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ORIGINAL ARTICLE

# Disorganisation of basement membrane zone architecture impairs melanocyte residence in vitiligo

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## Abstract

**The basement membrane zone is the interface between the epidermis and dermis, and it is disrupted in several skin conditions. Here, we report the results of a comprehensive investigation into the structural and molecular factors of the basement membrane zone in vitiligo, a dermatological disorder characterised by depigmented patches on the skin. Using electron microscopy and immunofluorescence staining, we confirmed abnormal basement membrane zone morphology and disrupted basement membrane zone architecture in human vitiliginous skin. Furthermore, we identified elevated expression of matrix metalloproteinase 2 (MMP2) in human dermal fibroblasts as a key factor responsible for basement membrane zone matrix degradation. In our *in vitro* and *ex vivo* models, overexpression of MMP2 in fibroblasts led to basement membrane zone disruption and melanocyte disappearance. Importantly, we reveal that the loss of melanocytes in vitiligo is primarily linked to their weakened adhesion to the basement membrane, mediated by binding between integrin β1 and laminin and discoidin domain receptor 1 and collagen IV. Finally, inhibition of matrix metalloproteinase 2 expression reversed depigmentation in a mouse model of vitiligo. In conclusion, our research shows the importance of basement membrane zone integrity in melanocyte residence and offers new avenues for therapeutic interventions to address this challenging skin condition.**



**Keywords:** fibroblast; melanocyte; basement membrane; vitiligo; MMP2

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*No conflicts of interest were declared.*

## Introduction

The basement membrane zone (BMZ) is a critical superstructure in the skin that connects and separates the epidermis and the dermis [1,2]. It is highly specialised, maintaining the integrity of the skin and serving as an architectural link and a functional continuum between the epidermis and the underlying dermis [3]. The BMZ is composed of four distinct layers: the basal cell layer, the lamina lucida, the lamina densa, and the sublamina densa (superficial papillary dermis) [4]. The BMZ's molecular and structural specialisation is crucial for intertissue interactions, including epithelial–fibroblast, epithelial–muscle, and epithelial–nerve interactions [5].

Differentiated epidermal melanocytes are specialised, pigment-producing cells derived from the neural crest. Under usual homeostatic conditions, they localise in the basal layer of the epidermis where they are maintained in a quiescent state and rarely proliferate, keeping a lifelong stable ratio of 1:5 with basal keratinocytes [6]. Melanocyte homeostasis is intricately regulated by epidermal keratinocytes, the surrounding basement membrane (BM) stroma, and nearby dermal cells [7]. Cellular physiology and pathophysiology depend on cell–cell and cell-matrix interactions, and differentiated epidermal melanocytes are strictly localised at the BM and cannot survive if they leave the basal layers, except in nevi or melanomas [8]. Perturbations in this delicate balance can lead to intriguing pigmentary disorders (for example, freckles, melasma, vitiligo) [6,9].

Vitiligo is a puzzling disorder characterised by selective loss of epidermal melanocytes by an unknown mechanism [10]. Vitiligo was first described 3,500 years ago and is a common skin disorder with an incidence of 1–2% worldwide [11,12]. The most striking histological

feature of vitiligo is the disappearance of melanocytes in the epidermis. The cellular and molecular mechanisms underlying melanocyte disappearance have attracted much interest from cell biologists and dermatologists. So far, many theories regarding the cause of melanocyte disappearance have been proposed, including autoimmune mechanisms, neural deficiencies, impaired oxidation–reduction status, and reduced melanocyte adhesion [13,14]. A consensus on the true mechanism remains elusive. Autoreactive $CD8^+$ T cells have been implicated in melanocyte destruction [13,15], but a deeper exploration should be conducted to examine the involvement of other cutaneous cells and the complexities of the microenvironment surrounding melanocytes in mediating their loss.

The BM is a thin sheet of highly specialised extracellular matrix [1,2] located between the epidermis and the dermis that participates in diverse cellular functions including adhesion, proliferation, differentiation, and survival [3]. Destruction of BM extracellular matrix macromolecules is seen in diseases such as arthritis [16], periodontal disease [17], and dermatological disorders [18]. Several studies have also noticed abnormal BM morphology in lesional skin of patients with vitiligo [19–22]. However, further investigations of BM in vitiligo pathology remain limited.

Matrix metalloproteinase 2 (MMP2), also known as gelatinase A or type IV collagenase, plays a crucial part in the dynamics of the BM through degrading key components, such as type IV collagen and laminin [23]. Although several studies have focused on MMP2 expression in vitiligo [24–26], in-depth investigations into its specific role in the pathogenesis are still insufficient.

Given that the BM provides structural support for keratinocytes and melanocytes, it is reasonable to hypothesise that structural abnormalities of the BM may have an impact on melanocyte disappearance in vitiligo. Therefore, in this study, we examined the precise mechanisms behind melanocyte loss from the BM in vitiligo, integrating observations of MMP2 involvement and BM integrity to better understand the disease's pathology.

## Materials and methods

### Human skin specimens

Frozen biopsies were obtained from lesional and perilesional skin of well-defined patients with nonsegmental vitiligo ($n = 11$, seven patients in a progressive state and four patients in a stable state) (supplementary material, Table S1) and from corresponding sites on healthy donors ($n = 5$). More details are presented in the Supplementary materials and methods. Written informed consent was obtained from all participants prior to study inclusion. The study was approved by the Medical Ethics Committee of Osaka Metropolitan University (No. 4152). All procedures involving human subjects were in accordance with the Helsinki Declaration of 1975, as revised in 1983.

### Fluorescence immunohistochemistry

Human skin tissue and reconstructed 3D skin equivalent were embedded in OCT compound (Sakura Finetek, Tokyo, Japan) and were cut into 6-μm sections for fluorescence immunohistochemistry staining and analyses. Details are presented in Supplementary materials and methods.

### Electron microscopy and pre-embedding immunoelectron microscopy

Skin punch biopsies (1-mm in diameter) were fixed for electron microscopy. Pre-embedding gold-enhancement immunogold labelling was performed as detailed previously [27] with a slight modification. Details are presented in Supplementary materials and methods.

### Western blot

Fresh skin punch biopsies were processed for protein extraction as described previously [28]. Details are presented in Supplementary materials and methods.

### Cell culture and establishment of MMP2-overexpressing cells

Human dermal fibroblasts and C57BL/6J mouse dermal fibroblasts were cultured and then transfected with an MMP2-expressing episomal vector. Details are presented in Supplementary materials and methods.

### Reconstructed 3D full-thickness human skin equivalents

A reconstructed 3D full-thickness human skin-equivalent model was generated as described previously [29] with modifications. Details are presented in Supplementary materials and methods.

### *Ex vivo* human skin explant culture

Human skin from the lateral abdomen was obtained with the informed consent of patients undergoing abdomen-reduction surgery, in accordance with the ethical guidelines of the Medical Ethics Committee of Osaka Metropolitan University (No. 4152). Details are presented in Supplementary materials and methods.

### *In situ* proximity ligation assay

Multicolour *in situ* proximity ligation assay (PLA) was performed using the Duolink PLA Multicolour Reagent Pack (DUO96000, Millipore Sigma, Burlington, MA, USA) as described previously [30]

with slight modifications. Details are presented in Supplementary materials and methods.

### Mice and *in vivo* experimental procedures

Experiments were conducted in accordance with the 'Guiding principles for the care and use of animals in the field of physiological sciences' (http://physiology.jp/wp-content/uploads/2015/07/Guiding-principles.pdf) and the experimental protocol used in this study was approved by the Committee for Animal Experiments at Osaka Metropolitan University (permit No. 18042). More details are presented in the Supplementary materials and methods.

### Statistical analysis

Experiments were repeated at least three times. Data are presented as mean ± SD. One-way analysis of variance with Dunnet's test or unpaired Student's *t*-test (two-tailed) was used for statistical analyses. A $p$ value of less than 0.05 indicated statistical significance. All statistical analyses for this study were performed using GraphPad Prism version 8.0.0 for Windows (GraphPad Software, Boston, MA, USA).

## Results

### BMZ architecture is disorganised in vitiligo

To investigate the BMZ in vitiligo, we performed an ultrastructural analysis using skin biopsy specimens from five healthy donors and five patients with generalised vitiligo (two stable and three progressive cases). We observed abnormal BM morphology in specimens from patients with both stable and progressive vitiligo. Specifically, the BM of healthy skin contained intact lamina densa with high electron density, whereas the BM of vitiliginous skin contained branched and fragmented lamina densa, some of which were multilayered (Figure 1A). Moreover, the anchoring fibrils, which are usually evenly distributed in healthy skin, were scattered and distributed in both the upper and lower portions of the branched lamina densa in vitiliginous skin (Figure 1A, red asterisks).

Next, we used immunofluorescence staining to investigate the expression and localisation of BM proteins. The BM in healthy skin showed standard morphology with a thin, continuous linear band of collagen IV and a typical number and distribution of melanocytes. In contrast, vitiliginous skin displayed abnormal BM morphologies, including thickening, severe disruption, and

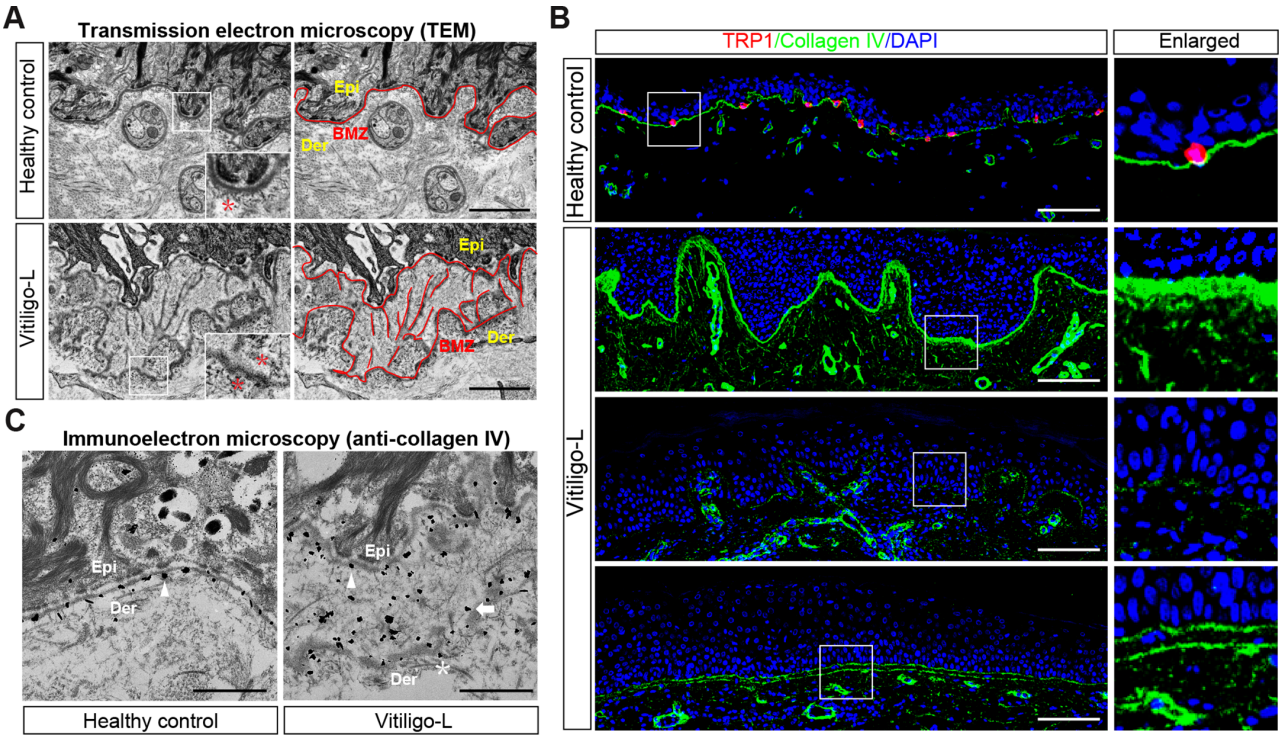


**Figure 1.** Disorganisation of basement membrane zone architecture in vitiligo. (A) Representative ultrastructural images of ultrathin sections of skin from five healthy controls and five patients with generalised vitiligo. The right panel shows the areas of epidermis (Epi), dermis (Der), and basement membrane zone (BMZ, red line). In the left panel, the area of the white rectangle is shown enlarged at the lower right, and anchoring fibrils (red asterisk) are shown. Vitiligo-L: lesional vitiliginous skin. Scale bars: 2 μm. (B) Representative immunofluorescence images of skin specimens from five healthy controls and 11 patients with generalised vitiligo stained with anti-collagen IV (green) and anti-tyrosinase-related protein 1 (TRP1, red) antibodies and DAPI (blue). The areas in the white rectangles are shown enlarged in the corresponding right panels. Scale bars: 100 μm. (C) Representative immunoelectron microscopy images of frozen sections stained with anti-collagen IV antibody. White arrowhead: collagen IV-labelled gold-particle deposits close to the lamina densa; white arrow: collagen IV-labelled gold-particle deposits close to the branched structure of the lamina densa; white asterisk: collagen IV-labelled gold-particle deposits close to the layered structure of the lamina densa. Scale bars: 1 μm.

multilayering, accompanied by the disappearance of melanocytes (Figure 1B).

We further investigated the ultrastructural localisation of collagen IV using immunoelectron microscopy. In healthy skin, we observed intact BM with collagen IV-labelled gold-particle deposits (Figure 1C, white arrowhead in left panel) located close to the lamina densa in a continuous arrangement. However, in vitiliginous skin, we observed conspicuous structural alterations of the BM, with collagen IV-labelled gold-particle deposits scattered across a wide area not only close to the usual lamina densa area (Figure 1C, white arrowhead in right panel) but also near the branched (Figure 1C, white arrow) and multilayered (Figure 1C, white asterisk) structures of the lamina densa. These results reveal a disrupted BMZ architecture in lesional vitiliginous skin.

## MMP2 expression is elevated in the dermis of vitiliginous skin

To investigate the cause of BM structure disruption, we measured the expression levels of MMP2 and MMP9, the major proteolytic enzymes capable of degrading BM collagen, in skin sections from patients with vitiligo. We found that vitiliginous skin had significantly elevated MMP2 expression in the upper dermis compared with healthy controls (Figure 2A,B).

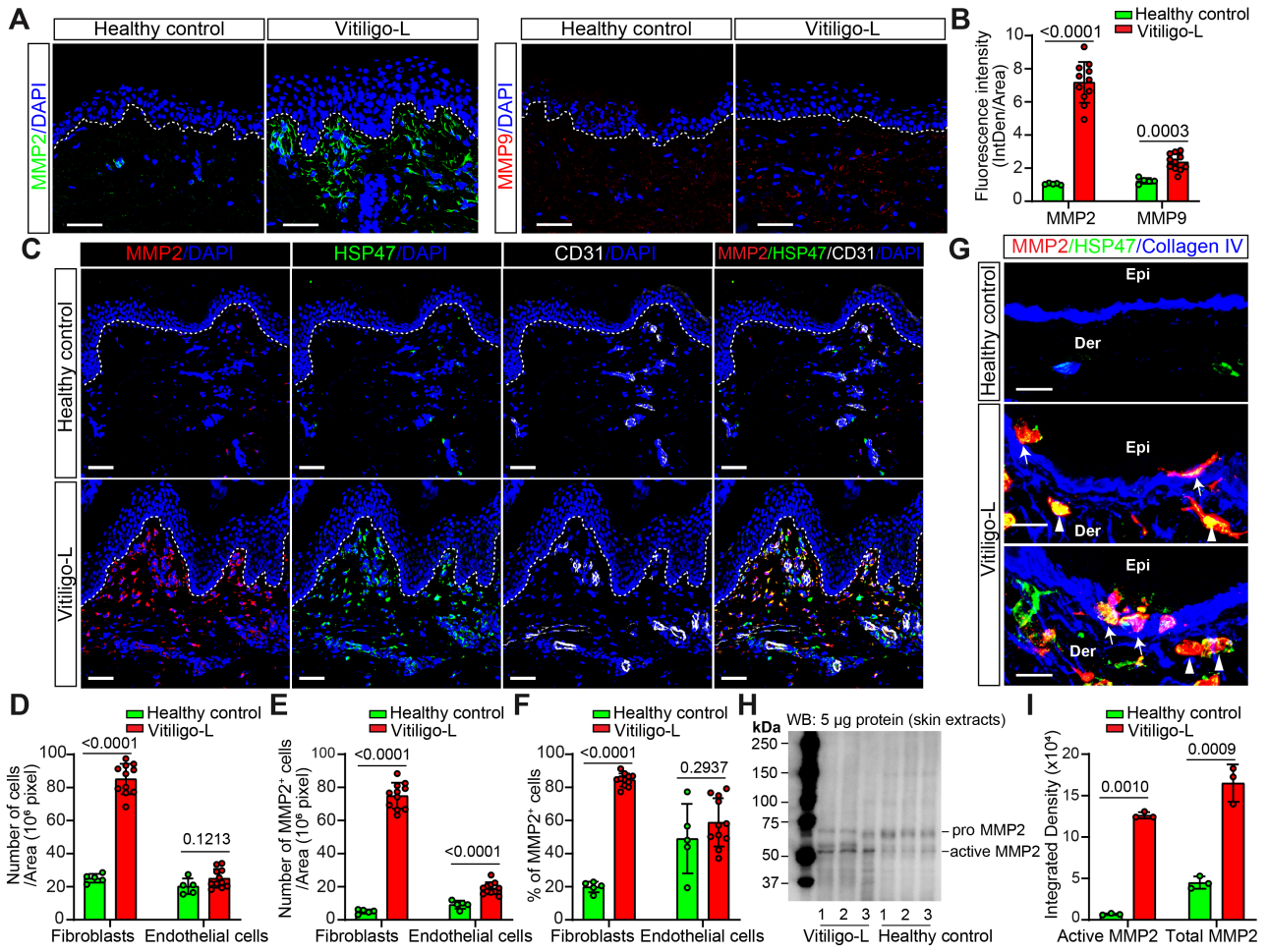


**Figure 2.** Matrix metalloproteinase 2 (MMP2) expression is elevated in the dermis of vitiliginous skin. (A) Representative immunofluorescence images of frozen sections of skin from five healthy controls and 11 patients with generalised vitiligo. MMP2 is stained green, and MMP9 is stained red. Vitiligo-L: lesional vitiliginous skin. (B) Quantitation of MMP2 and MMP9 expression by fluorescence intensity measurement. IntDen/area: integrated density/area. (C) Representative immunofluorescence staining of MMP2 (red), the fibroblast marker HSP47 (green), and the endothelial cell marker CD31 (white) with DAPI (blue) in skin from healthy controls and patients with vitiligo. (D) Quantitation of fibroblast and endothelial cell numbers per $10^6$ pixels in healthy and vitiliginous skin from the immunofluorescence staining data in C. (E) Quantitation of $MMP2^+$ fibroblast and $MMP2^+$ endothelial cell numbers per $10^6$ pixels from the immunofluorescence staining data in C. (F) The percentages of $MMP2^+$ fibroblasts among all fibroblasts and $MMP2^+$ endothelial cells among all endothelial cells in healthy and vitiliginous skin from the immunofluorescence staining data in C. (G) Representative immunofluorescence staining of $MMP2^+$ fibroblasts distributed around the basement membrane (BM). MMP2 is stained red; HSP47 is stained green; and collagen IV, the main component of the BM, is stained blue. White arrowhead: $MMP2^+$ fibroblasts close to the BM; white arrow: $MMP2^+$ fibroblasts that have passed through the BM. (H) Western blot analyses showing expression of the pro- and active forms of MMP2 in lysates from three healthy skin specimens and three lesional vitiliginous skin (vitiligo-L) specimens. (I) Quantitation of active MMP2 and total MMP2 (the total of active and pro MMP2) expression from the western blot data in H. Nuclei are counterstained with DAPI (blue) in A and C. Scale bars in A and C: 50 μm; in G: 20 μm. Data in B, D, E, F, and I are shown as mean ± SD.

In human skin, MMP2 is primarily produced in fibroblasts [31] and vascular endothelial cells [32]. To identify the main contributors to increased MMP2 levels in vitiliginous skin, we conducted immunolocalisation studies on healthy and vitiligo-affected skin. Frozen sections from five healthy controls and 11 patients with vitiligo were stained with anti-MMP2 antibodies, along with anti-HSP47 (fibroblast marker) and anti-CD31 (endothelial cell marker) antibodies. Fibroblast numbers were significantly higher in the vitiliginous skin than in healthy skin, while endothelial cell numbers showed no significant difference (Figure 2C,D). Moreover, MMP2-positive fibroblast numbers in vitiliginous skin were almost four times higher than in healthy skin (Figure 2E). As a percentage of the total number of MMP2-positive cells, MMP2-positive fibroblasts in vitiliginous skin were nearly four times more abundant than in healthy skin, with no significant difference in MMP2-positive endothelial cells (Figure 2F).

To investigate the localisation of MMP2-positive fibroblasts in relation to the BM, we conducted immunofluorescent labelling using anti-MMP2 antibodies and co-stained with anti-HSP47 and anti-collagen IV (BM marker) antibodies. Interestingly, MMP2-positive fibroblasts in vitiliginous skin were found in proximity to the BM (Figure 2G, white arrowheads) and were observed traversing the BM to reach the epidermis (Figure 2G, white arrows).

Next, we examined the activity of MMP2 by western blot using skin lysates from three patients with vitiligo (one stable and two progressive cases) and three healthy controls. MMP2 was highly expressed and mainly in its active form in vitiliginous skin, in contrast to healthy skin, in which it was slightly expressed and mainly in the inactive form (Figure 2H,I). These findings indicate that increased MMP2 expression by dermal fibroblasts in vitiliginous skin may contribute to BM destruction and, subsequently, melanocyte disappearance.

## Overexpression of MMP2 in dermal fibroblasts results in melanocyte disappearance

To determine whether damage to the BM precedes melanocyte disappearance, we developed an *in vitro* 3D skin-equivalent model. In this model, half of the collagen gel in the dermis layer was seeded with mock-transfected fibroblasts (mock-Fbs) and half with MMP2-overexpressing fibroblasts (MMP2-Fbs) (Figure 3A). Once the structured collagen gel was established, melanocytes and keratinocytes were uniformly seeded on top of the gel. After 1 week of air–liquid interface cell culture, macroscopically pigmented skin developed on the part of the gel prepared with mock-Fbs, whereas no pigmentation developed on the part of the gel prepared with MMP2-Fbs (Figure 3B). The 3D skin equivalents prepared with MMP2-Fbs had damaged BM and a lack of melanocytes, while those prepared with mock-Fbs had a typical BM morphology and distributed melanocytes in the basal layer (Figure 3C).

We further created an *ex vivo* subcutaneous injection model, in which mock-Fbs or MMP2-Fbs were subcutaneously inoculated into cultured, full-thickness, fresh skin explants. To track the injected fibroblasts, we labelled them with PKH fluorescence cell-membrane labelling dye (Figure 3D). Skin explants injected with MMP2-overexpressing fibroblasts displayed BM disruption and melanocyte disappearance (Figure 3E). These results indicate that increased MMP2 expression by dermal fibroblasts is a key factor in melanocyte disappearance in vitiligo.

## Melanocyte disappearance results from decreased adhesion to the BM

We performed immunofluorescent staining to further examine the disappearance of melanocytes from the damaged BM. Immunofluorescent staining revealed that melanocytes in depigmented 3D skin equivalents tended to be located at the suprabasal layer, detached from the BM, and were negative for cleaved caspase-3, suggesting that apoptotic events were not involved in their disappearance (supplementary material, Figure S1A).

We examined adhesion patterns between melanocytes and the BM matrix using *in situ* PLA, an antibody-based technology that enables the visualisation of protein–protein interactions. Specifically, we examined integrin β1–laminin binding and discoidin domain receptor 1 (DDR1)–collagen IV binding in 3D skin equivalents. In *in situ* PLA, when a pair of specific primary antibodies (DDR1 and collagen IV; integrin β1 and laminin) are in close proximity, complementary DNA strands on a corresponding pair of secondary antibodies with PLA probes engage in rolling circle amplification to generate a single fluorescence signal *in situ*, indicating the presence of the corresponding protein–protein interaction (supplementary material, Figure S1B). This assay revealed decreased interactions of DDR1–collagen IV and integrin β1–laminin in 3D skin equivalents prepared with MMP2-overexpressing fibroblasts compared with mock-Fbs (supplementary material, Figure S1C,D). Abnormal adhesion between melanocytes and the BM was implicated in 3D skin equivalents prepared with the MMP2-Fbs.

Immunofluorescence staining detected melanocyte detachment from the BM in perilesional skin of patients with vitiligo (Figure 4A). Expression levels of DDR1, collagen IV, integrin β1, and laminin were not significantly different between healthy and perilesional vitiliginous skin (Figure 4B,C,F). We used *in situ* PLA and co-immunohistochemistry staining of melanocytes using anti-tyrosinase-related protein 1 (anti-TRP1) antibodies to visualise adhesions between melanocytes and the BM matrix (Figure 4D,E, white rectangle in lower right panel). In healthy skin melanocytes, integrin β1–laminin interactions were more predominant compared with DDR1–collagen IV interactions.

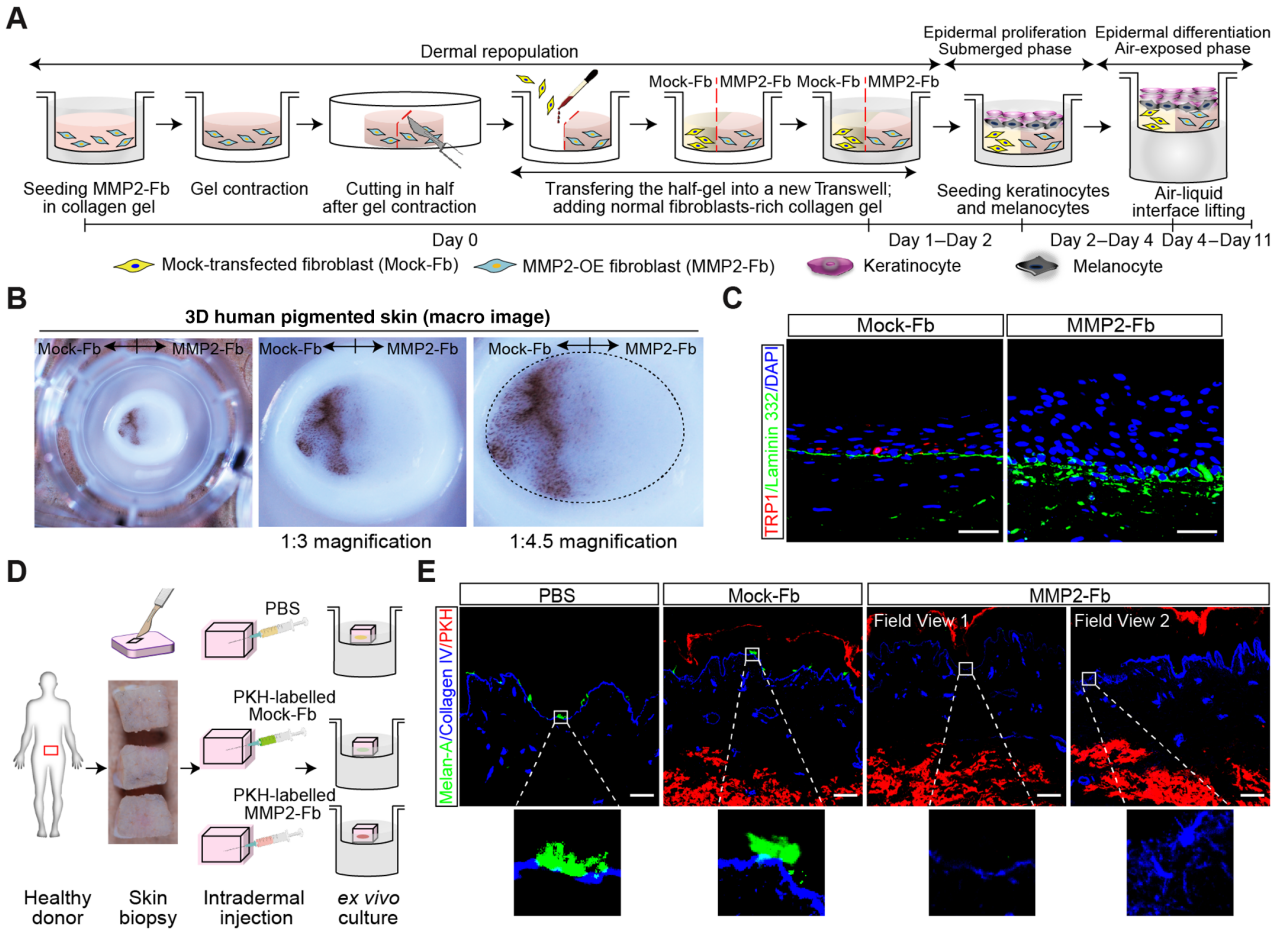


Figure 3. Overexpression of matrix metalloproteinase 2 (MMP2) in dermal fibroblasts results in melanocyte disappearance in 3D full-thickness human skin equivalents and human *ex vivo* cultured skin explants. (A) The steps for making the 3D full-thickness human skin-equivalent model. OE: overexpression. (B) Representative macroscopic images of skin equivalents on day 11. (C) Representative immunofluorescence staining of the melanocyte marker tyrosinase-related protein 1 (TRP1, red) and the basement membrane (BM) marker laminin 332 (green) in skin equivalents. Nuclei are counterstained with DAPI (blue). (D) The steps for making the human *ex vivo* cultured skin explant model. (E) Representative immunofluorescence staining of collagen IV (blue) and Melan-A (green) in cultured human skin explants. Intradermally injected fibroblasts are labelled with PKH fluorescent dye (red). The white rectangles outline the areas of melanocytes and BM, and the enlarged details are shown in the corresponding lower panels. Scale bars in C and E: 50 μm.

However, both interactions were significantly diminished in perilesional vitiliginous skin melanocytes (Figure 4E,G). These findings suggest that melanocyte loss in vitiligo may be linked to a reduced adhesion pattern between melanocytes and the BM (Figure 4H).

Unlike melanocyte detachment, keratinocyte detachment (*i.e.* blistering) was not observed in depigmented 3D skin equivalents and perilesional vitiliginous skin, despite weakened focal-adhesion signal (integrin β1–laminin binding) around basal keratinocytes (supplementary material, Figure S1C and Figure 4E). Hence, we investigated hemidesmosome protein expression and binding signals. Expression levels of integrin α6 and laminin were not different in healthy and lesional vitiliginous skin (supplementary material, Figure S2A,C). However, collagen XVII and collagen IV levels were significantly elevated in the lesional vitiliginous skin compared with healthy skin (supplementary material, Figure S2B,C). Using *in situ* PLA, we examined hemidesmosome adhesion signals between basal keratinocytes and the BM matrix (supplementary material, Figure S2D). In basal keratinocytes of healthy skin, integrin α6–laminin interactions predominated over collagen XVII–collagen IV interactions (supplementary material, Figure S2E,F). In contrast, basal keratinocytes of vitiliginous skin had a marked reduction in integrin α6–laminin interaction, while collagen XVII–collagen IV interaction notably increased (supplementary material, Figure S2E,F). These results suggest altered adhesion patterns between basal keratinocytes and the BM (supplementary material, Figure S2G).Compensatory enhanced hemidesmosome adhesion, possibly facilitated by collagen XVII binding to collagen IV, may underlie the absence of skin blistering.

## Decreased melanocyte adhesion to the BM is caused by abnormal BM architecture

The expression levels of DDR1, collagen IV, integrin β1, and laminin were similar between vitiliginous and healthy skin. However, vitiliginous skin had decreased DDR1–collagen IV and integrin β1–laminin

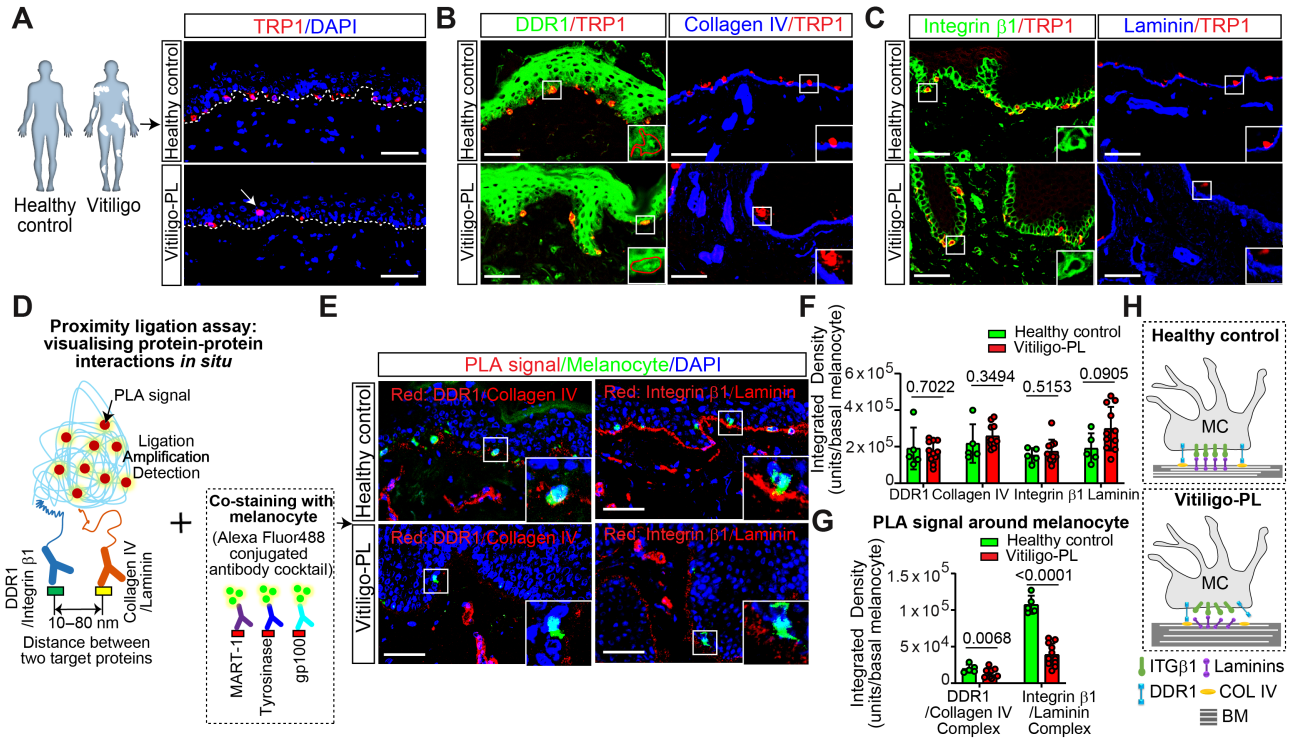


**Figure 4.** Melanocyte disappearance results from decreased adhesion to the basement membrane in vitiliginous skin. (A) Representative immunofluorescence staining of tyrosinase-related protein 1 (TRP1, red) in healthy skin and perilesional vitiliginous skin (vitiligo-PL). Nuclei are counterstained with DAPI (blue). A detached melanocyte is shown with a white arrow. (B) Representative immunofluorescence staining of discoidin domain receptor 1 (DDR1, green), collagen IV (blue), and TRP1 (red) in healthy skin and vitiligo-PL. Enlarged details of the areas in the white rectangles are shown on the lower right. The red lines circling the areas in the enlarged images indicate melanocytes. (C) Representative immunofluorescence staining of integrin β1 (green), laminin (blue), and TRP1 (red) in healthy skin and vitiligo-PL. Enlarged details of the areas in the white rectangles are shown on the lower right. (D) Schematic diagram of the proximity ligation assay (PLA) and melanocyte antibody (MART-1, Tyrosinase and gp100) cocktail immunofluorescence co-staining. (E) The interactions between DDR1 and collagen IV and between integrin β1 and laminin are visualised in red fluorescence using *in situ* PLAs. Melanocytes are visualised in green. Nuclei are counterstained with DAPI (blue). Enlarged details of the areas in the white rectangles are shown on the lower right. (F) Quantitation of the immunofluorescence signal around melanocytes in B and C. (G) Quantitation of the fluorescence intensity around melanocytes in PLAs in E. (H) Illustration of melanocyte (MC)-basement membrane (BM) adhesion in healthy skin and vitiligo-PL. COL: collagen; ITG: integrin. Scale bars in A, B, C, and E: 50 μm. Data in F and G are shown as mean ± SD.

interactions (Figure 4). To investigate the cause of these reduced interactions, we assessed the physical distance between melanocytes and the BM using transmission electron microscopy (Figure 5A). As basal cells typically attach to the lamina densa of the BM, we measured the width of the lamina lucida (the distance between the basal cell plasma membrane and the lamina densa) in both healthy and vitiliginous skin. Surprisingly, we found no difference between healthy skin and vitiliginous skin (Figure 5A,C).

To further explore the decreased DDR1–collagen IV and integrin β1–laminin bindings in vitiliginous skin, we performed immunoelectron microscopy. In healthy skin, laminin, the primary adhesion molecule responsible for melanocyte binding to the BM, was clearly distributed along and confined to the lamina densa. However, in lesional and perilesional vitiliginous skin, laminin was diffusely spread within the lamina densa and beneath the disorganised BM architecture (Figure 5B). Moreover, the amount of laminin confined in the lamina lucida was significantly reduced in vitiliginous skin compared with healthy skin (Figure 5B,D). These findings suggest that the disorganised arrangement of the BMZ may contribute to the loss of melanocytes, which rely on laminin for anchoring through the integrin β1 protein (Figure 5E).

## Inhibition of MMP2 reverses depigmentation in a novel mouse model of vitiligo

We used B6.Cg-Tg(KRT14-Kitl)1Takk/TakkRbrc transgenic mice, characterised by retained melanocytes in the epidermis, to investigate the impact of BM degradation on skin pigmentation. Murine mock-Fbs and MMP2-Fbs were subcutaneously transplanted into the back skin of B6.Cg-Tg(KRT14-Kitl)1Takk/TakkRbrc mice (Figure 6A). Subcutaneous transplantation of MMP2-Fbs in these mice resulted in vitiligo-like depigmentation (Figure 6B), with disrupted BM structure and absence of epidermal melanocytes (Figure 6C). Co-injection of MMP2-Fbs with MMP2 inhibitors (SB-3CT or tanomastat) reversed the depigmentation (Figure 6D–F), supporting the crucial role of MMP2 in BM disruption and melanocyte loss. These findings

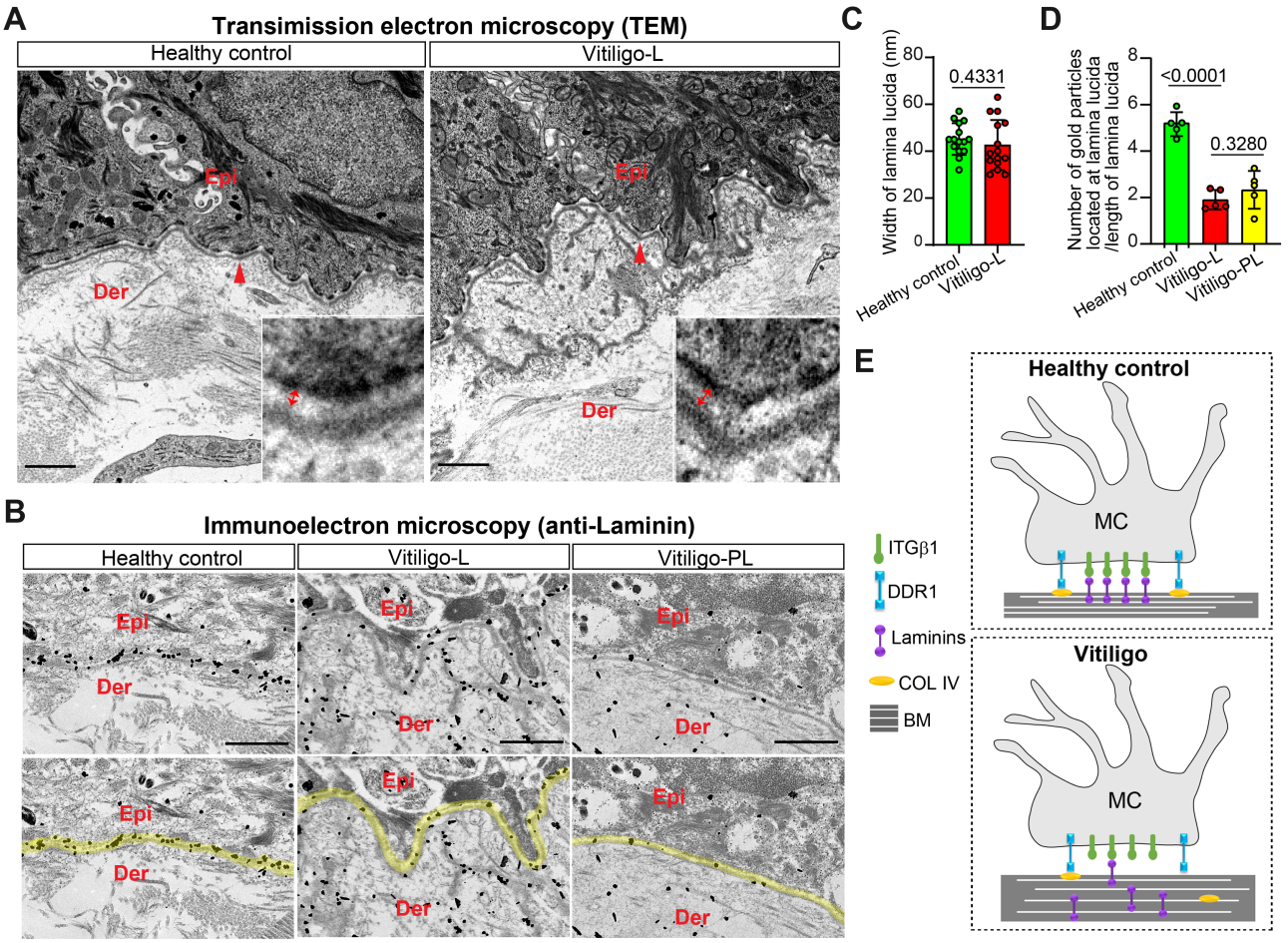


Figure 5. The decreased adhesion of melanocytes to the basement membrane is due to abnormal basement membrane architecture. (A) Representative ultrastructural images of ultrathin sections of skin from five healthy controls and five patients with generalised vitiligo. Vitiligo-L: lesional vitiliginous skin. Enlarged images of the areas indicated by the red arrowheads are shown on the lower right. The red double arrow indicates the lamina lucida. Epi: epidermis; Der: dermis. Scale bars: 1 μm. (B) Representative immunoelectron microscopy images of ultrathin skin sections stained with anti-laminin antibody. The area of the lamina lucida is outlined in yellow. Vitiligo-PL: perilesional vitiliginous skin. Scale bars: 1 μm. (C) Quantitation of the width of the lamina lucida in A. Three measurements were performed per section. (D) Quantitation of the ratio of the number of laminin gold particles located at the lamina lucida to the length of the lamina lucida. (E) Illustration of melanocyte (MC)-basement membrane (BM) adhesion in healthy skin and vitiliginous skin. DDR: discoidin domain receptor 1; COL: collagen; ITG: integrin. Data in C and D are shown as mean ± SD.

establish the B6.Cg-Tg(KRT14-Kitl)1Takk/TakkRbrc mouse model as a valuable tool for studying vitiligo pathogenesis and highlight MMP2 inhibition as a potential therapeutic approach for vitiligo treatment.

## Discussion

The BM of vitiliginous skin can be thickened [19,20], damaged [21], or branched [22], but it is unknown how the BM structure is disrupted in vitiligo and what role BM disruption plays in the pathophysiology of vitiligo. We confirmed abnormal BM structure in vitiliginous skin and that the disorganised BMZ prevents melanocytes from adhering to the BM via integrin β1–laminin binding. Moreover, we discovered that the disorganised BM architecture is caused by increased MMP2 expression in dermal fibroblasts. MMP2-Fbs implanted in 3D skin equivalents and *ex vivo* cultured skin explants showed BM structural disruption and melanocyte detachment. B6.Cg-Tg(KRT14-Kitl)1Takk/TakkRbrc mice developed vitiligo-like skin lesions after subcutaneous inoculation with MMP2-Fbs, and treatment with MMP2 inhibitors successfully reduced the depigmentation in these mice.

Melanocyte floating and defective melanocyte adhesion in vitiligo have been described previously [33,34], but most studies have focused on melanocyte–keratinocyte adhesion. Few investigations have looked at melanocyte–BM adhesion. Cells in the basal layer of the epidermis are bound to the BM mainly through integrins, but the mechanistic details of melanocyte adhesion to the BM are still unknown. Melanocytes have been reported to express integrin β1 both *in vitro* and *in vivo* [35]. In addition, DDR1, a membrane protein, was recently reported to bind collagen IV and mediate the adhesion of melanocytes to BM [36].

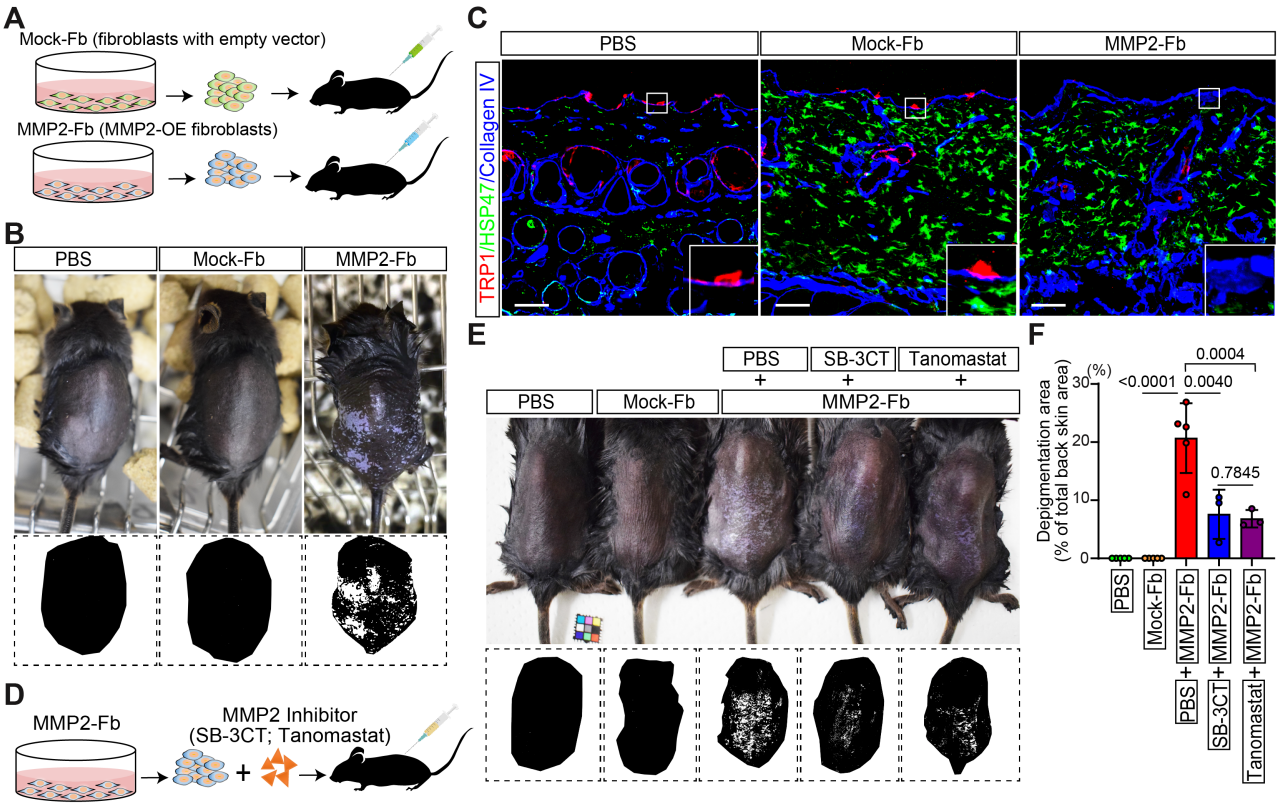


**Figure 6.** Subcutaneous inoculation of mice with matrix metalloproteinase 2 (MMP2)-overexpressing fibroblasts induced a vitiligo phenotype that could be reversed by inhibition of MMP2. (A) The steps to make the subcutaneous fibroblast inoculation mouse model. OE: overexpression. (B) Macro appearance of the mice at 3 weeks after inoculation with fibroblasts. The bottom panels show the corresponding threshold binarisation images of the back skin of the mice. (C) Representative immunofluorescence staining of tyrosinase-related protein 1 (TRP1, red), HSP47 (green), and collagen IV (blue) in mouse skin sections. Enlarged details of the areas in the white rectangles are shown in the corresponding lower panels. Scale bars: 50 μm. (D) The experimental procedure to treat mice with MMP2 inhibitor. (E) Macroscopic images of the mice 2 weeks after subcutaneous inoculation. The bottom panels show the corresponding threshold binarisation images of the back skin of the mice. (F) Quantitation of the percentage of depigmentation area on the back skin of the mice. Data in F are shown as mean ± SD.

Furthermore, some studies reported that loss of melanocytes in vitiligo resulted from decreased DDR1 expression in melanocytes [37,38]. We verified melanocyte detachment in vitiligo but found that neither the expression levels of melanocyte adhesion molecules nor the expression levels of BM proteins were decreased. Instead, immunoelectron microscopy, histoimmunofluorescence staining, and PLA analyses revealed the reduction in melanocyte adhesion, and the reduction was due to disorganisation of the BMZ architecture. These findings imply that the detachment of melanocytes in vitiligo is caused by an aberrant spatial distribution of adhesion molecules in the BM.

In contrast to the decreased adhesion and detachment of melanocytes, we did not observe any detachment of keratinocytes (*i.e.* blistering). The attachment of melanocytes to the BM is considerably weaker than that of keratinocytes and is mediated by integrin β1–laminin binding [35] and DDR1–collagen IV binding [36]. It is well known that there are no hemidesmosome complexes in melanocytes, as these structures are exclusive to keratinocytes [39]. The presence of intact hemidesmosomes in basal keratinocytes was confirmed by our ultrastructural observations of vitiliginous skin. The histoimmunofluorescence staining conducted on vitiliginous skin demonstrated the presence of upregulated collagen XVII in basal keratinocytes, indicating a compensatory response. Therefore, a powerful compensating mechanism in keratinocytes may enable these cells to adhere to the BM via collagen XVII and other molecules in hemidesmosomes, even when the distribution of laminin is aberrant.

MMP2 and MMP9 are the main MMPs involved in breaking down BM components [40,41]. Our study found that MMP2 expression was dramatically increased in the dermis of vitiliginous skin compared with healthy skin, and MMP9 expression was slightly increased as well, regardless of whether the vitiligo was stable or progressive. Recent research revealed decreased E-cadherin expression by melanocytes [34] and increased MMP9 expression in the epidermis of lesional vitiliginous skin [42], suggesting that reduced E-cadherin levels in melanocytes and MMP9-induced breakdown of E-cadherin on keratinocytes and melanocytes may cause melanocyte detachment in vitiligo. Our results show, however, that for melanocytes to inhabit the skin's basal layer, they must adhere not only to neighbouring keratinocytes but also to the

BM. In addition, MMP9 should affect not only cell–cell adhesion but also BM assembly.

MMP levels are elevated in various tissues in autoimmune disorders [43], including brain tissue in autoimmune encephalomyelitis [44,45], cerebrospinal fluid in multiple sclerosis [46], synovial fluid in rheumatoid arthritis [47], and salivary glands in Sjogren's syndrome [48]. In the present study, MMP2 expression was increased in dermal fibroblasts of vitiliginous skin compared with healthy skin, but the underlying reason is unknown. There are many reports on paracrine modulation of MMP2 expression. Numerous cytokines that are increased in lesional vitiliginous skin, including TGF-β [49,50], TNF-α [51,52], IL-1β [52,53], IL-6 [53], and IFN-γ [54] have been reported to stimulate MMP2 expression. Destruction of extracellular matrix macromolecules is seen in other diseases [16–18], and invading inflammatory cells exacerbate the pathogenesis of those diseases. These invading immune cells are thought to play an aetiologic role through their ability to produce cytokines that induce MMP expression in resident mesenchymal cells [55].

Damage to the epidermal BM alters interactions between epidermal keratinocytes and the extracellular matrix [56]. Epidermal keratinocytes interact with extracellular matrix components such as proteolytic fragments or cleavage sites (neoepitopes) in the surrounding stroma, which affects epidermal homeostasis and keratinocyte phenotypes [2,56,57]. Under pathological conditions, extensive BM destruction results in enhanced release of cytokines in the epidermis [56–58]. There is emerging evidence that keratinocytes in lesional vitiliginous skin are genetically [59], metabolically [60,61], and biologically [62] distinct from keratinocytes in nonvitiliginous skin. We found that disruption of the BMZ architecture altered the expression pattern of adhesive molecules in basal keratinocytes (supplementary material, Figure S2), and the cytokines TGF-β1, TNF-α, IL-1β, IL-6, and IFN-γ were highly expressed in the epidermis of vitiliginous skin lesions (supplementary material, Figure S3). These cytokines might induce MMP2 expression in dermal fibroblasts via paracrine regulation. Hence, there may be a vicious cycle (supplementary material, Figure S4) in which MMP2 destroys BM; the consequent disruption of the BM alters the phenotype of keratinocytes, resulting in cytokine production, which in turn upregulates MMP2 expression in fibroblasts. When the usual BM healing process is interrupted, a pathological vicious cycle of cytokine and protease release occurs, leading to the development of a permanent and intractable pathological skin phenotype, such as stable vitiligo. The vicious cycle described here may explain why melanocytes cannot return to the BM for a long time after they disappear, but it remains unclear what triggers the initial event in the cycle. As the body's first line of defence, the NOD-like receptor protein 1 (NLRP1) and NOD-like receptor protein 3 (NLRP3) inflammasomes are activated in response to various external stimuli, such as oxidative stress, UV radiation, chemical agents, extracellular ATP, metabolic impairment and microbial dysbiosis, and the concentration of IL-1β, IL-6, and TNF-α consequently increases in the epidermis [63,64]. The infiltrating lymphocytes and other inflammatory cells detected in the early stage of vitiligo may also be capable of producing these cytokines. Ultimately, the cytokines may induce MMP2 expression in dermal fibroblasts and initiate the vicious cycle of vitiligo development. Nonetheless, the pathogenesis of vitiligo likely involves a convergence of genetic, environmental, and metabolic factors, along with autoimmune responses [65]. Further research is required to examine how these elements integrate with our hypothesis concerning the role of MMP2 and other cellular components in the disease's development.

In our study, increased MMP2 expression was evident in protein extracts from vitiligo lesions but not from healthy skin. Notably, the prevalent form of MMP2 in these extracts was the active 62-kDa variant, indicating localised activation of the latent (pro) form within the dermis of affected skin. The activation of pro-MMP2 is influenced by various factors, including tissue inhibitors of metalloproteinases 2, membrane type 1 MMP, organomercurials, MMP3, and MMP7 [23]. Additional investigations are necessary to establish the potential involvement of these factors in MMP2 activation within vitiliginous skin.

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## Author contributions statement

FY, LY, TS, and IK designed the research. FY, LY, YK and SL performed the research. FY and LY performed data validation and analysis. FY, LY, YT, TS, TN, KN, SS, SI, DT and IK interpreted the data. YT, TS, TN, KN, SS, SI, DT and IK searched the literature. All authors were involved in writing the paper and had final approval of the submitted and published versions.

## Data availability statement

All data are included in the article and supplementary files, or are available from the corresponding author upon reasonable request.

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## SUPPLEMENTARY MATERIAL ONLINE

**Supplementary materials and methods**

**Figure S1.** Melanocyte disappearance results from decreased adhesion to the basement membrane in the 3D skin-equivalent model

**Figure S2.** Compensatory enhanced hemidesmosome adhesion in the basal keratinocytes of vitiliginous skin

**Figure S3.** Increased cytokine expression in the epidermis of vitiliginous skin

**Figure S4.** Summary illustration of matrix metalloproteinase 2 (MMP2)-induced melanocyte disappearance in vitiligo

**Table S1.** Clinical characteristics of the patients