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Keratinocyte-derived Laminin-332 Promotes Adhesion and Migration in Melanocytes and Melanoma*

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Melanocytes are highly motile cells that play an integral role in basic skin physiological processes such as wound healing and proper skin pigmentation. It has been postulated that surrounding keratinocytes contribute to melanocyte migration, but underlying mechanisms remain rather vague so far. In this study, we set out to analyze the specific potential contribution of keratinocyte components to melanocytes and melanoma cell migration-related processes. Our studies revealed that A375 human melanoma cell attachment, spreading, and migration are interestingly better supported by HaCaT keratinocyte extracellular matrix (ECM) than by self-derived A375 ECM. Moreover, HaCaT ECM caused increased integrin α6 expression, adhesion-mediated focal adhesion kinase phosphorylation, and focal adhesion formations. Similar effects were confirmed in human melanocytes. Furthermore, we found that keratinocyte-derived soluble factors did not appear to significantly contribute to these processes. Specific extrinsic factors that promoted melanoma migration were attributed to keratinocyte-derived laminin-332, whereas alternative ECM component such as laminin-111 and fibronectin functions appeared to have insignificant contributions. Taken together, these studies implicate extrinsic laminin-332 in promoting the high mobility property and perhaps invasiveness inherently characteristic of, and that are the menace of, melanocytes and melanomas, respectively.

Melanocytes, which are present in the skin, hair, eyes, and ears, synthesize melanin via a process known as melanogenesis (1), and thus play a key role in the pigmentary system of the skin in the body. These cells are located in the bottom layer of the skin (basal epidermis), where they comprise 5–10% of the total cells. The major cells comprising the epidermis are keratinocytes, which are organized into the basal cell layer, spinous cell layer, granular cell layer, and keratinized squames (2). The keratinocytes in the basal cell layer gradually differentiate, proliferate, and migrate upward to form the primary protection of the body from the outside environment.

There is a close and important functional association between melanocytes and keratinocytes. Melanocytes transfer mature melanosomes to neighboring keratinocytes, resulting in visible skin pigmentation (3) and protecting the keratinocytes from the deleterious effects of UV light (4). Therefore, melanocytes play an important role in keratinocyte functions. Reciprocally, keratinocytes mediate melanocyte functions via several pathways, including cell-cell adhesion, cell-matrix adhesion, and paracrine signaling (5). Normal melanocytes maintain cell-cell adhesion with keratinocytes by expressing cell-cell adhesion proteins such as E-cadherin, desmoglein 1, and connexins (6). In turn, keratinocytes secrete many paracrine factors to melanocytes, including α-melanocyte-stimulating hormone (α-MSH),[2] adrenocorticotrophic hormone, endothelin-1, -2, and -3, FGF-2, and hepatocyte growth factor, all of which regulate the proliferation and differentiation of epidermal melanocytes (7). For example, α-MSH, as a ligand of melanocortin receptor 1, stimulates melanogenesis via the generation of cAMP. In addition, α-MSH can also enhance melanocyte proliferation, confer anti-inflammatory effects, and suppress the migration/invasion of melanoma cells (8–10).

Adhesion between skin cells and the surrounding matrix is regulated by various keratinocyte-produced extracellular matrix (ECM) factors, including fibronectin, laminin, and collagen (11, 12). In particular, the laminins are involved in the regulation of melanocytes. Laminin-332 is known to initiate hemidesmosome formation and support stable attachment of the epidermis to the dermis (13). Laminin-332 also enhances keratinocyte migration during wound healing (14), stimulates tumor growth and invasion, and enhances the formation of lamellipodia by tumor cells (15). In general, the ECM regulates cell behavior by influencing cell proliferation, survival, morphology, migration, and differentiation. In the case of malignant cancers, the ECM also regulates invasion and metastasis. Tumor cells from melanoma, a malignant tumor that arises from mutant melanocytes, reportedly use receptor-mediated recognition of various ECM molecules to move through the basement membrane (16). Therefore, both soluble factors and keratinocyte-derived ECM factors seem to be involved in the functional regulation of melanocytes.

Because keratinocytes regulate various melanocytes behaviors, including proliferation, melanin synthesis, and dendritogenesis and because the ECM regulates various cell behaviors, we hypothesized that keratinocytes-derived ECM factors might

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[2] The abbreviations used are: α-MSH, α-melanocyte-stimulating hormone; ECM, extracellular matrix; FAK, focal adhesion kinase; HaCaT ECM, HaCaT-derived ECM.

act as important regulators of melanocytes. Here, we demonstrate that laminin-332, a component of basement membrane, plays a crucial role in the adhesion and migration of melanocytes and melanoma.

EXPERIMENTAL PROCEDURES

Materials—The mAb against GAPDH was purchased from AbFrontier (Korea). The polyclonal antibody against laminin γ2 chain and the mAbs against fibronectin and β-actin were purchased from Santa Cruz (Santa Cruz, CA). The phosphorylation site-specific polyclonal antibody against FAK (Tyr(P)^{397}) and was purchased from Abcam (Cambridge, UK). The polyclonal antibody against integrin α2 was purchased from Santa Cruz. The polyclonal antibodies against FAK, integrin α4 and a6 were purchased from Cell Signaling (Beverly, MA). The monoclonal antibody against paxillin was purchased from Upstate Biotechnology, Inc. (Waltham, MA). Laminin and fibronectin were purchased from Upstate. Collagen type I and laminin-332 were purchased from Abcam.

Cell Culture and Transfection—The A375 and SK-MEL-5 human melanoma cell lines, B16F10 mouse melanoma cell line, and the HaCaT human keratinocyte cell line were maintained in DMEM (WelGene, Daegu-si, Korea), supplemented with 10% (v/v) FBS (Hyclone) and gentamicin (50 μg/ml; Sigma) at 37 °C in a humidified 5% CO_2 atmosphere. The primary human epidermal melanocyte was purchased from Modern Cell & Tissue Technologies, Inc. The human epidermal melanocyte was maintained in melanocyte growth medium-4 (Lonza), supplemented with FBS, rh-Insulin, GA-1000 (gentamicin sulfate amphotericin-B), calcium chloride, phorbol 12-myristate 13-acetate, bovine pituitary extract, hydrocortisone, and rh-FGF B at 37 °C in 5% CO_2 in a humidified atmosphere. Transient transfections were carried out using Lipofectamine 2000 (Invitrogen) according to the provided instructions.

RNA Extraction and RT-PCR—Total RNA was extracted from cells and analyzed on 1% agarose gel to verify RNA integrity. Total RNA was reverse transcribed, and aliquots of the resulting cDNA were amplified using the following primers: human integrin α2, 5′-GCATTGAAAACACTCGAT-3′ (forward) and 5′-TCGGATCCCAAGATTTTCTG-3′ (reverse); human integrin α3, 5′-TGGGAGCTGTTTATTGGTCG-3′ (forward) and 5′-GGGCCTAGAGGTGGAGTTCT-3′ (reverse); human integrin α4, 5′-TCGAATAAAGGATTGGTTTG-CAT-3′ (forward) and 5′-AAATGTTGCATGGAATATAC-GGG-3′ (reverse); human integrin α5, 5′-CCTCCCAATTTC-AGACTCCC-3′ (forward) and 5′-ACAAGGGTCCTTCACA-GTGC-3′ (reverse); human integrin α6, 5′-GACTTGAAAGA-AATGGTGAATGC-3′ (forward) and 5′-TAGCACCTGTTT-GCTGTTGC-3′ (reverse); human LAMC2, 5′-TGGAGAAC-GCTGTGATAGGTGTCG-3′ (forward) and 5′-TGTGTAAG-TCTTGGTGAGCCCAC-3′ (reverse); human fibronectin, 5′-CCGTGGGCAACTCTGTC-3′ (forward) and 5′-TGCGG-CAGTTGTCACAG-3′ (reverse); and human collagen type I, 5′-TCGGCGAGAGCATGACCGATGGAT-3′ (forward) and 5′-GACGCTGTAGGTGAA GCGGCTGTT-3′ (reverse).

After an initial denaturation at 94 °C for 5 min, the samples were subjected to 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 60 s. The reaction products were analyzed on 1% agarose gels.

siRNA—Human LAMC2-, FN-, ITGA3-, and ITGA6-specific siRNA oligonucleotides were designed. The sequences were as follows: LAMC2 siRNA, 5′-GCAAAGAGGAUCAAA-CAAAUU-3′ (sense) and 5′-UUUGUUUGUAUCCUCUUUG-CUU-3′ (antisense); FN siRNA, 5′-GCUGAAGACACAAGG-AAAUUU-3′ (sense) and 5′-AUUUCCUUGUGUCUUCAG-CUU-3′ (antisense); and ITGA3 siRNA, 5′-CCUACUACUUC-GAGAGGAAUU-3′ (sense) and 5′-UUCCUCGAAGUAGUA-GGUU-3′ (antisense); and ITGA6 siRNA, 5′-GAGUAUGAA-UUCAGGGUAAUU-3′ (sense) and 5′-UUACCCUGAAUUC-AUACUCUU-3′ (antisense). Scrambled siRNA (siGENOME nontargeting siRNA 2) was purchased from Dharmacon (Abbott Park, IL) and used as a control.

Immunoblotting—The cultures were washed twice with PBS, and the cells were lysed in radioimmune precipitation assay buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 10 mM NaF, and 2 mM Na_3VO_4) containing a protease inhibitor mixture (1 mg/ml aprotinin, 1 mg/ml antipain, 5 mg/ml leupeptin, 1 mg/ml pepstatin A, and 20 mg/ml phenylmethylsulfonyl fluoride). The lysates were clarified by centrifugation at 13,000 × *g* for 15 min at 4 °C, denatured with SDS sample buffer, boiled, and analyzed by SDS-PAGE. The resolved proteins were transferred to polyvinylidene difluoride membranes (Amersham Biosciences) and probed with the appropriate antibodies. The signals were detected by enhanced chemiluminescence (Animal Genetics Inc., Suwon-si, Korea).

Cell Spreading Assay—ECM molecules (*e.g.* gelatin, fibronectin, laminin-111, collagen type I, and laminin-332) were diluted in serum-free medium (1 μg/cm^2), added to the above described ECM-bearing plates, and incubated at 25 °C for 1 h. The plates were then washed with PBS and blocked with 0.2% heat-inactivated BSA for 1 h. After washing with PBS, the cells were plated to the ECM-coated plates and incubated for various periods at 37 °C in 5% CO_2.

Preparation of Keratinocyte-derived ECM—Keratinocyte-derived ECM was prepared according to the method of Rodeck *et al.* (17). Briefly, HaCaT cells grown at confluence in tissue culture plates were detached with 0.05% trypsin and 1 mM EDTA in PBS. The detached cells were removed, and the adherent ECM was washed with PBS and treated with 0.1 mg/ml soybean trypsin inhibitor (Invitrogen). The plates were then washed three times with PBS, blocked with 0.2% heat-inactivated bovine serum albumin (BSA) for 1 h, and washed three more times with PBS. Alternatively, HaCaT cells grown in tissue culture plates were removed by sequential extraction with 1% Triton X-100 (in PBS), 2 M urea (in 1 M NaCl), and 8 M urea (in 1 M NaCl) (18). For cell spreading assays, A375 cells were plated to the matrix-coated plates and incubated for various periods at 37 °C in 5% CO_2.

Immunofluorescence Analysis—The cells were plated to 12-well plates containing coverslips and fixed with 3.5% paraformaldehyde for 10 min. After being washed with PBS, the cells were blocked with 0.5% BSA and incubated overnight with an anti-laminin γ2, anti-fibronectin, or anti-collagen type I antibody at 4 °C. After being washed with PBS, the cells were incubated with an FITC-conjugated goat anti-mouse or a Texas

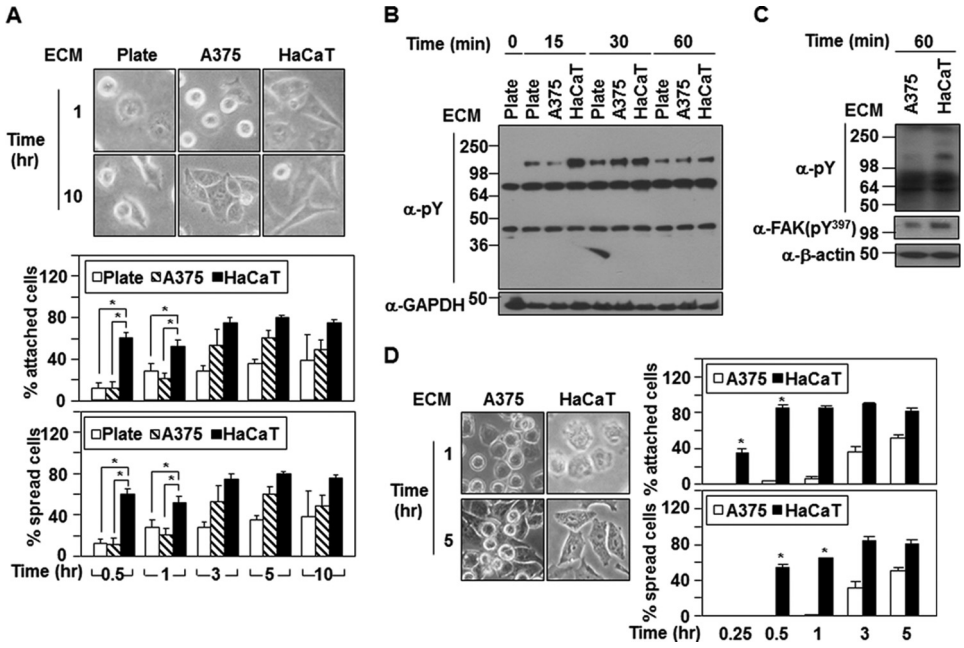

FIGURE 1. **Keratinocyte-derived ECM promotes the spreading of melanoma cells.** *A*, A375 cells (1×10^5 cells/well in 12-well plates) were distributed to tissue culture plates without or with ECMs prepared by removing either HaCaT or A375 cells with 0.05% trypsin and 1 mM EDTA in PBS. The cells were incubated at 37 °C in serum-free DMEM, and digital photographs were taken using a phase contrast microscope at the indicated time points (*top panel*). Attached and spread cells were counted. The results shown reflect the mean percentages of attached and spread cells per field ± S.E. from three independent experiments (*bottom panel*). *, $p < 0.01$; **, $p < 0.05$ *versus* plate or A375 ECM. *B*, A375 cells plated on the different ECMs were incubated for the indicated time periods. Total cell lysates were analyzed by Western blotting with an anti-phosphotyrosine (*α-pY*) antibody. Each blot was then stripped and reprobed with an anti-GAPDH (*α-GAPDH*) antibody as a loading control. *C*, A375 cells plated on the different ECMs were incubated for 60 min. Total cell lysates were analyzed by Western blotting with either anti-phosphotyrosine (*α-pY*) or anti-phospho-FAK (*α-FAK(pY^{397})*) antibodies; β-actin (*α-β-actin*) was detected as a loading control. *D*, A375 cells were distributed to tissue culture plates with ECMs prepared by removing either HaCaT or A375 cells with 1% Triton X-100. Digital photographs were taken using a phase contrast microscope at the indicated time points (*left panel*). Attached and spread cells were counted. The results shown reflect the mean percentages of attached and spread cells per field ± S.E. from three independent experiments (*right panel*). *, $p < 0.01$; **, $p < 0.05$ *versus* A375 ECM.

Red-conjugated goat anti-rabbit antibody for 1 h at 25 °C. The coverslips were then mounted on glass slides, and the slides were observed by fluorescence microscopy.

Transwell Migration Assay—Fibronectin or laminin-332 was coated to each well of a Transwell plate (Costar; 8.0-μm pore size), and then the membranes were allowed to dry at 25 °C for 1 h. The Transwell plates were assembled in a 24-well plate, and the lower chambers were filled with FBS-containing medium. The cells (1×10^5) were added to each upper chamber with serum-free medium, and the plates were incubated at 37 °C in 5% CO_2 for 24 h. The cell that had migrated to the lower surface of the filters were stained with 0.6% hematoxylin and 0.5% eosin and counted.

Monitoring Cell Adhesion and Migration—Cell adhesion and migration were monitored using the xCELLigence system (Roche Applied Science). For determination of cell adhesion, E plate 16 (Roche Applied Science) assemblies were coated with ECM molecules and seeded with cells (2.0×10^4 cells/well). Each plate was then assembled on the RTCA DP analyzer, and data were gathered at 5-min intervals for 5 h at 37 °C in 5% CO_2. The data were analyzed using the provided RTCA software. To examine cell migration, laminin-111, laminin-332 and fibronectin were added to each well of a CIM plate 16 (Roche Applied Science; 8-μm pore size), and the membranes were allowed to dry at 25 °C for 1 h. The lower chambers were filled with fresh medium containing 10% FBS or with serum-free medium. The upper chambers were filled with serum-free medium (30 μl/well), and the plate was incubated at 37 °C in 5% CO_2 for 1 h. The background was measured using a RTCA DP analyzer. The cells were added to each well, and the plate was incubated at 25 °C. After 30 min, the CIM plate was assembled onto the RTCA DP analyzer, and cell migration was assessed at 5-min intervals for 20 h at 37 °C in 5% CO_2. The obtained data were analyzed using the provided RTCA software.

Statistical Analysis—The data are represented as the means from three independent experiments. Statistical analysis was performed using an unpaired Student's *t* test. A *p* value less than 0.01 or 0.05 was considered statistically significant.

RESULTS

Keratinocyte-derived ECM Promotes the Adhesion and Spreading of Melanoma Cells—To investigate the effect of keratinocyte-derived ECM on melanocytes, we first performed spreading assays with A375 human melanoma, a malignant tumor that arises from mutant melanocytes. When detached and replated, A375 cells attached and spread faster on HaCaT-derived ECM (HaCaT ECM) than on their own ECM (A375 ECM). Approximately 60% of A375 cells attached and spread within 30 min on HaCaT ECM, whereas more than 90% of these cells remained unattached after 30 min on A375 ECM (Fig. 1*A*). At 1 h post-plating, the A375 cells incubated on the HaCaT ECM had a more cylindrical morphology and flatter, sheet-like spreading compared with those incubated on the A375 ECM; these morphological differences could be seen for up to 10 h. Consistent with our observation of increased cell adhesion, overall tyrosine phosphorylation was much higher in total cell

lysates from A375 cells incubated on HaCaT ECM than in those incubated on A375 ECM (Fig. 1*B*). Notably, phosphorylation of FAK at tyrosine 397, which is a key signaling event during cell adhesion (19), was also increased in total cell lysates from A375 cells on HaCaT ECM (Fig. 1*C*). We also obtained similar results in A375 cells on HaCaT ECM, which was prepared by removing cells using 1% Triton X-100 (Fig. 1*D*). These data suggest that keratinocyte-derived ECM molecules can regulate melanoma cell function.

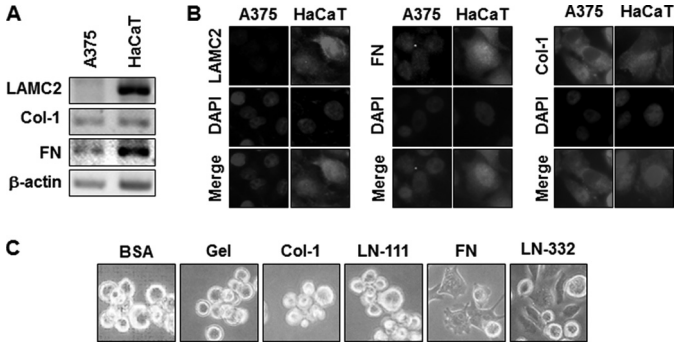

FIGURE 2. **HaCaT cells produce laminin-332.** *A*, total RNA was extracted from exponentially growing A375 and HaCaT cells, and the mRNA expression levels of laminin γ2 chain (*LAMC2*), collagen-1 (*Col-1*), and fibronectin (*FN*) were analyzed by RT-PCR, using β-actin as a loading control. *B*, either A375 and HaCaT cells were cultured on coverslips and immunostained with either anti-fibronectin (*FN*), anti-laminin γ2 chain (LAMC2) or anti-collagen type I (Col-1) antibodies. The results were visualized with either an FITC-conjugated goat anti-mouse antibody or a Texas Red-conjugated goat anti-rabbit antibody. DAPI was used to stain nuclei. *C*, A375 cells (1×10^5 cells/well in 12-well plates) were seeded to the indicated ECM-coated plates. After the cells were incubated at 37 °C for 3 h, digital photographs were taken under a phase contrast microscope, as described in Fig. 1*A*. Heat-inactivated BSA was used as the control.

Keratinocyte-derived Laminin-332 Promotes the Adhesion and Spreading of Melanoma Cells—Keratinocytes express and deposit various ECM proteins, including fibronectin and laminin. To investigate the difference between A375 ECM and HaCaT ECM, we examined the mRNA expression levels of some key molecules in both cell lines (Fig. 2*A*). Although both cell lines expressed similar amounts of collagen type I, HaCaT cells expressed much more fibronectin than A375 cells. Interestingly, the mRNA for LAMC2, which encodes the laminin γ2 chain of laminin-332, was expressed only in HaCaT cells. Consistent with this finding, immunocytochemical studies revealed that laminin-332 protein expression was higher in HaCaT cells *versus* A375 cells (Fig. 2*B*). Accordingly, we next investigated which keratinocyte-derived ECM proteins could regulate the adhesion and spreading of melanoma cells (Fig. 2*C*). Plating of A375 cells on wells coated with different ECM proteins revealed that these cells attached and spread well on plates coated with fibronectin and laminin-332, but not gelatin, collagen type I, or laminin-111. When we compared the adhesion and spreading of A375 cells on these proteins with that on the HaCaT ECM (Fig. 3*A*), we obtained broadly similar results. More than 50% of the A375 cells attached and spread on the HaCaT ECM within 30 min, and most of the cells had attached and spread within 1 h. Although only 50% of the cells spread on wells coated with fibronectin or laminin-332, and maximal spreading required 5 h, the mor-

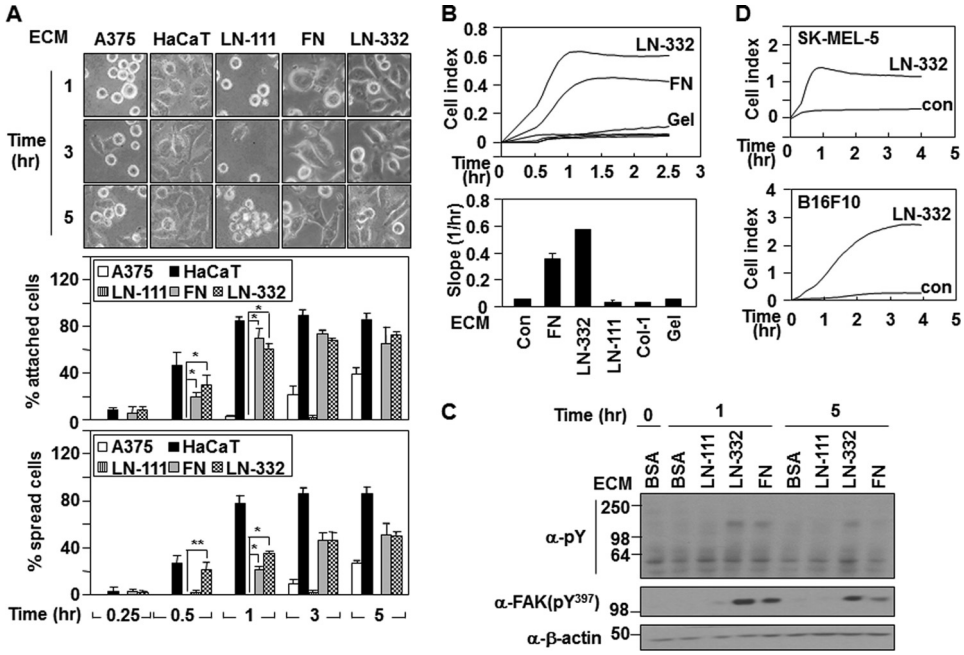

FIGURE 3. **Laminin-332 enhances the adhesion and spreading of melanoma cells.** *A*, A375 cells (1×10^5 cells/well in 12-well plates) were distributed to the indicated ECM-coated plates in serum-free DMEM. After incubation at 37 °C for the indicated periods, the cells were digitally photographed under a phase contrast microscope (*top panel*), and attached or spread cells were counted. The results are shown as the mean percentages of attached and spread cells per field ± S.E. from three independent experiments (*bottom panel*). *, $p < 0.01$; **, $p < 0.05$ *versus* LN-111. *B*, A375 cells (2.0×10^4 cells/well) were seeded in duplicate to the indicated ECM-coated E plates; a noncoated well was used as a control. Cell adhesion curves were monitored using the xCELLigence system (*top panel*). The rates of cell adhesion over 1 h were analyzed using the RTCA software (*bottom panel*). *C*, A375 cells were plated on the different ECMs and incubated for the indicated time periods. Total cell lysates were analyzed by Western blotting with the anti-phosphotyrosine (α-*pY*) or anti-phospho-FAK (α-*FAK(pY397)*) antibodies, with β-actin (α-*β-actin*) detected as the loading control. *D*, SK-MEL-5 (*top panel*) and B16F10 (*bottom panel*) cells (2.0×10^4 cells/well) were seeded in duplicate to the laminin-332-coated E plates; a noncoated well was used as a control. Cell adhesion curves were monitored using the xCELLigence system.

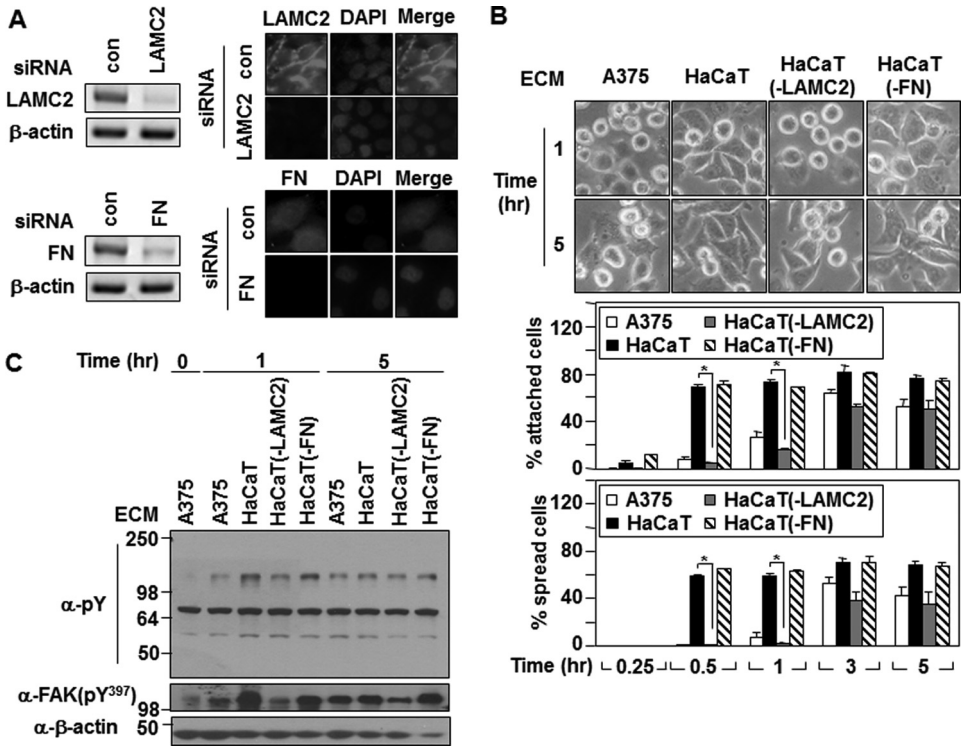

FIGURE 4. **Keratinocyte-derived laminin-332 is crucial for the adhesion and spreading of melanoma cells.** *A*, HaCaT cells were transfected with siRNAs targeting fibronectin (*FN*) or laminin γ-chain (*LAMC2*). The expression levels of the target mRNAs were analyzed by RT-PCR, with a control (*con*) siRNA used as the siRNA control and β-actin detected as the loading control (*left panel*). Both cells were cultured on coverslips and immunostained with either anti-fibronectin (*FN*) or anti-laminin γ2 chain (*LAMC2*) antibodies. The results were visualized with either an FITC-conjugated goat anti-mouse antibody or a Texas Red-conjugated goat anti-rabbit antibody. DAPI was used to stain nuclei (*right panel*). *B*, A375, HaCaT, or HaCaT cells transfected with siRNA targeting either laminin γ2 chain (*HaCaT(−LAMC2)*) or fibronectin (*HaCaT(−FN)*) were cultured to confluence. The cells were then removed, the ECM beds were prepared, and A375 cells (1×10^5 cells/well in 12-well plates) were seeded to the ECM-coated plates. After being incubated at 37 °C for the indicated periods, the cells were digitally photographed under a phase contrast microscope (*top panel*), and attached or spread cells were counted. The results are given as mean percentages of attached and spread cells per field ± S.E. from three independent experiments (*bottom panel*). *, $p < 0.01$; **, $p < 0.05$ *versus* HaCaT ECM. *C*, A375 cells were plated on the indicated ECMs as described in *B* and incubated for the indicated time periods. Total cell lysates were analyzed by Western blotting with anti-phosphotyrosine (*α-pY*) and anti-phospho-FAK (*α-FAK(pY^{397})*) antibodies; β-actin (*α-β-actin*) was detected as the loading control.

phologies of the spread cells were very similar to those of the cells spread on the HaCaT ECM. These data suggest that fibronectin and laminin-332 can regulate the attachment and spreading of melanoma cells.

Next, we monitored the adhesion rates of A375 cells on the various ECM proteins in real time, using the xCELLigence system. Our results revealed that the A375 cells attached and spread most effectively on laminin-332 (Fig. 3*B*). Growth on laminin-332 also enhanced the tyrosyl phosphorylation of FAK at 397 (Fig. 3*C*). Similarly, laminin-332 promoted the adhesion of human SK-MEL-5 and mouse B16F10 melanoma cells (Fig. 3*D*).

To further investigate the potential involvement of fibronectin and laminin-332 in the regulation of melanoma cell adhesion, we used unique 21-bp siRNA sequences targeted against fibronectin and laminin γ2 chain to knock down the expression levels of these ECM proteins. HaCaT cells transfected with the siRNA constructs showed decreased mRNA and protein expression of the targeted proteins (Fig. 4*A*). Interestingly, A375 cells plated on HaCaT ECM derived from laminin-332 knockdown cells showed decreased attachment and spreading. In contrast, A375 cells attached and spread well on HaCaT ECM derived from fibronectin knockdown cells (Fig. 4*B*). Consistent with these findings, tyrosyl-397 phosphorylation of FAK of A375 cells was reduced in HaCaT ECM derived from laminin-332 knockdown cells but not fibronectin knockdown cells (Fig. 4*C*). These results suggested that laminin-332 is crucial for melanoma cell attachment and spreading on keratinocyte-derived ECM.

Integrin α6 Regulates the Laminin-332-mediated Adhesion of Melanoma Cells—Integrins are important cell surface receptors that bind to ECM proteins, and integrins α6β1, α6β4, and α3β1 are known receptors for laminin-332 (20–22). Therefore, we hypothesized that integrins could be crucial receptors for laminin-332-mediated melanoma cell attachment and spreading. To examine this possibility, we investigated expression of integrin α subunits in A375 cells (Fig. 5). Both A375 and HaCaT cells expressed integrin α2, α3, α4, and α6, and integrin α5 was expressed only in A375 cells (data not shown but refer to Fig. 5*A*). Interestingly, adhesion on HaCaT ECM caused altered mRNA and protein expression of integrin α6 (Fig. 5*A*). Furthermore unique siRNA sequences targeted against integrin α6 that effectively reduced its mRNA expression significantly blocked the adhesion and spreading of A375 cells on HaCaT ECM (Fig. 5*B*). However, those effects were not seen in A375 cells transfected with siRNA of integrin α3 (si-ITGA3; Fig. 5*C*). These findings indicate that integrin α6 participates in the laminin-332-directed adhesion of melanoma cells to keratinocyte-derived ECM.

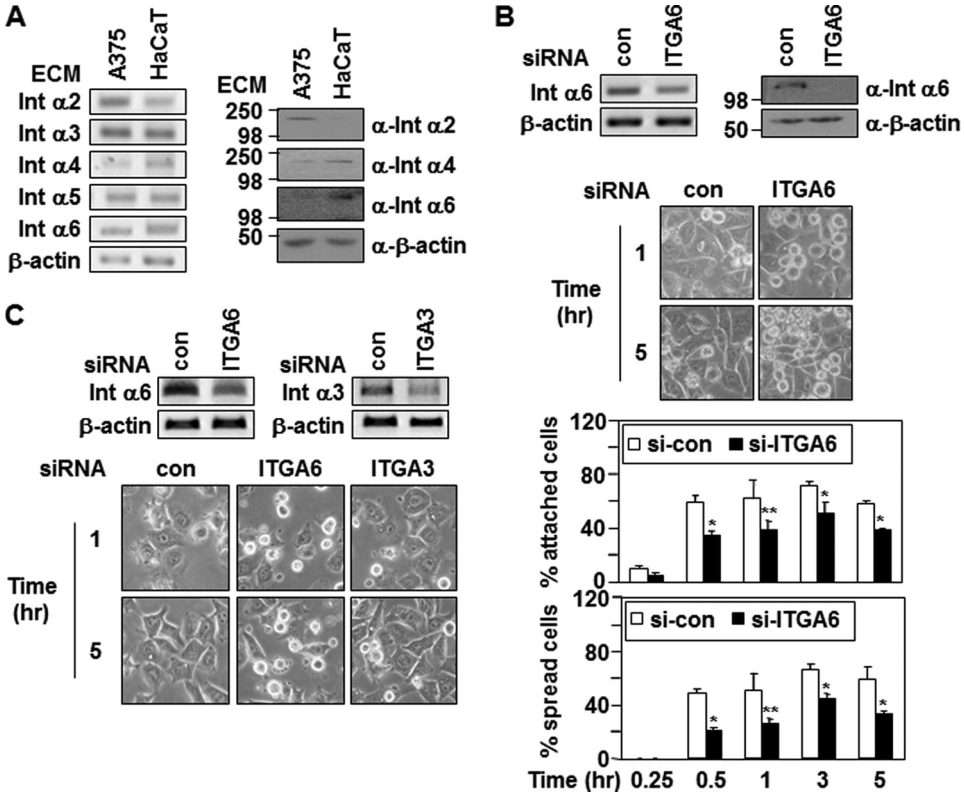

FIGURE 5. **Integrin α6 regulates melanoma cell spreading on laminin-332.** *A*, A375 cells were plated on A375 ECM or HaCaT ECM and incubated at 37 °C for 5 h, and the mRNA expression levels of the various integrin (*Int*) subunits were analyzed by RT-PCR. β-Actin was used as the control (*left panel*). A375 cells were plated on A375 ECM or HaCaT ECM and incubated at 37 °C. After 7 h, total cell lysates were analyzed by Western blotting with the indicated anti-integrin antibodies (*right panel*). *B*, A375 cells were transfected with control or integrin (*Int*) α6-targeting siRNAs. The mRNA expression level of the target protein was analyzed by RT-PCR. β-Actin was used as the control. The protein expression of the integrin α6 was analyzed by Western blotting with anti-integrin α6 (*α-Int α6*) antibody (*top panel*). A375 cells transfected with the indicated siRNAs were seeded on HaCaT ECM. After the indicated time periods, the cells were digitally photographed under a phase contrast microscope (*middle panel*), and attached or spread cells were counted. The results are given as the mean percentages of attached and spread cells per field ± S.E. from three independent experiments (*bottom panel*). *, $p < 0.01$; **, $p < 0.05$ *versus* si-control. *C*, A375 cells were transfected with control or integrin α6- or integrin α3-targeting siRNAs. The mRNA expression level of the target protein was analyzed by RT-PCR. β-Actin was used as the control (*top panel*). A375 cells transfected with the indicated siRNAs were seeded on HaCaT ECM. After the indicated time periods, the cells were digitally photographed under a phase contrast microscope (*bottom panel*).

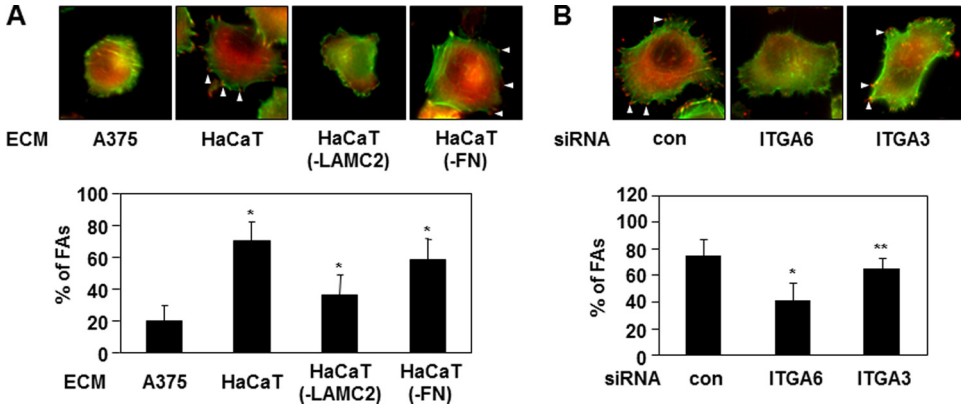

FIGURE 6. **Laminin-332 promotes focal adhesion formation in HaCaT cells.** *A*, either A375 or HaCaT cells transfected with siRNA targeting either laminin γ2 chain (*HaCaT(−LAMC2)*) or fibronectin (*HaCaT(−FN)*) were cultured on coverslips to confluence. The cells were then removed, the ECM beds were prepared, and A375 cells (3×10^5 cells/well in 12-well plates) were seeded to the ECM-coated plates. After being incubated at 37 °C for the 1 h, the cells were fixed and immunostained with anti-paxillin antibody (Texas Red) and phalloidin (FITC-conjugated) (*top panel*). The number of cells positive for focal adhesions were counted (*bottom panel*). *, $p < 0.01$; **, $p < 0.05$ *versus* A375 ECM. *B*, A375 cells (3×10^5 cells/well in 12-well plates) transfected with siRNA targeting ITGA6 or ITGA3 were plated on HaCaT ECM-coated coverslip. After being incubated at 37 °C for the 1 h, the cells were fixed and immunostained with anti-paxillin antibody (Texas Red) and phalloidin (FITC conjugated) (*top panel*). The number of cells positive for focal adhesions were counted (*bottom panel*). *, $p < 0.01$; **, $p < 0.05$ *versus* control (*con*). The *arrowheads* point to focal adhesion sites.

Laminin-332 Promotes Focal Adhesion Formation in Melanoma Cells—Because focal adhesions serve as the mechanical linkages to the ECM, we next investigated focal adhesion formation of melanoma cells on laminin-322 (Fig. 6). Although ~70% of A375 cells on HaCaT ECM showed focal adhesion formation, focal adhesion formation was significantly reduced

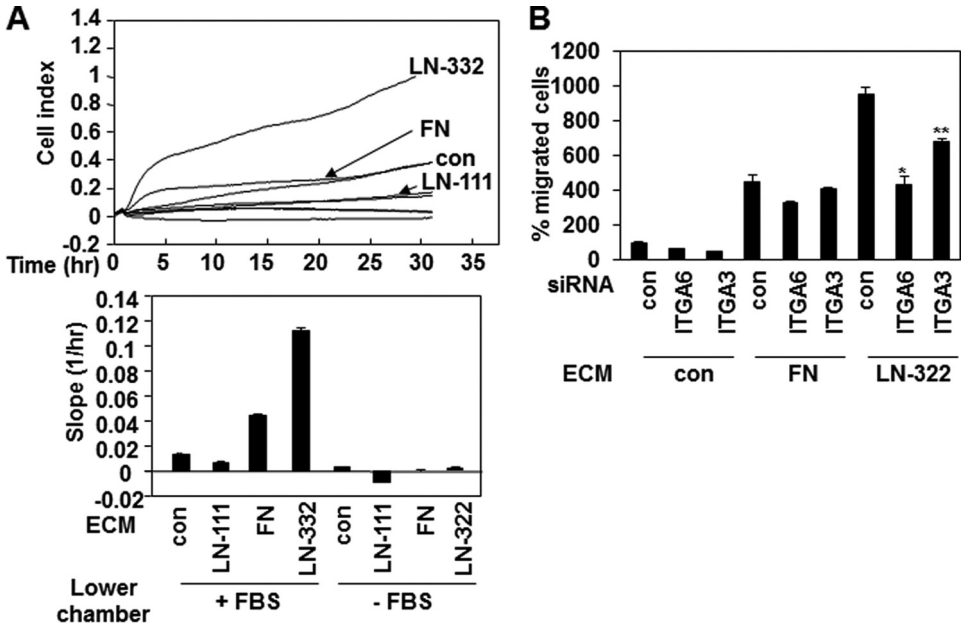

FIGURE 7. **Laminin-332 stimulates the migration of melanoma cells.** *A*, A375 cells (1.5×10^4 cells/well) were seeded in duplicate to the upper chambers of CIM plates coated with the indicated ECMs; a noncoated well was used as the control (*con*). The lower chambers were filled with medium containing 10% FBS (+*FBS*), and migration curves were monitored using the xCELLigence system (*top panel*). The migration rates over 5 h were analyzed using the RTCA software (*bottom panel*). *B*, A375 cells (1×10^5 cells/well) transfected with the indicated siRNAs were seeded in the upper chambers of coated with the indicated ECMs; a noncoated well was used as the control. The lower chambers were filled with medium containing 10% FBS. After 24 h, migrated cells were stained with hematoxylin and eosin. *, $p < 0.01$; **, $p < 0.05$ *versus* si-control.

in A375 cells plated on HaCaT ECM derived from laminin-332 knockdown cells. In contrast, focal adhesion formation was slightly decreased in A375 cells on HaCaT ECM derived from fibronectin knockdown cells (Fig. 6*A*). Similarly, focal adhesion formation was decreased in integrin α6, but not α3, and knocked down A375 cells plated on HaCaT ECM, compared with control (Fig. 6*B*). These results suggest that keratinocyte-derived laminin-332 promotes focal adhesion formation in melanoma cells.

Laminin-332 Enhances the Migration of Melanoma Cells—Because cell-ECM adhesion is correlated with cell migration (23), we next used the xCELLigence system to investigate whether keratinocyte-derived laminin-332 regulates the migratory ability of melanoma cells. As expected, A375 cells showed better migration on laminin-332 over the other tested ECM proteins (Fig. 7*A*). Consistently, siRNA sequences targeted against integrin α6 significantly reduced migration of A375 cells on laminin-332 (Fig. 7*B*).

Laminin-332 Promotes Adhesion and Migration of Melanocytes—We next investigated whether laminin-332 was involved in the regulation of adhesion and migration of human melanocytes (Fig. 8). Like melanoma cells, human melanocytes attached and spread most effectively on laminin-332 among ECMs tested (Fig. 8*A*). In addition, laminin-322 enhanced the tyrosyl phosphorylation of FAK at 397 (Fig. 8*B*) and migration of human melanocytes (Fig. 8*C*). Consistently, siRNA sequences targeted against integrin α6 significantly inhibited the migration of A375 cells (Fig. 8*D*). These results demonstrate that laminin-332 promotes the adhesion and migration of melanocytes.

Keratinocyte-derived Soluble Factors Are Not Essential for the Adhesion of Melanoma Cells—Because both the ECM and soluble factors are known to regulate cell functions (7, 15), we investigated whether soluble keratinocyte-derived factors might be involved in the adhesion of melanoma cells. A375 cells were plated to tissue culture plates in the presence of conditioned medium from HaCaT cells. In contrast to ECM, the conditioned medium did not significantly affect the adhesion and spreading of A375 cells (Fig. 9*A*). Similarly, conditioned medium from HaCaT cells did not affect the adhesion and spreading of A375 cells plated on either A375 ECM or HaCaT ECM (Fig. 9*B*). These results suggest that soluble keratinocyte-derived factors are not essential for the adhesion and spreading of melanoma cells.

DISCUSSION

In both normal skin and melanoma, keratinocytes regulate melanocytes through various means, including cell-cell interactions, cell-matrix interactions, and paracrine factor production. Here, we show that keratinocytes regulate melanoma cells through laminin-332. It is known that laminin-332, which is a component of the basement membrane, mediates the firm attachment of basal keratinocytes to the basement membrane (24–26). Because melanocytes also reside within the basal layer of the epidermis and attach to the basement membrane, it is plausible for laminin-332 to regulate the adhesions of both keratinocytes and melanocytes. Indeed, because there are relatively few melanocytes in the epidermis, and melanocytes do not make significant amount of laminin-332 (11), the need for laminin-332-mediated attachment may form part of the basis for the cooperation between melanocytes and keratinocytes. Here, we show that keratinocyte-derived laminin-332 can regulate melanoma cell adhesion. A375 human melanoma cells attached and spread more effectively on keratinocyte-de-

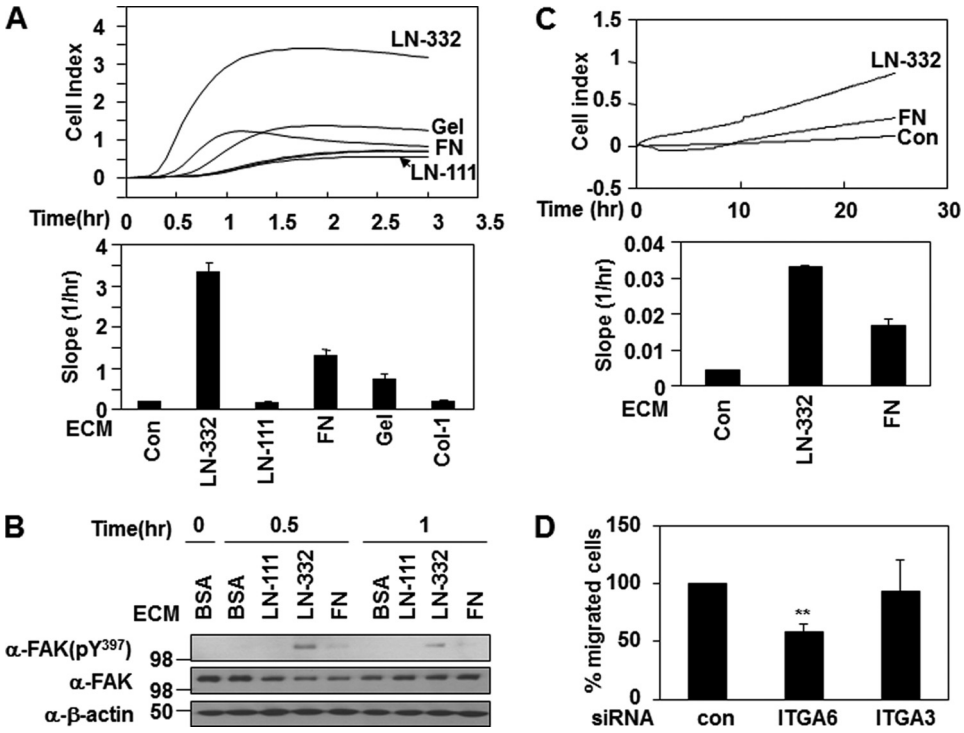

FIGURE 8. **Laminin-332 promotes adhesion and migration of melanocytes.** *A*, melanocytes (2.0×10^4 cells/well) were seeded in duplicate to the indicated ECM-coated E plates; a noncoated well was used as a control (*Con*). Cell adhesion curves were monitored using the xCELLigence system (*top panel*). The rates of cell adhesion over 1 h were analyzed using the RTCA software (*bottom panel*). *B*, melanocytes (5×10^5 cells/well; six wells) were plated on the indicated ECMs and incubated for the indicated time periods. Total cell lysates were analyzed by Western blotting with anti-phospho-FAK (*α-FAK*(pY^{397})) and anti-FAK (*α-FAK*) antibodies; *β*-actin (*α-β-actin*) was detected as the loading control. *C*, melanocytes (2×10^4 cells/well) were seeded in duplicate to the upper chambers of CIM plates coated with the indicated ECMs; a noncoated well was used as the control. The lower chambers were filled with medium containing 0.5% FBS, and migration curves were monitored using the xCELLigence system (*top panel*). The migration rates over 25 h were analyzed using the RTCA software (*bottom panel*). *D*, melanocytes (1×10^5 cells/well) transfected with the indicated siRNAs were seeded in the upper chambers of coated with the LN-332. The lower chambers were filled with medium containing 0.5% FBS. After 24 h, migrated cells were stained with hematoxylin and eosin. **, $p < 0.05$ *versus* si-control.

rived ECM than their own ECM (Fig. 1). Furthermore, laminin-332, which was differentially expressed in keratinocytes *versus* melanoma cells (Fig. 2), was found to be crucial for the attachment and spreading of melanoma cells on the keratinocyte-derived ECM (Figs. 3 and 4).

Laminin-332 is a ligand for a number of receptors, including integrins $\alpha6\beta1$, $\alpha6\beta4$, $\alpha3\beta1$, and syndecan (13). Here, we show that the adhesion on laminin-332 caused altered expression of integrin $\alpha6$ and that siRNA-mediated knockdown of integrin $\alpha6$ diminished the ability of laminin-332 to enhance the attachment and spreading of A375 cells (Fig. 5). Because integrin $\alpha6\beta4$ is known to be down-regulated in melanoma cells (27), it is likely that integrin $\alpha6\beta1$ mediates the laminin-332-mediated adhesion of melanoma cells. Oikawa *et al.* (27) demonstrated that laminin-332 stimulated migration of melanoma cells through integrin $\alpha3\beta1$ and $\alpha6\beta1$, and Tsuji *et al.* (28) reported that integrin $\alpha3$ mediated laminin-332-mediated melanoma cell migration and invasion on laminin-332-coated membrane. Because we found that integrin $\alpha6$ regulates laminin-332-mediated cell adhesion and migration better than integrin $\alpha3$ (Figs. 7 and 8), integrin $\alpha6$ may play a major role in the regulation of laminin-332-mediated cell adhesion.

Previously, laminin-332 was reported to stimulate the migration of keratinocytes during wound healing (14), tumor growth/invasion, and the formation of lamellipodia in tumor cells (15). Consistent with a previous report that laminin-332 is able to promote melanoma cell migration, as shown in a chemotaxis assay with soluble laminin-332 and a haptotaxis assay with laminin-332-coated membranes (27, 28), we herein show that laminin-332 stimulates the migration of melanoma cells (Fig. 7). Thus, keratinocytes appear to regulate melanoma cell functions. Like melanoma, keratinocyte-derived laminin-332 plays a crucial role in regulating melanocyte functions. Keratinocytes express laminin-332 during the human skin wound healing process, and laminin-332 stimulates the migration of keratinocytes (14, 29) and melanocytes across the wound bed (30, 31). Therefore, keratinocyte-derived laminin-332 is expected to enhance melanocyte migration for wound repair.

The migration of melanocytes is an important event in repigmentation of vitiligo, a common skin disorder in which white spots appear on the skin. Narrow band UVB light phototherapy has been widely used for the treatment of vitiligo (32). A previous study showed that conditioned medium from human keratinocytes exposed to UVB enhanced proliferation but did not affect migration of human melanocytes (33). However, UVB stabilizes HIF1-α to increase production of laminin-332 from keratinocytes (29, 34). Therefore, it was considered likely that laminin-332 derived from keratinocytes regulates melanocytes during UVB phototherapy treatment. In the present study, we showed that laminin-332 directly regulated adhesion and

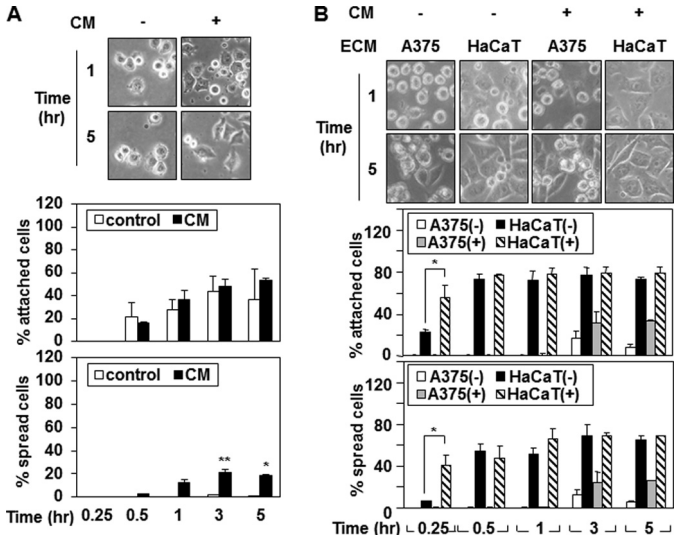

FIGURE 9. **Keratinocyte-derived soluble factors are not essential for regulating adhesion of melanoma cell.** *A*, A375 cells (1×10^5 cells/well) were seeded to 12-well tissue culture plates in the presence of conditioned medium (*CM*) from HaCaT cells. After the plates were incubated at 37 °C for the indicated time periods, the cells were digitally photographed under a phase contrast microscope (*top panel*), and attached or spread cells were counted. The results are given as the mean percentages of attached and spread cells per field ± S.E. from three independent experiments (*bottom panel*). *, $p < 0.01$; **, $p < 0.05$ *versus* control. *B*, A375 cells (1×10^5 cells/well) were distributed to plates coated with A375 ECM or HaCaT ECM and treated with conditioned medium from HaCaT cells. After the cells had been incubated at 37 °C for the indicated time periods, photographs were taken as described for *A* (*top panel*), and attached or spread cells were counted. The results are given as mean percentages of attached and spread cells per field ± S.E. from three independent experiments (*bottom panel*). *, $p < 0.01$ *versus* HaCaT(−).

migration of human melanocytes (Fig. 8). These data clearly support the notion that keratinocytes regulate melanocyte functions. Therefore, keratinocyte-derived laminin-332 seems to play a critical role in the adhesion-related functions of both melanocyte and melanoma cells. Several matrix metalloproteinases, such as matrix metalloproteinases 2, 3, 13, 14, and 20, have been shown to degrade laminin γ2 chain and induce epithelial cell migration (35–37). Thus, future work may be warranted to determine whether the laminin-332-mediated stimulation of melanoma cell migration is associated with matrix metalloproteinases.

In addition to ECM molecules, keratinocytes produce various soluble factors that have been reported to regulate melanocytes, including α-MSH, FGF-2, hepatocyte growth factor, and stem cell factor (7). Interestingly, however, keratinocyte-derived soluble factors did not significantly affect the adhesion of melanoma cells under our experimental conditions (Fig. 9). Thus, in the context of adhesion, the ECM seems to be the major regulator of melanoma cell behavior. However, we herein used more than physiological amounts of ECM, and soluble factors were found to slightly enhance cell adhesion during the early stages of spreading (Fig. 9). Thus, it remains formally possible that soluble factors could be involved in cell adhesion *in vivo*.

In summary, keratinocytes play critical roles in regulating melanoma and melanocyte functions. Here, we show that various keratinocyte-derived ECM proteins, particularly laminin-332, are involved in regulating the adhesion, spreading, and migration of melanoma and melanocytes *in vitro*. In contrast, keratinocyte-derived soluble factors are not essential for the adhesion of melanoma cells. Further studies will be required to clarify the precise regulatory mechanisms underlying the cross-talk between keratinocytes and melanoma cells.

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Keratinocyte-derived Laminin-332 Promotes Adhesion and Migration in Melanocytes and Melanoma

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