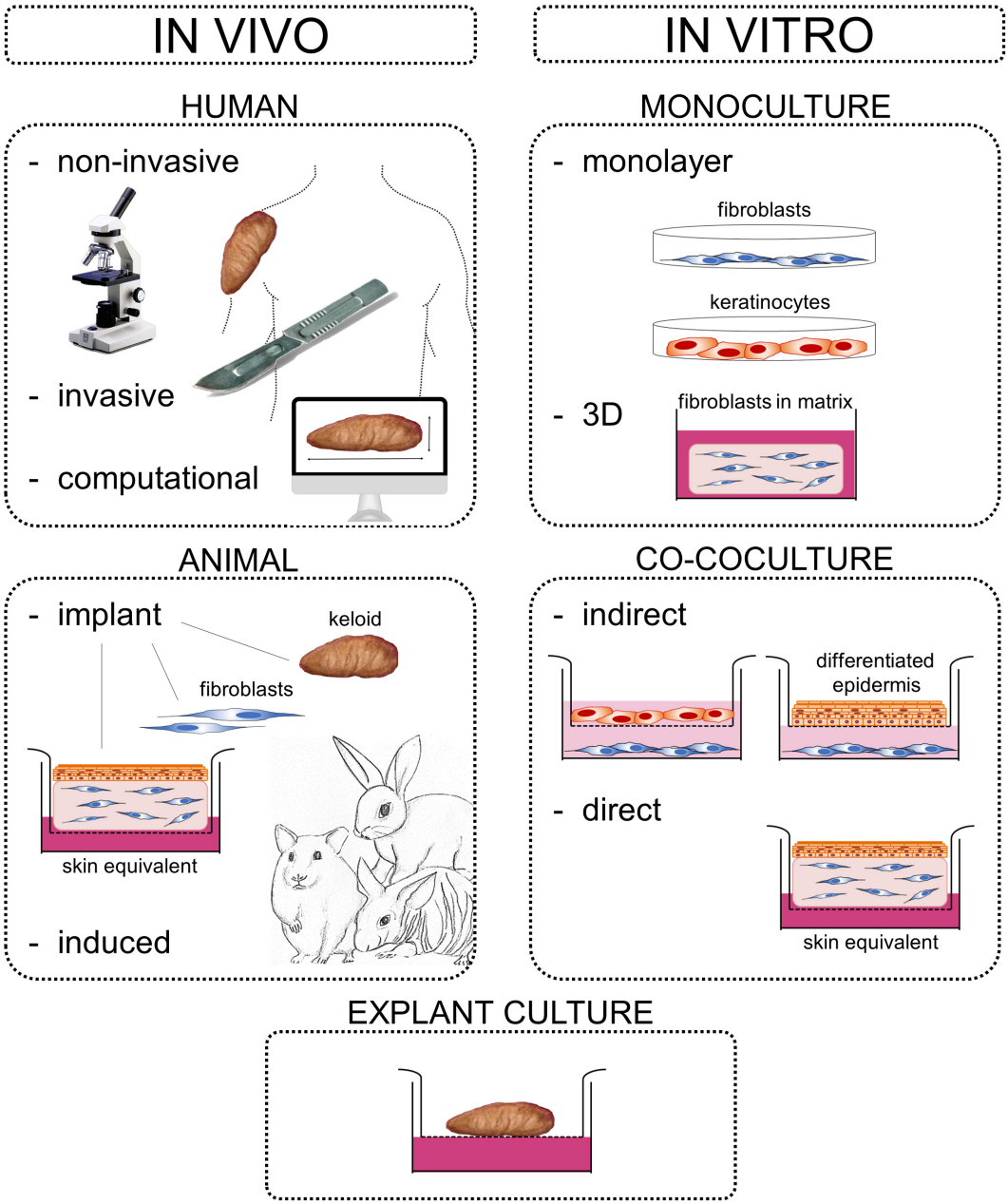

FIGURE 3 | Keloid scar models. Overview of available keloid scar models. Keloid models can be further subdivided into *in vivo* and *in vitro* models, which can be of human or animal origin. Human *in vivo* models include non-invasive testing methods (e.g., imaging and microscopy) and invasive methods, such as serial biopsy or inducing keloid formation by wounding. Animal *in vivo* models include implantation of keloid tissue fragments, fibroblasts or full thickness skin equivalents, as well as inducing keloid scar development by irritation or wounding. *In vitro* keloid models range from simple monolayers to 3D structures and co-culture systems. Indirect co-culture systems include monolayer keloid fibroblasts combined with either monolayer keratinocytes or a fully differentiated epidermis. Lastly, explant cultures are a combination of *in vivo* and *in vitro* as the method involves the maintaining of keloid tissue fragments in *in vitro* culture. Figure first published in Limandjaja (2019), used with permission.

keloid development remain largely speculative for the time being, but the changing blood flow likely affects the skin cellular constituents in ways that promote fibrotic processes (Huang and Ogawa, 2014). Angiotensin-converting enzyme (ACE) was proposed as a potential common mechanism for the pathogenesis of both keloids and hypertension, but Stewart and Glass (2018) found no correlation between plasma ACE levels and keloids or hypertension. Nevertheless, antihypertensives such as ACE-inhibitors (e.g., captopril), calcium-channel blockers (e.g., verapamil) have been able to reduce keloid symptoms and are part of the current therapeutic arsenal for keloid treatment. Huang and Ogawa (2014) reviewed the evidence for the

participation of systemic hypertension in hypertrophic and keloid scar pathogenesis and although they could not establish a strong causal relationship, hypertension was considered a likely risk factor for abnormal scarring.

MODELS OF KELOID SCARRING

Keloid scar models can largely be divided into *in vivo* and *in vitro* models, **Supplementary Tables S4A–E** and **Figure 3** give an overview of the currently available keloid scar models.

In vivo Keloid Models

In vivo keloid models are of the human or animal variety. Currently available *in vivo* human keloid models (**Supplementary Table S4A**) can be further subdivided into non-invasive, invasive, and computational models. Non-invasive *in vivo* modeling generally comprises live imaging whereby certain tissue characteristics are visualized. Invasive keloid modeling varies from the relatively minor FDG injections to measure glucose metabolism (Ozawa et al., 2006), to serial biopsies to evaluate keloid development over time (Lin et al., 2015) and even includes attempts to induce keloid formation in known keloid formers by wounding (Mancini and Quaife, 1962). Despite their value especially in the clinical setting for follow-up evaluation, human *in vivo* models post-1962 are inherently limited in the ability to manipulate experimental variables and yield primarily observational data. While Lebeko et al. (2019) saw particular merit in the temporal data acquired from serial keloid biopsy analysis as an *in vivo* '4D model' in their review on keloid models, it is important to recognize the potential risk of exacerbating the existing keloid scars by continual provocation with biopsy-associated injury.

In animal models, keloid formation is either induced or involves the implantation of human keloid tissue (see **Supplementary Table S4C**). Unfortunately, inducing keloid scar formation has proven virtually impossible (Seo et al., 2013) and more often than not, hypertrophic scars developed instead of keloids (Morris et al., 1997; Khorshid, 2005). Implanting human keloid cells or tissue fragments into animal models has been more successful (Hillmer and Macleod, 2002) and resulted in the development of a palpable nodule-like mass. While implanted keloid tissue was generally able to retain the keloid-specific keloidal collagen even within the animal model, the use of already established keloid tissue does not allow us to study *de novo* keloid development. However, when tissue culture techniques were combined with IL-6 exposure to implant a keloid fibroblasts-hydrogel suspension into nude mice, the resultant mass not only showed the greatest increase in growth, but also gave rise to *de novo* keloidal collagen formation (Zhang Q. et al., 2009). The appeal of an *in vivo* animal model is obvious, but there is an important limitation to the use of any animal for the study of keloid scarring, in that keloids occur exclusively in humans (Tuan and Nichter, 1998; Yang et al., 2003). Though exuberant granulation tissue on horse limbs has often been posed as the equine version of keloid tissue, a recent study (Theoret et al., 2013) comparing keloids and exuberant granulation tissue has shown they are not in fact one and the same. Fundamental differences in essential skin physiology (hair follicle density, epidermal, and dermal thickness) and wound healing mechanisms between rodents and humans (Perez and Davis, 2008; Ramos et al., 2008) pose additional important limitations. The *in vivo* microenvironment in animal models may therefore provide a less than human-like exposure to the keloid cell population.

Keloid Explant Models

Keloid tissue explants do not necessarily require implantation into an animal model in order to survive and be used as a keloid model in and of itself, they can be maintained in culture after removal from the human body for up to 6 weeks (Duong et al., 2006; Bagabir et al., 2012b) (see **Supplementary Table S4B**). Various culture methods have been tried, but keloid morphology appears best conserved in explants embedded in collagen gel that are cultured air-exposed (Duong et al., 2006; Bagabir et al., 2012b; Mendoza-Garcia et al., 2015). Unfortunately, relying on fresh keloid tissue for this explant model system has its obvious logistical issues with limited availability and potential inter- and intralesional variability between samples. Another important limitation of this model type is the absence of circulatory system, as is the case with all *in vitro* models to date. Ultimately, this model system appears best suited for the testing of therapeutics (Syed et al., 2012, 2013a,b) rather than studying the pathogenetic mechanisms underlying keloid formation, as there is very limited ability to manipulate any experimental variables.

In vitro Co-culture Keloid Models

As skin physiology, immunology and wound healing is markedly different in animals (Hillmer and Macleod, 2002; Ramos et al., 2008; Seo et al., 2013; Seok et al., 2013) and since keloids are exclusive to humans (McCauley et al., 1992; Yang et al., 2003), there is a need for relevant human *in vitro* models (see **Supplementary Tables S4D, E**). What is more, *in vitro* co-culture systems allow for extensive experimental manipulation, such as the development of heterotypic models combining normal keratinocytes with keloid fibroblasts to study the individual contribution of keloid fibroblasts to keloid formation.

Indirect co-cultures consist of double chamber systems with keratinocytes seeded (as a monolayer or differentiated epidermis) onto an upper porous transwell insert and fibroblasts cultured in a monolayer on the underlying bottom well (see **Supplementary Table S4D**). These indirect co-cultures are simple-to-execute experiments that are particularly suited to study paracrine interactions, as previously discussed in 'Abnormal Keratinocyte-Fibroblast Interactions'. However, the monolayer fibroblast cultures and the artificial separation between keratinocytes and fibroblasts, bear little resemblance to the true *in vivo* situation.

Direct co-cultures represent the most *in vivo*-like *in vitro* culture model and include either mixed monolayer cultures or full thickness skin equivalents comprised of keloid-derived cells (see **Supplementary Table S4E**). The full thickness skin equivalents comprised entirely of keloid-derived keratinocytes and fibroblasts most closely resemble the native keloid, but thus

far, these have only been developed in the context of implantation into an animal model (Supp et al., 2012b; Lee Y.-S. et al., 2015) and/or subjected only to limited experimental analysis (Supp et al., 2012a). Overexpression of ECM components, particularly collagen I, was the common denominator in these keloid models. Recently, we were also able to reconstruct keloids *in vitro* with keloid-derived keratinocytes and fibroblasts, using a collagen-elastin scaffold (Limandjaja et al., 2018a). Compared with *in vitro* reconstructed normal skin, the keloid model showed a trend of increased dermal thickness, increased α-SMA and p16 expression, reduced HGF secretion and reduced collagen type IV α2 chain, hyaluronan synthase 1 and matrix metalloproteinase 3 gene expression. More importantly, the keloid model behaved differently from similarly reconstructed hypertrophic scars, and was able to demonstrate intralesional heterogeneity within keloids (Limandjaja et al., 2018b). In other words, a relatively simple *in vitro* keloid model was already able to demonstrate certain intrinsic abnormalities in keloid-derived keratinocytes and fibroblasts and in doing so, identified certain aspects of keloid behavior which could not have been deduced from *ex vivo* biopsy analysis alone. In our opinion, this both illustrates and validates the use of skin tissue engineering as an important adjunct to furthering keloid scar research.

Ultimately, there is no single universal keloid model that is able to satisfactorily answer every experimental objective (Hillmer and Macleod, 2002; Marttala et al., 2016). Overall, animal models are better suited for therapeutic testing and particularly for safety testing prior to human administration (Hillmer and Macleod, 2002; Marttala et al., 2016), while tissue culture systems are best suited to study keloid pathogenesis (Hillmer and Macleod, 2002; Marttala et al., 2016). Given the exclusive occurrence of keloids in humans (Tuan and Nichter, 1998; Yang et al., 2003) and other serious limitations of using animal models for wound healing studies, as well as the fact that research on its pathogenesis cannot rely solely on (immunohistochemical) analysis of an intact native specimen; it seems pertinent to focus our efforts on further advancing tissue culture models - in particular the *in vitro*, organotypic full thickness keloid skin equivalents. Once proven faithful to actual *in vivo* keloid biology, using this model to study keloid formation will eventually aid in the development of superior treatment methods by uncovering new mechanisms of pathogenesis. In addition, such a model would have the added benefit of serving as a test object for future therapeutic modalities without the complication of ethical objections (Yang et al., 2003).

THE KELOID DISORDER

The International Classification of Disease (ICD) represents the international standard for reporting diseases and health conditions and in the most recently released ICD-11 (2018), keloids have been grouped under 'fibromatoses and keloids,' "*a heterogenous group of disorders characterized by increased deposition of fibrous tissue in the skin and subcutaneous tissues.*" This is departure from the previous ICD-10 (2016) in which keloids were still categorized under 'hypertrophic disorders of skin,' and is the result of recent international agreement to refer to keloids as part of a 'keloid disorder' rather than a 'keloid scar.' This change in ICD classification and nomenclature reflects growing consensus among keloid researchers and clinicians alike that keloids more closely resemble a benign, fibrous tumor than scar tissue and can best be described in terms of a fibrotic disorder.

By their very definition of continued, invasive horizontal growth (Burd and Huang, 2005), keloids clearly share certain characteristics of cancerous growths. Lu et al. (2007b) have previously described keloids as behaving "*clinically as non-metastatic malignancies, although histologically they are benign.*" Similarly, Ladin et al. (1998) coined keloids 'incomplete malignancies' as keloid fibroblasts are still apoptosis-responsive to *in vitro* treatment despite overall reduced levels of apoptosis. The term refers to the manner in which keloids show some, but not all the changes associated with tumourigenesis. The absence of metastasis despite their invasive growth and the aforementioned cancer-like characteristics, separates keloids from true malignant tumors and lends further support to the 'incomplete malignancy' term coined by Ladin et al. (1998).

The change in nomenclature reflected in the newest edition of the ICD may seem like an exercise in semantics, but it should be noted that the cosmetic association with the word 'keloid scar' significantly complicates both insurance coverage for treatment as well as funding for research. This is not to say this nomenclature change must result in an altogether ban on the association of the word 'scar' with keloids, merely that we shift the focus away from a purely cosmetic association, toward broadening our perspective of keloids for the fibroproliferative disorders that they are and in this way recognize the severity of this fibrotic disorder.

Keloid research remains plagued by significant controversy and contradictory findings, which has been highlighted throughout this manuscript where possible. Much of this could be resolved by the standardization of study designs and general research approach. Important considerations for future research include (i) inclusion of a description of the keloid phenotype (based on growth pattern), (ii) clarification of the location within the keloid from which samples were taken (e.g., growing/bulging area versus more quiescent region; peripheral versus central areas), (iii) inclusion of scars of similar maturation stage (at least 1 year old). Lastly, the inclusion of both hypertrophic and normotrophic scars would be a valuable addition to keloid research, as this provides a more comprehensive view of the entire scarring spectrum.

AUTHOR CONTRIBUTIONS

GL, SG, and FN conceived the manuscript outline. GL performed the literature study and wrote the manuscript with input from all the authors. SG, FN, and RS supervised, helped shape the manuscript, and offered final critical revision.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00360/full#supplementary-material

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USP15 Enhances Re-epithelialization Through Deubiquitinating EIF4A1 During Cutaneous Wound Repair

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Re-epithelialization is a fundamental process in wound healing that involves various cytokines and cells during cutaneous barrier reconstruction. Ubiquitin-specific peptidase 15 (*USP15*), an important member of the deubiquitinating enzymes (DUBs), removes ubiquitin chains from target proteins and maintains protein stability. However, the dynamic role of USP15 in epithelialization remains unclear. We aimed to investigate the regulatory function of USP15 in re-epithelialization. An excisional wound splinting model was established to evaluate the re-epithelialization rate in *Usp15* knockout (KO) mice. Coimmunoprecipitation (Co-IP) and mass spectrum analyses were performed to identify USP15-interacting proteins. RNA-sequencing was performed for transcriptome analysis in keratinocytes and uploaded into NODE database (http://www.biosino.org/node, accession numbers: OEP000770 and OEP000763). First, a significant delay in epithelialization was observed in the *Usp15* KO mice. Moreover, inhibition of cell migration and proliferation was observed in the USP15-silenced keratinocytes (HaCaTs). Moreover, we revealed for the first time that USP15 could interact with eukaryotic initiation factor 4A-1 (EIF4A1), thereby promoting translational efficacy in keratinocytes, which is essential for keratinocyte proliferation and migration. Conclusively, the USP15-EIF4A1 complex significantly accelerated re-epithelialization in wound healing. These observations helped elucidate the function and mechanisms of USP15 in modulating re-epithelialization in wound healing, providing a promising target for refractory wound treatment.

Keywords: epithelialization, USP15, deubiquitinating, post-translational modification, EIF4A1

Abbreviations: DUBs, deubiquitinating enzymes; ECM, extracellular matrix; EIFA1, eukaryotic initiation factor 4A-1; HDFs, human dermal fibroblasts; PTM, post-translational modification; R-SMADs, receptor-regulated SMADs; TBR1, TGF-β type I receptor; TGF-β, transforming growth factor-β; USP15, ubiquitin-specific peptidase 15; USP4, ubiquitin-specific protease 4.

INTRODUCTION

Wound healing is a complicated biological processes, involving the spatial and temporal synchronization of various cells and cytokines, with a distinct pattern in inflammatory, proliferative and remodeling phases (Tellechea et al., 2019). Deficiencies of homeostasis in the local microenvironment, including alterations in local physical forces, oxygen content, chemokines secretion and extracellular matrix remodeling, leads to impaired wound healing (Zhang et al., 2019). Delayed wound healing results in pain, infections, financial expenditures and loss of physical function, which affect over 40 million people (Miura et al., 2019). However, there has been no standard therapeutic approach to halt refractory wounds progression so far.

Keratinocytes in the basal layer of the skin play a major role in the maintenance of tissue homoeostasis and in skin repair in response to a cutaneous injury (Deshayes et al., 2018). During the re-epithelialization process, epithelial precursor cells migrate from the edges of the wound, proliferate and differentiate to create a new epidermis (Deshayes et al., 2018). Previous studies have demonstrated that keratinocyte function is essential in wound repair. For example, mice expressing human S100A2 exhibited delayed cutaneous wound repair, and the p53-S100A2 feedback loop impairs re-epithelialization in wound healing (Pan et al., 2018). Therapeutically, adipose stem cell-derived exosomes combined with hyaluronic acid significantly accelerated wound healing through promoting epithelialization and angiogenesis (Liu et al., 2019).

Ubiquitin-specific peptidase 15 (*USP15*), an important member of deubiquitinating enzymes (DUBs), removes ubiquitin chains from target proteins and promotes protein stability (Villeneuve et al., 2013). A previous study has shown that USP15 interacts with receptor-phosphorylated SMAD proteins (R-SMADs) and deubiquitinates transforming growth factor-β (TGF-β) receptor 1 (TBR1), thereby promoting protein stability (Inui et al., 2011). In addition, USP15 participates in diverse pathophysiological processes, such as regulating neuroinflammation, T cell activation and tumourigenesis (Eichhorn et al., 2012; Zou et al., 2014; Torre et al., 2017). Our previous study showed that USP15 plays a key role in the activation of the TGF-β signaling pathway in human dermal fibroblasts (HDFs) (Zhao et al., 2019). However, the role of USP15 in keratinocytes and re-epithelialization remains unclear.

Our research thus aimed to investigate the dynamic function of USP15 in re-epithelialization. USP15 was mainly distributed in keratinocytes, and a significant delay in epithelialization was observed in the *Usp15* knockout (KO) mice. In addition, inhibition of cell migration and proliferation was observed in the USP15-silenced keratinocytes. Moreover, for the first time, we revealed that USP15 could interact with and deubiquitinate eukaryotic initiation factor 4A-1 (EIF4A1), thereby promoting translational efficacy in keratinocytes. Taken together, these observations help elucidate the function of the USP15-mediated modulation of re-epithelialization during wound healing, providing a novel promising target for refractory wound treatment.

MATERIALS AND METHODS

Usp15 Knockout Mice

The animal experiments were approved by the Independent Committee of Shanghai Ninth People's Hospital and conducted in accordance with the guidelines established by the National Health and Family Planning Commission of China. *Usp15* wt and *Usp15*−/− mice with a C57BL/6 background were maintained and bred under standard pathogen-free conditions and genotyped as previously described (Zhao et al., 2019). Eight- to ten-week-old male *Usp15* wild-type mice and *Usp15*−/− control littermates (offspring from heterozygote breeding) were used for the experiments. Only healthy mice without any inflammatory bowel disease were included in the study.

Excisional Wound Splinting Model

Mice were anesthetized by an intraperitoneal injection of Ketanest/Rompun. The back was shaved, and two full-thickness cutaneous in 6mm diameter were generated using a standard biopsy punch. The 2 wounds on the back of each animal were at least 10 mm apart from each other. The wound was then sutured and fixed by a glued rubber ring. The mice were sacrificed on appropriate days, and an area of 8 mm in diameter, which included the complete epithelial margins, was excised. The wounds were bisected in the caudocranial direction, and the tissue was either fixed overnight in 4% paraformaldehyde or immediately frozen in liquid nitrogen. Histological analysis was performed on serial sections from the central portion of the wound.

Immunofluorescence (IF) and Immunohistochemistry (IHC)

Wound beds surrounded by a margin of non-wounded skin were collected at days 0, 1, 3, 5, and 7 post wounding. The samples were fixed overnight in 4% paraformaldehyde at 4°C. The tissues were processed through graded ethanol solutions and embedded in paraffin blocks using standard protocols. The tissue sections (6 μm) were stained with haematoxylin and eosin. For the IHC and IF assays, the sections were incubated with primary antibody against USP15 (Abcam, ab4850, 1:200) and EIF4A1 (Abcam, ab31217, 1:200) diluted in blocking solution overnight at 4°C. After incubation with horseradish peroxidase-conjugated secondary antibody (IHC) or IF (anti-rabbit, 546 nm; anti-mouse, 488 nm), the sections were counterstained with haematoxylin and developed with diaminobenzidine.

Western Blot Analysis

Cells were harvested at indicated times and rinsed twice with PBS. The cell extracts were prepared using lysis buffer and centrifuged at 13,000 *g* for 30 min at 4°C. The protein samples were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) in 4–20% (wt/vol) polyacrylamide gels and transferred to polyvinylidene fluoride membranes. After the membranes were blocked with 5% BSA for 2 h at room temperature, they were incubated with 1.0 μg/mL antibody in 5% BSA overnight at 4°C. The membranes were then incubated

with a secondary antibody conjugated to horseradish peroxidase. The signals were detected by electrochemiluminescence reagent. Protein bands were visualized in Amersham Imager 600 detection system (GE Chalfont, United Kingdom).

RNA-Sequencing (RNA-seq)

Total RNA was extracted from the keratinocyte cell line (HaCaT) after silencing USP15 and EIF4A1 using TRIzol reagent (Invitrogen, Carlsbad, CA, United States). We confirmed the RNA integrity by using a 2100 Bioanalyzer (Agilent Technologies, United States). We measured the RNA concentration in a Qubit 2.0 fluorometer by using the Qubit RNA Assay Kit (Life Technologies, Carlsbad, CA, United States). We prepared the libraries from 100 ng of total RNA using an Illumina TruSeq RNA Sample Prep Kit (San Diego, CA, United States). The libraries were sequenced using the Illumina HiSeq 2500 platform (San Diego, CA, United States). The mRNA levels of the unigenes identified using TopHat v2.0.9 and Cufflinks were normalized by the Fragments Per Kilobase of exon model per Million mapped reads (FPKM), and the log2-fold changes between two samples were tested statistically to determine whether an individual gene's expression was altered significantly. We used the criteria of false discovery rate (FDR) < 0.01 and fold changes <0.25 or >4.0 (<-2 or >2 in log2 ratio value, p-value < 0.05) to identify the differentially expressed genes. The value of gene expression was listed in **Supplementary Table S2** (after silencing USP15) and **Supplementary Table S3** (after silencing EIF4A1).

Cell Counting Kit-8 (CCK-8) Assay

Cell proliferation was assessed by Cell Counting Kit8 (Dojindo, Tokyo, Japan) following the manufacturer's protocals. Briefly, cells were seeded in triplicate in 96-well plates at a density of 2000 cells/100 μL. CCK-8 solution was added at the indicated time points to detect the absorbance at 450 nm.

Colony Formation Assay

A volume of 2 mL of complete DMEM medium containing 2000 cells was placed in each well of a six-well plate. The plate was stained with 0.25% crystal violet after 2 weeks.

Transwell Assay

A 24-well transwell system with polycarbonate filters (8-μm pores, Millipore, Burlington, MA, United States) was used. The upper compartment contained 10,0000 cells suspended in the appropriate medium with 2% FBS which was harvested 24 h later; the lower chamber contained 10% FBS. The upper cells in the chamber were removed, whereas those that migrated to the other side were stained with 0.25% crystal violet and photographed.

Wound Healing Assays

Cells were seeded into 6-well dishes and grown to confluence. A sterilized 1-mL pipette tip was used to generate a scratch through the diameter, and the debris was washed away. A total of 8 areas were selected randomly in each well at 40x magnification, and the cells in three wells of each group were quantified in each experiment.

Coimmunoprecipitation

For silver staining and mass spectrum (MS) analysis, coimmunoprecipitation was performed according to the Pierce™ Co-Immunoprecipitation Kit (Thermo Fisher, United States, 26149). Briefly, 20 μg of anti-USP15 antibody (Proteintech, United States, 14354-1-AP) was coupled to the resin. Cellular lysate was then prepared using IP lysis buffer (25 mM Tris-HCl pH 8.0, 200 mM NaCl, 5 mM $MgCl_2$ and 1 mM DTT) and incubated with coupled resin at 4°C overnight. The resins were then washed with IP lysis buffer and eluted with IP elution buffer (0.2 M glycine, pH 3.0). Other coimmunoprecipitation experiments were performed using the Pierce™ Classic Magnetic IP/Co-IP kit (Thermo Fisher, United States, 88804). The lysate was first prepared by suspending the cells in IP lysis buffer for 15 min at 4°C. Immunoprecipitation was performed with 1 mg of protein and 10 μg of anti-EIF4A1 antibody in 500 μL of IP lysis buffer at 4°C overnight. The reaction mixtures were incubated with Protein A and Protein G Magnetic Beads (50 μL) at 4°C for 1 h on a rotator. The immunoprecipitated complexes were washed twice with IP wash buffer. The washed beads were incubated with 5× reduction loading buffer and boiled at 100°C for 5 min. The proteins released from components of the complexes were examined by SDS-polyacrylamide gel electrophoresis (PAGE) and western blotting with anti-USP15 antibodies.

MS Analysis

Half of each peptide sample was separated and analyzed with a Nano-HPLC coupled to a Q-Exactive mass spectrometer (Thermo Finnigan). Separation was performed using a reversed-phase column (100 μm, ID × 15 cm, Reprosil-Pur 120 C18-AQ, 1.9 μm). The mobile phases were H_2O with 0.1% FA and 2% ACN (phase A) and 80% ACN and 0.1% FA (phase B). Separation of the sample was executed with a 120-min gradient at a 300 nL/min flow rate. Gradient B was as follows: 8–35% for 92 min, 35–45% for 20 min, 45–100% for 2 min, 100% for 2 min, 100–2% for 2 min and 2% for 2 min. Data-dependent acquisition was performed in the profile and positive mode with the Orbitrap analyser at a resolution of 70,000 (200 m/z) and m/z range of 350–1400 for MS1; for MS2, the resolution was set to 17,500 (200 m/z). The top 10 most intense ions were fragmented by higher energy collisional dissociation (HCD) with a normalized collision energy (NCE) of 28% and isolation window of 2 m/z. The dynamic exclusion time window was 30 s.

Cell Cycle Analysis

A total of 10^6 cells were collected and fixed overnight in 70% ethanol at −20°C. The fixed cells were centrifuged and washed three times with 10 mL of PBS, resuspended in 500 μL of propidium iodide (BD-Pharmingen, United States, 550825) and incubated in the dark at room temperature for 15 min. The cells were analyzed by flow cytometry.

Ubiquitination Assay

The lysate was first prepared by suspending the cells in IP lysis buffer for 30 min at 4°C. Immunoprecipitation was

performed with 1 mg of protein and 10 μg of anti-EIF4A1 antibody in 500 μL of IP lysis buffer at 4°C overnight. The reaction mixtures were incubated with Protein A and Protein G Magnetic Beads (50 μL) at 4°C for 1 h on a rotator. The immunoprecipitated complexes were washed twice with IP wash buffer. The washed beads were incubated with 5× reduction loading buffer and boiled at 100°C for 5 min. The proteins released from components of the complexes were examined by SDS-polyacrylamide gel electrophoresis (PAGE) and western blotting with anti-Ub antibodies (Cell Signalling Technology, #3933).

Statistical Analysis

The results are expressed as the mean ± SEM. Unpaired *t*-tests were conducted to identify significant differences ($p < 0.05$) in the wound-healing and scratch assay experiments. GraphPad Prism (GraphPad Software, San Diego, CA, United States) software was used for this analysis.

RESULTS

Delayed Re-epithelialization in the *Usp15* Knockout Mice

First, we tested USP15 expression in the full layer of human cutaneous tissue. Both IF (**Figure 1A**) and IHC (**Figure 1B**) showed that the USP15 protein signal was enriched in keratinocytes, while other cells presented weak USP15 expression. Furthermore, *Usp15* knockout mice were used in our study. Similarly, Usp15 was also distributed in the keratinocytes in full layer of mouse cutaneous tissue while presented weak signal in Usp15 KO mice (**Figure 1C**). We then confirmed that the USP15 protein expression in keratinocytes was indeed silenced in the *Usp15* knockout mice (**Figure 1D**, lanes 7–12). We then established an excisional wound splinting model and observed a significant delay in re-epithelialization in the *Usp15* knockout mice (**Figures 1E,F**). In the wound healing process, the gap (white dashed line) would be closed by the proliferation and migration of keratinocytes (green dashed line). Furthermore, we examined the epithelium gap of the wound model was significantly increased in *Usp15* knockout mice (**Figure 1G**).

Loss of USP15 Attenuated the Proliferation and Migration of Keratinocytes

We next explored the regulatory function of USP15 in keratinocytes. After transfecting lentiviruses with short hairpin sequences for USP15 (shUSP15) and the negative control (shNC), we observed the EGFP signal after transfection (**Supplementary Figure S1A**, panels 2–4). Moreover, we observed a significant reduction in the mRNA (**Supplementary Figure S1B**, upper panel) and protein expression of USP15 (**Supplementary Figure S1B**, lower panel). Transwell assays demonstrated that the upper layer cells transferred through an 8 μm hole decreased after inhibition of USP15 (**Figures 2A,B**). The wound healing assay showed a significant delay in the wound recovery rate in the USP15-silenced group (**Figures 2C,D**). Moreover, the cells also formed fewer and smaller colonies in the USP15 knockdown group than the control group (**Figure 2E**). The CCK-8 assay proved that the cellular proliferative rate was suppressed in the USP15-inhibited cells (**Figure 2F**). Flow cytometric assays showed a decreased percentage of cells in S phase after suppression of USP15 expression (**Figure 2G**). Notably, the difference of two shRNAs in flow cytometric assay could be due to different silencing efficiency of USP15. In addition, it could be also referred from unexpected 'off-target' silencing by shRNAs, which is reason why we performed two shRNAs in our study. Although it is different between two shRNAs groups, however, compare to control group, they all presented with decreased percentage of S phage and increased percentage of G0/G1 phase. More importantly, re-generated keratinocyte of *Usp15* KO cutaneous tissue presented decreased positive rate of Ki67 than wild-type group (**Figure 2H**). Taken together, these experiments showed that USP15 was vital in the proliferation and migration of keratinocytes, either *in vitro* or *in vivo*.

Transcriptome Profiling in the USP15-Silenced Cells

To further explore the detailed mechanism of the regulatory role of USP15 in keratinocytes, we performed transcriptional screening after USP15 inhibition. An RNA-seq assay demonstrated that USP15 silencing led to the upregulation of 425 genes and downregulation of 475 genes in HaCaT cells (**Figure 3A**, fold change > 1.5, $p < 0.05$, accession number: OEP000770)[1]. Gene Ontology (GO) analyses and Circos plots showed that the main differentially expressed genes were associated with epidermal processes, such as cornification, skin epidermal development and keratinocyte differentiation (**Figure 3B**), while upregulated genes were mainly associated with transcriptional regulation (**Supplementary Figure S2A**), such as nucleosome assembly, H3K27 tri-methylation and RNA polymerase II guided transcription. The Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis demonstrated that the top upregulated pathways were associated with immunity and regulation of carcinogenesis (**Figure 3C**), while downregulated pathways were associated with infection and metabolic regulation (**Supplementary Figure S2B**). Notably, our group previously demonstrated that USP15 could activate TGF-β signaling pathway activity in dermal fibroblasts. However, in keratinocytes, the TGF-β signaling pathway was not downregulated after USP15 was silenced (**Supplementary Figure S3A**). Moreover, through a Gene Set Enrichment Analysis (GSEA), we found that the translation-related process was significantly downregulated after suppressing USP15 (**Figure 3D**).

USP15 Interacted With EIF4A1

To underline the detailed mechanism of USP15 in translational regulation, we performed coimmunoprecipitation, and the lysate was identified by silver staining (**Figure 4A**). A mass spectrum

[1]http://www.biosino.org/node

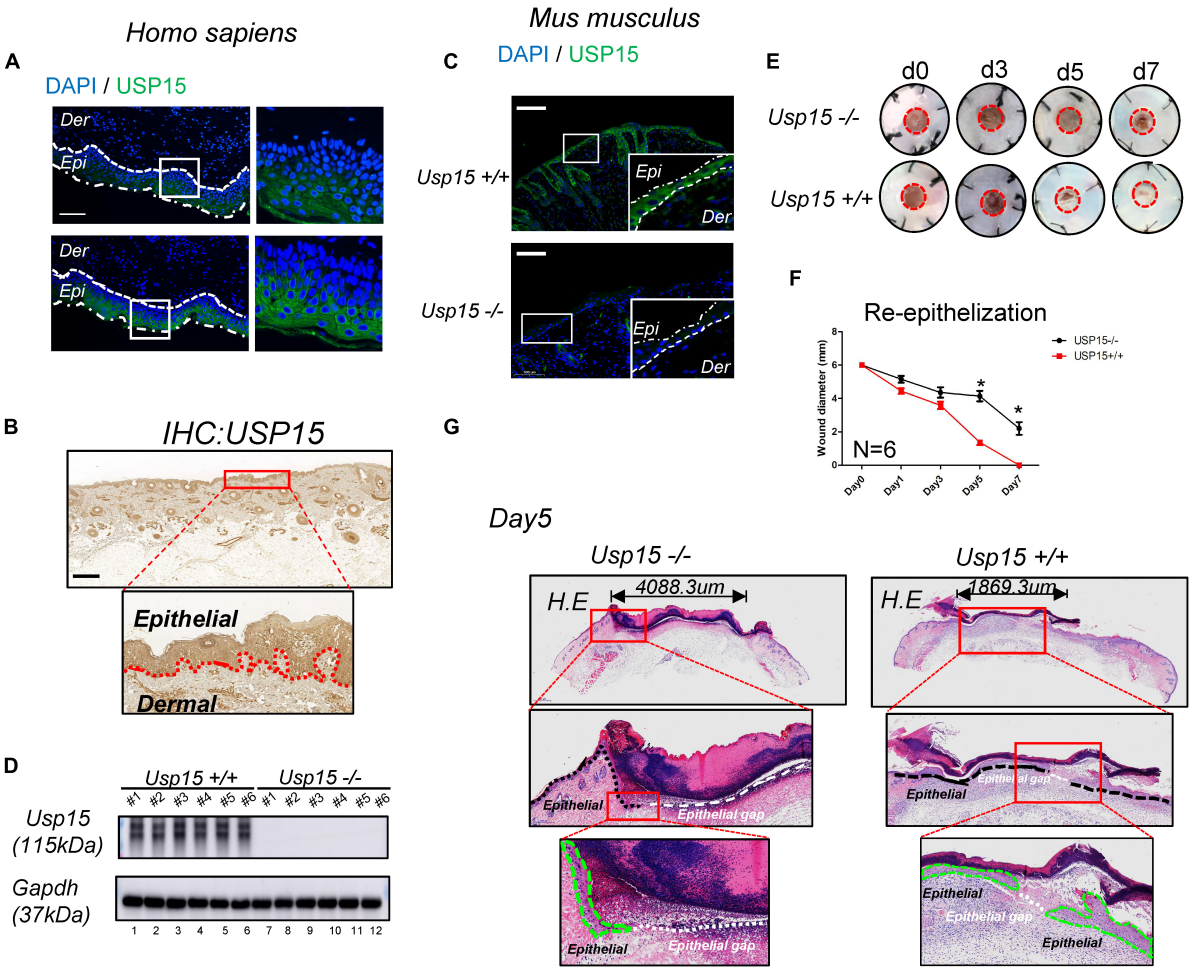


FIGURE 1 | Delayed re-epithelialization in USP15–/– mice. **(A,B)** Immunofluorescence **(A)** and immunohistochemistry **(B)** of USP15 in human normal full-layer cutaneous tissue. Green: USP15; Blue: DNA; Scale bar: 50 μm. **(C)** Immunofluorescence of Usp15 in mouse normal cutaneous tissue. Green: Usp15; Scale bar: 50 μm. **(D)** Western blot assays were performed to measure Usp15 expression in the *Usp15*–/– animals and their wild-type littermates. Numbers of mice in each group: 6. **(E,F)** An excisional wound splinting model was established to evaluate the re-epithelialization rate in the USP15 knockout (KO) mice. Red circle: 6 mm diameter. *$p < 0.05$. Numbers of mice in each group: 6. **(G)** Haematoxylin-eosin staining revealed the epidermal gap in the excisional wound splinting model of the USP15 KO mice and their wild-type littermates. Black dash line: basal cell. Green dash area: regenerated epithelial. White dash line: the gap between regenerated epithelium of the wound area.

analysis identified 18 proteins that could specifically bind USP15 (**Figure 4B** and **Supplementary Figure S4A**). These 18 proteins were identified in three replicates of mass spectrum using anti-USP15, however, no signal was detected in anti-IgG group (**Supplementary Figure S4B**). GO assays showed that these specific binding proteins were mainly distributed in the ribosome (**Figure 4C** and **Supplementary Figure S5A**). Furthermore, through a protein interaction network, we found that EIF4A1 may play a central role in USP15-guided translational regulation (**Figure 4D**). In addition, a western blot assay proved that the USP15 protein could be pulled down in the anti-EIF4A1 group, which indicated a direct interaction between EIF4A1 and USP15 (**Figure 4E**). It should be explained that EIF4A1 protein was 48 kDa, which is similar to IgG heavy chain (50 kDa). We were unable to distinguished these two bands after IP assay if we used USP15 as a bait. Moreover, after silencing USP15, the global protein USP15 has been largely reduced (**Figure 4F**, lane 1) and weaker interaction signal was then identified (**Figure 4F**, lane 4).

USP15 Deubiquitinated EIF4A1 and Enhanced Its Stability *in vitro* and *in vivo*

Since USP15 could interact with EIF4A1, we then investigated whether EIF4A1 expression could be regulated by USP15. We found that the mRNA level of EIF4A1 remained unchanged after silencing USP15 (**Figure 5A**). However, a significant reduction in the EIF4A1 protein level was observed after knocking down USP15 (**Figure 5B**, lanes 2–3). These results indicated that USP15 could enhance EIF4A1 expression at the post-transcriptional level. Since USP15 is a DUB, we tested the ubiquitination level after silencing USP15. As expected, the ubiquitination level of EIF4A1 was significantly upregulated

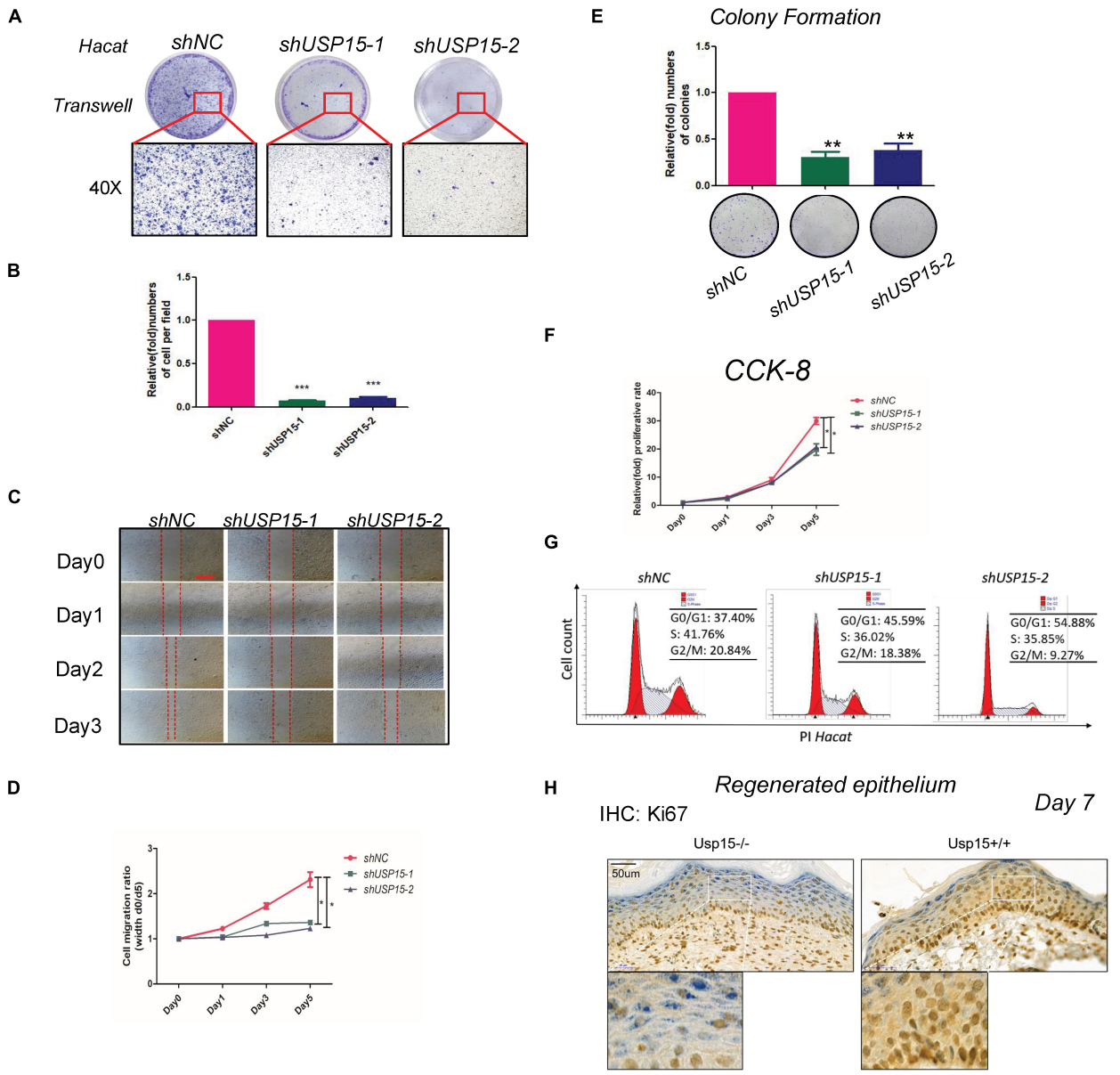


FIGURE 2 | Loss of USP15 inhibited proliferation and migration *in vitro*. **(A)** A transwell assay was performed to determine the migratory ability of the *USP15*-silenced keratinocytes (HaCaT). **(B)** Quantification of the numbers of transferred cells. The colony number in the empty vector group was set as 100%. All experiments were performed in triplicate, and the relative cell numbers are shown as the mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. **(C)** A wound healing assay was performed to determine the migratory ability of the *USP15*-silenced keratinocytes (HaCaT). Scale bar: 50 μm. **(D)** Quantification of the wound recovery rate in the wound healing assay. All experiments were performed in triplicate, and the relative cell migration ratio is shown as the mean ± SEM. $*p < 0.05$. **(E)** A colony formation assay was performed to determine the colony formation ability of the USP15-silenced cells. The colony number in the shNC group was set as 100%. All experiments were performed in triplicate, and the relative colony formation rates are shown as the mean ± SEM. $*p < 0.05$, $**p < 0.01$. **(F)** A CCK-8 assay was performed to determine the proliferative rate of the USP15-silenced cells. The OD value of Day 0 was set as 100%. $*p < 0.05$. **(G)** Cell cycle analysis by flow cytometry was performed to determine the percentage of cells in the different cell cycle phases. The *X*-axis represents the FL2 channel-captured PI staining signals, and the *Y*-axis represents the cell counts. **(H)** Immunohistochemistry of Ki67 in the regenerated keratinocyte in *Usp15* knockout mice and their wild-type littermates at 7 days after establishing excisional wound splinting model. Scale bar: 50 μm.

after inhibiting USP15 (**Figure 5C**). We then overexpressed Usp15 in HaCaT cells. We observed a significant increase of Usp15 expression in both mRNA (**Supplementary Figure S6A**) and protein (**Figure 5D**, 1st panel) level. Moreover, we have observed EIF4A1 protein expression was significantly upregulated (**Figure 5D**, 2nd panel) while the RNA expression of EIF4A1 remain unchanged (**Supplementary Figure S6B**) which demonstrated that USP15 could interact with and deubiquitinate EIF4A1, thus promoting EIF4A1 protein stability. Furthermore, a weak fluorescent signal of EIF4A1 was observed in the

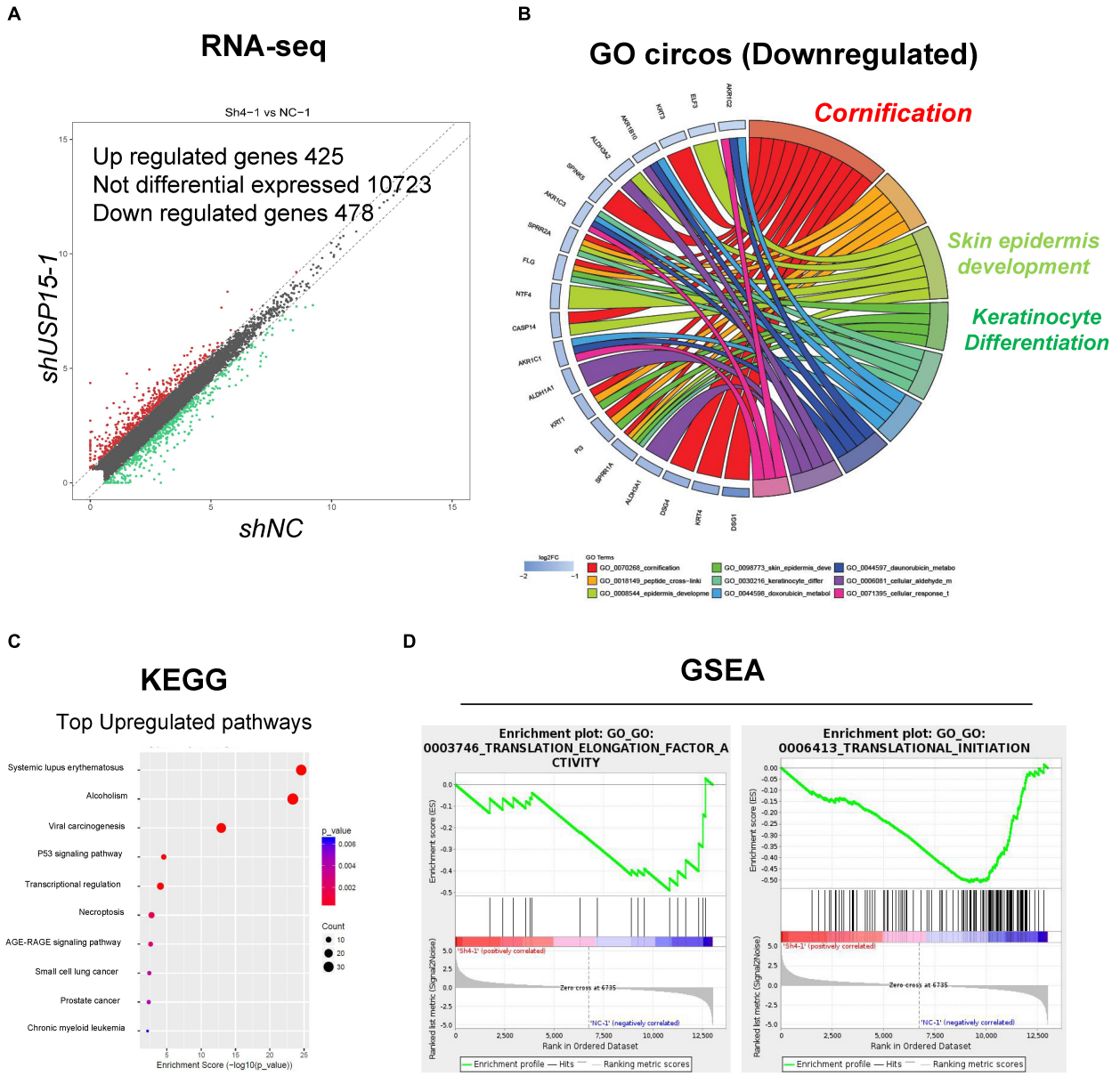
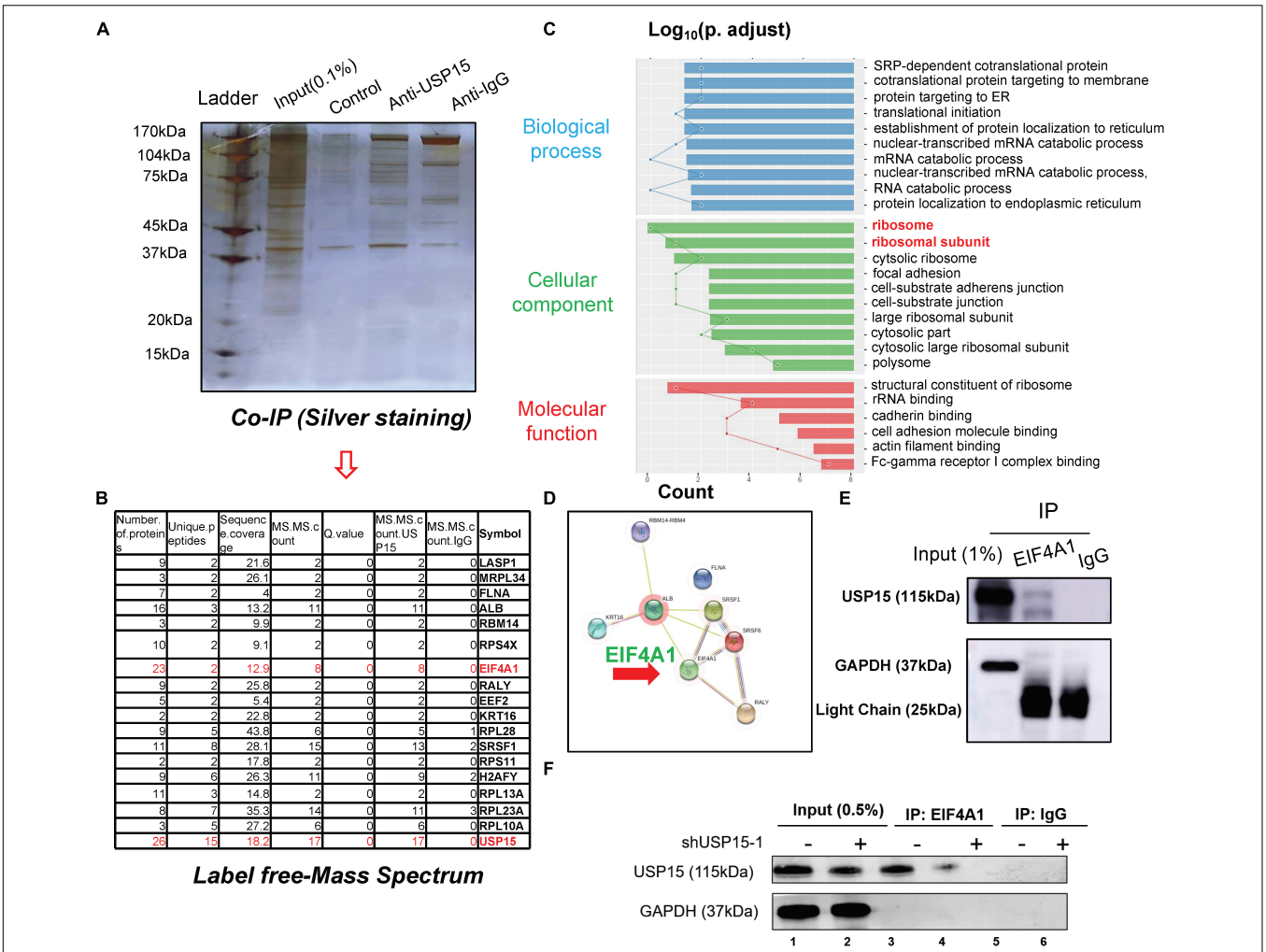


FIGURE 3 | Transcriptional profiling in the USP15-silenced cells. **(A)** Volcano plots of differentially expressed genes. The red points denote the significantly upregulated genes, and the green points denote the significantly downregulated genes. Fold change > 1.5, $p < 0.05$. **(B)** A Gene ontology analysis and a Circos plot demonstrated that the differentially expressed genes were mainly associated with epidermal processes. The GO assay was performed in http://geneontology.org/. **(C)** A Kyoto Encyclopedia of Genes and Genomes assay illustrated pathways mainly involved in USP15 silencing. The KEGG assay was performed in https://www.kegg.jp/. **(D)** A Gene Set Enrichment analysis was performed to illustrate the translation-related pathway intensity after USP15 silencing. The GSEA assay was performed in https://www.gsea-msigdb.org/gsea/index.jsp.

USP15−/− mice, indicating that USP15 could also promote EIF4A1 protein stability *in vivo* (**Figure 5E**).

EIF4A1 Promoted the Proliferation, Migration and Translation of Keratinocytes

EIF4A1 is a key factor in translational initiation; however, the role of EIF4A1 in keratinocytes remains unclear. Thus, we inhibited EIF4A1 expression with two siRNAs in HaCaT cells. Both siRNAs resulted in an ∼70% mRNA knockdown efficacy (**Figure 6A**). In addition, the EIF4A1 protein level was significantly downregulated after silencing EIF4A1 (**Figure 6B**). Furthermore, the EIF4A1-silenced cells presented a slower proliferative rate and formed smaller colonies than the control cells (**Figures 6C,D**). Moreover, both wound healing assays (**Figures 6E,F**) and Transwell assays (**Figure 6G**) demonstrated that cellular migration was significantly attenuated after



Number. of.proteins	Unique.peptides	Sequence.coverage	MS.MS.count	Q.value	MS.MS.count.USP15	MS.MS.count.IgG	Symbol
9	2	21.6	2	0	2	0	LASP1
3	2	26.1	2	0	2	0	MRPL34
7	2	4	2	0	2	0	FLNA
16	3	13.2	11	0	11	0	ALB
3	2	9.9	2	0	2	0	RBM14
10	2	9.1	2	0	2	0	RPS4X
23	2	12.9	8	0	8	0	EIF4A1
9	2	25.8	2	0	2	0	RALY
5	2	5.4	2	0	2	0	EEF2
2	2	22.8	2	0	2	0	KRT16
9	5	43.8	6	0	5	1	RPL28
11	8	28.1	15	0	13	2	SRSF1
2	2	17.8	2	0	2	0	RPS11
9	6	26.3	11	0	9	2	H2AFY
11	3	14.8	2	0	2	0	RPL13A
8	7	35.3	14	0	11	3	RPL23A
3	5	27.2	6	0	6	0	RPL10A
26	15	18.2	17	0	17	0	USP15

FIGURE 4 | The interactome analysis of USP15. **(A)** A coimmunoprecipitation assay was performed to identify the USP15-interacting proteins. The eluted protein was identified by silver staining. **(B)** A mass spectrum analysis was performed, and 18 specified USP15-interacting proteins were identified. **(C)** A Gene Ontology assay demonstrated that the differentially expressed genes were mainly distributed in ribosomes. The GO assay was performed in http://geneontology.org/. **(D)** A protein interaction network assay was performed to identify the central proteins in the USP15 interactome. This assay was performed in https://string-db.org/. **(E)** An immunoprecipitation assay was performed to illustrate the interaction between EIF4A1 and USP15. EIF4A1 was used as a bait. The GAPDH protein presented a strong band in input group while IgG light chain only presented in IP group. **(F)** An immunoprecipitation assay was performed after silencing USP15. EIF4A1 was used as a bait. The GAPDH protein presented a strong band in input group while absent in IP group.

knocking down EIF4A1. Most importantly, a transcriptome analysis was performed and demonstrated that there were 319 upregulated and 356 downregulated genes after silencing EIF4A1 (**Figure 7A**, see footnote 1, accession number: OEP000763). These altered genes were mainly involved in cellular starvation, cytoskeleton/actin remodeling and the notch signaling pathway (**Figure 7B**). Moreover, a significant reduction in the translation process was observed after interfering with EIF4A1, which indicates the essential role of EIF4A1 in translation (**Figure 7C**).

DISCUSSION

Re-epithelialization plays a vital role in wound healing, which involves numerous cytokines and cells during cutaneous barrier reconstruction (Mazzalupo et al., 2002; Jackow et al., 2016). USP15, an important DUB, removes ubiquitin chains from target proteins and promotes protein stability (Padmanabhan et al., 2018). Here, we demonstrated for the first time that a keratinocyte-expressed protein, USP15, deubiquitinated EIF4A1 and prevented its degradation, thereby enhancing the translation process and accelerating re-epithelialization both *in vitro* and *in vivo* (**Figure 7D**). These observations shed light on the novel regulatory role of USP15 in the re-epithelialization of wound healing, providing a promising target for refractory wound treatment.

Notably, USP15, a vital DUB that can remove ubiquitin from proteins, has been shown to be involved in DNA repair, TGF-β signaling pathways and mitophagy (Cornelissen et al., 2014). For instance, USP15 was reported to regulate transcription and DNA

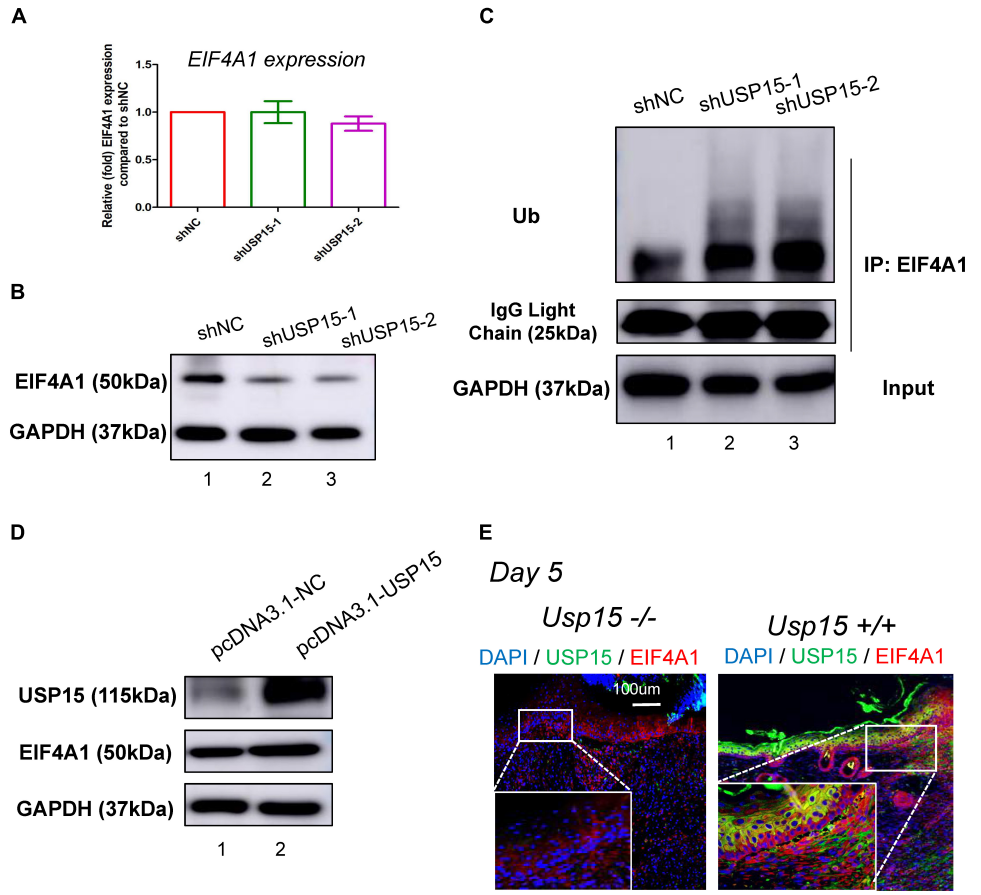


FIGURE 5 | USP15 removed ubiquitin from EIF4A1. **(A)** Real-time PCR was performed to measure the EIF4A1 mRNA expression in the USP15-silenced keratinocytes (HaCaT). **(B)** A western blot assay was performed to identify the EIF4A1 protein expression in the USP15-silenced keratinocytes (HaCaT) cells. **(C)** A coimmunoprecipitation assay was performed to measure the ubiquitination level of EIF4A1 after inhibiting USP15. **(D)** A western blot assay was performed to identify the EIF4A1 protein expression in the USP15-overexpressed keratinocytes (HaCaT) cells. pcDNA3.1 was used as vector for overexpression. pcDNA3.1-NC was referred as control. **(E)** Immunofluorescence of USP15 and EIF4A1 in the *Usp15* knockout mice and their wild-type littermates. Scale bar: 200 μm.

repair via deubiquitinating the histone H2B (Long et al., 2014). Our group previously demonstrated that USP15 could directly accelerate wound healing by stabilizing TBR1 and maintaining the TGF-β signaling pathway in fibroblasts (Zhao et al., 2019). However, the role of USP15 in keratinocytes was different from that in fibroblasts. In our study, we found that the TGF-β signaling pathway remained unchanged in keratinocytes, which indicated a novel function of USP15.

To explore the target protein of USP15, we performed a multi-omics analysis. RNA-seq demonstrated that USP15 was highly associated with translational regulation. Coimmunoprecipitation and MS analyses revealed that the interactome of the USP15 protein was mainly distributed in the ribosome, and EIF4A1 was a key factor in translational initiation. Here, we revealed for the first time that USP15 could enhance the translation process, indicating a novel function of the USP15/EIF4A1 complex.

Translation initiation is a rate-limiting and highly regulated process that requires diversified coordinated action of eukaryotic initiation factors (EIFs) (Wolf et al., 2010). The DEAD-box helicase eIF4A1 has been proven to unwind structured RNA elements within the 5′ untranslated region (5′UTR) to facilitate ribosome binding (Modelska et al., 2015; Peters et al., 2018). Alterations in EIF4A1 activity-modulating proteins expression have been observed in the tumourigenesis of melanoma (Joyce et al., 2017), breast cancer (Stoneley and Willis, 2015), and pancreatic cancer (Ma et al., 2019). However, the role of EIF4A1 in re-epithelialization remains unclear. Here, for the first time, we demonstrated that EIF4A1 was essential in the translational regulation, proliferation and migration of keratinocytes, which indicated a novel molecular function in wound healing and a promising target of refractory wounds.

Mostly, translational initiation factor family were 'house-keeping genes' and initiation of translation is the rate-limiting step in protein synthesis in all living cells (Mejias-Navarro et al., 2020). These factors have been proven to play a vital role in wound healing, tumorigenesis, cell stemness and epithelial-mesenchymal transition, etc. For example, elF2a is involved in DNA damage repair and could regulate autophagy in tumors (Arasi et al., 2019). elF3 family modulate of the hypoxia

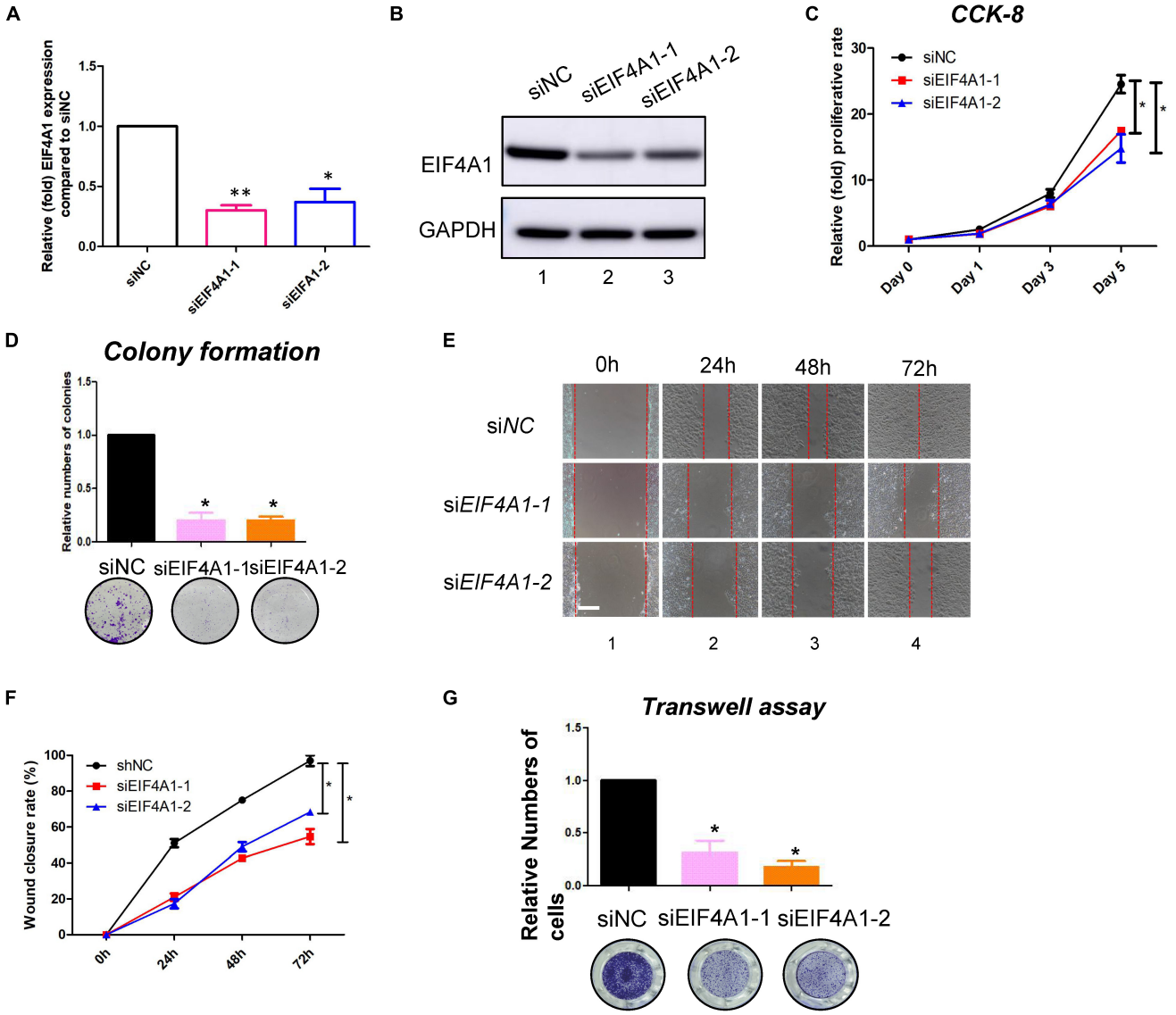


FIGURE 6 | EIF4A1 promoted the cell growth and migration in keratinocytes. **(A)** Real-time PCR was performed to measure the EIF4A1 mRNA expression in the EIF4A1-silenced HaCaT cells. Scramble-siRNAs served as a negative control (siNC). *$p < 0.05$, **$p < 0.01$. **(B)** A western blot assay was performed to identify the EIF4A1 protein expression in the EIF4A1-silenced HaCaT cells. GAPDH was referred as endogenous control. **(C)** A CCK-8 assay was performed to determine the proliferative rate of the EIF4A1-silenced cells. The OD value of Day 0 was set as 100%. *$p < 0.05$. **(D)** A colony formation assay was performed to determine the colony formation ability of the EIF4A1-silenced cells. The colony number in the shNC group was set as 100%. All experiments were performed in triplicate, and the relative colony formation rates are shown as the mean ± SEM. *$p < 0.05$. **(E)** A wound healing assay was performed to determine the migratory ability of the EIF4A1-silenced keratinocytes (HaCaT). Scale bar: 50 μm. **(F)** Quantification of the wound recovery rate in the wound healing assay. All experiments were performed in triplicate, and the relative cell migration ratio is shown as the mean ± SEM. *$p < 0.05$. **(G)** A transwell assay was performed to determine the migratory ability of the EIF4A1-silenced cells. Quantification of the numbers of transferred cells is shown in the upper panel. The colony number in the empty vector group was set as 100%. All experiments were performed in triplicate, and the relative cell numbers are shown as the mean ± SEM. *$p < 0.05$.

inducible factors (HIFs) and suppress tumorigenesis. elF4e has been identified to be involved in cutaneous wound healing (Schwarz et al., 2002). Moreover, EIF4A1 was identified to be significantly upregulated in a proteomic investigation of human burn wounds by 2D-difference gel electrophoresis (Wolf et al., 2010; Joyce et al., 2017). Conclusively, the initiation factors are 'house-keeping genes,' which are vital in protein homeostasis, it is hard to choose another initiation factor as control. We would like to further validate the role of other initiation factors in wound healing, which would warrant an individual study.

Skin wound healing requires diverse coordinated interactions across various cells, such as macrophages, activated T/B lymphocytes and fibroblasts (Schmidt and Horsley, 2013; Rognoni and Watt, 2018). To date, our group has only uncovered the functions of USP15 in fibroblasts and keratinocytes. Further

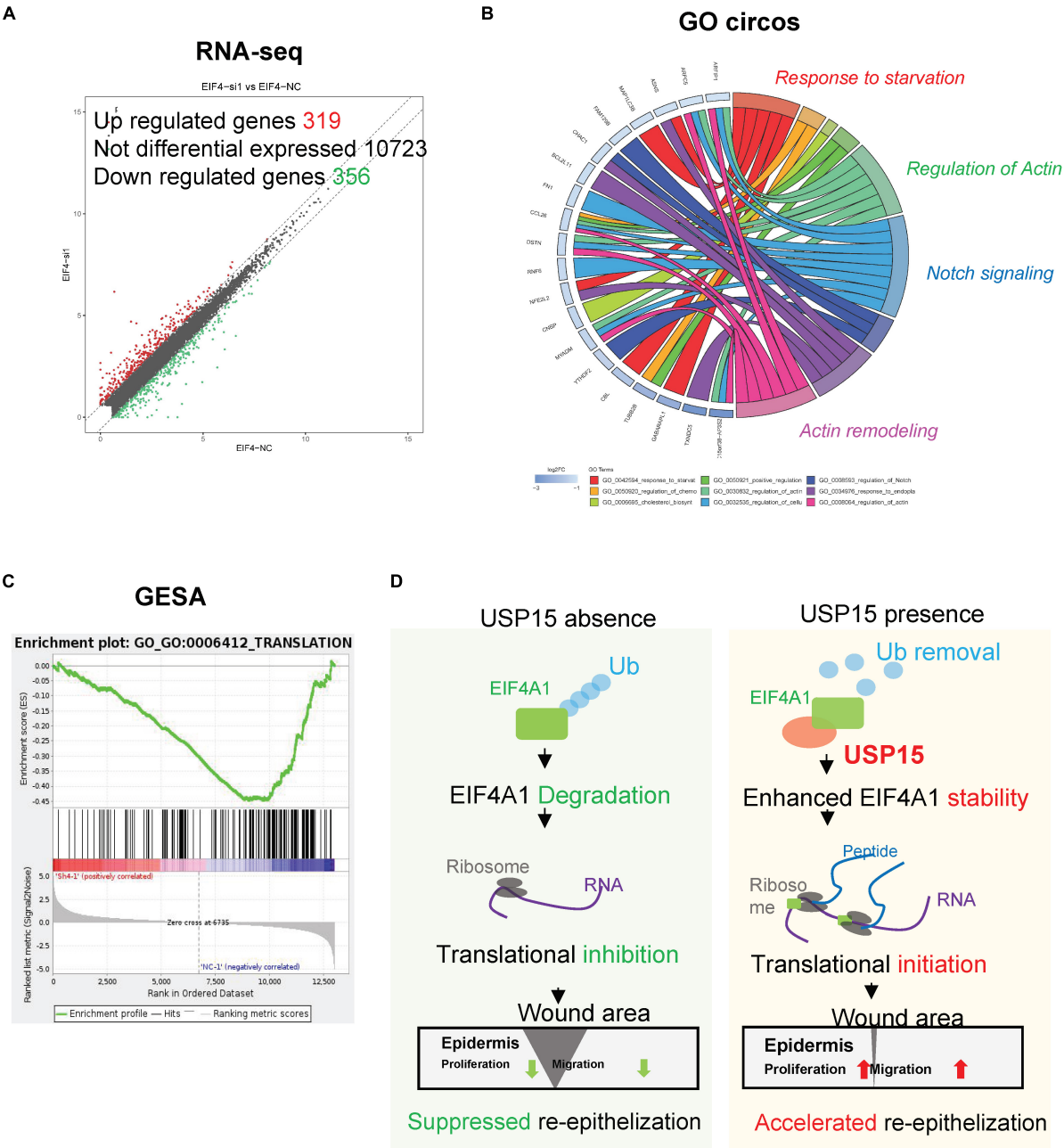


FIGURE 7 | Transcription profiling in the EIF4A1-silenced cells. **(A)** Volcano plots of differentially expressed genes. The red points denote 319 significantly upregulated genes, and the green points denote 356 downregulated genes. Fold change > 1.5, $p < 0.05$. **(B)** A Gene Ontology assay and Circos plot demonstrated that the differentially expressed genes were mainly associated with cytoskeleton remodeling. The GO assay was performed in http://geneontology.org/. **(C)** A Gene Set Enrichment analysis illustrated that the translation signaling pathway was significantly inhibited after EIF4A1 silencing. This assay was performed in https://string-db.org/. **(D)** Schematic of the study. Silencing *USP15/EIF4A1* attenuates the translation initiation process and results in dysfunctional re-epithelialization. USP15 directly deubiquitinates EIF4A1 to promote wound healing in keratinocytes.

investigations could explore the regulatory roles of USP15 in other cells. In addition, mice and humans show a fundamental difference in cutaneous wound healing (Dunn et al., 2013). Further studies should be performed to explore the possibility of treating chronic refractory wounds through supplementation with recombinant *USP15* protein or USP15-carrying adenovirus.

Thus, we concluded that silencing *USP15/EIF4A1* attenuates the translational initiation process and then results in dysfunctional re-epithelialization. Mechanistically, our research proves that USP15 directly deubiquitinates EIF4A1 to promote wound healing in keratinocytes. Because USP15 harbors druggable enzymatic activity as a member of the DUBs, it is

considered a potential therapeutic target with vital clinical applications (He et al., 2017). Recombinant DUBs or DUB-based virus-related therapy could further be applied to accelerate re-epithelialization, whereas small molecule inhibitors that target DUBs may become a promising intervention for cutaneous overhealing-associated diseases, such as hypertrophic scars and keloids (Lee et al., 2010; Piao et al., 2015). Further experiments on the effects of overexpressing *USP15* (such as recombinant protein or virus-related therapy) will be performed in the near future to assess the function of USP15 in the treatment of refractory wounds.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: RNA-seq data has been uploaded to National Omics Data Encyclopedia (NODE) database (http://www.biosino.org/node/project, accession number: OEP000770 for USP15 silencing and OEP000763 for EIF4A1 silencing).

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee of Shanghai Ninth People's Hospital.

AUTHOR CONTRIBUTIONS

YXZ and XH designed and performed the experiments and drafted the manuscript. YXZ and ZZ were responsible for the sample collection and data analysis. YFZ revised the manuscript. GZ provided the mice. QL, TZ, and GZ discussed and approved the manuscript. All the authors approved this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00529/full#supplementary-material

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MINI REVIEW
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Macrophage Dysregulation and Impaired Skin Wound Healing in Diabetes

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Monocytes (Mo) and macrophages (Mϕ) play important roles in normal skin wound healing, and dysregulation of wound Mo/Mϕ leads to impaired wound healing in diabetes. Although skin wound Mϕ originate both from tissue resident Mϕ and infiltrating bone marrow-derived Mo, the latter play dominant roles during the inflammatory phase of wound repair. Increased production of bone marrow Mo caused by alterations of hematopoietic stem and progenitor cell (HSPC) niche and epigenetic modifications of HSPCs likely contributes to the enhanced number of wound Mϕ in diabetes. In addition, an impaired transition of diabetic wound Mϕ from "pro-inflammatory" to "pro-healing" phenotypes driven by the local wound environment as well as intrinsic changes in bone marrow Mo is also thought to be partly responsible for impaired diabetic wound healing. The current brief review describes the origin, heterogeneity and function of wound Mϕ during normal skin wound healing followed by discussion of how dysregulated wound Mϕ numbers and phenotype are associated with impaired diabetic wound healing. The review also highlights the possible links between altered bone marrow myelopoiesis and increased Mo production as well as extrinsic and intrinsic factors that drive wound macrophage dysregulation leading to impaired wound healing in diabetes.

Keywords: monocytes, macrophages, diabetes, wound healing, bone marrow, inflammation, hematopoietic stem, progenitor cells

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INTRODUCTION

Skin wound healing involves distinct but overlapping phases of hemostasis, inflammation, proliferation, and remodeling (Eming et al., 2014). Immediately following injury, platelet aggregation is important to achieve coagulation and hemostasis in the wound. The inflammatory phase is induced by pro-inflammatory mediators released by injured tissues, and is critical for controlling infection, clearing necrotic debris, and induction of the wound healing process (Zhang and Mosser, 2008; Koh and DiPietro, 2011; Eming et al., 2017). Next, the proliferative or tissue formation phase involves proliferation of a number of cell types to form a provisional connective tissue matrix, new blood vessels, and epithelial closure. Finally, during the remodeling phase the

Abbreviations: CMP, common myeloid progenitor, GMP, granulocyte macrophage progenitor; DIO, diet-induced obese; HSC, hematopoietic stem cells; HSPC, hematopoietic stem and progenitor cell; IL-1R1, interleukin-1 receptor 1; Mo/Mϕ, monocyte/macrophage; PPARγ, peroxisome proliferator-activated receptor γ; RAGE, receptor for advanced glycation end products; STZ, streptozotocin.

newly formed tissues are remodeled to improve their integrity (Eming et al., 2014). A diverse set of cells such as platelets, mast cells, neutrophils, monocytes (Mo), macrophages (Mϕ), lymphocytes, keratinocytes, fibroblasts, and endothelial cells all contribute to the process of skin wound healing (Canedo-Dorantes and Canedo-Ayala, 2019).

Among all cell types Mo/Mϕ play critical roles in each phase of wound repair through host defense, tissue debridement and cell regulatory functions (Goren et al., 2009; Mirza et al., 2009; Lucas et al., 2010; Boniakowski et al., 2017). Studies using *LysM-Cre/DTR* genetically modified mice that allow for inducible depletion of Mo/Mϕ by diphtheria toxin (DT) administration provide strong evidence that these cells are required for normal wound healing, promoting angiogenesis, collagen deposition, and closure (Goren et al., 2009; Mirza et al., 2009; Lucas et al., 2010).

Properly regulated numbers and phenotypes of Mo/Mϕ are crucial for efficient wound repair, and the dysregulation of either may lead to impaired wound healing. For example, increased numbers of wound Mo/Mϕ have been shown to be associated with impaired wound healing in diabetes (Mirza and Koh, 2011; Bannon et al., 2013; Barman et al., 2019b). Similarly, an impaired transition from pro-inflammatory into pro-healing wound Mo/Mϕ phenotypes and reduced phagocytic ability contribute to chronic inflammation and impaired wound healing in diabetes (Mirza and Koh, 2011; Bannon et al., 2013; Mirza et al., 2013, 2014; Gallagher et al., 2015; Yan et al., 2018; Barman et al., 2019b). This brief review considers the origin, heterogeneity and function of wound Mϕ during normal wound healing followed by discussion of how dysregulation of numbers and phenotypes of wound Mϕ may lead to impaired diabetic wound healing. The review also highlights the possible links between altered bone marrow myelopoiesis, wound macrophage dysfunction and impaired wound healing, and finally highlights gaps in the current literature, whose filling could lead to new therapeutic interventions for diabetic wounds.

ORIGIN OF SKIN WOUND Mϕ

Skin wound Mϕ originate both from tissue resident Mϕ and infiltrating Mo with significantly larger contribution from the latter (Davies et al., 2013; Malissen et al., 2014; Minutti et al., 2017; Burgess et al., 2019). Dermal Mϕ are likely early responders to skin wounding via recognition of damage associated molecular pattern (DAMP) molecules or pathogen associated molecular pattern (PAMP) molecules (Davies et al., 2013; Malissen et al., 2014; Minutti et al., 2017). These tissue-resident Mϕ originate from yolk sac but are replenished by fetal liver-derived Mo in the embryo and by bone marrow Mo after birth. The major functions of these Mϕ are maintenance of skin homeostasis and integrity, tissue repair, and stress response (Tamoutounour et al., 2013; Ginhoux and Guilliams, 2016; Yanez et al., 2017). In addition, Langerhans cells, which are epidermal dendritic cells but share Mϕ markers such as MHC-II, F4/80 and CD14 also play important roles in wound healing (Malissen et al., 2014; Minutti et al., 2017). Langerhans cells originate both from the yolk sac during primitive hematopoiesis and fetal liver-derived Mo during definitive hematopoiesis. However, in contrast to dermal Mϕ, Langerhans cells are maintained by self-replication without any replenishment from bone marrow monocyte pool (Merad et al., 2002; Hoeffel et al., 2012, 2015; Gomez Perdiguero et al., 2015; Ginhoux and Guilliams, 2016).

Skin wounding induces a rapid, large infiltration of inflammatory Mo ($CCR2^{+}Ly6C^{+}$) into wounds followed by conversion of the Mo into Mϕ ($Ly6C^{-}F4/80^{+}$) as healing progresses (Koh and DiPietro, 2011; Willenborg et al., 2012; Crane et al., 2014; Rodero et al., 2014; Wynn and Vannella, 2016; Barman et al., 2019a,b). Blood Mo are thought to be the main source of wound Mo/Mϕ and a rapid decrease in $CD11b^{+}CD115^{+}Ly6C^{hi}$ blood Mo 4–6 h post wounding correlates in time with the increase of inflammatory Mo in skin wound Mo (Rodero et al., 2014). After infiltrating wounds, novel recent findings demonstrate that inflammatory Mo/Mϕ ($Ly6C^{hi}F4/80^{-/lo}$) proliferate rapidly peaking on day 6 post-wounding. In contrast, the majority of mature wound Mϕ ($Ly6C^{-}F4/80^{+}$) remain at resting G0 phase indicating that proliferation of infiltrating inflammatory Mo followed by their differentiation into mature Mϕ results in wound Mϕ expansion (Pang et al., 2019). In addition, several studies have demonstrated that bone marrow-derived Mo contribute to skin wound Mϕ and that similar to other tissue injuries such as myocardial infarction and hindlimb ischemia, skin wounding also promotes bone marrow monopoiesis in mice (Ishida et al., 2008; Sager et al., 2015; Fang et al., 2018; Barman et al., 2019a). However, unlike myocardial infarction, skin wounding-induced monopoiesis in bone marrow occurs independently of IL-1R1 signaling (Sager et al., 2015; Barman et al., 2019a). Altogether, these data clearly suggest that there is a communication between skin wounding and bone marrow for increased Mo production which may be critical for normal wound healing.

Mϕ SUBSETS IN SKIN WOUND HEALING

During the early phases of healing, wound Mo/Mϕ exhibit a pro-inflammatory or "classically activated" M1-like phenotype, which gradually transitions into a healing-associated or "alternatively activated" M2-like phenotype; it is well documented that such transition of phenotypes is essential for normal wound healing (Willenborg et al., 2012; Mirza et al., 2013, 2014; Kimball et al., 2018). In addition to the M1/M2 or related classification schemes, other phenotypically distinct Mϕ subsets have been reported to play important roles during skin wound healing. For example, dermal Mϕ identified as $CD64^{+}$, $MERTK^{+}$, and $CCR2^{-/low}$ influence wound healing by their highly phagocytic nature (Malissen et al., 2014). A recent study has demonstrated two distinct Mϕ subsets in skin wounds which differ in both function and origin and are distinguishable by surface CX3CR1 staining. $CX3CR1^{hi}$ Mϕ were derived from tissue resident Mϕ and were predominantly alternatively activated, whereas $CX3CR1^{-/lo}$ wound Mϕ were derived from recruited Mo and exhibited both classical and alternative activation states (Burgess et al., 2019). Another subset of tissue resident Mϕ known as skin trans endothelial radio-resistant anti-inflammatory Mϕ

(STREAM) were found to be located in perivascular regions and constitutively express an anti-inflammatory transcriptional profile. Interestingly, these Mϕ were resistant to polarization toward inflammatory phenotypes under inflammatory stimuli, hence appearing to be critical for tissue repair and regeneration (Barreiro et al., 2016). Similarly, another report described $CD11b^{+}F4/80^{+}CD206^{+}CD301b^{+}$ wound Mϕ to be critical for reparative mechanism which are increased during the proliferative phase of wound healing (Shook et al., 2016). These data suggest that there are functionally distinct Mϕ subsets in skin wounds which play critical roles at different stages of wound healing, however, how the reported Mϕ subsets may be related to each other, how each is regulated and their precise roles in healing remain to be determined.

Mϕ FUNCTIONS IN NORMAL WOUND HEALING

Tissue-resident dermal Mϕ are likely among the earliest responders to skin injury, helping to induce the inflammatory response via release of hydrogen peroxide resulting in recruitment of blood neutrophils and monocytes (Davies et al., 2013; Malissen et al., 2014; Minutti et al., 2017). During early wound healing, Mo/Mϕ help to clear the wound of contaminating microbes as well as apoptotic neutrophils and cellular debris via phagocytosis (Meszaros et al., 1999, 2000; Silva, 2010; Soehnlein and Lindbom, 2010; Chen et al., 2015). The importance of Mϕ in such wound debridement is supported by studies targeting macrophage peroxisome proliferator-activated receptor γ (PPARγ), which plays a role in efferocytosis (Chen et al., 2015). PPARγ KO mice exhibit increased accumulation of apoptotic neutrophils in wounds and impaired wound healing, indicating impaired clearance of apoptotic cells. Further, treatment with a PPARγ agonist reduced accumulation of apoptotic neutrophils in wounds and improved healing (Chen et al., 2015).

Another important function of Mϕ is to regulate the activity of other wound cells via the production and release of many different cytokines and growth factors. Early after tissue injury, Mϕ release numerous inflammatory mediators including IL-1β, TNF-α, IL-6, and others to amplify the inflammatory response (Barrientos et al., 2008). In addition, wound Mo/Mϕ are an important source of growth factors such as VEGF, which is critical for angiogenesis and tissue growth (Stockmann et al., 2011; Willenborg et al., 2012). Later in the healing process, Mϕ secrete other growth factors such as TGF-β, FGF, and IGF-1 that induce cell proliferation and protein synthesis which are critical for healing (Hunt et al., 1984; Rappolee et al., 1988). Finally, Mϕ have been shown to be involved in collagen degradation during tissue remodeling phase of repair (Madsen et al., 2013; Roch et al., 2014; Wang et al., 2017). A study that depleted Mϕ in *LysM-Cre/DTR* mice at different stages of wound healing supports the notion that Mϕ change functions throughout the healing process – loss of Mϕ during early stages of healing leads to reduced epithelialization, granulation tissue formation and wound contraction whereas Mϕ depletion during the mid-phase abrogates transition of wound tissues from regeneration to maturation phase (Lucas et al., 2010). Collectively, these studies demonstrate that Mϕ play diverse roles throughout each stage of wound healing and thus are integral components of wound repair.

Mo/Mϕ DYSREGULATION AND IMPAIRED WOUND HEALING IN DIABETES

Diabetes is a metabolic disorder leading to low-grade systemic inflammation which is known to have a significant impact on the immune system (Esser et al., 2014). Dysregulated metabolic pathways and host immune response contribute to impairments in each phase of wound healing, ultimately causing delayed wound healing in diabetes (Patel et al., 2019). Along with various other factors, alterations in both the number and phenotype of wound Mo/Mϕ likely contribute to impaired wound healing in diabetes. The number of infiltrating Mo is found to be higher in the wounds of leptin receptor mutant ($Lepr^{db}$) db/db type 2 diabetic mice early after wounding (Bannon et al., 2013; Barman et al., 2019b). Following such increased monocyte accumulation, the macrophage subsets ($Ly6C^{+}F4/80^{+}$ and $Ly6C^{-}F4/80^{+}$) are also increased significantly in db/db mouse wounds at later time points suggesting a persistent Mo/Mϕ response in diabetic wounds (Mirza and Koh, 2011; Gallagher et al., 2015; Kimball et al., 2018; Barman et al., 2019b). Further, a recent study has shown that the proportion of early wound Mϕ differentiated from infiltrating bone marrow-derived monocytes in db/db diabetic mice are increased whereas wound Mϕ derived from tissue resident Mϕ remain unaltered as compared to non-diabetic wounds (Burgess et al., 2019). Impaired wound healing in high-fat-diet induced obese (DIO) pre-diabetic mice is also associated with persistent accumulation of inflammatory Mo/Mϕ in non-healing wounds (Gallagher et al., 2015; Kimball et al., 2018). Prolonged infiltration of blood $Ly6C^{hi}$ Mo is thought to be responsible for the sustained accumulation of inflammatory Mo/Mϕ in the skin wounds of DIO mice. Such extended infiltration of inflammatory Mo may contribute to the observed defect in the transition from $Ly6C^{hi}$ into $Ly6C^{lo}$ Mo/Mϕ phenotypes (Kimball et al., 2018) (**Figure 1A**). In contrast to these reports, there are also reports of decreased numbers of wound Mo/Mϕ early after wounding in diabetic mice attributed in part to an early impairment of chemotaxis into the wound (Wood et al., 2014; Yan et al., 2018). The discrepancy in wound macrophage numbers between studies could result from technical differences in the assessment of wound Mϕ and deserves further study.

Wound Mo/Mϕ in diabetic mice persistently express high levels of M1-like Mϕ markers such as NOS2, TNF-α, IL-1β, MMP9, and low levels of M2-like Mϕ markers such as Arginase 1, CD206, CD36 (Mirza and Koh, 2011; Bannon et al., 2013; Mirza et al., 2013, 2014). Diabetic wound-derived Mϕ also exhibit decreased expression of pro-healing factors such as IGF-1, TGF-β and VEGF (Mirza and Koh, 2011; Mirza et al., 2013, 2014). Similar to diabetic mice, wound biopsies from human

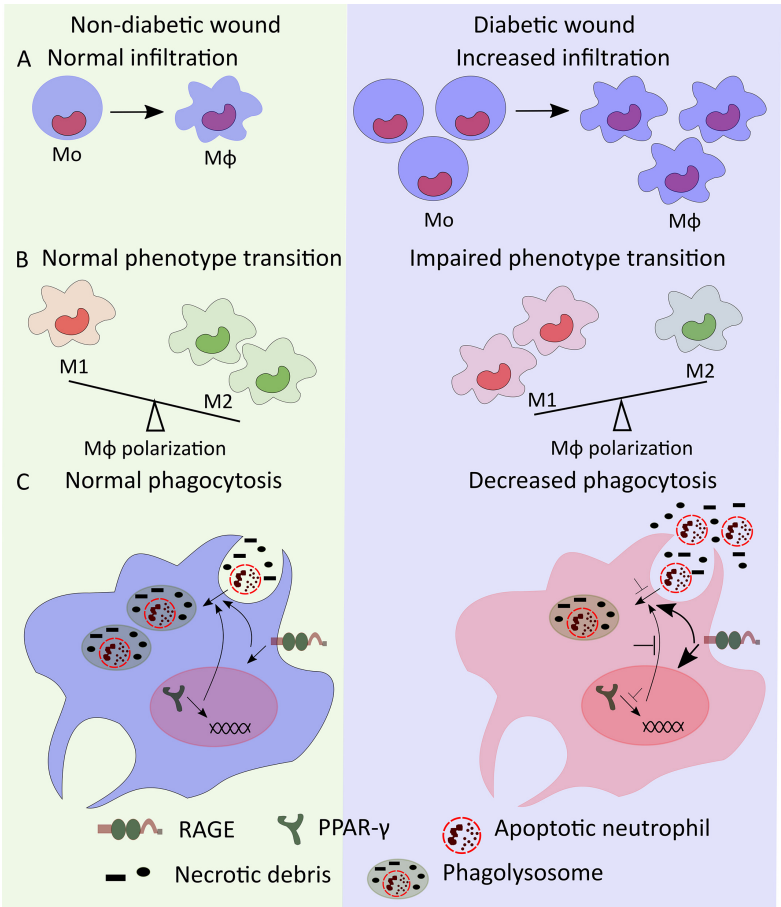

FIGURE 1 | Mo/Mϕ dysregulation in diabetic wound. **(A)** High numbers of bone marrow-produced Mo may lead to increased number of diabetic wound Mϕ. **(B)** Mϕ transition from M1- to M2-like phenotypes is impaired in diabetic wounds resulting in increased accumulation of M1-like wound Mϕ. **(C)** Decreased PPAR-γ and increased RAGE signaling reduce the phagocytic ability of wound Mϕ decreasing phagocytosis of apoptotic neutrophils and necrotic debris thus leading to increased accumulation of neutrophils and necrotic debris in diabetic wounds.

diabetic foot ulcers also show increased proportion of M1-like Mϕ ($CD68^+$ and $IL\text{-}1\beta^+$) and decreased proportion of M2-like Mϕ ($CD163^+$, $CD206^+$, and $Arginase\text{-}1^+$), respectively (**Figure 1B**) (Bannon et al., 2013; Mirza et al., 2013; Gallagher et al., 2015). The sustained pro-inflammatory phenotype of diabetic wound Mϕ likely helps to drive a persistent pro-inflammatory microenvironment in diabetic wounds characterized by increased levels of IL-1β, IFN-γ, TNF-α, and IL-12 as well as decreased levels of pro-healing factors such as IGF-1, TGF-β1, VEGF, and IL-10 (Mirza and Koh, 2011; Bannon et al., 2013; Mirza et al., 2013). Together, these reports indicate that dysregulation of Mϕ polarization likely plays critical roles in impaired diabetic wound healing in both mice and humans.

There is also consensus in the literature that wound Mo/Mϕ in diabetic mice and humans show impaired phagocytosis that may contribute to impaired wound healing (Khanna et al., 2010; Pavlou et al., 2018). Wound Mϕ in diabetic mice exhibit reduced phagocytosis resulting in increased accumulation of apoptotic cells in the wounds and a sustained pro-inflammatory microenvironment (Khanna et al., 2010). In addition, reduced PPAR-γ expression in diabetic wound Mϕ and improved wound healing in diabetic mice with topical wound treatment with PPAR-γ agonist is consistent with the idea that PPAR-γ-mediated macrophage clearance of apoptotic wound neutrophils may play an important role in wound healing (Khanna et al., 2010; Chen et al., 2015; Mirza et al., 2015). In another report, antibody mediated topical inhibition of receptor for advanced glycation end products (RAGE) signaling showed reduced number of neutrophils in diabetic wounds in association with enhanced phagocytosis by Mϕ and improved wound healing further supporting the notion that increased neutrophil accumulation in diabetic wounds results from reduced phagocytic ability of Mϕ which is closely associated with impaired wound healing in diabetes (**Figure 1C**) (Wang et al., 2017). Together, these reports suggest that decreased efferocytosis by wound Mϕ is critical for impaired wound healing in diabetes.

Dysregulated Mϕ activity in diabetic wounds impairs processes critical for normal wound healing. For example, Mϕ are known to play important roles in neovascularization

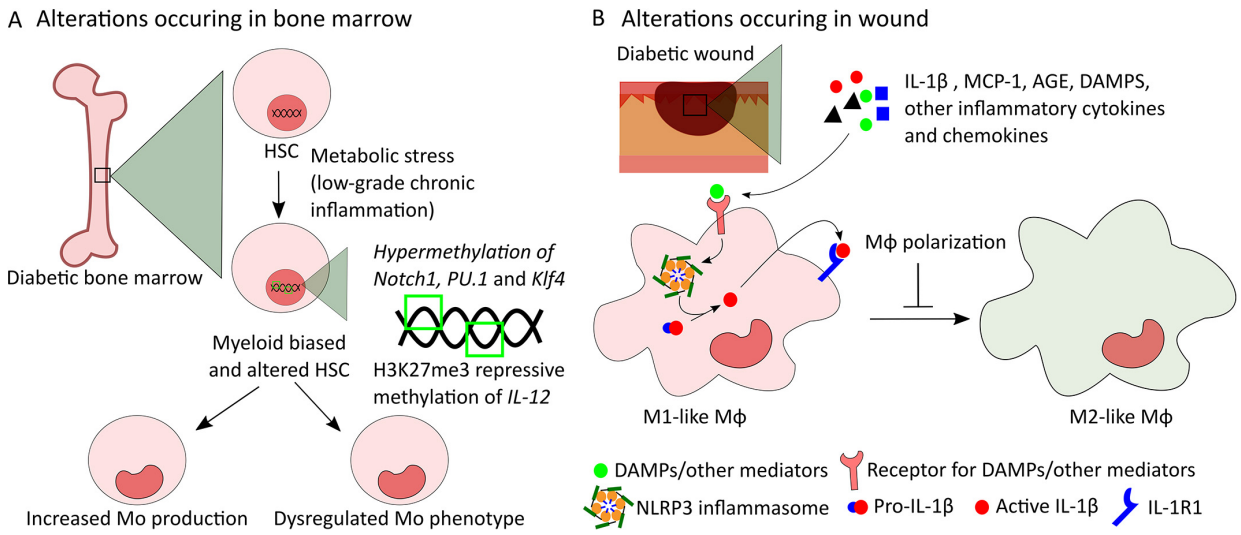

FIGURE 2 | Potential mechanisms of alterations in bone marrow-derived Mo and in wound Mϕ in diabetes. **(A)** Low-grade chronic inflammation caused by metabolic stress induces myeloid bias in diabetic HSCs leading to increased production of Mo. In addition, intrinsic modifications of HSCs caused by epigenetic changes such as hypermethylation of *Notch1*, *PU.1*, and *Klf4* genes and H3K27me3 repressive methylation of *IL-12* gene can be passed down to Mo altering their phenotype. **(B)** Increased levels of IL-1β, MCP-1, AGE, DAMPs, and other inflammatory cytokines and chemokines in wound microenvironment induce NLRP3 and IL-1R1 signaling and other inflammatory pathways in wound Mϕ leading to dysregulated polarization of Mϕ from M1 to M2-like phenotype in diabetic wounds.

during wound healing, and decreased production of VEGF-A as well as reduced VEGFR1 signaling by diabetic wound Mϕ likely contribute to impaired angiogenesis in diabetic wounds (Stockmann et al., 2011; Willenborg et al., 2012; Okizaki et al., 2016; Okonkwo and DiPietro, 2017; Gurevich et al., 2018; Okonkwo et al., 2020). Mϕ likely also play important roles in the impaired maturation and remodeling of the vasculature in diabetic wounds. Altogether, the available data suggest that dysregulation of wound Mo/Mϕ both in terms of numbers and phenotypes plays critical roles in impaired diabetic wound healing.

DIABETES-INDUCED ALTERATION OF Mo PRODUCTION

Several reports have shown that diabetes increases monopoiesis in the bone marrow of STZ-induced and Akita (*Ins2*^{Akita}) type 1 diabetic mice where pancreatic β cells are destroyed by toxic effects of STZ or misfolded insulin (Nagareddy et al., 2013), DIO pre-diabetic mice (Singer et al., 2014), and db/db type 2 diabetic mice at steady state (Nagareddy et al., 2014; Barman et al., 2019b). STZ-induced diabetes and DIO mice also exhibit extramedullary myelopoiesis in the spleen resulting in increased number of splenic Mo during homeostasis (Vasamsetti et al., 2018). Furthermore, diabetic patients have been shown to display elevated number of circulating inflammatory Mo ($CD14^+CD16^-$) indicating diabetes-induced enhanced monopoiesis in human (Vasamsetti et al., 2018). However, skin wounding-induced Mo expansion in mouse bone marrow is not further augmented by diabetes (Barman et al., 2019b). Collectively, these data suggest increased steady-state monopoiesis in diabetes likely contributes to enhanced wound Mo/Mϕ and impaired wound healing (**Figure 2A**).

Increased monopoiesis in diabetes is associated with modification of the hematopoietic stem and progenitor cell (HSPC) compartment (Ferraro et al., 2011; Gallagher et al., 2015; Lee et al., 2018; Vasamsetti et al., 2018; Barman et al., 2019b). Recently, bone marrow-derived myeloid progenitors (LK, $Lin^-cKit^+Sca\text{-}1^-$ cells) from db/db mice have been shown to be intrinsically modified to produce increased number of Mo upon IL-1β or M-CSF challenge *in vitro* corroborating enhanced potential of diabetic myeloid progenitors to Mo production (Barman et al., 2019b). Alterations of the HSPC niche, which regulates HSPC maintenance, mobilization and differentiation, likely play an important role in the alterations of HSPC phenotypes in diabetes (Ferraro et al., 2011; Lucas et al., 2013; Morrison and Scadden, 2014; Boulais and Frenette, 2015; Vasamsetti et al., 2018; Albiero et al., 2019). Increased level of sympathetic nerves in the HSC niche is believed to induce the alterations in niche components in diabetes (Ferraro et al., 2011; Vasamsetti et al., 2018).

Importantly, increased IL-1R1 signaling in myeloid restricted bone marrow progenitors may be responsible for increased monopoiesis in obese and pre-diabetic mice (Nagareddy et al., 2014). In these studies, IL-1β produced by adipose Mϕ is thought to mediate communication between adipose tissue and bone marrow. However, diabetic db/db recipient mice transplanted with *Il1r1*$^{-/-}$ or WT donor cells showed no difference in myeloid cell output indicating that IL-1R1 signaling is likely not involved in diabetes-associated increased myelopoiesis in the db/db mouse model (Barman et al., 2019b). These seemingly disparate roles of IL-1R1 signaling in increased

myelopoiesis in different animal models of pre-diabetes and diabetes deserve further study.

In addition, RAGE has also been shown to be associated with increased monopoiesis in other models of diabetes such as STZ-induced and Akita diabetic mice (Nagareddy et al., 2013). Neutrophil-derived S100A8/A9 mediated activation of NF-kB-dependent RAGE signaling in common myeloid progenitors (CMPs) which in turn induces GMP proliferation via growth factors leads to increased monopoiesis in these diabetic mice (Nagareddy et al., 2013). Taken together, these data suggest that IL1-R1 and RAGE signaling may act as intrinsic drivers to promote monopoiesis in pre-diabetic and diabetic mice.

WOUND MICROENVIRONMENT AND BONE MARROW PROGENITOR MODIFICATIONS INFLUENCE DIABETIC WOUND Mϕ

Mϕ phenotypes in diabetic wounds can be altered both through local effects mediated by the wound microenvironment and through epigenetic modifications that may occur in bone marrow progenitors that are passed down to macrophage progeny (Mirza et al., 2013, 2014; Gallagher et al., 2015; Yan et al., 2018). There is strong evidence in the literature supporting the importance of the local microenvironment in determining macrophage phenotypes. First, the ability of diabetic wound conditioned medium to induce M1-like phenotypes in bone marrow-derived Mϕ *in vitro* supports the hypothesis that diabetic wound microenvironment may play important roles in determining Mϕ phenotypes (Mirza et al., 2013, 2014). Studies showing that local modification of diabetic wound microenvironment by blocking IL-1β or RAGE, or inhibiting inflammasome pharmacologically can shift wound Mϕ toward pro-healing phenotypes further support the notion that wound microenvironment has significant effect on dysregulation of Mϕ phenotypes in diabetic wounds and that sustained activation of NLRP3 (NLR family, pyrin domain-containing 3) inflammasome or of the RAGE pathway in wound Mo/Mϕ is likely involved (**Figure 2B**) (Mirza et al., 2013, 2014; Wang et al., 2017). Further, the level of MCP-1 is found to be higher in the wounds of DIO mice and antibody mediated inhibition of this chemokine during the inflammatory phase of wound healing normalizes the number of inflammatory wound Mo/Mϕ and improves wound healing (Kimball et al., 2018). Together, these data suggest that wound microenvironment may play important roles in dysregulated Mϕ functions in diabetic wounds.

Intrinsic modifications of bone marrow progenitors that are passed down to Mo/Mϕ may also contribute to dysregulated Mϕ phenotypes in diabetic wounds (Bannon et al., 2013; Gallagher et al., 2015; Yan et al., 2018; Davis and Gallagher, 2019). For example, differential responses to classical and alternate activation *in vitro*, amplified pro-inflammatory phenotypes and sustained potential to produce inflammatory tissue Mϕ observed in bone marrow-derived cells from different diabetic mouse models suggest that diabetes functionally alters progenitors in the bone marrow which may lead to dysregulated Mϕ phenotypes in diabetic wounds (Nagareddy et al., 2014; Singer et al., 2014). Additional studies indicated that epigenetic modifications of HSPCs may be involved in such phenotypic alterations in diabetic bone marrow progenitors (Gallagher et al., 2015; Yan et al., 2018; Davis and Gallagher, 2019). For example, epigenetic modification of HSPCs by means of decreased repressive histone methylation mark H3K27me3 at the IL-12 gene promoter has been shown to be passed down to wound Mϕ in DIO mice resulting in increased pro-inflammatory wound Mϕ and impaired wound healing (Gallagher et al., 2015). In addition, transplantation of diabetic hematopoietic stem cells (HSCs) from db/db into WT mice has been shown to cause delayed wound healing in association with sustained accumulation of M1-like wound Mϕ indicating that dysregulation of diabetic wound Mϕ phenotypes happens via a HSC autonomous mechanism (Yan et al., 2018). Epigenetic modification of myeloid lineage associated genes such as *Notch1*, *PU.1* and *Klf4* by DNA methyltransferase 1 (Dnmt1) mediated hypermethylation may induce such alterations in diabetic HSCs (**Figure 2A**) (Yan et al., 2018). Altogether, these data suggest both wound microenvironment-mediated alteration of wound Mϕ and epigenetic modification of HSPCs as potential mechanisms of dysregulated Mϕ phenotypes in diabetic wounds.

CONCLUSION, IMPLICATIONS, AND FUTURE DIRECTIONS

In summary, both sustained increases in the number of wound Mo/Mϕ and dysregulation of their phenotype, caused both by intrinsic alterations in bone marrow progenitors and by a pro-inflammatory wound microenvironment, lead to impaired wound healing in diabetes (Mirza et al., 2013, 2014; Nagareddy et al., 2014; Singer et al., 2014; Gallagher et al., 2015; Yan et al., 2018). Improved understanding of factors that regulate wound Mo/Mϕ numbers and phenotype have led to new therapeutic interventions attempting to normalize the Mϕ response in mice targeting the NLRP3 inflammasome/IL-1β and RAGE pathways; these findings await translation to humans (Mirza et al., 2013; Wang et al., 2017). Another approach for normalizing Mϕ phenotypes in non-healing wounds could be altering epigenetic modifications of genes associated with dysregulated Mϕ phenotype (Gallagher et al., 2015; Yan et al., 2018). However, more comprehensive knowledge on epigenetic changes that drive persistent inflammation in diabetic wound Mo/Mϕ will be useful to specifically target relevant genes.

Studies showing that diabetes-associated increased monopoiesis at steady-state contributes to increased Mϕ accumulation in diabetic wounds suggest the possibility that targeting monopoiesis may help normalizing wound Mϕ accumulation and improve diabetic wound healing (Barman et al., 2019b). However, much remains to be learned about the regulation of monopoiesis during wound healing.

Lastly, several studies have highlighted the importance of Mo subsets in health and disease (Wolf et al., 2019). Heterogenous Mo/Mϕ populations have also been identified in various tissues

such as lung (Mould et al., 2019), aorta (Cochain et al., 2018), and heart (Dick et al., 2019) under diseased conditions. However, our knowledge of the heterogeneity of skin wound Mϕ is lacking, especially during impaired healing. Multiplex analyses such as single cell RNA-sequencing, multiparameter flowcytometry, and imaging mass cytometry will be helpful to acquire knowledge on Mϕ subsets present during wound healing, and how these are regulated during normal and impaired wound healing which may, in turn, provide insight into new approaches for manipulating inflammation and improving healing.

AUTHOR CONTRIBUTIONS

PB and TK have made a substantial, direct and intellectual contribution to the work. Both authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Recent Advances in the Controlled Release of Growth Factors and Cytokines for Improving Cutaneous Wound Healing

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Bioengineered materials are widely utilized due to their biocompatibility and degradability, as well as their moisturizing and antibacterial properties. One field of their application in medicine is to treat wounds by promoting tissue regeneration and improving wound healing. In addition to creating a physical and chemical barrier against primary infection, the mechanical stability of the porous structure of biomaterials provides an extracellular matrix (ECM)-like niche for cells. Growth factors (GFs) and cytokines, which are secreted by the cells, are essential parts of the complex process of tissue regeneration and wound healing. There are several clinically approved GFs for topical administration and direct injections. However, the limited time of bioactivity at the wound site often requires repeated drug administration that increases cost and may cause adverse side effects. The tissue regeneration promoting factors incorporated into the materials have significantly enhanced wound healing in comparison to bolus drug treatment. Biomaterials protect the cargos from protease degradation and provide sustainable drug delivery for an extended period of time. This prolonged drug bioactivity lowered the dosage, eliminated the need for repeated administration, and decreased the potential of undesirable side effects. In the following mini-review, recent advances in the field of single and combinatorial delivery of GFs and cytokines for treating cutaneous wound healing will be discussed.

Keywords: growth factors, cytokines, tissue regeneration, wound healing, biomaterials, controlled release

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INTRODUCTION

Wound healing and tissue regeneration are complex cellular processes that involve an interplay of growth factors (GFs), chemokines, cytokines, and other signaling molecules (Castano et al., 2018; Rodrigues et al., 2018; Brazil et al., 2019). There have been numerous discoveries in the field that enlighten the mechanism of wound healing, and with these scientific advancements, different technological solutions are being suggested. However, a complete understanding of the tissue regeneration mechanisms following wound injury has yet to be deciphered. Worldwide prevalence of skin and subcutaneous diseases are among the most common diseases with just over 600 million people affected in 2015 (Vos et al., 2017). Wound care has a significant economic impact on the world healthcare system and in the United States alone, the cost is $50 billion per

year (Gainza et al., 2015b). Medical treatment often cannot completely re-epithelialize the injured site, leaving a scar (Coentro et al., 2018; Monavarian et al., 2019). The scar is a collection of fibrotic cells that in some cases, limit the normal functions of the organ and body (Rippa et al., 2019). Researchers have shown that specific GFs and cytokines play an essential role in cutaneous injury repair and the topical administration of some of them has already proven to significantly enhance wound closure. These GFs include, but are not limited to, epidermal GF (EGF), fibroblast GF (FGF), platelet-derived GF (PDGF), transforming GF-beta (TGF-β), and vascular endothelial GF (VEGF) protein families (Yamakawa and Hayashida, 2019). Growth factors involved in wound healing can be delivered to promote and expedite the process of tissue regeneration. However, the direct application of GFs alone was shown to be ineffective due to the hostile environment at the wound site, subsequent loss of bioactivity of the GFs, and poor skin penetration (Yamakawa and Hayashida, 2019). Additionally, a single injection cannot provide prolonged stimulation of wound healing due to the complexity of the process and repetitive administration can even lead to undesired side effects such as tumorigenesis (Park et al., 2017). Thus, various biomaterials have been used to deliver cytokines and GFs in a controlled fashion. To be effective, biomaterials should be biocompatible, biodegradable, and mimic the composition of the ECM (Mir et al., 2018; Saghazadeh et al., 2018; Suarato et al., 2018; Sultankulov et al., 2019b). They must also be able to bind GFs and cytokines, stabilize them, and provide sustainable release (Elviri et al., 2017; Dellacherie et al., 2019; Wang W. et al., 2019). Apart from that, it has been shown that biomaterials themselves can promote tissue regeneration due to antibacterial, moisturizing, and ECM mimicking properties. This mini-review summarizes recent advances in the development of biomaterials loaded with wound healing stimulating and modulating molecules.

HYDROGELS

One of the most commonly used biomaterials is hydrogel, a polymer network capable of absorbing water and serving as a scaffold for drug delivery, which has been widely used in wound healing (Li and Mooney, 2016; Dimatteo et al., 2018; Daly et al., 2019). Since the end of the 20th century, FGF family proteins have been attractive in drug development and one have been clinically approved for wound treatment (Hui et al., 2018a). Basic FGF (bFGF) incorporated into a bioinspired hydrogel demonstrated a sustained release of GF over time, increased fibroblast proliferation *in vitro,* as well as enhanced wound closure in the scratch assay, and promoted wound healing in the full-thickness skin incision murine wound model (Zhang et al., 2018). In addition, bFGF released from hydrogel promoted higher fibroblast and extracellular protein density. It is important to note that hydrogels were used as a drug delivery system to stabilize the embedded GFs from rapid protease degradation at ECM, and served as a supportive material to enhance wound healing. In the case of acidic FGF (aFGF)-Carbomer 940 hydrogel, higher stability of aFGF in comparison to bolus aFGF, which was used in a non-diabetic murine wound model, resulted in accelerated cell proliferation, neo-vascularization, and wound healing in type 2 diabetic rats (Hui et al., 2018b). In some studies, the hydrogel was incorporated with other drug delivery systems such as microspheres and liposomes (Xu et al., 2017, 2018; Shamloo et al., 2018). This double layer system promoted the sustained release of the incorporated bFGF for a longer period of time, resulting in enhanced wound closure, organized collagen deposition, vascularization, granulation, myofibroblast formation, and reduced inflammation. The proposed mechanism for increased neovascularization was through bFGF induced VEGF expression and better healing was also due to deeper permeation of bFGF in the wounded area (Xu et al., 2017, 2018). Another research group demonstrated that hyaluronan/collagen hydrogel containing heparin-binding EGF-like GF enhances wound healing in an organ culture model of the porcine skin (Thönes et al., 2019). Heparin also enabled sustained release of stromal cell-derived factor-1 (SDF-1) that accelerated wound healing and increased vascularization in rats (Yao et al., 2020). In order to maximize protection from degradation, Devalliere et al. (2017) constructed genetically fused proteins from elastin-like peptide (ELP) with keratinocyte GF (KGF) or ARA290 (Erythropoietin derivative), an apoptosis-protective molecule, loaded them into fibrin hydrogel and tested their combinatorial effect on skin wounds of diabetic mice (Devalliere et al., 2017). The results demonstrated improved wound healing by facilitating blood vessel formation, epithelialization, and granulation tissue formation. It was suggested that ELP promoted dermis regeneration and limited accessibility of the GFs for protease degradation and collectively ELP-KGF stimulated keratinocytes migration and proliferation, whereas ELP-ARA290 assisted in inhibiting tissue apoptosis.

Another approach is to use hydrogel in its liquid phase for better coverage of the injured site. One group used a heparin-poloxamer consisting hydrogel, which is liquid at 4°C and solid at body temperature. The time of the topical administration was just enough to cover the wounded area tightly prior to formation of the gel substance. Wu et al. (2016b) also used a thermo-sensitive hydrogel to show that the binding interaction between drug and scaffold is crucial by comparing the effectiveness of hydrogel loaded with aFGF or bFGF. In addition to overall improvement in wound healing, the authors demonstrated that the structural differences between GFs of the same family affected the efficiency of tissue regeneration. Even though both GFs were linked to the material via their heparin-binding domain, aFGF demonstrated to be more effective than bFGF. An interesting approach took advantage of the increased temperature at the inflammation site for controlled delivery of VEGF using a thermosensitive microneedle hydrogel. This study demonstrated enhanced wound healing, granulation, collagen deposition, angiogenesis, and reduced inflammation (Chi et al., 2020). Similarly, other groups invented a self-healing type of hydrogel that behaved as a liquid during syringe injection and solidified upon wound coverage (Chen G. et al., 2017; Chen et al., 2019). The self-healing properties of the hydrogel particles

were attributed to the imine and acylhydrazone bonds (Chen et al., 2019). The use of this type of hydrogel, with loaded bFGF, enhanced the formation of granulation tissue, increased angiogenesis, and inhibited inflammation and activity of the pro-inflammatory factors, TNFα and IL-6 (Chen G. et al., 2017). Other studies evaluated the effect of an injectible hydrogel in complex with silver and bFGF on infected wounds. The results demonstrated the advantages of a composite by observing improved wound closure, collagen deposition, vascularization, re-epithelialization, granulation, and keratinocytes migration. In addition, reduced inflammation was associated with the anti-inflammatory M2 but not pro-inflammatory M1 macrophages (Xuan et al., 2020).

The natural wound healing process never comes with a single molecule orchestrating the activity of the downstream effectors, but rather a complex interplay of all components at specific stages (Rousselle et al., 2018). A further improvement toward the simulation of the programmed molecular "game" of the living organism is the delivery of two or more active molecules. Several groups have used combinations of different GFs or GFs with active molecules (Yang et al., 2017; Park et al., 2018; Yoo et al., 2018). Yoo et al. (2018) studied the combination of EGF and bFGF incorporated into hydrogel. Increased rate of wound closure, decreased number of pro-inflammatory cells, enhanced re-epithelialization, and granulation tissue formation were associated with the dual effect of EGF and bFGF. It was suggested that EGF promoted fibroblast migration, while bFGF was responsible for enhanced collagen deposition, granulation, and re-epithelialization by secreting TGF-α and IL-1 (Yoo et al., 2018). Another group demonstrated enhanced wound healing by dual delivery of Substance-P and TGF-β1 loaded microparticles in the injectable hydrogel to the irradiated skin of mice. Specifically, nude mouse panniculus adiposus and carnosus layers were thicker and repaired faster in the dual delivery group compared to the control group. It's been proposed that the drug's effect was mediated by the migration of mesenchymal stem cells (Park et al., 2018). Thus, thermo-sensitive hydrogels alone, or in combination with microvesicles, have demonstrated the ability to enhance wound healing by protecting proteins from protease degradation and promoting gradual release of the drugs over an extended period of time. Moreover, studies showed that dual drug delivery was more effective than a single drug incorporated hydrogel. **Table 1** summarizes the effects of GFs and cytokines on wound healing.

NANOPARTICLES

Nanoparticles possess a wide range of applications, including usage in drug administration (Patra et al., 2018). A number of papers have been published that use drug loaded nanoparticles in treating wounds. For example, fibrin nanoparticles loaded with KGF enhanced wound closure and cell migration *in vivo* by binding KGF to its receptor located on the keratinocyte (Muhamed et al., 2019). Other groups showed the advantage of nanoparticles based delivery over GF/cytokine treatment alone. Application of the hyaluronic acid microparticles loaded with EGF to the wound area enabled a similar effect of bolus EGF administration with significantly lower concentration. Moreover, accelerated migration of inflammatory cells during the inflammation phase enhanced wound healing (Kang et al., 2017). KGF linked gold nanoparticles (GNP) exhibited enhanced wound healing and re-epithelialization properties in comparison to KGF administration alone. There was an acceleration in keratinocyte migration and proliferation, but no significant promotion in granulation layer formation, which contributes to extensive fibrosis (Pan et al., 2018). Furthermore, the same research group investigated the effect of KGF loaded GNPs on a diabetic mouse wound model and confirmed previous findings. In addition, they confirmed that KGF-GNP specifically binds to KGF receptor and even better than KGF alone, possesses a higher level of resistance to harsh environments, and increases levels of collagen I, α-SMA, and TGF-β1, which are associated with the wound healing process (Li et al., 2019).

Non-diabetic and diabetic murine skin wounds treated with VEGF164 loaded poly(lactic-co-glycolic acid) nanoparticles showed enhanced wound closure accompanied by increased re-epithelialization and granulation but not wound contraction. The suggested mechanism was via VEGFR2 activation which is known to be a mediator of VEGF for angiogenesis through further stimulation of p38/MARK pathway protein, kinase B for apoptosis inhibition and cell proliferation (Chereddy et al., 2015; Aday et al., 2017). Gainza et al. (2015a) used a porcine wound healing model and demonstrated that wound healing was enhanced by the application of EGF loaded lipid nanoparticles in terms of enhanced wound closure, collagen deposition, angiogenesis, and reduced inflammation and number of myofibroblasts. However, the study shows that bolus EGF treatment also resulted in enhanced wound healing and the authors explain the advantages of nanostructure linked GF by reduced concentration (Gainza et al., 2015a).

Another example of a drug delivery system with promising therapeutic applications are nanofibers. Nanofibrous coating is a porous dressing that better simulates an ECM structure (Gainza et al., 2015b; Chen S. et al., 2017). One group used granulocyte colony-stimulating factor (G-CSF) incorporated into chitosan nanoparticles and further mixed with poly(ε-caprolactone) (PCL) nanofibers. Moreover, to increase cell attachment, the scaffold was coated with collagen I. *In vivo* experiments demonstrated improved wound closure, scar reduction, fibroblasts maturation, as well as increased collagen density and a decreased amount of neutrophils (Tanha et al., 2017).

OTHER DRUG DELIVERY SYSTEMS

The last section is dedicated to other developed or advanced drug delivery systems for wound healing. Both chitosan and collagen mentioned above were also used in biofilm composition, which is another type of drug delivery system. For the mechanical stability of the structure, authors used grapheme oxide (GO) rather than nanofibers by considering its physicochemical properties

TABLE 1 | Effects of growth factors and cytokines on wound healing.

Material	Growth factor/cytokine	Effect	References
Hydrogel	bFGF	Increased fibroblast proliferation *in vitro,* enhanced wound closure in the scratch assay, and promoted wound healing *in vivo*	Zhang et al., 2018
	bFGF	Enhanced wound closure, organized collagen deposition, vascularization, granulation, myofibroblast formation, and reduced inflammation	Xu et al., 2017, 2018
	bFGF	Enhanced the formation of granulation tissue, increased angiogenesis, and inhibited inflammation and activity of the pro-inflammatory factors, TNFα and IL-6	Chen G. et al., 2017
	bFGF	Enhanced wound closure, collagen deposition, vascularization, re-epithelialization, granulation, keratinocytes migration, and reduced inflammation	Xuan et al., 2020
	aFGF	Accelerated cell proliferation, neo-vascularization, and wound healing	Hui et al., 2018b
	aFGF or bFGF	Improved wound healing	Wu et al., 2016b
	EGF and bFGF	Increased rate of wound closure, decreased number of pro-inflammatory cells, enhanced re-epithelialization, and granulation tissue formation	Yoo et al., 2018
	SDF-1	Accelerated wound healing and increased vascularization	Yao et al., 2020
	VEGF	Enhanced wound healing, granulation, collagen deposition, angiogenesis, and reduced inflammation	Chi et al., 2020
	EGF-like growth factor	Enhanced wound healing in an organ culture model of the porcine skin	Thönes et al., 2019
	ELP-KGF and ELP-ARA290	Improved wound healing by facilitating blood vessel formation, epithelialization, and granulation tissue formation	Devalliere et al., 2017
	Substance-P and TGF-β1	Enhanced wound healing, thicker panniculus adiposus and carnosus layers	Park et al., 2018
Nanoparticle	KGF	Enhanced wound closure and cell migration	Muhamed et al., 2019
	KGF	Enhanced wound healing and re-epithelialization, accelerated keratinocyte migration and proliferation	Pan et al., 2018
	KGF	Enhanced wound healing, bioactivity, and increased levels of collagen I, α-SMA, and TGF-β1	Li et al., 2019
	EGF	Accelerated migration of inflammatory cells during the inflammation phase, enhanced wound healing	Kang et al., 2017
	EGF	Enhanced wound closure, collagen deposition, angiogenesis, and reduced inflammation and number of myofibroblasts	Gainza et al., 2015a
	VEGF164	Enhanced wound closure accompanied by increased re-epithelialization and granulation but not wound contraction	Chereddy et al., 2015
Nanofiber	G-CSF	Improved wound closure, scar reduction, fibroblast maturation, as well as increased collagen density and decreased amount of neutrophils	Tanha et al., 2017
Biofilm	bFGF	Enhanced healing, reduced pro-inflammatory cell accumulation, promoted granulation layer formation	Liu et al., 2017
	EGF	Accelerated wound healing via stimulating keratinocyte differentiation and reducing inflammation	Kim et al., 2016
	VEGF and PDGF-BB	Enhanced angiogenesis, granulation, and keratinocyte proliferation	Almquist et al., 2015
Coacervate	bFGF	Enhanced wound closure, angiogenesis, collagen deposition, granulation, cell proliferation in the wound area, re-epithelialization, and hair follicle formation	Wu et al., 2016a
	IL-10 and TGF-β3	Accelerated wound closure, enhanced re-epithelialization, angiogenesis, collagen I distribution, and reduced hypertrophic scar formation	Park et al., 2019
Fibrin matrix	VEGF-A165 and PDGF-BB	Enhanced vascularization, suppressed migration of the neutrophils to the wounded area, and attracted $Ly6C^{+}CD11b^{+}$ monocytes	Ishihara et al., 2018
Collagen matrix	EGF or bFGF	Accelerated wound healing, re-epithelialization, neovascularization, and collagen deposition	Choi et al., 2018
Lyotropic liquid crystal	EGF	Reduced inflammation, increased wound closure and re-epithelialization	Zhou et al., 2019
	VEGF	Enhanced vascularization	Wang B. et al., 2019
Cryogel	IL-10, TGF-β1, VEGF and bFGF	Improved the regenerative process on a murine internal splint wound model, including neovascularization, wound closure, granulation, and re-epithelialization	Jimi et al. (2020)

and an increasing interest for wide applications in biomedicine. A modified collagen-chitosan biofilm, which was loaded with bFGF, showed enhanced healing in full-thickness wounds in Sprague-Dawley rats by reducing pro-inflammatory cell accumulation and promoting granulation layer formation (Liu et al., 2017). Deeper penetration was also responsible for wound acceleration in hyaluronate (HA) conjugated EGF film administered on a rat wound model. Mechanistically, HA elevated β-defensin 2 activity stimulated keratinocytes differentiation, whereas the increased levels of TGF-β contributed to cellular proliferation, and diminished levels of TNF-α and IL-1 were associated with reduced inflammation (Kim et al., 2016). The concentration dependent effect was observed using VEGF with PDGF-BB embedded multilayer film that enhanced angiogenesis, granulation (combinatorial effect), and keratinocyte proliferation (Almquist et al., 2015).

One example is self-assembled hydrophobic vehicle particles composed of different materials collectively named as coacervates. Heparin composed coacervate, which was loaded with bFGF, enhanced wound closure, angiogenesis, collagen deposition, granulation, cell proliferation in the wound area, re-epithelialization, and hair follicle formation. These results were associated with increased levels of TGF-β1, CD31, and α-SMA. Additionally, VEGF activity was also promoted by bFGF in the first 7 days post-skin injury consistent with other reports (Wu et al., 2016a). Another group used heparin for dual delivery of GFs utilizing nanofibers coated with coacervate made of poly (ethylene argininyl aspartate diglyceride) and heparin. IL-10 and TGF-β3 bind to heparin via its negatively charged groups (Park et al., 2019). The combinatorial delivery of IL-10 with TGF-β3 on a rat skin wound model showed accelerated wound closure, enhanced re-epithelialization, angiogenesis, collagen I distribution, and reduced hypertrophic scar formation. TGF-β can mediate its effect through activation of Smad proteins (Landén et al., 2016).

Ishihara and colleagues demonstrated that GFs can also specifically bind to laminin, an ECM component, and then promote wound healing. VEGF-A165 and PDGF-BB selectively bind to the heparin-binding domain of the laminin embedded into the fibrin matrix. The complex further enhanced vascularization, suppressed migration of the neutrophils to the wounded area, and attracted Ly6C + CD11b + monocytes (Ishihara et al., 2018). Another distinctive approach was achieved by Choi and colleagues via the addition of disulfide bonds to EGF and bFGF. Moreover, the authors separately incorporated these GFs into a collagen based matrix and demonstrated accelerated wound healing, re-epithelialization, neovascularization, and collagen deposition in type I and type II diabetic mice wound models (Choi et al., 2018).

Lyotropic liquid crystals (LLC) possess gelation properties as injectable hydrogels and form a solid gel-like substance upon exposure to liquid at the wounded area. Several studies observed reduced inflammation, increased wound closure and re-epithelialization with EGF loaded LLC, and well developed blood vessels with VEGF loaded LLC using the skin wound excisional mice models in both cases (Wang B. et al., 2019; Zhou et al., 2019).

Based on our previously published studies on successful simultaneous incorporation of perivascular stem cell secreting three GFs, VEGF, MCP-1, and IL-6, into heparin coacervate (Mansurov et al., 2017), and the use of a composite cryogel for GF delivery (Sultankulov et al., 2019a), our group tested the sequential topical application of cryogel that was loaded with four GFs/cytokines. The sequential targeted delivery of cryogel released IL-10 and TGF-β1 that was followed by the delivery of VEGF and bFGF significantly improved the regenerative process on a murine internal splint wound model, including neovascularization, wound closure, granulation, and re-epithelialization (Jimi et al., 2020).

CONCLUSION

A number of studies demonstrated that clinically approved GFs affect the proliferation stage of wound healing by regulating tissue regenerating and immunomodulatory signaling pathways. However, the low stability and hence short time of the protein's bioactivity at the site of wound injury limits the effectiveness of bolus administration. Development of biocompatible materials has significantly enhanced tissue regeneration and wound healing. Biomaterials composed of a hydrogel, nanoparticles, nanofibers, cryogel, and a combination of other molecules served as a drug delivery system by protecting GFs/cytokines from protease degradation and promoting sustained release over an extended period of time. The major challenges for drug loaded biomaterials are mechanical strength and porosity for simulation of the ECM, ability to gradually release the linked cargos and biodegradability. In addition, some research groups demonstrated that the combinatorial effect of the simultaneous delivery of two or more GFs/cytokines promotes tissue regeneration even further, thus revealing the advantage of a combinatorial effect in contrast to single drug administration. Overall, the rapidly growing field of drug delivery systems for wound healing has an important value for their therapeutic application in medicine.

AUTHOR CONTRIBUTIONS

AS contributed to the conception of the study and edited the manuscript. AN, AJ, and SJ wrote the sections of the manuscript. All authors read and approved the submitted version.

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Highly Water-Preserving Zwitterionic Betaine-Incorporated Collagen Sponges With Anti-oxidation and Anti-inflammation for Wound Regeneration

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A core problem in wound healing – with both fundamental and technological significance – concerns the rational design of bioactive and moist microenvironments. Here, we design a new class of zwitterionic betaine-incorporated collagen sponges (BET@COL) with integrated anti-oxidation and anti-inflammatory properties for promoting wound healing in a full-thickness wound model. The presence of zwitterionic betaine in a 3D network structure of collagen enables tightly bound and locked water molecules inside sponges via ionic solvation and confinement effect, while the integration of this amino acid also empowers the sponge with anti-oxidation and anti-inflammatory functions. *In vitro* results demonstrated that BET@COL collagen sponges strongly preserved water content up to 33.78 ± 0.78% at the 80th min at 37°C (only 0.44 ± 0.18% in control), and also exhibited high cell biocompatibility. Further, BET@COL collagen sponges with different betaine contents were applied to a full-thickness cutaneous wound model in mice, followed by a systematical evaluation and comparison of the effect of preserved water on wound healing efficiency *in vivo*. The optimal BET@COL collagen sponges were able to maintain high water content (e.g., moist microenvironment), suppress oxidative stress, improve anti-inflammation, all of which impose synergetic healing effects to promote wound closure, granulation formation, re-epithelization, collagen deposition and angiogenesis. This work demonstrates a new material as a promising candidate for wound dressing.

Keywords: wound dressing, zwitterionic betaine, collagen, oxidative stress, water preserving

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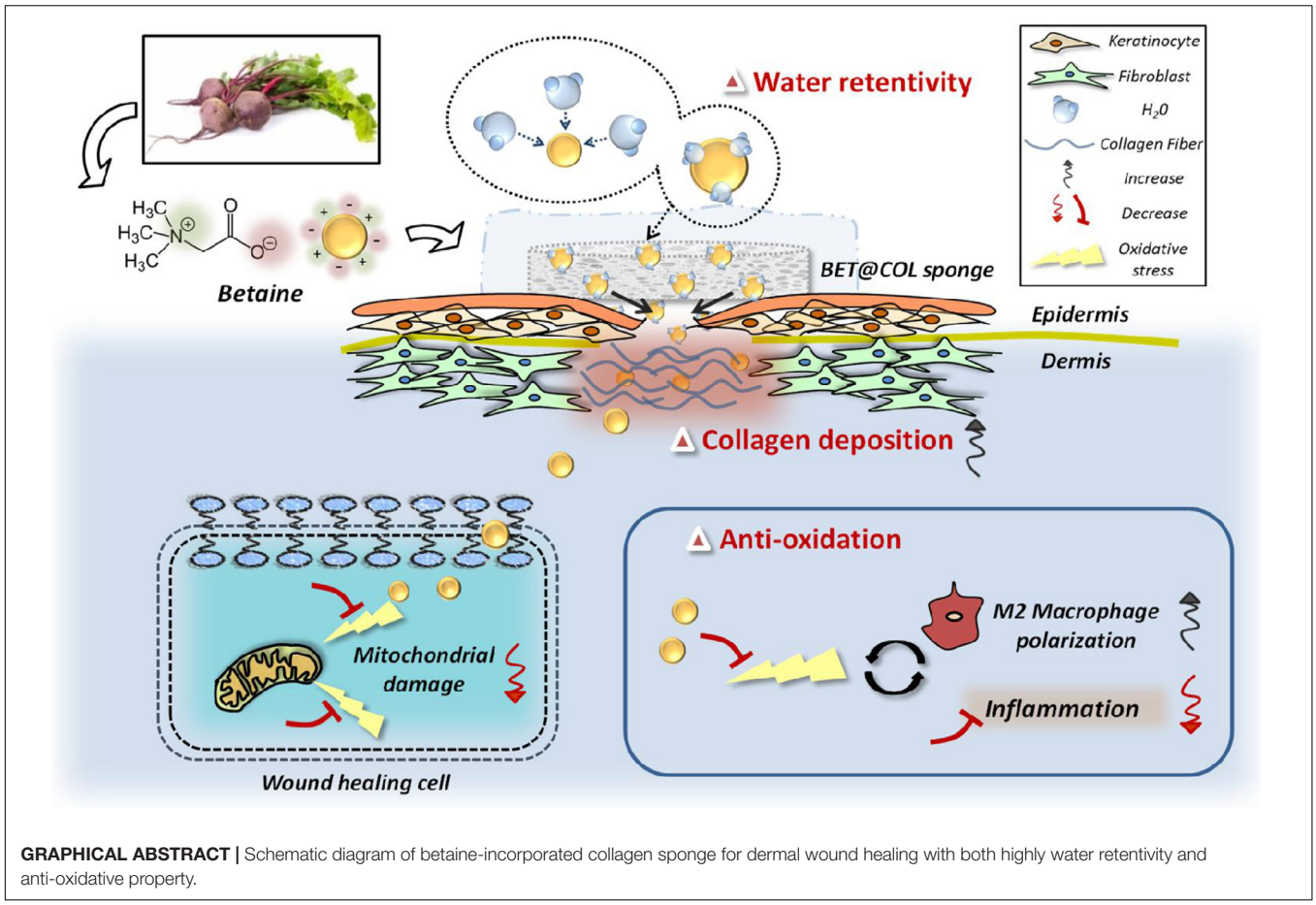

GRAPHICAL ABSTRACT | Schematic diagram of betaine-incorporated collagen sponge for dermal wound healing with both highly water retentivity and anti-oxidative property.

INTRODUCTION

Dermal wound healing is a sophisticated process mainly involving several largely overlapping phases including inflammation, proliferation and remodeling. It is generally accepted that water plays a very important role in maintaining the biofunctions of 90% human soft tissues (not limited to human skin) (Warner et al., 1988; Ousey et al., 2016). Specifically, while human skin contains approximately 30% of water, water content can go up to 70% in the viable epidermis and drop to 15–30% at a junction between the stratum granulosum (SG) and the stratum corneum (SC). Thus, water is not simply and uniformly distributed in human skin, and this water distribution becomes even more complex and dynamic around wound tissues. When a cutaneous wound has just happened, the skin rapidly responds to this external damage by introducing growth factors and proteinases to mediate the healing process, which begin to gather and accumulate fluid exudates (Atiyeh et al., 2002). Afterward, as the healing proceeds, a provisional extracellular matrix (ECM) starts to form as the primary component of wound microenvironment, followed by a long period of remodeling to complete the whole healing process (Warner et al., 1988; Ousey et al., 2016). A moist wound microenvironment was previously demonstrated to facilitate the process of re-epithelialization by benefiting keratinocyte migration through preventing scab formation (Winter, 1962; Hinman and Maibach, 1963). In addition, it provides wounds with more lasting contact with uninfected wound fluid, which favors the proliferation of healing-associated cells (Katz et al., 1991). Additionally, super-hydrated (e.g., only saline solution) environment could accelerate the wound healing process especially at the inflammatory and proliferative phases as compared to dry wounds (Dyson et al., 1988). Conversely, a dry healing environment caused by simple exposure of the wound to the air or by traditional gauze dressing coverage was reported to dehydrate the wound site, cause adhesion and retard the healing process (Cho and Lo, 1998). Therefore, new types of moisture-retentive materials have been developed to create an ideal moist healing environment to efficiently promote wound healing, such as hydrogels (Fonder et al., 2007). Numerous studies have been performed with special focus on the development of water-retentive hydrogels (e.g., gelatin-based hydrogels) for wound dressings (Zhao et al., 2016, 2017; Sun et al., 2017).

However, it is still a challenge to generate water transportation and preservation from simple hydrogels. While hydration promotes wound healing via re-epithelialization, there is still a of clear definition to determine the "optimal" or "balanced" moist environment for wounds, due to the complex nature of skin (Elias and Wakefield, 2011). Therefore, development of novel long-term water preserving dressing materials to efficiently cope with

wound exudates and the moist environment, would be a desirable approach for dermal wound application. It was also found that oxidative damage might be one of the most detrimental consequences of dehydration, e.g., oxidation level in yeast cells increased for more than 10-fold after dehydration, indicating that water depletion generates more oxidative stress (Pereira Ede et al., 2003; Franca et al., 2007). These findings suggest a close relationship between water preservation and cellular anti-oxidation during wound repair. Additionally, it has been found that in the wound fluid of mice, along with the inflammation period, oxidative stress occurs with the dramatically increasing production of hydrogen peroxide [H_2O_2, a member of the reactive oxygen species (ROS) family] (Roy et al., 2006). It should also be noticed that at the inflammation phase of wound repair, an imbalance between ROS and its detoxification system would occur, leading to a disproportionately high level of oxidative stress in the wound site (Hameedaldeen et al., 2014). This oxidative stress has mutual promoted effects with the inflammatory reaction and plays a critical role in the wound healing process (Lan et al., 2013). In the inflammatory phase, the "respiratory burst" caused by neutrophil infiltration leads to the production of free radicals, which results in mitochondrial and DNA damage, lipid peroxidation, the inactivation of free radical scavenger enzymes and cell apoptosis or necrosis (Babior, 1978; Lennon et al., 1991; Wiseman and Halliwell, 1996; Cadenas and Davies, 2000). Thus, attenuating oxidative stress response turns out to be an effective strategy for the treatment of acute and chronic wounds, and the development of drugs with anti-oxidative stress function, topically applied to dermal wounds, show a promising future (Cao et al., 2018; Zhu et al., 2018). For all these reasons, multifunctional wound dressings have been under intensive investigations. In recent years, a variety of synthetic material-based wound dressings were fabricated with salutary effects including adhesive, antibacterial, and antioxidant properties for improving healing outcome (Li et al., 2019; Liang et al., 2019; He et al., 2020).

Considering both water preserving and anti-oxidative stress properties, the naturally originated compound, zwitterionic betaine, was considered for its special properties. As a small N-trimethylated amino acid, betaine widely exists in plants, animals and microorganisms (Craig, 2004). It is extensively recognized as an organic osmolyte, and in plants serves as a protector against drought, high salinity and osmotic stress (Yancey et al., 1982). Betaine is particularly characterized by its lasting moisture-retentive ability and is widely used in skin care products due to its chemical structure featuring a hydrophilic head which attracts water molecules (Wattanaploy et al., 2017). Moreover, previous studies suggested that betaine helped improve the hydration state of the epithelium (Nicander et al., 2003). It is noteworthy that, except for its water preserving function, in mammals, the anti-oxidative effect of betaine has been revealed by numbers of studies. Systematic administration of betaine was reported to decrease lipid peroxide levels and increase glutathione (GSH) levels, which indicated its anti-oxidative ability (Balkan et al., 2004). Betaine supplementation also alleviated liver fibrosis by inhibiting both oxidant and inflammatory processes (Tsai et al., 2015; Bingul et al., 2016). A wide range of evidence has shown that systematic administration of betaine restrained the oxidative stress of the target organ, while its local application on dermal skin has seldom been studied.

Herein, the anti-oxidation effects and mitochondria protection of betaine against oxidative damage on NIH 3T3 fibroblasts were first investigated. Then, a varied amount of betaine was loaded into collagen sponges (initially obtained from rat tail tendon) to form BET@COL dressing. With the addition of betaine, there was a significant increase in the water-preserving properties of collagen sponges. Then this series of BET@COL with varied betaine loadings was applied to the full-thickness wound model of C57BL/6 mice. Results showed that, the BET@COL (4 mg) group exhibited the best wound enclosure rate, granulation formation and collagen deposition. Therefore, this BET@COL dressing with satisfactory therapeutic effects on dermal wounds regeneration as a hydrogel substitute showed great potential in future practical clinical use for acute and chronic wound care.

MATERIALS AND METHODS

Materials and Reagents

Dulbecco's modified Eagle's medium (DMEM), phosphate buffer saline (PBS), penicillin and streptomycin were purchased from Gibco BRL, Invitrogen Corp., (Carlsbad, CA, United States). Fetal bovine serum (FBS) was obtained from Hyclone, Thermo Scientific (United States). Cell counting kit-8 (CCK8) reagent, bovine serum albumin (BSA), DAPI, hematoxylin and eosin dyes, RIPA lysis buffer (P0013B) and Phenylmethanesulfonyl fluoride (PMSF, ST506) were purchased from Beyotime® Biotechnology (China). Betaine and hydrogen peroxide (H_2O_2) were obtained from Sigma-Aldrich LLC (United States). Triton X-100 and Masson's trichrome staining kit were purchased from Solarbio Science & Technology Co., Ltd (China). Primary rabbit monoclonal to heme oxygenase-1 (HO-1, ab68477), rabbit polyclonal antibody to cytokeratin (ab9377), mouse polyclonal antibody to CD68 (ab955), rabbit polyclonal antibody to CD163 (ab182422), donkey anti-rabbit IgG Alexa Fluor® 647-conjugated secondary antibody (ab150075), donkey anti-rabbit IgG Alexa Fluor® 488-conjugated secondary antibody (ab150073) and donkey anti-mouse IgG Alexa Fluor® 647-conjugated secondary antibody (ab150111) were obtained from Abcam (United Kingdom). Goat anti-rabbit (H + L) HRP secondary antibody was obtained from Bioworld Technology (United States). FITC labeled Phalloidin (40735ES75) and JC-1 mitochondrial membrane potential assay kit (40706ES60) were obtained from Yeasen Biotech. Co., Limited (China). Goat anti-rabbit horseradish peroxidase-conjugated secondary antibody was purchased from Pierce Biotechnology (United States). Bicinchoninic acid (BCA) reagent was obtained from Thermo (United States). Polyvinylidene fluoride (PVDF) membrane and fat free milk were purchased from Bio-Rad (United States). Tween 20 was obtained from Aladdin Chemistry Co., Ltd (China).

Cell Lines and Cell Cultures

A mouse fibroblast cell line NIH 3T3 was purchased from American Type Culture Collection and cultivated in DMEM containing 10% FBS, 100 unit/mL penicillin and 100 μg/mL streptomycin in a controlled incubator at 37°C with an atmosphere of 5% CO_2.

Cell Viability Assays

To measure the effects of betaine on cell viability, NIH 3T3 fibroblast cells were seeded at 8.0 × 10 (Atiyeh et al., 2002)cells per well in 96-well plates and cultured in serum containing DMEM mentioned above for 24 h. Then the origin medium was replaced by fresh medium (100 μL/well) with the addition of betaine at various concentration of 0, 2, 5, 10, 50, and 100 mM, respectively. After 12 h treatment and wash with PBS, fresh medium (100 μL/well) containing 10% CCK8 reagent was added to each well for another incubation of 2 h at 37°C. The cell viability was determined by measuring the absorbance at 450 nm using a microplate reader (Molecular Devices, SoftMax® Pro 5, United States).

To measure the protective effects of betaine on cells against oxidative damage, NIH 3T3 fibroblasts cells were seeded and cultivated for 24 h as described above. Then the origin medium was replaced by fresh medium (100 μL/well) with the addition of betaine at the concentration of 0, 2, and 5 mM, respectively. After betaine pretreatment at 37°C for 5 h, the medium was then replaced with fresh medium (100 μL/well) with the addition of 50 μM H_2O_2 for another incubation of 4 h at 37°C. At the end of incubation, each well was washed with PBS and fresh medium (100 μL/well) containing 10% CCK8 reagent was added for another incubation of 2 h at 37°C. Finally, the absorbance was determined at 450 nm.

Cell Immunofluorescence

Thr NIH 3T3 Fibroblast cells were seeded at 2.0 × 10 (Ousey et al., 2016)cells per well in 6-well plates and cultured for 24 h followed by the replacement of medium by fresh medium (4 mL/well) with 0 and 2 mM betaine, respectively. After betaine pretreatment at 37°C for 5 h, the medium was replaced with fresh medium (4 mL/well) with 50 μM H_2O_2 for another incubation of 4 h at 37°C. The medium was then carefully removed and washed 3 times with PBS. Cells were fixed with 4% paraformaldehyde for 30 min at 4°C, and incubated in 5% BSA containing 0.1% Triton X-100 for 40 min at 37°C. After that, cells were incubated with a rabbit monoclonal antibody to HO-1 (1:200) diluted in PBS containing 1% BSA overnight at 4°C. Cells were then thoroughly washed with PBS and incubated with a donkey anti-rabbit IgG Alexa Fluor® 647-conjugated secondary antibody (1:1000) and FITC labeled Phalloidin (1:100) diluted in PBS at 37°C for 1 h in the dark. Finally, cells were stained with DAPI to visualize the nuclei. Fluorescence images were captured using a Nikon confocal laser microscope (Nikon, A1 PLUS, Tokyo, Japan). Image-Pro Plus 6.0 software was used to count positive fluorescent samples in each fluorescent image, followed by further statistical analysis.

Western Blotting

The total cell lysates of NIH 3T3 fibroblasts after different treatments were collected with RIPA lysis buffer (with 1% PMSF) for 15 min on ice. After centrifugation (12,000 × *g*, 10 min, 4°C), the supernatant was collected and the protein concentration of which was then quantified using BCA reagents. After that, equal amounts of protein samples from each group were separated through a Bis-Tris polyacrylamide gel (12%) under 80 V and transferred to a PVDF blotting membrane, which was then blocked with 5% skimmed milk in TBST (10 mM Tris–HCl, 100 mM NaCl and 0.1% Tween 20) for 1.5 h at room temperature on a rotary shaker and then incubated with a rabbit monoclonal antibody to HO-1 (1:1000) diluted in TBST overnight at 4°C. The membrane was washed with TBST three times and incubated with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:8000) for 2 h at room temperature on a rotary shaker. The protein bands on the PVDF membrane were visualized using ChemiDicTM XRS + Imaging System (Bio-Rad), and the signal intensities of which were quantified using ImageJ software. Band intensities were normalized to GAPDH.

Mitochondrial Membrane Potential Examination

The JC-1 mitochondrial membrane potential assay kit was used to test mitochondrial membrane potential △Ψm. Briefly, NIH 3T3 Fibroblast cells were pretreated with 2 mM betaine for 3 h, followed by a 100 μM H_2O_2 stimulation for 1 h. 20 min before the termination of stimulation, the cells of positive control group were incubated with 2 mL medium containing 2 μL carbonyl cyanide 3-chlorophenylhydrazone (CCCP, 10 mM) which induces cell apoptosis. Then cells were washed with PBS 3 times and cell suspensions were obtained after trypsinization. 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) solution from JC-1 mitochondrial membrane potential assay kit was then used to treat cells for 20 min at 37°C. Cells were then washed with JC-1 staining buffer solution for three times. After that, cells were transferred to a black 96-well plate (100 μL per well) and fluorescence of the mitochondria monomers (excitation wavelength: 485 nm; emission wavelength: 535 nm) and aggregates (excitation wavelength: 550 nm; emission wavelength: 600 nm) were determined using a microplate reader (Molecular Devices, SoftMax® Pro 5, United States). The △Ψm status of mitochondria was presented by the ratio of red to green fluorescence. For mitochondria observation, cells after treatment were incubated with JC-1 solution for 20 min at 37°C, washed with JC-1 staining buffer solution for three times, fixed with 4% paraformaldehyde for 30 min at 4°C and finally stained with DAPI to visualize the nuclei. Fluorescence images were captured using a Nikon confocal laser microscope (Nikon, A1 PLUS, Tokyo, Japan).

Collagen Sponge Preparation

The type I collagen was extracted from the tails of SD rats provided by the Laboratory Animals Center of Wenzhou Medical University. First, rat tails were rinsed and sterilized with 75%

ethanol. Then the tendons in the tails were carefully extracted, cut into small pieces and placed at 4°C for 48 h. After tendons became dry, they were dissolved in acetic acid (0.1 M) for 5 days. After that, the suspension was centrifuged and put in tissue culture dishes with its pH adjusted to 7.0. Then it was frozen at −80°C and freeze-dried to form collagen sponges. The collagen sponges were punched into round pieces with a diameter of 7 mm and then disinfected with ultraviolet radiation for *in vivo* application.

The inner spongy structure of collagen sponges was observed using a scanning electron microscopy (SEM, VEGA3 TESCAN). Firstly, the collagen sponges were dried in liquid nitrogen. Secondly, they were gold sputtered for 60 s under high-vacuum conditions by a Desk II gold sputter coater (Denton Vacuum, Morristown, NJ, United states). The inner structure was finally observed and captured.

Water Preservation Examination

40 μL 0.9% saline or 0.2 g/mL betaine solution was added to round collagen sponges 7 mm in diameter. After absorption, each piece of collagen sponge was immediately weighed (recorded as W_0) and placed in an incubator chamber at 37°C. At each determined time point (the Nth minute), each collagen sponge containing a different solution was weighed (recorded as W_N) and recorded for further analysis of water preservation. The weight of non-evaporable solute (betaine or sodium chloride) was recorded as W_S. Water preservation was calculated as following:

$$\text{Water Preservation (\%)} = (W_N\text{-}W_S)/(W_0\text{-}W_S) \times 100\%$$

In vitro Betaine Release Profile

First, BET@COL with different drug loads (2, 4, and 8 mg) were immersed in 400 μL saline, respectively. At each determined time point (0.5, 1, 2, 3, 4, 5, and 6 days) the saline of each group was collected for further concentration detection and fresh saline was added. All the samples were stored at 4°C before detection. Then, we prepared betaine solutions at gradient concentrations of 0.3, 0.6, 1.2, 1.5, 1.8, 2.4, 3.0, 4.2, and 5.4 mg/mL as standards. The betaine amount was detected by high performance liquid chromatography (HPLC) using an HPLC System (Agilent 1100, United States) with the mobile phase containing CH_3CN (solvent A) and H_2O (solvent B) (85:15, v/v) at a flow rate of 0.7 mL/min at 30°C on a Merck Purospher@ STAR RP-18 endcapped (5 μm) Hibar@ RT 250-4.6 HPLC column (Darmstadt, Germany). The wavelength of the evaluation was 195 nm. Three independent samples were tested in each group (n = 3). After the detection of standards, a calibration curve was drawn, by which the betaine amounts of the samples were calculated.

In vivo Wound Healing Study

In this study, we used male C57BL/6 mice (8–10 weeks, obtained from the Laboratory Animals Center of Wenzhou Medical University). All experiments were performed in accordance with international ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals. Mice were individually anesthetized via intraperitoneal injection with 4% chloral hydrate and the dorsal skin was shaved and sterilized with ethanol. After stitching silicone rings with an internal diameter of 8 mm and thickness of 0.5 mm on the dorsal skin to prevent skin contraction, two round full-thickness wounds with a diameter of 6 mm were created by a biopsy punch (Acuderm® Inc., Fort Lauderdale, FL, United States) per mouse. Mice were randomly divided into five groups: group 1 was applied with 0.9% saline (the control group), group 2 with free betaine solution (4 mg), group 3–5 with BET@COL with different drug loads (2, 4, and 8 mg), similar to the dose-administration used in the previous report (Mahibalan et al., 2016). After application, wounds were covered with a sheet of 3M Tegaderm Film (3M Health Care, Germany) and medical bandages. Dressings were changed per week. We photographed the wounds on day 7, 10, 14, 17, and 20 post surgery and measured the wounds areas using Image-Pro plus. On day 7 and 20, mice were sacrificed after anesthesia and the full-thickness wound tissues were harvested, fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at a thickness of 5 μm using a microtome (LEICA RM2235, Germany) for further investigation.

Histopathological Examination

Hematoxylin and eosin (H&E) staining and Masson's trichrome staining were performed here. First, samples were dewaxed in xylene for 30 min and rehydrated using gradient ethanol. Second, for H&E staining, samples were submerged for 5 min in hematoxylin, 3 min in PBS and then stained with eosin for 2 min. For Masson's trichrome staining, samples were stained with A1:A2 (1:1) for cell nuclei visualization for 5 min and submerged for 3 s in acid alcohol for differentiation, 5 min in ponceau acid fuchsin solution to stain fibrous tissue, 1 min in 2% acetic acid solution, 30 s in phosphomolybdic acid solution for differentiation, 20 s in aniline blue and 5 min in distilled water. Finally, after dehydration of gradient ethanol and 15 min in xylene, slides were mounted with neutral resin. The stained sections were photographed using a Nikon microscope (Nikon, Tokyo, Japan).

Immunohistochemistry and Immunofluorescence

For immunohistochemical staining, after deparaffinization and rehydration, the sections were incubated in 3% H_2O_2 for inactivation of the endogenous peroxidase for 15 min and in 5% BSA for blockage of the non-specific binding sites at 37°C for 30 min. For immunohistochemical staining, the sections were incubated with a rabbit polyclonal antibody to cytokeratin (1:300) diluted in PBS containing 1% BSA at 4°C overnight. After being thoroughly washed with PBS, the sections were incubated with a goat anti-rabbit (H + L) HRP secondary antibody (1:1000) diluted in PBS at 37°C for 60 min, then counterstained with hematoxylin, mounted with neutral resin and finally photographed using a Nikon microscope (Nikon, Tokyo, Japan). For immunofluorescence staining, after deparaffinization and rehydration, the sections were incubated in 5% BSA for blockage of the non-specific binding sites at 37°C for 30 min, followed by incubation with a rabbit monoclonal antibody to HO-1 (1:200), a mouse polyclonal antibody to CD68 (1:200) and a rabbit polyclonal antibody to CD163 (1:200) diluted in PBS containing 1% BSA at 4°C overnight. After being thoroughly

washed with PBS, the sections were incubated with a donkey anti-rabbit IgG Alexa Fluor® 488-conjugated secondary antibody (1:1000), a donkey anti-mouse IgG Alexa Fluor® 647-conjugated secondary antibody (1:1000), and a donkey anti-rabbit IgG Alexa Fluor® 647-conjugated secondary antibody (1:1000) diluted in PBS at 37°C for 60 min in the dark, according to the primary antibodies. The sections were then stained with DAPI, mounted with antifade mounting medium and photographed using a Nikon confocal laser microscope (Nikon, A1 PLUS, Tokyo, Japan). Image-Pro Plus 6.0 software (Nikon, Tokyo, Japan) was used to count positive fluorescent samples in each fluorescent image, followed by further statistical analysis.

Statistical Analysis

All data were expressed as mean ± standard error (SE). Statistical differences were performed using one-way analysis of variance (ANOVA) followed by Tukey's test with GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, United States). For all tests, $^{*}p$-value < 0.05, $^{**}p$-value < 0.01, $^{***}p$-value < 0.001.

RESULTS AND DISCUSSION

In vitro Effects of Betaine on NIH 3T3 Fibroblasts

To affirm the cyto-compatibility of betaine on fibroblasts, one of the main cell types of the skin which also plays an indispensable part during the wound process (Takeo et al., 2015), NIH 3T3 fibroblasts were treated with betaine at various concentrations of 0, 2, 5, 10, 50, and 100 mM. The consequent graph of cell viability measured by CCK8 assay shown in **Figure 1A** suggested that betaine exerted no toxic effect to NIH 3T3 fibroblasts. At the relatively low concentrations, betaine exhibited slight

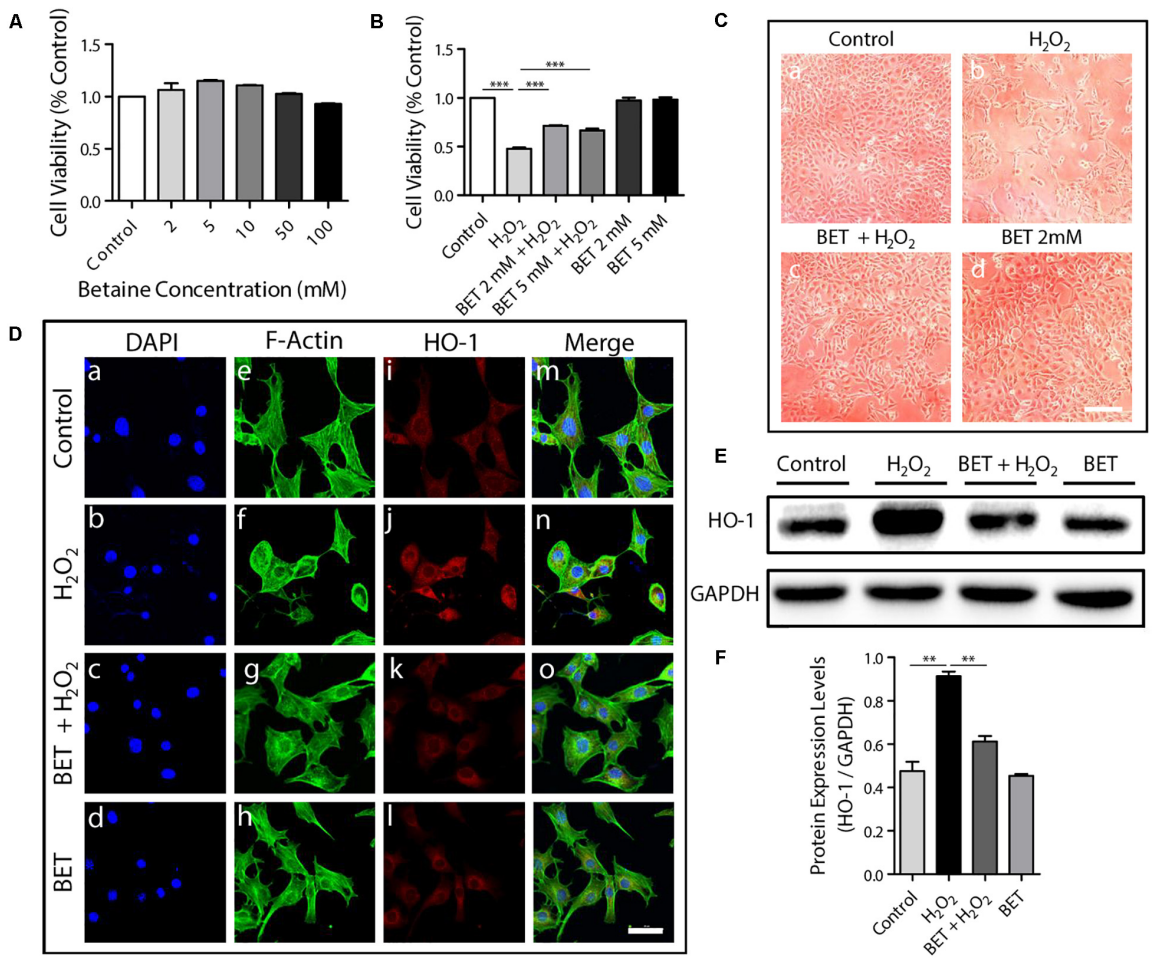

FIGURE 1 | The cyto-compatibility and anti-oxidative stress property of betaine *in vitro*. **(A)** NIH 3T3 cell viability after 12 h' betaine treatment of different concentration, $n = 6$. **(B)** NIH 3T3 cell viability after 4 h' H_2O_2 (50 μM) exposure with or without pretreatment of 5 h' betaine (2 mM and 5 mM), $n = 6$. **(C)** NIH 3T3 cell morphology observed by optical telescope: **(a)** control **(b)** after 4 h' H_2O_2 (50 μM) exposure **(c)** after 4 h' H_2O_2 (50 μM) exposure with pretreatment of 5 h' betaine (2 mM). Scale bar: 200 μm. **(d)** after 5 h' betaine treatment. **(D)** HO-1 immunofluorescence staining of NIH 3T3 cells after 4 h' H_2O_2 (50 μM) exposure with or without pretreatment of 5 h' betaine (2 mM). The figures share the same scale bar of 50 μm. **(E,F)** HO-1 expression analysis by Western blot, $n = 3$. NIH 3T3 cells were treated as described in **(D)**. BET: betaine. Statistical differences were performed using ANOVA. $^{***}p < 0.001$, $^{**}p < 0.01$.

effects of promoting cell proliferation. To mimic the oxidative stress damage which occurs at the wound site, we conducted H_2O_2-induced oxidative damage assays on NIH 3T3 fibroblasts *in vitro*. For exploring the protective effects of betaine against oxidative stress, NIH 3T3 fibroblasts were pretreated with 0, 2, and 5 mM betaine in medium for 5 h, followed by the addition of 50 mM H_2O_2 for 4 h. We should note that cell biocompatibility was tested under normal physiological condition, while cell protection tests against oxidative stress were performed by using H_2O_2 to treat cells. Additionally, upon H_2O_2 stimulation, even the low-dose betaine groups (2 mM and 5 mM) both showed significant protective effects, indicating the excellent cell protective effect under oxidative stress of betaine. While the 5 mM group showed the slightly lower cell viability than the 2 mM group, no statistical significance was observed between the two groups ($p = 0.0611$). As seen in **Figure 1B**, H_2O_2 stimulation caused an obvious cell death with only 47.7 ± 2.6% living cells compared with the untreated cell, which was significantly rescued by the pretreatment of 2 mM betaine (71.3 ± 1.2%) and 5 mM betaine (66.6% ± 4.0%), respectively. The photographs in **Figure 1C** visually displayed the cell morphology and density variation under different treatment, which was in accordance with the result in **Figure 1B**, further indicating that betaine served as an effective protector of cells resisting oxidative stress *in vitro*.

Heme oxygenase-1 (HO-1) expression was reported to be induced by stimuli including oxidative stress and thus was considered as the indicator of oxidative stress levels (Schipper et al., 2006). To further verify betaine's effects upon oxidative stress, HO-1 was used as an intracellular oxidative stress levels detector. Immunofluorescence staining of HO-1, counterstained with F-actin (stained by FITC-labeled Phalloidin) for cytoskeleton of NIH 3T3 fibroblasts was shown in **Figure 1D**. The H_2O_2 group exhibited obviously higher fluorescence intensity of HO-1 in contrast with the control group with cell shrinkage (**Figures 1D,b**). However, with the pretreatment of 2 mM betaine, HO-1 expression was reduced compared with the H_2O_2 group whereas the cell morphologies were much closer to the untreated group (**Figures 1D,c**), indicating a good antioxidant effect of betaine on NIH 3T3 cells. Then HO-1 protein expression was determined by western blotting as shown in **Figures 1E,F**. The 4 h exposure to H_2O_2 caused a significant increase of HO-1 expression in NIH 3T3 fibroblasts by 1.92-fold ($p < 0.01$) compared with control cells. Cells pretreated with 2 mM betaine exhibited a slighter increase of HO-1 expression when exposed to H_2O_2 by 1.29-fold. Thus, betaine pretreatment contributed to a decrease of HO-1 expression in cells exposed to H_2O_2, suggesting that the betaine had protected the cells from oxidative damage by lowering the oxidative stress level of cells suffering oxidative damage, which also can explain the increased cell viability in **Figure 1B**.

Oxidative stress response has significant effects on mitochondria, which influences mitochondrial membrane

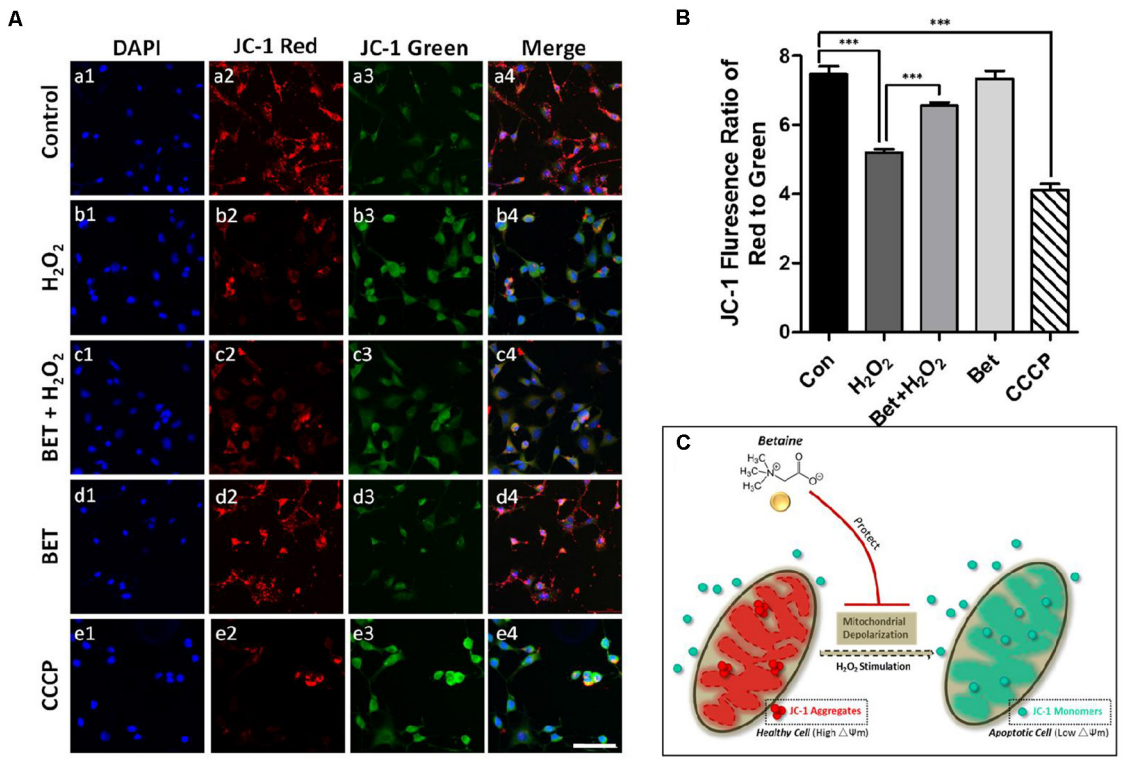

FIGURE 2 | Betaine protected the mitochondria in NIH 3T3 cells against oxidative damage. **(A)** Fluorescence staining of JC-1 for mitochondria membrane potential. Red: mitochondrial aggregates. Green: mitochondrial monomers. Scale bar = 100 μm. **(B)** The ratio of red to green fluorescence, $n = 3$. CCCP: positive control. BET: betaine. **(C)** Schematic showing the mitochondrial protective effect of BET against H_2O_2 damage. Statistical differences were performed using ANOVA, $^{***}p < 0.001$.

potential, leading to mitochondrial depolarization (Lieven et al., 2003). During the wound healing process, the inevitable elevated oxidative stress reaction, along with inflammation, induces cell apoptosis, during which mitochondria plays an important role (Granville et al., 2001). In order to investigate mitochondrial status change in wound healing-associated cells under oxidative stress stimulus, we performed examinations of mitochondrial membrane potential ($\triangle\Psi$m) using JC-1 staining. In healthy cells, mitochondria have a high $\triangle\Psi$m, shown as red stained aggregates formed by JC-1 dye. While in cells with mitochondria damage, $\triangle\Psi$m is lower and JC-1 will exhibit green fluorescence as monomers. As observed in **Figures 2A,a1–a4**, NIH 3T3 fibroblasts in the control group had a high intensity of red fluorescence with a relatively low level of green fluorescence, indicating a high $\triangle\Psi$m of mitochondrion. After H_2O_2 (100 μM) exposure for 1h, a significant reduction in red fluorescence could be seen. Meanwhile, the loss of $\triangle\Psi$m caused a strong green fluorescence (b1-b4), whereas the pretreatment with betaine attenuated $\triangle\Psi$m loss resulted from H_2O_2 stimulus, indicated by a brighter red fluorescence (c1-c4). Additionally, free betaine treatment exerted minimal effects on $\triangle\Psi$m in fibroblasts (d1-d4). CCCP (carbonyl cyanide m-chlorophenylhydrazone) is a protonophore uncoupling agent for oxidative phosphorylation and a potent inducer for increasing membrane proton conductance of $\triangle\Psi$m (Kasianowicz et al., 1984; Livingston et al., 2019; Soutar et al., 2019), thus CCCP is served as a positive control for comparison (e1-e5). A more accurate measure of $\triangle\Psi$m change after different treatments was performed by calculating the ratio of red to green fluorescence, the low level of which represented the loss of $\triangle\Psi$m and a damaged mitochondria condition. In **Figure 2B**, in assistance with images in **Figure 2A**, the exposure to H_2O_2 led to a dramatic decrease of fluorescence ratio (5.21 ± 0.10) compared to the control cells (7.48 ± 0.23), which was measured using a microplate reader. While betaine pretreatment significantly rescued the loss of $\triangle\Psi$m with an elevated ratio of 6.56 ± 0.08 ($p < 0.001$). The mitochondrial protective effect of BET against H_2O_2 damage was presented in the diagram in **Figure 2C**.

Characterization of BET@COL

In order to provide a supportive moisturizing scaffold for wound regeneration, we encapsulated betaine into a thin collagen material derived from rat tail collagen (**Figures 3A,a**). The SEM results (**Figures 3A,b**) showed an inner loose spongy construction of collagen, which was speculated to have good absorbency to achieve better drug load and the wound exudation absorption as well. We next investigated the water preserving ability of betaine in collagen sponges by comparing to that of 0.9% saline-containing collagen sponges. An equivalent volume of betaine solution and saline was evenly added to the collagen sponge for a complete absorption, followed by dehydration at 37°C. Weight of the collagen sponges of two

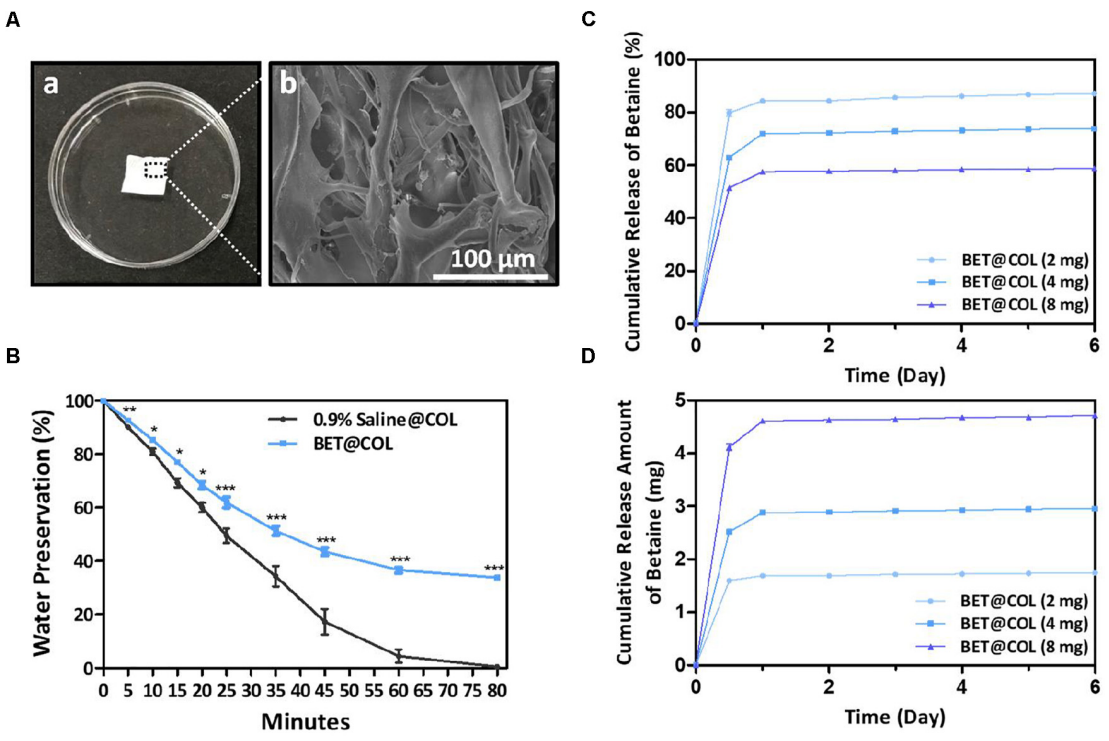

FIGURE 3 | Characteristics and betaine release of BET@COL. **(A)** Photographs of thin collagen sponge dressing and its inner instruction **(a)** of collagen sponge dressings by SEM **(b)**. Scale bar: 100 μm. **(B)** Water preservation percentage of BET@COL, $n = 3$. **(C,D)** *In vitro* release profile of BET@COL. The cumulative release percentage **(C)** and the cumulative release amount **(D)** of betaine of different drug load (2 mg, 4 mg, and 8 mg) measured by HPLC, $n = 3$. BET, betaine; COL, collagen. Statistical differences were performed using ANOVA. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

groups were obtained at different time points and the water preservation (%) was calculated and shown in **Figure 3B**. At each time point, the betaine group showed significantly higher water preservation percentage compared with the saline group, indicating an excellent water preserving function of betaine. On the 80th min, the saline containing collagen sponge had only 0.44 ± 0.18% water preservation, while the betaine group remained 33.78 ± 0.78% ($p < 0.001$). Thus the addition of betaine endowed the collagen sponge with significantly improved water retentivity, which could provide a much more moist environment on the wound surface in favor of epithelial crawling.

Next, after absorbing betaine solutions containing various drug amounts of 2, 4, and 8 mg, BET@COL were investigated for betaine release rate by HPLC at determined time points. The calibration curve for betaine was found to have good linearity over the range of 0.6–5.4 mg/mL ($r = 0.997$). **Figure 3C** showed the cumulative release percentage of betaine. All the groups reached the release peak on day 1, with the maximum cumulative release of 87.2 ± 0.1% (2 mg), 73.9 ± 0.1% (4 mg) and 59.0 ± 0.1% (8 mg) during the whole releasing process. The cumulative release amounts of betaine were 1.7 ± 0.003 mg (2 mg), 3.0 ± 0.004 mg (4 mg) and 4.7 ± 0.01 mg (8 mg) presented in **Figure 3D**. In our acute wound model experiments, the "burst release" of betaine in the early phase helps to alleviate the inflammatory response through its anti-oxidative stress effect. Additionally, this *in vitro* release is likely different from the release profile *in vivo* at wound sites. It is expected that during the wound healing process, the collagen sponge would be further hydrolyzed by wound collagenases (Chattopadhyay and Raines, 2014), so as to gradually release the rest of the betaine entrapped in the sponge for the purpose of maintaining the gradual healing. According to **Figures 3C,D**, as the incorporated betaine increased, the cumulative release percentage of betaine decreased, but the cumulative release amounts of betaine increased. This could be due to the strong intermolecular electrostatic interactions between zwitterionic betaine pairs and between betaine and collagen matrix, which could tighten the sponge networks and thus impose an additional barrier for releasing betaine from the sponge (Zheng et al., 2018; McCoy et al., 2019).

The Wound Healing Promoting Effects of BET@COL

Since the protective effects against oxidative damage and the water retentivity of betaine *in vitro* were confirmed as mentioned above, we performed animal experiments to investigate its effects on the dermal wound healing process. Representative photographs of differently treated wounds at determined time points were shown in **Figure 4A**. Overall, all BET@COL groups with different drug loads exhibited accelerated wound closure,

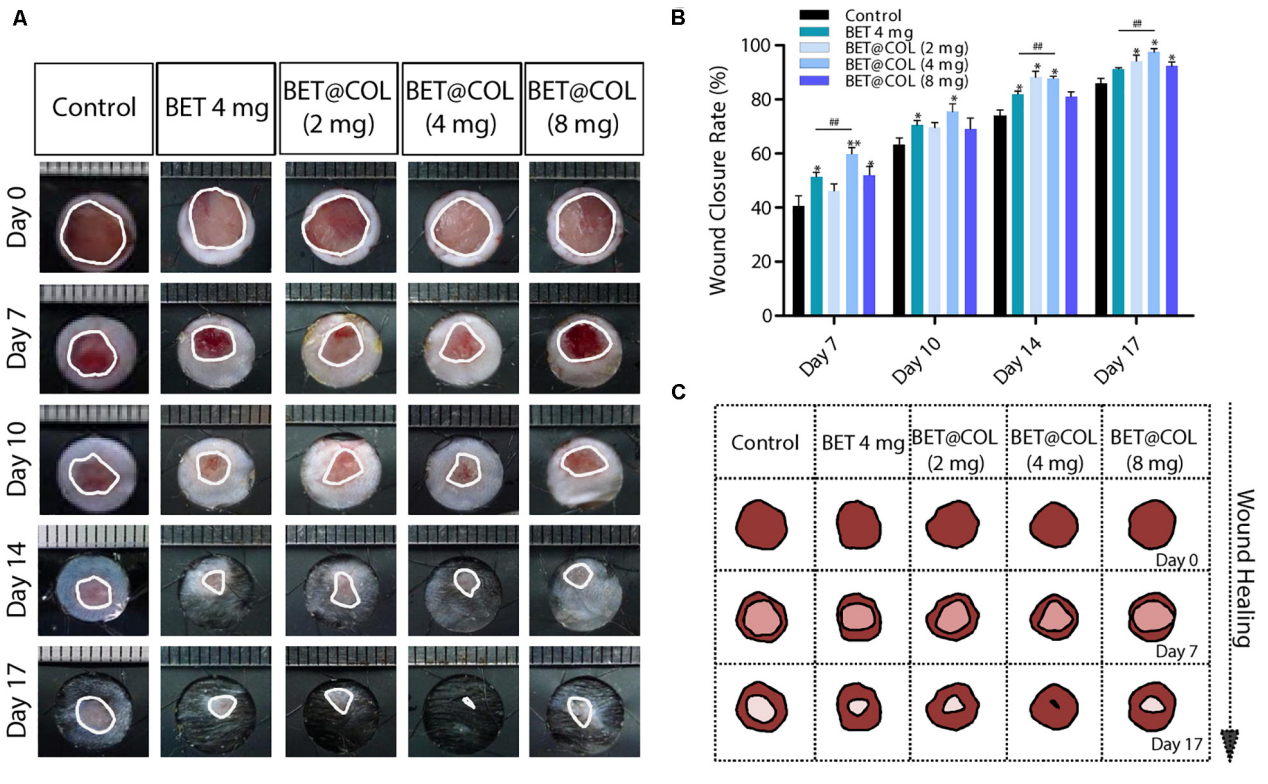

FIGURE 4 | BET@COL significantly resurfaced dermal full-thickness wound. **(A)** Representative pictures of the macroscopic wound healing processes of the control, free betaine solution treated and BET@COL treated wounds. The rulers in the pictures are shown in millimeters. **(B)** Statistical wound closure rates of the groups mentioned above, $n > 6$. **(C)** Schematic diagram of the traces of *in vivo* wound closure over 17 days. BET, betaine; COL, collagen. Statistical differences were performed using ANOVA. $^{**}p < 0.01$, $^{*}p < 0.05$, compared to control group, $\#\#p < 0.01$.

among which the BET@COL (4 mg) group showed the fastest wound closure rate, suggesting 4 mg as an appropriate drug amount loaded in collagen sponges to achieve better wound healing (**Figure 4B**). Especially on day seven post-surgery, the healing rate of the control group was 40.73 ± 3.64%, while the BET@COL (4 mg) showed a significantly accelerated rate of 59.92 ± 2.33% ($p < 0.01$). At the end of the observation (day 17), the BET@COL (4 mg) group also had the highest wound enclosure rate of 97.56 ± 1.25% ($p < 0.01$), which was shown as an almost complete wound resurfacing in **Figure 4A**. In order to make a comparison with the BET@COL (4 mg) group, free betaine solution (4 mg) was also applied to the wounds. Comparing the BET@COL (4 mg) group with the free betaine (4 mg) group, on day 7, 14, and 17 post wounding, the BET@COL (4 mg) group showed a significantly accelerated healing rate ($p < 0.01$), indicating an additional pro-healing function of the collagen. Additionally, the free betaine solution (4 mg) treated group significantly promoted wound closure on day 7, 10, and 14 in contrast with the untreated group. The BET@COL (2 mg and 8 mg) groups exhibited slight healing promoting effects with no general statistical significance. The overall wound healing trends during the observation period in the five groups were illustrated in the schematic diagram in **Figure 4C**.

The histological constructions of the dermal wounds undergoing different treatment were observed by histological staining on wound tissue sections (**Figure 5**). The re-epithelialization of the wounds on day 7 was observed by the immunohistochemical staining of cytokeratin, a marker indicating epidermis and hair follicles (**Figures 5A,b1–b5**). The BET@COL (4 mg) group exhibited the shortest epidermal gap (1.46 ± 0.06 mm) compared with 2.11 ± 0.16 mm of the control group ($p < 0.01$). The wounds treated with free betaine solution (4 mg) also showed an accelerated epidermal regeneration, with an epidermal gap of 1.71 ± 0.09 mm remaining (**Figure 5B**). The above observations of epidermis crawling were approximately consistent with the macroscopic

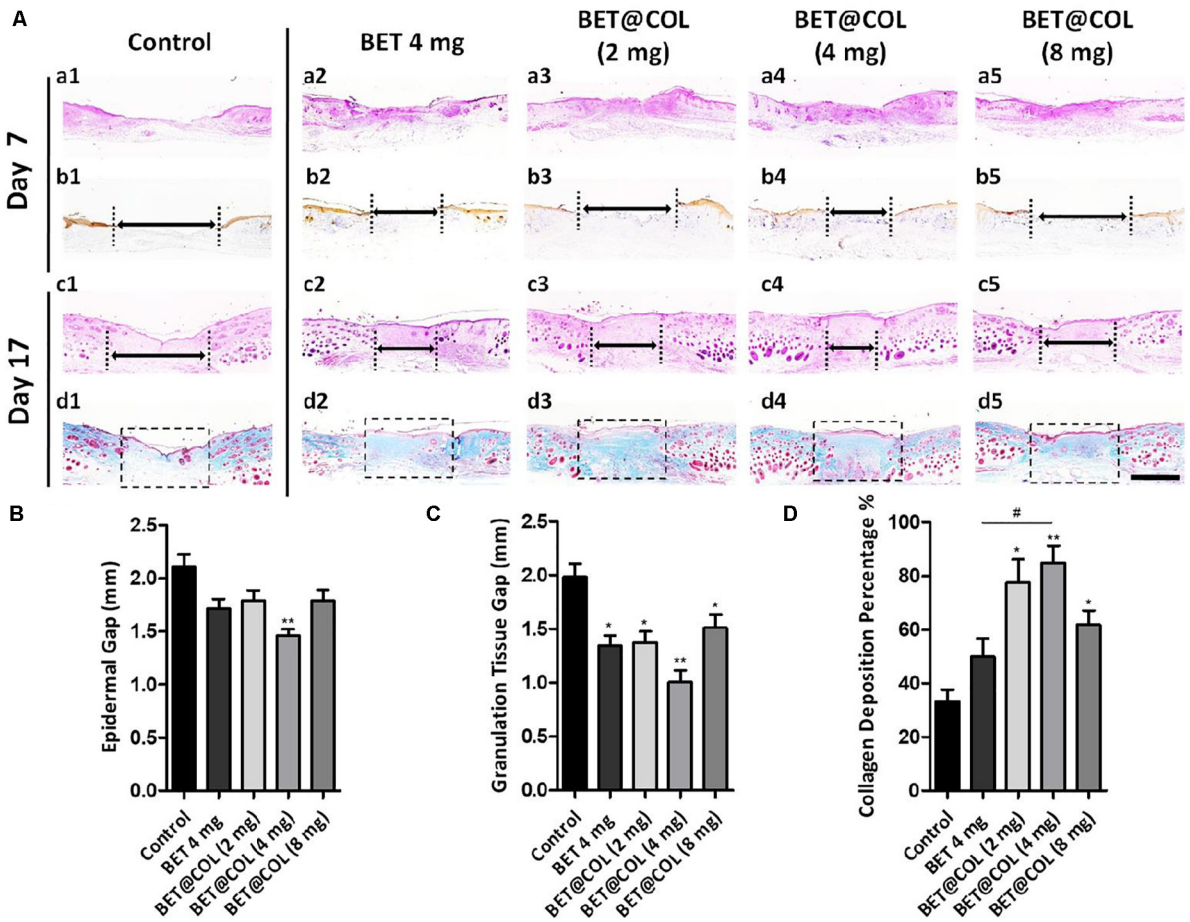

FIGURE 5 | Histological analysis of wound sections on days seven and 17 post treatment. **(A) (a1–a5, c1-c5)** Representative pictures of H&E (Hematoxylin and Eosin) staining of the control, free betaine solution treated and the BET@COL treated wound tissue sections on day seven and day 17. **(b1–b5)** Immunohistochemical staining of cytokeratin of the groups mentioned above on day seven. **(d1–d5)** Masson's trichrome staining of the groups mentioned above on day 17. Double-headed arrows indicated an epidermal gap **(b1–b5)** or granulation tissue gap **(c1–c5)**. Dotted boxes indicated collagen deposition sites **(d1–d5)**. Scale bar is 1 mm. **(B)** Length of epidermal gap on day seven, $n > 3$. **(C)** Granulation tissue gap on day 17, $n > 3$. **(D)** Collagen deposition percentage on day 17, $n > 3$. BET, betaine; COL, collagen. Statistical differences were performed using ANOVA. $^{**}p < 0.01$, $^{*}p < 0.05$, compared to control group, $\#p < 0.05$.

wound enclosure rate in **Figure 4**, which may be resulting from the moist wound environment created by BET@COL together with the therapeutic effects of betaine. Granulation tissue is the newly formed construction in the wound bed which is mainly composed of connective tissues and blood capillaries and finally remodeled into mature skin construction (Ali et al., 2016). The granulation tissue gaps on day 17 were measured to evaluate the deep tissue regeneration and the statistical result was shown in **Figure 5C**. The control group remained an unhealed gap of 1.98 ± 0.12 mm, while the BET@COL (4 mg) group accelerated granulation tissue development from both wound edges, leaving a narrow gap of only 1.01 ± 0.11 mm ($p < 0.001$). Other BET@COL treated groups also showed significant reduction, which was 1.37 ± 0.10 mm for the BET@COL (2 mg) group ($p < 0.01$) and 1.51 ± 0.12 mm for the BET@COL (8 mg) group ($p < 0.05$). For the free betaine (4 mg) group, significantly shorten granulation gap was also observed (1.34 ± 0.10 mm, $p < 0.01$), which was wider than the BET@COL (4 mg) group. Furthermore, the effects of BET@COL on collagen deposition on day 17 were analyzed by Masson's trichrome staining, by which collagen and nuclei were marked as blue and muscle marked as red (**Figures 5A,d1–d5**). It was observed that the wounds treated with BET@COL produced obviously more and denser collagen fibers, and the statistical result confirmed that the BET@COL (4 mg) group had a significantly higher collagen deposition percentage of 84.87 ± 6.33% compared with the control group (33.33 ± 4.26%, $p < 0.01$) and the free betaine (4 mg) group (49.95 ± 6.80%, $p < 0.05$). Moreover, the free betaine solution treatment showed no significant difference in collagen deposition compared with the control group, while there appeared a considerable increase in the BET@COL (4 mg) group by 2.55-fold compared with the control group, and the BET@COL groups with a drug loading of 2 mg and 8 mg also showed significantly enhanced collagen deposition in wounds. Furthermore, we used the immunohistochemical staining of α-SMA (α-smooth muscle actin) (Yoshizawa et al., 2015; Seo et al., 2018), a marker for smooth muscle cell and mature vascularization, to evaluate angiogenesis and vascular maturation on 7-day tissue sections (**Supplementary Figure S1**). As shown by red arrows in **Supplementary Figure S1**, the wounds treated by BET@COL dressings exhibited a higher density of newly formed blood vessels and larger lumen sizes than both the control and BET (4 mg) groups. Among BET@COL dressings, the BET@COL (4 mg) dressing showed the best angiogenesis, as evidenced by the highest density of blood vessels and the largest vessel size (**Supplementary Figures S1B,C**). The angiogenesis

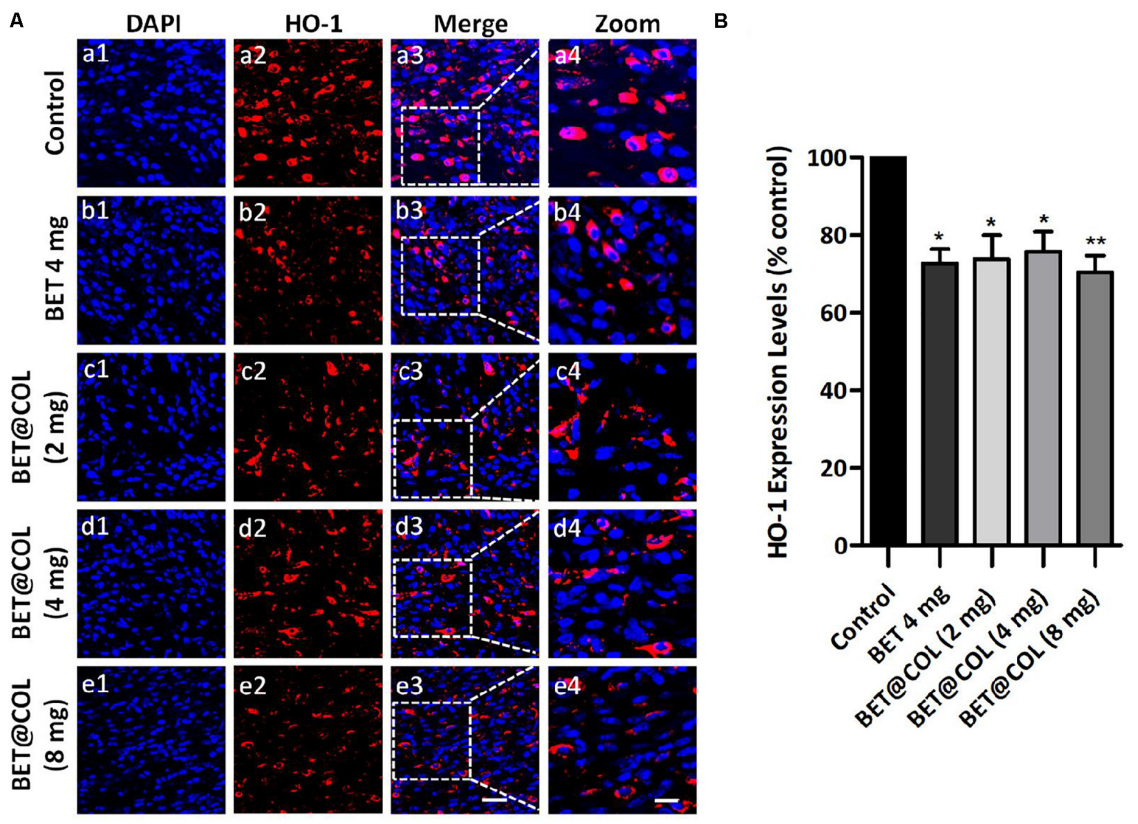

FIGURE 6 | Betaine ameliorates oxidative stress level at wound sites. **(A)** Immunofluorescence photomicrographs of heme oxygenase-1 (HO-1) in wounds for the control group, the free betaine solution (4 mg) group and BET@COL (2 mg, 4 mg, and 8 mg) treated group on day seven. Scale bars: 20 μm [10 μm in magnified images **(a4–e4)**]. **(B)** Quantitative result of immunofluorescence, $n = 5$. BET, betaine; COL, collagen. Statistical differences were performed using ANOVA. $^{**}p < 0.01$, $^{*}p < 0.05$, compared to control group.

promoting effect of BET@COL sponges was attributed to a synergistic combination of water-preserved, anti-oxidation, and anti-inflammatory property of zwitterionic betaine in collagen sponge. Specifically, (i) the moister environment created by the BET@COL sponges contributes to the accelerated dermal/wound bed repairs, including epithelialization, cell proliferation, ECM synthesis, and angiogenesis (Rousselle et al., 2019); (ii) a moister environment also prolongs the retention of bFGF and VEGF at wound sites to promote neovascularization; (iii) betaine can effectively suppress the inflammatory responses by minimizing oxidative stress at wound sites, thus boosting the subsequent collagen deposition and angiogenesis (Eming et al., 2009). In conclusion, with the beneficial effects exerted by betaine, in addition with the support of collagen, BET@COL accelerated wound regeneration by prompting re-epithelialization, granulation formation, collagen deposition and angiogenesis.

BET@COL Restrained Oxidative Stress and Inflammatory Response Upon Wound Healing Process

The *in vitro* anti-oxidative stress and mitochondria-protecting effects of betaine were confirmed on NIH 3T3 fibroblasts, and we wondered if the application of betaine on dermal wounds had such protective effects against oxidative damage caused by skin injuries. We performed immunofluorescence staining of HO-1, an indicator of oxidative stress levels described above, on wound tissue sections on day seven (**Figure 6A**). The control group exhibited a relatively higher intensity of HO-1 expressing cells in wound center, indicating a stronger oxidative stress response, which was significantly reduced to lower levels after the administration of betaine. Compared with the control group, the free betaine solution (4 mg) treated wounds exhibited 72.65 ± 3.64% ($p < 0.05$) and the BET@COL groups showed decreased levels of 73.80 ± 6.16% ($p < 0.05$), 75.66 ± 5.12% ($p < 0.05$) and 70.44 ± 4.24% ($p < 0.01$) for drug loadings of 2, 4, and 8 mg, respectively (**Figure 6B**). Thus, we speculated that betaine speeded up wound closure and histological regeneration partially by attenuating oxidative stress and protecting cells from oxidative damage.

The inflammation phase is a critical part of the wound healing process, while the exaggerated and prolonged inflammatory response has been proposed to be pathogenic mechanism of chronic wounds. During the inflammation period, macrophages infiltration is an indispensable link, mainly consisting of M1 macrophages (the pro-inflammatory phenotype) and M2 macrophages (the anti-inflammatory phenotype). The polarization toward M2 macrophages prompted the resolution of inflammation and benefited wound healing (Yin et al., 2013; He et al., 2017). As mentioned above, inflammation is closely coupled to oxidative stress. The two responses promote

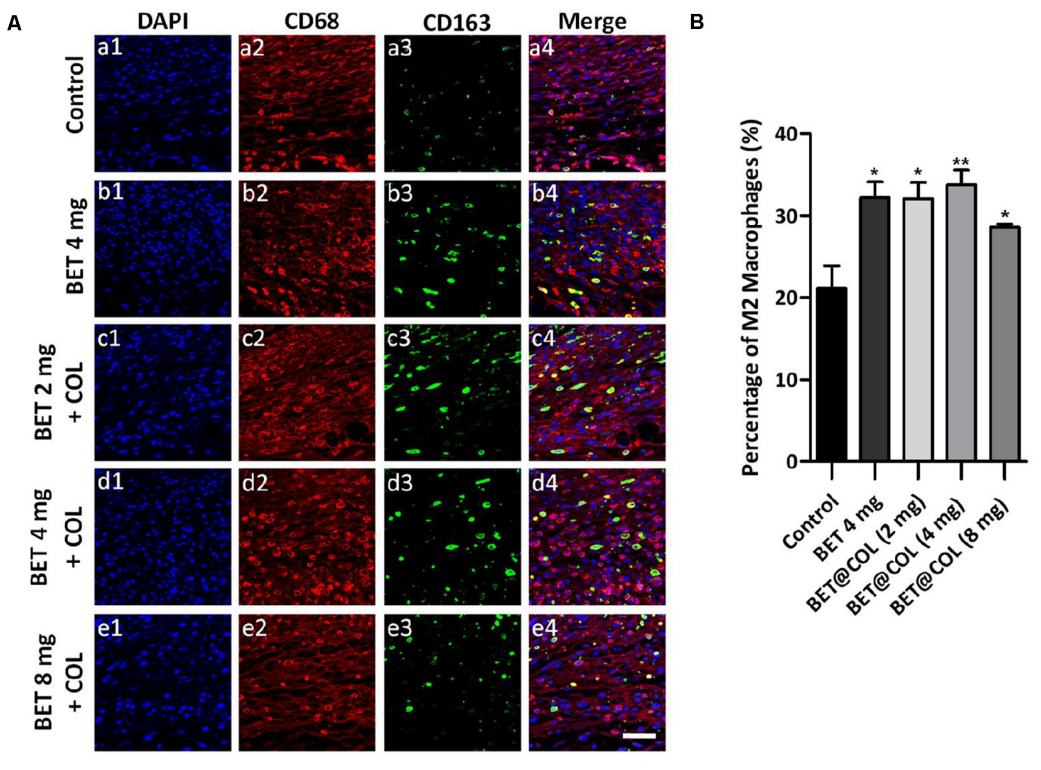

FIGURE 7 | Betaine promoted M2 macrophages polarization in wounds on day seven. **(A)** Fluorescence photomicrographs of M2 macrophages of the control, free betaine solution treated and BET@COL treated wounds. Scale bar is 50 μm. **(B)** Quantitative result of immunofluorescence staining, $n > 5$. BET, betaine; COL, collagen. Statistical differences were performed using ANOVA. $^{**}p < 0.01$, $^{*}p < 0.05$, compared to control group.

each other with complicated underlying mechanisms. We assumed that the alleviation of oxidative stress levels by betaine would reduce the inflammatory response in the wounds in the meantime. In order to verify our assumption, we evaluated the inflammation levels by calculating the percentage of M2 macrophages. CD68 was chosen as a marker of macrophages for all subtypes and CD163 for M2 macrophages (Labonte et al., 2014). As illustrated in **Figure 7A**, the control group had a low level of CD163 expressing cells while the free betaine (4 mg) solution and BET@COL treated groups exhibited higher levels. Significantly increased levels of M2 macrophages by 1.59-fold in BET@COL (4 mg) ($33.76 \pm 1.82\%$, $p < 0.01$) and by 1.52-fold in free betaine solution (4 mg) group ($32.20 \pm 1.93\%$, $p < 0.01$) compared with the untreated group ($21.17 \pm 2.74\%$) were demonstrated in **Figure 7B**, indicating enhanced polarization of M2 macrophages and a restraint of inflammatory response by betaine application.

CONCLUSION

A moist and bioactive microenvironment plays a critical role in determining wound healing fate. While zwitterionic betaine has demonstrated its superior water binding and antifouling properties, the less efforts and progress have been made to study its effect on dermal wound healing. In this work, we proposed to incorporate betaine into BET@COL collagen sponges to improve their water retention ability, multiple cell biocompatibility *in vitro*, and wound healing efficiency *in vivo*. First, betaine alone demonstrated its superior cell biocompatibility to reduce oxidative damage on NIH 3T3 fibroblasts with an improved survival rate when exposed to H_2O_2, and more specifically, to significantly prevent mitochondria damage caused by H_2O_2 stimulation in NIH 3T3 fibroblasts. Further, upon incorporation of betaine into collagen sponges and applying the resultant BET@COL collagen sponges to a full-thickness wound mice model, the sponges demonstrated their accelerated wound healing efficiency as evidenced by enhanced wound closure, accelerated granulation tissue formation, increased collagen deposition and improved new blood vessel formation. Such high wound healing efficiency is likely attributed to the betaine-induced high water retention, low oxidative stress and low inflammation. The results indicate that the betaine-based materials could hold promise for wound dressing applications.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee of Wenzhou Medical University and followed the International Ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals.

AUTHOR CONTRIBUTIONS

HH, JZ, and JW proposed and designed the project. AC, YA, WH, and TX synthesized and fabricated sponges. AC, YA, MY, and SL performed cell and tissue tests. AC, YA, SL, and XX performed mice model. All authors participated in result analysis and discussion and manuscript writing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00491/full#supplementary-material

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REVIEW
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The Role of an IL-10/Hyaluronan Axis in Dermal Wound Healing

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Scar formation is the typical endpoint of postnatal dermal wound healing, which affects more than 100 million individuals annually. Not only do scars cause a functional burden by reducing the biomechanical strength of skin at the site of injury, but they also significantly increase healthcare costs and impose psychosocial challenges. Though the mechanisms that dictate how dermal wounds heal are still not completely understood, they are regulated by extracellular matrix (ECM) remodeling, neovascularization, and inflammatory responses. The cytokine interleukin (IL)-10 has emerged as a key mediator of the pro- to anti-inflammatory transition that counters collagen deposition in scarring. In parallel, the high molecular weight (HMW) glycosaminoglycan hyaluronan (HA) is present in the ECM and acts in concert with IL-10 to block pro-inflammatory signals and attenuate fibrotic responses. Notably, high concentrations of both IL-10 and HMW HA are produced in early gestational fetal skin, which heals scarlessly. Since fibroblasts are responsible for collagen deposition, it is critical to determine how the concerted actions of IL-10 and HA drive their function to potentially control fibrogenesis. Beyond their independent actions, an auto-regulatory IL-10/HA axis may exist to modulate the magnitude of $CD4^+$ effector T lymphocyte activation and enhance T regulatory cell function in order to reduce scarring. This review underscores the pathophysiological impact of the IL-10/ HA axis as a multifaceted molecular mechanism to direct primary cell responders and regulators toward either regenerative dermal tissue repair or scarring.

Keywords: dermal scarring, fetal wound healing, inflammation, IL-10, extracellular matrix, hyaluronan, T lymphocytes

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CLINICAL SIGNIFICANCE

Dermal wound healing is an intricate process, driven by fibrotic mechanisms that cause scarring at the site of injury. Though the underlying mechanisms of wound healing are not fully understood, caring for wounds is a large part of our healthcare system. More than 100 million people in developed countries are estimated to develop scars annually, largely resulting from medical

procedures (Bayat et al., 2003). In 2012, more than 35 million surgeries were performed in the United States and over 300 million worldwide, each resulting in at least one dermal wound. Furthermore, millions of these patients suffer from diabetes or related autoimmune disorders, leading to poor healing and chronic wounds (Weiser et al., 2016) with an estimated annual cost of care approaching $100 billion (Sen, 2019). Even in those without underlying conditions, scars reduce the biomechanical strength, elasticity, and integrity of the skin and impair its function (Scott Adzick et al., 1985; Corr and Hart, 2013). Scars also contribute to pain and psychological distress, as patients have reported feelings of anxiety regarding the appearance of the scar or the memories surrounding the instigating event (Brown et al., 2008).

The high morbidity of scar formation is not unique to the skin but is common in post-inflammatory reactions of other organs. For instance, scar tissue can be deposited in place of cardiac tissue after a myocardial infarction, which then interferes with synchronous cardiac function. Similarly, interstitial pulmonary fibrosis, a progressive condition defined by deposition of scar tissue in the lung interstitium, is caused by post-inflammatory fibrotic reactions. Renal fibrotic diseases, such as crescentic glomerulonephritis, also emphasize the systemic nature of the scar formation processes (Sziksz et al., 2015; Steen et al., 2019). Taken in a broader context, it has been postulated that fibrosis accounts for almost 50% of worldwide mortality (Wynn, 2004). Due to the high incidence and the high-risk morbidity of fibrosis, it is imperative to elucidate the mechanisms that lead to fibrosis rather than regeneration after injury so that new therapies can be developed to widely address the physical and psychosocial consequences of aberrant fibrosis.

Unlike fetal and mucosal tissues, which physiologically heal scarlessly, postnatal skin inevitably undergoes fibrotic wound healing, which begs the question of what determines the difference between regenerative and fibrotic tissue repair (Leavitt et al., 2016). In contrast to postnatal skin, fetal tissues and adult mucosal tissues feature lower inflammatory responses underscored by reduced immune cell recruitment and activation, lower transforming growth factor (TGF) -β1 levels (Whitby and Ferguson, 1991), and increased vascular maturity and keratinocyte proliferation (Glim et al., 2014; DiPietro, 2016); all of which are consistent with regenerative wound healing. Fetal skin exhibits lower infiltration of macrophages, mast cells, dendritic cells, and T lymphocytes (Adolph et al., 1993; Cowin et al., 1998; Wulff et al., 2012; Walraven et al., 2016), leading to reduced levels of pro-inflammatory interleukin (IL)-6 and IL-8 (Leung et al., 2012). On the contrary, fetal skin has elevated levels of the anti-inflammatory cytokine IL-10 (Leung et al., 2012). Moreover, fetal skin is rich in type III collagen arranged in a "basket weave" pattern, allowing it to have more elasticity, whereas adult skin has a densely packed type I collagen matrix (Kathju et al., 2012). The fetal extracellular matrix (ECM) is also uniquely characterized by increased amounts of high molecular weight (HMW) hyaluronan (HA), which has anti-inflammatory and anti-fibrotic functions. Interestingly, IL-10 has been shown to promote HMW HA synthesis in dermal fibroblasts (Balaji et al., 2017), which in turn has been shown to promote IL-10 production in lymphocytes (Bollyky et al., 2011). This suggests a possible biologic feedback loop between fibroblasts and lymphocytes, communicated by IL-10 and HMW HA, that can drive the postnatal wound healing response toward either a fibrotic, scar-forming phenotype or a regenerative phenotype.

The present review aims to address the morbidity of fibrosis as it relates to differences between tissue types and the contribution of immune cell responders to ECM remodeling, with specific notes on the role of IL-10 and HA in modulating fibroblast response to injury and the potential physiological impact of T lymphocyte driven regulation.

STAGES OF DERMAL WOUND HEALING

Dermal wound healing can be divided into four stages: hemostasis, inflammation, proliferation, and remodeling (Barnes et al., 2018; Larouche et al., 2018). The initial response to injury is the formation of a platelet plug for hemostasis, followed by the infiltration of primary immune cell responders. During the proliferative phase, granulation tissue, containing a highly vascularized network of ECM components and growth factors, is formed as keratinocytes and fibroblasts, respectively, are recruited to form an epithelial layer and deposit a new ECM. Myofibroblasts then contract and re-approximate the wound edges, and the temporary fibronectin and type III collagen matrix is replaced with a type I collagen-based scar (Rodero and Khosrotehrani, 2010). **Table 1** summarizes the main cell types involved in wound healing and their functions.

Scarring is dictated by both the inflammatory and proliferative phases; since the skin plays a major role as a barrier against external pathogens, robust immune cell recruitment is necessary to prevent infection. However, inflammation also triggers fibrosis — the hallmark of the proliferative phase (Wick et al., 2013). Much of the cutting-edge research in the field focuses on the two main players in scarring: inflammation and ECM remodeling. By reciprocally regulating each other's signaling, inflammatory reactions and ECM deposition can direct the cells at the site of injury to mold the structure of newly formed tissue.

INFLAMMATORY RESPONSE IN WOUND HEALING

Cellular Response

Upon injury, much of the pro-inflammatory environment is created by the innate immune response, which is crucial to fight potential infection and activate wound healing. The initial response is dominated by neutrophils for the first 1–2 days in order to limit infection (Kim et al., 2008). This is an important stage for increasing the secretion of inflammatory factors along with the recruitment of other leukocytes against target pathogens (Devalaraja et al., 2000). Interestingly, data from neutrophil depleted mice revealed accelerated epithelialization, suggesting that neutrophils slow wound healing. However, neutrophils do

TABLE 1 | Role of various cell types in regulating healing.

Cell type	Role	References
Neutrophils	- Limit local infection - Secrete pro-inflammatory factors to increase immune response - Recruit leukocytes - Reduce rate of epithelialization	Devalaraja et al., 2000, Dovi et al., 2003
M1 macrophages	- Secrete pro-inflammatory cytokines - Present antigens to T cells - Associated with chronic wounds - Activate M2 macrophages late into response	Deiters et al., 2004, Ishida et al., 2008, Delavary et al., 2011, Willenborg and Eming, 2014
M2 macrophages	- Scavenge cellular debris - Increase extracellular matrix (ECM) synthesis - Secrete transforming growth factor (TGF)-β1 to recruit fibroblasts - Secrete interleukin (IL)-10 - Secrete VEGF for angiogenesis - Accelerate wound closure	Kovacs and DiPietro, 1994, Gordon, 2003, Lucas et al., 2010, Rodero and Khosrotehrani, 2010, Kathju et al., 2012, Willenborg and Eming, 2014, Okizaki et al., 2015
Fibroblasts	- Form ECM at injury site - Secrete growth factors, including TGF-β - Deposit collagen - Secrete hyaluronan (HA)	Gabbiani, 2003, Wang et al., 2011, Wight and Potter-Perigo, 2011, Rayahin et al., 2015, Avery et al., 2018, Barnes et al., 2018
Myofibroblasts	- Contract wound by applying tensile strength to ECM components - Promote type I collagen deposition	Gabbiani, 2003

T lymphocytes	- Effector T cells
	  - Increase inflammatory cytokines, target damaged cells
	- $CD4^+$ T cells
	  - Release anti-inflammatory cytokines, including IL-10, and promote macrophage M2 polarization
	  - Prevent macrophage / neutrophil infiltration
	  - Increase microvascularization
	- Tregs
	  - Dampen immune response
	  - Reduce macrophage M1 polarization
	- Tr1 cells
	  - Secrete IL-10
		Gawronska-Kozak et al., 2006, Roncarolo et al., 2006, Bollyky et al., 2011, Zaiss et al., 2013, 2015, Strbo et al., 2014, Weirather et al., 2014, Arpaia et al., 2015, Lei et al., 2015, Mariani et al., 2019, Wang et al., 2019

B lymphocytes	- Produce humoral antibody response - Activate T cell response - Accelerate wound closure in chronic wounds - Associated with slowed wound closure in severe combined immune deficient (SCID) mice	Strbo et al., 2014, Sîrbulescu et al., 2017, Wang et al., 2019

not appear to directly affect the dermal structure and collagen deposition (Dovi et al., 2003), emphasizing the multifactorial nature of scarring.

Other primary immune cell responders involved in fibrogenic wound healing include macrophages (Lucas et al., 2010; Barnes et al., 2018; Larouche et al., 2018), which secrete cytokines to drive cell recruitment and differentiation and scavenge cell debris at the site of injury via phagocytosis. The actions of macrophages are necessary for angiogenesis, granulation tissue formation, and rapid would closure during the early phases of healing (Mirza et al., 2009). Furthermore, phagocytosis by macrophages upholds the transition of the wound into an anti-inflammatory environment focused on stabilizing the matrix to prevent further damage (Wick et al., 2013). The plasticity of macrophages has been noted by their capability to differentiate into distinct subsets mainly represented by active pro-inflammatory macrophages (M1) seen early at the site of injury and anti-inflammatory macrophages (M2) seen later at the recovery stage (Gordon, 2003; Willenborg and Eming, 2014; Dal-Secco et al., 2015; Chuang et al., 2016). While M1 macrophages are activated by bacterial products, such as lipopolysaccharide (LPS) or inflammatory cytokines (Delavary et al., 2011), M1 to M2 polarization has been mainly attributed to the anti-inflammatory cytokines IL-10 and IL-4. The cytoprotective significance of these molecules is supported by evidence showing that M1 to M2 macrophage transition was delayed when cytokine function was blocked, contributing to delayed or absent collagen redistribution (Dal-Secco et al., 2015).

As stated previously, M1 macrophages are important in propagating the inflammatory response and removing harmful products resulting from skin damage, such as bacterial components and debris from damaged cells. By acting as antigen presenting cells, macrophages can also activate adaptive immunity via T lymphocyte differentiation and function (Delavary et al., 2011). Later during the proliferative phase, M1 macrophages undergo M2 polarization, initiating anti-inflammatory cytokine secretion, prominently IL-10 (Willenborg and Eming, 2014). This allows wounds to heal without inflammation-induced damage and prevents late hemorrhaging (Lucas et al., 2010). The M1 pro- to M2 anti-inflammatory transition is triggered by the phagocytosis of cellular debris and fibroblast activation (Leibovich and Ross, 1975). Specifically, the M2c subset plays an important role in removing cellular debris by upregulating cell surface receptors, stimulating ECM synthesis, and promoting angiogenesis (Rodero and Khosrotehrani, 2010; Willenborg and Eming, 2014). In that context, M2 macrophages release growth factors, including vascular endothelial growth factor (VEGF) and TGF-β1, that are important for neovascularization and granulation tissue formation (Gordon, 2003; Lucas et al., 2010; Kathju et al., 2012). These cytokines recruit both keratinocytes and fibroblasts, upregulate collagen deposition, and elicit pro-fibrotic healing (Kovacs and DiPietro, 1994; Delavary et al., 2011).

Macrophages have been shown to impact the healing of chronic wounds as evidenced by their depletion delaying fibroblast infiltration and collagen deposition, ultimately holding back wound closure (Ishida et al., 2008; Rodero and Khosrotehrani, 2010). Concomitantly, macrophages can accelerate wound closure by further differentiating into fibroblasts to enhance the production of ECM components

(Sinha et al., 2018), a phenomenon also observed in chronic wounds of diabetic mice. Prolonged inflammation and lack of M1 to M2 macrophage conversion is characteristic of chronic wounds (Mirza and Koh, 2011), where the reduced expression of M2 genes and slowed wound closure can be rescued by the exogenous addition of TGF-β1 (Okizaki et al., 2015). The presence of factors that specifically recruit and activate macrophages, such as macrophage chemoattractant protein (MCP)-1, were linked to faster wound epithelialization in diabetic mice (Deiters et al., 2004; Niebuhr et al., 2008). This supports the notion that both recruitment and effective M1 to M2 macrophage polarization can impact the formation of new tissue and wound closure.

Differences between adult and fetal skin macrophage levels provide insight into their role in fibrosis. Fetal skin was shown to have fewer inflammatory cells overall and contained a greater percentage of M2 macrophages relative to adult skin (Walraven et al., 2016). This is also the case in both healthy and wounded oral mucosal tissue (Glim et al., 2015). Another difference is the type of TGF-β secreted by macrophages. Fetal skin has higher levels of TGF-β3, which is associated with reduced collagen deposition and scarless healing (Bullard et al., 2003). By contrast, TGF-β1 is the main type present in adult tissues and is a major mediator of scarring. Furthermore, the introduction of TGF-β1 to fetal skin leads to fibrosis, whereas its depletion from adult skin prevents fibrosis (Shah et al., 1995; Sullivan et al., 1995).

As demonstrated by these studies, the temporal localization of various immune cell types to a wound can impact healing by instigating an inflammatory or anti-inflammatory microenvironment. In addition to the recruitment of other responders, one of the main roles of these immune cells is the secretion of cytokines, which play an integral role in determining the presence and extent of scarring.

Cytokine Response

The importance of inflammation at the site of injury has led to a focus on better understanding the regulation of regenerative healing by cytokines. During the initial response, two of the cytokines responsible for propagating inflammation are IL-6 and IL-8, which have been directly correlated to an increased number of macrophages in the wound (Avazi et al., 2019). In the presence of IL-6 and IL-8, both fibroblasts and epithelial cells have a diminished migratory capacity and secrete increased amounts of pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α (Basso et al., 2016). Conversely, the relative lack of an inflammatory environment in their absence contributes to the phenotype of scarless healing. In contrast to adult wounds where IL-6 and IL-8 are dominant and present for longer periods after injury (Liechty et al., 1998, 2000a), fetal skin has significantly lower levels of these inflammatory cytokines (Leung et al., 2012). Strikingly, the application of IL-6 to fetal wounds causes scarring in this naturally scarless environment (Liechty et al., 2000a).

Just as pro-inflammatory cytokines are linked to fibrosis, the opposite is also true of the protective activity of anti-inflammatory cytokines against fibrosis. Namely, IL-10 is an anti-inflammatory cytokine that consistently demonstrates to be a major mediator in preventing scars (Gordon et al., 2008; Peranteau et al., 2008). The function of IL-10 was initially studied in T cell differentiation, as it is released by the helper T lymphocyte (Th2) subset to counter pro-inflammatory cytokines secreted by the Th1 subset. It has therefore been associated with diseases caused by a presence of Th2 cells, including allergies and asthma (Delavary et al., 2011). IL-10 is ubiquitously expressed and secreted by many cell types in addition to lymphocytes, including granulocytes (such as neutrophils), endothelial cells, keratinocytes, and mast cells. Expression by these alternate cell types can in turn influence both the innate and adaptive immune responses (Moore et al., 2001). IL-10 also plays a role in preventing chronic inflammatory diseases, such as ulcerative colitis and Crohn's disease. Mutations in IL-10 or the IL-10 receptor have been associated with severe enterocolitis that originates from altered hematopoietic stem cell signaling and loss of regulatory T cell function, which leads to hyper-active effector $CD4^+$ cell responses (Glocker et al., 2011; Shouval et al., 2014). The cytokine has also been considered as a therapeutic option for chronic Crohn's disease and psoriasis and for acute encephalitis and other central nervous system diseases, as it has been shown to be effective in reducing inflammation (O'Garra et al., 2008).

IL-10 exerts its anti-inflammatory actions by transducing signals through a tetramer cell surface receptor composed of the IL-10Rα and IL-10Rβ subunits (Shi et al., 2014). Once bound, downstream signaling goes through the janus kinase - signal transducer and activator of transcription (JAK-STAT) pathways, which ultimately leads to the phosphorylation and activation of the STAT3 transcription factor (Kotenko et al., 1997; Walter, 2014; Sziksz et al., 2015). This pathway is an important gatekeeper as IL-6 also in part signals via STAT3. The balance of these factors influences the pro or anti-inflammatory cytokine milieu (Ouyang et al., 2011). IL-10 also reduces the infiltration of immune cell responders via inhibition of p38 MAPK and deactivation of HuR, an mRNA stabilizer (Rajasingh et al., 2006). For example, in a myocardial infarction model, IL-10 destabilizes mRNA encoding TNF-α, leading to less harmful left ventricular remodeling and reduced apoptosis of cardiac cells after injury (Krishnamurthy et al., 2009). Current research posits a role for IL-10 in mediating the metabolism of other cell types. For example, IL-10 induces macrophages to transition their metabolism from glycolysis to oxidative phosphorylation (Ip et al., 2017), which can drive M2 macrophage polarization (Galván-Peña and O'Neill, 2014). In the context of wound healing, this transition can mitigate M1 macrophage subset-driven inflammation at the injury site and promote effective wound healing (Willenborg and Eming, 2014).

IL-10 also facilitates the transition from the inflammatory to the proliferative phase by modulating the type and number of primary immune cell responders that migrate to a site of injury and regulating the expression of cytokines (Willenborg and Eming, 2014). The overexpression of IL-10 reduces the expression of pro-inflammatory mediators such as IL-6, MCP-1, and heat shock protein 47 (Peranteau et al., 2008). When given prophylactically, IL-10 decreases the number of pro-inflammatory cells at the wound site (Gordon et al., 2008; Peranteau et al., 2008), while concomitantly acting on macrophages to prevent the expression of inflammatory signals, such as TNF-α (Bogdan et al., 1991).

Evidence that IL-10 plays a role in ECM remodeling during injury stems from studies that show how scarring is caused by the transformation of fibroblasts to myofibroblasts, the deposition of collagen, and the reduction in matrix metalloprotease (MMP) activity. In these studies, IL-10 exerts a protective action against scar tissue formation by downregulating collagen production (Wangoo et al., 2003). This is true in different types of tissue, including models of pulmonary fibrosis and myocardial infarction (Nakagome et al., 2006; Rajasingh et al., 2006), in which IL-10 influences proteolytic enzymes to lyse the ECM and decrease macrophage TGF-β1 expression to prevent fibrosis (Nakagome et al., 2006; Shi et al., 2013, 2014).

Elevated IL-10 baseline levels are required for fetal skin to heal scarlessly, and the loss of this cytokine in fetal tissues has been associated with increased levels of inflammation in wounds and fibrotic wound repair (Liechty et al., 2000b). Accordingly, IL-10 and IL-4 deficient mice showed increased inflammatory monocytes, neutrophils, and macrophages, in conjunction with high deposition of thick collagen fibers. Treatment with IL-10 led to reduced inflammatory cell numbers and restoration of normal skin architecture and strength, which included randomly oriented collagen fibers, and clinically reduced scar size and redness (Gordon et al., 2008; Leung et al., 2012; Kieran et al., 2013; Morris et al., 2014). In support of these collective studies, **Figure 1** demonstrates that overexpression of IL-10 in adult dermal wounds confers scarless healing (Gordon et al., 2008; Peranteau et al., 2008; Leung et al., 2012). IL-10 upholds this regenerative phenotype through mechanisms associated with accelerated dermal healing and by interacting with fibroblasts to modulate ECM remodeling. Specifically, IL-10 crosstalk with the ECM underscores the relevance of the interaction between inflammatory responses and ECM-driven modulation of fibrosis.

ROLE OF THE EXTRACELLULAR MATRIX AND HYALURONAN IN WOUND HEALING

Extracellular Matrix

The ECM comprises a network of structural and functional molecules that surround cells and provide a scaffold for growth and physical connectivity, which is essential to transduce environmental cues through growth factor signaling and drive inter- and intra-cellular reactions. Briefly, the ECM structure includes proteins, such as collagen fibrils, elastin, and fibronectin, which enable cell adhesion and provide tissue strength. Interspersed within the ECM are proteoglycans and glycosaminoglycans, which facilitate scaffold formation and ensure compressive strength (Briquez et al., 2015). At the core of ECM remodeling are MMPs, which play a key proteolytic role to cleave the structure at times of post-injury tissue repair (Kular et al., 2014). The ECM is also a source of growth factors and, therefore, its integrity in maintaining and releasing the required growth factors is crucial to enable healing (Briquez et al., 2015).

At the time of initial injury, the ECM scaffold is damaged, eliciting the influx of cells, cytokines, and growth factors that are necessary for the initial inflammation and repair responses. During this response, fibroblasts are stimulated by platelet derived growth factor (PDGF) and are responsible for creating a new ECM structure. A temporary ECM is built, containing fibrin, fibrinogen, and fibronectin to facilitate fibroblast adherence and secretion of proteoglycans and glycosaminoglycans (Wight and Potter-Perigo, 2011). This enhanced ECM traps inflammatory cells, thus creating a localized inflammatory microenvironment. After the formation of the granulation tissue, fibroblasts secrete growth factors, including TGF-β and fibroblast growth factor, and differentiate into myofibroblasts to contract the wound. Finally, fibroblasts remodel the provisional ECM by replacing immature type III collagen with type I collagen (Wight and Potter-Perigo, 2011; Avery et al., 2018; Barnes et al., 2018).

Differences in wound healing can be attributed to ECM composition; for instance, higher levels of type VI collagen in the lungs cause faster epithelialization than type I collagen (Mereness et al., 2018). Type VI collagen also impacts the fibroblast response in the skin by reducing the rate of fibroblast migration from the wound (Theocharidis et al., 2016). Further, the differentiation of fibroblasts into myofibroblasts is also dictated by the ECM, as high levels of fibronectin in the wound prevent early differentiation, while the expression of type I collagen later in healing induces differentiation into myofibroblasts (Avery et al., 2018). The ECM composition also differs in early gestational skin. Fetal skin, which heals scarlessly, has higher levels of type III collagen and HA, in broad contrast to adult skin where type I collagen predominates (Sawai et al., 1997; Lovvorn et al., 1999). Adult skin also has an increased overall collagen content and increased stiffness at baseline as compared to that of fetal skin (Kular et al., 2014).

Though a large percentage of healthy skin ECM is composed of type I collagen, there are significant structural differences between the ECM of healthy and scarred skin (Eming et al., 2014), which can be defined by the orientation of collagen fibers. Namely, collagen in healthy skin has a basket-weave pattern that provides high tensile strength, whereas collagen in scar tissue is arranged in thick parallel bundles (van Zuijlen et al., 2003). The collagen bundle formation in scar tissue is dependent on TGF-β1, which prevents collagen degradation and enhances the maturation of type III collagen into type I (Eming et al., 2014).

An association also exists between the tensile strength of the ECM and the forces needed to contract the wound. Specifically, fetal skin has thin collagen fibers that are under low stress, but adult skin is made up of thicker bundles of collagen that are physiologically under higher stress (Barnes et al., 2018). This causes an increased mechanical load on ECM components of adult skin, leading to decreased cell apoptosis, increased expression of cell survival genes, and hypertrophic scar formation during the proliferative healing phase (Aarabi et al., 2007). The change in phenotype is driven by immune responses, as increased mechanical forces in the wound lead to T lymphocyte activation through IL-4 and IL-13, and recruitment of macrophages and fibroblasts to the injury site (Wong et al., 2011). These results demonstrate a link between the ECM and immune responses in wound healing, and in fact, the ECM

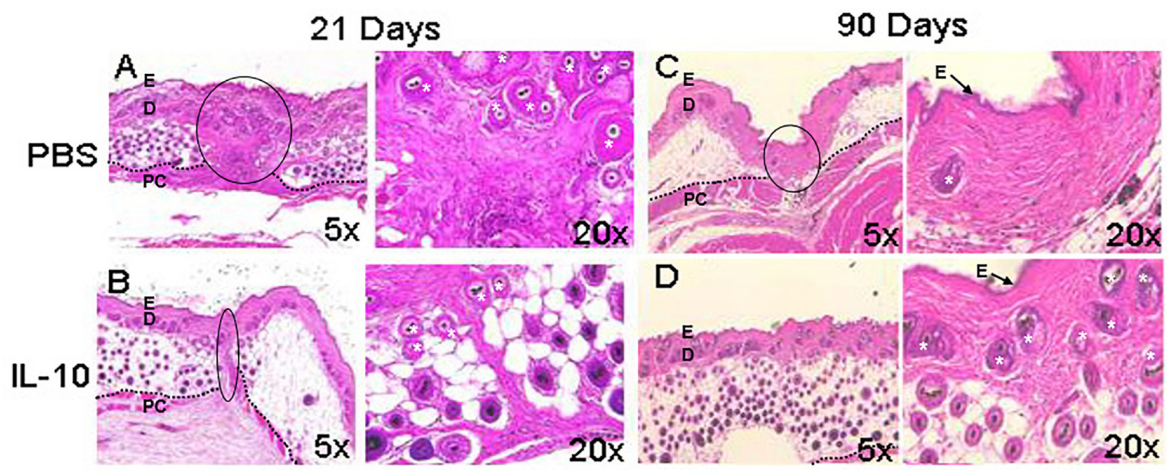

FIGURE 1 | Wound healing phenotype in response to IL-10. The overexpression of interleukin (IL)-10 was accomplished using an adenoviral vector in mouse dermal wounds. Compared to PBS treated controls, IL-10 reduced scar formation. After 21 days, PBS led to the formation of a dense collagen matrix with well defined scar **(A)**, whereas IL-10 prevented formation of a defined scar **(B)**. At 90 days, the PBS treated wound shows mature scar tissue that is distinct from the surrounding skin **(C)**. IL-10 led to the generation of elements of dermal tissue, with reticular collagen and hair follicles, similar to surrounding uninjured tissue **(D)**. E, epidermis, D, dermis, *, hair follicle, PC, panniculus carnosus. The black dotted line indicates the separation between the dermis and deeper structures. The black solid line indicates scar. Images from Gordon et al. (2008), with permission.

molecule HA has been identified as a central link in ECM-inflammatory cell crosstalk.

Hyaluronan

Despite its simple disaccharide chain structure of repeating D-glucuronic acid and DN-acetylglucosamine residues, HA has multifaceted functions (Cyphert et al., 2015). Its charged structure allows it to attract large amounts of water, providing compressibility and lubrication to tissues, including joints and the skin. HA has also been shown to transduce either pro- or anti-inflammatory signals and induce or abrogate critical cell functions, including differentiation, proliferation, migration, and invasion (Cyphert et al., 2015). Which of these seemingly bipolar cues are activated depends largely on the molecular mass of HA, which can then interact with specific receptors and other ECM components to result in regeneration or fibrosis (Cyphert et al., 2015). The molecular weight of HA is determined by a complex balance of synthesis by hyaluronic acid synthase isoforms (HAS1-3) and degradation by hyaluronidase enzymes (HYAL1-4). The final HA molecular weight variants are generally classified into low molecular weight (LMW) HA (< 0.5 MDa) and HMW HA (> 1.2 MDa) variants, each with distinct effects on wound healing (Stern et al., 2006). HA with a molecular weight above 1.2 MDa (HMW HA) reduces the activity of pro-inflammatory cytokines associated with LPS and macrophage infiltration, such as IL-1α, IL-6, and TNF-α. However, when LMW HA variants were applied, increased pro-inflammatory cytokine activity was observed (McKee et al., 1996; Neumann et al., 1999). These results can potentially be explained by differences in the binding affinity of HA molecular weight variants to cognate HA receptors (CD44, RHAMM, HARE, LYVE1, layilin, TLR2, and TLR4), which could change how the multi-dimeric structures cluster at the cell membrane and transduce either pro- or anti-inflammatory signals (Hardwick et al., 1992; Carreira et al., 2001; Prevo et al., 2001; Jiang et al., 2005). However, more work is needed to elucidate the specific signaling pathways activated by LMW or HMW HA.

Injury increases the production of HA by upregulating HAS expression, while simultaneously increasing its degradation into a LMW variant by upregulating hyaluronidase expression and the presence of oxidative stress (Powell and Horton, 2005; Ruppert et al., 2014). This is important during the early inflammatory phase of wound healing, as it facilitates robust post-wounding immune responses. Prior data support a mechanism by which LMW HA acts as a damage associated molecular pattern (DAMP) and interacts with toll-like receptors (TLR) 2 and 4 on immature dendritic cells to induce the release of the inflammatory IL-1β, TNF-α, IL-6, and IL-12 cytokines. Release of these cytokines recruits neutrophils to the site of injury and drives T lymphocyte differentiation (Termeer et al., 2002; Jiang et al., 2005; Scheibner et al., 2006; Ruppert et al., 2014). Binding of LMW HA to TLR4 on antigen presenting cells also leads to dendritic maturation (Termeer et al., 2002). This signaling cascade can be enhanced by fibroblasts, which further enable the responses to DAMPs and production of IL-6, IL-8, and MCP-1 to escalate the state of inflammation (Wang et al., 2011; D'Arpa and Leung, 2017).

High molecular weight HA has shown beneficial effects during wound healing, including the reduction of inflammatory cytokine expression after ultraviolet damage of keratinocytes and the recruitment of primary immune cell responders to the site of injury after smoke inhalation (Huang et al., 2010; Hašová et al., 2011). It has been proposed that HMW HA exerts its anti-inflammatory/anti-fibrotic effects by countering the interaction of LMW HA with TLRs and preventing signaling downstream of the TLR-2 receptor (Scheibner et al., 2006). Through this process, HMW HA prevents the accumulation of advanced glycation end

products, which can cause pro-inflammatory cytokine expression (Neumann et al., 1999).

The impact of HMW HA on immunity during wound healing stems from its direct interaction with primary immune cells through CD44, a receptor expressed by neutrophils, T lymphocytes, macrophages and dendritic cells (Powell and Horton, 2005). For instance, neutrophil and macrophage migration to lung tissues in the presence of LPS is significantly reduced by HMW HA/CD44 interactions (Liang et al., 2007) and the pro-inflammatory cells are more likely to undergo apoptosis when HMW HA is produced (He et al., 2013). Similarly, dendritic cells exhibit a slower maturation pattern in the presence of HMW HA, which reduces the activation of adaptive immunity (Gebe et al., 2017). In contrast to LMW HA, HMW HA directs M1 to M2 resident macrophage polarization, increasing phagocytic capacity and IL-10 expression (He et al., 2013). Remarkably, HMW HA can even reverse the M1 phenotype, reduce NOS2 and IL-12β, and increase IL-10 levels when added to LPS-activated M1 macrophages (Rayahin et al., 2015). In sum, CD44/HMW HA interactions prevent fibrotic scarring, a concept that is strongly supported by targeted *in vivo* inactivation of CD44 (KO), resulting in wounds with greater accumulation of type 1 collagen and fibrillar collagen (Govindaraju et al., 2019).

Consistent with its anti-inflammatory and anti-fibrotic effects, HMW HA is being considered as a therapeutic option to accelerate wound healing in chronic or impaired wounds. For example, application of exogenous HMW HA to diabetic wounds, which have lower baseline HA levels than healthy controls, improved healing by increasing neovascularization and TGF-β1 levels (Galeano et al., 2011). Also, the addition of HMW HA to the skin of a healthy mouse increased the rate of re-epithelialization in wound healing (Ramos-Torrecillas et al., 2015), thus supporting the benefits of HMW HA in reducing post-injury scarring. Fetal wounds have higher basal amounts of HMW HA relative to adult skin wounds, which directly prevent scarring by reducing the recruitment of fibroblasts and collagen deposition (Mast et al., 1992).

REGULATION OF FIBROBLASTS BY IL-10 AND HYALURONAN

As previously discussed, both IL-10 and HMW HA can interdependently reduce pro-fibrotic wound healing, but their respective contributions to scar prevention is intertwined with their regulation of fibroblast responses to injury. Fibroblasts are the main cell type involved in new ECM production and wound closure. The myofibroblast subset, in particular, has contractile properties that can place the ECM network under stress to close the wound. In a fibrotic wound, myofibroblasts remain in the wound site for a longer period, providing them time to mature in the presence of fibronectin and TGF-β1 and synthesize α-SMA and collagen I (Gabbiani, 2003).

Since fibroblasts are responsible for forming the ECM, the regulation of their differentiation and acquired phenotypes is crucial to modulating fibrosis. Fetal fibroblasts experience slower metabolism and apoptosis than adult fibroblasts, but show increased migration and invasion (Ellis et al., 1997; Balaji et al., 2015). However, adult fibroblasts recapitulate this fetal phenotype in response to IL-10 and HA treatment in a dose dependent manner. Of note, both IL-10 and HA are required to recapitulate the migration and invasion capabilities of fetal fibroblasts in the adult counter parts. This was demonstrated when adult fibroblasts treated with IL-10 in the presence of an HA synthase inhibitor showed no improvement in migratory function (Balaji et al., 2015). IL-10 also controls the HA content of the ECM by inducing fibroblast deposition of HMW HA, which directs regenerative healing (Balaji et al., 2017). Indeed, fetal fibroblasts produce an HA rich pericellular matrix as a result of the high physiologic concentration of IL-10 in fetal tissues. A similar pattern of HA in the ECM of adult skin can be established by upregulating HAS1-3 with the addition of IL-10, as shown in **Figure 2** (King et al., 2013a; Balaji et al., 2017).

STAT3, a molecule downstream of the IL-10 - IL-10R signaling, is necessary to produce high HMW HA concentrations and achieve regenerative scarless wound healing. Briefly, when IL-10 binds to its receptor, the JAK1-STAT3 pathway is activated, and STAT3 is phosphorylated to yield an active form that translocates into the nucleus and acts as a transcription factor (Riley et al., 1999; Walter, 2014). This process prevents the release of inflammatory cytokines that have been implicated in fibrosis and increases the presence of HMW HA to accelerate healing. The necessity of STAT3 in IL-10 anti-inflammatory signaling was demonstrated in STAT3 deficient macrophages, where the presence of LPS led to TNF-α production, irrespective of IL-10 treatment (Riley et al., 1999). Additionally, STAT3 is important to promote vascularization at the site of injury and enable improved healing (Krishnamurthy et al., 2009).

As previously stated, STAT3 activation is increased upon IL-10 binding to its cognate receptor (King et al., 2013a; Balaji et al., 2017). This activation proved to be beneficial in hypertrophic scars, where the addition of IL-10 similarly increases phosphorylated STAT3 (pSTAT3) and reduces expression of collagen and myofibroblast markers, including Col1, Col3, and α-SMA (Shi et al., 2014). Conversely, inhibition of IL-10 leads to lower levels of pSTAT3 and reduced nuclear localization of STAT3, which are associated with less HA production and significantly slower fetal wound healing (King et al., 2013b). These collective data support a STAT3-dependent mechanism in wound healing; namely, that STAT3 activation by IL-10 signaling induces HMW HA production and promotes vascularization to enable faster wound closure and attenuated scarring.

ROLE OF LYMPHOCYTES IN WOUND HEALING

Much of the research to date has focused on the role of inflammation, ECM, and the innate immune system in driving wound healing. Innate immune cells and their cytokine products are known to play a large role in determining whether a wound will heal via regenerative or fibrotic tissue repair. However, cells of the adaptive immune system can also drive pro- and

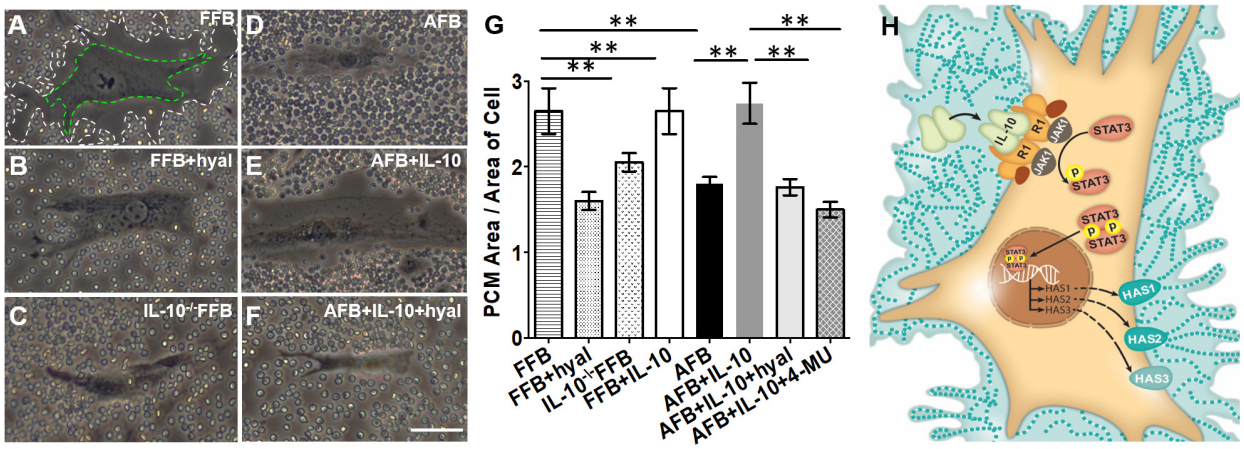

FIGURE 2 | Distribution of hyaluronan (HA) in pericellular matrix (PCM) of fibroblasts in the presence of IL-10. Phase contrast imaging of fibroblasts shows differences in PCM area between fetal and adult fibroblasts. White dotted lines indicate the border of the PCM. Green dotted lines indicate the border of the cell body. Around fetal fibroblasts, there is a dense HA PCM **(A)**, which significantly decreases in the presence of hyaluronidase **(B)** or the absence of IL-10 **(C)**. In adult fibroblasts, the HA matrix is typically smaller **(D)**, but increases to a size similar to that of the fetal fibroblast in the presence of IL-10 **(E)**. This effect is reversed by the presence of hyaluronidase **(F)**. The quantification of the area of HA rich PCM relative to the area of the cell in adult and fetal fibroblasts is shown in **(G)**. **(H)** Demonstrates the pathways by which IL-10 increases the HA PCM. FFB, fetal fibroblast; AFB, adult fibroblast; HAS, hyaluronan synthase; HYAL, hyaluronidase; 4-MU, 4-methylumbelliferone. $^{**}p < 0.01$; scale bar, 50 μm **(A–F)**. Figure from Balaji et al. (2017), with permission.

anti-inflammatory responses to regulate the direction of post-injury wound healing (Strbo et al., 2014; Nosbaum et al., 2016; Larouche et al., 2018).

Following an acute response to injury, adaptive immunity is activated to provide antigen-specific responses. Dendritic cells are specialized cells that capture, process, and present antigens to inactive/naïve B or T lymphocytes. Upon antigen and cognate co-stimulatory signaling, B lymphocytes are activated to provide the humoral production of antigen-specific antibodies. Concurrently, T lymphocytes further differentiate into either effector $CD4^+$ Th lymphocyte subsets to drive immune responses or cytotoxic $CD8^+$ T lymphocyte subsets to clear aberrantly developed, damaged or infected cells. Both $CD4^+$ and $CD8^+$ subsets are under tight control by regulatory T cells, which modulate all immune responses (Strbo et al., 2014).

While both B and T lymphocytes have roles in the development of fibrosis, the significance of T lymphocytes in wound healing can be readily demonstrated in immune deficient models such as athymic mice and severe combined immune deficient (SCID) mice. The athymic mouse models comprise a variety of immune deficient strains that lack functional mature T lymphocytes. Early studies using athymic mice highlight the importance of temporal changes in T cell levels—their early presence is necessary for restoring the biomechanical strength of skin, whereas later introduction of T lymphocytes reduces skin integrity (Barbul et al., 1989). A common denominator among most of these models is the low levels of collagen, high levels of HA, and increased tensile strength of the skin, similar to the profile seen in fetal skin (Gawronska-Kozak et al., 2006). These observations differ in SCID mice, which lack both B and T lymphocytes, where wound healing is accelerated. These mice show faster granulation tissue formation, epithelial growth, and collagen deposition; however, this leads to increased scarring. **Figure 3** demonstrates how the reconstitution of lymphocytes, specifically $CD4^+$ Th lymphocytes, prevents macrophage and neutrophil infiltration and collagen deposition, while increasing microvascular formation. The end result is less inflammation and fibrosis (Wang et al., 2019). On the other hand, the adoptive transfer of B cells in animal models with chronic wounds accelerates wound closure, increases fibroblast infiltration and TGF-β1 expression, and decreases cell apoptosis within the wound space (Sîrbulescu et al., 2017). Though the pathophysiological significance of B and T lymphocytes in post-injury tissue repair have not been specifically defined, the outcomes from their presence within wounds highlight the complexity and multifactorial nature of immune responses to wound healing.

The role $CD4^+$ Th lymphocytes is more nuanced, as $CD4^+$ cells can further differentiate into Th1 or Th2 subsets. Th1 cells are responsible for cellular immune responses while Th2 cells are important in humoral responses. There is a constant interplay between the levels of these cell types, where the cytokines secreted by one suppress the other, allowing the immune response to be targeted to a specific type of pathogen (Romagnani, 2000; Wynn, 2004). These subsets also have opposing roles in the fibrotic response (Wynn, 2004). Cytokines from the Th1 response, such as interferon-γ, create an anti-fibrotic healing profile (Wynn et al., 1995; Widney et al., 2000). However, cytokines from Th2 cells, most importantly IL-13 (Chiaramonte et al., 1999; Saito et al., 2003), accelerate wound healing, but do so at the cost of increased collagen expression and fibrosis (Kumar et al., 2002; Sandler et al., 2003). Interestingly, a predominance of Th2 cells consistently correlates with M1 to M2 macrophage polarization and Treg activation during wound healing, which strongly suggests a role for T lymphocytes in the resolution of

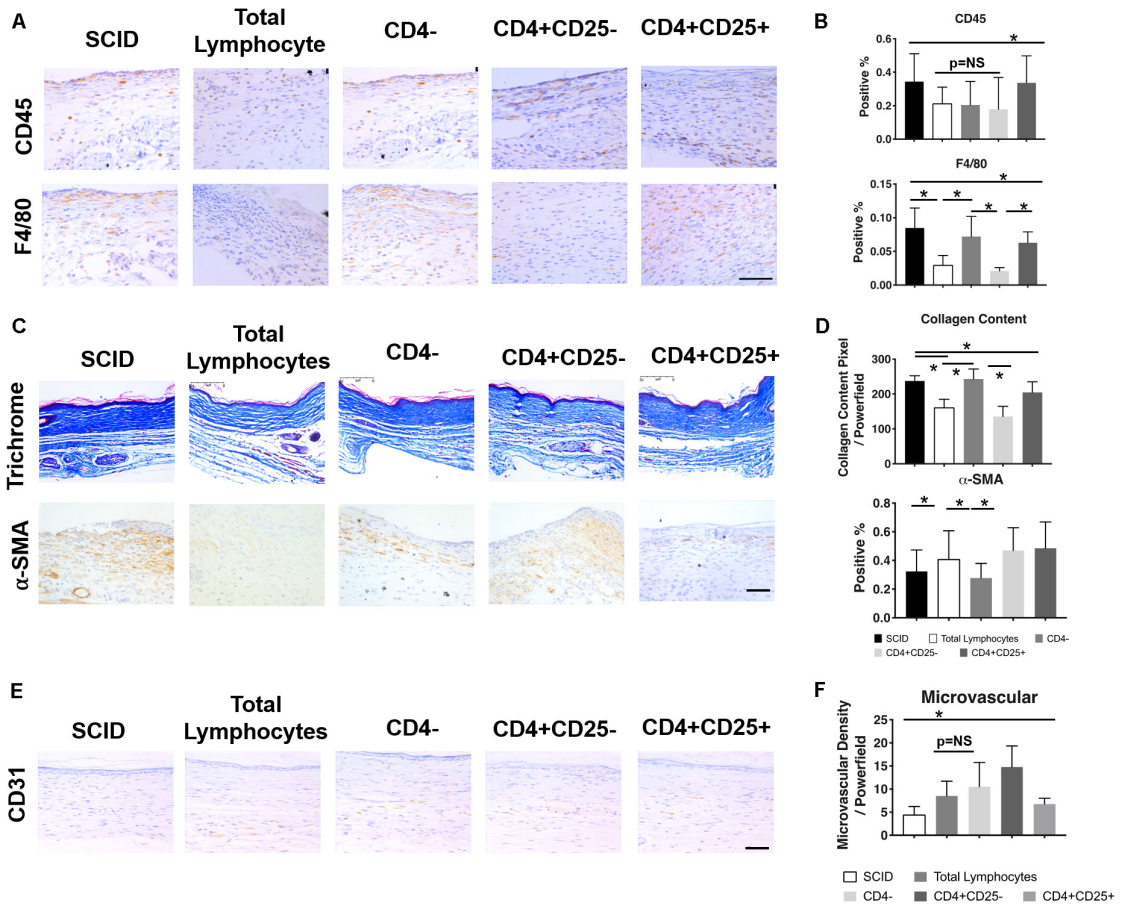

FIGURE 3 | Relationship between T lymphocytes and scarring in severe combined immune deficient (SCID) mice. IHC images **(A)** and quantification **(B)** of $CD45^+$ leukocytes and $F4/80^+$ macrophages show changes in their levels after the introduction of total lymphocytes, $CD4^-$ lymphocytes, $CD4^+CD25^-$ lymphocytes, and $CD4^+CD25^+$ lymphocytes. Similar changes with the introduction of T lymphocytes were seen in collagen content and the presence of myofibroblasts in the trichrome and α-SMA stained tissue, respectively **(C,D)**. Generally, a reduction in pro-inflammatory/pro-fibrotic cells and collagen deposition was observed after the introduction of specific T cell subsets. There was also an increase in microvascularization after the introduction of particular lymphocyte subsets **(E,F)**. $^*p < 0.05$; scale bars, 75 μm **(A)**, 200 μm **(C,F)**. Figure from Wang et al. (2019), with permission.

inflammation and wound regeneration (Mariani et al., 2019). In fact, there is emerging evidence of a dynamic interplay with innate immunity supported by the observation that early neutrophil signaling contributes to subsequent Th2 influence on macrophage M2 differentiation to promote scarless tissue repair (Tacchini-Cottier et al., 2000; Chen et al., 2014; Ma et al., 2016; Mariani et al., 2019). Therefore, the balance between the cytokine profile and immune cell presence is crucial in guiding healing.

$CD4^+$ T Regulatory Lymphocytes

Regulatory T cells comprise a subset of T lymphocytes that are known to modulate the magnitude of immune responses to uphold immune tolerance and control the direction of inflammatory outcomes. Recent studies show that there are distinct subsets of regulatory cells, namely: (1) Tregs characterized by $CD25^+CD4^+$ markers that express the transcription factor Foxp3 and (2) Tr1 cells distinguished by their lack of Foxp3 expression (Pellerin et al., 2014). The presence of Foxp3 propagates a Treg response, which in turn increases tolerance to antigens by directly interacting with effector immune cells and altering cytokine profiles (Roncarolo et al., 2006; Wu et al., 2006; Zhao et al., 2006; Burzyn et al., 2013; Roh et al., 2015). Though both cell types secrete IL-10 to modulate the immune response (Vieira et al., 2004; Hossain et al., 2013), Tr1 cells are antigen specific (Gregori et al., 2010) and employ IL-10 as their primary mechanism of suppressing antigen presenting cell and T cell activity (Gregori et al., 2012).

Tregs and their anti-fibrotic properties are dependent on HA binding via its receptor CD44, which is upregulated on activated T cells and increases Treg signaling. Treg binding of HA via CD44 is associated with an upregulation of Foxp3 and lower proliferation of the immune cells typically involved in an inflammatory response (Bollyky et al., 2007). Furthermore, the increased expression of Foxp3 can act as a signal of Treg activation and the transition toward a reduced immune response (Bollyky et al., 2009).

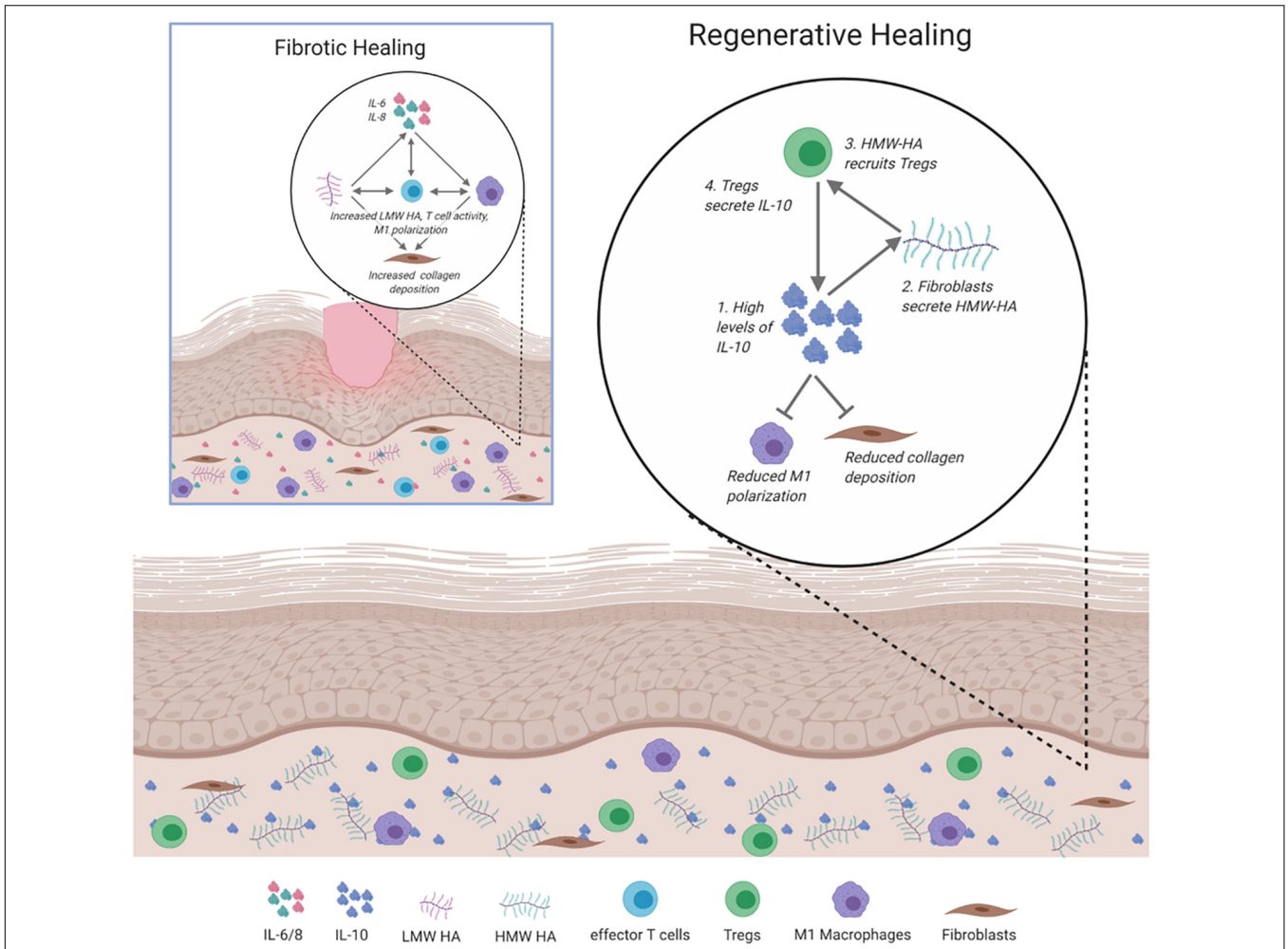

FIGURE 4 | Signaling and cellular regulation pathways involved in fibrotic and regenerative healing. In fibrotic healing, inflammatory signals, including IL-6 and IL-8 activate M1 macrophages to propagate the inflammatory response, activate effector T cells, and recruit fibroblasts to deposit collagen. This is supported by low molecular weight (LMW) HA. Regenerative healing is characterized by reduced inflammation due to the presence of IL-10, HMW HA, and Tregs. IL-10 induces fibroblasts to secrete HMW HA, which results in the increased presence of Tregs and reduced inflammatory macrophage polarization and collagen deposition.

HMW HA, but not LMW HA, also induces differentiation into Tr1 cells, which regulate inflammation via IL-10 (Bollyky et al., 2011).

The capacity of Tregs to remain at the site of the wound has been attributed to their upregulation of the epithelial growth factor receptor (EGFR), which readily binds the mast cell derived ligand amphiregulin (Zaiss et al., 2013; Mariani et al., 2019), enabling access to the wounded tissue for cytoprotective T lymphocytes. Here, Tregs can expand, continue to increase the EGFR ligand, and thus induce the proliferation and differentiation of regenerative tissue repair cell mediators, including progenitor stem cells. When the wound healing response transitions away from inflammation, Tregs are recruited to reduce the presence of $CD45^+$ leukocytes at the site of injury by secreting anti-inflammatory cytokines and by stimulating receptors on T cells that prevent activation. Additionally, by expressing CTLA4, Tregs reduce T lymphocyte interaction with antigen presenting cells, therefore decreasing their co-stimulation (Lei et al., 2015).

Previous studies sustain that $CD4^+$ Th2 lymphocytes influence the anti-inflammatory/fibrotic M1 to M2 transition and regenerative healing, which is supported by an interactive crosstalk with regulatory cells. In this way, Tregs can become the deciding factor to direct homeostatic resolution of inflammation during post-injury tissue repair by increasing the release of anti-inflammatory IL-10 at the wound microenvironment and reducing or even abrogating fibrogenic activity. Tregs can also potentiate anti-inflammatory M1 to M2 macrophage conversion, and can persist and increase their functionality to compensate for the absence of other $CD4^+$ T lymphocyte subsets (Zaiss et al., 2013, 2015; Arpaia et al., 2015; Mariani et al., 2019).

Since Tregs promote anti-inflammatory reactions, it would be expected that they would reduce fibrosis in wound healing, which has been observed in multiple tissue models (Li et al., 2018). For example, in the skin, the presence of Foxp3+

Tregs increased granulation tissue formation and the rate of wound closure (Nosbaum et al., 2016). In cardiac muscle after a myocardial infarction, the presence of Tregs prevented the differentiation of macrophages toward an M1 phenotype (Weirather et al., 2014). In lung tissue, the presence of Tregs led to reduced fibroblast recruitment to the wound (Garibaldi et al., 2013). This data supports the well-established pattern that a reduction in inflammation and maintaining the appropriate time scale for the recruitment of different cell types can improve healing and attenuate scarring.

IL-10 Hyaluronan Axis Regulating Lymphocyte and Fibroblast Crosstalk

These observations discussed so far on inflammation and ECM responses during wound healing support the role of IL-10, HA, $CD4^+$ T cells and fibroblasts in regulating fibrosis. IL-10 promotes the decrease of fibrotic cytokines and regulates the fibroblast phenotype, but also exerts its functions in an HA dependent manner. For instance, IL-10 has the capacity to induce fibroblast differentiation and function to mirror the phenotype and physiological features of fetal fibroblasts and achieve the synthesis and secretion of increased levels of HMW HA (Balaji et al., 2017). Furthermore, HMW HA stimulates $CD4^+$ Th1 to Th2 lymphocyte polarization, via CD44 receptor signaling, to drive regenerative tissue repair. The HMW HA - CD44 axis can also facilitate the expansion and functional performance of the anti-inflammatory, regenerative Treg subset. Through an upregulation of Foxp3 expression, these cells reduce the presence of primary inflammatory cell responders to dermal injury (Bollyky et al., 2007). Tr1 cells also secrete IL-10, which provides positive feedback to auto-regulate the Th lymphocyte repertoire (Bollyky et al., 2011). These $CD4^+$ lymphocyte subsets, such as Th2, Tr1, and $Foxp3^+$ Treg lymphocytes, are in part characterized by IL-10 production. Taken together, these findings may represent the underpinnings of a potential lymphocyte-fibroblast feedback loop. That is, the loop wherein IL-10 promotes HMW HA synthesis in fibroblasts and HMW HA regulates $CD4^+$ lymphocyte IL-10 expression to direct dermal wound healing outcomes. Recent data have indicated a direct crosstalk between $CD4^+$ T lymphocytes and fibroblasts to regulate ECM formation (Gaucherand et al., 2017). Future studies will further elucidate this mechanism and use this knowledge to leverage the potential development of new regenerative therapeutics for dermal wound repair.

CONCLUSION

The complex interplay between the inflammatory responses, ECM composition, and immunity is inherently intertwined in directing how skin wounds heal. The key players that balance pro- or anti-fibrotic responses to injury are summarized in **Figure 4**.

Understanding the framework of mechanisms that coordinate cellular and molecular interactions to result in regenerative or fibrotic wound healing is critical to inform innovative approaches to tissue repair. Ultimately, this knowledge can be leveraged to design and develop next-generation therapeutics to reduce fibrotic responses, and potentially restore the complete integrity of the damaged skin. Initial studies have been performed in humans with local doses of IL-10 at the site of the wounds, leading to reduced fibrosis (Kieran et al., 2013). Similar results have also been obtained in animal models treated with HA and in hypertrophic scars of humans after failed corticosteroid treatments (Mustoe et al., 2002; Hu et al., 2003). The outcomes of ongoing therapies, in addition to studies on fibrosis, underscore the contribution of different cell types, cytokines, and environmental conditions, which include feedback loops between IL-10, HMW HA, and $CD4^+$ lymphocytes. Consideration of all of these factors will be essential to successfully treat or prevent fibrosis and change clinical practice.

AUTHOR CONTRIBUTIONS

SK conceptualized the review. KS drafted the article. All authors discussed the results, and critically edited the article. All authors contributed to the article and approved the submitted version.

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REVIEW
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Mesenchymal Stem Cells Adaptively Respond to Environmental Cues Thereby Improving Granulation Tissue Formation and Wound Healing

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Granulation tissue formation constitutes a key step during wound healing of the skin and other organs. Granulation tissue concomitantly initiates regenerative M2 macrophages polarization, fibroblast proliferation, myofibroblast differentiation with subsequent contraction of the wound, new vessel formation, and matrix deposition. Impaired granulation tissue formation either leads to delayed wound healing or excessive scar formation, conditions with high morbidity and mortality. Accumulating evidence has demonstrated that mesenchymal stem cell (MSC)-based therapy is a promising strategy to ameliorate defects in granulation tissue formation and to successfully treat non-healing chronic wounds. In this review we give an updated overview of how therapeutically administered MSCs ensure a balanced granulation tissue formation, and furthermore discuss the cellular and molecular mechanisms underlying the adaptive responses of MSCs to cue in their direct neighborhood. Improved understanding of the interplay between the exogenous MSCs and their niche in granulation tissue will foster the development of MSC-based therapies tailored for difficult-to-treat non-healing wounds.

Keywords: mesenchymal stem cells, environment sensing, wound healing, granulation tissue, scarring, myofibroblasts, proliferation, inflammation

INTRODUCTION

Cutaneous wound healing is characterized by three distinct yet overlapping phases including inflammation, proliferation, and remodeling (Singer and Clark, 1999; Wlaschek and Scharffetter-Kochanek, 2005; Eming et al., 2007). Granulation tissue formation is the hallmark of the proliferation phase. During granulation tissue formation, the polarization of reparative M2 macrophages, proliferation and differentiation of contractile myofibroblast, wound contraction, new vessel formation, and matrix deposition are concomitantly initiated (Gabbiani et al., 1971; Flanagan, 1998; Gabbiani, 1998; Hinz and Gabbiani, 2003; Hinz, 2007, 2016; Hinz et al., 2012; Jiang and Rinkevich, 2020). Recent studies revealed that myofibroblasts in granulation tissue derived

from Engrailed-1 (En1)-positive lineage by lineage tracing studies (Rinkevich et al., 2015; Jiang et al., 2018) or from a subset that expresses delta like non-canonical Notch ligand 1 (Dlk-1) as the surface marker (Driskell et al., 2013). The initial cells and extracellular matrix proteins in the granulation tissue are mainly contributed by the subcutaneous fascia that underneath the dermal layer (Correa-Gallegos et al., 2019). Aberrations in granulation tissue formation result in non-healing chronic wounds or excessive scarring (Wynn and Ramalingam, 2012; Hu et al., 2018), both constitute major concerns for global healthcare.

Mesenchymal stem cells (MSCs) are multipotent progenitor cells residing in different tissues including skin (Hocking and Gibran, 2010; Jiang and Scharffetter-Kochanek, 2015). Apart from self-renewal and their differentiation capacity into several lineages, their paracrine impact on distinct resident and recruited cells is essential for tissue homeostasis and wound repair. Their relationship to En1 lineage positive fibroblasts or Dlk-1 positive fibroblasts is currently unresolved. Employing single cell sequencing analysis combined with *in vivo* tracing analysis and genetic deletion of quiescence or other markers have recently allowed to better define the origin and the lineage hierarchy of mesenchymal progenitors and stem cells in tissue and organ regeneration (Scott et al., 2019; Soliman et al., 2020).

MSCs have emerged as a potentially promising therapy for wound healing disorders of the skin and other organs. So far, adult tissue-derived MSCs have not entered clinical routine as fully approved products for the treatment of non-healing wounds, likely due to the unresolved hurdles with homogeneous isolation, and reliable and reproducible potency (Galipeau and Sensebe, 2018; Martin et al., 2019). However, there is ample evidence that MSCs enhance wound healing in preclinical wound models when applied on acute wounds (Li et al., 2006; Jiang et al., 2013), and more importantly to chronic wounds (Falanga et al., 2007; Javazon et al., 2007; Dash et al., 2009; Akita et al., 2010; Jimenez et al., 2012). MSCs promote granulation tissue formation, angiogenesis, and re-epithelialization, leading to accelerated wound closure. Moreover, MSCs establish a regenerative rather than fibrotic microenvironment, resulting in improved healing quality (Jiang and Scharffetter-Kochanek, 2015).

Accumulating evidence suggests that MSCs are endowed with the capacity for environmental sensing during granulation tissue formation and are able to mount adaptive responses, a superior property compared to conventional chemical-based or recombinant growth factor-based therapies (Basu et al., 2018; Vander Beken et al., 2019; Jiang et al., 2020b; Munir et al., 2020). This adaptive property allows for refinement of MSC-based therapies in near future. The application of MSCs at high numbers into wounds is currently the gold standard over manipulation of MSCs in their endogenous niche. In fact, endogenous MSCs are either depleted in chronic wounds or are themselves part of the pathogenic event (Lim et al., 2019) and, in consequence, cannot contribute to proper healing. In this review, we give recent cellular and molecular insights into how therapeutically applied MSCs behave as an environment sensing adaptive drugstore in granulation tissues during skin wound healing.

FUNCTIONAL ROLES OF MSCs IN REGULATING GRANULATION TISSUE FORMATION

MSCs Improve Fibroblast Proliferation and Myofibroblast Activation

Impaired fibroblast proliferation leads to deficient granulation tissue formation. Fibroblast senescence, insufficient transforming growth factor beta 1 (TGF-β1) and platelet-derived growth factor (PDGF) signaling is known to result in defects of granulation tissue formation, which substantially delays the onset of proliferation phase. This is consistently observed in aging-related chronic wounds (Jiang et al., 2020a), diabetic foot ulcers (Falanga, 2005), pressure ulcer (Stanley et al., 2005), and chronic venous ulcers (Hasan et al., 1997; Agren et al., 1999).

The initial upsurge of MSC research was focused on their differentiation and transdifferentiation potential to replace the damaged tissue. Sasaki and colleagues reported that bone marrow-derived MSCs (BM-MSCs) are recruited into the wounds and contribute to wound repair by transdifferentiation into keratinocytes, endothelial cells and pericytes (Sasaki et al., 2008). Later on, accumulating evidence suggest that the engraftment efficiency and integration rate of exogenous MSCs in the repaired wounds are low. The contribution of MSCs to granulation tissue is predominantly through their trophic, paracrine and immunomodulatory functions by secretion of growth factors, proangiogenic factors, cytokines and chemokines, such as TGF-β, insulin-like growth factor-1 (IGF-1), prostaglandin E2 (PGE2), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF), basic fibroblast growth factor (FGF-2), vascular endothelial growth factor (VEGF), angiopoietin-1, stromal cell-derived factor-1 (SDF-1), erythropoietin, monocyte chemoattractant protein-1 (MCP-1). More details are reviewed in Murphy et al. (2013); Jiang and Scharffetter-Kochanek (2015); Hu et al. (2018).

MSCs transplanted into full-thickness wounds augment proliferation of fibroblasts, and activate the fibroblast-to-myofibroblast transition. In physiological wounds, adipose tissue-derived MSCs (AT-MSCs) delivered by a medical-grade silicone carrier coated by plasma polymerization with a thin layer of acrylic acid (ppAAc) accelerate wound healing by promoting granulation tissue formation featured with higher numbers of alpha-smooth muscle actin $(\alpha\text{-SMA})^+$ myofibroblasts and $CD31^+$ vessel endothelial cells (Jiang et al., 2013). The ppAAc coating preserves the cellular properties of MSCs grown on the carrier and possesses the high delivery efficiency of MSCs when the carrier is applied topically to the wounds (Jiang et al., 2013).

Pathological conditions with defective granulation tissue formation often lead to chronic wounds. Patients with leukocyte adhesion syndrome type I (LAD-1), which is due to mutations in the *CD18* gene encoding the common β chain of β2 integrins, typically show impaired wound healing with reduced wound contraction and, in consequence, life-threatening infections (Anderson and Smith, 2001). The follow-up study with

CD18-deficient ($CD18^{-/-}$) mice, closely mimicking human LAD-1, revealed that delayed healing of full-thickness excisional wounds was due to severely impaired formation of granulation tissue and impaired wound contraction (Scharffetter-Kochanek et al., 1998). In this model, $CD18^{-/-}$ neutrophils cannot bind to endothelial cells, thus cannot extravasate from vessels into the wound site. In addition, $CD18^{-/-}$ neutrophils are resistant to apoptosis (Coxon et al., 1996; Weinmann et al., 2003). Therefore, the number of apoptotic neutrophils at the wound site was dramatically reduced. In addition, due to the loss of β2 integrins (CD18 deficiency), macrophages are unable to adhere to and subsequently phagocytose apoptotic neutrophils. Lacking this stimulus, the wound macrophages are incapable of secreting TGF-β1 (Peters et al., 2005). In consequence, the myofibroblast activation was substantially dysregulated with significantly reduced expression of the myofibroblast markers α-SMA and fibronectin in $CD18^{-/-}$ wounds (Peters et al., 2005). Recently, we have demonstrated that the xenotransplantation of human AT-MSCs to $CD18^{-/-}$ wounds are able to restore the low TGF-β1 concentration in the wound microenvironment of $CD18^{-/-}$ mice. Through the release of human TGF-β1, transplanted MSCs promotes the proliferation and myofibroblast differentiation from resident mouse fibroblasts in wounds, profoundly enhances wound contraction and vessel formation, rescues granulation tissue formation, and accelerates wound healing in the $CD18^{-/-}$ model (Jiang et al., 2020b). Several other frequently occurring chronic wounds are affected by a persistent inflammation phase and an almost complete lack of granulation tissue formation. Some of them will be discussed in more detail in section "MSCs Function as Guardians of Inflammation During Granulation Tissue Formation."

MSCs Reduce Contraction and Improve the Quality of Wound Healing

By contrast to conditions with reduced wound contraction, "over-healing" with hyperproliferation of fibroblasts and/or persistence of myofibroblasts in the granulation tissue cause contracture, fibrosis, and scarring frequently occurring in hypertrophic scars and keloid scars (Wynn and Ramalingam, 2012; Ud-Din and Bayat, 2020). MSC-based therapy can ameliorate fibrosis and promote tissue regeneration through suppressing wound contraction and tension (Uysal et al., 2014), reducing the number of myofibroblasts at the end of the proliferation stage (Stoff et al., 2009), and increasing wound tensile strength (McFarlin et al., 2006).

TNF-stimulated gene-6 (TSG-6) released from BM-MSCs is responsible for the reduction of granulation tissue formation at late stage of wound healing. MSC-derived TSG-6 suppresses the release of TNF-α from activated macrophage and concomitantly induces a switch from a high to an anti-fibrotic low TGF-β1/TGF-β3 ratio, which results in lower expression of α-SMA and less collagen deposition in the granulation tissue. Therapeutically administered MSCs also influence the collagen fiber arrangement in the restoration tissue of healed wounds. Compared to densely packed and disorganized thick collagen fibers in control scars, collagen fibers in MSC-injected scars are thinner and organized in a basket-weave pattern, which significantly enhance the tensile strength of the healed wounds (Qi et al., 2014). Human BM-MSCs engraftment inhibited the hypertrophic scarring in a rabbit ear hypertrophic scar model, through increased secretion of TSG-6 from MSCs when they undergo apoptosis (Liu et al., 2014). Interestingly, polymorphisms in the *TSG-6* gene enhance susceptibility to hypertrophic scar and keloid formation. Also, TSG-6 expression is significantly reduced in hypertrophic scars and keloids (Tan et al., 2011). Prostaglandin E2 (PGE2) has been suggested to be another anti-scarring molecule that upregulated in transplanted BM-MSCs in wounds (Nemeth et al., 2009). It reprograms T cell and macrophage responses, and subsequently induces IL-10 release and prevents excessive collagen deposition (Liechty et al., 2000). In addition, nitric oxide produced by MSCs in the wound can scavenge ROS and may prevent tissue fibrosis (Ferrini et al., 2002; Jackson et al., 2012). Of note, a recent study reveals that grafting AT-MSCs enriched fat reduces radiation-induced skin fibrosis and joint contracture, also reduces skin stiffness, increases skin elasticity, and decreases the proportions of pro-fibrotic $CD26^{+}Dlk1^{+}$ fibroblasts in the irradiated skin (Borrelli et al., 2020).

MSCs Function as Guardians of Inflammation During Granulation Tissue Formation

Prolonged inflammation in wounds profoundly hinders granulation tissue formation and delays wound healing. For example, the defect in the transition of pro-inflammatory M1 to anti-inflammatory M2 leads to persistent release of TNF-α and hydroxyl radical from over-activated M1 macrophages. This persistent oxidative attack induces senescence of wound fibroblasts and causes non-healing or chronic wounds, often occurs in venous leg ulcers, peripheral occlusive arterial, pressure and diabetic ulcers (Mirza and Koh, 2011; Sindrilaru et al., 2011; Mirza et al., 2013; Krzyszczyk et al., 2018). Senescent fibroblasts adopt a senescence associated secretory phenotype (SASP) with persistent release of pro-inflammatory cytokines, chemokines and matrix-degrading metalloproteinases, among them TNF-α, IL-1 and IL-6, which in consequence, fuel a vicious cycle of M1 macrophage activation further delaying the switch to M2 macrophages and granulation tissue formation (Mirza and Koh, 2011; Sindrilaru et al., 2011).

The anti-inflammatory property enforces the most prominent beneficial effects of MSC-based therapies, which has been discussed in detail in a few excellent reviews (Prockop and Oh, 2012; Wang et al., 2016; Regulski, 2017; Munir et al., 2018). Despite the initial lung entrapment, exogenously administered MSCs migrate along cytokine gradients toward inflamed tissue and home to sites of injury, where they suppress inflammation in their local environments (Rustad and Gurtner, 2012). Therefore, they are ideally suited for the therapy of chronic wounds which are characterized by unrestrained and prolonged inflammation.

In physiological (acute) wounds, MSCs suppress the TNF-α-dependent inflammation and thereby increase anti-inflammatory

M2 macrophage numbers (Jiang et al., 2013), through MSC-derived TSG-6 (Qi et al., 2014). Moreover, injection of dermal MSCs expressing ATP binding cassette subfamily B member 5 (ABCB5) into an iron overload chronic wound model in mice, closely mimicking chronic venous leg ulcers in patients, the release of interleukin-1 receptor antagonist (IL-1RA) from ABCB5$^+$ MSCs was observed to fundamentally dampen inflammation and to shift the prevalence of unrestrained pro-inflammatory M1 macrophages toward repair promoting anti-inflammatory M2 macrophages at the wound site (Vander Beken et al., 2019). The good manufacturing practices (GMP)-grade ABCB5$^+$ MSCs fulfill the regulatory requirements to be employed for the treatment of chronic wounds in humans (Tappenbeck et al., 2019). Of note, clinical phase IIa studies have recently been initiated (EudraCT numbers: 2015-000399-81, 2017-000233-31, and 000234-57) with promising results of the first studied patients suffering from CVU.

MSCs also ameliorate unrestrained neutrophil activation as reported in a mouse model of vasculitis. This beneficial MSC effect is enforced by augmenting intercellular adhesion molecule-1 (ICAM-1) on macrophages to engulf apoptotic neutrophils and, furthermore, by detoxifying the reactive oxygen species (ROS)-rich environment through enhanced release of soluble extracellular superoxide dismutase (SOD3) from injected AT-MSCs (Jiang et al., 2016, 2017a,b).

MSCs Support Immune Response to Clear Infections

An interesting phenomenon has been observed during the MSC-based trials that under immunosuppressive regimens, the infection rates are not increased (Regulski, 2017). In line with that, cumulative data indicate that the immunoregulatory function of MSCs reveals high plasticity. In contrast to the strong immunosuppressive effect of MSCs on allogenic responses and persistent sterile inflammation, MSCs do not appear to suppress immune cell functionality in the presence of infectious agents, but rather facilitate the clearance of infections and accelerate wound repair.

The immunoregulatory fate of MSCs can be switched in accordance with the dynamics of inflammation, which depends on the strength of activation of the immune system, the types of inflammatory cytokines present (Wang et al., 2014). *In vitro*, the plasticity of mouse BM-MSC-mediated immunomodulation in response to fluctuations in inflammation levels has been demonstrated by the titration of IFN-γ and TNF-α (Li et al., 2012). Moreover, antigen-pulsed human BM-MSCs stimulated with a low dose of IFN-γ act as antigen-presenting cells and can thus activate antigen-specific cytotoxic CD8$^+$ T cells (Chan et al., 2006). During viral infection, human BM-MSCs do not inhibit cytotoxic T lymphocyte (CTL) functions, but rather facilitate CTL responses by releasing interferon-gamma (IFN-γ) (Kang et al., 2005). In mice suffering from chronic *Staphylococcus aureus* infection with biofilm formation, therapeutically administered human BM-MSCs, when combined with antibiotics, significantly reduce bacterial numbers at the wound site and improve wound healing. The beneficial MSC effects on wound healing is due to the secretion of antimicrobial peptides and triggering the bactericidal functions of neutrophils such as phagocytosis and neutrophil extracellular trap (NET) formation (Chow et al., 2020). Recent clinical trials indicate that autologous transplantation of MSCs could vastly improve outcomes for patients suffering from multidrug resistant strains of *Mycobacterium tuberculosis* (Skrahin et al., 2014, 2016). MSC-based therapy has also demonstrated beneficial effects against parasite infections such as Malaria (Souza et al., 2015) and *Schistosoma japonicum* (Xu et al., 2012).

A simplified scheme elucidating the modulatory roles of MSCs on myofibroblast activation and immune response during granulation tissue formation is summarized in **Figure 1**.

CELLULAR AND MOLECULAR MECHANISMS UNDERLYING THE ADAPTIVE RESPONSES OF MSCs

It is fascinating that MSCs can adaptively restore and attenuate defects in granulation tissue formation independent of whether myofibroblast proliferation is pathologically reduced or enhanced. Similarly, MSCs adaptively regulate the immune response depending on the requirements of the tissue and the organism. The molecular mechanisms underlying these seemingly reciprocal responses of MSCs have started to be unraveled in the recent decade. As to the question why MSCs have evolved sensing mechanisms and the ability to raise distinct adaptive responses to changing environmental cues is currently not fully understood. An interesting possibility could be that stem cells with regenerative potential and self-renewal capacity to maintain and recover tissue homeostasis would profit from protecting their blueprint, the DNA. Controlling noxious oxidative attacks, inflammation and infection by sensing and shaping their neighborhood would very much support DNA protection. Here we give an overview of how MSCs adaptively respond to trophic factors, inflammation, oxidative and mechanical stresses (**Figure 2**).

MSCs Act as Rheostat of Trophic Factors to Re-establish Tissue Homeostasis

TGF-β signaling is the most important pathway that determines granulation tissue formation and wound healing outcome (Desmouliere et al., 1993; Margadant and Sonnenberg, 2010; Lichtman et al., 2016; Jiang and Rinkevich, 2020). Deficiency in TGF-β1 expression as in CD18$^{-/-}$ (Peters et al., 2005) or dysregulated TGF-β1 signaling as in diabetic foot ulcers (Badr et al., 2012; Zhang et al., 2016) and chronic venous leg ulcers (Sindrilaru et al., 2011) result in chronic non-healing wounds, whereas excessive TGF-β1 leads to contracture and scarring as seen in hypertrophic scars and keloid scars (Jagadeesan and Bayat, 2007; Chalmers, 2011; Wynn and Ramalingam, 2012). Therefore, supplementation of not too much or too little TGF-β1 would be the preferred ideal situation. This, however, is technically difficult to achieve with recombinant TGF-β1 protein,

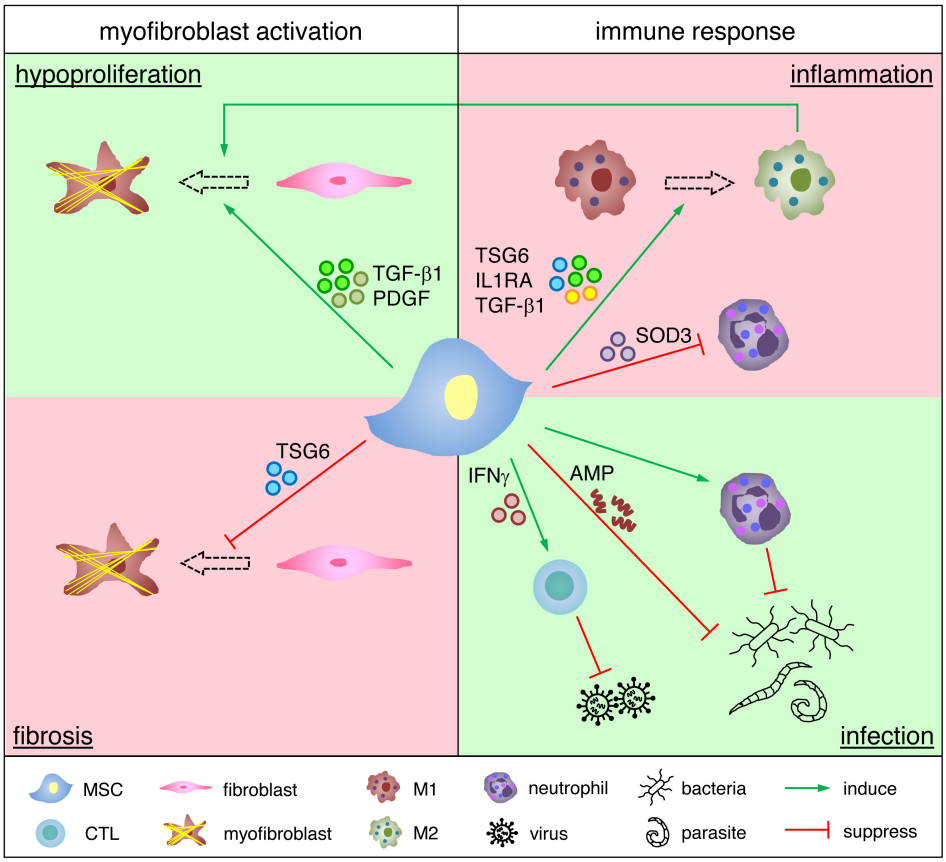

FIGURE 1 | Functional roles of MSCs in regulating granulation tissue formation. MSCs promote fibroblast proliferation and myofibroblast differentiation in a hypoproliferative environment, whereas they suppress myofibroblast activation and reduce myofibroblast numbers in a fibrotic environment. MSCs induce M1 to M2 transition and inhibit neutrophil activation in an over-inflamed environment, whereas they support cytotoxic T lymphocytes (CTL) and neutrophils for the clearance of infectious agents. Due to space limitation only selected examples of how MSCs impact on granulation tissue are depicted.

in particular as the requirement for TGF-β1 dynamically changes during tissue repair and may not be the same in different wounds and different individuals. Recently, we have shown that human AT-MSCs are endowed with the unique sensing capacity for environmental TGF-β1 levels *in vitro* and *in vivo* at the wound site.

The sensing of actual TGF-β1 concentrations in the environment is initiated by binding of TGF-β1 to TGFβ receptors on MSCs. The activation of TGF-β receptors either suppress or upregulate the expression level of microRNA-21 (miR-21), depending on the TGF-β1 concentrations at the wound site. In murine LAD-1 wounds that represent an excellent model for chronic wounds with a profound TGF-β1 deficient state, activation of TGFβ receptors induces miR-21, which blocks the translation of Smad7, a negative regulator of TGF-β signaling. This, in consequence, promotes enhanced TGF-β1 release from MSCs. By contrast, overactivation of TGFβ receptors on MSCs at high TGF-β1 concentrations down-regulates miR-21 and, thus, no longer inhibits Smad7 translation, eventually suppressing the release of TGF-β1 from MSCs (Jiang et al., 2020b). The signaling circuit of TGF-β receptor – miR-21 – Smad7 – TGF-β1 adapts the release of MSC-derived TGF-β1 exactly to the demands at the wound site (**Figure 2**).

Pattern Recognition and Danger Molecule Sensing

When a robust immune response is necessary to clear invading pathogens or to initiate repair responses, this is mainly initiated by a large amount of pathogen-associated molecular pattern (PAMP) and/or danger-associated molecular pattern (DAMP) at the wound site or in the endogenous environment of MSCs.

At the injury site, therapeutically applied MSCs have the capacity to mount an adaptive response by sensing PAMP via toll-like receptors (TLRs). The activation of TLR4 signaling in human AT-MSCs by the bacterial wall component lipopolysaccharide (LPS) increases the release of CXCL-5 (GCP2), CXCL-6 (ENA78), IL-1β and IL-8, and thereby augments the recruitment and activation of neutrophils and macrophages. Due to phagocytosis of increased numbers of apoptotic neutrophils, macrophages release more TGF-β1 and subsequently promote a strong myofibroblast-driven wound contraction and profoundly accelerate wound healing (Munir et al., 2020). In another study,

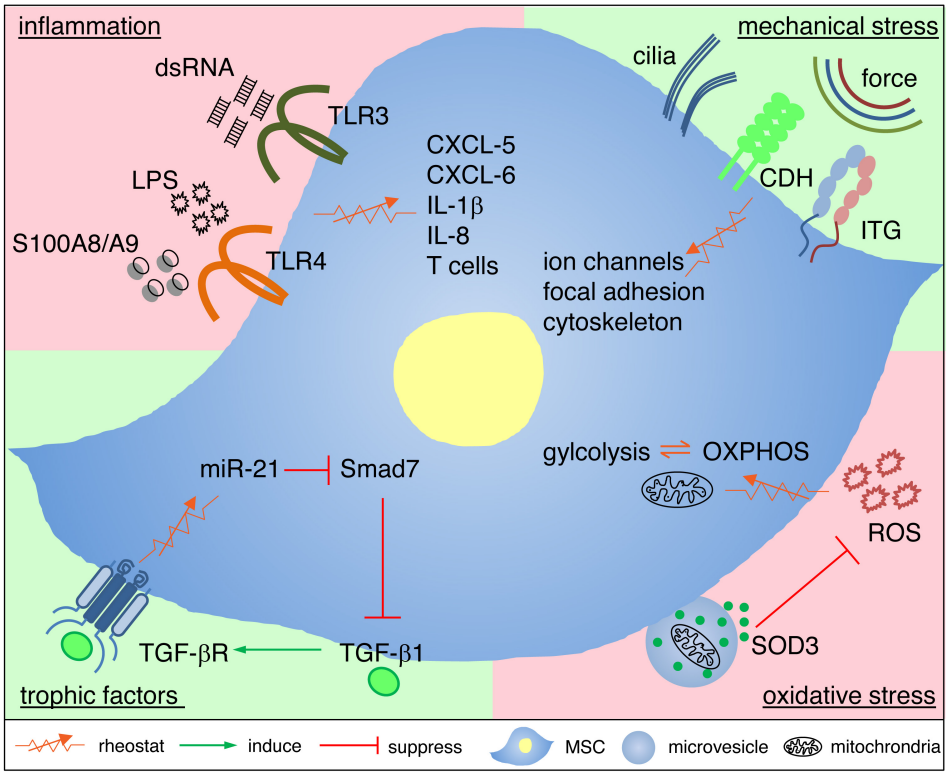

FIGURE 2 | Cellular and molecular mechanisms underlying adaptive responses of MSCs. MSCs utilize rheostatic (sensing) mechanisms to respond to the local environment in granulation tissue with deficient or excessive trophic factors, inflammatory mediators, oxidative stress or mechanical stress. This adaptive response to cues from the microenvironment depends on distinct intracellular signaling, changes of the transcriptome and metabolic reprogramming. Adaptive MSC responses, in consequence, rebalance the partly hostile microenvironment, and thus protect themselves by rebalancing their niche, and this favors a shift to healing and tissue homeostasis. For further details see main text. CDH, cadherins; ITG, integrins; OXPHOS, oxidative phosphorylation, ROS, reactive oxygen species.

Liotta and colleagues have shown that ligation of TLR3 and TLR4 on human BM-MSCs leads to the temporary inhibition of MSCs to suppress T cell proliferation. This is achieved by downregulation of the Notch ligand Jagged-1 expressed on the surface of MSCs, and thus blocking MSC's suppressive instructions to the interacting T cells through their Notch receptors (Liotta et al., 2008). The authors hypothesize that this is an effective mechanism to block the immunosuppressive activity of MSCs and therefore to restore an efficient T-cell response in the course of dangerous infections sustained by double-stranded RNA viruses or Gram-negative bacteria. Of note, activation of TLRs do not affect the immunophenotype or differentiation potential of MSCs (Liotta et al., 2008).

Although S100A8/A9 (MRP8/14), a DAMP and an endogenous TLR4 ligand, primed AT-MSCs also lead to accelerate wound healing, the adaptive response of MSCs to S100A8/A9 is quite different when compared to that to LPS priming (Basu et al., 2018). Changes in the transcriptome of LPS primed MSCs enhance mainly the numbers and the activity of neutrophils, and thereafter in the second instance macrophages are activated following engulfment of apoptotic neutrophils with enhanced release of TGF-β1 (Munir et al., 2020). By contrast, in S100A8/A9 primed MSCs mainly a transcriptome responsible for "the big clean up" of tissue debris at the wound site was observed with increased expression of MSCs genes which upon release of the encoded proteins enhance the phagocytotic capacity of macrophages. In addition, genes enhancing niche protection and energy homeostasis most likely play a major role in accelerating wound healing after injection of S100A8/A9 primed MSCs (Basu et al., 2018). It would be interesting to tease out how MSCs are able to mount distinct adaptive responses in their transcriptome through TLR4 in a ligand-specific manner.

TLR signaling in MSCs is regulated by various miRNAs (Abdi et al., 2018). For example, high miR-21 expression can enhance TLR signaling by its transcriptional suppression of Wnt (Fafian-Labora et al., 2017), whereas let-7b miRNA suppresses TLR signaling by directly targeting TLR4 (Ti et al., 2015). The interplay between TLR pathway and miRNAs adds further complexity and flexibility to TLR signaling of licensed MSCs in response to their changing environment.

Adaptive Metabolic and Antioxidant Responses

In their native environment, MSCs are in a quiescent state characterized by low proliferation and high multi-potentiality. In this state, MSC metabolism is primarily maintained by glycolysis (Katajisto et al., 2015). Upon wounding, rapid proliferation for

tissue repair and the demand for enhanced ATP production in MSCs depends significantly more on oxidative phosphorylation (Pattappa et al., 2011). This metabolic shift, likely mediated through hypoxia-inducible factor (Ma et al., 2009), regulates the global MSC secretome and exosome biosynthesis (Yuan et al., 2019), and thereby can tightly control MSCs and their paracrine response to environmental cues at the injury site.

Impaired mitochondrial function has been associated with chronic wounds featured by persistent inflammation and impaired granulation tissue formation (Peppa et al., 2009). Particularly, in diabetic foot ulcers, fibroblasts and endothelial cells bearing damaged mitochondria are prone to apoptosis, which further impairs the cellularity of granulation tissue, angiogenesis and perfusion of the newly forming restoration tissue with oxygen and nutrients (Berlanga-Acosta et al., 2013). MSCs are able to respond to such mitochondria malfunction under stress or injury by transferring mitochondria to damaged and stressed cells at the wound site (Murray and Krasnodembskaya, 2019). The mitochondria transfer between MSCs and target cells can be uni-directional (Court et al., 2020) or bi-directional (Figeac et al., 2014) and occurs via tunneling nanotubes or microvesicles, depending on local environmental cues.

It was first reported in 2006 that mitochondria and mitochondrial DNA (mtDNA) can be actively transferred from human BM-MSCs to ethidium bromide pretreated recipient cells with mutated and depleted mtDNA, to rescue the aerobic respiration in recipient cells (Spees et al., 2006). The *in vivo* evidence that the transfer of intact mitochondria can contribute to tissue repair was provided several years later. BM-MSCs attached to LPS-injured mouse alveolar epithelial cells in a connexin-43 gap junction-dependent manner, and transfer intact mitochondria by forming nanotubes and microvesicles. The mitochondrial transfer resulted in increased alveolar ATP concentrations and contribute to tissue repair (Islam et al., 2012). Under oxidative stress, MSCs employ the arrestin domain-containing protein 1 (ARRDC1)-mediated microvesicles for transfer of healthy mitochondria to macrophage. This mitochondria transfer results in enhanced bioenergetics in macrophages, while decreasing intracellular oxidative stress in MSCs (Phinney et al., 2015). Similarly, in response to an inflammatory environment, human MSCs from varying sources including bone marrow, menstrual blood and umbilical cord, have been shown to transfer mitochondria to T cells. Surprisingly, it results in increased glycolysis in T cells and induce differentiation and activation of $CD25^{+}FoxP3^{+}$ regulatory T cells (Court et al., 2020). Such adaptive responses of MSCs restrict inflammation, reduces tissue damage and expedites the repair machinery.

Another mechanism of an adaptive MSC response to protect against elevated oxidative stress in the local environment is the release of SOD3, a soluble form of superoxide dismutase, which detoxifies superoxide anion radicals in their niche. Therapeutically injected AT-MSCs utilize this mechanism to suppress unrestrained neutrophil activation and to alleviate severe tissue damage in a murine immune-complex mediated vasculitis model. In consequence, AT-MSCs reduce the superoxide anion concentrations in the microenvironment and consequently prevented enhanced neutrophil death, neutrophil extracellular trap formation and spillage of matrix degrading neutrophil elastase, gelatinase and myeloperoxidase (Jiang et al., 2016).

Sensing Mechanical Stress

MSCs sense mechanical stimuli such as tissue stiffness, compression, tension and hydrostatic pressure. These parameters are often changed in granulation tissue during wound healing and are abnormal in pathological scars and contractures (Delaine-Smith and Reilly, 2012; Steward and Kelly, 2015).

The adhesion of MSCs to matrix and other cells are key for the mechanotransduction of MSCs. Therefore, integrins responsible for cell-matrix binding, and cadherins responsible for cell-cell binding, are hypothesized to function as mechanosensors. For instance, β3 integrin mediates myogenic differentiation of BM-MSCs on soft matrix (Yu et al., 2013), whereas α2 integrin enforces osteogenic differentiation on stiff substrate (Shih et al., 2011). Cadherin-2 is mandatory for myogenesis of MSCs (Gao et al., 2010), and Cadherin-11 is involved in establishing a profibrotic niche with active TGF-β (Lodyga et al., 2019).

The engagement of integrins and cadherins triggers downstream events including ion channel opening for Ca^{2+} influx (Mizuno, 2005; Liedert et al., 2008), focal adhesion assembly with focal adhesion kinase (FAK) activation (McBeath et al., 2004) and cytoskeleton remodeling (Lv et al., 2015), to promote different cellular responses and MSC differentiation. Recently, microtubule-based organelles called primary cilia extending dynamically from human BM-MSCs have been observed. They serve as "multifunctional antenna" that sense both chemical and mechanical signals (Hoey et al., 2012a,b).

MSCs not only respond to mechanical cues, but can also acquire a memory of stiffness of the local environment, even 2 weeks after the initial stimulation. The memory of stiffness is mediated by miR-21, under the control of myocardin-related transcription factor-A (MRTF-A) (Li et al., 2017). This is interesting as miR-21 is also required for sensing and responding to environmental TGF-β1 (Jiang et al., 2020b), and TGF-β1 regulates collagen synthesis and tissue contraction both contributing to stiffness.

It is worthy to note that the current knowledge of molecular details of the mechanosensing is mainly derived from *in vitro* systems with 2D or 3D MSC cultures. It is known that 2D culture especially with stiff plastic surface drastically changes the cellular properties of MSCs, including morphology, migration mechanism, differentiation capacity, gene expression profile and subsequent secretome. Engineered 3D culture systems that eliminate apical-basal polarization while still paying attention to both cellular access of soluble nutrients and physical constraints on cell morphology and proliferation are closer to the mechano-environment of the *in vivo* condition (Smith et al., 2017; Zhou et al., 2017; Doolin et al., 2020). However, *in vivo* situations are far more complex and clinically relevant given the unresolved fibroproliferative conditions of keloid and scar formation. Therefore, *in vivo* studies which further advance our understanding how MSCs sense and integrate

downstream signals from multiple mechanosensors, and how MSCs subsequently respond to shape their surrounding matrix will be of major interest for future investigations.

A DISRUPTED MSC NICHE LEADS TO LOSS OF REGENERATIVE POTENTIAL AND PROMOTES SCARRING

Tissue resident MSCs are mainly found at perivascular locations in different organs including skin (Crisan et al., 2008; Dulauroy et al., 2012; Yamanishi et al., 2012; Lemos and Duffield, 2018; Vander Beken et al., 2019). This location allows MSCs to rapidly detect local cues within the vessels and adjacent tissue, including the occurrence of PAMPs and DAMPs or the reduction in O_2 concentrations. Recently, it became evident that if the endogenous tissue resident MSCs is forced to leave their homeostatic niche, or in case they cannot adequately deal and adapt to the changing environment, they become a source of myofibroblasts in fibrotic diseases (El Agha et al., 2017; Lemos and Duffield, 2018). This is a very important and clinically relevant notion which imposes high responsibility on medical doctors to consider the original niche of stem cells before transplanting them in a therapeutic intent into a non-fitting environment. In fact, inconsiderate injection of AT-MSCs into the eye to treat aging-related macular degeneration, the major cause of blindness in the elderly, resulted in enhanced formation of myofibroblast. Due to their contractile properties, the myofibroblasts tear off the retina with a sudden and result in vision loss (Kuriyan et al., 2017).

Two very recent studies have identified a subpopulation of perivascular MSCs in the heart and muscle, which are characterized by the expression of PDGFRα, stem cell antigen-1 (Sca-1, Ly6A) and the tumor repressor hypermethylated in cancer 1 (Hic1) (Scott et al., 2019; Soliman et al., 2020). Both studies have shown that $Hic1^+$ MSCs play important roles in the homeostasis and repair of heart and skeletal muscle, differentiate into different cell types including fibroblasts and are kept in a resting state by Hic1 (Kim and Braun, 2020). The transcription repressor Hic1 is required for maintaining quiescence, since deletion of Hic1 leads to activation and expansion of MSCs, and subsequently leads to pathological fibrosis of heart (Soliman et al., 2020) and skeleton muscle (Scott et al., 2019), respectively. The exact molecular targets of Hic1 remain elusive.

In skin, dermal $ADAM12^+$ MSCs, or $Dlk1^+$ MSCs at a preferred perivascular niche are responsible for the maintenance of tissue turnover and homeostasis (Desmouliere et al., 1993; Driskell et al., 2013). However, during skin wounding, in a hypoxic and myofibroblast-inductive microenvironment, with high level of platelet-derived serotonin (Dees et al., 2011), Th2 cytokines IL4/IL-13 (Kodera et al., 2002), or connective tissue growth factor (CTGF, CCN2) (Lee et al., 2010), these endogenous dermal MSCs subpopulation may contribute to skin fibrosis and scarring through activation of 5-hydroxytryptamine receptor 2B (5-HT2B), IL-4 receptor alpha (IL-4Rα) (Nguyen et al., 2020), and TGF-β1 (Lipson et al., 2012) pathways, respectively.

Endogenous MSCs in an environment where they are incapable to detoxify enhanced oxidative stress become damaged themselves and likely lose their reparative capacity. For instance, MSCs isolated from patient with atherosclerosis have impaired mitochondrial function, and lost their immunosuppressive function against T cell proliferation (Kizilay Mancini et al., 2018). MSCs reprogram their metabolic activity from glycolysis during tissue homeostasis to oxidative phosphorylation during wound repair, resulting in accumulation of cytotoxic metabolic byproducts. This adaptive alteration reduces the metabolic fitness of endogenous MSCs, and decreases the basal autophagy and mitophagy rate, and subsequently leads to higher rate of cellular senescence and reduced regenerative potency (Ho et al., 2017; Singh et al., 2017).

Taking the consideration of the fact that perivascular dermal MSCs comprise only about 0.3–2.5% of total mesenchymal cells in the skin (Chen et al., 2007; Haniffa et al., 2007; Vander Beken et al., 2019), the number of endogenous MSCs is not sufficient to shape the hostile environment. However, when administered at the wound site or into tissue at high numbers, the adaptive responses of MSCs are able to restore the niche to a balanced state to facilitate high quality wound repair and possibly skin regeneration.

MODIFYING SCAFFOLD TO SIMULATE WOUND ENVIRONMENT TO POTENTIATE MSCS EFFICACY

MSC delivery using polymer-based biomaterials as scaffold has been the subject of intense investigation. Scaffold materials may be of natural, synthetic, or composite origin and engineered using a multitude of approaches including porogen leaching, electrospinning, molecular self-assembly, and phase separation. Scaffold can provide mechanical support and act as a niche for MSCs to improve their survival and paracrine activity that eventually promote angiogenesis, reepithelialization and wound healing. The advantages of using scaffold for MSC-based therapy has been nicely summarized in the recent reviews (Yildirimer et al., 2012; Dash et al., 2018; Hu et al., 2018). For example, the AT-MSC-seeded elastin scaffold demonstrated rapid cell proliferation with deposition of new ECM. Experimentation with the seeded scaffold *in vivo* with a murine excisional wound model revealed greater would closure (Machula et al., 2014).

On the other hand, the seeded MSCs have shown to adapt and modify the material property of scaffolds, such as porosity, surface topographies, and stiffness. For example, AT-MSCs on the electrospun fibers produced significantly higher levels of anti-inflammatory and pro-angiogenic cytokines compared to those cultured on microplates (Su et al., 2017). The authors concluded that the topography of fibrous scaffolds can provide unique microenvironment to modulate the paracrine function of AT-MSCs. The underlying molecular mechanism is not clear yet, although upregulated NFκB signaling in MSCs has been demonstrated.

Current scaffold research focus on the enhancement of MSC survival, proliferation and differentiation. However, that may

not be exclusively essential for the beneficial effects on wound healing. In the authors' opinion, the secretome released from MSCs continuously adapting to microenvironmental changes is key to facilitate reparative or regenerative processes needed for tissue homeostasis. Therefore, modification of scaffolds may be developed to simulate the microenvironment of the target tissue, which may induce the adaptive responses of the seeded MSCs. Such pre-conditioning of MSCs may potentiate their therapeutic efficacy. Some attempts have moved toward this direction, for example, modifications to collagen scaffolds enriched with biomacromolecules such as glycosaminoglycans (GAGs) or laminin or hyaluronic acid have been generated to replicate the wound ECM environment (Liu et al., 2008; Catanzano et al., 2015). More detailed bioactive cues mimicking disease-specific niche such as changes in fluid composition, mechanical stress, cell density, and nutrient levels could be considered for scaffold design, aim to not only promote wound repair but also scarless regeneration.

CONCLUSION AND FUTURE PERSPECTIVES

The therapeutic potential of MSC-based therapy for chronic wounds is largely dependent on the ability of MSCs to release soluble factors and in a paracrine manner modulate granulation tissue formation rather than to engraft and differentiate/transdifferentiate into host tissue. Regardless of the caveats in their identity or source, MSCs have been reported to have beneficial effects on both accelerating wound healing and reducing scarring. The advantage of an MSC-based therapy over a recombinant growth factor cocktail-based therapy relies on the unique capacity of MSCs to sense and re-establish a reparative and regenerative local environment in response to changing requests of the wound.

The studies we have discussed here collectively indicate that MSCs are licensed to exert their reparative capacity after stimulation with environmental cues, such as PAMP and/or DAMP through TLRs, or growth factors and/or inflammatory mediators through respective receptors. This licensing-to-execution mode of responses makes MSCs highly suited to be therapeutically exploited as "smart adaptive drugstore" that perfectly meets to the specific and changing demands of defined wounds.

The here discussed original studies focus on MSCs from a particular tissue source, commonly BM-MSCs, AT-MSCs or skin connective tissue derived MSCs; however, MSCs are present in almost all types of mesenchymal tissues. It is an open question whether MSCs of different tissue origins all possess environmental adaptation capacity, or MSCs from one particular source are supreme in eliciting adaptive response, therefore better suited for clinical application. In addition, whether allogenic MSCs are as good as autologous MSCs in adaptive responses remain elusive. Moreover, we need to keep in mind that the essential role of MSCs in pathology of chronic wound healing disorders is not attributed to MSCs alone but is unique and directly linked to the disease-specific microenvironment. Therefore, the adaptive response of MSCs cannot be assumed as a general phenomenon, but require careful validation for distinct MSCs populations and, importantly, in a disease-specific manner.

Although current data clearly demonstrate the adaptive responses of therapeutically administered MSCs in wound healing, such roles of endogenous MSCs have yet to be investigated, due to the lack of specific markers to trace and monitor MSCs *in vivo*. It is certain that research into the mystery of tissue-resident MSCs will bring new insight into the roles of these unique cells in physiological and various pathophysiological conditions. The new knowledge we acquire from the endogenous licensing signals would feedback to optimize the preparation protocols of MSCs tailored for the clinical treatments of chronic wounds and even to shift the healing outcome from scarring toward skin regeneration.

AUTHOR CONTRIBUTIONS

DJ and KS-K wrote the manuscript and prepared the figures. Both authors contributed to the article and approved the submitted version.

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Gene Expression Profile of Isolated Dermal Vascular Endothelial Cells in Keloids

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Wound healing is a complex biological process, and imbalances of various substances in the wound environment may prolong healing and lead to excessive scarring. Keloid is abnormal proliferation of scar tissue beyond the original wound margins with excessive deposition of extracellular matrix (ECM) and chronic inflammation. Despite numerous previous research efforts, the pathogenesis of keloid remains unknown. Vascular endothelial cells (VECs) are a major type of inductive cell in inflammation and fibrosis. Despite several studies on vascular morphology in keloid formation, there has been no functional analysis of the role of VECs. In the present study, we isolated living VECs from keloid tissues and investigated gene expression patterns using microarray analysis. We obtained 5 keloid tissue samples and 6 normal skin samples from patients without keloid. Immediately after excision, tissue samples were gently minced and living cells were isolated. Magnetic-activated cell sorting of VECs was performed by negative selection of fibroblasts and $CD45^+$ cells and by positive selection of $CD31^+$cells. After RNA extraction, gene expression analysis was performed to compare VECs isolated from keloid tissue (KVECs) with VECs from normal skin (NVECs). After cell isolation, the percentage of $CD31^+$ cells as measured by flow cytometry ranged from 81.8%–98.6%. Principal component analysis was used to identify distinct molecular phenotypes in KVECs versus NVECs and these were divided into two subgroups. In total, 15 genes were upregulated, and 3 genes were downregulated in KVECs compared with NVECs using the *t*-test (< 0.05). Quantitative RT-PCR and immunohistochemistry showed 16-fold and 11-fold overexpression of *SERPINA3* and *LAMC2*, respectively. SERPINA3 encodes the serine protease inhibitor, α1-antichymotripsin. Laminin γ2-Chain (LAMC2) is a subunit of laminin-5 that induces retraction of vascular endothelial cells and enhances vascular permeability. This is the first report of VEC isolation and gene expression analysis in keloid tissue. Our data suggest that *SERPINA3* and *LAMC2* upregulation in KVECs may contribute to the development of fibrosis and prolonged inflammation in keloid. Further functional investigation of these genes will help clarify the mechanisms of abnormal scar tissue proliferation.

Keywords: keloid, wound healing, vascular endothelial cells, LAMC2, SERPINA3

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INTRODUCTION

Normal wound healing is a complex process composed of multiple sequential, albeit overlapping, phases (hemostasis, inflammatory, proliferative, and remodeling/maturation) that involve finely tuned interactions between many molecular, cellular, physiological, biochemical and mechanical factors (Berry et al., 1998; Wong et al., 2012; Aoki et al., 2019). When the remodeling/maturation phase is deranged, scars can start to grow and spread abnormally, thus resulting in the pathological scars known as keloids and hypertrophic scars (Diegelmann and Evans, 2004). Pathologically, these abnormal scars are characterized by excessive deposition of extracellular matrix (ECM) (Amadeu et al., 2004). To date, the mechanisms involved in the remodeling/maturation phase of normal and abnormal wound healing have been relatively poorly researched. This lack of knowledge has hampered the development of strategies that can prevent abnormal scar formation and/or effectively treat these scars once they have formed (Lee and Jang, 2018).

Multiple cell types play key roles in normal and abnormal wound healing: of particular interest are immune cells, fibroblasts, myofibroblasts, vascular endothelial cells (VECs), and mural cells (vascular smooth muscle cells and pericytes). In relation to immune cells, it is thought that abnormal prolongation of the inflammation into the scar remodeling phase is a crucial mechanism that underlies pathological scar formation and progression (Brissett and Sherris, 2001; Ogawa, 2017). Similarly, fibroblasts and myofibroblasts, which lay down ECM, are major players in the excessive fibrosis that characterizes pathological scarring. Finally, VECs and their extensive interactions with the surrounding mural cells appear to play key inductive roles in scar pathogenesis: there is now considerable evidence that their abnormal functions and interactions with immune and fibrotic cells may promote both the inflammation and ECM deposition in abnormal scarring. For example, in keloids, the metabolism of the ECM is profoundly abnormal, resulting in 'keloidal collagen', which are thick and complex collagen bundles that eventually become extremely interconnected (Ehrlich et al., 1994; Matsumoto et al., 2017). These pathological changes are accompanied by deranged angiogenesis, as shown by low capillary density, microvessel occlusion and luminal flattening, and a chaotic microvessel architecture characterized by relatively large capillaries with few connections with the smaller capillaries. Despite the relatively high blood flow in keloids (as shown by Doppler), it is likely that this faulty angiogenesis leads to local hypoxia that in turn promotes aberrant ECM metabolism (Tuan et al., 2008; Kurokawa et al., 2010). This hypothesis is supported by the fact that two effective treatments for keloids, namely, radiotherapy and laser therapy, act by suppressing angiogenesis and by destroying the poorly functioning blood vessels; the latter promotes the function of the stable blood vessels and thereby eliminates hypoxia (Park et al., 2012; Ogawa and Akaishi, 2016).

There are also other indirect lines of evidence that suggest that VEC dysfunction plays an important role in keloid pathogenesis (Ogawa and Akaishi, 2016). For example, patients with hypertension tend to have worse keloids than nonhypertensive patients (Arima et al., 2015). Moreover, patients with keloids have lower endothelial function than patients without keloids, as measured by reactive hyperemia peripheral arterial tonometry (Noishiki et al., 2017). It is also possible that the familial tendency of keloids and the fact that black people develop keloids much more readily than Caucasians relate to the presence of one or more single nucleotide polymorphisms that promote VEC dysfunction: one recent candidate is a variant of the N-acylsphingosine amidohydrolase-1 (ASAH1) gene, which is expressed by VECs and mural cells (Santos-Cortez et al., 2017).

Despite these observations, there are few functional analyses of the VECs from keloids. This reflects technical difficulties associated with isolating endothelial cells from tissues with excessive fibrosis. As a result, the roles of VECs in keloid formation and progression and their underlying molecular mechanisms remain poorly understood. We recently found a method for successfully isolating live endothelial cells from keloid tissues: the tissue is first gently digested and the resulting free cells are separated by magnetic sorting. The aim of this study was to analyze the gene expression profiles of keloid VECs to identify the factors that could contribute to (i) keloid formation and (ii) pathological interactions between angiogenesis, inflammation, and fibrosis.

MATERIALS AND METHODS

Patients and Tissues

Tissue samples were obtained during surgical procedures that were performed between May 2016 and October 2016 at Nippon Medical School Hospital, Tokyo, Japan. Keloid tissues were obtained from the central region of surgically resected keloids. Normal skin samples were obtained from the 'dog ear' tissues (bulges of excess skin) that were left after reconstructive surgery and that had to undergo additional resection in patients without keloids or any systemic complications. The study was approved by the Institutional Review Board of Nippon Medical School, Tokyo, Japan and adhered to the principles of the Declaration of Helsinki and its revisions (27-02-560). Written informed consent was obtained from each study participant.

Tissue Dissociation

The resected tissue specimens were placed in Dulbecco's modified Eagle's medium (DMEM; Fujifilm Wako, Osaka, Japan) supplemented with Antibiotic-Antimycotic (ABAM; Thermo Fisher Scientific, Waltham, MA, United States). The specimens were transported to the laboratory and cut into 1 × 2-cm with full thickness. The VECs were then isolated by washing the tissues three times with DMEM supplemented with ABAM, mincing the tissues into approximately 3-mm^3 pieces with scissors, and digesting the mix with Skin Dissociation Kit (Miltenyi Biotech, Bergish Gladbach, Germany) according to the manufacturer's instructions. Briefly, this process involved placing the tissue mix in a gentleMACS C tube together with DMEM containing 1% bovine serum albumin (BSA), Enzyme P, Enzyme D, and Enzyme A (Miltenyi Biotech). The mixture was then allowed to digest for approximately 1 hour in a GentleMACS Octo Dissociator with Heaters (Miltenyi Biotech). Subsequently, the

cells were squeezed through 100-μm Cell Strainers (Corning, Corning, NY, United States) and harvested by centrifugation (300 *g*, 10 min, 4°C). Thereafter, PEB buffer composed of phosphate-buffered saline (PBS), pH 7.2, 0.5% BSA, and 2 mM EDTA was added to the cells and mixed well.

Magnetic Sorting of VECs

The isolated cells were sorted by the Miltenyi Biotech magnetic sorting system as shown in **Figure 1**. The first step was to add Anti-CD45 MicroBeads and Anti-Fibroblast MicroBeads (the latter are conjugated to monoclonal mouse anti-human fibroblast antibodies and mainly target CD44) (Miltenyi Biotech) to the cells and to incubate the mixture at room temperature for 10 min. Anti-CD31-PE antibodies (Miltenyi Biotech) were then added, followed by incubation at 4°C in the dark. Subsequently, the cell suspension was subjected to magnetic sorting using LS Columns (Miltenyi Biotech) that had been washed three times with PEB. The cells that passed through the column were collected and centrifuged (300 *g*, 10 min, 4°C), after which the supernatant was removed. Anti-PE-MicroBeads (Miltenyi Biotech) and PEB were then added to the cells and mixed well. The resulting cell suspensions were loaded onto thrice-washed LS Columns and the cells that passed through were discarded. After removing the column from the magnet, the cells were washed out into a tube and then, to increase the purity of the VECs, passed through another LS column. Thereafter, the resulting suspension was immediately placed in RNAlater (Thermo Fisher Scientific). Some of the suspension was analyzed by flow cytometry using FACScan (BD Biosciences, Franklin Lakes, NJ, United States) to determine the purity of the VECs.

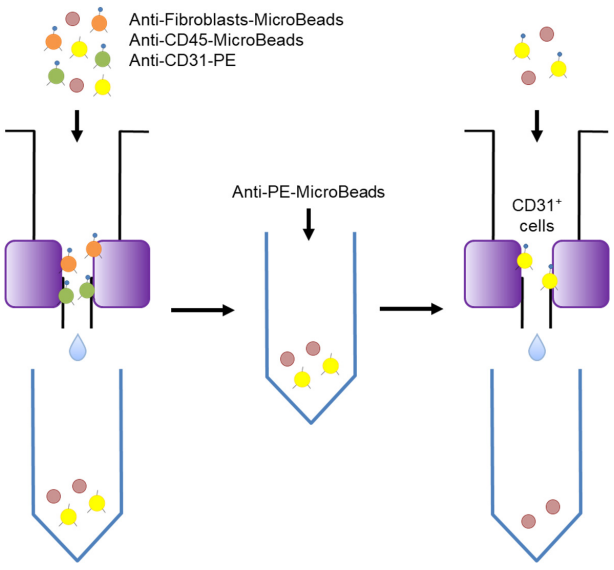

FIGURE 1 | Schematic depiction of the magnetic sorting procedure used to isolate the skin vascular endothelial cells. Magnetic sorting was performed in two steps that separated the CD31-positive cells from the fibroblasts and CD45-positive cells.

RNA Extraction

Vascular endothelial cells RNA was isolated by using RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The integrity and overall quality of the total RNA were checked by using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, United States).

Microarray Analysis

Vascular endothelial cells gene expression analysis was performed by using Agilent SurePrint G3 Human Gene Expression 8 × 60 K ver3.0 (Agilent Technologies) according to the manufacturer's instructions (One-Color Microarray-Based Gene Expression Analysis Protocol Version Jan. 2012; Agilent Technologies). In brief, cyanine-3 (Cy3)-labeled cRNA was prepared from 75 ng RNA by using the One-Color Low Input Quick Amp labeling kit (Agilent Technologies. Dye incorporation and cRNA yield were checked with a NanoDrop spectrophotometer (Thermo Fisher Scientific). For hybridization, 600 ng of Cy3-labeled cRNA was fragmented at 60°C for 30 min. On completion of the fragmentation reaction, 25 μl of 2X Agilent hybridization buffer was added to the fragmentation mixture and hybridized for 17 h at 65°C in a rotating Agilent hybridization oven. After hybridization, the microarrays were washed with GE Wash Buffer. Slides were scanned after washing on the Agilent Technologies Microarray scanner. The fluorescence intensities on scanned images were extracted and preprocessed by Agilent Feature Extraction software (v10.7.3.1). The raw signals were normalized using the percentile shift normalization method; the value was set at 75th percentile and log-transformed. Universal Human Reference RNA (Agilent Technologies) was used as the control.

Quantitative RT-PCR (qRT-PCR)

cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). qRT-PCR assays were performed using an ABI Prism 7500 System (Applied Biosystems, Foster City, CA, United States) with Applied Biosystems TaqMan Gene Expression Assays (SERPINA3, Hs00153674_m1; LAMC2, Hs01043717_m1; GAPDH, Hs03929097_g1) and TaqMan Gene Expression Master Mix (Thermo Fisher Scientific). For each primer set, the optimal dilution was determined and melting curves were used to determine the amplification specificity. GAPDH served as the internal control after it was found to validate several expression assays. Relative expression was calculated by using the $2^{-\Delta\Delta Ct}$ method with correction for different amplification efficiencies.

Immunohistochemistry

Formalin-fixed paraffin-embedded slices of the keloid and normal tissues were baked for 30 min at 58°C, deparaffinized in xylene, rehydrated through graded alcohol, and antigen-retrieved at 98°C in a water bath for 30 min in sodium citrate buffer (10 mM sodium citrate, pH 6.0). The slides were then immersed in 0.3% hydrogen peroxide diluted in methanol for 20 min to block endogenous peroxidase activity. Subsequently, they were

preincubated with 5% bovine serum albumin (BSA) at 15–20°C for 15 min to reduce non-specific reactions. Thereafter, the slides were incubated with anti-SERPINA3 (1:50, Abcam, Cambridge, MA, United States) or anti-LAMC2 (1:200, GeneTex, Irvine, CA, United States) antibody. The slides were sequentially incubated with a secondary antibody, Histofine Simple Stain MAX-PO (MULTI) (Nichirei Biosciences, Tokyo, Japan) at 15–20°C for 30 min, followed by staining with DAB (Dako, Glostrup, Denmark). Finally, the slides were counterstained with Mayer's hematoxylin, dehydrated in graded concentrations of ethanol, and mounted.

Lipopolysaccharide (LPS) Stimulation of a Dermal VEC Line

The HMEC-1 cell line is derived from human dermal microvascular endothelium. It was purchased from ATCC (Manassas, VA, United States) and cultured in MCDB131 medium supplemented with 10 ng/mL epidermal growth factor, 1 μg/mL hydrocortisone, 10 mM glutamine, and 10% fetal bovine serum (FBS). LPS from E. coli O111 was purchased from Fujifilm Wako (Osaka, Japan). The HMEC-1 cells were plated in a 24-well plate at densities of 4×10^4 cells/well and grown until sub-confluency. The cells were then cultured with or without 1 μg/ml LPS for 15 h (Dayang et al., 2019), after which the cells were harvested for RNA extraction and qRT-PCR.

Effect of Laminin-5 on Dermal Fibroblast Proliferation and Apoptosis

Laminin-5 consists of α, β, and γ subunits; the γ subunit is encoded by LAMC2 (Hashimoto et al., 2006). Normal human dermal fibroblasts (NHDFs) were purchased from TaKaRa (Shiga, Japan) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS. They were plated in 96-well plates at density of 1×10^4 cells/well and grown for 24 h. The cells were then treated with or without 0.5 μg/ml of human recombinant laminin-5 (also known as laminin-332) (Oriental Yeast, Tokyo, Japan) in DMEM containing 2% FBS for 15 h (Baba et al., 2008). Cell proliferation and apoptosis were assessed by using ApoLive-Glo Multiplex Assay (Promega, Madison, WI, United States) according to the manufacturer's instructions. Thus, the cells were incubated with Viability Reagent for 30 min at 37°C and fluorescence was measured at the wavelength set of 400Ex/505Em. The cells were then incubated with Caspase-Glo 3/7 Reagent for 30 min at room temperature and luminescence was measured.

Statistical Analysis

Comparisons between the two groups in terms of continuous variables were performed with Student's *t*-test (if the data were normally distributed) or with the non-parametric Mann-Whitney-Wilcoxon (if the data were not normally distributed). Statistical significance was set at $p < 0.05$, unless otherwise specified. Statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R 2.13.0 (R Foundation for Statistical Computing, Vienna, Austria). More precisely, EZR is a modified version of R commander (version 1.6-3) that was designed to add statistical functions that are frequently used in biostatistics.

RESULTS

Efficiency and Accuracy of VECs Isolation and RNA Extraction

The magnetic sorting system shown in **Figure 1** successfully and efficiently isolated live VECs from keloid and normal skin samples. Total RNAs were then obtained from the VECS of five keloid samples (KVECs) and six normal skin samples (NVECs). The clinical characteristics of the patients, the purity of the isolated VECs (as measured by flow cytometry), and the integrity of the extracted RNAs are presented in **Table 1**. VEC purity ranged from 81.8% to 98.6%. Representative flow cytometric plots and histograms are shown in **Figure 2**. RNA integrity (as expressed by RNA Integrity Number) ranged between 6.4 and 9.4.

Microarray Analysis Followed by Hierarchical Clustering and Principal Component Analysis

The VEC RNAs were subjected to microarray analysis followed by hierarchical clustering and principal component analysis (PCA): this allowed us to visualize significant gene expression differences between the KVECs and NVECs. Hierarchical clustering showed that the KVECs and NVECs largely fell into two separate clusters: the exception was one KVEC sample (K5), which formed a subcluster that branched out from the main KVEC cluster with two NVEC samples (N10 and N11). Thus, the mRNA expression profiles of the KVECs were largely different from those of the NVECs (**Figure 3A**). PCA then showed that the KVECs were located a marked distance away

TABLE 1 | Characteristics of the patients, vascular endothelial cell (VEC) purity, and RNA integrity.

Sample No.	Range of age	Area	VECs (% of cells in the sample)	RIN
Keloids				
K-1	26–30	Shoulder	97.5	8.6
K-2	60–65	Anterior chest	93.4	8.8
K-3	60–65	Lower abdomen	98.6	9.4
K-4	20–25	Upper limb	98.3	9.3
K-5	56–60	Lower abdomen	98.5	8.3
Normal skin samples				
N-6	76–80	Eyelid	85.9	7.5
N-7	70–75	Thigh	96.8	6.4
N-8	60–65	Dorsal	95.5	9.2
N-9	76–80	Eyelid	97.4	9.1
N-10	70–75	Eyelid	81.8	6.8
N-11	60–65	Lower abdomen	93.9	7.0

RIN, RNA integrity number.

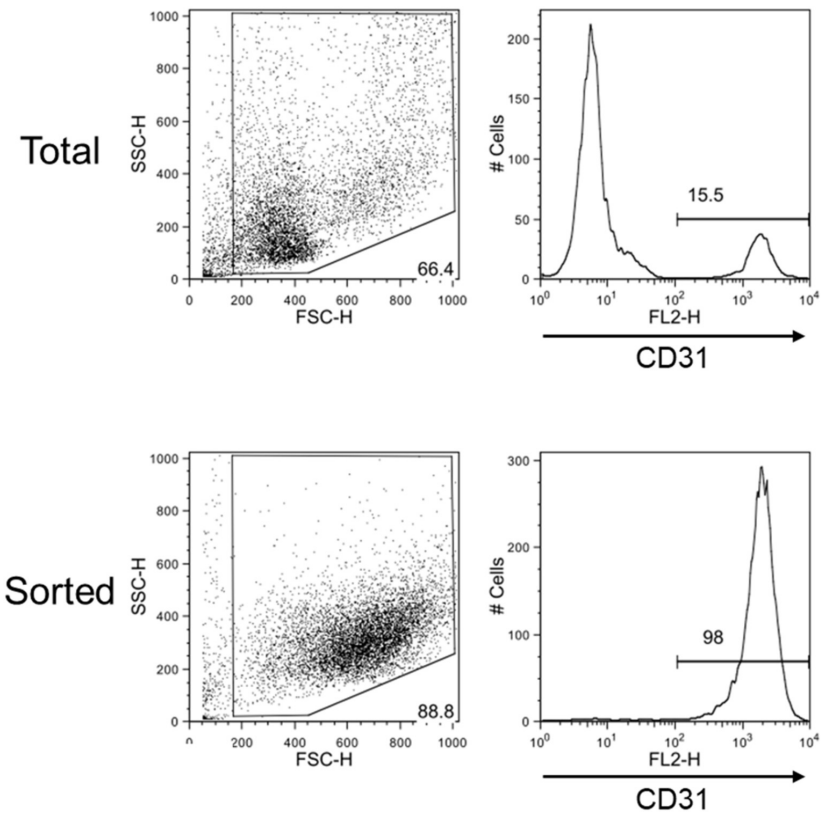

FIGURE 2 | Flow cytometric evaluation of the efficiency with which skin vascular endothelial cells were isolated. Representative flow cytometry plots and histograms of total dispersed cells before sorting (upper) and after sorting (lower) are shown. Very pure CD31-positive cell samples were obtained by magnetic sorting.

from the NVECs (**Figure 3B**, upper plot); this trend was confirmed by a different 3-dimensional representation of the data (**Figure 3B**, lower plot). Thus, while there was substantial sample variability, the KVECs clearly had different molecular phenotypes compared to the NVECs.

Extraction of Genes and Network Analysis

Relative to NVECs, 15 and three genes in KVECs were respectively up- and down-regulated (as determined by Welch's *t*-test; all $p < 0.05$) (**Table 2**). **Table 3** lists the 10 genes that exhibited the greatest up- or downregulation in KVECs relative to NVECs. Of these, we focused on two factors, SERPINA3 and LAMC2, which are known to be involved in ECM metabolism and inflammation, respectively (Aumailley, 2013; Li et al., 2018). SERPINA3 and LAMC2 exhibited 16- and 11-fold upregulation in the microarray analysis, respectively (**Table 3**). To visualize whether these genes play an important role in KVEC gene regulation, we performed network analysis with the 74 and 80 genes that were, respectively, up- and down-regulated in KVECs by more than 5-fold. Both SERPINA3 and LAMC2 were located near the center of the network, which suggests that they may play important roles in KVEC phenotype (**Figure 4**).

Validation of Microarray Data Regarding SERPINA3 and LAMC2 mRNA Expression

We performed qRT-PCR to verify the microarray data. Given the relatively large variation between samples, qRT-PCR was performed using RNA extracted from six NVECs and seven KVECs: thus, two additional KVEC samples from different patients were added to the five KVEC samples that were used to generate the microarray analysis data. The two additional KVEC samples were from patients aged 36–40 years whose keloids were on the anterior chests (VEC purities were 98.8% and 99.1%). The qRT-PCR analysis confirmed that the KVECs had significantly higher mRNA expression levels of both SERPINA3 and LAMC2 compared to NVECs (**Figures 5A,B**).

Histological Validation of SERPINA3 and LAMC2 Protein Expression in Blood Vessels of Skin Samples

Three keloids and three normal skin specimens were obtained by surgery from a separate cohort of patients. The keloid samples were obtained from patients whose keloids were on the lower abdomen (age 56–60 years) and anterior chests (age 61–65 and 76–80 years). The three non-keloid samples were from patients aged 11–15, 21–25, and 71–75 years. Immunohistochemical

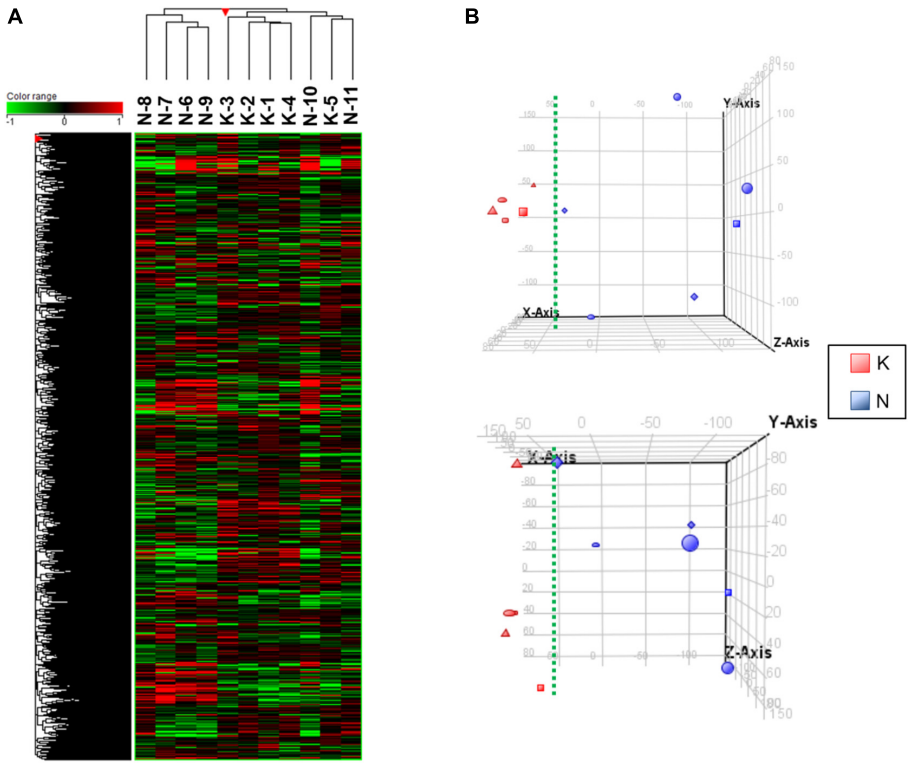

FIGURE 3 | Hierarchical clustering and principal component analysis (PCA) of the vascular endothelial cell (VEC) samples. **(A)** The expression patterns of all samples are shown in the heatmap. The sample numbers of the normal VECs (NVECs) and keloid VECs (KVECs) are indicated at the top. On hierarchical clustering, the KVECs and NVECs generally fell into two separate clusters. **(B)** PCA plots that visualize the distribution of KVECs and NVECs in terms of gene expression pattern. The KVECs were distributed close to each other and away from the NVECs. As a result, the KVECs could be divided from the NVECs by the indicated green broken line. The upper and lower plots indicate the distributions from two different 3-dimensional viewpoints.

TABLE 2 | Genes that were expressed at significantly ($p < 0.05$) higher or lower levels in KVECs compared to in NVECs (as determined by using Welch's *t*-test).

Probe Name	Gene Abbreviation	Gene Name	Corrected p-value
Upregulated			
A_23_P103256	CFHR3	complement factor H-related 3	0.010
A_33_P3304170	PIK3CG	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit gamma	0.010
A_23_P41145	FAM3D	family with sequence similarity 3, member D	0.018
A_33_P3367692	CFH	complement factor H	0.018
A_19_P00808586	LINC01085	long intergenic non-protein coding RNA 1085	0.018
A_33_P3286349	DNAAF3	dynein, axonemal, assembly factor 3	0.018
A_33_P3318288	CFH	complement factor H	0.018
A_23_P114740	CFH	complement factor H	0.025
A_32_P35969	CHRNA7	cholinergic receptor, nicotinic, alpha 7 (neuronal)	0.026
A_23_P49559	GPR142	G protein-coupled receptor 142	0.026
A_23_P79251	EHD3	EH-domain containing 3	0.026
A_33_P3333158	CPXM2	carboxypeptidase X (M14 family), member 2	0.026
A_23_P124927	RGS14	regulator of G-protein signaling 14	0.035
A_23_P57110	SLC52A3	solute carrier family 52 (riboflavin transporter), member 3	0.042
A_33_P3245631	TTC39A	tetratricopeptide repeat domain 39A	0.050
Downregulated			
A_33_P3245290	AQP7P1	aquaporin 7 pseudogene 1	0.020
A_21_P0005172	LINC00472	long intergenic non-protein coding RNA 472	0.026
A_22_P00017310	MIR99AHG	mir-99a-let-7c cluster host gene (non-protein coding)	0.044

KVEC, keloid vascular endothelial cell; NVEC, normal vascular endothelial cell.

TABLE 3 | Genes that were highly up- or down-regulated in KVECs compared to in NVECs.

Probe Name	Gene Abbreviation	Gene Name	FC
Upregulated			
A_33_P3211198	NCMAP	noncompact myelin associated protein	30.51
A_23_P137797	RYR2	ryanodine receptor 2 (cardiac)	18.51
A_23_P2920	SERPINA3	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	16.08
A_23_P160214	TTC39A	tetratricopeptide repeat domain 39A	12.57
A_23_P160968	LAMC2	laminin, gamma 2	11.01
A_19_P00323692	XIST	X inactive specific transcript (non-protein coding)	10.86
A_33_P3399788	SERPINA3	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	10.81
A_23_P69154	FAM198A	family with sequence similarity 198, member A	10.75
A_23_P94902	KCTD8	potassium channel tetramerization domain containing 8	10.54
A_23_P29773	LAMP3	lysosomal-associated membrane protein 3	10.44
Downregulated			
A_23_P324384	RPS4Y2	ribosomal protein S4, Y-linked 2	−40.26
A_33_P3224331	DDX3Y	DEAD (Asp-Glu-Ala-Asp) box helicase 3, Y-linked	−23.37
A_23_P259314	RPS4Y1	ribosomal protein S4, Y-linked 1	−21.81
A_23_P15786	KRT25	keratin 25, type I	−19.70
A_32_P98072	TCHH	trichohyalin	−15.51
A_23_P99044	KRT71	keratin 71, type II	−15.21
A_23_P97141	RGS1	regulator of G-protein signaling 1	−13.98
A_23_P78201	KRT35	keratin 35, type I	−13.10
A_24_P234768	HTR4	5-hydroxytryptamine (serotonin) receptor 4, G protein-coupled	−11.24
A_23_P362694	FDCSP	follicular dendritic cell secreted protein	−10.19

KVEC, keloid vascular endothelial cell; NVEC, normal vascular endothelial cell.

analysis of the six samples showed that all of the keloids had greater protein expression of both SERPINA3 and LAMC2 in the blood vessels than the normal skin samples. Representative images are shown in **Figures 6A,B**. These results are consistent with the microarray data.

Inflammation-Induced Dysfunction Increases SERPINA3 and LAMC2 Expression in VECs but LAMC2 Does Not Affect Fibroblast Proliferation

To assess whether SERPINA3 and LAMC2 could participate in keloid pathogenesis, we asked whether activation with LPS changes the SERPINA3 and LAMC2 expression exposed to it, blood vessels exhibit hyperpermeability, and VECs adopt proinflammatory leukocyte-recruiting phenotypes, including the production of proinflammatory cytokines, chemokines, and adhesion molecules (Wang L. et al., 2017; Dayang et al., 2019). Indeed, LPS treatment of the VEC line with LPS caused it to upregulate SERPINA3 and LAMC2 expression (**Figure 7A**). This is consistent with the upregulated expression of these genes in KVECs (**Figures 5**, **6**).

Since secreted LAMC2 promotes the proliferation and survival of cancer cells (Garg et al., 2014; Sato et al., 2014), and excessive fibroblast proliferation and insufficient apoptosis play an important role in keloid pathogenesis (Funayama et al., 2003), we speculated that LAMC2-overexpressing VECs could secrete LAMC2 and thereby promote local fibroblast proliferation and survival. However, we observed that the LAMC2-treated fibroblasts did not exhibit increased proliferation or resistance to apoptosis (**Figure 7B**).

DISCUSSION

Keloids and hypertrophic scars are caused by genetic and environmental factors; the environmental factors can be divided further into local and systemic factors. In terms of genetic factors, keloids associate with single nucleotide polymorphisms; in addition, people with dark skin are 15-fold more likely to develop keloids than people with lighter skin (Miller and Nanchahal, 2005; Nakashima et al., 2010). The local factors that promote pathological scar development are, like the genetic factors, well-studied and include the elastic tension on the skin and mechanical stress (Aoki et al., 2020). Much less well-understood are the systemic factors that associate with keloidogenesis; these include adolescence, pregnancy (Park and Chang, 2012; Krakowski et al., 2016), and endothelial dysfunction. With regard to the latter factor, mounting evidence now suggests that disturbed endothelial function may in fact play a key role in initiating and augmenting the pathogenic activities of other important cellular players in pathological scarring. For example, our recent studies show that poor endothelial function and hypertension associate with keloid development and aggravation (Arima et al., 2015; Noishiki et al., 2017). These clinical observations led us to analyze the gene expression in VECs from keloid patients, with the aim of identifying local cell interactions or microenvironments that could be exploited therapeutically.

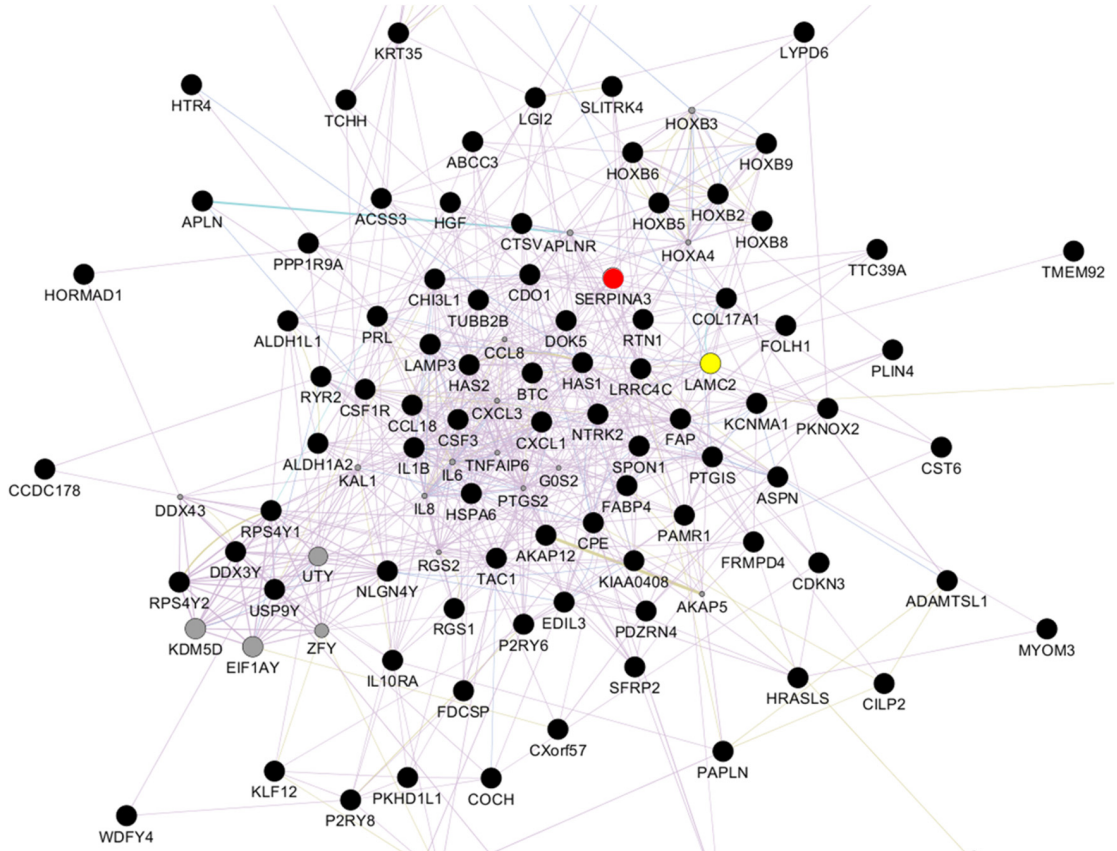

FIGURE 4 | Network analysis with the 74 and 80 genes that were, respectively, up- and down-regulated by more than 5-fold in keloid vascular endothelial cells. SERPINA3 (red) and LAMC2 (yellow) are located near the center of the network.

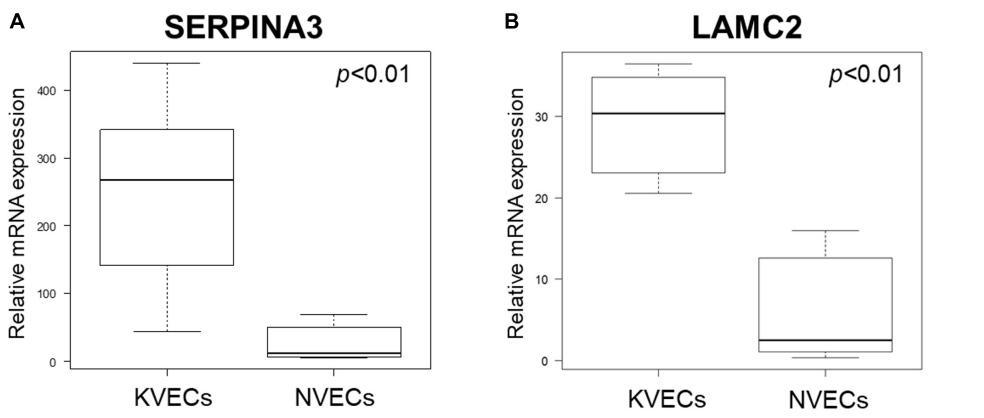

FIGURE 5 | Validation of the microarray data by quantitative RT-PCR. The relative mRNA expression levels of SERPINA3 **(A)** and LAMC2 **(B)** in keloid vascular endothelial cells (KVECs) (n = 7) and normal vascular endothelial cells (NVECs) (n = 6) are shown. All values in this figure represent the mean ± SD.

The proper maintenance of vascular integrity requires that VECs interact with and adhere to the ECM. Along with endothelial cell proliferation and migration, these interactions between VECs and the ECM also play an important role in the angiogenesis that participates in crucial processes such as osteogenesis, tumor growth, and wound healing (Langen et al., 2017; Lugano et al., 2018; Bi et al., 2019). It is known that during the aberrant wound healing that leads to pathological scarring, VEC proliferation and new blood vessel formation may be shaped by immune and other factors such as endothelin-1, vascular endothelial growth factor, platelet-derived growth factor, and transforming growth factor-β. Notably, VECs may also

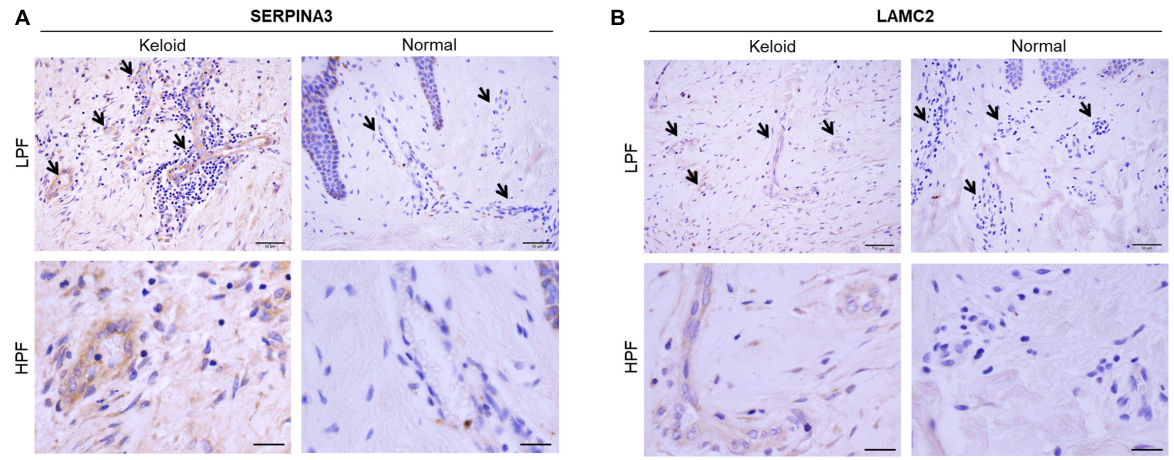

FIGURE 6 | Immunohistochemical validation of **(A)** SERPINA3 and **(B)** LAMC2 protein expression in the blood vessels of keloid and normal skin tissues. Representative histological images are shown. Arrows indicate the blood vessels in the dermis. LPF, low-power field, HPF, high-power field. Scale bars: LPF, 50 μm, HPF, 20 μm.

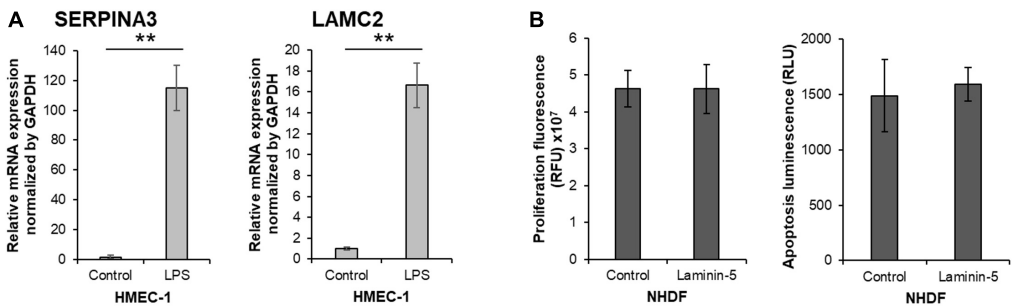

FIGURE 7 | *In vitro* study on the biological functions of SERPINA3 and LAMC2 in vascular endothelial cells and fibroblasts. **(A)** A human dermal vascular endothelial cell line (HMEC-1) was treated with or without 1 μg/ml lipopolysaccharide (LPS) for 15 h (n = 6). The relative mRNA expression levels of SERPINA3 and LAMC2 in the cells was determined. **(B)** Normal human dermal fibroblasts (NHDF) were treated with or without 0.5 μg/ml laminin-5 for 15 h (n = 10). The relative cell proliferation and apoptosis was measured. All values in this figure represent the mean ± SD. ** $p < 0.01$.

secrete the latter factor, thereby activating nearby fibroblasts and initiating fibrotic reactions (Kiya et al., 2017; Wang X.Q. et al., 2017). This pathogenic role of VECs is supported by our finding that the abundant hyalinized keloidal collagen that characterizes keloids appear to arise from the perivascular area (Matsumoto et al., 2017). Based on these reports, we postulated that when inflammation occurs, VECs may play important roles in regulating the behavior of other cell types and tissue homeostasis. In the present study, we identified the genes that are up- or down-regulated in KVECs and then focused on SERPINA3 and LAMC2, both of which are sharply up-regulated in KVECs and thus may contribute to keloid pathogenesis.

The serpin family consists of a functionally diverse set of proteins that are named after their function as serine proteinase inhibitors (Potempa et al., 1994). Serpins play important roles in inflammation, immunity, coagulation, dementia, and cancer and can be potential biomarkers and therapeutic targets for disease (Heit et al., 2013). SERPINA3 is also known as α1-antichymotrypsin: it is an acute phase reactive protein and a serpin family member. Although its biological role in wound healing remains unknown, SERPINA3 appears to reduce leukocyte protease-mediated tissue damage (Gooptu and Lomas, 2009). Serpins also inhibit neutrophil-derived cathepsin G, which is a potent regulator of inflammatory processes and is directly involved in the degradation of ECM molecules that are essential for skin repair (Capodici et al., 1989), including collagen (Cipriani et al., 2018; Ito and Nagata, 2019), elastin (Ma et al., 2013), and fibronectin (Chang et al., 2012; Li et al., 2017). Furthermore, SERPINA3 is a potent inhibitor of matrix metalloproteinase type 9 activation in human wound healing (Han et al., 2008; Reiss et al., 2009). Our data showed that SERPINA3 is upregulated by 16-fold in KVECs compared to NVECs; this upregulation in VECs was observed at both the mRNA and protein level. Notably, we also found that when normal VECs become inflamed and dysfunctional due to exposure to LPS, their expression of SERPINA3 rises significantly. Thus, given its roles in inflammation, SERPINA3 may also act in keloid pathogenesis by altering local immune responses.

Laminins are large heterotrimers that constitute the basement membrane in ECM. They are composed of α, β, and γ

subunits (Yurchenco et al., 2018). VECs attach to several ECM components, including fibronectin, collagen, and laminin (Kubota et al., 1988). Laminins not only directly affect leukocyte migration, they also indirectly influence endothelial barrier function by modulating endothelial barrier properties (Song et al., 2017). LAMC2 is the short arm of Laminin-5. It shapes cell motility and growth, thereby affecting morphogenic events (Navdaev et al., 2008; Garg et al., 2014). The targeted degradation of laminin-5 and LAMC2 by matrix metalloproteinases promote cell migration (Giannelli et al., 1997; Cheng et al., 2009). Significantly, LAMC2 induces the shrinkage of VECs, thereby enhancing vascular permeability *in vitro* and *in viv*o (Sato et al., 2014). Our data showed that KVECs have 11-fold upregulated expression of LAMC2. Moreover, as with SERPINA3, LPS treatment of normal VECs increased their LAMC2 expression. These findings together with the known activities of LAMC2 suggest that LAMC2 overexpression in KVECs may encourage endothelial hyperpermeability, which in turn may prolong the inflammatory stage, thereby promoting keloid formation and progression. Since LAMC2 promotes the proliferation and survival of cancer cells (Garg et al., 2014; Sato et al., 2014), and keloids are characterized by excessive fibroblast proliferation and resistance to apoptosis (Funayama et al., 2003), we also asked whether LAMC2 secretion by VECs could promote keloid fibroblast proliferation and downregulate apoptosis. However, culturing normal fibroblasts with LAMC2 had no effect on these properties. Further studies on the potential underlying mechanisms are warranted.

Because keloids are elastic fibrotic lesions that are formed by excessive deposition of ECM components, it is hard to digest keloids and isolate cells with small populations such as VECs. While VECs have been isolated from soft organs or VECs-rich tumors (Mao et al., 2016; Liu et al., 2017; Crouch and Doetsch, 2018), only one study has reported the isolation of VECs from (hypertrophic) scars (Wang et al., 2008). However, recent technological advances, namely, the application of gentle digestion followed by magnetic sorting, allowed us to isolate VECs from keloid tissues. The gentle digestion contributes to the high cell survival rates and the two magnetic cell sorting-based isolation steps that, respectively, removed the many CD45-positive cells and fibroblasts and positively selected the CD31-positive VECs led to very pure VECs populations. In the future, similar recent advances in this technology will be used to isolate and analyze targeted single cells.

This study has some limitations. First, not all VECs in keloids express CD31. Second, the up- and down-regulated genes do not simply reflect the activities of KVECs: rather, they are likely to reflect the sum of not only KVEC behavior but also systemic and local inflammatory factors. More functional experiments in which these genes are knocked out or down are required.

CONCLUSION

This is the first study to analyze the expression profiles of VECs from keloids. It showed that two genes involved in immune cell, ECM, and endothelial regulation, namely, SERPINA3 and LAMC2, are highly upregulated in KVECs compared to in NVECs. These findings may not only help to clarify how keloids develop but they may also be useful targets for anti-keloid therapies that are effective at different stages after onset. For example, inhibiting LAMC2 early after onset may prevent the vascular permeability and prolongation of inflammation that drives keloid growth. Similarly, inhibiting SERPINA3 in well-established keloids may promote immune responses and the degradation of the ECM, thereby reducing the keloid mass and/or preventing its further outgrowth. More detailed functional analyses of the VECs in keloids are warranted.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the microarray datasets submitted to Gene Expression Omnibus (GEO)-NCBI and are accessible through GEO Series accession number GSE121618.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Institutional Review Board of Nippon Medical School, Tokyo, Japan. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

NM collected, assembled, analyzed, interpreted the data, and wrote the draft of manuscript. MA designed the study, analyzed, interpreted the data, and wrote the manuscript. YO, KK, SE, and TD analyzed and interpreted the data. SA and RO supervised the study and performed critical review. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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A New Animal Model for Pathological Subcutaneous Fibrosis: Surgical Technique and *in vitro* Analysis

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Fibrosis is a condition that affects the connective tissue in an organ or tissue in the restorative or responsive phase as a result of injury. The consequences of excessive fibrotic tissue growth may lead to various physiological complications of deformity and impairment due to hypertrophic scars, keloids, and tendon adhesion without understating the psychological impact on the patient. However, no method accurately quantifies the rate and pattern of subcutaneous induced hypertrophic fibrosis. We, therefore, devised a rodent excisional model to evaluate the extent of fibrosis with talc. Tissue specimens were set on formalin, and paraffin sections for histological, immunohistochemical, and molecular analysis talc was used to induce the fibroproliferative mechanism typical of hypertrophic scars. This pathway is relevant to the activation of inflammatory and fibrotic agents to stimulate human hypertrophic scarring. This model reproduces morpho-functional features of human hypertrophic scars to investigate scar formation and assess potential anti-scarring therapies.

Keywords: pathological fibrosis, wound model, animal model, scar, talc

INTRODUCTION

Keloids, tendon adhesion and other diseases resulting from excessive connective tissue formation to a pathological degree are challenging undertakings for plastic and orthopedic surgeons who are not able to guarantee satisfactory outcomes.

Fibrosis is a condition that occurs in connective tissue and tissue as a typical physiological consequence of the reparative mechanism that follows injury. Hypertrophic fibrosis increases the tissue volume due to an anomalous propagation of fibroblast cells and aggregation of extracellular elements such as collagen. The augmented volume of fibrotic tissue may lead to important physical and psychological disabilities and impairments (Brown et al., 2008; Finnerty et al., 2016; Zhang et al., 2018). The healing process of a tissue injury is characterized by three concurring mechanisms: inflammation, proliferation and remodeling (Reinke and Sorg, 2012).

These phases occur at onset of the lesion with hematoma, movement of cells from the surrounding tissue and the activation of inflammatory mediators. During the proliferative phase, new blood vessels form from pre-existing vessels followed by the deposition of extracellular

compounds. During remodeling the extracellular matrix is characterized by the development of elements and affections, which consequently lead to the deposition of proteoglycan and collagen associated to scarring and rigidity in the joints (Gurtner et al., 2008).

Hypertrophic scars of the dermis are caused by the aggregation of collagen cross-links and are responsible for tissue hardening as well as an excess of collagen in the area above the surrounding skin or the area around the initial wound (Sidgwick and Bayat, 2011). Kischer and Brody (1981) defined collagen nodules as the structural unit of hypertrophic scars and keloids (Liu et al., 2012). The nodules are devoid of mature scars and present an advanced organization and precise orientation of significantly dense fibroblasts and unidirectional collagen fibrils. Moreover, a hypertrophic scar possesses a rich vasculature (in relation to scar age) which differentiates it from normal skin. The scar besides, presents an elevated mesenchymal cell density, and a deep epidermal cell layer. Keloidal scars consist of type III (early) or type I (late) collagen differing according to the maturity (Lee and Jang, 2018).

Flexor tendon reconstruction is a challenging issue if the injury presents in zone II of the hand. Common occurrences are adhesions involving the areas between the tendon and the surrounding tissue and limitations in the range of motion of the finger with subsequent contraction of the joint. Histologically, tendon fascicles contain type I collagen fibers and spiraling bundles of mature fibroblasts or tenocytes that are long and narrow. Adhesions between the tendon and the surrounding tissue area is common in injuries that mainly heal extrinsically, conversely to intrinsic cellular healing which will present with fewer adhesions and reduced density (Mass and Tuel, 1991). However, adhesion formation is also related to tendon suture, sheath damage and immobilization following surgery which are inevitable consequences of the injury itself and the reconstruction phase (Elliot et al., 2007; Riccio et al., 2010).

Clinical examples of hypertrophic fibrosis also include Ledderhose's syndrome, Peyronie's disease, or more frequently Dupuytren's disease. Histological analyses have shown a higher number of collagen III deposits compared to collagen I deposits, and an increase in collagen hydroxylation and glycosylation, myofibroblasts and myoglobin proteins. Despite our extensive knowledge regarding the biology of cytokines, growth factors and the altered expression of several genes, the etiology and pathogenesis of the disease are still unclear (Bisson et al., 2004; Chen et al., 2011) with gray areas especially in the biology and treatment of proliferative fibrotic tissue. The lack of literature is partly a result of inaccurate, inaccessible, and unreproducible animal models for the study of hypertrophic fibrosis (Huang et al., 2013).

The role of the various cell types and cytokines have been analyzed in hypertrophic scarring via *in vitro* studies investigating cell lines and primary cell culture. However, the efficacy of the procedure is seriously restricted to the differing conditions in controlled cultures and the wound-healing contexts considering, in particular, the numerous and intricate relations among types of cells, cytokines and extracellular matrix components. The search for optimal animal studies are underway but are currently being disputed (Dohi et al., 2019), due to the phylogenetic differences in wound healing (Ramos et al., 2008). Rodent and human skin differ considerably as in hair covering density—rodent hair has a shorter growth cycle compared to human hair; dermal papillae/apocrine gland variations; the differences in the panniculus carnosus (in humans it will rapidly aid in wound contraction) (Wong et al., 2011).

Attempts to develop animal models have always been a challenging and not always successful enterprise but despite the drawbacks numerous experimental animal models have indeed evolved. In particular, Morris et al. (1997) studied proliferative scar progress in a rabbit ear abrasion and transplantation of human proliferative scar tissue (Morris et al., 1997). Moreover, Aksoy et al. (2002) developed scar hypertrophy in guinea pigs by irritation caused by coal tear application (Aksoy et al., 2002). Wound healing was also investigated by Galiano et al. (2004) and Jimi et al. (2017a) observing that wound contraction was related to skin mobility in animals As for tendon adhesion, two models are mostly used: the removal of the flexor sheath with exposure of the underlying flexor digitorum profundus and superficialis system, or the association of a complete or partial tendon laceration. New Zealand White Rabbit toes are commonly adopted as well as chicken hind-paws or canine forepaws (Porat et al., 1980; de Wit et al., 2008; Taguchi et al., 2009).

Thoracoscopic talc poudrage (2.5–10 g) has been successfully used as a treatment option in reiterated pleural effusions due to the ability of talc to promote an extensive inflammatory reaction involving coagulation parameters and fibroblast proliferation (Marchi et al., 2007; Moreno-Merino et al., 2012). Therefore we hypothesized that subcutaneous talc installation could induce a chronic inflammatory reaction that promoted an irregular expansion of fibroblast cells and extracellular component aggregation resulting in the formation of a hypertrophic scar. Wound models with subcutaneous talc instillation have not been widely used, therefore we assessed an alternative procedure to correctly monitor epidermal reconstruction following subcutaneous talc instillation, to determine a standard evaluation of dermal remodeling and renewal.

Literature is currently lacking in a set assessment tool regarding subcutaneous induced hypertrophic fibrosis. The aim herein is to describe the surgical technique and to assess histologically, a new animal model for hypertrophic fibrosis.

We propose to redesign the pathological mechanism that underpin hypertrophic scarring with the use of talc.

MATERIALS AND METHODS

We conducted the study according to European and Italian Law on animal experimentation and all policies and procedures conformed to 86/609/CEE directives. Forty-eight male Wistar rats (340 ± 60 g/BW) were subjects of the study (Experimental Animal Models for Aging Units Research Department, I.N.R.C.A./I.R.R.C.S., Ancona, Italy). This animal experiment was approved by the Ethic Committee (No.1CHPL/08-13). Inbred, genetically identical rats were used. The study subjects were equally

distributed into four groups. Animals were maintained in single cages and regulated for temperature and moisture level, adequate supplies of water and food were available at all times.

Surgical Procedure and Sample Preparation

We administered ketamine (40 mg/ Kg) and intramuscular xylazine (5 mg/kg) to anesthetize the rats setting them face down on a warm pad. We used this combination for a constant and reliable level of immobilization and anesthesia (Fish et al., 2008). A monolateral cutaneous incision of 2 cm in length was performed, on a randomized side, in the dorsal paravertebral region just below the scapula (**Figure 1A**). This position prevents rats from interfering (i.e., biting or scratching) with the surgical treatment. After the cutaneous incision a 2 × 2 cm squares pocket was executed between the subcutaneous connective layer and the Dorsalis Magnus muscle fascia (**Figure 1B**); we also implemented scraping without cutting the underlying Dorsalis Magnus muscle fascia using a repeated passage of a surgical blade. Talc at different concentrations was injected into the cavity through a sterile syringe (**Figure 1C**). Skin closure was performed with close attention to preventing talc diffusion or flow out (**Figure 1D**).

We randomly assigned 48 rats to four groups to endeavor optimal talc concentration for a hypertrophic scar.

GROUP A: Control group, only the subcutaneous pocket and fascia scratch were performed.

GROUP B: Treatment group, 1 ml of sterile saline solution + 16 mg of SteritalcF4 was instilled into the subcutaneous pocket.

GROUP C: Treatment group, 1 ml of sterile saline solution + 160 mg of SteritalcF4 was instilled into the subcutaneous pocket.

GROUP D: Treatment group, 1 ml of sterile saline solution + 300 mg of SteritalcF4 was instilled into the subcutaneous pocket (Marchi et al., 2007).

We administered an antibiotic therapy of 75 mg/Kg of oxytetracycline daily for 6 days and Carprofen 0.4 mg/kg at 12-h intervals on the first day post-surgery. The animals were sacrificed by anesthetic surplus; in each group, 4 rats were sacrificed 2 weeks after surgery, 4 weeks after surgery and 6 weeks after surgery. The experimental sites were dissected, and full thickness samples including the overlying skin and partial underlying muscle were collected and fixed in formaldehyde 4% and embedded in paraffin.

Gross Examination

All rats were clinically examined by a single operator at the time of 2, 4, and 6 weeks. The following parameters were assessed at each examination stage: presence of infection, wound dehiscence, size of the palpable fibrosis area and level of adherence to the palpation between skin and muscle plane. The latter parameter referring to stiffness, was assigned a value between 0 and 10, where 0 stands for no adherence; 10 stands for fixed skin to the muscle plane.

Histological and Immunohistochemical Evaluation

For histological analyses all the specimens were sectioned (5 μm) and were stained with haematoxylin-eosin and Masson's trichrome. Antibodies were used for immunohistochemical assessment. All specimens were examined by two masked assessors via light microscopy (Nikon Eclipse 600, Milan, Italy) and NIS-Elements Microscope Imaging Software (Nikon).

To verify our hypotheses two researchers performed masked microscopic examinations to analyse the cellular response in terms of neovascularization, fibrosis and inflammation Specifically, we analyzed 3 slides per sample using light microscopy at 20× for initial magnification. Three sections of a specimen comprised each slide and we examined five fields per tissue section. A semi-quantitative investigation was used to compare Group A and B for the specific cell types: A Polymorphic Nuclear Cells (a cell with a nucleus lobed into segments and cytoplasmic granules, i.e., granulocytes); phagocytic cells (large mononuclear cells, i.e., macrophages and monocyte-derived giant cells); non-phagocytic cells (small mononuclear cells, i.e., lymphocytes, plasma cells and mast cells.); fibroblasts; endothelial cells; elastic fibers; and collagen fibers. Assessments were all conducted blindly and hence scored as follows—absence (score 0), scarce presence (score 1), moderate presence (score 2), and profuse presence (score 3). We conducted a minimum of three assessments and expressed the values accordingly—mean ± Standard Deviation.

Real-Time PCR Array Analysis

Total RNA from biopsies was removed with the RNeasy Mini Kit (Qiagen Gmbh, Hilden, Germany), DNase digestion using the RNase-Free DNase Set (Qiagen). 800 ng of total RNA from all specimens was reverse transcribed using an RT2 First Strand kit (Qiagen Sciences, Germantown, MD USA). Real-time PCR was conducted compliant with instructions within the Rat Wound Healing RT2 Profiler PCR array (SABiosciences, Frederick, MD, USA) employing RT2 SYBR Green ROX FAST Mastermix (SABiosciences).

We performed thermal cycling and fluorescence detection via Rotor-Gene Q 100 (Qiagen), analyzing data with Excel-based PCR Array Data Analysis templates (SABiosciences). We reported the results as an expression of every target gene in the specimens collected after treatment in comparison with pre-treatment specimens in Group A and Group B.

Statistical Analysis

Values were indicated as the mean standard error. We conducted Least square Linear regression for assessment using a computer-aided statistics program (SPSS 16.0 software, SPSS Inc., Chicago, IL, USA). A $P < 0.05$ was considered statistically significant. One-way analysis of variance (ANOVA) was used for data analyses. Repeated-measures ANOVA with a *post-hoc* analysis using Bonferroni's multiple comparison was performed. T tests were used to determine significant differences ($p < 0.05$). Repeatability was calculated as the standard deviation of the difference between measurements. All testing was performed in SPSS 16.0 software

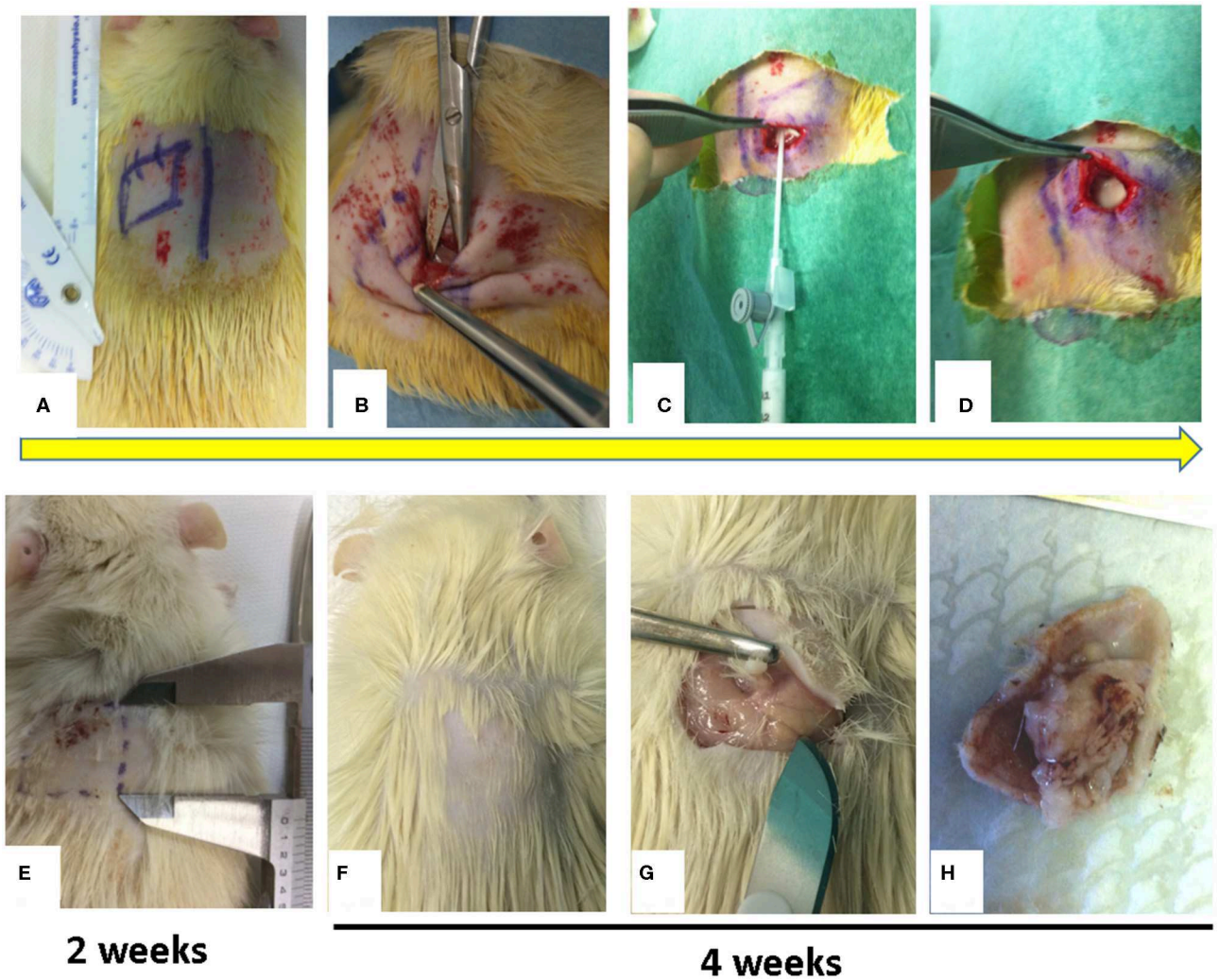

FIGURE 1 | Figure showed a sample of surgical procedure. **(A)** Monoliteral cutaneous incision in the dorsal paravertebral region; **(B)** Pocket preparation between subcutaneous connective layer and the Dorsalis Magnus muscle fascia; **(C)** Injection at different concentrations of talc into the pocket; **(D)** Skin closure of pocket to avoid talc overflow; **(E)** at 2 weeks, palpatory, in the injection site there is no subcutaneous granuloma formation (the rats were not sacrificed); **(F–H)** at 4 weeks, palpatory, in the injection site, there is subcutaneous granuloma formation and after sacrificing the rat, evidence of subcutaneous granuloma formation is observed.

(SPSS Inc., Chicago, Illinois, USA) (license of the University of Padua, Italy).

RESULTS

Gross Examination

No cases of infection in the surgical site nor dehiscence in the surgical wounds were observed. On clinical examination, 2 weeks after surgery, none of the four groups of rats showed subcutaneous fibrosis on palpation nor minimal adherence (**Figure 1E**). The rats were thus not sacrificed. At both 4 and 6 weeks after surgery in groups A and B no development of a clinically palpable fibrotic area was noted and the adherence score was always <5. On the contrary, the animals in groups C and D after 4 weeks of surgery showed a clear area of fibrosis and the development of consistent adhesions. In particular when we compared groups C and D, a gross examination revealed a stiff, fixed and thicker lesion in group D compared to group C. The size of the fibrotic area and the score of adhesions in each group were stable over time with no alteration between 4 and 6 weeks (**Figures 1F–H**).

Representative views of fibrotic volume and scores are displayed in **Table 1**. A small area was evident between the skin and the muscle tissue, which corresponded only to the injection site. Talc-injected rodents exhibited strongly induced fibrosis within 4 weeks. Notably, increasing doses of talc triggered an increasing tissue reaction. These data suggest adequacy of the one-shot injection procedure and the efficiency of talc as an inducer of murine fibrosis.

Histological and Histomorphometric Analysis

H&E staining and Masson's trichrome staining were conducted to clarify histological differences of adhesion (fibrosis) strength. In the ininjured control H&E stain sample (**Figures 2A–C**),

TABLE 1 | Gross examination.

Animal No.	Dose of talc	2 weeks time				4 weeks time				6 weeks time			
		Palpable area of fibrosis	Stiffness of the area	Infection	Wound dehiscence	Palpable area of fibrosis	Stiffness of the area	Infection	Wound dehiscence	Palpable area of fibrosis	Stiffness of the area	Infection	Wound dehiscence
1	0 mg	No palpable fibrosis	2	No	No	No palpable fibrosis	3	No	No	Sacrificed at 4 weeks			
2	0 mg	No palpable fibrosis	2	No	No	No palpable fibrosis	2	No	No	No palpable fibrosis	2	No	No
3	0 mg	No palpable fibrosis	3	No	No	No palpable fibrosis	3	No	No	Sacrificed at 4 weeks			
4	0 mg	No palpable fibrosis	2	No	No	No palpable fibrosis	2	No	No	No palpable fibrosis	2	No	No
5	0 mg	No palpable fibrosis	1	No	No	No palpable fibrosis	2	No	No	Sacrificed at 4 weeks			
6	0 mg	No palpable fibrosis	3	No	No	No palpable fibrosis	3	No	No	No palpable fibrosis	3	No	No
7	0 mg	No palpable fibrosis	3	No	No	No palpable fibrosis	4	No	No	Sacrificed at 4 weeks			
8	0 mg	No palpable fibrosis	2	No	No	No palpable fibrosis	2	No	No	No palpable fibrosis	2	No	No
9	0 mg	No palpable fibrosis	3	No	No	No palpable fibrosis	3	No	No	Sacrificed at 4 weeks			
10	0 mg	No palpable fibrosis	3	No	No	No palpable fibrosis	3	No	No	No palpable fibrosis	3	No	No
11	0 mg	No palpable fibrosis	3	No	No	No palpable fibrosis	4	No	No	Sacrificed at 4 weeks			
12	0 mg	No palpable fibrosis	4	No	No	No palpable fibrosis	4	No	No	No palpable fibrosis	4	No	No
13	16 mg	No palpable fibrosis	3	No	No	No palpable fibrosis	4	No	No	Sacrificed at 4 weeks			
14	16 mg	No palpable fibrosis	2	No	No	No palpable fibrosis	2	No	No	No palpable fibrosis	2	No	No
15	16 mg	No palpable fibrosis	2	No	No	No palpable fibrosis	3	No	No	Sacrificed at 4 weeks			
16	16 mg	No palpable fibrosis	3	No	No	No palpable fibrosis	4	No	No	No palpable fibrosis	4	No	No
17	16 mg	No palpable fibrosis	3	No	No	No palpable fibrosis	3	No	No	Sacrificed at 4 weeks			
18	16 mg	No palpable fibrosis	4	No	No	No palpable fibrosis	4	No	No	No palpable fibrosis	4	No	No
19	16 mg	No palpable fibrosis	3	No	No	No palpable fibrosis	4	No	No	Sacrificed at 4 weeks			
20	16 mg	No palpable fibrosis	4	No	No	No palpable fibrosis	5	No	No	No palpable fibrosis	5	No	No
21	16 mg	No palpable fibrosis	5	No	No	No palpable fibrosis	5	No	No	Sacrificed at 4 weeks			
22	16 mg	No palpable fibrosis	3	No	No	No palpable fibrosis	4	No	No	No palpable fibrosis	5	No	No
23	16 mg	No palpable fibrosis	3	No	No	No palpable fibrosis	3	No	No	Sacrificed at 4 weeks			
24	16 mg	No palpable fibrosis	2	No	No	No palpable fibrosis	3	No	No	No palpable fibrosis	2	No	No
25	160 mg	No palpable fibrosis	2	No	No	2 cm^2	6	No	No	Sacrificed at 4 weeks			
26	160 mg	No palpable fibrosis	3	No	No	4 cm^2	7	No	No	4 cm^2	7	No	No
27	160 mg	No palpable fibrosis	4	No	No	2 cm^2	5	No	No	Sacrificed at 4 weeks			
28	160 mg	No palpable fibrosis	4	No	No	2 cm^2	5	No	No	2 cm^2	5	No	No
29	160 mg	2 cm^2	4	No	No	3 cm^2	4	No	No	Sacrificed at 4 weeks			
30	160 mg	2 cm^2	5	No	No	3 cm^2	7	No	No	3 cm^2	7	No	No
31	160 mg	No palpable fibrosis	2	No	No	2 cm^2	5	No	No	Sacrificed at 4 weeks			
32	160 mg	No palpable fibrosis	3	No	No	4 cm^2	6	No	No	4 cm^2	6	No	No
33	160 mg	No palpable fibrosis	2	No	No	4 cm^2	4	No	No	Sacrificed at 4 weeks			
34	160 mg	No palpable fibrosis	3	No	No	4 cm^2	7	No	No	4 cm^2	7	No	No
35	160 mg	No palpable fibrosis	2	No	No	2 cm^2	7	No	No	Sacrificed at 4 weeks			
36	160 mg	No palpable fibrosis	3	No	No	3 cm^2	6	No	No	3 cm^2	7	No	No
37	300 mg	2 cm^2	4	No	No	4 cm^2	7	No	No	Sacrificed at 4 weeks			
38	300 mg	No palpable fibrosis	4	No	No	2 cm^2	8	No	No	3 cm^2	8	No	No
39	300 mg	No palpable fibrosis	3	No	No	3 cm^2	6	No	No	Sacrificed at 4 weeks			
40	300 mg	2 cm^2	3	No	No	4 cm^2	5	No	No	4 cm^2	5	No	No
41	300 mg	No palpable fibrosis	3	No	No	2 cm^2	8	No	No	Sacrificed at 4 weeks			
42	300 mg	No palpable fibrosis	3	No	No	3 cm^2	8	No	No	3 cm^2	8	No	No
43	300 mg	No palpable fibrosis	2	No	No	3 cm^2	7	No	No	Sacrificed at 4 weeks			
44	300 mg	2 cm^2	5	No	No	4 cm^2	7	No	No	4 cm^2	6	No	No
45	300 mg	2 cm^2	5	No	No	4 cm^2	8	No	No	Sacrificed at 4 weeks			
46	300 mg	No palpable fibrosis	4	No	No	3 cm^2	6	No	No	3 cm^2	6	No	No
47	300 mg	No palpable fibrosis	3	No	No	2 cm^2	7	No	No	Sacrificed at 4 weeks			
48	300 mg	2 cm^2	4	No	No	4 cm^2	7	No	No	4 cm^2	7	No	No

we observed occurrences of loose connective tissue rich in fibroblasts and with smooth collagen fibers as well as reticular fibers. In the section obtained by incision and scratch (i.e., control) connective tissue was moderately loose but, at higher magnification, the main presence of fibroblasts and leucocytes) was evident (**Figures 2D–F**). A reactive vascular component and a more fibrous connective tissue with irregular thick collagen, fibers, were well-defined in the lesions obtained by incision and injection of 16 mg/ml of talc (**Figures 2G–I**); a high level of talc crystal was observed in the lesions obtained by incision and injection of 160 mg/ml, and the presence of multigiant cells was noted at a higher magnification (**Figures 2L–N**). On the contrary, we noted the presence of fibrotic capsules around the talc with collagen nodules in the lesions obtained by incision and injection of 300 mg/ml of talc and, at higher magnification several macrophages were evident (**Figures 2O–Q**).

Talc crystals and elastic fibers in the adhesion tissues were then stained blue using Masson's trichrome and analyzed by sections from incision and injection of 160 and 300 mg/ml. The presence of multigiant cells was evident together with a leukocyte infiltration mainly represented by macrophages, was also detected (**Figure 3**). On the contrary, the histological analysis performed on control lesion sections showed the presence of relatively loose connective tissue, rich in fibroblasts and smooth collagen fibers as well as reticular fibers with several vascular structure and leucocytes (**Figures 3A–C**).

Immunohistochemical staining (data not shown) on collagen type I, III, and IV demonstrated a rise in the collagen type I-III ratio compared to normal murine skin with alphaSMA-positive myofibroblasts. These data indicated talc-induced inflammation and its contribution to the development of adhesion (fibrosis) tissue and irruption of the immune cells.

The ingression of macrophages as well as myofibroblasts and elastic fiber deposits, were observed in the murine model at high concentration of talc. These findings show the rodent model as an example of foreign-body-induced adhesion tissues in humans.

Signs of an inflammatory condition (**Figure 4**) were detected in all groups after treatment: high infiltration of granulocytes, macrophages, polymorphic nuclear cells (PMNs) and non-polymorphic nuclear cells, high fibroblasts and endothelial cells, few elastic fiber and high collagen fibers were present as well as polymorphic nuclear cells (PMNs) and non-phagocytic cells (lymphocytes).

Real-Time PCR Array Analysis

We performed real-time PCR array analysis to assess the primary molecules within the wound healing phase.

ECM components were specifically analyzed such as cellular adhesion proteins, remodeling enzymes, inflammatory cytokines, and growth factors (**Figure 5**). As for the ECM components, we observed that the treated samples (B, C, D) a possessed a higher expression of collagens compared to the control samples. The most relevant rise was associated to collagen type 5 and 3 mRNA relative expression. Concerning the remodeling enzymes, we noted a remarkable rise in treatment transcription regarding the matrix metalloproteinases (MMPs).

The analysis of the inflammatory cytokines revealed that the treated samples displayed a more extensive expression in the inflammatory interleukin (IL) 1 and a lower expression in anti-inflammatory cytokines (IL10). We reported, besides, significant levels of growth factor transcripts, such as the fibroblast growth factor (FGF) 7, FGF10, transforming growth factor (TNF) A, and vascular endothelial growth factor (VEGF).

DISCUSSION

Wound healing in adult mammalians is poorly understood but three overlaying phases are certainly involved—inflammation, proliferation/tissue formation, and remodeling—that results in the formation of a scar (Takeo et al., 2015). Mature scars are largely composed of fibroblasts and type I collagen. This fibrotic "patch" response to injury restores tissue integrity but fails to recapitulate the form and function of the native tissue. Research is currently focusing on the processes of underlying fibrosis to curtail the mechanism and promote regenerative healing (Padmanabhan et al., 2019). Fibrosis in response to injury is not limited to the skin but occurs in almost all adult tissues, specifically idiopathic pulmonary fibrosis (IPF) may be a consequence of myocardial infarction Fibrosis is most apparent in the skin and in dysregulated form will lead to the generation of hypertrophic scars or keloids (Padmanabhan et al., 2019).

Many of the models of hypertrophic scarring consider the pathological features of the hypertrophic scar as opposed to the reproduction process that results in the development of the hypertrophic scarring. Extensive studies have been conducted to define hypertrophic scars and keloids as individual disorders as far as histologic uniqueness and clinical features (Cameron et al., 2014) are concerned.

To the best of our knowledge, consensus is lacking as regards the association of collagen subtypes to hypertrophic scar tissue and the role of key cell types or well-known cytokines as TNF alfa, IL1, IL6, IL10, FGF (Komi et al., 2019). As a consequence, the attention dedicated to the hypertrophic scar phenotype would shift to the fibroproliferative mechanism yielding hypertrophic scarring in an adequate animal model."

The present study may be the first to describe a rodent model of subcutaneous fibrosis. We considered a simple animal model to assess the novel injection administration, to evaluate besides, the adhesion-promoting compounds as well as defining the temporal sequence and correct dosage that may guarantee an efficient adhesion formation.

Histologically, adhesion tissues (subcutaneous fibrosis) presenting with dense, uniform collagen bundles, and perivascular infiltration as well as vascular wall enlargement are typical histologic features observed in subcutaneous inflammation in humans. Immunohistochemical investigations have previously demonstrated that infiltration at a cellular level will primarily contain inflammatory interleukin. Our model generated histopathologic descriptions regarding human hypertrophic scarring, attributing features such as dermal

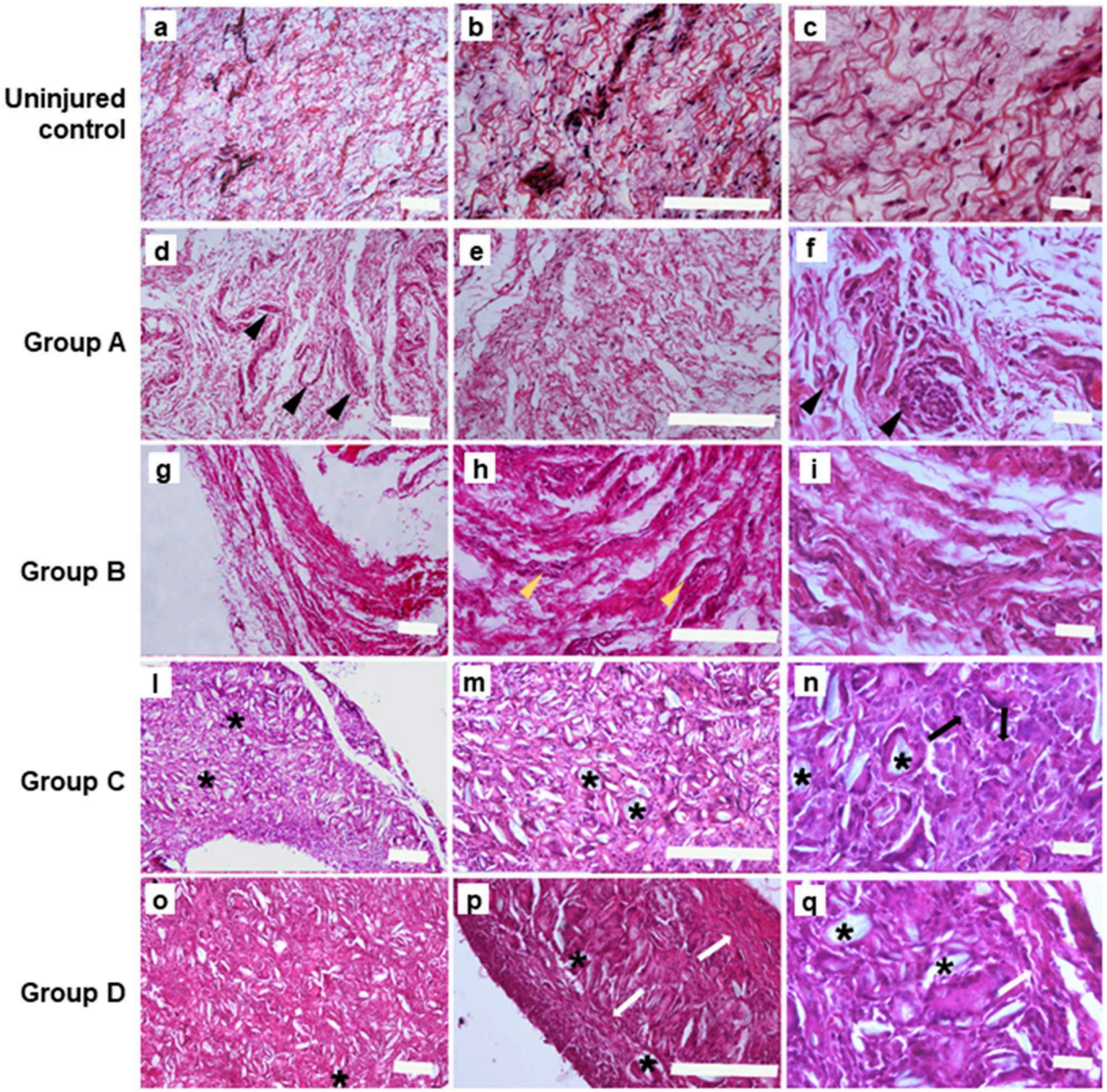

FIGURE 2 | Hematoxylin&Eosin staining performed on the samples at 4 weeks. **(A–C)** Uninjured lesions (Scale bars: a, b 100 μm; c 20 μm); **(D–F)**. Group A: control lesions obtained only by incision and milling, note the presence of leucocytes (pointed arrow) (Scale bars: d, e 100 μm; f 20 μm); **(G–I)**. Group B: lesions obtained by incision an injection of 16 mg/ml of talc (Scale bars: g, h 100 μm; i 20 μm); in **(H)** yellow pointed arrows depict the reactive vascular component; **(L–N)**. Group C: lesions obtained by incision with 160 mg/ml of talc injection; note the presence of talc crystal (*) and multigiant cells (->) (Scale bars: l, m 100 μm; n 20 μm); **(O–Q)**. Group D: Lesions obtained by incision with injection of and 300mg/ml of talc, note the presence the presence of fibrotic capsule (white arrow) around the talc residuals (*) (Scale bars: o, p 100 μm; q 20 μm).

inspissation owing to collagen deposits, parallel collagen fiber orientation, collagen whorls and inflammatory cell infiltrates. The modifying composition of the extracellular matrix along with collagen fibrillogenesis are important factors that concern fibrotic disease (Karsdal et al., 2013), there is modification in the collagen I to collagen III association within hypertrophic scarring. This could be due to the high concentration of MMPs while in hypertrophic scarring less MMPs are active but TIMPs are expressed at higher levels, this results in accumulation of collagen. However, investigations regarding the modifications are inconsistent (Xue and Jackson, 2015).

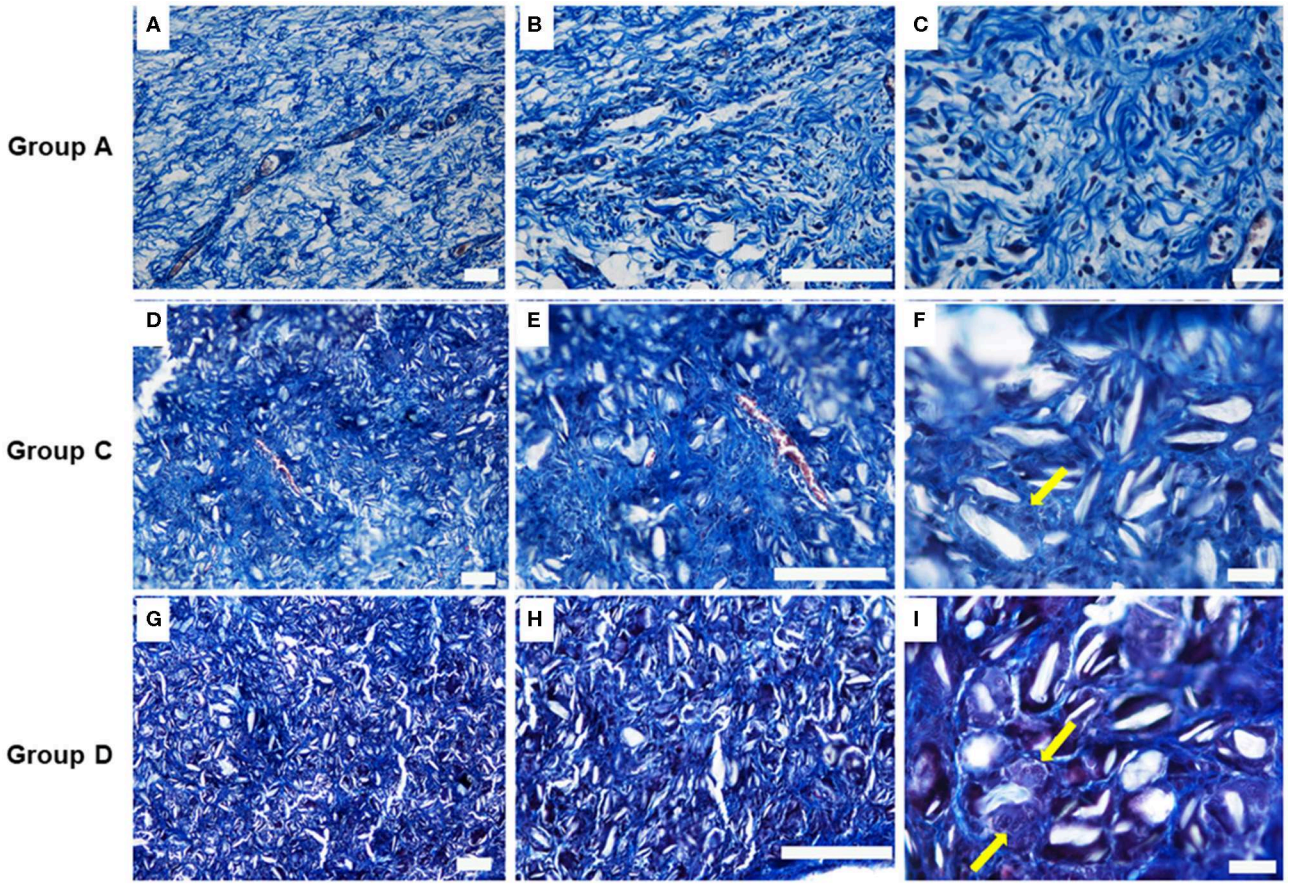

FIGURE 3 | Masson's trichrome stain images on the samples at 4 weeks. **(A–C)** Group A: control lesions (Scale bars: a, b 100–c 20 μm); **(D–F)** Group C: Lesions obtained by incision with injection of 160 mg/ml of talc (Scale bars: d, e 100–f 20 μm); **(G–I)** Group D: Lesions obtained by incision with injection of 300 mg/ml of talc (Scale bars: g, h 100–i 20 m). Note the presence at higher magnification, of multigiant cells (yellow arrows).

Interestingly, our observations suggested the efficient adhesive potential of talc as regards the subcutaneous cavity. Moreover, this potential increased with dose increments. We reported successful adhesion tissue in all animals at 4 weeks subsequent to talc administration. Fibrotic volume was greater in the high-dose groups than in the low-dose groups. However, no significant differences were observed regarding adhesive strength of tissue at 4 and 6 weeks. We recorded greater volume and strength of adhesion tissue at 4 compared to the 2-weeks interval subsequent to injection, but we reported no further change between the 4 and 6 weeks interval. These findings suggest that the 4-weeks time course sufficed to promote the highest rate of adhesions. Furthermore, indications suggest that 300 mg of talc administration for a duration of 4 weeks was sufficient to enhance fibrotic tissue in rats.

At the onset of a cutaneous injury, the accumulation of phagocytic cells as macrophages induce a release of proinflammatory and immunomodulatory mediators that has been evaluated by means of gene expression. Our results confirmed the expression of TNF-α and inflammatory mediators, including VEGF, interleukin (IL)-6, and IL-1, that contribute to increase of endothelial permeability and vasodilation, and facilitate migration of inflammatory cells (mainly monocytes and neutrophils to the site of injury). Moreover, we evaluated the stimulation of fibroblast proliferation during the proliferative phase via IL-10, and basic fibroblast growth factor (bFGF) to produce a new extracellular matrix (ECM) such as collagene type 5 and 3.

During cutaneous wound healing, matrix metalloproteinases remodel the ECM from type III to type I collagen while myofibroblasts mediate wound contraction. During dysregulated fibrosis, cells have been shown to excessively deposit ECM, as in the treated samples herein that showed a greater expression of collagens compared to the control group. We, reported besides, an important rise in collagen type 5 and 3. Driskell et al. demonstrated that reticular fibroblasts in the lower dermis were primarily responsible for fibrosis (Driskell et al., 2013). The present study is in line with the literature (Liu and Zhang, 2008; Corriveau et al., 2009), regarding reports of increased collagen type 3 and type 5 deposition in early fibrosis and increased collagen type I expression in the late stages of the

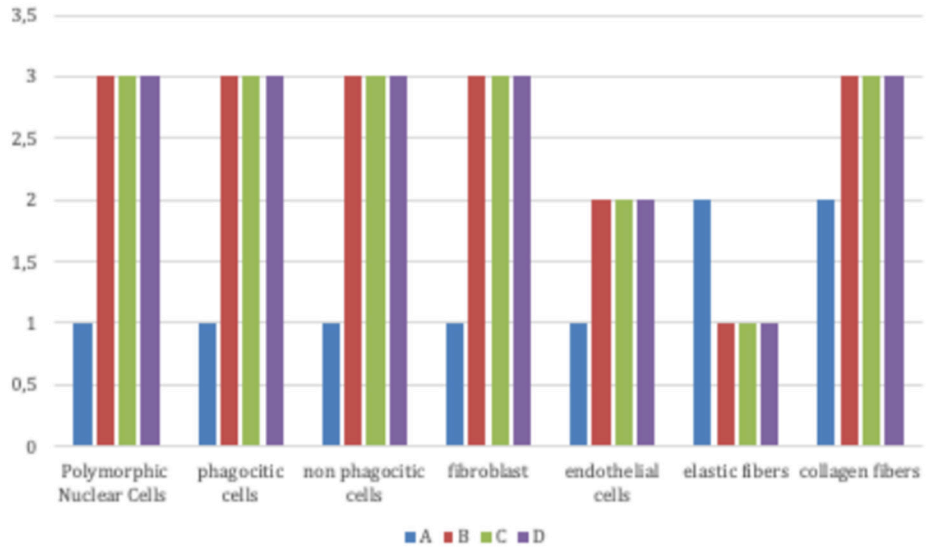

FIGURE 4 | Signs of inflammatory condition in all groups (Group A: control lesions; Group B: 16 mg/ml talc injection; Group C: 160 mg/ml talc injection; Group D: 300 mg/ml talc injection) at 4 weeks: high infiltration of granulocytes, macrophages, polymorphic nuclear cells (PMNs) and non-polymorphic nuclear cells, high fibroblasts and endothelial cells, few elastic fiber, and high collagen fibers were present.

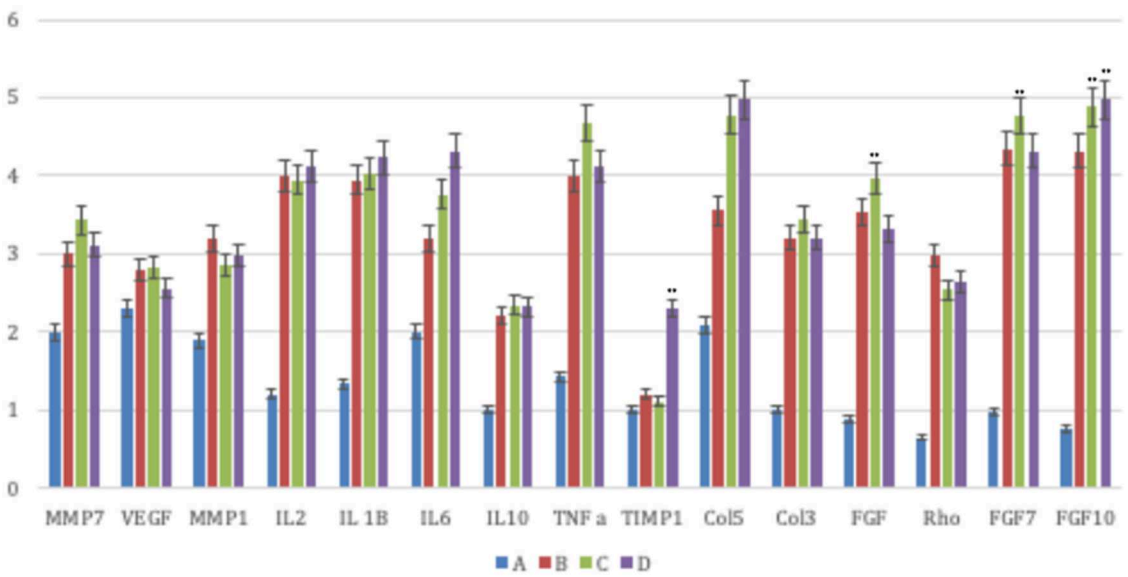

FIGURE 5 | The principal molecules involved in the wound healing process were investigated by means of a real-time PCR array analysis in all groups (Group A: control lesions; Group B: 16 mg/ml talc injection; Group C: 160 mg/ml talc injection; Group D: 300 mg/ml talc injection) at 4 weeks. Regarding the ECM components, the treated samples showed a greater expression of collagens (type 5 and 3 mRNA) compared to control. Concerning the remodeling enzymes, all matrix metalloproteinases (MMPs) displayed a significant increase. Concerning the inflammatory cytokines the treated samples exhibited a greater expression of inflammatory interleukin (IL) 1 and a lesser expression of anti-inflammatory cytokines (IL10). Moreover, they showed high levels of growth factor transcripts, such as the fibroblast growth factor (FGF) 7, FGF10, transforming growth factor (TGF) A, TGFB1, and vascular endothelial growth factor (VEGF). Repeated-measures ANOVA with a *post-hoc* analysis using Bonferroni's multiple comparison. *T* tests were used to determine significant differences ($p < 0.05$). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Repeatability was calculated as the standard deviation of the difference between measurements.

disease. These findings demonstrated that the overproduction of collagen type 5 in particularly severe skin-thickening cases, may justify occurrences of skin fibrosis. Moreover, we may confirm the importance of over-expression in collagen type 5 and type 3 in fibrosis. The increased amounts of collagen type V and type III mRNA expression observed herein indicate

transcription regulation most likely attributable to TGF-beta1. Investigations have demonstrated that TGF-beta signalizing is able to increase the expression of collagen in the skin and dermal fibroblast cultures (Sargent et al., 2010). The treatment samples of our study revealed increased levels of growth factors such as FDF7, FGF10, TGF-beta, and VEGF. In particular, VEGF showed a profibrotic effect by multiple mechanisms (Tanaka et al., 2019). The use of talc enhanced the fibroproliferative pathway typical of hypertrophic scarring resulting in scars that directly activated inflammatory and fibrotic mediators fundamental to the human hypertrophic scarring process which is devoid of epidermal injury. In addition, proinflammatory factors, for example interleukin (IL)-1α, IL-1β, IL-6, and tumor necrosis factor-α are overexpressed in keloid, which proposes that the gene expression of these factors in the skin are responsive to trauma. Chronic inflammation, can be favored resulting in invasive keloid growth. Furthermore, the overexpression of proinflammatory factors indicates that keloids and hypertrophic scars are inflammatory conditions of skin, particularly of the reticular dermis (Ogawa, 2017). Based on these considerations, it is obvious that medical doctors cannot regulate the genetic risk factors of a pathological scar. Nevertheless, they can use a number of treatments that, remarkably, work by reducing inflammation like hyaluronic acid (Gao et al., 2019; Riccio et al., 2019), dermal micrograft (Svolacchia et al., 2016; Jimi et al., 2017b). The etiology of skin fibrosis is unknown, albeit investigations proposing unknown antigens as the cause of T-cell activation thus provoking the above-mentioned mechanisms of disease onset. The analysis of inflammatory cytokines in our treated samples revealed a higher expression of inflammatory interleukin (IL) 1 and a lower rate of anti-inflammatory cytokines (IL10). For this reason, Park et al. (2018) demonstrated the contribution of IL-1 in fibrosis by prompting IL-6 and TGF-beta expression in skin fibroblasts yielding a higher fibrotic expression in induced murine models. Moreover, this fibrosis model could help physicians to identify the most useful type of treatment for different clinical conditions of fibrosis such as cellulite and hypertrophic fibrosis that include Ledderhose's syndrome, Peyronie's disease, or more frequently Dupuytren's disease.

CONCLUSION

Hypertrophic scarring may be generated via a new model specifically with the use of talc to enhance dermal fibroproliferation. The innovative technique is able to reveal morpho-functional features of human hypertrophic scars to investigate scar formation and assess potential anti-scarring therapies. The talc model may be adopted to better understand the complexities that regard the formation of hypertrophic scars and to foresee preclinical use of anti-scarring treatment.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by I.N.R.C.A./I.R.R.C.S. Ethical Committee (No. 1CHPL/08-13).

AUTHOR CONTRIBUTIONS

AM contributed conception and design of the study. AM, MM-B, VR, FO, and BZ performed experiments. NZ organized the database. FD performed the statistical analysis. AM and FD wrote the first draft of the manuscript. FD and BZ wrote sections of the manuscript. AM, FD, and MR approved final version of manuscripts. All authors contributed to manuscript revision, read, and approved the submitted version.

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frontiers in Cell and Developmental Biology

REVIEW
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Primary Ciliary Signaling in the Skin—Contribution to Wound Healing and Scarring

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Primary cilia (PC) are solitary, post-mitotic, microtubule-based, and membrane-covered protrusions that are found on almost every mammalian cell. PC are specialized cellular sensory organelles that transmit environmental information to the cell. Signaling through PC is involved in the regulation of a variety of cellular processes, including proliferation, differentiation, and migration. Conversely, defective, or abnormal PC signaling can contribute to the development of various pathological conditions. Our knowledge of the role of PC in organ development and function is largely based on ciliopathies, a family of genetic disorders with mutations affecting the structure and function of PC. In this review, we focus on the role of PC in their major signaling pathways active in skin cells, and their contribution to wound healing and scarring. To provide comprehensive insights into the current understanding of PC functions, we have collected data available in the literature, including evidence across cell types, tissues, and animal species. We conclude that PC are underappreciated subcellular organelles that significantly contribute to both physiological and pathological processes of the skin development and wound healing. Thus, PC assembly and disassembly and PC signaling may serve as attractive targets for antifibrotic and antiscarring therapies.

Keywords: primary cilia, cell signaling, scar formation, wound healing, inflammation, fibroblast, myofibroblast

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1. INTRODUCTION

The wound healing cascade progresses through the partially overlapping stages of hemostasis, inflammation, repair/proliferation, and remodeling (Almine et al., 2012). Failure of the cascade to progress through these stages gradually and in due order delays wound healing and can lead to excessive scarring or the formation of chronic wounds and ulcers (Landén et al., 2016). In the hemostasis phase, activated platelets induce formation of a fibrin clot and trigger the release of growth factors, such as platelet-derived growth factor (PDGF) and transforming growth factor β-1 (TGFβ-1). Production of growth factors, cytokines, and chemokines throughout the inflammatory stage results in coordinated activation of the immune system (Liu et al., 2003). The inflammatory phase is characterized by the migration of leukocytes into the injured skin. The coordinated activities of these cells help in clearing microbes, foreign material, and cell debris from the wound, setting the stage for the proliferation phase.

During the wound repair/proliferation phase, fibroblasts, and endothelial cells of blood vessels in the wound bed start to proliferate and form a highly vascularized fibrotic tissue that is rich in extracellular matrix (ECM) components to fill the tissue defect. Also, epithelial cells within or surrounding the wound proliferate and begin to migrate to re-surface the wound.

Due to the uneven, granulated appearance of the surface, it is called granulation tissue. It is a mixture of vascular structures, fibroblasts, macrophages, collagen bundles, fibronectin, and hyaluronic acid. Granulation tissue can be found also in chronic wounds with delayed epithelialization (Martin and Nunan, 2015). In the repair/proliferation phase, the formation of granulation tissue improves the structure and function of the wounded skin (Schultz and Wysocki, 2009). Granulation tissue cells produce TGF-β 1 and TGF-β 2, which in turn induce fibroblasts to proliferate and synthesize the provisional ECM. In hypertrophic scars, this fibroblast response is left unchecked, causing excessive cell proliferation and ECM production (Penn et al., 2012).

Upon the transition from granulation tissue to a scar, in the remodeling phase, the abundant type III collagen that dominates the proliferation stage ECM is largely replaced by type I collagen through the balanced activity of matrix metalloproteinases and their tissue inhibitors (Singer and Clark, 1999). This leads to the formation of a relatively acellular scar. Disturbances in the balance between collagen synthesis and degradation also impair normal healing and can contribute to the development of pathological scars (Sussman and Bates-Jensen, 2007). Moreover, the inflammatory signals in the forming scar are sustained, the scar tissue continues to grow forming hypertrophic scars and keloids. While a hypertrophic scar extends outward from the skin surface and does not surpass the limits of the original tissue defect, the keloid tissue spreads into the wound edges (Ogawa, 2017).

The aim of this review is to summarize the current understanding of primary cilia (PC) functions and their main signaling pathways associated with inflammation, scar formation, and wound healing under normal and pathological conditions. We collected literature across species, cell, and tissue types as well as disease models to provide further insight into the possible roles of the PC in skin.

2. PRIMARY CILIA

2.1. History of Primary Cilia

Cell membrane protrusions that carry out essential functions are present in archaea, bacteria, and eukaryotes. The motile hairlike flagella of archaea and bacteria and the motile cilia of eukaryotes share structural similarities (Fisch and Dupuis-Williams, 2011). They are comprised of a 9+2 axoneme, a circular structure consisting of nine microtubule doublets enclosing a central pair of microtubules (Fisch and Dupuis-Williams, 2011). In addition to these motile cilia, the mammalian ciliary subtypes include motile embryonal nodal cilia with a 9+0 axoneme, sensory nonmotile kinocilia with a 9+2 axoneme and PC (Satir and Christensen, 2008). PC are singular and nonmotile, and have a 9+0 axoneme thus lacking central microtubule pair.

The primary cilium was first discovered in the renal epithelium and thyroid gland in 1898 by Zimmerman, who named it "Zentralgeissel" or central flagellum (Zimmermann, 1898). In 1962, the name "primary cilium" was introduced by Sorokin (1962). They described PC in the central nervous system (Sorokin, 1962), and later on fibroblasts and smooth muscle (SM) cells (Sorokin, 1968).

The sensory functions of PC (Pedersen et al., 2006; Singla and Reiter, 2006; Berbari et al., 2009) were initially already suggested by Zimmermann (1898). However, until their association with human disorders in the twenty-first century, PC were largely neglected. Ciliopathies, which are syndromes with genetic alterations in genes coding for PC-associated proteins, include polycystic kidney disease, Bardet-Biedl syndrome (BBS), Joubert syndrome, Meckel Gruber syndrome, Ellis-van Creveld syndrome, and Jaune syndrome. More attention was given to PC after Huangfu et al. (2003) associated them with the Hedgehog (Hh) pathway that regulates cell proliferation and differentiation and has implications in cancer (Gupta et al., 2010; Sari et al., 2018). The cell-membrane-covered axoneme is comprised of nine microtubule doublets (9+0 axoneme) that extend from the basal body (Ishikawa and Marshall, 2011) and protrude into the extracellular space (Adams, 2010; Hoey et al., 2012).

2.2. Structure of Primary Cilia

A primary cilium consists of three parts: (1) the basal body, (2) the transition zone, and (3) the axoneme (Ishikawa and Marshall, 2011; Seeger-Nukpezah and Golemis, 2012) (**Figure 2**).

The membrane of the primary cilium is enriched in specific signaling molecules, such as transmembrane receptors and phosphoinositides that are essential for chemical sensing and signaling (Anvarian et al., 2019). The transition zone is located between the basal body and the axoneme and contains protein complexes that, together with gatekeeper structures such as Y-links and basal body transition fibers, control the incoming and outgoing stream of ciliary proteins (Garcia-Gonzalo and Reiter, 2017). The base of the cilium is flanked by membrane invaginations called ciliary pockets that are important for the internalization of specific signaling molecules. **Figure 1** shows a crosscut section of a PC with its subsections together with proteins involved in PC maintenance.

2.3. Assembly and Disassembly of Primary Cilia

Ciliogenesis is tightly orchestrated by cell cycle progression (Prescott, 1970; Sánchez and Dynlacht, 2016) (**Figure 2**). The assembly of a primary cilium starts after the end of mitosis

Abbreviations: BBS, Bardet-Biedl syndrome; BMP, bone morphogenetic protein; ECM, extracellular matrix; EMT, epithelial-to-mesenchymal transition; EMyT, epithelial-myofibroblast transition; GPCRs, G-protein-coupled receptors; Hh, Hedgehog; IFT, intraflagellar transport; IGF-1, insulin-like growth factor 1; IL, Interleukin; IS, the immunological synapse; MSC, mesenchymal stromal cells; PC, primary cilia; PDGF, platelet-derived growth factor; RTKs, receptor tyrosine kinases; SM, smooth muscle; SMAD, small mothers against decapentaplegic; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; VSMCs, vascular smooth muscle cells.

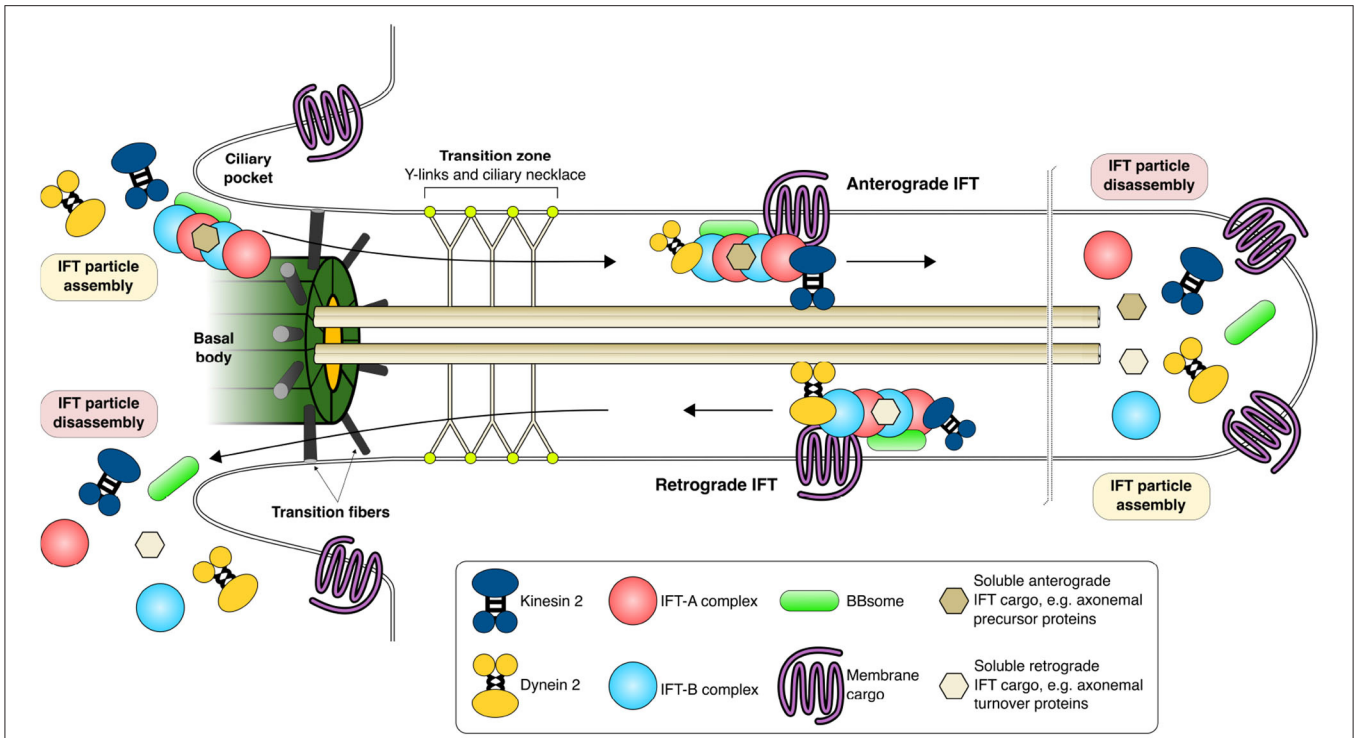

FIGURE 1 | Intraflagellar transport and functional maintenance of the primary cilium. IFT particles are assembled in the cytoplasm and are transported through the transition zone coupled to IFT trains, multi-megadalton complexes composed of IFT-A and -B proteins, kinesin, dynein, BBsome proteins as well as soluble and membrane cargoes. Anterograde IFT carries cargo toward the tip of the primary cilia whereas retrograde IFT carries cargo away from the primary cilia toward the cytoplasm. Kinesins are motor proteins that move along the axoneme in anterograde IFT while dyneins participate in retrograde IFT. The primary cilia is formed from a modified mother centriole (basal body) from which the nine pairs of axonemal tubules grow and extend out from the cell surface covered by the plasma membrane. The plasma membrane composition of the primary cilia is specifically maintained and guarded by the transition fibers and transition zone (Y-links and ciliary necklace) through which only specific proteins and their cargo are allowed. References for this figure (Cole and Snell, 2009; Basten and Giles, 2013; Nachury, 2014; Taschner and Lorentzen, 2016; Eguether and Hahne, 2018; Wingfield et al., 2018).

(Sánchez and Dynlacht, 2016). Small cytoplasmic vesicles (preciliary vesicles) originating from the Golgi complex and endosomal recycling compartment are first transported by specific kinesins to the distal end of the mother centriole to form a larger ciliary vesicle (Sorokin, 1962; Schmidt et al., 2012; Kobayashi et al., 2014; Lu et al., 2015b). From within this vesicle, the membrane-surrounded axoneme grows to extend out of the cell surface, covered by the plasma membrane (Gilula and Satir, 1972).

As the axonemal structure grows in length, the assembled microtubules are stabilized through acetylation of tubulin by specific tubulin acetyltransferases (α-tubulin K40 acetyltransferase, αTAT, and MEC-17) (Leroux, 2010). It has been suggested that this process is activated by the Aurora-A centrosomal kinase (AURKA) that controls mitotic entry via activation of cyclin-dependent kinase 1 (CDK1) B (Marumoto et al., 2005; Pugacheva et al., 2007). The disassembly of PC by AURKA requires the activity of histone deacetylase 6 (HDAC6). Mirvis et al. (2019) demonstrated that PC disassembly through these molecular signals could occur through at least two mechanisms: through gradual degradation and incorporation of the axoneme into the cell or through whole-cilium shedding (deciliation) with the axoneme being excised at its base.

Altogether, the formation, maintenance, and degradation of the primary cilium are highly regulated processes. The dynamic interrelationship between the assembly and disassembly processes determines the length of the cilium (Keeling et al., 2016). Relative to the length of the cilium, the cell's ability to sensitively detect extracellular cues is either increased or decreased. PC length can affect mechanotransduction, the process by which cells transduce mechanical forces into biological signals (Spasic and Jacobs, 2017) or cause defects in signal processing (Canterini et al., 2017).

2.4. Signaling Pathways

PC guide such fundamental cellular functions as proliferation and differentiation and thereby contribute to organ development, tissue homeostasis, and repair as well as the regulation of inflammation (Pazour et al., 2000; Huangfu et al., 2003; Marshall and Nonaka, 2006; Singla and Reiter, 2006; Berbari et al., 2009; Green and Mykytyn, 2014). Signaling through Wnt Gerdes et al. (2007), Notch (Ezratty et al., 2011), Hh (Huangfu et al., 2003), G-protein-coupled receptors (GPCRs) (Schou et al., 2015), TGF-β (Clement et al., 2013), and insulin-like growth factor-1 (IGF-1) (Yeh et al., 2013) has been associated with PC. All these signaling

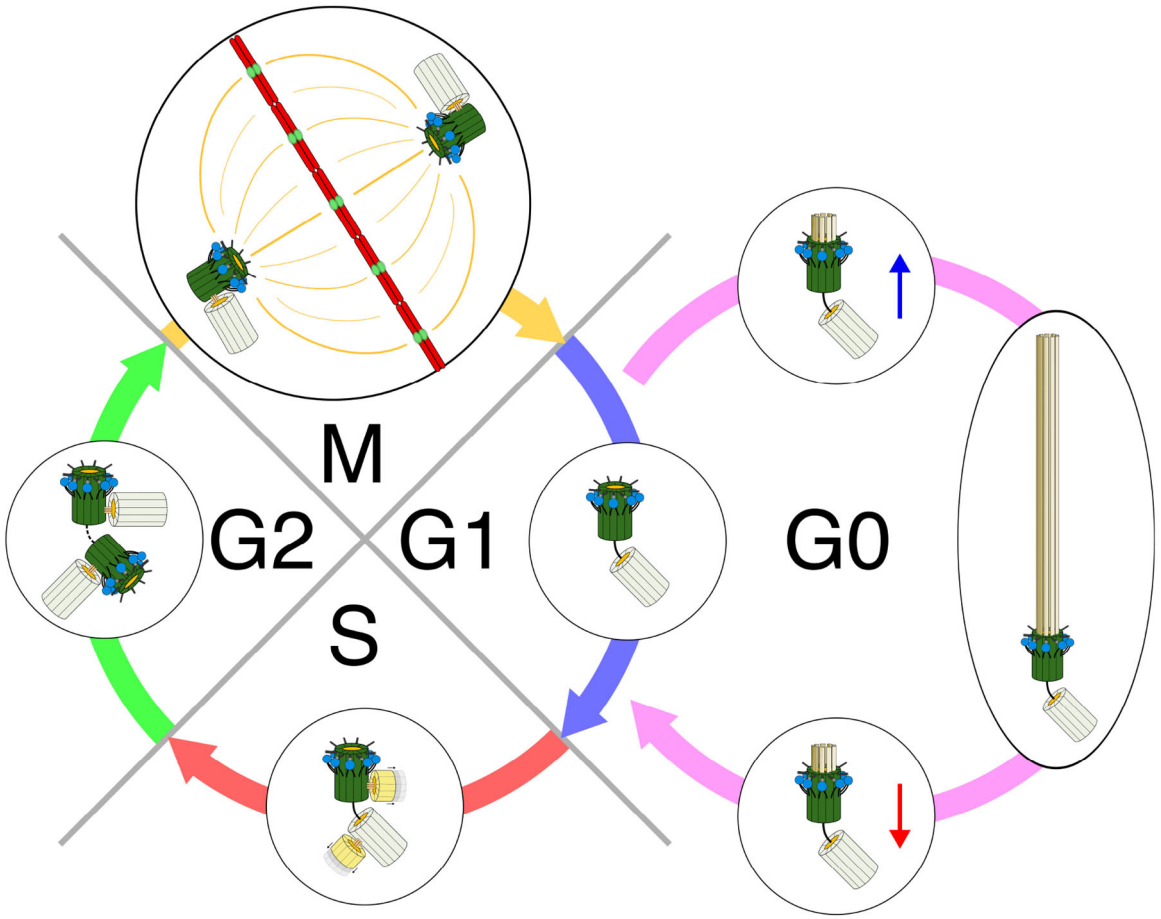

FIGURE 2 | Primary cilia, centrioles, and cell cycle. Early G1—Centrioles are disengaged from their orthogonal orientation. The mother centriole with its distal appendages and its accompanying daughter centriole with its pericentriolar protein-rich matrix form the basal body for ciliation. G0—Basal body (BB) migrates to the plasma membrane. Plasma membrane invaginates and centriolar elongation progresses in ciliary vesicles. The BB anchors to and fuses with the cell membrane. Axonemal growth is initiated. A functional primary cilia is formed. Its length and function are dynamically controlled by constant turnover as well as both cellular and environmental cues. Late G1—Before re-entry to the cell cycle, the first wave of primary cilia disassembly occurs. Ciliary shortening is required to allow the cell to enter the S-phase. S—Both mother and daughter centrioles duplicate to form new mother-daughter pairs. Duplication occurs in an orthogonal orientation from the existing centrioles. The newly formed centrioles become the new daughter centrioles, with a new mother centriole established from the old daughter for its respective cell after division. G2—The second wave of cilia disassembly and resorption occurs and is finalized in order for the cell to enter mitosis. The new mother centriole (old daughter) acquires its distal and sub-distal appendages. M—Centrioles in their engaged, orthogonal orientation form the centrosomes for mitosis and subsequent cell division. Redrawn using information from the following: Ishikawa and Marshall (2011), Basten and Giles (2013), and Wang and Dynlacht (2018).

pathways are important for skin development and contribute to wound repair (Bielefeld et al., 2013).

The primary cilium acts as a signaling hub that coordinates the activity of numerous signaling pathways (Nishimura et al., 2019), including in addition to the above-mentioned signaling pathways signaling transmitted by and associated with bone morphogenetic protein (BMP) (Clement et al., 2013), planar cell polarity (Ross et al., 2005), platelet-derived growth factor receptorα (PDGFRα) (Schneider et al., 2005), receptor tyrosine kinases (RTKs) (Christensen et al., 2012), Hippo (Yu and Guan, 2013), NFkappaB (Karin, 1999), mTOR (Pala et al., 2017), and ECM receptors (McGlashan et al., 2006). In many cases, the primary cilium integrates simultaneous inputs, thus fine-tuning and integrating signals from different sources into an orchestrated cellular response. Examples of signaling cascades associated with PC are presented in **Figure 3**.

2.4.1. TGF-β

TGF-β/BMP signaling plays a crucial role in cell proliferation, migration, differentiation, apoptosis, ECM remodeling, immune functions, and tumor metastasis (Guo and Wang, 2009), and is one of the major signaling pathways associated with myofibroblast differentiation and epithelial-mesenchymal transformation (Thannickal et al., 2003).

Of the three TGFβ isoforms, TGFβ-1 is the main signaling molecule in most tissue types and pathological processes, including skin and cutaneous wound healing (Wang, 2001; Barrientos et al., 2008). In the skin, TGFβ-1 is expressed in

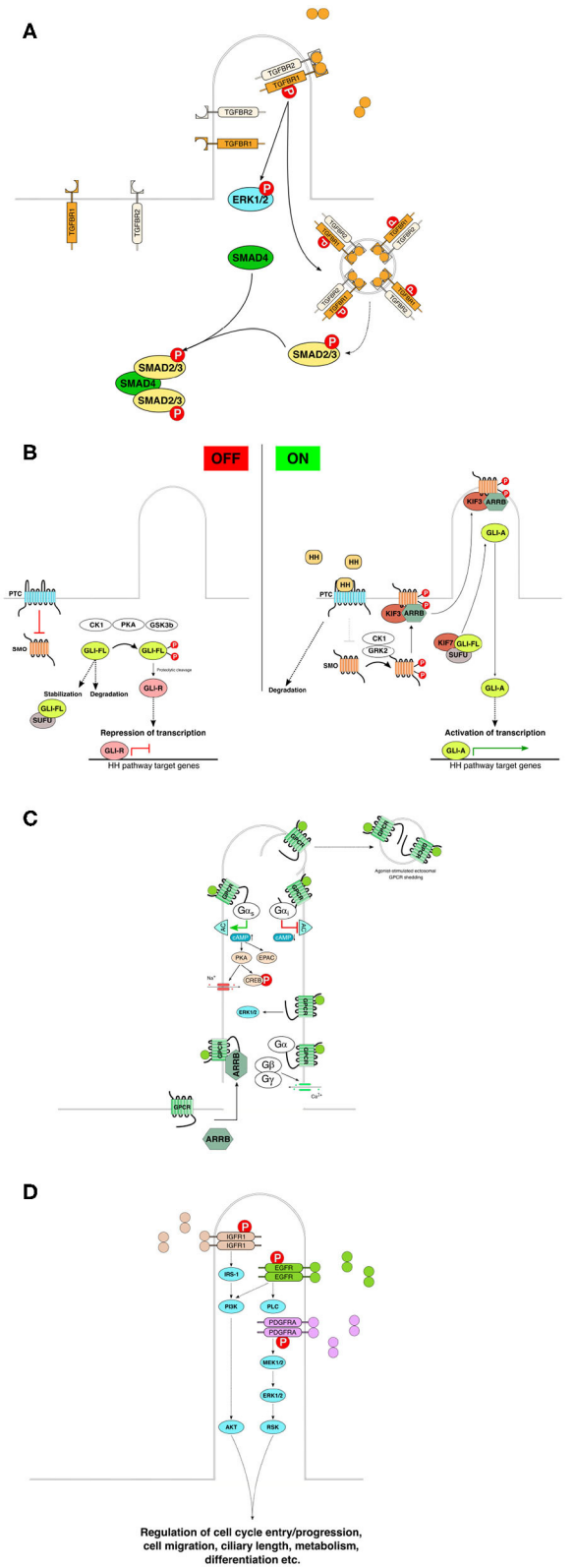

FIGURE 3 | Basic components and rough characteristics of each indicated signaling pathway in relation to primary cilia. **(A)** TGFbeta receptors

(Continued)

FIGURE 3 | accumulate in the primary cilia, and ligand binding to the TGFBR2 faciliates recruitment of TGFBR1 to form the heterodimeric receptor complex. Subsequent phosphorylation and activation of the receptor leads to downstream activation of the SMAD family of transcription factors by active receptor transport and aggregation in endosomal vesicles for downstream signal enhancement. **(B)** Mammalian Hedgehog signaling is tightly linked to the primary cilia. In the absence of signal, the pathway is maintained inactive or in a repressive state through the inhibitory action of Patched on Smoothened, and through the proteolytic cleavage or SUFU-mediated stabilization of GLI family of transcription factors. Upon activation by Hedgehog pathway ligands (Sonic Hedgehog, Indian Hedgehog or Desert Hedgehog) the inhibitory activity of PTC on SMO is abrogated and SMO can, with the help of beta-arresin and kinesin 3, translocate to the primary cilia. Active SMO then facilitates dissociation of GLI from its regulator SUFU leading to activation of GLI and GLI-A-induced transcription of HH pathway response genes in the nucleus. **(C)** The primary cilia-associated G-protein-coupled receptors are transported to the primary cilia assisted by e.g., beta-arrestin. Ligand stimulation leads to receptor activation and activation of downstream signaling depending on the receptor's coupling to various types of G-proteins. Ligand-induced shedding of G- proteins in ectosomes from the primary cilia denotes an additional level of regulation or suppression of G-protein signaling. **(D)** Receptor tyrosine kinase pathways are also associated with the primary cilia. For example, IGF, EGF, and PDGF utilize primary cilia for signaling. Ligand binding results in downstream activation of specific kinase cascade activations leading to activation of transcription factors and intracellular effectors that participate in the regulation of various cellular processes. Redrawn using information from the following: Ruel and Thérond (2009), Ishikawa and Marshall (2011), Christensen et al. (2012), Hilgendorf et al. (2016), Bangs and Anderson (2017), Christensen et al. (2017), and Anvarian et al. (2019). BBsome, Bardet-Biedl syndrome protein complex; IFT, intraflagellar transport; PTC, patched; SMO, smoothened; SUFU, suppressor of fused homolog; GLI-FL, glioma-associated oncogene (zinc finger protein GLI) full-length; GLI-A, activated form of GLI; GLI-R, repressor form of GLI; CK1, casein kinase 1; PKA, protein kinase A; GSK3b, glycogen synthase kinase 3 beta; HH, hedgehog; GPRK2, G-protein- coupled receptor kinase 2; ARRB, beta-arrestin; KIF, kinesin superfamily protein; TGFB, transforming growth factor beta; TGFBR, TGFB receptor; SMAD, family of transcription factors homologous to *C. elegans* SMA and MAD families; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase kinase; IGFR, insulin-like growth factor receptor; EGFR, epidermal growth factor receptor; PDGFRA, platelet-derived growth factor receptor alpha; IRS, insulin receptor substrate; PI3K, phosphoinositide 3-kinase; PLC, phospholipase C; AKT, protein kinase B; RSK, ribosomal protein S6 kinase; GPCR, G-protein-coupled receptor; AC, adenylate cyclase; cAMP, cyclic adenosine monophosphate; CREB, cAMP-responsive element-binding protein; EPAC, rap guanine nucleotide exchange factor, exchange protein directly activated by cAMP; G alpha s, G-protein alpha subunit, stimulatory; G alpha i, G-protein alpha subunit, inhibitory; Gbeta, gamma, G-protein beta and gamma subunits, respectively; RTK, receptor tyrosine kinase.

the stratum granulosum and stratum corneum, while TGFβ-2 and -3 are expressed in the supra-basal layers, suggesting that each TGF-β isoform has a different function in keratinocyte proliferation and differentiation (Gold et al., 2000; Cho et al., 2004). While TGF-β-1 and -2 promote scar tissue formation, TGF-β-3 reduces scar formation (Lin et al., 1995; Shah et al., 1995). However, the TGF-β-1 and -2 receptors are present both in fetal and adult dermal tissues (Helmo et al., 2013). Soo et al. (2003) suggested that increased levels of TGF-β-3 expressed early in fetal wounds may compete with TGF-β-1 and -2 to bind to the type II receptor and, moreover, that an anti-scar effect of TGF-β-3 is seen after the early TGF-β-3 induction in fetal wounds or after early application to adult wounds.

There is an increasing body of evidence that PC play an important role in both canonical and non-canonical TGF-β/BMP signaling and, more importantly, in fine-tuning the balance of these pathways (Anvarian et al., 2019) (**Figure 3A**). It has been shown that in an inactive state, the TGF-β receptors accumulate at the tip of the primary cilium (Clement et al., 2013).

TGF-β/BMP signaling is induced via activation of heterotetrameric type I (RI) and type II (RII) receptor complexes that act as serine/threonine kinases. Upon ligand binding, the receptors are translocated to the base of the cilium and are internalized via clathrin-dependent endocytosis. The activation of TGF-β receptors leads to phosphorylation and activation of transcription factors, small mothers against decapentaplegic (SMAD) 2/3 (Huang and Chen, 2012; Clement et al., 2013). Activated SMAD2/3 bind to and induce the nuclear translocation of a related molecule SMAD4 and the formation of a transcriptionally active complex with SMAD4 regulating thereby gene expression (Clement et al., 2013). Also, clathrin-independent extracellular regulated kinase 1/2 (ERK1/2) activation by TGF-β receptors is located at the ciliary base (Clement et al., 2013). The exact molecules that are involved in the trafficking of TGF-β receptors along primary cilium are not yet described. Nevertheless, the trafficking of Ras-related protein Rab-11A (RAB11), which is involved in endosomal recycling of TGF-β receptors is impaired by the loss of the mother centriole protein centrosomal protein of 128 kDa (CEP128) that coordinates the localization of GF-β receptors, resulting in impairment of TGF-β signaling (Mitchell et al., 2004; Westlake et al., 2011; Mönnich et al., 2018). Non-canonical TGF-β/BMP signaling involves, for example, activation of extracellular signal-regulated protein kinase (ERK)1/2, which in turn activates MAP kinase (Clement et al., 2013).

Interestingly, the negative feedback regulator of TGF-β signaling, SMAD7, and the E3 ubiquitin-protein ligase SMURF1 also localize to the base of the primary cilium and have been suggested to thereby limit excessive TGF-β/BMP signaling (Clement et al., 2013; Heldin and Moustakas, 2016; Miyazawa and Miyazono, 2017; Koefoed et al., 2018).

2.4.2. Wnt/Catenin

The wnt-PCP pathway has been implicated in the regulation of cell morphology, migration, and oriented cell division and has been shown to involve Ryk, Ror family kinases, and the Vangl protein (Green et al., 2014; Yang and Mlodzik, 2015). Many of the core components of the Wnt pathway have been found in PC, and several proteins implicated in different stages of the Wnt signaling cascade have been found to localize to the base of PC (Corbit et al., 2008; Chen et al., 2011; Lancaster et al., 2011).

There are three major types of Wnt signaling: the β-catenin pathway, which is considered the canonical Wnt pathway, and the non-canonical planar cell polarity (PCP) and Wnt/Ca2+ pathways (MacDonald et al., 2007; Semenov et al., 2007; Houschyar et al., 2015). Wnt signaling or the Wnt/β-catenin pathway plays an essential role in skin development and maintenance (Logan and Nusse, 2004; Fuchs, 2007; Clevers and Nusse, 2012). It has also been closely associated with tissue regeneration and repair. The ligands of this pathway include a number of vertebrate homologs of the Drosophila wingless (wnt) gene that bind to the frizzled family of receptors and numerous coreceptors (MacDonald and He, 2012; Niehrs, 2012). Binding to the Frizzled (Fzd) receptor, which leads to complex formation with the coreceptors LRP5/6 and Disheveled (DVL). As a result, β-catenin is stabilized and translocates to the nucleus to regulate gene expression (Kim et al., 2013; Sineva and Pospelov, 2014).

There are conflicting data regarding the role of ciliary transport in the regulation of Wnt signaling. On the one hand, it has been shown that the primary cilium acts as an inhibitor of Wnt signaling, as the loss of the key molecular motors Kif3A, intraflagellar transport 88 (IFT88), and Ofd1 was associated with augmented signaling in response to Wnt3A (Corbit et al., 2008). On the other hand, it was shown that Kif3A might inhibit Wnt signaling independent of the primary cilium (Kim et al., 2016), leaving much to be investigated in future studies.

2.4.3. Notch

The Notch signaling pathway regulates the development and homeostasis of many types of tissues, such as the nervous system, the vascular system, the hematopoietic system, somites, the muscle, the skin, and the pancreas (Hansson et al., 2004). The Notch signaling pathway controls epidermal differentiation (Watt et al., 2008), and it has also been suggested that the Notch pathway is associated with wound repair (Thélu et al., 2002; Chigurupati et al., 2007; Outtz et al., 2010).

In mammals, there are four different Notch receptors that are activated by ligands belonging to the family of Delta-like and Jagged proteins (Brittan and Wright, 2007). Ligand binding induces cleavage of the intracellular domain, which enters the nucleus and activates transcription of target genes (Moore and Alexandre, 2020). A small amount of evidence has indicated that loss of the key transport proteins Kif3a and IFT88 results in disruption of Notch signaling in cultured keratinocytes and the intact epidermis. However, the exact mechanism by which PC regulate Notch signaling is not yet clear (Ezratty et al., 2011).

2.4.4. Hedgehog

The Hh signaling pathway is involved in tissue development, homeostasis, and repair as well as in regulating morphogenesis of the skin during embryogenesis (Ingham and McMahon, 2001; Bielefeld et al., 2013).

There are three Hh proteins in mammals, namely, Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh) (Tasouri and Tucker, 2011) (**Figure 3B**). Shh plays a major role in skin development and maintenance, while Ihh is involved in cartilage formation and Dhh plays a role in gonadal morphogenesis (Bitgood and McMahon, 1995; Boras-Granic et al., 2006). In addition to its role in normal skin homeostasis, Hh signaling is the main player in the development of the most widespread human malignancy—basal cell carcinoma (Kasper et al., 2012).

The central signaling transducer is the 7-transmembrane molecule Smoothened (Smo), which is under constant inhibition of the 12-transmembrane molecule Patched (Ptch), which acts as a Hh receptor. As a result, the transcriptional regulator GLI3 is processed via protein kinase A-mediated phosphorylation

into a repressor form that suppresses the transcription of Hh target genes (Mukhopadhyay and Rohatgi, 2014). Upon ligand binding, Ptch inhibition of Smo is relieved, resulting in the accumulation of transcriptionally active forms of GLI2 and subsequent activation of target gene expression (Kasper et al., 2012). The exact mechanism of signal transduction from Ptch to Smo is not clear; however, a role for cholesterol or cholesterol derivatives, such as oxysterols, has been suggested (Kinnebrew et al., 2019).

The non-canonical Hh signaling pathway involves the activation of GLI transactivators independently of Smo. Most of the key events of Hh signaling are coordinated by the primary cilium. Ptch is localized in caveolin-1-containing membrane lipid rafts at the base of the primary cilium together with SMURF1 and SMURF2 (Yue et al., 2014). Caveolin-1 accumulation at the base of the cilium is dependent on the molecular motor protein KIF13B, and both of these proteins have a considerable impact on Hh signaling (Schou et al., 2017). Upon Hh ligand binding, Ptch1 is cleared from the membrane due to its ubiquitination; consequently, Smo molecules are transported into the ciliary membrane from other parts of the cell (Corbit et al., 2005; Rohatgi et al., 2007).

It has been demonstrated that interaction between Smo and a ciliary protein, Evc, that is localized close to the transition zone is crucial for Hh signal activation (Dorn et al., 2012). As a result, GLI2 and GLI3 are transported by an the IFT protein Tg737 to the tip of the cilium. Loss of this protein alters the correct processing of both active and repressive forms of GLI1 and GLI3, suggesting that the processing events themselves may take place in the tips of the cilia (Haycraft et al., 2005). KIF7, an atypical kinesin, is also involved in the transport and correct processing of the GLI proteins at the tips of cilia, potentially via modulating by modulating the ciliary architecture (Liem et al., 2009; He et al., 2014). Another ciliary protein, GPR161, that belongs to the GRCRs, acts as a repressor for of Hh signaling by promoting the processing of GLI3 into the repressive form, most likely by activation of PKA via cAMP upregulation. Interestingly, the ciliary localization of GPR161 is dependent on IFT-A complex components, most notably Tulp3, which is an adaptor protein that is responsible for the transport of specific GPCRs into the cilium (Mukhopadhyay et al., 2013).

It appears that PC retrograde trafficking of Hh pathway complexes is important for proper Hh signaling, as disruption of IFT 25 and 27 results in the accumulation of Ptch-1 and Smo in the primary cilium and the disruption of Hh signaling (Keady et al., 2012; Eguether et al., 2014). The primary cilium may also play a role in non-canonical Hh signaling. It has been shown that the IFT protein IFT80 can repress activation of the GTPase RhoA by Shh, balancing the canonical and non-canonical Hh signaling pathways (Yuan et al., 2016).

2.4.5. G-Protein-Coupled Receptors

The primary cilium functions as a hub for GPCR signaling (**Figure 3C**). GPCRs, the largest class of proteins, regulate numerous functions in the cells. The GPCR repertoires of various cell types differ. For example, the majority of neurons located in the mammalian brain have cilia that are enriched in specific GPCRs. GPCRs recruit G-protein heterotrimers consisting of Gα, Gβ, and Gγ subunits. Signal transduction through GPCRs involves the replacement of GDP by GTP on the Gα subunit. Gα and Gβ γ subunits dissociate, and the activated Gα subunit stimulates or inhibits adenyl cyclases (ACs) or activates phospholipase-C (PLC).

The plethora of signaling events that are affected by Gβ γ subunits is only beginning to emerge. Canonically Gβ γ signaling affects the activity of not only potassium and calcium ion channels and PLC but also PI3K, MAP kinases, and some AC isoforms. Hydrolysis of bound GTP to GDP reassociates the Gα and Gβ γ subunits and thus ends their signaling activity (Khan et al., 2013; Hilger et al., 2018). Defects in cilia, as seen in the case of ciliopathies, also affect the nervous system (Guemez-Gamboa et al., 2014). Inactivating mutations in several genes encoding ciliary proteins result in defects in GPCR accumulation in cilia. For example, disruption of a gene encoding a BBS complex (BBSome) protein disrupts the ciliary localization of somatostatin receptor subtype 3 (SSTR3), melanin-concentrating hormone receptor 1 and neuropeptide Y (NPY) receptor subtypes 2 and 5 (NPY2R and NPY5R) (Berbari et al., 2008; Loktev and Jackson, 2013; Green et al., 2016).

In the case of SSTR3, its ciliary localization is dependent on the adaptor protein β-arrestin (Green et al., 2016). The lack of NPY2R and NPY5R in mouse hypothalamic neurons results in excessive food intake, as signaling via these receptors is required for sensing the neuropeptide Y signal that controls sufficient food intake (Loktev and Jackson, 2013). Obesity is also a symptom of melanocortin 4 receptor (MC4R) mutations that impair its ciliary localization in both humans and mice, suggesting that ciliary localization is essential for the functionality of this GPCR receptor (Davenport et al., 2007; Siljee et al., 2018).

2.4.6. Receptor Tyrosine Kinases

RTKs form a prominent class of signal transducers that can be subdivided into several subclasses depending on their specific properties (Lemmon and Schlessinger, 2010). RTKs act as receptors for a wide variety of biomolecules. Most RTKs homodimerize upon ligand binding, which induces autophosphorylation and activation of the RTK subunits.

Subsequently, the activated RTK complex phosphorylates downstream targets that include different intracellular kinases (the MAP kinase family, PI3K-AKT pathway kinases, PL Cγ, and others), which exert the effects induced by specific ligand molecules (Crudden et al., 2018). Signaling through PDGFRα, insulin receptor (IR), and IGF-1 receptor (IGF-1R) is tightly connected to PC (Christensen et al., 2012) (**Figure 3D**).

2.4.7. PDGFRα

PDGFRα signaling is disrupted by inactivation of the IFT88 and IFT172 transporters, which also causes aberrant cilium assembly (Schneider et al., 2010). Furthermore, IFT20, another ciliary protein, stabilizes the CBL E3 ubiquitin ligases that target PDGFRα for degradation and thereby limit PDGF signaling. Inactivation of IFT20 results in ciliary collapse, inactivation of CBL proteins, and excessive uncoordinated signaling from PDGFRα (Schmid et al., 2018). Ciliary IGF-1R signaling is

crucial for adipogenesis. While IGF-1R is localized to the ciliary membrane, its primary targets, insulin receptor substrate 1 (IRS-1) and AKT kinase, reside at the base of the cilium, facilitating coordinated signal transduction and activation of adipogenic differentiation upon receptor activation (Zhu et al., 2009).

3. PRIMARY CILIA IN SKIN CELLS AND WOUND HEALING

3.1. Primary Cilia, Inflammation, and Skin Immune Cells

Neutrophils are the first cells to be recruited from the circulation to the site of injury in the early stage of inflammation (Sinno and Prakash, 2013). As such, neutrophils have a relatively short lifespan, but the survival signals, such as cytokines, chemokines, and growth factors, in the wound environment can prolong their lifespan. Activated neutrophils produce several proinflammatory and proangiogenic mediators, for example, vascular endothelial growth factor (VEGF), TNF-α, and interleukin 1 (IL-1), to recruit more immune cells and enhance the innate response as well as to promote the progression of wound healing to the inflammatory stage. Further, because neutrophils produce reactive oxygen species and antimicrobial peptides that are essential for wound decontamination in the early inflammatory phase, the failure of subsequent neutrophil apoptosis leads to the persistence of these factors in the wound. Such aggravation of the inflammatory phase can provoke delays in wound healing (Landén et al., 2016). Macrophage activity, on the other hand, can counteract these signals and promote neutrophil apoptosis (Meszaros et al., 2000). Circulating monocytes infiltrate the wound and differentiate into macrophages to facilitate wound healing. Macrophages phagocytose and clear apoptotic neutrophils. This process, called efferocytosis, induces macrophages to produce factors that help limit inflammation and aid wound healing to promote progression to the proliferative phase (Koh and DiPietro, 2011). Macrophages and monocytes are pivotal cells in antimicrobial immune defense and the repair of tissue damage repair. The absence of macrophages leads to impaired wound healing (Mirza et al., 2009), and it has been shown that macrophages play important roles during the different stages of the wound repair process (Lucas et al., 2010).

There seems to be an association between macrophages and the response to injury in case when ciliary functions in tissues are damaged (Zimmerman et al., 2017). Data from primary ciliary dyskinesia (PCD), a genetic disorder in which mutations cause defects in all cilia, has shown that dysfunctional cilia reduce infection resistance and that the inflammatory response is aggravated by PCD monocytes (Cockx et al., 2017).

3.2. Mast Cells

Mast cells are multifunctional and highly granulated immune cells of the myeloid lineage that synthesize and store various mediators of inflammation (Krystel-Whittemore et al., 2016). They are located in mucosal and epithelial tissues throughout the body, specifically in areas below the epithelium, surrounding blood cells, SM, mucous, and hair follicles (da Silva et al., 2014; Krystel-Whittemore et al., 2016). Mast cells in the skin are activated by antigens through IgE binding and produce histamine, which is an essential chemical mediator of inflammation, among other bioactive substances (Kawamoto and Masuko, 2013).

There is increasing interest in mast cells due to their potential role in wound healing (Trautmann et al., 2000; Masatoshi et al., 2002; Chisholm and Greene, 2011). Histamine stimulates the proliferation of fibroblasts derived from both normal skin and keloid and scar tissue (Russell et al., 1977). Furthermore, the proinflammatory cytokine TNF-α is produced by mast cells (Walsh et al., 1991). It has been shown that TNF-α induces fibroblast proliferation and collagen metabolism, and it also increases the length of PC. This may play a critical role in wound healing (Tharp, 1989).

3.3. T Cells

T cells or T lymphocytes are pivotal leukocytes in the immune system and regulators of inflammation (Eagar and Miller, 2019). Circulating naïve T cells are activated when they encounter their cognate antigens, leading to cell proliferation and differentiation into effector T cells (Takamura, 2018). It has been shown that T cell activation is induced in the context of peptide-major histocompatibility complex ligands at the surface of antigen-presenting cells (APCs) via the T cell receptor. The immunological synapse (IS) is a highly specialized interface that forms between a T cell and an APC (Grakoui et al., 1999).

T cells are known to lack PC. Despite this fact, T cells possess certain cilia-like functions at the IS, a cell-cell junction between T cells and antigen-presenting cells, and they continue to express the proteins involved in ciliogenesis and use these proteins to build the IS (Cassioli and Baldari, 2019). There is increasing interest in the role of the IS since T cells may express several common proteins that are present in both the IS and PC. Although the IS and PC are structurally different, many ciliary proteins are recognized as active participants in IS-related functions in non-ciliated T cells (Cassioli and Baldari, 2019). The IS and PC still have limited structural and functional similarities, for instance, providing sensing and signaling platforms in cells.

However, PC are not generally found in hematopoietic cells (Cassioli and Baldari, 2019). Cells of hematopoietic origin still maintain some functions of PC, such as the activity of certain signaling pathways (Finetti et al., 2011).

3.4. Langerhans Cells

Langerhans cells (specialized dendritic cells, DCs), and CD8+ cytotoxic T cells (Maibach and Honari, 2014) reside in the skin's superficial epidermal layer (Clayton et al., 2017). There are also various specialized immune cells in the dermis, such as antigen-presenting dermal DCs, T cells, B cells, NK cells, mast cells, monocytes, and macrophages (Pasparakis et al., 2014).

Langerhans cells are bone-marrow-derived antigen-presenting immune cells located in the basal and suprabasal layer of the epidermis (Lombardi et al., 1993). Langerhans cells have characteristic features of dendritic cells, and the presence of PC in both Langerhans and dendritic cells has been shown (Toriyama et al., 2020). Granulocyte macrophage-colony stimulating factor

(GM-CSF) is a hematopoietic growth factor that is mainly produced by immature Langerhans cells and stimulates the formation of PC in granulocytes and macrophages, activating the differentiation of granulocytes and macrophages from bone marrow precursor cells. Furthermore, PDGFRα localizes to PC, and PDGFα signaling promotes the proliferation of dendritic cells (Toriyama et al., 2020).

Interestingly, in the inflamed epidermis of atopic dermatitis patients, which has reduced barrier function, the number of cells with PC was increased, and atypically ciliated Langerhans cells and keratinocytes were found (Toriyama et al., 2020). As Langerhans cells present antigens to lymphocytes similar to macrophages, PC that regulate their activity are essential for the immune functions of the skin (Marks and Miller, 2013).

4. PRIMARY CILIA AND NEURONAL REGULATION OF CUTANEOUS IMMUNITY AND INFLAMMATION

It has been suggested that mechanical stress, which involves skin stretching, may result in the formation or aggravation of various cutaneous inflammatory disorders including pathological scars, atopic dermatitis, and psoriasis via stimulation of mechanosensitive nociceptors on sensory nerves in the skin (Akaishi et al., 2008; Choi and Di Nardo, 2018; Malakou et al., 2018). These sensory nerves release neuropeptides, such as substance P (SP) and calcitonin gene-related peptide (CGRP). As the nerve endings have contacts with virtually all epidermal and dermal cell types (Scholzen et al., 1998), the signals emanating from these nerves cause the activation of endothelial and vascular smooth muscle cell (VSMCs), leading to vasodilation and permeabilization of vessels (Akaishi et al., 2008). In addition, histamine release from mast cells is induced by SP (Columbo et al., 1996), which also further contributes to vasodilation and the permeabilization of vessels. These reactions are considered to result in neurogenic inflammation, in which central stimulation of sensory nerves evokes antidromic impulses that induce vasodilation and plasma extravasation and result in a local inflammatory response (Holzer, 1998; Brookoff, 2009), which contributes to the formation or aggravation of pathological scars, atopic dermatitis, and psoriasis (Akaishi et al., 2008). Furthermore, SP causes the production of TNF-α and IL-6 in mast cells (Azzolina et al., 2003), and these proinflammatory factors are associated with the appearance of pathological scars. Increased levels of SP stimulate the release of various other proinflammatory cytokines such as TNF-α, IFN-γ in the case of atopic dermatitis, and psoriasis (Remröd et al., 2007; Choi and Di Nardo, 2018). Neuropeptides and numerous cells also stimulate TGF-β, whose signaling pathway is coordinated by PC, and nerve growth factor (NGF) (Akaishi et al., 2008). NGF is thought to be one of the mediators of mechanical tension signals in hypertrophic scarring (Xiao et al., 2013). The expression of NGF is increased in the skin of patients with atopic dermatitis, and this seems to contribute to aggravation of the disease (Dou et al., 2006).

It has been observed that sensory neurons are associated with wound repair, and depletion of cutaneous sensory innervation is related to a delay of the wound healing process (Maggi et al., 1987; Carr et al., 1993; Smith and Liu, 2002; Barker et al., 2006). Further, sensory nerves seem to control physiological and pathological processes in the skin through the activation of target cells, which express receptors for neuromediators (Kruger, 1996; Roosterman et al., 2006). Thus, dorsal root ganglia or sensory neurons may not only receive and transduce mechanical and noxious stimuli but also play a valuable role in wound repair. However, the exact mechanisms are not yet known. Interestingly, exogenous administration of CGRP and SP is thought to promote wound closure (Engin, 1998; Delgado et al., 2005; Toda et al., 2008; Rook et al., 2009), and CGRP in particular has been observed to improve epithelial proliferation in some studies (Seike et al., 2002; Yu et al., 2009).

5. PRIMARY CILIA, GRANULATION TISSUE FORMATION, AND WOUND HEALING

5.1. Endothelial Cells

In endothelial cells, PC are essential for sensing of blood flow shear stress (Chen et al., 2017). They also contribute to sensing of pH and oxygen (Anvarian et al., 2019).

Endothelial cell responses to FSS through PC help maintain barrier function, blood vessel wall permeability, and vascular tone (Jones et al., 2012; Peng et al., 2019). PC are also involved in endothelial cell inflammatory signaling, as their loss promotes vascular inflammation (Dinsmore and Reiter, 2016), and their length is controlled by cytokine stimuli, as mentioned above (Dummer et al., 2018). Defects in endothelial PC cause disorders of blood fluid-induced responses and result in vascular dysfunctions, such as hypertension, aneurysm, and atherosclerosis (Pala et al., 2018).

The pro-and antiangiogenesis phases of the wound healing process require precise sequential orchestration, and if poorly controlled, they can promote fibrotic scar formation (DiPietro, 2016). Interestingly, Chen et al. (2017) demonstrated that FFS sensing and Notch signaling through endothelial cell PC are required for arterial maturation and recruitment of vascular mural cells/pericytes in zebrafish, suggesting that endothelial PC may play a crucial role in controlling angiogenesis during wound healing.

5.2. Vascular Smooth Muscle Cells

Although not directly in contact with luminal flow and shear stress in intact arteries, VSMCs, and adventitial fibroblasts can be exposed to transmural interstitial flow (Shi and Tarbell, 2011). PC respond to mechanical deflection and integrin activation with an increase in intracellular calcium concentrations (Lu et al., 2008). Mechanosensing in VSMCs not only induces changes in contraction and cell morphology but also leads to altered production of cytokines and other vasoactive and pro-/anti-inflammatory signaling molecules (Shi and Tarbell, 2011).

BBSome in vascular smooth muscle were recently demonstrated to contribute to the control of vascular function and stiffness (Reho et al., 2019). The deletion of a gene encoding

a critical BBSome component, Bbs1, resulted in vascular dysfunction, increased contractility, and reduced relaxation (Reho et al., 2019).

Furthermore, in contrast to non-ciliated cells, ciliated VSMCs showed more efficient migration in wound repair (Lu et al., 2008). Lu et al. (2008) found out that ciliary redistribution following wounding is regulated through the ciliary integrin-ECM interaction, as ciliary resettlement was decreased after β1-integrin blockade. Since VSMCs and the ECM are essential for regulating vascular tissue homeostasis, differentiation, and wound repair, cilium-mediated functions may be pivotal for maintaining vascular functional integrity (Raines, 2000). As compression therapy (discussed below) is one of the most widely used methods to prevent or reduce the appearance of scars in wound patients (discussed below), the mechanosensing via PCs may have important implications in both pathogenesis and also the treatment of such scars.

5.3. Fibroblasts

Dermal fibroblasts are dominant components of the skin's dermal layer, which resides underneath the epidermis. Based on anatomical location and structural and functional differences, these fibroblasts are divided into superficial (papillary) and deep dermal (reticular) fibroblasts (Brown and Krishnamurthy, 2018) as well as hair follicle-associated fibroblasts that reside in the immediate vicinity of hair follicles (Jahoda and Reynolds, 2001). However, these fibroblast populations only scratch the surface of fibroblast heterogeneity. Guerrero-Juarez et al. (2019) discovered at least 12 different fibroblast populations in murine skin wounds using single-cell resolution RNA sequencing.

Phenotypic differences between fibroblasts result in differences in ECM production and organization, growth factor production, and contributions to inflammatory responses (Doane and Birk, 1991). Fetal skin has been observed to heal scarlessly in mammals until a certain gestational age (Colwell et al., 2005). However, the exact mechanism underlying scarless fetal wound healing is not known, there are significant differences in the ECM, the inflammatory response, cellular mediators, gene expression, stem cell function, and fibroblast phenotypes between fetal and postnatal wounds (Shizuru et al., 2005; Larson et al., 2010). Collagen production is more tightly regulated in fetal fibroblasts (Gosiewska et al., 2001). A low level of TGF-β-1 has also been suggested to be associated with reduced scar formation (Shah et al., 1994).

PC regulate signaling pathways that are necessary for fibroblast migration during wound healing (Christensen et al., 2008). Signaling via PDGFRα regulates reorganization of the cytoskeleton and coordinates cell migration during wound healing (Schneider et al., 2010; Christensen et al., 2013). Moreover, fibroblast signaling through PDGFα has been shown to contribute to increased deposition of collagen and fibronectin, suggesting that PC signaling may guide dermal remodeling (Horikawa et al., 2015). PC in fibroblasts are increasingly recognized as a major regulator of ECM deposition in health and disease (Teves et al., 2019; Villalobos et al., 2019; Collins and Wann, 2020).

Although the frequency of PC on keloid or hypertrophic scar fibroblasts has been shown not to differ from that on normal fibroblasts (Strugnell et al., 1996), keloid fibroblasts show increased levels of PDGF receptors and demonstrate increased migration and proliferation in response to PDGFR ligand stimulation (Haisa et al., 1994). It is known that PC-associated PDGFR signaling activates the downstream JAK/STAT pathway, especially STAT1 and STAT3 (Vignais et al., 1996). Recently, Lee et al. (2019) implicated the STAT3 signaling pathway in keloid pathogenesis via RNA sequencing analysis of normal and keloid fibroblasts. Although these results indirectly suggest that fibroblast PC signaling could be involved in pathological scarring, direct experimental evidence to prove or disprove their role in this process is lacking.

Mechanical force applied to fibroblasts causes them to respond with signal transduction by the process of mechanotransduction. The mechanical force, or their stimuli, are then transduced into biochemical and gene expression signaling pathways. Thus, the stimuli alter the cellular function or induce apoptosis. Mechanical stimulation can also be converted into chemical signaling in cells such as fibroblasts, increasing fibroblast fibrotic gene expression, and increasing apoptosis. It has been suggested that cellular adhesions, which serve as mechanoreceptors in general, may also play an essential role in scar modulation after pressure therapy (Atiyeh et al., 2013).

5.4. Myofibroblasts

5.4.1. Myofibroblasts and Wound Healing

In the maturation or remodeling phase of wound healing, increased mechanical tension and TGF-β signaling stimulate fibroblasts to differentiate into myofibroblasts, a highly contractile mesenchymal cell type. Myofibroblasts were initially described in granulation tissue during open wound healing as modulated fibroblasts present in all organs and numerous physiological conditions (Kalluri and Zeisberg, 2006; Hinz et al., 2012) or as modified fibroblasts that possessed characteristics of SM cells, such as bundles of microfilaments (Gabbiani et al., 1971). Myofibroblast phenotypes are modified by TGF-β, inflammatory factors, and immune cells, such as different macrophage phenotypes, ECM stiffness, and aging (Avery, 2017; Shook et al., 2018). Classically, myofibroblasts are identified by the expression of α-SMA, which promotes their contractility (Hinz et al., 2003). Myofibroblasts and their specific subpopulations can be identified by the expression of fibroblast activation protein alpha (FAPα), a membrane-bound serine protease, Pdgfra, stem cell antigen-1 (Sca1), integrin alpha-8 (Itga8), CD34, and dipeptidyl peptidase-4 (Dpp4, CD26) (Avery, 2017; Mah et al., 2017; Shook et al., 2018). Myofibroblasts with high FAP expression represent a reactive phenotype induced by low ECM stiffness and ECM fibronectin. These cells have been suggested to contribute to ECM synthesis and proteolysis, whereas SMA-expressing myofibroblasts exhibit a contractile phenotype induced by high ECM stiffness and type I collagen (Avery, 2017). The dynamic and multifaceted nature of myofibroblast phenotypes is further reflected by the term proto-myofibroblast, which was coined to describe an α-SMA-negative transitional phenotype that may also reflect

(Tomasek et al., 2002; Hinz and Lagares, 2020) the reversibility of the myofibroblast phenotype (Tomasek et al., 2002; Nagaraju et al., 2019).

Myofibroblasts contribute to wound healing in various ways. They have the ability to secrete ECM molecules, such as collagen type I and collagen type III, and exert their contractile properties (Tomasek et al., 2002). The contractile apparatus of the myofibroblast comprises actin, myosin, and related proteins, such as alpha-smooth muscle actin (α-SMA), that are also found in other SM-expressing cells (Darby et al., 1990; Hinz et al., 2003; Van Caam et al., 2018). Myofibroblasts also form specialized adhesion structures with the ECM, which facilitate contraction of damaged tissue areas (Van Caam et al., 2018). Normally, during the resolution phase of tissue repair, myofibroblasts undergo apoptosis (Klingberg et al., 2013). Evasion of myofibroblast apoptosis, on the other hand, results in fibrosis and fibrotic disease (Hinz and Lagares, 2020). For instance, systemic sclerosis or scleroderma is an autoimmune disorder that typically results in fibrosis of the skin. Fibrosis is the hallmark of scleroderma and is described as excess deposition and accumulation of ECM in the dermis (Jinnin, 2010). Reduced apoptosis is evident, for example, in hypertrophic scars after burn injury (van der Veer et al., 2009; Liu et al., 2013).

It has been demonstrated that in such cases, collagen type I is replaced by collagen type III, which is typically present in remodeling tissues but not in normally healing wounds (Gabbiani et al., 1976). Therefore, the formation of granulation tissue accompanies the modulation of fibroblasts toward myofibroblasts during wound healing (Gabbiani, 2003). The progression from granulation tissue to scar tissue is known to involve the disappearance of myofibroblasts (Desmouliere et al., 1995). Hypertrophic scars or keloids appear when granulation tissue cells are not removed (Desmouliere et al., 1995) (see below). Further, most myofibroblasts express SM proteins, e.g., α-SMA and desmin. In general, α-SMA-expressing myofibroblasts are absent in keloids and normal scars but present in hypertrophic scars (Koese and Waseem, 2008).

5.4.2. Myofibroblast Transformation

TGF-β signaling progresses through the formation of a heterotetrameric receptor confirmed to include type I and II TGF-β receptors (Huang and Chen, 2012). The assembly and activation of the receptor complex upon ligand binding results in phosphorylation and activation of the SMAD transcription factors SMAD2/3 (Huang and Chen, 2012; Clement et al., 2013).

Epithelial-myofibroblast transition (EMyT) is a unique and advanced type of Epithelial-to-mesenchymal transition (EMT), in which epithelial cells transdifferentiate into myofibroblasts that synthesize ECM proteins and produce contractile fibers (Radisky et al., 2007). TGF-β is known to be an inducer of EMT and EMyT (Xu et al., 2009). On the other hand, some studies have shown that the activation of TGF-β signaling alone is not sufficient to stimulate EMyT (Masszi et al., 2004; Sahin and Gungor, 2008). Moreover, it has also been suggested that a specific defect in PC activates EMT without TGF-β and exacerbates TGF-β-induced EMT (Han et al., 2018). Rozycki et al. indicated that the formation of the myofibroblast phenotype was followed by the absence of PC, regardless of whether the precursors were epithelial or mesenchymal cells. Further, the transition to the myofibroblast state is essential for deciliation because TGF-β is able to induce myofibroblast transition in mesenchymal cells (Rozycki et al., 2014).

Interestingly, PC have been demonstrated to regulate myofibroblast transition (Egorova et al., 2011). Rozycki et al. (2014) showed that PC of fibroblasts or epithelial cells are initially required for myofibroblast transition and growth but are subsequently lost upon acquisition of the myofibroblast phenotype. The EMyT and the fibroblast-to-myofibroblast transition are controlled by TGF-β signaling (Cigna et al., 2012). Regulation of PC signaling, assembly, or disassembly may thus have important therapeutic implications for pathologies that involve dysregulation of myofibroblast differentiation or persistence.

Myofibroblasts undergo apoptosis (Gabbiani, 1996), which is associated with TGF-β signaling (Zhang and Phan, 1999). TGF-β stimulates the synthesis of α-SMA and activates the production of collagen type I, which suggests that TGF-β regulates the tissue shape via modulation of ECM formation. TGF-β also initiates EMT and EMyT and thereby contributes to the regulation of wound healing. Moreover, PC undergo major alterations during EMyT (Rozycki et al., 2014). Amendt et al. (2002) showed that the expression of the dominant-negative type II TGF-β receptor in keratinocytes causes increased keratinocyte proliferation in the wound, which leads to strongly accelerated re-epithelialization.

These findings support the notion that TGF-β reduces re-epithelialization by repressing keratinocyte proliferation. Hence, reducing TGF-β signaling may contribute to better wound healing. It has also been suggested that the absence of PC causes dysregulated cell displacement during wound healing, leading to a reduced rate of wound healing and defects in wound closure (Schneider et al., 2010). Thus, PC are indispensable for wound healing and the formation of granulation tissue.

5.5. Keratinocytes

Keratinocytes are the major cell type of the skin's outer layer, the epidermis (Lee et al., 2013). The programmed process of keratinocyte renewal and differentiation generates and maintains the multilayered structure of the epidermis (Pincelli and Marconi, 2010). The epidermis spans from the self-renewing stem cell compartments to the cornified outermost layers and constitutes the protective barrier of the body (Simpson et al., 2011; Lu et al., 2015a). Epidermal stem cells reside in the basal layer of the interfollicular epidermis and in skin appendages (Evans and Kaufman, 1981; Molofsky et al., 2004; Cichorek et al., 2013). These cells are essential for wound healing (Raja et al., 2007; Pastar et al., 2008) and skin regeneration after injury (Burgoyne et al., 2009; Dehkordi et al., 2019).

The formation of PC on keratinocytes is linked to differentiation (Croyle et al., 2011), whereas the disruption or loss of PC leads to keratinocyte hyperproliferation (Ezratty et al., 2011). Because the balance between keratinocyte proliferation and differentiation governs epidermal homeostasis, PC-associated signaling can greatly influence keratinocyte fate

decisions and thus maintenance of the epidermal structure (Choi et al., 2016).

5.6. Bone Marrow-Derived Cells

Mesenchymal stromal cells (MSC) originate from the bone marrow are able to differentiate into various mesenchymal cell lineages; they are widely used in cell-based therapeutic approaches, including regeneration of damaged connective tissue (Ozawa et al., 2008). PC are present on MSCs. Interestingly, tumor necrosis factor-α (TNF-α) seems to activate the dose-dependent depletion of PC in MSCs, which suggests that PC can be a biological marker for the tumor-supporting properties of MSCs (Vézina et al., 2014).

5.7. Adipose Cells

Adipocyte precursor cells are activated and populate the wound during the proliferative phase of acute wound healing (Schmidt and Horsley, 2013; Guerrero-Juarez et al., 2019). Macrophages can contribute to the recruitment of these cells to the wound (Guerrero-Juarez et al., 2019). Adipocyte precursors, in turn, contribute to the recruitment of fibroblasts to the wound and promote dermal regeneration and remodeling (Schmidt and Horsley, 2013).

Schmidt and Horsley (2013) speculate that the activation of adipocyte precursors is driven by the interplay between immune cells and that intercellular signaling between fibroblasts and adipocytes could be mediated by PDGF and BMP signaling. Since these signaling pathways are governed by PC, these results further suggest a central role of signaling through preadipocyte and fibroblast PC in the control of wound healing. Dysregulated intercellular communication among adipocytes, immune cells, and fibroblasts may thus contribute to scarring through uncoordinated stimuli that regulate cell proliferation or ECM deposition.

The obesity phenotype of ciliopathies may be associated with these pathways since PC are related to Hh and Wnt signaling (Sen et al., 2009). BBS is a rare ciliopathy characterized by obesity, retinal dystrophy, postaxial polydactyly, and renal dysfunction (Forsythe and Beales, 2013). A dysfunctional BBSome protein complex controlling IFT transport of cargo, for example, receptor proteins, to and from the PC (Wingfield et al., 2018) can cause defects in ciliary signaling pathways, such as Wnt and Hh, that contribute to dysfunctional differentiation of adipocytes (Liu and Lechtreck, 2018). In preadipocytes, the BBSome is required for ciliogenesis (Sen et al., 2009).

6. OTHER SKIN CELLS WITH LESS ESTABLISHED ROLES IN WOUND HEALING AND SCARRING

6.1. B Cells

B cells, also called B lymphocytes, are a subclass of leukocytes that play an essential role in pathogen-specific immunity by producing antibodies (Stollar, 1998). Furthermore, they can also act as antigen-presenting cells (Rodríguez-Pinto, 2005). There has been increasing interest in B cells due to their potential role in the cutaneous immune system. *In vivo* and clinical studies have demonstrated that B cells have proinflammatory and suppressive roles in inflammatory skin disorders (Egbuniwe et al., 2015).

The IS is associated with B cell activation, as B cells can be activated by APCs, and B cell receptor signaling starts to form the IS when B cells contact membrane-bound antigens (Carrasco et al., 2004; Huang et al., 2005). The IS also works as a platform for B cells to recognize pathogenic antigens on APCs, similar to the T cell IS (Huang et al., 2005). Under certain conditions, immortalized T and B cells may form an initial primary cilium. However, PC do not exist in normal mature hematopoietic cells *in vivo*. Hence, in these cells, the expression of PC-related proteins may be severely reduced or even absent (Cassioli and Baldari, 2019).

6.2. Melanocytes

In the human skin, neural crest-derived melanocytes are distributed throughout the basal layer of the epidermis (Barbieri et al., 2014). The ratio of melanocytes to keratinocytes varies from 1:4 to 1:10 depending on the location (Norris, 2012).

Extrinsic factors, such as ultraviolet radiation, the cellular microenvironment, hormones, and inflammation, modulate the degree of skin pigmentation (Costin and Hearing, 2007; Arndt et al., 2013). The skin pigment melanin is synthesized in melanosomes within melanocytes. Melanosomes are delivered to keratinocytes via the dendrites of melanocytes (melanocyte-to-keratinocyte transfer). Melanin protects the inner microenvironment of keratinocytes, including their DNA, from UV radiation-induced damage (Wu and Hammer, 2000; Bowman and Marks, 2018). Genetic determinants regulate the quantity of melanin produced by melanocytes and the size of the melanosomes that are transferred to keratinocytes (Bessou-Touya et al., 1998). Although the association between skin pigmentation and PC remains unclear, PC are indeed found on melanocytes. However, they are frequently lost in melanomas (Kim et al., 2011; Le Coz et al., 2014). Sensing and signaling through PC may thus contribute to the functions of differentiated melanocytes.

6.3. Merkel Cells

Merkel cells are located in the basal layer of the epidermis, accounting for <5% of the total epidermal cell population (McGrath and Uitto, 2010). They are essential for neuroendocrine functions, and due to their synapse-like contacts with neurons, these cells are instrumental for sensing pain and mechanical stimuli such as light touch and hypotonia-induced cell swelling (Abraham and Mathew, 2019). The surface of Merkel cells is characterized by numerous microvilli that participate in sensory functions (Toyoshima et al., 1998), but the role of PC remains unclear. However, the PC-associated Hh signaling cascade guides cells to the skin sensory lineage (Xiao et al., 2016), contributing to the formation of the touch dome, a sensory organ comprised of Merkel cells and specialized keratinocytes.

7. FIBROSIS, KELOIDS, AND HYPERTROPHIC SCARS

Fibrosis is a pathological process that results from chronic inflammatory reactions caused by, for instance, tissue injury. Fibrosis is characterized by excess accumulation of ECM and can lead to permanent scarring e.g., keloids, and hypertrophic scars in the skin (Wynn, 2008). The inflammatory response is aggravated by increased accumulation, enhanced production, or failure to remove stimulating factors; if unregulated, inflammation can drive fibrosis (Wynn and Ramalingam, 2012). Moreover, fibrosis is known to evoke persistent myofibroblast activation and inflammation, which can cause severe organ dysfunction (Hinz and Lagares, 2020).

Keloids are pathological scars that are described as a benign fibroproliferative dysfunction characterized by abnormal excessive deposition of collagen during wound healing. Keloids extend beyond the borders of the original wound and invade the normal skin, which distinguishes these scars from other pathological hypertrophic scars (Lee et al., 2004). Keloids are common in African, Spanish, and Asian populations, with an incidence ranging from 4.5 to 16%, and cause itching, pain, and a burning sensation (Niessen et al., 1999). Keloids can occur within years, and more likely after an inciting stimulus such as dermal injury or an inflammatory process (Limandjaja et al., 2020); however, they are most often found on the chest, shoulders, upper back, and back of the neck, where skin tension is higher than in other areas of the body (Ogawa et al., 2012; Liu et al., 2020). In addition, keloids tend to occur on the earlobes as a result of ear piercing, burns, or surgical procedures. The earlobes consist of tissue with little to no tension, and Chike-Obi et al. (2009) suggested that keloids can also form in areas with minimal tension due to the proliferation of dermal elements after injury. Keloids do not regress, unlike hypertrophic scars (Murray, 1994), and the exact pathogenic mechanism of keloid formation is still unclear. Various mechanisms of healing disorders seem to be associated with keloid formation.

Ogawa (2017) suggested that keloids and hypertrophic scars are derived from an abnormal inflammatory reaction in the skin since proinflammatory factors, including IL-1α, IL-1β, IL-6, and TNF-α, are associated with keloid formation. Additionally, disorders of vascular cells can cause pathological scars since inflammation stimulates excessive angiogenesis, endothelial disorder, and vascular hyperpermeability (Huang and Ogawa, 2020). It has been proposed that mechanical tension promotes the dysregulation of cell proliferation and apoptosis during wound healing and may result in keloids and hypertrophic scars (Pozos, 2014; Harn et al., 2019; Ogawa, 2019).

Myofibroblasts create specialized adhesion structures with the ECM, which causes tension in their surroundings and contracts damaged tissue. Generally, expansion of the ECM is caused by increased skin tension, resulting in stiffer tissue. Harn et al. (2019) suggested that keloids form differently than hypertrophic scars and other normal scars because of the variation in scar sensitivity to skin tension. PC and the ECM are functionally closely connected, and defects in PC are associated with deregulated fibrosis. Thus, we hypothesize that defects in PC may facilitate not only the development of ciliopathies (Seeger-Nukpezah and Golemis, 2012) but also the formation of keloids. Moreover, keloids contain remarkably increased and disorganized collagen bundles. Fibroblasts in keloids respond abnormally to growth factors and stimuli arising from the ECM during wound healing, and the delay of fibroblast apoptosis in keloids seems to be associated with uncontrolled production of excessive amounts of collagen (Huang et al., 2013).

Hypertrophic scars can occur within 4–8 weeks after injury and occur in up to 90% of burn patients (Oosterwijk et al., 2017). Hypertrophic scars are more common than keloids. Hypertrophic scars are a fibrotic disorder resulting from unchecked proliferation of fibrous tissue following injury to the skin. They are described as raised, erythematous, itchy lesions (Peacock, 1970). Unlike keloids, hypertrophic scars ultimately regress and occur mainly around joint areas- the elbows and knees (Niessen et al., 1999; Butzelaar et al., 2016). The recurrence rates of hypertrophic scars are lower than those of keloids after excision (Gauglitz et al., 2011). Hypertrophic scars and keloids have excessive collagen content. While hypertrophic scars contain well-organized type III collagen positioned in parallel to the epidermal surface and abundant myofibroblasts, keloids contain disorganized type I and III collagen with no myofibroblasts (Slemp and Kirschner, 2006). Both scar types show overexpression of numerous fibroblast-specific proteins, such as fibronectin (Sephel and Woodward, 2001), which may sustain pathological wound healing or promote downregulation of the mechanisms that terminate the wound healing process.

8. PRIMARY CILIA-TARGETED ANTI-FIBROTIC THERAPEUTIC APPROACHES

The intricate mechanisms involved in PC assembly and disassembly, as well as the molecules and pathways involved in their regulation, constitute some of the many possible targets for therapeutic interventions that involve signaling through the PC. Examples of such targets include suppressors of PC formation (mitostatin, NDEL1, CP110 KIF24, and INPP5E), facilitators of PC resorption (AURKA, HDAC6, CDC20, and DYNLT1) and promoters of ciliogenesis (MST1/2, the CRL3-KCTD17 complex, PLK4, NPHP6, and Rab8a) (Walz, 2017).

Another level of regulation could involve proteins and pathways associated with PC function. These functional regulators include IFT proteins of, for example, the BBSome that transport cargo to and from PC, kinesins, and dyneins as well as the IFT-A and -B complexes (Ishikawa and Marshall, 2011; Basten and Giles, 2013).

Corticosteroids are the first-line clinical treatment of keloids and hypertrophic scars. Fibroblast production and proliferation capabilities are affected by corticosteroids. They inhibit fibroblast growth and cause fibroblast degeneration, and have been shown to affect the length of PC (Khan et al., 2016). Corticosteroids seem to also induce an increase in the production of basic fibroblast growth factor and additionally a decrease in the observed

production of TGF-β1 by human dermal fibroblasts, endogenous VEGF, and IGF-1 (Roques and Téot, 2008). As glucocorticoids act as general suppressors of inflammation the role of ciliary signaling modulation in scar suppression is not yet clear.

In addition to corticosteroids, many other drugs and small molecules have also been shown to affect the length of cilium (Khan et al., 2016). These include compounds that increase cyclic AMP, such as phosphodiesterase inhibitors or activators of cAMP production, for example, forskolin (Miyoshi et al., 2011) or cholera toxin (Hirst, 2001). Moreover, among environmental factors, hypoxia exerts cell type-dependent regulatory effects on ciliogenesis (Miyoshi et al., 2011), possibly through pVHL signaling (Thoma et al., 2007).

Therapeutic approaches to induce myofibroblast apoptosis or sensitize myofibroblasts to apoptosis are currently under intensive investigation, and several drugs are undergoing phase II/III studies for the treatment of fibrotic diseases (Hinz, 2020). Recently, Choi et al. (2019) demonstrated that PC can protect neuronal cells from apoptosis and that treatment with ciliobrevin D, a drug that inhibits dynein function and disrupts the formation of PC, sensitizes these cells to apoptosis. Their results indicating that the pharmacological modification of PC affects cellular programmed cell death pathways suggest that PC may serve as an attractive target to induce myofibroblast apoptosis for the treatment of fibrosis and pathological scars.

As PC have prominent roles in a number of intracellular pathways several potential approaches to suppress pathological sar formation that targets these pathways can be envisaged. One possibility is to interfere with or inhibit myofibroblast transdifferentiation via different PC-associated signaling pathways, such as TGF-β regulated by a CTGF-dependent pathway in concert with either EGF or IGF-2 (Grotendorst et al., 2004). Moreover, ligands of the Wnt/beta-catenin-pathway can upregulate TGF-β signaling and induce myofibroblast differentiation.

Blocking the canonical Wnt receptor (β-Catenin signaling route) or interfering with this signaling pathway through PC could offer a target to decrease myofibroblast transformation.

The role of PC in mechanotransduction and the associated downstream signaling pathways, including TGF-β/Smad, mitogen-activated protein kinase (MEK), RhoA/ROCK, Wnt, and TNF-α could also act as therapeutic targets.

It has been suggested that disruptions in cellular mechanotransduction can cause impaired wound healing and scar formation (Jaalouk and Lammerding, 2009). Mechanotransduction is also one of the mechanisms through which Negative Pressure Wound Therapy (NPWT), also called vacuum-assisted closure (VAC) exerts its therapeutic effects in wound treatment (Huang et al., 2014). Further, since PC length is thought to be critical to cellular mechanotransduction (Spasic and Jacobs, 2017), treatments that modify the length of PC could possibly be utilized to enhance the effects of NPWT.

Increasing mechanical tension is key to the development of scar tissues through biochemical signals (Barnes et al., 2018), and it is possible to reduce scar formation by modifying these signals, including neutralization of TGF-β or addition of TGF-β3 (Shah et al., 1995). There are more techniques to reduce mechanical forces/tension to treat pathological scars, such as silicone gel sheets and paper tape. Silicone gel sheets reduce tensile stresses in the wound (Akaishi et al., 2010). Moreover, they are thought to decrease TGF-β and TGF-β2 expression in fibroblasts (Kuhn et al., 2001). Paper tape also seems to reduce scarring by reducing wound tension (Rosengren et al., 2013). Clinically, there are reports that paper tape reduces scar volume significantly and reduces mechanical tension, which results in minimal scar formation (Atkinson et al., 2005; Rosengren et al., 2013). A meta-analysis also indicated that pressure therapy was effective for hypertrophic scar patients, improving pigmentation, and redness and even reducing scar coloration (Ai et al., 2017).

Pressure therapy, also known us compression therapy represents the standard care for preventing and treating hypertrophic scars after burn injury. For example, elastic bandages or compression garments are used as compression therapy. It has been suggested that pressure may affect directly cellular scar components (Li-Tsang et al., 2010). There seems to be an increase in ECM rigidity produced by compression garments leads to a higher level of mechanoreceptor activity and increased cellular apoptosis (Atiyeh et al., 2013). Given the mechanosensory functions of PC, it seems possible that they may contribute to these effects of pressure therapy.

9. CONCLUSIONS

Our understanding of the role of PC in regulating the activity of a number of signaling pathways has improved considerably due to the growing interest in PC. Here, we reviewed the roles of different key PC signaling pathways and the related cells and discussed their possible impacts on scar formation and wound healing. As PC have central roles in many pathways that regulate the cutaneous wound healing process, their dysfunction is hypothesized to cause aberrant wound healing and repair resulting in the appearance of pathological scars. However, this hypothesis needs further study. Understanding and exploring this mechanism may help to promote new breakthroughs and the development of effective medical solutions that can alleviate pathological scars.

AUTHOR CONTRIBUTIONS

MH and EK drafted the paper. RO, VJ, HL, and JV reviewed and edited the manuscript. All authors read and approved the final manuscript.

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REVIEW
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Collagen-Derived Di-Peptide, Prolylhydroxyproline (Pro-Hyp): A New Low Molecular Weight Growth-Initiating Factor for Specific Fibroblasts Associated With Wound Healing

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Many cells and soluble factors are involved in the wound healing process, which can be divided into inflammatory, proliferative, and remodeling phases. Fibroblasts play a crucial role in wound healing, especially during the proliferative phase, and show heterogeneity depending on lineage, tissue distribution, and extent of differentiation. Fibroblasts from tissue stem cells rather than from healthy tissues infiltrate wounds and proliferate. Some fibroblasts in the wound healing site express the mesenchymal stem cell marker, p75NTR. In the cell culture system, fibroblasts attached to collagen fibrils stop growing, even in the presence of protein growth factors, thus mimicking the quiescent nature of fibroblasts in healthy tissues. Fibroblasts in wound healing sites proliferate and are surrounded by collagen fibrils. These facts indicate presence of new growth-initiating factor for fibroblasts attached to collagen fibrils at the wound healing site, where the collagen-derived peptide, prolyl-hydroxyproline (Pro-Hyp), is generated. Pro-Hyp triggers the growth of p75NTR-positive fibroblasts cultured on collagen gel but not p75NTR-negative fibroblasts. Thus, Pro-Hyp is a low molecular weight growth-initiating factor for specific fibroblasts that is involved in the wound healing process. Pro-Hyp is also supplied to tissues by oral administration of gelatin or collagen hydrolysate. Thus, supplementation of gelatin or collagen hydrolysate has therapeutic potential for chronic wounds. Animal studies and human clinical trials have demonstrated that the ingestion of gelatin or collagen hydrolysate enhances the healing of pressure ulcers in animals and humans and improves delayed wound healing in diabetic animals. Therefore, the low molecular weight fibroblast growth-initiating factor, Pro-Hyp, plays a significant role in wound healing and has therapeutic potential for chronic wounds.

Keywords: collagen, fibroblast, Pro-Hyp, collagen peptide, wound healing, p75NTR, mesenchymal stem cells

INTRODUCTION

Cutaneous wound healing is a complex and highly regulated process with many cells and soluble factors working together during this process (Han and Ceilley, 2017; Stunova and Vistejnova, 2018; Cañedo-Dorantes and Cañedo-Ayala, 2019). The cutaneous wound healing process is generally divided into three overlapped phases: inflammatory, proliferative, and remodeling or maturation phases. The inflammatory phase starts shortly after hemostasis and is triggered by the activation of adhered platelets. Chemokines and cytokines, which are released from the activated platelets and damaged tissue, recruit neutrophils and monocytes to the wound sites (Golebiewska and Poole, 2015). Neutrophils, which appear in the wound 4 h after injury and decrease during the subsequent weeks, eliminate pathogens by phagocytosis and the release of bactericidal reactive oxygen species and peptides (Brinkmann et al., 2004). The infiltration of monocytes starts the second day and the monocytes are changed to macrophages. Macrophages increase and reach maximum levels during the proliferative process. Macrophages scavenge tissue debris and the remaining neutrophils by phagocytosis and secrete protein growth factors and cytokines that promote proliferation and migration of the cells involved in wound healing (Wynn and Barron, 2010; Ploeger et al., 2013). Circulating lymphocytes migrate to the wound healing sites early after injury and remain up to the last phase. Approximately 3 days after the initial injury, the proliferative phase starts. This phase is responsible for the lesion closure. Fibroblasts infiltrate to the wound sites and proliferate and produce extracellular compounds such as collagen, which form the basis of granulation tissue. The granulation tissue provides the basis for re-epithelization and some fibroblasts differentiate into myofibroblasts, initiating wound contraction (Tomasek et al., 2002). To supply nutrients to the granulation tissue, the induction of angiogenesis occurs (Greaves et al., 2013). After ∼2–3 weeks, the proliferative phase is followed by the remodeling or maturation phase to achieve tensile strength through contraction, reorganization, degradation, and resynthesis of the extracellular matrix (Xue and Jackson, 2015). The cells involved in the previous phases are removed by apoptosis. Thus, the granulation tissue is gradually remodeled and changed to scar tissue consisting of less cellular and vascular components and rich in collagen fibers, resulting in an ∼70–80% tensile strength of the uninjured skin (Stunova and Vistejnova, 2018). Aberrant wound healing may result in a chronic wound or non-healing wound, which is a burden to the patient, caregiver, and medical system. Aberrant wound healing is often occurs under diabetic and malnutrition conditions (Han and Ceilley, 2017).

In wound healing process, fibroblasts play a significant role especially during the proliferative phase. Fibroblasts migrate, proliferate, and produce extracellular matrix compounds in response to protein growth factors, such as epidermal growth factor, insulin, interleukin-1β, tumor necrosis factor-α, transforming growth factor-β1, platelet-derived growth factor, and fibroblast growth factors (Ejiri et al., 2015; Cañedo-Dorantes and Cañedo-Ayala, 2019). In cell culture systems, fibroblasts proliferate on plastic substrate in the presence of fetal bovine serum (FBS), which is rich in protein growth factors and fibronectin. The removal of low molecular weight compounds from FBS does not affect the growth of fibroblasts on the plastic substrate (Asai et al., 2020a). Fibroblasts have to attach to the substrates for survival. In tissues, fibroblasts attach to collagen fibrils directly using collagen receptors such as α1β1 and α2b1 integrins (Zeltz and Gullberg, 2016) and indirectly via fibronectin using α5β1 integrin (Wu et al., 1993). In cell culture systems using non-coated culture plates, fibroblasts are attached to the plate surface via fibronectin, which is present in FBS and promote cell proliferation (Hayman and Ruoslahti, 1979). However, some researchers have used collagen gel-coated plates for the cultivation of fibroblasts (Yoshisato et al., 1985; Nishiyama et al., 1989; Kono et al., 1990; Shigemura et al., 2009; Asai et al., 2020a,b). The collagen solution becomes to be polymerized at neutral pH after incubation at 37°C. During this process, collagen molecules assemble into collagen fibrils (Yoshisato et al., 1985). Fibroblasts that are attached to the collagen fibrils get in a quiescent state even in the presence of FBS. The denatured collagen also forms gel but does not assemble into the fibrils, and cannot inhibit the growth of fibroblasts. A higher concentration of collagen gels induce a robust inhibitory effect on fibroblast proliferation (Yoshisato et al., 1985; Kono et al., 1990). Therefore, the inhibitory effect of collagen gel on fibroblast growth cannot be solely attributed to softness of collagen gels compared to plastic plate, while it is known that extracellular mechanical stress affect the growth and differentiation of cells, including fibroblasts (Jansen et al., 2017). Thus, fibroblasts growth is controlled not only by protein growth factors but also by interaction with collagen fibrils. Growth regulatory mechanisms of fibroblast settled on collagen fibrils are thus vital for understanding the *in vivo* wound healing process. Nevertheless, this topic has received limited attention many years up to now.

Normal fibroblasts present in a quiescent state all over the connective tissues in the body under physiological condition. The suppression of proliferation of fibroblast cultured on collagen gel could therefore mimic the quiescent characteristics of fibroblasts in healthy tissue (Yoshisato et al., 1985; Kono et al., 1990). However, fibroblasts in collagen matrixes start to proliferate after wounding, in which, additional factor(s) are required for initiating fibroblast proliferation. However, rational information is still limited for the fibroblasts under such context. Our group have previously addressed; after total skin excision in mice, collagen di-peptide, prolyl-hydroxyproline (Pro-Hyp), which is generated in the granulation tissue after wounding (Jimi et al., 2017) and Pro-Hyp can act as a trigger for growth initiation in the fibroblasts cultured on collagen gel (Asai et al., 2020a,b; Shigemura et al., 2009).

The present study does not aim to review cutaneous wound healing processes comprehensively. Instead, the present mini review is thus focusing especially on the function of a new low molecular weight growth-initiating factor, Pro-Hyp, in wound healing process and discuss its therapeutic potential for chronic wounds in the end.

GENERATION OF COLLAGEN-DERIVED DI-PEPTIDES IN TISSUES

Collagen is a major extracellular matrix protein and has a triple helical domain and small globular domains. Collagen forms a molecular family consisting of different gene products. Collagen molecular species are referred to as "Type" with Roman numerals (Van der Rest and Garrone, 1991). Types I, II, III, V, and XI collagens form collagen fibrils and are referred to as fibril-forming collagens. Other collagen types are classified as non-fibrillar collagen, which play essential biological functions; however, their contents are far lower than that of the fibrillar collagens (Van der Rest and Garrone, 1991). Type I collagen is the primary collagen in the skin, bone, and tendons. Type III and V collagens co-exist with Type I collagen. Type II collagen is the primary collagen in cartilage with minor Type XI collagen. Collagen molecules consist of three subunits referred to as the α chain. Each α chain is designated by Roman numerals (Type) and Arabic numerals (subunit number). The major collagen molecules in the skin, Types I and III collagens, are designated as $[\alpha1(I)]_2\alpha2(I)$ and $[\alpha1(III)]_3$, respectively.

Collagen consists of post-translationally modified amino acids, hydroxyproline (Hyp), and hydroxylysine (Hyl), which play significant roles in stabilizing the triple helix structure and the inter- and intra-molecular cross-links of collagen molecules, respectively (Rappu et al., 2019). Prolyl residues especially in the Y position of Gly-X-Y motif in collagen are frequently changed to a hydroxyprolyl residue by prolyl hydroxylase. Each collagen subunit has ∼80–100 Hyp residues depending on the collagen type and animal species. The Gly-Pro-Pro motif is most abundant in Gly-X-Pro- motifs (43, 23, and 37 repeats) in the predicted sequences of human mature α1(I), α2(I), and α1(III) chains, respectively (ExPASy, Entry numbers P02452, P08123, P02461). Thus, the Gly-Pro-Hyp motif is present most abundantly in major collagens in the skin.

The triple helix structure of collagen resists most proteinases, except for collagenases such as matrix metalloproteinases (MMPs)-1 and−8 (Jabłońska-Trypuć et al., 2016). Collagenase fragments with a triple helix structure can collapse to a denatured form with a globular structure at body temperature. This denatured form of collagen fragments can be degraded by other endoproteinases and exopeptidases into amino acids and oligopeptides (Sunada and Nagai, 1983).

Pro-Hyp increases in the blood of patients who have bone metastasis cancer (Mazzi et al., 1996; Inoue et al., 2001). The Pro-Hyp motif is most abundant in major collagens, and Pro-Hyp resists human plasma peptidases (Iwai et al., 2005). Therefore, Pro-Hyp is liberally released by the degradation of endogenous collagen presented in tissues.

Pro-Hyp was also found to be generated in the granulation tissue at wound healing site of mouse (C57BL/6) skin after excision and did not increase in the skin of the healthy tissues of the same animal (Jimi et al., 2017). The infiltration of NIMP-R14- and MMP-8-positive neutrophils into the granulation tissue was also observed. The generation of Pro-Hyp in the granulation tissue of the skin of the db/db mouse, which has a mutation in the gene encoding the leptin receptor and is susceptibile to obesity, insulin resistance, and Type 2 diabetes, was significantly lower than that in the wild-type mouse (C57BL/6) during all periods of wound healing (day 1–8) (Jimi et al., 2017). Decreased infiltration of NIMP-R14- and MMP-8-positive neutrophils into the granulation tissue and lower levels of blood granulocyte colony-stimulating factor (G-CSF) were also observed in db/db mice. The topical application of recombinant human G-CSF onto the wound of the db/db mice increased the number of infiltrated neutrophils and Pro-Hyp in the granulation tissue (Jimi et al., 2017). Therefore, neutrophils play a significant role in the generation of Pro-Hyp in granulation tissue by the excretion of collagenase (MMP-8) as illustrated in **Figure 1**.

Pro-Hyp, Leu-Hyp, and Gly-Pro-Hyp were significantly increased in the mouse ear with dermatitis, whereas these peptides did not increase in other ear without dermatitis in the same animal (Kusubata et al., 2015; Sato et al., 2019). Among these, Pro-Hyp was the most abundant; however, isotope-labeled Pro-Hyp was specifically cleaved in the ear with dermatitis. Thus, very rapid synthesis and degradation of Pro-Hyp occurs in tissues under inflammation.

Taken together, these data suggest that collagen-derived tri- and di-peptides are generated by the degradation of major collagen in tissues under inflammation where extracellular matrix remodeling occurs. Pro-Hyp is a major compound owing to its high peptidase resistance and the abundance of Pro-Hyp motifs in the major collagen types I and III in the skin.

FOOD-DERIVED COLLAGEN PEPTIDES IN BODY

Collagen in food material is denatured by heat treatment and converted to gelatin. Gelatin is soluble in hot water; therefore, hot water can extract gelatin from animal, bird, and fish skin as well as their bones. Gelatin is easily degraded by food-grade proteases, which produce peptides with molecular weights of 1,000–5,000 Da (Iwai et al., 2005; Osawa et al., 2018). This gelatin hydrolysate is referred to as collagen hydrolysate or collagen peptide, which is used as a food ingredient.

At least 12 collagen-derived peptides, e.g., Pro-Hyp, Hyp-Gly, Pro-Gly, Glu-Hyp, Ala-Hyp, Ile-Hyp, Leu-Hyp, Phe-Hyp, Ser-Hyp-Gly, Ala-Hyp-Gly, Gly-Pro-Hyp, and Pro-Hyp-Gly, have been identified in human blood after the ingestion of collagen hydrolysate and meat rich in collagen (Iwai et al., 2005; Ohara et al., 2007; Ichikawa et al., 2010; Shigemura et al., 2011, 2018; Asai et al., 2019). Hydroxyproline containing peptides have been shown to significantly increase after the ingestion of collagen hydrolysate in a dose-dependent manner (Shigemura et al., 2014). In some cases, it can reach ∼100 μM or more (Iwai et al., 2005; Ohara et al., 2007), which is much higher than the previously reported values for food-derived peptides in human blood (Matsui et al., 2002; Foltz et al., 2007). In all cases, Pro-Hyp was most abundant in human blood plasma, accounting for ∼50% of the total collagen peptides in the human blood. Thus, the ingestion of collagen hydrolysate can supply

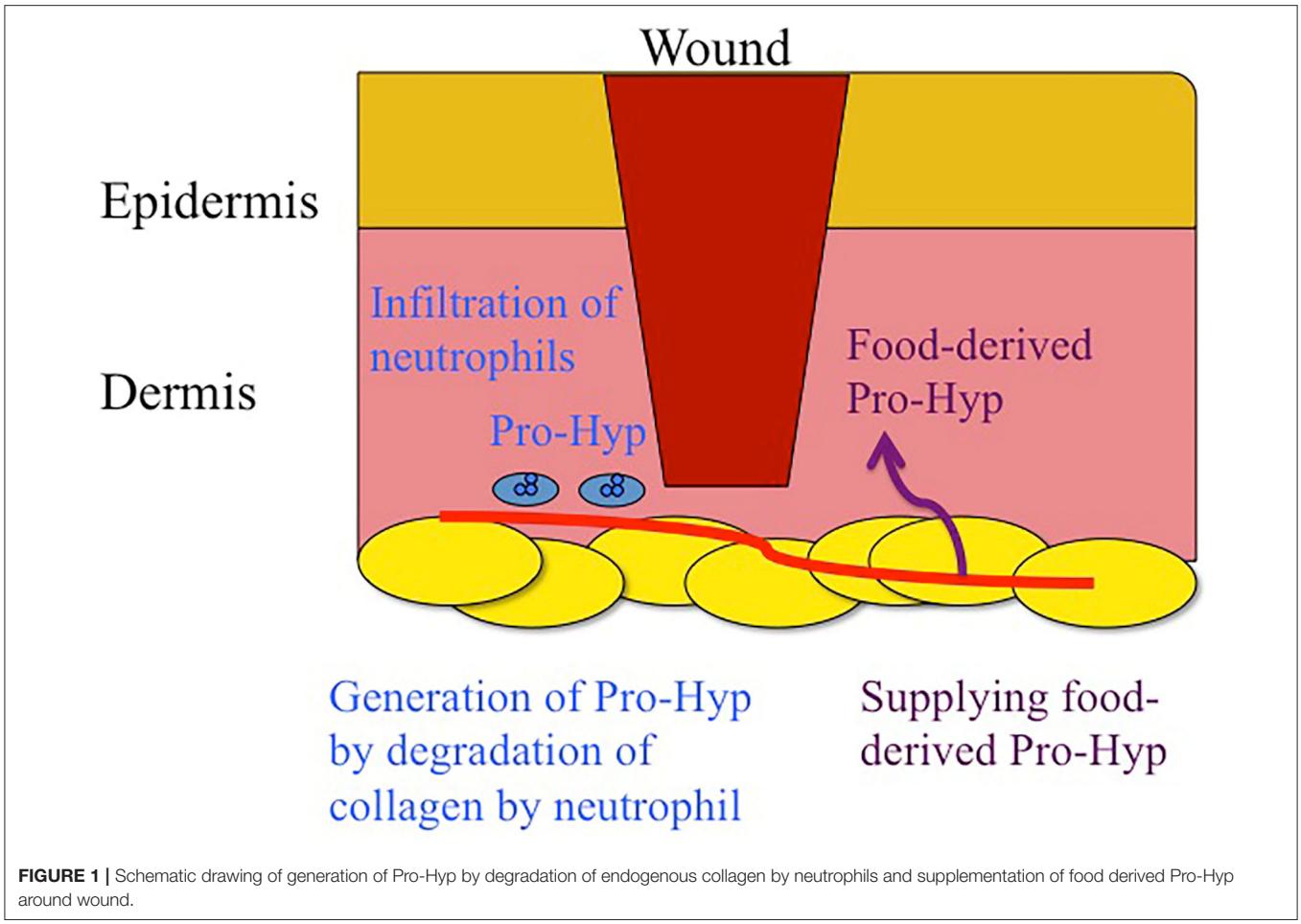

FIGURE 1 | Schematic drawing of generation of Pro-Hyp by degradation of endogenous collagen by neutrophils and supplementation of food derived Pro-Hyp around wound.

collagen peptides, which have the same structure as endogenous collagen peptides in wound healing sites and inflammatory tissues (**Figure 1**).

Approximately 24 h after the ingestion of collagen peptides, the plasma Pro-Hyp level returned to its initial level, with some of the Pro-Hyp excreted in the urine (Taga et al., 2019). Animal study using [^{14}C] Pro-Hyp demonstrated that radioactivity was observed in rat skin fibroblasts, femur chondrocytes, and synovial cells 30 min after the ingestion of [^{14}C] Pro-Hyp (Kawaguchi et al., 2012). Thin layer chromatography analysis revealed that [^{14}C] Pro-Hyp in cartilage was metabolized to other modified peptides and amino acids; however, it was not identical to proline. Some of the orally administered Pro-Hyp was incorporated into these cells and further metabolized; however, the metabolites formed were not identified.

Pro-Hyp–LOW MOLECULAR WEIGHT FIBROBLAST GROWTH-INITIATING FACTOR

In general, collagen-coated plates are utilized for acquiring stabilized growth and differentiation in cultured cells. Fibroblasts attached to collagen fibrils-assembled collagen gel, however, stop their growth even in the presence of FBS (Yoshisato et al., 1985; Kono et al., 1990; Shigemura et al., 2009; Asai et al., 2020a,b). However, contradict results have been reported (Nishiyama et al., 1989; Asai et al., 2020a). To clarify the contradiction, Asai et al. (2020a) demonstrated that in fibroblasts cultured on collagen gels, the cells could proliferate in the medium containing an FBS, although, when low molecular weight compounds <6,000 Da were eliminated from the FBS, fibroblast growth could cease on collagen gel, however, the fractionalized FBS could not stop cellular proliferation on a plastic substrate. The medium was supplemented with conventional low molecular weight nutrients such as amino acids, glucose, vitamins, and minerals. Thus, the compound(s) responsible for the switch of proliferation on/off on collagen gel culture are present in the low molecular weight fraction in FBS, while growth factors and attachment factors including fibronectin still remained in the fractionated FBS. Using the fractionated FBS, the effect of Pro-Hyp, an endogenous and food-derived collagen di-peptide, was examined on the growth of fibroblasts cultured on collagen gel. Consequently, Pro-Hyp (200 μM) stimulated proliferation of fibroblasts cultured on the collagen gel (Asai et al., 2020a,b). This small peptide is, therefore, a low molecular weight

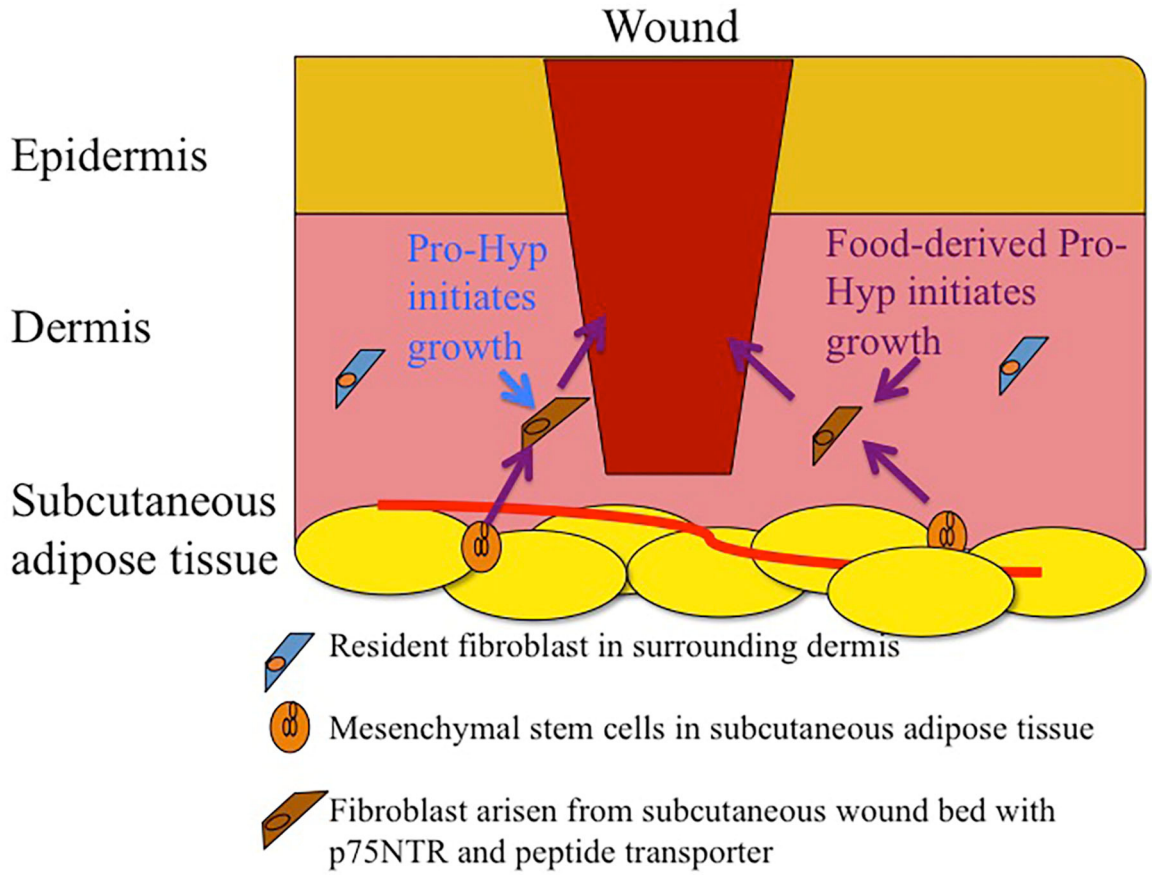

FIGURE 2 | Schematic drawing of infiltration of fibroblasts arisen from subcutaneous wound bed mesenchymal stem cells rather than fibroblasts in healthy dermis. Endogenous and food-derived Pro-Hyp initiates growth of the fibroblasts arisen from subcutaneous wound bed.

fibroblast growth-initiating factor (LMW-FGIF). Pro-Hyp was also contained in relatively high levels (up to approximately 45 μM) in commercially available FBS, but it depended upon suppliers, brands, and lots (Asai et al., 2020a). The presence of Pro-Hyp in FBS at different levels has been a stumbling block for the detection and identification of LMW-FGIF using the cell culture system. There is the possibility that other collagen-derived peptides could be classified as LMW-FGIF, although their contents might be lower than those of Pro-Hyp. The authors recommend that the low molecular weight compounds should be removed from commercially available FBS or use of serum-free and defined media when seeking other LMW-FGIF when using a cell culture system.

HETEROGENEITY OF SKIN FIBROBLASTS IN ASSOCIATION WITH WOUND HEALING

Fibroblasts are an ill-defined cell groups. Many cell surface markers for fibroblasts have been proposed. These markers are expressed temporally on fibroblasts depending on the ontogeny process and their distribution in tissues (Driskell et al., 2013). Some markers, such as platelet-derived growth factor receptor and some cytoskeletons, are present in all fibroblasts, while these markers are also present in non-fibroblast cells. Thus, there is no universal cell surface marker that is specific for fibroblasts. Adult skin fibroblasts show heterogeneity and plasticity (des Jardins-Park et al., 2018; Stunova and Vistejnova, 2018; Cañedo-Dorantes and Cañedo-Ayala, 2019). Dermal fibroblasts have been shown to arise from at least two different lineages: the upper dermal and lower lineage. In the adult skin, mesenchymal stem cells are located in hair follicles (Ge et al., 2016) and subcutaneous adipose tissue (Yamamoto et al., 2007). Fibroblasts, which migrate to the wound site and form granulation tissue during the proliferative phase, have been shown to arise from the subcutaneous wound bed (lower lineage) rather than from the surrounding healthy dermis (Rossio-Pasquier et al., 1999; Geer et al., 2004; Driskell et al., 2013). Thus, fibroblasts differentiated from mesenchymal stem cells rather than resident fibroblasts in the healthy dermis play a significant role in the proliferative phase of the wound healing process as illustrated in **Figure 2**. The International Society for Cellular Therapy (2006) defined mesenchymal stem cells as being able to adhere to plastic plates, differentiate into osteoblasts, adipocytes, or other cell

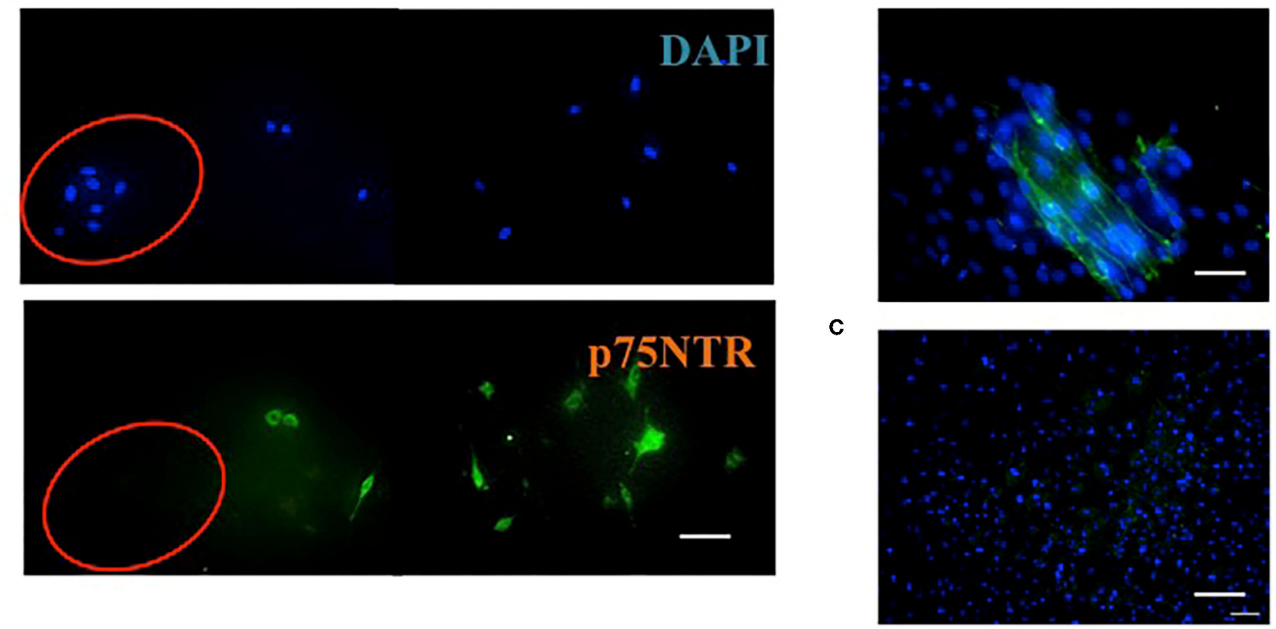

FIGURE 3 | Immunocytochemistry of cells on the outside of mouse skin after incubation for 1 day **(A)**, 2 weeks **(B)**, and 4 weeks **(C)**. A: Cells were stained with DAPI (blue) or antibodies against p75NTR (green). Cells in the red circle are keratinocytes; the rest are fibroblasts. **(B,C)** Fibroblasts were stained with DAPI (blue) and antibodies against p75NTR (green). Scale bar = 50 μm. This figure was adapted with permission (Asai et al., 2020b).

types; and express CD73, CD90, and CD 105 (Dominici et al., 2006). However, fibroblasts in some cell lines from different tissues also express these marker proteins (Denu et al., 2016). Thus, low-affinity nerve growth factor receptor (p75NTR or CD271) has been used as mesenchymal stem cell markers in bone marrow, adipose tissue, and the dermis (Tomellini et al., 2014; Álvarez-Viejo et al., 2015; Pincelli, 2017). p75NTR was initially discovered in neuronal cells (Fabricant et al., 1977). p75NTR-positive cells are present around the bulge region of hair follicles of healthy mouse, which co-express Sox-2, nestin, and S100β, all makers for nerve-terminal-associated neural crest precursor cells (Johnston et al., 2013). Some of these cells have been suggested to be associated with axons that are sprouted into the regenerating dermis after skin injury (Johnston et al., 2013). However, p75NTR is also expressed in various non-neuronal cell types, such as fibroblasts and macrophages, under inflammatory conditions (Trim et al., 2000; Nakamura et al., 2006; Palazzo et al., 2012; Meeker and Williams, 2014). These studies have shown that p75NTR-positive cells play an important role in wound healing and accumulate in the granulation tissue, whereas only a small number of p75NTR-positive cells are observed in healthy skin (Iwata et al., 2013).

Huang et al. (2013) reported that fibroblast-like cells, which were obtained from trypsinized human skin, did not express p75NTR after 4–5 days of cultivation in DMEM containing 10% FBS. In contrast, as shown in **Figure 3**, fibroblasts, which migrated from the piece of skin in the same medium, expressed p75NTR and p75NTR-positive fibroblasts declined after few day cultivation (Asai et al., 2020b). Thus, fibroblasts differentiated from mesenchymal stem cells temporally expressed p75NTR but rapidly lost p75NTR after growth.

Pro-Hyp, LMW-FGIF, proliferates p75NTR-positive fibroblasts cultured on the collagen gel but not p75NTR-negative fibroblasts (Asai et al., 2020b). Liquid chromatography-tandem mass spectrometry analysis revealed that Pro-Hyp was incorporated into mouse skin fibroblasts; however, the incorporation was suppressed after prolonged cultivation (Asai et al., 2020b). The incorporation of Pro-Hyp into p75NTR-positive and -negative fibroblasts was examined by using fluorescein isothiocyanate (FITC)-labeled Pro-Hyp because FITC-labeled and non-labeled di-peptides pass through the same peptide transporter 1 (PepT1) (Abe et al., 1999). The fluorescence of the FITC-labeled Pro-Hyp was only observed in the p75NTR-positive fibroblasts (**Figures 4A–C**) and the fluorescence was weak on the nuclei (**Figure 4D**). Thus, the FITC-labeled Pro-Hyp was incorporated into the cytosol rather than being bound on the cell surface (Asai et al., 2020b). p75NTR-positive fibroblasts specifically incorporate Pro-Hyp, which might trigger the proliferation of fibroblasts cultured on collagen gel. Even the primary cultured fibroblasts, however, changed their nature within a few days, which makes it difficult to elucidate the response of fibroblasts to LMW-FGIF and the interaction between extracellular matrixes using a cell culture system. The expression of p75NTR and the ability to incorporate di-peptides are useful biomarkers for distinguishing fibroblast subpopulations in cell culture systems.

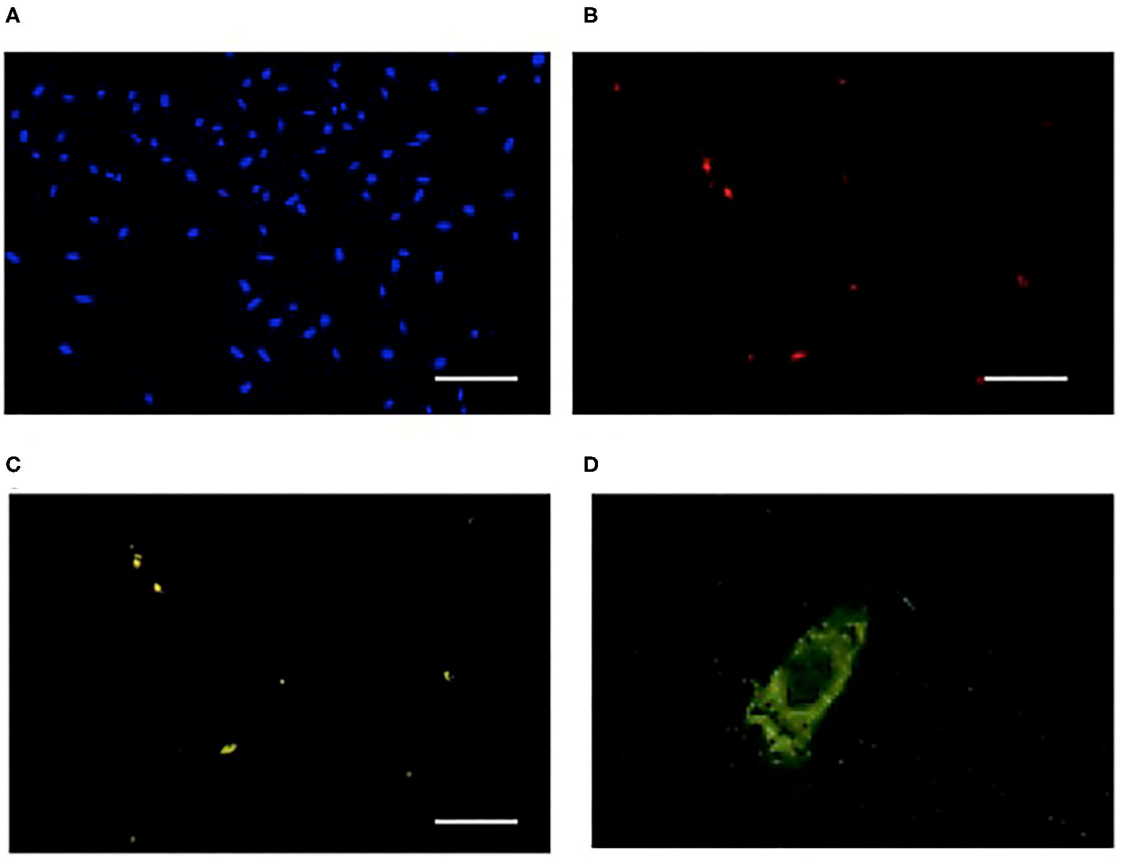

FIGURE 4 | Incorporation of FITC-labeled Pro-Hyp into fibroblasts. FITC-labeled Pro-Hyp was specifically incorporated into 75NTR-positive fibroblasts. Fibroblasts outside of the skin after 4 days of incubation were used. Cells were stained with DAPI in **(A)**, antibody against p75NTR (red) in **(B)**, fluorescence of FITC-labeled Pro-Hyp (green) in **(C)**. Scale bar = 50 μm. High magnification picture of fibroblast incorporating FITC-labeled Pro-Hyp in **(D)**. This figure was adapted with permission (Asai et al., 2020b).

INFLUENCE OF THE SUPPLEMENTATION OF COLLAGEN PEPTIDES ON WOUND HEALING

Diabetic patients often suffer from chronic wounds, the mechanism of which is not fully understood (Han and Ceilley, 2017). The generation of Pro-Hyp in the granulation tissue of a db/db mouse, a diabetic model animal, was smaller than that in a normal mouse (Jimi et al., 2017). Pro-Hyp is crucial for the proliferation of p75NTR-positive fibroblasts cultured on collagen gel. Some animal studies have also demonstrated that the oral administration of fish gelatin and collagen hydrolysate improved the delayed wound healing in diabetic rats and mice, respectively (Zhang et al., 2011; Xiong et al., 2020). Therefore, supplementation of collagen hydrolysate or gelatin has therapeutic potential for chronic wounds in diabetic patients. To the best of our knowledge, human clinical trials have not been undertaken to prove the therapeutic effect of collagen hydrolysate on chronic wounds in diabetic patients.

Pressure ulcers are another chronic wound with several animal studies suggesting that supplementation with collagen hydrolysate improves the healing of pressure ulcers (Nakao et al., 2013). Human clinical trials using placebo controls have demonstrated that supplementation with collagen hydrolysate enhances healing of pressure ulcers (Lee et al., 2006; Sugihara et al., 2015, 2018; Yamanaka et al., 2017). The Japanese Society of Pressure Ulcers Guideline Revision Committee (2016) has cited collagen hydrolysate in their therapeutic guidelines. In these studies, Pro-Hyp, which is derived from orally administered collagen hydrolysate, was considered to play a crucial role in enhancing the healing of pressure ulcers. Sugihara et al. (2018) demonstrated that low molecular weight collagen hydrolysate (average molecular weight: 1,200 Da) that was rich in Pro-Hyp exerted better therapeutic effects on patients with pressure ulcers than the conventional collagen hydrolysate (5,000 Da). The ingestion of collagen hydrolysate with low molecular weight has been shown to increase blood collagen peptide levels compared to that with high molecular weight (Ichikawa et al., 2009). However, different blood collagen peptide levels were observed after ingestion of the same dose of collagen hydrolysates with similar average molecular weights made from different sources (Ohara et al., 2007). Therefore, the therapeutic effect of collagen

hydrolysate depends on its source and preparation method, and formulated collagen hydrolysate, which can provide reproducible Pro-Hyp levels in the blood after ingestion, is required for wound healing therapy.

Food-derived Pro-Hyp circulates in the blood system and is potentially delivered to all tissues. However, an *in vitro* study indicated that p75NTR-negative fibroblasts in healthy tissue did not respond to Pro-Hyp (Asai et al., 2020b). No adverse effects due to abnormal proliferation of fibroblasts have been observed after long-term administration of collagen hydrolysate in healthy volunteers (Shigemura et al., 2018) and pressure ulcer patients (Sugihara et al., 2015, 2018; Yamanaka et al., 2017). Thus, supplementation with collagen hydrolysate does not generate severe adverse effects on non-wound tissue.

An animal study suggested that the supplementation of collagen hydrolysate has the potential to promote healing of wounds after cesarean section (Wang et al., 2015). Yasueda et al. (2016) reported that the supplementation of collagen hydrolysate reduced hospital stays after colon cancer surgery. These preliminary reports suggest that supplementation with collagen hydrolysate might enhance the healing of surgical wounds. However, the effects of food-derived Pro-Hyp on the growth of cancer cells and cancer-associated fibroblasts have not been examined. In addition, there is limited data on the effect of the ingestion of collagen hydrolysate on carcinogenesis in animal models. The oral administration of a collagen fraction to hamsters with chemo-induced pancreatic duct carcinoma (0.4% in the diet) for 50 days did not show exacerbation (Kitahashi et al., 2006). The effects of Pro-Hyp on carcinogenesis in other animal models should be examined before it is used to promote surgery recovery in cancer patients.

CONCLUSION

Human clinical trials and animal studies have demonstrated that the ingestion of gelatin and collagen hydrolysate enhances wound healing, especially diabetes-induced chronic wounds and pressure ulcers. After the ingestion of collagen hydrolysate, Pro-Hyp increases in human blood. Pro-Hyp is also generated by the degradation of endogenous collagen in wound healing sites as summarized in **Figure 1**. Endogenous and food-derived Pro-Hyp can enhance wound healing by stimulating the growth of p75NTR-positive fibroblasts in the wound healing site without adverse effects on healthy tissue because it does not significantly affect quiescent p75NTR-negative fibroblasts in healthy tissue (**Figure 2**). The small collagen peptide, Pro-Hyp, is a low molecular weight growth-initiating factor for fibroblasts and plays a crucial role in wound healing by initiating the proliferation of fibroblasts with mesenchymal stem cell marker, p75NTR and peptide transporter(s).

AUTHOR CONTRIBUTIONS

KS: conceptualization and writing—original draft. SJ: supervising, reviewing, and editing. TA: reviewing and editing. All authors contributed to the article and approved the submitted version.

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