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Involvement of PDGF-BB in the Perivascular Localization of Mesenchymal Stem Cells in Human Skin

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Abstract

Somatic stem cells play crucial roles in homeostasis and regeneration of adult tissues. In contrast to the epidermis, several studies have analyzed the exact nature and localization of stem cells in the human dermis. We aimed to elucidate the effect of Platelet-derived Growth Factor (PDGF)-BB on the perivascular existence of Mesenchymal Stem Cells (MSCs), and to analyze the effect of MSCs on dermal homeostasis. We applied a 3D laser scanning confocal microscope to visualize the sites of PDGF-BB deposition. Additionally, we analyzed the interactions between MSCs and the microvasculature in the dermis using several *in vitro* experimental systems including 3D skin equivalents. PDGF-BB accumulation was apparent in the periendothelial portion of the microvasculature in the dermis. MSC migration and colony formation was stimulated by PDGF-BB. Moreover, a significant increase in *PDGFB* gene expression was observed upon addition of MSC culture supernatant, whereas there was a drastic decrease in Intercellular Adhesion Molecule 1 (ICAM-1) expression. In addition, the production of the dermal components was enhanced in 3D skin equivalents harboring a tubular-like network induced by MSCs. These results indicate that PDGF-BB functions as a key molecule for the existence of MSCs, resulting in an appropriate interaction with the dermal microvasculature.

Introduction

Mesenchymal Stem Cells (MSCs) are multipotent stem cells, which are isolated from the bone marrow, adipose and other tissues including skin [1-5]. Numerous *in vitro* studies have demonstrated that MSCs are advantageous for tissue repair and regeneration [6]. However, the *in vivo* nature of MSCs is still poorly understood. Recent studies have proven that MSCs are involved in the niche of Hematopoietic Stem Cells (HSCs) in the bone marrow [7,8]. MSCs supply maintenance and quiescence factors during bone marrow homeostasis [9]. Moreover, not only the endosteum, but also the vascular niches are involved in the regulation of HSCs in the bone marrow [10]. Consistent with the existence of the vascular niches in the bone marrow, several reports have demonstrated that MSCs are localized in the perivasculature of various tissues [5,11-13]. However, unlike bone marrow, the nature of stem cell niches in other organs remains obscure.

In general, Platelet-derived Growth Factor (PDGF)-BB promotes the proliferation and migration of mesenchymal cells during wound healing [14]. PDGF-BB plays a crucial role in the recruitment of mural cells (smooth muscle cells or pericytes) during neovascularization or adult tissue repair [15-17]. PDGF-BB/PDGFR-β signaling is involved in the blood-brain-barrier of the central nervous system [18,19]. Although PDGFR- β is profoundly expressed in MSCs [20,21], the roles of the PDGF-BB/PDGFR- β signaling in the behaviors of MSCs have not been fully explored, yet. In a previous study, we have reported that human adult dermis contains MSCs in the perivasculature [5]. To better understand the regulatory mechanisms controlling skin homeostasis and regeneration, further investigation of the MSCs' roles in the dermis are required. In the present study, we attempted to determine whether PDGF-BB meditates the perivascular localization and/or the behaviors of dermal MSCs. In addition, the influence of MSCs on dermal homeostasis was investigated using 3D skin equivalents.

Materials and Methods

Antibodies and Reagents

Antibodies against the following proteins were used for indirect immunofluorescent microscopy: sheep polyclonal anti-human CD31 (AF806; R&D Systems, Minneapolis, MN, USA), mouse anti-human intercellular adhesion molecule (ICAM; AF796; R&D Systems), rabbit polyclonal anti-human PDGF-BB (ab9704; Abcam, Cambridge, UK). PDGF receptor beta neutralizing antibody was purchased from R&D Systems (AF385) for function blocking. The following reagents were used in this study: 3, 3'-diaminobenzidine (DAB; Thermo Fisher Scientific, Waltham, MA, USA), Hoechst 33342 (Thermo Fisher Scientific), paraformaldehyde (PFA; Nacalai Tesque, Kyoto, Japan) and phytohemagglutinin (PHA; Wako Pure Chemical Industries, Osaka, Japan). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA). Additionally, for analyses of 3D skin equivalents, the following antibodies were applied: mouse anti-human fibrillin-1 (MAB2502; Merck KGaA, Darmstadt, Germany), mouse anti-human fibronectin (M010; Takara Bio, Otsu, Japan), mouse anti-human filaggrin (Sc-66192; Santa Cruz Biotechnology, Dallas, TX, USA) and rabbit polyclonal anti-human Tenascin C (AB19011; Merck KGaA).

Cell Culture

Human skin fibroblasts (Kurabo Industries, Osaka, Japan) were cultured in DMEM containing 10% FBS (Thermo Fisher Scientific) and antibiotic-antimycotic solution (Nacalai Tesque). Human epidermal keratinocytes (Kurabo Industries) were cultivated in Epilife medium (Thermo Fisher Scientific). Human umbilical vein endothelial cells (HUVECs; Lonza, Basel, Switzerland) were cultured in Endothelial Growth Media (EGM-2, Lonza). Type I collagen-coated dishes (Asahi glass, Tokyo, Japan) were used for keratinocytes and HUVEC culture. Human MSCs derived from adipose tissue (Thermo Fisher Scientific) were expanded using a low-serum MSC medium (MesenPro RS medium: Thermo Fisher Scientific). All cell types were used within the first five passages.

RT-PCR

Total RNA of cultured cells was prepared using ISOGEN reagent (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. Then, it served as the template for reverse transcription using Superscript III (Thermo Fisher Scientific), followed by PCR amplification with Ex Taq polymerase (Takara Bio). The cDNA was also used as a template for quantitative PCR using FastStart DNA Master PLUS SYBR Green I Kit and a LightCycler (Roch Diagnostics, Mannheim, Germany). Human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal standard.

The PCR primers used in this study were as follows: for human *GAPDH*,

5 -gagtcaacggatttggtcgt-3 and 5 -tgggatttccattgatgaca-3 (201 bp); for human *ICMA-1*,

5 -agcttctcctgctctgcaac-3 and 5 -aatccctctcgtccagtcg-3 (106 bp); for human *PDGFA*,

5 -ctaagcatgtgcccgagaag-3 and 5 - tcgtaaatgaccgtcctggt-3 (100 bp); for human *PDGFB*,

5 -cctggcatgcaagtgtga-3 and 5 -ccaatggtcacccgattt-3 (108 bp).

Immunostaining

Scalp tissue pieces were embedded in an Optimal Cutting Temperature (OCT) compound, and then immediately frozen with a dry-ice/hexane mixture. To detect immunoreactivity in a stereoscopic view, 50 m sections were subjected to double-immunofluorescence staining. Samples were fixed with 4% PFA at 4°C overnight, followed by incubation with a serum-free blocking reagent (Agilent Technologies, Santa Clara, CA) at room temperature for 1 h. Antibody mixtures of rabbit anti-human PDGF-BB (1:200) combined with sheep anti-human CD31 (10µ g/m) were applied to the tissue sections, and then incubated at 4°C overnight. After washing with TBST for more than 2 h, the samples were incubated with the appropriate secondary antibody conjugated to Alexa Fluor® 488 or 594 (Thermo Fisher Scientific) at 4°C overnight. Confocal microscopy analysis was performed with Pascal and IMARIS (Zeiss, Oberkochen, Germany) after being briefly fixed with 4% PFA. In addition, immunocytochemical detection was performed as previously described [5]. The primary antibodies, anti-CD31 and anti-ICAM-1, were used at 10 µg/ml. Signals were visualized by HRP-conjugated secondary antibodies (Agilent Technologies) with DAB chromogen.

Enzyme-linked Immunosorbent Assay (ELISA)

The PDGF-BB protein content in HUVEC culture supernatants was determined using Quantikine Immunoassay systems for human PDGF-BB (R&D Systems). Briefly, HUVECs were seeded at 40,000 cells per well, and cultured under low-serum growth conditions (HuMedia-EG2: Kurabo) in 24-well tissue-culture plates coated with type I collagen (Asahi glass). Culture supernatants collected 48 h after seeding were subjected to PDGF-BB ELISA according to the supplier's instructions.

Colony-forming Assay

To confirm the Colony-forming Units of Fibroblasts (CFU-F), cells were plated at 50 cells/10-cm dish. After 14 days of cultivation at 37°C in a humidified 5% CO_2/95% air incubator, the cells were fixed with methanol for 5 min, dried briefly and stained

with Giemsa solution (Nacalai Tesque) for 10 min. The dishes were washed with large volumes of water. Colonies more than 1 mm in diameter were then counted to assess CFU-F efficiency.

Transwell Assay

Transwell inserts coated with fibronectin (FluoroBlok Cell Culture Inserts: BD Biosciences, San Jose, CA, USA) were used for the chemotactic migration assay. Inserts were placed in 24-well tissue-culture plates, and then 3,000 primary MSCs were added into the insert with serum-free MSC medium (StemPro MSC SFM: Thermo Fisher Scientific), while serum-free MSC medium with or without PDGF isoforms was added into the lower part of the tissue-culture plate. Cells were allowed to migrate to the bottom side of the insert membrane overnight. Cells that migrated to the bottom side of the membrane were fixed with methanol and stained with Hoechst 33342, followed by manual counting.

Tube Formation Assay

A commercially available kit was used for the tube formation assay according to the manufacturer's instructions (In Vitro Angiogenesis Assay Kit: Merck KGaA). Before the assay, MSCs and HUVECs were labeled with a red fluorochrome (PKH26: Sigma-Aldrich) and a green fluorochrome (PKH67: Sigma-Aldrich), respectively. HUVECs suspended in EGM-2 medium were plated on Extracellular Matrix (ECM) pre-coated 8-well chamber slides (BD Biosciences). After cellular network structures were partially developed by 5-6 h, 1,000 MSCs labeled with PKH26 were allowed to self-assemble into networks for 4 h in serum-free MSC medium (StemPro MSC SFM: Thermo Fisher Scientific). To visualize MSCs migrating toward networks, time-lapse images were collected every 10 min using a laser scanning confocal microscope.

3D Skin Equivalents

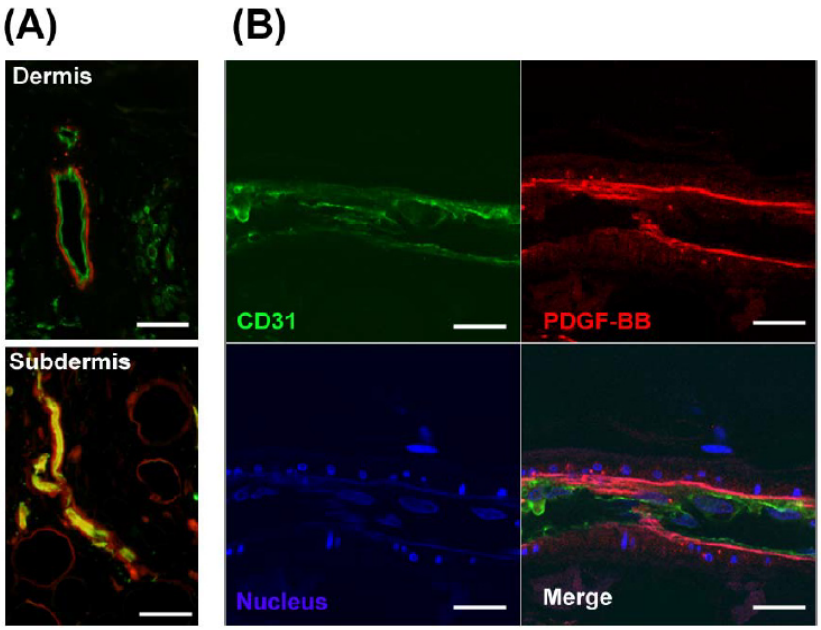
3D skin equivalents were prepared according to a slightly modified method described in a previous report [22]. Briefly, keratinocytes were cultured on top of a dermal equivalent consisting of type I collagen and fibroblasts in a 3D fashion. Only the keratinocytes' layer was exposed to air, to form a cornified layer using Epilife/DMEM (1:1) supplemented with 5% FBS. The medium was changed every other day. To evaluate the MSCs' effects on 3D skin equivalents, a cell mixture of HUVECs (2×10^5) and MSCs (2×10^5) was applied to develop a tubular-like network on the dermal equivalent using EGM-2/MesenPro (1:1) medium before overlaying the keratinocytes.

Results

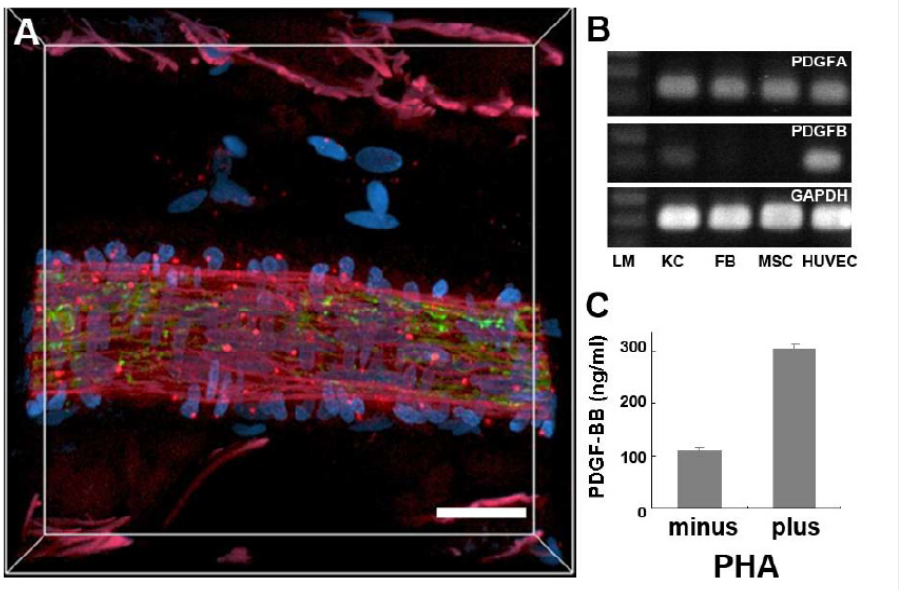
PDGF-BB Deposition in the Periendothelial Portion of Dermal-subdermal Microvasculature

Previously, we found that MSCs are localized in the perivasculature of the dermis, similar to subdermal adipose tissues [5,13]. We initially performed immunofluorescence analysis to investigate the localization of PDGF-BB in the human dermal-subdermal portion. When human scalp skin tissues were subjected to immunostaining with PDGF-BB antibody, we clearly detected PDGF-BB immunoreactivity in the dermal microvasculature (Figure 1A, upper panel). The microvasculature of the subdermal adipose tissue was also positive for PDGF-BB immunostaining (Figure 1A, lower panel). It is well known that the PDGF-B chain is retained on the cell surface through binding to heparan sulfate proteoglycans with a retention motif [23]. In addition, PDGF-BB can act in the cell-bound form proximate to the producing cell [24]. For instance, PDGF-BB secreted from endothelial cells and retained within the extracellular spaces binds to PDGFR-β on pericytes, which is involved in the maintenance of the blood-brain-barrier in the central nervous system [25]. Consistent with these properties, 3D confocal images clearly demonstrated that PDGF-BB deposited in the periendothelial portion adjacent to CD31 (Figure 1B). Moreover, fluorescent projection images clearly demonstrated that the dermal microvasculature was enclosed with PDGF-BB immunoreactivity (Figure 2A).

We next evaluated the expression level of the *PDGFA* and *PDGFB* genes *in vitro*. The expression level of *PDGFA* was similar among the four cell types, keratinocytes, fibroblasts, MSCs and HUVECs (Figure 2B, upper panel). In contrast, *PDGFB* was predominantly expressed in HUVECs. Keratinocytes expressed *PDGFB* at a low level, but fibroblasts and MSCs did not (Figure 2B, middle panel), suggesting that PDGF-BB is possibly derived from endothelial cells in the human dermal microvasculature. Indeed, as shown in Figure 2C, PDGF-BB was detected in the culture supernatant of HUVECs with increased levels after PHA treatment, resembling macrophages [26]. Overall, the results suggest that PDGF-BB derived from endothelial cells and deposited in the periendothelial space is involved in the perivascular localization of MSCs in human dermal-subdermal microvasculature.



Figures 1(A,B): Periendothelial localization of PDGF-BB. Human scalp tissues were subjected to immunofluorescence double-staining with PDGF-BB (red) and CD31 (green). **(A)** The immunoreactivity of PDGF-BB was observed adjacent to that of CD31 both in the dermis and subdermis. **(B)** Confocal microscopy view of dermal blood vessels demonstrated an apparent existence of PDGF-BB in the periendothelial portion. Scale bar = 100 µm (upper panel in A), 50 µm (lower panel in A) and 25 m (B).

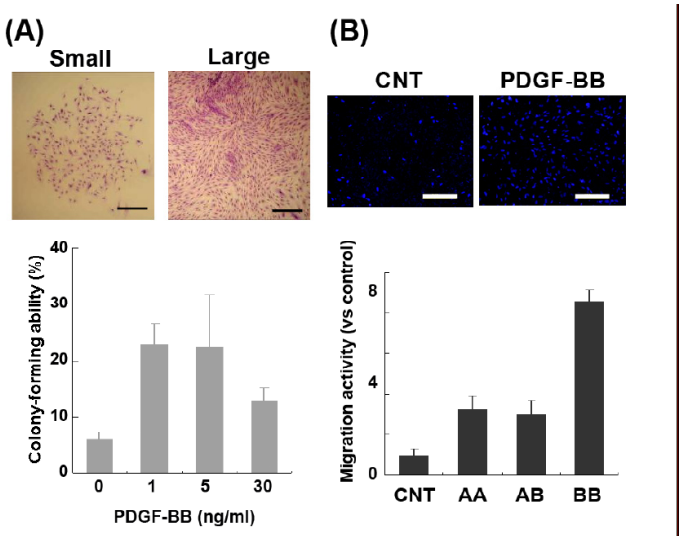


Figures 2(A-C): Endothelia production of PDGF-BB. **(A)** Fluorescent projection image of the dermal blood vessels was obtained from confocal acquisition of a Z-stack of a 50-µm-thick human scalp section after PDGF-BB immunofluorescence staining. PDGF-BB (red), CD31 (green) and Hoechst 33342 (blue). Scale bar = 25 µm. **(B)** Quantitative PCR analysis demonstrated a dominant gene expression of *PDGFB* in HUVECs, in contrast to an equal gene expression of *PDGFA* and *GAPDH*. **(C)** PDGF-BB production was confirmed by ELISA of HUVEC supernatants. There was a 3-fold increase in PDGF-BB production in HUVEC after phytohemagglutinin stimulation.

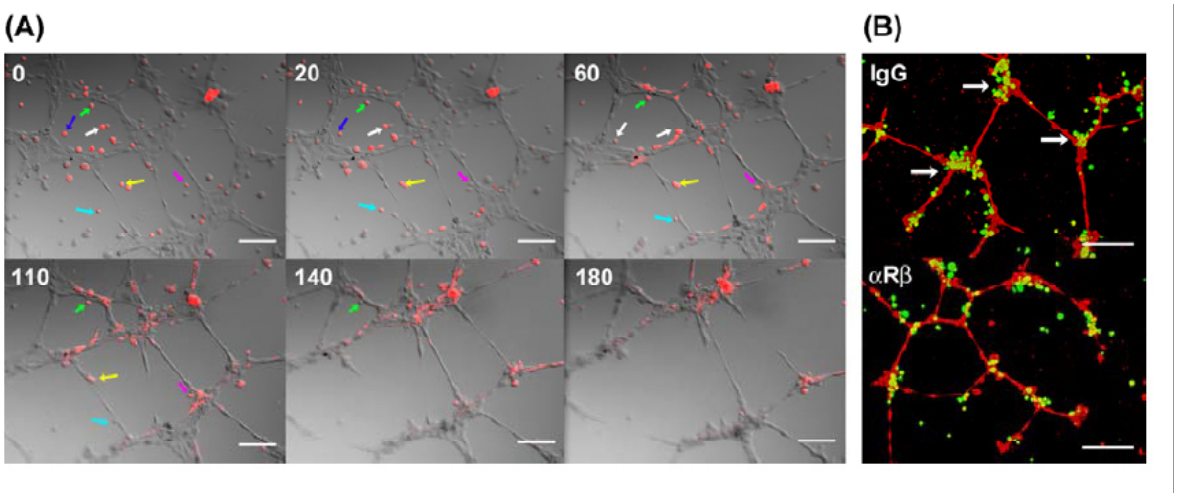
Effects of PDGF-BB on MSC Behaviors

The observations described above led us to examine the effects of PDGF-BB on MSC behaviors. It is well known that PDGF-BB stimulates the proliferation of various mesenchymal cell types, e.g., fibroblasts, pericytes and smooth muscle cells. Here, we assessed the effect of PDGF-BB on the self-renewal capacity of MSCs. The CFU-F assay revealed that PDGF-BB markedly stimulated the colony-forming ability of these cells (Figure 3A). Interestingly, a lower dose of PDGF-BB (1 or 5 ng/ml) resulted in higher CFU-F ability compared with a higher dose of PDGF-BB (30 ng/ml).

We next investigated the chemotactic effects of PDGF-BB on MSCs by the transwell migration assay. Surprisingly, contrary to the CFU-F ability, the chemotaxis of MSCs was accelerated by PDGF-BB at 30 ng/ml, but not at 5 ng/ml (data not shown). Furthermore, comparison of the three classic PDGF isoforms (PDGF-AA, AB and BB) revealed that the MSC migration was markedly accelerated by PDGF-BB compared with PDGF-AA and AB (Figure 3B). Next, we examined the recruitment of MSCs into the microvasculature using time-lapse confocal microscopy *in vitro*. When MSCs were applied on human tubular-like networks reconstructed *in vitro*, they moved toward the networks, attached to the surface, and then accumulated at the branch points within a few hours (Figure 4A). Furthermore, in this assay, PDGFR- was inhibited to elucidate whether PDGF-BB was involved or not. As expected, the accumulation of MSCs at the branch points tended to decline upon neutralization by PDGFR-β antibodies (Figure 4B). These results support the idea that PDGF-BB derived from endothelial cells regulates MSC recruitment and self-renewal in the human dermal-subdermal portion.



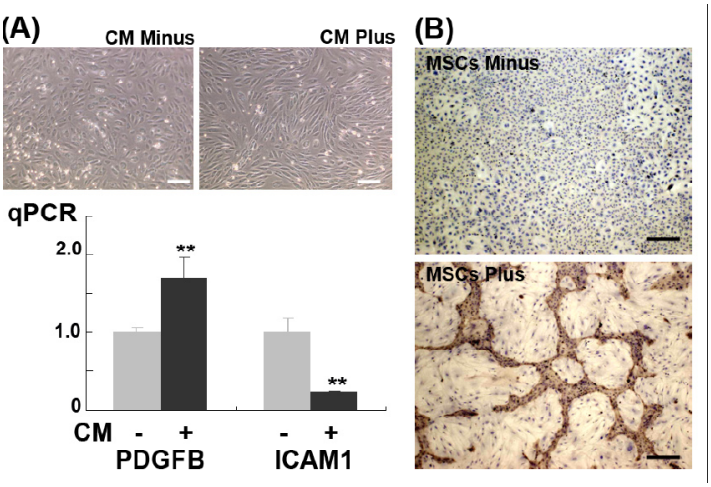
Figures 3(A,B): The effect of PDGF-BB on MSCs was examined *in vitro*. **(A)** MSCs showed colony-forming ability. A large colony with more than 1-mm diameter was counted as positive. Addition of PDGF-BB ligands at 1 or 5 ng/ml increased the colony-forming ability by 3-4-fold. Scale bar = 200 µm. **(B)** The migration ability was markedly enhanced in the presence of 30 ng/ml PDGF-BB (7-fold increase). Scale bar = 200 µm.



Figures 4(A,B): Migration of MSCs to tubular-like networks. **(A)** Time-lapse images demonstrate the migration of MSCs (red) toward the network in serum-free conditions. Colored arrows designate the same cells at each point (0-180 min). Scale bar = 200 µm. **(B)**The number of MSCs (green) attached to the branch (arrows in IgG control) decreased in the presence of anti-PDGFR- antibody (R). Scale bar = 200 µm.

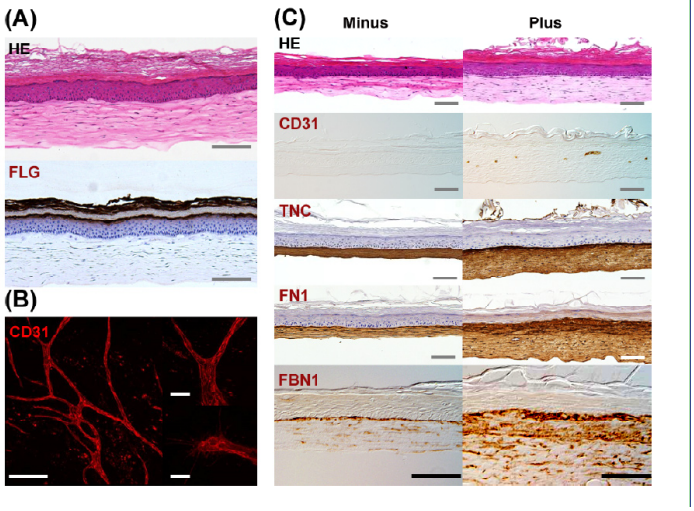
Influence of MSCs on Endothelial Cell Behaviors and Dermal Homeostasis

Subsequently, we investigated the influence of MSCs on endothelial cell behaviors. When MSC-conditioned medium was applied to HUVECs after reaching confluence, the apical-basal polarity of HUVECs was more apparent (Figure 5A, upper right panel) compared with the fresh medium control (Figure 5A, upper left panel). Using quantitative PCR, we found that the gene expression of *PDGFB* was significantly upregulated in HUVECs treated with MSC-conditioned medium, while ICAM-1 expression was suppressed (Figure 5A, lower panel). Thereafter, we co-cultured HUVECs and MSCs. Within only 48 h of incubation, tubular-like networks positive for CD31 immunostaining were formed, compared with the negative control (Figure 5B).



Figures 5(A,B): The effects of MSCs on HUVEC were examined *in vitro*. **(A)** The supernatants of MSCs sustained the inactive condition of HUVEC, characterized by their flattened morphology. Quantitative PCR analysis revealed that the MSC supernatants upregulated *PDGFB* and downregulated ICAM-1. Scale bar = 100 µm. **(B)** Addition of MSCs resulted in the assembly of tubular-like networks comprised of CD31-positive cells. Scale bar = 200 µm.

We further analyzed the roles of MSCs in dermal homeostasis using a 3D skin equivalent system. Before testing the effects of MSC addition, we verified the morphology and differentiation markers of skin equivalents. At day 14 of air-liquid interface exposure, a well-organized epidermal layer was formed above the dermal layer (Figure 6A, H&E). The stratum corneum was also observed as a flattened and non-nucleated layer at the top of the constructs. In addition, when keratin 10 or fillagrin localization was investigated by immunostaining, keratin 10 showed a broad pattern within the suprabasal layer (data not shown), while fillagrin was essentially restricted to the granular layer (Figure 6A, FLG). Then, using this technique, we confirmed the formation of tubular-like networks on the surface of dermal equivalents without an overlay of keratinocytes (Figure 6B). Additionally, we evaluated the effects of tubular-like networks in 3D skin equivalents. Evidently, after 14 days of cultivation, the dermal portion with networks was much thicker than that without networks, while the epidermal portion was almost indistinguishable (Figure 6C, H&E). CD31 immunoreactivity was obvious just beneath the epidermal layer of the 3D skin equivalents with networks (Figure 6C, CD31). In addition, tenascin C and fibronectin were profoundly distributed in the upper region (Figure 6C, TNC or FN1). Furthermore, fibrillin1, which is a component of elastic fibers, also accumulated in the upper region according to the addition of MSCs (Figure 6C, FBN1). These data suggest that MSCs localized at the periendothelial area play important roles in dermal homeostasis by influencing the microvasculature.



Figures 6(A-C): The roles of MSCs in dermal homeostasis were examined using 3D skin equivalents. **(A)** After a 21-day air-liquid interface exposure, the restricted expression of fillagrin (FLG) in the granular layer was confirmed in the well-organized epidermal layer constructed above the dermal layer. Scale bar = 100 µm. **(B)** A network positive for CD31 was constructed on the surface of dermal equivalents prior to the overlying with keratinocytes. Tip-like structures were confirmed as well as branching points. Scale bar = 100 µm (left) or 20 m (right). **(C)** 3D skin equivalents with a CD31-positive network retained a thicker dermal portion with a dominant accumulation of tenascin C (TNC), fibronectin (FN1) and fibrillin1 (FBN1). Scale bar = 100 µm or 50 µm (bottom panels).

Discussion

Our data support a model in which PDGF-BB is involved in a perivascular niche within the human skin to regulate MSC behaviors. Using *in vivo* and *in vitro* experiments, we determined the role of PDGF-BB in the perivasculature of the human skin. We clearly demonstrated the periendothelial deposition of PDGF-BB, consistent with the localization of MSCs. Essentially, PDGF-

BB promoted self-renewal and chemotaxis of MSCs. In addition, MSCs affected the behavior of endothelial cells such as tubular-like formation and gene expression. Future investigations analyzing the role of MSCs in skin homeostasis may uncover their extensive function in the skin, as implied by the upregulation of dermal components in 3D skin equivalents.

MSCs in other tissues have been shown to form niches for other cell types. HSC engraftment is supported by MSCs within the perivascular portion of the bone marrow [1]. In addition, the stromal vascular fraction of the adipose tissue is a rich source of not only preadipocytes but also MSCs [2]. Moreover, it has been reported that MSCs originate from the perivascular portion of various human organs [3,11]. As well as the perivascular localization, dominant PDGFR-β expression highlights an association between pericytes and MSCs, despite the lack of analysis on PDGF-BB/PDGFR-β signaling in MSCs [20,21]. In contrast, MSC-like cells such as Skin-derived Precursor Cells (SKPs) or multilineage-differentiating stress enduring (Muse) cells have not been uncovered about not only their localization but also their nature in the mammalian dermis [27,28]. Combined with our data demonstrating that MSCs are a component of the dermal vascular niche, these studies emphasize the significance of the vascular niche for the maintenance of MSCs within individual organs.

Based on the deposition of PDGF-BB ligands in the perivascular space, the dominant expression of the *PDGFB* gene in endothelial cells, and the induced colony-forming ability of MSCs by PDGF-BB ligands, we propose that endothelial cells produce and secrete PDGF-BB to sustain MSCs adjacent to the dermal-subdermal vasculature. The crucial roles of PDGF-BB have been thoroughly investigated in the behavior of pericytes in vascular biology [29-31]. For instance, mice lacking PDGF-B display defects in the blood-brain-barrier, resulting in increased permeability, namely a deficiency in pericyte ensheathment [18,19]. In addition, endothelial-specific PDGF-B transgenic mice showed a loss of function in the retina associated with reduced pericyte coverage [32]. As expected, we showed perivascular deposition of PDGF-BB ligands in the vasculature of human dermis and subdermal fat. Also, the expression of the *PDGFB* gene was profoundly detected in cultured endothelial cells compared with other cell types. These observations correlated well with the C-terminal binding motif of PDGF-BB, largely restricting its distribution and availability *in vivo* to the area near the site of production [33]. Moreover, we revealed that the colony-forming ability of MSCs was markedly enhanced by PDGF-BB stimulation. This result was compatible with a previous study reporting about human bone marrow stromal precursors [34]. In that report, PDGF-BB demonstrated a significant ability to support the colony growth of stromal precursors. Taken together, periendothelial deposition of PDGF-BB ligands derived from the endothelium works to sustain MSCs at the perivasculature, leading to the maintenance of their function in human skin.

The effects of PDGF-BB are complicated because soluble or cell surface-bound PDGF-BB ligands activate the two different PDGFR- cohorts in human pericytes, resulting in distinct biological endpoints [35]. PDGFR activation depends on the concentration of PDGF-BB ligands in fibroblasts [36]. In addition, PDGFs promote the migration of multiple mesenchymal cell types such as fibroblasts, smooth muscle cells and MSCs [37]. Moreover, PDGF-BB is involved not only in the maintenance of vasculature, but also in mural cell recruitment during angiogenesis [16-18,38]. In fact, we observed that PDGF-BB at a high-dose strongly stimulated the migration of MSCs compared with the PDGF-AA or AB isoforms. Additionally, blocking PDGFR- led to a decreased recruitment of MSCs to the endothelial tube *in vitro*. These results were supported by a previous report that showed PDGFR- activation in fibroblast and pericyte recruitment during cutaneous wound healing [14]. Collectively, our current analysis suggests that PDGF-BB is also associated with the recruitment of MSCs around blood vessels, similar to other tissues.

A recent study has revealed that MSCs played crucial roles in the perivascular niche of HSCs in the bone marrow by providing maintenance and quiescence factors for HSCs [39]. In this study, we found that MSCs affected the behaviors of endothelial cells, for instance the formation of capillary-like networks positive for PECAM1/CD31. Similarly, previous studies have demonstrated network formation *in vivo* by co-operation of endothelial cells with MSCs [40,41]. In addition, the marked downregulation of ICAM-1 by MSC-conditioned medium is consistent with ICAM-1 suppression on resting endothelium [42]. Moreover, we demonstrated that a supplement of MSC-conditioned medium resulted in a significant upregulation of the *PDGFB* gene in endothelial cells. The effects of mural cells on PDGF-BB expression in endothelial cells were not fully examined, in contrast to the effects of PDGF-BB on pericyte behavior [43,44]. Further substantial studies, therefore, should be performed to verify the roles of MSCs in gene expression of endothelial cells. Our data also suggest that MSCs contribute to the homeostasis of the ECM in the dermis by stabilizing the vasculature. In general, the degradation of the ECM is relatively rapid in *in vitro* 3D skin equivalents [45]. However, we found a remarkable increase in thickness and fibronectin deposition in the dermal tissue harboring the artificial capillary networks induced by MSCs in 3D skin equivalents. Recently, several studies have reported the effect of human bone marrow MSCs in organotypic culture systems [46,47]. In addition, autocrine fibronectin showed a dynamic influence on MSCs in a 3D culture system [48]. These observations mimicked the roles of MSCs in wound healing or injury conditions. It remains to be determined whether MSCs or mature vasculature is responsible for the ECM homeostasis of the dermis under physiological conditions.

In summary, we report that the periendothelial portion of the human skin is a site of PDGF-BB expression, correlating with the perivascular localization of MSCs. Essentially, self-renewal and chemotaxis of MSCs were markedly stimulated in the presence of PDGF-BB. In addition, MSCs affected the behaviors of endothelial cells such as tube-formation and gene expression. Furthermore, MSCs contributed to the homeostasis of the ECM in the dermis *in vitro*. These findings suggest that PDGF-BB plays crucial roles in the behavior of MSCs in the microvasculature, leading to maintenance of dermal homeostasis. Our study also raises the possibility that MSCs are a useful tool for tissue engineering and cell therapy.

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Disclosure of Potential Conflicts of Interest

The authors are employees of Shiseido Company, Ltd.

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