INFLAMMATION, STEM CELLS AND WOUND HEALING IN SKIN AGING

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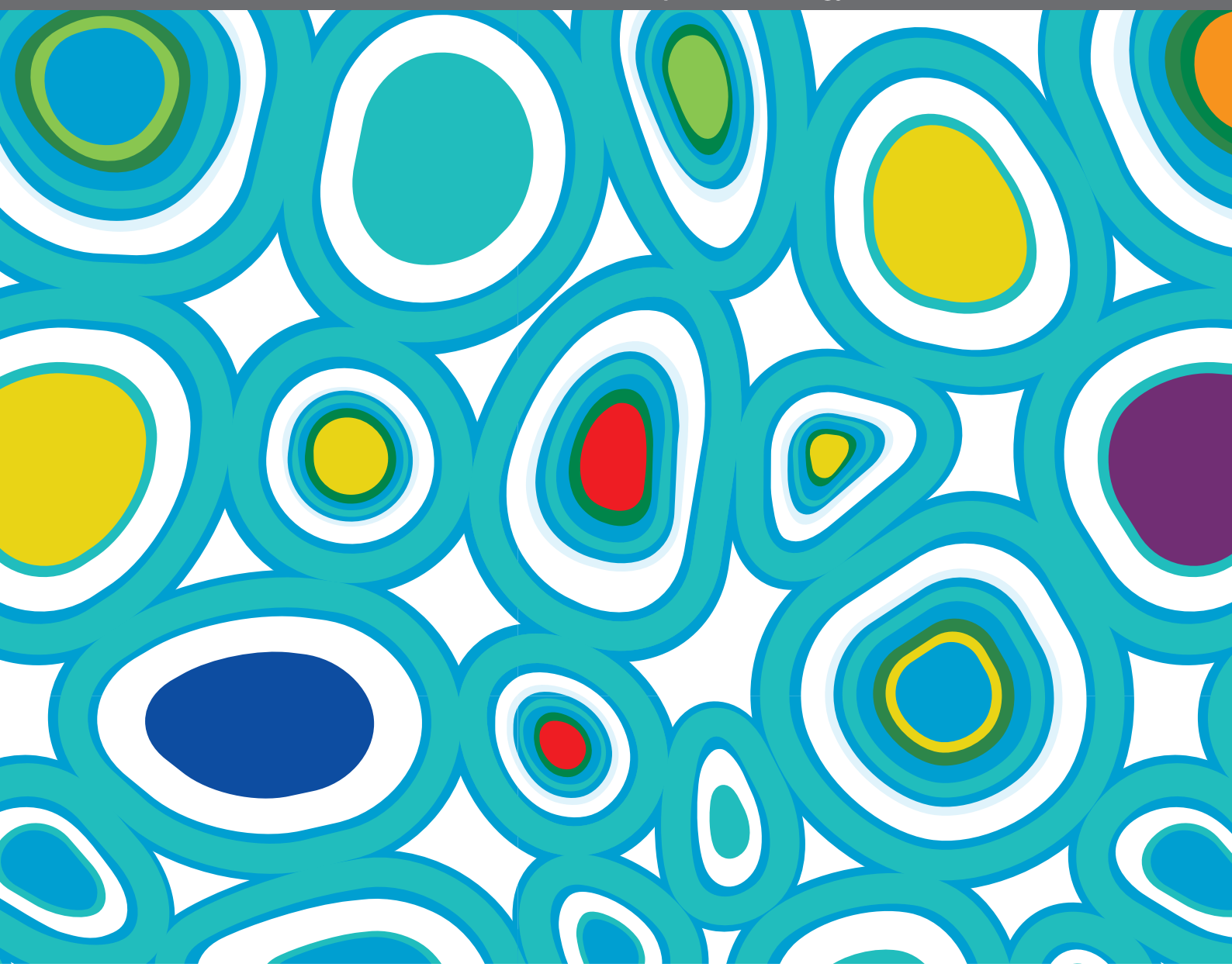

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INFLAMMATION, STEM CELLS AND WOUND HEALING IN SKIN AGING

Topic Editors:
Ji Li, University of Cagliari, Italy
Wen-Hui Lien, Université Catholique de Louvain, Belgium
Mingxing Lei, Chongqing University, China

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EDITED AND REVIEWED BY
Valerie Kouskoff,
The University of Manchester,
United Kingdom

*CORRESPONDENCE
Ji Li,
lydia.1208@hotmail.com
Wen-Hui Lien,
wen-hui.lien@uclouvain.be
Mingxing Lei,
mingxing@cqu.edu.cn

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Editorial: Inflammation, stem cells and wound healing in skin aging

Mingxing Lei[1]*, Wen-Hui Lien[2]* and Ji Li[3,4,5]*

[1]111 Project Laboratory of Biomechanics and Tissue Repair, Key Laboratory of Biorheological Science and Technology of the Ministry of Education, College of Bioengineering, Chongqing University, Chongqing, China, [2]de Duve Institute, Université catholique de Louvain, Brussels, Belgium, [3]Department of Dermatology, Xiangya Hospital, Central South University, Changsha, China, [4]Hunan Key Laboratory of Aging Biology, Xiangya Hospital, Central South University, Changsha, China, [5]National Clinical Research Center for Geriatric Disorders, Xiangya Hospital, Central South University, Changsha, China

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Editorial on the Research Topic
Inflammation, stem cells and wound healing in skin aging

Introduction

Skin aging is the most recognizable consequence of senescence, mainly manifested as epidermal and dermal thinning, reduced elasticity, wrinkle formation, skin relaxation, abnormal skin pigmentation, wound healing disorders, hair graying, and pilosebaceous unit degeneration (Kohl et al., 2011; Kanaki et al., 2016; Gu et al., 2020). It is believed that the extrinsic skin aging primarily arises from UV-light exposure, whereas several factors are shown to induce intrinsic skin aging, including cellular senescence and the shortening of telomeres, mutations of mitochondrial DNA, oxidative stress, genetic mutations, and decreased levels of hormones, such as estrogen and progesterone (Kohl et al., 2011). Overall, skin aging is a highly complex but incompletely understood process, and despite great progress in recent years, many mysteries of aging mechanisms remain unsolved.

Skin is in direct contact with the environment outside the body and is stimulated by various external factors, such as UV radiations, microorganisms, etc. External and internal stimuli trigger the immune response of skin cells, causing a series of inflammatory reactions that induce inflammatory skin diseases, such as psoriasis, rosacea, atopic dermatitis, etc. Long-term chronic inflammation may induce skin cell senescence, and the compromised stem cell activity and wound responses are the consequence of skin aging (Figure 1). Therefore, a deep understanding of physiological regulation and pathological mechanisms of skin aging helps to advance the regenerative biology field and future clinical applications. Here we organize this Research Topic with a collection of original research and review articles that explore skin

aging-related inflammation, stem cell activity, and wound healing. This collection aims to provide new insights into skin aging.

Skin aging

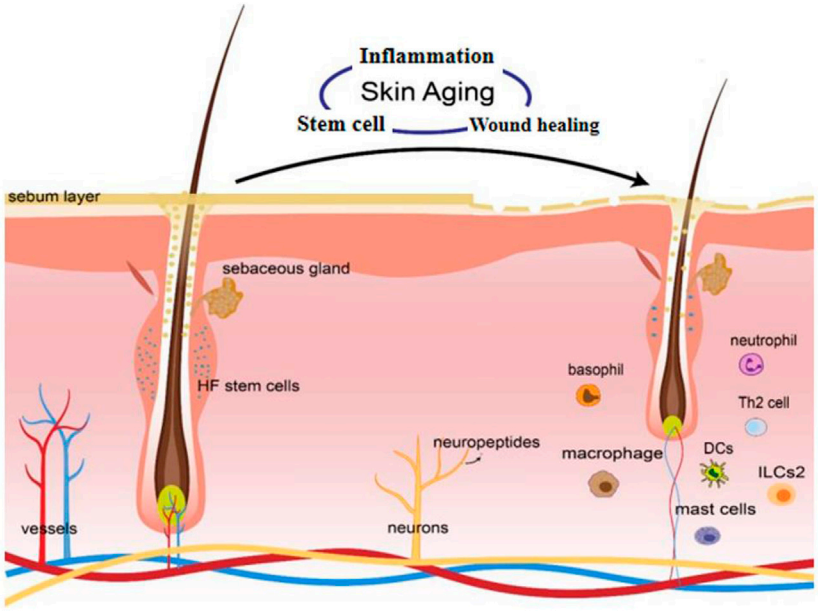
Skin aging occurs under various circumstances, and light damage is considered to be the main exogenous cause. With the progress of aging, inflammation appears, and various profound structural and functional changes take place one after another (Hsu et al., 2014), all of which play either a positive or a negative regulatory role on aging in feedback. The two articles in this Research Topic discuss the latest viewpoints on skin aging in relation to light damage and skin structure. Light damage is an important risk factor for photoaging. Wang *et al.* found that HSP27 could play a protective role in UV irradiation-induced skin photoaging by stimulating autophagy and reducing reactive oxygen species (ROS) production; therefore, it may serve as a potential therapeutic target for photoaging (Wang et al.). Furthermore, the dermal extracellular matrix (ECM) constitutes the main framework of the dermis, and its composition changes greatly with skin aging. Li *et al.* analyzed the composition of dermal ECM in decellularized skin scaffolds of different age groups using a quantitative proteomics approach, and identified the regulatory pattern of ECM in the process of aging. Their results provide new clues for biomaterials that can be utilized in skin regeneration (Li et al.). Other than the above-mentioned aspects, many other factors are also involved in skin aging and anti-aging. Here we discuss three major factors that play crucial roles in skin aging, including inflammation, stem cells, and wound healing.

Inflammation and skin aging

With aging, the whole body is progressing into a chronic low inflammatory state, which in turn accelerates aging in feedback by enhancing oxidative stress, DNA damage, and stem cell aging. The mini-review by Chen *et al.* summarized the relationship between type 2 inflammation and skin immunosenescence, and brought up the idea that skin inflammation and skin aging could regulate each other. They showed that chronic low levels of proinflammatory factors released by senescent cells could induce skin immunosenescence and inflammation, and suggested that it is promising to ameliorate inflammatory skin diseases by delaying skin immunosenescence (Chen et al.). The existing studies show that aging-related inflammation results in various diseases, such as hypertension (Liberale et al., 2022), diabetes (Bharath et al., 2020), and so on. In the skin, aging leads to functional damage of immune cells, fibroblasts, keratinocytes, etc., and then consequently causes chronic inflammation and immune diseases. Hu *et al.* revealed the dysregulation of invariant natural killer T (iNKT) cells in the pathogenesis of psoriasis and proposed suginumumab that targets a key factor of iNKT cells, IL-17, as a therapeutic drug (Hu et al.). Chang *et al.* reviewed the innate immune disorders in the pathogenesis and progression of vitiligo, including the early activation of NK cells, dendritic cells, and the involvement of various T cells, and proposed that immunomodulatory therapy is critical for vitiligo (Chang et al.). The senescence-associated secretory phenotype (SASP), involving high levels of inflammatory cytokines, chemokines, and matrix metalloproteinases, is considered to be the primary cause of the harmful effects of senescent cells. This strongly suggests the unavoidable effect of inflammation in inducing and promoting aging (Picardo et al., 2015). At present, it is generally believed that neuroimmune interactions play an increasingly important role in aging-related inflammation and are also the basis of the pathogenesis of these immune diseases. Zhu, Y. *et al.* described the mutual regulation of neural and immune systems in skin, and analyzed the neuroimmune mechanisms of various inflammatory skin diseases (Zhu et al.).

Stem cells and skin aging

The decrease in the number and activity of stem cells is an inevitable change during skin aging, which leads to age-related alopecia and delayed wound healing. As the primary skin appendage, the hair follicle relies on hair follicle stem cells (HFSCs) to regenerate hair during skin homeostasis. HFSCs and hair follicle organoids are major models for studying hair-related diseases and skin aging (Lei and Chuong 2016; Lei et al., 2017). Six papers in this Research Topic highlighted the regulation and mechanisms in the development and degeneration of the pilosebaceous unit that consists of the hair follicle and sebaceous gland. Hou *et al.* systematically reviewed the signaling pathways and neuroendocrine changes during sebaceous gland differentiation and aging, and summarized the prevention and treatment measures against sebaceous gland aging (Hou et al.). Lin *et al.* delineated the morphological development, cycle, and molecular regulation of hair follicles (Lin et al.). Lyu *et al.* provided a comprehensive summary of the molecular mechanisms regulating hair follicle degeneration during aging. They outlined how nutrient sensing, metabolic reprogramming, altered mitochondrial activity, and epigenetic regulation affect hair regeneration, and affirmed the dominant role of the tissue microenvironment in regulating aging epithelial stem cell function (Lyu and Ge). Vikhe Patil *et al.* summarized the expression and function of peptidylarginine deiminases (PADIs), enzymes that convert



Skin aging	Inflammation
Chen, Yang et al. 2022; Wang, Li et al. 2022; Li, Li et al. 2021; Hou, Wei et al. 2022	Hu, Chen et al. 2022 ; Chang, Lee et al. 2021; Zhu, Duan et al. 2022
Stem cells	**Wound healing**
Lin, Zhu et al. 2022; Vikhe Patil; Mak et al. 2021; Chen, Fan et al. 2021; Zhang, Chen et al. 2021; Lyu and Ge 2022	Pang, Zhu et al. 2022; Park 2021

FIGURE 1
Inflammation, stem cells and wound healing in skin aging is characterized by thinned epidermal and dermal, damaged skin barrier, smaller hair follicles, fewer HF stem cells, and minimized DP. Skin aging leads to impaired stem cell activity, chronic inflammation, and compromised wound healing, and is also associated with neuroimmune and inflammatory disorders.

amino acid arginine to citrulline, in the hair follicle stem cell lineage and inflammatory alopecia, and provided a comprehensive perspective on how citrullination modulates hair follicle regeneration and contributes to inflammatory alopecia (Patil et al.). Chen *et al.* revealed a new function of dermal white adipose tissue (dWAT) in regulating hair follicle development during aging, and pointed out that the massive inflammatory infiltration of aging dWAT may be a central factor hindering hair follicle regeneration (Chen et al.). Zhang *et al.* generalized the trophic and regulatory effects of the follicular sympathetic nerves and their neuropeptides in hair follicle immunity and growth (Zhang et al.).

Wound healing and skin aging

Impaired wound healing in the elderly imposes significant pressure on clinic treatment. With aging, the skin is vulnerable to various damages due to the destruction of its barrier function and the degeneration of stem cells. Pang *et al.* revealed a crucial role of Keratin 17 in epidermal barrier repair, in which its expression is upregulated upon acute disruption of the epidermal barrier to promote lipid metabolism *via* increasing the nuclear transport of SREBP-1 and PPARγ (Pang et al.). Park *et al.* affirmed the similarities between embryonic skin development and adult skin repair processes. In this mini-review, the author summarized and compared the differences in cellular components, neighboring tissue status, and surrounding environment between the two, and provided clues for the repair of skin damage (Park).

In conclusion, the findings and ideas presented in this Research Topic provide insights into inflammation, stem cell activity, and wound healing in skin aging. With the cutting-edge experimental techniques and increasingly interdisciplinary approaches employed in this Research Topic, we have witnessed strong progress in the field. We hope that this Research Topic will pave a new way to elucidate the new mechanism of aging-related skin inflammatory diseases, hair regeneration, and wound healing, to help us advance the process of regenerative biology, and to guide the development

and clinical application of anti-inflammatory and anti-aging drugs in the future.

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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Recent Progress in the Understanding of the Effect of Sympathetic Nerves on Hair Follicle Growth

Jiarui Zhang†, Ruosi Chen†, Lihong Wen, Zhexiang Fan, Yilong Guo, Zhiqi Hu and Yong Miao**

Department of Plastic and Aesthetic Surgery, Nanfang Hospital, Southern Medical University, Guangzhou, China

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Edited by:
Mingxing Lei,
Chongqing University, China

Reviewed by:
Pietro Gentile,
University of Rome Tor Vergata, Italy
Bing Zhang,
Westlake University, China

***Correspondence:**
Zhiqi Hu
huzhiqidr@163.com
Yong Miao
doctormiao0371@163.com

† These authors have contributed equally to this work

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Clinical observation and experimental studies have long suggested that the perifollicular nerves have nutritional and regulatory effects on the growth, development, and physiological cycle of hair follicles (HFs), even though the concrete mechanism remains obscure. Recently, with the progress of immunohistochemistry and molecular biology techniques, more innovation has been made in the study of the follicular sympathetic nerves and its nerve-effect factor norepinephrine affecting hair follicle stem cells. This review highlights the progress in the regulation of the sympathetic nervous system toward the growth of HFs.

Keywords: sympathetic nerves, hair follicles, norepinephrine, hair follicle stem cells, hair growth

INTRODUCTION

Immature hair follicles (HFs) are divided into three enclosed epithelial cylinders as highly sensitive, dynamic micro-organs developed from the embryonic epidermis. The largest cylinder in the center forms the hair shaft (fiber), and the outermost cylinder forms the outer root sheath (ORS), which separates the intact structure from the dermis. In the middle, the inner root sheath (IRS) shapes the hair shaft and induces its outward growth (Paus and Cotsarelis, 1999). Mature HFs contain the principal cell types and experience a repetitive growth cycle comprising alternating phases of growth (anagen), degeneration (catagen), and resting (telogen), which are affected by many growth factors, cytokines, hormones, and various neuropeptides (Dry, 1926; Stenn and Paus, 2001; Schneider et al., 2009; Geyfman et al., 2015).

Presenting normally as an indispensable part of the physiological environment of HFs, the sympathetic nerves play an important role in the growth of HFs.

Our review focuses on the biological characteristics of the sympathetic nerves, along with the application of its nerve-effect factor, norepinephrine (NE), in HF growth.

Abbreviations: HF, hair follicle; ORS, outer root sheath; IRS, inner root sheath; APM, erector muscle; β2-AR/ADRB2, β2-adrenocorticoid receptor; POMC, proopiomelanocortin; ACTH, adreno-cortico-tropic-hormone; HPA, hypothalamic-pituitary-adrenal stress axis; AA, alopecia areata; NE/NA, norepinephrine/noradrenaline.

SYMPATHETIC NERVES DISTRIBUTION IN HFS

HFs undergo various stages of morphogenesis, which is consistent with a certain state of innervation (Paus et al., 1997). From the beginning, the nerve bundles gradually infiltrate the subepidermal interstitial layer and build up an increasingly denser innervation network with the development of the hair cycle. In general, HFs are dominated by the autonomic nervous system, which differentiates into three species: the first one so-called the subepidermal neural-plexus (SEP) stays above the aponeurosis. Under the epidermis there comes with the second layer named subcutaneous neural-plexus (SCP). There are deep dermal neural-plexus (DDP) between the dermis and the developing subcutaneous tissues (Paus et al., 1997). As a branch of the autonomic nervous system all over these aspects, the sympathetic nerves are critical for maintaining body physiology under steady state (Karemaker, 2017). Simultaneously, compact vascular networks synchronize the creating sequence and spatial distribution with the nerves between the epidermis, aponeurosis, dermis, and subcutaneous tissue. It suggests a parallel regulation mechanism in space and time from the beginning of the development of nerves in HFs.

Located between blood vessels and HFs, the sympathetic nerves form a tight connection with the arrector pili muscles (APMs) and vascular smooth muscle. In the dermis, sympathetic nerves converge as a single nerve fiber. Enwrapped and penetrated by sympathetic nerves, APMs provide stable anchors that maintain sympathetic innervations to the HFs. Meanwhile, such a reticular network at the dominant site surrounds the hair follicle stem cells (HFSCs) and forms synapse-like connections with HFSCs. Adjacent to the site where APM inserts into the connective tissue sheath of HFs, sympathetic nerve fibers intermingle but extend beyond the APMs, thus composing a spatial connection with the HFSCs at different positions throughout the outer bulge region and the hair germ (Botchkarev et al., 1997, 1999).

In addition, sympathetic fibers are wrapped by the endoneurium composed of specialized collagen and Schwann cells. As the sympathetic nerves approaches HFSCs, the bundled endoneurium opens only on the side that faces HFSCs, exposing nerve fibers to HFSCs, thus preferably enhancing the diffusion of neurotransmitters such as NE-containing vesicles toward HFSCs. This suggests that the sympathetic nerves innervate not only APMs, but also HFSCs in the extra-follicular bulge area and hair germ layer (Shwartz et al., 2020; **Figure 1**).

CORRELATIVE CHANGES IN THE SYMPATHETIC NERVES AND SYMPATHETIC NEUROPEPTIDES IN HFS

Because of the insufficiency of melanocytes in the epidermis, the pigmentation in the trunk of C57BL/6 mice depends completely on the melanin-producing melanocytes in their HFs, whereas the production of melanin in HFs only remains active during their growth period. Also, the color-synchronized growth phases of C57BL/6 mice allow for the isolation and analysis of HFs at specific stages induced by hair extraction (Chase and Eaton, 1959). Therefore, evaluating the skin color conversion of C57BL/6 model macroscopically would be convenient to identify the cycle of HFs—anagen (black), catagen (gray), and telogen (pink) phases. It provides an ideal system for exploring the interaction between sympathetic nerves and the HF cycle.

Considering the characteristics of uniform hair growth waves induced by shaving the back of C57BL/6 mice, Muller-Rover et al. (2001) and Peters et al. (2001) used immunofluorescence and neuronal markers to rearrange the periodic development of dorsal nerves in C57BL/6 mice. Peters found that the general innervation had significant changes and remodeling at specific phases of the hair cycle. The plasticity of HFs innervation in C57BL/6 relied on hair cycle—the amount of autonomic rhythmic nerves amplified significantly during the early anagen, while plummeting in the catagen and reaching the lowest level in the telogen (Steinkraus et al., 1996; Slominski et al., 1998, 2007; Peters et al., 2002). The hair embryo is immunoreactive to most of the neuronal markers. During the period of mouse fetal development at E14/E15 with the mature dorsal root nerve, we could not detect morphologically distinguishable hair embryo formation in the dorsal skin nor the nerve fiber bundles (Steinkraus et al., 1996; Slominski et al., 1998, 2007; Peters et al., 2002).

These findings suggest that the formation and sequential appearance of various types of nerve endings, including sympathetic nerves, are determined by HFs rather than programmatically produced by the dorsal root ganglia (Steinkraus et al., 1996; Slominski et al., 1998, 2007; Peters et al., 2002).

Botchkarev et al. (1999) observed the sympathetic localization of HFs in experimental C57 mice. Compared with the telogen phase, the formation of sympathetic adrenergic and tyrosine hydroxylase (TH) nerve fibers in the dermis and subcutaneous tissue of synchronized HFs increased sharply in the early anagen phase, but decreased steeply in the catagen phase (Botchkarev et al., 1999; Slominski et al., 1999; Kim et al., 2011)—TH antigen is a specific marker of adrenergic nerves (Ljungberg and Johansson, 1993).

Proopiomelanocortin (POMC) is an upstream regulator of the sympathetic nerves in the skin that regulates the secretion of various hormones, including adrenocorticotropic hormone (ACTH), melanocyte-stimulating hormone (MSH), and β-endorphin. The skin not only serves as the target of POMC but also expresses the POMC gene to promote the secretion of related peptides (Paus et al., 1999). The transcription and translation of the POMC gene in the skin of C57BL/6 mice are hair cycle-dependent, which shoot up dramatically in the anagen, but decay in the catagen, and rest in the telogen (Paus et al., 1999). With the application of reversed-phase high- performance liquid chromatography combined with a specific radioimmunoassay, various hair cycle-related sympathetic neuropeptides could be observed in the skin of C57/BL6 mice: the concentration of ACTH was low in the early anagen but was boomed in two steps. The first rapid rise appeared in the early anagen, followed by the

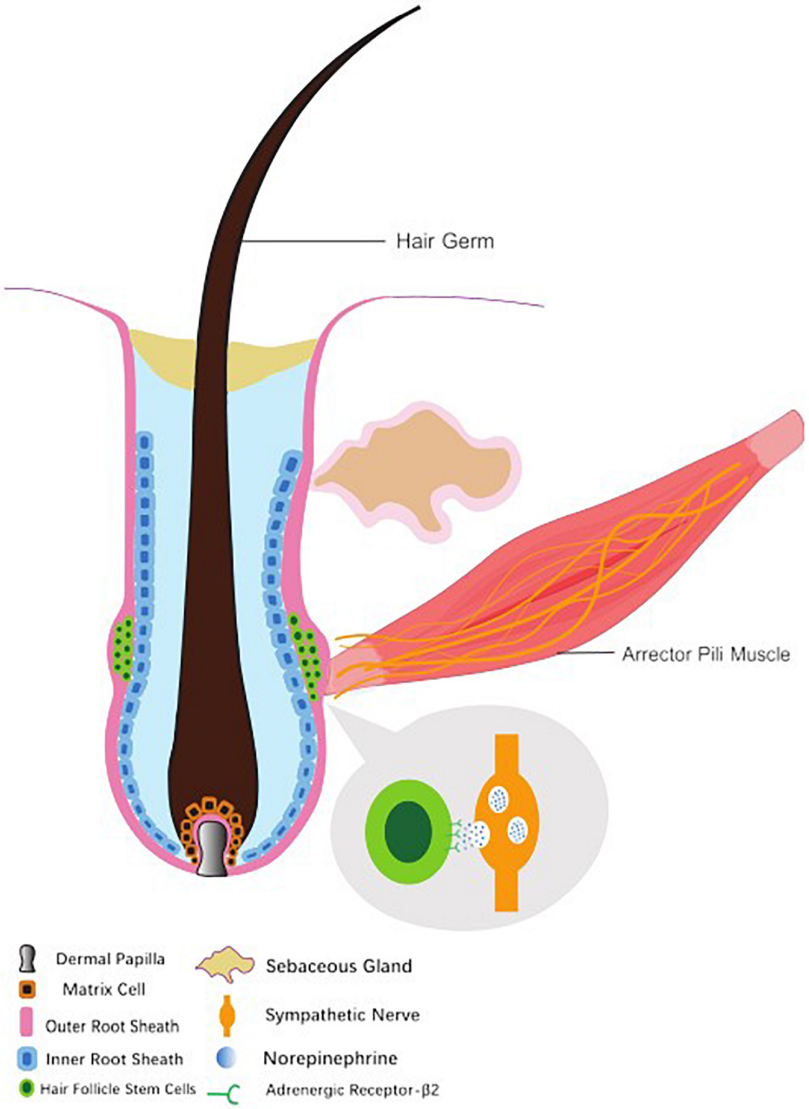

FIGURE 1 | Hair follicle structure, hair follicle stem cell and accessory organs. The arrector pili muscle provides as an anchor to maintain sympathetic innervation to hair. The sympathetic nerves form synapse-like connections with hair follicle stem cells and regulate them through vesicles containing norepinephrine.

second slow climb out in anagen VI. While in the catagen, the concentration of ACTH descended rapidly to the lowest level in the telogen (Slominski et al., 2001).

These changes are accompanied by a parallel expression of adrenergic neurotrophic factor and beta 2-adrenoceptor (β2-AR), -the specific adrenergic receptor on HFs. Likewise, keratinocytes containing β2-AR can be gradually detected in the non-follicular circulating epithelium (especially in the isthmus and bulge region) during the early anagen (Slominski et al., 2001). Moreover, a significant increase in the number of TH-IR nerve fibers accompanied by exocrine norepinephrine secretion was detected by histochemistry (Slominski et al., 2001). During the catagen, they degenerated to a feeble level in the proximal inner root sheath (IRS) and became undetectably in the telogen (Slominski et al., 2001).

In summary, the periodic physiological distribution of sympathetic nerves in HFs and the diversity of adrenoceptor expression show that the growth of sympathetic nerves is related to the growth cycle of HFs. Furthermore, searching for the periodic expression level of sympathetic nerve-related peptides and upstream and downstream regulatory factors in HFs exploit new ideas regarding of the effect of sympathetic nerves on HF growth and the treatment of pathological situations of HFs.

The Effect of Sympathetic Nerves on HF Stress System

As a unique and sensitive mini-organ, the HF represents not only the target of stress hormones and autoimmune responses, but can also secrete related stress factors that affect

skin conditions (Welch, 1992; Pacak and Palkovits, 2001). The systematic biological response of organisms to external stressors (or classical stress responses) mainly activates the central hypothalamic-pituitary-adrenal (HPA) stress axis, which in turn releases corticotropin-releasing hormone (CRH) to activate the receptor (CRH-R) in the hypothalamus. The downstream target organ-adrenal gland gives out adrenocorticoid-derived peptide (POMC), and the sympathetic nerves produce adrenocorticoid into the blood circulation (Ito et al., 2005). The stress response also comprises autonomic sympathetic regulation of target organs and the immune system: the sympathetic neuropeptides affect the function of the immune system, while the immune system regulates the function of the central nervous system by releasing cytokines. In organ culture and microdissection of HFs, CRH up-regulates the expression of upstream sympathetic molecules, such as POMC, ACTH, and α-MSH peptides. These molecules promote the secretion of cortisol by HFs, which accumulate and exocrine in the keratinocytes of ORS in HFs, and then inhibit the expression of various stressor hormones in ORS. This suggests that HFs have a similar classical HPA axis negative feedback regulation effect (Ito et al., 2005; **Figure 2**).

Recently studies have shown that a functional stress response system acts independently of the central HPA axis in the HFs, which performs and coordinates the peripheral stress response (Arck et al., 2006; Ito, 2010). Emerging research have suggested that stress is a trigger of common dermatoses—deficiency of the stress system may influence both the HF immune and hormone systems, leading to the induction of autoimmune hair diseases such as alopecia areata (AA) (Alexopoulos and Chrousos, 2016). Psoriasis episodes are often preceded by stressful decreased expression of CRHR-1 which was observed in psoriatic epidermis or dermis, whereas increased levels of CRH were found in the serum of patients with psoriasis. Of note, the development of mast cells in the hair follicle is stimulated by CRH (Basavaraj et al., 2011). Stress also plays a significant role in exacerbating and perpetuating the itch-scratch cycle in atopic dermatitis. Psychological distress stimulates the HPA axis of sympathetic system, which in turn increase secretion of inflammatory mediators that play a significant role in disturbing the skin barrier (Rampton, 2011). What's more, stress may suppress the activation of the cutaneous HPA axis through glucocorticoids and, thereby, reduce melanogenesis to account for vitiligo (Pang et al., 2014). The sympathetic nerves are stimulated by stressors, which can regulate a variety of HF stress responses by releasing NE, which is the key regulatory point of the HF local stress system (Webster et al., 2002; Botchkarev, 2003; Takenaka et al., 2017). Prospectively, stress caused by the sympathetic nerves around the HFs might be a research focus in dermatology.

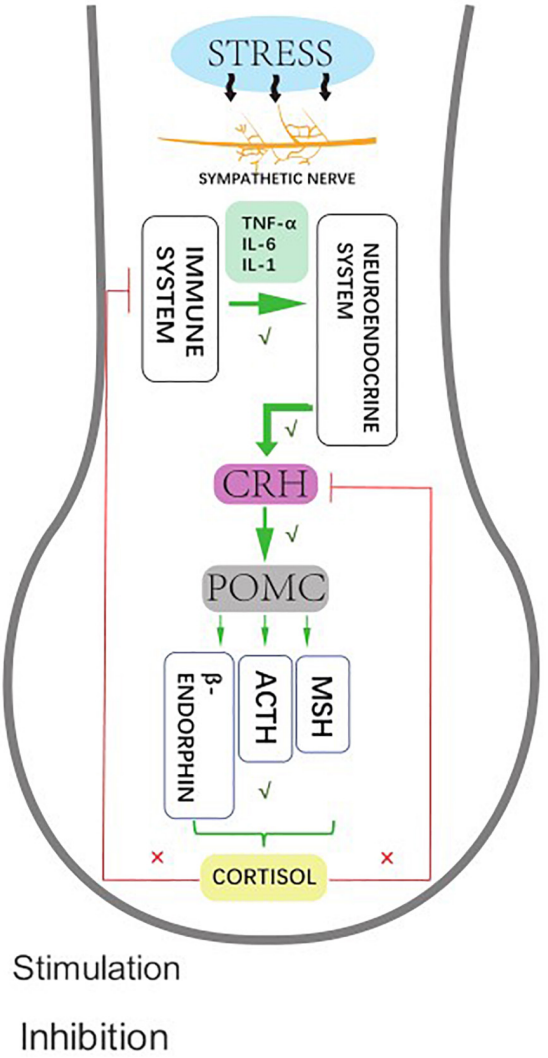

FIGURE 2 | Human hair follicles display HPA axis-like regulatory feedback systems. Affected by hair stress-sensors, the sympathetic nerve activity elevates to different degrees, showing neuroendocrine production and release of corticotropin releasing hormone (CRH), which leads to signal transduction pathway enhances the production and secretion of the anterior pituitary-derived POMC peptides, ACTH, and β-endorphin. Meanwhile, the glucocorticoid receptor agonist cortisol down-regulates follicular CRH expression as an adaptive response to stabilize and restore the general homeostasis.

The Effect of Sympathetic Nerves on Hair Follicle Immune System

Early studies have shown that both primary and secondary lymphoid tissues are innervated by postganglionic sympathetic neurons, which secrete NE as primary neurotransmitters, while immune cells express adrenergic receptors, which bind to epinephrine and norepinephrine, enabling the immune system to respond directly to signals from the autonomic nervous system (Nance and Sanders, 2007). When the pattern recognition receptors (PRRs) on the HF sympathetic nerves are stimulated by pathogen-related molecular patterns (PAMPs), the sympathetic neurons suddenly secrete NE.

NE can regulate a variety of immune functions, both systematic and local. The most prominent is the anti-inflammatory effect of NE on congenital and acquired cells. Studies have shown that the adrenergic receptor signals on T cells regulate their transport to surrounding tissues and secondary lymphoid organs (Nakai et al., 2014). They proposed that NE

not only inhibits the secretion of inflammatory cytokines and cytolytic activity of mice and human effector $CD8^+$ T cells, but also indirectly limits the extent of $CD8^+$ T-cell initiation by inhibiting the cross-presentation of dendritic cells (Estrada et al., 2016). In addition, the mechanism of NE-mediated inhibition of tumor necrosis factor-alfa (TNF-α) secretion induced by lipopolysaccharide from pathogenic bacteria involves the junction molecule beta-inhibitory protein, which is regulated by the post-translation of adrenergic signals. The beta-inhibitory protein blocks the activation of immune transcription factor-NFκB (Takenaka et al., 2017). Didem has shown that NE inhibits the secretion of various pro-inflammatory cytokines induced by pathogenic stress and rapidly induces the expression of the anti-inflammatory cytokine IL-10 by specifically binding to ADRB2 (Agac et al., 2018).

THE EFFECT OF SYMPATHETIC NERVES ON HAIR FOLLICLE GROWTH

The sympathetic nerves play an important role in the skin nutrition (Agac et al., 2018). Clinically, the experimental subcutaneous injection of the sympathetic chemical toxin 6-hydroxydopamine (6-OHDA) into neonatal mice selectively damaged the terminal nerves containing NE in skin. Subsequently, the morphological interruption of mice HFs was observed. However, isoproterenol, a sympathetic β-adrenoceptor agonist, could partially restore hair growth and even restore HF growth and development (Asada-Kubota, 1995). The premature development of HFs appeared after the induction of over innervation of adrenergic nerves in the denervated skin, which indicates that the sympathetic nerves have an important effect on normal hair growth (Crowe et al., 1993). As the researchers found a substantial reorganization of follicular sympathetic adrenergic cutaneous innervation accompanied by the HF regeneration cycle in mice, great attention was paid on the profound changes in NE secretion (Crowe et al., 1993). After using guanidine to selectively deplete the NE reserve of sympathetic nerves endings, hair growth was inhibited. Then, the cycle does not recover until the adrenaline level is restored (Kong et al., 2015).

In tumor therapy, the local application of NE skin patch to neonatal rats before high-dose radiation injury or high-dose systemic chemotherapy injury can prevent traumatic alopecia after radiotherapy and chemotherapy (Soref and Fahl, 2015; **Figure 3**).

This phenomenon not only discusses the general strategy of local application of epinephrine or NE to prevent alopecia caused by cancer treatment, but also efficiently reflects that epinephrine may play an important role in the maintenance of normal hair growth.

Murphy et al. (1998) and Grando et al. (2006) reported that hypodermic injection of epinephrine leads to premature hair growth in the lower back of C57BL/6 mice, suggesting that sympathetic neurotransmitters promote hair growth in a low concentration-dependent manner. The sympathetic nerves innervate into the arrector pili muscles of HFs as part of the HFSC niche and regulates a small portion of the SCs in the upper bulge region by providing ligands for sonic hedgehog (SHH) (Fan et al., 2018; Chen et al., 2020). Phenomena mentioned above state that the sympathetic nerves exert positive influence on hair growth and has considerable research prospects.

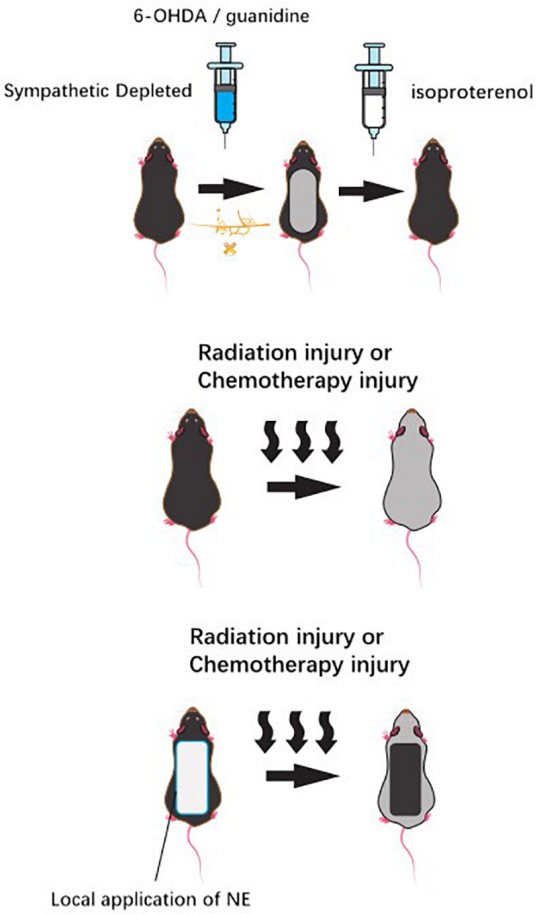

FIGURE 3 | Chemical sympathetic nerve depletion via administration of the neurotoxin 6-hydroxydopamine (6-OHDA) or guanidine to mice inhibited HF growth, while usage of isoproterenol recovered the hair growth. Likely, topical application of norepinephrine to the back skin of rats before a large radiation insult or large systemic chemotherapy insult can prevent the alopecia that was seen in vehicle control animals.

Recently, the new concept of the sympathetic regulation of the HF triad has been proposed, where the arrector pili muscles (APMs) and sympathetic nerves form a dual-component niche to modulate hair follicle stem cell (HFSC) activity, which means sympathetic neurons directly regulate stem cells with NE through synaptic-like structures, while the APMs maintain sympathetic innervation of stem cells. Such a tri-lineage unit formed in the skin is stimulated by cold, causing not only sympathetic excitation and contraction of pili muscles, manifesting as goosebumps, but also activates HFSC. In the absence of NE signaling, HFSCs enter deep quiescence by down-regulating the cell cycle and metabolism and up-regulating the resting regulatory factors Foxp1 and Fgf18. With the progression of the hair cycle, descendants of HFSCs secrete SHH to guide

the formation of APM sympathetic niches, which control the regeneration of adult HFs (Fan et al., 2018; Chen et al., 2020). The triad system provides a powerful persuasion for the theory of the sympathetic nerves affecting hair growth.

SIDE EFFECTS OF SYMPATHETIC NERVES OVER-ACTIVATION ON HAIR DEPIGMENTATION

Researchers have reported that a low concentration of 750 nM NE injected into the back of C57BL/6 mice significantly promotes the proliferation of corneal epithelial cells, while 100 μM NE has a toxic inhibitory effect (Asada-Kubota, 1995; Agac et al., 2018). We suspect that the accumulation of norepinephrine brought by sympathetic nerves over-excitation plays a biological role in a concentration-dependent manner. It might be applied to HF melanocyte stem cells affected by the norepinephrine accumulation, leading to the fallout of hair pigmentation.

Related to the clinically observed phenomenon called "overnight white-head," which means the rapid destruction of hair pigmentation, a study indicated that acute stress leads to hair graying through the rapid depletion of melanocyte stem cells. Loss of pigmentation was independent of immune attack or adrenal stress hormones but resulted from activation of the sympathetic nerves, which changes the melanocyte niche. The motionless melanocyte stem cells proliferate and differentiate rapidly, then migrate outward from the normal survival area, and become permanently exhausted, causing irreversible whitening of black hair (Zhang et al., 2020).

Abnormal sympathetic nerves distribution was observed in the skin lesions of patients with alopecia areata (Tatu et al., 2019). In this regard, we assume that abnormalities in sympathetic nerves distribution or morphology might follow androgenetic alopecia (AGA) or senile alopecia, both of which are worthy of further study.

DISCUSSION

A large amount of experimental data has confirmed the reciprocal communication between the sympathetic nerves and HFs, which provides novel insights into the mechanisms of physiology progression on hair growth. Shwartz et al. discovered that the sympathetic nerves secreted NE to activate hair growth of mice (Tatu et al., 2019), and Zhang proposed the stress hormone corticosterone regulates hair follicle stem cell (HFSC) quiescence and hair growth in mice (Choi et al., 2021). At present, it has not been established whether neurogenesis and cross-talk of neuropeptides are coincidental or prerequisites in the development of human hair growth. Moreover, the anomaly of either the characteristic configuration or the distribution of the sympathetic nerves is unknown for incurring the specific dysfunction of HFs in different hair diseases such as androgen alopecia, folliculitis, or senile alopecia. Studies have described the phenomenon of the sympathetic nerves directly activate HFSCs with NE to proliferate in mice. However, the mechanism of the sympathetic nerves affecting hair growth of human is not clear in clinical studies. Hence, the promising correlation of the sympathetic nerves and human HFs cannot be ignored and further investigations are recommended.

The action of HFSCs is controlled by its niche. This microenvironment incorporates the daughter cells of the HFSCs in the bulge, which enact their self-recovery ahead of schedule and in the late anagen stages (Tanimura et al., 2011). Maintaining the niche of HFSCs is vital for HF homeostasis and harm fix. Divisions of HFSCs are not frequent in mature HFs, and their greater part are in a lethargic state. As such, it is vital to comprehend the components of their activation and induction, which will allow for the use of multipotent growth factors in hair regrowth. Their use is complicated by the fact that the expression of receptors on the various growth factors and the effect of the microenvironment may vary. Not all target points in HFSCs therapy have been distinguished (Gentile and Garcovich, 2019).

Without the sympathetic signaling, HFSCs enter deep quiescence by down-regulating the cell cycle and metabolism while up-regulating quiescence regulators Foxp1 and Fgf18. During development, HFSCs progeny secretes Sonic Hedgehog (SHH) to direct the formation of this APM-sympathetic nerve niche, which in turn controls HFs regeneration (Tatu et al., 2019).

Hair growth was also regulated prevalently by the Wnt biomolecular pathway and ERK activation in which HFSCs and growth factors are involved. Specifically, it has been additionally demonstrated that Wnt/β-catenin signaling is necessary for the growth and upkeep of the mesenchymal stem cells (MSCs) (Huelsken et al., 2001; Tsai et al., 2014). The survival and growth of MSCs relies on signs transmitted by the HFSCs, for instance, the TGF-β or the Wnt pathway (Huelsken et al., 2001; Tsai et al., 2014). Likewise, HFSCs are influenced by MSCs in the dermal papilla, which are in close contact with the germinal matrix that are isolated by the basal membrane. They appear to be part of vital significance in the activation of hair growth and in signal transmission during recovery (Rompolas and Greco, 2014). Specifically, Gentile innovatively obtained autologous micrografts containing MSCs from centrifugation of a punch biopsy of the scalp, and used mesotherapy gun accessible for mechanical and controlled MSCs infiltration to treat the affected area of the patients with AGA (Gentile et al., 2020). It is considerable whether the norepinephrine combined with stem cell therapy could better play the synergistic effect of hair growth promotion.

If the sympathetic nerves support hair growth, sympathetic neuropeptides must be secreted at a certain level to maintain the stability of the internal environment of HFs. However, the understanding of the aspect of sympathetic neuropeptides in both stable physiological state and pathological conditions of HFs is still limited.

Recently, the use of autologous platelet-rich plasma (PRP) was determined to provide substantial help in hair regeneration owing to the platelets' ability to advance neo-angiogenesis, cell expansion, and separation (Cervelli et al., 2009, 2013). Platelet-rich plasma (PRP) are rich in growth factors, of which are engaged in hair bio-molecular activity (Cervelli et al., 2012). DPCs have shown improved proliferation, improved Bcl-2 and

FGF-7 levels, activated ERK and Akt proteins, and up-regulation of β-catenin when cultured in an initiated PRP-enhanced growth medium (Li et al., 2012). Each of these elements decidedly impacts hair growth. However, there are too many protocols for the preparation of PRP depending on the different times for centrifugation and RPM used, the number of platelets, the accessibility of growth factors, and chemokines. Thus, it is hard to evaluate which kind of PRP planning is better for clinical indications (Dhurat and Sukesh, 2014). Compared with the PRP, the sympathetic nerves may exert the parallel positive influence on hair regeneration by norepinephrine. The current knowledge in biology, the limits of past translational research will provide a strong basis to advance viable clinical approaches for regenerative aims in hair tissue engineering. Larger, randomized, double-blinded, controlled trials are needed to optimize cell administration protocols and to confirm the early observations of promising clinical outcomes.

Considering the short half-life of neuropeptides and the fast regulation of sympathetic nerves, local application of related drugs may reduce the side effects associated with systemic administration. In recent years, drug carriers such as liposomes, exosomes, and extracellular vesicles (EVs), have been used to effectively deliver neuropeptides into the skin. In a clinical model review, micro-needling is a minimally invasive dermatological procedure in which fine needles are rolled over the skin to puncture the stratum corneum. This therapy is used to induce collagen formation, neovascularization and growth factor production of treated areas. Micro-needling has been used in a wide range of dermatologic conditions, including AGA and alopecia areata (Fertig et al., 2018). Neuropeptides from sympathetic nerves are easily accessible in clinical institution, which facilitates relevant experiments combined with micro-needling on hair growth or wound healing. More exploration of the precise cellular and molecular transmission of the sympathetic neuropeptides in the circumstances of inflammation, dysplasia and atrophy in HFs opens up breaking ground.

Thus, given these factors, the concrete mechanism of sympathetic nerves taking effect on hair growth will be key to elucidating the complex regulation of HFs, and will facilitate the identification of druggable molecular targets for hair disease intervention. It makes sense for further experimental or clinical studies to investigate the translational value or clinical applications of the sympathetic nerves in hair follicles.

AUTHOR CONTRIBUTIONS

JZ was responsible for manuscript writing. RC, LW, ZF, and YG were responsible for manuscript modifying. YM was responsible for the conception and design. ZH was responsible for the final approval of the manuscript. RC was responsible for the figure preparation and manuscript modifying. All authors read and approved the final manuscript.

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Emerging Role of Dermal White Adipose Tissue in Modulating Hair Follicle Development During Aging

Jian Chen†, Zhe-Xiang Fan†, De-Cong Zhu†, Yi-Long Guo, Ke Ye, Damao Dai, Zhi Guo, Zhi-Qi Hu, Yong Miao and Qian Qu**

Department of Plastic and Aesthetic Surgery, Nanfang Hospital of Southern Medical University, Guangzhou, China

Hair follicle stem cells are extensively reprogrammed by the aging process, manifesting as diminished self-renewal and delayed responsiveness to activating cues, orchestrated by both intrinsic microenvironmental and extrinsic macroenvironmental regulators. Dermal white adipose tissue (dWAT) is one of the peripheral tissues directly adjacent to hair follicles (HFs) and acts as a critical macroenvironmental niche of HF. dWAT directly contributes to HF aging by paracrine signal secretion. However, the altered interrelationship between dWAT and HF with aging has not been thoroughly understood. Here, through microdissection, we separated dWAT from the skin of aged mice (18 months) and young mice (2 months) in telogen and depilation-induced anagen for transcriptome comparing. Notably, compared with young dWAT, aberrant inflammatory regulators were recapitulated in aging dWAT in telogen, including substantial overexpressed inflammatory cytokines, matrix metalloproteinases, and prostaglandin members. Nonetheless, with anagen initiation, inflammation programs were mostly abolished in aging dWAT, and instead of which, impaired collagen biosynthesis, angiogenesis, and melanin synthesis were identified. Furthermore, we confirmed the inhibitory effect on hair growth of CXCL1, one of the most significantly upregulated inflammation cytokines in aging dWAT. Besides this, we also identified the under-expressed genes related to Wnt signaling fibroblast growth factor family members and increased BMP signaling in aging dWAT, further unraveling the emerging role of dWAT in aging HFs malfunction. Finally, we proved that relieving inflammation of aging dWAT by injecting high-level veratric acid stimulated HF regenerative behavior in aged mice. Concomitantly, significantly decreased TNF-a, CCL2, IL-5, CSF2, and increased IL10 in dWAT was identified. Overall, the results elaborated on the complex physiological cycling changes of dWAT during aging, providing a basis for the potential regulatory effect of dWAT on aging HFs.

Keywords: aging, hair follicle, dermal white adipose tissue, inflamm-aging, microenvironment

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Mingxing Lei,
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Zhili Deng,
Central South University, China
Ohsang Kwon,
Seoul National University, South Korea

****Correspondence:***
Yong Miao
13265031995@163.com
Qian Qu
15521263230@163.com

†These authors share first authorship

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INTRODUCTION

Progressive deterioration in the regenerative potential of stem cells is a hallmark of aging, which results in the failure to maintain proper tissue homeostasis. Hair follicles (HFs) are independent autonomous stem cell niches and undergo continuous regenerative cycling during their lifespan. With aging, HF have diminished self-renewing capacity, manifesting as cycling defects and poor

responsiveness to activating stimuli. HF cycling slows down (**Figure 1A**) with aging and gradually turns into senescent alopecia. Proper homeostasis between inhibiting signals and activating signals underlies continued HF growth. With aging, inhibitory signals transcend the activatory signals, becoming the dominant environmental factor for HFs (Lei and Chuong, 2016).

The aberrant repressive signalings come from both the intrinsic niche microenvironment and extrinsic macroenvironment. In intrinsic factors, transcription nuclear factor of activated T cells, cytoplasmic 1 (Nfatc1) is a downstream target of BMP signaling, and its upregulation in aging HFs considerably contribute to the HF aging process (Keyes et al., 2013). Besides, fork-head box C1 (Foxc1) (Lay et al., 2016; Wang L. et al., 2016), LIM homeobox 2 (Lhx2) (Folgueras et al., 2013), collagen type XVII alpha 1 (Col17a1) (Matsumura et al., 2016) are all crucial to aging properties of HFs. Regarding macroenvironmental factors, multiple cues from varied tissues are integrated to govern HF maintenance. Surrounding adipose tissues, blood vessels (Mecklenburg et al., 2000; Yano et al., 2001; Xiao et al., 2013), nerves (Brownell et al., 2011; Zhang et al., 2020), adjacent cells [epidermal cells (Doles et al., 2012) and immune cells (Chen et al., 2015; Wang et al., 2019, 2017)] are all involved in the extra-follicular macroenvironment regulators. Among these regulators, dermal white adipose tissue (dWAT) acts as a significant niche supporting HF development and its malfunction is directly responsible for HF aging (Guerrero-Juarez and Plikus, 2018).

Traditionally, adipose tissue performs basic nutritional and metabolic functions in the body. But in the skin, dWAT also plays an important tissue-specific role: it provides essential factors modulating periodic HF growth, responds to skin bacterial infection, and promotes wound healing (Zwick et al., 2018). The tight relationship between dWAT and HF has been established by various mouse models with defects in dWAT. Mice lacking Early B cell factor 1 (Ebf1-/-) lost adipocyte precursors in their skin, resulting in telogen retention and failure in anagen re-entry (Festa et al., 2011). Diacylglycerol acyltransferase (DGAT) 1/2 deficient mice indicated impaired triglyceride metabolism and reduced dWAT, resulting in a destroyed skin barrier and hair loss (Chen et al., 2002; Stone et al., 2004). Peroxisome proliferator-activated receptor-gamma (PPARγ) serves as a dominant regulator of adipocytes, and its ablation leads to total lipoatrophy and temporary postnatal delayed HF morphogenesis (Sardella et al., 2017). In terms of mechanism, it has been proved that during late catagen/early telogen, high levels of BMP2 derived from mature adipocytes in dWAT maintains HF in a quiescent, refractory telogen state (Plikus et al., 2008). Nonetheless, in late competent telogen, adipocyte progenitors produce platelet-derived growth factor subunit-α (PDGFa), directly stimulating a new HF cycle (Plikus et al., 2008).

More importantly, in the aging process of HF, dWAT was also proved to exert noticeable functions. Aging dWAT overexpressed inhibiting signals Bmp2, Dkk1, and Sfrp4 during early anagen but decreased activating signal follistatin during late telogen and early anagen, causing repressive HF cycling behavior in aging mice. After transplanting aging skin to young donor mice, defective HF growth in aging mice was partially recovered under the modulation of young dWAT (Chen et al., 2014). dWAT has a well-established role in HF aging but the prise interplay between it and HF in aging phenotype remained largely unclear. Such being the case, to probe more deeply into the role changes of dWAT during aging, we separated dWAT in telogen/anagen of elderly/young mice for transcriptome analysis and further exploration.

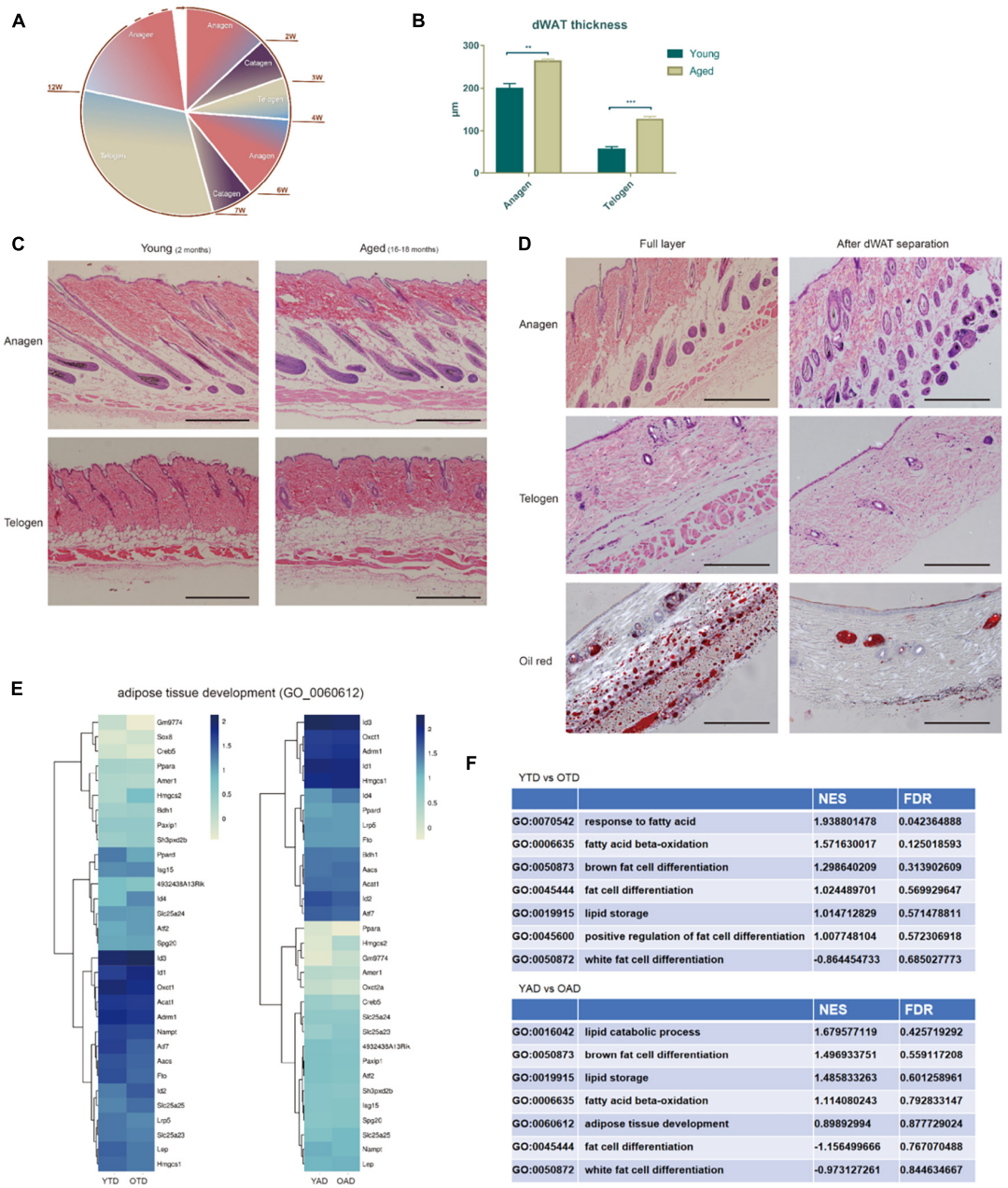
RESULTS

Hair Growth Wanes as Aging Progresses With Dermal White Adipose Tissue Rewiring

Most HFs in old mice are in refractory telogen, which hardly initiates proliferative machinery into action. Moreover, in contrast to synchronous anagen activation in young mice, aged HF wave propagation becomes increasingly asynchronous and fragmented. To interrogate HF cycling-dependent variations between aged (16–18 months) and young (2 months) mice, we induced a new hair growth cycle *via* depilation (hair plucking using a mixture of wax and rosin). As a result, young mice entered anagen within 8–10 days post-depilation, whereas old mice took 12–13 days for anagen entry, characterized by more sparse hairs than in young mice. Upon depilation stimuli, aged HFs exhibit activation more synchronously, mimicking the response fashion of young mice, though over a longer period. This result indicated that the responsiveness of HFs to tissue-regenerating cues were still retained with aging and could be activated by exogenous stimulus.

Dermal white adipose tissue regenerates in parallel with the hair cycle, which preceed adipogenesis in anagen and lipolysis in telogen (Zwick et al., 2018). First, we compared the thickness of dWAT of old and young mice at anagen/telogen. Intriguingly, we found that aging dWAT thickness increased at both anagen and telogen. Especially at telogen, the thickness of aged dWAT is much higher than that of young dWAT (**Figures 1B,C**). The results were different from the traditional view that dWAT thickness decreases with aging. After repeating a many slices we confirmed that the actual thinning tissue in the aging skin was the dermis rather than the dWAT, and the thinner dWAT may be caused by the cutting angle of the HE slice. Coincidentally, we found that Salzer et al. also identified increased thickness of dWAT and thinner dermis in aging mice in 2018 (Salzer et al., 2018). Using Single-cell transcriptomics, they proved that skin fibroblasts acquired an adipogenic profile with aging and transformed into adipogenic lineage cells, resulting in an increase in the number of adipogenic lineage cells and thickened dWAT (Salzer et al., 2018).

Aiming to further explore cycling behavior differences of dWAT between aged and young mice, we processed a transcriptome analysis of dWAT in the telogen/anagen phase of old and young mice. Old mice (18 months) and young mice (2 months) at telogen were selected and were respectively used for new anagen induction *via* hair plucking. Since dWAT is a mix of various types of cells (adipocyte lineage, immune cells,



YTD vs OTD

		NES	FDR
GO:0070542	response to fatty acid	1.938801478	0.042364888
GO:0006635	fatty acid beta-oxidation	1.571630017	0.125018593
GO:0050873	brown fat cell differentiation	1.298640209	0.313902609
GO:0045444	fat cell differentiation	1.024489701	0.569929647
GO:0019915	lipid storage	1.014712829	0.571478811
GO:0045600	positive regulation of fat cell differentiation	1.007748104	0.572306918
GO:0050872	white fat cell differentiation	-0.864454733	0.685027773

YAD vs OAD

		NES	FDR
GO:0016042	lipid catabolic process	1.679577119	0.425719292
GO:0050873	brown fat cell differentiation	1.496933751	0.559117208
GO:0019915	lipid storage	1.485833263	0.601258961
GO:0006635	fatty acid beta-oxidation	1.114080243	0.792833147
GO:0060612	adipose tissue development	0.89892994	0.877729024
GO:0045444	fat cell differentiation	-1.156499666	0.767070488
GO:0050872	white fat cell differentiation	-0.973127261	0.844634667

FIGURE 1 | Aging dWAT undergoes structural remodeling with HF defective growth. **(A)** Decelerated hair cycling with aging. **(B,C)** dWAT thickness significantly increased with aging. Comparing to young mice, the dermis thickness decreased while the dWAT thickness increased at both anagen and telogen in aged mice. $^{**}P < 0.01$, $^{***}P < 0.001$. $n = 10$ **(D)** By microdissection, the subcutaneous adipose tissue and panniculus carnosus were in turn removed, and dWAT was then harvested by slightly scraping from the bottom of HFs. **(E)** Unsupervised clustering of OAD/YAD, OTD/YTD samples based on GO signature—adipose tissue development. No significant difference was indicated between each comparison. Dark blue represents high expression level and yellow represents low expression level. **(F)** GSEA analysis of adipose-related terms all exhibited no significant enrichment. All terms' FDR exceeds 0.05. NES, Normalized Enrichment score. FDR, False discovery rate.

vascular cells, and so on), we chose microdissection to separate complete dWAT from mouse skin. After carefully removing the subcutaneous adipose tissue and panniculus carnosus in turn, dWAT were harvested by slightly scraping from the bottom of HFs (**Figure 1D**). Therefore, four groups were enrolled in this study: OTD (old mice, telogen, dWAT), YTD (young mice, telogen, dWAT), OAD (old mice, anagen, dWAT), YAD (young mice, anagen, dWAT).

Concerning the increased dWAT thickness of aged mice, we speculated whether there was excessive adipogenesis in aging dWAT. Therefore, we then conducted gene ontology (GO) and gene set enrichment analysis (GSEA) of these data. However,

based on the results, we found no significant difference in adipose tissue development between aging and young dWAT at both anagen and telogen (**Figures 1E,F** and **Supplementary Figure 1**). This result is in line with the most recent study by Salzer showing that the thickness of dWAT significantly increasing with aging (Salzer et al., 2018) is mainly due to the replenishment of other transformed adipogenic lineage cells (aging dermal fibroblasts) rather than enhanced development of the original adipose tissue.

Screening Aging-Related Dermal White Adipose Tissue Changes With Hair Follicles Cycling

After performing differential expression analysis for pairwise comparisons—YTD vs OTD and YAD vs OAD, we found that the number of differentially-expressed genes (DEGs) in the YTD vs OTD comparison is much higher than that in YAD vs OAD (856 vs 248, **Figure 2A**). In old mice, HFs are trapped in long-period telogen and seldom initiate anagen spontaneously. In a previous section, we found that aged HF still retained regenerative abilities for anagen induction upon stimuli, underscoring the main difference between aged and young HF was in telogen. In agreement with this, when comparing aged dWAT with young dWAT, more differences were identified in telogen than anagen.

To broadly determine the biological function changes of dWAT with HF cycling during aging, we performed GO enrichment analysis respectively for upregulated and downregulated DEGs of the above comparisons. Consequently, in anagen, transcripts for proteins involved with "collagen fibril organization," "melanin biosynthetic process," "blood vessel development," and "cell adhesion" were the top functional categories of genes decreased in OAD (**Figure 2B**), which was consistent with the GSEA conclusions (**Figure 2C**). In anagen, the expansion of dWAT was accompanied by tissue reconstruction with collagen synthesis (Yamamoto and Yamauchi, 1999), vascularization (Yano et al., 2001), and so on. Nonetheless, aging dWAT exhibited important molecular features of impaired collagen biosynthesis, angiogenesis, and melanin synthesis while in anagen (**Figure 2D**), which indicated great structural and compositional remodeling and a reduced capacity for tissue regeneration in aging dWAT. On the one hand, these discoveries suggested the function degradation of cells in dWAT with aging, and on the other hand, ECM remodeling, in turn, disturbed the homeostasis of cells inside dWAT and neighboring skin cells, such as HF cells.

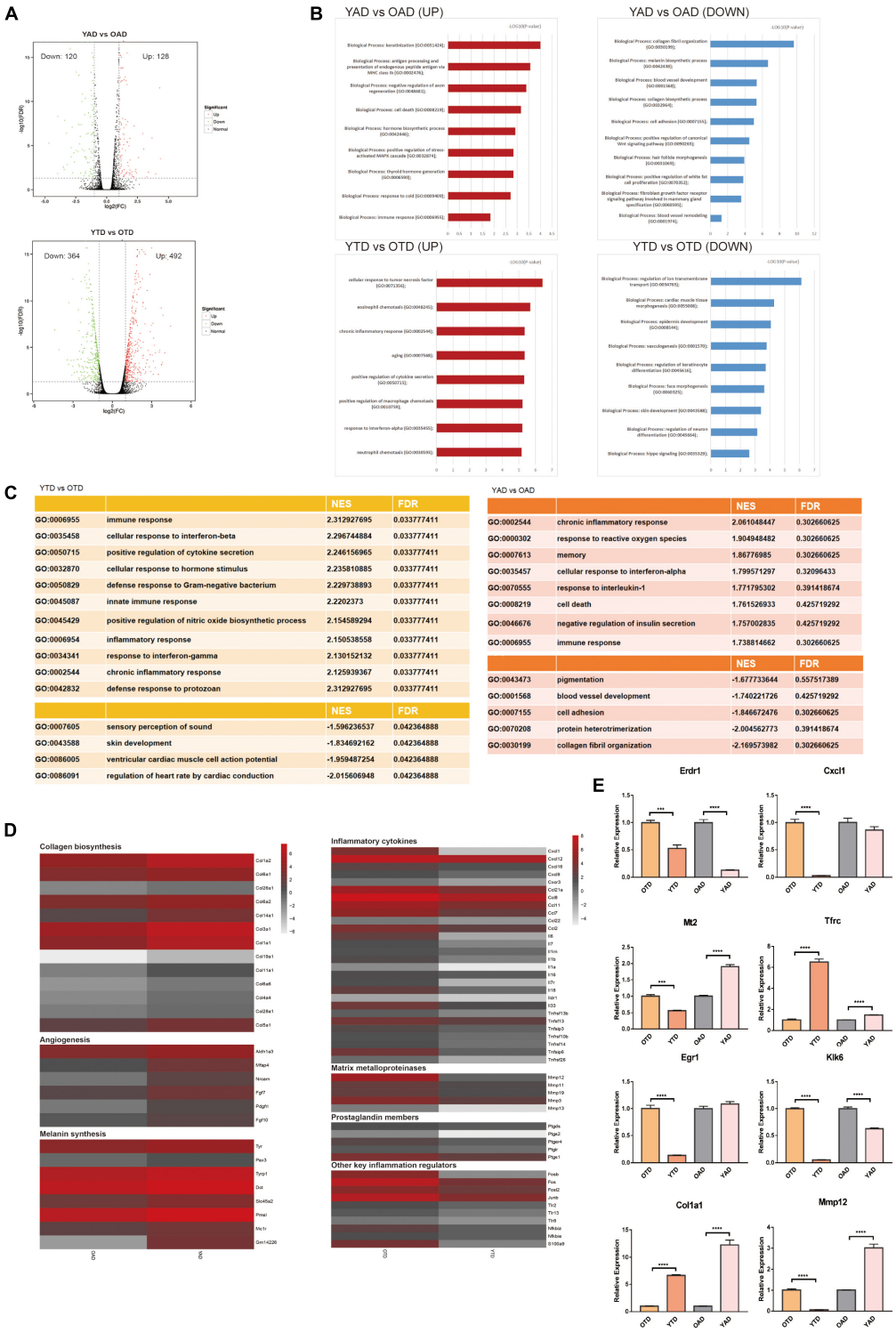
In telogen, the level of inflammation-related genes was massively increased in aged dWAT. The top upregulated biological progress GO terms in YTD vs OTD which were congruously enriched in inflammation activities were: "cellular response to tumor necrosis factor," "eosinophil chemotaxis," "chronic inflammatory response," and "positive regulation of cytokine secretion" (**Figure 2B**). GESA results also proved significant enriched activated inflammation programs in OTD (**Figure 2C**). We therefore concluded that inflammatory infiltration was the most striking feature distinguishing OTD from YTD. Specifically, substantial inflammatory cytokines and genes related to matrix metalloproteinases, prostaglandin biosynthesis, positive immune regulation, and activator protein 1 (AP-1) family were all included in the most predominant upregulated DEGs in OTD (**Figure 2D**).

Besides, normal basic biological functions were also altered in aged dWAT at telogen. Downregulation of "regulation of ion transmembrane transport," "epidermis development," "regulation of keratinocyte differentiation," and "skin development" supported the idea that reprogrammed aged dWAT failed to provide essential growth signals to neighboring skin cells (**Figures 2B,C**). Finally, to validate this RNA-Seq data, differential expression levels of some significant signature genes were further evaluated by RT-qPCR in samples obtained from independent biological replicates (**Figure 2E**).

Aggravated Inflammation of Aging Dermal White Adipose Tissue During Telogen Perturb Tissue Homeostasis

A continuous, low-grade inflammation typical of aging, which has been termed as "inflamm-aging," perpetrates adverse tissue structural and functional remodeling in age-related diseases (Xia et al., 2016; Lu et al., 2021). Comparing with YTD, we found that substantial inflammatory cytokines [such as chemokines, interleukins, and tumor necrosis factor-alpha (TNFalpha)] significantly elevated during telogen in OTD. To further demonstrate the aberrant expression levels of inflammatory regulators in OTD, we selected some important inflammation-related signature DEGs, including CXCL1, MMP12, EGR1, and SPP1, which all were most significantly overexpressed in OTD, to perform immunofluorescence. Obviously, we obtained very similar results showing that these proteins in OTD assume overexpression significantly compared with that in YTD, and their distribution was mainly concentrated in dWAT (**Figures 3A–C**), and which was consistent with the results of qRT-PCR (**Figure 3D**). Furthermore, CXCL1 and MMP12 both indicated high intravascular expression in OTD. Hence, a significantly aggravated inflammation infiltration of aging dWAT during telogen was identified.

Next, using KEGG Pathway enrichment analysis, we found that the TNF signaling pathway was one of the most significant activated pathways in OTD (**Supplementary Figure 2**). In addition to TNF signaling, many chemokines and interleukins were upregulated markedly in OTD. The accumulation of these cytokines would exert chemotactic activities for macrophages (Cx3cl1, Ccl11, Ccl2), neutrophil (Il1b, Cxcl9, Ccl2, Cxcl1), and eosinophil (Ccl8, Ccl11, Ccl2, Ccl7), highlighting the locally advanced inflammation response in aging dWAT during telogen (**Figure 2D**). In obesity, adipocytes produced several chemokines (Cxcl2, Cxcl12, Ccl2, etc.) recruiting macrophage and neutrophils, causing systemic inflammation, insulin resistance, and the dysfunction of local endothelial cells (Gouranton et al., 2011; Rouault et al., 2013; Kim et al., 2014). Therefore we deduced that in aging dWAT, concentrated secretion of inflammatory cytokines in telogen would play a key role in HF disability. To verify the secretion of the above cytokines in OTD, we used a mouse cytokine/chemokine 23-plex array panel. As a result, significantly upregulated protein levels



YTD vs OTD

		NES	FDR
GO:0006955	immune response	2.312927695	0.033777411
GO:0035458	cellular response to interferon-beta	2.296744884	0.033777411
GO:0050715	positive regulation of cytokine secretion	2.246156965	0.033777411
GO:0032870	cellular response to hormone stimulus	2.235810885	0.033777411
GO:0050829	defense response to Gram-negative bacterium	2.229738893	0.033777411
GO:0045087	innate immune response	2.2202373	0.033777411
GO:0045429	positive regulation of nitric oxide biosynthetic process	2.154589294	0.033777411
GO:0006954	inflammatory response	2.150538558	0.033777411
GO:0034341	response to interferon-gamma	2.130152132	0.033777411
GO:0002544	chronic inflammatory response	2.125939367	0.033777411
GO:0042832	defense response to protozoan	2.312927695	0.033777411

		NES	FDR
GO:0007605	sensory perception of sound	-1.596236537	0.042364888
GO:0043588	skin development	-1.834692162	0.042364888
GO:0086005	ventricular cardiac muscle cell action potential	-1.959487254	0.042364888
GO:0086091	regulation of heart rate by cardiac conduction	-2.015606948	0.042364888

YAD vs OAD

		NES	FDR
GO:0002544	chronic inflammatory response	2.061048447	0.302660625
GO:0000302	response to reactive oxygen species	1.904948482	0.302660625
GO:0007613	memory	1.86776985	0.302660625
GO:0035457	cellular response to interferon-alpha	1.799571297	0.32096433
GO:0070555	response to interleukin-1	1.771795302	0.391418674
GO:0008219	cell death	1.761526933	0.425719292
GO:0046676	negative regulation of insulin secretion	1.757002835	0.425719292
GO:0006955	immune response	1.738814662	0.302660625

		NES	FDR
GO:0043473	pigmentation	-1.677733644	0.557517389
GO:0001568	blood vessel development	-1.740221726	0.425719292
GO:0007155	cell adhesion	-1.846672476	0.302660625
GO:0070208	protein heterotrimerization	-2.004562773	0.391418674
GO:0030199	collagen fibril organization	-2.169573982	0.302660625

FIGURE 2 | Overview of dWAT alterations with aging at anagen and telogen. **(A)** Volcano Plots of DEGs in YTD vs OTD and YAD vs OAD. **(B)** GO enrichment analysis respectively for upregulated and downregulated DEGs in YTD vs OTD and YAD vs OAD. **(C)** GSEA analysis indicated the most significantly enriched terms in YTD vs OTD and YAD vs OAD. However, in YAD vs OAD, all terms' FDR exceeds 0.05, which only represents general trends. **(D)** Heat map of genes related to collagen biosynthesis, angiogenesis, and melanin synthesis with significant changes in YAD vs OAD, and inflammation-related genes with significant changes in YTD vs OTD. Genes expressed at low levels are in gray, and genes expressed at high levels are in red. **(E)** qRT-PCR validation of selected significant DEGs (Klk6 and Erdr1are significant UP_DEGs and Col1a1and Mt2 are significant DOWN_DEGs in YAD vs OAD; Cxcl1, Egr1, Mmp12 are significant UP_DEGs and Tfrc is significant DOWN_DEG in YTD vs OTD). For each experimental data point, $n = 8$. $^{***}P < 0.001$, and $^{****}P < 0.0001$.

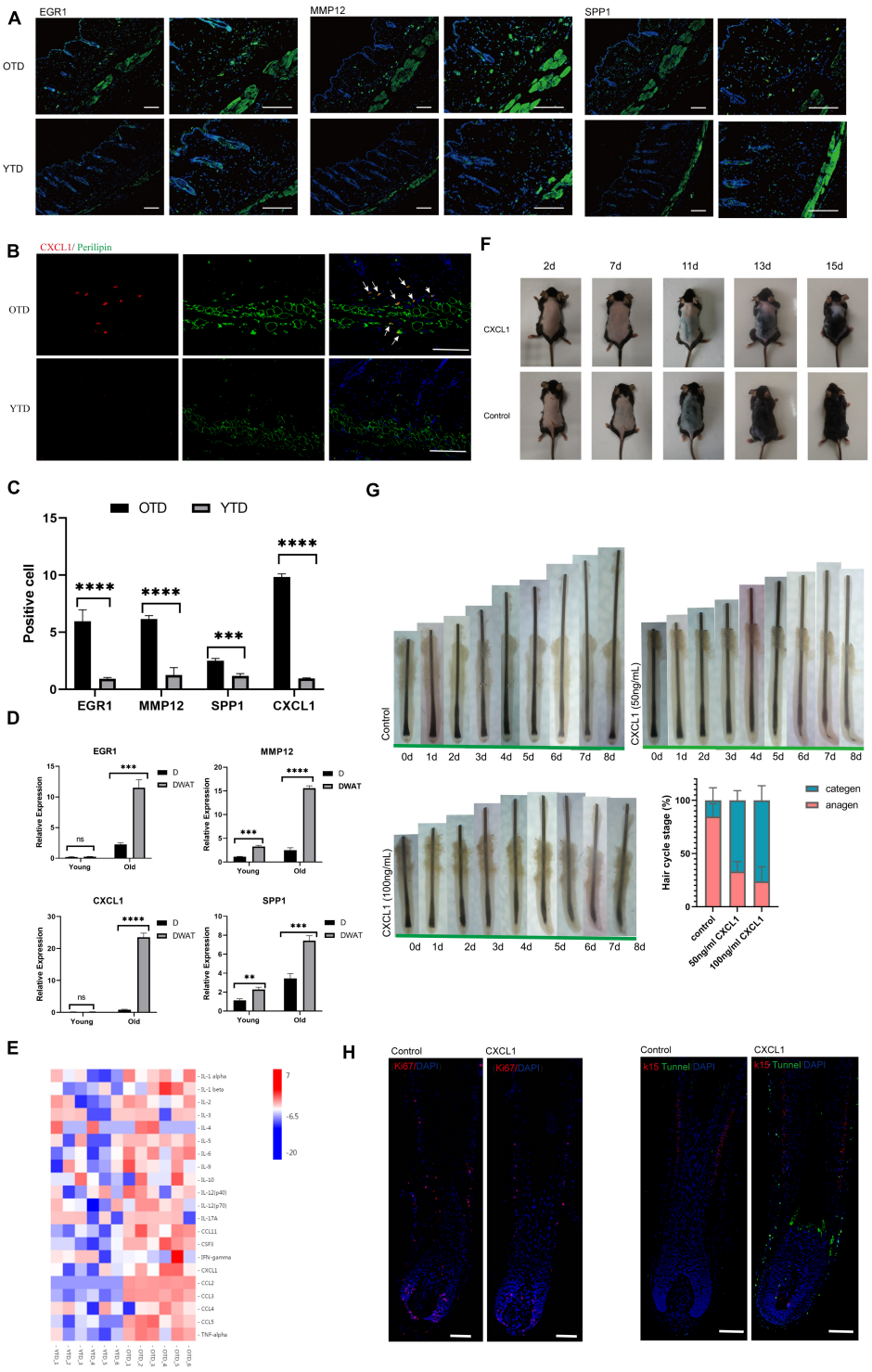


FIGURE 3 | Aberrant inflammatory infiltration in aging dWAT during telogen. **(A–C)** Immunostaining images showed enhanced expression of crucial inflammation regulators EGR1, CXCL1, MMP12, SPP1 in OTD. Their distribution was mainly concentrated in dWAT. CXCL1 co-located with perilipin, a marker of mature adipocytes. Scale bars = 100 μm. **(D)** The relative mRNA expression level of the differential genes EGR1, CXCL1, MMP12, SPP1 in dermal tissues and dWAT. $n = 8$. **(E)** Significant upregulated protein levels of 21 inflammatory cytokines were found in OTD by a mouse cytokine/chemokine 23-plex array panel. $n = 6$. **(F)** CXCL1 injection resulted in delayed HF activation. **(G)** Organ culture assays of hair follicle. Human isolated hair follicles cultured with CXCL1 for 8 days in 24 well plates and histomorphometric analysis of hair follicle stages after organ cultured 4 days. Addition of CXCL1 50 and 100 ng/mL promoted hair follicle entry categen. For each experimental data point, $n = 12$. **(H)** Immunofluorescence of Day 3 cultured hair follicle showing apoptosis and decreased proliferation signaling appeared significantly in the CXCL1 group. K15, a specific marker of HFSC, was co-stained with apoptosis signal using tunnel test kit as well. Scale bars = 200 μm. $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

of 21 cytokines were found in OTD, including TNFa, CXCL1, CCL2, and interleukins (**Figure 3E**), highlighting the enhanced production of these inflammatory cytokines in OTD and their critical role in HF modulation.

Previous studies proved an age-associated increase of inflammatory cytokines derived from the epidermal compartment contributed to aging phenotypes of HFSCs (increased number, impaired function, and inability to tolerate stress) (Doles et al., 2012). Likewise, it was plausible that the inflammation signaling of aged dWAT during telogen would inevitably destabilize HFSCs maintenance. We have already verified the massively increased secretion of inflammatory cytokines in OTD, and here we further summarized specific functional mechanisms of these known inflammatory mediators during HF development in **Table 1**, which suggests various possible negative regulation mechanisms of inflammatory factors on HFs.

Furthermore, we found that CXCL1 was one of the top significant upregulated DEG in OTD, and its overexpression in aging dWAT was further confirmed by immunofluorescence and cytokine array panel. CXCL1 co-localized with perilipin, a marker of mature adipocyte, in dWAT (**Figure 3B**). To investigate whether CXCL1 evokes alopecia, C57 mice received an intradermal injection (500 ng in 500 μl) every other day for a total of five times. Observably, the CXCL1 group indicated a delayed telogen-anagen transition (**Figure 3F**). In order to identify that CXCL1 had direct inhibitory effects on hair growth, we performed organ culture assays with isolated human hair follicles in the presence of CXCL1 (50ng/mL and 100ng/mL) or PBS. As a result, CXCL1 inhibited hair growth and promoted hair categen entry *in vitro* (**Figures 3E,F**). CXCL1 induced most hair follicles started to enter categen in the third day at 50 ng/mL or in the second day at 100 ng/mL, while most hair follicles in the control group were still in anagen status until the eighth day (**Figure 3G**). Using immunofluorescence, we also found that apoptosis in the CXCL1 group rose significantly in the hair matrix and connective tissue sheath, in addition, CXCL1 decreased the Ki67 signaling (**Figure 3H**). These results suggested that CXCL1 could promote cell apoptosis and prevent the cells' proliferation of hair follicles. The inhibition function of CXCL1 on HFs underlined its negative role in the process of aging dWAT regulating HFs. Thus, we inferred that infiltrated inflammation factors of aging dWAT played an inhibitory role together, resulting in the hindrance of HF growth.

Wnt, FGF, and BMP Signaling Altered in Aging Adipogenic Macroenvironment

It is well known that dWAT can secrete some critical regulator molecules that participate in the regulation of HF homeostasis, including BMPs (Plikus et al., 2008), PDGF (Festa et al., 2011), DKK1 and Sfrp4 (Chen et al., 2014), and HGF (Nicu et al., 2021). Here, in addition to the identified inflamm-aging of dWAT, we wondered if other essential signalings supporting HF growth were also extensively reprogrammed. Therefore, we analyzed and summarized the expression level of the regulatory signal molecules of HF. Consequently, we found, Wnt signaling pathway, FGFs, as well as the BMP signaling pathway all indicated abnormal alterations in aging dWAT (**Figure 4A**).

Canonical WNT signaling was one of the most crucial regulators of dWAT and HF development. In the data, repressed WNT signaling in both anagen and telogen of aging dWAT was reported. Active WNT signaling was a vital step for HF to initiate new anagen (Greco et al., 2009). Comparing with YAD, obvious downregulation of WNT10b and Wnt8a, and upregulation of DKK2 indicated inhibited WNT signaling in OAD. In accordance with anagen, though with a lower expression level, WNT signaling of telogen also downregulates in OTD relative to YTD, including downregulation of WNT3, WNT3a, and LEF1, and upregulation of WNT inhibitors DKK3 and Sfrp4 (**Figure 4A**). Sfrp4 has been proven previously to be upregulated in aging dWAT, leading to HF regeneration retention (Chen et al., 2014). All of these results underscored an age-dependent repression of WNT signaling in dWAT, which might be partially responsible for the diminished self-renewing capacity of aging HF.

Furthermore, we noted a significant change in FGFs in aging dWAT, especially during anagen (**Figure 4A**). FGF5, an essential inhibitor of HF growth, which induced HF regression and promoted the transition from anagen to catagen (Hebert et al., 1994; Higgins et al., 2014), now indicated elevated expression in aging dWAT during anagen, which may contribute to the retarded HF growth. Additionally, downregulation of FGF7 and FGF10 was detected in OAD as well. As major HF regulators, FGF7 and FGF10 peaked at anagen V, playing a crucial role in stimulating HF development (Kawano et al., 2005; Iino et al., 2007). Taken together, the results presented showed that the aging dWAT secreted an increased level of inhibitory FGF signal, FGF5, and a decreased level of positive FGF signal, FGF10, and FGF7, which makes it difficult for aging HFs to activate and enter anagen.

Besides, it was well established that enhanced BMP2 expression of adipocytes resulted in a prolonged refractory telogen (Plikus et al., 2008). In our results, BMP2 also increased in both anagen and telogen, but not at significantly elevated levels (**Figure 4A**). Besides, BMP4, which was also released by dWAT and had an unfavorable effect on HF growth, indicated upregulation in OTD. Likewise, Noggin, a typical antagonist of BMP signaling, was also downregulated in OTD. Finally, we conducted immunofluorescence and proved the overexpression of BMP2 and FGF5 in aging dWAT, which may contribute to the impaired cycling behavior in aging mice (**Figure 4B**).

Veratric Acid Facilitates Aging HF Regrowth by Mitigating Dermal White Adipose Tissue Inflammation

Collectively, based on the bioinformatics analysis, the current results unveiled aggravated inflammation and abnormal expression levels of HF regulatory signals of aging dWAT, all of which may play a negative role in the control of aging HF homeostasis. Here in the study, we focused on the dWAT inflammation. Then we ascertained whether the inflammation relief of aging dWAT would alleviate the aging HF retardation

TABLE 1 | | Functional mechanisms of known inflammatory mediators in HF development and specific expression patterns in aging dWAT.

Cytokines	Properties related to HF	References	Expression patterns in dWAT_FPKM		
			Gene	OTD	YTD
TNF-a	Have significant positive association with AA; is required for normal cell death of HF and anagen-catagen transition in mice; Injection of TNF-a leads to apoptosis of HF bulb matrix keratinocytes in mice. TNF-a also inhibits HF elongation in a dose-dependent manner. K14-TNF alpha transgenic mice exerted impaired HF growth.	Soma et al., 1998; Ruckert et al., 2000	Tnfaip6	9.95549	1.71115
			Tnfrsf13b	1.87504	0.630668
			Tnfaip8l2	7.895	5.12489
			Tnfsf13	11.0502	5.9425
			Tnfaip3	3.83357	1.64293
			Tnfrsf10b	2.64808	1.14413
			Tnfrsf11a	2.01242	1.15726
			Tnfrsf1b	10.7332	6.5328
			Tnfsf10	9.14176	5.90121
			Tnfrsf14	2.82864	1.09449
			Tnfsf12	39.4577	29.5722
			Tnfrsf26	1.20813	0.199513
IL-6	IL-6 KO mice resulted in STAT3 pathway activation and enhanced wound-induced hair neogenesis; DP cells secreted IL-6 in response to dihydrotestosterone.		Il6st	111.495	72.9043
			Il6	6.19491	0.224645
			Il6ra	7.08773	5.37911
IL-1a/b	Act as a crucial mediator inducing HF regression. Upregulation of IL1 leads to diminished and atrophic hair follicles; IL-1a and IL-1b inhibit HF growth in organ culture; IL1 transgenic mice were characterized by hair loss and focal skin inflammation.		Il1a	0.514973	0.0371823
			Il1b	3.36946	0.739462
			Il1rap	2.6094	2.00408
			Il1r1	11.1733	7.76998
			Il1r2	9.15598	6.54273
			Il1rn	2.50242	1.32742
			Il1rl2	5.44209	3.64348
IL16	Its polymorphisms may play a role in AA;		Il16	3.20473	1.97227
IL-7	IL-7 derived from HF are required for homeostasis of CD4(+) and CD8(+) skin-resident memory T cells		Il7	2.87597	0.598882
			Il7r	2.72528	0.162671
IFN-gamma	An important inducer of catagen in HF; its upregulation can result in the collapse of immune privilege of HF		Ifnar2	36.3087	23.0931
			Ifngr1	55.3079	40.3651
Cxcr3	Overexpressed on alopecic effector T cells and its ablation prevents AA onset; Deficiency in cxcR3 impaired the patterning of primary hair placodes.		Cxcr3	1.09397	0.289984
CXCL9/10/11	Significantly upregulated in AA lesions and promoted AA progression.		Cxcl9	2.91259	1.33173
			Cxcl10	7.33465	4.84593
			Cxcl11	0.0975968	0.0488607
Cxcl1/2	Cxcl1/2 are associated with AA susceptibility		Cxcl1	15.1405	0.108917
			Cxcl2	0.781783	0.158399
Ccl2	Plucking HFs released Ccl2 to signal to neighboring unplucked HFs, activating HFSCs for regeneration; the isthmus of HF expressed CCL2 to promote the recruitment of Langerhans cells into skin	Chen et al., 2015	Ccl2	15.5977	5.95203
CCL8	HF bugle region produced CCL8 which inhibit the recruitment of Langerhans cells into the skin		Ccl8	269.611	87.7888

in telogen. We chose veratric acid (VA), a major benzoic acid derivative from plants and fruits, which has been proven to exhibit antibacterial, anti-inflammatory, and anti-oxidant activities (Choi et al., 2012; Shin et al., 2013; Ran et al., 2014), to perform intraperitoneal injection in aged mice. Finally, after 30 days, we noted that the gross appearance of hairs of the VA group became thicker, darker, and more lustrous than that of the control group (**Figure 5A**). Moreover, more anagen HFs in the VA group were identified based on histological H&E, corroborating activated HF growth (**Figure 5A**). Quantitative analysis showed that VA treatment increased the hair weight of old mice, and had no significant effect on young mice (**Figure 5B**). Meanwhile, increased Ki67 signaling was identified in the VA group (**Figures 5C,E**). Altogether, VA treatment can effectively facilitate aging HF regrowth. To further confirm whether VA reduced the inflammatory factor level of dWAT, the cytokine/chemokine 23-plex array panel was used for the test. The results further showed that the inflammatory cytokines were significantly reduced in the dWAT of the VA group, including TNF-a, CCL2, and IL-5. CSF2 and IL10 were increased (**Figure 5D**). However, the level of CXCL1 showed no obvious change (data not shown). There is some research confirming that VA could inhibit the generation of some inflammation cytokines, including interleukin-6 (IL-6), interleukin-8(IL-8), and interferon-γ (IFN-γ) (Choi et al., 2012; Shin et al., 2013; Wang Q. B. et al., 2016); so it is plausible that VA mainly downregulated the level of TNF-a, CCL2, and IL-5. CSF2 and upregulated the level of IL10 in aging dWAT, relieving the negative effect of these inflammatory factors on aging HFs and boosting HF regrowth.

We also tested the expression level of essential signalings for HF growth. Consequently, BMP signaling (BMP2/4) has no change after VA treatment (data not shown), but the enhanced level of β-catenin in HFs and decreased level of FGF5 of

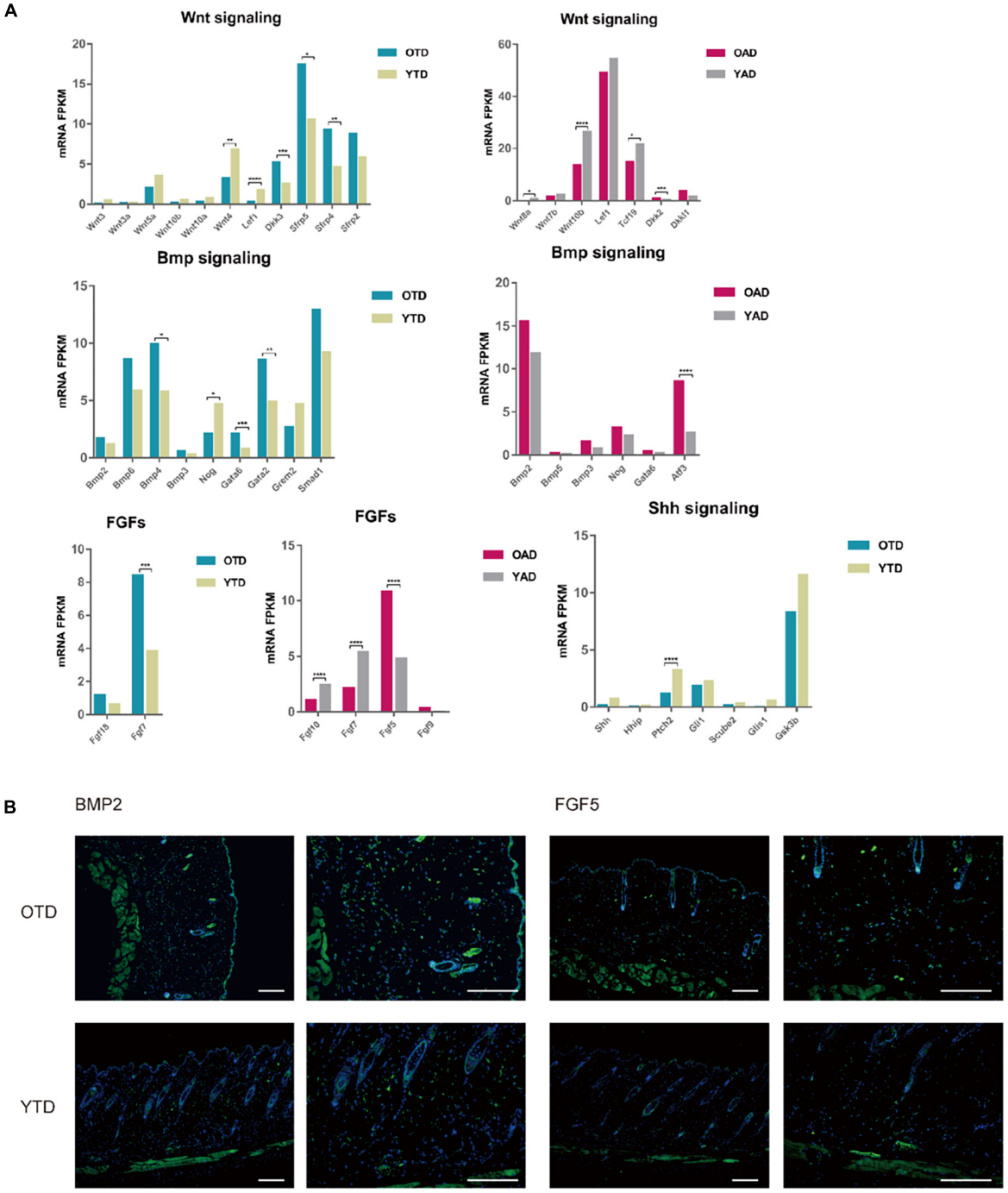


FIGURE 4 | Aging dWAT negatively modulated HF behaviors. **(A)** Essential growth signaling for HF development indicated abnormal expressions in aging dWAT, including WNT, BMP, and FGFs. SHH-related signaling showed little changes. **(B)** BMP2 and FGF5 indicated overexpression in aging dWAT. Scale bars = 100 μm. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

HFs and dWAT were proven (**Figures 5E,F**). Therefore, these findings suggested that the anti-inflammation effect of VA in dWAT was capable of finally attenuating the aging HFs dysfunction by activating β-catenin signaling and repressing the level of FGF5.

DISCUSSION

The local microenvironment plays a dominant role in determining cell behaviors. As an indispensable soil for HF growth, dWAT is closely bound up with HF homeostasis.

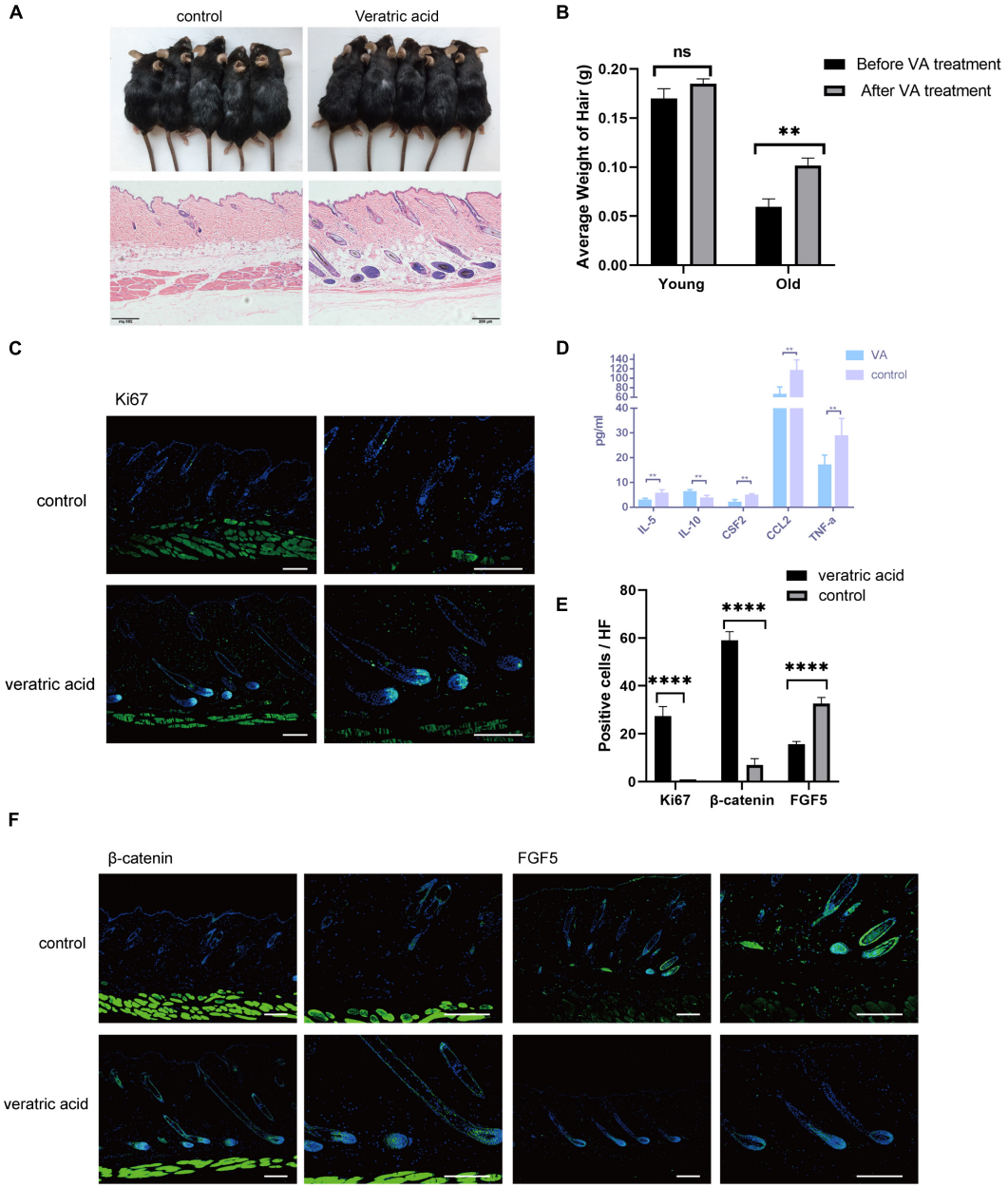


FIGURE 5 | VA injection stimulated HF regrowth by lowering dWAT inflammation. **(A)** VA injection stimulated HF regenerative behavior in aged mice. **(B)** The weight of the back skin hair in the old mice after VA treatment was significantly heavier than that before VA treatment, while no difference was found in young mice. $n = 10$. **(C)** Increased ki67 signaling was identified in the VA group. Scale bars = 100 μm. **(D)** Decreased levels of TNF-a, CCL2, IL-5, CSF2, and increased IL-10 were proved in dWAT after VA treatment. **(E)** Quantification of positive cells in each hair follicle about ki67, β-catenin, and FGF5. **(F)** Increased β-catenin signaling and reduced FGF5 level were identified in the VA group. Scale bars = 100 μm. $n = 6$. ns, Not Significant. $^{**}P < 0.01$, and $^{***}P < 0.0001$.

Interpreting the interaction alterations between dWAT and HFs with aging is not only essential for understanding the involved aging mechanisms but also supply a promising therapeutic approach of senescent alopecia from the angle of dWAT targeting treatment.

Previous work has demonstrated that aged progenitor cells could be rejuvenated by exposure to a young systemic environment by establishing parabiotic pairings between young and aged mice (Conboy et al., 2005). In contrast, the regenerative potential of the progenitor cells of young animals was remarkably impaired after exposure to aging systemic milieu (Conboy et al., 2005). Furthermore, autologous skin graft transplantation experiments also proved that a young donor dWAT microenvironment could partially restore the regenerative cycling behavior of aged HFs (Plikus et al., 2008; Chen et al., 2014). In this study, we found that aging

dWAT renovated tissue microenvironment, manifesting as inflammatory infiltration, ECM degrading, impaired collagen biosynthesis, angiogenesis, and melanin synthesis. This was accompanied by altered essential signalings (Wnt, Bmp) and growth factors (FGFs) for HF regulation. All these aging phenotypes reflect the function deterioration and tissue reconstruction of aging dWAT, and meanwhile, these altered factors are integrated to determine aging HF cell development (**Figure 6**).

In our profiling, plentiful collagen-related genes (Col1a1, Col3a1, Col6a6, Col5a1, Col4a4, etc.) occupied a large proportion of the decreased DEGs of OAD vs YAD. Collagen synthesis was mainly coupled to the anagen stage of the hair cycle (Plikus et al., 2008; Chen et al., 2014). Correspondingly, decreased expression of collagen-related genes in OAD indicated impaired the ability of collagen secretion with aging. Similarly, dermal vascularity increased with anagen and regressed during categen and telogen, which was orchestrated by HFs (Mecklenburg et al., 2000; Stenn and Paus, 2001). Perifollicular vascularization offered optimal growth nutrients facilitating HF growth and was positively related to HF size (Yano et al., 2001; Ozeki and Tabata, 2002). Deficiency of vascularization was also an important contributory factor of androgenetic alopecia (Chew et al., 2016). Impaired angiogenesis was tightly associated with the development of many age-related disorders (Ungvari et al., 2013; Roca et al., 2014) and also involved in OAD. Besides, decreased expression of key genes related to melanin synthesis in OAD was also identified, including Pmel, Mc1r, Slc45a2, and other melanocyte linage markers. This result might have two explanations: (1) adipose tissue had the function of melanin production and generated more melanin in obesity-related inflammation (Randhawa et al., 2009). The antioxidant and

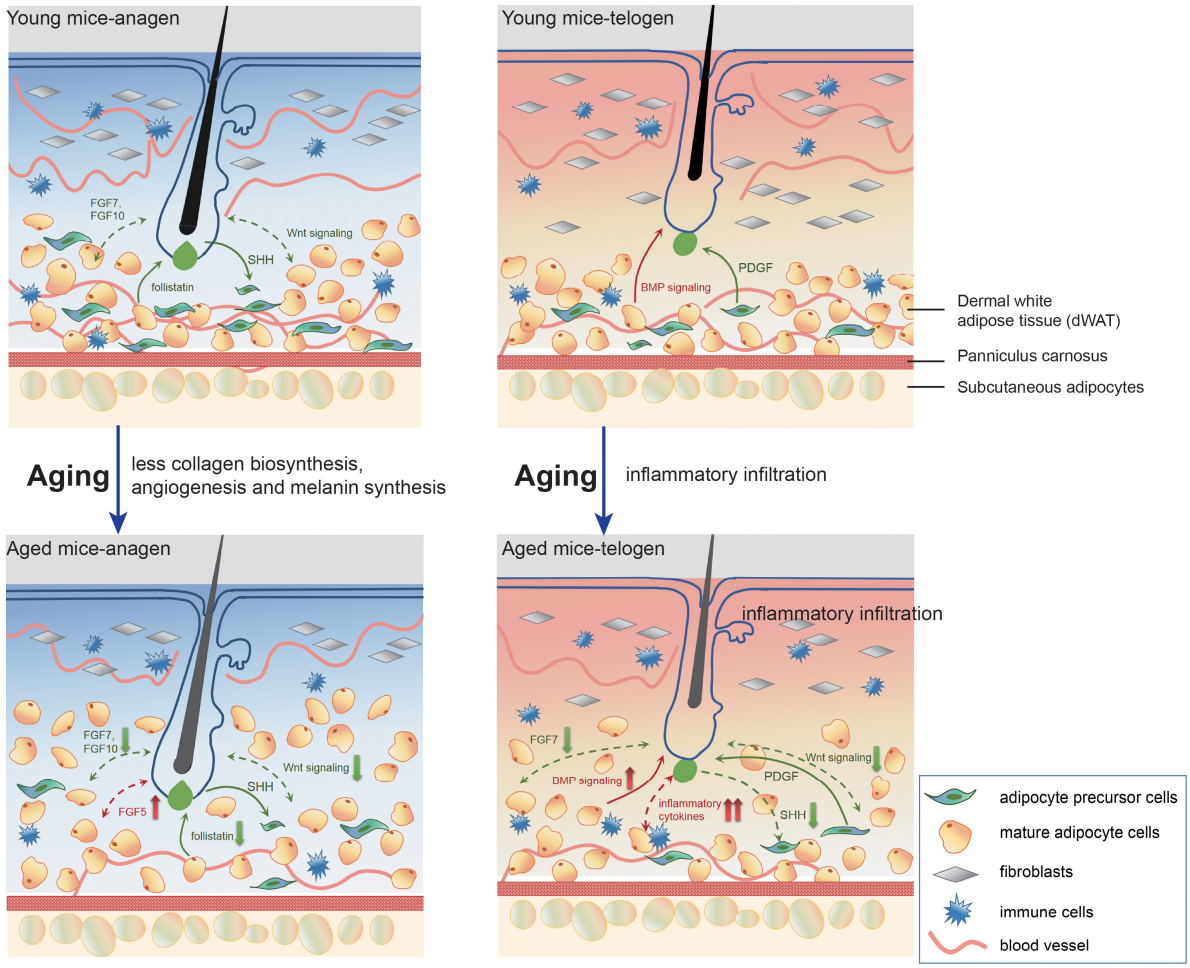


FIGURE 6 | A model of pathological interrelationship alterations between dWAT and HF with aging. With aging, dWAT undergoes structural remodeling: the thickness increases with the replenishment of other transformed adipogenic lineage cells (aging dermal fibroblasts) (Salzer et al., 2018). Meanwhile, during anagen, the impaired ability of paracrine signals secretion of aging cells in dWAT resulted in deficient collagen biosynthesis, angiogenesis, and melanin synthesis. Besides, crucial regulators for HF development also indicated abnormal expression: including follistatin (Chen et al., 2014), WNT signalings, and FGFs. During telogen, remarkably, aberrant inflammation recapitulated aging dWAT, which caused a serious bottleneck for HF cycling. Concomitantly, altered BMP, WNT signalings, and FGFs also underscored the emerging role of dWAT in aging HFs malfunction. The green arrows represent positive effects and the red one represents negative effects. The solid and dashed lines denote published results and our results respectively.

anti-inflammatory properties of melanin supported a hypothesis that melanogenesis plays an important role in abating oxidative stress and inflammation of adipose tissue (Randhawa et al., 2009). Here we speculated that aging weakened the melanogenesis of dWAT, causing increased oxidative stress and inflammation; (2) HF melanogenesis was strictly coupled to anagen, ceased during categen, and was absent in telogen (Slominski et al., 1994). Impaired melanogenesis here might also come from the unavoidable mixed hair bulbs in the isolated tissues and was in agreement with the extensively reduced melanin production of graying HFs. Collectively, all these phenomena could be attributed to the decrease in paracrine signals secretion of aging cells in dWAT. It is noticeable, however, there is not a one-way communication between HF and dWAT. Aging HFs might also have significant implications on the defective performance of aging dWAT, which is worth deeper exploration.

The most prominent pathological characteristic of aging dWAT was aberrant inflammatory infiltration in telogen. We have identified increased expression levels of substantial inflammatory cytokines, TNFa, CXCL1, CCL2, interleukins, as well as significant inflammation regulators, including MMP12, EGR1, and SPP1 in OTD. Moreover, we found that compared to the epidermis and dermis layer, these inflammation regulators are mainly concentrated in dWAT. It raised the tantalizing possibility that in aging dWAT, continuous production of inflammatory cytokines caught HF in prolonged telogen and suppressed HF cycling behavior. Among these inflammation regulators, heightened TNF production was an essential part of the inflammatory microenvironment of aging diseases (Slominski et al., 1994). The organ culture system has confirmed that TNFalpha markedly inhibited HF elongation in a concentration-dependent manner, resulting in morphological abnormalities and bulb matrix cells apoptosis (Soma et al., 1998; Ruckert et al., 2000). Here we focused on CXCL1, one of the top DEGs in OTD, which showed a significantly high intravascular expression in aging dWAT. Using an *in vivo* mouse model, we proved that intradermal injection of CXCL1 delayed HF cycling behavior. Meanwhile, organ culture with CXCL1 revealed similar results. In the data, we noted that the level of CXCL1 did not downregulate in anagen (**Figure 2E**). Nonetheless, its negative role was not significant at anagen. There may be two reasons. One is because the expression level of other activation signals exceeds the level of CXCL1, so the HFs are activated. Another reason may be because the anagen in our study was induced by depilation, which may be accompanied by upregulation of inflammatory factors activating HFs to enter anagen (Chen et al., 2015). Furthermore, the CXCL1 in OAD may be released by the plucked hair and may play an inductive role in anagen initiation rather than an inhibitory role. Overall, our data support a scenario in which overexpression of CXCL1 during telogen played a detrimental role in aging HF growth, trapping HF in telogen. Similarly, other inflammatory cytokines may also contribute to the aging HF retardation. We deduced that the synergistic effect of various inflammation regulators in dWAT triggered the dysfunctional growth of HFs.

Dermal white adipose tissue serves as an integral part of the skin and its stimulated inflammatory response in telogen with aging may result from the local microenvironment of the skin or systemic inflammation. In our study, we do not explore the detailed source of inflammation. From our perspective, the primary factor propelling basic aging processes was senescent cells in dWAT. Genes related to matrix metalloproteinases (MMPs), prostaglandin biosynthesis, positive immune regulators were included in the most predominant upregulated DEGs in OTD. With aging, senescent cells can secrete a complex combination of factors, including diverse cytokines, chemokines, growth factors, MMPs, and lipids, and this was referred to as the senescence-associated secretory phenotype (SASP) (Herranz and Gil, 2018; Di Micco et al., 2021), which is consistent with the phenotype of aging dWAT revealed by sequencing data. A new study has proven transplanting senescent adipose cells into young mice caused persistent physical dysfunction, including tissue inflammation (Xu et al., 2018). Our findings coincided with the possible contribution of SASP of the senescent cells to the inflamm-aging of dWAT. Therefore, like CXCL1, it can be inferred that the overexpression of these inflammation factors may be the consequence of SASP of dWAT. Meanwhile, the secretion of cytokines will recruit macrophages and may further secrete MMPs to degrade ECM. Besides, the degraded ECM also leads to the abnormal expression of inflammation factors. There are studies confirming that ECM can affect the secretion and expression of CXCL1 (Wang et al., 2018). So in conclusion, it can be inferred that both the overexpression of inflammation factors and ECM remodeling in aging dWAT are the result of changes in the microenvironment caused by aging, and the interplay of them further reinforced each other. Therefore, senescent cells, which are the source of SASP, are the most important targets. Recently, emerging studies supported selective elimination of senescent cells as a crucial therapeutic method for treating degenerative aging disorders (Baar et al., 2017; Childs et al., 2017; Jeon et al., 2017; Xu et al., 2018). These conclusions also indicated promising therapeutic approaches to ameliorate aging phenotype in dWAT.

With aging, a previous study proved that adipocyte cells overexpressed Bmp2, Dkk1, and Sfrp4, resulting in defective HF regeneration (Chen et al., 2014). Likewise, our results also identified aberrant expression of Bmp signaling and repressed Wnt signaling in aging dWAT in both anagen and telogen. Besides, some vital growth factors (FGFs) for HFs also downregulated in aging dWAT, highlighting the important contribution of the paracrine factors of dWAT to the diminished self-renewal of aging HFs. These aberrant signalings could serve as targets for the regulation of HF aging. However, since that the role of aggravated inflammation of aging dWAT on aging HFs has not been identified, we try to trigger HFs activation by temporarily lessening the inflammation activity. After high-level VA injection in aged mice, defective HF growth in aged mice was partially rescued, along with a significant downregulation of TNF-a, CCL2, IL-5, CSF2, and upregulation of IL10 in dWAT, further underscoring that targeting dWAT inflammation could activate aging HF cycling behavior. Moreover, VA treatment also activated β-catenin signaling and downregulated FGF5 expression level, but had no effect on BMP signaling. Altogether, VA injection alleviated the inflammation level of aging dWAT inflammation, interacting with other downstream signaling for HF growth, and finally induced aging HF regrowth.

Our study provides a detailed and direct portrait of dWAT alterations with aging and identifies its potential regulatory effect on aging HF. The most dominant feature of aging dWAT distinguishing young dWAT was massive inflammatory infiltration during telogen, which inevitably brought out deleterious influence and might be the core factor retarding HF regeneration. Moreover, reducing dWAT inflammation was capable to encourage HF cycling in aged mice. Nowadays, the crucial role of dWAT in HF regulation has been attracting more and more attention. Based on our study, future mechanistic work is needed for delineating the exact interplay between dWAT and HF with aging, which may provide new avenues for developing therapeutic agents to stimulate aging HF growth.

MATERIALS AND METHODS

Ethics Approval and Consent to Participate

All experiments reviewed and approved by The Ethics Committee of Nanfang Hospital, Southern Medical University.

Mice

To perform the aging study, young (2 months at telogen), and aged mice (16–18 months at telogen) C57BL/6 were utilized in this study. Mice were all provided by the Experimental Animal Center at Southern Medical University (Guangzhou, China). The experimental procedures were performed with protocols approved by the Institutional Animal Care and Use Committee.

Sample Preparation for Transcriptome Sequencing

Using microdissection to separate dWAT tissue from skin. DWAT harvest from 20 young mice (2 months) and 20 aged mice (18 months) at telogen and 20 young mice (2 months) and 20 aged mice (18 months) at depilation-induced anagen were used for the transcriptome sequencing. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, United States) and the RNA quality and quantity were evaluated by using Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent, CA, United States) with RIN number > 7.5. Qualified RNAs were used for further transcriptome sequencing analysis.

RNA Library Construction and Sequencing

The library preparations were sequenced on an Illumina Hiseq 2500 platform and the details of library construction and sequencing are the same as in our previous study (Miao et al., 2018).

Hair Follicle Organ Culture

Hair follicles in the anagen VI stage from normal human scalp skin in 12 persons were isolated as previously described (Philpott, 1999). Then the hair follicles were cultured for 8 days in Williams E medium supplemented with 2 mmol/L L-glutamine, 10 ng/mL hydrocortisone, 10 lg/mL insulin, and an antibiotic/antimycotic mixture at 37°C in 5% CO2 and 95% air in a humidified incubator as described previously (Philpott et al., 1994). To explore the role of CXCL1 in hair growth, the media were supplemented with recombinant human CXCL1 (Proteintech) at day 0. The normal medium was used as a control.

Quantitative PCR Analysis

For qPCR validation of DEGs, we used another eight young mice (2 months) and eight aged mice (18 months) at telogen and depilation-induced anagen. RNA was prepared using the cDNA Reverse Transcription Kit (Promega, A5001, Madison, WI, United States). Aliquots of RNA were analyzed in triplicate by using qRT-PCR performed according to standard protocols by ABI Step One Plus system (Applied Biosystems, Foster, CA, United States) by GoTaq® qPCR Master Mix (Promega, A6001, Madison, WI, United States). The melting curve was performed to verify the specificity of PCR amplification. RNA was normalized to expression levels of GAPDH. All data were analyzed by unpaired Student *t* test and were presented as the mean ± standard error. The Primer Sequences of qRT-PCR are as follow:

Mus-GAPDH-F	ACCCCCAATG TGTCCGTCGT	Mus-Klk6-QF	AATGTAGTGACAC AGAGCACAGAAC
Mus-GAPDH-R	AGCCCAAGATG CCCTTCAGTGG	Mus-Klk6-QR	AACCACACCCA TAGAGTAGGATTG
Mus-Cxcl1-QF	CAAGTAACGGA GAAAGAAGACAGAC	Mus-Mmp12-QF	CAAAGTCAATAA TGTACCCCACC
Mus-Cxcl1-QR	AGGACCCTCAAA AGAAATTGTATAG	Mus-Mmp12-QR	CCATAGAGGGA CTGAATGTTACG
Mus-Col1a1-QF	TCTCTGGTCT CCAGGGTCCTC	Mus-mt2-QF	GGGCTGCATCT GCAAAGAG
Mus-Col1a1-QR	TCCGTCTTTG CCAGGAGAAC	Mus-mt2-QR	AAAGGCTAGGC TTCTACATGGTC
Mus-Egr1-QF	CTCCTTCAGC ACCTCAACTGG	Mus-Tfrc-QF	AATCAAAATGTGA AGCTCATTGTG
Mus-Egr1-QR	CTTCCCTCCTGT GCTTTTATGTC	Mus-Tfrc-QR	TGGGCTCCTACTA CAACATAACG
Mus-Erdr1-QF	CGAAAGCACA CACGTAGAAGC	Mus-Spp1-QF	TCACTCCAATC GTCCCTACA
Mus-Erdr1-QR	CTTCCTCCGTG AGAATCGCT	Mus- Spp1-QR	CTGGA ACATCG TATGGGTA

Immunofluorescent Staining and Confocal Microscopy

The samples were fixed in 4% paraformaldehyde, dehydrated, then embedded in paraffin and sectioned (4–6 μm).

All procedures were performed according to the standard protocol. Antibodies and dilutions used were: CXCL1 (1:100, proteintech), MMP12 (1:50, proteintech), SPP1 (1:50, Abcam), EGR1 (1:50, Abcam). β-catenin (1:250,Abcam), BMP2 (1:100, Abcam), FGF5 (1:100, Abcam), ki67 (1:250,Abcam), Secondary antibodies: Alexa Fluor-488 (Abcam). Nuclei were stained using 4 diamidino-2-phenylindole (DAPI). Proliferation and apoptosis of hair follicles were assessed by Ki67/TUNEL

immunostaining as reported before (Foitzik et al., 2006). Imaging was performed using Zeiss LSM 880 confocal microscope (Zeiss, Oberkochen, Germany).

CXCL1/Veratric Acid Treatment

CXCL1 (500 ng) was dissolved in 500ul 0.9% NaCl and intradermally injected in the dorsum of 6–7 week C57 mice (n = 10 per group). The control group (n = 10 per group) were injected with 500 μl solvent. The injection treatment was conducted every other day for a total of five times.

Veratric acid (80 mg/kg) dissolved in 2% DMSO + 40% PEG + 5% Tween + H_2O, and were injected in 26 aged C57 mice (16 months) once per day for 30 days, the control group are 20 of the same aged mice (16 months), which intraperitoneally received an equal volume of 2% DMSO + 40% PEG + 5% Tween + H_2O per day for 30 days as well. The mice were killed for further experimentation at 30d.

Luminex Cytokine Panels

A mouse Bio-Plex cytokine/chemokine 23-plex array panel based on Luminex technology was used for the quantification of inflammatory cytokines/chemokines in dWAT. DWAT harvest from six young mice (2 months) and six aged mice at telogen were analyzed in the panel. Besides, dWAT in six mice from the VA group and six mice from the control group were also analyzed in the panel. Data were measured on Bio-Plex 200 System and calculated by Bio-Plex Manager 6.0 and 6.1 software.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, GSE134893.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committee of Nanfang Hospital, Southern Medical University. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by The Ethics Committee of Nanfang Hospital, Southern Medical University.

AUTHOR CONTRIBUTIONS

JC: design, methodology, writing, data collection, and analysis. Z-XF and D-CZ: methodology, data collection, and design. Y-LG and KY: methodology and formal analysis. DD and ZG: data curation and formal analysis. Z-QH: study design and methodology. YM: funding acquisition, supervision, and writing—review and editing. QQ: project administration, funding acquisition, design, supervision, data collection and analysis, and writing—review and editing. All authors agreed with the manuscript content.

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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.2021.728188/full#supplementary-material

Supplementary Figure 1 | Unsupervised clustering of OAD/YAD, OTD/YTD samples based on other adipose-related GO signatures.

Supplementary Figure 2 | KEGG enrichment analysis respectively for upregulated and downregulated DEGs in YTD vs OTD and YAD vs OAD.

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Time-Resolved Extracellular Matrix Atlas of the Developing Human Skin Dermis

Mansheng Li[1†], Xiao Li[1†], Binghui Liu[1], Luye Lv[2], Wenjuan Wang[3], Dunqin Gao[4], Qiyu Zhang[4], Junyi Jiang[1], Mi Chai[5], Zhimin Yun[6], Yingxia Tan[6], Feng Gong[6], Zhihong Wu[4], Yunping Zhu[1,7], Jie Ma[1]* and Ling Leng[4]**

[1]State Key Laboratory of Proteomics, Beijing Proteome Research Center, National Center for Protein Sciences (Beijing), Beijing Institute of Life Omics, Beijing, China, [2]Institute of NBC Defense, Beijing, China, [3]Department of Dermatology, Chinese PLA General Hospital, Beijing, China, [4]Stem Cell and Regenerative Medicine Lab, State Key Laboratory of Complex Severe and Rare Diseases, Department of Medical Science Research Center, Translational Medicine Center, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China, [5]Department of Plastic and Reconstruction Surgery, Chinese PLA General Hospital, Beijing, China, [6]Department of Stem Cell and Regenerative Medicine Laboratory, Institute of Health Service and Transfusion Medicine, Beijing, China, [7]Basic Medical School, Anhui Medical University, Anhui, China

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Edited by:
Mingxing Lei,
Chongqing University, China

Reviewed by:
Wang Wu,
Chongqing University, China
Lynn Yap,
Duke-NUS Medical School, Singapore
Jinran Lin,
Fudan University, China

***Correspondence:**
Ling Leng
lengling@pumch.cn
zhenlinger@126.com
Jie Ma
majie729@163.com
Yunping Zhu
zhuyunping@gmail.com

[†]*These authors have contributed equally to this work*

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Skin aging is a physiological issue that is still relatively poorly understood. Studies have demonstrated that the dermal extracellular matrix (ECM) plays important roles in skin aging. However, the roles of the changes in ECM characteristics and the molecules that are secreted to the extracellular space and are involved in the formation of the dermal matrix from birth to old age remain unclear. To explore the way in which the ECM microenvironment supports the functions of skin development across different age groups is also poorly understood, we used a decellularization method and matrisome analysis to compare the composition, expression, and function of the dermal ECM in toddler, teenager, adult, and elderly skin. We found that the collagens, glycoproteins, proteoglycans, and regulatory factors that support skin development and interact with these core ECM proteins were differentially expressed at different ages. ECM expression markers occurring during the process of skin development were identified. In addition, our results elucidated the characteristics of ECM synthesis, response to skin development, and the features of the ECM that support epidermal stem cell growth *via* the basement membrane during skin aging.

Keywords: decellularization, extracellular matrix, matrisome, skin development, skin aging

INTRODUCTION

The human skin is a complex organ comprising a range of tissues that act in harmony to create a protective barrier against environmental stresses such as heat, solar ultraviolet light, irradiation, and pathogens, and regulates the body's temperature and degree of water loss (Quan and Fisher, 2015). The human skin consists of two major layers. The upper layer, or *epidermis*, is a multilayered epithelium that continually undergoes terminal differentiation. Underneath the *epidermis* lies the dermis, which is enriched with dermal fibroblasts, vascular connective tissue, and dense extracellular matrix (ECM). Dermal collagen constitutes the bulk of the skin, making up 90% of the skin by dry weight (Uitto, 1986). The mechanical properties and functioning of the skin depend on the composition, structure, and organization of the dermis (Haake et al., 2001).

The human skin undergoes a natural aging process in response to intracellular and external stresses, and damage from environmental sources (Naylor et al., 2011). A study of 1,713 American women aged over 50 years highlighted the psychological suffering associated with aging (Hofmeier et al., 2017). Aging is generally believed to be related to the alterations of the dermal ECM (Gunin et al., 2010). On the basis of their structural and functional features, ECM components are divided into the "core matrisome", made up of collagens, proteoglycans, and glycoproteins, and "matrisome-associated" components, comprising ECM-bound proteins and carbohydrates. These carbohydrates include glycosaminoglycan (GAG) chains, which are not linked to a core protein but are bound by anionic charge binding to proteins. They include regulatory signals and secreted factors bound to one or more core matrisome components (termed the "matrisome") (Naba et al., 2016). During the first 10 years of childhood, the dermis develops at the cellular level (Gunin et al., 2010). In fetuses, *in utero* and toddler, the skin transiently has the functional characteristic of wound healing with only slight scarring (Krejčí et al., 2015). Although there have been several studies on the ECM in young and aging skin, including investigations of the roles of collagens, the elastic fiber network, proteoglycans, and GAGs, (Ge et al., 2020; Mccabe et al., 2020), the composition and function of the ECM over the course of skin development remain unclear.

Most previous findings have been based on the expression of ECM proteins in cells or whole tissues, (Kaur et al., 2019; Ewald, 2020; Ge et al., 2020), rather than those secreted from cells, due to a lack of effective methods with which to obtain the complete bioactive scaffold while preserving the ECM components. We previously developed a method for skin decellularization and used it to investigate differences in the composition and expression of whole tissue ECM and secreted ECM (Leng et al., 2020; Liu et al., 2020). Our strategy facilitates the application of tissue engineering principles to skin bio matrix scaffold materials, promising to accelerate, and enhance tissue regeneration. Using this method, we identified the extracellular matrisome composition of human, pig, and rat skin. On the basis of the conclusions drawn from this work, we have analyzed the pathological features of the matrisome in skin keloids, providing a useful model for the diseases and identifying potential targets for therapy (Zhang et al., 2021). In this study, we used the same method, combining a quantitative proteomics approach to the analysis of ECM composition in skin decellularized bioscaffolds of different ages and aiming to establish a new standard of "young and active" skin ECM composition and to discover new clues regarding tissue engineering for skin regeneration.

MATERIALS AND METHODS

Sample Preparation

A total of ten skin tissue samples from healthy individuals of four different ages, i.e., toddler (1–3 years old), teenager (8–18 years old), adult (30–50 years old), and elderly (>60 years old), were used in this study. All skin samples were collected from the abdomen, thigh, or back of the donors, and provide by the Department of Dermatology, PLA General Hospital, China. Written informed consent was obtained from all the participants. Detailed characteristics of these healthy individuals are provided in **Supplementary Table S1**. This study was approved by the Medical Ethics Committee of Chinese PLA General Hospital (NO. S2018-123-02).

Preparation of Decellularized Skin Scaffolds

A 3- to 6-mm-thick segment of skin tissue was cut using an electric skin picker. Skin tissues were rinsed with cold phosphate-buffered saline (PBS), followed by delipidation using phospholipase A2 combined with sodium deoxycholate for 4 h in a shaker at 37°C until the tissue segments became oyster white. The surface of the skin was scraped gently and carefully using the back of a scalpel to remove the *epidermis*. The decellularized dermal samples were placed in sterilized 1.5 ml microcentrifuge tubes. Then, samples were rinsed using 3.4 M NaCl for 1 h followed by a final wash with PBS containing nucleases (10 μg/ml DNase, 5 μg/ml RNase) at 37°C for 1 h. Finally, the decellularized scaffolds were flash-frozen for proteomics analysis.

Protein Extraction, Digestion, and Liquid Chromatography–Tandem Mass Spectrometry (LC–MS/MS) Analysis

Decellularized human skin scaffolds were homogenized, and proteins were extracted using 1 ml Protein Extraction Reagent Type 4 (Sigma-Aldrich) for every 125 mg of pulverized tissue. After centrifugation at 4°C and 14,000 × *g* for 10 min, the supernatants were reduced by adding 1 × protease inhibitors tributylphosphine to a final concentration of 5 mM, followed by vortexing for 10 min at room temperature. After centrifugation at room temperature and 14,000 × *g* for 30 min, supernatants were transferred to a clean tube and stored at −80°C. For peptide extraction, 25 μg samples (from the supernatants) were solubilized in 10 mM DTT at 37°C for 4 h. Samples were added with 1 M IAA and kept in the dark for 1 h. All samples were collected, and the suspension was removed after centrifugation for 12,000 × *g*. Thereafter, 100 μl UA was added and the suspension was removed after centrifugation twice. NH_4HCO_3 (50 mM) was added to the samples, and the supernatants were removed after centrifugation at room temperature and 12,000 × *g* for 5 min. A final digestion was performed by incubating with trypsin at a ratio of 1:50 enzyme/substrate, at 37°C overnight. After centrifugation at 14,000 × *g* for 30 min, the supernatants were transferred to clean tubes for the LC–MS/MS analysis. The peptide mixtures were analyzed using Q-Exactive mass spectrometer (Thermo Fisher Scientific) equipped with an Easy-nLC nanoflow LC system (Thermo Fisher Scientific).

MS/MS Data Identification and Bioinformatics Analysis

Raw MS files were analyzed using the MaxQuant software (version 1.6.5.0) (Cox and Mann, 2008) against the human

UniProt database (https://www.uniprot.org/, accessed on May 13th, 2020) and contaminants database. Peptide identification was performed using a precursor mass tolerance of up to 4.5 ppm and a fragment mass tolerance of 20 ppm. Cysteine carbamidomethylation was set as the fixed modification, and N-terminal acetylation and methionine oxidations were used as variable modifications. Up to two missed cleavages were allowed, and trypsin was set as the enzyme specificity. Automatic target and reverse database searches were used, and a false discovery rate of 1% at both the peptide and protein levels was allowed. Protein quantification was performed based on the intensity-based absolute quantification method (Schwanhausser et al., 2011) embedded in MaxQuant.

All bioinformatics analyses were performed using the R language and statistical environment, version 3.6.3. The quantification values of identified proteins were normalized by taking the fraction of total, followed by multiplication by 10^6. To test for significant differences in the expression of proteins between the four different age groups, one-way ANOVA (analysis of variance), or Kruskal–Wallis tests were performed. The R package clusterProfiler (version 3.12.0) (Yu et al., 2012) was used to annotate the identified proteins according to the Biological Processes, Cellular Components, and Molecular Functions defined by the Gene Ontology (GO) (Ashburner et al., 2000). KEGG pathway analysis was also performed (Ogata et al., 1999) (https://www.kegg.jp/kegg/pathway.html). Tissue expression information regarding the significant proteins was retrieved and integrated from the online tool DAVID (https://david.ncifcrf.gov/) (Huang Da et al., 2009). ECM proteins, including six categories of core ECM (collagens, proteoglycans, and ECM glycoproteins) and ECM-associated proteins (ECM regulators, ECM-affiliated proteins, and secreted factors), were annotated using MatrisomeDB (http://matrisomeproject.mit.edu/) (Naba et al., 2016). Heat maps of the ECM protein quantitation values were constructed using the R package ComplexHeatmap (version 2.0.0) (Gu et al., 2016). The basement membrane (BM) located ECM proteins were confirmed by the GO's Cellular Components annotation retrieved from DAVID. We also used the circlize package (version 0.4.11) (Gu et al., 2014) to circularly visualize the upregulation and downregulation of ECM proteins in human skin dermis with aging.

Immunochemical Staining

Samples were fixed in 4% formaldehyde overnight at 4 °C and then processed using a dehydration gradient. After tissues were embedded in paraffin, sections were cut to a thickness of 4 μm for hematoxylin and eosin staining, immunohistochemistry and immunofluorescence analysis. The sections were deparaffinized and heated with a microwave to boil for at least 12 min with antigen retrieval buffer, removed by endogenous catalase in 0.3% H_2O_2 for 30 min after cooling. Then, sections were blocked with normal horse serum in Tris-buffered saline for 1 h and blocked with Avidin/Biotin Blocking Kit, stained with antibodies ab11575 (anti-laminin antibody LAMC3, Abcam, dilution: 1:200), ab6586 (COL4A1, Abcam, dilution: 1:1,000), 13530-1-AP (NID2, Proteintech, dilution: 1:200), ab97779 (MMP2, Abcam, dilution: 1:400), and 11060-1-AP (ANXA5, Proteintech, dilution: 1:200) overnight at 4°C. After staining with a secondary antibody, the sections were colored. For immunofluorescence, skin sections were analyzed for COL6A1 and COL12A1 expression using antibodies against 17023-1-AP (COL6A1, Proteintech, Sankt Leon-Rot, Germany, dilution: 1:200) and 19727-1-AP (COL12A1, Proteintech, dilution: 1:200), and SLPI against sc-373802 (SLPI, Santa Cruz Biotechnology, Inc., United States). Samples were then incubated with secondary antibodies (SA00009-2, Proteintech) for 1 h at room temperature and counterstained with DAPI. Images were taken at ×20 and ×40 magnification and analyzed using Volocity Demo (×64) (PerkinElmer, Waltham, MA).

RESULTS

Skin Proteome Profile of Different Ages

To investigate the ECM composition of different ages, decellularized skin scaffolds were obtained using the decellularized method established by our group previously (Leng et al., 2020; Liu et al., 2020; Zhang et al., 2021). Especially, the decellularized skin scaffolds remained the BM structure (**Figure 1A** and **Supplementary Figure 1A**). Next, Raman spectroscopy was used to analyze skin tissues of different ages and found that the decellularized samples, including the BM and dermis (DM), could be easily distinguished from native skin tissues (**Supplementary Figure S1B–E**). Quantitative proteomics is an important method for the identification of the ECM components secreted into, and located in, the extracellular space (Leng et al., 2020; Liu et al., 2020). To investigate the molecular basis of skin aging, we used a quantitative proteomics approach to analyze the composition of the ECM in skin decellularized bioscaffolds of different ages. The overall workflow is shown in **Figure 1A**. We used these data to establish a secreted "ECM map" and a developmental pattern of the dermal ECM during skin development (**Supplementary Figure S2A–C**). The pairwise Pearson's correlation coefficients of repeat experiments with the samples from the same age group show good reproducibility, with a high positive correlation (correlation coefficient: 0.69–0.94) (**Supplementary Figure S2B**). Annotated by the MatrisomeDB, ECM proteins are divided into "core matrisome" proteins including collagens, glycoproteins and proteoglycans, as well as "matrisome associated" proteins including ECM-affiliated proteins, ECM regulators, and secreted factors. A total of 263 ECM proteins of six types were identified in the study, with 213, 223, 233 and 180 ECM proteins in toddlers, teenager, adult, and elderly skin tissues respectively (**Figures 1B,C**, **Supplementary Figure S2C** and **Supplementary Table S2**). There were 152 ECMs commonly identified in skin tissues of all four age stages with an overlap rate only about 57.8% (**Figure 1C**). Besides, ECM glycoproteins, ECM regulators, ECM-affiliated proteins, and

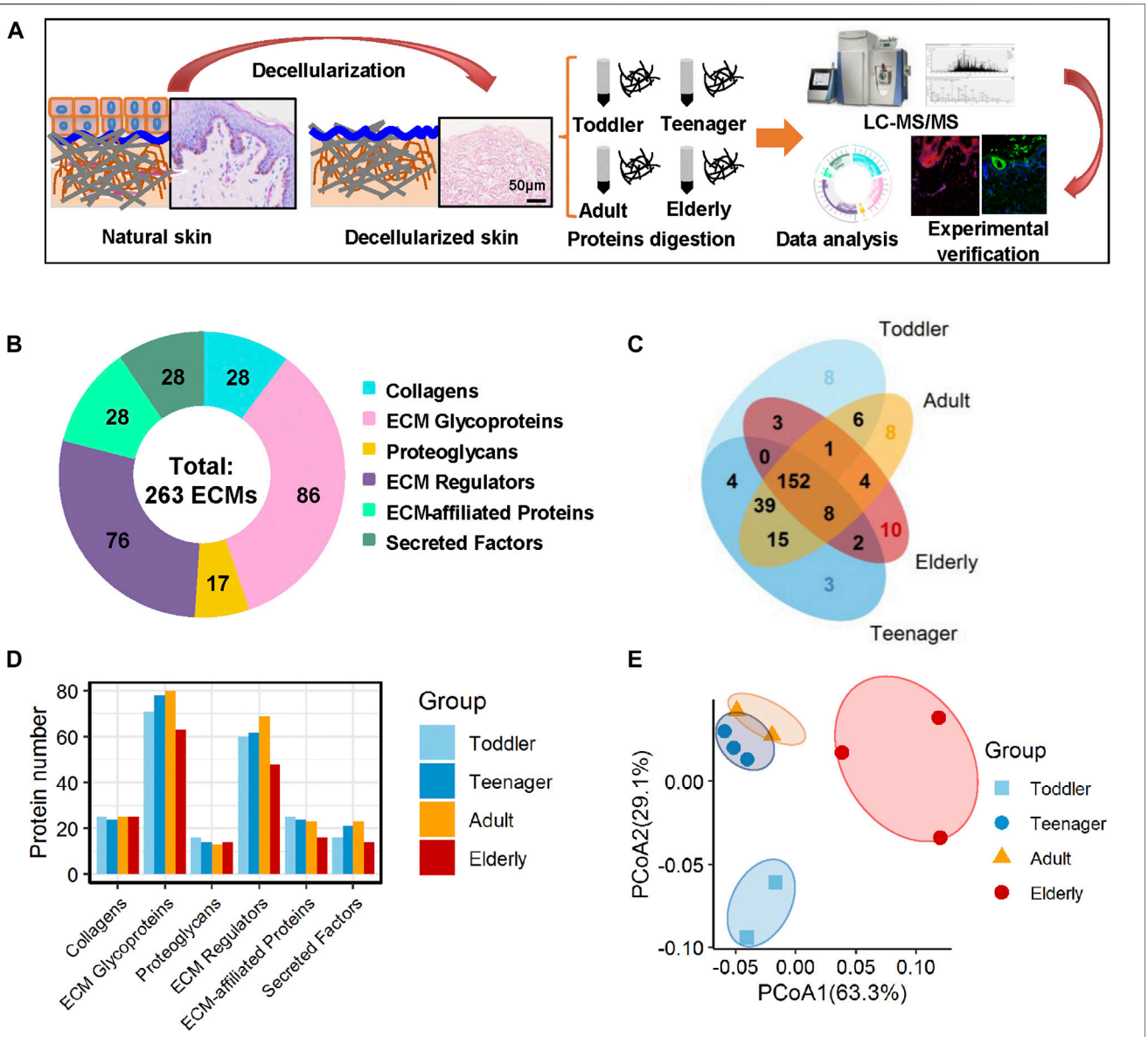

FIGURE 1 | Matrisome profile of the human skin dermis. **(A)** General workflow of the whole experimental design, including decellularization, mass spectrometry-based quantitative proteomics, bioinformatics analyses, and experimental verification. **(B)** Six types of ECM proteins (collagens, ECM glycoproteins, proteoglycans, ECM regulators, ECM-affiliated proteins, and secreted factors) expressed at four ages (toddler, teenager, adult, and elderly). Pie charts represent the proportion of the six components (collagens, ECM glycoproteins, proteoglycans, ECM regulators, ECM-affiliated proteins, and secreted factors) that make up the matrisome in the human skin dermis. **(C)** Overlap of ECM proteins in the skin scaffolds of four ages (toddler, teenager, adult, and elderly). **(D)** Histogram shows the number of the six ECM components identified at four age stages. **(E)** PCoA analysis of the samples at four ages (toddler, teenager, adult, and elderly) based on their ECM profile. The technical repeats were produced for each sample, represented by different colored points in the figure.

secreted factors in the elderly were present at lower levels than those in the other three age groups (**Figure 1D**). Principal coordinates analysis (PCoA) was performed to explore the similarities and dissimilarities of the samples in the four age modules based on the ECM characteristics, and the results suggest that the samples from the four groups differed greatly (**Figure 1E**). To identify representative ECM characteristics at different ages and understand their biological significance, we performed differential expression analysis on all ECM at the four different ages (**Figures 2A,B** and **Supplementary Table S2**). Biological process analysis revealed that the skin of toddlers was mainly associated with collagen fibril organization, glycosyl metabolism, and chondrocyte and *epidermis* development. Skin from the teenager stage mainly exhibited hemidesmosome assembly, cell-matrix adhesion, cell junction assembly, and response to growth factors. For adults,

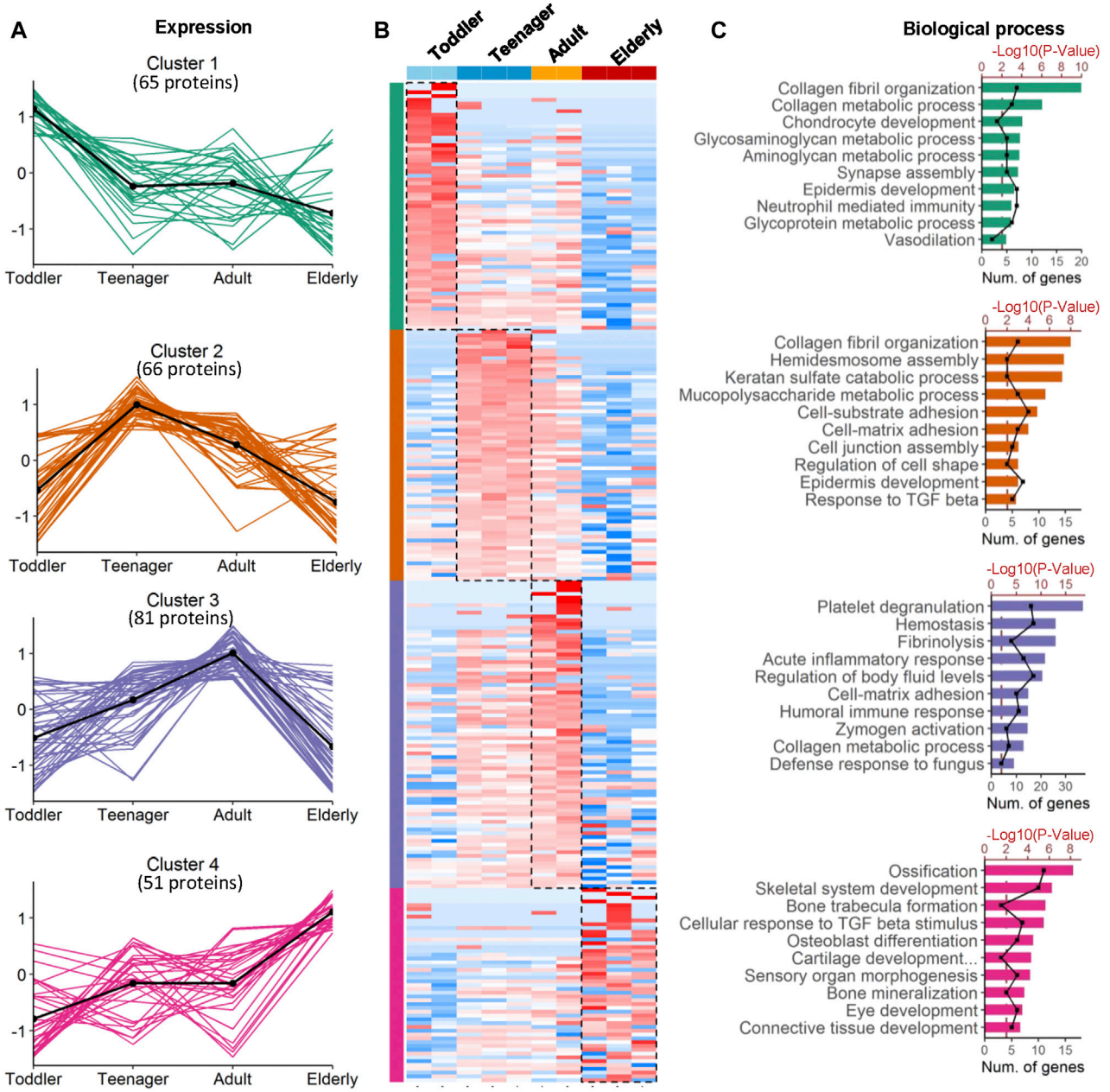

FIGURE 2 | Coexpression analysis of age-specific ECM protein modules. **(A)** Four protein modules revealed ECM specificity based on age stage. Cluster 1–4 are the protein groups highly expressed in toddler, teenager, adult and elderly, respectively. **(B)** Heatmap analysis of ECM identified in samples from skin bioscaffolds of four ages (toddler, teenager, adult, and elderly) according to log_2 normalized protein intensity. Red and blue boxes indicate proteins with increased and decreased abundance, respectively. Green, purple, and rose bars correspond to the proteins enriched in skin bioscaffolds from toddler, teenager, adult, and elderly. **(C)** Clusters of proteins associated with similar biological process were grouped according to the degree of enrichment.

the highly expressed proteins of skin were mainly associated with acute inflammatory responses, wound healing, and defense against fungi. Elderly skin mainly expressed protein concerned with the process of ossification, bone mineralization, and connective tissue development (**Figure 2C**), which may be one of the reasons for the lack of mechanical elasticity in elderly skin. These results indicated that the ECM matrisome microenvironment at the four ages reflects the developmental characteristics of the skin tissue.

Skin Core Matrisome Features at Different Ages

Collagens are the principal structural component of the cross-linked networks of the ECM. During skin development, the

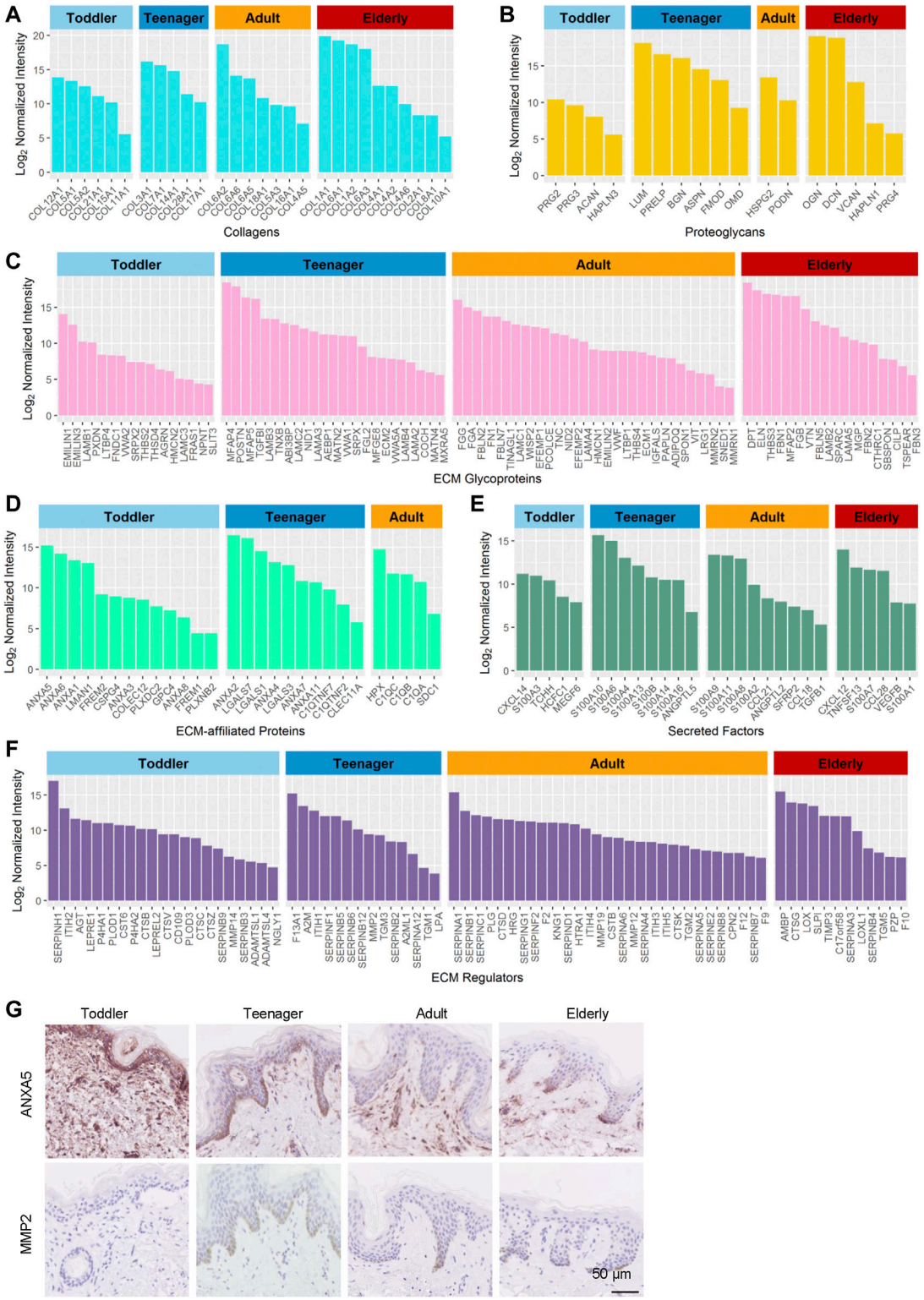

FIGURE 3 | Highly expressed proteins of the six ECM components in the human skin dermis at four ages (toddler, teenager, adult, and elderly). **(A–F)** Histograms show highly expressed proteins of the collagen ECM, proteoglycan ECM, ECM glycoprotein, ECM-affiliated protein, secreted factor ECM, and ECM regulator, respectively. **(G)** Immunohistochemistry of ANXA5 and MMP2 in the skin tissues of toddler, teenager, adult, and elderly (scale bar represents 50 μm).

proportions of the fibril-associated collagens COL12A1, COL21A1 and COL11A1, (Ricard-Blum, 2011), as well as the BM collagens COL5A1/2 and COL15A1, were higher in toddler bioscaffolds than in any other stage of life (**Figure 3A** and **Supplementary Table S3**). The fibril-forming collagen COL3A1, the fibril-associated collagen COL14A1, and the BM collagens COL7A1, COL28A1, and COL17A1 were highly expressed in teenager bioscaffolds, whereas the short-chain collagen COL6A2/5/6, the fibril-forming collagen COL5A3, the micro fibril collagen COL16A1, and the BM collagens COL18A1 and COL4A5 were highly expressed in adult bioscaffolds. The skin of the elderly is generally believed to have decreased levels of collagens. (Quan and Fisher, 2015). However, we found that the short-chain collagens COL6A1, the fibril-associated collagens COL1A1/2, and the BM collagens COL4A2/6, COL2A1, and COL8A1, as well as a cartilage-specific collagen, COL10A1, were in elder bioscaffolds.

Proteoglycans are interspersed among collagen fibrils in the ECM and provide structural strength. They can store and release biologically active soluble signals by binding to sulfated carbohydrate chains of GAGs (Farach-Carson et al., 2014). Our results revealed that toddler bioscaffolds showed the highest expression of the hyaluronic acid binding proteoglycans, including ACAN and HAPLN3 (**Figure 3B**), which may generate a large osmotic swelling pressure, conferring skin stiffness and resistance to deformation (Juhlin, 1997), as well as skin hydration and viscoelasticity (Hernández et al., 2011). A small leucine-rich proteoglycans (LUM), and two bone-related ECMs (ASPN and OMD) were found highly expressed in teenager bioscaffolds. Five proteoglycans, including BGN, DCN, VCAN, HAPLN1, and PRG4, were found highly expressed in bioscaffolds from elderly donors. Proteoglycans can also influence the rate of assembly, size, and structure of collagen fibrils (Schonherr and Hausser, 2000; Kadler et al., 2008), indicating that highly expressed age-specific proteoglycans can affect the skin by regulating collagens.

Glycoproteins are major components of the noncollagenous core of the matrisome components of the skin ECM, regulating cell proliferation, migration, differentiation, and wound healing (Pankov and Yamada, 2002; Rousselle et al., 2019). Our results indicated that most of the highly expressed glycoproteins in toddler bioscaffolds were related to the member of the elastin microfibril family (EMILIN3), regulators of transforming growth factor beta (THSD4 and LTBP4), and tissue development (NPNT and FRAS1) (**Figure 3C**). Highly expressed glycoproteins in teenager skin were involved in elastic fiber assembly (MFAP4 and TNXB) and the formation of filamentous networks (MATN4). Angiogenesis-associated ECM proteins (ADIPOQ, THBS4, MMRN1, and MMRN2), neuronal development (FBLN7) were highly expressed in adult skin. In elderly skin, highly expressed glycoproteins were involved in bone mineralization (MGP) and response to transforming growth factor beta (FBN1 and CILP). These results indicated that the mechanical and functional properties provided by the different compositions of these core matrisomes at different ages may determine skin aging.

Regulatory Matrisomes Are Essential for Skin Function

In addition to the core matrisome proteins, the composition and regulation of matrisome-associated proteins, such as ECM-affiliated proteins, regulators, and secreted factors, were studied in the aging dermis. Our results revealed that 23, 20, 15, and 14 matrisome-associated proteins, most of which are regulators of cell attachment and growth (Martin et al., 1984; Naba et al., 2012), were highly expressed in toddler, teenager, adult, and elderly bioscaffolds, respectively (**Supplementary Table S3**). In toddler skin, the highly expressed matrisome-associated proteins included placental anticoagulant proteins (ANXA5/8) and ECM associated proteases (CTSV, ADAMTSL1/4, PLOD3, P4HA1/2, and NGLY1) (**Figures 3D–F**). Meanwhile, the placental anticoagulant protein (ANXA4), proteins specifically expressed in keratinocytes (LGALS3/7 and TGM1/3), C1q tumor necrosis factor-related proteins (C1QTNF2/C1QTNF7), cell cycle and differentiation regulators (S100A10/14 and S100B), and protease inhibitors (SERPINB12, SERPINF1, and SERPINA12) are highly expressed in teenager skin. In adult scaffolds, the matrisome-associated proteins with high expression level were mainly associated with blood coagulation (KNG1 and F12), inflammation (SDC1, S100A2/8, CCL18/21, and TGFB1), fibrinolysis (HRG), hyaluronic acid regulation (ITIH4/5), and the inhibition of proteases (SERPINB8, SERPINC1, SERPINF2, and SERPINE2). In older scaffolds, those proteins were mainly involved in inflammation (CXCL12, S100A7, TNFSF13, CCL28, VEGFB, S100A1, AMBP, and SLPI) and aging (LOXL1). Among these proteins, the protein ANXA5 with high expression in toddler and MMP2 with high expression in teenager were selected for the verification of the expression patterns during aging using immunohistochemistry staining (**Figure 3G**). These results indicated that anticoagulant and cell development-associated matrisome-associated components were highly expressed in toddler and teenager. Wound healing and aging associated matrisome-associated components were mainly expressed in adult and elder bioscaffolds.

Functional Matrisome Composition of EpSCs Niche

Aging is related not only to the dermal ECM but also to the state of Epidermal stem cells (EpSCs) (Stern and Bickenbach, 2007). The BM is a major component of the natural stem cell niche of basal EpSCs and provides necessary complex stimuli that affect the behavior of basal cells (Leng et al., 2020). BM components in samples from the four age categories were analyzed to evaluate how they support EpSC function throughout skin development. Our results revealed that 13, 14, 12, and 12 BM ECM proteins were specifically highly expressed in the skin of toddler, teenager, adult, and elderly, respectively (**Figure 4A** and **Supplementary Table S4**). Toddler scaffolds were mainly enriched in ECM proteins involved in the process of cell fate determination (FREM1/2), cell adhesion and migration-related ECM (FREM2 and LAMC3), and BM maintenance ECM (COL5A1, VWA2, and

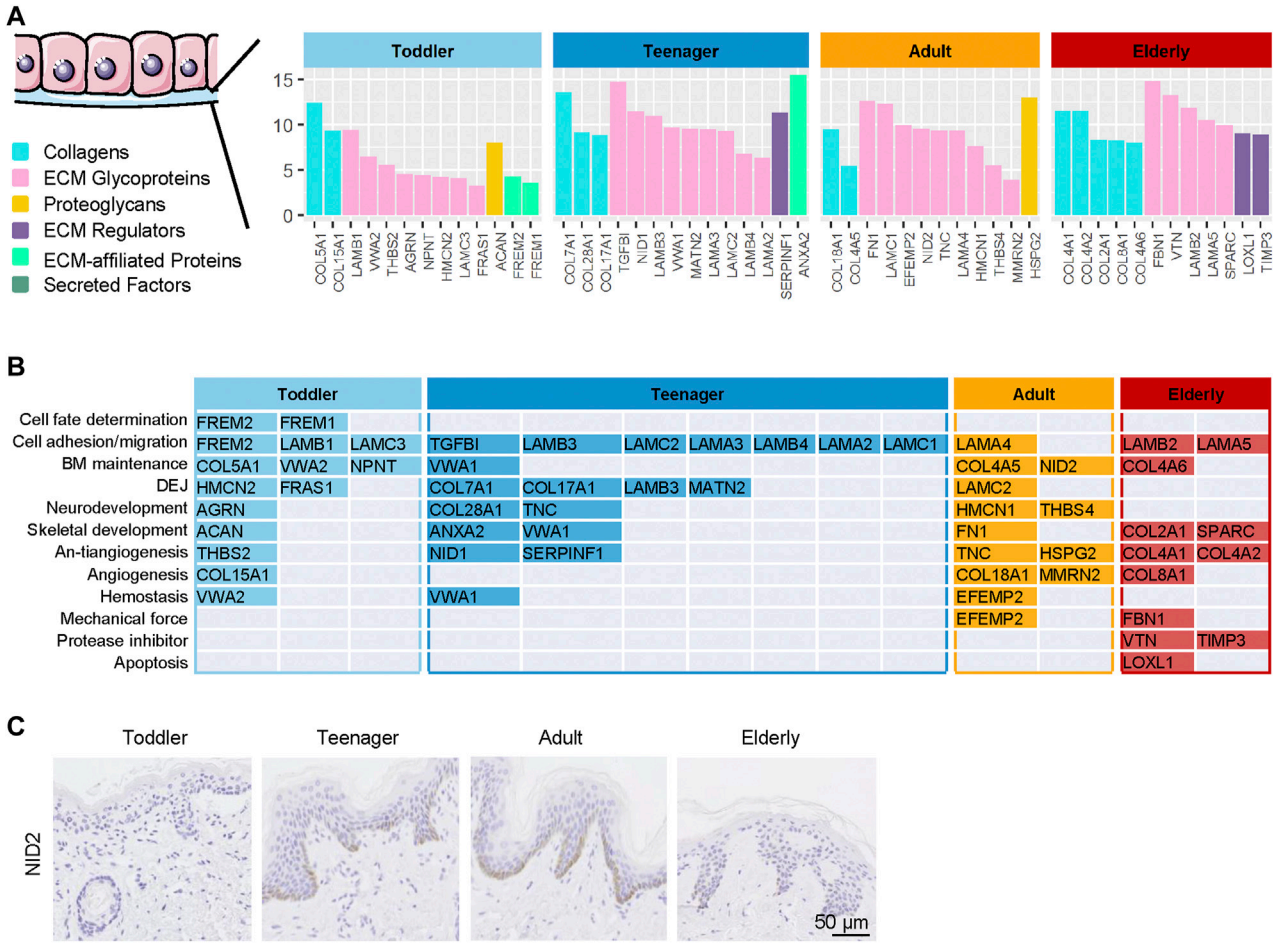

FIGURE 4 | | Highly expressed basement membrane (BM) proteins in the six ECM components in the human skin dermis from four ages (toddler, teenager, adult, and elderly). **(A)** Six types of ECM proteins of BM (collagens, ECM glycoproteins, proteoglycans, ECM regulators, ECM-affiliated proteins, and secreted factors) expressed at four ages (toddler, teenager, adult, and elderly). **(B)** Functional analysis of BM located ECM from four ages (toddler, teenager, adult, and elderly). **(C)** Immunohistochemistry of NID2 in the skin tissues of toddler, teenager, adult, and elderly (scale bar represents 50 μm).

NPNT) (**Figure 4B**). Teenager bioscaffolds were mainly enriched in the proteins of cell fate determination-related ECM (LAMA3, LAMB3/4, and LAMC2) and dermal–epidermal junctions (DEJ) (COL7A1, COL17A1, LAMB3, and MATN2). Among these, mutations in COL7A1 and COL17A1 were associated with dystrophic epidermolysis bullosa (Burgeson, 1993; Dang and Murrell, 2008). In addition, COL17A1 is an important anchoring fibril related to the hemidesmosome complexes, but it was also identified as a novel EpSC marker (Bilousova and Degregori, 2019; Liu et al., 2019). Neurodevelopment and antiangiogenesis-related ECM were relatively abundant in teenager, adult, and elderly scaffolds. We found two protease inhibitors (VTN and TIMP3) and an apoptosis-related ECM protein (LOXL1) in elder scaffolds. These results indicated that the ECM composition of the BM, which is in direct contact with EpSCs, differs according to the stage of skin development. NID2, a high expressed protein in adult, was selected for the verification of the expression patterns during aging using immunohistochemistry staining (**Figure 4C**). These different EpSCs stimuli may explain the differences in EpSCs activity at different ages.

Regulatory Pattern of ECM in the Process of Aging

Here we focused on the ECM proteins that gradually increased or decreased from toddler to elderly. Markers of epidermal development from EpSCs to mature keratinocytes have been well studied (Gonzales and Fuchs, 2017), whereas ECM markers for dermal development have not. We identified 24 ECM proteins upregulated with age and 26 ECM proteins downregulated with age among the four age groups (**Figure 5A** and **Supplementary Table S2**). Our results indicated that during skin development, ECM expression of collagen catabolic processes, protease activity, apoptotic signaling, immune cell migration, and defense response to microorganisms increased gradually (**Figure 5B** and **Supplementary Figure S3A**). These findings indicted that cell vitality, the ability to resist external microorganisms, and ECM

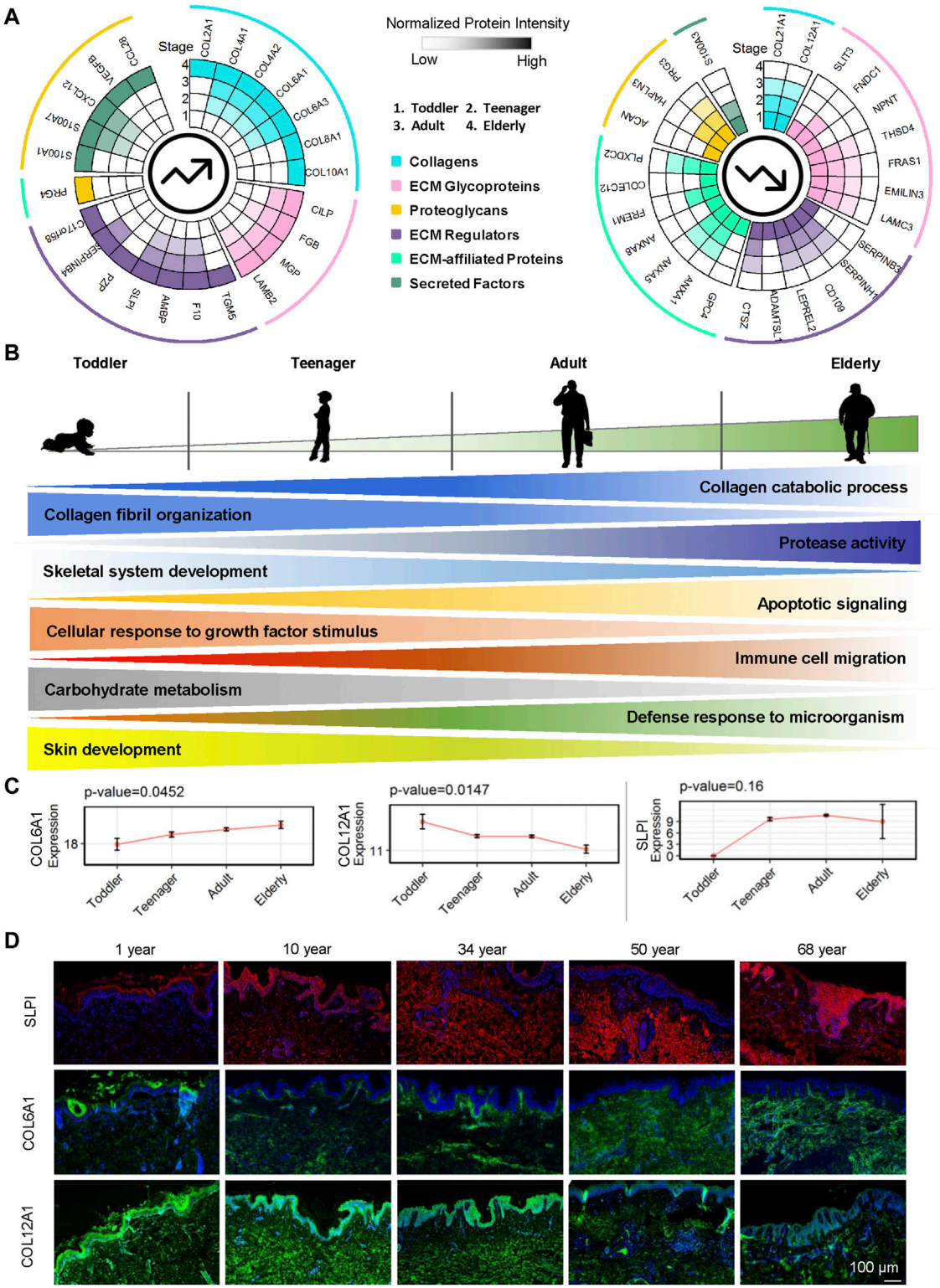

FIGURE 5 | Roles of ECM proteins in human dermal development. **(A)** Upregulated and downregulated ECM proteins in the human skin dermis at four ages (toddler, teenager, adult, and elderly). Each circle represents an age stage. Blue, pink, yellow, purple, green, and cyan lines correspond to the six components (collagens, ECM glycoproteins, proteoglycans, ECM regulators, ECM-affiliated proteins, and secreted factors) of the skin ECM. **(B)** Schematic representation of dermal ECM function evolution during human skin development and aging. **(C)** Expression of COL6A1, COL12A1, and SLPI in skin scaffolds of four ages (toddler, teenager, adult, and elderly). **(D)** Immunofluorescence of SLPI, COL6A1, and COL12A1 identified in the human skin dermis (scale bar represents 50 μm).

remodeling are decreased as skin ages, resulting in increased susceptibility to skin infection and injury. Aging and relaxation of the skin appear to increase microorganism invasion and inflammatory factor production and enable the skin to continue undergoing wound healing, resulting in the formation of scars, which may also cause accelerated skin aging. With age, collagen fibril organization, skeletal system development, cellular response to growth factor stimulus, carbohydrate metabolism, and skin development decreased gradually (**Figure 5B** and **Supplementary Figure S3B**). For example, COL6A1, SLPI, and AMBP were gradually upregulated whereas COL12A1, COL21A1, FRAS1, ANXA5, and CD109 were downregulated over the course of the lifetime (**Figure 5C** and **Supplementary Figure S4**). COL6A1, COL12A1, and SLPI were selected for the verification of the expression patterns during aging using immunofluorescence staining (**Figure 5D**). COL6A1 was found to be specifically located around the BM of the epidermal–dermal junction in 10-year-olds, and then, with increasing age, its level of expression gradually increased in the dermis. By contrast, the collagen bundles of COL12A1 were highly expressed in 10- and 34-year-old dermis, and their expression decreased significantly with age.

DISCUSSION

Developmentally, extracellular matrices, composed of core ECM and ECM associated regulators, provide a tissue framework and cell niche that provides a micro- and macroarchitecture to help in driving cell behavior and function. Decellularized ECM derived from organs or tissues using physical and chemical treatments were used to study cell-matrix interactions based on their characteristics of complex composition, formation of vascular networks, and unique, tissue-specific architecture. Naturally derived biomaterials have physical and chemical effects that collectively form a foundation that can be used for the bioengineering of tissue bionic construction and that directly promotes tissue regeneration through secreted factors (Sadtler et al., 2016). Better understanding of ECM composition should lead to increases in their use *in vitro* and *in vivo* and should lead to the development of more robust tools for bioengineering and regenerative medicine strategies.

Alterations to the dermal ECM composition and organization are major drivers of aging of human skin. The biology of human skin aging can be viewed largely as a disorder of the dermal matrisome. However, our current knowledge regarding the impact of aging on the human skin matrisome is very limited. In this study, we used a tissue engineering method combined with a quantitative proteome technique to analyze the ECM composition and the function of human skin tissues during aging. Collagen is the main component of human skin. We found that the BM collagens were the main collagens expressed in an age-specific manner in toddler and teenager skin and short-chain and fibril-associated collagens were more abundant in adult and elderly skin. The proportions of these collagens may determine, to a great extent, the different physical structure and mechanical properties of skin at different ages. For example, we found that COL12A1 was highly expressed in toddler dermal skin, and as the skin aged, COL12A1 almost disappeared from the skin, indicating its important role in the physical support of the skin structure. Proteoglycans associated with skin stiffness and resistance to deformation were most abundant in toddler skin, and elastin micro fibril related glycoproteins were found in high levels in toddler and teenager skin, an observation which may explain why young people have more elastic skin.

ECM factors associated with skin development, such as growth factors and tissue developmental glycoproteins, were enriched in toddler skin. Keratinocytes specifically expressed in ECM were mainly in teenager skin. Epidermal stem cell proliferation and angiogenesis-related proteoglycans, and angiogenesis and neuronal development-related glycoproteins were found mainly in adult skin. We found that wound healing associated ECM, such as fibrinogen-related glycoproteins and blood coagulation-related regulators, as well as immunocyte stimulation and inflammation-related proteoglycans, was enriched in adult skin. Ossification and fibrosis-related glycoproteins and inflammation and aging-related regulators were enriched in elderly skin. In our recently work (Zhang et al., 2021), the dermal ECM components of keloids tissues have been identified. By comparing the data with the keloids ECMs, we found some of the components, e.g., SLPI, THBS3, show both high expression trends in keloids and in elderly skin, indicating these ECMs could share similar functions in elderly and keloids skin tissue. For example, SLPI is a secretory inhibitor, which can promote the immune response by protecting the epithelial surface from the attack of endogenous proteolytic enzymes. Our results showed this protein was highly expressed in the elderly skin, which may lead to increasing immune response and aging of elderly skin. Thus, SLPI could be used as a potential marker of skin diseases with abnormal immune response.

CONCLUSION

Our study is the first to use a decellularized method combined with proteomics tools to investigate the molecular differences and development characteristics of the dermal matrix during skin aging. The time-resolved ECM atlas constructed in this study identifies the ECM components with different types in tollder, teenager, adult and elderly, providing a new standard of age-specific skin ECM composition to discover new clues regarding tissue engineering for skin regeneration. In addition, the age-specific functions of different types of ECM proteins during skin aging were systematically analyzed, providing a comprehensive understanding of age-related changes in human skin ECM proteins and potential value in predicting other disease state in skin tissues.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: http://www.proteomexchange.org/, PXD016440.

ETHICS STATEMENT

This study was reviewed and approved by the Medical Ethics Committee of Chinese PLA General Hospital (No. S2018-123-02). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

Conceptualization: LL, JM, YZ, ML, and XL; Data Curation: LL, JM, ML, and XL; Formal Analysis: LL, JM, ML, XL, and BL; Funding Acquisition: YZ, FG, YT, and ZW; Investigation: LL, JM, ML, XL, and LLv, DG, QZ, and ZY; Resource: WW, MC, ZY, and LLv; Software: ML, XL; Visualization: LL, JM, and ML; Writing Original Draft: LL, JM; Writing Review and Editing: LL, JM, ML, and XL.

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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.2021.783456/full#supplementary-material

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REVIEW
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A Hairy Cituation – PADIs in Regeneration and Alopecia

*Kim Vikhe Patil, Kylie Hin-Man Mak and Maria Genander**

Department of Cell and Molecular Biology, Karolinska Institutet, Stockholm, Sweden

In this Review article, we focus on delineating the expression and function of Peptidyl Arginine Delminases (PADIs) in the hair follicle stem cell lineage and in inflammatory alopecia. We outline our current understanding of cellular processes influenced by protein citrullination, the PADI mediated posttranslational enzymatic conversion of arginine to citrulline, by exploring citrullinomes from normal and inflamed tissues. Drawing from other stem cell lineages, we detail the potential function of PADIs and specific citrullinated protein residues in hair follicle stem cell activation, lineage specification and differentiation. We highlight PADI3 as a mediator of hair shaft differentiation and display why mutations in *PADI3* are linked to human alopecia. Furthermore, we propose mechanisms of PADI4 dependent fine-tuning of the hair follicle lineage progression. Finally, we discuss citrullination in the context of inflammatory alopecia. We present how infiltrating neutrophils establish a citrullination-driven self-perpetuating proinflammatory circuitry resulting in T-cell recruitment and activation contributing to hair follicle degeneration. In summary, we aim to provide a comprehensive perspective on how citrullination modulates hair follicle regeneration and contributes to inflammatory alopecia.

Keywords: citrullination, hair follicle, alopecia, inflammation, stem cell, epigenetics, Peptidylarginine deiminase

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Srikala Raghavan,
Institute for Stem Cell Science and Regenerative Medicine (inStem), India

****Correspondence:***
Maria Genander
maria.genander@ki.se

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INTRODUCTION

Hair follicles (HFs) undergo phases of destruction and regeneration throughout the lifespan of an organism. Regeneration and hair formation depend on balanced stem cell renewal and differentiation, integrating transcriptional and epigenetic regulation with microenvironmental niche-derived cues. Failure to coordinate signaling, or respond to inflammatory signals, deregulates hair follicle lineage progression, abrogates regeneration, and commonly leads to alopecia. Posttranslational modifications are enzymatically catalyzed amino acid alterations, which constitute a non-genetic mechanism for modulating protein function. Whereas our understanding of how posttranslational modifications affects stem cell lineage progression and disease progression is far from complete, extensive work identifying histone modification "codes" acting to maintain cell states as well as to ensure proper lineage progression, suggest that the impact of protein modifications requires further investigation.

Here, we focus on how citrullination contributes to hair follicle lineage progression during regeneration, and how citrullination-mediated inflammation acts to perpetuate disease progression in inflammatory alopecia. We hope that this work will inspire scientists to explore new citrullination paved avenues towards the understanding of skin and skin disease.

Peptidylarginine Deiminases Catalyse Citrullination

Protein citrullination, or deimination, is the conversion of the positively charged amino acid arginine to neutral citrulline by replacement of the arginine side chain imine with an ureido

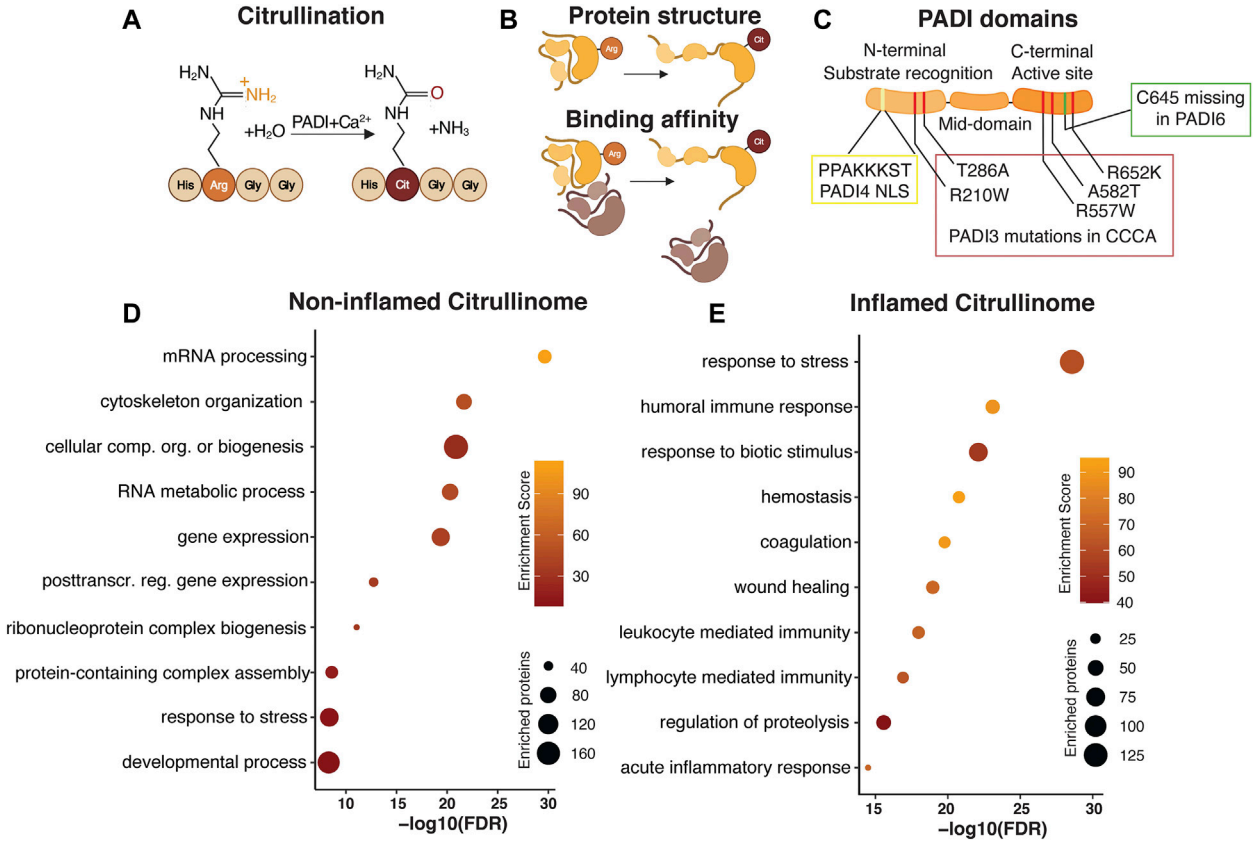

FIGURE 1 | Citrullination as a dynamically regulated posttranslational modification. Citrullination, the Peptidylarginine deiminase (PADI) dependent conversion of arginine to citrulline **(A)** is an irreversible posttranslational modification. PADIs require the presence of calcium for the enzymatic activity, which acts to replace the amide group of the arginine side chain with oxygen, yielding ammonia as a side product. Replacing the positively charged arginine with neutral citrulline alters the protein conformation and binding affinity **(B)**. PADI enzymes are highly conserved with an N-terminal domain mediating substrate recognition, a middle domain, and a C-terminal domain where the active site resides. The N-terminal domain in PADI4 contains a nuclear localization signal (NLS). Human *PADI3* mutations associated with CCCA are mapped to both the N- and C-terminal domains. Cysteine 645, essential for active site catalytic activity is missing in PADI6 **(C)**. Gene Ontology analysis of published human citrullinomes display distinct enrichment profiles in non-inflamed compared to inflamed tissue **(D,E)**.

group (**Figure 1A**). Peptidylcitrulline, the presence of the non-essential amino acid citrulline, is thus not a product of translation but generated *via* enzymatic alteration of an existing peptide.

Catalysing citrullination are the highly conserved, calcium dependent, Peptidylarginine deiminase enzymes. There are currently five different mammalian PADIs described, namely PADI1, 2, 3, 4, and 6. PADIs generally require calcium binding to bring about conformational changes that generate the active cleft and enzymatic activity. Once active, PADI enzyme activity alters the overall charge, conformation and function of the target protein (**Figure 1B**) (Vossenaar et al., 2003). Although PADIs display up to 95% of sequence homology (Mechin et al., 2007) they show distinct tissue expression, localization and dimerization ability (Vossenaar et al., 2003), all which collectively affects protein substrate specificity. Whereas PADI1, 3 and 6 localizes to the cytoplasm, PADI2 and PADI4 can shuttle to the nucleus. In addition, PADI2/3/4, but not PADI1, attain full enzymatic activity only after a head-to-tail (N-to-C domain) homodimerization (Arita et al., 2004; Lee C.-Y. et al., 2017; Saijo et al., 2016; Slade et al., 2015). Interestingly, PADI6 lacks several calcium binding sites as well as the active cleft amino acid cysteine 645, which are conserved in the other family members (Witalison et al., 2015) and is unable to citrullinate substrates which are readily citrullinated by the other PADIs *in vitro* (Knuckley et al., 2010) suggesting that the lack of C645 renders PADI6 enzymatically inactive (**Figure 1C**). It is however possible that PADI6 has different substrate preferences or requires additional cofactors than calcium to function (Raijmakers et al., 2007). PADI6 homodimerization is yet to be confirmed.

The catalysation of citrullination commonly either antagonizes or facilitates other types of posttranslational modifications, such as methylation and acetylation (Cuthbert et al., 2004; Denis et al., 2009), thereby extending the functional impact of citrullination well beyond single arginine residue modification. Interestingly, whereas other posttranslational modifications are reversible, no de-citrullinating enzyme is yet described,

suggesting that removal of citrullination is linked to protein turnover.

Mining the Citrullinome to Understand Function

Most work detailing citrullination have reported the identification of single citrullination residues, and successfully elucidated the functional impact of the isolated modification on that particular protein in a specific context. In more recent attempts to identify cellular processes influenced by the enzymatic action of PADIs, the citrullinome, or proteome-wide citrullination signature, of distinct cell types, organs and disease conditions have been characterized by mass spectrometry. Focusing on human citrullinomes, we analysed all citrullinated proteins identified in normal non-inflamed cells and tissues (Lewallen et al., 2015; Lee et al., 2018; Tanikawa et al., 2018) and compared their functional classification to citrullinated targets found in inflammatory disease (370 and 251 unique proteins respectively) (van Beers et al., 2013; Tutturen et al., 2014; Tilvawala et al., 2018). Selecting for the 10 most enriched, and unique, gene ontology terms reveal striking differences in biological processes associated with, and hence likely to be influenced by, citrullination. Whereas citrullination in non-inflamed tissue is associated with, but not limited to, mRNA processing, gene expression and cytoskeletal organization, protein citrullination in inflammatory disease is centred around stress and immune responses (**Figures 1D,E**). It is interesting that the biological processes linked to citrullination are distinct in normal and inflamed tissue, hinting at key cellular functions where citrullination is required for maintaining cell identity in non-inflamed tissue, as well as identifying aspects of citrullination-dependent biology which could be explored for therapeutic purposes during inflammation. Collectively, this meta-analysis indicates that the citrullinome is not a static entity, but rather dynamically defined, relying on context and cell type specific determinants to distinguish the biological processes influenced by citrullination.

How PADIs Find Their Targets – What we Know About Substrate Specificity

Despite the abundance of protein arginine, not all proteins, and far from all arginine residues become citrullinated. How PADI enzymes determine substrate specificity, and if substrate specificity is altered during disease, like inflammatory alopecia, is largely unknown.

PADI1/3 mediated citrullination in the HF is associated with remodeling of intermediate filaments, usually by affecting filament polymerization, susceptibility to cross-linking or proteolytic enzymes and dimerization abilities (Kizawa et al., 2008; Briot et al., 2020), functionality required for hair shaft differentiation. Interestingly, many PADI1/3 known substrates belong to the S100-fused protein family (filaggrins, hornerin, trichohyalin and S100A3), hinting at an overlapping preference based on function and/or structure for substrate specificity (Tarcsa et al., 1997; Knuckley et al., 2010). However, *in vitro* citrullination of S100A3 demonstrates that PADI1 and PADI3 citrullinate distinct S100A3 arginine residues (Kizawa et al., 2008), suggesting that PADI1/3 citrullination specificity is more complex than general affection for S100-fused proteins. If PADI enzyme substrate specificity is altered during the development of inflammatory alopecia is a matter of speculation, however pro-inflammatory cytokines have been demonstrated to inhibit epidermal PADI1 expression, subsequently reducing citrullination of KRT1 and contributing to remodeling of the inflamed epidermis (Padhi et al., 2021). In addition, proteomic analyses demonstrate epidermal specific citrullination profiles in atopic dermatitis (Winget et al., 2016), suggesting that alterations of both enzyme and substrate abundance are common features of skin inflammation.

Whereas arginine residues on all histones tails have been shown to be citrullinated (Cuthbert et al., 2004; Wang et al., 2004; Hagiwara et al., 2005), only H1 and H3 residues (H1R54 and H3R2/R8/R26) are jointly citrullinated by PADI2 and PADI4 (Christophorou et al., 2014; Falcao et al., 2019). Similarly, only a minority of PADI2 and PADI4 non-histone targets are shared (Tanikawa et al., 2018), suggesting that the function of PADI enzymes is largely non-redundant. Clues to enzyme specificity come from work identifying an RG/RGG motif in a subset of PADI4 targets (Tanikawa et al., 2018) indicating that binding motifs determining enzyme target specificity may exist.

Functionally, the outcome of histone citrullination is diverse. Citrullination of linker histone H1 (H1R54) result in chromatin de-condensation and transcriptional activation (Christophorou et al., 2014; Guertin et al., 2014), whereas H3 citrullination (H3R2, R8, R17 and R26) is associated with both transcriptional repression and activation, likely by counteracting activating methylation marks or by recruitment of additional histone-modifying enzymes (see **Figure 2**). Furthermore, PADI2/4 mediated citrullination of transcription factors (Kolodziej et al., 2014; Ghari et al., 2016; Sun et al., 2017; Sun et al., 2019) or epigenetically active enzymes (Lee et al., 2005; Christophorou et al., 2014; Deplus et al., 2014) allows PADIs to influence cell states independent of histone citrullination.

Skin immune cell infiltration is a hallmark of inflammatory alopecia. Activated neutrophils and T-cells express enzymatically active PADI2 and PADI4 which contributes to several aspects of the inflammatory process. For example, H3R8 citrullination at pro-inflammatory TNF-α and IL-8 promoters drive cytokine expression (Sharma et al., 2012) in activated T-cells. In addition, direct citrullination of IL-8 and TNF-α proteins modulates their pro-inflammatory effect (Proost et al., 2008; Moelants et al., 2013) highlighting the many ways cell state and type can influence citrullination specificity.

Even though we do not fully understand PADI enzyme substrate specificity, it is clear that individual enzymes display distinct substrate preferences. Although tissue and disease-associated alterations in the citrullinome correlate to enzyme activity and substrate abundance, aspects of substrate recognition may be context-dependent, relying on, for example, a tissue-specific set of co-factors.

Citrullination and Stem Cell Renewal

Here we aim to delineate the function of PADIs from a hair follicle-centric perspective, using the wealth of existing hair follicle stem cell (HFSC) expression profiling as the starting point for understanding the impact of citrullination on HFSC lineage progression. A functional role for PADIs in epidermal differentiation is well established and comprehensively summarized elsewhere (Cau et al., 2018; Mechin et al., 2020).

As hair follicles (HFs) initiate regeneration, primed hair germ stem cells are the first to divide, followed by proliferation of bulge HFSCs (Greco et al., 2009). Whereas profiling of resting HFs reveals little evidence of *Padi* expression, mRNAs of both *Padi3* and *Padi4* are found in activated hair germ and bulge stem cells (Lien et al., 2011) and *Padi3/4* chromatin accessibility is increased in regenerating compared to resting hair germ (Adam et al., 2018), suggesting that PADI expression, and potentially function, is coupled to HFSC activation and self-renewal. Whilst there is no functional genetic evidence available for the role of *Padi3/4* in HFSCs, *Padi4* is part of the hematopoietic stem cell self-renewal gene signature (Nakashima et al., 2013; Young et al., 2021), and *Padi4* KO mice display increased hematopoietic stem cell proliferation (Young et al., 2021) indicating a PADI4-dependent program maintaining the stem cell pool by restricting proliferation and self-renewal. Work in embryonic stem cells reveal that PADI4 is required for maintaining pluripotency and loss, or chemical inhibition, of PADI4 leads to arrested embryo development, reduced expression of pluripotency genes and skewing of fate towards differentiation (Kan et al., 2012; Christophorou et al., 2014; Zhang et al., 2016). It is possible that the presence of PADI3/4 in activated HFSCs act complementary to restrict proliferation and maintain the stem cell pool during hair follicle regeneration.

Interestingly, PADI4 associates with, and citrullinates, *de novo* DNA methylases DNMT3A and DNMT3B (Christophorou et al., 2014; Deplus et al., 2014). DNMT3A and DNMT3B are found in HFSCs located in the bulge and in the outer root sheet during hair follicle regeneration (Rinaldi et al., 2017; Joost et al., 2020). Whereas HFSC expression of *Dnmt3a/b* increases during telogen-to-anagen transition (Lien et al., 2011), no significant *Dnmt3a/b* expression is found in progenitor or differentiated lineages of the regenerating HF, indicating that the function of DNMT3A/B is restricted to HFSCs. DNA methylation signatures of quiescent and activated HFSCs are distinct and sufficient to define the two stem cell states (Bock et al., 2012), suggesting that the DNMT3 activity is dynamically regulated during HFSC activation and subsequent initiation of HF growth. PADI4 mediated citrullination of arginine residues in the DNMT3A nuclear localization signal leads to increased DNMT stability, methyltransferase activity (Deplus et al., 2014; Sun et al., 2017; Zeng et al., 2020) and likely affects DNMT3A binding affinity to other proteins (Guo and Fast, 2011; Stadler et al., 2013), indicating that PADI4 could influence global DNA methylation patterns by targeting DNA methylases in activated HFSCs.

Loss of DNMT3A in quiescent HFSCs fails to induce HFSC activation (Chen et al., 2021). However, human epidermal stem cells devoid of either DNMT3A or DNMT3B display reduced self-renewal and increased differentiation upon grafting (Rinaldi et al., 2016). It is possible that *de novo* methylation is largely dispensable in quiescent HFSCs, however required when HFSCs are challenged to self-renew during HF regeneration. If so, PADI4-mediated citrullination in renewing HFSCs could act to modulate and/or physically direct DNMT3 methylase activity, ensuring balanced stem cell renewal and proliferation during initiation of hair follicle regeneration.

Expression and Function of PADIs in Regenerating Hair Follicles

Activation and expansion of hair germ stem cells leads to the formation of the hair bulb, consisting of progenitor cells which specify into subpopulations of lineage committed progenitors, most of which eventually exit the cell cycle and fuel the differentiated hair lineages. Single cell RNA-sequencing place *Padi1*, *Padi3* and *Padi4* in discrete cell lineages during hair differentiation (Joost et al., 2020). Whereas *Padi1/3* expression is mapped to the differentiated inner root sheath (IRS) and medulla lineages, *Padi4* is preferentially expressed in the cortical hair shaft and medulla lineages (Adam et al., 2018; Joost et al., 2020). In addition, *Padi3* and *Padi4* are turned on in the hair bulb progenitor population, likely before lineage commitment (Nachat et al., 2005; Adam et al., 2018; Joost et al., 2020) and judging from the mutually exclusive expression pattern in IRS and cortical lineages, it is possible that PADI3 and PADI4 are involved in the specification of hair shaft progenitors.

Interestingly, *Padi3* and *Padi4* chromatin is bound by combinations of BMP and WNT lineage effectors in HF progenitor and committed lineage cells. Whereas PADI3 is induced in response to Vitamin D Receptor and LEF1 (independent of β-Catenin activation) (Palmer et al., 2008), LEF1 ChIP-sequencing in HF progenitor cells identifies *Padi4*, but not *Padi3*, as a LEF1 target gene (Adam et al., 2018). Furthermore, pSMAD1/5 binding sites are located to both *Padi3* and *Padi4* promoters (Genander et al., 2014), indicating that aspects of WNT signaling, in cooperation with BMP is required for fine-tuning of *Padi3* and *Padi4* expression in the regenerating hair follicle. Collectively, these data suggest that expression of PADI3/4 is under the control of specific subsets of hair follicle lineage effectors, potentially acting to further refine or lock lineage specification by mediating PADI expression.

Analogously, PADI2 skews effector T-cell specification towards a Th17 fate at the expense of Th2 (Kawalkowska et al., 2016; Sun et al., 2019), mechanistically by citrullination of arginine residue R330 located in the DNA binding domain of the transcription factor GATA3. Citrullinated GATA3 fails to bind DNA efficiently, resulting in reduced expression of GATA3 target genes and subsequent Th lineage fine-tuning (Sun et al., 2019) (**Figure 3**). Interestingly, GATA3 is required for IRS progenitor specification (Kaufman et al., 2003; Genander et al., 2014) indicating that co-expression of PADI4 and GATA3 in a subset of progenitor cells could act to inhibit the transcriptional activity of GATA3, hence favoring hair shaft over IRS lineage specification.

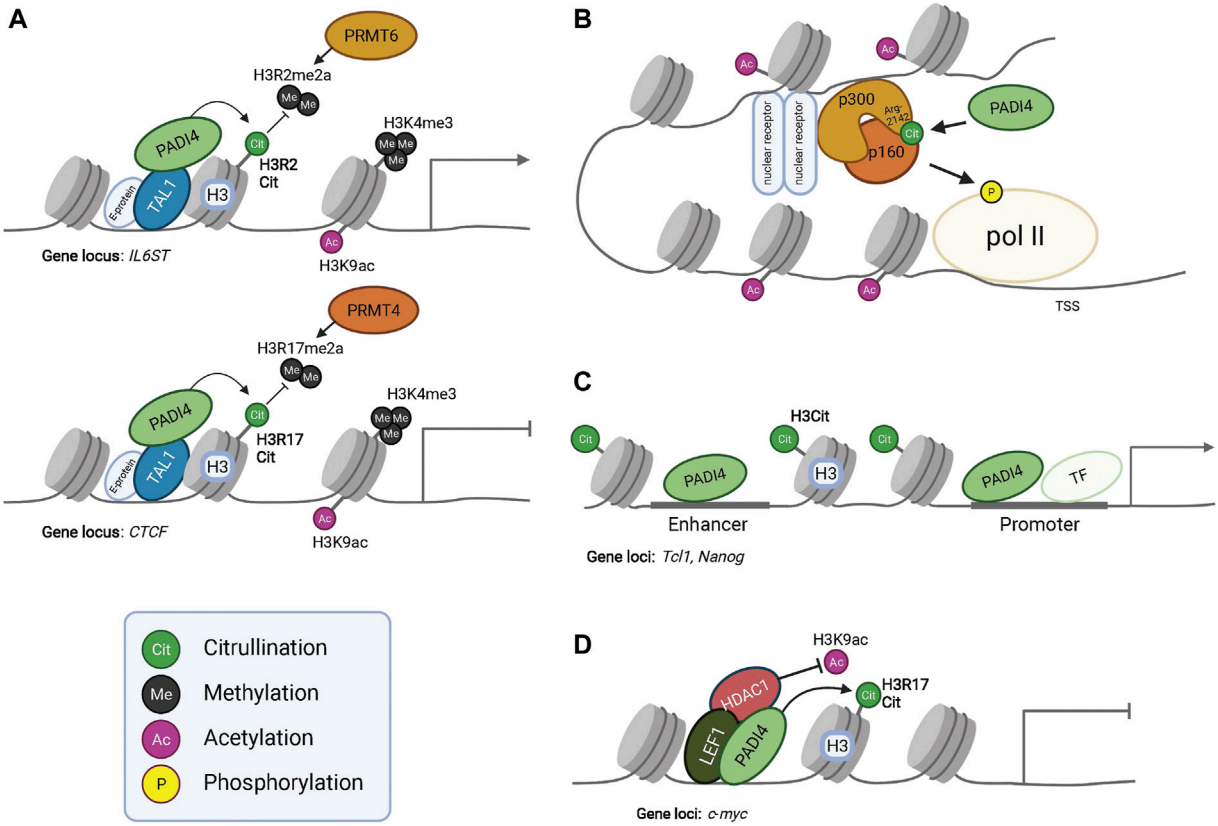

FIGURE 2 | PADI dependent modulation of epigenetic and gene expression programs. PADI4 interacts with transcription factor TAL1 and citrullinates histone H3 to regulate gene expression. Citrullination of H3R2 results in antagonism of PRMT6 mediated methylation at this residue, resulting in the activation of IL6ST transcription **(Upper panel** in **A)**. Citrullination of H3R17 antagonizes PRMT4 mediated methylation and represses gene transcription of CTCF **(Lower panel** in **A)**. PADI4 can in this way function both as a repressor or activator of gene expression, given the context and binding partners. However, PADI4 always antagonizes the function of PRMTs. The active transcription mark H3K9ac is present in both conditions but at much lower levels at the CTCF locus, indicating interplay between histone tail modifications for transcriptional regulation. **(B)** Citrullination of acetyltransferase p300 at residue R2142 by PADI4 facilitates its dimerization with p160, leading to histone acetylation and eventually phosphorylation of Polymerase-II (pol II) for activated gene transcription. Additionally, citrullination of R2142 antagonizes its methylation by PRMT4 (CARM1) (not shown), exemplifying the regulatory effects of the subtle yet significant post-translational modifications. **(C)** Occupancy of PADI4 in combination with histone H3 citrullination at the enhancer and promoter of *Nanog* and *Tcl1* enable gene expression. **(D)** PADI4 can interact with HDAC1 and together bind to LEF1 at the *c-myc* transcriptional start site to repress gene expression. During PADI4-deficiency, HDAC1 fails to bind LEF1 and *c-myc* repression is alleviated.

In addition to citrullination of transcription factors and consequently modulating their binding affinity towards DNA, other co-factors or their subcellular localization (Christophorou et al., 2014; Ghari et al., 2016; Sun et al., 2017; Zhang et al., 2011), PADI4 can, on its own, act as a transcriptional regulator. For example, PADI4 interacts with transcription factor TAL1 at gene promoters in a leukemic cell line (**Figure 2A**) (Kolodziej et al., 2014) acting to facilitate activation as well as silencing of lineage determining genes, likely by recruiting distinct co-factors at different subsets of genes.

PADIs in Hair Follicle Lineage Differentiation

Although *Padi1/3* are co-expressed in the differentiated inner root sheath and medulla lineages, *Padi1* expression is significantly lower than that of *Padi3,* identifying PADI3 as the likely main contributor of protein citrullination in the IRS and medulla. PADI1/3 protein localization correlates well with mRNA expression, as well as global citrullination pattern in both human and mouse hair follicles (Nachat et al., 2005; Kizawa et al., 2008; Palmer et al., 2008), supporting the spatial resolution of the transcriptional profiling. It is not clear what regulates *Padi1* expression in the hair follicle, however *in vitro* data suggests Vitamin D signaling to be upstream of *Padi1* transcription in the epidermis (Hu et al., 2014; Padhi et al., 2021).

Functionally, PADI3 has been shown to citrullinate trichohyalin (TCHH) (Rogers et al., 1977; Tarcsa et al., 1996; Tarcsa et al., 1997), a structural protein abundantly expressed in the IRS and medulla. It is believed that PADI1/3-mediated citrullination of trichohyalin provide mechanical strength and enable air entrapment for thermal insulation in the IRS and medulla, respectively (Steinert et al., 2003). Citrullination allows trichohyalin solubilization from granules and subsequent transglutaminase-3 (TGM3) mediated crosslinking to keratins, further contributing to the hair shaft structure (Tarcsa et al., 1997). Other established citrullinated proteins expressed in the HF include the shared PADI1/3 structural protein target S100A3

(Kizawa et al., 2008) also associated with hair shaft differentiation.

Human *PADI3* mutations are linked to uncombable hair syndrome, manifesting as frizzy and fair hair resistant to combing flat (Basmanav et al., 2016), and Central centrifugal cicatricial alopecia (CCCA), a type of scarring alopecia found predominantly in women of African ancestry (Malki et al., 2019). *PADI3* mutations affect both protein folding and enzymatic activity, which manifests functionally (**Figure 1C**). Uncombable hair syndrome is caused by the lack of crosslinking of intermediate filaments in the IRS (Tarcsa et al., 1997) and CCCA patients display reduced levels of both trichohyalin and S100A3, suggesting that reduced PADI3-mediated citrullination affects both target function and stability in the human hair shaft.

In contrast to the enrichment of PADI3 found in the IRS, *Padi4* is preferentially expressed in the cortical hair shaft lineage together with the medulla (Adam et al., 2018; Joost et al., 2020). Whereas PADI4 is associated with self-renewal and stemness in some systems, nuclear PADI expression is also positively linked to differentiation. PADI2 expression drives oligodendrocyte differentiation, and expression is increased with differentiation (Falcao et al., 2019). Similarly, differentiated macrophages display higher PADI2/4 levels compared to the undifferentiated U937 monocyte cell line (Lai et al., 2019). Considering that PADI2/4 can have common targets (Tanikawa et al., 2018), it is possible that PADI2 and PADI4 have redundant functions in systems where co-expressed. In contrast, PADI4 is not reported to be able to compensate for PADI3, likely due to their mutually exclusive cellular localization, suggesting that PADI1/3 and PADI4 have unique targets and hence functions in hair follicle lineage differentiation.

PADIs as Modulators of the Epigenetic Landscape

In addition to directly citrullinating DNMTs and affecting DNA methylation patterns, emerging evidence suggest that PADI4, and to some extent PADI2, can through various mechanisms impinge on the epigenetic landscape by modulating enhancer availability as well as histone modifications. Here we delineate a PADI4 mediated epigenetic interplay relevant for hair follicle regeneration.

To allow cell state specific combinations of key transcription factors to drive hair follicle lineage progression, HFSC fate transitions require dynamic remodeling of enhancers (Adam et al., 2015). Interestingly, Lee et al. demonstrated that the enhancer assembly of p300 acetyltransferase transcriptional coactivator complex is controlled by the counteracting effects of PADI4-mediated citrullination and PRMT4-mediated methylation, at arg-2142 residue of p300 (Lee et al., 2005). Methylation of arg-2142 by PRMT4 blocked co-factor p160 binding to p300, thereby inhibiting the acetylation activity of p160/p300 and transcription of target genes. In contrast, citrullination of p300 restored p160 binding *in vitro* and activated target gene transcription (**Figure 2B**) (Shang et al., 2000).

Although the HF might not rely on the steroid receptor p160 as a transcriptional coactivator, the above finding indicates citrullination of p300, a general acetyltransferase, controls both chromatin organization and transcriptional regulation by interfering with co-factor binding. It is possible that LEF1, in accordance with its role in keeping cell-type specific enhancers active during HFSC lineage commitment and lineage diversification (Adam et al., 2018), could act as the HF specific p300 co-activator.

Additionally, ChIP-qPCR in mouse embryonic stem cells revealed that PADI4 can target to enhancers. Occupancy of PADI4 was detected at the enhancer of *Tcl*, *Nanog*, and *Kit*, and H3 citrullination of these loci was sharply reduced upon treatment with the PADI4 inhibitor Cl-amidine, strongly suggesting that PADI4 is an enhancer-associated protein, and its occupancy is functional (**Figure 2C**) (Christophorou et al., 2014). Taken together, these convergent lines of evidence point to a role of PADI4-mediated enhancer regulation.

Deacetylation Act Cooperatively With Citrullination to Regulate Gene Expression

In addition to being a possible transcriptional target of LEF1 and pSMAD1/5, PADI4 has the potential to bind directly to, and influence the function of, LEF1 in progenitor cells as well as differentiated HF lineages. Although LEF1 is known to act as a transcriptional activator in the HF, binding of LEF1 to the histone deacetylase HDAC1 generally leads to LEF1-mediated gene repression (Billin et al., 2000).

Considering the broad HDAC1 expression pattern and general histone and non-histone deacetylase activity during hair follicle growth (Joost et al., 2020), loss of HDAC1 would likely impinge on multiple aspects of hair follicle regeneration. Indeed, loss of HDAC1 (alone, or in combination with HDAC2 to reduce redundancy), affects all epidermal lineages (LeBoeuf et al., 2010; Winter et al., 2013; Hughes et al., 2014). HDAC1 devoid hair follicles formed properly during morphogenesis but failed to enter the first resting phase (Winter et al., 2013; Hughes et al., 2014) resulting in progressive cyst formation and hair follicle atrophy.

Molecular examination revealed that both HFSC and progenitor markers were downregulated, and no hair follicle differentiation markers were expressed in the absence of HDAC1. In contrast, differentiated cysts expressed Involucrin (IVL), a marker normally associated with the epidermal lineage. Interestingly, HDAC1 binds to promoters of epidermal differentiation genes in epidermal progenitor cells (Winter et al., 2013) and upregulation of differentiation markers in the absence of HDAC1 activity correlates with increased histone acetylation levels. Ectopic expression of epidermal differentiation markers in HDAC1 devoid HF cysts suggests that HDAC1 suppresses (epidermal) lineage fate also in hair follicles.

PADI4 is described to interact with HDAC1 in several cell types (Denis et al., 2009; Nakashima et al., 2013) and gene manipulation experiments demonstrate that histone citrullination and deacetylation activity are functionally linked

in regulating promoter activity. For example, PADI4 and HDAC1 simultaneously bind the pS2 promoter, resulting in acquisition of H3 arginine citrullination, loss of H3 arginine methylation and RNA polymerase II binding during the pS2 transcriptional clearing phase. In contrast, knockdown of *Padi4* and *Hdac1* by RNAi resulted in reduced H3 citrullination, increased H3 acetylation, and acquisition of H3R2 and H4R3 dimethylation marks. Interestingly, downregulation of *Hdac1* alone resulted in less pronounced epigenetic alterations, demonstrating that PADI4 and HDAC1 work cooperatively to favor citrullination and deacetylation of the pS2 promoter (Denis et al., 2009). In support of the synergistic functionality of citrullination and deacetylation protein modification, recent work demonstrate compensation on a transcriptional level – knock down of *Hdac1/2* leads to transcriptional upregulation of *Padi4*, whereas loss of *Padi4* upregulates *Hdac1/2* expression (Winter et al., 2013; Liu et al., 2018).

Given the restricted HF expression of PADI4, it is possible that PADI4/HDAC1 function as a transcriptional repressor complex to prevent aberrant expression of lineage unrelated genes in the hair follicle, potentially by interacting with lineage specific transcription factors such as LEF1. In hematopoietic stem cells, PADI4/LEF1/HDAC1 co-occupy the upstream region of the *c-Myc* transcriptional start site and silence gene expression *via* citrullination and deacetylation (**Figure 2D**) (Nakashima et al., 2013). However, in the absence of PADI4, HDAC1 fails to associate with LEF1 and *c-Myc* repression is alleviated. Considering LEF1's known role in HS lineage specification and differentiation (Merrill et al., 2001; Adam et al., 2018), it is plausible to speculate that a PADI4/LEF1/HDAC1 complex would act to restrict hair shaft specification and/or differentiation by sequestering and functionally transforming the gene regulatory activity of LEF1. Nevertheless, the possibility of PADI4/HDAC1 co-regulation of hair shaft genes should not be ruled out. Although PADI4/HDAC1 has only been reported to repress transcription, this complex may also support gene expression in a context-dependent manner, such as in cooperation with transcriptional coactivators at hair shaft gene loci.

PADIs as Modulators of Histone Methylation

In addition to the ability of PADI4 to contribute to the epigenetic landscape by modulating histone acetylation and DNA methylation, a growing number of studies reports functional interplay between histone arginine citrullination and methylation, either by competing for the same arginine or through citrullination-dependent methylation on lysine residues. In addition, PADI4 was the first enzyme identified participating in reversing histone mono-methylation through deamination (Cuthbert et al., 2004; Hidaka et al., 2005; Raijmakers et al., 2007; Yanming et al., 2004). Therefore, the function of PADI4 generally opposes arginine methyltransferases, such as those in the Protein arginine methyltransferases (PRMT) family (**Figure 2A**). Several PRMTs are expressed broadly in the regenerating hair follicle (Joost et al., 2020), where they methylate distinct arginine residues on both histone tails and transcriptional coactivators, but PRMT1 is the only family member whose function has been investigated in the skin.

PRMT1 is prominently expressed in both the HF and epidermal progenitor cells (Bao et al., 2017; Joost et al., 2020) and is required for maintaining epidermal progenitor cells by supporting the expression of proliferation genes and silencing genes associated with differentiation, either by activating H4R3me2 or by functioning as a transcriptional co-factor. Loss of PRMT1 leads to depletion of the self-renewing epidermal progenitor population due to premature differentiation. PADI4 expression in hair follicle progenitor and hair shaft lineage cells could act to counterbalance PRMT1-induced H4R3me2 marks, favoring differentiation over proliferation and thereby modulating either progenitor pool size or induction of differentiation in the hair shaft lineage.

In addition, PRMT5-mediated dimethylation of H3R2 and H3R8 act to antagonize H3K27me3 repressive mark deposited by Polycomb-repressive complex (PRC) 2 (Liu et al., 2020). Both normal and leukemic hematopoietic cells deficient in *Prmt5* gained H3K27me3 globally, resulting in downregulation of targeted genes. These observations suggest PRMT5 sustains gene expression and cell proliferation by counteracting PRC2-mediated H3K27me3 transcriptional repression in the context of hematopoietic cells. In the regenerating hair follicle, PRC-mediated H3K27me3 repression acts as a transcriptional switch, governing the differential expression of HFSC and progenitor cell genes to enforce maintenance as well as transition between stem cell and progenitor states (Lien et al., 2011). Since the catalytic activity of the PRC2 complex is PRMT5 independent and histone modifications have been shown to have an allosteric effect on histone modifiers (Moritz and Trievel, 2018), PRC2-catalyzed H3K27me3 may be impaired by the presence of H3R2me2s or H3R8me2s deposited by PRMT5. PADI4 would hence act to counteract PRMT-mediated transcriptional programs by directly antagonizing arginine dimethylation, thereby indirectly preventing ectopic activation of PRC2-silenced genes.

Alopecia – Inflammation Mediated Hair Loss

Considering the complexities of hair follicle formation, it is perhaps surprising that hair follicles are at all able to faithfully regenerate. In addition, hair follicle regeneration is continuously challenged by injurious and infectious insults. Despite this, an infection rarely elicits systemic responses but is contained at the site of transgression, a feature likely stemming from several intricate defence mechanisms employed by the hair follicle epithelium (Christoph et al., 2000; Paus et al., 1998). Failure to respond to injury or contain infection does however, through different mechanisms, commonly lead to hair follicle loss, or alopecia. Inflammatory alopecia results in the loss of hair follicles and/or hair shafts (Bolduc et al., 2016a, b; Harries and Paus, 2010). Inflammation is mediated by an innate (neutrophilic) and adaptive (lymphocytic) cellular component and it is likely that the

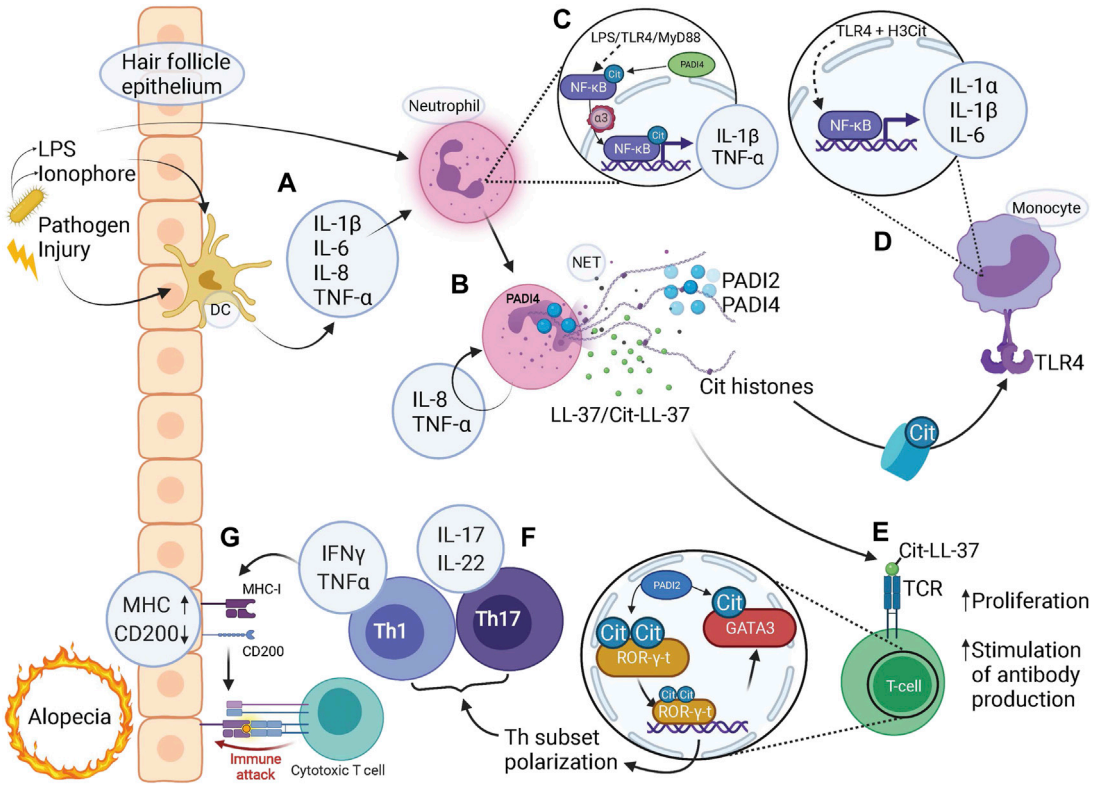

FIGURE 3 | Citrullination-mediated inflammation contributes to alopecia. **(A)** Microbial or damaging insults stimulate tissue residing dendritic cells (DC) to release pro-inflammatory cytokines which in turn activates neutrophils. Pathogen-derived LPS and ionophores can also act to directly stimulate neutrophils *via* TLR4-binding or influx of calcium, respectively. **(B)** NETs released *via* PADI4-dependent pathways by activated neutrophils contain citrullinated (Cit) histones, PADI2, PADI4, LL-37 and citrullinated LL37 (along with granular components such as MPO and NE, not shown). **(C)** LPS binding to TLR4, acting *via* MyD88, activates NF-κB signaling. Citrullination of NF-κB by PADI4 enhances its interaction with importin-α3 which mediates nuclear translocation of NF-κB, with expression of pro-inflammatory target genes IL-1β and TNF-α. **(D)** Citrullinated histones bind to TLR4 and *via* NF-κB signaling elicit a strong inflammatory response by release of pro-inflammatory cytokines IL-1α, IL-1β, and IL-6. **(E)** Citrullinated (Cit)LL-37 generated during NET release is a strong TCR agonist, which enable increased T-cell proliferation and facilitates T-cell stimulated production of anti-cit/native cross-reactive LL-37 autoantibodies. **(F)** Citrullination of transcription factors ROR-γ-t and GATA3 in T-helper (Th) cells skews cell fate towards Th1 and Th17 subsets with associated pro-inflammatory cytokine profiles. **(G)** A pro-inflammatory milieu coerces the hair follicle epithelium to upregulate MHC class-I to present autoantigens as well as downregulate "no-danger" signal CD200, resulting in a collapse of immune privilege. Given the activated state of the adaptive immune system, represented by T-cells, an immune attack is imminent, bringing about either destruction or exhaustion of HFSCs resulting in alopecia. **(A–G)** Note the several steps at which pro-inflammatory cytokines are produced to facilitate an inflammatory environment, involving both the innate and adaptive immune system either on their own or in combination, highlighting the contribution of citrullination at all levels of immunity and inflammation.

interface between the innate and adaptive immunity is critical in the development of alopecia (**Figure 3**).

Upon microbial invasion, epithelial cells in the hair follicle release antimicrobial peptides (Reithmayer et al., 2009) together with pro-inflammatory cyto- and chemokines to attract immune infiltration and clear invasion (**Figure 3A**). Additionally, hair follicle bulge and bulb epithelium reside in a state of relative immune privilege, expressing low levels of MHC class I as to prevent the presentation of self-antigens to autoreactive T-cells (Ito et al., 2004), while also producing immunosuppressant molecules such as TGF-β2 and cortisol (Paus et al., 2005; Anzai et al., 2019). Hair follicle stem cells also express CD200, functioning as a "no-danger" signal (Rosenblum et al., 2006) by promoting anti-inflammatory and immune-tolerance signaling to CD200R expressing T-cells and macrophages (Rosenblum et al., 2004).

Collapse of the immune privilege is a likely contributory mechanism leading to alopecia. Lesioned human scalp shows upregulation of MHC class I and II (Harries et al., 2010; Paus and Cotsarelis, 1999) and downregulation of CD200 (Rosenblum et al., 2004; Rosenblum et al., 2006) in the bulge (HFSCs), corroborated by the deletion of CD200 that in a mouse model resulted in peri-follicular immune cell infiltration and subsequently alopecia. Furthermore, pro-inflammatory cytokine INF-γ stimulation leads to MHC-I upregulation in the bulge, bulb, and matrix regions (Ito et al., 2004), potentiating a breakage of tolerance. The source of IFN-γ is predominantly activated T-cells and macrophages, emphasising the requirement of a pro-inflammatory environment preceding the lymphocytic infiltrate observed in alopecia (**Figure 3G**). Nonetheless, once the immune privilege is compromised, the

hair follicle's defence strategies are limited – premature initiation of catagen, dystrophic anagen (shedding of hair shaft), or replacement of epithelium with fibrous tissue – resulting in the onset of alopecia (Paus and Cotsarelis, 1999; Harries and Paus, 2010). Destructive immune assault to the bulge results in irreversible alopecia, such as Central centrifugal cicatricial alopecia (CCCA), due to depletion of the HFSC pool (Jaworsky et al., 1992; Pozdnyakova and Mahalingam, 2008; Al-Refu et al., 2009). In contrast, alopecia areata is characterized by an immune infiltrate residing in the bulb progenitor cell region (Gilhar et al., 2007; Gilhar et al., 2012), sparing HFSCs and allowing for hair follicle regeneration once inflammation resolves.

Mutations Affecting Citrullination Linked to Alopecia

Interestingly from a citrullination point of view, a human genetic variant of *PTPN22* (*Protein Tyrosine Phosphatase Non-Receptor Type 22*) is associated with severe forms of alopecia areata (Kemp et al., 2006; Betz et al., 2008), a finding which was further corroborated by two independent genome-wide association studies, which identified susceptibility loci in *PTPN22* in alopecia areata patients (Martinez-Mir et al., 2007; Petukhova et al., 2010). Physical interaction of PTPN22 with PADI4 in immune cells sequesters PADI4 enzymatic activity, whereas PTPN22-deficiency results in hypercitrullination and a higher propensity for neutrophil activation to form neutrophil extracellular traps (NETs, discussed below) (Chang et al., 2015). Additionally, the C1858T *PTPN22* polymorphism associated with alopecia areata (Betz et al., 2008; Petukhova et al., 2010) was shown to be directly linked with the inability of PTPN22 to suppress PADI4 activity, and enhance inflammation-stimulated NET formation in rheumatoid arthritis (Chang et al., 2015). Apart from calcium, PTPN22 is thus far the only identified biological regulator of PADI4 activity.

As previously discussed, human *PADI3* mutations are associated with Central centrifugal cicatricial alopecia (CCCA), a scarring alopecia developing in scalp regions exposed to repetitive mechanical stress and as a consequence, inflammation (Malki et al., 2019). In contrast to the mechanism described for PTPN22 in directly affecting PADI4 activity in neutrophils, mutations in *PADI3* reduce the citrullinating activity in the hair follicle itself, thereby affecting protein target stability. CCCA patients display lower expression of trichohyalin and S100A3, known PADI3 targets in the hair follicle, which in turn affects the hair structure and resistance to mechanical stress of the hair shaft. Carriers of *PADI3* mutations hence display increased susceptibility to inflammation-induced hair loss due to intrinsic structural hair shaft defects caused by reduced citrullination.

Neutrophil Activation – Role of PADI4

Neutrophils are principal effectors of the innate immune system and are the first immune cells to migrate to a site of infection or damage (Kolaczkowska and Kubes, 2013). Neutrophils act to kill microorganisms as well as modulating the immune response predominantly through the release of protein containing neutrophilic granules (Papayannopoulos, 2018), and Neutrophil Extracellular Traps (NETs), a mesh-like structure composed of decondensed chromatin containing histones and antimicrobial agents (Brinkmann et al., 2004). NETs function to physically immobilize and neutralize invading pathogens but have also been shown to contribute to the pathology of autoimmune and inflammatory diseases (Garcia-Romo et al., 2011; Knight et al., 2013; Lowes et al., 2014; Lee K. H. et al., 2017; Chiang et al., 2019; Dinallo et al., 2019; Fert-Bober et al., 2020; Yang et al., 2021).

Although citrullinated histones have long been considered a hallmark of NETs (Brinkmann et al., 2004), and PADI4 an essential driver of NET formation (Wang et al., 2009; Li et al., 2010; Hemmers et al., 2011; Lewis et al., 2015; Thiam et al., 2020), opposing studies showed that NETs could form without the presence of citrullinated histones, suggesting NET formation can be PADI4-independent (Parker et al., 2012; Konig and Andrade, 2016; Kenny et al., 2017). More recent work delineating the molecular mechanisms of NET formation revealed distinct PADI4 dependent and independent pathways generating NETs, stemming from the type of activating agent stimulating the neutrophil to generate NET (Boeltz et al., 2019; Rosazza et al., 2021).

Nevertheless, neutrophils do express high levels of PADI2 and PADI4 (Asaga et al., 2001; Darrah et al., 2012; Zhou et al., 2017), and NET-associated citrullination is highly correlated with autoinflammatory disease (Kessenbrock et al., 2009; Khandpur et al., 2013; Van Avondt and Hartl, 2018; Castanheira and Kubes, 2019) indicating that whether PADI-activity is essential for NET formation or not, citrullination contributes to NET-associated inflammation.

Citrullination Orchestrates a Self-Perpetuating Inflammatory Environment

It is likely that even minor inflammatory events cause tissue residing macrophages or mast cells to release proinflammatory cytokines such as IL-1β, IL-6, IL-8, TNF-α and IFN-γ, which in addition to pathogen-derived ionophores and lipopolysaccharides (LPS) (Thiam et al., 2020), are able to stimulate PADI4 activity and NET formation in neutrophils (**Figure 3A**) (Neeli et al., 2008; Liu et al., 2017; Papayannopoulos, 2018; Schon and Erpenbeck, 2018; Klopf et al., 2021). Once stimulated, neutrophils themselves induce expression of proinflammatory IL-8 and TNF-α, further fuelling neutrophil activation and NET release in a positive feed-back loop (**Figure 3B**).

The expression of pro-inflammatory cytokines is known to be regulated by NF-κB signaling, a transcriptional effector whose activity is influenced by direct citrullination in immune cells, as well as indirectly by the presence of citrullinated H3 in NETs. Interestingly, LPS-induced neutrophil activation leads to endogenous NF-κB

dependent expression of pro-inflammatory TNF-α and IL-1β (**Figure 3C**) (Sun et al., 2017). In this study, PADI4 was found to citrullinate NF-κB at four sites within the p65 Rel homology domain. Citrullination enhanced p65 interaction with Importin α3, facilitated p65 nuclear localization and augmented transcription of target genes IL-1β and TNF-α.

Additionally, Tsourouktsoglou et al. (2020) were able to generate NETs both in the presence and absence of PADI4. Fragmented nucleosomes expulsed during NET formation in neutrophils were sequestered by monocytes, bound Toll-like receptor (TLR) 4 and activated NF-κB-dependent expression of pro-inflammatory cytokines IL-1β, IL-1α, and IL-6. Interestingly, PADI4-mediated H3 citrullination increased H3 binding to TLR4 and potentiated the pro-inflammatory environment. In contrast, PADI4-deficiency reduced inflammation although NETs were still generated (**Figure 3D**). These proinflammatory cytokines are in turn potent activators of NF-κB expression (Shao et al., 2019), indicating the importance of self-perpetuating mechanisms in establishing a pro-inflammatory environment.

The Antimicrobial Peptide LL-37 is Found in NETs and Contributes to Inflammation

The Cathelicidin antimicrobial peptide LL-37 is found in NETs where it binds DNA, forming an immunogenic complex insensitive to DNAse I (Pinegin et al., 2015), thereby preventing efficient chromatin clearing and contributing to the pro-inflammatory effects of NETs (Hakkim et al., 2010). LL-37 has been shown to trigger T-cell activation and subsequent IFN-γ and IL-17 production (Lande et al., 2014) as well as directly stimulate IFN-γ release in plasmacytoid dendritic cells (Lande et al., 2011), further expanding the inflammatory environment.

PADI2 and PADI4 localized to NETs readily citrullinates LL-37 (Casanova et al., 2020; Chapman et al., 2019) (**Figure 3B**). Citrullinated LL-37 displays impaired microbicidal effects (Al-Adwani et al., 2020; Kilsgard et al., 2012), and is unable to lower the production of IL-6 and IL-8 in virus-infected epithelial cells, (Casanova et al., 2020), suggesting that citrullinated LL-37 is less effective at decreasing an inflammatory environment. Furthermore, work in systemic lupus erythematosus (SLE) (Lande et al., 2020) demonstrate that citLL-37 acts as a stronger T cell receptor (TCR) agonist and generates a more pronounced proliferative response in T-cells at lower concentrations compared to native LL-37, suggesting that the levels of citLL-37 generated during NET release would be sufficient to induce an autoreactive T-cell response (**Figure 3E**). Furthermore, T cells exposed to NETs are more prone to mediating an adaptive immune response (Tillack et al., 2012), placing citrullination-associated features at the interface between innate and adaptive immunity.

PADIs as Regulators of T-Cell Polarization and Cytokine Profile

Activated T-cells are important mediators of hair follicle destruction (Cetin et al., 2009; Bertolini et al., 2020). In addition to being targets of citrullination-dependent activation signaling emanating from neutrophils, T-cells themselves express both PADI2 and PADI4 (Liu et al., 2018; Sun et al., 2019), which functionally contribute to the inflammatory T-cell response (**Figure 3E**).

In a murine SLE model, the deletion of either *Padi2* or *Padi4*, or treatment with PADI-inhibitor Cl-amidine, resulted in decreased Th1 and Th17 cell numbers and production of associated cytokine IFN- γ, while Th2 cell numbers were increased (Kawalkowska et al., 2016; Liu et al., 2018), suggesting that PADI activity modulates Th subset polarization and subsequently the inflammatory cytokine profiles. Mechanistically, PADI2 skew Th polarization towards Th17 fate by simultaneous citrullination of key transcription factors GATA3 and ROR-γ-t. Citrullination of a single arginine residue in GATA3 weakens its DNA-binding abilities, whereas conversely, DNA binding capacity is enhanced by citrullination of ROR-γ-t (**Figure 3F**) (Sun et al., 2019), thereby balancing target gene expression and effectively Th cell fate. Interestingly, Th17 cells and associated cytokine profile have been reported to be enriched within lesions of alopecia areata and correlated with disease severity (Tanemura et al., 2013; Han et al., 2015; Loh et al., 2018). The exact involvement of Th17 cells and the contribution of citrullination within such lesions requires further investigation.

Just as activated neutrophils produce pro-inflammatory TNF-α and IL-8, stimulation of Jurkat T-cells results in increased TNF-α and IL-8 expression through a citrullination dependent mechanism. Sharma et al. showed antagonizing binding of the transcriptional repressor HP1α and PADI4 at *Tnf-α* and *Il-8* promoter regions (Sharma et al., 2012). Citrullination of H3R8 (H3Cit8) disabled binding of HP1α at H3K9me3 residues. Stimulation of the Jurkat cells resulted in increased levels of H3Cit8+K9me3 dually marked histones, and increased expression of TNF-α and IL-8, a phenomenon which was reversed by PADI4 inhibition through Cl-amidine treatment. It is possible that PADI4 antagonizes HP1α-mediated repression of *Tnf-α* and *Il-8* promoters also in activated neutrophils, however this remains to be determined.

TNF-α expression is additionally regulated by NF-κB in a citrullination dependent manner (as discussed above). However the potential synergies between histone citrullination at the *Tnf-α* promoter and direct citrullination of a TNF-α transcriptional effector remains to be investigated. What is clear is however that transcriptional activity within T-cell populations can be fine-tuned by intrinsic citrullination, positioning citrullination as a means to achieve a plethora of actions in both innate and adaptive immunity.

Citrullination – More Than Meets the Eye

Despite an increasing number of reports delineating the protein expression of PADI enzymes, the tools used to identify PADIs and their enzymatic activity are still underdeveloped, adding up to a discordant and sometimes even contradictory picture. Here we have chosen to rely predominantly on mRNA expression for detailing cell populations that express PADI enzymes in the hair follicle and inflammation. In cases where protein characterization is available, mRNA and protein expression profiles largely overlap. However challenging to visualize, there is reason to

believe that the function of these elusive proteins is far more complex than first perceived. Our current understanding of citrullination suggests that the functional outcome of citrullination is context dependent and dynamically regulated. The deiminase activity of PADIs is thus employed by most cells, in one way or the other, to achieve an array of functions.

In view of citrullination as a stable, and possibly irreversible, modification it is intuitive to localize PADIs to differentiated IRS and medulla lineages, where citrullination acts to mediate structural rigidity of the hair shaft. However, citrullinated proteins are also linked to progenitor proliferation and immune cell activation, cell types continuously responding to changing environmental cues, indicating that citrullination can be used by cells in a more multifaceted way than so far appreciated. Functionally, citrullination is seldom an end in itself, but rather acts in conjunction with other types of posttranslational modifications, be it on histones or otherwise. As such, citrullination seems to be a precision tool used to fine-tune the molecular functionalities a cell requires to maintain the complex interplay of protein and gene regulation, normally and in disease.

Recent findings have unveiled the broad implications PADIs may have on stem cell maintenance and differentiation in diverse stem cell niches. In the HFSC lineage, PADI expression correlate with HFSC activation, progenitor specification and hair shaft lineage differentiation, positioning PADIs at key HF lineage transition points. Whereas PADI1 and PADI3 in the regenerating hair follicle are currently exclusively associated with citrullination of structural hair proteins, work detailing the PADI1 and PADI3 HF cell state specific citrullinomes would likely provide new insights into the HF specific role of PADI1/3, in differentiating as well as self-renewing cell populations. Taking into account the redundancy of PADI1 and PADI3 in the HF, it is likely that establishment of a mouse line genetically devoid of both PADI1/3 expression would be required to grasp the full functional magnitude of these enzymes in the regenerating hair follicle.

In contrast to the relatively narrow functionality linked to PADI1 and PADI3, the implications of PADI4 expression in the hair follicle cast a wider web. PADI4 has a known role in regulating stem cell renewal and specification in stem cell lineages, and expression in activated HFSCs and progenitor cells suggests PADI4 could act analogously in HFs. The ability to citrullinate DNA methylases, modulate the function of key HF lineage determining transcription factors, synergizing with histone deacetylases and antagonizing arginine methylases indicates that the action of PADI4 in the regenerating hair follicle could be complex and modulate all stages of regeneration. Although aspects of epigenetic regulation of HFSC lineage progression is well established, how citrullination impinges on the HF epigenetic landscape is completely unknown. Delineating potential PADI4 dependent fine-tuning of the epigenetic landscape suggest an overall role in maintaining self-renewal and restricting differentiation.

Considering the complex function of PADIs in the HFSC lineage, it is perhaps not surprising that PADIs target a variety of pro-inflammatory signaling pathways in both neutrophils and T-cells in the skin. Paradoxically, citrullination of cytokines associated with neutrophil-mediated inflammation such as IL-8, CXCL-10, CXCL-11 and CXCL-12 reduces their respective inflammatory potency (Loos et al., 2008; Proost et al., 2008; Struyf et al., 2009). Moreover, citrullinated TNF-α show reduced ability to stimulate production of inflammatory cytokines by fibroblasts *in vitro* (Moelants et al., 2013). However tempting to speculate that citrullination, in addition to its established pro-inflammatory role could act as an inflammatory antagonist, citrullination of these chemokines were investigated *in vitro* without addressing the source of PADIs or the involvement of NETs. It therefore remains to be determined if chemokine citrullination occurs in an attempt to negatively balance the inflammatory environment initially employed to attract and activate neutrophils and NET formation.

Although the lion's share of citrullination-dependent inflammatory events leading up to hair follicle stem cell destruction in alopecia is mediated through immune cells, it is likely that alterations in epithelial PADI expression contributes to disease progression. NF-κB was shown to bind an intronic enhancer element of *PADI1* and was found to be crucial for its expression in human keratinocytes (Ying et al., 2010). Although evidence suggests that PADI1 activity is diminished in psoriatic skin (Ishida-Yamamoto et al., 2000), and IL-22 driven hyperproliferation in psoriatic keratinocytes downregulate PADI1 (Padhi et al., 2021), it is enthralling to envision an epithelial interdependency between PADI enzymes and NF-κB signaling, especially given the broad interconnectedness these have been shown to display in immune cells.

Additionally, it is noteworthy that human *PTPN22* mutations associated with alopecia areata and rheumatoid arthritis, also negatively regulates lymphocyte activation by reducing the PTPN22 phosphatase activity (Wu, 2006). Although PTPN22 inhibition of PADI4 activity is phosphorylation-independent, removal of either mode of PTPN22 suppressive abilities, be it *via* TCR signaling by phosphorylation or physical interaction with PADI4, both lead to immune cell activation. It would be of interest to investigate if the blockage of PTPN22 phosphorylation (and in this way activation of TCR signaling) would correlate to citrullination within the regulatory transcriptional landscape brought about by TCR signaling, either *via* citrullination of transcription factors or histones.

Given the contribution of PADIs to autoinflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus and psoriasis, it is likely that the pathogenesis of alopecia follows similar disease trajectories. Both immune cell involvement and the cytokine profile driving the pro-inflammatory environment in alopecia match those observed in citrullination-associated autoimmune diseases. Although there are obvious gaps in our understanding of how PADIs directly and indirectly influence hair follicle regeneration normally and during inflammatory alopecia, drawing from other stem cell niches or inflammatory diseases enables identification of common mechanisms likely applicable to the hair follicle. Continuous technological and methodological development will undoubtedly further our understanding of citrullination in the skin, normally and during inflammatory disease.

AUTHOR CONTRIBUTIONS

KVP, KH-M, and MG conceptualised and wrote the manuscript. KVP made the meta-analysis. KVP and KH-M made the illustrations.

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Vitiligo: An Autoimmune Skin Disease and its Immunomodulatory Therapeutic Intervention

Wei-Ling Chang[1,2]*, *Woan-Ruoh Lee*[3,4], *Yung-Che Kuo*[1] and *Yen-Hua Huang*[1,2,5,6,7,8,9,10]*

[1]TMU Research Center of Cell Therapy and Regeneration Medicine, Taipei Medical University, Taipei, Taiwan, [2]International Ph.D. Program for Cell Therapy and Regeneration Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan, [3]Graduate Institute of Medical Sciences, Taipei Medical University, Taipei, Taiwan, [4]Department of Dermatology, Taipei Medical University Shuang Ho Hospital, New Taipei City, Taiwan, [5]Department of Biochemistry and Molecular Cell Biology, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan, [6]Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, Taipei, Taiwan, [7]TMU Research Center of Cancer Translational Medicine, Taipei Medical University, Taipei, Taiwan, [8]Center for Reproductive Medicine, Taipei Medical University Hospital, Taipei Medical University, Taipei, Taiwan, [9]Comprehensive Cancer Center of Taipei Medical University, Taipei, Taiwan, [10]PhD Program for Translational Medicine, College of Medical Science and Technology, Taipei Medical University, Taipei, Taiwan

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Edited by:
Mingxing Lei,
Chongqing University, China

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Wei Xu,
Chongqing Hospital of Traditional Chinese Medicine, China
Marina Gomzikova,
Kazan Federal University, Russia

***Correspondence:**
Wei-Ling Chang
smolljade12@tmu.edu.tw
Yen-Hua Huang
rita1204@tmu.edu.tw

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Vitiligo is a chronic autoimmune depigmenting skin disorder characterized by patches of the skin losing functional melanocytes. Multiple combinatorial factors are involved in disease development, among which immune T cells play a prominent role. The immune cells implicated in melanocyte destruction through adaptive immunity include $CD8^+$ cytotoxic T cells and regulatory T cells, and aberrantly activated skin-resident memory T cells also play a role in melanocyte destruction. Over the past several years, major progress in understanding vitiligo pathogenesis has led to the development of targeted therapies. Janus kinase (JAK) inhibitors, which share the similar mechanism that autoactivates $CD8^+$ T cells in chronic inflammatory diseases, have been reported to have therapeutic significance in vitiligo. Recently, immunomodulatory therapeutic interventions in vitiligo have been emerging. Mesenchymal stem cells (MSCs) regulate cytokine secretion and the balance of T-cell subsets, which makes them a promising cell-based treatment option for autoimmune diseases. The induction of MSC-mediated immunomodulation is complicated and occurs by contact-dependent mechanisms and soluble extracellular vesicle (EV) mediators. EVs released from MSCs contain various growth factors and cytokines with anti-inflammatory effects in the skin immune response. Here, we summarize and discuss the progress to date in targeted therapies that immunomodulate the niche environment of vitiligo, from the clinical trial of JAK inhibitors to the potential of MSCs and MSC-EVs. The available information was collected to highlight the need for further research into the treatment of vitiligo.

Keywords: vitiligo, autoimmune skin disorder, skin-resident memory T (TRM) cell, janus kinase, stem cell therapy

INTRODUCTION

Vitiligo is an autoimmune skin disorder characterized by white patches of skin losing functional melanocytes, the pigment-producing cells of the skin. Vitiligo is a common skin disorder with an incidence rate of 0.1–2% worldwide, and it has no sex bias (Kruger and Schallreuter, 2012). Vitiligo has a significant impact on patients' quality of life and self-esteem and predisposes them to an increased risk of sunburn and skin cancer.

Vitiligo is a multifactorial disorder that combines genetic susceptibility, the generation of inflammatory mediators from environmental triggers, and autoimmune responses (**Figure 1**) (Schallreuter et al., 2008; Bergqvist and Ezzedine, 2021). Attracted by proinflammatory cytokines, autoreactive $CD8^+$ T cells that destroy the pigment-producing cells of melanocytes through the mediation of interferon-gamma (IFN-γ) signaling are believed to promote the apoptosis of melanocytes in vitiligo. Recent studies have found a subset of T cells called skin-resident memory T (T_{RM}) cells, which do not circulate but reside at the white spot site of previous lesions. Therefore, T_{RM} cells are responsible for vitiligo relapse, the most difficult part of treatment.

The treatment of vitiligo is still one of the most difficult dermatological challenges. Actual treatments rely on the use of topical steroids or topical calcineurin inhibitors and are more effective when combined with phototherapy. However, relapse occurs in ~40% of patients with vitiligo within 1 year after stopping treatment (Cavalie et al., 2015). Ideally, management of vitiligo focuses on stopping the immune destruction of melanocytes, halting depigmentation, stimulating repigmentation, and preventing relapses. Mesenchymal stem cells (MSCs) could induce melanocyte regeneration and have immunomodulatory properties that balance T-cell subsets (Weiss and Dahlke, 2019). Over the past several years, major progress in the understanding of vitiligo pathogenesis (Bergqvist and Ezzedine, 2020) and the application of MSC/other cellular therapies have led to the development of targeted therapies that are now being tested (Esquivel et al., 2020; Ha et al., 2020).

This review provides an update on the pathogenesis of vitiligo. We summarize the progress to date of MSC-based therapy and discuss its potential for new therapeutic interventions for the chronic autoimmune disease vitiligo.

PATHOGENESIS OF IMMUNITY IN VITILIGO

Initiators of Oxidative Stress and Immunity

Oxidative stress is considered one of the most crucial initiators of vitiligo. Environmental factors such as ultraviolet radiation accelerate the generation of reactive oxygen species and activate the unfolded protein response (UPR), which initiate several inflammatory mediators. Under oxidative stress, UPR activation causes stressed melanocytes and stressed keratinocytes to release several inflammatory mediators that are reported to be biomarkers of vitiligo, such as interleukin (IL)-1β, C-X-C chemokine ligand 9 (CXCL9), CXCL10, and CXCL16. Stressed melanocytes release CXCL12 and CCL5, which are mediated in T-cell homing to the skin in vitiligo (Rezk et al., 2017). Stressed keratinocytes produce CXCL16, which recruits $CD8^+$ T cells (Li et al., 2017). This proinflammatory factor further causes the activation of natural killer (NK) cells and chemoattracts melanocyte-specific $CD8^+$ T cells to melanocytes and causes their apoptosis. Last, oxidative stress alters the WNT pathway, which is involved in melanocyte differentiation by decreasing WNT expression (Regazzetti et al., 2015).

By sensing stress signals released from melanocytes and keratinocytes, innate immune cells, including NK cells and dendritic cells (DCs), are activated early in vitiligo (Xie et al., 2016). Exosomes act as the mediator of communication between stressed melanocytes and immune cells (Wong et al., 2020). These exosomes deliver vitiligo target antigens to nearby DCs and stimulate the immune system. We discuss the details of the effect of exosomes later.

Role of $CD8^+$ T Cells

In vitiligo, adaptive immune activation is responsible for killing melanocytes specifically. Patchy infiltration of T cells occurs adjacent to melanocytes, especially in the leading edge of vitiligo depigmentation (van den Boorn et al., 2009). Serum frequency of melanocyte-specific $CD8^+$ T cells is higher in patients with vitiligo than in their healthy counterparts, and the frequency is related to disease severity (Richmond et al., 2013).

$CD8^+$ T cell mediates melanocyte apoptosis in various ways (Chen et al., 2021). $CD8^+$ T cells produce several cytokines, including the proinflammatory cytokine IFN-γ. After the binding of IFN-γ to its receptor, the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway activates and stimulates the transcription of the chemokine ligands CXCL9 and CXCL10, which create a positive feedback loop for T-cell recruitment and function (Bergqvist and Ezzedine, 2021). Both CXCL9 and CXCL10 share a single receptor: CXCR3. Melanocyte-specific autoreactive T cells in patients with vitiligo express CXCR3 in both the blood and in lesional skin (Harris, 2016). In a mouse model, CXCR3-depleting antibodies reduce autoreactive T-cell numbers and reverses vitiligo (Richmond et al., 2017). Therefore, IFN-γ is thought to have one of the central roles to promote autoreactive $CD8^+$ T-cell recruitment for vitiligo pathogenesis.

Role of Skin-Resident Memory T Cells

Vitiligo is thought to be a skin memory disease because clinical observations show that relapse occurs essentially at the same location as a previously depigmented spot (Cavalie et al., 2015), implying that autoimmune memory plays a crucial role in the recurrence of vitiligo lesions.

Memory T cells were thought to be circulatory and to enter the tissues only to clear infection. Recent research has defined another pool of memory T cells called T_{RM} cells. T_{RM} cells do not circulate but reside in specific tissues to provide rapid protective immunity against reinfecting pathogens, and they recruit effector cells from the circulation (Jiang et al., 2012; Mueller and Mackay, 2016; Richmond et al., 2019). Of the memory T cells present in healthy skin tissue, 20–60% of the

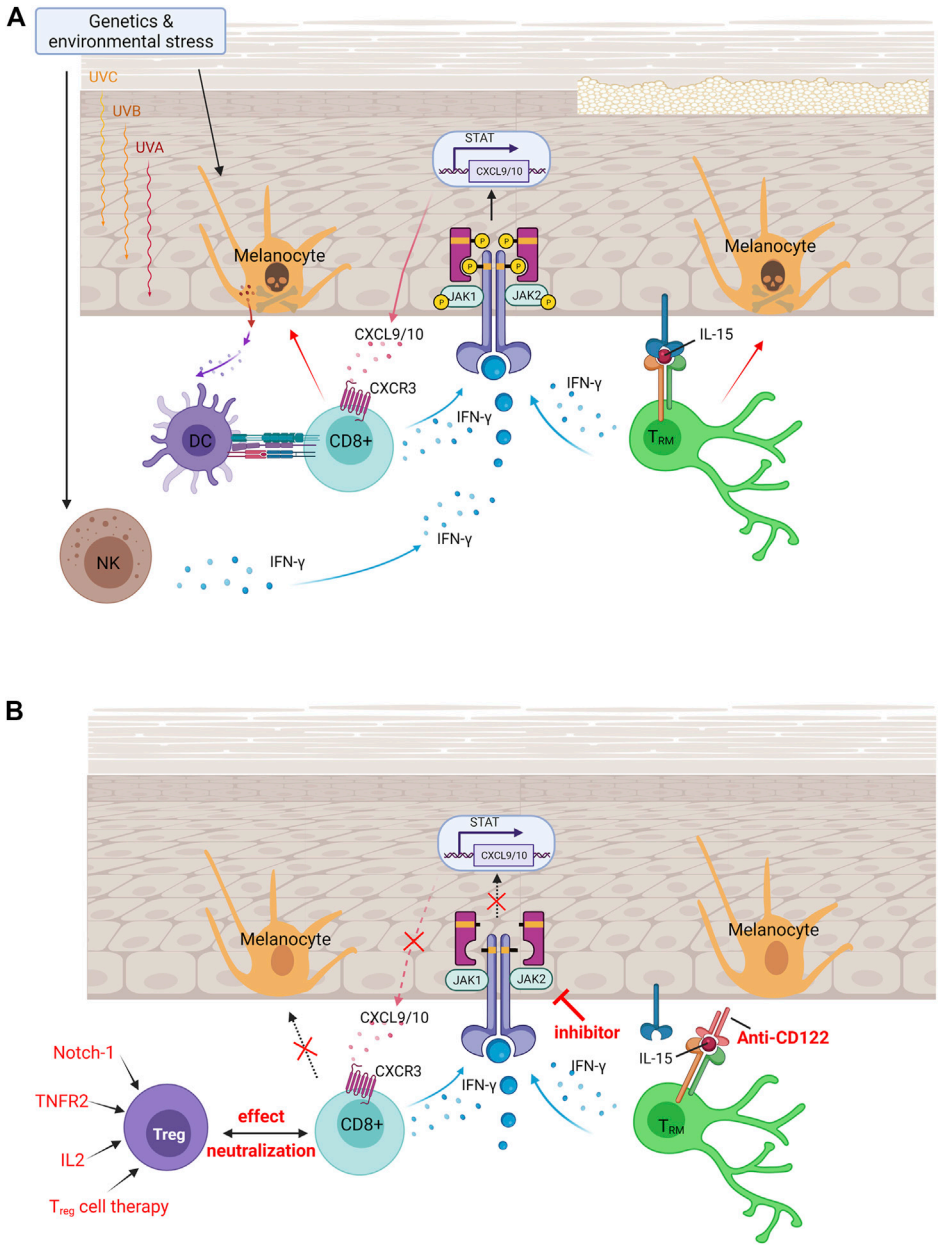

FIGURE 1 | Vitiligo pathogenesis and currently available treatments for vitiligo. **(A)** Pathologies of vitiligo. In vitiligo, melanocytes are more susceptible to oxidative stress, which in turn targets antigens to nearby dendritic cells and induces their maturation into efficient antigen-presenting cells. Upon endogenous or exogenous stress, NK cells produce IFN-γ that induces the production of chemokines. Binding of IFN-γ to its receptor activates the JAK/STAT pathway and leads to T-cell recruitment and function, which induces melanocyte apoptosis. Established vitiligo is maintained by T_{RM} cells, which remain long-lived in the skin through IL-15–dependent signaling. **(B)** Immunomodulated therapeutic intervention in vitiligo. Immunomodulatory therapies are currently available treatments for vitiligo, including the increase of T_{reg} cells to neutralize effector $CD8^+$ T-cell function, small molecule–targeted drugs of JAK inhibitors to block IFN-γ–CXCR3–CXCL9/10 signaling axis, and anti-CD122 antibody (IL-15 receptor subunit) to decrease IFN-γ production and deplete autoreactive $CD8^+$ T_{RM} cells. DC, dendritic cells; NK, natural killer cells; T_{RM}, skin-resident memory T cells; JAK, Janus kinase; IFN-γ, interferon-gamma; CXCL, chemokine (C-X-C motif) ligand; CXCR, chemokine (C-X-C motif) receptor; STAT, signal transducer and activator of transcription; T_{reg}, regulatory T cells; IL-2, interleukin 2; IL-15, interleukin 15; TNFR2, tumor necrosis factor receptor 2.

population are T_{RM} cells, revealing the high variation among healthy individuals (Boniface et al., 2018). In vitiliginous skin, the population of T_{RM} cells is high (up to 93%), consistent with their role as memory cells that persist after inflammation elimination. Functional $CD8^+$ skin T_{RM} cells were found in vitiligo, suggesting that T_{RM} cells are responsible for long-term maintenance and potential relapse of vitiligo (Boniface et al., 2018; Richmond et al., 2019).

To facilitate the effect of T_{RM} cells, appropriate cytokine niches expressing IL-12, IL-15, IL-33, IFN-β, tumor necrosis factor (TNF)-α, and transforming growth factor (TGF)-β are formed in tissues (Mackay et al., 2013). Many studies have found that IL-15 supports T_{RM} cell development, maintenance, and survival (Mackay et al., 2015). IL-15–deficient mice showed reduced T_{RM} cell formation through the reduction of Bcl-2 expression, a prosurvival molecule, in $CD103^+$ T_{RM} cells (Mackay et al., 2013). Both human and mouse autoreactive T_{RM} cells in vitiligo express high levels of the CD122 subunit of the IL-15 receptor in the blood and lesional skin. Melanocyte-specific T_{RM} cells cluster and produce high IL-15 levels in the vitiliginous skin (Malik et al., 2017), and antibody blockade of IL-15 signaling durably reverses depigmentation (Richmond et al., 2018). Therefore, IL-15 signaling was thought to be a therapeutic target for vitiligo.

Role of Regulatory T Cells

Regulatory T cells (T_{reg} cells) are a subpopulation of T cells with tolerance to self-antigens, and they help prevent autoimmune diseases. T_{reg} cells isolated from the peripheral blood of patients with vitiligo are impaired, suppressing the proliferation and activation of $CD8^+$ T cells *in vitro* (Lili et al., 2012). The exosomal pathway mediates the activation of $CD8^+$ T cells, as well as the T_{reg} cells balance which is associated with the disruption of autoimmune tolerance in vitiligo (Wong et al., 2020). The expression of immune profile including transcription factor T-beta, TBX21 (T-Box Transcription Factor 21), CXCR3, and CCR5 (C-C Motif Chemokine Receptor 5) in T_{reg} is believed to support the formation of resident memory T cells (Ferreira et al., 2020). Current studies based on single-cell RNA sequencing of human vitiligo reveals that CCL5-CCR5 cytokine signaling serve as a chemokine circuit between effector $CD8^+$T cells and T_{reg} cells (Gellatly et al., 2021). Therefore, modulating the immune response is considered as a therapeutic target for the treatment of vitiligo.

IMMUNOMODULATORY THERAPEUTIC INTERVENTION IN VITILIGO

Vitiligo is a chronic disease that requires lifelong therapy with immune mediators, phototherapy, or skin grafting. However, ~40% of patients with vitiligo experience relapse within 1 year after stopping treatment (Cavalie et al., 2015). The advancement of technology has enabled the development of alternative target therapies for vitiligo. Recently, immunomodulation of JAK signaling, T_{RM} cells, and T_{reg} cells has become a promising treatment for vitiligo.

The effect of the IFN-γ–CXCL9/CXCL10–CXCR3 axis on the killing of melanocytes by $CD8^+$ T cells is significant. In this axis, keratinocytes sense IFN-γ and generate CXCL9/CXCL10 mediated by JAK and associated STAT. JAK is a family of four proteins: JAK1, JAK2, JAK3, and TYK2. After stimulation by specific cytokines, JAKs form a heterodimer with various combinations, activating different STATs (Angelini et al., 2020). JAK inhibitors are emerging as a new class of small molecule–targeted drugs for the treatment of rheumatoid arthritis, a chronic inflammatory disorder that primarily affects joints. In dermatology, considerable progress has been made in emerging topical and systemic JAK inhibitor treatments (Solimani et al., 2019). A report showed that a patient who had both alopecia areata, a common immunological cause of hair loss, and vitiligo experienced some hair regrowth and repigmentation after treatment with a JAK inhibitor targeting JAK1/2, ruxolitinib (Harris et al., 2016). This is a potential therapy for vitiligo because such chronic inflammatory disorders exhibit similar pathogenesis, as they are both IFN-γ–driven and dependent on $CD8^+$ T cells (Bertolini et al., 2020). The latest phase 2 trial showed that the JAK inhibitor ruxolitinib was effective in vitiligo treatment, with high clinical relevance and therapeutic significance (Rosmarin et al., 2020). Another pan-JAK inhibitor, tofacitinib, also shows repigmentation ability when combined with phototherapy for stimulating melanocytes, providing a higher treatment effect (Liu et al., 2017). The literature has shown that the most significant repigmentation is seen on the face, whereas patches located on the trunk or lower extremities are not prominent. This raises an interesting issue regarding the regional specificity of the vitiligo lesion site, some of which is in a bilateral symmetric distribution (Bergqvist and Ezzedine, 2021). Another point worth noting is that much of the regained pigment regressed after discontinuing ruxolitinib, whereas much of the regrown hair was maintained (Harris et al., 2016). This indicates that although these autoimmune diseases exhibit similar pathogenesis, JAK inhibitors exert different effects on clinical manifestations. On the basis of the complexity of JAK signaling, toxicities that may limit a strong and ubiquitous JAK blockade should be considered.

The rapid, but not durable, repigmentation effect of JAK inhibitors indicates the most troublesome recurrence symptoms of vitiligo. JAK inhibitors exert their action by abrogating the chemotaxis of cytotoxic cells instead of by removing T_{RM} cells that remain long-lived in the skin through IL-15–dependent signaling. Subsequent studies have therefore investigated therapeutic treatment targeting IL-15 signaling. Treatment with anti-CD122 antibody, a subunit of the IL-15 receptor on human and mouse T_{RM} cells, has been shown to decrease IFN-γ production and to deplete autoreactive $CD8^+$ T_{RM} cells in mice with established vitiligo (Richmond et al., 2018).

T_{reg} cells have immunosuppressive properties; hence, their expansion may be an efficient strategy. T_{reg} expansion can be achieved through ectopic expression of its regulators IL-2, TNF receptor 2, and Notch-1 (Mukhatayev et al., 2021). IL-2 is required for the differentiation of T_{reg}; conversely, T_{reg} expresses the high-affinity IL-2 receptor to collect IL-2. TNF receptor 2 is highly expressed on T_{reg} cells, and, in graft-versus-host disease, stimulated TNF receptor 2 is reported to efficiently expand natural T_{reg} cells. Notch-1 inhibition enhances T_{reg} populations and suppressive function in transplantation. Another possible option is T_{reg} cell therapy, in which T_{reg} cells are harvested from the patient's circulation to expand them *in vitro* and transfer the expanded T_{reg} cells back to the patient. The challenge is the scarcity of T_{reg} cells in the blood and the slow rate of expansion *in vitro* when applied in clinical

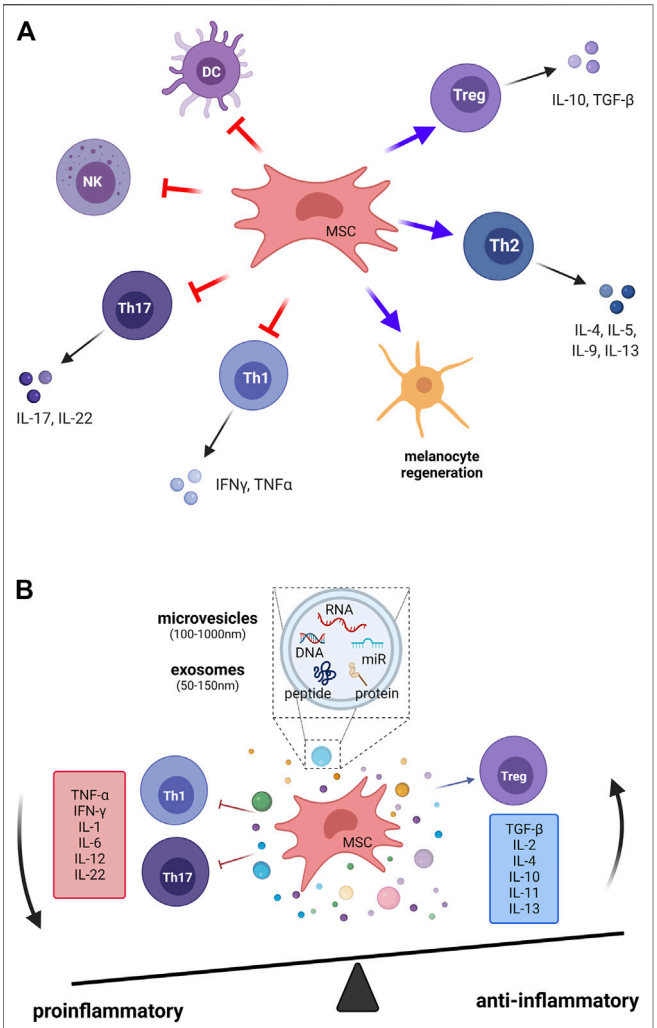

FIGURE 2 | The immunomodulatory effects of mesenchymal stem cells in vitiligo. The induction of MSC-mediated immunomodulation is complicated through **(A)** cell–cell contact–dependent mechanisms and **(B)** several soluble mediators that are secreted from MSCs. Treg, regulatory T cells; Th1, T helper 1 cell; Th2, T helper 2 cell; Th17, T helper 17 cell; DCs, dendritic cells; NK, natural killer cells; MSC, mesenchymal stem cell; IL, interleukin; IFN-γ, interferon-gamma; TNF-α, tumor necrosis factor-alpha; TGF-β, transforming growth factor-beta.

treatment (Sakaguchi et al., 2020). To improve the number of T_{reg} cells, elevation of the recruitment of T_{reg} cells to the epidermis through gene gun treatment–induced CCL22 overexpression is the currently preferred option to delay vitiligo progression (Eby et al., 2015). To improve the quality of T_{reg} cells, the emerging method available is chimeric antigen receptor (CAR) T cell therapy. Modified T_{reg} cells are engineered *in vitro* to recognize a specific target antigen and are then returned to the patient to stimulate intracellular signaling to increase the T-cell response to gain higher function than the nonspecific bystander T_{reg} cells (Mukhatayev et al., 2021). With the help of CAR technology, abundant antigen-specific T_{reg} cells can be acquired through *in vitro* expansion, and after adding specificity to T_{reg} cells, T cells with specificity can be transformed into T_{reg} cells (Rosado-Sanchez and Levings, 2020). However, the transduced T_{reg} cells may still function as cytotoxic T cells due to instability. Further technological developments may improve the quality and reliability of the CAR T_{reg} cell production for clinical application.

IMMUNOMODULATORY EFFECTS OF MSCs AND THEIR EXTRACELLULAR VESICLES

Mesenchymal Stem Cell

MSCs show promise as a type of cell-based therapy option for autoimmune diseases based on their immunomodulatory properties. We summarize the clinical trials of cell therapy in autoimmune-related skin diseases (**Supplementary Table S1**). The induction of MSC-mediated immunosuppression is complicated and occurs through contact-dependent mechanisms and several soluble mediators (**Figure 2**). Although the exact mechanisms that mediate the immunomodulatory effects of MSCs are still not fully understood, cell-based MSC therapy remains prevalent because MSCs can be applied in patients with vitiligo in several ways, including stopping immune destruction, stimulating repigmentation, and preventing relapses.

Studies have demonstrated that MSCs suppress immune reactions and mediate complicated mechanisms that change the T-cell subset polarization from proinflammatory (Th1, Th17) subsets to anti-inflammatory Th2 (Lu et al., 2009; Duffy et al., 2011) and T_{reg} cells (Ghannam et al., 2010). On the one hand, in MSC-treated animals, the levels of inflammatory cytokines, including IL-17, IFN-γ, IL-2, and TNF-α, produced by Th1 and Th17 are significantly reduced. On the other hand, the anti-inflammatory cytokines IL-10 and TGF-β are highly expressed on T_{reg} cells, which directly suppress responder T cells (Chatterjee et al., 2014; Ujiie, 2019). These cytokines lead to tissue damage and vitiligo pathology. To sum up, MSCs regulate cytokine secretion and the balance of T-cell subsets, which stop the immune destruction of melanocytes.

Regarding the cell–cell interaction aspect, DC and NK cells are also important targets for the immunomodulatory activity of MSCs. MSCs affect the differentiation, migration, maturation, and antigen presentation of DCs, which leads to triggering of tolerogenic T cells (Jiang et al., 2005; English et al., 2008). Adult MSCs inhibit IL-2–induced NK cell activation (Spaggiari et al., 2006).

In addition to their immunomodulatory properties, MSCs promote human melanocyte proliferation and resistance to apoptosis through the PTEN pathway in vitiligo (Zhu et al., 2020). It has been reported that dermal mesenchymal stem cells (DMSCs) could significantly inhibit the skin-homing activity of $CD8^+$ T lymphocytes (Zhou et al., 2013). Clinical trial shows that transplantation of cell suspension containing DMSCs resulted in excellent response in patients with vitiligo to extent of repigmentation (Thakur et al., 2019). Adipose tissue-derived mesenchymal stem cells (ADSCs) have a multipotential nature that can differentiable into melanocytes under the presence of

beta FGF and melanocyte growth factor (Zhang P. et al., 2014). The potential application of ADSCs in vitiligo can take the advantages from that the ADSCs can increase the proliferation of melanocytes under the presence of beta FGF and melanocyte growth factor, inhibit the production of proinflammatory cytokines, and protect against apoptosis (Owczarczyk-Saczonek et al., 2017).

MSCs isolated from different regions show phenotypic heterogeneity (Chen et al., 2020). To achieve their therapeutic benefit, a careful evaluation of appropriate cell sources and consistent protocols and dosages should be conducted in the future.

Extracellular Vesicles

Current evidence shows that in soluble mediators, the released extracellular vesicles (EVs) from MSCs exert immunomodulatory effects. EVs are heterogeneous lipid bilayer–surrounded vesicles, and they act as mediators of intercellular communication. The recognition of exosomes by target cells is specific and occurs through surface receptors. MSCs derived EVs have shown the immunosuppressive role on different immune cells, including B cells, T cells, dendritic cells, and macrophages (Gomzikova et al., 2019). MSC exosomes could induce anti-inflammatory cytokines expression, and could activate M2-like monocytes to induced T_{reg} polarization (Zhang B. et al., 2014). Morrison et al. found that MSC derived EVs promote an anti-inflammatory and highly phagocytic macrophage phenotype via extracellular vesicle mitochondrial transfer (Morrison et al., 2017). Recent studies have reported that EVs are associated with skin physiology in the cutaneous microenvironment. Adipocyte-derived stem cell–conditioned media can activate hair growth, and microinjury using a fractional laser or microneedling can also induce wound healing and hair regeneration (Lee et al., 2021). The significantly reduction of mRNA expression of various pro-inflammatory cytokines in alleviate atopic dermatitis skin lesions on mice model by the ADSC-derived exosomes (Cho et al., 2018) provides the potential cell-free therapy of ADSC-derived exosomes in other immunological skin disease, such as vitiligo. We summarize the studies of extracellular vesicles for autoimmune-related skin diseases (**Supplementary Table S2**). Here, we highlight two aspects of the role of exosomes in vitiligo: the immunomodulation effects and the interaction of melanocytes and keratinocytes through exosomes (50–150 nm of EVs). First, the exosomal pathway is crucial for the regulation of $CD8^+$ T cells and T_{reg} cells (Wong et al., 2020), which is key to the pathological conditions for vitiligo. Studies of human umbilical cord MSC-derived exosomes have shown the inhibition of the proliferation of $CD8^+$ T cells and the increase in the proportion of T_{reg} cells (Liu et al., 2015). Exosomes derived from multiple sclerosis can disrupt T_{reg} cell homeostasis by suppressing the insulin-like growth factor 1 receptor and TGF-β receptor 1 (Kimura et al., 2018). The conditioned media from human umbilical cord blood–derived MSCs contain various growth factors and cytokines with anti-inflammatory effects in the skin immune response, as analyzed by RT-PCR and ELISA (Kim et al., 2020). Second, EVs function as a new mode of signaling in intercellular communication through carrying mediator vesicles (Stahl and Raposo, 2019). Both melanocytes and keratinocytes can secrete exosomes for mutual interaction. Synthesis of melanin in melanocytes occurs in organelles called melanosomes, and melanin is distributed to the epidermis through the transport of melanosomes to adjacent keratinocytes for skin pigmentation through exosomes (D'Mello et al., 2016). Furthermore, exosomes carrying selected microRNAs from murine keratinocytes are targeted to melanocytes and induce the inhibition of melanogenesis by altering gene expression and enzyme activity in melanocytes (Kim et al., 2014; Liu et al., 2019). As intercellular communicators, signaling EVs must interact with the target cell with high fidelity. However, little is known about how signals are transmitted to the intracellular target. More investigation on the specific target fidelity of EVs will improve the therapeutic strategies for vitiligo and other skin regenerative medicine interventions.

DISCUSSION

Vitiligo is a chronic depigmenting skin disorder, for which current management strategies have limited efficacy, emphasizing the need for improved treatment options. Similar to other autoimmune diseases, including rheumatoid arthritis in which immune cells attack joints, and alopecia areata that attacks the hair bulb, immune cells target melanocytes in vitiligo through similar immune cell populations and cytokines to activate the JAK signaling pathway. JAK inhibitors are emerging as a new class of small molecule–targeted drugs for the treatment of chronic inflammatory diseases, including rheumatoid arthritis, alopecia areata, and vitiligo. The latest phase 2 trial showed that ruxolitinib cream was effective in vitiligo treatment, with high clinical relevance and therapeutic significance (Rosmarin et al., 2020). Although the value of different JAK inhibitor specificities across disease states remains to be defined, JAK inhibitors are an effective and safe alternative treatment for vitiligo.

Compared with previous relatively rudimentary treatment with immuosuppressants or steroids that have various side effects and with which relapse is common, novel immunomodulatory therapies can balance the immune mechanisms by changing stem cells. Stem cells have anti-inflammatory and immunomodulatory properties and have potential as a targeted therapy for vitiligo. MSC-derived extracellular vesicles, which accompany exosomes that act as potent drug carriers by delivering cargo to target cells, are an emerging management approach. Moreover, because MSCs often require invasive procedures, approaches that only require them to be cultured *in vitro* and use of their released EVs may have increased scalability and yield per MSC batch. EVs are easier to preserve and transfer, have lower immunogenicity, and therefore are safer for therapeutic administration.

We propose that the method of MSCs administration depends on the lesional pattern of Vitiligo. Generalized pattern is the common type of vitiligo (80–95%) in which multiple spots on the skin that are found on both sides of the body. Segmental vitiligo affects only one area of the body, which affects about 5% of adults

and 20% of children. In the case of generalized vitiligo, intravenous administration could be applied because MSCs can affect systematically. It has been reported that MSCs migrate in response to inflammatory mediators and home to injured sites (Chapel et al., 2003; Zhuang et al., 2021). The supported examples could be found when using ADSCs to treat chronic wound (phase I, NCT02961699). In the case of segmental vitiligo, administrated MSCs can be given on the lesion site by intradermal injection (Kandil, 1970), microneedling, or spray-on (**Supplementary Table S1**). Depending on different considerations, there may have several ways for MSC-derived extracellular vesicles administration, including intravenous injection and inhalation for systematic effects, as well as *in situ* injection or ointment for local lesion sites. The biodistribution of administrated MSCs is important for the safety and efficacy of cell therapy. Several factors that can affect the pharmacokinetics of the administered MSCs, including cell size, cell source, immunological features, and immunogenic reactions (Zhuang et al., 2021). Future directions will be focused on the real-world efficacy and safety of this new class of immunomodulatory therapies.

AUTHOR CONTRIBUTIONS

W-LC conceived the idea, wrote the manuscript, and created the figures and tables. Y-CC review and created the table. W-RL review and edited the manuscript. Y-HH conceived, review, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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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.2021.797026/full#supplementary-material

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Keratin 17 Is Required for Lipid Metabolism in Keratinocytes and Benefits Epidermal Permeability Barrier Homeostasis

Bingyu Pang†, Zhenlai Zhu†, Chunying Xiao, Yixin Luo, Hui Fang, Yaxing Bai, Zhongbin Sun, Jingyi Ma, Erle Dang and Gang Wang**

Department of Dermatology, Xijing Hospital, Fourth Military Medical University, Xi'an, China

The epidermal barrier refers to the stratum corneum, the uppermost layer of the skin, and constitutes the first line of defense against invasion by potentially harmful pathogens, diminishes *trans*-epidermal water loss, and plays a crucial role in the maintenance of skin homeostasis. Keratin 17 (K17) is a type I epithelial keratin with multiple functions, including in skin inflammation, epithelial cell growth, protein synthesis, and tumorigenesis. However, the relationship between K17 and the skin barrier has yet to be systematically investigated. In this study, we found that acute disruption of the epidermal permeability barrier led to a rapid increase in epidermal K17 expression *in vivo*. *Krt17* gene deficiency in mice resulted in decreased expression of lipid metabolism-related enzymes and antimicrobial peptides, while also delaying epidermal permeability barrier recovery after acute disruption. Adenovirus-mediated overexpression of K17 enhanced, whereas siRNA-mediated knockdown of *Krt17* inhibited, the expression of fatty acid synthase (FASN) and that of the transcription factors SREBP-1 and PPARγ *in vitro*. We further confirmed that K17 can facilitate the nuclear transportation of SREBP-1 and PPARγ and promote lipid synthesis in keratinocytes. This study demonstrated that K17 contributes to the restoration of the epidermal permeability barrier *via* stabilizing lipid metabolism in keratinocytes.

Keywords: epidermal barrier, Keratin 17, lipid metabolism, fatty acid synthase, sterol regulatory element-binding protein 1, peroxisome proliferator-activated receptor gamma

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Ji Li,
Xiangya Hospital, Central South University, China

Reviewed by:
Yuling Shi,
Tongji University, China
Sung-Jan Lin,
National Taiwan University, Taiwan

****Correspondence:***
Erle Dang
del-5527@163.com
Gang Wang
wanggangxjyy@163.com

†These authors have contributed equally to this work

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INTRODUCTION

The epidermal barrier is primarily constituted by the stratum corneum (SC), which is the outer layer of the skin, and represents a robust barrier against external environmental stressors as well as a water-tight barrier that prevents *trans*-epidermal water loss (TEWL). The loss of structural and biophysical homeostasis can provoke or aggravate chronic skin disorders (Egawa and Kabashima, 2016; Yosipovitch et al., 2019). The epidermal barrier is comprised of protein-enriched corneocytes embedded in an intercellular lipid matrix, called the "brick and mortar" model. The cellular complement is immersed in an intercellular lipid "mortar" composed of ceramides, cholesterol, and free fatty acids (FFAs) (Nemes and Steinert, 1999; Jia et al., 2018). Aberrant key steps in lipid metabolism in keratinocytes can result in alterations to barrier lipid components and thereby weaken epidermal barrier functionality (Akiyama, 2017). However, the precise mechanisms underlying the regulation of lipid metabolism in keratinocytes and the maintenance of skin homeostasis remain poorly understood.

Keratin 17 (K17), a type I epithelial keratin intermediate filament protein, is widely distributed in basal cells of complex epithelia, including hair follicles, sebaceous glands, fingernails, and eccrine sweat glands (Kurokawa et al., 2011). Physiologically, K17 plays a key role in maintaining normal hair follicle functions; accordingly, mice deficient for *Krt17* exhibit severe alopecia after birth resulting from TNF receptor-mediated apoptosis (McGowan et al., 2002). Although K17 is undetectable in normal *epidermis*, it is highly expressed in some skin disorders, in which it accelerates keratinocyte proliferation and promotes inflammation (Jiang et al., 2017; Yang et al., 2017; Yang et al., 2018). Moreover, *Krt17*-null mouse embryos show delayed wound closure due to decreased AKT/mTOR signaling activity, thereby revealing a critical role for K17 in skin repair (Kim et al., 2006). Nevertheless, whether and how K17 contributes to epidermal barrier function remains unknown.

Recently, our group noticed that K17 expression was upregulated in the *epidermis* after barrier perturbation, and altered lipid metabolism in keratinocytes K17. This suggested that K17 plays a critical role in epidermal barrier repair. In this study, we assessed the role of K17 in epidermal barrier function using *Krt17* knockout mice and then validated the findings in HaCaT cells. The results showed that K17 expression was upregulated following acute disruption of the epidermal barrier, an effect that promoted the recovery of the skin barrier *via* the modulation of lipid metabolism. These findings offer a novel viewpoint on the biological role of K17 and suggest a new therapeutic strategy for regulating epidermal barrier function.

MATERIALS AND METHODS

Mice and Treatment

Krt17 knockout mice in a C57BL/6J background were kindly provided by Prof. Pierre A. Coulombe (Johns Hopkins University, Baltimore, MD, United States). Female mice, 6–8 weeks old, were used in the experiments. All experimental procedures were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Review Committee for the Use of Animals of the Fourth Military Medical University. In the acute barrier abrogation model, barrier permeability was disrupted in mice by repeated applications of cellophane tape on the shaved back until a 10-fold increase in TEWL levels was achieved (Tsai et al., 1994). SC hydration and TEWL were measured immediately (0 h) and at 3 and 6 h after barrier disruption using a multifunctional skin physiology monitor (MPA10, Courage-Khazaka Electronic GmbH). The skin was collected 6 h after acute disruption, and the *epidermis* was either separated from the dermis by heat separation (Man et al., 2015) or fixed in formalin for histopathological analysis. The recovery rate was calculated as follows:

$$Recovery\ rate = \frac{TEWL\ (0\ h) - TEWL\ (3\ h\ or\ 6\ h)}{TEWL\ (0\ h)} \times 100\%$$

Cell Culture and Transfection

Human HaCaT keratinocytes (American Type Culture Collection, Manassas, VA, United States) were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, United States) supplemented with 10% fetal bovine serum (Gibco) and maintained at 37°C in a humidified atmosphere containing 5% CO_2. HaCaT cells were transfected either with pEGFP-N1-K17 or short interfering RNA (siRNA) targeting K17 using Lipofectamine 3,000 (Invitrogen; California; United States). The siRNA sequences for K17 are listed in **Supplementary Table S1**.

RNA extraction and Real-Time Quantitative PCR Analysis

Total RNA was extracted using Trizol reagent (Takara, Tokyo, Japan) and purified using chloroform/isopropanol/ethanol. The extracted RNA (1 μg/10 μl reaction) was converted to cDNA using the Prime Script RT Master Mix Kit (Takara). Quantitative real-time PCR (qPCR) was performed using SYBR Premix Ex Taq II (Takara) on a Chromo4 Continuous Fluorescence Detector with a PTC-200 DNA Engine cycler (Bio-Rad, CA; United States). The cycling conditions were as follows: 95°C for 2 min, followed by 45 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 10 s, and extension at 72°C for 15 s. Relative quantification was performed using the ΔΔCT method. All reactions were run in triplicate for at least three independent experiments. Data are expressed as a percentage of control (setting controls as 100%). The sequences of the primers used are listed in **Supplementary Table S2**.

Immunofluorescence and Confocal Microscopy

Biopsies obtained from the skin of the back of the mice were fixed in 12% formaldehyde solution and embedded in paraffin. For immunofluorescence (IF) staining, cells or skin biopsy specimens were permeabilized with 0.5% Triton X-100 for 10–15 min at room temperature. After washing three times with PBS, the cells or skin biopsy specimens were incubated with primary antibodies targeting K17 (sc-393002; Santa Cruz Biotechnology), SREBP-1 (IgG-2A4; BD Biosciences), or PPARγ (81B8; Cell Signaling Technology) at 4°C overnight, washed three times with PBS, and then incubated with Cy3-, fluorescein isothiocyanate (FITC)- (for cells transfected with siRNA and tissue slices), or Alexa Fluor 647-conjugated (for cells transfected with pEGFP-N1-K17) secondary antibodies (ab6939, ab6785, and ab150075, respectively; Abcam); nuclei were counterstained with Hoechst 33,258 (Solarbio Technology; Beijing; China). The samples were observed and imaged using a confocal microscope (LSM880; Carl Zeiss; Germany).

Western Blot Analysis

Western blot was performed as previously described (Jian et al., 2011) using the following antibodies: anti-K17 (ab53707; Abcam), anti-FASN (ab128856; Abcam), anti-SREBP-1 (PA1-337; Invitrogen), anti-PPARγ (81B8; Cell Signaling Technology), anti-β-tubulin (10068-1-AP; Proteintech), anti-β-actin (66009-1-Ig, Proteintech), anti-GAPDH (60004--1-Ig; Proteintech), and anti-lamin-A/C (sc-7292; Santa Cruz Biotechnology). Band intensities were quantified using Image Lab version 5.2.1 (Bio-Rad). Relative band intensities were normalized to that of the loading control.

Co-Immunoprecipitation

After the respective experiments, treated cells were subjected to co-IP assays [anti-K17 (sc-393002; Santa Cruz Biotechnology) or anti-IgG

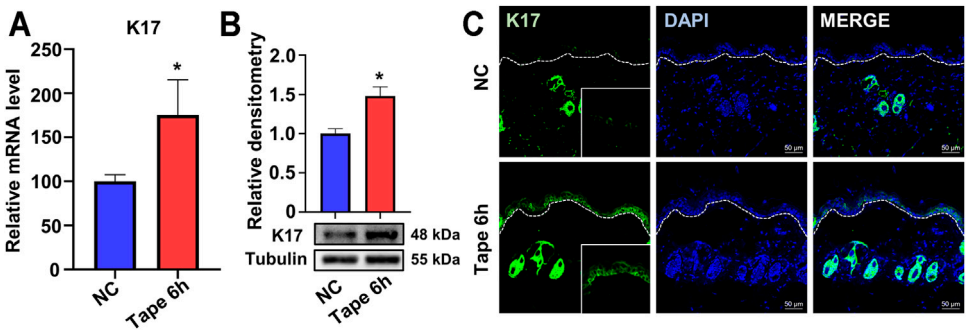

FIGURE 1 | The expression of keratin 17 was upregulated after acute epidermal disruption in wild-type mice. **(A)** The mRNA levels of keratin 17 (*Krt17*) in the *epidermis* 6 h after tape stripping as detected by real-time quantitative PCR (RT-qPCR). **(B)** The protein levels of K17 as determined by Western blot; quantification was based on three independent experiments. **(C)** Immunofluorescence staining for K17 (green). *$p<0.05$.

(TA-02; Origene, MD, United States) antibodies] using Protein A/G PLUS-Agarose (sc-2003C; Santa Cruz Biotechnology) according to the instructions of the manufacturer. Whole-cell lysates were purified in lysis buffer and incubated with anti-K17 or anti-IgG antibody on a rocker platform for 3 h followed by incubation with Protein A/G PLUS-Agarose at 4°C overnight. After four PBS washes, the supernatant was discarded and the pellets were resuspended in 1× electrophoresis sample buffer for immunoblot analysis.

Oil Red O Staining

Transfected HaCaT cells or frozen sections were fixed in 4% paraformaldehyde (PFA) for 10 min at room temperature and incubated in 0.5% Oil Red O (O8010-5; Solarbio) solution for 30 min. Tissue sections were then experienced a counter staining for nuclei with hematoxylin for 2 min. Images of cells were recorded using a FV-1000S confocal microscope (Olympus). The stained areas were detected and quantified (adjusted to cell quantity) using the ImageJ v1.8.0_172 (NIH, United States). Biopsies were scanned by NDP view system (Hamamatsu, Japan).

Statistical Analysis

The data were analyzed using the unpaired, two-tailed Student's *t*-test or one-way analysis of variance in GraphPad Prism v.8.0 (GraphPad Software, La Jolla, CA, United States). Each experiment was performed at least three times. Values of $p < 0.05$ were considered statistically significant.

RESULTS

The Expression of Keratin 17 was Upregulated Following the Disruption of the Epidermal Permeability Barrier in Wild-Type Mice

To explore the changes in K17 expression after skin barrier disruption, acute disruption of the epidermal permeability barrier was instigated by repeated tape stripping on the backs of wild-type (WT) mice. Compared with control, undisrupted *epidermis*, *Krt17* mRNA levels were significantly upregulated 6 h after tape stripping as revealed by RT-qPCR (**Figure 1A**) and further confirmed by Western blotting (**Figure 1B**). Consistent with this, IF staining showed a marked accumulation of K17 in the *epidermis* of the test group; in contrast, K17 expression was barely detectable in normal interfollicular *epidermis* (**Figure 1C**). These results indicated that K17 could be induced following acute insult to the epidermal permeability barrier.

The Lack of *Krt17* Delayed Epidermal Permeability Barrier Recovery

To clarify the effect of K17 on the epidermal permeability barrier, skin barrier function and recovery after injury were investigated in *Krt17* knockout mice. TEWL serves as a reliable readout of permeability barrier status *in vivo* (Hu et al., 2017). No differences in basal TEWL levels (**Figure 2A**) or SC hydration status (**Figure 2B**) were detected between WT and *Krt17* knockout mice. Subsequently, we compared the TEWL levels between the two groups of mice immediately (0 h after tape stripping) and 3 and 6 h after tape stripping-induced epidermal barrier disturbance. We found that the TEWL levels of WT mice showed a marked decline, whereas those of *Krt17*-null mice exhibited little change (**Figure 2C**). Additionally, compared with WT mice, the recovery rate of *Krt17* knockout mice was significantly delayed at 6 h after skin disruption (**Figure 2D**). Oil Red O staining was also performed to illustrate the lipid matrix. It was clearly demonstrated that lipid staining of *Krt17* knockout mice dorsal skin at steady state exhibited a moderate reduction compared with WT mice. Moreover, epidermal lipids were slightly increased 6 h after tape stripping in WT mice, whereas those of *K17* null mice had a declined intensity compared with that of untreated skin samples, which is probably due to the delayed lipid generation. The staining results illustrated an insufficient lipid production in *Krt17* knockout mice *epidermis* after barrier disruption (**Figure 2E**). Taken together, these results suggested that K17 is essential for skin barrier recovery rather than its maintenance at steady state.

The Relative Expression Levels of Genes Encoding Lipid Synthesis-Related Enzymes Were Downregulated in the Epidermis of *Krt17* Knockout Mice

To determine the effect of K17 on key genes associated with epidermal function, the relative expression levels of a plethora of

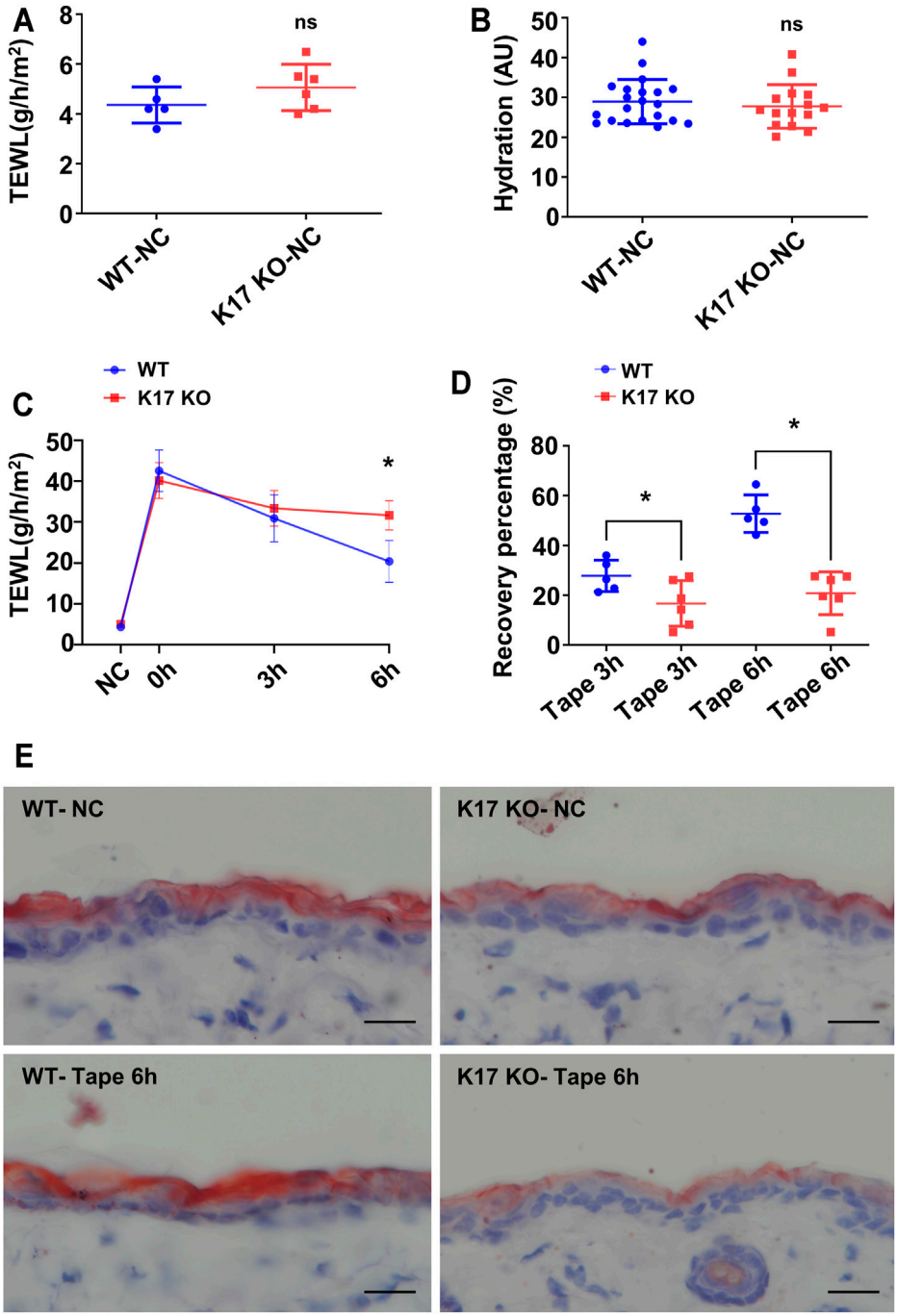

FIGURE 2 | The ability of the epidermis to repair itself was impaired in keratin 17-null mice. **(A)** *Trans*-epidermal water loss (TEWL) in keratin 17 (*Krt17*) knockout and wild-type (control) mice before tape stripping. **(B)** The basal levels of epidermal hydration in *Krt17* knockout and wild-type (control) mice. **(C)** TEWL monitoring at 0, 3, and 6 h after permeability barrier disruption. **(D)** Recovery rate at 3 and 6 h. **(E)** Oil Red O staining of biopsies at steady state and 6 h after disruption. Scale bars = 20 μm*$p < 0.05$.

genes related to epidermal barrier function, including proliferation, differentiation, lipid metabolism, and antimicrobial activities, among others (Mizutani et al., 2009; Svoboda et al., 2016; Zhong et al., 2016; Danso et al., 2017), were assessed by RT-qPCR. The results showed that the mRNA expression levels of genes associated with keratinocyte proliferation, differentiation, SC hydration, and tight junctions were comparable between *Krt17* knockout and WT mice (**Supplementary Figures S1A–E**). However, the expression levels of genes encoding the antimicrobial peptides S100A8,

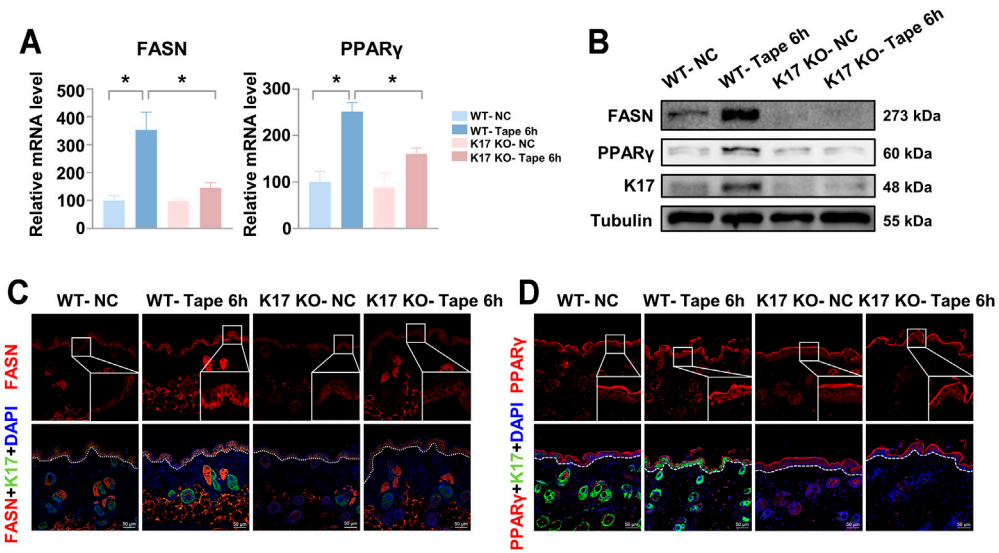

FIGURE 3 | Keratin 17 knockout abolishes the upregulation of fatty acid synthase (FASN) (epidermal lipid synthesis enzyme) and peroxisome proliferator-activated receptor gamma (PPARγ) (transcription factor) expression after epidermal barrier disruption *in vivo*. The shaved skin in the backs of keratin 17 (*Krt17*) knockout and wild-type (control) mice was disrupted using tape stripping. The skin was collected for analysis 6 h after tape stripping. **(A)** Quantitative real-time PCR results of the relative mRNA expression levels of the epidermal lipid synthesis enzyme, FASN and the transcription factor, PPARγ. Data were normalized to non-tape-stripped wild-type controls (controls were set as 100%). Data are representative of at least three independent experiments and each group consisted of three mice. **(B)** The protein levels of FASN, PPARγ, and K17 were measured in each group of mice. Tubulin served as the loading control. **(C,D)** Immunofluorescence staining of FASN and PPARγ. Scale bars = 50 μm. Results are shown as means ± SEM. $^*p < 0.05$.

S100A9, and LL-37 were markedly reduced in *Krt17*-null mice compared with those in WT mice (**Supplementary Figure S1F**). Importantly, relative to the WT controls, *Krt17* knockout mice exhibited a significant reduction in fatty acid synthase (*Fasn*) and peroxisome proliferator-activated receptor gamma (*Pparg*) levels after tape stripping (**Figure 3A**). Western blot and immunofluorescent staining results were consistent with those obtained by qPCR analysis (**Figures 3B–D**). Combined, these findings demonstrated that *Krt17*-null mice display lower mRNA expression levels of genes encoding lipid metabolism meditators and antimicrobial peptides.

Keratin 17 Regulates Fatty Acid Synthase and Peroxisome Proliferator-Activated Receptor Gamma Expression in Keratinocytes

To confirm the results obtained *in vivo*, HaCaT cells were transfected with siRNA targeting KRT17 (si-K17) following which the mRNA and protein expression of FASN and PPARγ was analyzed by RT-qPCR and Western blot, respectively. We found that the expression of both FASN and PPARγ was downregulated in K17-depleted cells at both the mRNA (**Figure 4A**) and protein (**Figure 4B**) levels. HaCaT cells were also transfected with a K17 overexpression plasmid (pEGFP-N1-K17). As shown in **Figures 4C, D**, the overexpression of K17 led to the induction of both the mRNA and protein expression of FASN and PPARγ. These findings indicated that K17 positively regulates FASN and PPARγ expression in keratinocytes. Subsequently, we sought to determine the overall influence of K17 on lipid metabolism using Oil Red O staining. The results clearly demonstrated that, compared with the controls, lipid staining area was greater in HaCaT cells transfected with pEGFP-N1-K17 (**Figure 4E**) and smaller in si-K17-transfected cells (**Figure 4F**). Taken together, these results suggest that K17 exerts positive effects on lipid metabolism *via* the modulation of FASN and PPARγ expression.

Keratin 17 Promotes the Nuclear Localization of Sterol Regulatory Element-Binding Protein 1 and Peroxisome Proliferator-Activated Receptor Gamma, Thereby Inducing the Expression of Lipid Synthesis-Related Enzymes

Several studies have demonstrated that sterol regulatory element-binding protein 1 (SREBP-1) is a transcription factor for a series of lipid metabolism-related enzymes such as acetyl-CoA carboxylase (ACC), stearoyl-CoA desaturase-1 (SCD-1), and, notably, FASN (Shimano and Sato, 2017). Accordingly, we sought to determine whether a correlation exists between K17 expression and SREBP-1 and PPARγ protein levels. The Western blotting results showed that inhibiting K17 using siRNA resulted in the downregulation of full-length SREBP-1 (flSREBP-1) and nuclear SREBP-1 (nSREBP-1), whereas the overexpression of K17 led to a reduction in flSREBP-1 levels but an increase in the levels of cleaved SREBP-1 (**Figures 5A, B**). As shown in **Figure 5C**, nuclear staining for SREBP-1 and PPARγ was barely discernable following K17 knockdown, whereas the opposite was observed

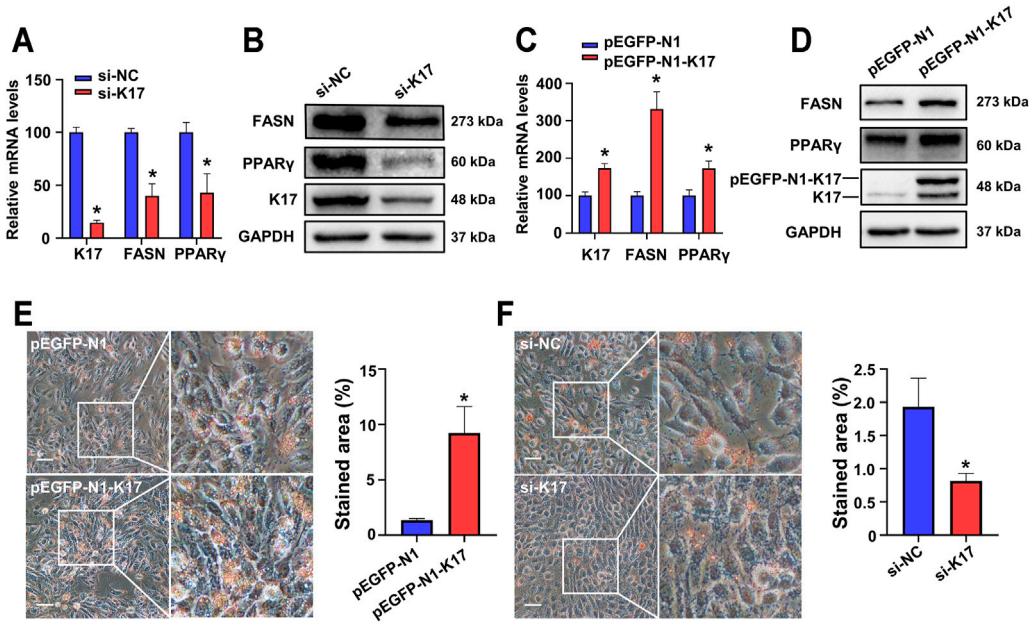

FIGURE 4 | K17 in keratinocytes maintains and regulates FASN and PPARγ expression. **(A,B)** The mRNA and protein levels of K17, FASN, and PPARγ in the keratinocyte cell line HaCaT were measured after transfection with K17 siRNA. **(C,D)** The mRNA and protein levels of K17, FASN, and PPARγ in HaCaT cells were measured after transfection with pEGFP-N1-K17. Data are expressed as means ± standard error of the mean of three independent experiments. **(E,F)** Lipid droplets in HaCaT cells transfected with pEGFP-N1-K17 or K17 siRNA were stained with Oil Red O and visualized by light microscopy at ×400 magnification. Scale bars = 50 μm *$p < 0.05$.

when K17 was overexpressed (**Figure 5D**), as determined by IF. These results were further confirmed by Western blot analysis of separated cytoplasmic and nuclear fractions obtained from treated cells (**Figure 5E**).

Co-immunoprecipitation using anti-K17 antibody was then performed to determine whether K17 can potentially bind to SREBP-1 and PPARγ (**Figure 5F**). The results suggested that K17 expression was positively correlated with nuclear-localized SREBP-1 and PPARγ and that K17 directly interacted with SREBP-1 and PPARγ and directed their nuclear translocation.

DISCUSSION

In the present study, we found that K17 expression was upregulated following tape stripping-induced skin barrier disruption. We further found that, in comparison with WT mice, *Krt17* knockout mice exhibited delayed barrier recovery and reduced FASN and PPARγ expression levels during barrier reconstruction. Additionally, our data showed that K17 influenced the subcellular localization of SREBP-1, a key transcription factor for FASN and PPARγ, leading to changes in lipid metabolism in keratinocytes.

K17 is only weakly expressed in normal human *epidermis*, but is inducible under conditions of stress, such as after wounding, viral infection, tumor growth, and skin diseases (Kim et al., 2006; Jin and Wang, 2014; Mikami et al., 2015; Yang et al., 2017). Our data further confirmed this, in that we found that epidermal barrier disruption, a less severe insult, is sufficient to induce the expression of K17. Multiple roles have been ascribed to K17 to date. The loss of K17 protein leads to a dose-dependent delay in the closure of embryonic skin wounds, highlighting the critical role of K17 in re-epithelialization during skin repair (Mazzalupo et al., 2003). Studies have shown that K17 can bind to adaptor protein 14-3-3δ and thereby facilitate keratinocyte proliferation and protein synthesis through phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling (Kim et al., 2006). In addition to cell proliferation, high K17 expression levels have also been linked with inflammatory skin diseases, such as psoriasis and atopic dermatitis. We have previously documented the roles of K17 in attracting inflammatory cytokines, promoting T-cell infiltration, and the thickening of the *epidermis* in psoriasis, as well as the associated molecular mechanisms (Shi et al., 2011; Jin and Wang, 2014; Yang et al., 2017; Yang et al., 2018). In the present study, we found that acute barrier disruption resulted in increased expression of K17 at both the mRNA and protein levels and that K17 deficiency compromised barrier repair. Our data further revealed a novel function for K17 in modulating lipid metabolism in keratinocytes. The delayed wound healing observed in *Krt17*-null mice from an early stage suggests that the protective role of K17 in the maintenance of barrier homeostasis may involve at least two distinct mechanisms, namely, cell proliferation and lipid synthesis. These results establish K17 as an essential regulatory factor in restructuring irritated *epidermis*.

Extracellular lipids in the *epidermis* mainly refer to lipids of the SC, primarily including ceramides, FFAs, and cholesterol. Variations in the fatty acid profile have been found to

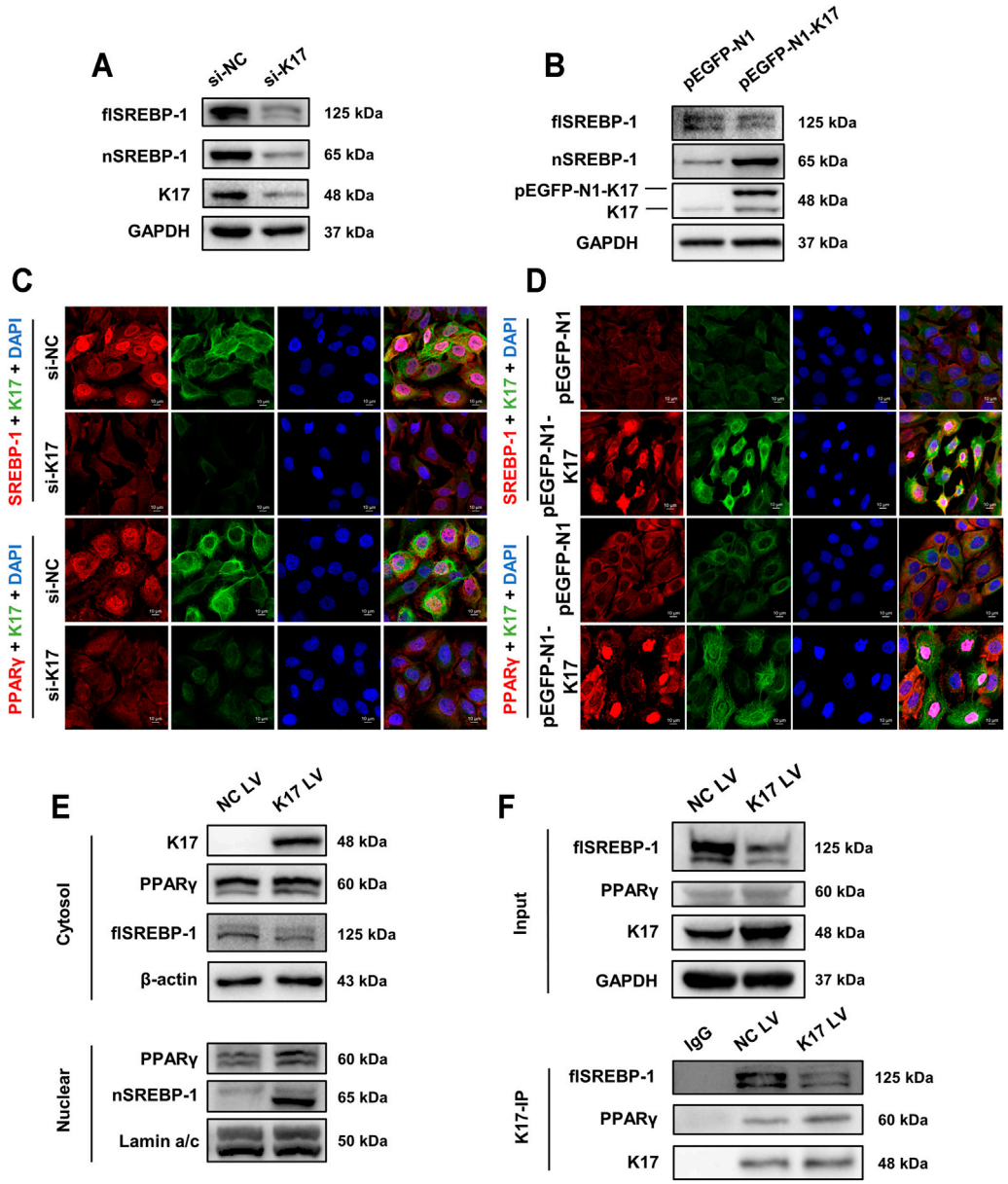

FIGURE 5 | K17 alters the subcellular localization of SREBP-1 and PPARγ. **(A,B)** The protein levels of full-length SPEBP-1 (flSREBP-1) and nuclear SPEBP-1 (nSREBP-1) in HaCaT cells were measured after transfection with K17 siRNA or pEGFP-N1-K17. **(C,D)** Immunofluorescence analysis of FASN and PPARγ subcellular localization in HaCaT cells after transfection with K17 siRNA or pEGFP-N1-K17. Scale bars = 10 μm. **(E)** HaCaT cells were treated with pCMV6-XL5-K17 (K17 LV) and a nuclear fraction was prepared. SREBP-1 and PPARγ protein was quantitated by Western blot. Lamin A/C and β-actin were used as loading controls for nuclear and cytosol fractions, respectively. **(F)** Cells were co-immunoprecipitated with anti-K17 antibody and SREBP-1 and PPARγ proteins were detected by Western blot. Data are expressed as means ± standard error of the mean from three independent experiments. *$p < 0.05$.

correlate with skin disorders (van Smeden et al., 2014; Kiezel-Tsugunova et al., 2018; Bhattacharya et al., 2019). The level of FASN, a key enzyme required for *de novo* fatty acid biosynthesis, increases rapidly following permeability barrier disruption and promotes epidermal homeostasis through several mechanisms (Ottey et al., 1995). FASN can enhance cell proliferative ability and maintain membrane synthesis (Vazquez-Martin et al., 2008). Furthermore, blocking the enzymatic activity of FASN can decrease phospholipid production, inhibit cell proliferation, and alter the metabolite profile in cancer cell lines (Jones and Infante, 2015). In addition, FASN is known to regulate and integrate with several signaling pathways, including the protein kinase C (PKC) and the PI3K/AKT/mTOR pathways, both of which are involved in cell growth and protein synthesis (Menendez, 2010; Benjamin et al., 2015). FASN inhibition can also hinder protein modifications by hampering palmitoylation, which is essential for skin barrier integrity (Ventura et al., 2015; Chen et al., 2020). Apart from the increased level of FASN, FASN

expression was downregulated in *Krt17*-null mice following permeability barrier disruption. Our data indicated that K17 is a contributor to the epidermal barrier by regulating the expression of FASN, a key lipid synthesis enzyme, thereby affecting multiple processes related to FASN activity.

SREBP-1 is a key and well-characterized transcription factor for FASN. SREBP-1 is expressed as two isoforms, SREBP-1a and SREBP-1c. The SREBP-1a isoform was reported to be the main isoform expressed in human keratinocytes, where it plays a role in epidermal barrier function (Shimano and Sato, 2017). SREBP-1 has been demonstrated to function as an important node in the promotion of cell proliferation mediated by AMPK-dependent p70 ribosomal S6 kinase-1 (S6K1) (Düvel et al., 2010). Another study also highlighted the importance of SREBP activation in lipogenesis and cell development (Xu et al., 2020). More directly, liver X receptor (LXR), a SREBP-1 agonist, can stimulate lipid synthesis, lamellar body secretion, and post-secretory lipid processing, mechanisms that account for its ability to improve epidermal permeability barrier homeostasis (Man et al., 2006). It has also been shown that SREBP-1 upregulation results in increased expression of genes associated with lipid compounds and lamellar body formation in keratinocytes (Yokoyama et al., 2009). Independently of its transcriptional level, SREBP-1 proteolytic cleavage and subsequent translocation into the nucleus are required for its proper functioning (Eberlé et al., 2004). Mutants of sterol regulatory element-binding factor (*SREBF-1*), which encodes SREBP-1, display impaired cleavage and the absence of nuclear translocation. Genetic alterations in SREBP-1 also lead to the development of ichthyosis follicularis alopecia and photophobia (IFAP) syndrome, which is partially characterized by ichthyosis follicularis (Wang et al., 2020). These observations highlight the underlying influence of SREBP-1 in the lipid matrix of the permeability barrier and skin barrier functioning. As mentioned above, K17 has been reported to facilitate keratinocyte proliferation and protein synthesis through AKT/mTOR signaling (Kim et al., 2006). This pathway is a well-characterized modulator of SREBP-1 signaling through controlling the nuclear entry of lipin 1 (Peterson et al., 2011). Our data also verified the positive relationship between K17 expression and AKT/mTOR signaling activity (**Supplementary Figure S2**). Thus, it is likely that K17 triggers AKT/mTOR signaling and mediates SREBP promoter activity and its nuclear protein abundance. Here, we found that SREBP-1 cleavage and nuclear translocation were inhibited under K17 deficiency, which led to a delay in barrier repair. Parallel to the indirect modulation of SREBP-1 by AKT/mTOR signaling, we found that K17 interacts directly with flSREBP-1 *in vitro*. That K17 has been recently identified inside the nucleus of epithelial cells and was reported to exert direct effects on cell proliferation and gene expression suggests that K17 may be directly involved in SREBP-1 nuclear translocation (Depianto et al., 2010; Hobbs et al., 2016; Nair et al., 2021). The results of the present study provide further evidence that SREBP-1 signaling plays a crucial role in epidermal differentiation and skin barrier reordering and highlight the regulatory effect of K17 in this process. However, the precise molecular mechanisms underlining the direct interaction and signaling pathways between K17 and SREBP-1 require further exploration.

PPARs comprise a crucial set of transcription factors that control lipid metabolism, skin barrier permeability, inflammation, and cell proliferation and differentiation (Sertznig and Reichrath, 2011). Among the three PPAR isoforms, PPARγ is the main functional isoform in mammalian skin, especially during keratinocyte differentiation (Ramot et al., 2015). After acute disruption of the permeability barrier by either tape stripping or extraction of barrier lipids with repeated acetone treatment, recovery of permeability barrier function was shown to be accelerated in animals treated topically with PPARγ agonists (Man et al., 2006). One mechanism underlying this effect was reported to be that topical treatment with PPARγ activators increased cholesterol, fatty acid, and sphingolipid synthesis in the *epidermis* by inducing the mRNA expression of the corresponding enzymes (Schmuth et al., 2008). PPARγ activation also markedly stimulates the mRNA expression of ABCA12 in human keratinocytes in a dose- and time-dependent manner (Jiang et al., 2008). ABCA12, a member of the ABC superfamily of proteins, facilitates the delivery of sphingolipids to lamellar bodies in keratinocytes. Here, evidence obtained both *in vitro* and *in vivo* suggested that increased K17 expression resulted in a corresponding induction of PPARγ expression. Thus, we uncovered a significant role for K17 in promoting lipid metabolism through regulating the PPARγ signal and, perhaps, also maintaining the regular formation of a protective lipid matrix in the skin.

In conclusion, our data indicated that altered K17 levels may serve as an indicator of *epidermis* impairment. Our results further underlined that K17 plays an indispensable role in lipid metabolism in keratinocytes, as well as in the reconstruction of the skin barrier, *via* the modulation of SREBP-1 and PPARγ. Our findings suggest that the role of keratins may not be limited to structural support, but may also include the modulation of cellular metabolism. In addition, we propose that the upregulation of K17 serves primarily as a compensatory, protective response to barrier defects, thereby ensuring survival. However, the prominent proinflammatory effect resulting from an increase in K17 levels may also promote the deterioration of the epidermal defect, and in turn, lead to a further increase in K17 expression. Overall, our study provides a novel perspective regarding the role played by K17 in the pathology of skin diseases characterized by epidermal disruption.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the Review Committee for the Use of Animals of Fourth Military Medical University.

AUTHOR CONTRIBUTIONS

BP, ZZ, ED, and GW conceptualized the study. BP wrote the manuscript. BP, ZZ, CX, YL, HF, YB, ZS, JM, ED, and GW contributed to the generation and/or analyses of the data. All authors read the manuscript and contributed to the discussions and 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.2021.779257/full#supplementary-material

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Building vs. Rebuilding Epidermis: Comparison Embryonic Development and Adult Wound Repair

Sangbum Park[1,2,3]*

[1]Institute for Quantitative Health Science and Engineering (IQ), Michigan State University, East Lansing, MI, United States, [2]Division of Dermatology, Department of Medicine, College of Human Medicine, Michigan State University, East Lansing, MI, United States, [3]Department of Pharmacology and Toxicology, College of Human Medicine, Michigan State University, East Lansing, MI, United States

Wound repair is essential to restore tissue function through the rebuilding of pre-existing structures. The repair process involves the re-formation of tissue, which was originally generated by embryonic development, with as similar a structure as possible. Therefore, these two processes share many similarities in terms of creating tissue architecture. However, fundamental differences still exist, such as differences in the cellular components, the status of neighboring tissues, and the surrounding environment. Recent advances in single-cell transcriptomics, *in vivo* lineage tracing, and intravital imaging revealed subpopulations, long-term cell fates, and dynamic cellular behaviors in live animals that were not detectable previously. This review highlights similarities and differences between adult wound repair and embryonic tissue development with a particular emphasis on the epidermis of the skin.

Keywords: wound repair, development, skin, epidermis, tissue-resident immunity

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Wen-Hui Lien,
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Aiko Sada,
Kumamoto University, Japan
Rajeev Kumar Pandey,
The Johns Hopkins Hospital, United States
Thomas S. Lisse,
University of Miami, United States

***Correspondence:**
Sangbum Park
spark@msu.edu

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INTRODUCTION

The epidermis is the topmost layer of the skin. It plays an important role in protecting our bodies from the surrounding environment such as temperature, pH extremes, pathogens, UV, and mechanical stress. The skin epithelium builds a rigid structure by forming cellular junctions between cells such as desmosomes, adherens, and tight junctions. While stratified epithelium acts as physical barriers, tissue-resident immune cells perform immunological barrier functions. These immune cells surveil the surface of the skin and protect our body from diverse insults such as pathogens and tumors by mediating immunity and tolerance. The epidermis is an ideal model system to investigate the repair process because its position as the outermost protective layer of the body means it is highly prone to injury. This review will discuss how the epidermal barrier is built during development and how the rebuilding process restores the damaged barrier after injury. In addition, direct comparison of the same cellular components during these two distinct processes will provide broad insights into epidermal regeneration.

Physical and Immunological Barriers of the Epidermis

The skin epithelium has a highly organized structure (**Figure 1**). Undifferentiated epithelial cells are located at the bottommost basal layer (also known as stratum basale). The basal layer cells gradually and continuously differentiate from bottom to top. Basal layer cells are anchored to the basement membrane *via* hemidesmosomes and focal adhesions. Only basal epithelial cells can self-renew during homeostasis postnatally. The human epidermis has a rete ridge structure because the uneven

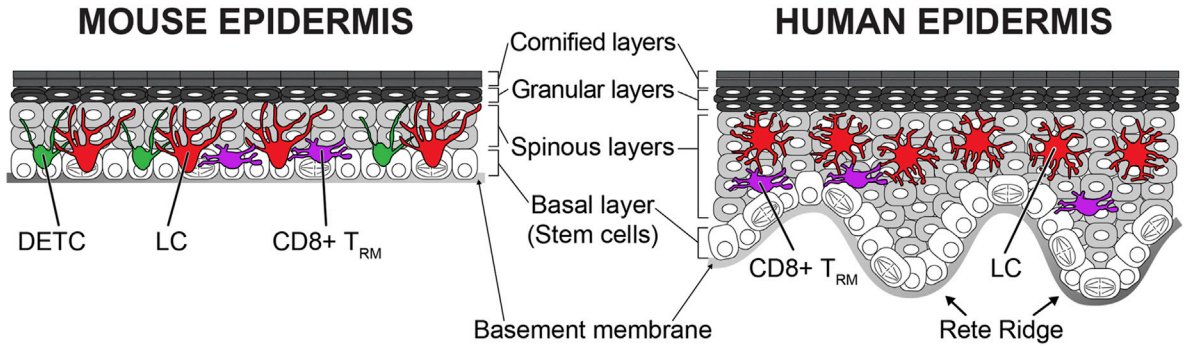

FIGURE 1 | Structure and cellular components of mouse and human epidermis. Highly organized epithelial cells composed of stratified epithelium in the epidermis. The bottommost basal layer cells are attached to the basement membrane. Epithelial stem cells are in the basal layer and differentiate upward. Both mouse and human epidermis have four different layers (basal, spinous, granular, and cornified layers). The human epidermis is much thicker than the mouse epidermis and has an undulated rete ridge structure. Both undifferentiated and differentiated epithelial cells form cellular junctions to build the solid stratified structure. The stratified epithelium acts as physical barriers from outer environment. There are tissue-resident immune cells within the epidermis. Langerhans cells (LCs), dendritic epidermal T cells (DETCs), and $CD8^+$ tissue-resident memory T cells (T_{RM}s) exist in the mouse epidermis, but humans do not have the DETCs. These immune cells continuously surveil possible infections and perform immunological barrier functions.

epidermal thickness is undulated. Depending on the spatial distribution, basal epithelial cells show different characteristics. Cells located at the bottom of the rete ridge express high levels of integrin $\alpha 6$ and kertin15, and cells at the top of the rete ridge express high levels of integrin β1, MCSP, and Lrig1 (Jones et al., 1995; Li et al., 1998; Legg et al., 2003; Webb et al., 2004; Jensen and Watt, 2006). It remains controversial which cells have higher stemness, but a transplantation study suggested that some cells have the capacity for long-term self-renewal. Transplantation of human epidermis in a patient with junctional epidermolysis bullosa showed that there are a limited number of long-lived stem cells. These stem cells have the potency to self-renew in the long term and to generate progeny (Hirsch et al., 2017). As aging progresses, the skin epidermis loses the rete ridge structure and becomes flat (Giangreco et al., 2010). Similar to aged human epidermis, mouse skin does not have rete ridges and remains flat throughout life. There are several hypotheses about stem cells in the basal layer in the mouse epidermis. Conclusions differ depending on the experimental designs (lineage tracing with different mouse lines, label-retention, and intravital imaging) and region of skin (back, tail, ear, and paw) (Gonzales and Fuchs, 2017). The label retention approach with the H2B-GFP pulse-chase system exhibited stem cells spatially organized depending on the position of scale/interscale regions and blood vessels in the mouse back and tail epidermis (Gomez et al., 2013; Sada et al., 2016). Lineage tracing by Cre recombinase [keratin 14 promoter (K14)-CreER, involucrin promoter (Inv)-CreER] demonstrated that quiescent stem cells are stochastically positioned in the epidermis of the mouse tail skin and that these cells contribute the long-term homeostasis and wound repair (Mascré et al., 2012). However, lineage tracing with a different CreER marker [Cyp1a1 promoter (Ah)-CreER] in the tail skin supports a stochastic model (Clayton et al., 2007). Intravital imaging from the ear and paw also showed that all basal epithelial cells equally possess the potency to self-renew and differentiate with random fate decisions (stochastic) (Doupé et al., 2010; Rompolas et al., 2016). Additional studies are needed to elucidate the stemness of basal epithelial cells in the epidermis from both humans and mice. Since mouse skin is more experimentally tractable, the rest of this review will summarize what is known about the development and the wound repair from mouse studies.

Basal epithelial cells differentiate by delaminating from the basement membrane and moving sequentially through the upper spinous, granular, and cornified layers. Differentiating cells change not only position but also cellular characteristics. During differentiation, basal epithelial cells stop expressing the integrins that form focal adhesions and hemidesmosomes (Fuchs and Raghavan, 2002). The expression of P-cadherin also disappears, but E-cadherin is continuously maintained to form adherens junctions. Adhesion *via* desmosomes is also sustained, but isotypes of the desmocollins and desmoglein, which constitute the desmosomes, are gradually changed (Simpson et al., 2011). Microtubule organization also changes during differentiation. Microtubules are rearranged from a centrosomal array in the basal layer to cortical localization in the suprabasal layers. The microtubule rearrangement during the differentiation might be related to the inactivation of the centrosome which blocks cell cycle progression of suprabasal cells (Lechler and Fuchs, 2007; Muroyama and Lechler, 2017). Basal epithelial cells express keratins 5 and 14, which are gradually replaced with keratins 1 and 10 during differentiation (Simpson et al., 2011). It was reported that keratin 10 expression is already detectable in cells located in the basal layer (Schweizer et al., 1984). More recently, several studies expanded on this finding by showing more globally that the transcriptional program associated with differentiation (including keratin 10 but also other things like Desmoglein 1, suprabasin, and Krtdap) (Haensel et al., 2020; Wang et al., 2020; Cockburn et al., 2021). This suggests that the differentiation

process is initiated in the basal layer before delamination occurs. The spinous layers (also known as stratum spinosum) are the layers following the basal layer. The spinous layers provide a strong barrier function by establishing a strong intercellular connection and thick keratin bundles (Fuchs, 2009). The granular layers (also known as stratum granulosum) are the upper layers of the spinous layers. Cells in the granular layers form intercellular tight junctions which prevent penetration of outer pathogens and body water loss (Fuchs, 2008). The granular layer cells have keratohyalin granules within as their nomenclatures suggest (Goleva et al., 2019). The granules are membraneless protein deposits, and their function is still unclear. Recently, it was revealed that filaggrin assembles keratohyalin granules *via* liquid–liquid phase separation (Quiroz et al., 2020). The cornified layers (stratum corneum) are the outmost layers of the epidermis. These are dead cells that are composed mainly of keratin and cell membrane. There are lipids composed of ceramides, cholesterol, and fatty acids between cells (Lippens et al., 2005). The cornified cells and intercellular lipids protect our skin by regulating water loss, acidic pH, permeability, and skin microbiome (Del Rosso and Levin, 2011).

There are three epidermis resident immune cells, Langerhans cells (LCs), dendritic epidermal T cells (DETCs), and $CD8^+$ tissue-resident memory T cells (T_{RM}s) (Chong et al., 2013). These immune cells form the first-line barrier from the environment and work as sentinels by surveilling the surface and neighboring cells of the epidermis (**Figure 1**). LCs are well-known antigen-presenting cells. LCs express tight junction proteins and extend their dendrites upward to interact with gap junctions in the granular layer (Kaplan, 2017). Mature LCs can penetrate tight junctions with the tip of their dendrites and capture antigens on the skin surface (Kubo et al., 2009; Ouchi et al., 2011). LCs become activated after antigen uptake. The expression of MHC-II, CD80, and CD86 is increased in activated LCs (Rattis et al., 1996). Although LCs are static during homeostasis, activated LCs gain high mobility. Activated LCs mobilize by disconnecting from epithelial cells by decreasing E-cadherin and EpCam expression (Brand et al., 2019). Interestingly, the inhibition of E-cadherin or EpCAM in LCs does not promote the exit of LCs from the epidermis (Ouchi et al., 2016; Brand et al., 2019). These data suggest that adhesion molecules do not directly regulate the migration of LCs in the absence of inflammation. Activated LCs migrate to the lymphatic vessels by using C-X-C motif chemokine receptor 4 (CXCR4) and CC chemokine receptor 7 (CCR7) and enter the lymphatic system (Ohl et al., 2004; Ouwehand et al., 2008). Eventually, these cells migrate into the lymph nodes to present antigen to naive T cells (Otsuka et al., 2018).

The dendritic epidermal T cells (DETCs) are $\gamma\delta$ T cells in the epidermis. These cells exist only in the mouse epidermis but not in the human epidermis (Nielsen et al., 2017). The majority of T cells have classical $\alpha\beta$ T-cell receptors (TCRs), whereas DETCs have $\gamma\delta$ TCRs similar to intestinal intraepithelial lymphocytes (IELs) (Vandereyken et al., 2020). The $\gamma\delta$ T cells are classified as innate-like lymphocytes with the NK cells. Therefore, DETCs have characteristics from both innate and adaptive immunities (Bennett et al., 2015). DETCs show innate pattern recognition and NKG2D ligand expression, like innate immune cells, and express a rearranged TCR, like adaptive immune cells (MacLeod and Havran, 2011). DETCs have a dendritic morphology and the tip of their dendrites contact tight junctions and surveil the outer environment like LCs (Chodaczek et al., 2012). Although TCR ligands of DETCs are still unknown, there are several pieces of evidence indicating that DETCs react to bacterial infection, tumor cells, and stress signals from neighboring epithelial cells (Hayday, 2009; MacLeod and Havran, 2011; Komori et al., 2012; Vermijlen et al., 2018). Unlike dynamic IELs, DETCs are more static, similar to LCs during homeostasis (Hoytema van Konijnenburg et al., 2017). The difference in the behaviors of these two $\gamma\delta$ T-cell types could be dictated by the structural and environmental differences between the skin and the intestine. The skin is composed of stratified epithelium with a dry surface, whereas the intestine is a simple squamous epithelium and has many nutrients on the surface which favors the microbiome (Grice and Segre, 2011; Byrd et al., 2018; Fan and Pedersen, 2021). Recently, it has been shown that LCs and DETCs actively maintain their regular distribution during homeostasis (Park et al., 2021). Remarkably, these immune cells recover regular distribution within a few days after local perturbation with laser ablation and after global depletion with diphtheria toxin-induced cell death. It is revealed that dendritic interaction is important for maintaining a regular distribution, but the underlying mechanisms remain unclear. Interestingly, depletions of one immune population do not impact the other's patterning (Park et al., 2021). These data suggest that although LCs and DETCs have a similar tiling pattern, these cells maintain the pattern independently through different mechanisms.

Immunological memory is a key factor of adaptive immunity for long-term protection. The skin also has memory function from tissue-resident memory T (T_{RM}) cells (Hirai et al., 2021). In the epidermis, $CD8^+$ T_{RM} cells are predominant, whereas $CD4^+$ T_{RM} cells mainly exist in the dermis (Szabo et al., 2019). After infection, $CD8^+$ T_{RM} cells repopulate the infected epidermis and are maintained by IL-7, IL-15, and TGF-β (Ariotti et al., 2012; Adachi et al., 2015; Dijkgraaf et al., 2019). These cells have a memory to a specific antigen (Hirai et al., 2021). After antigen recognition, these cells massively proliferate and differentiate into cytotoxic effector T cells (Menares et al., 2019). In contrast to LCs and DETCs, $CD8^+$ T_{RM} cells are mobile within the epidermis during homeostasis (Ariotti et al., 2012; Zaid et al., 2014). The higher mobility enables $CD8^+$ T_{RM} cells to cover the epidermis with lower density than LCs and DETCs. Interestingly, a territory of $CD8^+$ T_{RM} cells can overlap with LCs but not with DETCs (Zaid et al., 2014). This suggests that $CD8^+$ T_{RM} cells and DETCs have a repulsive interaction. This exclusion could indicate functional redundancy between T cells.

Formation and Repair of the Skin Epithelium

During embryogenesis, skin epidermis originates from the ectoderm (Koster and Roop, 2007). A single layer of surface ectoderm, which expresses keratins 8 and 18, is formed on the basement membrane. These cells differentiate to keratins 5 and 14 positive epidermal lineage cells by BMP and p63 signaling (Lutz et al., 2010). p63 knockout studies revealed that p63 is a master

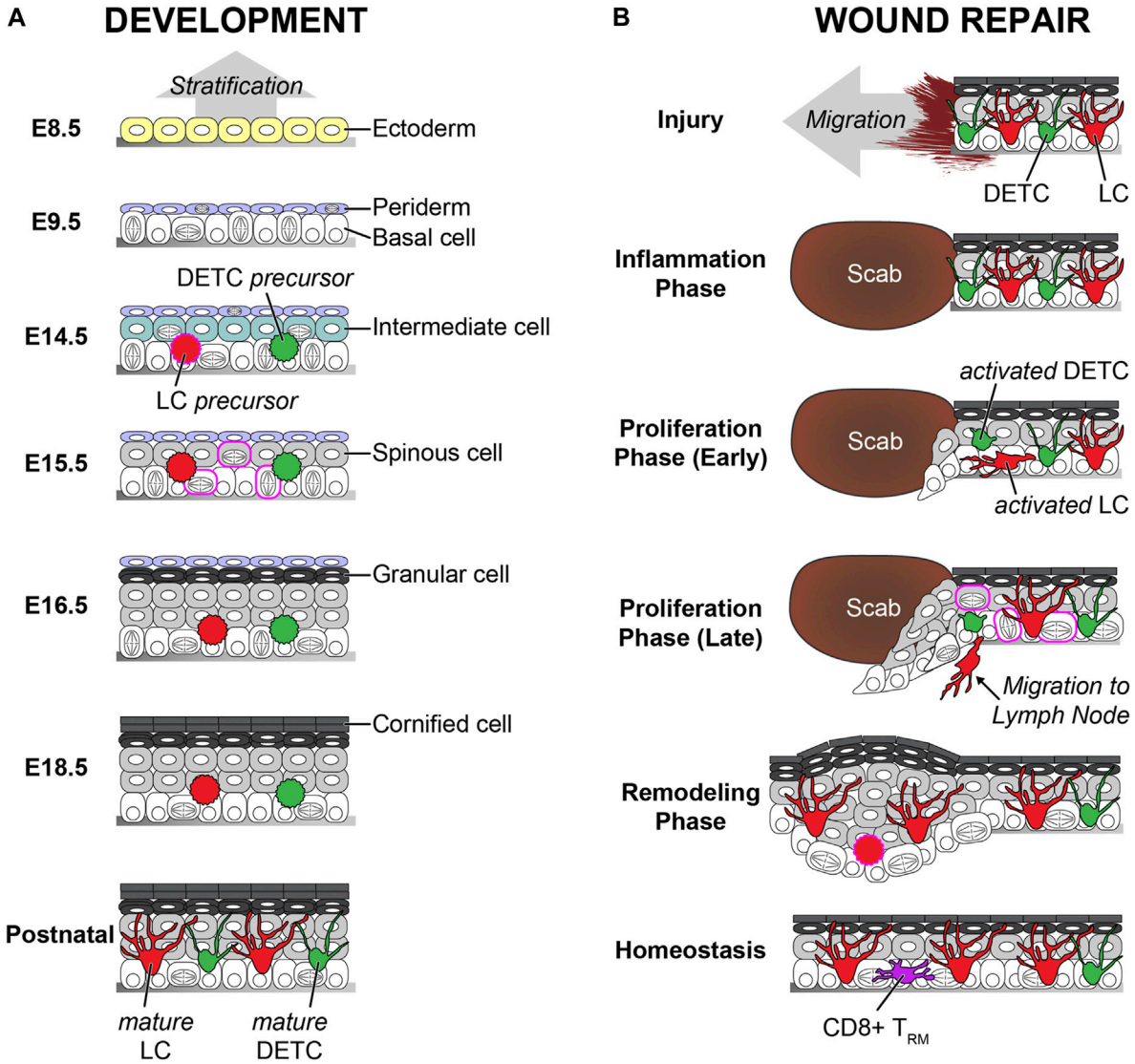

FIGURE 2 | Comparison of the development and the wound repair of the epidermis. **(A)** The vertical stratification builds the epidermis during the development. Ectoderm-derived single layer cells on the basement membrane differentiate to the basal layer cells. Basal layer cells make the periderm on the top and the intermediated layer between the basal layer and the periderm. The intermediate cells change to spinous cells. The spinous cells differentiate into granular cells. The periderm sheds off at the later stage of the embryogenesis, and the granular cells differentiate to the cornified cells before birth. Both precursors from LCs and DETCs are seeded in the epidermis at the early embryogenesis. These immature cells increase the number by proliferation burst and change to dendritic morphology by maturation after birth. The embryonic days (E) are based on mouse embryogenesis. **(B)** The lateral migration rebuilds the damaged epidermis during the wound repair. The inflammation phase is initiated right after the injury. Next, the re-epithelialization starts during the proliferation phase. Both undifferentiated basal and differentiated spinous cells migrate together. Proliferation is dramatically increased. Migrating cells and cells in the spinous layer show cell division during re-epithelialization, which are not observed during homeostasis. The epidermis becomes thick after the re-epithelialization, and the thickness gradually decreases during the remodeling phase. Both DETCs and LCs are activated after the inflammation phase. The activated DETCs become round, and the activated LCs lose contact with the tight junctions. The activated LCs leave the epidermis to migrate to the lymph node. LC density is low right after the re-epithelialization, but LCs recover the normal density during the remodeling phase. The DETCs do not exist in the neo-epidermis, and $CD8^+$ TRMs replace the territory of the DETCs. Similar features between the development and the wound repair are labeled in magenta. During the wound repair, the proliferation rate is dramatically increased, and even asymmetric and spinous divisions are observed like the development. In addition, there is a possibility that LC precursors repopulate on the neo-epidermis like the development.

regulator of epidermal stratification (Mills et al., 1999; Yang et al., 1999). An additional layer, called periderm, is also formed above the basal layer at this time point. The periderm layer is transient during embryogenesis and acts as a protective barrier by preventing immature adhesion between epithelial cells during development (Richardson et al., 2014). Asymmetric cell divisions of basal cells make an intermediate layer. Cells in the intermediate layer become the spinous layer (Lechler and Fuchs, 2005). Differentiation of spinous layer cells creates a granular layer above the spinous layer. The periderm layer sheds off in the

late stage of embryogenesis, and the cornified layers are formed on the top. To build the fully differentiated epidermis by using a 3D *ex vivo* skin organ culture system, keratinocytes need to be incubated at the air–liquid interface (Carlson et al., 2008). In contrast, full-term neonates have a fully developed epidermis with the cornified layers, despite the epidermis being exposed to amniotic fluid (Oranges et al., 2015) (**Figure 2**).

Wound repair in adult skin is a complex process in which diverse cell types participate. Generally, it is divided into three phases: inflammation, proliferation, and remodeling (Gurtner et al., 2008). First, the inflammation phase starts immediately after the injury. Immune cells, such as neutrophils and macrophages, are recruited at the site of injury and clean up dead cells and bacteria. Next, the proliferation stage follows inflammation as cells in the skin reconstitute the damaged structure of the skin, including epithelium, extracellular matrix, and blood vessels. After skin injury, adjacent epithelial cells seal the damaged area *via* re-epithelialization, also known as epidermal repair. Abnormal re-epithelialization enhances the opportunity for additional infections which lead to the development of severe diseases, such as non-healing chronic wounds (Eming et al., 2014). Whereas the single basal layer forms stratified epithelium *via* vertical differentiation during development, and the damaged epidermis is restored by the lateral movement of adjacent epithelial cells. Chemical cues from infected microbiome, dead cells, and immune cells, as well as physical cues from the empty space, initiate the migration of epithelial cells near the wound area within a few hours after injury (Ben Amar and Wu, 2014; Pastar et al., 2014; Chester and Brown, 2017; Krzyszczyk et al., 2018). The contribution of the epidermal basal and suprabasal layers has been long debated. Recently, intravital imaging revealed that both basal and suprabasal cells actively migrate with dynamic lamellipodial movements (Park et al., 2017). Migration tracking analysis identified that the speed of migration is vertically "gradient." The bottom basal layer cells are fast, and middle spinous layer cells are slow. The top granular layer cells do not move, which could be due to the solid cell–cell adhesion *via* tight junctions. The participation of differentiated cells is crucial because inhibiting the migration of suprabasal cells delays wound closure dramatically (Park et al., 2017). This could be because of the lateral movement of the migrating cells, as well as because of the urgency to reduce the exposure to the external environment. During the re-epithelialization, proliferation is dramatically increased like the development after the initiation of migration to supply cells to the wound. Previously, it was considered that proliferation cannot occur in migrating cells. However, the proliferation and migration territories are partially overlapped, and cells in the overlapped territory, also known as a mixed zone, can perform both proliferation and migration at the same time. In addition, cells in the suprabasal layers can proliferate during wound repair similar to epidermal development (Richardson et al., 2014; Park et al., 2017; Damen et al., 2021). It remains unclear whether undifferentiated cells can be found in the upper layers because of premature delamination or because differentiated cells can dedifferentiate during wound repair. Finally, the last phase of the wound repair is remodeling. Since differentiation was increased during re-epithelialization, the epidermis is thicker than the normal epidermis before injury (Aragona et al., 2017; Park et al., 2017). The thick epidermis on the wounded area, also known as a neo-epidermis, slowly becomes thinner overtime during the remodeling. It is still unclear how the epidermal thickness is regulated and whether remodeling dermis impacts the epidermal thickness. Since apoptosis plays a crucial role to maintain epidermal homeostasis and to remodel psoriatic epidermis, increased apoptosis might be involved in thickness reduction (Weatherhead et al., 2011; Riwaldt et al., 2021). Alternatively, an enhanced differentiation rate could lead to faster shedding of cells from the skin's surface, reminiscent of the periderm elimination during the development (**Figure 2**).

Several signaling factors play important roles in the development and in the wound repair. For example, calcium signaling and reactive oxygen species (ROS) are essential for the differentiation of the epidermis during development (Hamanaka et al., 2013; Bikle et al., 2014). These factors regulate collective migration and enhance proliferation of epithelial cells during wound repair (Hoffmann and Griffiths, 2018; Tu et al., 2019). In terms of the epidermal growth factor receptor (EGFR) signaling, EGFR KO mice the show abnormal epidermal structure during the development (Mascia et al., 2013). However, the EGFR is related to the migration and the proliferation of epithelial cells during wound repair, rather than the structural abnormalities (Repertinger et al., 2004). Collectively, these data suggest that signaling factors impact both the development and the wound repair, but regulatory mechanisms can be different depending on the circumstances of the tissue.

Tissue-Resident Immune Cells in Development vs. Injury

During development, LCs are derived from two different sources. The first wave of precursors comes from the yolk sac, and the second wave of precursors comes from the fetal liver (Hoeffel et al., 2012). Since the yolk sac–derived LC precursors are mostly replaced by LC precursors from fetal liver, the latter is predominant in the adult epidermis (Hoeffel et al., 2012). The LC precursors in the fetal skin do not show dendritic morphology, and their number is much lower than LCs in the adult skin. The proliferation of LCs is dramatically increased, and LCs become mature within 1–2 weeks after birth (Doebel et al., 2017). Morphological and functional maturation, together with increased cell numbers, establish the regular immune network to create the immunological barrier (Deckers et al., 2018). Recently, single-cell RNA-seq and mass cytometry data revealed that there are two subsets of LCs, which are phenotypically and functionally distinct, in the human skin (Liu et al., 2021). However, it is unclear whether these subsets are dependent on their fetal origins.

Although mature LCs in the adult skin are fully differentiated, they can self-renew within the tissue much like tissue-resident macrophages (Doebel et al., 2017). Multicolor fate mapping demonstrated that mature LCs maintain their network within the epidermis without replenishment from precursors in the steady state (Ghigo et al., 2013). Therefore, about 5% of total

LCs proliferate during homeostasis (Chorro et al., 2009). However, once immunological damage occurs, such as LC depletion, by using genetic mouse models with diphtheria toxin, UV irradiation, or inflammation, outer precursor cells repopulate the damaged area and differentiate to mature LCs (Kaplan, 2017). Similar to the developmental LC seeding, two distinct types of precursors repopulate at different time points (Seré et al., 2012). The first wave is from Gr-1^{hi} monocytes 1–2 weeks after damage, and LCs from these monocytes stay short term in the epidermis. The second wave is for long-term repopulation by bone marrow–derived precursors a few weeks after damage. LCs also recover their network after the skin injury. Previous studies showed that LCs are located on the neo-epidermis within 2–5 days. However, it is still unclear whether LCs repopulate during or after re-epithelialization like the development. Given the short time of repopulation, there is a possibility that LCs in the epidermis migrate together with epithelial cells during the re-epithelialization.

DETCs originate only from the fetal thymus during development (Havran and Jameson, 2010). Precursor cells maturate into DETCs in the epidermis and can self-renew in the adult skin during homeostasis like LCs (Gentek et al., 2018). However, in contrast to LCs, there are no additional precursors for replenishment in adults. Therefore, DETCs are not found in the damaged area after wound repair, but CD8$^+$ T_{RM} covers the region instead (Zaid et al., 2014; Sandrock et al., 2018). Although DETCs do not participate in the reconstitution of the immunological barrier, activated DETCs near the wound edge directly contribute to re-epithelialization by enhancing proliferation with the secretion of keratinocyte growth factors (Jameson et al., 2002). Mobility and long-term fate of activated DETCs within the repairing epithelium need to be identified in the future.

CONCLUSION

The epidermis is composed of diverse cell types, and each cell type has a specialized barrier function to protect our body. The proper cellular function can be exerted within the spatially organized structure. Wound repair is the process of restoring the organization in an orderly manner to regain pre-existing architecture. The epidermal architecture is initially built during embryonic development. Therefore, wound repair and embryonic development share a common goal of building/rebuilding a functional tissue and several common features of processes. Although the damaged skin is built well *via* the repairing process, it still has limitations compared to developmental skin formation such as scarring and loss of appendages. Comprehensive understanding of tissue creation by development and by the repair processes will provide new therapeutic strategies for more efficient healing.

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Dysregulated Peripheral Invariant Natural Killer T Cells in Plaque Psoriasis Patients

Yifan Hu[1,2,3†], *Youdong Chen*[2,3†], *Zeyu Chen*[2,3†], *Xilin Zhang*[1,3], *ChunYuan Guo*[1,3], *ZengYang Yu*[2,3], *Peng Xu*[2,3], *Lei Sun*[1,3], *Xue Zhou*[2,3], *Yu Gong*[2,3], *Qian Yu*[2,3]* and *Yuling Shi*[1,2,3]*

[1]Department of Dermatology, Shanghai Skin Disease Hospital, Tongji University School of Medicine, Shanghai, China, [2]Department of Dermatology, Shanghai Tenth People's Hospital, Tongji University School of Medicine, Shanghai, China, [3]Institute of Psoriasis, Tongji University School of Medicine, Shanghai, China

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***Correspondence:**
Qian Yu
yuervictory@163.com
Yuling Shi
shiyuling1973@tongji.edu.cn

†These authors have contributed equally to this work

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Background: Psoriasis is a common immune-mediated skin disease that involves T-cell-mediated immunity. Invariant natural killer T (*i*NKT) cells are a unique lymphocyte subpopulation that share properties and express surface markers of both NK cells and T cells. Previous reports indicate that *i*NKT cells regulate the development of various inflammatory diseases. IL-17 is a key cytokine in the pathogenesis of psoriasis and a key therapeutic target. Secukinumab is a fully human IgG1κ antibody that targets IL-17A, thereby antagonizing the biological effects of IL-17.

Objective: To explore the expression of *i*NKT cells in psoriasis patients and the effect of secukinumab on them.

Methods: We examined the frequencies of *i*NKT cells, Tregs, naïve and memory $CD4^+$and $CD8^+$T cells in the PBMCs as well as their cytokine production in a cohort of 40 patients with moderate-to-severe plaque psoriasis and 40 gender- and age-matched healthy controls. We further collected peripheral blood of another 15 moderate-to-severe plaque psoriasis patients who were treated with secukinumab and evaluated the proportion of *i*NKT cells in the PBMCs at baseline and week 12.

Results: The frequencies of conventional $CD4^+$ T cells, $CD8^+$ T cells, and Tregs in the PBMCs were comparable between psoriasis patients and healthy controls, but the frequencies of Th17 cells, Tc1 cells and Tc17 cells were increased in psoriasis patients. The frequency of peripheral *i*NKT cells and $CD69^+$ *i*NKT cells was significantly decreased in psoriasis patients. Both *i*NKT2 cells and *i*NKT17 cells were increased in psoriasis patients, but the ratio of *i*NKT2 cells vs *i*NKT17 cells was significantly reduced in psoriasis patients. After receiving secukinumab, the proportion of *i*NKT cells in the PBMCs of patients was increased, while the proportion of *i*NKT17 cells was decreased.

Conclusion: Dysregulated *i*NKT cells may be involved in the pathogenesis of psoriasis and secukinumab may play a regulatory role on *i*NKT cells.

Keywords: psoriasis, iNKT cells, IFN-γ, IL-4, IL-17

INTRODUCTION

Psoriasis is a common inflammatory skin disease that tends to recur frequently and presently has no cure. The incidence of psoriasis varies worldwide, and its prevalence rate in China is 0.47% (Ding et al., 2012). The development of psoriasis primarily involves T-cell-mediated immunity, and the interleukin (IL)-23/T helper 17 (Th17) cell axis plays an essential role in the pathogenesis of psoriasis (Boehncke and Schon, 2015).

Natural killer T cells (NKT cells) are a unique lymphocyte subpopulation that shares immune properties and expresses surface markers of both natural killer (NK) cells and T cells. Upon activation, NKT cells rapidly produce Th1, Th2, and Th17 cytokines (Brennan et al., 2013). In general, NKT cells are divided into type I (invariant NKT [*i*NKT]) and type II (non-*i*NKT) cells (Godfrey et al., 2004). *i*NKT cells express an invariant TCRα chain consisting of Vα14/Jα18 paired with a limited range of TCRβ chains in mice or Vα24/Jα18 paired with Vβ11 in humans (Koseki et al., 1991; Arase et al., 1992; Lantz and Bendelac, 1994). *i*NKT cells demonstrate CD1d restriction, and α-GalCer is the first CD1d-presented lipid antigen for *i*NKT cells (Kawano et al., 1997). Therefore, immunostaining with α-GalCer-loaded CD1d tetramers could be useful for precise identification of *i*NKT cells (Kawano et al., 1997).

Based on cytokine production and transcription factor expression, *i*NKT cells can be differentiated into at least three subsets: Th1-like *i*NKT cells (*i*NKT1) that secrete interferon-γ (IFN-γ) and express T-bet (Townsend et al., 2004); Th2-like *i*NKT cells (*i*NKT2) that produce Interleukin-4 (IL-4) and are dependent on the transcription factors PLZF, GATA3, and IRF4 for development (Kim et al., 2006; Lee et al., 2013); and Th17-like *i*NKT cells (iNKT17) that secrete Interleukin-17 (IL-17) and express ROR-γt (Coquet et al., 2008).

*i*NKT cells are a type of key immunoregulatory T cell. *i*NKT cells have been reported to be involved in the development of various inflammatory diseases. They participate in the control of inflammatory bowel disease, allograft tolerance, and regulation of atopic eczema (Seino et al., 2001; Fuss et al., 2004; Simon et al., 2009; Tsuruyama et al., 2012). Previous research showed that the population of NKT cells increases significantly in psoriatic lesions (Bonish et al., 2000; Cameron et al., 2002; Ottaviani et al., 2006; Zhao et al., 2008). In contrast to the accumulation of NKT cells in psoriatic plaques, a few studies have documented decreased proportions and compromised immune activities of NKT cells in the peripheral blood of psoriasis patients (Van Der Vliet et al., 2001; Koreck et al., 2002; Werner et al., 2011). On the contrary, Langewouters *et al.* found an increase in the number of circulating $CD94^{+}CD161^{+}$ NKT cells in psoriasis patients (Langewouters et al., 2008). It has been confirmed that human *i*NKT cells can produce IL-17 in a pro-inflammatory environment (Moreira-Teixeira et al., 2011). Mars *et al.* discovered that *i*NKT cells played an important role in limiting the development of the Th17 lineage and provided a natural barrier against Th17 responses in EAE mouse model (Mars et al., 2009). Keunhee's study also showed that *i*NKT cells can suppress Th17 cell differentiation (Oh et al., 2011). However, the cell surface markers utilized to identify *i*NKT cells in the aforementioned studies, for example, CD3, CD161, and CD94, were not specific to *i*NKT cells. In the present study, we used CD1d tetramers, which are exclusive markers of *i*NKT cells, to accurately identify *i*NKT cells and evaluate their immune functions in psoriasis patients.

Secukinumab is a fully human IgG1κ antibody that targets IL-17A, thereby antagonizing the biological effects of the cytokine. In 2015, secukinumab was approved by the European Medicines Evaluation Agency (EMEA) and the U.S. Food and Drug Administration (FDA) for marketing in Europe and the United States for the treatment of adult moderate-to-severe plaque psoriasis. We also evaluate the proportion of *i*NKT cells in the PBMCs of psoriasis patients treated with secukinumab at baseline and week 12, and analyze whether there is a difference in the proportion of *i*NKT cells before and after treatment.

TABLE 1 | Details of moderate-to-severe plaque psoriasis patients.

Characteristics	Healthy Control (N = 40)	Psoriasis Patients (N = 40)
Age, years, mean (SD)	40.1 ± 10.05	40.85 ± 9.81
Female sex, n (%)	45	45
Body mass index	23.87 ± 2.44	26.34 ± 4.21
Disease duration, years, mean (SD)	-	16.63 ± 9.21
PASI score, mean (SD)	-	18.88 ± 7.63
PGA score, mean (SD)	-	2.25 ± 0.43
BSA (%), mean (SD)	-	39.53 ± 5.59
DLQI score, mean (SD)	-	11.86 ± 7.17

MATERIALS AND METHODS

Patients

This study was approved by Shanghai Tenth People's Hospital Ethics Committees (IRB approval number: 2013-RES-14). We collected the peripheral blood of 40 moderate-to-severe plaque psoriasis patients and 40 gender- and age-matched healthy controls from December 2017 to December 2019. The disease severity of psoriasis patients was assessed using the psoriasis area and severity index (PASI) score. Patients' PASI score were all ≥10 when the blood samples were drawn (**Table 1**). We also collected peripheral blood of another 15 moderate-to-severe plaque psoriasis patients who were treated with secukinumab before and after the 12 weeks of treatment. Another 15 gender- and age-matched healthy controls' peripheral blood were also collected. All the participants had no other autoimmune diseases, systemic diseases, malignant tumor or active infections, and had not received systemic therapy for at least 4 weeks or topical therapy for at least 2 weeks. All the procedures were in accordance with the tenets of the Declaration of Helsinki for research involving human subjects. Informed consent was obtained from all the participants, and their clinical information and peripheral blood samples were collected for analysis.

Treatment and Assessments

15 patients received subcutaneous secukinumab 300 mg at Week 0, 1, 2, 3, 4. After that, they received subcutaneous secukinumab

300 mg every 4 weeks for maintenance treatment. During the treatment, patients should not take any other drugs or physical therapy that may affect the evaluation of efficacy, such as calcineurin inhibitors, glucocorticoids, vitamin D3 derivatives, acitretin, methotrexate, cyclosporine, other biological agents, PUVA and NB-UVB. Patients were recommended to use moisturizing cream daily.

PASI, PGA and BSA score were used for patients' efficacy assessment.

Isolation of Peripheral Blood and Flow Cytometry

PBMCs were freshly separated from human peripheral blood using Ficoll-Paque Plus (Catalog# 17-1440-03, GE Healthcare) according to the manufacturer's recommendations. PBMCs were treated *in vitro* with Cell Stimulation Cocktail (Catalog# 00-4970-03, eBioscience) for 5 h to detect cytokine secretion. To identify dead cells, the cells were first stained with Fixable Viability Stain 780 (Catalog# 565,388, BD Biosciences) for 15 min at room temperature. Subsequently, the cells were stained for 30 min with surface marker antibodies in phosphate-buffered saline containing 2% fetal bovine serum at 4°C. For detecting intracytoplasmic cytokines (IC), the cells were fixed with IC Fixation Buffer (Catalog# 00-8222-49, eBioscience) for 30 min at 4°C. For analyzing intranuclear transcription factors, the cells were fixed with Fixation/Permeabilization Diluent and Concentrate (Catalog# 88-8824-00, eBioscience) at 4 °C for 40 min. After fixation, the cells were stained with intracellular antibodies in Permeabilization Buffer (Catalog# 00-8333-56, eBioscience) at 4 °C for 30 min.

To analyze $CD4^+$ T cells, $CD8^+$ T cells, regulatory T cells (Treg), and *i*NKT cell frequencies and immunofunctions, PBMCs were stained with the following anti-human antibodies: APC-conjugated α-GalCer:CD1d tetramer (NIH tetramer facility, United states), FITC-conjugated anti-CD4 (Catalog# 11-0048-42, eBioscience), APC-conjugated anti-CD4 (Catalog# 17-0049-42, eBioscience), PE-conjugated anti-CD4 (Catalog# 12-0048-42, eBioscience), PE-conjugated anti-CD8 (Catalog# 12-0086-42, eBioscience), PerCP/Cy5.5-conjugated anti-CD8 (Catalog# 301,032, eBioscience), PE-conjugated anti-IL-17A (Catalog# 12-7179-42, eBioscience), APC-conjugated anti-IFN-γ (Catalog# 17-7319-82, eBioscience), PE/cyanine 7 (Cy7)-conjugated anti-IL-4 (Catalog# 25-7049-82, eBioscience), PE-conjugated anti-CD25 (Catalog# 12-0259-42, eBioscience), APC-conjugated anti-forkhead box P3 (FOXP3) (Catalog# 17-4777-42, eBioscience), PE/Cy7-conjugated anti-CD3 (Catalog# 300,420, BioLegend), FITC-conjugated anti-CD69 (Catalog# 11-0699-42, eBioscience), FITC-conjugated anti-CD45RA (Catalog# 11-0458-42, eBioscience), PE/Cy7-conjugated anti-CD45RO (Catalog# 25-0457-42, eBioscience), FITC-conjugated anti-GATA3 (Catalog# 53-9966-42, eBioscience), PE-conjugated anti-ROR-γt (Catalog# 12-6988-82, eBioscience), PerCP/Cy5.5-conjugated anti-T-bet (Catalog# 644,805, Biolegend), and FITC-conjugated anti-IFN-γ (Catalog# 11-7319-82, eBioscience).

Data were acquired on a FACS Canto II (BD Biosciences) and analyzed using the FlowJo software (Tree Star).

Statistical Analysis

Data are presented as mean ± SD and shown as dot plots of individual samples. Statistical significance was assessed with a two-tailed paired student's *t*-test. Correlation analysis was performed using the Pearson correlation test. All statistical analyses were performed using the GraphPad Prism software. For all cases, significant differences were considered at p values < 0.05.

RESULTS

Normal Distribution of Circulating $CD4^+$ T Cells, $CD8^+$ T Cells and Tregs in Psoriasis Patients

First, we examined peripheral conventional T cells by flow cytometry. As shown in **Figures 1A, 1B**, the percentages of $CD4^+$ and $CD8^+$ T cells in the PBMCs were comparable between psoriasis patients and healthy controls. There was also no significant difference in the percentages of naïve ($CD45RA^+$) and memory ($CD45RO^+$) T cells within either the $CD4^+$ T cell or $CD8^+$ T cell subsets between psoriasis patients and healthy controls (**Figures 1C,D**). We further analyzed T-cell activation based on the expression of CD69. As shown in **Figures 1E,F**, a higher number of CD69-positive $CD4^+$ T cells were found in the psoriasis patients than in the healthy controls. However, no significant differences were observed in the proportion of CD69-positive $CD8^+$ T cells between psoriasis patients and healthy controls. Similarly, no significant difference was detected in the proportion of circulating $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs (**Figure 1G,H**, gating strategy in **Supplementary Figure S1**). Thus, our results demonstrated normal distribution of peripheral conventional T cells and overactivation of conventional $CD4^+$ T cells.

Increase in Th17 Cells, Tc1 and Tc17 Cells in the PBMCs of Psoriasis Patients

Next, we examined the production of IFN-γ, IL-4, and IL-17 by conventional T lymphocytes. There was no significant difference in the percentages of Th1 and Th2 cells between psoriasis patients and healthy individuals (**Figures 2A,B**). Notably, the proportion of Th17 cells was significantly augmented in the PBMCs of psoriasis patients (**Figures 2A,B**), as reported in previous studies (Furue et al., 2019). Moreover, the proportions of circulating T cytotoxic 1 (Tc1) and Tc17 cells were significantly upregulated in psoriasis patients (**Figures 2C,D**).

Decrease in the Frequency of *i*NKT Cells in the PBMCs of Psoriasis Patients

*i*NKT cells have been shown to play a crucial role in the development of autoimmune diseases (Bendelac et al., 2007). The α-GalCer-loaded CD1d tetramer is the best reagent currently available to accurately distinguish *i*NKT cells in terms of specificity and sensitivity (Berzins et al., 2011). As depicted in

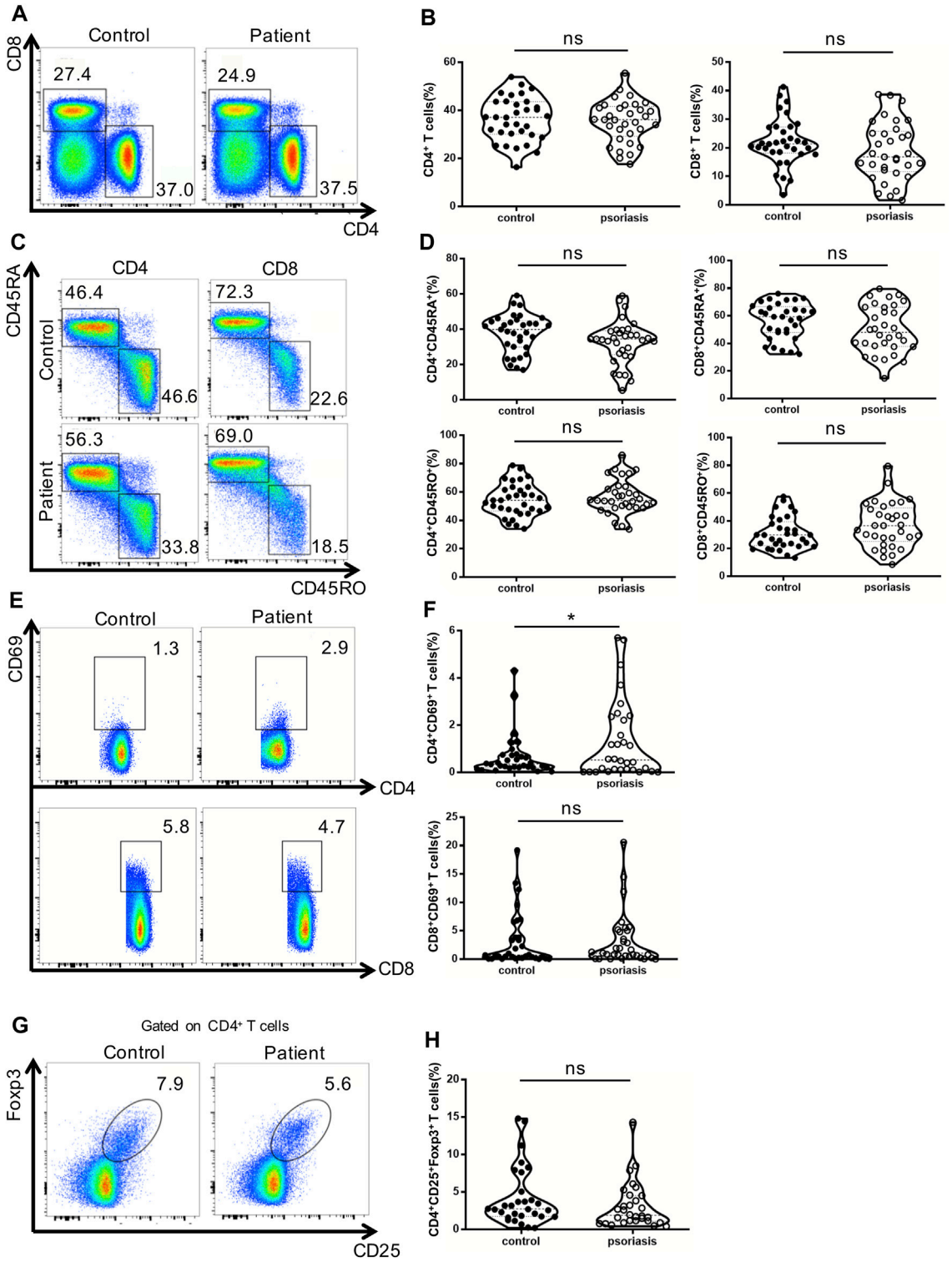

FIGURE 1 | Distribution of conventional T cells and Tregs in the peripheral blood. **(A)** Flow cytometry analysis of $CD4^+$ and $CD8^+$ T cells in PBMCs from moderate-to-severe plaque psoriasis patients and healthy controls. **(B)** Summary plots showing individual results of the frequency of $CD4^+$ and $CD8^+$ T cells in moderate-to-severe plaque psoriasis patients versus healthy controls. **(C)** Flow cytometry analysis of memory (CD45RO+) and naïve (CD45RA+) $CD4^+$ and $CD8^+$ T cells in PBMCs from moderate-to-severe plaque psoriasis patients and healthy controls. **(D)** Summary plots showing individual results of the frequency of CD45RA+ and CD45RO + $CD4^+$ and $CD8^+$ T cells in moderate-to-severe plaque psoriasis patients versus healthy controls. **(E)** Flow cytometry analysis of CD69 expression in $CD4^+$ and $CD8^+$ T cells in PBMCs from moderate-to-severe plaque psoriasis patients and healthy controls. **(F)** Summary plots showing individual results of the frequency of $CD69^+$ $CD4^+$

(*Continued*)

FIGURE 1 | T cells and CD69 $^{+}$ CD8^{+} T cells in moderate-to-severe plaque psoriasis patients versus healthy controls. **(G)** Flow cytometry analysis of CD4^{+}CD25 $^{+}$ Foxp3+ Tregs in PBMCs from moderate-to-severe plaque psoriasis patients and healthy controls. **(H)** Summary plots showing individual results of the frequency of CD4^{+}CD25 $^{+}$ Foxp3+ Tregs in psoriasis patients versus healthy controls. Data show mean +SEM. *p*-values were determined by paired Student's t-test. ns, no significance, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$.

Figures 3A,B, the proportion of *i*NKT cells in the PBMCs from psoriasis patients was lower than that in healthy controls. This indicates that a defect in *i*NKT cells might be involved in the development of psoriasis. However, there was no correlation between the proportion of *i*NKT cells and PASI score in psoriasis patients (**Supplementary Figure S2**).

Increase in *i*NKT2 and *i*NKT17 Cell Sublineages in Psoriasis Patients

Human mature *i*NKT cells can be divided into functionally distinct CD4^{+} and CD4^{-} subsets. CD4^{+} *i*NKT cells produce both Th1 and Th2 cytokines, whereas the CD4^{-} *i*NKT subset mainly exhibits a Th1 cytokine profile (Gumperz et al., 2002). To investigate whether *i*NKT cells from psoriasis patients exhibited phenotypic abnormalities, we first analyzed the proportion of the CD4^{+} *i*NKT subset in the PBMCs, but no significant differences were detected between psoriasis patients and healthy controls (**Figures 3C,D**).

We further analyzed the sublineages of *i*NKT cells (**Figures 3E–G**). While the Mean Fluorescence Intensity (MFI) of T-bet^{+} *i*NKT cells (*i*NKT1) remained unaltered, the MFI of GATA3^{+} *i*NKT cells (*i*NKT2) and ROR-γt^{+} *i*NKT cells (*i*NKT17) were significantly increased in psoriasis patients. Moreover, we found that the ratio of GATA3^{+} *i*NKT cells vs ROR-γt^{+} *i*NKT cells decreased and the ratio of ROR-γt^{+} *i*NKT cells vs T-bet^{+} *i*NKT cells increased in psoriasis patients, suggesting that there may be imbalance of *i*NKT cells sublineages in psoriasis (**Figure 3H**).

Decrease in *i*NKT Cell Activation and Increased IL-4- and IL-17-Producing *i*NKT Cells in Psoriasis Patients

CD69 has been utilized as a cell-surface marker of *i*NKT cell maturation and activation. The percentage of CD69^{+} *i*NKT cells was reduced in the PBMCs of psoriasis patients, suggesting that *i*NKT cells are less activated in psoriasis patients competed to healthy controls (**Figures 4A,B**). In addition, the MFI of IL-4- and IL-17-producing *i*NKT cells were significantly increased in psoriasis patients, whereas no significant difference was detected in the MFI of IFN-γ-producing *i*NKT cells between psoriasis patients and healthy controls (**Figures 4C–E**). This was in accordance with our findings for the *i*NKT cell sublineages. Furthermore, there was no significant difference in the ratio of IL-17-producing *i*NKT cells vs IFN-γ-producing *i*NKT cells and the ratio of IL-4-producing *i*NKT cells vs IFN-γ-producing *i*NKT cells between psoriasis patients and healthy controls (**Figure 4F**).

Increase in the Frequency of *i*NKT Cells in Psoriasis Patients After Receiving Secukinumab

Secukinumab is effective in the treatment of moderate and severe plaque psoriasis. Patients' characteristics, PASI, PGA and BSA score before and after the treatment are shown in **Table 2**. We further analyzed the proportion of *i*NKT cells in the PBMCs of psoriasis patients before and after they treated with secukinumab, and found an increase of *i*NKT cells after the treatment (**Figures 5A,B**). Moreover, we found a decrease of *i*NKT17 subset in *i*NKT cells after the treatment, while *i*NKT1 and *i*NKT2 subset remained no change (**Figures 5C–E**).

DISCUSSION

In the present study, we isolated mononuclear cells from peripheral blood and detected the expression of *i*NKT cells, conventional T cells and their cytokine production, as well as Treg cells by flow cytometry. Our study represents a large-scale systemic analysis of the basic immunophenotypes of psoriasis patients with strictly matched healthy individual controls. We found that there were no differences between psoriasis patients and the healthy controls with regard to the percentages of conventional CD4^{+} and CD8^{+} T cells, naïve, memory CD4^{+} and CD8^{+} T cells and active CD8^{+} T cells. However, an increase in CD4^{+} T cell activation was found in psoriasis patients. IL-17A is a key cytokine that participates in the pathogenesis of psoriasis. Biologic agents targeting IL-17A or IL-17RA have been demonstrated to have considerable clinical impact in the treatment of psoriasis, and this further proves the vital role of IL-17A in psoriasis (Leonardi et al., 2012; Papp et al., 2012; Van De Kerkhof et al., 2016). Marcel *et al.* showed that the proportion of IL-17A-producing CD8^{+} T cells in the blood of psoriasis patients correlates with their PASI score (Teunissen et al., 2014). In our study, we found that the Th17 and Tc17 populations were significantly increased in the PBMCs of psoriasis patients, in accordance with previous studies (Teunissen et al., 2014; Dainichi et al., 2018). We also found that the population of Tc1 cells was significantly increased in psoriasis patients. However, there were no differences in the percentages of Th1, Th2, and Tc2 cells between psoriasis patients and healthy controls. Treg cells are a subset of T cells that can suppress the inflammation induced by other T cells in autoimmune diseases (Jorn Bovenschen et al., 2011; Barbi et al., 2014; Deng et al., 2016; Ma et al., 2018). Several studies have shown the decreased number and impaired suppressive capacity of Treg cells in autoimmune diseases (Read et al., 2000; Balandina et al., 2005; Zhou et al., 2009; Bailey-Bucktrout et al., 2013; Nakagawa et al., 2016). But research results of Treg cells in

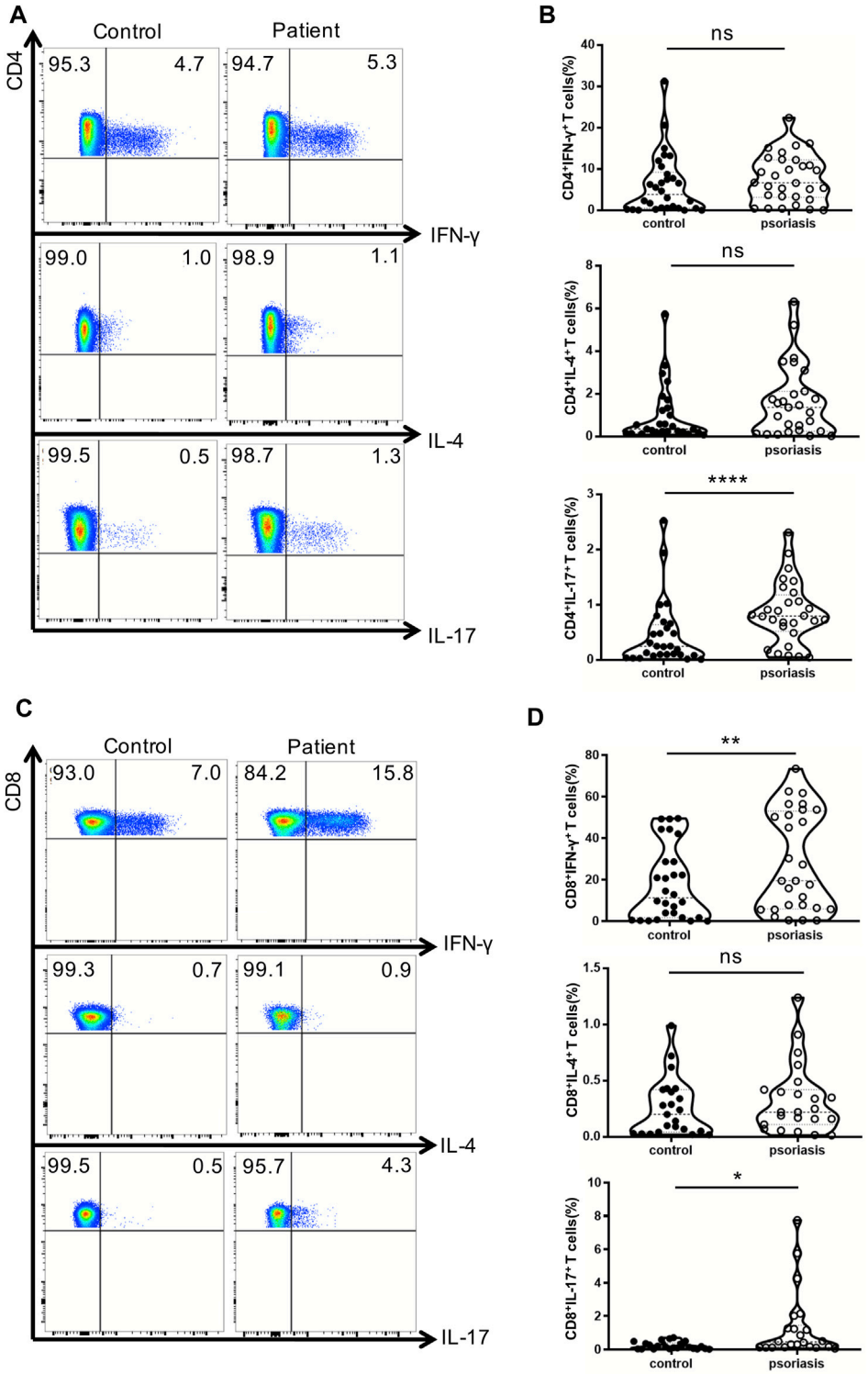

FIGURE 2 | Cytokine-producing T cells from peripheral blood. PBMCs isolated from moderate-to-severe plaque psoriasis patients and healthy controls were stimulated with Cell Stimulation Cocktail for 5 h. The IFN-γ-, IL-4- and IL-17-producing T cells were determined by intracellular staining and flow cytometry analysis. **(A)** The proportion of IFN-γ–,IL-4- and IL-17-producing $CD4^+$ T cells in psoriasis patients versus healthy controls. **(B)**Summary plots showing individual results of the frequency of IFN-γ–,IL-4- and IL-17- producing $CD4^+$ T cells in psoriasis patients versus healthy controls. **(C)**The proportion of IFN-γ–,IL-4- and IL-17-producing $CD8^+$ T cells in psoriasis patients versus healthy controls. **(D)**Summary plots showing individual results of the frequency of IFN-γ-,IL-4- and IL-17- producing $CD8^+$ T cells in psoriasis patients versus healthy controls. Data show mean +SEM. *p*-values were determined by paired Student's t-test. ns, no significance, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$.

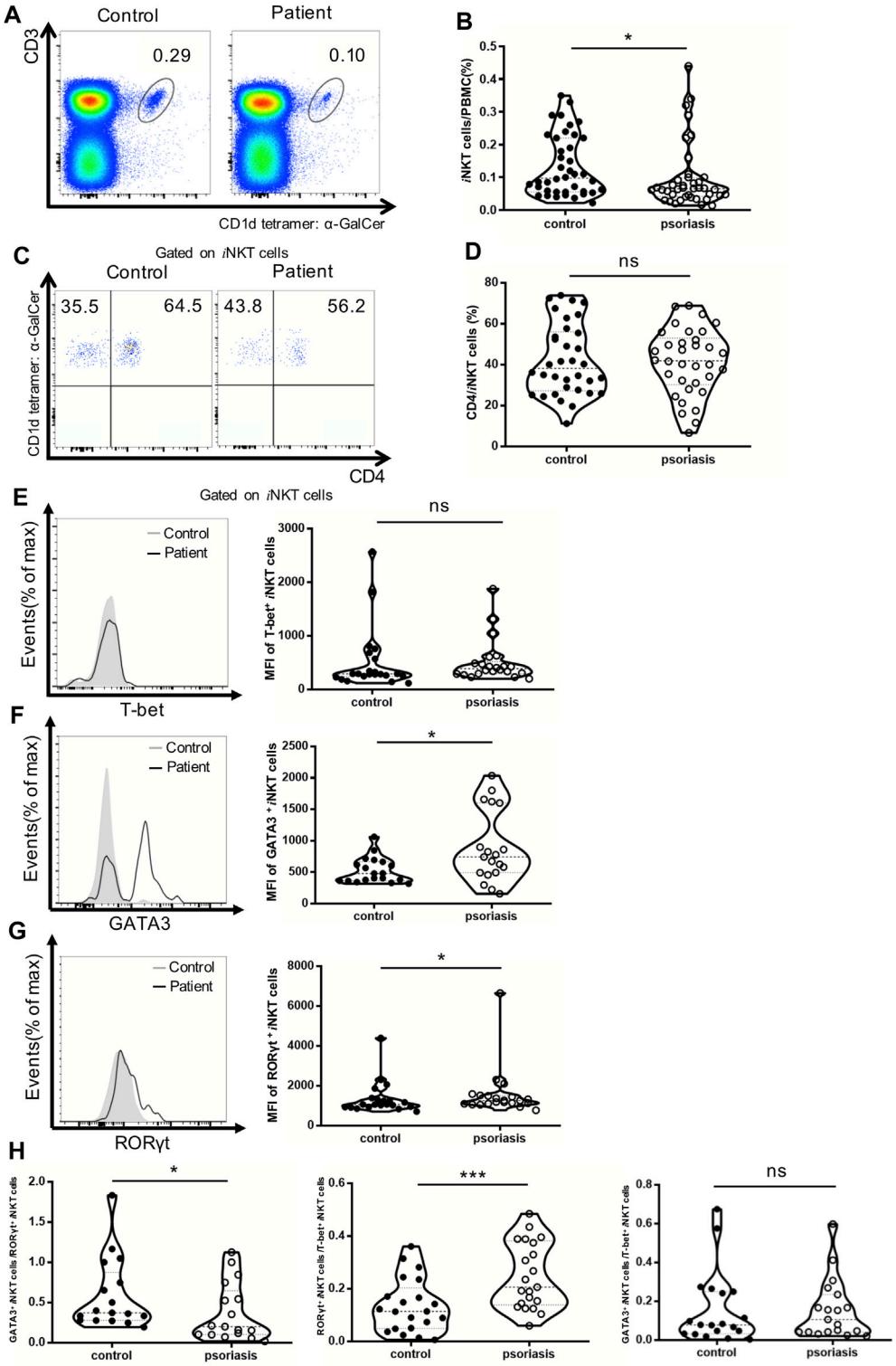

FIGURE 3 | *i*NKT cells frequency and cell subsets in PBMCs of psoriasis patients. **(A)** Representative FACS dot plots for iNKT cells from psoriasis patients and healthy controls. **(B)** Summary plots showing individual results of iNKT cell frequency in psoriasis patients versus healthy controls. **(C)** Representative FACS dot plots for iNKT cell CD4 expression in psoriasis patients and healthy controls. **(D)** Summary plots showing individual results of the frequency of $CD4^+$ iNKT cells in psoriasis patients versus healthy controls. **(E)** Representative histogram and summary plots showing individual results of the MFI for T-bet + iNKT cells in psoriasis patients and healthy controls. **(F)** Representative histogram and summary plots showing individual results of the MFI for GATA3+ iNKT cells in psoriasis patients and healthy controls. **(G)** Representative histogram and summary plots showing individual results of the MFI for RORγt + iNKT cells in psoriasis patients and healthy controls. **(H)** Summary plots showing individual results of GATA3+ iNKT cells/ RORγt + iNKT cells, RORγt + iNKT cells/T-bet + iNKT cells and GATA3+ iNKT cells//T-bet + iNKT cells in psoriasis patients versus healthy controls. Data show mean +SEM. *p*-values were determined by paired Student's t-test. ns, no significance, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$.

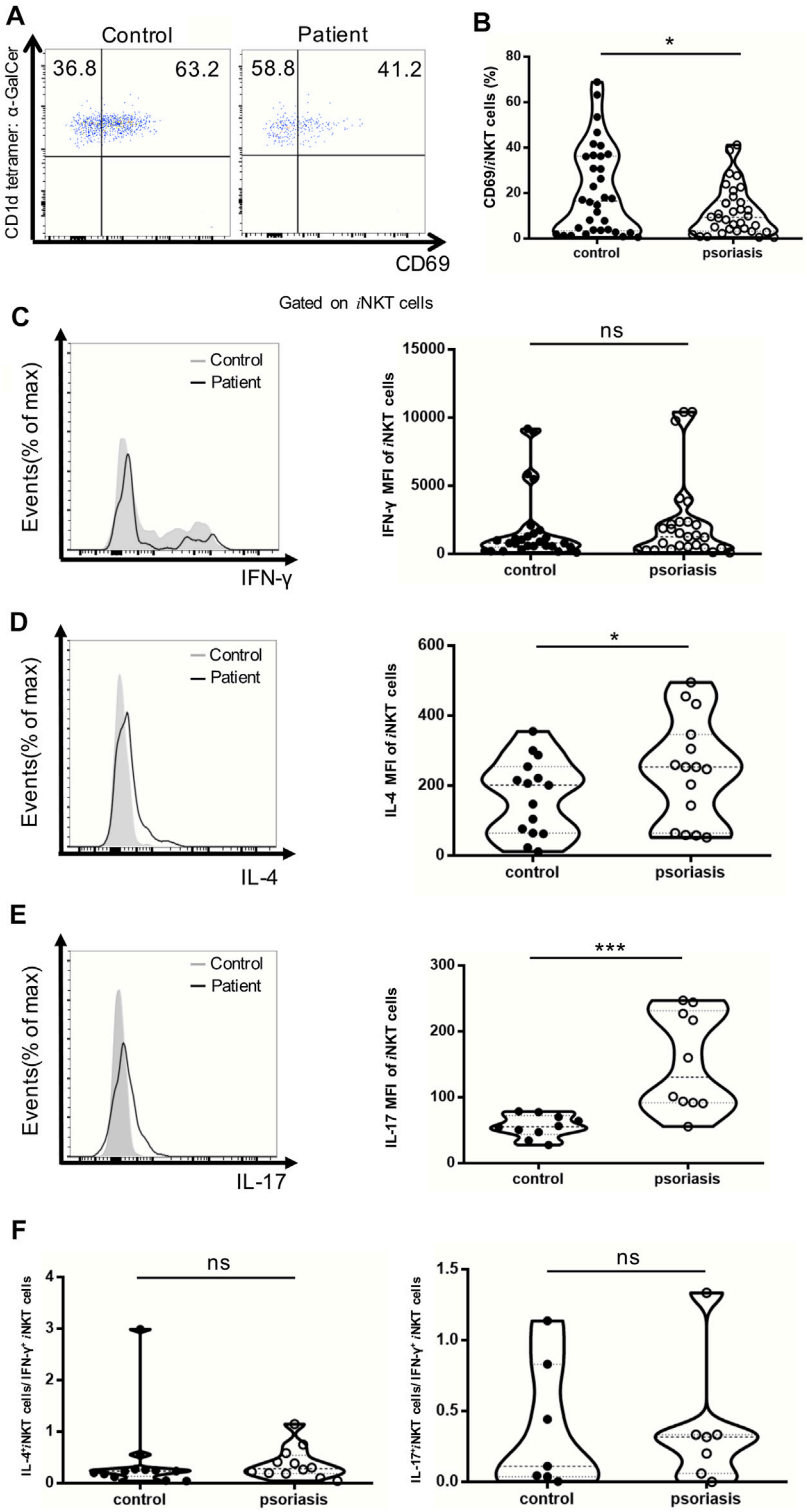

FIGURE 4 | *i*NKT cells activation status and cytokine production in PBMCs of psoriasis patients. Intracellular IFN-γ and IL-4 production of circulating *i*NKT cells was analyzed after stimulation with Cell Stimulation Cocktail for 5 h. **(A)** Representative FACS dot plots for iNKT cell CD69 expression in psoriasis patients and healthy controls. **(B)** Summary plots showing individual results of the frequency of CD69 $^{+}$ iNKT cells in psoriasis patients versus healthy controls. **(C)** Representative histogram and summary plots showing individual results of the MFI of IFN-γ-producing iNKT cells in psoriasis patients versus healthy controls. **(D)** Representative histogram and summary plots showing individual results of the MFI of IL-4-producing iNKT cells in psoriasis patients versus healthy controls. **(E)** Representative histogram and summary plots showing individual results of the MFI of IL-17-producing iNKT cells in psoriasis patients versus healthy controls. **(F)** Summary plots showing individual results of IL-4-producing *i*NKT cells/IFN-γ-producing *i*NKT cells and IL-17-producing *i*NKT cells/IFN-γ-producing *i*NKT cells in psoriasis patients versus healthy controls. Data show mean +SEM. *p*-values were determined by paired Student's t-test. ns, no significance, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$.

TABLE 2 | Details of moderate-to-severe plaque psoriasis patients treated with secukinumab.

Patient characteristics	Baseline (N = 15)	Week 12 (N = 15)
Age, years, mean (SD)	36.53 ± 12.08	36.76 ± 12.08
Female sex, n (%)	26.7%	26.7%
Body mass index	23.7 ± 4.59	23.18 ± 4.18
Disease duration, years, mean (SD)	12.73 ± 5.52	12.96 ± 5.52
PASI score, mean (SD)	18.81 ± 9.78	0.82 ± 0.71
PGA score, mean (SD)	2.27 ± 0.44	0.67 ± 0.47
BSA (%), mean (SD)	33.67 ± 26.19	2.4 ± 2.3
DLQI score, mean (SD)	14.27 ± 5.57	2.93 ± 3.49

peripheral blood of psoriasis varies a lot. Furuhashi found that the number of Treg cells in the PBMCs of severe psoriasis patients with PASI >12 decreased significantly, and the number of Treg cells increased after phototherapy (Fabio et al., 2018). Karamehic's study showed that the number of $CD4^+CD25^+$Treg cells in the PBMCs of psoriasis patients was significantly lower than that of healthy control, but there was no significant correlation with PASI score (Dainichi et al., 2019). In the meantime, the results of multiple studies have shown that the number of Treg cells in the peripheral blood of psoriasis patients is not significantly different from that of healthy control or is more than healthy control (Dantas et al., 2016; Zhang et al., 2016). In our study, we found that the number of $CD4^+$ $CD25^+$ $FoxP3^+$ Treg cells was also not altered in psoriasis patients, but we did not conduct in-depth research on Treg cells.

As mentioned earlier, in the present study, we used CD1d-tetramer staining, a specific method for *i*NKT identification, to accurately determine the percentage of *i*NKT cells. A significant decrease in peripheral blood *i*NKT cells was observed in psoriasis patients. Interestingly, we found that the proportion of *i*NKT cells increased in patients treated with IL-17A inhibitor secukinumab. Combined with the increased NKT cells in psoriatic lesions reported in previous studies (Bonish et al., 2000; Cameron et al., 2002; Ottaviani et al., 2006; Zhao et al., 2008). We speculated that the *i*NKT cells in the PBMCs have accumulated in psoriatic plaques. And when the lesions subside, *i*NKT cells may come back to peripheral blood.

Human *i*NKT cells can be segregated into $CD4^+$ and $CD4^-$ subsets according to their phenotypic and functional characteristics (Gumperz et al., 2002). $CD4^+$ *i*NKT cells produce both Th1 and Th2 cytokines, whereas the $CD4^-$ subset exhibits a Th1 cytokine profile. However, we found no difference in $CD4^+$ and $CD4^-$ subsets between psoriasis patients and healthy controls. We also examined the sublineages of

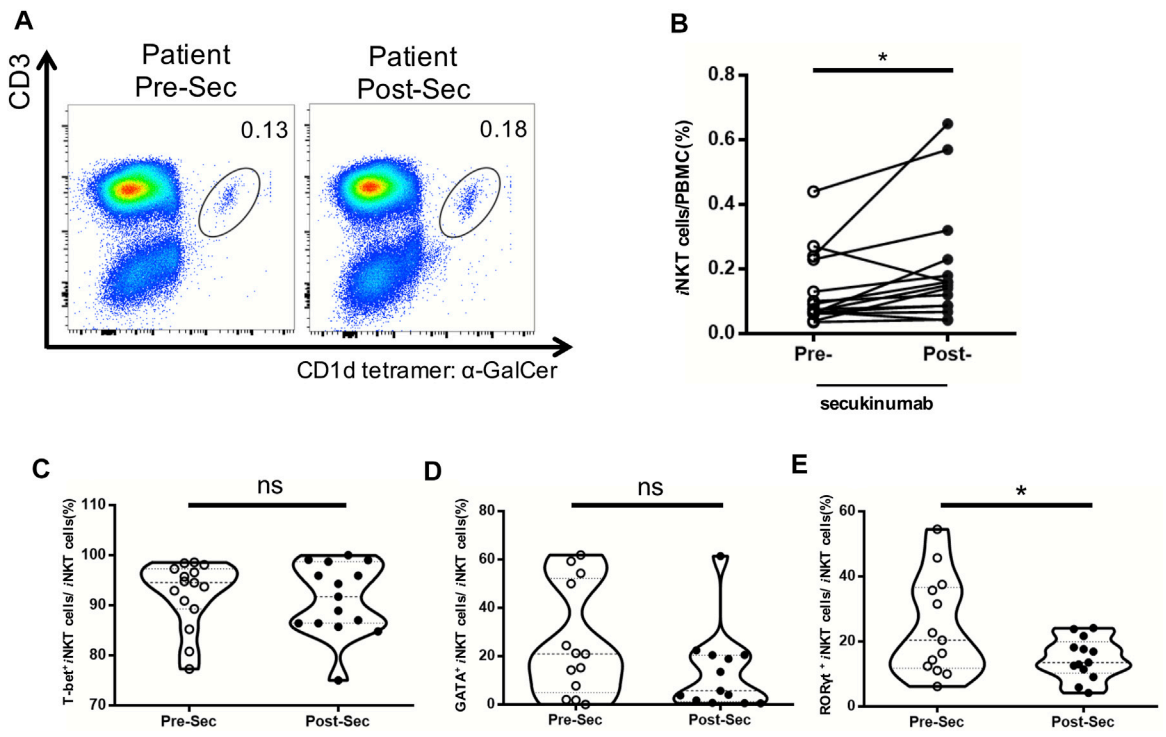

FIGURE 5 | Increased frequency of *i*NKT cells in psoriasis patients treated with secukinumab. PBMC were isolated from psoriasis patients (n = 15) at baseline untreated (Week 0) and 12 Weeks post treatment with secukinumab. **(A)** Representative FACS dot plots for *i*NKT cells from psoriasis patients before and after secukinumab treatment versus healthy controls. **(B)** Summary plots showing individual results of *i*NKT cell frequency in psoriasis patients before and after secukinumab treatment. **(C)** Summary plots showing individual results of T-bet$^+$ *i*NKT cells frequency in psoriasis patients before and after secukinumab treatment. **(D)** Summary plots showing individual results of GATA3$^+$ *i*NKT cells frequency in psoriasis patients before and after secukinumab treatment. **(E)** Summary plots showing individual results RORγt$^+$ *i*NKT cells frequency in psoriasis patients before and after secukinumab treatment. Data show mean +SEM. *p*-values were determined by paired Student's t-test. ns, no significance, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$.

*i*NKT cells and found that the MFI of GATA3$^+$ *i*NKT cells and RORγt$^+$ *i*NKT cells were significantly increased in psoriasis patients. We further analyzed the ratio of GATA3$^+$ *i*NKT cells vs ROR-γt$^+$ *i*NKT cells and found a decrease in psoriasis patients. Besides, the ratio of ROR-γt$^+$ *i*NKT cells vs T-bet$^+$ *i*NKT cells increased in psoriasis patients, which indicated that *i*NKT cells are more likely to differentiate into ROR-γt$^+$ *i*NKT cells in psoriasis. We also found a decrease of *i*NKT17 subset in *i*NKT cells after secukinumab treatment in psoriasis patients. This indicated a potential interaction between IL-17 and *i*NKT cells. Perhaps the decreased proportion of *i*NKT17 cells might be a counterbalance to the administration of IL-17A agonist. We cannot exclude the possibility that the alternation in *i*NKT cells might correlate with disease remission caused by secukinumab treatment. To explore the inner mechanism, we will stimulate *i*NKT cells with IL-17 to examine their interactions in our future research.

Although *i*NKT cells constitute only a small fraction of lymphocytes, their ability to rapidly secrete large amounts of cytokines, make them an important regulator of the Th1, Th2, and Th17 cytokine balance in immune responses. In our study, we found a decrease in the CD69$^+$ subset in psoriasis patients. This indicated that psoriasis patients may have less activated *i*NKT cells than healthy controls. But we also found that *i*NKT cells in psoriasis patients secreted higher levels of IL-4 and IL-17, which is consistent with the increase observed in the sublineages of *i*NKT cells in psoriasis patients. In our opinion, the reason why *i*NKT cells in psoriasis patients secreted higher levels of IL-4 and IL-17 mainly lies in the increase of *i*NKT2 and *i*NKT17 subsets. And the inflammatory environment in psoriasis patients may be the reason for *i*NKT functional lineage differentiation shift. Also, the IFN-γ producing *i*NKT1 may express higher CD69 than *i*NKT2 and *i*NKT17. While the proportion of *i*NKT2 and *i*NKT17 augmented in psoriasis patients, the CD69 expression decreased relatively. *i*NKT cells anergy may also be a reason for the decrease of CD69$^+$*i*NKT cell in the PBMCs of psoriasis patients, although it's not the primary mechanism. It has also been reported that Treg cells suppress NKT cell tumoricidal function by inducing more CD4$^-$ NKT cell anergy and less CD4$^+$ NKT cell anergy (Ihara et al., 2019). Therefore, there may be interaction between Treg cells and NKT cells in psoriasis, which is also the direction of our further research.

We didn't found difference in the ratio of IL-17-producing *i*NKT cells vs IFN-γ-producing *i*NKT cells and the ratio of IL-4-producing *i*NKT cells vs IFN-γ-producing *i*NKT cells between psoriasis patients and healthy controls, which indicated there were no imbalance between them. But this result still needs to be verified on more samples.

Based on the increase in Th17 and Tc17 levels, lower proportion and level of activation of *i*NKT cells, increase in the population of *i*NKT17 cells and higher proportion of *i*NKT cells after secukinumab treatment in psoriasis patients, we speculate that dysregulated *i*NKT cells may be involved in the pathogenesis of psoriasis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Shanghai Tenth People's Hospital Ethics Committees. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YH, YC, ZC, XiZ, CG, ZY, PX, LS, XuZ, YG, and QY conducted the experiments. YS planned the study and evaluated the results. YH, YC, and ZC analyzed the results. YH wrote the paper. YS reviewed and verified the original manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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MINI REVIEW
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Skin Immunosenescence and Type 2 Inflammation: A Mini-Review With an Inflammaging Perspective

*Bangtao Chen[1], Jing Yang[1], Yao Song[2], Daojun Zhang[2] and Fei Hao[2]**

[1]Department of Dermatology, Chongqing University Three Gorges Hospital, School of Medicine, Chongqing University, Chongqing, China, [2]Department of Dermatology, The Third Affiliated Hospital of Chongqing Medical University, Chongqing, China

Skin-resident stromal cells, including keratinocytes, fibroblasts, adipocytes, and immune cells including Langerhans cells, dendritic cells, T cells, and innate lymphoid cells, and their functional products work in concert to ensure the realization of skin barrier immunity. However, aging-induced immunosenescence predisposes the elderly to pruritic dermatoses, including type 2 inflammation-mediated. Inflammaging, characterized by chronic low level of pro-inflammatory cytokines released from senescent cells with the senescence-associated secretory phenotype (SASP), may drive immunosenescence and tangle with type 2 inflammatory dermatoses. The present mini-review summarizes current evidence on immunosenescence and type 2 inflammation in the skin and further focuses on future needs from an inflammaging perspective to clarify their complexity.

Keywords: immunosenescence, inflammaging, skin aging, dermatosis, mini-review and challenges

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****Correspondence:***
Fei Hao
haofei62@medmail.com.cn

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INTRODUCTION

The skin is the largest active immune organ, covering the body's outermost layer and performing the function of resisting external stimulus, thus maintaining skin homeostasis. Skin barrier inevitably undergoes characteristically immunological declines with advancing age, termed skin immunosenescence. Higher incidences of many dermatoses such as infectious diseases, non-communicable autoimmune diseases, and cutaneous malignancies, and more pathological states such as unspecific itchiness and delayed wound healing are observed in the elderly alongside immunosenescence (Farage et al., 2009). Senescent cells remain senescence-associated secretory phenotype (SASP) secreting low-level pro-inflammatory cytokines including CRP, IL-1β, IL-6, and TNF-α, which is usually referred to inflammaging (Lopes-Paciencia et al., 2019; Fitsiou, et al., 2021). Type 2 inflammatory dermatosis such as atopic dermatitis (AD), chronic spontaneous urticaria (CSU), and bullous pemphigoid (BP) frequently affect the elderly and are presumed to be correlated with skin immunosenescence. Moreover, the diseases affecting the elderly are prone to more severity, therapeutic resistance, and longer duration. The current mini-review focuses on skin immunosenescence and type 2 inflammation and present future needs from an inflammaging perspective, promising better management of type 2 inflammatory dermatosis in the elderly (**Figure 1**).

SKIN BARRIER IMMUNITY

Skin-resident stromal cells, including keratinocytes, fibroblasts, and adipocytes, and immune cells including Langerhans cells (LCs), dendritic cells (DCs), T cells, and innate lymphoid cells (ILCs)

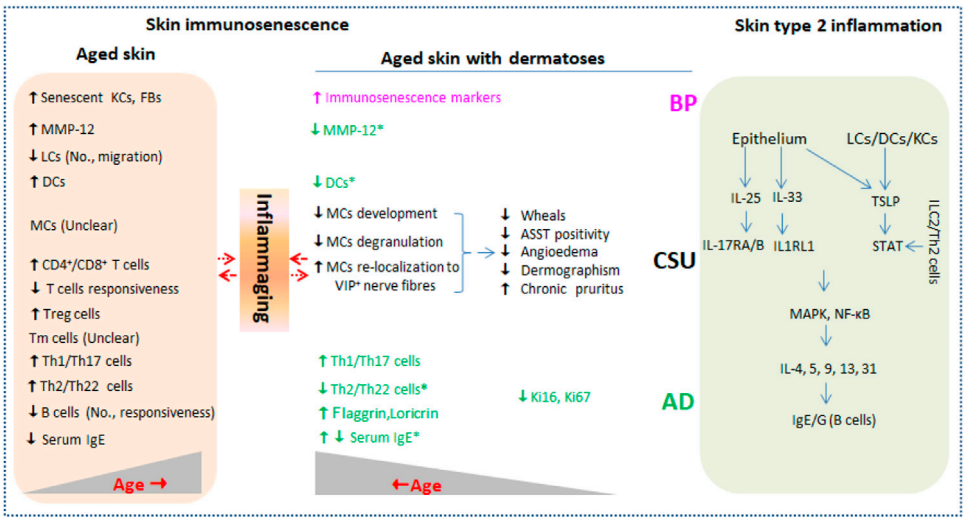

FIGURE 1 | Current evidence of immunosenescence in aged skin with or without type 2 inflammation dermatoses. Type 2 inflammation dermatoses such as AD, CSU, and BP are driven by key cytokines including IL-25, IL-33, and TSLP released from damaged epithelium and LCs/DCs (right column). Skin barrier inevitably undergoes characteristically immunological changes (skin immunosenescence) during aging in healthy individuals (left column) and in individuals affected by type 2 inflammation dermatoses. Inflammaging that is characterized by low level of pro-inflammatory cytokines including CRP, IL-1β, IL-6, and TNF-α produced by senescent skin cells may be in complex interaction with the two conditions. No., number; KCs, keratinocytes; FBs, fibroblasts; LCs, Langerhans cells; DCs, dendritic cells; MCs, mast cells; ILC2, innate lymphoid cell 2; Tm, memory T cells; Ig, immune globulin; MMP-12, matrix metalloproteinase 12; ASST, autologous serum skin test; TSLP, thymic stromal lymphopoietin; STAT, signal transducer and activator of transcription; IL1RL1, IL-1 receptor-like 1; AD, atopic dermatitis; CSU, chronic spontaneous urticaria; BP, bullous pemphigoid.

together with their functional products ensure the realization of skin barrier immunity. The cells mentioned above work synergistically or antagonistically upon harmful environmental exposures challenge, leading to reinforced or compromised networks protecting the skin against damage or causing dermatosis, respectively.

Stromal Cells

Epidermal keratinocytes express and secrete antimicrobial ribonuclease RNase 7 and antimicrobial peptides adrenomedullin, β-defensins, and cathelicidin upon recognizing pathogenic components *via* its constitutive expressions of toll-like receptors (TLRs) on the cellular surface (Miller and Modlin, 2007; Köllisch, et al., 2005). In addition, keratinocytes function in the presentation of antigen from $CD4^+$ to $CD8^+$ T cells and promote tissue repair *via* chemokine (IL-1β, IL-8, and CCL20)-mediated leukocyte recruitment during early wound healing (Black, et al., 2007; Li, et al., 2017; Sperling, et al., 2012). They also serve as key sites for UVB-catalyzed production of active vitamin D3 (Zbytek, et al., 2008). Dermal fibroblasts are not only key to supporting wound healing through the secretion and remodeling of extracellular matrix (ECM) but also essential for facilitating innate immune response to microbial infections by secreting cytokines and chemokines with involvement of toll-like receptor activation (Ghetti, et al., 2018; Cole, et al., 2018; Haniffa, et al., 2007; Bautista-Hernández, et al., 2017). Interestingly, fibroblasts were uncovered to inhibit T-cell proliferation and induce the production of immunoregulatory cytokines such as IL-10 (Haniffa, et al., 2007). Moreover, adipocytes differentiated from dermal fibroblasts upon *Staphylococcus aureus* infection can also produce the antimicrobial peptide cathelicidin (Zhang, et al., 2015).

Immune Cells

LCs, the mononuclear phagocyte within the epidermis, not only produce antimicrobial peptide hBD3 and initiate a local immune response mainly by presenting antigens to T cells (Ferris, et al., 2013; Atmatzidis, et al., 2017; Pilkington, et al., 2018a), but also migrate to skin regional lymph nodes for enhancing immune response to exogenous antigens and promoting tolerance to self-antigens (West and Bennett, 2018; Atmatzidis, et al., 2017).

DCs and macrophages, the mononuclear phagocyte located in the dermis, are also the sentinels of the innate immunity working similarly to epidermal LCs. Dermal DCs comprise $CD1c^+$ or $CD141^+$ myeloid and plasmacytoid forms, while the latter is hardly observed in steady-state skin (Collin and Bigley, 2018). Compared with their blood counterparts, normal dermal DCs associated with T-cell proliferation displayed an activated phenotype with increased expression of co-stimulatory receptors (McLellan, et al., 1998). Dermal macrophages are specifically labeled with CD163, and the cells also contribute to wound and nerve healing by suppressing inflammation upon tissue injury (Kolter, et al., 2019).

The same as skin LC and DC, B cells found in healthy skin are integral for presenting antigen at low concentration to T cells (Geherin, et al., 2012). Moreover, skin B cells also modulate inflammation response by secreting pro- or anti-inflammatory mediators (Debes, and McGettigan, 2019).

Skin-resident T cells derived from T cells differentiated and matured in the thymus *via* migration through the lymphatic or

circulatory system. Phenotypically, 80%–90% of the skin T-cell pool is memory T (Tm) cells, and the remaining is recirculating T cells (Nguyen, et al., 2019). Tm cells have stronger immune surveillance against reinvasions, and expressions of CD69 and CD103 on cell surface commonly characterize this type of T cells (Mackay, et al., 2013). The number of $CD4^+$ Tm cells is three and six times that of $CD8^+$ Tm cells in the epidermis and dermis, respectively (Watanabe, et al., 2015). With a memory skin-resident phenotype inducing immune tolerance, $Foxp3^+$ regulatory T cells (Tregs) are in close proximity to hair follicles where skin commensal-metabolized short-chain fatty acid sodium butyrate or UVB light increases $Foxp3^+$ expression in non-Tregs or drive $Foxp3^+$ Tregs proliferation (Schwarz, et al., 2017; Yamazaki, et al., 2014; Scharschmidt, et al., 2017). Overall, αβ T cells dominate in the skin as in circulation (Nielsen, et al., 2017).

Cutaneous ILCs located in the epidermis and dermis are newly identified immune cells whose function is not fully understood, but influxes of ILC2 in AD and LC1/3 in psoriatic plaques were demonstrated (Brüggen, et al., 2016; Akdis, et al., 2020). Neutrophils are seldom in the skin, while they can infiltrate the skin upon exposure to a harmful stimulus (Rijken, et al., 2005). In addition, allergens or inflammatory irritants can induce the release of histamine and inflammatory mediators from cutaneous mast cells (MCs), mediating wheals and itch onset (Otsuka, and Kabashima, 2015).

SKIN IMMUNOSENESCENCE

The skin goes roughly through stages of immaturity, maturation, and decline over lifespan as with all other organs. Although incredibly durable, aging still causes skin structure and function changes, termed skin aging. This process is usually exaggerated by extrinsic exposures such as UVR. Morphologic and related functional changes in chronologically or intrinsically aged skin were summarized in a review conducted by Zouboulis, and Makrantonaki, (2011). In particular, immunosenescence contributes to the increased susceptibility to skin disorders with malignancies, infections, and autoimmunity in the elderly. Skin immunosenescence refers to declines in function or number of all skin cells responsible for immune surveillance (Corsini, et al., 2009). Senescent cells, promoted by telomere shortening and genome instability, remain SASP secreting low level of pro-inflammatory cytokines including IL-1β, IL-6, and CRP, thus altering the skin's microenvironment (Lopes-Paciencia, et al., 2019; Fitsiou, et al., 2021). Presumably, skin inflammaging characterized by chronic low-level inflammation is believed to be the main driver for remodeling the immunological response in senescent skin cells (Ghosh, and Capell, 2016).

Stromal Cell Senescence

Dermal senescent fibroblasts accumulated with age and displayed SASP rich, thus maintaining inflammaging phenotype (Wlaschek, et al., 2021). Such changes contribute to disruptions of collagen homeostasis, delayed wound healing, and increased likelihood of skin tumorigenesis; however, its antibacterial immunity loss caused by aging has been associated with impaired adipocyte differentiation (Zhang, et al., 2019; Wasko, and Horsley, 2019). Compared with fibroblasts, the impact of accumulated senescent keratinocytes in the epidermis on inflammaging or antibacterial immunity is limited due to its higher turnover rate (Pilkington, et al., 2021).

Immune Cell Senescence

In aged skin, decreased proliferation of *in situ* LC progenitors causes a reduced number of LCs, and LCs are also less able to migrate from the epidermis in response to harmful stimulus due to the declined availability of local IL-1β, which collectively contributes to impaired skin barrier integrity and diminished antimicrobial and tumor cell defense (Pilkington, et al., 2018b). In addition, LC-mediated skin barrier perturbation may facilitate the onset of skin inflammaging by initiating cytokine release from cutaneous cells (Wittmann, et al., 2014).

To some extent, the state of thymus and T cells in circulation are implicated in many dermatoses, which also reflect the profiles of skin-resident T cells. In geriatric individuals, circulating T cells in total number remain unchanged, accompanied by reduction of naive T cells due to thymic involution and increase of Tm cells since the prolonged exposure to external substances over the lifespan (Thomas, et al., 2020). However, little is known regarding the changes in skin-resident Tm cells during aging. A higher ratio of $CD4^+$ to $CD8^+$ T cells was found in aged skin than in young skin, indicating a more severe pro-inflammatory response phenotype (Zuelgaray, et al., 2019). Cytokines during inflammaging can be Th2 pattern dominant with an increased incidence of allergic diseases and Th1 pattern dominant with a higher frequency of chronic infections and neoplastic diseases. It was reported that Tregs numbers and immunosuppressive receptor PD-1 increased in aged skin, thus causing reactivation of infectious diseases or skewing inflammatory microenvironment by suppressing both Th1 and Th2 responses (Lages, et al., 2008). Additionally, a diminished response of T cells to specific antigens in advanced age may collectively explain why the chronic low-level inflammation characterizes the state of inflammaging (Bektas, et al., 2017).

Aging-related changes in skin B cells are similar to skin T cells except that B cells from the elderly are less efficiently stimulated. Thus, antibody generation decreases, and immune response to vaccines and antigens is weakened (Pinti, et al., 2016).

IMMUNOSENESCENCE AND TYPE 2 INFLAMMATORY DERMATOSIS

Type 2 inflammation phenotypes in skin and circulation are usually in traffic with each other and remain consistent. IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) released from damaged epithelium directly activate the production of IL-4, 5, 9, 13, and 31 from ILC2 and Th2 cells, thus characterizing type 2 inflammation immunity (Akdis, et al., 2020). Both IL-25 and IL-33 can activate MAPK and NK-κB signaling pathways *via* binding to IL-17RA/B and IL-1 receptor-like 1 (IL1RL1), respectively (Akdis, et al., 2020; Deng, et al., 2021). By activating signal

transducer and activator of transcription (STAT), skin LC/DC-derived and keratinocyte-derived TSLP are critical for Th2-type immune responses and mediating pruritus exacerbation, respectively (Kim et al., 2013). In addition, humoral immunity characterized by allergen-specific antibody IgE or autoantigen-specific autoantibody IgG matured by IL-13 and IL-4 is also involved in type 2 inflammatory dermatosis such as AD, CSU, and BP (Gandhi, et al., 2016).

Atopic Dermatitis

Globally, 10% of adults and 1%–3% of elderly populations are troubled by AD (Lloyd-Lavery, et al., 2019; Williamson, et al., 2020). Moreover, the increasing predisposition of late AD development in older adults is due to exposure-induced epidermal barrier malfunction and immunosenescence-caused chronic itch in advanced age. The core of AD is skin inflammation involving IgE produced by B cells and inflammatory mediators of T-cell origin, while Th2 cytokines dominate in the inflammation milieu (Tanei, and Hasegawa, 2016; Tanei, et al., 2013). Th2/Th22 cytokines in skin increase during aging in healthy individuals, while the opposite phenomenon is observed in older AD patients (Bocheva, et al., 2021; Gittler, et al., 2012). With age progressing, Th1- and Th17-related mediators in lesioned and non-lesioned skin in individuals suffering from AD are markedly increased, as observed in healthy adults (Bocheva, et al., 2021; Gittler, et al., 2012). Zhou et al. showed that inflammatory DCs in the skin and cutaneous expression of matrix metalloproteinase 12 (MMP-12) were reduced in both affected and unaffected skin in AD with aging (Agrawal, et al., 2012; Zhou, et al., 2019). Reduction in specific and total serum IgE with aging in patients with allergic rhinitis, asthma, or insect allergy implies a decreasing proportion of extrinsic atopy among older adults; however, the association between serum IgE and aging in AD patients remains inconsistent (Zhou, et al., 2019; Mediaty, and Neuber, 2005). In addition, aging-related increment in terminal keratinocyte differentiation markers (filaggrin and loricrin) and decrement in epidermal hyperplasia markers (Ki16 and Ki67) were also observed in AD (Zhou, et al., 2019), which might be attributable to attenuation in the Th2/Th22 cytokine axes (Boniface, et al., 2005); moreover, it reveals a critical role for crosstalk between immune cell senescence and stromal cell-mediated immunity impairment in severity of geriatric AD.

Chronic Spontaneous Urticaria

Traditionally, CSU is an allergic dermatosis mediated by degranulation and histamine released from skin MCs or basophils (Bracken, et al., 2019). As mounting CSU patients show antihistamine resistance, it is supposed to be T cell-mediated with emerging evidence that concentrations of circulating cytokines released from Th1/Th2 and Th17 cells correlated positively with disease severity in our previous study (Chen, et al., 2018). Kay et al. added the finding that increased expressions of IL-25, IL-33, and TSLP in skin wheals of patients with CSU further accurately characterize the pathogenesis and categorize it as type 2 inflammatory dermatosis (Kay, et al., 2015; Vadasz, and Toubi, 2015). In retrospective investigations performed in localized areas, older CSU patients made up 9.4%–25% of the CSU population. Furthermore, fewer wheals, lower rates of ASST positivity, angioedema, and dermographism, and more comorbidities were reported in elderly patients with CSU diagnosis (Chen, et al., 2012; Magen, et al., 2013). The atypical symptoms are pertinent to aging-related immunosenescence. For one thing, stromal-cell functional impairment with aging was proved to cause a decline in MC development (Tsuboi et al., 2012). For another, skin MCs accumulated while their degranulation capability was reduced with aging. Furthermore, they re-localize to the papillary dermis, where MCs keep in closer proximity to macrophages and VIP^+ nerve fibers while the association with dermal vasculature is weakened (Pilkington, et al., 2019). Unfortunately, little is known regarding alterations of number, function, and crosstalk among MCs, basophils, and T and B cells in elderly individuals with CSU.

Bullous Pemphigoid

BP, an autoimmune blistering dermatosis in the elderly mediated by IgG autoantibodies to skin hemidesmosome proteins (BP180 and/or BP230) and activation of complement component C3, is characterized by urticarial plaques, tense blisters, and intractable pruritus (Bağcı, et al., 2017). In BP development, autoreactive T cells work cooperatively. Increased circulating Th2 cells and IL-4 promote B-cell proliferation, antibody production, and immunoglobulin class-switching, while skin-resident Th17 cells and IL-17 activate local neutrophil-mediated inflammatory response, thus causing tissue damage (Fang, et al., 2020; Boehncke, and Brembilla, 2019). In recent studies, specific anti-BP180/230 IgE in BP were detected by immunoassays; furthermore, positive associations between IgE content and Th2 cell-specific cytokines IL-4/-13 and symptomatic disease phenotypes were shown (Cozzani, et al., 2018; Messingham, et al., 2019). The finding that IgE-driven BP promises the therapeutic regimes using Th2 inhibitors in BP-affected frail patients with good safety and ideal effectiveness. It was reported that disease clearance or satisfactory response was achieved in 12 of 13 BP patients (an average age of 76.8 years) treated with Dupilumab, an IL-4 receptor alpha antagonist with the property of inhibiting IL-4/-13 signaling and IgE secretion (Abdat, et al., 2020). Immunosenescence-related aging is conceivably responsible for the increased incidence of BP in the elderly (Pietkiewicz, et al., 2016; Yaar, and Gilchrest, 1987). However, fewer studies focus on the effect of immunosenescence or inflammaging on pathophysiological characteristics of BP, and only a meeting paper uncovered increased markers of immunosenescence in BP patients (Noe, et al., 2015).

FUTURE NEEDS

Too many questions regarding skin immunosenescence and type 2 inflammations need to be answered.

Firstly, existing studies fail to provide direct and strong evidence for the involvement of immunosenescence in type 2

inflammatory dermatosis. Distinctive clinical features and incidences of type 2 inflammatory dermatosis between the young and the elderly are observed, and the difference is often thought to be caused by aging-related changes including immunosenescence, but direct evidence remains insufficient. As evidenced by the recent discovery of TH2-interacting fascial fibroblasts (TIFFs) in mouse and human skin, skin-resident or -infiltrating immune cells and stromal cells are complexly interacting and influence each other throughout life (Boothby, et al., 2021). They undergo structural and functional alterations simultaneously, but overall, inflammaging phenotype characterizes the skin microenvironment during normal aging. In aged individuals with type 2 inflammatory dermatosis, the relationship of the skin microenvironment with inflammaging and changes to cutaneous immunity is more complex due to repeated scratching caused by uncontrolled itchiness. Therefore, it is far-fetched to conclude that a specific pathophysiological change is independently caused by a specific senescent cell alone or the disease itself. In particular, further exploration of the associations between remolding of senescent fibroblast-released ECM and type 2 inflammation in aged skin will provide new insights into strategies used in related dermatosis.

Secondly, could skin immunosenescence contribute to systemic immunosenescence or *vice versa* from the perspective of inflammaging? A shining shared feature of type 2 inflammatory dermatoses in the young or elderly is the presence of Th2 cytokines in circulation and the lesions, and inflammation state in lesions is proposed to be orchestrated by systemic inflammation phenotype (Rafei-Shamsabadi, et al., 2019; Pezzolo and Naldi, 2020). Given this, the reverse argument is worth further considering, especially in the elderly with impaired skin barrier as epidermal abnormality in AD has been proposed to drive systemic inflammation (Elias and Steinhoff, 2008). Hu et al. showed that tape stripping-induced epidermal dysfunction led to an age-associated increase in levels of circulating inflammatory cytokines in mice (Hu, et al., 2017), and Ye et al. also provided the evidence that correction of epidermal function by emollient lowered systemic inflammaging measured by circulating levels of IL-6 and TNF-α in chronically aged human (Ye, et al., 2019). These studies may collectively support the thesis that epidermal dysfunction-mediated immunosenescence could contribute to the onset or severity of type 2 inflammatory dermatosis with systemic inflammation involved in the elderly. However, more investigations are warranted to confirm the thesis by untangling their cause and effect.

On the contrary, the complexity of systemic and tissue inflammaging can also be witnessed in the efficacy of anti-inflammaging agents. Anti-inflammaging drugs indeed hold promise for increasing healthy aging, and much effort aimed at slowing aging by targeting inflammaging has been conducted (Partridge, et al., 2020; Suggs, et al., 2014). Rapamycin, metformin, and various botanicals showed delaying the aging process by inhibiting cellular senescence dependent or independent of their anti-inflammaging properties (Partridge, et al., 2020; Suggs, et al., 2014). For example, topical rapamycin, an FDA-approved agent, showed no beneficial effects in inflammaging (Correia-Melo, et al., 2019) but improved histological appearance of aged skin by reducing fibroblast senescence and increasing collagen VII (Chung, et al., 2019; Qin, et al., 2018). As such, the correction of the impaired skin barrier by anti-inflammaging agents preventing or mitigating systemic inflammaging is meaningful and easily articulable.

Thirdly, other intrinsic drivers of skin immunosenescence or inflammaging should also be identified in terms of the organism as a whole. Changes to gut and skin microbiota, mitochondrial damage-associated molecular patterns (DAMPs), abnormal activity of coagulation and fibrinolysis, complements, and vitamin D3 deficiency during aging have also been linked with type 2 inflammation dermatoses (Nakahara, et al., 2021; Sánchez-Borges et al., 2018; Hashimoto, et al., 2020). Whether the correction of abnormalities benefits improvement of related dermatoses through immunosenescence retardation remains to be further investigated.

Lastly, skin immunosenescence can be partly determined with flow cytometry and immunohistochemistry by frequency assessments of senescent immune cells due to their end-stage differentiated and cell-specific markers; however, no techniques are available for assessing inflammaging caused by indicated senescent cells *in vivo*. It appears that all immune- or non-immune-senescent cells possess SASP properties releasing low levels of IL-1β, IL-6, TNF-α, and CRP. These pro-inflammation mediators that can shuttle through skin and circulation are non-specific for SASP-centered inflammaging. More than that, they can be transiently modulated by acute or persistently modulated by chronic inflammatory diseases, including type 2 inflammation dermatoses in young or older populations. Meanwhile, immunosenescence and inflammaging in the skin are mutually regulated, but they do not always parallel, especially for senescent cells in the end stage. Herein, screening of reasonable indicators for inflammaging in the elderly with and/or without inflammation dermatosis *via* longitudinal data from large samples is expected.

AUTHOR CONTRIBUTIONS

FH designed the study; BC and YS wrote the manuscript; DZ and JY revised the manuscript.

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HSP27 Protects Skin From Ultraviolet B -Induced Photodamage by Regulating Autophagy and Reactive Oxygen Species Production

Zi-Yue Wang[1†], Ang Li[1†], Xin Huang[2], Gen-Long Bai[1], Yu-Xin Jiang[1], Ruo-Lin Li[1], Chuan Liu[1], Zhu-Yuan Wen[1], Ping Wang[1] and Ai-Jun Chen[1*]*

[1]Department of Dermatology, The First Affiliated Hospital of Chongqing Medical University, Chongqing, China, [2]Prescriptions Department, College of Traditional Chinese Medicine, Chongqing Medical University, Chongqing, China

Ultraviolet (UV) irradiation has been well documented to be linked with almost all skin problems we know, and both dermis and epidermis may be affected to varying degrees by UV irradiation. Every time when exposed to sunlight without protection, our skin will step closer to photoaging, leading to irreversible consequences ultimately. Heat shock protein 27 (HSP27) is a vital protein involved in cell growth, autophagy, apoptosis, drug resistance, tumor genesis and metastasis. Evidence suggests that the organism is subjected to various internal and external environmental stresses (heat, oxidative stress, organic toxicants, etc.), and HSP27 with high expression has protective function. However, the expression of HSP27 in coping with UV irradiation have not been examined thoroughly. In this study, photodamage models were developed through different doses of UVB irradiation in human epidermal keratinocytes (HEKs) (30 mJ/cm^2), human dermal fibroblasts (HDFs) (150 mJ/cm^2) and mouse skin (2,700 mJ/cm^2). HSP27 knockdown decreased cell viability and increased the incidence of UVB-induced reactive oxygen species (ROS) production. We got consistent results *in vivo* and vitro. Compared with that in the UVB group, the expression of LC3B was significantly lower, while the expression of p62 was significantly higher in the UVB + si-HSP27 group. It was also revealed that HSP27 knockdown reduced the expressions of some antioxidants, such as superoxide dismutase (SOD) and catalase (CAT), which accelerated UVB-induced ROS release. Moreover, histological results showed that epidermis was thickened and collagen fibers were disorganized in the UVB + si-HSP27 group. These findings have demonstrated that HSP27 might play a photoprotective role in the UVB-induced skin damage process by maintaining the normal autophagy and antioxidant level. It is implied that HSP27 could be a potential therapeutic target of photodamage. However, determination of the definitive mechanism requires further exploration.

Keywords: Hsp27, UVB, photodamage, autophagy, ROS

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Xia Lei,
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Yong Yang,
Chinese Academy of Medical Sciences and Peking Union Medical College, China
Sheikh Tasduq Abdullah,
Indian Institute of Integrative Medicine (CSIR), India

***Correspondence:**
Ping Wang
wang_ping@hospital.cqmu.edu.cn
Ai-Jun Chen
cajhx@aliyun.com

†These authors have contributed equally to this work

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INTRODUCTION

Sunshine not only brings us convenience, but is also likely to endanger our health (Micka-Michalak et al., 2019). Skin, as our first and the largest line of defense in contact with the outside world, resists the adverse effects of harmful environmental factors. This is the reason why it may be hurt originally, resulting in a variety of diseases one after another. Among all the environmental factors affecting skin health, ultraviolet (UV) in sunlight is undoubtedly the one that we have the greatest exposure to. Its photodamage to skin (including cell death, photoaging, carcinogenesis, etc. (Jobe et al., 2018)) is conclusive and noteworthy. UVA (315–340 nm) and UVB (280–320 nm) are the main action spectra of skin photodamage. Although the latter is mostly absorbed by keratinocytes in epidermis due to its short wavelength, it can still reach dermis, where fibroblasts are located. The intensity of photodamage caused by UVB is 800–1,000 times that of the same dose of UVA (Syed et al., 2013).

Autophagy, a self-protection mechanism widely existing in eukaryotic cells, maintains cell homeostasis through realization of the cell metabolism (Guo et al., 2019). It plays a crucial role in the process of cell survival and death. Ultraviolet in sunlight is one of the external factors regulating autophagy (Sample & He, 2017). Although the specific mechanism is unclear, existing research shows that in terms of either keratinocytes or fibroblasts, UVB exposure can cause autophagy changes in either keratinocytes or fibroblasts (Chen et al., 2018; J. A.; Zhang et al., 2020). Inhibited autophagy may accelerate cell aging, and activated autophagy may have powerful anti-aging effect (J. Zhang et al., 2016). At the same time, some scholars believe that UVB irradiation causes photodamage by producing ROS (Cavinato & Jansen-Durr, 2017). Excessive ROS not only induces DNA damage and aggravates the possibility of carcinogenesis (Panich et al., 2016), but also disrupts the balance of oxidation/antioxidant system, impeding the production of the endogenous antioxidants such as SOD and catalase CAT, thereby reducing the skin's ability to defend against ROS mediated photodamage (Choi et al., 2017).

HSP27 is an important member of the Small Heat Shock Proteins (sHSPs) family. In addition to its well-known molecular chaperone and anti-apoptotic effects, many studies have shown that there is a link between HSP27 and autophagy to some extent. Liu et al. (Liu et al., 2018) found that HSP27 was involved in cytoplasmic chaperone-mediated autophagy (CMA) induced by exogenous Annexin A1 (ANXA1) mimic peptide-Ac2-26 of microglia, providing a promising targeted therapeutic approach for improvement of cerebral apoplexy. Other relevant studies have proved that Phosphorylated-HSP27 (p-HSP27) is closely related to the dynamic regulation of cytoskeleton. It modulated the autophagy process by means of controlling the transport of autophagosome skeleton and the binding and degradation of lysosomes (R. Zhang et al., 2015). In another study by Liang et al. (Liang et al., 2018), it was observed that the autophagy stimulated by HSP27 promoted the anti-cancer effect of curcumin in colon cancer cells. Nonetheless, whether HSP27 can participate in the occurrence and development of UVB mediated skin photodamage by adjusting autophagy still remains unclear. The purpose of this study is to investigate the effect of HSP27 on UVB-induced acute skin photo-damage and its potential mechanism.

MATERIALS AND METHODS

Cell Culture

All procedures were approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University, and all patients signed the written informed consent. HEKs and HDFs were derived from the healthy skin tissue samples of patients who underwent biopsies at the First Affiliated Hospital of Chongqing Medical University between December 2019 and May 2021. The samples cut into pieces of 0.5*0.5 cm size were isolated with 0.5% trypsin (Sigma, United States) under sterile conditions, with epidermis and dermis being separated. Digested with 0.25% trypsin (Gibco, United States) and free ethylenediamine tetracetic acid (EDTA), the epidermis cultured in the defined-keratinocyte serum-free medium (DK-SFM) was supplemented with keratinocyte growth factors (Millipore, United States) at 37°C in a 5% CO_2 incubator. Dermis was digested by using type II collagenase (Sigma, United States) and maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco, United States) containing 10% fetal bovine serum (FBS, Biological Industries, Israel) and 1% penicillin-streptomycin (Beyotime Biotechnology, China) at 37°C in a 5% CO_2 atmosphere. The cells grew generally from tissues after about 1 week, and were harvested when 70–80% confluency was reached. In this study, at the first two passages, keratinocytes were used, and at the second two passages, fibroblasts. The medium was altered every other day or every 3 days.

Animals Feed

The 8-week-old C57BL/6 mice (20 ± 2 g, n = 15) were provided by the Animal Experimental Center of Chongqing Medical University. With the conditions of ambient temperature of 23 ± 2°C, relative humidity of 60%, and the alternation of day and night for 12/12 h, the mice were free to obtain food and water. All animal experiments complied with the ethical standards stipulated by the Experimental Animal Ethics Committee of the First Affiliated Hospital of Chongqing Medical University.

UVB Irradiation

The UVB irradiation dose of cells was determined according to the results of pre-experiment, and the modeling method in animal experiments referred to the study of Sajo et al. (Sajo et al., 2017). During the entire process of the experiments, the photodamage models were established by using the UVB lamps with an emission wavelength between 290 and 320 nm (TL20/12, Philips, Netherlands). The irradiation intensity (mW/cm^2) was measured by the UVB radiometer (Photoelectric Instrument Factory of Beijing Normal University, China), and then the irradiation time was adjusted according to the intensity.

In vitro, HEKs and HDFs were irradiated once at 30 mJ/cm^2 and 150 mJ/cm^2, respectively. Note that HDFs needed to be

removed from the culture medium, washed with phosphate-buffered saline (PBS, Gibco, United States) three times, and covered with a thin layer of PBS to be irradiated. The control group was exposed to equal conditions without UVB radiation. *In vivo*, the shaved ear skin of the mice was exposed to 1,350 mJ/cm^2 UVB radiation once a day in 2 days.

Cell Viability Calculation

The viability of cells treated with different interventions was assessed by using the CCK-8 kit (MCE, United States) according to the manufacturer's protocol. 100 μl of cells were inoculated in 96-well plates. After diverse treatments, 10 μl of CCK-8 solution was added to each well for sufficient reaction. The absorbance at 450 nm was measured by using Multiskan Spectrum (ThermoFisher Scientific, United States).

An Analysis of Quantitative Reverse Transcription Polymerase Chain Reaction

24 h after UVB irradiation, extracted from cells and tissues by the RNAiso Plus Kit (TaKaRa, Japan), the total RNA was transformed into the single strand cDNA by using the RT Master Mix for qPCR (MCE, United States). Eventually, reverse transcription polymerase chain reaction was accomplished with The SYBR Green qPCR Master Mix (MCE, United States).

The primer sequences for targeting genes were listed as follows:

Cells: p62, forward: 5′-TGAGTCCCTCTCCCAGATGCT-3′, reverse: 5′-GGGGGATGCTTTGAATACTGG-3'; HSP27, forward: 5′-CCCACCCAAGTTTCCTCCT-3′,reverse: 5′-GGC AGTCTCATCGGATTTTG-3'; LC3B, forward: 5′-AAGGCGCT TACAGCTCAATG-3′,reverse: 5′-ACACTGACAATTTCATCC CGA-3'; CAT, forward: 5′-ACCTGTGAACTGTCCCTACCG-3′,reverse: 5′-TCATTGGCAGTGTTGAATCTCC-3'; SOD1, forward: 5′-CGAGCAGAAGGAAAGTAATGG-3′,reverse: 5′-CCAAGTCTCCAACATGCCTC-3'; SOD2, forward: 5′-CCTGG AACCTCACATCAACG-3′,reverse: 5′-CAACGCCTCCTGGTA CTTCTC-3'; ACTB, forward: 5′-AGAAAATCTGGCACCACAC CT-3′,reverse: 5′-GATAGCACAGCCTGGATAGCA-3'.

Animals: p62, forward: 5′-ACTACCCCAGAAAGTTCCAGC-3′,reverse: 5′-TTTCCCGACTCCATCTGTTC-3'; HSP27, forward: 5′-GGCATTTGGACACGGAAGT-3′,reverse: 5′-GGGCTCAAC TCTGGCTATCTC-3'; LC3B, forward: 5′-GCTAACCAAGCCTTC TTCCTC-3′,reverse: 5′-TGCTGTCCCGAATGTCTCC-3'; CAT, forward: 5′-CATTCAGAAGAAAGCGGTCAA-3′,reverse: 5′-TTC TCAGCGTTGTACTTGTCCAG-3'; SOD1, forward: 5′-GGGAAC CATCCACTTCGAGC-3′,reverse: 5′-TCCTGCACTGGTACAGC CTTG-3'; SOD2, forward: 5′-CTGGACAAACCTGAGCCCTAAG-3′,reverse: 5′-TTGGACTCCCACAGACACGG-3'; GAPDH, forward: 5′-GACATCAAGAAGGTGGTGAAGC-3′,reverse: 5′-GAAGGTGGAAGAGTGGGAGTT-3'.

Western Blotting Analysis

After 24 h' exposure, proteins were isolated from cells by using RIPA lysis buffer (Beyotime Biotechnology, China) including 1% phenylmethylsulfonyl fluoride (PMSF, MCE, United States). Besides, the proteins of mice skin were extracted by T-PER™ Tissue Protein Extraction Reagent (ThermoFisher Scientific, United States). The total protein concentrations were measured by applying BCA Protein Assay Kit (Beyotime Biotechnology, China). Approximately 40 μg of every protein sample was separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose (NC) filter membranes. Then, the membranes were blocked in 7% skimmed milk in TBST for 1 h at room temperature and incubated with primary antibodies listed below at 4°C overnight: HSP27 (1:1,000, Abcam, United States), p-HSP27 (1:1,000, Cell Signaling Technology, United States), β-actin (1: 2,000, Cell Signaling Technology, United States), SOD1 (1: 1,000, Cell Signaling Technology, United States), p62 (1:1,000, Bimake, United States), LC3B (1:1,000, Bimake, United States), CAT (1:1,000, Bimake, United States), SOD2 (1:1,000, Bimake, United States), and GADPH (1:1,000, Bimake, United States). Washed with TBST 3 times, the membranes were then incubated with horseradish peroxidase (HRP)-linked secondary antibodies (1:5,000, Cell Signaling Technology, United States) for 1 h at room temperature. Finally, the protein bands were imaged by using the SuperSignalTM West Femto Maximum Sensitivity Substrate Kit (ThermoFisher Scientific, United States).

siRNA Transfection

The siRNA (GenePharma, China) sequences were used to restrain the expression of cells and animals HSP27.

In all experiments, cells with considerable viability were selected to be transfected by the sequences, 5′-GGACGAGCA UGGCUACAUCTT-3' (sense strand) and 5′-GAUGUAGCC AUGCUCGUCCTT-3' (antisense strand). Inoculated in 6-well plates and pre-cultured for 24 h, the cells were transfected until they were completely attached to the plates. Sequentially, growth factor-free (HEKs) and serum-free (HDFs) medium containing siRNA and HiPerFect Transfection Reagent (Qiagen, Germany) were used to replace the complete medium. After 6 h of incubation, the aforesaid media were replaced with the complete medium. Subsequent experiments were performed after 24 h of culture.

The sequences exerted on animals HSP27 knockdown were 5′-UUCUCCGAACGUGUCACGUTT-3' (sense strand) and 5′-ACGUGACACGUUCGGAGAATT-3' (antisense strand). After 1-week adaptive feed, siRNA was injected locally in one ear of the mice randomly selected. Succeeding experiments were implemented 24 h later.

Reactive Oxygen Species Levels Detection

The cells were inoculated in 6-well plates and pre-cultured for 24 h, with the complete medium being replaced with 1 ml of growth factor-free (HEKs) and serum-free (HDFs) medium containing DCFH-DA (Beyotime Biotechnology, China). Incubated at 37°C in a 5% CO_2 incubator for 20 min, the cells were washed with the growth factor-free (HEKs) and serum-free (HDFs) medium for three times and then irradiated with UVB lamp. After the irradiation, the

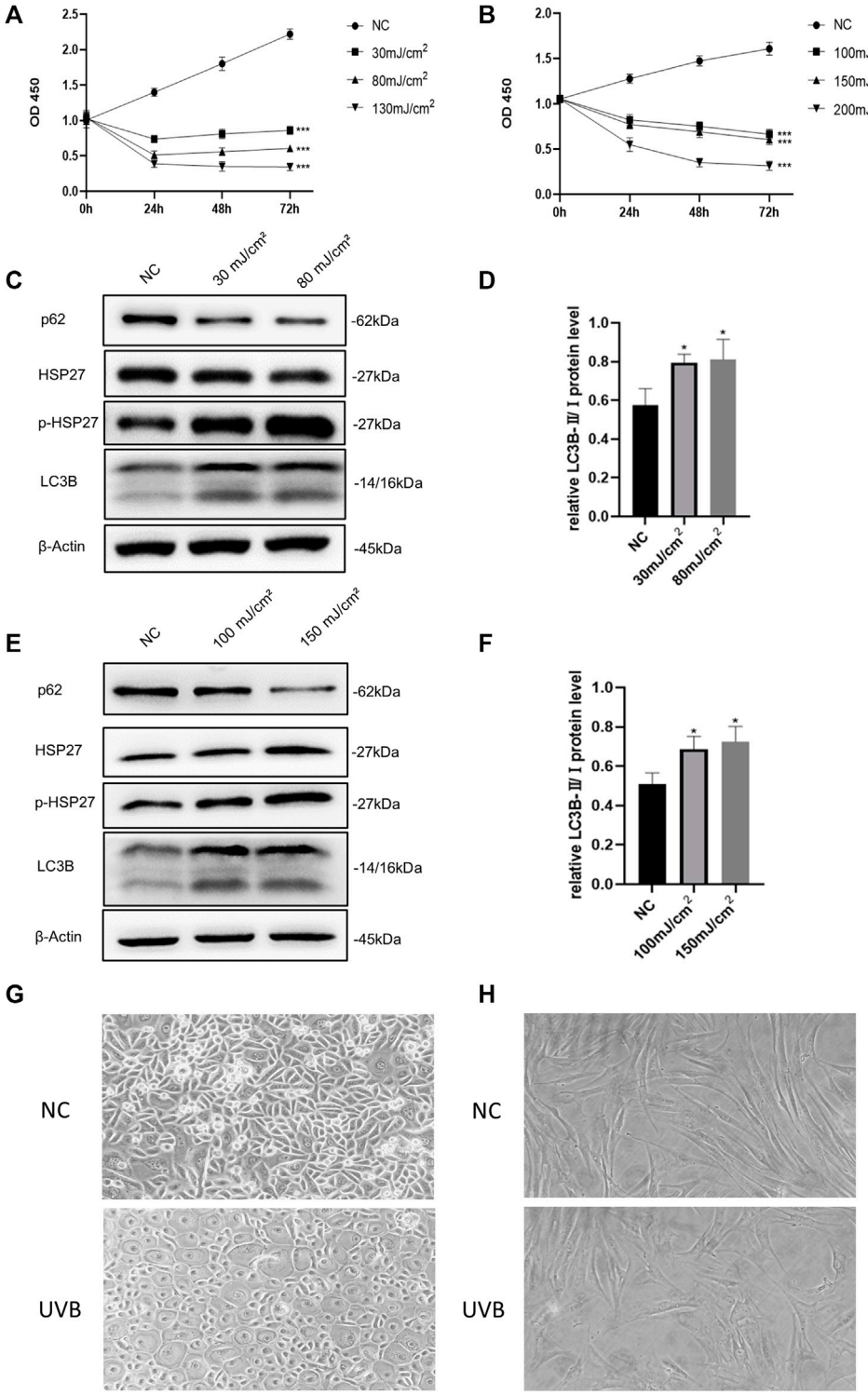

FIGURE 1 | Effects of UVB irradiation on cell viability and autophagy. **(A)** The proliferation of HEKs was detected by the CCK-8 method at 0, 24, 48 and 72 h after different doses of UVB irradiation. **(B)** The proliferation of HDFs was detected by the CCK-8 method at 0, 24, 48 and 72 h after different doses of UVB irradiation. **(C)** HSP27, p-HSP27, p62, and LC3B protein expressions were detected by using Western blotting in HEKs at 24 h post-UVB, regarding β-actin as a reference. **(D)** The LC3B-**II**/**I** protein expressions of HEKs at 24 h post-UVB. **(E)** HSP27, p-HSP27, p62, and LC3B protein expressions were detected by using Western blotting in HDFs at 24 h post-UVB, regarding β-actin as a reference. **(F)** The LC3B-**II**/**I** protein expressions of HDFs at 24 h post-UVB. **(G)** Morphological variations of HEKs at 24 h after UVB irradiation was observed by microscope (200×). **(H)** Morphological variations of HDFs at 24 h after UVB irradiation was observed by microscope (200×). All the results were expressed as mean ± SD, and the experiment was repeated at least three times (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$).

labeled cells were washed twice with PBS and digested with 0.25% trypsin (Beyotime Biotechnology, China). All the samples were resuspended in 500 μl of PBS, and then analyzed by the flow cytometry and CYTExpert (Beckman Coulter, United States).

Histological Analysis

24 h after the last irradiation, the ears of mice were cut, fixed in 4% paraformaldehyde solution for 24 h, and then were dehydrated and embedded by paraffin to section for hematoxylin-eosin (H&E) staining, Masson-trichrome staining, and immunohistochemical labeling severally. All stained skin specimens were observed under a microscope, and images were collected for subsequent analysis by ImageJ (National Institutes of Health, United States).

Statistical Analysis

Statistical analysis was conducted by using Graphpad Prism 8.0 (Graphpad Software, Inc., United States) and IBM SPSS Statistics 22.0 (IBM Corporation, United States), and the error bars of data were shown as mean ± standard deviation (SD). The differences between the two groups were analyzed by using the Student's t-test. With respect to more groups, one-way analysis of variance (ANOVA) was chosen to test the differences. $p < 0.05$ was considered statistically significant. All the results were obtained from at least three independent experiments.

RESULTS

UVB Irradiation Inhibited Human Epidermal Keratinocytes and Human Dermal Fibroblasts Viability and Activated Autophagy

Based on previous studies, we selected different radiation dosages to construct photodamage models of HEKs and HDFs induced by UVB irradiation (Hwang et al., 2018; Park et al., 2018). According to the results of CCK-8 detection (**Figures 1A,B**), UVB irradiation obviously led to a significant decrease in the activity of skin cells. When the dosages increased to 130 mJ/cm^2 (HEKs) and 200 mJ/cm^2 (HDFs), the cells viability decreased by more than 60%, seriously influencing the proliferation ability. Therefore, these two groups were excluded in the subsequent experiments. As shown in **Figures 1C–F**, compared with the control group, although the protein level of HSP27 was not significantly varied after UVB irradiation, its phosphorylated form (p-HSP27) expression was notably elevated along with the increase of dosage of radiation. Furthermore, the expression level of LC3B-II/I in both cells was enhanced, and the expression level of p62 was expressively decreased, indicating a probable relation between HSP27 and autophagy. Hence 30 mJ/cm^2 (HEKs) and 150 mJ/cm^2 (HDFs) were picked out as the radiation dosages for the development of the photodamage model of cells. Meanwhile, the morphology of both types of cells changed dramatically with UVB exposure (**Figures 1G,H**). HEKs altered from small polygons to larger quasi-circular ones, while HDFs became thicker, shorter and irregular in shape, confirming the success of establishment of the photodamage model.

Heat Shock Protein 27 Knockdown Hindered UVB Irradiation and Induced Autophagy of Human Epidermal Keratinocytes and Human Dermal Fibroblasts

To explore the effect of HSP27 on UVB-induced photodamage, we successfully constructed HSP27 low expression models by means of siRNA (**Figures 2A,B,I,J**). The proliferation ability of cells was evaluated by the CCK-8 test, showing that the growth of UVB + si-HSP27 group was significantly inhibited, compared with the group of cells irradiated by UVB alone (**Figures 2C,K**). We also found that after HSP27 gene knockdown, the protein or mRNA expression level of HEKs and HDFs exhibited p62 accumulation and LC3B-II/I reduction (**Figures 2D–H,L–P**). These results suggested that HSP27 might act a photoprotective part in UVB induced photodamage by promoting autophagy.

Heat Shock Protein 27 Knockdown Exacerbated UVB-Induced Reactive Oxygen Species Amassing and Attenuated the Antioxidant Capacity of Human Epidermal Keratinocytes and Human Dermal Fibroblasts

In an animal experiment related to cardiac senescence, researchers found that specific motivation of the myocardial HSP27 expression could resist the cardiac functional damage caused by senescence, interfering the production of ROS in cells (Lin et al., 2016). For the sake of determining whether this phenomenon existed in the skin photodamage induced by UVB irradiation, we firstly contrasted the ROS levels before and after UVB irradiation. As illustrated in **Figures 3A,B,G,H**, UVB irradiation could distinctly attract the production of ROS in HEKs and HDFs. In addition, in the UVB + si-HSP27 group, the ROS level was aggrandized even more. Then, we probed the variations of some intracellular antioxidants. Unscrambling the western blotting and RT-qPCR outcomes (**Figures 3C–F,I–L**), the production of CAT, SOD1 and SOD2 were lessened in post-irradiation cells with inhibited HSP27, compared to the mere UVB irradiation group. CAT mainly decreased at mRNA level of HDFs while SOD decreased at both protein and mRNA levels of these 2 cells, demonstrating that the latter was more likely to weaken the antioxidant state of cells. Therefore, we speculated that HSP27 might also have a photoprotective impact on cells by suppressing UVB-induced oxidative stress response and ROS production.

Heat Shock Protein 27 Knockdown Aggravated Skin Photodamage Treated by UVB in Mice

In animal experiments, the expression of HSP27 was increased in the tissues with UVB exposure, and siRNA was also applied to

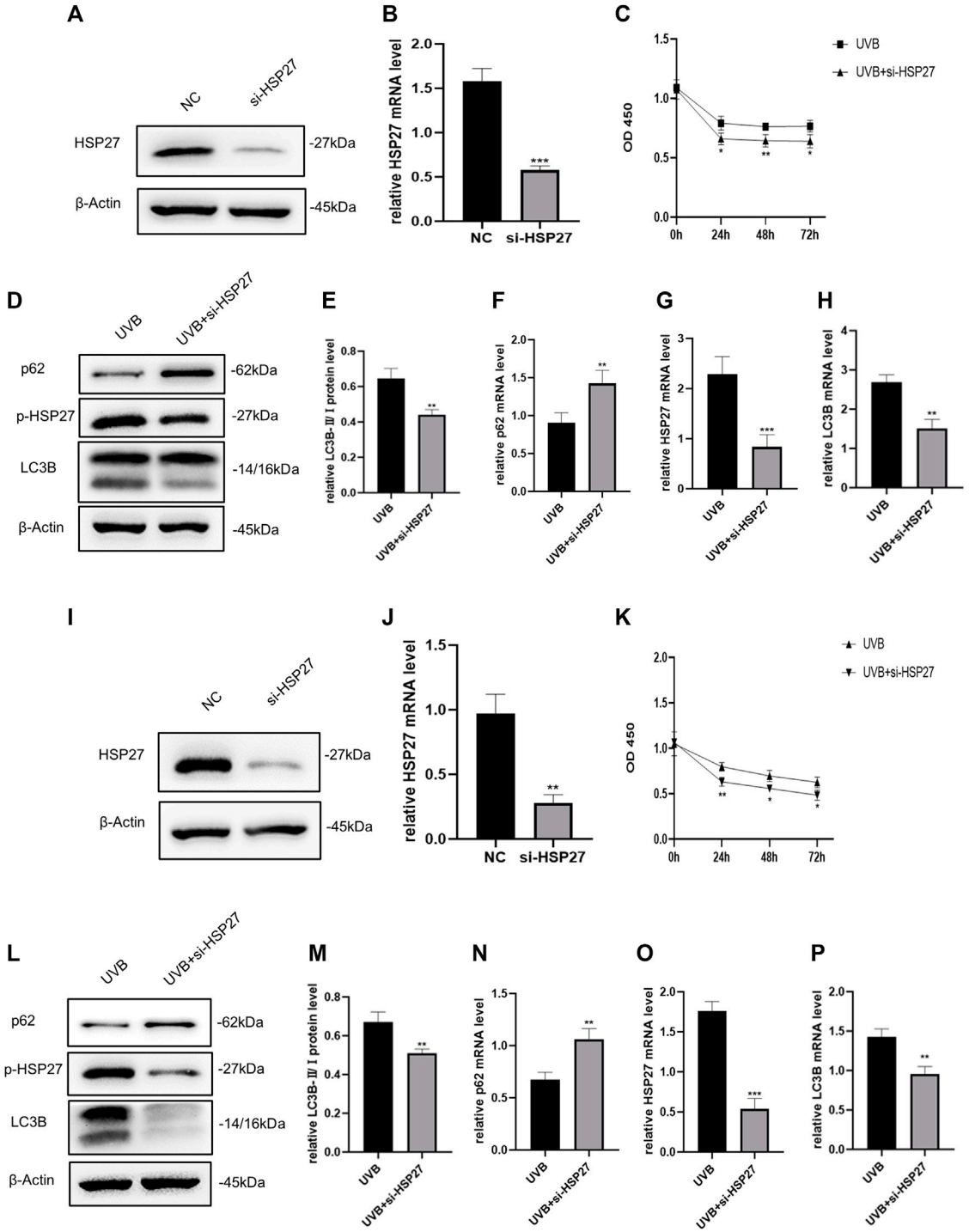

FIGURE 2 | Effects of HSP27 knockdown on cell activity and autophagy under UVB irradiation. **(A,D)** HSP27, p-HSP27, p62 and LC3B protein expressions were detected by using Western blotting in HEKs after HSP27 gene knockdown, regarding β-actin as a reference. **(B,F–H)** HSP27, p62 and LC3B mRNA levels were detected by using RT-qPCR in HEKs after HSP27 gene knockdown, quantified by using ACTB as a reference. **(C)** The proliferation ability of HEKs was detected by the CCK-8 method at 0, 24, 48 and 72 h after UVB irradiation in the control group and the si-HSP27 group. **(E)** The LC3B-II/I protein expressions of HEKs after HSP27 gene knockdown. **(I,L)** HSP27, p-HSP27, p62 and LC3B protein expressions were detected by using Western blotting in HDFs after HSP27 gene knockdown, regarding β-actin as a reference. **(J,N–P)** HSP27, p62 and LC3B mRNA levels were detected by using RT-qPCR in HDFs after HSP27 gene knockdown, quantified by using ACTB as a reference. **(K)** The proliferation ability of HDFs was detected by the CCK-8 method at 0, 24, 48 and 72 h after UVB irradiation in the control group and the si-HSP27 group. **(M)** The LC3B-II/I protein expressions of HDFs after HSP27 gene knockdown. All the results were expressed as mean ± SD, and the experiment was repeated at least three times (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$).

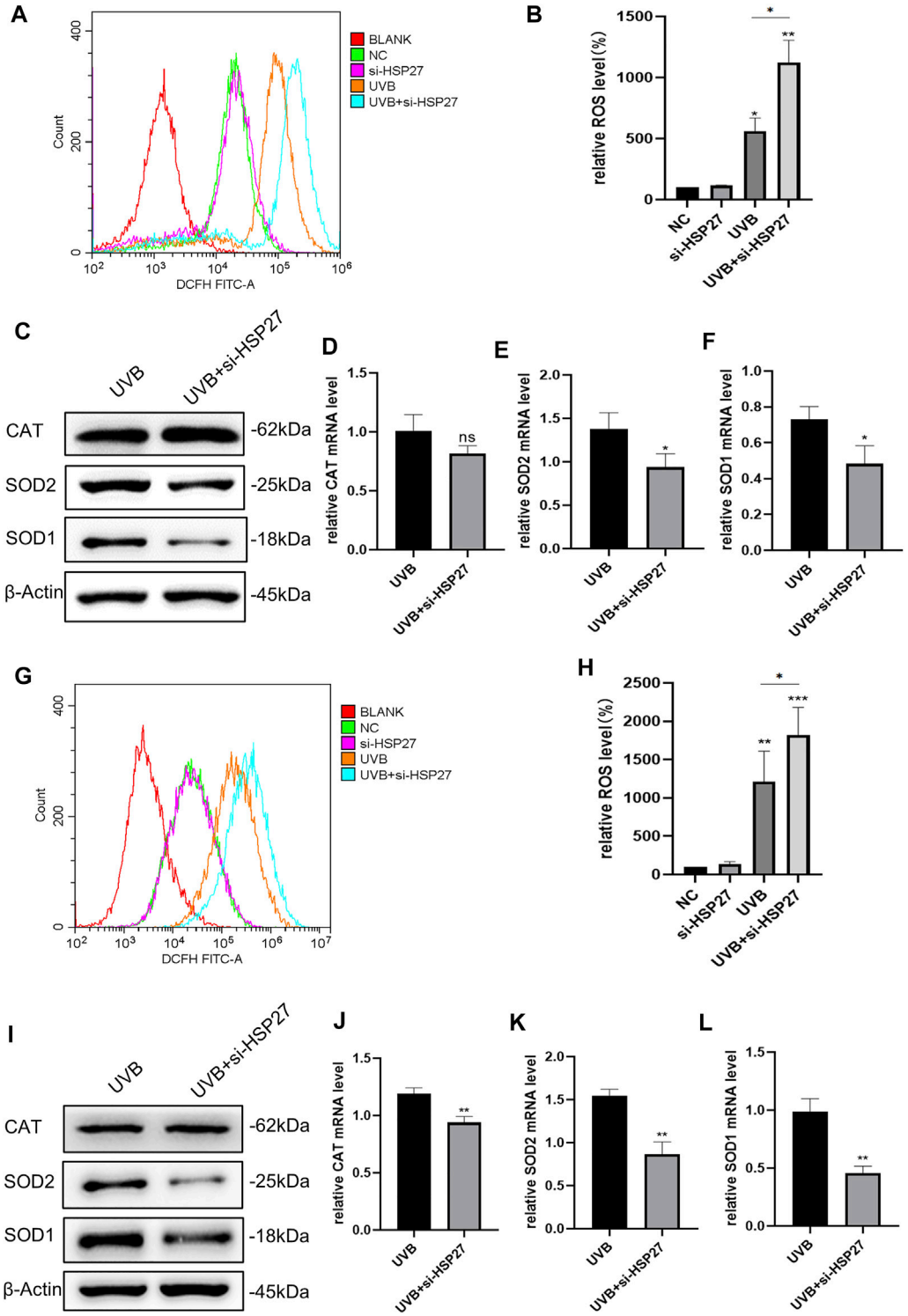

FIGURE 3 | Effect of HSP27 knockdown on the antioxidant capacity of cells irradiated with UVB. **(A,B)** Alterations of ROS level in HEKs after HSP27 gene knockdown. **(G,H)** Alterations of ROS level in HDFs after HSP27 gene knockdown. **(C)** CAT, SOD1 and SOD2 protein expressions were detected by using Western blotting in HEKs after HSP27 gene knockdown, regarding β-actin as a reference. **(I)** CAT, SOD1 and SOD2 protein expressions were detected by using Western blotting in HDFs after HSP27 gene knockdown, regarding β-actin as a reference. **(D–F)** CAT, SOD1, SOD2 mRNA levels were detected by using RT-qPCR in HEKs after HSP27 gene knockdown, quantified by using ACTB as a reference. **(J–L)** CAT, SOD1, SOD2 mRNA levels were detected by using RT-qPCR in HDFs after HSP27 gene knockdown, quantified by using ACTB as a reference. All the results were expressed as mean ± SD, and the experiment was repeated for at least three times (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$).

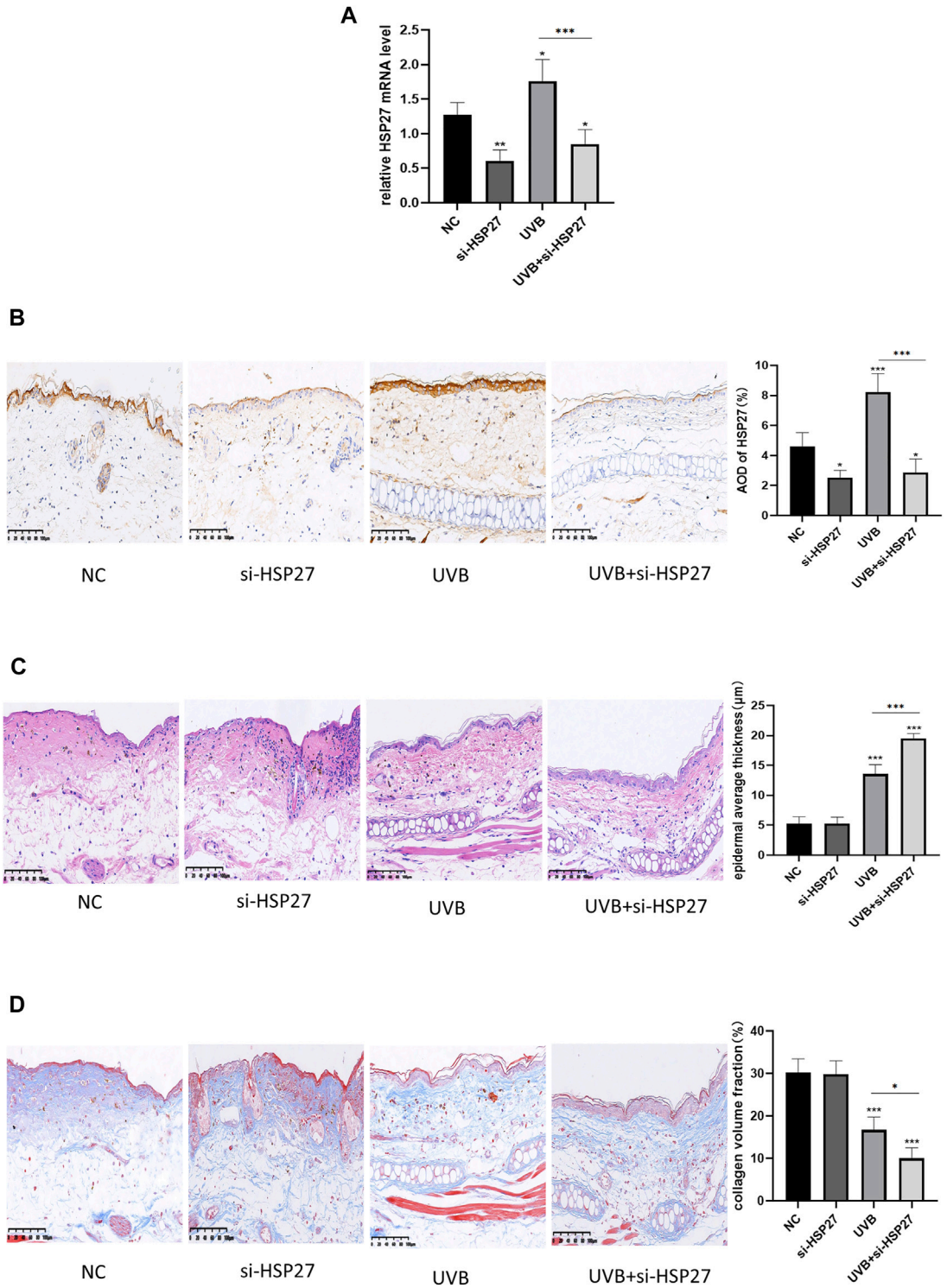

FIGURE 4 | Effects of HSP27 knockdown on UVB-induced skin pathological changes in mice. **(A)** HSP27 mRNA level was detected by using RT-qPCR after HSP27 gene knockdown, quantified by using GAPDH as a reference. **(B)** Immunohistochemical marker for HSP27 results and quantification of Average Optical Density (AOD). **(C)** H&E staining results and quantification of epidermal average thickness. **(D)** Masson-trichrome staining results and quantification of collagen volume fraction. All the results were expressed as mean ± SD, and the experiment was repeated at least three times (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$).

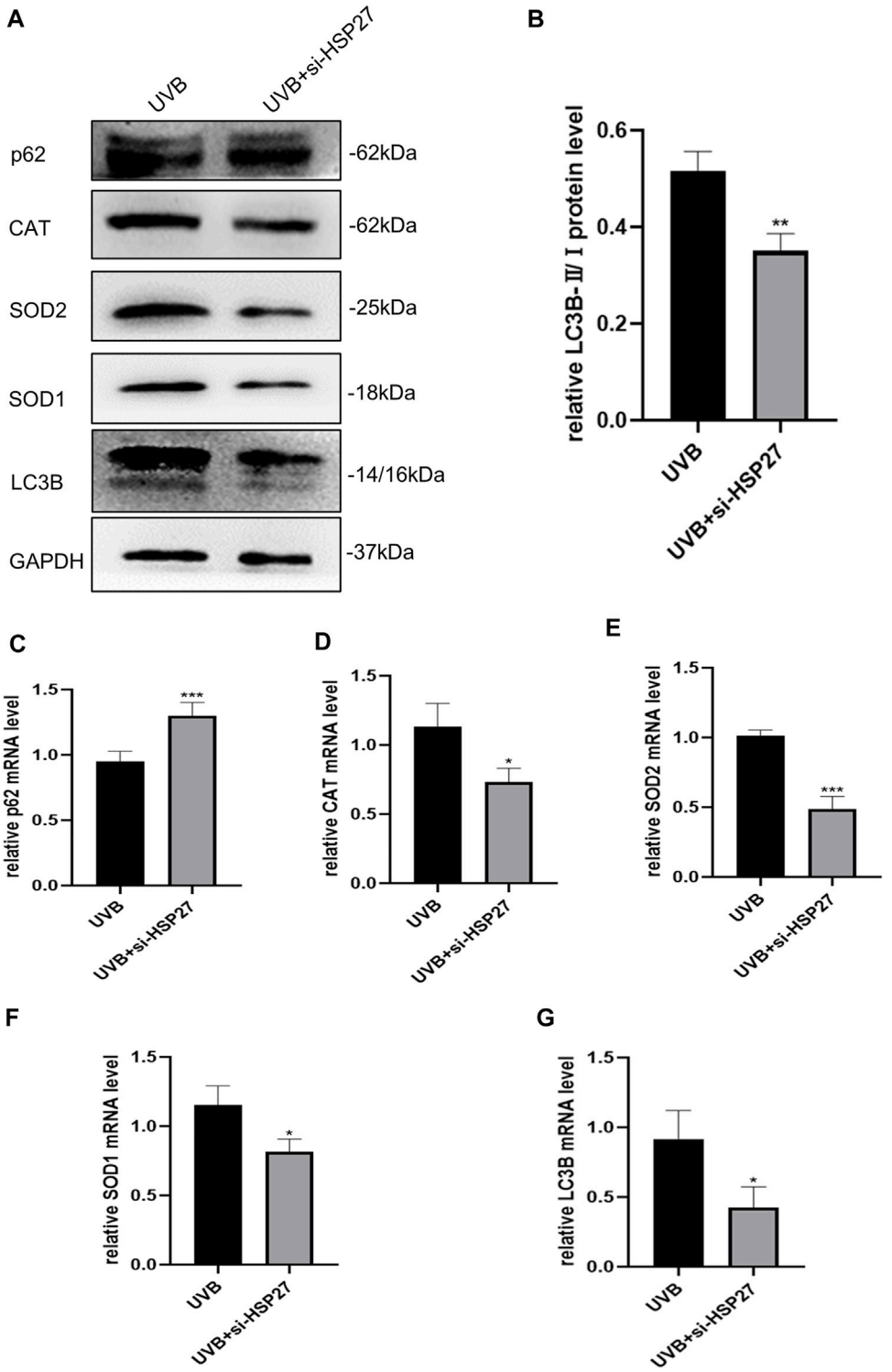

FIGURE 5 | Effects of HSP27 knockdown on skin autophagy and antioxidant capacity of mice irradiated with UVB. **(A)** p62, LC3B, CAT, SOD1 and SOD2 protein expressions were detected by using Western blotting after HSP27 gene knockdown, regarding GAPDH as a reference. **(B)** The LC3B-II/I protein expressions after HSP27 gene knockdown. **(C–G)** p62, LC3B, CAT, SOD1 and SOD2 mRNA levels were detected by using RT-qPCR after HSP27 gene knockdown, quantified by using GAPDH as a reference. All the results were expressed as mean ± SD, and the experiment was repeated at least three times (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$).

specifically down-regulate the expression of HSP27 in mice ear skin (**Figures 4A,B**). Studies illustrated that epidermal incrassation and differentiation in connective tissue were prevalent in photodamage skin (Tak et al., 2020). H&E and Masson-trichrome staining were used to assess the histopathologic changes of mice skin after 24 h of the last UVB irradiation. As presented in **Figures 4C,D**, compared with those in the non-irradiated groups, the epidermis was thickened, cells were disordered and collagen fibers were loose, reduced, fractured and disorganized in the irradiated group. Apart from that, the above changes were more remarkable in the UVB + si-HSP27 group than those in the UVB group.

Heat Shock Protein 27 Knockdown Alleviated the Autophagy and Antioxidant Capacity of Mice Skin Treated by UVB Irradiation

In order to determine whether the role of HSP27 in UVB-induced photodamage was consistent *in vivo* and vitro experiments, the autophagy related indexes p62 and LC3B and antioxidant indexes CAT, SOD1 and SOD2 of mice skin tissues were also examined. Fortunately, similar results were acquired in both protein levels (**Figures 5A,B**) and mRNA levels (**Figures 5C–G**). The mice co-treated with UVB irradiation and HSP27 downregulation manifested an apparent increase of p62, while visible decrease in the expressions of LC3B, CAT, SOD1 and SOD2, compared to the UVB group. This further revealed that HSP27 was perhaps beneficial to preventing skin from UVB-caused photodamage by regulating autophagy level and antioxidant capacity of the organism.

DISCUSSION

It is well known that either acute exposure or chronic repeated exposure to UV light will lead to various skin lesions, such as sunburn, chronic actinic dermatitis, and even induce carcinogenesis (Sharma et al., 2018). At the same time, the absorption of radiation by skin chromophores or the formation of ROS can give rise to premature skin senescence, pigmentation and collagen loss (Silva et al., 2017). Due to the differences in the degree of clinical manifestations and the recovery speed of the body, most researchers pay more attention to the chronic photodamage caused by UV light, especially the consequences of photoaging (Zhao et al., 2018; Rui et al., 2019; Yang et al., 2019). However, the progress in preventing the acute photodamage induced by UV radiation is also required to be explored. Although UVB is mainly absorbed by keratinocytes in the epidermis, it can still reach the upper dermis, and generate pathological variations of the dermis through signaling pathways (Takeuchi & Runger, 2013). On the grounds of reviewed literature and previous studies of our team, we initially irradiated HEKs with 30 mJ/cm^2, 80 mJ/cm^2 and 130 mJ/cm^2, and HDFs with 100 mJ/cm^2, 150 mJ/cm^2 and 200 mJ/cm^2. Subsequently, considering the diversification in cell activity, autophagy activity and HSP27 expression level, a new UVB-induced photodamage model *in vitro* was proposed.

As a molecular chaperone, HSP27 can assist in protein folding, aggregation, transportation and stabilization. Beyond that, it is capable of restraining apoptosis through caspase-dependent and caspase-independent pathways (Acunzo et al., 2012). Given previous outcomes of our group, we revealed that rapamycin, an autophagy activator, might potentially to preserve skin fibroblasts from UVA-induced photoaging by inhibition of p53 and p-HSP27 and counteract the collagen destruction operated by HSP27 knockdown (Bai et al., 2021). Interestingly, in our trials, p-HSP27 expression modulation synchronizing with the autophagy activation was discovered in either photodamage models of HEKs and HDFs *in vitro* or these models of C57BL/6 mice *in vivo*. In addition, HSP27 knockdown would exacerbate the destruction of collagen resulting from UVB exposure. These phenomena manifested that HSP27 might protect the skin from photodamage caused by UVB irradiation by promoting autophagy.

UVB exposure will destroy the antioxidant defense system, resulting in oxidative stress (Wang et al., 2016). A large number of ROS, such as superoxide anion radical (·O_2-), hydrogen peroxide (H_2O_2) and hydroxy radical (-OH), etc. are generated in the cell (Di Meo et al., 2016). The ROS amount produced by keratinocytes and fibroblasts is related to UV radiation in a dose-dependent manner. Oxidative stress reaction induced by ROS generates lipid peroxidation and DNA injury, resulting in photodamage to the skin (Hideg et al., 2013). Other studies proved that HSP27 had a certain antioxidant capacity. For example, VB-037, a quinoline derivative, restrained ROS level by controlling the HSP27 and P38/JNK signaling pathways to improve neuron damage and neuroinflammation, providing a promising direction for exploring therapeutic candidates for Alzheimer's disease (Chiu et al., 2019). In addition, based on analysis of clinical samples from coronary artery disease (CHD) patients and the atherosclerosis mice models, researchers detected that HSP27 exerted an enormous function on constraining the generation of ROS and the progression of atherosclerosis by inhibiting the mitochondrial apoptosis pathway of CHD (H. L. Zhang et al., 2019). Our experimental results also attested this perspective. The knockdown of HSP27 would lead to excessive production of ROS, and reduce the antioxidant capacity of cells after UVB irradiation. On the basis of this, we deduced that HSP27 could play a photoprotective role by inhibiting the oxidative stress response.

Autophagy is essentially a natural process of self-renewal and self-protection in organism. With the help of the lysosome, damaged, senescent and dying organelles and cytoplasm are devoured, degraded and recycled to balance the intracellular environment. When the cell metabolism or stimulation increases, autophagy will be activated, and the oxidative stress is one of its powerful factors (Wollert, 2019). The p62 protein encoded by *SQSTM1* gene, which not only plays an indispensable role in autophagy, but also an effective target gene of the antioxidant system. For autophagy, p62 operates through the ubiquitin-proteasome pathway, binding to ubiquitinated products and targeting and transporting them to the autophagosome. While binding to lysosomes, the autophagosome contents, including p62, will be degraded (Kumsta et al., 2019). In

terms of antioxidant, p62 is better known as a natural activator of Nrf2-antioxidant response element (ARE) transcription pathway. p62 binds to kelch-like ech associated protein 1 (Keap1), leading to dissociation of Keap1 and Nrf2. Then Nrf2 is released and accumulated in the cytoplasm, promoting its binding to the specific sequence ARE. And then a series of the downstream genes were activated to regulating the expression of several antioxidant enzymes including SOD1, SOD2 and CAT. (Bartolini et al., 2018). In addition, recent studies have found that under the induction of PPARGC1A, p62 had anti-aging effect by activating autophagy and upregulating the expression of antioxidant proteins. (Salazar et al., 2020). In this study, autophagy was up-regulated, and the antioxidant system was also activated by UVB radiation. Whether p62 or other antioxidant pathways serve as a bridge between these two events will be one of the focuses of our future research.

Taken together, these findings demonstrated that HSP27 might protect cells or tissues from the adverse effects of UVB irradiation by stimulating autophagy and reducing ROS production, offering proofs that HSP27 might be an effective therapeutic target for the prevention of UVB irradiation-induced skin lesions. Regrettably, we have not yet determined the specific pathways by which HSP27 regulates autophagy and ROS levels to exert its anti-photodamage function, and the existence of correlation between UVB-induced autophagy activation and the occurrence of oxidative stress. In future studies, we will make efforts to clarify the definite mechanism of HSP27 photoprotection.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The ethics committee of the first affiliated hospital of Chongqing medical university. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by The ethics committee of the first affiliated hospital of Chongqing medical university.

AUTHOR CONTRIBUTIONS

Z-YW and AL performed all experiments, analysed data, and accomplished the manuscript. C-AJ, PW and XH provided technical guidance. G-LB, Y-XJ, R-LL, CL and Z-YW reviewed the literature. Z-YW and AL contributed equally to this work. All authors examined and approved the final manuscript.

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REVIEW
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Neuroimmune Interaction: A Widespread Mutual Regulation and the Weapons for Barrier Organs

Yan Zhu[1,2†], *Shixin Duan*[1,2†], *Mei Wang*[1,2†], *Zhili Deng*[1,2,3*] and *Ji Li*[1,2,3*]

[1]Department of Dermatology, Xiangya Hospital, Central South University, Changsha, China, [2]Hunan Key Laboratory of Aging Biology, Xiangya Hospital, Central South University, Changsha, China, [3]National Clinical Research Center for Geriatric Disorders, Xiangya Hospital, Central South University, Changsha, China

Since the embryo, the nervous system and immune system have been interacting to regulate each other's development and working together to resist harmful stimuli. However, oversensitive neural response and uncontrolled immune attack are major causes of various diseases, especially in barrier organs, while neural-immune interaction makes it worse. As the first defense line, the barrier organs give a guarantee to maintain homeostasis in external environment. And the dense nerve innervation and abundant immune cell population in barrier organs facilitate the neuroimmune interaction, which is the physiological basis of multiple neuroimmune-related diseases. Neuroimmune-related diseases often have complex mechanisms and require a combination of drugs, posing challenges in finding etiology and treatment. Therefore, it is of great significance to illustrate the specific mechanism and exact way of neuro-immune interaction. In this review, we first described the mutual regulation of the two principal systems and then focused on neuro-immune interaction in the barrier organs, including intestinal tract, lungs and skin, to clarify the mechanisms and provide ideas for clinical etiology exploration and treatment.

Keywords: neuroimmune crosstalk, nerve, immune, barrier organ, neuropeptide, neurotransmitter

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Mingxing Lei,
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Reviewed by:
Bing Zhang,
Westlake University, China
Jinwei Zhang,
Chongqing Hospital of Traditional Chinese Medicine, China

***Correspondence:**
Zhili Deng
dengzhili@csu.edu.cn
Ji Li
liji_xy@csu.edu.cn

[†]These authors have contributed equally to this work and share first authorship

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1 INTRODUCTION

Adapting to environmental changes and maintaining homeostasis requires the involvement of both the nervous system and immune system (Veiga-Fernandes and Pachnis, 2017; Zhang et al., 2021). The nervous system dominates tissues or organs mainly through a variety of neurotransmitters, while the immune system resists pathogens by phagocytosis and immune-active substances. According to research in recent years, the two systems can be linked together as a whole through certain agents, such as neurotransmitters, cytokines, hormones, etc (Chavan et al., 2017; Reardon et al., 2018). Neurotransmitter receptors are distributed on some immune cells, which are the foundation of neuromodulation of the immune system. And there are also cytokine receptors on nerve endings, which are the essential pathway for immunomodulation of the nervous system. The nervous system speeds up the immune response, and the immune system correspondingly helps transfer information to the nervous system. Neuro-immune interaction enriches the body's response to the environment. However, under certain circumstances, neuro-immune interactions are the driving factors for the onset and progression of numerous diseases (Kabata and Artis, 2019; Tanaka and Okusa, 2019; Scheiblich et al., 2020).

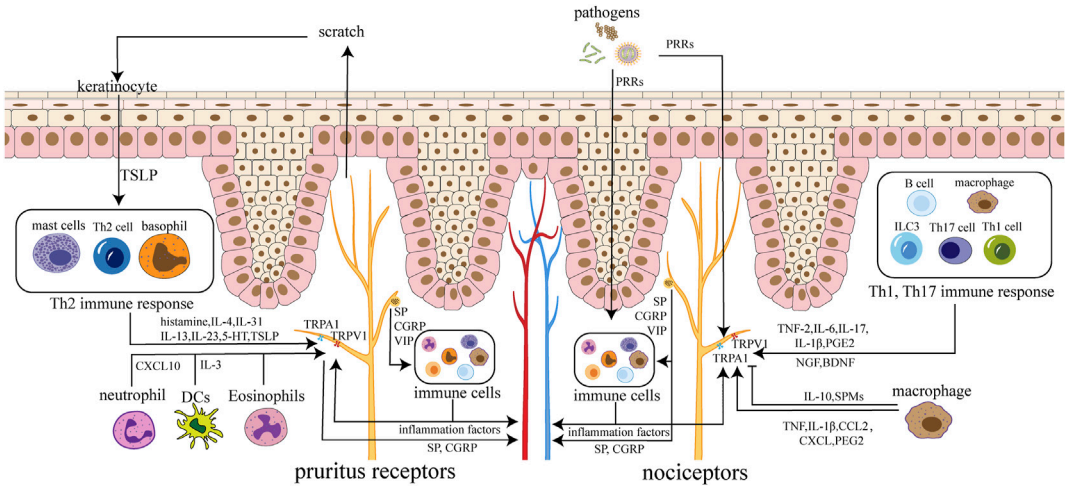

FIGURE 1 | Neuroimmune circuits in skin. Immune cells activate receptors and channels on sensory neurons in the skin by secreting cytokines, chemokines, and lipids, leading to itching and pain. Nerve endings can also regulate immune cells by releasing SP, CGRP, VIP and other neuropeptides. Neuropeptides and factors can act on the skin and blood vessels, causing inflammation and edema. Pathogens can stimulate nociceptors directly or by inflammatory cytokines from immune cells. Pruritus receptors' activation cause scratch, which leads to the destruction of keratinocytes with TSLP released, triggering amplification effect. Nociceptors are mainly involved in Th1 and Th17 immune responses, while pruritus receptors are mainly involved in Th2 immune responses.

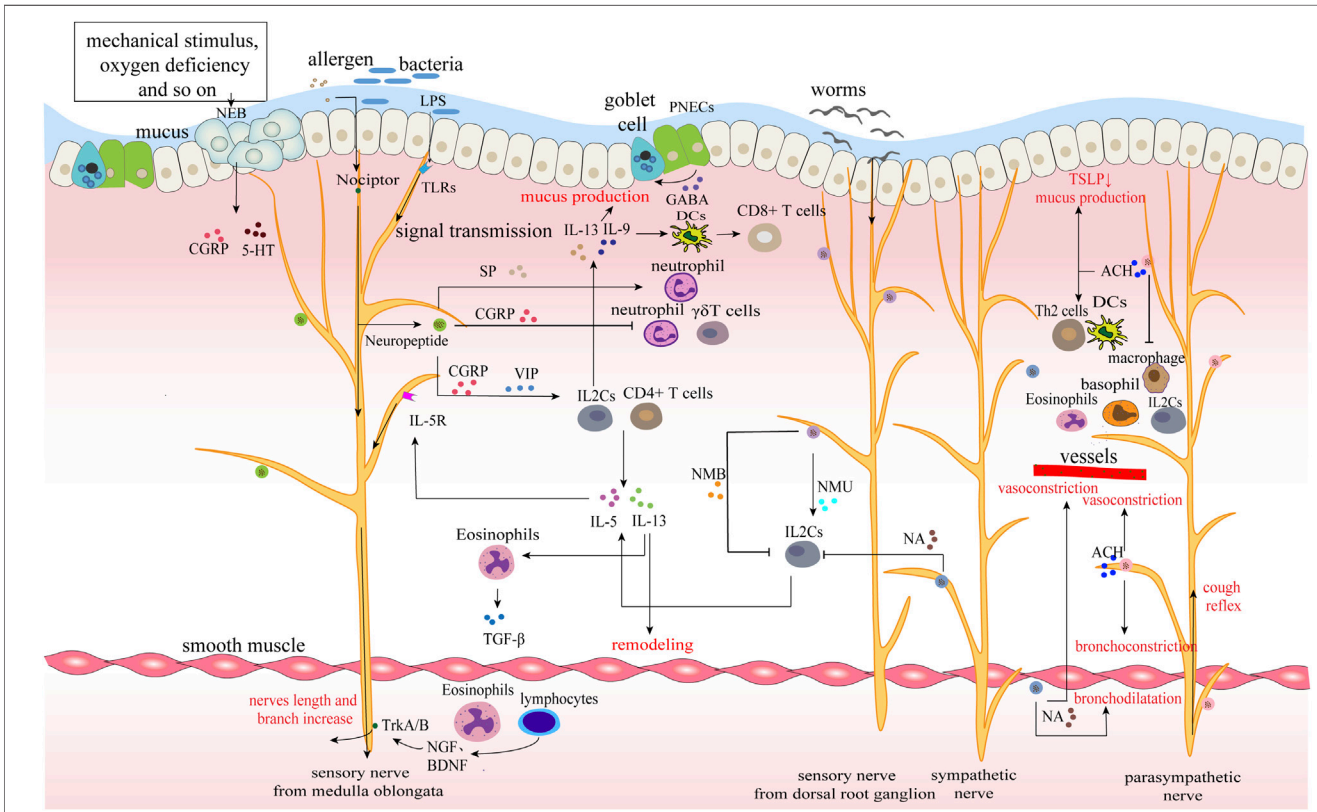

FIGURE 2 | Neuroimmune circuits in lungs. The epithelium, nerves and immunity of the lung together constitute the airway barrier. Various allergens, bacteria and parasites stimulate and interact with different nerves to upload signals and regulate immunity. For example, LPS of bacteria combines with TLRs of sensory nerves to promote the release of neuropeptides, while worms act on sympathetic nerves to secrete neurotransmitters and mainly regulate ILC2s. Immune cells transmit immune signals to the nerve by releasing cytokines, forming a closed-loop of nerve and immunity. Moreover, immune cells synthesize nerve growth factor to promote nerve growth and increase nerve exposure in the airway.

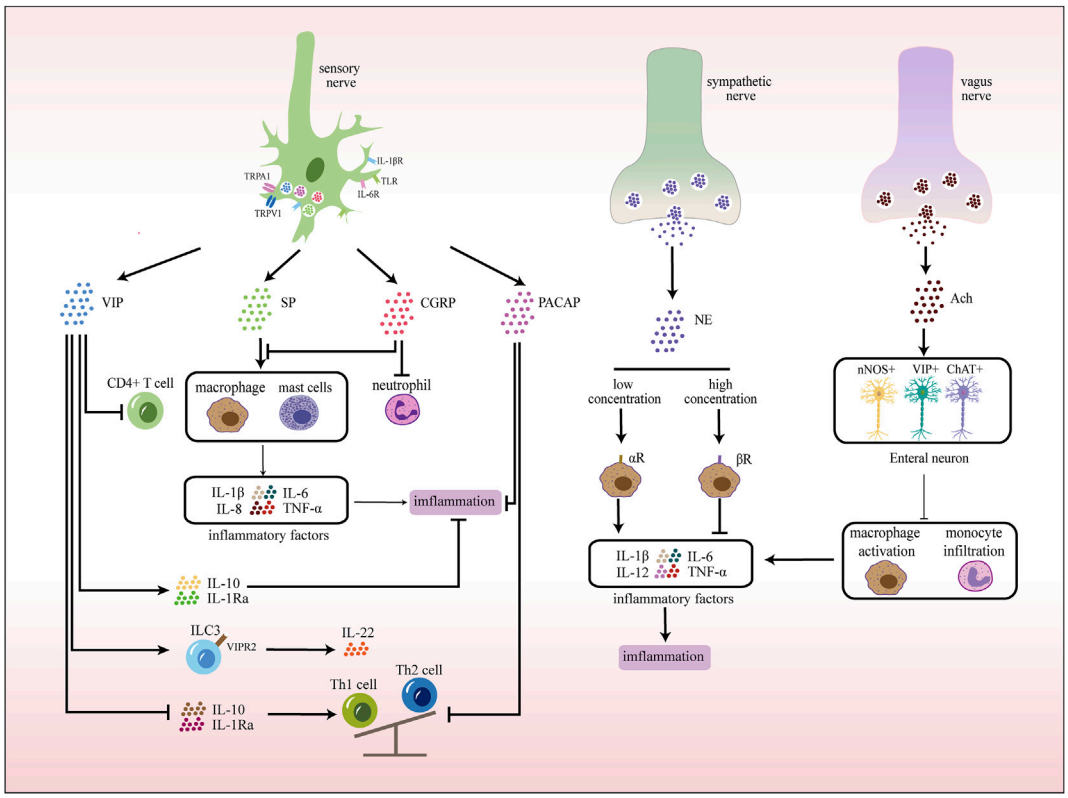

FIGURE 3 | Neuroimmune communication in the intestinal tract. In the gut, sensory nerves express receptors that recognize various stimuli, pathogens, and cytokines. Activated sensory nerves release different neurotransmitters that interact with immune cells. In addition, exogenous sympathetic and vagus nerves also participate in inflammatory responses by releasing corresponding neurotransmitters.

For the past few years, there is increasing evidence about the mutual regulation of nerve and immune systems, which reveals that nerves regulate the generation, maturation, and release of immune cells (Elenkov et al., 2000), and immunity is involved in neural development (Cowan and Petri, 2018; Vainchtein et al., 2018). As the first line of defense, the gut-skin-lung barrier plays an important role in clearing harmful substances, resisting pathogen invasion, and maintaining homeostasis, and its dysfunction leads to the occurrence and progression of diseases. What's more, there are dense nerve innervation and resident immune cell groups on the barrier, and the neuro-immune interaction occupies a more significant position in the pathogenesis of diseases. For example, neuropeptides secreted by sensory nerves trigger neurogenic inflammation and lead to allergic diseases (Sousa-Valente and Brain, 2018), while sympathetic and parasympathetic nerves produce neurotransmitters binding to different receptors on immune cells for immune regulation (Kenney and Ganta, 2014). Cytokine receptors on nerve endings collect information of immune status and transmit feedback signs.

At present, the mechanism of neuro-immune interactions remains unclear, and individualized treatment is hard to practice. Further basic research and clinical trials are needed to find out the pattern of neuroimmune-related disease and provide a more rational therapeutic regimen.

2 THE MUTUAL REGULATION OF NERVOUS SYSTEM AND IMMUNE SYSTEM

From the embryonic period, the development of the immune and nervous systems is regulated by each other. Immune cells are almost regulated by nerves from differentiation to function execution. And nerves perform hormone-mediated immune regulation through the hypothalamic-pituitary-adrenal (HPA) axis. The immune system also influences nerve growth and regulates the HPA axis through various immunoactive substances.

2.1 Neural Regulation of Immune Cell Production

Immune cells are originated from hematopoietic stem cells (HSCs) in the bone marrow, differentiate to the lymphoid stem cell stage which differentiate into pro-B and pro-T cells, and finally become a variety of immune cells in the bone marrow and thymus. However, studies have found that the nervous system regulates the generation, migration, and differentiation of HSCs directly or through substances such as neurotransmitters and neuropeptides (Agarwala and Tamplin, 2018).

The formation of HSCs occurs at the embryonic day 27–40, which refers to endothelial-to-hematopoietic transition (EHT) of

hematopoietic endothelial cells of the embryonic dorsal aorta. Studies have found that hypoxic stress induces central neural crest cells to produce 5-hydroxytryptamine (5-HT) neurotransmitter signals in the embryonic zebrafish. 5-HT further affects the formation of distant embryonic hematopoietic stem and progenitor cells (HSPCs) *via* the HPA axis and local glucocorticoid (Kwan et al., 2016). Similarly, central cholinergic signals increase the secretion of catecholamines (CAs) and γ-aminobutyric acid (GABA) through the hypothalamic-pituitary-adrenal (HPA) axis and peripheral neurons, promoting the proliferation and mobilization of immature $CD34^+$ HSCs (Spiegel et al., 2007; Pierce et al., 2017; Shao et al., 2021). In addition, peripheral neurons secrete CAs and calcitonin gene-related peptide (CGRP) to drive granulocyte colony-stimulating factor (G-CSF)-induced mobilization of HSCs (Katayama et al., 2006; Maryanovich et al., 2018; Gao et al., 2021).

2.2 Neural Regulation of Immune Cell Maturation and Release

The nervous system communicates with lymphatic tissues or organs through sensory and autonomic nerves. Among them, the thymus, as a central immune organ, receives dual innervation of sympathetic and parasympathetic nerves. Immunofluorescent staining of mouse thymus shows the inside nerve fibers distribution and indicates that T cell maturation is regulated by nerves (Al-Shalan et al., 2019). Lymph nodes are mainly supplied by sensory and sympathetic nerves. There are extensive and close contacts between nerve fibers and various dendritic cells in mouse lymph nodes (Hu et al., 2019). Sensory neurons detect the immune state of peripheral lymph nodes through nociceptors and undergo remodeling induced by inflammation, playing a vital role in inflammatory signal transduction and immune regulation (Huang et al., 2021). In the spleen, sensory nerves release neuropeptides to regulate the maturation, distribution, and function of immune cells (Felten et al., 1987; Jung et al., 2017), and denervation of the mouse spleen impairs plasma cell formation during T-cell-dependent immune responses (Zhang et al., 2020). The sympathetic nerve regulates the production of CXCL13 to organize the aggregation and distribution of immune cells in the spleen's white pulp (Murray et al., 2017). Spleen cells of α7-nAChR knockout (α7-KO) mice produce more TNF-α and IL-6 (Fujii et al., 2017), suggesting that vagal nerve stimulation relieves systemic inflammation and prevents organ damage by inhibiting the production of cytokines in the spleen by acetylcholine (Ach) (Bassi et al., 2020).

2.3 Effect of the Immune System on Nerve

In bidirectional neuroimmune communication, immunogenic signals also act on neurons. As an important signal transduction factor of the immune system, cytokines not only coordinate the immune response but also mediate the signal exchange between the immune system and the nervous system, affecting the normal development of the nervous system and the activity of the neuroendocrine axis.

The early life inflammatory cytokine environment including maternal immune activation *in utero*, perinatal systemic inflammation, and childhood infection, represented by IL-1β, IL-6, TNF-α and IFN-γ, can affect the normal development of the nervous system (Jiang et al., 2018). This is probably due to the interaction of cytokines and major histocompatibility complex I (MHC-I) on neurons, and thus, negatively regulating synaptic plasticity and synaptic pruning (Paolicelli et al., 2011; Schafer et al., 2012). In addition, ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), cardiotrophin-1 (CT-1), and IL-6 constitute a group of structurally related cytokines that is linked to neuronal nutrition and neuronal development through the NF-kb dependent pathway (Middleton et al., 2000).

Various cytokines have been shown to affect HPA axis activity *in vivo*. IL-1 increases adrenocorticotropic hormone (ACTH) and Glucocorticoid (GC) levels by influencing the secretory activity of the hypothalamus (Dunn, 2000). Moreover, various hematopoietic cytokines, such as TNF-α, IL-6, LIF, oncostatin M (OM), CNTF, interleukin-1 (IL-11), CT-1, etc., synergically enhance IL-1 mediated ACTH and corticosterone secretion to a certain extent (Turnbull and Rivier, 1999).

3 NEUROIMMUNE CROSSTALK IN SKIN, LUNG, AND INTESTINE

Skin, lung, and intestine share common anatomical features, including dense innervation and abundant resident immune cells. Nerve endings express cytokine receptors, and receptors for neuropeptides and neurotransmitters are also widely distributed on immune cells. Nerves and immune cells are close to each other in space. These points laid a physiological foundation for local neuro-immune interaction.

3.1 Neuroimmune Crosstalk in the Skin

The skin consists of epidermis, dermis, and subcutaneous tissue. Various inflammatory factors, neurotransmitters, neuropeptides, and receptors mediate neuroimmune communication between the epidermis, dermis, and appendages to regulate local skin homeostasis (Kobayashi et al., 2019) (**Figure 1**). The neuroimmune crosstalk of the skin plays a vital role in skin diseases such as psoriasis, atopic dermatitis, allergic dermatitis (Trier et al., 2019; Wang and Kim, 2020).

The nerves in the skin include the sensory, motor and autonomic nerves. Sensory nerves are divided into Aβ-fibers, Aδ-fibers, and C-fibers according to diameter and incoming velocity. C-fibers, including nociceptors and itch receptors, terminate in the epidermis and communicate most closely with neuroimmune (Lumpkin and Caterina, 2007). C-fibers are divided into peptide-energetic neurons and non-peptide-energetic neurons. The peptide-energetic neurons are marked by TRPV1 channels and can secrete neuropeptides, while the NP2 subpopulation of non-peptide-energetic neurons can also express TRPV1. The autonomic nerves of the skin are distributed only in the dermis, and most of them come from cholinergic sympathetic nerve fibers, which are involved in the blood and lymphatic circulation of the skin and the regulation of skin accessory organs.

TABLE 1 | The role of neuropeptide in cutaneous neuroimmune crosstalk.

Substance	Main biological effect
SP	Activates MCs (INVALID CITATIONb; Subramanian et al. (2016); Asadi et al. (2012); Theoharides et al. (2010); Lim et al. (2017)
CGRP	Increases vascular permeability Krämer et al. (2005)
	Induces MCs degranulation Krämer et al. (2005)
	Promotes T helper 2 cells (Th2) responses suppressing T helper 1 cells (Th1) responses Krämer et al. (2005)
	Shifting langerhans cells (LCs) to type 2 responses Krämer et al. (2005)
NGF	Promotes nerve growth and secretion of SP and CGRP (Joachim et al. (2007); Roggenkamp et al. (2012); Blake et al. (2019a)
	Promotes immune cell activation and migration Skaper, (2017)
	Inhibits hair growth under stress (Peters et al., 2004)
VIP	Induces MCs degranulation Subramanian et al. (2016)
	Inhibits Th1 responses and nhances Th2 and T helper 17 cells (Th17) responses Ding et al. (2012)
	Increases vascular permeability Helyes et al. (2015)
NPY	Activates MCs Paus et al. (2006)
	Increases vascular permeability Paus et al. (2006)
Chemokine Like Family Member 4 (TAFA4)	Promotes macrophage production of IL-10, anti-inflammatory Hoeffel et al. (2021)
Norepinephrine	Controls innate immunity by stimulating T cells to secrete acetylcholine Rosas-Ballina et al. (2011)

TABLE 2 | The reaction of immune cells to the nervous system.

Substances	Main biological effect
Histamine	Induces itch by TRPV1 neurons Peters et al. (2004)
5-HT	Activates HTR7: 5-hydroxytryptamine receptor 7 (HTR-7) to induce itch Morita et al. (2015)
IL-31	Causes itch by activating endothelin-1-responsive neurons to promote brain natriuretic peptide (BNP) synthesis and release Meng et al. (2018)
	Activates TRPV1+ neurons (Cevikbas et al., 2014; Xu et al., 2020a)
	Promotes GRP release to induce itch Sakata et al. (2019)
IL-1	Promotes SP release Baral et al. (2019)
	Causes pain receptor sensitization Baral et al. (2019)
IL-33	Causes itch Franke et al. (2021)
	Increases the release of vascular endothelial growth factor (VEGF) and TNF from MCs with SP Theoharides et al. (2010); Taracanova et al. (2017)
IL-4, IL-13	Induces chronic itch Oetjen et al. (2017)
IL-6	Causes pain receptor sensitization (Baral et al. (2019)
IL-10	Inhibits pain neuron activation Baral et al. (2019)
IL-17	Induces TRPV4 expression to mediate mechanical hyperalgesia Segond von Banchet et al. (2013)
	Causes pain receptor sensitization Baral et al. (2019)
TNF	Causes pain receptor sensitization Baral et al. (2019)

The autonomic nerves of the skin can secrete and release neurotransmitters such as acetylcholine, neuropeptides such as NPY, galanin, CGRP, and VIP, and neuromodulators such as tyrosine hydroxylase (Roosterman et al., 2006).

When sensing external noxious stimuli, skin sensory nerves regulate immunity by releasing neuropeptides, neurotransmitters, and other substances (**Table 1**) (Gouin et al., 2017). Immune cells also modulate sensory nerve activity by releasing inflammatory factors. Skin sensory nerves are divided into peptidergic neurons and non-peptidergic neurons. Peptidergic neurons marked by the transient receptor potential vanilloid1 (TRPV1) channel release a variety of neuropeptides, mainly Substance P (SP) and CGRP, occupying a leading position in skin neuroimmune (Roosterman et al., 2006). Mas-related G-protein-coupled receptor D-expressing (MrgprD) neurons in non-peptidergic neurons also inhibit mast cells (MCs) activation by releasing glutamate, thereby maintaining skin homeostasis (Zhang et al., 2021). The autonomic nerves of the skin are distributed only in the dermis, and they mainly consist of cholinergic sympathetic nerve fibers. The autonomic nerve participate in the blood and lymph circulation of the skin and the regulation of skin accessory organs by releasing neurotransmitters such as acetylcholine and neuropeptides (Roosterman et al., 2006).

Immune cells such as MCs, macrophages, neutrophils, basophils, T cells, and keratinocytes of the skin also act on neurons by releasing various active substances (**Table 2**).

3.1.1 Immune Crosstalk of Pruritus Receptors

Nerve endings that transmit itch and pain communicate most closely with immune cells and are highly associated with inflammation (Wang and Kim, 2020).

Most pruritus receptors are non-peptidergic (NP) neurons, consisting of three subgroups: NP1, NP2, and NP3. Each

subgroup expresses different receptors and channels. Itch-inducing cytokines are often associated with type 2 cell responses. Keratinocytes release cytokines such as transient receptor potential ankyrin 1 (TSLP), which activate downstream type 2 immunity (Tamari et al., 2021). Type 2 immune cells release histamine, cytokines (IL-4, IL-31, IL-13, IL-33, 5-HT, TSLP), 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), and other itch factors to activate TRPV1 and transient receptor potential ankyrin 1 (TRPA1), which in turn activate Voltage-gated Na (+) (NaV) channels. Thus, action potentials are generated, and the signals are sent to the brain to generate itch. Itch causes scratching behavior, which make keratinocytes damaged to release TSLP, activating downstream pathways and forming a vicious circle (Wilson et al., 2013a; Mack and Kim, 2018).

Itch is closely related to MCs. In pathological conditions such as psoriasis and epidermal hyperplasia, MCs are increased and located near the primary afferent nerve endings that transmit itch and pain sensations, and there is bidirectional solid crosstalk between them (Voss et al., 2021). On the one hand, MCs release various mediators to activate pruritus receptors through direct or indirect pathways; on the other hand, MCs also generate nerve growth factor (NGF) to promote the growth of sensory nerve fibers (Leon et al., 1994). Furthermore, innervation is also necessary for MCs function. Passive cutaneous anaphylaxis (PCA)-induced degranulation of MCs is significantly reduced under denervation of sensory neurons (Siebenhaar et al., 2008).

Other immune cells are also involved in the generating of itch. Neutrophils activate C-X-C motif chemokine receptor 3 (CXCR3) by upregulating the expression of C-X-C Motif Chemokine Ligand 10 (CXCL10), leading to sensory nerve activation (Walsh et al., 2019). In wounds, dendritic cells, especially type 2 conventional dendritic cells (cDC2s), are the primary source of IL-31. IL-31 induces the expression of TRPV1 and Interleukin 31 Receptor A (IL-31RA) in neurons through the Jak1-Stat3 pathway, thereby increasing the sensitivity of itch sensory neurons (Xu et al., 2020a). Eosinophils, which rise rapidly after skin exposure to toxicants, mediate inflammation and are also able to alter local innervation and promote itch by increasing SP expression (Lee et al., 2015).

3.1.2 Immune Crosstalk of Painful Nerve Endings

Immune cells release cytokines (such as TNF-α, IL-6, IL-17A, IL-1β, NGF, prostaglandin E2), lipids, and other factors. These factors directly activate neurons and lead to the production of pain. And they also indirectly cause pain sensation by causing sensitization of central or peripheral neurons (Cook et al., 2018). Cytokines involved in pain are often associated with type 1 or 17 cell responses.

Macrophages are closely connected with pain perception. After tissue injury or infection, macrophages release various chemokines and lipid mediators. These factors bind to receptors on nociceptors, leading to increased activity of TRPA1/TRPV1 and voltage-gated sodium channels of nociceptors through activation of the downstream mitogen-activated protein kinases (MAPK) pathway and protein kinase A (PKA), resulting in increased pain sensitivity. The activation of nociceptors lead to the release of SP, which binds to macrophage neurokinin-1 receptor (NK-1R), leading to the infiltration of macrophage cells, forming positive feedback (Chen et al., 2020a). In addition, monocytes and macrophages also play an analgesic effect by releasing anti-inflammatory factors such as IL-10 and specialized pro-resolving mediators (SPMs) (Ji et al., 2016). Macrophages also express neurotrophic factors such as NGF and brain derived neurotrophic factor (BDNF), which maintain neuronal growth and survival (Blake et al., 2019a).

Keratinocytes are also closely related to pain receptors. Under physiological conditions, keratinocytes play analgesic and rewarding roles by being able to release β-endorphin, a proopiomelanocortin (POMC)-derived peptide (Fell et al., 2014). However, when keratinocytes are damaged, it leads to hyperalgesia. Pain neurons also release neuropeptides, leading to further activation of keratinocytes, which promotes the development of inflammation (Talagas et al., 2020).

Pain neurons modulate immune cell activity by releasing mediators such as neuropeptides and even miRNA-rich exosomes (McMahon et al., 2015; Simeoli et al., 2017; Baral et al., 2019; Huang et al., 2021). Activation of nociceptors also initiates or enhances neurogenic inflammation (Michoud et al., 2021). During infection, pathogens activate pain receptors through pattern recognition receptors (PRRs), resulting in the release of neuropeptides and the activation of immune cells. And they also directly activate the immune system to activate pain neurons through inflammatory factors. The nervous system and the immune system activate each other and work together to strengthen the defenses (Chiu, 2018).

Pain also leads to neuron-mediated immunosuppression. Nav1.8 is a sodium channel, mainly expressed in C-fibers of sensory neurons in the dorsal root ganglia. During HSV-1 infection, Nav1.8 + nociceptors inhibit skin neutrophil infiltration, limit tissue damage, control dendritic cells (DCs) responses to antigen presentation, and induce the initiation of $CD8^+$ T cells (Filtjens et al., 2021).

3.1.3 Neuroimmune Crosstalk in Cutaneous Host Defense

Skin nerves play an integral role in regulating host defenses. Pathogen molecules such as endotoxin and flagellin directly activate receptors on sensory neurons to induce pain or itch to modulate immune responses (Chiu et al., 2013a; Chiu et al., 2013b). In response to nociceptive stimuli, inflammatory factors, and pathogen molecules, TRPV1+ neurons specifically activate the type 17 immune response by releasing CGRP to stimulate DCs to produce IL-23. IL-23 prompts γδT cells to produce IL-17 and IL-22 cytokines, which in turn recruit neutrophils and monocytes to enhance host defense (Cohen et al., 2019). This means that nerve fibers in the inflamed skin can activate the immune defenses of the uninfected skin, enabling it to fight against the potential threat of infection (Kashem et al., 2015a; Cohen et al., 2019). This pathway enhances the skin's defense against C. Albicans (Kashem et al., 2015b) and leads to psoriatic dermatitis (Riol-Blanco et al., 2014). During HSV-1 infection, Nav1.8 + nociceptors are activated to inhibit skin neutrophil infiltration, limit tissue damage, and control DC responses to

affect antigen presentation and induce the initiation of $CD8^+$ T cells (Filtjens et al., 2021).

Nevertheless, it is worth noting that neuroimmune does not just boost defenses. Pathogens also promote self-transmission by suppressing pain perception through neuroimmune pathways or activating pain to suppress the immune system (Baral et al., 2019). In necrotizing fasciitis, *Streptococcus* pyogenes secrete streptolysin S (SLS) to activate neurons to generate pain and cause pain neurons to release CGRP. CGRP inhibits the recruitment of neutrophils (Chiu et al., 2013a) and the release of TNF-α from macrophages, which reduces immune bactericidal effect (Chiu, 2018).

3.1.4 Neuroimmune Crosstalk in Skin Inflammation

Skin neurogenic inflammation is caused by the neuropeptides released from sensory nerve endings, manifested as vasodilation, plasma extravasation, edema, cell infiltration, and enhanced nociception (Peters et al., 2005; Sorkin et al., 2018). Neurogenic inflammation promotes skin healing and immune defense but may increase pathological immune responses such as anaphylaxis.

The neuroimmune interaction between peripheral nerve endings and MCs is critical in neurogenic inflammation. After peripheral nerve stimulation, various factors are produced to drive MCs degranulation, which in turn recruits immune cells such as monocytes, eosinophils, and neutrophils (Kulka et al., 2008). SP activates immune cells, causing the release of leukotrienes through the lipoxygenase pathway. Leukotrienes cause plasma extravasation, leading to neurogenic edema (Le Filliatre et al., 2001). SP and CGRP can also act on vascular endothelial cells and smooth muscle cells, increasing vascular permeability and increasing plasma extravasation (Saria, 1984; Brain and Williams, 1989).

Neuropeptides such as SP and CGRP released from nerve endings activate MCs to release mediators such as histamine, leading to the aggregation of inflammatory cells. In turn, MCs and immune cell product activate neurons (Siebenhaar et al., 2008; Steinhoff et al., 2018). Neural and immune promote each other, which forms a vicious circle leading to the generation of neurogenic inflammation (Rosa and Fantozzi, 2013). Interactions between neuropeptides, neuropeptide receptors, and neuropeptide-degrading enzymes are key for the progression of neurogenic inflammation (Cattaruzza et al., 2009).

3.1.5 Neuroimmune Crosstalk in Skin Allergic Diseases

In allergic skin reactions, allergens directly activate TRPV1+ sensory neurons, and results in itch and pain (Perner et al., 2020). TRPV1+ nerve fibers colocalize closely with MRGPRB+ (MAS Related GPR Family Member D) MCs in the skin. During allergy, activated TRPV1+ nerve fibers activate MRGPRB2 on MCs through SP, leading to the release of inflammatory factors (Serhan et al., 2019a; Serhan et al., 2019b). SP also activate MRGPRA1(Mas-related G-protein coupled receptor member A1) on CD301b + DCs, causing their migration to dLN to initiate Th2 cell differentiation and induce skin type 2 inflammation (Perner et al., 2020). The recruited inflammatory cells produce a large number of cytokines, which further activate neurons to generate itch and pain with releasing neuropeptides. Allergens can also directly activate basophils to produce leukotrienes, especially Leukotriene C4 (LTC4) (Chen et al., 2020b). Leukotrienes lead to acute pruritus in atopic dermatitis (AD) through CysLTR2 (Cysteinyl Leukotriene Receptor 2) receptors on a subset of NP3 sensory neurons (Wang et al., 2021). Itch induces scratching behavior, which leads to the release of TSLP from damaged skin keratinocytes. TSLP leads to the aggravation of type 2 inflammation, thus forming a vicious circle (Wilson et al., 2013b; Buhl et al., 2020). Keratinocytes are also capable of secreting NGF and glial cell-line-derived neurotrophic factor (GDNF) to induce neurite outgrowth and increase CGRP + sensory fibers, increasing nerve fiber density in the epidermis of patients with atopic eczema (Roggenkamp et al., 2012).

3.2 Neuroimmune Crosstalk in the Lungs

In the lungs, sensory nerves are distributed in every layer of the airway, especially in the mucosal layer of the airway. Their nerve endings form some special structures, such as nociceptors and neuroepithelial cell body (NEB) (Gelb and Nadel, 2016; Mazzone and Undem, 2016). Sympathetic and parasympathetic nerves enter the lung with blood vessels and bronchus and are distributed around smooth muscle, glands and vessels (**Figure 2**). Dense nerve distribution facilitates the regulation of airway tension, mucus secretion, and cough reflex, and resident immune cells make a timely immune response to external stimuli. Epithelial cells, neurons, and immune cells interact to maintain the relative homeostasis of the lungs.

Sensory innervation of the respiratory tract mainly comes from the vagus branch from nodose/jugular ganglia and the somatosensory afferent nerves comes from neurons in the dorsal root ganglion (Camp et al., 2021). Pulmonary sensory nerve endings express Toll-like receptors (TLRs, a type of pattern-dependent recognition receptor) that recognize pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides (LPS, a component of the cell wall of gram-negative bacteria), and transmit signals to the central nervous system (CNS). The CNS makes a comprehensive response and sends signals through the vagus nerve to increase the expression of acetylcholine (Ach), promote cough reflex and regulate immune defense (Chen et al., 2020b; Yamada and Ichinose, 2018a; Jung et al., 2018). Also, pulmonary sensory nerve fibers constitute special nociceptors, which, under the stimulation of allergens and pathogens, on the one hand, cause cough, pain, and bronchoconstrictive reflex for initial resistance, on the other hand, induce neurogenic inflammation through calcium-mediated neuropeptide release (**Table 3**) (Chen et al., 2020b; Baral et al., 2018). This process is clinically common in allergic diseases, asthma, chronic obstructive pulmonary diseases (COPD), etc., Meanwhile, cytokine receptors such as IL-5R are also expressed in the sensory nerve endings, receiving cytokine signals in the inflammatory response, producing positive feedback and enhancing immunity (Huh and Veiga-Fernandes, 2020). In addition, another sensor, the NEB, is also distributed in the airway. They are compact structures within the mucous membrane of the airway and are innervated by vagal afferent

TABLE 3 | The role of neuropeptide in pulmonary neuroimmune crosstalk.

Substance	Main biological effect
SP	Promotes bronchoconstriction Lo et al. (2018)
	Increases mucus secretion Talbot et al. (2020)
	Increases the release of cytokines from MCs (Thapaliya et al. (2021); Wang et al. (2022)
	Promotes immune migration, increases neutrophil adhesion and phagocytic activity, and stimulates the virulence of *Staphylococcus aureus* Mashaghi et al. (2016)
CGRP	Promotes bronchoconstriction and vasodilation Lo et al. (2018)
	Stimulates ILC2s and downstream immune responses Sui et al. (2018)
VIP	Relaxes airway smooth muscle and pulmonary resistance vessels Szema et al. (2017)
	Increases gland secretion McMahon et al. (2020)
NMB	Inhibits the type II inflammatory response (Chen et al., 2020b; Inclan-Rico et al., 2020)
NMU	Activates ILC2s and amplifies il-25-induced allergic inflammation (Wallrapp et al. (2017); Xu et al. (2020b)
	Promotes Th2 cytokine production and type 2 inflammatory tissue response Cardoso et al. (2017)

fibers. The apical surface of NEB is exposed to the airway, responding to a series of stimuli such as hypoxia, hypercapnia, mechanical stretching, norepinephrine, etc., and producing several bioactive mediators, including bell bombesin, CGRP, and 5-HT (**Table 3**) (De Virgiliis and Di Giovanni, 2020; Mahmoud et al., 2021). Sensory nerves from the dorsal root ganglion can not only release neuromedin U (NMU) to promote the proliferation of ILC2 and type 2 inflammatory response (Cardoso et al., 2017; Klose et al., 2017; Wallrapp et al., 2017), but also secrete neuromedin B (NMB) to inhibit ILC2 response, eosinophilia, and mucus production in the stage of allergic inflammation and worm infection, forming a bidirectional regulation (Chen et al., 2020b; Inclan-Rico et al., 2020).

Cholinergic parasympathetic nerves originate from the vagus nucleus of the medulla oblongata and function through the release of ACh (Huang et al., 2019). Binding to M1/M3 receptors, Ach causes a pro-inflammatory effect, promoting bronchoconstriction, mucus secretion and vasodilation, and participating in the cough reflex, which is closely related to asthma, and atopic diseases (Cazzola et al., 2021). However, the effect of Ach on the N receptor is mostly inflammation-resisting. Immune cells express α7 nicotinic acetylcholine receptor (α7nAChR), which binds to Ach to suppress the release of inflammatory cytokines. This specific pathway is known as the cholinergic anti-inflammatory pathway (Yamada and Ichinose, 2018a). For smokers, nicotine, the main component of cigarettes, is the ligand of nicotinic acetylcholine receptor (nACHR), therefore possesses anti-inflammatory properties (Bosmans et al., 2017).

Sympathetic innervation of the lungs originates from the sympathetic ganglion generated from the upper thoracic segment of the spinal cord, and acts on bronchial vessels and submucosal glands through norepinephrine (NE), mainly regulating bronchodilation and mucus secretion under pressure (De Virgiliis and Di Giovanni, 2020). NE inhibits the release of cytokines through β2 adrenergic receptors (β2AR) of immune cells and plays an anti-inflammatory role.

In addition, there are several special cells in the lungs, which are significant in neuroimmune, leading to many pathological states. Pulmonary neuroendocrine cells (PNECs) are endodermal-derived airway epithelial cells innervated by sensory nerves, which are enriched in airway branch points and collect external environmental information from the air (Xu et al., 2020b). PNECs release neuropeptides and neurotransmitters under hypoxia, hypercapnia and nicotine stimulation to regulate inflammatory response, and mucus secretion of airways (Garg et al., 2019). Neuroairway associated macrophage (NAM) is a newly discovered special mesenchymal tissue-resident macrophage, which is closely related to neuronal projection in the airway. It is found that NAM reduces inflammatory damage in influenza virus infection, but the specific mechanism remains unclarified (Ural et al., 2020). Whether NAM participates in the resistance of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection and its possible clinical application is currently a research hotspot (De Virgiliis and Di Giovanni, 2020). Eosinophils and lymphocytes in the lungs synthesize neurotrophic factors (NGF and BDNF) that bind to receptors (TrkA/B) to increase nerve length and branch points, exposing nerve endings to lumen for storage and release of neurotransmitters and neuropeptides from these TRPV1 + fibers. It increases mucus secretion, collagen deposition, and smooth muscle hyperplasia (Pavón-Romero et al., 2021).

3.2.1 Neuroimmune Crosstalk in the Pulmonary Immune Defense Response

It has been found that sensory nerves play a major role in pulmonary infectious diseases (releasing neuropeptides to initiate immune defense (**Table 3**)). In the case of *Staphylococcus aureus*, SP stimulates bacterial virulence and promotes the migration, adhesion, and phagocytic activity of neutrophils. TRPV1+ afferent nerve in nociceptor modulates protective immunity by releasing cGRP, inhibiting neutrophil recruitment and γδT cell-mediated defense (Baral et al., 2018; Camp et al., 2021). In helminth infection, NEB can release NMU and NMB to bidirectionally regulate the immune response. NMU activates the second group of innate lymphocytes (ILC2)-mediated type 2 inflammatory response and aggravates inflammatory damage (Cardoso et al., 2017; Xu et al., 2020b). NMB inhibits ILC2 proliferation and reduces inflammation (Inclan-Rico et al., 2020).

Many studies on SARS-CoV-2 suggested that its approach to human host cells is mainly mediated by transmembrane protein angiotensin-converting enzyme 2 (ACE2) and transmembrane

protease serine 2 (TMPRSS2), and these receptors are highly expressed in airway epithelium and pulmonary parenchyma (De Virgiliis and Di Giovanni, 2020). Patients are often accompanied by cerebral injury symptoms, such as headache, nausea and vomiting, confusion of consciousness, epilepsy, etc., (Acharya et al., 2020). Studies have revealed that SARS-CoV-2 spreads to the choroid plexus through blood transmission and the breakthrough blood-brain barrier to infect the CNS (Matsuda et al., 2004; Desforges et al., 2019; Fagre et al., 2021). In Corona Virus Disease 2019 (COVID-19), upregulated inflammatory mediators interact with receptors expressed on sensory neurons to activate sensory neurons and release neuropeptides, leading to vasodilation, immune cell recruitment, and neurogenic inflammation (Song et al., 2021). In addition, the newly discovered NAM is proved to relieve inflammation in influenza and has the possibility of fighting the inflammatory storm in severe cases of COVID-19 (Schneider et al., 2014).

3.2.2 Neuroimmune Crosstalk in Pulmonary Inflammation

COPD is a classic type of pulmonary airway inflammation characterized by persistent respiratory symptoms and airflow restriction. We take COPD as an example to illustrate the neuroimmune crosstalk common in pulmonary inflammation.

COPD is developed from chronic bronchitis and emphysema to the stage of continuous airflow limitation in pulmonary function examination, and its incidence and prevalence remain high in China. Increased activity of the cholinergic system in COPD results in airway smooth muscle contraction leading to airflow restriction (Yamada and Ichinose, 2018b). The development of COPD goes through a long inflammatory progression stage, during which the time for Ach release is prolonged, the expression of M1R and M3R in airway structure is increased, and the pro-inflammatory effect of Ach through the M receptor is enhanced (Blake et al., 2019b). While α7nAChR inhibits the release of inflammatory cytokines by inflammatory cells (including macrophages and dendritic cells) and has a pro-inflammatory effect during the inflammatory stage (Hollenhorst and Krasteva-Christ, 2021). During acute inflammation, neuropeptides released by sensory nerve endings modulate the progression of inflammation. SP increases adherent aggregation and phagocytic activity of neutrophils. VIP reduces the contraction response of airway smooth muscle and reduces the degree of airflow restriction (**Table 3**) (Camp et al., 2021).

3.2.3 Neuroimmune Crosstalk in Asthma

Asthma is a recurrent disease characterized by chronic airway inflammation and airway hyperresponsiveness. Allergens enter the airway and activate T cells through antigen presentation. Activated helper Th2 cells secrete cytokines such as interleukin to directly activate MCs and eosinophils, leading to chronic airway inflammation. B cells can also be activated to produce specific IgE, which binds to MCs and eosinophils to cause smooth muscle contraction. Several neuroimmune processes are involved in the development of asthma.

IL2C is important in allergic diseases. It is regulated by a variety of neuropeptides and neurotransmitters and produces massive cytokines to regulate the immune response. Nociceptors, NEB, and PNECs innervated by sensory nerves receive allergen stimulation and immediately induce the secretion of neuropeptides (VIP, CGRP, SP, etc.,) to cause neurogenic inflammation (**Table 3**) (Mou et al., 2021). VIP and CGRP enhance the activity of ILC2, TH2, and other cells (Nassenstein et al., 2018), and promote the production of inflammatory factors. SP promotes the degranulation of MCs, release of pro-inflammatory cytokines, and immune migration, increasing the adhesion of neutrophils to bronchial epithelial cells and neutrophils phagocytic activity. In addition, CGRP directly activates ILC2s at a close spatial distance under allergen stimulation, not only triggering downstream type 2 immune response but also promoting ILC2s to produce IL-5, recruiting eosinophils, and aggravating allergic reactions (Camp et al., 2021). PNECs amplify inflammation by releasing CGRP and the neurotransmitter GABA. Among them, GABA induces the hyperplasia of airway epithelial goblet cells and causes excessive secretion of airway mucus (Sui et al., 2018).

The parasympathetic nerve mainly mediates smooth muscle contraction and cough reflex through Ach, exacerbating dyspnea in asthmatic patients (Gosens and Gross, 2018). Ach binds to muscarinic acetylcholine receptor (mAChR) on dendritic cells to promote the polarization of TH2 cells and binds to MCs to increase their activity and aggravate airway edema and exudation (Bosmans et al., 2017). Ach acts on N receptors of immune cells to reduce the production of TSLP, thereby inhibiting the activity of ILC2s, promoting the immunosuppression of Treg cells, reducing the degranulation and cytokines of MCs, and cutting down the release of histamine and migration of eosinophils from basophils. In addition, a7nAChR inhibits the production and release of inflammatory factors on immune cells to cause a cholinergic anti-inflammatory effect, which relieves asthma airway to a certain extent. For example, Ach combined with a7nAChR on ILC2s reduces the proliferation of ILC2, suppresses the production of IL-5 and IL-13, and aggravates ILC2-mediated airway hyperresponsiveness (AHR) (Yamada and Ichinose, 2018a). Macrophage a7nAChR inhibits the inflammatory mediators, such as TNF-α and IL-1β, to produce and reduce inflammatory damage by binding Ach. Anticholinergic and muscarinic receptor antagonists are applied to relieve bronchoconstriction in asthma (Bosmans et al., 2017).

Under stress, NE produced by sympathetic nerve activation binds to β2AR can not only relax smooth muscle but also inhibit ILC2 mediated type 2 inflammation and mucus production, alleviating asthma symptoms. Studies have found that β2AR agonists effectively relax smooth muscle relaxation, inhibit immune cell recruitment, and cytokine release (Voisin et al., 2017).

3.3 Neuroimmune Crosstalk in the Intestinal Tract

Neurons in the intestinal tract are divided into the extraintestinal nerves and the enteric nervous system (ENS)

TABLE 4 | Regulation of intestinal neuropeptides on immunity.

Neuropeptide	Role in gut
SP	Activates the NF-KB pathway of target cells (macrophages, MCs, etc.) Promote the release of pro-inflammatory factors IL-1 β, IL-6, IL-8, TNF-α Shimizu et al. (2008)
CGRP	Antagonizes the proinflammatory effect of SP Engel et al. (2012b) Promotes prostacyclin (PGI2) synthesis, reduces TNF-α production, and inhibits neutrophil aggregation Okajima and Harada, (2006)
VIP	Reduces the number of cells expressing TLR2 and TLR4, and decreases CD4+T cells in lesions Arranz et al. (2006) Inhibits the production of CXCL10 and promotes the production of C-C motif chemokine 22 (CCL22), thereby shifting the immune response to Th2 and reducing Th1 cell infiltration in inflammatory lesions Delgado et al. (2004a) Inhibits the production of pro-inflammatory cytokines and promotes the production of IL-10 and IL-1 receptor antagonists (IL-1Rα), thereby mediating anti-inflammatory effects Delgado et al. (2004b) Activates the secretion of interleukin-22 (IL-22) in group 3 innate lymphoid cell (ILC3s) to promote the mucosal barrier function Seillet et al. (2020)
PACAP	Anti-inflammatory effect Promotes the balance of Th1/Th2 reaction Azuma et al. (2008)

by their location (Furness, 2012). Extraintestinal innervation includes the sympathetic nerve, vagus nerve, dorsal root nerve and nociceptive nerve. Neurons of ENS are mainly located in the submucosa and muscular layer, and together with glial cells form a ganglion network surrounding the intestinal tube, which is spatially divided into two layers: the intramuscular Austenian plexus and the submucosal plexus (SMP) (Furness, 2008; Schemann et al., 2020). ENS forms reflex circuits with the external sympathetic and parasympathetic nervous systems that innervate all intestinal layers, mediating intestinal motility, epithelial secretion, and vascular regulation (Benarroch, 2007; Schneider et al., 2019). Similarly, the intestinal tract is also rich in resident immune cells, which are mostly clustered in the lamina propria and muscularis of the mucosal area, including innate immune cells (macrophages, MCs, NK cells, innate lymphocytes, γδT cells, etc.) and adaptive immune cells (T cells, B cells). This anatomical intimacy between neurons and immune cells provides the basis for neuro-immune interaction (**Figure 3**).

Gut sensory nerves receive nutrients, mechanical stretching, lumen pressure, and immune stimulation to carry gut sensory signals from the gut to the brain stem and spinal cord (Spencer and Hu, 2020). They recognize harmful and inflammatory stimuli in three ways:

1) PRRs, such as TLRs, to recognize PAMPs produced by invading bacteria or viruses during infection (Barajon et al., 2009; Burgueño et al., 2016);
2) Cytokine receptors, to recognize factors secreted by immune cells (IL-1β、IL-6、IL-10 and TNF-α, etc.,) (Hughes et al., 2013);
3) Danger signal receptors, especially fast electrical channels (TRPV1, TRPV4, TRPA1, and TRPM8), to recognize oxidative stress products (ATP, uric acid, hydroxynonenal, etc.,) during harmful stimuli (heat, acid, and chemicals, etc.,), inflammation and tissue damage (Reinshagen et al., 1994).

Activated sensory nerves, on the one hand, produce reverse axonal reflex. It promotes the release of neuropeptides (SP, CGRP, VIP, etc.,) from nerve terminals on resident immune cells, leading to chemotaxis and activation of neutrophils, macrophages, MCs and lymphocytes (**Table 4**). At the same time, these neuropeptides can directly act on vascular endothelial cells, thereby promoting vascular dilation and increasing capillary permeability, resulting in plasma extravasation and edema. On the other hand, the activated nervous system produces cytokines to participate in immune activities (Kermarrec et al., 2019; Jarret et al., 2020).

The immune system also acts as a counterforce to the intestinal nervous system. Cytokines are the main signaling molecules, which regulate the intestinal nerve density and function. For example, TNF-α and IL-1β promote neurite growth by increasing glial secretion of GDNF (INVALID CITATIONa), while IL-1β and IL-6 modulate synaptic sensitivity and transmission efficiency (Xia et al., 1999).

3.3.1 Neuroimmune Crosstalk in Intestinal Host Defense

As mentioned above, sensory nerves densely distributed in the gut sense PAMPs and products and actively participate in host defense by releasing neuropeptides (SP, CGRP, VIP, etc.,) (Lai et al., 2017; Mao et al., 2013). On the one hand, these neuropeptides interact with immune cells to mediate neurogenic inflammation, intestinal contraction and mucus secretion, and is important for pathogen clearance (**Table 5**). On the other hand, due to the similar structure to antibacterial peptides (AMP), neuropeptides act on the negatively charged cell membrane of bacteria, change the membrane structure through depolarization, produce physical pores, and play an antibacterial role (Ganz, 2003; Hansen et al., 2006).

In addition, the intestinal nervous system can produce cytokines directly involved in host defense. Intestinal intermuscular plexus neurons produce IL-18 and stimulate goblet cells to secrete AMP and mucus, thereby enhancing the protective effect of intestinal mucus barrier and mediating protection against bacterial infection (*Salmonella*) (Jarret et al., 2020).

However, neuro-immune bi-directional communication during host defense is a double-edged sword. Persistent low-grade inflammation of the intestinal tract after infection leads to

TABLE 5 | The role of neuropeptides in intestinal host defense.

Neuropeptide	Role	Pathogen
SP	Activates macrophages, promotes IL-12 production, and further induces IFN-γ to mediate pathogen clearance (Kincy-Cain and Bost. (1996); Kincy-Cain and Bost. (1997) Promotes secretory immunoglobulin (S-IgA) response Walters et al. (2005)	*Salmonella*
CGRP	Reduces the density of intestinal micro pleated cells (M cells) and maintains the level of small intestinal filamentous bacteria (SFB), mediating *salmonella* infection Lai et al. (2020)	*Salmonella*
VIP	Promotes ILC3 recruitment and enhances IL-22 signaling, mediating protection against bacterial infections Yu et al. (2021)	C.rodentium

sensitization and loss of intestinal neurons, which is an important pathogenic factor of intestinal dysfunction after infection. There is an increased risk of irritable bowel syndrome after intestinal infection (Halvorson et al., 2006; Spiller and Garsed, 2009). Many patients have long-term abdominal pain after infection, which may be related to chronical sensitization of TRPV1+ neurons in the intestinal tract (Balemans et al., 2017). In addition, the non-classical inflammasome NlrP6 and caspase-11 (Casp11) -dependent pathway mediates rapid and sustained loss of intestinal neurons in the persistent hypoinflammatory state of postinfection, which is associated with intestinal motility disorders (Matheis et al., 2020).

In contrast, neuroimmunity in the gut also mediates anti-inflammatory responses and tissue damage repair during homeostasis. Tyrosine hydroxylase+ (TH+) neurons signal to myoclastic macrophages (MM) *via* β2AR to polarize MM into a tissue-protective phenotype (Gabanyi et al., 2016). During the intestinal pathogen infection, these macrophages protect intestinal neurons from Casp11-dependent death by expressing arginase and protective polyamines, which are important tissue-protective effects during postinfection and homeostasis (Matheis et al., 2020; Ahrends et al., 2021).

3.3.2 Neuroimmune Crosstalk in Inflammatory Bowel Disease

During intestinal inflammation, activated neurons release neurotransmitters and neuropeptides that play a pro-inflammatory or anti-inflammatory role and regulate inflammatory and immune states.

The effect of adrenergic neurons on inflammation depends on receptor binding ability. At low concentrations, NE binds *a* -adrenergic receptors, while at higher concentrations, NE primarily interacts with β receptors (Straub et al., 2006; Willemze et al., 2019). *a* receptors have pro-inflammatory effects and can promote the production of downstream pro-inflammatory cytokines, which play a significant role in intestinal defense function (Green et al., 2003). Blocking α2-adrenergic receptors down-regulate TNF and IL-1β and improve 2,4,6-trinitrobenzenesulfonic acid (TNB)-induced colitis (Bai et al., 2009). In contrast, several experiments have shown that exogenous NE can act on β receptors and exert dose-dependent anti-inflammatory effects.

They can inhibit the expression of macrophage inflammatory cytokines IL-1β, IL-6, IL-12, TNF-α in the inflammatory state, thereby alleviating colon inflammation and edema symptoms (Willemze et al., 2019; Mallesh et al., 2021). In the inflammatory state of the intestinal mucosa, semaphorin 3C (SEMA3C) and other sympathetic rejection factors are highly expressed, the intestinal sympathetic nerve fibers are lost (Straub et al., 2008), and the synthesis and storage of NE, DA and 5-HT are blocked (Magro et al., 2002). Besides, the density of *a* receptors is increased and of β receptors is decreased (Martinolle et al., 1993; Blandizzi et al., 2003). All this evidence suggests that imbalance of sympathetic energy transmission may be a part of IBD.

The vagus nerve has an important protective role in IBD, including acute recurrence of experimental colitis and chronic colitis (Ghia et al., 2006; Ghia et al., 2007; Liu et al., 2020). A nationwide cohort study in Sweden showed that patients undergoing vagotomy had an increased risk of developing IBD and that vagotomy was positively associated with later IBD (Liu et al., 2020). In the intestinal tract, the vagus nerve inhibits monocyte infiltration and macrophage activation, and reduces the release of related inflammatory factors (IL-1β, TNF-α, INF-γ, IL-18, etc.,), thereby inhibiting early acute inflammatory response and improving mucosal epithelial integrity (Ghia et al., 2006; Ghia et al., 2007). In addition, sacral nerve stimulation has been shown to improve colonic inflammation and enhance colonic barrier function by enhancing parasympathetic nerve activity and modulating ENS and immune system activity in mice (Tu et al., 2020). Conversely, vagal inhibition and decreased acetylcholine levels increase susceptibility to intestinal inflammation (Ghia et al., 2008). The activity of NF- kB in colonic mucosa and the expression of inflammatory factors such as IL-1β and TNF-α in colonic macrophages of mice with vagal nerve resection were significantly increased (Ghia et al., 2006; O'Mahony et al., 2009).

Sensory neuro-immune communication is also important in the development and progression of IBD (Kihara et al., 2003; Kimball et al., 2004; Engel et al., 2011; Engel et al., 2012a). Multiple pieces of evidence have confirmed the pro-inflammatory role of TRPV1+ and TRPA1+ neurons in experimental colitis. TLR4 co-localization with TRPV1 has been observed in a variety of primary afferent neurons, including DRG. In the course of colitis, the expression of TLR4 in DRG is increased, which upregulates TRPV1 expression and TRPV1 current density, mediates inflammatory pain, and regulates inflammatory state (Shen et al., 2017; Wu et al., 2019; Esquerre et al., 2020). Furthermore, the severity of colitis was positively correlated with the gradient of TRPV1 positive neurons in the colon (Engel et al., 2012a). This may be due to the activation of sensory neurons by inflammatory mediators that sensitize TRPV1 and TRPA1 channels, promote Ca2+ channel opening, and release pro-inflammatory

neuropeptide SP, leading to pro-inflammatory events (Engel et al., 2011; Engel et al., 2012a; Bertin et al., 2014). TRPM8+ neurons play a protective role in acute colitis (innate immune cell-mediated) (Ramachandran et al., 2013; de Jong et al., 2015). TRPM8 is located in mucosal epithelial cells and high-threshold colonic sensory neurons (Austroy's plexus) (Ramachandran et al., 2013), which may co-express TRPV1 and have the ability to cross-desensitize TRPV1 (Brain and Williams, 1989). Activation of TRPM8 blocks TRPV1-mediated calcium signaling, thereby inhibiting inflammation (Ramachandran et al., 2013).

3.3.3 Neuroimmune Crosstalk in Food Allergy

Food allergy is mainly manifested as hypersensitivity reaction (type I) caused by the interaction between submucosal sensory neurons and MCs. It is characterized by increased mucosal secretion and intense contraction of muscle tissue, leading to abdominal pain and diarrhea symptoms (Schemann and Camilleri, 2013; Yashiro et al., 2021).

In allergic reactions, specific IgE binding to FCεR1 on the MC surface recognizes the allergen, degranulates after activation, and releases mixed mediators (histamine, prostaglandin, leukotriene, 5-HT, cytokines, complement, etc.,) (Liu et al., 2003; Van Nassauw et al., 2007). Histamine is an important component of mixed media. On the one hand, it acts on H2 receptors in the postsynaptic membrane of intrinsic primary afferent neurons, resulting in a long-lasting and slow excitatory postsynaptic potential effect, which enhances the synchronous prolongation of the intestinal wall and secretory activity of mucosal epithelial cells (Nemeth et al., 1984). On the other hand, histamine and prostaglandin can inhibit sympathetic nerve activity, which forms inhibitory synapses with submucosal nerves, and increase the excitability of submucosal secreted motor neurons. After that, submucosal motor neurons release VIP and Ach to promote the secretion of water, electrolytes and mucus in the mucosa (Blandizzi et al., 2000; Liu et al., 2003).

In addition, intramuscular gut neurons themselves also express FcepsilonR1s (FcεRIs) (van der Kleij et al., 2010), which are directly activated by IgE during allergic reactions, leading to muscle contraction and diarrhea symptoms, and the production of histamine to further activate MCs and aggravate food allergic reactions (Yashiro et al., 2021).

4 CONCLUSION

The immune system and nervous system can sense various stimuli inside and outside the body and communicate with each other, helping to maintain host health and homeostasis. Skin, lung, and intestine are the barrier organs of the nervous system and immune cells densely distributed. There are more and more evidences of the existence of neuroimmune units and the close interaction between neuroimmune cells. Elucidating the mechanisms of neuroimmune interaction may have important implications for maintaining and reconstructing tissue homeostasis and discovering new therapeutic targets for barrier organ infectious diseases, inflammatory diseases and allergic diseases.

It is important to note that most of current research methods in the study of neuroimmune drugs promote, physical injury, chemical stimulation, electrical stimulation, etc., while providing preliminary evidence of neuroimmune signal can influence each other, but neuroimmune interaction of group autonomy mechanism is still unclear, and on the study of the neuroimmune cells of the organ is still insufficient. In the future, combined advanced technologies such as tissue-specific optogenetics, chemical genetics and intercellular labeling systems are expected to further explore the cellular intrinsic mechanisms of neuro-immune interactions.

AUTHOR CONTRIBUTIONS

YZ, SD, and MW were the main authors of the review, and completed the collection and analysis of relevant literature and materials, as well as the writing of the first draft of the paper. ZD and JL were the architects and directors of the project and directed the writing of the paper. All authors have read and agreed to the final text.

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REVIEW
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Morphogenesis, Growth Cycle and Molecular Regulation of Hair Follicles

*Xiangyu Lin, Liang Zhu and Jing He**

Department of Pathology and Pathophysiology, Shanghai Skin Disease Hospital, School of Medicine, Tongji University, Shanghai, China

As one of the main appendages of skin, hair follicles play an important role in the process of skin regeneration. Hair follicle is a tiny organ formed by the interaction between epidermis and dermis, which has complex and fine structure and periodic growth characteristics. The hair growth cycle is divided into three continuous stages, growth (anagen), apoptosis-driven regression (catagen) and relative quiescence (telogen). And The Morphogenesis and cycle of hair follicles are regulated by a variety of signal pathways. When the signal molecules in the pathways are abnormal, it will affect the development and cycle of hair follicles, which will lead to hair follicle-related diseases.This article will review the structure, development, cycle and molecular regulation of hair follicles, in order to provide new ideas for solving diseases and forming functional hair follicle.

Keywords: hair follicles, morphogenesis, hair cycling, WNT, BMP

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Reviewed by:
Weiming Qiu,
General Hospital of Central Theater Command, China
Ping Wu,
University of Southern California, United States
Wang Wu,
College of Bioengineering, Chongqing University, China

****Correspondence:***
Jing He
hejing76@126.com

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1 INTRODUCTION

As the first barrier against external environmental damage, the skin is composed of three layers. The first is the outermost epidermis, consisting of cycling keratinocytes that pile up and transitions into outer layers of dead, cornified keratinocytes that provide the protection against environmental insult and loss of moisture. The second layer underlying the epidermis is the dermis, which contains the skin appendages, including hair follicles, sebaceous glands (SGs), eccrine glands, and apocrine sweat glands. Nails, which are appendages found at the ends of digits, also arise from the dermis. Finally, the deepest layer of the skin underlying the dermis is the subcutaneous tissue, which consists of insulating adipose tissue and connective tissue that connects the skin to the tissue underneath the skin. Blood vessels and nerves in the subcutaneous tissue provide the source of capillaries and nerve endings that penetrate into the dermis and interact with the appendages (Stephens, 2022).

Hair follicles, as one of the important skin appendages, plays an irreplaceable role in skin function and in the process of skin regeneration. The hair follicle is a unique skin structure found in mammals, and is essentially a small organ formed by the interaction between epidermis and dermis. Hair follicles contain many components and have complex, fine structures. They have a high capacity of self-renewal, and display a periodic growth cycle that takes place continually throughout the life span of mammalian organisms. The hair follicle is rich in stem cell populations that contribute not only to hair growth and regeneration but also contribute to skin regeneration after injury. Thus, hair follicles can serve as important models for tissue regeneration and systems biology research (Ma et al., 2017). The growth of hair follicles and activity of these stem cells is highly regulated by various signaling pathways. Hair growth is affected by many factors such as age, climate, environment, and health status, and these factors can influence the development of hair follicle tumors, alopecia areata, and other related diseases.

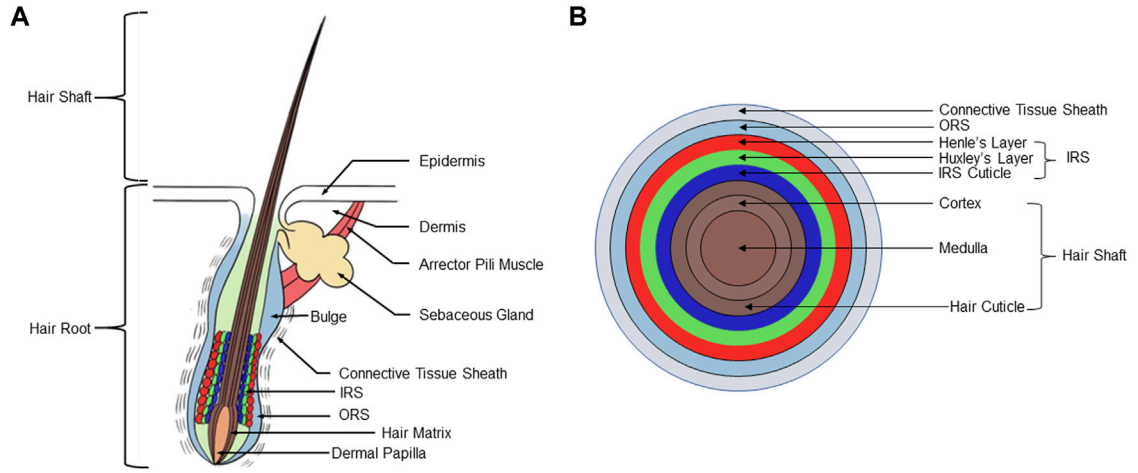

FIGURE 1 | Structure of hair follicle. **(A)** A human scalp hair follicle (anagen VI): the permanent (infundibulum, isthmus) and anagen associated (suprabulbar and bulbar area) components of the hair follicle. **(B)** Schematic diagram of the concentric layers of the hair follicle bulb, including hair shaft, IRS, outer root sheath, and connective tissue sheath. IRS, inner root sheath.

2 STRUCTURE OF HAIR FOLLICLES

As the largest organ of the human body, the skin is mainly composed of epidermis and dermis (Souto et al., 2022) (**Figure 1A**). The epidermis can be further divided into sublayers consisting of, from external to basal, the stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum, and the stratum basale. The dermis is located immediately beneath the stratum basale and consists of papillary layer and reticular layer. Subcutaneous tissue beneath the dermis, also termed the hypodermis, mainly includes loose connective tissue and adipose tissue. Although the shape and size of hair follicles may vary considerably depending on their specific location in the body, they all have the same basic structure (Morita et al., 2021) (**Figure 1B**). The hair follicles run obliquely in the skin. On the obtuse side of the skin surface, there is a bundle of smooth muscle connecting the hair follicle and the papillary layer of the dermis, called the arrector pili muscle (APM). APM is innervated by the sympathetic nervous system. When the APM contracts, it erects the hair and promotes secretion from associated sebaceous gland (SG). The hair follicle is divided into four regions from top to bottom: infundibulum, isthmus, suprabulbar region, and the bulb. The region from the opening of the hair follicle to the opening of the SG is called the infundibulum, the region from the opening of the SG to the attachment of the APM is called the isthmus. Beneath the isthmus, starting at the attachment site of the APM, is the suprabulbar region, which terminates in an enlarged, spherical structure called the bulb (Carrasco et al., 2019).

The composition of cells in the upper half of the hair follicle, the infundibulum, and isthmus, is relatively constant. However, the isthmus does contain a population of stem cells that can help to re-populate the epidermis during wound healing. Studies have identified a population of $Gli1^{+}Lgr6^{+}$ cells in the isthmus which can contribute to formation of wound epithelium, and can provide a source of long-lasting epithelial precursors in healed epidermis (Snippert et al., 2010; Huang S. et al., 2021). The bulge region is located at the junction of the APM and the ORS. Cotsarelis et al. (1990) based on experiments labeling skin hair follicle cells with 3H-TdR, were the first to propose that hair follicle stem cells (HFSCs) are contained in the hair follicle bulge region. HFSCs have typical stem cell characteristics, are highly proliferative, and are critical for the maintenance of hair growth and renewal. Studies by Hsu et al. (2011) show that the periodic growth of hair follicles depends on the maintenance of HFSCs, which can participate in the formation of hair follicles, the maintenance of SGs, and the renewal of the epidermis. For example, $krt15^{+}$ HFSCs of the bulge can provide progeny to help rapidly populate the wound epithelium and repair the epidermis (Ito et al., 2005; Yu et al., 2020). Festa et al. (2011) have found that the formation of fat *in vivo* is synchronized with the activation of HFSCs, and the number of subcutaneous adipose precursor cells reaches a peak during the activation of HFSCs.

Composition of the lower region of the hair follicle is much more variable, including differentiated epithelial cells, hair matrix, and dermal papilla (DP) (O'Sullivan et al., 2021). The bulb is located at the lowest end of the hair follicle and is the active growth center of the hair. Hair follicles are obliquely rooted in the dermis, and the dermis plays a key role in supplying nutrients for hair follicle growth and development (Lee et al., 2020). The upwardly-directed indentation in the bottom of the bulb is the DP, formed by an intrusion of connective tissue, which contains a rich supply of capillaries and nerve endings (Park et al., 2018). The DP supplies nutrition for hair growth and maintenance of the follicle. The DP is a multicellular tissue structure formed by the aggregation of dermal cells, which plays an important role in inducing hair growth (Ge et al., 2020). Dermal stem cells are a kind of skin stem cells with self-renewal ability, dermal cup and lower dermal sheath harbors dermal stem cells which regenerate a new dermal sheath and repopulates cells into the DP during hair

cycling (Rahmani et al., 2014). Injury of hair follicles was shown to recruit more dermal stem cell progeny to become DP cells (Sparks et al., 2019). Matsuzaki et al. showed that mouse dermal papilla cells (DPCs) cultured *in vitro* still retain the ability to induce hair follicle formation *in vivo*, which provides an experimental basis for hair follicle reconstruction (Matsuzaki and Yoshizato, 1998). Oliver's study found that after removing the lower third of the hair follicles of mouse vibrissa, the DP will regenerate and produce vibrissa, but if more of the hair follicles are removed, vibrissa will not regenerate (Oliver, 1966). If more than one-third of the lower part of the hair root is removed from the hair follicles, the vibrissa can be regenerated after the hair papilla is implanted into the base of the hair follicles (Oliver, 1967).

The hair matrix cells are located in the upper part and lateral side of the DP, and melanocytes are scattered between them. DPCs can induce the formation of epithelial components such as inner root sheath (IRS) and medulla of the shaft by interacting with their surrounding hair matrix cells (Limbu and Higgins, 2020). From the upper section of the bulb to the upper part of the hair follicle, it presents a concentric circle-shaped layer, which is divided into three parts from inside to outside: hair shaft (HS), IRS, outer root sheath (ORS). The HS is the part exposed to the skin and is composed of keratinocytes. From inside to outside, the layers of the shaft are the medulla, cortex, and hair cuticle (Watanabe et al., 2021). The IRS consists of the IRS cuticle, Huxley's layer, Henle's layer, and companion layer from inside to outside (Watanabe et al., 2021). The ORS is produced by the Malpighian layer of epidermis (Nilforoushzadeh et al., 2020). The IRS and the ORS are collectively called the epithelial sheath, which belongs to the epidermal component of the hair follicle. The outermost layer of the hair follicle is called the connective tissue sheath, and is also known as the dermal sheath. This layer is derived from the mesenchymal hair follicle dermis component, and is composed of three layers of collagen fibers arrayed in different directions (Martino et al., 2021). The connective tissue sheath is an important material basis for maintaining and regenerating dermal papillae, and is a necessary structure for hair follicle regeneration (Heitman et al., 2020). SGs are located in the dermis of the skin, and their ducts open between the isthmus and infundibulum of the hair follicle (Geueke and Niemann, 2021).

3 MORPHOGENESIS OF HAIR FOLLICLES

Hair follicles are micro organs-formed by the interaction between epidermis and dermis (Ji et al., 2021). Thus, these structures are composed of cells that arise from two different embryonic tissue sources, ectoderm and mesoderm (Ji et al., 2021). Epidermal stem cells and neural crest stem cells are derived from ectoderm (Yang et al., 2019; Soto et al., 2021), while mesenchymal stem cells arise from mesoderm (Schaefer et al., 2020). Epidermal-derived cell lines include SG cells and keratinocytes. Keratinocytes can further differentiate into IRS cells, ORS cells, and hair cells (Morgan et al., 2020). Mesenchymal-derived cell lines include fibroblasts and connective tissue sheath cells (Wang et al., 2018; Hernaez-Estrada et al., 2022). Neural crest cells can form melanocytes, which are the source of hair pigment (Gacem et al., 2020).

The development of hair follicles is essentially a three-step process: induction, organogenesis, and cytodifferentiation, and these three steps include eight stages (Carbonnel et al., 2020; Schmidt-Ullrich and Paus, 2005) (**Figure 2**). During embryonic development, the morphogenesis of hair follicles depends on the regulation of a series of signals between dermis and epidermis. They mediate the interaction between dermal and epidermal cells, induce the orderly proliferation and differentiation of the two cell populations, and guide the cells to finally form HS, root sheath, and DP (Mapar et al., 2021). In the first stage, when signals inducing hair follicle cell generation are emitted from dermal cells, epithelial cells receiving dermal cell signals gradually thicken and form hair follicle basal plates (Zhao et al., 2021). The hair follicle basal plates will send out relevant signals to induce a large number of dermal cells to aggregate under the hair follicle basal plates, and a brand-new hair follicle will be generated at this aggregation point. In the second stage, when a critical density of dermal cells converge under the hair follicle basal plates, the dermal cells will send a signal to induce the hair follicle basal plate to expand downwards, so that the hair follicle structure can enter the dermis and form hair buds. Hair buds deep in the dermis gradually become columnar structures, and a large number of dermal fibroblast cells accumulate at the end (Paus et al., 1999). After entering the third stage, the hair buds are deeply sunk in the dermis layer, and keratinocytes are arranged in a columnar shape around the hair buds. In the fourth stage, the hair buds continue to thicken, and dermal cells converge under the basal plate of hair follicles to form dermal papillae. The formation of a hair bulge occurs in the fifth stage. At the same time, the DP induces the proliferation of hair matrix cells, which further differentiate into HS and IRS (Houschyar et al., 2020). In the sixth stage, hair follicle accessory organs and complete hair follicle structures differentiated from epithelial cells have been formed. In the seventh stage, the tip of the hair enters the hair canal, and the SGs are fixed on the wall of the hair follicle. In the eighth stage, the hair follicles have completed their development, and the HSs pass through the surface of the epidermis (Lee et al., 2018).

During the morphogenesis of mouse hair follicles, induction includes the first stage, which occurs on the 14th day of embryonic development. Organogenesis includes the second to the fifth stage, the second stage occurs at day 15.5, and the third to fifth stage occurs between days 16.5–17.5. Finally, cytodifferentiation takes place from stage 6 to stage 8, which occurs at 18.5 days of embryonic development (Schmidt-Ullrich and Paus, 2005) (**Figure 2**).

4 HAIR FOLLICLE CYCLING

Hair follicles go through regular growth cycles throughout the whole life process, and the changes that occur during the cycle are mainly changes in the morphology and structure of dermal papillae at the bottom of hair follicles, the formation of new HS, and the shedding of old hair. This cycle is divided into three

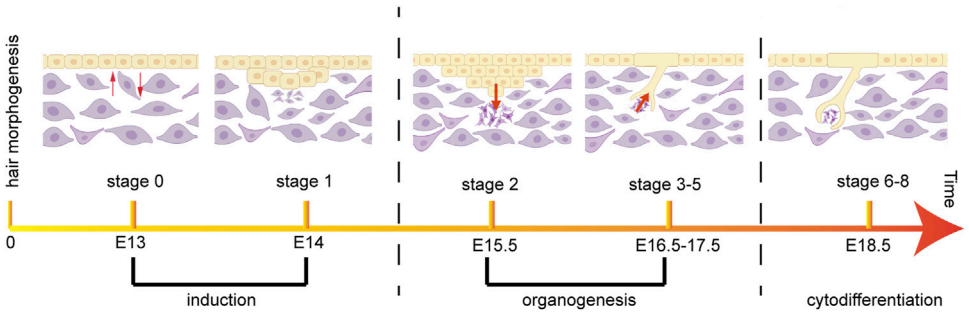

FIGURE 2 | Morphogenesis and timing of hair follicle during mouse embryonic development. The most important developmental stages of mouse pelage hair follicles are divided into induction, organogenesis and cytodifferentiation.

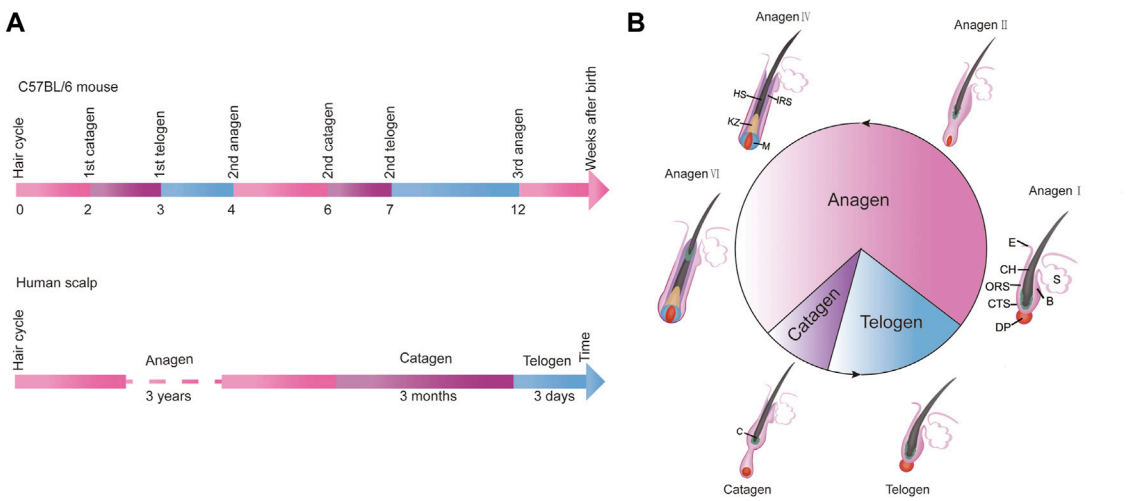

FIGURE 3 | The growth cycle of hair. **(A)** The time-scale for the hair cycle in female C57BL/6 mice during the first 14 weeks after birth (the upper part); The time-scale for the hair cycle in humans (the lower part). **(B)** The morphology of hair follicles at different stages of the hair cycle. Anagen: growth phase, catagen: regression phase, telogen: resting phase.

stages: anagen, catagen, and telogen (Shin et al., 2020). Under typical conditions, the time scale of each cycle is relatively constant and precise. For example, C57BL/6 mice have a precise time scale for the occurrence of anagen, catagen, and telogen of hair follicles. Newborn mice enter catagen in the second week after birth, telogen in the third week, and the anagen in the fourth week (Chen et al., 2019) (**Figure 3A**). On the scalp of an adult, anagen lasts for approximately 3 years, followed by a catagen of about 3 weeks, and then a telogen of about 3 months (Grymowicz et al., 2020; Oh et al., 2016) (**Figure 3B**). Of course, the progress and timing of the three stages of the hair follicle growth cycle can also be affected by many factors: genetic background, environmental factors, gender factors, nutritional factors, and others (Muller-Rover et al., 2001). The hair growth cycle of different strains of mice was different, and the skin color of C57BL/6 mice changed with different hair follicle growth periods. Temperature and light can also affect the growth of hair follicles. Studies have shown that red light at 650 nm can promote the proliferation of human hair follicle cells and significantly delay the transition of hair follicles from anagen to telogen (Yang et al., 2021). The influence of gender factors on hair growth cycle is mainly regulated by hormones, and androgen has a high influence on hair growth and cycle (Grymowicz et al., 2020). At the same time, the regular growth cycle of hair can not be separated from the nutrition supply and regulation of peripheral nerves of hair follicles (Zhang J. et al., 2021).

4.1 Anagen

The anagen stage is the most active period of hair follicle growth, at which time the hair grows rapidly and forms a complete HS (Suen et al., 2020). The proliferation of secondary hair bud cells near the DP marks the beginning of anagen, and the hair follicles penetrate into the subcutaneous tissue. The bulb cells proliferate rapidly, the HS and IRS cells begin to differentiate, and the morphology and volume of DPCs and bulbs become larger (Vishlaghi and Lisse, 2020). Histologically, hair follicles during anagen are slender and straight, and the follicles are oriented at an angle so that the hair can be laid flat on the surface of the body. The keratinocyte progenitor cells in the matrix migrate to the top of the hair follicle and differentiate into HS and IRS cells. When

HS cells enter terminal differentiation, they will bind closely with cysteine-rich hair keratin to form 10 nm bundle-like filaments. This cross-linking gives the HS a characteristic tensile strength and flexibility. The IRS can also be keratinized, which can support and guide the growth of HS in the process of HS differentiation. During anagen, the cell cycle of highly proliferative stromal cells is about 18 h (Harland, 2018). The time of growth cycle determines the length of hair and is related to the continuous proliferation and differentiation of stromal cells at the base of hair follicles (Morgun and Vorotelyak, 2020).

4.2 Catagen

The typical characteristics of hair follicles entering catagen are that HS stops growing, cell proliferation and differentiation ability begins to decline, cells begin to undergo apoptosis, and hair follicles rapidly degenerate. Apoptosis occurs in the epithelial cells of hair matrix and ORS, and the volume of DP becomes smaller (Nicu et al., 2020). The DPCs are resistant to apoptosis due to their expression of the anti-apoptotic protein BCL-2 (Nan et al., 2020). During catagen, the degeneration of hair follicles is highly regulated, and a large number of keratinocytes in hair follicles begin to undergo programmed death (Bak et al., 2020). At this stage, melanin production in hair follicles stops and melanin cells in some hair follicles also begin to undergo apoptosis (Bejaoui et al., 2020). By the end of the catagen stage, the hair follicles have atrophied and the DPCs have begun to condense and move upward to the lower part of the bulge area. If the DPCs of a follicle fails to move to a position underneath the bulge area during catagen, the hair follicle no longer undergoes cyclic growth, and this ultimately results in hair loss, which has been confirmed in humans and mice with hair loss gene mutations (Choi et al., 2021; Zhang Y. et al., 2021). The time for humans to first enter catagen occurs in the uterus, while mice enter catagen about 17 days after birth (Paus and Foitzik, 2004).

4.3 Telogen

After the catagen stage, the hair follicle enters telogen, when the biological activity of the hair follicle is the weakest and the HS falls off. However, expression and activity of the relevant regulatory factors in the hair follicle governing its cyclical growth will be significantly enhanced to prepare for the beginning of the next anagen. During telogen, the DPCs migrate to the lower part of the bulge, so that the DPCs can interact directly with the stem cells in the bulge. DPCs are essential for the activation of stem cells and the initiation of new hair cycles. After activation, HFSCs proliferate and the number of HFSCs reaches a critical value, the next anagen stage of hair follicles begins (Kim et al., 2022). In the hair cycle of mice, the first telogen is very short, lasting only 1–2 days; the second telogen lasts more than 2 weeks, starting about the 42nd day after birth (Hwang et al., 2021) (**Figure 3C**).

5 THE MOLECULAR REGULATION OF HAIR FOLLICLE MORPHOGENESIS AND CYCLING

A variety of different molecular signaling pathways are involved in governing hair follicle development and cycling, such as the canonical WNT and BMP signaling pathways. Additionaly, miRNAs can also contribute to the regulation of morphogenesis and regeneration of hair follicles. Different signal pathways and factors combine to form a complex molecular regulatory network, the activity of which results in the proper morphogenesis and regeneration of hair follicles.

5.1 Signaling Pathways in the Morphogenesis and Cycle of Hair Follicles

Signaling pathways regulate hair follicle morphological development and cycles strictly. And when disturbed, hair follicle-related diseases will develop. WNT, BMP, EDAR, and Sonic hedgehog (Shh) are considered the main pathways involved in regulating follicle morphogenesis, while other pathways are thought to influence morphogenesis as well (Rishikaysh et al., 2014). When the ligand, receptor and signal transduction molecules of these signal pathways are abnormal, the development of animal hair follicles will be affected, leading to changes in hair growth. Genes reported to promote the early morphogenesis of hair follicles include WNT/β-catenin, WNT10b, LEF1, and EDAR as expressed in the epidermis; and WNT/β-catenin, WNT5a, LEF1, and Noggin as expressed in the dermis. Genes believed to inhibit the early morphogenesis of hair follicles include DKK4 and BMP2 as expressed in the epidermis; and DKK1, BMP4, and BMP7 as expressed in the dermis (Albrecht et al., 2021; Huang J. et al., 2021).

5.1.1 WNT Signaling Pathway

The WNT pathway is one of the most important signaling pathways regulating hair follicle morphogenesis and cycle. It is also the earliest known signaling pathway to initiate the induction of hair follicle development by regulating the formation of the basal plate (Zhao et al., 2022). Canonical WNT signaling pathway mainly includes WNT protein, cell surface Frizzled receptor family, Dishevelled (DSH) receptor family protein, β-catenin, and axin/GSK-3/APC complex.

WNT is a secretory glycoprotein with more than 20 related family members. Its secretion is mediated by wntless (Wls), which is a transmembrane transporter. Although the role of Wls in the induction of hair follicle development is still unclear, it has been found to exist in embryonic epithelium and hair follicles after hair formation (Huang et al., 2012). Studies have found that the WNT family can be divided into primary WNT and secondary WNT. The primary WNT includes WNT3, WNT4, and WNT6. WNT3a is only expressed in bulge and decreased in expression during catagen, and not is expressed at all during telogen (Li et al., 2021; Xing et al., 2018). Guo et al. (2012) found that melanocytes in hair follicles express both WNT3a and β-catenin proteins at the same time. It is speculated that they play an important role in the proliferation, differentiation, and pigment deposition of hair follicle melanocytes. The secondary WNT includes WNT2, WNT7b, WNT10a, and WNT10b. Primary WNT is necessary for the induction of hair follicles, while secondary WNT mainly plays a role in the development of hair follicles (Nicu et al., 2021; Zhang W. et al., 2021). WNT10b mainly plays a role in the mammalian hair follicle cycle and is highly expressed during

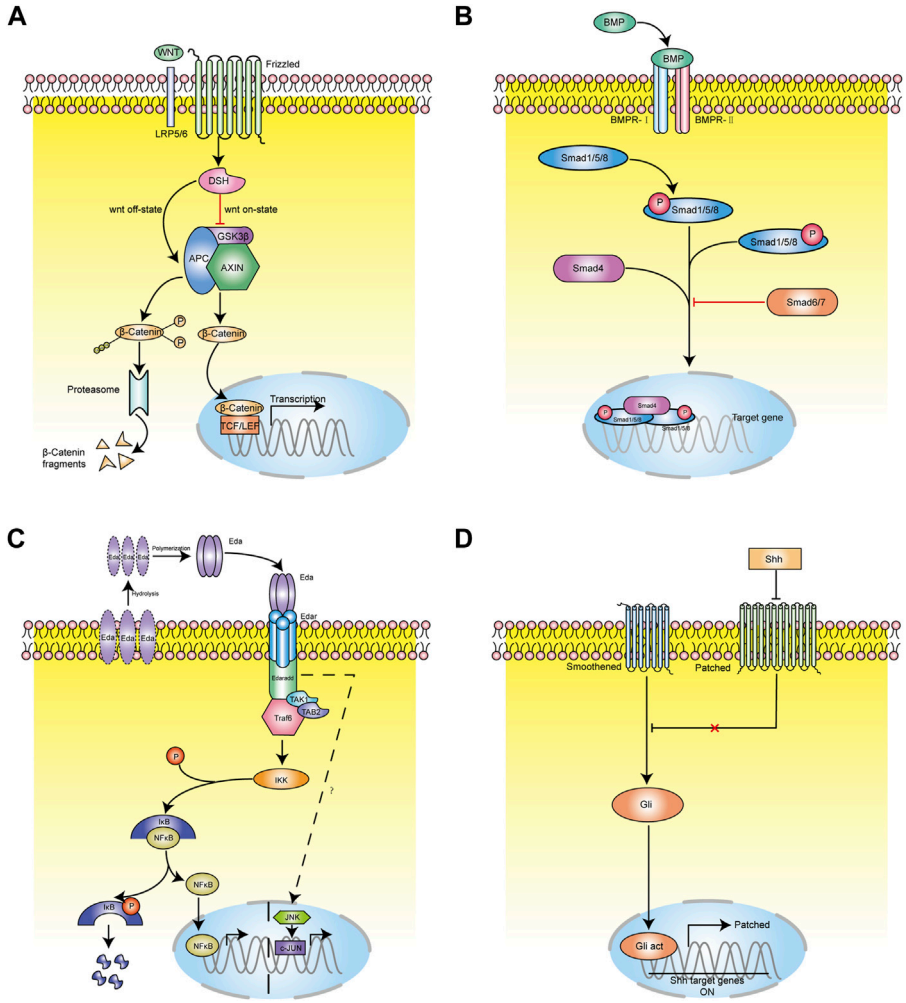

FIGURE 4 | The molecular regulation of hair follicle morphogenesis and cycling. **(A)** WNT signaling path mode diagram. Schematic drawing illustrating canonical WNT signaling pathway mainly includes WNT protein, cell surface Frizzled receptor family, Dishevelled (DSH) receptor family protein, β-catenin, and axin/GSK-3/APC complex. β-catenin plays a key role in the canonical WNT signaling pathway. **(B)** BMP signaling pathway mode diagram. The classical BMP signaling pathway is that ligand BMP binds to phosphorylated serine and threonine receptors and is transported into cytoplasm. In cytoplasm, BMP combines with Smad1/5/8 and phosphorylates the C terminal of Smad1/5/8. The phosphorylated Smad1/5/8 combines with Smad4 and transports to the nucleus. **(C)** EDAR signaling path mode diagram. EDAR signaling pathway is mainly composed of EDA ligand, transmembrane receptor EDAR (including EDAA1 and EDAA2 subtypes), and intracellular binding protein EDARARR. **(D)** SHH signaling path mode diagram. Ptch binds to Smo to inhibit Smo activity. In the presence of Hh, the binding of Ptch1 to Hh protein eliminates the inhibitory effect on Smo, and Smo transmits signals to downstream Gli transcription factors through a complex transduction process.

anagen of the hair follicle, thus promoting epithelial differentiation and early development of hair follicles (Bai et al., 2021). During telogen, overexpression of WNT10b can induce hair follicles to change from refractory phase to inductive phase, thus entering anagen (Hawkshaw et al., 2019; Liu et al., 2021). WNT3a is mainly expressed in root sheath progenitor cells, bulbs, hair bulges, epidermis, melanocytes, and melanin stem cells in hair follicles. Chen et al. (2015) found that the expression of tumor necrosis factor α (TNF- α) increased after hair removal, while TNF- α related peptides could significantly stimulate keratinocytes to express WNT3, WNT10a and WNT10b. TNF- α promotes hair regeneration by activating the NF- κB signaling pathway and finally activating the WNT signaling pathway. β-catenin is an important mediator of the canonical WNT signaling pathway (**Figure 4A**). It is mainly expressed in ORS, IRS, hair matrix, HS, and other structures of hair follicles. DPCs, hair matrix cells, and ORS cells express β-catenin at high levels (Zhao et al., 2022). β-catenin is the core signal transduction factor in WNT signaling pathway. During hair follicle regeneration, β-catenin expressed in HFSCs in the hair germ and bulge activates the LEF/TCF complex, which further initiates the transcription of downstream target genes c-myc and cyclinD1 involved in cell cycle control and apoptosis, thus promoting the activation, proliferation, and directional differentiation of HFSCs (Lin et al., 2015). WNT signaling protein is mainly expressed during hair follicle anagen, decreased during catagen, and inactivated during telogen.Zhu et al. shows that LncRNA H19 plays a role by directly down-

regulating the expression of WNT inhibitors DKK1, Kremen2 and SFRP2, and inducing miR-29a to activate WNT signal, thus forming a new regulatory feedback loop between H19 and miR-29a to maintain hair follicle induction potential. LncRNAH19 maintains the hair follicle induction ability of dermal papilla cells by activating WNT pathway, which may be a target for the treatment of androgenic alopecia (Zhu et al., 2020). Blimp1 is both a target and a mediator of key dermal papilla inductive signaling pathways including transforming growth factor-β and WNT/β-catenin (Telerman et al., 2017).

5.1.2 BMP Signaling Pathway

The BMP pathways is another key signalling related to hair follicle morphogenesis and cycle. Mou et al. (Telerman et al., 2017) found that in skin tissue culture, treatment with BMP led to the formation of hair follicle base plate, while treatment with the BMP antagonist Noggin could increase the density of hair follicle base plate, which further proved the inhibitory effect of BMP on hair growth. Studies have found that the use of Noggin (a BMP antagonist) in mouse skin can significantly shorten the refractory period and promote hair regeneration, so BMP may play a role as an inhibitory signal of hair growth (Plikus et al., 2008). BMP is a family of secreted glycoproteins, belonging to the transforming growth factor (TGF) superfamily, and are multifunctional growth factors. It activates signal transduction by binding with BMP receptors (**Figure 4B**) (Monsivais et al., 2021). A serine-threonine kinase receptor, which form an active kinase heterotetrameric receptor complex after combination with BMP. This activated kinase complex phosphorylates the C-terminus of Smad1/5/8 (Infarinato et al., 2020), which subsequently binds to Smad4 and is transported to the nucleus. With the cooperation of other transcription factors, the transcription of target genes of Smad1/5/8 is initiated to regulate the proliferation and differentiation of HFSCs (Olsen et al., 2020). Smad6 and Smad7 are inhibitory Smad proteins, which block BMP signal transduction by competing with Smad4 to bind Smad1/5/8, and can also cause ubiquitin degradation of Smad1/5/8 (Chen et al., 2021). The BMP signaling pathway acts on the refractory period and regeneration period in telogen, during which BMP has different signal activity intensity. Moreover, Plikus et al. (2017) found that myofibroblasts can reprogramming to adipocytes during wound healing by the participation of new hair follicles. And this process triggers the BMP signaling pathway, thus further activating the expression of adipocyte transcription factors during development.The BMP pathway has been reported to play a positive role in determining the glandular fate during the induction stage of eccrine sweat gland. additionaly, the eccrine sweat glands were converted to hair follicle-like structures in Bmpr1a conditional knockout mice (Lu et al., 2016).

5.1.3 EDAR Signaling Pathway

EDAR is also one key signalling pathway related to the development and cycle hair follicle. It is mainly composed of EDA ligand, transmembrane receptor EDAR (including EDAA1 and EDAA2 subtypes), and intracellular binding protein EDARARR (**Figure 4C**) (Wang et al., 2020). EDAR and EDA belong to the TNF superfamily. EDAR contains extracellular ligand binding N-terminal, single transmembrane domain, and intracellular death domain (Schuepbach-Mallepell et al., 2021). Its death domain can specifically bind to the extracellular domain of intracellular binding protein EDARARR, initiate signal transduction, and regulate the transcription of downstream target genes (Wohlfart and Schneider, 2019). In the hair follicle cycle of wild-type mice, the expression of EDA, EDAR, and EDARADD reaches the peak at the end of anagen, decreases from the end of catagen to the middle stage, and was the lowest in telogen (Fessing et al., 2006). At the end of anagen, Eda-A1 was expressed in hair matrix, IRS, and ORS. In the middle stage of catagen, the expression of Eda-A1 decreased sharply and was only expressed in the hair buds of secondary hair follicles. EDAR was expressed in the hair matrix and the IRS at the middle stage of the anagen, but at the end of anagen, the expression of EDAR in the IRS and the ORS increased rapidly. At the beginning of catagen, EDAR was expressed only in the IRS and ORS, but at the end of catagen, EDAR only appeared in the hair buds of secondary hair follicles (Gomez et al., 2013).

5.1.4 Shh Signaling Pathway

Shh is a small secreted glycoprotein that is frequently involved in inducing cell proliferation, cell fate determination, and patterning in a number of developing tissues. Transmembrane proteins Patched (Ptch) and Smoothened (Smo) are two transmembrane proteins components of Shh signaling (**Figure 4D**) (Morinaga et al., 2021). In the absence of Shh protein, Ptch binds to Smo to inhibit Smo activity. In the presence of Shh, the binding of Ptch1 to Shh protein eliminates the inhibitory effect on Smo, and Smo transmits signals to downstream Gli (glioma-associated oncogene homologue) transcription factors through a complex transduction process, activating Gli and allowing transport of Gli into the nucleus (Sun et al., 2021). After activated Gli enters the nucleus, it initiates the transcriptional expression of downstream Cyclin D1 and N-myc genes (Sigafoos et al., 2021).

Shh participates in the morphogenesis of hair follicles during embryonic development. The DPCs of Shh mutant mice were reduced in number, and these mice lacked normal hair follicles so they could not maintain normal hair morphology (Lim et al., 2018). Dermal expression of Shh is critical for maturation of the DP and maintaining expression of DP-specific genes during morphogenesis (Woo et al., 2012). Shh continues to participate in the regulation of follicle cycling in adults by promoting the transition of hair follicles from telogen to anagen (Gao et al., 2019; Zhang X. et al., 2021). Studies using an anti-Shh monoclonal antibody that disrupts Shh activity show destruction of hair follicles during anagen and subsequent hair loss. This supports the idea that the Shh signaling pathway plays a key role in the hair growth of mice (Choi, 2018; Zhang X. et al., 2021). Shh secreted from perifollicular nerve endings is also important for the maintenance of $Gli1^{+}Lgr6^{+}$ stem cells present in the follicle that can contribute to epidermal healing after wound formation (Brownell et al., 2011). The varied effects of Shh in the touch dome to the ligand source, with locally produced Shh acting as a morphogen essential for lineage

specification during development and neural Shh regulating postnatal touch dome stem cell maintenance (Xiao et al., 2016).

5.2 miRNAs Regulating Hair Follicle Morphogenesis

MiRNA is expressed in the skin and hair follicles of mammals and plays an important role in regulating the development and regeneration of hair follicles (Andl and Botchkareva, 2015; Hochfeld et al., 2017; Horsburgh et al., 2017). Mice lacking dicer enzyme could not form normal miRNA, resulting in the formation of hair bud-like cysts (Yi et al., 2006) in the epidermis. The miR-24 can affect the differentiation of mouse HFSCs by inhibiting Tcf-3 during the hair follicle anagen. The hair of mice with ectopic expression of miR-24 becomes thinner and these mice develop serious defects in hair follicle development (Amelio et al., 2013). The expression of miR-22 during catagen and telogen is higher than that in anagen. miR-22 regulates hair follicle cyclical changes and affects the formation of IRS and HS by inhibiting the expression of transcription factors DLX3, FOXN1, and HOXC13 (Cai et al., 2020; Yuan et al., 2021). MiR-125b can inhibit the expression of target genes Blimp1 and Vdr, resulting in the inhibition of the differentiation of HFSCs and promoting stem cell renewal (Zhang et al., 2011). BMP4 has an inhibitory effect on hair follicle development, and the expression of miR-21 can weaken this effect (Xiong et al., 2022).

6 PERSPECTIVES

The morphogenesis and grow cycling of hair follicles involve many cells and molecules. These signaling molecules are not independent, and various studies have shown that they are formed into complex regulatory network. Therefore, the search for key signaling molecules that control these processes has become a major focus of hair research. Currently, treatment with certain medications, over-expression or inhibition of endogenous genes and increasing the secretion of extracellular vesicles (EVs) are the main strategies to promote hair growth, which involve a variety of signaling pathways and molecules. All these findings provide new ideas for the clinical treatment of hair follicle related diseases such as alopecia areata. Despite significant advances in this field, what are the key activators that promote the transformation of hair follicle telogen and anagen stages through signaling pathways and what is the molecules mechanism of hair growth promotion through HFSCs and dermal stem cells are still unclear. Moreover, although the transplantation of potential cell mixtures, hair follicle organoid construction *in vitro*, reprogramming induction and the establishment of a drug delivery system do contribute to the formation of hair follicles, the construction of functional hair follicles with normal cycling activity is still a great challenge for the hair research field. Therefore, further studies are still needed to on the activation and maintenance of hair cycling in the next phase.

AUTHOR CONTRIBUTIONS

XL was responsible for manuscript writing and the figure preparation. LZ was responsible for the figure modification. JH was responsible for the design and final approval of the manuscript. All authors read and approved the final manuscript.

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REVIEW
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Toward Elucidating Epigenetic and Metabolic Regulation of Stem Cell Lineage Plasticity in Skin Aging

*Ying Lyu and Yejing Ge**

Department of Cancer Biology, The University of Texas MD Anderson Cancer Center, Houston, TX, United States

Skin is the largest organ in human body, harboring a plethora of cell types and serving as the organismal barrier. Skin aging such as wrinkling and hair graying is graphically pronounced, and the molecular mechanisms behind these phenotypic manifestations are beginning to unfold. As in many other organs and tissues, epigenetic and metabolic deregulations have emerged as key aging drivers. Particularly in the context of the skin epithelium, the epigenome and metabolome coordinately shape lineage plasticity and orchestrate stem cell function during aging. Our review discusses recent studies that proposed molecular mechanisms that drive the degeneration of hair follicles, a major appendage of the skin. By focusing on skin while comparing it to model organisms and adult stem cells of other tissues, we summarize literature on genotoxic stress, nutritional sensing, metabolic rewiring, mitochondrial activity, and epigenetic regulations of stem cell plasticity. Finally, we speculate about the rejuvenation potential of rate-limiting upstream signals during aging and the dominant role of the tissue microenvironment in dictating aged epithelial stem cell function.

Keywords: skin aging, stem cell lineage plasticity, inflammaging, epigenetics, metabolism

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Brian C. Capell, University of Pennsylvania, United States
Ramiro Iglesias-Bartolome, Center for Cancer Research (NIH), United States

****Correspondence:***
Yejing Ge
YGe1@mdanderson.org

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INTRODUCTION

As the body's largest organ, skin harbors a cadre of cell types. First and foremost, epithelial cells serve as the fundamental units of our barrier; they reside in the interfollicular epidermis and pilosebaceous unit, the latter including the sebaceous gland and the hair follicle (Hardy, 1992; Fuchs, 1995; Paus and Cotsarelis, 1999; Watt, 2001; Lopez-Pajares et al., 2013). Fibroblasts in the dermis secrete bulk extracellular matrix in the tissue for not only structural support but also mediate signaling (Gurtner et al., 2008; Sennett and Rendl, 2012; Heitman et al., 2019; Plikus et al., 2021; McAndrews et al., 2022). Subcutaneous adipocytes and dermal pre-adipocytes exhibit remarkable lineage plasticity to mediate metabolic and signaling regulations (Horsley and Watt, 2017). Immune cells including those of the innate and adaptive systems provide tissue surveillance (Allison and Havran, 1991; Merad et al., 2008; Heath and Carbone, 2013), support repair and regeneration (DeStefano and Christiano, 2014; Ali et al., 2017; Eming et al., 2017), and communicate with the microbiome (Nakatsuji and Gallo, 2012; Byrd et al., 2018; Kobayashi et al., 2019). Oxygen and nutrients are exchanged via the endothelial and lymphatic vasculature, sensations are conducted through the intertwining neuronal network, ultraviolet radiation protection is afforded by melanocytes, and hair follicles are erected by arrector pili muscles; all of these cell types exert their respective functions while maintaining crosstalk with juxtaposed epithelial stem cells (Nishimura et al., 2005; Brownell et al., 2011; Fujiwara et al., 2011; Chang et al., 2013; Gur-Cohen et al., 2019; Shwartz et al., 2020; Zhang et al., 2020).

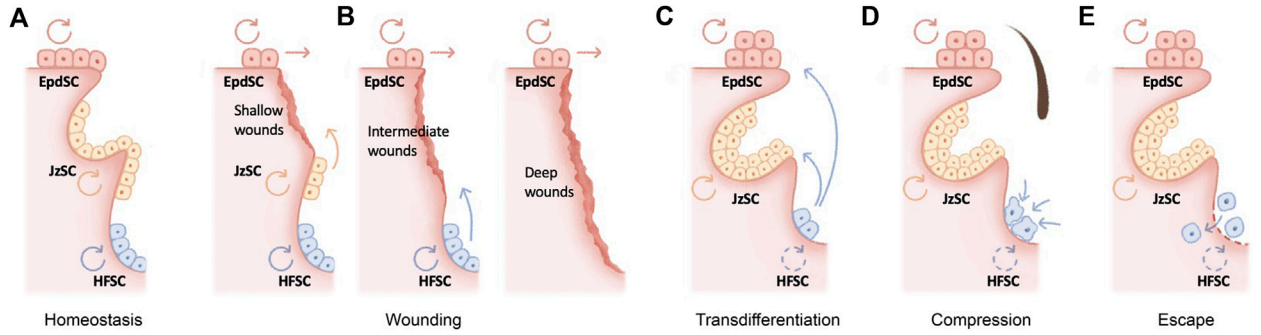

FIGURE 1 | Molecular mechanisms underlying hair follicle miniaturization in aging **(A)** The hair follicle as a major skin appendage is maintained by hair follicle stem cells (HFSCs) residing in the bulge, an anatomic location beneath the sebaceous gland and isthmus of the pilosebaceous unit. HFSCs fuel the cyclic regeneration of hair follicles during homeostatic hair growth, whereas epidermal stem cells (EpdSC) and junctional zone stem cells (JzSCs) maintain the epidermis and junctional zone, respectively **(B)** HFSCs drive both follicular and epidermal regeneration during wound repair. Depending on the wounding depth, different populations of stem cells are induced to contribute to wound repair **(C–E)** Several cellular and molecular mechanisms have been recently proposed to explain the fate of aging HFSCs in murine models, including epidermal transdifferentiation **(C)**, mechanical compression *in situ* **(D)**, and escape into dermis **(E)**.

The skin epithelium is maintained by its resident stem cells, harboring the capacity for long-term self-renewal and multi-lineage differentiation. The hair follicle as a major skin appendage is maintained by hair follicle stem cells (HFSCs) residing in the bulge, an anatomic location beneath the sebaceous gland and isthmus of the pilosebaceous unit (Cotsarelis et al., 1990) (**Figure 1A**). HFSCs fuel the cyclic regeneration of hair follicles during homeostatic hair growth (Morris et al., 2004; Tumbar et al., 2004; Greco et al., 2009; Hsu et al., 2011). They contribute to both follicular and epidermal regeneration during transplantation and wound repair (Taylor et al., 2000; Blanpain et al., 2004; Claudinot et al., 2005; Ito et al., 2005; Levy et al., 2005), and they initiate skin squamous cell carcinomas upon oncogenic transformation (Lapouge et al., 2011). Likewise, exhibiting remarkable plasticity, several HFSC populations adjacent to the bulge contribute to wound repair and tumorigenesis (Nijhof et al., 2006; Jaks et al., 2008; Jensen et al., 2009; Snippert et al., 2010; Page et al., 2013; Donati et al., 2017; Ge et al., 2017; Ge and Fuchs, 2018) (**Figure 1B**).

Aging in the skin is graphically pronounced and includes a wrinkly surface due to dermal extracellular matrix atrophy and gray hair owing to a loss of melanocytes. Accompanying these overtly notable signs, the old skin manifests many hallmarks of aging (Lopez-Otin et al., 2013) such as stem cell exhaustion, genotoxic stress, metabolic deregulation, and epigenetic erosion. In our current essay, we focus on skin aging, especially in the context of stem cell function and the origin and consequence of lineage deregulation. Whenever applicable, we compare mammalian skin to model organisms and adult stem cell of other tissues and organs. Overall, we entertain the idea that skin is an ideal system to understand and tackle organismal aging.

MOLECULAR MECHANISMS UNDERLYING HAIR FOLLICLE MINIATURIZATION

Stem cells decline over time in number and/or in activity across organs and tissues. Curiously, this functional decline is often accompanied by skewed lineage output. For example, aging in the hematopoietic system is signified by pronounced myeloid lineage expansion at the expense of lymphoid cells (Sudo et al., 2000) (Cho et al., 2008; Florian et al., 2013). Aged skeletal stem cells have decreased bone- and cartilage-forming potential but produce more stromal lineages, leading to not only bone fragility but also hematopoietic skewing (Ambrosi et al., 2021). Secretory cell types dominate the aged intestinal stem cell output (Nalapareddy et al., 2017). The question of the fate of HFSCs during aging is a particularly intriguing one. Consistent with the lineage-skewing phenomenon at the organ level, hair follicles undergo miniaturization as HFSCs are diminished during aging, resulting in macroscopically sparse hair. In contrast, the epidermis and sebaceous gland undergo hyperplasia. Several cellular and molecular mechanisms have been recently proposed to explain the fate of aging HFSCs in murine models (**Figures 1C–E**).

In the skin, stem cell lineage infidelity where HFSCs expand their fate to regenerate the epidermis is observed during wound repair (Ge et al., 2017) (**Figure 1B**) and could be recapitulated upon the ablation of several key HFSC fate transcription factors in adult skin, including SOX9 (Kadaja et al., 2014) and NFIB/NFIX (Adam et al., 2020), suggesting a default HFSC response of epidermal differentiation under injury. In a more extreme scenario, epidermal cysts form in Notch signaling–deficient skin, suggesting blocked hair follicle differentiation (Yamamoto et al., 2003; Pan et al., 2004; Vauclair et al., 2005; Estrach et al., 2006; Demehri and Kopan, 2009). During aging, stress signals including DNA damage and a high-fat diet have been shown to drive epidermal conversion of HFSCs by compromising basement membrane integrity (Matsumura et al., 2016), altering stem cell symmetric divisions (Matsumura and Nishimura, 2021), and inducing oxidative stress (Morinaga et al., 2021), all of which contribute to the epidermal conversion of HFSCs and hair follicle miniaturization (**Figure 1C**).

On the other hand, deficiency of several HFSC quiescence regulators, such as LHX2 (Folgueras et al., 2013) or beta-

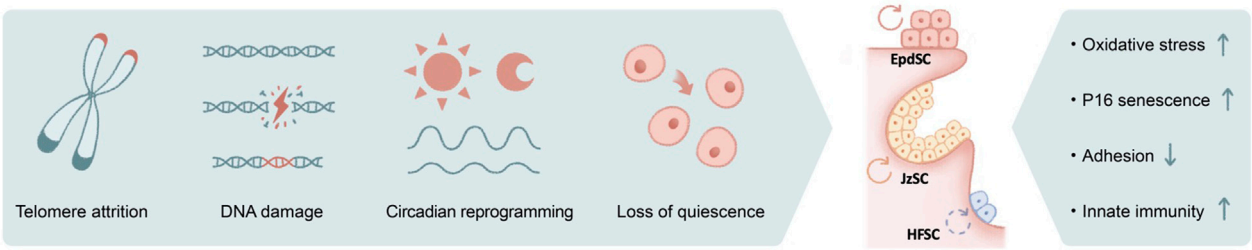

FIGURE 2 | The impact of genotoxic stress on skin aging. Numerous causes of genotoxic stresses including telomere attrition, DNA damage, circadian reprograming, and loss of quiescence could lead to stem cell functional decline in aging. The outcomes include increased oxidative stress, induction of P16 and cellular senescence, loss of cell adhesion, and activated innate immune pathways, among others.

catenin (Lien et al., 2014), appears to push HFSCs into the sebaceous gland lineage. In this regard, loss of BMP (Guha et al., 2004; Plikus et al., 2004; Qiu et al., 2011) or aberrant activation of LEF1 (Merrill et al., 2001; Niemann et al., 2002; Petersson et al., 2011), NOTCH1 (Estrach et al., 2006), and GLI2 (Allen et al., 2003; Gu and Coulombe, 2008) also manifests as ectopic sebaceous glands, although in some of these scenarios, it remains unclear whether sebaceous glands were mis-specified from HFSCs at the expense of hair follicles. Since sebaceous gland hyperplasia, like that of the epidermis, is a prominent signature of skin aging, it would be tempting to speculate that aged HFSCs also skew toward sebaceous gland differentiation, resulting in hair follicle miniaturization (**Figures 1B,C**).

An alternative model for the loss of aged HFSCs was recently proposed (**Figure 1D**): mechanical compression induces Piezo channel activation, calcium influx, and subsequently HFSC apoptosis (Xie et al., 2022). While the loss of inhibitory K6+ niche cells is known to cause HFSC activation (Hsu et al., 2011), in this context, mechanical cues play a dominant function, since the reinsertion of the hair shaft alone without the K6+ cells returned bulge HFSCs to quiescence (Xie et al., 2022). Corroborating this model, repetitive hair depilation induces hair follicle aging (Keyes et al., 2013; Lay et al., 2016), likely evoking mechanical compressions and the Piezo1-calcium-TNFα axis (Xie et al., 2022). Therefore, mechanical compressions induced HFSC apoptosis is likely a major contribution of HFSC exhaustion and hair follicle miniaturization during aging.

Remarkably, *via* intravital imaging, aged HFSCs have been shown to escape into the dermis (Zhang et al., 2021) (**Figure 1E**). This escape could be recapitulated by depletion of HFSC quiescence regulators FOXC1 (Lay et al., 2016; Wang et al., 2016) and NFATC1 (Keyes et al., 2013) in the hair follicles and is caused by the aging-associated loss of extracellular matrix proteins (Ge et al., 2020; Zhang et al., 2021). On the other hand, deregulation of the aging extracellular matrix due to niche stiffening underlies nuclear cytoskeletal remodeling and subsequently epigenome remodeling in the aging HFSCs (Koester et al., 2021), providing an important molecular link as to how the extracellular matrix niche determines aged HFSC fate (Ge et al., 2020; Koester et al., 2021).

IMPACT OF GENOTOXIC STRESS ON SKIN AGING

Telomeres shorten each time the genome duplicates, serving as a major driver for replicative aging. In mammals, telomerase expression is present and often abundant in adult stem cells (Sharpless and DePinho, 2004), including those of the skin (**Figure 2**). Among the defects in many highly proliferative organs, a signature phenotype of telomerase-deficient mice is alopecia and hair graying (Lee et al., 1998; Rudolph et al., 1999; Flores et al., 2005; Sarin et al., 2005). Likewise, in humans, mutations in the gene encoding for dyskerin, critical for telomere stability, lead to dyskeratosis congenita, a progressive bone-marrow failure syndrome characterized by abnormal skin pigmentation (Mitchell et al., 1999; Vulliamy et al., 2001).

Similar to telomere attrition, DNA damage–induced genotoxic stress due to replication error, mutagens, and reactive oxygen species (ROS) is well documented to accelerate aging (**Figure 2**). Among DNA repair pathways, it has been shown that non-homologous end joining (NHEJ) is specifically enhanced in quiescent hematopoietic stem cells (HSCs), whereas committed progenitors preferentially use homologous recombination as their repair mechanism (Mohrin et al., 2010). Similarly, quiescent bulge HFSCs are relatively protected against ionizing radiation, likely because of their elevated anti-apoptotic programs and enhanced NHEJ capacity (Sotiropoulou et al., 2010). Deficiencies in various NHEJ components (Difilippantonio et al., 2000; Gao et al., 2000) (Baker et al., 2004; Nijnik et al., 2007), spindle assembly checkpoint proteins (Baker et al., 2004), and DNA damage response pathways (Ito et al., 2004; Ruzankina et al., 2007) collectively contribute to premature aging of the hair follicles.

Disruption of the circadian clock results in pre-mature aging (Janich et al., 2011; Kondratov et al., 2006; Yu et al., 2021), and its associated molecular mechanisms are only beginning to emerge (**Figure 2**). It has been suggested the transit-amplifying cells of the hair follicles exhibit elevated sensitivity toward DNA damage during the day when their mitotic activities peak (Plikus et al., 2013), whereas the epidermis preferentially proliferates at night to avoid the high level of oxidative phosphorylation (with its byproduct ROS) during the day, presumably as a protective mechanism against genotoxicity (Stringari et al., 2015). In aged epidermal stem cells, arrhythmic and prolonged DNA replication combined with

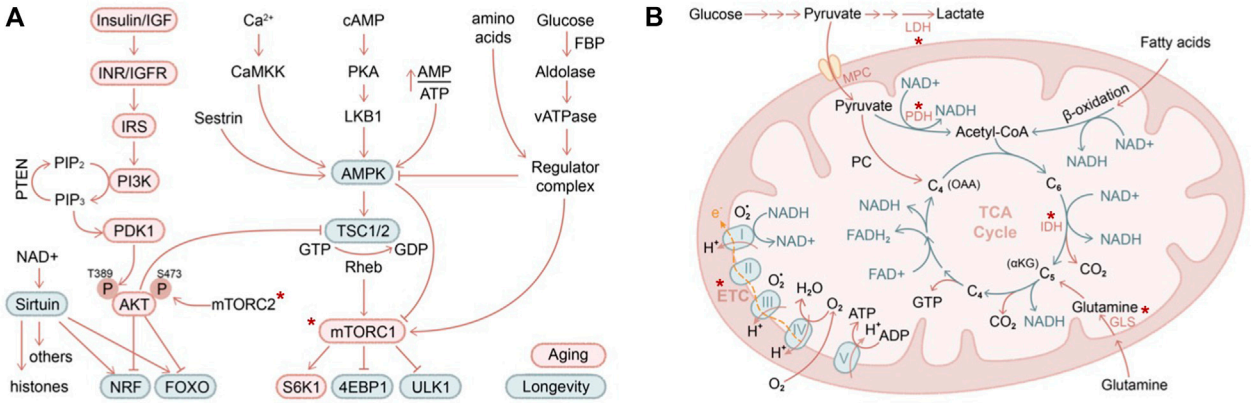

FIGURE 3 | Nutritional and metabolic regulation of skin aging **(A)** IIS (insulin/insulin-like growth factor) and AMPK/mTOR are two major pathways in regulating nutrients and metabolism that have been described in contributing to organismal aging (red circle) and longevity (green circle). Asterix indicates components of these pathways that have been demonstrated to contribute to skin aging **(B)** Mitochondrial biology is closely intertwined with aging biology. Highlighted in red are several key genes that have been shown to regulate skin stem cell aging *in vivo*. LDH, lactate dehydrogenase; PDH, pyruvate dehydrogenase; MPC, mitochondrial pyruvate carrier; GLS, glutaminase; IDH, isocitrate dehydrogenase (converting isocitrate into alpha-ketoglutarate). Asterix indicates components of these pathways that have been demonstrated to contribute to skin aging.

otherwise-normal oscillatory oxidative phosphorylation programs may therefore underlie the increased oxidative damage (Solanas et al., 2017). Nevertheless, complete disruption of the clock did not recapitulate selective rhythmic deregulation in a physiological aging setting, nor did circadian rewiring induced by a high-fat diet, suggesting the direct cause of aging-associated circadian reprogramming remains unclear (Solanas et al., 2017).

Furthermore, chronic inflammatory signals also contribute to DNA damage and genotoxicity. For example, IFNα stimulate HSCs to exit quiescence via IFNAR and STAT1 signaling, elevate mitochondrial ROS levels, and lead to DNA damage (Essers et al., 2009; Sato et al., 2009; Walter et al., 2015) (**Figure 2**). ROS may act upstream of DNA damage, evidenced by the observation that antioxidant treatment is able to suppress DNA damage and thus delay HSC aging (Essers et al., 2009; Walter et al., 2015). Genotoxic stress could further exacerbate ROS deregulation and subsequently activate p38MAPK and upregulate the expression of P16, resulting in HSC exhaustion (Ito et al., 2004) (Ito et al., 2006). In the case of HFSCs, DNA damage induces proteolysis of collagen XVII, a critical cell junction collagen, and induces stem cell differentiation (Matsumura et al., 2016). Likewise, ionizing radiation–induced DNA damage leads to melanocyte stem cell exhaustion and hair graying (Inomata et al., 2009). Strikingly, hair graying is completely suppressed in mice that are deficient in the double-stranded DNA-sensing cGAS/STING pathway (Dou et al., 2017), suggesting that the innate immunity pathway serves as a key mediator of radiation-induced aging.

NUTRITIONAL AND METABOLIC REGULATION OF SKIN AGING

Pioneering work has established several nutrient-sensing and energy-sensing pathways to be critically involved in lifespan extension across model organisms (**Figure 3A**), including insulin/insulin-like growth factor (IIS) (Kenyon, 2010), AMP-activated protein kinase (AMPK) (Herzig and Shaw, 2018), mammalian or mechanistic target of rapamycin (mTOR) (Wullschleger et al., 2006; Laplante and Sabatini, 2012), and sirtuin (Haigis and Guarente, 2006; Longo and Kennedy, 2006; Houtkooper et al., 2012) pathways. While mechanistic studies of these pathways in skin aging are rapidly emerging, in this section, we will review the historical aspects of these molecular mechanisms in various model organisms and adult stem cells in order to provide a broader context for skin aging.

In the IIS pathway, the selective ablation of signaling cascade components led to activation of downstream transcription factors FOXO (Lee et al., 2003; Murphy et al., 2003) and NRF (Sykiotis and Bohmann, 2008; Tullet et al., 2008), eliciting cellular protective programs against oxidative stress and promoting longevity (Tothova et al., 2007; Blackwell et al., 2015). AMPK senses cellular energy decline and activates a plethora of catabolic pathways while suppressing anabolic processes, promoting lifespan extension (Mair et al., 2011; Herzig and Shaw, 2018). The antidiabetic antineoplastic drug metformin exerts both AMPK-dependent (Ma et al., 2022) and AMPK-independent activities (Foretz et al., 2014) and, when used at a high dose, inhibits mitochondrial electron transport chain complex I (described further below), all of which may contribute to its utility in anti-aging (Zhou et al., 2001). Both IIS and AMPK pathways converge on mTOR. Nutrient and amino acid starvation inhibits mTOR activity, resulting in suppressed protein translation and enhanced autophagy via key mTOR targets S6K1 (ribosome protein S6 kinase B1), 4EBP1 (eukaryotic translation initiation factor 4E-binding protein 1), and ULK1 (unc-51 like autophagy activating kinase 1) (Wullschleger et al., 2006; Laplante and Sabatini, 2012). Sirtuins are NAD+ (nicotinamide adenine dinucleotide)–dependent deacetylases that act on both histone substrates to silence heterochromatin (Oberdoerffer et al.,

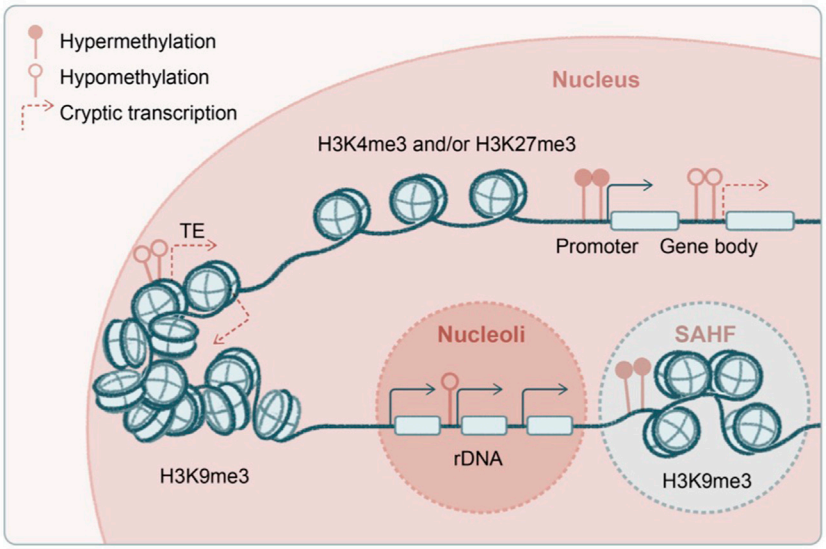

FIGURE 4 | Transcriptional and epigenetic noise in aging stem cells. Several proposed origins of the transcriptional and epigenetic noise in aged stem cells, including genome-wide hypomethylation and derepression of transposable elements, spurious transcription from the gene body, and deregulated transcription from ribosomal DNA (rDNA) at nucleoli. Global hypomethylation is accompanied by regional hypermethylation at gene promoters as well as formation of senescence-associated heterochromatin foci (SAHF). Some of the enzymes and post-translational histone modifications have been shown to regulate aging and longevity in a transgenerational fashion.

2008; Dang et al., 2009) and on non-histone substrates such as P53 (Luo et al., 2001; Vaziri et al., 2001), HSF1 (heat shock factor 1), FOXO (Brunet et al., 2004), and PGC1 (Rodgers et al., 2005), among others, to regulate aging.

Among these regulators, mTOR function has been genetically tackled in several adult stem cell, aging, and malignancy contexts. Hyperactivation of mTOR by deletion of *Pten* or *TSC1* exhausted neural stem cells (Bonaguidi et al., 2011) and HSCs (Yilmaz et al., 2006; Zhang et al., 2006; Gan et al., 2008; Chen et al., 2009), and the HSCs were transformed to become precursors of myeloproliferative disorder. On the other hand, loss of mTOR function via ablation of the mTORC1 component *Raptor* (regulatory-associated protein of mTOR) in mouse HSCs leads to non-lethal pancytopenia, splenomegaly, and accumulation of monocytoid cells. Raptor conditional knockout also compromised HSC regeneration and inhibited leukemogenesis evoked by *Pten* deficiency (Kalaitzidis et al., 2012). p53 and p16 mediate HSC exhaustion and serve as a roadblock to leukemic transformation upon *Pten* deletion (Lee J. Y. et al., 2010b). In adult but not childhood leukemia, deletion of the mTORC2 component *Rictor* blocked leukemogenesis and HSC depletion (Magee et al., 2012). Mechanistically, it has been shown that in *Drosophila* larvae, chronic stimulation of TOR (via constitutively active insulin receptor expression) induces ROS and activates JNK and FOXO, resulting in accumulation of Sestrin (a family of stress-sensing proteins) and activation of AMPK, facilitating autophagic clearance of damaged mitochondria, protein aggregates, or lipids (Lee J. H. et al., 2010a). Loss of Sestrin resulted in age-associated pathologies including triglyceride accumulation, mitochondrial dysfunction, muscle degeneration, and cardiac malfunction, which could be reversed by AMPK activation or TOR inhibition. In the skin, deletion of either *Mtor* itself or its complex components *Raptor* of mTORC1 or *Rictor* of mTORC2 led to skin barrier defects during development (Ding et al., 2016). Treatment with rapamycin (preferentially targeting mTORC1) reversed HFSC exhaustion induced by WNT1 overactivation (Castilho et al., 2009), whereas mTORC2 has been suggested to mediate glutaminase suppression in returning HFSCs to quiescence (Kim et al., 2020).

Calorie restriction or dietary restriction is widely known to ameliorate aging-associated decline of stem cells, such as HSCs (Chen et al., 2003; Warr et al., 2013; Tang et al., 2016), germline stem cells (Mair et al., 2010), and stem cells of the intestine (Regan et al., 2016; Akagi et al., 2018; Mihaylova et al., 2018), skeletal muscle (Cerletti et al., 2012), and skin (Forni et al., 2017; Solanas et al., 2017). Mechanistically, in the context of intestinal stem cells, calorie restriction enhances stem cell activity through a niche-dependent paracrine signal (Yilmaz et al., 2012). In HSCs, calorie restriction cell-autonomously preserves the functional autophagy-high and oxidative phosphorylation–low populations during aging (Warr et al., 2013; Ho et al., 2017). Similar to basal autophagy, chaperon mediated autophagy is also required for maintaining HSC function during aging (Dong et al., 2021). In aged neural stem cells, calorie restriction induced lysosome activation clears aggregates and restores stem cell activation (Leeman et al., 2018), and likewise, chaperon mediated autophagy is essential for preventing proteome collapse and neurodegenerations (Bourdenx et al., 2021). Calorie restriction also restores the transcriptional circadian rhythm of aged epidermal and muscle stem cells to their youthful level, such as replication and autophagy, respectively (Solanas et al., 2017).

By sensing energy demand and nutrient supply, stem cells adaptively tune the level of glycolysis (breakdown of glucose into pyruvate under aerobic conditions or lactate under anaerobic conditions) and oxidative phosphorylation pathways (including oxidation of pyruvate, glutamine, or fatty acid in mitochondria, TCA cycle, electron transport chain activity) (**Figure 3B**). It is commonly believed that long-term self-renewing stem cells reside in a hypoxic niche and downregulate mitochondrial activity (Mazumdar et al., 2010; Simsek et al., 2010; Takubo et al., 2010). Blocking the influx of glycolytic metabolites into mitochondria by overexpression of pyruvate dehydrogenase kinase (which inhibits pyruvate dehydrogenase and hence pyruvate oxidation) increases the long-term self-renewal capacity of HSCs (Simsek et al., 2010; Takubo et al., 2013), while deletion of mitochondrial pyruvate carrier (which transports pyruvate into mitochondria for oxidation) enhances the organoid-forming potential of intestinal stem cells (Schell et al., 2017). Likewise, in the skin, depletion of mitochondrial pyruvate carrier induced HFSC activation, whereas ablation of lactate dehydrogenase (which converts pyruvate to lactate during anaerobic glycolysis) blocked HFSC activation (Flores et al., 2017). Interestingly, glutamine oxidation is highly upregulated in HFSC progenies compared to HFSCs in organoid culture, and it has been proposed to promote HFSCs returning to quiescence at the end of the hair cycle (Kim et al., 2020). While oxidative phosphorylation and mitochondrial activity are unequivocally induced upon stem cell activation, whether glucose or glutamine influx contributes to the increased oxidation is challenging to directly determine *in vivo*. Tissue-specific depletion of glutaminase (which converts glutamine to glutamate), similar to the experiments performed on mitochondrial pyruvate carrier and lactate dehydrogenase, will likely provide further insights into this interesting question.

Mitochondrial biology has long been intertwined with aging biology (Beckman and Ames, 1998). Mice with mutated mitochondrial DNA polymerase Polg age prematurely (Trifunovic et al., 2004; Kujoth et al., 2005), inducing a differentiation block in tissues with high turnover rate including skin (Norddahl et al., 2011; Ahlqvist et al., 2012). Mice with epidermis-specific loss of TFAM (mitochondrial transcription factor A), required for the transcription of mitochondrial genes encoding electron transport chain subunits, showed impaired epidermal differentiation and hair follicle growth (Hamanaka et al., 2013; Kloepper et al., 2015). Indeed, electron transport chain activity declines in aging, and enhancing mitochondrial function by overexpression of PGC1 (Rera et al., 2011; Dillon et al., 2012) or supply of NAD+ (Gomes et al., 2013) delays aging. Consistently, during aging, telomere dysfunction compromises mitochondrial function *via* p53-mediated inhibition of PGC1 levels (Sahin and Depinho, 2010). In an interesting twist, it has been shown that mild mitochondrial stress prolongs lifespan in worms (Durieux et al., 2011) and yeast (Pan et al., 2011). The specific context under which these pathways operate and the cell types responding to these various manipulations likely play a major role in explaining these seemingly complex observations. It would be of future interest to examine how restoring ETC activity or inducing mild mitochondrial stress would affect skin ageing.

DECIPHERING TRANSCRIPTIONAL AND EPIGENETIC NOISE IN AGING STEM CELLS

A hallmark of aging-associated stem cell degeneration is transcriptional noise and epigenetic erosion, in which heterochromatin silencing and transcriptional fidelity appear crucial. Pioneering genetic and biochemical experiments have mapped out the central pathway for heterochromatin regulation (Jenuwein and Allis, 2001; Zhang and Reinberg, 2001; Grewal and Moazed, 2003; Jaenisch and Bird, 2003; Roeder, 2005; Workman, 2006; Kouzarides, 2007; Zaratiegui et al., 2007; Law and Jacobsen, 2010; Piunti and Shilatifard, 2016; Schuettengruber et al., 2017), a critical vulnerability during organismal aging. Epigenetic deregulations in aging have been extensively characterized in model organisms, where several parallels could be drawn into the mammalian systems including skin (**Figure 4**).

A key gene-silencing mechanism in mammals, DNA methylation is not conserved or has a very limited role in yeast, flies, and worms. Rather, histone modifications are heavily involved in transcriptional silencing in the latter. For example, during yeast replicative aging, decline of the deacetylase Sir2 results in acetylated histone 4 lysine 16 (H4K16ac) and increased transcription at subtelomeric regions (Dang et al., 2009), as well as enhanced ribosomal DNA (rDNA) transcription by RNA polymerase I and formation of extrachromosomal rDNA circles (Sinclair and Guarente, 1997) (H4K16ac and RNA polymerase I transcription factors are Sir2 substrates). Interestingly, aged HSCs exhibit global DNA hypomethylation that includes the rDNA regions (Sun et al., 2014), and they accumulate non-canonical gH2AX and ATR at nucleoli where rDNA transcription occurs, disrupting ribosome biogenesis (Flach et al., 2014). Notably, SIRT1 (the mammalian homolog of yeast Sir2) appears to contribute to repeat silencing and is involved in the DNA damage response in mammalian cells (Oberdoerffer et al., 2008), although its role in mammalian rDNA regulation is unknown. Since, unlike yeast, mammals use both DNA and histone methylation to mediate transcriptional repression, it will be interesting to see which of these pathways are involved in rDNA silencing during skin aging. Such effort will now be greatly facilitated by the recently completed draft of gapless human genome covering highly repetitive regions including rDNAs (Nurk et al., 2022).

Besides repetitive rDNA, another type of highly abundant repeats in the mammalian genome is transposons (**Figure 4**). The aging epigenome is shaped by global DNA hypomethylation, typically at gene-poor and late-replicating regions, accompanied by focal hypermethylation at genic regions, bearing strong resemblance to the cancer genome (Cruickshanks et al., 2013; Yuan et al., 2015). In yeast, decline of histone chaperones and global loss of histones during aging lead to transposon derepression and genome instability (Feser et al., 2010; Hu et al., 2014; O'Sullivan et al., 2010). Deficiencies in histone deacetylase SIRT6 in mice (Simon et al., 2019), exonuclease TREX1 in human cells (Thomas et al., 2017), and endonuclease AGO2 in flies (Li et al., 2013) have been shown to induce transposons in aging and inflammatory diseases. In a unique group of premature aging diseases, progeria and

laminopathies, lamin-associated domains become disintegrated (Kudlow et al., 2007), accompanied by global loss of H3K9me3 and structural changes in the heterochromatin (Scaffidi and Misteli, 2006; Zhang et al., 2015) and subsequently nuclear autophagy and cytosolic chromatin fragment–induced inflammation (Dou et al., 2015). Indeed, in keratinocytes, it has been shown that mechanical strain leads to H3K9me3 heterochromatin loss at the nuclear lamina and disruption of lineage gene expression during aging (Le et al., 2016; Koester et al., 2021), suggesting a viable hypothesis that deregulation of heterochromatin drives skin aging. Among H3K9me3 methyltransferases, G9a has been functionally examined in the mammalian epithelium. While G9a is minimally involved in homeostasis potentially because of functional redundancy, it is critical for tumorigenesis (Avgustinova et al., 2018; Avgustinova and Benitah, 2021).

Another source of spurious transcription in aging could be emanated from gene bodies (**Figure 4**). H3K36me3 methyltransferases are among the first discovered epigenetic regulators of longevity: Set2 in yeast (Carrozza et al., 2005; Sen et al., 2015) and Met-1 in worms (Hamilton et al., 2005; Pu et al., 2015). In these model systems, it has been shown that RNA polymerase II–associated methyltransferase deposits H3K36me3 along the gene body, recruiting histone deacetylase to suppress spurious transcription initiated from the gene body, and this transcriptional fidelity becomes compromised in aging. A similar mechanism has recently been described in mammalian cells (Neri et al., 2017; McCauley et al., 2021), in which DNA methyltransferase is likely recruited by H3K36me3 along the gene body to mediate the suppression of cryptic transcription (Dhayalan et al., 2010). The mammalian H3K36me3 is maintained by multiple methyltransferases, Setd2 and Nsd1/2/3, which exhibit both overlapping and distinct functions (Barral et al., 2022).

Both H3K9me3 and H3K36me3 are closely associated with DNA methylation and frequently exhibit interdependency. Epidermis-specific deletion of the *de novo* DNA methyltransferase Dnmt1 led to hair follicle miniaturization along with epidermal and sebaceous gland hyperplasia, a signature of premature aging in the skin (Li et al., 2012). Consistently, in the epidermis, Dnmt1 along with its foreshadowing E3 ubiquitin ligase Uhrf1 are required to maintain stem cell self-renewal (Sen et al., 2010; Mulder et al., 2012), loss of which results in autoinflammatory conditions in the skin (Beck et al., 2021). A related enzyme, Tet2, that catalyzes 5-hydroxymethylcytosine (5hmC) and promotes DNA demethylation, is essential to maintain epidermal differentiation (Boudra et al., 2021). Furthermore, deletion of the maintenance DNA methyltransferases Dnmt3a/b from epithelial cells largely spared skin homeostasis but contributed to malignant transformation (Rinaldi et al., 2016; Rinaldi et al., 2017). Interesting, deletion of mediator complex component Med1 (Nakajima et al., 2013) or the SWI/SNF-like BAF complex catalytic subunit Brg1 (Xiong et al., 2013) also elicits HFSC exhaustion and alopecia phenotype, although in the case of BAF complex, its function is context dependent and regulates either stem cell expansion or differentiation based on which lineage is examined (Bao et al., 2013; Mardaryev et al., 2014).

Another repressive histone post-translational modification proposed to regulate aging is the inactive or poised chromatin marker H3K27me3, which declines due to increased demethylase UTX-1 during aging (Jin et al., 2011; Maures et al., 2011). In this case, the impact of H3K27me3 loss on aging has been attributed to its specific regulation of the IIS (insulin/insulin-like growth factor) genes. In contrast, the excessive levels of the active chromatin marker H3K4me3 and its responsible methyltransferase, trithorax, has been shown to be detrimental to longevity (Greer et al., 2010; Greer et al., 2011). On the other hand, H3K4me3 demethylase promotes longevity in worms, with a remarkable transgenerational memory effect observed in this context (Greer et al., 2014). Mild mitochondrial stress-induced lifespan extension is associated with epigenetic remodeling via histone methyltransferases (Tian et al., 2016) and demethylases (Merkwirth et al., 2016). Remarkably, genome-wide mapping of 5mC and 5hmC in HSCs revealed extended regions of low-methylation canyons that are distinct from CpG islands and shores (Jeong et al., 2014). These canyons harbor either H3K27me3 or poised H3K27me3/H3K4me3 histone markers, whose borders are demarcated by 5hmC, and are deregulated upon Dnmt3a/Tet1 deletion or in aging (Jeong et al., 2014; Sun et al., 2014; Dixon et al., 2021).

In skin, histone methyltransferases of H3K27me3 (polycomb group) and H3K4me3 (trithorax group) regulate epidermal stem cell self-renewal and differentiation, respectively, during development and regeneration (Millar, 2011; Frye and Benitah, 2012; Perdigoto et al., 2014; Guan et al., 2020). For example, in PRC2 Ezh2 conditional knockout, P16 is derepressed, resulting in cell cycle arrest and epithelium hypoplasia (Ezhkova et al., 2009), similar to PRC1 deficiency (Cordisco et al., 2010; Luis et al., 2011). In contrast, the H3K27me3 demethylase promotes epidermal differentiation (Sen et al., 2008). Likewise, histone deacetylases HDAC1/2 (LeBoeuf et al., 2010) (Hughes et al., 2014) and HDAC3 (Szigety et al., 2020) have opposing functions in stem cell proliferation versus differentiation. On the other hand, the histone methyltransferase MLL4 (KMT2D) that catalyzes H3K4me1 is required to maintain epidermal homeostasis, whose loss resulted in the disruption of skin stratification and lipid metabolism (Lin-Shiao et al., 2018; Egolf et al., 2021). Interestingly, lack of Bmi1, a component of PRC1, leads to impaired mitochondrial function and to increased ROS and DNA damage, which could be reversed by antioxidant treatment or genetic disruption of Chk2 (Liu et al., 2009). These findings suggest that global chromatin disruption may override defects from specific gene deregulations, that it is not an individual gene or a few genes per se that elicit the phenotype. These molecular and cellular mechanisms are potentially extendable to aging.

Compared to global hypomethylation, equally noteworthy is the accompanying focal hypermethylation in aging chromatin (**Figure 4**). A prominent feature in cellular senescence is the formation of the senescence-associated secretory phenotype (SASP) on one hand (Coppé et al., 2010) and senescence-associated heterochromatin foci (SAHF) on the other (Narita et al., 2003; Zhang et al., 2005; Di Micco et al., 2011; Rai et al., 2014; Ricketts et al., 2015). Remarkably, a sparse hair coat in aged

mice is recovered upon FOXO4 peptide treatment, which blocks p53 nuclear localization and induces the apoptosis of senescent cells (Baar et al., 2017), supporting the functional significance of senescence in skin aging. SAHF has been suggested as a survival mechanism to "plug the hole" that assembles heterochromatin onto euchromatin regions, given the compromised nuclear lamina that otherwise is necessary to maintain the physiological heterochromatin. Alternatively, one could speculate these foci may titrate away rate-limiting factors (H3.3, methyltransferase, or metabolites required for transcriptional repression; see Discussion) from transposon repeats and heterochromatic regions, exacerbating the already-weakened epigenetic regulation during aging. Although this model has not been formally tested in aging, a similar titration- or competition-based inhibitory mechanism from H3K27M and H3K36M oncohistones has been shown in cancer (Lewis et al., 2013; Lu et al., 2016), leaving the field with the intriguing question of how the lineage specificity and gene selectivity is achieved in the context of global epigenetic deregulations.

DISCUSSION

While the identity of upstream signals that lead to defective epigenetic machinery in aging remains unclear, it appears safe to assume these factors would be rate-limiting. As mentioned, nutrients and metabolites, in addition to their impact on cell signaling transductions and mitochondrial biology, are appealing candidates that appear to dictate the aging transcriptome and epigenome. Many epigenetic enzymes that regulate methylation or acetylation exhibit K_M in the range of observed substrate concentrations in cells, suggesting rates of these reactions are highly sensitive to the cellular fluctuations of the corresponding metabolites, such as S-adenosyl methionine, acetyl-CoA, alpha-ketoglutarate, NAD+, and beta-hydroxybutyrate (Schvartzman et al., 2018), raising the intriguing question of the extent to which cellular metabolite deregulation shapes the aging epigenome. Indeed, it has been shown that in autophagy-deficient HSCs, DNA methylation and lineage gene expression are regulated by S-adenosyl methionine and alpha-ketoglutarate levels (Ho et al., 2017). Ascorbate and its downstream target TET2 dictate HSCs (Agathocleous et al., 2017) as well as (Boudra et al., 2021) epidermal function and tumorigenesis. NAD+ being the rate-limiting factor, competition between PARP-1 (also NAD+ dependent) and SIRT1 dictates cellular response in DNA damage and epigenome regulation (Bai et al., 2011) (Scheibye-Knudsen et al., 2014). RNA sequencing (RNA-seq) and assay for transposase-accessible chromatin with sequencing (ATAC-seq) at single cell level, and together with spatial transcriptomics provided unprecedented throughput and resolution of cell type, lineage trajectory, and tissue-level crosstalk in many biological contexts, and are likely to be powerful technologies in aging research. The current challenge lies in the metabolomics at a sub-cellular level and within the intact organism *in vivo*, both of which are critical to understand molecular determinants of aging.

Local tissue microenvironment plays a dominant role in dictating stem cell function, including aging. Aged dermal fibroblasts maintain their positional identity (Marsh et al., 2018) while manifesting adipogenic and inflammatory traits (Salzer et al., 2018; Mahmoudi et al., 2019). Secreted factors of BMP and WNT pathways enriched in adipocytes (Keyes et al., 2013; Chen et al., 2014) and pre-adipocytes (Festa et al., 2011) known to dictate hair follicle regeneration are disrupted in aged dermis (Chen et al., 2014; Chen et al., 2015). Skin-resident immune cells are drastically remodeled during aging (Giangreco et al., 2008; Doles et al., 2012), including regulatory T cells that are known to support hair follicle regeneration (Ali et al., 2017) and decline significantly in aged dermis (Ge et al., 2020). So are the arrector pili muscles and nerves (Brownell et al., 2011; Fujiwara et al., 2011; Shwartz et al., 2020) that provide niche input to HFSCs, both of which become dislodged in aged skin (Ge et al., 2020). Of significance, aged HFSCs can be rejuvenated by resident cell types and niche components of the young skin (Chen et al., 2014; Ge et al., 2020; Koester et al., 2021), suggesting that the tissue microenvironment drives stem cell function during aging. Future work will need to exploit the molecular identity, regulatory signals, and therapeutic potential of such rejuvenation factors.

AUTHOR CONTRIBUTIONS

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EDITED BY
Ji Li,
Xiangya Hospital, Central South University, China

REVIEWED BY
Wen-Chieh Chen,
Technical University of Munich, Germany
Hengguang Zhao,
Second Affiliated Hospital of Chongqing Medical University, China

*CORRESPONDENCE
Christos C Zouboulis,
christos.zouboulis@klinikum-dessau.de
Qiang Ju,
qiangju@aliyun.com

†These authors have contributed equally to this work

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Aging in the sebaceous gland

Xiaoxiao Hou[1,2,3†], Ziyu Wei[4†], Christos C Zouboulis[2*] and Qiang Ju[1*]

[1]Department of Dermatology, Renji Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai, China, [2]Departments of Dermatology, Venereology, Allergology and Immunology, Dessau Medical Center, Brandenburg Medical School Theodor Fontane and Faculty of Health Sciences Brandenburg, Dessau, Germany, [3]Berlin Brandenburg Center for Regenerative Therapies, Charite Universitatsmedizin Berlin, Berlin, Germany, [4]Genetic Skin Disease Center, Jiangsu Key Laboratory of Molecular Biology for Skin Diseases and STIs, Institute of Dermatology, Chinese Academy of Medical Sciences and Peking Union Medical College, Nanjing, China

Sebaceous glands (SGs) originate from hair follicular stem cells and secrete lipids to lubricate the skin. The coordinated effects of intrinsic and extrinsic aging factors generate degradation of SGs at a late age. Senescence of SGs could be a mirror of the late aging of both the human body and skin. The procedure of SG aging goes over an initial SG hyperplasia at light-exposed skin areas to end with SG atrophy, decreased sebum secretion, and altered sebum composition, which is related to skin dryness, lack of brightness, xerosis, roughness, desquamation, and pruritus. During differentiation and aging of SGs, many signaling pathways, such as Wnt/β-catenin, c-Myc, aryl hydrocarbon receptor (AhR), and p53 pathways, are involved. Random processes lead to random cell and DNA damage due to the production of free radicals during the lifespan and neuroendocrine system alterations. Extrinsic factors include sunlight exposure (photoaging), environmental pollution, and cigarette smoking, which can directly activate signaling pathways, such as Wnt/β-catenin, Notch, AhR, and p53 pathways, and are probably associated with the de-differentiation and hyperplasia of SGs, or indirectly activate the abovementioned signaling pathways by elevating the inflammation level. The production of ROS during intrinsic SG aging is less, the signaling pathways are activated slowly and mildly, and sebocytes are still differentiated, yet terminal differentiation is not completed. With extrinsic factors, relevant signaling pathways are activated rapidly and fiercely, thus inhibiting the differentiation of progenitor sebocytes and even inducing the differentiation of progenitor sebocytes into keratinocytes. The management of SG aging is also mentioned.

KEYWORDS

aging, sebaceous gland, differentiation, hyperplasia, stem cell

Introduction

With the development of the industrialized society, more and more people are concerned about skin aging. Due to endogenous and exogenous (mostly sun exposure) factors, the thickening of the stratum corneum, xerosis, wrinkles, and abnormal pigmentation occur. Several studies have elaborated on epidermal and dermal aging; however, the aging of sebaceous glands (SGs) has barely been studied (Zouboulis et al., 2016). Aging of SGs, especially in the light-exposed areas, starts with SG hyperplasia,

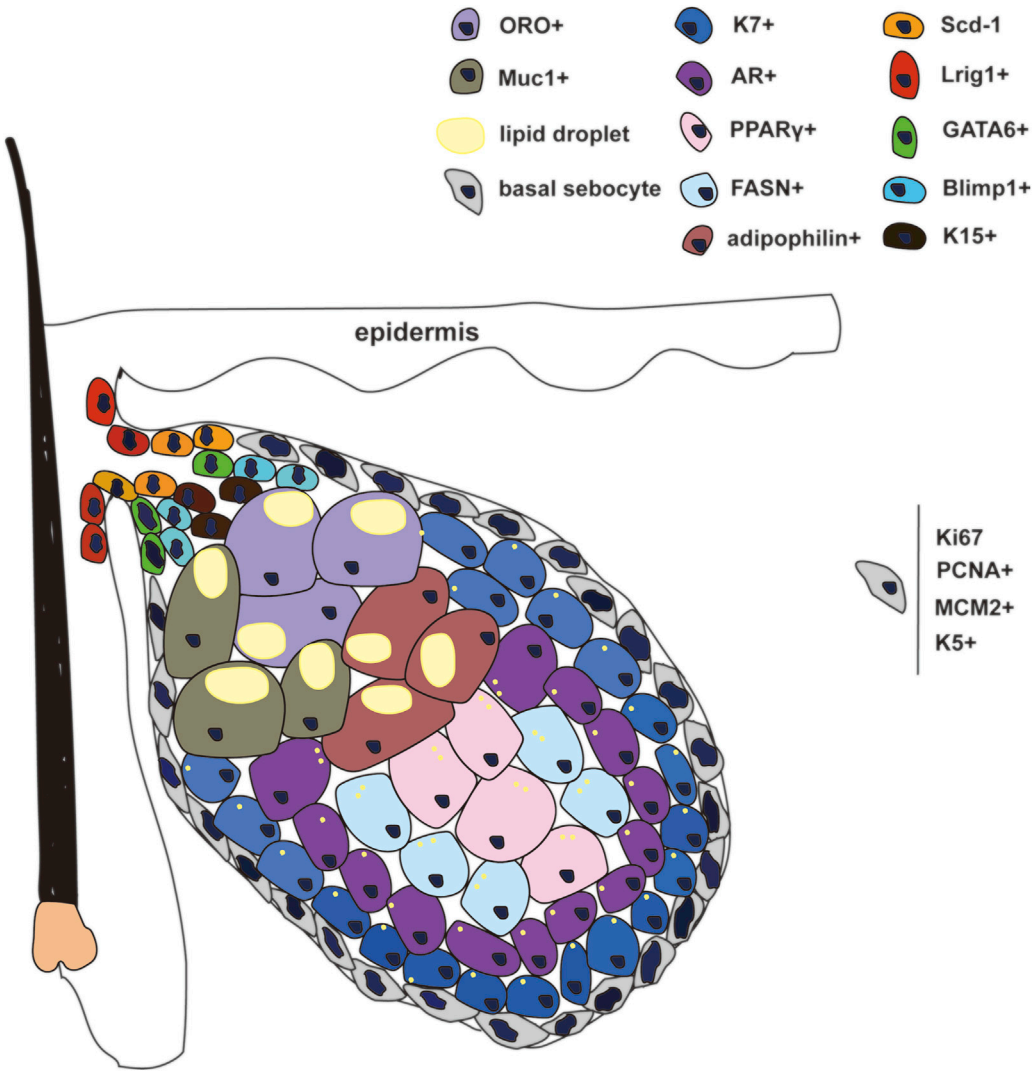

FIGURE 1
Different stages of cell pools and corresponding biomarkers in SG. Lrig1+, Scd-1, K15+, and GATA6+ cells represent the progenitor cells around the gland duct. Basal sebocytes can proliferate and differentiate. K7+ and AR+ cells represent the early differentiated sebocytes. PPARγ+ and FASN+ represent the differentiated sebocytes in the middle stage. ORO+ and Muc1+, and Adipophilin+ cells represent the terminally differentiated sebocytes.

followed by atrophy, decreased sebum secretion, and occasionally the development of SG carcinoma. In this review, we illustrate SG aging from the aspects of SG alterations, molecular signaling pathway modifications with aging, the multiple causes of SG aging, and the manifestations and treatment of SG aging disorders.

Stem cells, development, and differentiation of sebaceous glands

Embryologically, the epithelium and its appendages develop from the ectoderm. Stem cells of the ectoderm differentiate into the interfollicular epidermis (IFE), hair follicles (HFs), sweat glands, and SGs under modulator signaling pathways, including Wnt, ectodysplasin A receptor (EDAR), bone morphogenetic protein (Bmp), and Hedgehog pathways (Schmidt-Ullrich and Paus, 2005; Duverger and Morasso, 2014; Saxena et al., 2019; Sennett et al., 2015). As part of the pilosebaceous unit, the development of SGs is closely associated with the formation of HFs. The initiation of SG development occurs in the upper region of the HF (Paus et al., 1999). During the development of SGs, first sebocytes differentiate from Lrig1+ HF stem cells which migrate to the distal HF epithelium close to the IFE (Figure 1). Meanwhile, the expression of stearoyl CoA desaturase 1 (SCD1)

is detected concomitantly with the emergence of first sebocytes (Frances and Niemann, 2012). Lrig1 expression disappears once SCD1 is expressed in the SG progenitor cells. One cluster of SCD1-positive cells proceeds to the formation of two individual glands, and mature Lrig1-negative sebocytes are surrounded by Lrig1-positive stem cells. Those SCD1+ SG progenitor cells progress to proliferating basal cells anchored to the SG basement membrane. According to the results of Andersen et al. (2019), these progenitor cells undergo a defined process of random cell division and differentiation, which appears uncorrelated with the fate selection of neighboring cells, resulting in variable-sized SGs. Such a conclusion is opposed to the previous assumption that progenitor cells at the top of the gland replenish cells lost by differentiation at the basement membrane (Horsley et al., 2006). In the initial phase, B lymphocyte-induced maturation protein 1 (Blimp1)-positive cells represent a resident population of early differentiated sebocytes in mice as an intermediate stage between the progenitor and differentiated sebocytes, regulating the size and activity of SGs (Horsley et al., 2006; Kretzschmar et al., 2014). However, further studies have shown that Blimp1 is a terminal differentiation marker in human SGs (Magnusdottir et al., 2007). During the formation of SGs, keratin 15-positive cells are seen at the apical part of the SG, possibly representing SG precursors (Eisinger et al., 2010). The cells located at the basement membrane are positive for Ki67 (Andersen et al., 2019), proliferating cell nuclear antigen (PCNA) (Cottle et al., 2013), MCM2, and keratin 5 (Feldman et al., 2019). Basal sebocytes express the highest level of MYC in the SG. During maturation, MYC expression decreases, and SG proliferative cells progressively migrate and differentiate into the inner mass, from an early stage over middle stage to terminal differentiation, accumulating lipid droplets and eventually bursting to release lipids into the sebaceous duct. The early-stage differentiation markers are keratin 7 (K7) (de Bengy et al., 2019) and androgen receptor (AR) (Cottle et al., 2013). AR is highly expressed in the middle stage as well, and peroxisome proliferator-activated receptor gamma (PPARγ) and fatty acid synthase (FASN) are regarded as markers of middle-stage differentiation (Cottle et al., 2013). Terminally differentiated and mature sebocytes are oil red O (Feldman et al., 2019), melanocortin 5 receptor (MC5R), and mucin 1 (MUC-1) (Hinde et al., 2013; de Bengy et al., 2019), also known as the epithelial membrane antigen (EMA) and are adipophilin-positive (Hinde et al., 2013). Remarkably, K7 and MUC-1 are sebaceous markers in humans but not in murine SGs (Hinde et al., 2013). Homeostasis of SGs is maintained by the constant differentiation of sebocyte progenitor cells. Along with aging, progenitor cells are affected, and the SG differentiation was depleted (Zouboulis et al., 2008). Ki67 showed reduced expression in aged HFs in both human and mouse skin, revealing the diminished proliferation and regeneration of HFs (Chang et al., 2005; Ge et al., 2020). The protein level of PPARγ was found to be significantly downregulated in sebaceous glands based on the research that included 42 young and old human individuals (Elewa et al., 2015), indicating the reduced differentiation of sebocytes. However, in a mouse study, the activity of PPARγ and lipogenic genes such as acetyl-CoA carboxylase (Acc), fatty acid synthase (Fas), stearoyl-CoA desaturase 1 (Scd1), and sterol regulatory element-binding protein 1 (Srebp1) were elevated in an aging mouse model with chronic activation of p53 (Kim et al., 2014).

Molecular variations associated with sebaceous gland aging

Wnt/β-catenin signaling pathway

The Wnt/β-catenin pathway, an important pathway in regulating epidermal differentiation, increases the expressions of involucrin and cornifin in SGs, reduces the number of terminally differentiated sebocytes, downregulates sebum secretion, and is related to epidermal cyst formation (Lo Celso et al., 2008; Shang et al., 2021). Loss of β-catenin in mouse epidermis leads to the enlargement of SGs (Niemann et al., 2002; Lien et al., 2014). Furthermore, AR activation was verified to reduce β-catenin-dependent transcription in SZ95 sebocytes and induce sebocyte differentiation (Ebling et al., 1969; Rosignoli et al., 2003). With aging, the level of serum AR is downregulated, and the inhibition of the Wnt/β-catenin signaling pathway is reduced, resulting in reduced SG differentiation and decreased lipid secretion, turning to the hyperplasia of SG and the formation of epidermoma (Ceruti et al., 2018). This is similar to what we observed clinically in solar elastosis comedones (literally epidermomas) (Figure 2), which develop after prolonged sun exposure (Patterson et al., 2004).

Transforming growth factor-β

Transforming growth factor- (TGF-β) levels increase in dermal fibroblasts with aging (Gunin and Golubtzova, 2019). Interestingly, the significant activation of the TGF-β/Smad pathway in mouse skin-derived precursor supernatant after ultraviolet B (UVB) irradiation could alleviate the UVB irradiation damage (Li et al., 2020). This indicates that TGF-β may be an aging skin marker (Gunin and Golubtzova, 2019). Activation of the TGF-β signaling pathway has been found to downregulate sebocyte differentiation markers, such as fatty acid desaturase 2 (FADS2) and PPARγ, inhibit sebum secretion, and maintain the undifferentiated state of sebocytes (McNairn et al., 2013).

However, in fibroblasts, TGF-β was regarded as a rejuvenation marker during skin aging since it is a major regulator of the extracellular matrix, and reduction of TGF-β

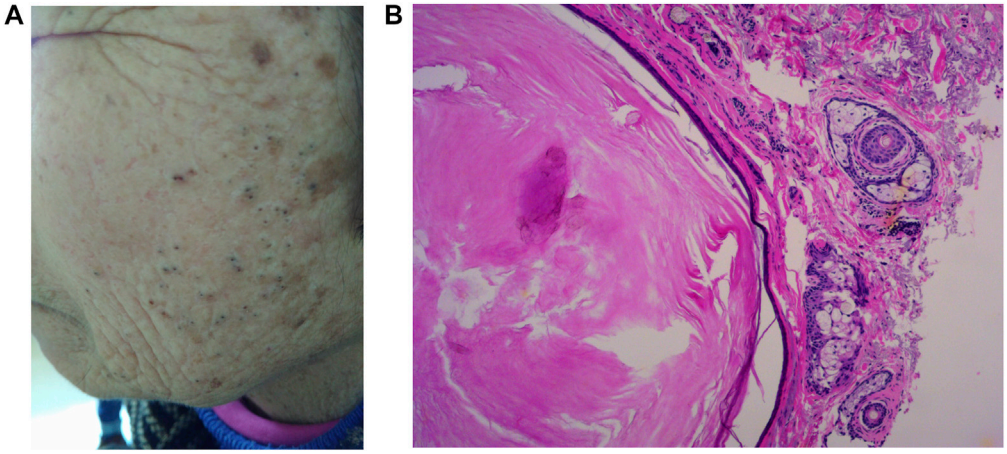

FIGURE 2
Favre–Racouchot disease (FRD) manifests as cutaneous atrophy and elastosis with keratinization of the pilosebaceous follicle and comedone formation and mainly affects the skin which is greatly exposed to sunlight **(A)**. Histology shows atrophic and keratinized SGs **(B)**.

was involved in the degradation of collagen and elastin fibers. In aged skin, activator protein-1 (AP-1) inhibits the TGF-β signaling pathway in fibroblasts and decreased the synthesis of collagen (Fisher et al., 2016). UV induced inhibition of the TGF-β signaling pathway by downregulating the TGF-β receptor type II (TbRII) and over-expressing Smad7 in human skin epidermis (Han et al., 2005).

p53

It has been demonstrated in several studies that the activation of p53 results in accelerated aging phenotypes in mice models (Tyner et al., 2002; Maier et al., 2004; Gannon et al., 2011), showing slow hair follicle cycling, epidermis thinning, reduced wound healing, and reduction of subcutaneous adipose lipid. Chronic activation of p53 can also lead to a decrease of Blimp1-positive sebocytes (sebaceous gland progenitor cells). Activation of p53 depletes the differentiation of sebaceous progenitor cells by activating PPARγ, resulting in the deplenishment of sebaceous progenitor cells, which in turn causes the atrophy of the entire sebaceous gland (Kim et al., 2014). It has also been reported that activation of p53 can inhibit c-MYC-induced sebaceous gland differentiation (Cottle et al., 2013) and attenuate the expression of insulin growth factor-1 receptor (IGF1R) (Werner et al., 1996) and AR (Shenk et al., 2001; Melnik, 2017), thus inhibiting the differentiation of sebocytes by suppressing the transactivation of PPARγ. In addition, p53 is mutated in 2/3 of sebaceous carcinomas (Kiyosaki et al., 2010), which is another manifestation of SG senescence.

Aryl hydrocarbon receptor

Environmental pollutants are believed to induce a range of skin conditions, including skin aging. Since they are natural ligands of the aryl hydrocarbon receptor (AhR), they usually disturb cell differentiation and lipogenesis. AhR signaling mediates cell apoptosis, oxidative stress, hyperpigmentation, and subcellular organelle dysfunction induced by particulate matter (PM) 2.5 in HaCaT keratinocytes (Piao et al., 2018; Shi et al., 2021). Correspondingly, Liu et al. have shown that a standard reference material of air pollution PM induced human skin keratinocyte and dermal fibroblast aging through cell growth inhibition and cell arrest, which could cause skin barrier damage and collagen degradation. The translocation of AhR into the nucleus, ERK, and c-Jun activation and aging-related gene transcription play a vital role in the aging process (Qiao et al., 2017). AhR was found to be expressed in SGs and immortalized sebocytes (Ju et al., 2011). Activation of AhR inhibits lipogenesis and alters sebocyte differentiation by reversing the differentiation lineage toward keratinocytes (Hu et al., 2016). Therefore, reduced numbers of terminally differentiated sebocytes and reduced sebum secretion occur. AhR was proven to modulate peptidoglycan (PGN)-induced expressions of tumor necrosis factor (TNF)-α and interleukin (IL)-8 in human SZ95 sebocytes, which intensified the inflammatory signaling in SGs (Hou et al., 2019). Elevated inflammation plays a vital role in skin aging. In general, sustained activation of AhR may lead to SG aging in terms of cell development and inflammation.

c-Myc

As a marker of basal proliferative sebocytes, c-Myc expression decreases along with the differentiation of sebocytes. Low levels of c-Myc activate AR, inhibit p53 activation, and promote SG differentiation and enlargement. High c-Myc activity induces p53 activation, thereby leading to SG proliferation and hyperplasia by blocking AR signaling (Lo Celso et al., 2008; Berta et al., 2010; Cottle et al., 2013). c-Myc mRNA and protein levels increased in SZ95 sebocytes incubated with elderly (60-year-old) female hormones for 5 days compared to sebocytes maintained in young (20-year-old) female hormones (Makrantonaki et al., 2006), which indicate that the differentiation ability decreased in the SG along with aging.

Hedgehog

Upregulation of the Hedgehog (Hh) pathway stimulates the proliferation of undifferentiated sebocytes, and there are crosstalks between Hh and Wnt-β-catenin signaling pathways (Niemann et al., 2003). The expression trend of Hh was consistent with that of β-catenin (Gat et al., 1998; Huelsken et al., 2001). Using the Hh inhibitor could reduce the cystic structures caused by aberrant activation of the Wnt-β-catenin signaling pathway (Shang et al., 2021). The Hh pathway also plays a vital role in basal cell carcinoma pathogenesis (Fania et al., 2020).

Notch

The Notch pathway has been reported to be involved in a variety of adult aging-related diseases, such as Alzheimer's disease, and cerebrovascular and cardiovascular diseases (Balistreri et al., 2016). *NOTCH2* was also found to be downregulated in aging sebocytes (Makrantonaki et al., 2006). Expressions of AR and PPARγ, as markers of early sebocyte differentiation, were detected unchanged even when the Notch pathway was knocked out; however, the expression of the terminal differentiation marker FASN was completely downregulated. Interestingly, SG cells rested at a stage of primary differentiation without progressing to full differentiation. Consequently, the accumulation of lipids started but stalled (Veniaminova et al., 2019).

Excluding the specific SG markers, genes involved in mitochondrial function, oxidative damage, and stress response showed altered expression in hormonally aged sebocytes, a fact that might lead to an increase in the accumulation of free radicals (Makrantonaki et al., 2006). Genes involved in the ubiquitin-proteosome pathway were downregulated, resulting in the accumulation of highly misfolded and damaged proteins. The expression of genes involved in cholesterol and fatty acid biosynthesis declined, contributing to the decrease in sebum amounts (Makrantonaki et al., 2006).

Changes in clinical features of sebaceous glands with aging

Sebum changes with aging

Sebaceous lipids are ubiquitously synthesized from sebocytes and secreted together with cell debris as sebum, contributing to ultraviolet protection, antioxidation, compound absorption, antibacterial effects, and skin hydration to protect the human skin (Zouboulis et al., 2016). Sebum secretion is relatively low in children as the level of circulating androgens including testosterone, dehydroepiandroster one sulphate (DHEAs), and insulin growth factor-1 begins to increase with adrenarche and further during puberty. The onset of puberty is often accompanied by a marked physiological increase in sebum, which is an important factor in the pathophysiology of acne vulgaris (Rocha and Bagatin, 2018). In elderly males, sebum production remains almost unchanged compared with that of young males even at the age of 80, while the sebum content in women begins to decline with menopause (Pochi et al., 1979; Zouboulis et al., 2022). In a large Chinese cohort, the skin surface sebum content was measured, and it was found that there was a peak at around the age of 40 years in females and 50 years in males, which could be some race/ethnic disparities. Meanwhile, the sebum content on the forehead in both males and females was higher than that on the forearm, and the level of sebum in males was always higher than that in females in different age groups (Man et al., 2009).

In addition, substantial changes in sebum composition occur with aging. As early as 1972, Cotterill et al. (1972) reported no significant differences in the sum of the percentages of triglycerides (TG) and free fatty acids (FFA) among different ages or sex, while the degree of hydrolysis varied considerably with age. Squalene is an unsaturated hydrocarbon produced by human SGs, and its content reached a maximum between the ages of 20 and 40 years in males, thereafter decreasing in the 41–60 age group. In addition, wax ester secretion rates reached their highest at the age between 15 and 35 and appeared to decline continuously throughout the elderly age range (Jacobsen et al., 1985). It should also be mentioned that photoaging could cause a range of changes in sebum components. Kim et al. (2010) observed that the levels of TG and FFA were significantly decreased in the epidermis of photoaged or acutely ultraviolet (UV)-irradiated human skin. Furthermore, they also demonstrated that triolein reduced basal and UV-induced metalloproteinase-1 (MMP-1) mRNA expression in cultured human epidermal keratinocytes, while various lipid synthesis enzyme inhibitors increased the MMP-1 expression significantly

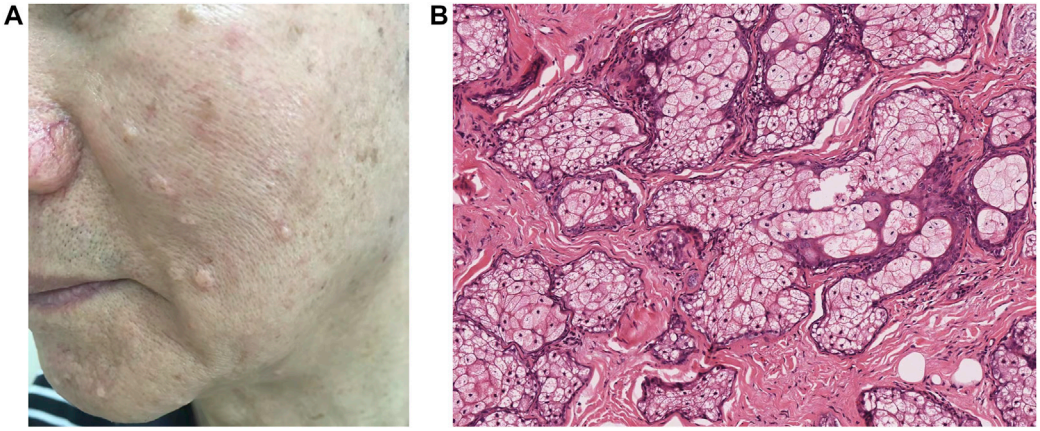

FIGURE 3
Skin-colored papules represent hyperplastic sebaceous glands disseminated on the face of an elderly patient. Clinically manifested as single or multiple pale yellow or skin color papules and nodules **(A)**, a great number of progenitor cells, and less mature sebocytes in histology **(B)**.

in a dose-dependent manner, hinting that TG and FFA may play important roles in photoaging of the human skin. In addition, free radicals generated by UV light could induce oxidative stress and promote the formation of squalene hydroperoxide and then cause the thickening of the epidermal layers, forming many deep crests on the skin surface and ultimately leading to skin wrinkling and photoaging in the human skin (Gat et al., 1998; Niemann et al., 2003) and hairless mouse skin (Chiba et al., 1999; Chiba et al., 2003). Moreover, UVB radiation can also affect lipid levels and lipid profiles *in vitro* and *in vivo* (Akitomo et al., 2003; Sato et al., 2017). Clinical research comparing all the differences in the sebum composition at the same time of individuals of different ages is still lacking.

With aging, skin becomes dryer and characterized by a lack of brightness of the skin surface, roughness, xerosis, desquamation, and pruritus, which is related to the decrease in sebum secretion and the reduced levels of epidermal and sebaceous lipids with age (Balin and Pratt, 1989).

Morphological and pathological changes of sebaceous glands with aging

The number of SGs basically remains unchanged, while the size of the SGs tends to initially increase with aging in the early stage (Plewig and Kligman, 1978; Fenske and Lober, 1986), especially in light-exposed skin (Zouboulis et al., 2016). However, in the late stage of aging or with excessive light exposure, the SGs would atrophy. Favre–Racouchot disease (FRD) is a typical disorder that mainly affects the elderly who are significantly exposed to sunlight. It manifests as cutaneous atrophy and elastosis with keratinization of the pilosebaceous follicle and the formation of pseudocomedones, which represent superficial epithelial tunnels (Helm, 1961; Patterson et al., 2004) (Figure 2A). The keratinization of the pilosebaceous follicle (Figure 2B) is assumed to be associated with the activation of Wnt/β-catenin, NOTCH, and p53 pathways, which leads to the proliferation and de-differentiation of sebocytes. There is probably a similarity in the pathogenesis of intrinsically aging-induced SG hyperplasia and photoaging-induced FRD.

The incidence of SG hyperplasia is 1% among healthy people, while in patients undergoing heart transplantation and taking immunosuppressive medications, it is 16% (de Berker et al., 1996). SG hyperplasia develops mainly in patients above 50 years, and it is mostly seen in the forehead and cheeks of elderly people, which may be related to the exposure of chronic sun exposure (Zouboulis and Boschnakow, 2001), and is clinically manifested as single or multiple pale yellow or skin color papules and nodules with a diameter of about 1–5 mm (Figure 3).

It is believed that the occurrence of SG hyperplasia in the elderly may be related to the decrease in androgen levels, which reduces the cellular turnover of sebocytes and subsequently leads to compensatory hyperplasia of SGs (Plewig and Kligman, 1978; Pochi et al., 1979; Fenske and Lober, 1986). In addition, the hormonal influence of insulin, thyroid stimulating hormone, and hydrocortisone may also increase sebocyte proliferation and contribute to SG hyperplasia (Fabiola Farci, 2022). At the same time, UV, especially UVA, may also cause SG hyperplasia in elderly patients and also induce the secretion of inflammatory cytokines including interleukins IL-1β and IL-8 in human

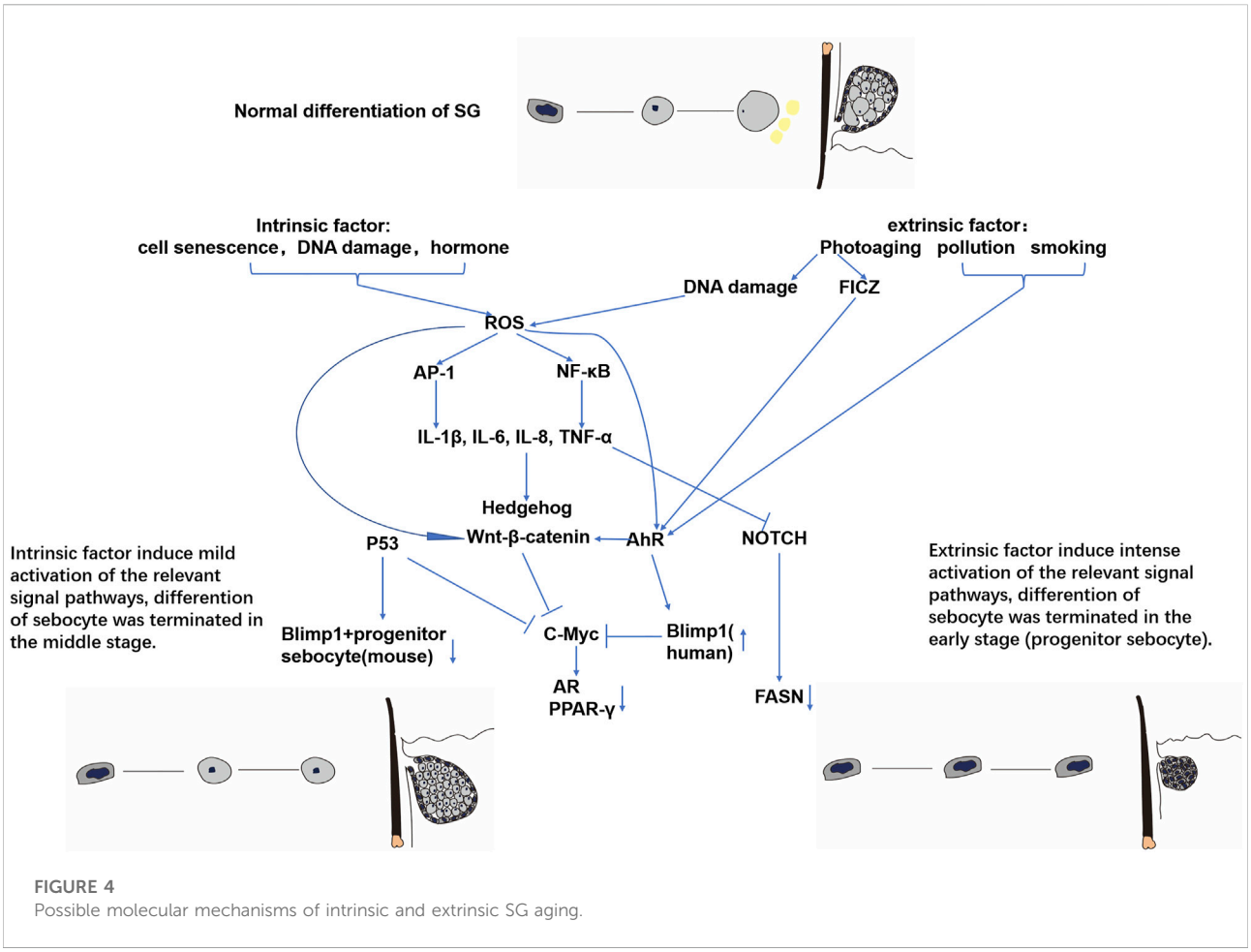

FIGURE 4
Possible molecular mechanisms of intrinsic and extrinsic SG aging.

sebocytes *in vitro* (Lee et al., 2013). However, opposing opinions have also been presented that chronic solar exposure was not a likely cause of the occurrence of SG hyperplasia and senile pseudocomedones (Kumar and Marks, 1987). Further studies are required to investigate how androgens and UV radiation interact with cellular turnover and differentiation of sebocytes then leading to SG hyperplasia.

The histological examination shows that numerous SGs filled with mature sebocytes are diffusely distributed in the superficial dermis, and the lobules open in the center of the dilated SG duct. The presence of four or more sebaceous lobules around a hair follicle has been suggested as a diagnostic criterion (Fabiola Farci, 2022). The prominent mature sebocytes present a vacuolized morphology and are rich in lipid vesicles. The basement membrane of SGs in young people tends to be thick, while the rim of basal cells in the elderly is much thinner. In addition, their fibers in the upper dermis are no longer elastic and manifest as distorted, thicker, and coagulated (Montagna and Carlisle, 1990; Zouboulis and Boschnakow, 2001).

Factors of sebaceous gland aging

Skin aging including SG aging can be classified as physiological (internal/chronological) aging and exogenous aging. The intrinsic factors that cause chronological aging include genetic, neuroendocrine system variation, and skin diseases.

Genetically, random processes lead to random cell senescence and DNA damage due to the production of free radicals, which also modify the inflammation status in the skin (Puizina-Ivic, 2008). Endogenous reactive oxygen species (ROS) are also heavily produced by mitochondria as they age (Chance et al., 1979). On the other hand, the neuroendocrine system varies along with the age, adrenal secretion of the steroid precursors dehydroepiandroster one (DHEA) and DHEAs, and hormones which are converted into androgen and estrogens and gradually decline over time (Herbert, 1995; Ferrari et al., 2000). (Figure 4).

Extrinsic factors, which might influence SGs' functions, include sunlight exposure (photoaging), pollution, smoking, and lifestyle factors such as diet, sleeping rhythm, and alcohol intake.

Photoaging

UVC (100–290 nm) is mostly blocked by the ozone layer, while UVB only penetrates into the epidermis and causes skin pigmentation. It has also been involved in photocarcinogenesis and skin-associated immunosuppression (Gilchrest, 1996; Benjamin et al., 2008). UVA is known to penetrate the dermis and is acutely responsible for skin erythema and mostly chronic skin damage (Gilchrest, 1996). Cumulative UVA is absorbed by cellular chromophores and generates ROS, including superoxide anion, hydrogen peroxide, and singlet oxygen, which could induce transcription factor activator protein-1 (AP-1) and nuclear factor kappa-B (NF-κB). The activation of AP-1 leads to the elevated expression of metalloproteases (MMPs), which could degrade collagen I and III. The activation of NF-κB upregulates the expression of a series of proinflammatory cytokines including IL-1β, TNF-α, IL-6, and IL-8. ROS production activates the Wnt/β-catenin pathway during mesenchymal stem cell aging (Zhang et al., 2013). Further research about the exact mechanism between ROS and the Wnt/β-catenin pathway in sebocytes needs to be conducted. It can be reasonably assumed that ROS and chronic inflammation could lead to SG hyperplasia through the activation of Wnt/β-catenin, NOTCH, Hedgehog, and p53 pathways.

Environmental pollution

An increasing number of studies have investigated the association between environmental pollution and skin aging (Li et al., 2015; Huls et al., 2016; Ding et al., 2017; Fuks et al., 2019). Pollutants include O_3, PM, nitrogen oxide (NO_2), cigarette smoke, and solid fuels, which can generate a substantial amount of polycyclic aromatic hydrocarbons (PAHs) and carbon monoxide (Vierkotter et al., 2010; Clark et al., 2013; Huls et al., 2016; Fuks et al., 2019). Mechanistically, these environmental pollutants generate free radicals on the skin including SGs, further activating nuclear factor erythroid-related factor 2 (Nrf2), AhR, AP-1, and NF-κB. On the other hand, 4-hydroxynonenal, the main product of oxidative stress after exposure to O_3, cigarette smoke, and PM, could directly regulate the activity of Nrf2, AP-1, PPARs, and AhR. As we mentioned earlier, upregulation of AhR could further reduce the lipid secretion of SGs by promoting sebocyte differentiation into keratinocytes. The evaluated inflammation through activation of AP-1 and NF-κB can induce the keratinization and hyperplasia signaling pathways in SGs. In an own previous study, benzo(a) pyrene (BaP), a compound found in cigarette smoke (Ortiz and Grando, 2012), has been shown to stimulate the secretion of IL-6 and reduce lipogenesis in SZ95 sebocytes (Sheikh et al., 2016; Hu et al., 2016). 2,3,7,8-Tetrachlorodibenzodioxin (TCDD) is the most potent compound of PAHs and the classic agonist of AhR, and its accumulation in sebum results in de-differentiation of sebocytes and dermal cyst formation by inhibition of the c-Myc signaling pathway and upregulates the Wnt pathway (Kretzschmar et al., 2014). TCDD could also enhance TNF-α and IL-8 secretion in PGN-treated sebocytes as well (Hou et al., 2019), further reinforcing SG aging.

Management of sebaceous gland aging

Prevention

Skin aging is a dynamic, multifactorial process, and the evidence level of the management of SG aging is still lacking. Several topical skin care products have been introduced in the prevention and treatment of SG aging, including sunscreens, anti-oxidants, vitamin C, and vitamin E (Zouboulis and Makrantonaki, 2011). Topical use of vitamins C and E improved wrinkles, skin tone, and texture, indicating their anti-aging and brightening effects of skin (Rattanawiwatpong et al., 2020; Jagdeo et al., 2021). In addition, eating a diet that is high in vegetables and fruits and avoiding cigarette smoking and pollution should also be noted (Farage KWM and Maibach, 2017). Recently, some randomized controlled studies had demonstrated that daily almond consumption may reduce wrinkle severity and improve skin pigmentation in postmenopausal women (Foolad et al., 2019; Rybak et al., 2021).

Treatment

Age-related natural hormone reduction is a common condition that can be typically treated with hormonal replacement therapy (HRT). Previous studies have confirmed that estrogen use was associated with a statistically significant decrease in the likelihood of senile dry skin and skin wrinkling (Dunn et al., 1997). Moreover, topically administered estradiol and methyl estradiolpropanoate (MEP) as in anti-aging cosmeceuticals with estrogen-like cutaneous effects have also been found to increase sebum levels and improve skin dryness in menopausal women (Callens et al., 1996; Draelos, 2018). Moreover, skin surface lipids have been shown to be increased in patients supplemented with both estrogen and progesterone, while estrogen alone has a sebum-suppressive action, hinting at the sebum secretion promoting effects of progesterone (Sator et al., 2001). Safety concerns have led to the application of HRT with bioidentical hormones at individualized doses tailored to each patient (Rosenthal et al., 2019). Apart from bioidentical hormones, newly discovered phytoestrogens from fermented soybean extracts have been found to improve skin hydration and viscoelasticity in rats without systemic toxicities (Rungseevijitprapa et al., 2021). However, it should be noted that HRT could increase the risk of breast, endometrial, and

ovarian cancers, so the doses should be tailored to each patient (Rees, 2011). Further studies should be conducted to provide more evidence in the treatment of SG aging.

SG hyperplasia is a relatively benign disorder, which does not usually require treatment. However, skin biopsies should be performed to differentially diagnose non-melanoma skin cancer (Salim et al., 2006). In addition, treatment can be conducted when skin lesions are unsightly and cause psychological distress for patients. Several treatment options exist including cryosurgery (Ataş and Gönül, 2017), photodynamic therapy (Horio et al., 2003), laser treatment (argon, carbon dioxide, or pulsed-dye laser) (Aghassi et al., 2000; Simmons et al., 2015), cauterization or electrodesiccation shaving or excision (Bader and Scarborough, 2000), topical treatments with chloroacetic or trichloroacetic acid, and systemic treatment with isotretinoin (Farage KWM and Maibach, 2017).

Summary

Research and discussions on skin aging focus on epidermal changes and degradation of dermal collagen. This review mainly discussed the alteration in lipid secretion and the changes in related molecular mechanisms of SGs in endogenous and exogenous aging. In the initial stage of aging, the differentiation of SGs is inhibited, and the proliferation is increased. Therefore, SGs show reduced lipid secretion and gland hyperplasia. In the late stage of aging with excessive photo exposure or environmental pollution, the over-activation of related molecular signaling pathways causes SG progenitor cells to differentiate into keratinocytes, which induces keratinization of pilosebaceous units, the most characteristic manifestation of Favre–Racouchot disease. There are still many unknown mechanisms in SG aging to be explored, and research needs to focus on the SG aging molecular mechanisms.

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XH and ZW conducted the literature search and drafted the manuscript. QJ and CCZ revised the manuscript. All authors have read and approved the manuscript.

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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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