Fibronectin-Containing Extracellular Vesicles Protect Melanocytes against Ultraviolet Radiation-Induced Cytotoxicity

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Skin melanocytes are activated by exposure to UV radiation to secrete melanin-containing melanosomes to protect the skin from UV-induced damage. Despite the continuous renewal of the epidermis, the turnover rate of melanocytes is very slow, and they survive for long periods. However, the mechanisms underlying the survival of melanocytes exposed to UV radiation are not known. Here, we investigated the role of melanocyte-derived extracellular vesicles in melanocyte survival. Network analysis of the melanocyte extracellular vesicle proteome identified the extracellular matrix component fibronectin at a central node, and the release of fibronectin-containing extracellular vesicles was increased after exposure of melanocytes to UVB radiation. Using an anti-fibronectin neutralizing antibody and specific inhibitors of extracellular vesicle secretion, we demonstrated that extracellular vesicles enriched in fibronectin were involved in melanocyte survival after UVB radiation. Furthermore, we observed that in the hyperpigmented lesions of patients with melasma, the extracellular space around melanocytes contained more fibronectin compared with normal skin, suggesting that fibronectin is involved in maintaining melanocytes in pathological conditions. Collectively, our findings suggest that melanocytes secrete fibronectin-containing extracellular vesicles to increase their survival after UVB radiation. These data provide important insight into how constantly stimulated melanocytes can be maintained in pathological conditions such as melasma.

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INTRODUCTION

Extracellular vesicles (EVs) are nanosized phospholipid-bilayer spherical particles that are secreted by most cells to facilitate intercellular communication (Bang and Thum, 2012; Simons and Raposo, 2009). Transcriptomic and proteomic profiling has revealed that EVs contain enzymes, receptors, structural and extracellular matrix proteins, transcription factors, and nucleic acids that perform diverse functions in recipient cells (Guescini et al., 2010; Kim et al., 2013a, 2014; Valadi et al., 2007). For example, EVs derived from stem cells are involved in tissue repair (Lai et al., 2010), whereas those derived from dendritic cells are important for antigen presentation and immune responses (Thery et al., 2002). Exosome-like vesicles derived from *Plasmodium falciparum*-infected erythrocytes facilitate communication within parasite populations (Regev-Rudzki et al., 2013), and tumor-derived EVs play a critical role in oncogenesis (Lima et al., 2011; Saleem and Abdel-Mageed, 2015) and favor the establishment of melanoma metastasis, possibly by upregulating TGF-β1 production and consequently down-modulating macrophage activation (Lima et al., 2009). A recent study also demonstrated that EVs derived from highly metastatic melanomas promoted the metastatic potential of primary tumors by signaling to bone marrow progenitors via the transfer of hepatocyte growth factor receptors (Peinado et al., 2012).

Fibronectin (FN) is a major extracellular matrix component that is involved in cell proliferation, migration, differentiation, and survival (Geiger et al., 2001; Kosmehl et al., 1996). FN is an EV protein (Deng et al., 2012; Kim et al., 2013a; Yu et al., 2006), as confirmed by multiple proteomic analyses (Kim et al., 2013a). The FN-containing EVs (FN-EVs) released from breast tumor cells in tumor tissue, where leukocytes participate in the induction of FN-EVs from tumor cells, promote tumor cell invasion and metastasis via activation of the focal adhesion kinase/Src-dependent signaling pathways (Deng et al., 2012). In EVs derived from trophoblasts and ovarian cancer cells, FN is a key molecule that induces the production of the proinflammatory cytokine IL-1β in macrophages (Atay et al., 2011). Furthermore, EVs from MDA-MB-231 breast cancer and U87 glioma cells confer the transformation phenotype to normal fibroblasts and epithelial cells by transferring tissue transglutaminase and FN, which induce signaling events that enhance cell survival and aberrant growth (Antonyak et al., 2011).

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Abbreviations: ACN, acetonitrile; CD81, cluster of differentiation 81; EV, extracellular vesicle; FN, fibronectin; PBS, phosphate buffered saline

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Melanocytes located in the basal layer of the skin epidermis secrete and transfer melanin-containing vesicles called melanosomes to neighboring keratinocytes to protect keratinocytes and the skin underneath them from UV radiation-induced damage (Hearing, 1993). Basal keratinocytes are highly proliferative and undergo a well-defined differentiation program that includes an apoptotic process, resulting in the continuous renewal of the epidermis. Unlike keratinocytes, melanocytes display low proliferative potential and are highly resistant to cell death because of their constitutive expression of the anti-apoptotic protein Bcl2, which leads to their sustained survival in the epidermis for decades (Rinnerthaler et al., 2014). Because of these characteristics, melanocytes are vulnerable to mutations that can occur over time as a result of repetitive UV exposure, which can cause hyperpigmentation disorders or the formation of melanomas. Given that melanocyte survival is pivotal for maintaining epidermal homeostasis because they prevent UV-induced DNA photodamage and tumorigenesis (Bertolotto, 2013; Luciani et al., 2011), it is critical for melanocytes to maintain their genomic stability, and other protection mechanism(s), besides BCL2, seem to be necessary. In this study, we examined the effect of melanocyte-derived EVs on the survival of melanocytes that were exposed to UVB radiation. We found that UVB enhanced the secretion of EVs that contained FN, which promoted the survival of UVB-irradiated melanocytes. Our findings provide important insight into melanocyte intercellular cooperation during protection against UVB-induced damage and the promotion of their survival under stressful conditions.

RESULTS

Human primary melanocytes release FN-EVs

Dynamic light scattering analysis of EVs from the cultured supernatants of human primary melanocytes revealed that they were homogeneous in size, with an average diameter of 195.10 ± 17.40 (Figure 1a). Proteomic analysis identified 262 proteins (Supplementary Table S1 online), 73% of which were included in the EVpedia (Kim et al., 2013a, 2014), compared with the 12% of EV proteins in the whole human proteome (UniProt). Among these proteins, EV-enriched proteins such as cluster of differentiation 81 (CD81) and heat shock protein 90 were identified, and their expression was further confirmed by western blot analysis (Figure 1b), wherein they showed enrichment in only the EV fraction and not in the medium control. Of the 262 proteins identified, 174 (66.4%) were annotated with at least one Gene Ontology cellular component term, and the composition of proteins related to the cell surface, lipid rafts, and the extracellular matrix (approximately 67.8%) was relatively high when compared with the composition of those in whole proteome (Figure 1c, Supplementary Table S2 online), implying the possible involvement of EVs in intercellular interactions and communication. The network analysis of the identified EV proteins, performed using the STRING database (version 9.05) (Franceschini et al., 2013) and the network-visualizing program Cytoscape (version 2.8.3) (Shannon et al., 2003), revealed a network of 39 proteins (Figure 1d). FN1 occupied a critical position in this network, that is, it had the highest centrality based on degree, betweenness, closeness, and radiality (Supplementary Table S1) among membrane proteins (blue nodes, Figure 1d). We next examined whether melanocyte-derived EVs contained FN by western blot analysis. To verify the FN signal, melanocytes were treated with forskolin, which is known to induce FN production (Dean et al., 1989). EVs isolated from mock-treated melanocytes contained FN, and the level of FN was increased by forskolin treatment (Figure 1e), suggesting that melanocytes inherently release FN-EVs. Confocal microscopy analysis demonstrated that FN colocalized with the EV marker CD81 on vesicular structures inside or on melanocyte dendrites (Figure 1f).

Fibronectin in extracellular vesicles is increased by UVB radiation

Because FN is secreted to promote cell survival, we investigated whether FN-EV secretion can be regulated. We treated melanocytes with UVB radiation, a major stressor of skin cells, and analyzed the release of EVs by western blot analysis using anti-EV marker (CD81 or heat shock protein 90) antibodies and determined the FN content in the EVs either by silver staining of SDS-PAGE gels or by western blot analysis. We found that the levels of CD81 and heat shock protein 90 were increased in the EVs isolated from UVB-treated melanocytes (Figure 2a, second and third panels) and that FN levels were increased in EVs (Figure 2a and b, first panels) but decreased in cell lysates (Figure 2b, second panel) without changes in mRNA expression (Figure 2c). Because the average sizes of the EVs isolated from UVB-treated or untreated melanocytes were similar (Figure 1a, Supplementary Figure S1 online), these data imply that UVB radiation may accelerate not only EV secretion but also the loading of FN from the intracellular pool into EVs. To confirm that FN is loaded into and secreted with EVs, we treated melanocytes with two different EV biogenesis inhibitors: the SMase inhibitor GW4869, which is the general inhibitor of EV secretion, or the p53 inhibitor pifithrin-μ, which inhibits EV secretion through a p53-dependent pathway (Yu et al., 2006), before UVB exposure. Both inhibitors decreased FN levels in EVs and levels of the EV marker CD81 (Figure 2d and e) but increased FN levels in cell lysates (Figure 2f and g), indicating that FN is loaded into and secreted with EVs after UVB radiation, probably through a p53-dependent pathway.

In the human skin epidermis, a single melanocyte is surrounded by 20–40 keratinocytes (Hoath and Leahy, 2003), and UVB radiation could also affect FN-EV secretion by these keratinocytes, which would contribute to the EV pool around the melanocytes in the skin. Therefore, we examined the release of FN-EVs by keratinocytes after UVB radiation. However, our results indicated that UVB did not stimulate FN-EV secretion by keratinocytes; moreover, the amount of FN in keratinocyte-derived EVs appeared to be different from the amount in melanocyte-derived EVs (Figure 3a). We used human dermal fibroblasts, which secrete FN to the basement membrane (Dean et al., 1989), to confirm FN signal and to investigate UV responsiveness. Similar to melanocytes, fibroblasts released EVs containing FN, as indicated by a single band above 98 kDa that was increased by UVA radiation, a major stressor of dermal fibroblasts (Pohl and Christophers, 1979) (Figure 3b). Our data indicate that in

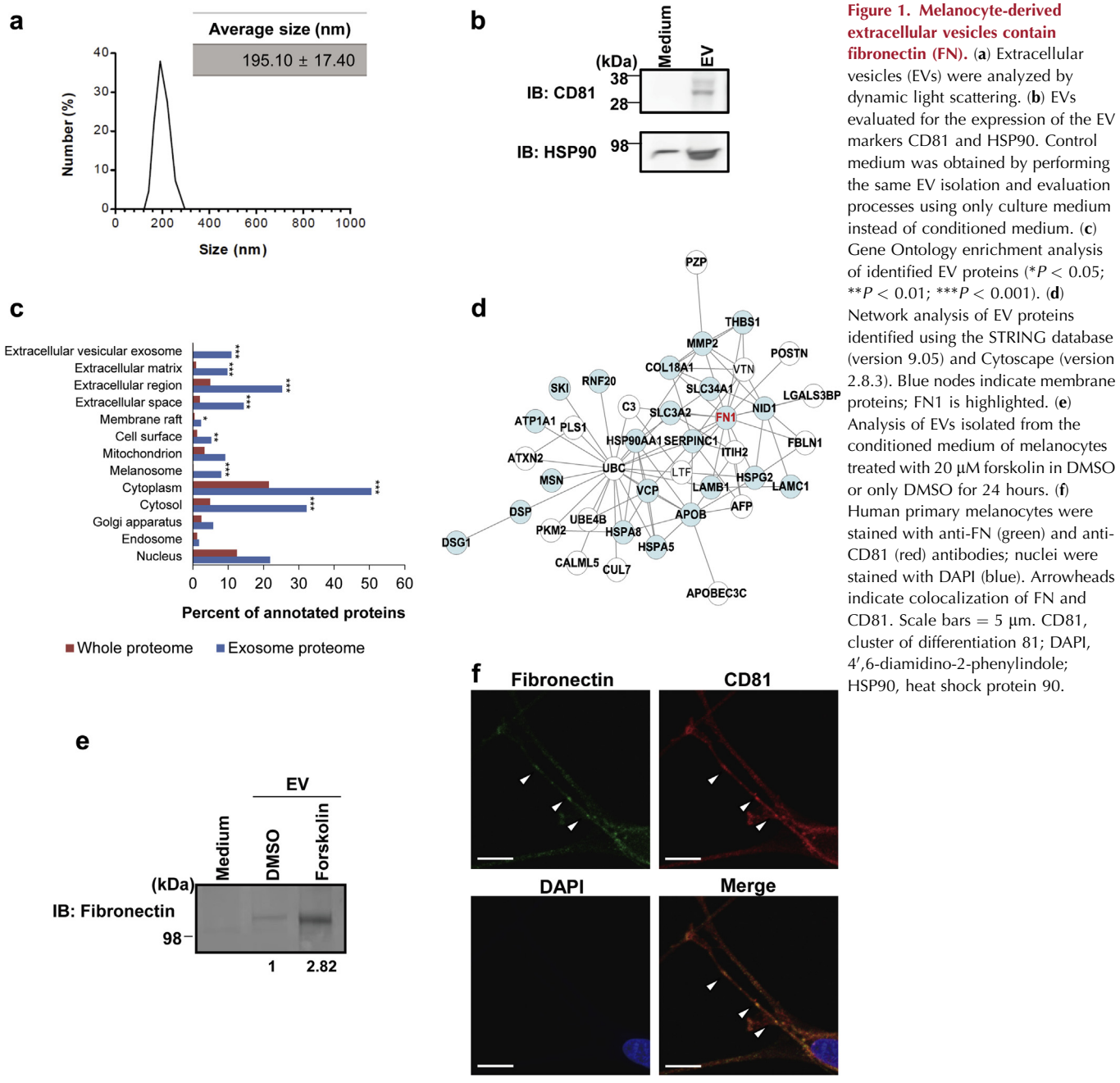

Figure 1. Melanocyte-derived extracellular vesicles contain fibronectin (FN). (**a**) Extracellular vesicles (EVs) were analyzed by dynamic light scattering. (**b**) EVs evaluated for the expression of the EV markers CD81 and HSP90. Control medium was obtained by performing the same EV isolation and evaluation processes using only culture medium instead of conditioned medium. (**c**) Gene Ontology enrichment analysis of identified EV proteins (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). (**d**) Network analysis of EV proteins identified using the STRING database (version 9.05) and Cytoscape (version 2.8.3). Blue nodes indicate membrane proteins; FN1 is highlighted. (**e**) Analysis of EVs isolated from the conditioned medium of melanocytes treated with 20 μM forskolin in DMSO or only DMSO for 24 hours. (**f**) Human primary melanocytes were stained with anti-FN (green) and anti-CD81 (red) antibodies; nuclei were stained with DAPI (blue). Arrowheads indicate colocalization of FN and CD81. Scale bars = 5 μm. CD81, cluster of differentiation 81; DAPI, 4′,6-diamidino-2-phenylindole; HSP90, heat shock protein 90.

the skin epidermis, keratinocytes and melanocytes release distinct types of FN-EVs and that only melanocyte-derived FN-EVs are upregulated by UVB radiation, suggesting a specific function for melanocyte-derived FN-EVs in melanocytes and in protecting the skin from UV-induced damage.

Fibronectin mediates the prosurvival effect of extracellular vesicles in UVB-irradiated melanocytes

Given that FN is implicated in cell survival and that melanocytes express the FN receptor $\alpha_5\beta_1$ integrin (Natali et al., 1995), we hypothesized that FN-EVs might play a role in melanocyte survival after UVB exposure. EVs isolated from the conditioned media of control cells (c-EVs) or UVB-exposed melanocytes (UV-EVs) were used to treat melanocytes, after which cell apoptosis assays were performed using a TUNEL system and cell viability tests were performed using crystal violet staining. After UVB radiation, apoptotic, TUNEL-positive cell death was observed in some melanocytes that were treated with c-EVs (Figure 4a and b, c-EV, arrowheads). However, TUNEL-positive nuclei were rarely detected in melanocytes that were treated with UV-EVs (Figure 4a and b, UV-EV). This difference disappeared when melanocytes were cultured with EVs derived from the cells pretreated with UV and GW4869 (Figure 4a and b, UV-EV vs. UV-EV/GW4869). In line with the results from the cell apoptosis assay, cell survival of UVB-irradiated melanocytes by UV-EVs, but not by UV-EV/GW4869, was significantly increased compared with that by c-EVs (Figure 4c). The effect of UV-EVs on cell survival was not observed when melanocytes were treated under normal conditions (Supplementary

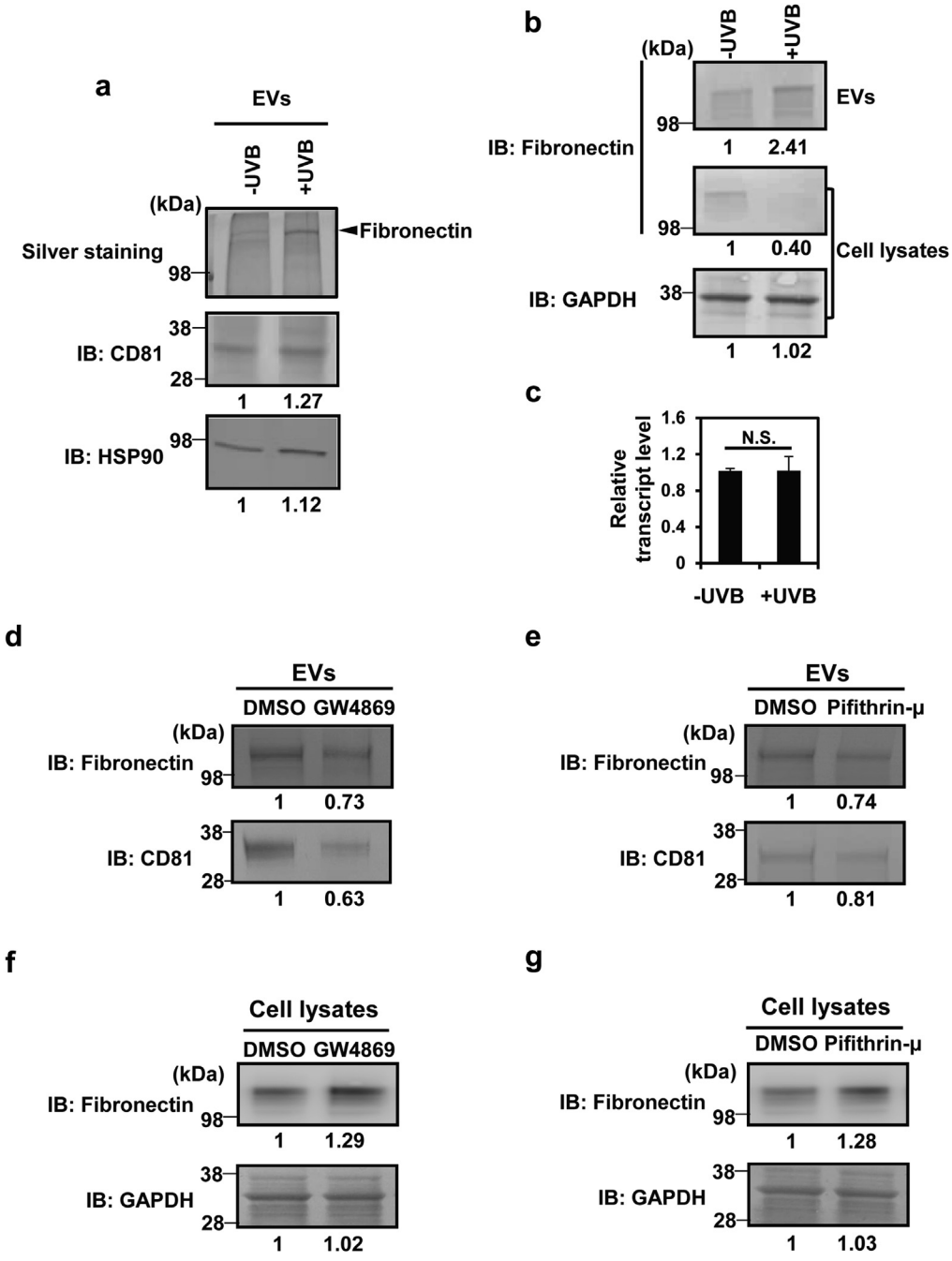

Figure 2. Fibronectin (FN) in extracellular vesicles is increased by UVB radiation in human primary melanocytes. A total of 5×10^6 human primary melanocytes (passage no. 3) were cultured on a 100-mm dish for 24 hours, irradiated with UVB (20 mJ/cm^2), and then incubated for 24 hours. (**a**) FN and extracellular vesicle (EV) markers (CD81, HSP90) were detected in EVs by silver staining and western blot analysis using each antibody. (**b**) FN levels in EVs and cell lysates were analyzed by western blot analysis using anti-FN antibodies. (**c**) FN mRNA expression was examined by RT-qPCR. The data are expressed as the mean ± SD of three independent experiments (N.S., not significant). (**d**–**g**) Human primary melanocytes were treated with 10 μM GW4869 (**d, f**) or 10 μM pifithrin-μ (**e, g**) for 1 hour before UVB exposure. After 20 mJ/cm^2 of UVB exposure, cells were incubated with each inhibitor for an additional 12 hours, and then the (**d, e**) EVs and (**f, g**) cell lysates were analyzed by western blot analysis using each antibody. CD81, cluster of differentiation 81; HSP90, heat shock protein 90; SD, standard deviation.

Figure S2a online). These data indicate that EVs or EV components derived from UVB-exposed melanocytes were involved in the protection of melanocytes from UV-induced cytotoxicity and in the increased survival of UVB-irradiated melanocytes. We also compared the morphology of UVB-irradiated melanocytes that were treated with c-EVs, UV-EVs, or UV-EV/GW4869. Melanocytes cultured with c-EVs after pretreatment with UVB appeared flat, demonstrated increased size, and extended multiple dendrites (Supplementary Figure S2b, c-EV), which are morphological characteristics observed in senescent melanocytes. However, when these melanocytes were cultured with UV-EVs, most of the cells showed a bipolar morphology, smaller size, and fewer dendrites (Supplementary Figure S2b, UV-EV), suggesting that they recovered from UVB stress. This recovery effect was not observed when cells were treated with UV-EV/GW4869 (Supplementary Figure S2b, UV-EV/GW4869). We examined whether the effects of UV-EVs on cell protection and survival were mediated by FN. Isolated UV-EVs were preincubated with anti-FN antibodies and then applied to UVB-irradiated melanocytes. Compared with the cells treated with control IgG-incubated UV-EVs, the melanocytes treated with the anti-FN antibody-incubated UV-EVs showed reduced survival (Figure 4d) and an increased number of condensed apoptotic nuclei (Supplementary Figure S3a online, arrowheads). This inhibitory effect on the promotion of cell survival was also observed when UVB-irradiated melanocytes were cultured with UV-EVs that

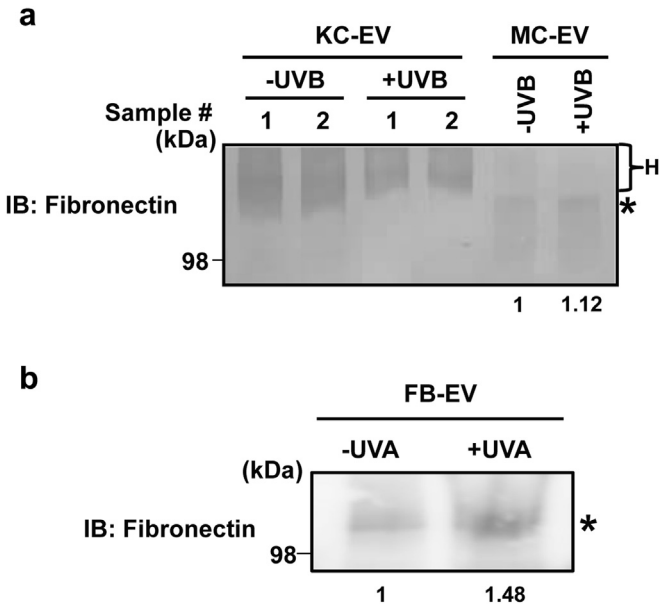

Figure 3. Extracellular vesicles from human primary keratinocytes contain higher molecular weight fibronectin, and fibronectin content did not increase in response to UVB radiation. (**a**) Human primary keratinocytes (KC) irradiated with UVB (30 mJ/cm^2) were cultured for 24 hours, and extracellular vesicles (EVs) isolated from conditioned medium were analyzed by western blot using an anti-fibronectin antibody. Nos. 1 and 2 represent different experiments. (**b**) Human primary fibroblasts (FB) irradiated with UVA (10 J/cm^2) were cultured for 24 hours, and EVs (FB-EV) isolated from conditioned medium were analyzed by western blot using an anti-fibronectin antibody. *, fibronectin; H, highly modified fibronectin; MC, melanocytes.

were preincubated with the FN inhibitor tetrapeptide H-Arg-Gly-Asp-Ser-OH (Supplementary Figure S3b). Overall, these results indicate that EVs increase the survival of UVB-irradiated melanocytes via FN.

Fibronectin is enriched around melanocytes in the hyperpigmented lesions of patients with melasma

Melasma is one of the most common patterns of hyperpigmentation in facial skin. One phenotypic characteristic of melasma is increased pigmentation that is accompanied by persistent hyperactivated melanocytes (Kim et al., 2012). We examined whether FN was enriched around melanocytes in melasma lesions. Skin specimens from patients with melasma, obtained from both hyperpigmented lesional skin and nonlesional skin, were stained with antibodies against FN and human melanoma black-45, a melanocyte marker. FN signal appeared to be dispersed throughout the dermis but concentrated around the basement membrane at the periphery of nonlesional skin (Figure 5a, FN; N#1, N#2, and N#3, arrowheads). This basement membrane-associated signal was decreased in the lesional skin (Figure 5a, FN; L#1, L#2, and L#3). Interestingly, the FN signal colocalized with melanocytes in the hyperpigmented lesions (80.6 ± 17.3%), but this was rarely observed in melanocytes in nonlesional skin (11.1 ± 19.2%) (Figure 5a, merged, boxed areas; Figure 5b). These results suggest that FN is enriched around melanocytes in hyperpigmented lesions, where it may increase melanocyte survival and, possibly, participate in their sustained activation.

DISCUSSION

In this study, we demonstrated that EVs derived from human primary melanocytes are involved in promoting melanocyte survival after UVB radiation and that FN is a key component underlying this EV function.

UV radiation damages DNA in the epidermal cells of the skin (Sinha and Hader, 2002). Melanocytes protect the skin against UV radiation by producing and transferring melanin to adjacent cells (Quevedo et al., 1965), and melanocyte survival is therefore essential for normal, healthy skin. Our data indicate that FN-EVs can be released from skin cells such as keratinocytes, fibroblasts, and melanocytes in vitro (Figures 2a and 3). However, FN-EVs released by keratinocytes are distinct from those released by melanocytes and fibroblasts, as shown by the different FN sizes, which could be due to different posttranslational modifications and responses to UV radiation. It was previously reported that FN could suppress apoptosis by binding to the $\alpha_5\beta_1$ integrin receptor in melanocytes (Natali et al., 1995; Scott et al., 1997). Given these data, the functional activity of FN or FN-EVs may be cell type specific in the skin epidermis, and it may be very important to melanocyte survival mechanisms that inhibit apoptosis. Although FN or FN-EVs are unlikely to be necessary for keratinocyte survival, given their highly proliferative and renewal capabilities, whether FN-EVs released from melanocytes can promote the survival of other cell types, for example, keratinocytes and fibroblasts, remains to be investigated.

Previously, it was reported that EVs, especially EVs derived from stem cells, exhibit cytoprotective properties under normal and stressful conditions. For example, induced pluripotent stem cell-derived EVs can promote the survival of cardiomyocytes, fibroblasts, and cardiac progenitor cells after acute myocardial ischemia/reperfusion injury (Wang et al., 2015), and mesenchymal stem cell-derived EVs improve hepatoprotection after toxicant-induced injury (Tan et al., 2014). In our study, we found that melanocyte-derived EVs also have the ability to protect melanocytes from UVB-induced cytotoxicity and to increase cell survival after UVB radiation. Finally, we identified FN as a key molecule in these processes. This notion is supported by the observed increase in FN-EVs released from UV-EV via either increased EV secretion or increased FN loading into EVs, and by results showing that blocking FN with a neutralizing antibody or an inhibitory peptide reduced the prosurvival effect of FN-EVs on UVB-irradiated melanocytes. Although FN-EVs play an essential role in melanocyte survival after UVB radiation, inhibiting EV secretion had a more pronounced effect on melanocyte survival than blocking FN, which suggests that other EV components may contribute to protecting cells against UVB. EVs contain transmembrane and soluble proteins, lipids, and genetic materials, including mRNAs and miRNAs, which may play important roles in EV-mediated cell-cell communication. Combining next-generation sequencing with proteomic analysis of EVs derived from UVB-exposed melanocytes may lead to the identification of other factors involved in melanocyte survival and help researchers better understand EV-mediated regulation of cellular responses to environmental stresses.

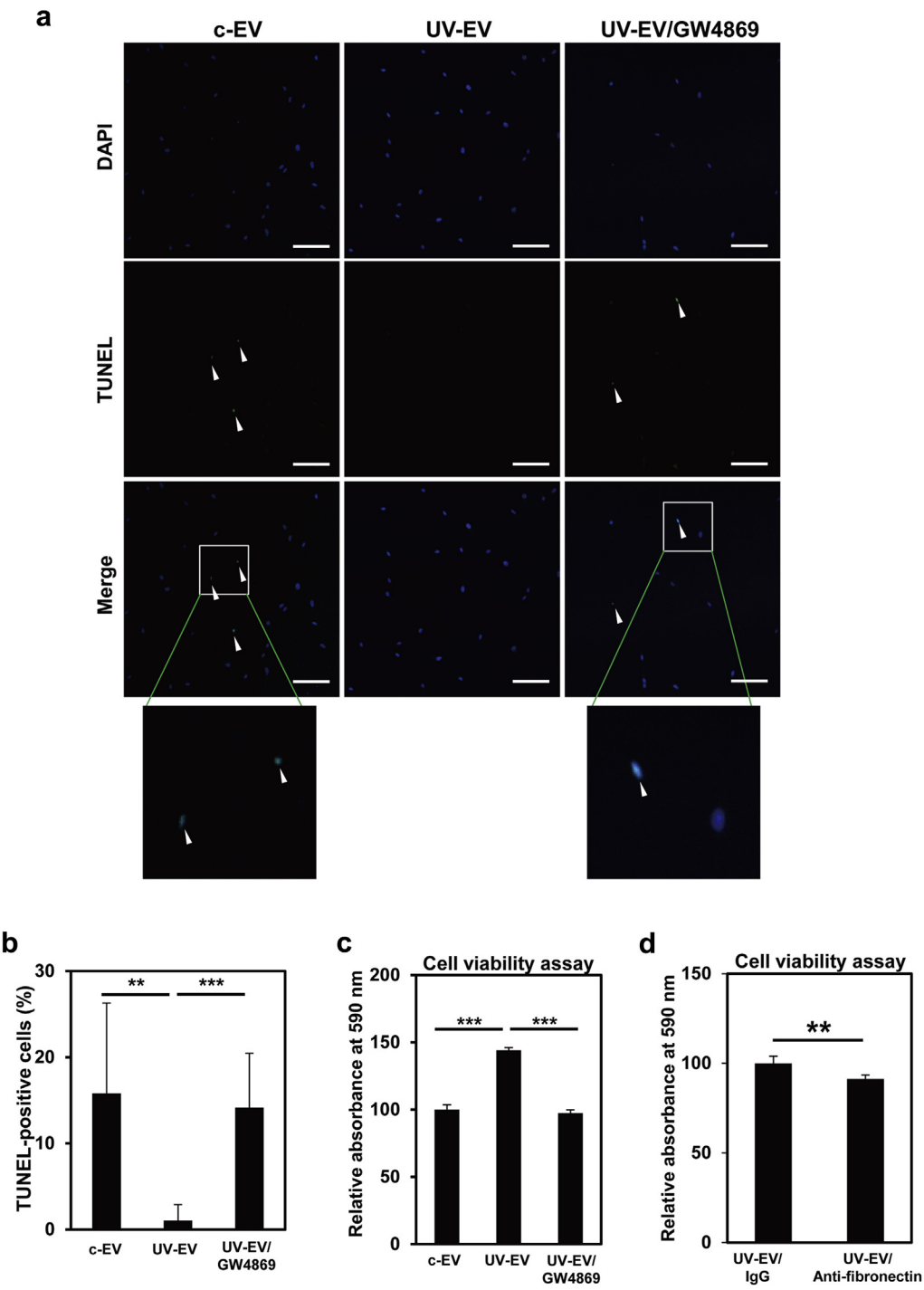

Figure 4. Fibronectin in extracellular vesicles from UVB-exposed melanocytes promotes melanocyte survival after UVB radiation. Each number of human primary melanocytes was plated, irradiated with UVB (20 mJ/cm^2), and then cultured with extracellular vesicles (EVs) from untreated (c-EV), UVB-treated (UV-EV), or UVB$^+$GW4869-treated (UV-EV/GW4869) melanocytes for 3 days. (**a**) Cell apoptosis assay. Cells were seeded at a density of 5×10^3 cells per well in two-well chamber slides. An apoptosis assay was performed using a DeadEnd Fluorometric TUNEL System. Nuclei (DAPI, blue) showing TUNEL-positive signal (green) are indicated by arrowheads. Boxed areas are magnified. Scale bars = 100 μm. (**b**) The quantification of apoptotic cells. After staining with TUNEL, five images per group were taken randomly, and TUNEL-positive cells were counted. The data are presented as the mean ± SD of three independent experiments (**$P < 0.01$; ***$P < 0.001$). (**c**) Cell viability assay. Cells were seeded at a density of 1×10^4 cells per well in six-well plates. Three days after treatment, cells were stained with 0.1% crystal violet, and the absorbance at 590 nm was measured after lysis with 10% acetic acid. The data are presented as the mean ± SD of three independent experiments (***$P < 0.001$). (**d**) Cell viability assay. Cells were seeded at a density of 1×10^4 cells per well in six-well plates. The next day, cells were irradiated with UVB (20 mJ/cm^2) and then cultured with UV-EVs, which had been preincubated with 10 μg of anti-fibronectin antibodies or control IgG, for 3 days and analyzed for cell survival. The data are presented as the mean ± SD of three independent experiments (**$P < 0.01$). DAPI, 4′,6-diamidino-2-phenylindole; SD, standard deviation.

It has been shown that melanocytes survive for decades in the human epidermis; however, hyperactivated, senescent, or genetically instable melanocytes (sensitive to UVB radiation or hormones) must undergo apoptosis to maintain epidermal homeostasis. Otherwise, these cells might cause hyperpigmentation disorders or melanomas. It remains to be investigated in detail why and how pigmentary disorders occur in normal skin. UV radiation and hormones, such as α-melanocyte-stimulating hormone and estrogen, are inducers of pigmentary disorders in many cases, and they can affect a relatively broad area. However, melanocytes in the affected area will respond differently depending on which genetic factors they express, where they are located, and how much and how often they are exposed to stimuli. As a result, there are differences in the amount of stimulation, migration, condensation, and melanin production that occurs in melanocytes, and this may be associated with the formation of hyperpigmented spots in normal skin. For example, genetic factors, such as the Wingless-type MMTV integration site family inhibitory factor-1 and estrogen-induced post-synaptic dentisty-95/discs larges/ZO-1 domain-containing-1 genes, are expressed differently in normal and melasma tissues and are involved in hyperpigmentation (Kim et al., 2012, 2013b). Considering that pigmentary disorders frequently

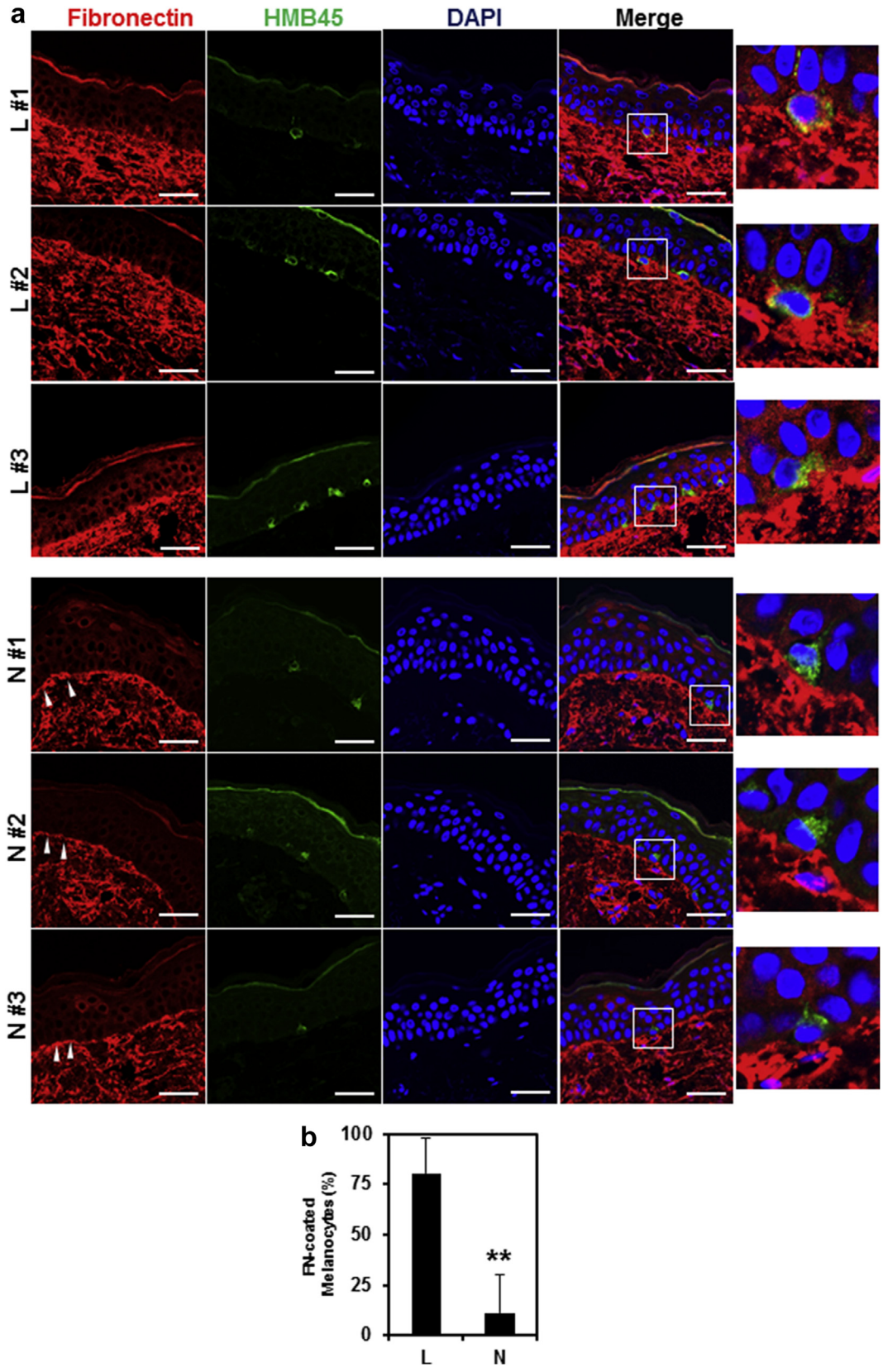

Figure 5. Fibronectin is enriched around melanocytes in the hyperpigmented skin areas of patients with melasma. (**a**) Hyperpigmented lesions (L) and normal (N) areas of human skin specimens obtained from three patients with melasma (#1, #2, and #3) were stained with anti-fibronectin (red) and anti-HMB45 (green) antibodies; nuclei were stained with DAPI (blue). Arrowheads indicate the border between the dermis and the epidermis. The inset is magnified. Scale bars = 50 μm. (**b**) Quantification of melanocytes coated or uncoated with fibronectin in the lesioned (L) and normal (N) skin areas (n = 3). $^{**}P < 0.01$. DAPI, 4′,6-diamidino-2-phenylindole; HMB, human melanoma black.

occur with age, the inherent ability of melanocytes to maintain long periods of survival may be directly involved in the generation of pigmentary disorders, when age is combined with other factors. In this regard, we suggest that the increased survival of melanocytes by FN-EVs may contribute to pigmentary disorders, as indicated by our result showing that FN-containing UV-EVs increased the survival of melanocytes exposed to UV radiation (Figure 4) and that FN was enriched around melanocytes in hyperpigmented lesional skin (Figure 5). We also treated melanocytes with estrogen and observed that similar to tyrosinase, FN mRNA was upregulated (Supplementary Figure S4 online). These results imply that FN provides a survival signal for melanocytes, even in pathological conditions induced by UV radiation or hormones and that this could be one reason for the maintenance of hyperactivated melanocytes. At the beginning of the experiment, we did not fully understand how the FN signals around the melanocytes in the lesional skin of patient's melasma are enhanced by either the increased FN expression level or the increased FN/FN-EVs secretion level. In our opinion, the latter is more probable in the lesional skin based on our in vitro experimental results. Additional studies investigating the effect of EV inhibitors on the pigmentary disorders might be helpful to weigh up the contribution of EV. Given that FN-EVs play a role as prosurvival factors in normal and pathological skins, the inhibition of FN-EV secretion could be one strategy to ameliorate hyperpigmentation disorders or melanomas because they promote the elimination of hyperactivated, senescent, and mutant melanocytes. On the basis of our previous study, where we showed that melanoblast-like cells are present in the human epidermis and that these cells can be differentiated into melanocytes (Cho et al., 2014), the normal human epidermis seems to possess a way to replenish melanocytes when necessary. Through this study, we found that FN-EV secretion was inhibited by GW4869, a general inhibitor of EV secretion that acts by blocking the classical EV biogenesis pathway, or pifithrin-μ, an inhibitor of the tumor suppressor activated pathway 6-dependent EV biogenesis pathway (Yu et al., 2006). The mechanism of action of these inhibitors inhibits the biogenesis of EVs and thereby downregulates the secretion of EVs. Only a few inhibitors have so far been shown to prohibit EV secretion, and no report has claimed to show a direct effect by inhibitors on pigmentary disorders. On the basis of our finding, we presumably think that EV signals including FN could increase around the melanocytes located in lesional sites of other pigmentary disorders (besides melasma) and also the use of these inhibitors could improve their symptoms in this aspect.

In conclusion, we have demonstrated that melanocytes release FN-EVs and that this release is increased in response to UVB radiation to promote melanocyte survival in the presence of UVB stress. We also observed that FN increased in the extracellular area around melanocytes in the hyperpigmented lesions of patients with melasma. These results suggest that melanocytes may function in intercellular cooperation via FN-EVs to support the maintenance of their population after UVB radiation. We also provide important insight into how melanocytes can survive for decades in the epidermis, which is consistently and repetitively exposed to UV radiation, and into how melanocytes in hyperpigmented lesional skin can be controlled.

MATERIALS AND METHODS

Patients

Three patients diagnosed with melasma were enrolled in this study after providing written informed consent. Three pairs of hyperpigmented and adjacent, normally pigmented skin specimens that were located on the lateral side of the upper cheek were biopsied with a 3-mm-diameter punch for immunohistochemical analysis. The study was approved by the Institutional Review Board of the Dongguk University Ilsan Hospital and was conducted according to the Declaration of Helsinki Principles.

Cell culture and materials

Normal human melanocytes (Cascade Biologics, Portland, OR) were maintained in M-254 medium (Cascade Biologics) containing human melanocyte growth supplement (Cascade Biologics). GW4869 (Santa Cruz Biotechnology, Santa Cruz, CA), pifithrin-μ (Sigma, St. Louis, MO), and β-estradiol (estrogen) (Sigma) were dissolved in DMSO. The FN inhibitor tetrapeptide H-Arg-Gly-Asp-Ser-OH (Santa Cruz Biotechnology) was dissolved in phosphate buffered saline (PBS).

EV isolation

For EV isolation, confluent melanocytes were washed with PBS and grown in M-254 medium for 48 hours. Conditioned medium was collected and centrifuged at 500 × *g* for 10 minutes, then at 3,000 × *g* for 20 minutes, and finally at 100,000 × *g* for 2 hours. The resulting EV-containing pellet was resuspended in PBS and stored at −80 °C. Alternatively, conditioned medium was clarified by filtration through 0.45-μm pore filters (Millipore, Billerica, MA) and concentrated using 100-kDa cutoff spin columns (Millipore). EVs were isolated using an ExoQuick-TC kit (SBI, San Jose, CA) according to the manufacturer's instructions. A volume of 20 μl of isolated EVs was analyzed by western blot and silver staining.

Dynamic light scattering analysis

EV diameter was measured using a Zetasizer Nano S instrument (Malvern Instruments, Worcestershire, UK) equipped with a 633-nm laser at a scattered intensity of 10 × 30 s.

In-gel protein digestion

EV proteins were resolved by SDS-PAGE. The SDS-PAGE gel was cut into 10 slices and subjected to in-gel digestion with trypsin (Promega, Madison, WI), as previously described (Bahk et al., 2004). Briefly, the gel slices were washed 4–5 times with 1:1 acetonitrile (ACN)/25 mM ammonium bicarbonate, pH 7.8, dehydrated in 100% ACN, and dried. After reduction in 10 mM dithiothreitol with 100 mM ammonium bicarbonate at 56 °C for 45 minutes and alkylation in 55 mM iodoacetamide with 100 mM ammonium bicarbonate at room temperature for 30 minutes in the dark, the slices were dried in 100% ACN and rehydrated in 325 mM ammonium bicarbonate containing 20 ng trypsin at 37 °C for 20 hours. The liquid was transferred to a new tube, and the remaining peptides were extracted from the gel with 50% (v/v) aqueous ACN containing 0.1% (v/v) formic acid at 30 °C for 40 minutes. The combined supernatants were evaporated and dissolved in 5% (v/v) aqueous ACN solution containing 0.1% (v/v) formic acid for mass spectrometry.

Identification of proteins by LC-MS/MS

The digested peptides were analyzed using reversed-phase capillary HPLC directly coupled to a Finnigan LCQ ion-trap mass

spectrometer (LC-MS/MS) as previously described (Zuo et al., 2001), with slight modifications. The peptides were bound to the trapping column for 10 minutes and eluted with a gradient of 5−80% (v/v) aqueous ACN containing 0.1% (v/v) formic acid at a flow rate of 0.2 μl/min for 50 minutes. Tandem MS was performed at m/z = 450−2,000 Da, and individual spectra were processed using TurboSEQUEST software (Thermo Quest, San Jose, CA). The obtained peak lists were used to query the MSDB or NCBI databases using the MASCOT program (http://www.matrixscience.com). Only significant hits were considered.

Network analysis

To build the interaction network of the identified EV-enriched proteins, the STRING database physical and functional protein interactions function (version 9.05) (Franceschini et al., 2013) and the network-visualizing program Cytoscape (version 2.8.3) (Shannon et al., 2003) were used. The centrality coefficients of each protein were calculated by Cytoscape.

UV irradiation

For UV irradiation, we used a Biosun UV irradiation system (Vilber Lourmat, Marnes-la-Valle-e, France). This instrument has a chamber for culture dishes, and temperature within the chamber did not exceed 30 °C during exposure. Melanocytes and keratinocytes were exposed to 20 and 30 mJ/cm^2 UVB, respectively, and fibroblast was exposed to 10 J/cm^2 UVA, as previously reported (Bin et al., 2013, 2014; Kim et al., 2013a; Shim et al., 2014).

Western blot analysis and silver staining

Cells or isolated EVs were lysed with lysis buffer (1% NP-40, 0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl, and 0.01 M $MgCl_2$) containing protease inhibitors (Sigma). Protein concentrations were determined using bovine carbonic anhydrase assays, and samples were resolved by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and probed with antibodies against FN and glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz Biotechnology), heat shock protein 90 (Cell Signaling Technology, Danvers, MA) and CD81 (SBI). For silver staining, SDS-PAGE gels were subjected to a Silver Stain Kit (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's instructions.

Confocal microscopy

Melanocytes were cultured on Lab-Tek chamber slides (Nunc, Penfield, NY), fixed with 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 in PBS containing 1% BSA for 5 minutes, and incubated with anti-FN and anti-CD81 antibodies. For immunohistochemistry, epidermal specimens were fixed in 4% paraformaldehyde and embedded in paraffin. After blocking with 3% BSA, the sections were incubated first with anti-FN and anti-HMB45 (GeneTex, Irvine, CA) antibodies and then with Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 594-conjugated goat anti-rabbit IgG secondary antibodies (Invitrogen, Carlsbad, CA), respectively. Cells were mounted and images were taken with a confocal laser-scanning microscope (LSM 700; Carl Zeiss, Jena, Germany).

Cell apoptosis and viability assays

For apoptosis assays, cells were fixed and stained using a DeadEnd Fluorometric TUNEL System according to the manufacturer's instructions (Promega), and images of TUNEL-positive cells were taken under a confocal microscope and used to count apoptotic cells. For cell viability assays, the cells were fixed with 4% paraformaldehyde in PBS for 15 minutes, washed with PBS, and stained with 0.1% crystal violet for 20 minutes. After being washed with PBS, the cells were dried and lysed with 10% acetic acid. The absorbance at 590 nm was measured using a spectrophotometer.

Quantitative real-time PCR

Total RNA was isolated using Trizol reagent (Invitrogen) and was reverse-transcribed into cDNA using ReverTra Ace (Toyobo, Osaka, Japan). FN gene expression analysis was performed using TaqMan Universal Master Mix and an Hs00365052_m1 TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA).

Statistical analysis

Two-tailed Student's *t*-tests were used to analyze differences between two groups. *P*-values less than 0.05 were considered statistically significant.

AUTHOR CONTRIBUTIONS

B-HB, TRL and E-GC conceived and designed the experiments. B-HB, D-KK, N-HK, E-JC and STK performed the experiments. B-HB, JB, YSG, A-YL, TRL, and E-GC analyzed the data. B-HB, JB, TRL, and E-GC wrote the paper.

CONFLICTS OF INTEREST

B-HB, E-JC, STK, TRL, and E-GC are all employees of Amorepacific Corporation.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2015.08.001.

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