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PROF. DESMOND JOHN TOBIN (Orcid ID : 0000-0003-4566-9392)

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E-Cadherin mediates UVR- and calcium-induced melanin transfer in human skin cells

Suman K. Singh¹, Richard Baker¹, Stephen K. Sikkink¹, Carine Nizard², Sylvianne Schnebert², Robin Kurfurst², Desmond J. Tobin¹*

¹Centre for Skin Sciences, School of Life Sciences, University of Bradford, Bradford, West Yorkshire, BD7 1DP, United Kingdom;
²LVMH Recherche, F45800 Saint Jean de Braye, France.
Corresponding author: d.tobin@bradford.ac.uk  Tel: (01274) 233585

Abstract:
Skin pigmentation is directed by epidermal-melanin units, characterized by long-lived and dendritic epidermal melanocytes (MC) that interact with viable keratinocytes (KC) to contribute melanin to the epidermis. Previously we reported that MC:KC contact is required for melanosome transfer, that this can be enhanced by filopodial and by UVR/UVA irradiation, which can up-regulate melanosome transfer via Myosin X-mediated control of MC filopodia. Both MC and KC express Ca²⁺-dependent E-cadherins. These homophilic adhesion contacts induce transient increases in intra-KC Ca²⁺, while ultraviolet radiation (UVR) raises intra-MC Ca²⁺ via calcium selective ORAI1 ion channels; both are associated with regulating melanogenesis.

However, how Ca²⁺ triggers melanin transfer remains unclear, and here we evaluated the role of E-Cadherin in UVR-mediated melanin transfer in human skin cells. MC and KC in human epidermis variably express filopodia-associated E-Cadherin, Cdc42, VASP and β-catenin, all of which were upregulated by UVR/UVA in human MC in vitro. Knockdown of E-cadherin revealed that this cadherin is essential for UVR-induced MC filopodia formation and melanin transfer. Moreover, Ca²⁺ induced a dose-dependent increase in filopodia formation and melanin transfer, as well as increased β-catenin, Cdc42, Myosin X, and E-Cadherin

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expression in these skin cells. Together these data suggest that filopodial proteins and E-Cadherin, which are upregulated by intracellular (UVR-stimulated) and extracellular Ca\(^{2+}\) availability, are required for filopodia formation and melanin transfer. This may open new avenues to explore how Ca\(^{2+}\) signalling influences human pigmentation.

**Key words:** melanocytes, keratinocytes, filopodial, melanin transfer.

**Introduction:**

Skin pigmentation, a critical phenotypic adaptation for ultraviolet radiation (UVR)-drenched terrestrial life, is dependent on the activity of cutaneous melanocytes (MC). This subpopulation of neural crest-derived cells migrates during embryogenesis to the integument's epidermis and hair follicles. There they engage in the rather special and still mysterious process of synthesizing melanin within MC-specific lysosome-related organelles called melanosomes, only to then transfer these granules to surrounding receptor keratinocytes (KC) of the epidermal melanin unit (KC) (1-5). The manner in which melanin granules are ‘donated’ to and accumulate in neighboring KC remains unclear, as does the process by which melanin distributes within the stratified epidermis to provide optimal protection. The sum of the available evidence suggests that multiple overlapping intracellular processes are involved in melanosome transfer, and that factors in MC and KC are involved in co-regulating this. Several hypotheses have been proposed including: (i) cytophagocytosis of MC dendrite tips (6) (ii) exocytosis of melanosomes and their subsequent uptake via phagocytosis into KC (7-9) (iii) shedding of melanosome-rich ‘packages’ by MC and their subsequent phagocytosis by KC (10-12) and most recently (iv) filopodia-mediated melanosome transfer (13-18). However the underlying regulatory and signalling pathways involved in melanin transfer remain poorly defined and this is the subject of the current study.

Originally proposed by Scott and colleagues in 2002 (13), we extended and developed the concept of the filopodial mode of melanin transfer to propose in 2010 a ‘filopodial-phagocytosis model’, to reveal an actual mechanism by which melanosomes can be finally donated to KC (16). MC are highly dendritic cells both *in situ* and in culture, and both dendrites and filopodia are important for melanosome transfer to KC (11,13,14,16,19). Autocrine or paracrine factors influence melanosome transfer, and it was demonstrated that alpha-melanocyte stimulating hormone (αMSH), Prostaglandin E2 (PGE2) and Bone morphogenetic proteins (BMPs) are all major participants in the response of MC to UVR, mediating the melanogenic response and melanin transfer to KC by promoting filopodial
melanin delivery (including maturation of melanosomes, filopodia formation, and broadening of filopodial diameter) (15, 20-22).

Our current view, which we develop here in this current study, is that direct cell-cell contact between MC and KC is a required for optimal melanosome transfer. Specifically, both MC and KC express E type (i.e., epithelial) cadherins, a family of glycoproteins expressed in the basal layer of epidermis and involved in MC-KC interaction (23). A role of E-cadherin in melanosome transfer is suggested by the loss of MC and KC contact in the acantholytic lesions of Darier's disease (24), which results in disrupted pigment transfer. These hypopigmented lesions exhibit ‘empty' KC, despite being surrounded by melanosome-filled MC dendrites (25). Cadherins are present over the entire cell surface, including filopodia and the lamellipodia leading edge. Their concentration at contact sites increase shortly after a cell makes contact with another cell, where they cluster to form higher order structures (26). Moreover, the extracellular domains of E-cadherin binds calcium (Ca\(^{2+}\)), which results in a conformational change that promotes the homophilic interaction with E-cadherin on an adjacent cell (27). Meanwhile, the intracellular domain of E-cadherin contains a highly-phosphorylated region vital for β-catenin binding, such that E-cadherin function depends on β-catenin regulating actin-containing cytoskeletal filaments (28). Initiation of E-Cadherin-mediated cell-cell attachment also activates the master filopodial regulator Cdc42 (29). Active Cdc42 inhibits β-Catenin degradation, and so can control many aspects of cell differentiation in skin (30). Moreover, cellular studies have suggested these proteins are important regulators of actin assembly and cell motility. For example, Ena/VASP proteins control filopodial dynamics in epithelia by remodeling the actin network in response to cadherin expression, and so provides an additional filopodial target for analysis in MC (31).

In the current study we address several fundamental questions relating to how E-cadherin mediates UV-induced melanin transfer, including: does E-cadherin regulate melanin transfer from MC to KC by altering MC filopodia formation? Does UVR and Ca\(^{2+}\) modulate the expression of major filopodial components like E-cadherin, β-catenin, Cdc42, MyoX and VASP to promote MC filopodia formation and melanin transfer to KC? Which signalling pathways (s) is/are used by UVR to regulate filopodia formation of MC and melanin transfer to KC in human epidermal skin cells?
Results and Discussion:

Expression of filopodia-associated proteins in human skin

Immunofluorescence analyses revealed intense plasma membrane and diffuse cytoplasmic expression of E-cadherin throughout the human epidermis (Fig. 1Ai). Both KC and MC exhibited E-cadherin expression. To investigate where filopodia-associated proteins are localised in human skin, tissue sections were immunostained with antibodies to β-catenin, Cdc42, VASP and (Fig. 1Aii-iv). β-catenin displayed marked cell surface expression in all layers of the epidermis with lower expression in the most superficial differentiated layers (Fig. 1Aii). Both KC and MC exhibited β-catenin expression. Thus, both E-cadherin and β-catenin expression were detected on the cell membrane of skin cells, reflecting their roles as adhesion proteins, their association with basement membrane zone desmosomes (32), and in the case of β-catenin a role as a nuclear transcription factor (30).

By contrast, the small Rho family GTPase Cdc42 displayed a diffuse cytoplasmic expression throughout the entire epidermis, including KC and MC (Fig. 1Aiii), with additional striking nuclear expression in many KC and MC, and along the basement membrane zone in direct contact with the dermis compartment of the skin. This small GTPase participates in cytoskeletal rearrangement to induce filopodia formation in human MC, and expression of this master regulator of filopodia formation is increased after UVR/UVA exposure (16). Our results indicate that normal human epidermis is a prominent location for the expression of this key regulator of signaling pathways that control a very diverse array of cellular functions, including those that regulate assembly and rearrangement of actin cytoskeleton to mediate cell-cell adhesion, communication (e.g., via filopodia) and migration (29).

It has previously been reported that Ena/VASP proteins control filopodial dynamics in epithelia by remodeling the actin network in response to cadherin expression (31). We therefore were keen to assess if VASP (vasodilator-stimulated phosphoprotein) protein was expressed by both KC and MC in normal human epidermis. VASP expression was highest in the basal and suprabasal layers of the epidermis, being concentrated in the perikaryon of the cells (Fig. 1Aiv), and was weaker and more diffuse in the differentiated KC of the upper epidermal layers. The elevated perinuclear/cytoplasmic expression of VASP in cells of the epidermal-melanin unit, places this key regulator at the site of most active MC:KC communication and interaction.
**UV regulates the expression of filopodia-associated proteins in human epidermal melanocytes in vitro**

Cellular E-cadherin molecules interact with the actin cytoskeleton via their intracellular domain and are enriched in the dense F-actin networks of filopodia (33). We have shown previously that UV can induce filopodia formation of MC, and also melanin transfer to KC, by up regulating the expression of filopodial-associated proteins MyoX and Cdc42 (16). To investigate the effect of UVR on E-Cadherin and on filopodia-associated proteins (including β-catenin, Cdc42 and VASP), cultured primary MC were irradiated with 25mJ/cm² for 6 hrs and protein expression were assessed by immunofluorescence analysis. Our data shows that E-Cadherin and all filopodia-associated proteins tested showed increased cytoplasmic and nuclear expression patterns after 25mJ/cm² UVR treatment (Fig. 1Bi-iv).

It has previously been reported that members of the Rho family can modulate E-cadherin function, and that E-cadherin can then activate Cdc42 expression, which demonstrates bi-directional interactions between the Rho- and E-cadherin signaling pathways (29). Therefore when MC E-Cadherin and Cdc42 are co-activated by UVR that pathways can then engage in bi-directional interactions to induce filopodia formation. Moreover, KC:MC intercellular contacts will activate the intracellular domain of E-cadherin to regulate various signaling proteins via E-cadherin’s multiple interaction sites, and form stable linkage with the actin cytoskeleton through β- and α-catenins (34). UVR is known to induce β-catenin expression in KC (35), and we report here that UVR upregulates β-catenin in human MC (Fig. 1Bii). Interestingly, the expression of an additional actin polymerization factor in filopodia, for example Ena/VASP involved in promoting long, unbranched actin filaments (36), was also induced by UVR in human MC (Fig. 1Biv).

**E-cadherin mediates UVR-induced melanocyte filopodia formation and melanin transfer in human skin cells in vitro**

Physical interactions between MC and KC plasma membranes are known to induce a transient intracellular Ca²⁺ signal in KC that is required for pigment transfer (37). However, the mechanism by which Ca²⁺ signaling triggers melanin transfer has not yet been clarified. Ca²⁺ regulation is crucial for melanogenesis, but given its key second messenger role in driving epidermal differentiation (38) may also influence filopodial protein expression/ filopodia formation, and so melanin transfer. Thus, we sought to determine whether Ca²⁺ may regulate filopodia formation in epidermal MC, and subsequently to promote melanin transfer to epidermal KC via its effect on E-cadherin.

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To investigate the effects of E-cadherin on melanin transfer and filopodial formation in vitro, siRNA knockdown of E-cadherin was used to investigate filopodia formation after UVR stimulation of MC. Treatment of MC with E-cadherin siRNA resulted in a marked reduction in filopodia formation as visualized by SEM compared to control-treated cells (Fig. 2A). This reduction in filopodial was also noted even after UVR stimulation. Thus, knockdown of Ca\(^{2+}\)-dependent E-cadherin demonstrated that E-cadherin is important for filopodia formation, and this occurs centrally as UVR-stimulation alone was not sufficient to recover filopodia formation in the absence of E-Cadherin.

To investigate how the reduction in filopodia formation affected melanosome transfer to KC, matched KC:MC co-cultures were established after E-cadherin knockdown in both MC and KC. E-cadherin knockdown resulted in a 50-fold reduction in melanosome transfer between the partner skin cell types, despite 25mJ/cm\(^2\) UVR stimulation (Fig. 2B,C) compared to control cultures. UVR stimulation of E-cadherin-intact resulted in an almost 100-fold increase in transfer. These findings suggest that E-cadherin expression and function is important for melanin transfer, and is also centrally involved in UVR-induced melanin transfer. Interestingly, Jiang and co-workers (39) have reported that UVR irradiation of human epidermis can result in precipitation of calcium in the upper epidermis, and increase cytosolic calcium in the lower dermis, reflecting alteration of the calcium gradient in the human epidermis (39). It is also possible that UVR-associated changes in epidermal calcium distribution may reflect a perturbation of the epidermal barrier induced by UVR irradiation.

**Ca\(^{2+}\) induced melanocyte filopodia formation and melanin transfer in human skin cells in vitro**

To confirm the role of cadherin in filopodia formation and in melanin transfer, we approached this more directly by evaluating the impact of different concentrations of calcium ions added extracellularly on MC filopodia formation and melanin transfer in MC:KC co-cultures. We chose to use Ca\(^{2+}\) ions as Ca\(^{2+}\) ions bind to the ectodomain of E-cadherin transmembrane glycoprotein in the extracellular space to activate E-Cadherin (28). Cells incubated with increasing Ca\(^{2+}\) concentrations (from 0.1 - 0.25 mM) increased both MC filopodia formation (Fig. 3A) and gp100-positive melanosome transfer in KC/MC co-cultures (Fig. 3B,C). However, higher concentrations of Ca\(^{2+}\) (i.e., 0.5 mM) produced only a marginal increase in both phenotypic effects, and Ca\(^{2+}\) at 1mM inhibited the cultures. These results confirmed that Ca\(^{2+}\) can indeed influence both filopodial formation and melanosome transfer in MC:KC co-culture in vitro. It is known that in the presence of Ca\(^{2+}\), E-Cadherin undergoes interaction with another E-Cadherin molecule on neighbouring cells to make MC:KC and in KC:KC active interaction sites possible, and ultimately triggering intracellular cell signaling to
promote filopodial proteins expression (28, 40) and thus promoting melanin transfer to KC via this route (among others). These findings concur with knowledge of gradients in calcium concentration that increase from 0.5mM (basal layer) to over 1.4mM in the upper epidermis (e.g., stratum granulosum). This gradient is critical for epidermal homeostasis (42). Specifically, normal epidermal homeostasis, in the context of KC, requires the expression of calcium binding proteins, like cadherins, to facilitate the terminal differentiation of KC (41). In psoriasis, where the barrier is defective, there is a global increase in calcium content (42).

We have found that high concentration of Ca\(^{2+}\) at 1mM exhibited the loss of the filopodia formation in MC (Fig. 3A) and subsequently there was inhibition in melanin transfer (Fig. 3B, C). It was shown by others that a rise in Ca\(^{2+}\) levels can cause two distinct, concentration-dependent effects separable by their different time courses: within the first 10 min, filopodia underwent significant elongation, while the second phase was characterized by a massive loss of filopodia (43).

**Ca\(^{2+}\) upregulates the expression of filopodia-associated proteins in human epidermal melanocytes in vitro**

To evaluate the effect of Ca\(^{2+}\) on E-Cadherin and filopodial protein expression (i.e., β-catenin, Cdc42, MyoX and VASP) in normal epidermal MC these cells were incubated with Ca\(^{2+}\)-free media for 24hrs, then incubated for another 24hrs in increasing concentrations of Ca\(^{2+}\) (0.1-1 mM). All filopodia-associated proteins tested showed a moderate increase in expression after Ca\(^{2+}\) treatment (Fig. s1i-iv). This was particularly marked at concentrations of Ca\(^{2+}\) from 0.1 mM-0.25 mM.

Extracellular Ca\(^{2+}\) gradients in skin, which are essential for keratinocyte differentiation (38,44), are also affected by UV irradiation. Specifically, UVR exposure can raise intracellular Ca\(^{2+}\) in MC through ORAI1 Ca\(^{2+}\) channels, and the expression of these channels have been shown to be involved in melanogenesis (45). These data suggest that Ca\(^{2+}\)-regulated proteins, like E-cadherin, are dependent on intracellular and external Ca\(^{2+}\) availability, and so these are key for inducing filopodia formation of MC and melanin transfer to KC in MC:KC co-culture. Ca\(^{2+}\)-induced upregulation of MC filopodial proteins also suggests that UVR stimulation of MC is also associated with a rise of Ca\(^{2+}\) in MC to induce the expression of major filopodial components like β-catenin, Cdc42, MyoX and VASP. Thus, we conclude that UVR exposure promotes filopodia formation in MC and subsequently induce ‘melanin transfer’ to KC. Thus, the regulation by Ca\(^{2+}\) of transmembrane proteins like E-Cadherin suggests the presence of positive feedback signal mechanism.
MAPK signalling is involved in UVR-induced filopodia formation and melanosome transfer

We were also interested to determine whether the observed UVR-induced effects on filopodia formation and melanin transfer were dependent on the activation of MAPK or phosphoinositide 3-kinases (PI3-K) pathways. Cells were incubated with specific inhibitors to ERK1/2 [PD98059 (PD)], p38 stress kinase [SB203580 (SB)] and PI3K [LY294002 (LY)] prior to UVR irradiation in order to investigate signalling pathways in filopodia formation. SEM analysis of MC, treated separately with the 3 signalling pathway inhibitors for 1hr prior to UVR irradiation with 25 mJ/cm\(^2\), showed that p38 & PI3K inhibition decreased UVR-stimulated filopodia formation, while ERK inhibition did not (Fig. 4A). It has been reported that UVR irradiation-induced melanogenesis is associated with the activation of ERK1/2 by upstream signals originating from reactive oxygen species or from activated tyrosine kinase receptors, rather than from damaged DNA (46). The current study also found that phosphorylation was not observed for c-Jun N-terminal kinases (JNK) or p38, indicating that ERK1/2 activation may be UVA-specific and not specifically needed for filopodia formation.

To evaluate the effect of MAPK inhibition on melanin transfer, co-cultures of MC:KC were incubated with SB, PD and LY with/without UVR treatment at 25mJ/cm\(^2\), followed by double immunofluorescence analysis of gp100 (MC lineage-specific marker) and cytokeratin expression for KC. The transfer of melanin between MC and KC was assessed (Fig. 4B,C) and results showed that inhibition of either p38 (by SB) or PI3K (by LY) significantly decreased melanin transfer. By contrast, ERK inhibition (PD) did not inhibit melanin transfer compared to basal control cells. Taken together these data suggest that ERK1/2 is not involved in UVR-induced melanin transfer or filopodia formation. The involvement of PI3-K in UVR-induced melanin transfer however, substantiates our proposed view of how MC filopodia interact with KC phagocytosis during the melanin transfer process (16). Here the motor protein MyoX, a recognised effector of phagocytosis, acts as a molecular link between PI3-K activation and pseudopodia extension during phagocytosis (47).

To further study the effects of UVR on p38 and phospho-p38 expression, MC were pre-treated with SB p38 kinase inhibitor (10µM) 1hr prior to UVR irradiation (Fig. s2). UVR irradiation in the absence of SB translocated p38 protein to the cell nucleus and increased the expression of both p38 and phosphorylated p38 (p-p38) compared to the basal status. While addition of SB reduced phospho-p38 to below basal levels (inhibiting phosphorylation of p38), no change in unphosphorylated p38 expression was detected. SB addition also decreased levels of nuclear p38 and phospho-p-38 levels overall (both nuclear and cytoplasmic expression) post-UVR irradiation (Fig. s2). The above results clearly show that

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UVR activates p38 MAPK signalling through phosphorylation of p38 and translocation of p38 into the nucleus in epidermal melanocytes.

It is possible that p38 MAPK activated by UVR may also induce E-Cadherin protein expression to stimulate filopodia formation and melanin transfer in human melanocytes, and there is evidence from other systems that p38 MAPK activation can induce of CDH1/E-Cadherin genes (e.g., colon cancer cells (48) and during mouse gastrulation (49).

UVR irradiation of human MC results in the p38 MAPK-dependent phosphorylation of CREB (Ca2+/cAMP response element binding protein), and the latter can then induce Mitf expression in these cells (50-52). Our demonstration here that UVR exposure activates p38 MAPK in human melanocytes (Fig s2) to regulate filopodia formation and melanin transfer could involve CREB stimulation of Mitf gene expression. Rab17, whose expression is regulated by Mitf, is known to induce filopodia formation in melanocytes and to increase melanosome concentration at the periphery of melanoma cells (53). It would be interesting to study whether p38 MAPK-mediated activation of Mitf in human MC, under the influence of UVR, is also involved in filopodia formation and melanin transfer. UVR also induced filopodia formation and subsequent melanin transfer in human MC:KC co-culture via the PI3K-pathway (Fig. 4), and it is of note that E-cadherin-mediated cell-cell adhesion has been recently reported to stimulate PI3K/Akt activation in human embryonic stem cells (54). Thus, UVR-induced filopodia formation and melanin transfer in human skin may be also dependent on the PI3K activation by E-Cadherin-mediated cell-cell adhesion.

In summary, we report that E-Cadherin can mediate UVR-induced melanin transfer, opening a new avenue to explore how Ca^{2+} signalling influences human pigmentation. Also relevant to studies of pigmentation is the observation that homophilic E-Cadherin cell–cell adhesion is redox-sensitive (55), a finding we previously have shown to be most markedly observed in vitiligo patients (56), which may implicate ROS-disrupted status of E-Cadherin function also in vitiligo pathogenesis (57). The latter study reported that E-Cadherin is required for melanocyte adhesion to the basal layer and these authors have developed 3D models to show that this can be disrupted in the context of both oxidative and mechanical stresses.

Material and Methods:

Materials: Calcium chloride (CaCl₂) was from Invitrogen, p38-specific inhibitor SB203580 was from Sigma, while inhibitors PD98059 (MEK) and LY294002 (PI3K) were from Cell Signaling technology, Inc. (Beverly, MA, USA). Antibodies to β-Catenin, MyoX, E-Cadherin,
Cdc42 antibody and E-Cadherin were from Abcam, (Cambridge, UK), while NKI/beteb from Monosan and cytokeratin were from Santa Cruz Biotechnology, (Santa Cruz, CA, USA).

**Matched epidermal melanocyte/keratinocyte co-culture:** Human abdominal skin was obtained with informed consent and local research ethics approval from normal healthy Caucasian donors with skin photo-type II (n=5, female 29-62y, average 52y) after elective plastic surgery. All cell culture reagents were from Invitrogen Ltd. (Paisley, UK) unless stated otherwise. Epidermal melanocytes(s) (MC) cultures were established as previously described (16) and grown in keratinocyte(s) (KC) serum-free medium (K-SFM) with Eagle’s minimal essential medium (EMEM) supplemented with 1% FBS, 1x non-essential amino acids, penicillin (100U/ml)/streptomycin (100µg/ml), 2mM L-glutamine, 5ng/ml basic fibroblast growth factor, and 5ng/ml endothelin-1 (Sigma, Dorset, UK).

Matched epidermal KC were established from the same biopsy specimen as MC above (17) and grown in K-SFM supplemented with 25µg/ml bovine pituitary extract (BPE), 0.2ng/ml rEGF, penicillin (100U/ml)/streptomycin (100µg/ml), and 2mM L-glutamine. Culture medium was replenished every second day. KC and MC were identified using anti-cytokeratin antibody (Abcam, Cambridge, UK) and melanocyte-specific NKI/beteb antibody (Monosan, Uden, Netherlands) to gp100 respectively. For co-culture studies, MC (passage 3) and KC (passage 2) were seeded onto Lab-Tek® chamber slides (ICN Biomedicals Inc., Aurora, OH, USA) at 4x10⁴ cells/well and in 1 MC to 10 KC ratio (17). Analysis of melanosome transfer was performed at 24h. For some experiments, MC or KC or MC:KC co-culture were treated with inhibitors SB203580 (SB, 10µM), PD98059 (PD, 10µM) and LY294002 (LY, 10µM) in the presence or absence of UVR.

**UV irradiation:** MC or MC:KC monocultures were irradiated with UVR as previously described (16). Briefly, cells were cultured in ‘starved’ medium lacking FBS and BPE (i.e. retaining bFGF and endothelin-1 for MC viability), temporarily submerged in PBS and irradiated with 25mJ/cm² UVR using a fluorescent UVB lamp (Waldmann UV6; emission 290–400nm, peak 313nm; Herbert Waldmann GmbH, Villingen-Schwenningen, Germany). The UVR used consisted of 66% UVB and 34% UVA. PBS was removed immediately after irradiation and replaced with fresh ‘starved’ media. Control cells were treated similarly but not irradiated. MC were analysed by scanning electron microscopy (SEM) and the melanosome transfer assay after 24 h UVR irradiation to evaluate filopodia and melanin transfer, respectively. Expression of the test proteins was assessed by double immunolabeling in MC monoculture after 25mJ/cm² UVR exposure.

**Cell treatments:** For calcium treatments cells were incubated in starved calcium-free media for 24h then incubated in media containing CaCl₂ (0.1mM, 0.25mM and 0.5mM for 24h).
MCs were analyzed by SEM after 24 h after treatment to evaluate filopodia status. MC:KC co-cultures were analyzed by melanin transfer assay after 24 h after treatment to evaluate melanin transfer. E-Cadherin, MyoX, VASP, Cdc42 and β-Catenin expression in MCs were analyzed by immunofluorescence and western blot at 24 h time point. MC:KC co-cultures established with MC or KC treated with either E-Cadherin siRNA or control siRNA were also exposed to 25 mJ/cm² UVR. Melanosome transfer was assessed at 24h post-UVR irradiation. For chemical inhibition, cells were treated with inhibitor for 1h before being irradiated. SB203580 (SB) and LY294002 (LY) and PD98059 (PD) were used at a concentration of 10 μM.

**Immunofluorescence of skin:** Ten micron sections were air-dried at room temperature (RT) for at least 1h, and then fixed in ice-cold acetone for 10 mins at -20°C. After equilibrating the slides were rinsed in PBS for 3 x 5 mins and sections isolated using a PAP Pen (Zymed, UK). Non-specific antibody binding was reduced by incubating in 10% donkey serum (Sigma-Aldrich, UK) diluted in PBS for at least 30 mins. Serum was poured off and followed by incubation in primary antibodies diluted in PBS containing 1% donkey serum overnight in a humidified slide chamber at 4°C. Excess primary antibody was rinsed by washing in PBS for 3 x 10 mins. Tissue was incubated in donkey Alexa-488 and 594 conjugated secondary antibodies (1:100 dilution, Invitrogen Molecular Probes, UK) for 1 hour at RT then rinsed in PBS for 4 x 10 mins. Slides were mounted for confocal microscopy under sealed coverslips in fluorescent mounting medium containing DAPI nuclear stain (VectorLabs, UK). Images were collected using the 365nm (DAPI) and 488nm (Alexa-488) and 543 (Alexa-594) channels on a Zeiss LSM confocal microscope by sequential line scanning. Images were processed using the LSM confocal image browser software (Zeiss, UK) and ImageJ (freeware).

**SEM assessment of cell morphology:** MC monoculture was prepared for SEM as described previously (16). Briefly, cells were fixed with 1% glutaraldehyde at 37 °C, post-fixed in 1% osmium tetroxide and 1% tannic acid as a mordant, dehydrated through a series of alcohol (20% to 70%), stained in 0.5% uranyl acetate, followed by dehydration (90% and 100%) before final dehydration in hexamethyl-disilazane (Sigma, Dorset, UK) and air-drying. Each slide was gold sputter-coated (EMITECH, K550) (Blazer 20 mA) for 10 min. Specimens were viewed under field emission SEM (FEI Quanta 400, Eindhoven, the Netherlands) at 10 keV.

**Immunofluorescence confocal microscopy:** Double-immunofluorescence staining in MC monocultures, MC:KC co-culture, and human skin cryosections was performed as described previously (16). Briefly, cells and tissue were fixed in ice-cold methanol for 10 min before air
drying and rehydration in PBS before blocking with 10% donkey serum (90 min) before overnight incubation at 4°C with E-Cadherin (1:50), MyoX (1:50), Cdc42 (1:50), VASP (1:200) and β-Catenin (1:50), followed by incubation with Alexa488-conjugated secondary antibody (1:100) (Invitrogen, Paisley, UK) for 1h. The second primary antibodies to cytokeratin (1:100) (Abcam, Cambridge, UK) or NKI/beteb (1:30), were applied for 1h followed by an Alexa594-conjugated secondary antibody (1:100) (Invitrogen, Paisley, UK). Slides were mounted in 4',6-diamidino-2-phenylindole (DAPI)-containing medium (Vector, Peterborough, UK) and imaged on a Zeiss LSM 510 confocal microscope (Carl Zeiss, Jena, Germany).

**siRNA Knockdown of E-Cadherin in MC:** MC monocultures or MC:KC co-cultures were transfected with siRNA according to the manufacturer’s instructions (Invitrogen, Paisley, UK). Briefly, 1d prior to siRNA treatment the cells were incubated at 37°C, 5% CO2 for 12h to allow cell attachment. The following synthetic siRNAs (Qiagen, West Sussex, UK) were used: *Felxitube Gene solution for E-Cadherin (CDH1), Entrez gene ID:999 (4siRNAs) (cat no. GS999; Detected transcript- NM_004360; length of transcript- 4815 bp). E-Cadherin siRNA (25nM) or control siRNA (25nM) (non-homologous to mammalian genome) was incubated with Lipofectamine 2000 (Invitrogen, Paisley, UK) for 20min to allow complex formation, before addition to co-cultures. Transfection medium was replaced after 12h with complete media and at 24h post siRNA transfection ‘knockdown’ was verified by immunofluorescence using antibodies against E-Cadherin (data not shown). For some experiments *E-Cadherin* siRNA-treated MC or *control* siRNA-treated MC were processed by SEM in order to test the siRNA effects on filopodia. MC:KC co-cultures were processed at 24 h by double labelling with gp100 (NKI/beteb) and cytokeratin antibody to detect melanosome transfer to KC.

**Quantitative analysis of melanosome transfer:** This was performed as previously described (17). Briefly, evaluation of melanosome transfer MC:KC co-cultures were performed by counting fluorescent gp100-positive spots within recipient KC in 5 random microscopic fields per well at 60x magnification in 3 independent experiments.

**Statistical analysis:** Statistical analysis was performed using Student’s paired t test. Quantitative data are presented as means ± SE for three separate experiments. Statistically significant differences are denoted with asterisks; *p< 0.01, **p< 0.001 and ***p< 0.0001.
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Conflict of Interest:

DJT, SKS, RB, SKSikkink are/were employees of the University of Bradford (UK). RK, CN and SS are employees of LVMH Recherche (France).

References


Legends

Figure 1: Normal adult human melanocytes express E-Cadherin and filopodial proteins Cdc42, β-Catenin and VASP in situ, which are upregulated by UVR in cultured epidermis melanocytes.

A: Double immunolabelling of normal human epidermis with anti-gp100 (NKI/beteb) (red) and (i) anti-E-cadherin antibody (green), (ii) anti-β-Catenin antibody (green), (iii) anti-Cdc42 antibody (green), (D) anti-VASP antibody (green). Left panel: Merge; Boxed area indicates high-power view of MC showing co-localization as a merged image (white, arrows). Right panel represent corresponding individual NKI/beteb immunoprobes. Scale = 60 μM

B: MC monoculture without (left panel) or with exposure to 25mJ/cm² UVR for 24h (right panel). MC were double immunolabelled with anti-NKI/beteb (red) (lower panels) and in upper panels (i) anti-E-cadherin antibody (green), (ii) anti-β-Catenin antibody (green), (iii) anti-Cdc42 antibody (green), (iv) anti-VASP antibody (green) to reveal increase in protein expression in response to UVR. Scale = 22 μM

Figure 2: Effect of E-Cadherin knockdown on melanocyte filopodia formation and melanin transfer to keratinocytes

A: (Upper panel) The dorsal surface of a MC treated with control siRNA: (i) basal condition, numerous filopodia are present, (ii) 25mJ/cm² UVR-treated MC filopodia are induced. (Lower panel). The dorsal surface of a MC treated with E-Cadherin siRNA (i, ii) exhibited an almost complete inhibition of filopodia formation irrespective of 25mJ/cm² UVR treatment. Scale bars: 50μM. High power views of the boxed regions are shown in right panels. Scale bars: 5μM.

B: Double-immunolabelling of MC/KC co-cultures for gp100 (NKI/beteb, green) and cytokeratin (red) revealed clear changes in number of transferred green fluorescent spots (i.e. melanin granules transferred to KC). MC/KC coculture established with control siRNA: (i) numerous gp100-positive spots are seen in KC in basal condition; (ii) increased numbers of gp100-positive spots are transferred after 25mJ/cm² UVR treatment. (iii, iv) MC/KC co-culture established with E-Cadherin-siRNA-treated cells exhibited reduced number of gp100-positive granules are transferred to KC irrespective of 25mJ/cm² UVR treatment. Scale bars: 10μM:

C: Quantification of melanosomes transferred to KC. Data are represented as means ± S.E. 20 cells/condition were assessed in each of 3 independent experiments. *p<0.01, **p<0.001.
Figure 3: Effect of Ca2+ on melanocyte filopodia formation and melanin transfer to keratinocytes

A: (Left panel) The dorsal surface of a MC treated with increasing concentration of Ca2+ induced filopodia in a dose dependent manner: (i) basal condition (Ca2+ free) (ii) 0.1mM Ca2+; (iii) 0.25mM Ca2+; (iv) 0.5mM Ca2+; (v) 1mM Ca2+. Scale bar: 20μM. High power views of the boxed regions are shown in right panels. Scale bar: 5μM.

B: (i) Double-immunolabelling of MC/KC cocultures for gp100 (NKI/beteb, green) and cytokeratin (red) revealed a clear increase in number of transferred green fluorescent granules (i.e. melanin granules transferred to KC) with increasing concentration of Ca2+: (i) basal condition (Ca2+-free) (ii) 0.1mM Ca2+; (iii) 0.25mM Ca2+; (iv) 0.5mM Ca2+; (v) 1mM Ca2+. Scale bar: 10μM.

C: Quantification of melanosomes/melanin granules transferred to KC. Data are represented as means ± S.E. 20 cells/condition were assessed in each of 3 independent experiments. *p<0.001.

Figure 4: Effect of MAPK inhibitors on melanocyte filopodia formation and melanin transfer to keratinocytes

A: (Left panel) The effect of specific kinase inhibitors on UVR-induced filopodia on the dorsal surface of MC (24h). MC were incubated with or without UVR (25mJ/cm2) in presence and absence of SB203580 (SB, 10µM), PD98059 (PD, 10µM) and LY294002 (LY, 10µM). Photographs revealed that SB & LY inhibition caused a decrease in UVR-stimulated filopodia formation, while PD inhibition did not. Scale bars: 20μM. High power views of the boxed regions are shown in right panels. Scale bars: 5μM.

B: The effect of specific kinase inhibitors on UVR-induced melanin transfer in MC/KC co-culture was assessed. Cells were incubated with or without UVR (25mJ/cm2) for 24h in presence or absence of SB203580 (SB, 10µM), PD98059 (PD, 10µM) and LY294002 (LY, 10µM). Cells were double-immunolabelled with anti-gp100 antibody (NKI/beteb, green) and anti-cytokeratin (red); and revealed clear changes in number of green fluorescent transferred to KC. Scale bar: 10μM.

C: Quantification of melanosomes transferred to KCs shown in Da. Data are means ± S.E; 20 cells/condition were assessed in each of 3 independent experiments. *p<0.001, **p<0.001, NS; Not-significant.
Figure s1 Effect of Ca²⁺ on filopodial protein expression in melanocytes

MC monoculture without (left panel) or with (left panel) exposure to 0.25mM Ca²⁺ for 24 h (right panel). MC were double immunolabelled with anti-NKI/beteb (red) (lower panel) and in upper panels (i) anti-E-cadherin antibody (green), (ii) anti-β-Catenin, (iii) anti-Cdc42 antibody (green), (iv) anti-MyoX antibody (green), (v) anti-VASP antibody (green) to reveal protein expression change in response to 0.25mM Ca²⁺. Scale bar: 10µM.

Figure s2: Effect of UVR on p38 MAPK activation in melanocytes

MC monocultures were treated with or without UVR (25mJ/cm²) in presence or absence of SB203580 (SB, 10µM) (2h). Cells were double-immunolabelled with anti-phosphop38 MAPK (green) or anti-p38 MAPK (green) and anti-gp100 antibody (red) to reveal nuclear translocation/activation of p38 MAPK. Scale = 20µm.
Figure 1:
Figure 2:

A

Basal

Ctrl siRNA

+ UVB (25mJ/cm²)

E-Cadherin siRNA

B

Basal

Ctrl siRNA

+ UVB (25mJ/cm²)

E-Cadherin siRNA

C

% Change of gp100 positive spots/KC vs Basal level

* P<0.01, ** P<0.001
Figure 4:

A

Basal

UVB (25mJ/cm²)

UVB + SB203580

UVB + PD98059

UVB + LY294002

B

Basal + UVB (25mJ/cm²)

Control

SB203580 p38 inhibition

PD98059 ERK inhibition

LY294002 PI3K inhibition

C

% Change of gp100 positive spots per cell

NS - Not significant, *P<0.01, **P<0.001