Endothelial Cells Promote Pigmentation through Endothelin Receptor B Activation

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Findings of increased vascularization in melasma lesions and hyperpigmentation in acquired bilateral telangiectatic macules suggested a link between pigmentation and vascularization. Using high-magnification digital epiluminescence dermatoscopy, laser confocal microscopy, and histological examination, we showed that benign vascular lesions of the skin have restricted but significant hyperpigmentation compared with the surrounding skin. We then studied the role of microvascular endothelial cells in regulating skin pigmentation using an *in vitro* co-culture model using endothelial cells and melanocytes. These experiments showed that endothelin 1 released by microvascular endothelial cells induces increased melanogenesis signaling, characterized by microphthalmia-associated transcription factor phosphorylation, and increased tyrosinase and dopachrome tautomerase levels. Immunostaining for endothelin 1 in vascular lesions confirmed the increased expression on the basal layer of the epidermis above small vessels compared with perilesional skin. Endothelin acts through the activation of endothelin receptor B and the mitogen-activated protein kinase, extracellular signal–regulated kinase (ERK)1/2, and p38, to induce melanogenesis. Finally, culturing of reconstructed skin with microvascular endothelial cells led to increased skin pigmentation that could be prevented by inhibiting EDNRB. Taken together these results demonstrated the role of underlying microvascularization in skin pigmentation, a finding that could open new fields of research for regulating physiological pigmentation and for treating pigmentation disorders such as melasma.

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INTRODUCTION

Pigmentation is a complex and a tightly regulated process. Microphthalmia-associated transcription factor (MITF) is the master gene of pigmentation and controls several key mechanisms in melanocytes such as melanogenesis, dendricity, and proliferation in response to environmental factors including UV radiation and to molecules produced by other cells in the skin. Activation of MITF induces expression of the key enzymes of melanogenesis, which are tyrosinase, dopachrome tautomerase (DCT), and tyrosinase-related protein 1, leading to the production of melanin. Numerous factors additionally provide the finer regulation of melanin pigment production and/or melanocyte growth and differentiation. Alpha-melanocyte-stimulating hormone and ACTH are the most potent activators of melanogenesis, whereas nitric oxide (NO) and some growth factors present in the circulation or secreted by keratinocytes act to varying degrees on melanogenesis and melanocyte growth, including basic fibroblast growth factor, KIT ligand, hepatocyte growth factor, endothelin 1 (Edn1), and some prostaglandins (Hirobe, 2005; Plonka *et al.*, 2009). Fibroblasts also have a key role in melanocytogenesis and melanogenesis. Palmoplantar fibroblasts express high levels of Dickkopf 1, which reduces melanocyte proliferation and differentiation by acting on MITF, explaining (at least partially) the lower pigmentation generally observed on human palms and soles (Yamaguchi *et al.*, 2007 and 2008). Fibroblasts also produce melanogenesis-associated factors that differ according to the skin type of the individual. One of these factors, known as neuregulin-1, is secreted by fibroblasts in black skin (skin type VI) and significantly increases the pigmentation of human melanocytes in culture (Choi *et al.*, 2010). Interestingly, fibroblasts also seem to be involved in melasma pathophysiology via their secretion of Wnt inhibitor factor-1 (Kang *et al.*, 2011; Kim *et al.*, 2013; Park *et al.*, 2014b).

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Abbreviations: DCT, dopachrome tautomerase; Edn1, endothelin 1; ERK, extracellular signal–regulated kinase; HMVEC, human dermal microvascular endothelial cell; MITF, microphthalmia-associated transcription factor; NHK, normal human keratinocyte; NHM, normal human melanocyte; NO, nitric oxide

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In addition to keratinocytes and fibroblasts, growing evidence also implicates endothelial cells in pigmentation (Plonka *et al.*, 2009). Indeed, melanocytes express receptors that can potentially be regulated by several factors secreted by endothelial cells such as vascular endothelial growth factor (VEGF), Edn1, NO, and leukotrienes (Kim *et al.*, 2005; Yamaguchi and Hearing, 2009). In addition, histological studies have clearly shown a significant increase in vascularization within melasma lesions compared with that in the surrounding healthy skin (Kim *et al.*, 2007). These results were subsequently confirmed by laser confocal microscopy examination (Kang *et al.*, 2010), although the significance of this increased vascularization in melasma remains poorly understood. However, a recent clinical report cited cases presenting with acquired telangiectatic and hyperpigmented macules (Park *et al.*, 2014a), for which the clinical and histological findings of skin hyperpigmentation localized above telangiectasias suggested a close relationship between melanocytes and endothelial cells.

In the current study, we therefore examined the role of dermal microvascular endothelial cells in regulating skin pigmentation.

RESULTS

Vascularization influences skin pigmentation *in vivo*

A total of 100 benign vascular skin lesions were assessed, comprising cherry angiomas, botriomycomas, spider angiomas, involutive infantile hemangiomas, capillaro-venous malformation, acquired bilateral telangiectatic macules, and leg telangiectasias. Using high-magnification digital dermatoscopy, we observed a mild to marked hyperpigmentation within and surrounding the vascular lesions compared with the surrounding skin in 89% of cases (Figure 1; Supplementary table 1 online). The hyperpigmentation was marked in 22% of cases. The association between the vascular lesions and the hyperpigmentation of the overlaying skin was significantly more frequent in dark skin types (III to V) compared with light ones (I and II; $P=0.021$) and in photoexposed areas compared with those located in photoprotected areas ($P<0.001$) (Supplementary Tables 1 and 2 online). No correlation was found between the size of vascular lesions and the overlying pigmentation (Supplementary Table 3 online). Analysis of the vascular lesions using laser confocal microscopy confirmed the increased pigmentation of the skin above the vascular proliferation compared with the surrounding skin (Supplementary Figure 1 online). We next analyzed tissue samples of benign vascular lesions of the skin, available in our biobank. Ten samples from cherry angiomas, botriomycomas, capillaro-venous malformation, and acquired bilateral telangiectasia were stained with hematoxylin eosin and Fontana–Masson staining to assess the vascularization and pigmentation, respectively. MITF staining was also performed to identify the melanocytes. For each sample, histological sections were analyzed in the center of the lesion and in the borders on the immediately surrounding, non-affected skin. The lesion sections showed an increased melanin content in the epidermis compared with the surrounding skin. However, there was no significant increase in melanocyte number with a mean ratio of melanocytes/keratinocytes of 0.128 and 0.122 in lesional and perilesional areas, respectively ($P=0.41$; Figure 2).

Microvascular endothelial cells increase the melanogenesis pathway in melanocytes

To further investigate the role of vascularization in skin pigmentation, we studied the effects of human dermal microvascular endothelial cells (HMVECs) on the melanogenesis pathway in normal human melanocytes (NHMs). In co-culture experiments with HMVECs and NHMs, we observed upregulation of the phosphorylated form of MITF in melanocytes after 30 minutes of co-culture (Figure 3a). After 3 days of co-culture, the expression level of tyrosinase and DCT was increased by approximately twofold (Figure 3b). This effect was also specific to the endothelial cells, because co-culturing of the NHMs with normal human keratinocytes (NHKs) under the same conditions produced no such effect (Supplementary Figure 2 online). We then investigated the potential role of endothelial cells in melanocyte proliferation. After plating the same number of NHMs for 7 days in a co-culture with HMVECs or alone, the melanocytes were counted. After 7 days of co-culture, the HMVECs induced a slight but a significant increase in melanocytes number (Figure 3c). Taken together, these results showed that the microvascular endothelial cells could promote melanogenesis and, to a lesser extent, enhance melanocyte proliferation.

Endothelin released by HMVECs upregulates melanogenesis signaling in NHMs

To understand how endothelial cells upregulate the melanogenesis pathway in melanocytes, we next investigated the factors released by endothelia by incubating NHMs with HMVEC-conditioned medium for 30 minutes and 3 days under the co-culture experiment conditions. The NHM starvation medium conditioned in the presence of endothelial cells (CM. HMVEC) induced similar phosphorylation of MITF after 30 minutes to that observed in the co-culture experiment with HMVECs (Figure 4a) and similarly increased tyrosinase and DCT expression after 3 days (Figure 4b). Many factors are produced and secreted by endothelial cells, but herein we focused on molecules that could potentially act on melanogenesis and melanocyte proliferation such as NO, VEGF, endothelin, and leukotriene. In our co-culture experiments, inhibition of NO with L-N^G-Nitroarginine methyl ester and 2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO; Supplementary Figure 3a online), VEGF receptor with VEGF receptor inhibitor IV and CBO-P11 (Supplementary Figure 3b online), and leukotriene with baicalein (Supplementary Figure 3c online) did not impair the phosphorylation of MITF. In contrast, inhibition of endothelin receptor with PD142893 inhibited the phosphorylation of MITF under our co-culture conditions (Figure 4c). Furthermore, we observed the same effects, with the same kinetics, on the phosphorylation of MITF and the levels of DCT and tyrosinase in melanocytes stimulated with Edn1 (Supplementary Figure 4 online). Analyzes of Edn1 secretion also showed that the HMVECs cultured in NHM starvation medium secreted fourfold more Edn1 than NHK, and no secretion was detected

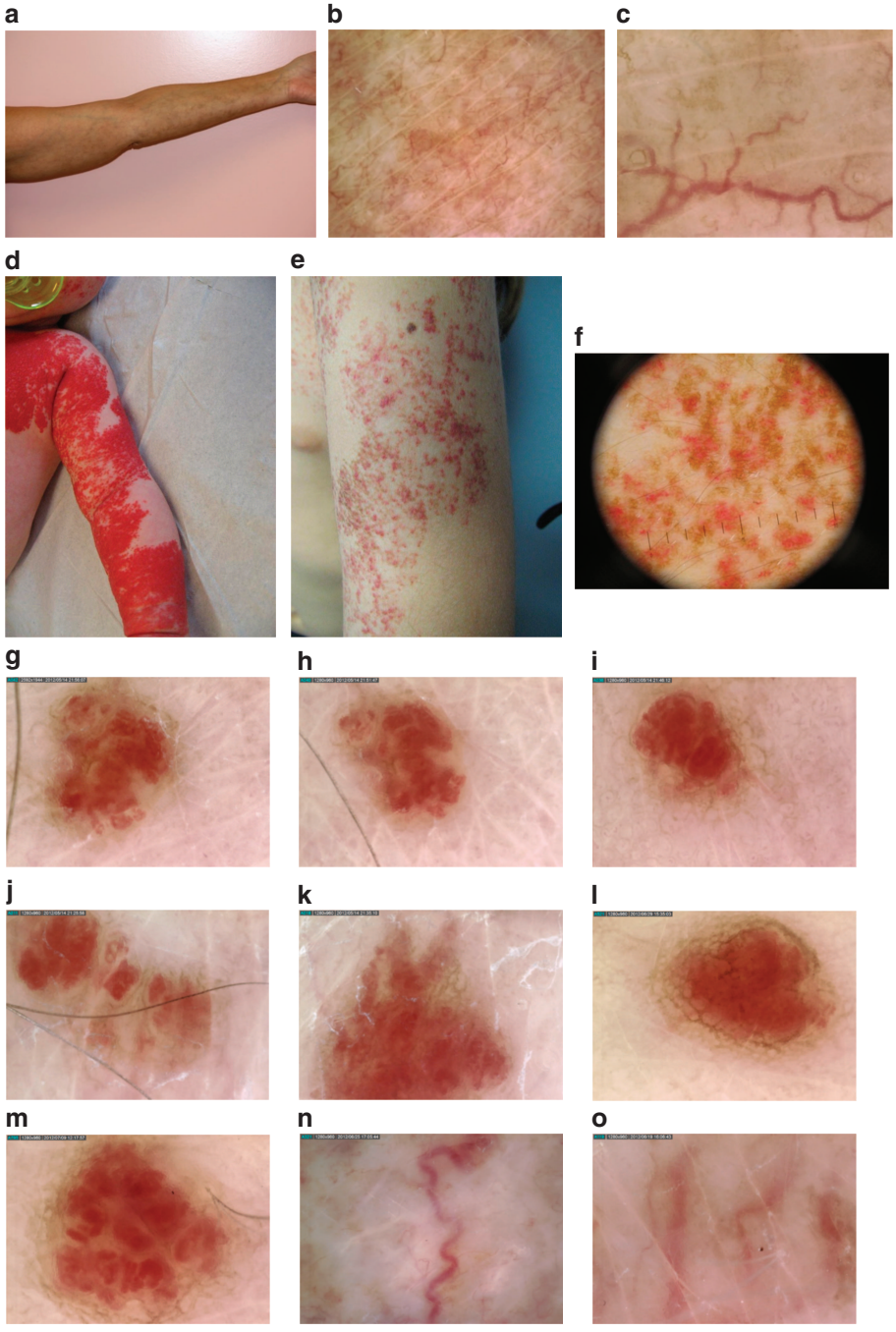

Figure 1. Clinical and epiluminescence dermatoscopy pattern of hyperpigmentation associated with vascularization. (**a**) Acquired bilateral telangiectatic macules. (**b**) Digital epiluminescence dermatoscopy of the lesions (×50). (**c**) Digital epiluminescence dermatoscopy of the lesions (×200). (**d**) Superficial congenital hemangioma in an infant of 2 months of age. (**e**) Clinical presentation at the age of 5 years with regression of the vascular component and hyperpigmentation localized only on the area of the hemangioma. (**f**) Digital epiluminescence dermatoscopy of the lesions (×10). Digital epiluminescence dermatoscopy (×200) of cherry angiomas (**g**–**m**) and leg telangiectasias (**n** and **o**).

for NHM (Figure 4d). Finally, to correlate these results *in vivo*, we assessed the expression of Edn1 in skin samples of vascular lesions compared with the perilesional skin and found increased Edn1 at the basal layers of the epidermis above vascular lesions (Figure 4e and h).

HMVECs induce melanogenesis via the activation of endothelin receptor B and the MAPK pathway via ERK1/2 and p38 in melanocytes

There are two subtypes of the endothelin receptor: EDNRA and EDNRB. In order to identify the receptor involved in this

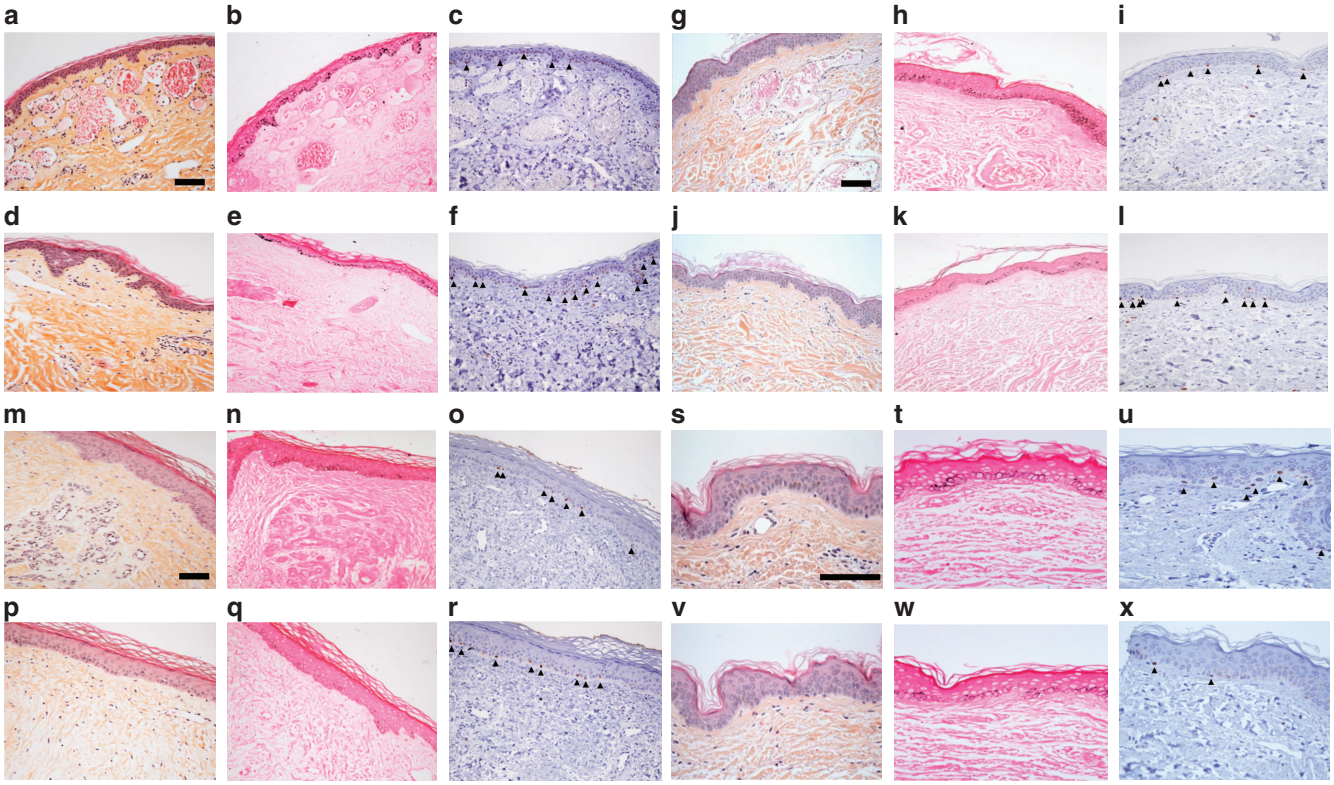

Figure 2. Histological analysis of vascular lesions. Cherry angioma with hematoxylin and eosin (HE) (**a**), Fontana–Masson (**b**) and microphthalmia-associated transcription factor (MITF) (**c**) staining. Perilesional normal skin of the same cherry angioma with HE (**d**), Fontana–Masson, (**e**) and MITF (**f**) staining. Capillary venous malformation with HE (**g**), Fontana–Masson, (**h**) and MITF (**i**) staining. Perilesional normal skin of the same capillary venous malformation with HE (**j**), Fontana–Masson, (**k**) and MITF (**l**) staining. Botriomycoma with HE (**m**), Fontana–Masson, (**n**) and MITF (**o**) staining. Perilesional normal skin of the same botriomycoma with HE (**p**), Fontana–Masson, (**q**) and MITF (**r**) staining. Acquired bilateral telangiectatic macules with HE (**s**), Fontana–Masson, (**t**) and MITF (**u**) stainings. Perilesional normal skin of the same lesion with HES (**v**), Fontana–Masson, (**w**) and MITF (**x**) staining. All the pictures were taken with ×200 magnification. Arrows designate MITF-positive cells. Scale bar = 50 μM.

process, we used specific inhibitors of EDNRA (BQ123) and EDNRB (BQ788). The inhibition of EDNRA showed no effect on MITF phosphorylation induced by endothelial cells, whereas the inhibition of EDNRB inhibited the effects of HMVECs on NHMs (Figure 5a). To determine whether Edn1 directly stimulates the melanocytes, we repeated the experiment using conditioned medium of HMVECs with or without BQ788. Treatment with BQ788 inhibited the phosphorylation of MITF induced by HMVEC-conditioned medium as observed for NHMs and HMVECs co-cultured in the presence of BQ788, showing that Edn1 released by HMVECs directly stimulates NHM (Figure 5b). The effect of EDNRB in this mechanism was further confirmed using siRNA knockdown of EDNRA and EDNRB expression. Specifically, decreasing EDRNB expression in NHMs inhibited the phosphorylation of MITF, but also the upregulation of tyrosinase induced by HMVECs, whereas silencing EDNRA had no effect on these processes (Figure 5c and d, Supplementary Figure 5 online).

In melanocytes, the stimulation of EDNRB activates protein kinase C, which, in turn, stimulates extracellular signal–regulated kinase (ERK)1/2 and p38, mitogen-activated protein kinases that are implicated in the phosphorylation of MITF and the upregulation of tyrosinase, respectively. We therefore also investigated the status of ERK1/2 and p38 in NHMs in co-culture with HMVECs and found that, after 30 minutes, ERK1/2 and p38 were phosphorylated (Figure 5e), with the phosphorylation of ERK also inhibited by siRNA EDNRB (Supplementary Figure 5 online). Furthermore, inhibition of the ERK pathway with U0126 inhibited the phosphorylation of MITF in NHMs co-cultured with HMVECs (Figure 5f), whereas the inhibition of p38 with SB203580 did not prevent this phosphorylation (Figure 5g). The increased tyrosinase and DCT after 3 days of co-culture was inhibited by U0126 and partially inhibited by SB203580, suggesting a role for both ERK and p38 activation in this mechanism (Figure 5h and i).

Taken together these results show that the microvascular endothelial cells secrete endothelin, which activates EDNRB on melanocytes and stimulates the ERK and p38 pathways. Activation of ERK leads first to the phosphorylation of MITF, whereas the activation of both ERK and p38 induces the subsequent upregulation of tyrosinase and DCT.

Microvascular endothelial cells increase the pigmentation of reconstructed epidermis through endothelin secretion

On the basis of the stimulation of melanogenesis by endothelial cells under co-culture conditions, we then assessed

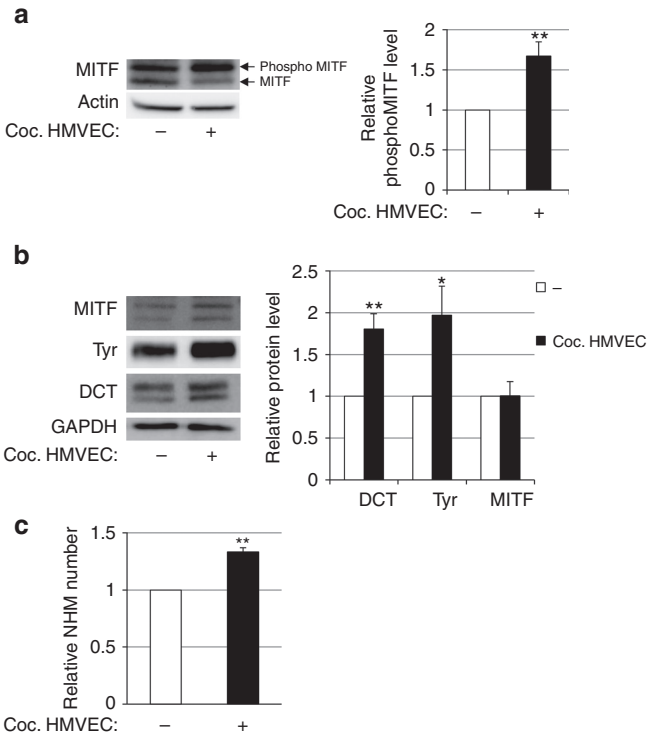

Figure 3. Microvascular endothelial cells increase the melanogenesis pathway in melanocytes. Normal human melanocytes (NHMs) are incubated with human dermal microvascular endothelial cells (HMVECs) in the transwell chamber during 30 minutes (**a**) or 3 days (**b**). The lysate of NHMs is analyzed by western blot with indicated antibodies and the relative protein level quantified ($n = 6$ and 8, respectively). The same number of NHMs is plated and incubated with HMVECs in the transwell chamber for 7 days (**c**). The HMVECs are changed every second day. After 7 days of co-culture, the NHMs are trypsinized and counted on Malassez cell ($n = 3$). $*P \leqslant 0.05$; $**P \leqslant 0.005$.

their effect on melanin synthesis in reconstructed epidermis composed of human keratinocytes and melanocytes cultured with or without HMVECs. After 3 weeks, the co-culture with HMVECs induced an increased pigmentation of the reconstructed epidermis that could be observed clinically (Figure 6a and b) and using Fontana–Masson staining (Figure 6c). This HMVEC-induced hyperpigmentation of the epidermis was prevented by the inhibition of EDNRB with BQ788 (Figure 6c).

DISCUSSION

In 1963 Fitzpatrick and Breathnach formulated the concept of the epidermal melanin unit, with melanocytes and keratinocytes working together to produce skin color. Forty years later, Jim Nordlund expanded this concept to a cutaneous troika involving keratinocytes, Langerhans cells, and melanocytes (Nordlund, 2007), with fibroblasts also recently implicated in regulating skin pigmentation (Yamaguchi *et al.*, 2007 and 2008; Choi *et al.*, 2010). Here, we demonstrated that the dermal microvascular endothelial cells also have a role in the complex regulation of skin pigmentation. In our experiments, endothelial cells, but not keratinocytes, could stimulate melanogenesis in melanocytes without UV stimulation. The absence or low stimulation of melanogenesis in our co-cultures with keratinocytes is not so surprising, and indeed all studies demonstrating a stimulating effect of keratinocytes on melanogenesis were conducted in the presence of UV stimulation (Duval *et al.*, 2001) or another pigmentation inducer such as Alpha-melanocyte-stimulating hormone (Lei *et al.*, 2002). We now show that endothelial cells have a role, through the secretion of endothelin, in upregulating key gene regulators of melanogenesis, MITF, tyrosinase, and DCT, without any UV stimulation. Activation of melanogenesis through activation of the MAPK pathway and phosphorylation of MITF is in accordance with previous reports (Sato-Jin *et al.*, 2008). However, the increased contrast in pigmentation between photo-exposed areas of the epidermis above vascular skin lesions and the perilesional skin led us to hypothesize that the stimulation of melanogenesis by endothelial cells might be even stronger after UV radiation. Further studies are warranted to confirm this hypothesis. Interestingly, in samples of the benign vascular lesions, the melanin contain was increased in the epidermis located above the microvascularization along with an increased expression of Edn1 in the basal layers of the epidermis, further confirming that Edn1 is produced *in vivo* by endothelial cells, reaches the basal layers of the epidermis, and is capable of stimulating melanogenesis. We additionally observed *in vitro* a slight increase in melanocyte proliferation when co-cultured with endothelial cells, although the number of melanocytes was not significantly increased in either reconstructed epidermis experiments or skin samples of benign vascular lesions. Thus, the proliferative effect of Edn1 appears limited, at least *in vivo*. Interestingly, it was recently reported that choroidal melanocytes could regulate uveal vascularization through the secretion of fibromodulin (Adini *et al.*, 2014), emphasizing the constant cross-talk between melanocytes and endothelial cells. Edn1 is also produced by proliferating endothelial cells or by cells involved in inflammatory processes. Thus, on the basis of the current study, it could be hypothesized that Edn1 also has a role in post-inflammatory hyperpigmentation. Although more pronounced in darker skin types and in photo-exposed area, our clinical and histological data show that the vascular component of the dermis influences the pigmentation of the skin *in vivo*. Clearly, the physiological function that endothelial cells have in skin pigmentation, beyond their role in pigmentation disorders, warrants further investigations. Indeed, the potential impact of vascularization on hyperpigmented lesions observed in acquired bilateral telangiectatic macules was suspected by the authors who reported this entity (Park *et al.*, 2014a). Now, our results confirm a role for endothelial cells but also rule out the implicated role of VEGF as initially hypothesized and show instead the key role of the released endothelin. The vascular component may also have a key role in melasma. Histological studies and confocal laser microscopy studies have clearly shown a significant increase in vascularization within melasma lesions compared with that in the surrounding healthy skin (Kim *et al.*, 2007; Kang *et al.*, 2010). Although the signification of this increased vascularization was unknown, studies using different therapeutic

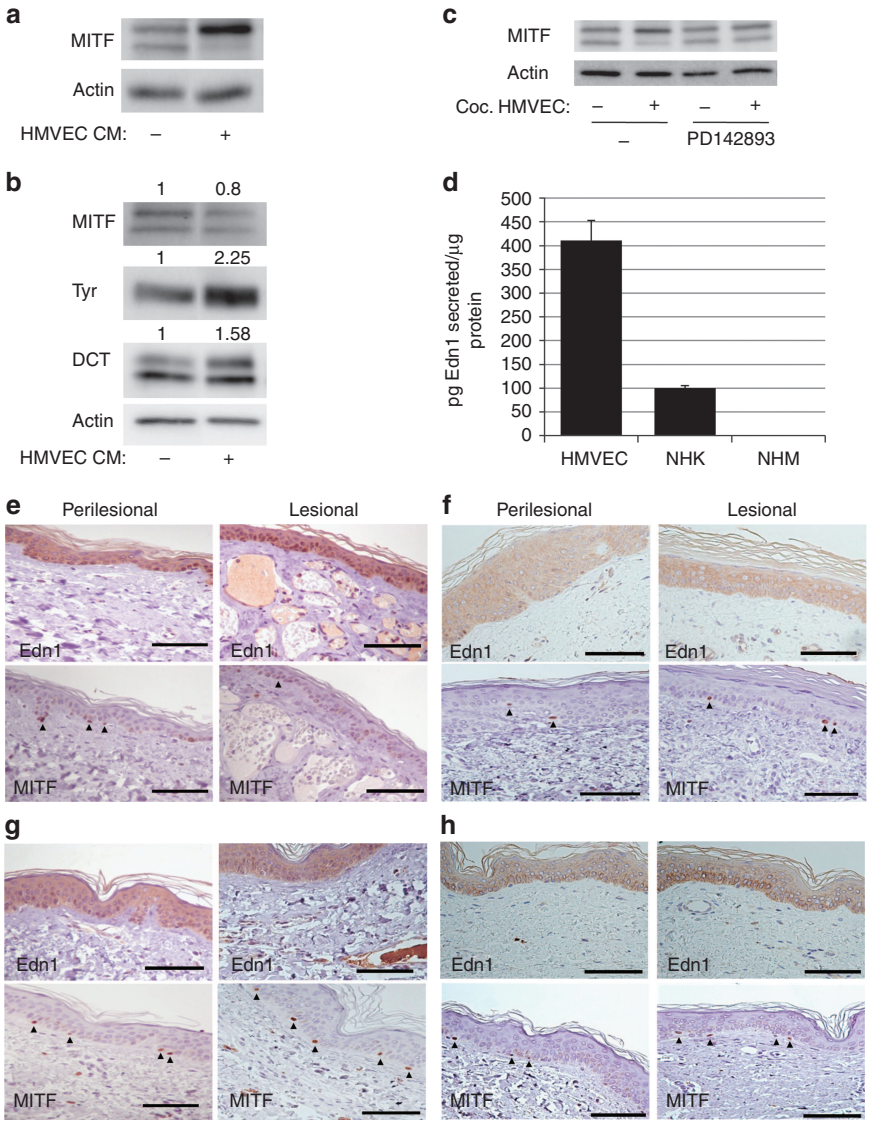

Figure 4. Endothelial cells secrete endothelin 1 implicated in the upregulation of the melanogenesis pathway in melanocytes. Human dermal microvascular endothelial cells (HMVECs) are incubated 24 hours with the normal human melanocytes (NHMs) starvation medium. NHMs are incubated with HMVEC-conditioned medium (HMVEC CM.) for 30 minutes (**a**) or 3 days and changed every day with new HMVEC CM. (**b**). NHMs and HMVECs are treated with the endothelin receptor antagonist PD142893 (5 µM) (**c**), 2 hours before the start of the co-culture with HMVECs for 30 minutes.The lysate of NHMs is analyzed by western blot with indicated antibodies. Numbers above the gels indicate levels of intensity compared with actin. The secretion of endothelin 1 by HMVECs, normal human keratinocytes (NHKs), and NHM in NHM starvation medium is measured using the ELISA method (**d**). The expression of endothelin 1 and microphthalmia-associated transcription factor (MITF) is analyzed in microvascular lesional skin sections compared with perilesional skin (**e**) (cherry angioma), (**f**) (botriomycoma), (**g**) (capillary venous malformation), (**h**) (acquired bilateral telangiectatic macules)). Arrows designate MITF-positive cells. Scale bar = 50 µM.

approaches, but sharing the same aim of targeting the vascular component of melasma, have been recently reported. A prospective comparative split face randomized study showed that the combination of stabilized Kligman's trio and pulsed dye laser (PDL) was significantly more effective than the stabilized Kligman's trio alone (Passeron *et al.*, 2011). Interestingly, the combination approach prevented, at least partially, the typical relapses after the summer period, whereas the cream alone did not. In support of these findings, additional data suggest a preventive role of targeting vessels in the relapses of melasma (Passeron, 2013). A different kind of approach also assessed the effect on melasma of tranexamic acid, an anti-fibrinolytic used to prevent and to treat some hemorrhagic events. The combined use of this agent topically and orally during 8 weeks decreased hyperpigmentation in melasma lesions, whereas histological examinations confirmed a decrease in melanin content and in vascularization (Na *et al.*, 2013). These pilot studies underline the potential interest in targeting the vascular component for treating melasma. However, using laser approaches to remove the vessels may promote post-inflammatory hyperpigmentation, especially in darker skin

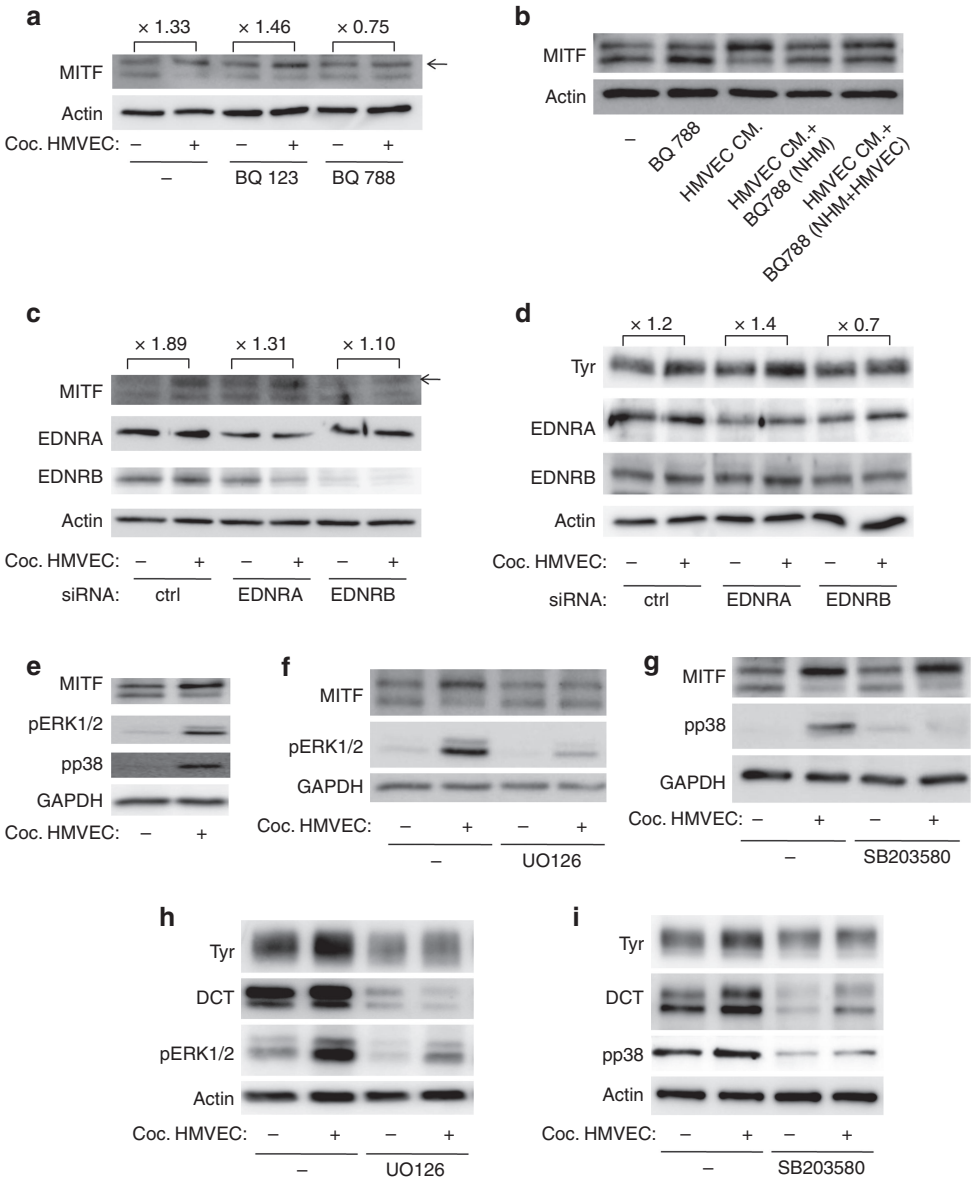

Figure 5. Endothelin acts through endothelin receptor B and mitogen-activated protein kinase (MAPK) extracellular signal–regulated kinase (ERK)1/2 and p38 to increase the melanogenesis pathway in melanocytes. Normal human melanocytes (NHMs) and human dermal microvascular endothelial cells (HMVECs) are pre-treated 2 hours with EDNRA and EDNRB inhibitors, respectively, BQ123 and BQ788 (2 μM), and put in co-culture for 30 minutes (**a**). NHMs are pre-treated 2 hours with EDNRB inhibitor (2μM) and incubated with HMVECs treated or not with BQ788-conditioned medium for 30 minutes (**b**). NHMs are transfected with siRNA directed against EDNRa, EDNRb, or siRNA-negative control. One (**c**) or 4 (**d**) days later, NHMs are incubated with HMVECs during 30 minutes (**c**) or 3 days (**d**). NHMs are incubated with HMVECs in the transwell chamber during 30 minutes (**e**). NHMs and HMVECs are stimulated with ERK1/2 inhibitor UO126 (10 μM) or p38 inhibitor SB203580 (10 μM) 2 hours before the addition of the HMVEC transwell chamber in the well for 30 minutes (**f** and **g**) or 3 days (**h** and **i**). The lysate of NHM is analyzed by western blot with indicated antibodies. Numbers above the gels indicate the levels of intensity compared with actin.

types (Passeron *et al.*, 2011), and the efficacy of tranexamic acid has still to be confirmed in a prospective comparative trial. Moreover, its effects are nonspecific and may induce side effects. By dissecting the pathway involved in the pigmentation associated with vascularization, we demonstrate here the key role of the EDNRB. Thus, developing topical agents to inhibit ENDRB activation on melanocytes may limit the impact of the underlying vascularization and provide, in combination with classic depigmenting agents, a powerful approach to treat melasma and prevent relapses.

MATERIALS AND METHODS

Patients

Consecutive patients presenting at least one benign vascular proliferation on the skin were included. Exclusion criteria were inflammatory skin disorders, photodermatoses, melasma, acquired brachial cutaneous dyschromia, post-inflammatory hyperpigmentation,

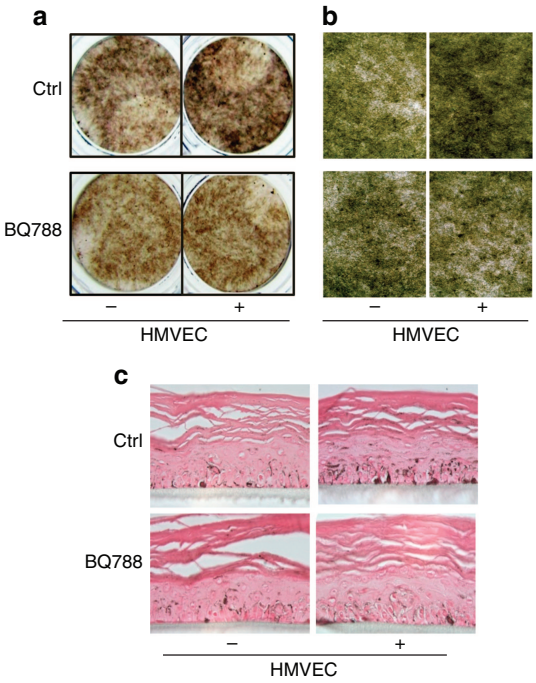

Figure 6. Endothelin released by endothelial cells increases the pigmentation in reconstructed epidermis. Reconstructed human pigmented epidermis in a transwell chamber is stimulated with EDNRb inhibitor BQ788 (2 μM) and incubated with human dermal microvascular endothelial cells (HMVECs) at the bottom of the well for 3 weeks. HMVECs are changed every other days, and BQ788 added every day. Reconstructed human pigmented epidermis is photographed in full size (**a**) or in ×20 magnification (**b**). Melanin quantity is determined by Fontana–Masson staining and observed at ×40 (**c**).

and concomitant medication with photosensitizing drugs. Cherry angiomas, botriomycomas, spider angiomas, involutive infantile hemangiomas, capillaro-venous malformation, acquired bilateral telangiectatic macules, and leg telangiectasias were included after written informed consent was granted. The patient age, gender, and skin type were noted together with the localization and size of involved lesions. As only noninvasive examination (epiluminescence dermoscopy) was performed and all the data were definitively anonymized, institutional review board approval was waived.

Assessment of pigmentation

A digital epiluminescence dermatoscope at ×200 magnification (Dinolite, Naarden, The Netherlands) was used for evaluating the vascular lesions and the pigmentation above the lesions. The same physician performed (HHG) all the evaluations. Pigmentation was assessed using a physician global assessment score that grades the difference in pigmentation within and in the infra millimeter border of vascular lesions compared with that in the surrounding skin. The contrast in pigmentation was scored as none, mild, moderate, or marked.

Vascular lesions were also assessed using laser confocal microscopy (Vivascope 1500, Caliber ID, Rochester, NY). Optical sections (Vivablock) of 4-mm width were acquired every 20 microns depth from the *stratum corneum* up to the superficial dermis. Confocal images were exported from the VivaScan Database to analyze using Confoscan to discern high- (bright) or low- (dark) intensity objects against circular and fixed-shape models. We thus detected optically hyperreflective keratinocytes surrounding an angioma cavity and less-pigmented keratinocytes located on the perilesional skin for the quantification of pigmentation.

Reagent and antibodies

We obtained antibodies against MITF and β-actin from Abcam (Cambridge, MA), pERK, and pp38 from Cell Signaling Technology (Beverly, MA), EDNRA, EDNAB, and GAPDH from Santa Cruz Biotechnology (Santa Cruz, CA), and tyrosinase and DCT from V. Hearing.

U0126 and SB203580, baicalein, VEGF receptor inhibitor IV, CBO-P11, and PTIO were purchased from Millipore (Billerica, MA), PD142893 from Enzo Life Science (Farmingdale, NY), and LNAME, endothelin 1, BQ123, and BQ788 from Sigma Aldrich (Saint Quentin Fallavier, France).

Cell culture

NHMs and NHKs were obtained from the foreskin of skin type IV children as described previously. Briefly, epidermal cells are obtained by overnight digestion of the skin in a dispase solution at 4°C followed by incubation of the epidermis in a trypsin/EDTA solution for 20 minutes at 37 °C. NHMs were isolated in MCDB 153 medium (Sigma Aldrich) supplemented with 2% FBS (Perbio; Helsingborg, Sweden), 5 μg ml^{-1} insulin (Sigma Aldrich), 0.5 μg ml^{-1} hydrocortisone (Sigma Aldrich), 16 nM TPA (Sigma Aldrich), 1 ng ml^{-1} FGF (Promega; Madison, WI), 15 μg ml^{-1} bovine pituitary extract (Invitrogen; Carlsbad, CA), 10 μM forskolin (Sigma Aldrich), and 20 μg ml^{-1} geneticin (Invitrogen) over 2 weeks. NHKs were isolated in KGM 2 medium (Promocell, Heidelberg, Germany). HMVECs were obtained from Invitrogen and grown in Cascade 131 medium supplemented with microvascular growth supplement on attachment factor-coated plates (Invitrogen). All cells were maintained at 37 °C in a 5% CO2 atmosphere.

Co-culture experiments

For the co-culture experiments, NHMs were seeded in six-well plates, whereas HMVECs and NHKs were seeded separately on 0.4 μM Transwell inserts(Becton Dickinson; East Rutherford, NJ).

Two days before experiments, NHMs were starved in MCDB 153 medium supplemented with 5 μg ml^{-1} insulin, 15 μg ml^{-1} bovine pituitary extract, and 2% fetal bovine serum to remove pro-pigmenting agents. HMVECs and NHKs were also incubated for 2 days in the NHM starvation medium to avoid medium-dependent effects on pigmentation. The co-cultures were initiated when a Transwell coated with HMVECs or NHKs was placed in a well containing cultured NHM.

Reconstructed human pigmented epidermis (RHPE) experiment

RHPE (skin type IV), obtained from SkinEthic (Lyon, France), is characterized by keratinocytes and melanocytes 3-D culture from foreskin disposed on a 0.4 μM transwell chamber that allows an air–liquid interface. According to SkinEthic procedure, RHPE are incubated 24 hours in RHPE growth medium before experiment.

For the HMVEC/RHPE co-culture experiment, HMVECs are seeded in 24-well plates and incubated 24 hours with RHPE growth factor before incubation with the RHPE. HMVECs are changed every second or third days during the 3 weeks of co-culture.

Histopathology

Each biopsy was fixed in 4% formalin and paraffin embedded. Morphological examination was carried out on sections (2-µm thickness) stained by hematoxylin and eosin or Fontana–Masson. Sections were heated for 20 minutes at 97 °C in low pH buffer solution for antigen retrieval (PT-link Dako device, Glostrup, Denmark). MITF immunolabeling (mouse mAb product code: M3621, Clone:D5, dilution 1/100, immunogen: Histidine fusion protein of the amino-terminal Taq-Sac fragment of human MITF cDNA, Dako France SAS, les Ulis,) and endothelin immunolabeling (goat polyclonal antibody, product code: ET-1 (N-8) sc-21625, dilution 1/200, Santa Cruz Biotechnology) were performed using a Dako autostainer according to the manufacturer's recommendations. After washing with phosphate-buffered saline, the sections were incubated with Envision FLEX+mouse (Linker)/HRP (DAKO) for 15 minutes at room temperature. After washing with phosphate-buffered saline, the sections were incubated with Envision FLEX/HRP (DAKO) for 20 minutes at room temperature, followed by revelation using the Envision System according to the manufacturer's recommendations, with DAB (diaminobenzidine) for 8 minutes as chromogen. Finally, the sections were counterstained in Mayer's hematoxylin and mounted in glycergel mounting medium (Dako). Melanocytes, identified by Mitf immunohistochemical staining, and basal keratinocytes were counted along the basement membrane, beside and away from the vascular lesion, within the entire length of each biopsy. We then set the ratio of keratinocytes/melanocytes. For each sample, at least 10 fields at ×400 magnification were studied.

Transient siRNA transfection

A total of 500,000 NHMs are used for reverse transfection. NHMs are trypsinized, resuspended in starvation medium, and transfected using INTERFER in (Polyplus Transfection; Illkirch, France) according to the manufacturer instructions. Briefly, siRNA-negative control, EDNRA and EDNRB (50 pmol) (#1-Thermo Fisher Scientific; Waltham, MA), (#2-Ambion; Foster City, CA) are incubated with INTERFER in 15 minutes and lay into the well before the addition of the cells. The experiments of 30 minutes of co-culture were conducted 4 days after the transfection, and the experiments of 3 days of co-culture were performed 1 day avec the transfection.

Statistical analysis

Statistical differences between groups were analyzed by Student's *t*-test. They were considered significant at $P<0.05$.

CONFLICT OF INTEREST

AM is an employee of Beiersdorf. The remaining authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Adini I, Ghosh K, Adini A *et al.* (2014) Melanocyte-secreted fibromodulin promotes an angiogenic microenvironment. *J Clin Invest* 124: 425–36

Choi W, Wolber R, Gerwat W *et al.* (2010) The fibroblast-derived paracrine factor neuregulin-1 has a novel role in regulating the constitutive color and melanocyte function in human skin. *J Cell Sci* 123:3102–11

Duval C, Regnier M, Schmidt R (2001) Distinct melanogenic response of human melanocytes in mono-culture, in co-culture with keratinocytes and in reconstructed epidermis, to UV exposure. *Pigment Cell Res* 14:348–55

Fitzpatrick TB, Breathnach AS (1963) The epidermal melanin unit system. *Dermatologische Wochenschrift* 147:481–9

Hirobe T (2005) Role of keratinocyte-derived factors involved in regulating the proliferation and differentiation of mammalian epidermal melanocytes. *Pigment Cell Res* 18:2–12

Kang HY, Bahadoran P, Suzuki I *et al.* (2010) *In vivo* reflectance confocal microscopy detects pigmentary changes in melasma at a cellular level resolution. *Exp Dermatol* 19:e228–33

Kang HY, Suzuki I, Lee DJ *et al.* (2011) Transcriptional profiling shows altered expression of wnt pathway- and lipid metabolism-related genes as well as melanogenesis-related genes in melasma. *J Investig Dermatol Symp Proc* 131:1692–700

Kim EH, Kim YC, Lee ES *et al.* (2007) The vascular characteristics of melasma. *J Dermatol Sci* 46:111–6

Kim EJ, Park HY, Yaar M *et al.* (2005) Modulation of vascular endothelial growth factor receptors in melanocytes. *Exp Dermatol* 14:625–33

Kim JY, Lee TR, Lee AY (2013) Reduced WIF-1 expression stimulates skin hyperpigmentation in patients with melasma. *J Investig Dermatol Symp Proc* 133:191–200

Lei TC, Virador VM, Vieira WD *et al.* (2002) A melanocyte-keratinocyte coculture model to assess regulators of pigmentation in vitro. *Anal Biochem* 305:260–8

Na JI, Choi SY, Yang SH *et al.* (2013) Effect of tranexamic acid on melasma: a clinical trial with histological evaluation. *J Eur Acad Dermatol Venereol* 27: 1035–9

Nordlund JJ (2007) The melanocyte and the epidermal melanin unit: an expanded concept. *Dermatol Clin* 25:271–81

Park JH, Lee DJ, Lee YJ *et al.* (2014a) Acquired bilateral telangiectatic macules: a distinct clinical entity. *JAMA Dermatol* 150:974–7

Park TJ, Kim M, Kim H *et al.* (2014b) Wnt inhibitory factor (WIF)-1 promotes melanogenesis in normal human melanocytes. *Pigment Cell Melanoma Res* 27:72–81

Passeron T (2013) Long-lasting effect of vascular targeted therapy of melasma. *J Am Acad Dermatol* 69:e141–2

Passeron T, Fontas E, Kang HY *et al.* (2011) Melasma treatment with pulsed-dye laser and triple combination cream: a prospective, randomized, single-blind, split-face study. *Arch Dermatol* 147:1106–8

Plonka PM, Passeron T, Brenner M *et al.* (2009) What are melanocytes really doing all day long...? *Exp Dermatol* 18:799–819

Sato-Jin K, Nishimura EK, Akasaka E *et al.* (2008) Epistatic connections between microphthalmia-associated transcription factor and endothelin signaling in Waardenburg syndrome and other pigmentary disorders. *FASEB J* 22: 1155–68

Yamaguchi Y, Hearing VJ (2009) Physiological factors that regulate skin pigmentation. *Biofactors* 35:193–9

Yamaguchi Y, Passeron T, Hoashi T *et al.* (2008) Dickkopf 1 (DKK1) regulates skin pigmentation and thickness by affecting Wnt/beta-catenin signaling in keratinocytes. *FASEB J* 22:1009–20

Yamaguchi Y, Passeron T, Watabe H *et al.* (2007) The effects of dickkopf 1 on gene expression and Wnt signaling by melanocytes: mechanisms underlying its suppression of melanocyte function and proliferation. *J Investig Dermatol Symp Proc* 127:1217–25