INTRODUCTION

The healing of wounds is an essential physiological process, and proceeds via overlapping and orchestrated phases, including hemostatic, inflammatory, proliferative, and remodeling phases, in which various cells, soluble factors, and extracellular matrix components are involved (Eming et al., 2007; Wells et al., 2016).

Therefore, wound repair is delayed in several circumstances, such as aging, diabetes, starvation, and immunocompromised states. In the clinical context, protracted healing of wounds may lead to severe complications requiring prolonged hospitalization, ablative operation, or even death of patients. Recently, negative pressure wound therapy has been found to aid healing for a variety of acute and chronic wounds (Peinemann and Sauerland, 2011). In skin wound healing, various substances, such as cytokines and growth factors, stimulate the migration and proliferation of dermal cells and keratinocytes to close the wounds (Borena et al., 2015; Coulombe, 2003). Growth factors, including platelet-derived growth factor, basic fibroblast growth factor, keratinocyte growth factor, and prostaglandin E1, are effective for cutaneous wound healing, and these recombinant or analog molecules are used as clinical drugs (Ortega et al., 1998; Werner and Grose, 2003; Zhang et al., 1994). Although lines of clinical evidence for the effectiveness of these therapies have accumulated, further medical treatments are eagerly anticipated.

The skin, especially keratinocytes, is continually exposed to UV rays, which was shown to inhibit keratinocyte motility in vitro and impair skin wound healing in C57BL/6 mice (Liu et al., 2015). Previous report showed that the exposure of keratinocytes to UV radiation leads to the intracellular production of 6-formylindolo[3,2-b]carbazole (FICZ), indicating that FICZ might be one of the effectors of UV radiation. FICZ exhibits high affinity for aryl hydrocarbon receptors (AhR) and is continually exposed to UV rays, which impairs wound healing. 6-Formylindolo[3,2-b]carbazole (FICZ) is a tryptophan photoprodator formed by UV exposure, indicating that FICZ might be one of the effectors of UV radiation. In contrast, treatment with tryptophan, the precursor for FICZ, promoted wound closure in keratinocytes. Therefore, the aim of our study was to determine the role of FICZ in wound healing. Here we showed that FICZ enhanced keratinocyte migration through mitogen-activated protein kinase/extracellular signal-regulated kinase activation, and promoted wound healing in various mouse models, including db/db mice, which exhibit wound healing impairments because of type 2 diabetes. Moreover, FICZ, the endogenous ligand of an aryl hydrocarbon receptor, accelerated migration even in the aryl hydrocarbon receptor knockdown condition and also promoted wound healing in DBA/2 mice, bearing a low-affinity aryl hydrocarbon receptor, suggesting that FICZ enhanced keratinocyte migration in a mitogen-activated protein kinase/extracellular signal-regulated kinase-dependent, but aryl hydrocarbon receptor-independent, manner. The function of FICZ might indicate the possibility of its clinical use for intractable chronic wounds.

Wound healing is an elaborate process composed of overlapping phases, such as proliferation and remodeling, and is delayed in several circumstances, including diabetes. Although several treatment strategies for chronic wounds, such as growth factors, have been applied, further alternatives are required. The skin, especially keratinocytes, is continually exposed to UV rays, which impairs wound healing. 6-Formylindolo[3,2-b]carbazole (FICZ) is a tryptophan photoprodactor formed by UV exposure, indicating that FICZ might be one of the effectors of UV radiation. In contrast, treatment with tryptophan, the precursor for FICZ, promoted wound closure in keratinocytes. Therefore, the aim of our study was to determine the role of FICZ in wound healing. Here we showed that FICZ enhanced keratinocyte migration through mitogen-activated protein kinase/extracellular signal-regulated kinase activation, and promoted wound healing in various mouse models, including db/db mice, which exhibit wound healing impairments because of type 2 diabetes. Moreover, FICZ, the endogenous ligand of an aryl hydrocarbon receptor, accelerated migration even in the aryl hydrocarbon receptor knockdown condition and also promoted wound healing in DBA/2 mice, bearing a low-affinity aryl hydrocarbon receptor, suggesting that FICZ enhanced keratinocyte migration in a mitogen-activated protein kinase/extracellular signal-regulated kinase-dependent, but aryl hydrocarbon receptor-independent, manner. The function of FICZ might indicate the possibility of its clinical use for intractable chronic wounds.

6-Formylindolo[3,2-b]Carbazole Accelerates Skin Wound Healing via Activation of ERK, but Not Aryl Hydrocarbon Receptor

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receptor (AhR) (Rannug et al., 1995). FICZ is a tryptophan oxidation product formed by exposure to UV or visible light, and is metabolized by CYP1A1, which is regulated through the FICZ/AhR pathway (Oberg et al., 2005; Wincent et al., 2012). FICZ is a major substrate for the CYP family, as mentioned above, and its hydroxylated metabolites are also good substrates for the sulfotransferase family, resulting in urinary excretion (Bergander et al., 2004; Wincent et al., 2009). Moreover, recent reports show that FICZ affects the expression of various genes via AhR, not merely CYP1A1 (Di Meglio et al., 2014; Morino-Koga et al., 2013). Despite various reports about the physical and chemical properties of FICZ, its association with wound repair remains unclear. In contrast, topical treatment of tryptophan, the precursor for FICZ, increased the rate of wound closure in keratinocytes and induced re-epithelialization in mice and patients (Bandeira et al., 2015; Barouti et al., 2015). Therefore, the aim of our study was to reveal the role of FICZ in wound healing.

RESULTS
FICZ promoted wound closure in keratinocytes and mouse models
After confirmed that FICZ dose-dependently induced CYP1A1 expression (Figure 1a, 1c), we performed in vitro scratch assay using the IncuCyte system (Essen Biosciences, Ann Arbor, MI) in an immortalized human...
keratinocyte cell line HaCaT and normal human epidermal keratinocytes to assess the effect of FICZ on wound closure in keratinocytes. Confluent monolayers of keratinocytes were scratched and incubated with 100 nM FICZ or DMSO (n = 6). (c) The relative wound area at 10 hours is shown. (d, e) HaCaT cells were treated with FICZ for 24 hours and the reaction products of (d) BrdU incorporation assay and (e) MTT assay were quantified (n = 5). (f, g) HaCaT cells were scratched and incubated with 100 nM FICZ or DMSO in the absence or (g) presence of 2 μM cytochalasin D (n = 6). All data are presented as mean ± SE (*P < 0.05, **P < 0.01, ***P < 0.001). FICZ, 6-formylindolo[3,2-b]carbazole; MTT, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; SE, standard error.

Figure 2. FICZ promotes keratinocyte migration, but not proliferation. (a–c) HaCaT cells were treated (a) without or (b) with 5 μg/ml mitomycin C for 2 hours. Cells were scratched and incubated with 100 nM FICZ or DMSO (n = 6). (c) The relative wound area at 10 hours is shown. (d, e) HaCaT cells were treated with FICZ for 24 hours and the reaction products of (d) BrdU incorporation assay and (e) MTT assay were quantified (n = 5). (f, g) HaCaT cells were scratched and incubated with 100 nM FICZ or DMSO in the absence or (g) presence of 2 μM cytochalasin D (n = 6). All data are presented as mean ± SE (*P < 0.05, **P < 0.01, ***P < 0.001). FICZ, 6-formylindolo[3,2-b]carbazole; MTT, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; SE, standard error.
FICZ did not affect scar formation

To check the effect of FICZ on scar formation, histopathological analyses were performed one month after wound healing. Clinically, no elevation of the scars above the surrounding normal skin was observed in FICZ- and vehicle-treated mice (data not shown). Hematoxylin and eosin and Elastica van Gieson stainings revealed that the epidermis was normally reconstructed and the collagen bundles and elastic fibers were regularly organized to form normotrophic scar in both mice (data not shown).

FICZ accelerated the migration, but not the proliferation, of keratinocytes

During the process of skin wound healing, keratinocytes proliferate and migrate from the edge of the wound toward the wound bed. We thus examined whether FICZ promoted the migration and/or proliferation of keratinocytes. We first treated HaCaT cells with mitomycin C to inhibit cell proliferation. FICZ significantly improved the rate of wound closure regardless of mitomycin C addition (Figure 2a–c). In this case, mitomycin C was biologically active because the wounded area was enlarged in its presence (Figure 2c). We then determined the effect of FICZ on cell proliferation using BrdU incorporation and 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assays. The rate of cell proliferation measured in these methods did not differ between the vehicle- and FICZ-treated cells (Figure 2d, 2e). In contrast, cell migration induced by FICZ was suppressed by cytochalasin D, an inhibitor of actin polymerization (Figure 2f, 2g). These results suggested that FICZ accelerated wound closure by promoting cell migration, but not cell proliferation.

Effect of FICZ was independent of AhR

Because FICZ is an endogenous ligand of AhR, we next assessed whether the typical exogenous AhR ligands,
benzo[a]pyrene and β-naphthoflavone, promoted wound closure in keratinocytes. Although both of them increased the expression of CYP1A1 mRNA in a concentration-dependent manner (Figure 3a, 3c), neither benzo[a]pyrene (Figure 3b) nor β-naphthoflavone (Figure 3d) accelerated the closure of the wounded areas. We then compared skin wound healing of DBA/2 mice, bearing a low-affinity AhR, with C57BL/6 mice, bearing a high-affinity AhR (Ema et al., 1994; Izawa et al., 2007; Okey et al., 2005). FICZ-containing ointment topically applied to the skin increased the expression levels of CYP1A1 and CYP1B1 mRNA in C57BL/6 mice (Figure 3e, 3g), whereas it did not significantly affect them in DBA/2 mice (Figure 3f, 3h). As shown in Figure 3i and 3j, FICZ significantly promoted wound healing compared with vehicle control in both C57BL/6 and DBA/2 mice. Thus, the effect of FICZ on wound closure might be AhR independent.

**FICZ accelerated wound closure in an AhR-independent manner**

To examine the involvement of AhR in FICZ-mediated skin wound healing, we first knocked down AhR using AhR-targeted small interfering RNA (siRNA). After the suppression of AhR protein levels had been confirmed (Figure 4a), IncuCyte scratch assay was performed to check the effect of FICZ on wound closure of AhR-silenced HaCaT cells. As shown in Figure 4b and 4c, FICZ accelerated the wound closure of HaCaT cells treated with AhR-targeted siRNA as well as control siRNA. We next blocked AhR activation in HaCaT cells using CH-223191, an AhR inhibitor. Although 10 μM CH-223191 significantly reduced FICZ-induced CYP1A1 mRNA expression (Figure 4d), CH-223191 did not inhibit the FICZ-induced promotion of wound closure of HaCaT cells as determined by IncuCyte scratch assay (Figure 4e, 4f). These results suggest that FICZ accelerated skin wound closure irrespective of AhR expression.
Figure 5. FICZ activates the MEK/ERK pathway in human keratinocytes. (a, b) HaCaT cells were scratched and treated with 100 nM FICZ or DMSO for 6 hours (n = 3). (c) HaCaT cells were scratched and treated with 100 nM FICZ in the presence or absence of 10 μM CH-223191, 10 μM U0126, or 50 μM PD98059 for 6 hours. (d–h) HaCaT cells were scratched and treated with 100 nM FICZ or DMSO for 6 hours (n = 3). All data are presented as mean ± SE. *P < 0.05, **P < 0.01. Akt, acutely transforming retrovirus AKT8 in rodent T-cell lymphoma; ERK, extracellular signal-regulated kinase; FICZ, 6-formylindolo[3,2-b]carbazole; JNK, c-Jun N-terminal kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; RAF, rapidly accelerated fibrosarcoma; SE, standard error.
FICZ accelerated cell migration via the activation of ERK, but not EGFR

Previous reports suggested that cell migration is mediated by various signaling pathways, including ERK, acutely transforming retrovirus AKT8 in rodent T-cell lymphoma (Akt), and c-Jun N-terminal kinase (JNK) (Fukumura et al., 2006; Huang et al., 2004). To reveal the molecular mechanism by which FICZ influences cell migration, we checked the levels of phosphorylated and total protein of signal molecules using unscratched and scratched keratinocytes. Compared with unscratched keratinocytes, FICZ significantly increased the ERK phosphorylation in scratched keratinocytes, but did not affect the phosphorylation levels of Akt and JNK (Figure 5a, 5b). FICZ-induced phosphorylation of ERK was suppressed by MEK/ERK inhibitors, U0126 and PD98059, but not by CH-223191, an AhR inhibitor (Figure 5c). To further investigate the involvement of ERK signaling on FICZ-induced cell migration, we examined the phosphorylation levels of ERK signaling upstream molecules including EGFR, rapidly accelerated fibrosarcoma, and MEK. FICZ significantly increased MEK and ERK phosphorylation, but did not affect the phosphorylation levels of EGFR and rapidly accelerated fibrosarcoma (Figure 5d–h). We next assessed cell motility induced by FICZ in the presence of various inhibitors. The acceleration of cell migration by FICZ was inhibited by U0126 (Figure 6a, 6b) and PD98059 (Figure 6c, 6d), but not by PD153035, an EGFR inhibitor (Figure 6e, 6f). Taken together, FICZ accelerated keratinocyte migration via the MEK/ERK signaling pathway, but not through EGFR.

DISCUSSION

Wound repair is an elaborate process composed of overlapping and orchestrated phases, including hemostatic, inflammatory, proliferative, and remodeling phases, in which various cells, soluble factors, and extracellular matrix components are involved (Eming et al., 2007; Wells et al., 2016). Re-epithelialization by the enhanced migration and proliferation of keratinocytes at the wound edge and the formation and contraction of granulation tissue mediated by activated fibroblasts and myofibroblasts during the proliferative phase are critical events for the closure of a wound (Coulombe, 2003). Here, we demonstrated that FICZ promoted skin wound healing by enhancing keratinocyte migration. FICZ, known as an endogenous high-affinity ligand for AhR, is formed from tryptophan in cells on exposure to UV radiation, and induces CYP1A1 expression through AhR activation, which results in hydroxylation and the elimination of FICZ in urine (Bergander et al., 2004; Rannug et al., 1995; Wincent et al., 2012). We found that the migration induced by FICZ was dependent on MEK/ERK pathway activation, but not AhR. FICZ accelerated cell migration even in the condition of AhR knockdown by siRNAs or an AhR inhibitor (Figure 4). Moreover, FICZ also promoted the wound healing in mice irrespective of the affinity of AhR (Ema et al., 1994; Izawa...
et al., 2007; Okey et al., 2005) (Figure 3i, 3j). On the other hand, it is crucial that keratinocytes proliferate and directionally migrate from the edges of the wound during wound healing, which is mediated by various signaling molecules, including Akt, JNK, and ERK (Kiwanuka et al., 2013; Squarize et al., 2010; Zhang et al., 2005). We found that FICZ increased ERK phosphorylation, but not that of Akt and JNK, and that MEK/ERK inhibitors attenuated the FICZ-induced acceleration of keratinocyte migration (Figures 5, 6). Interestingly, FICZ upregulated ERK phosphorylation only when keratinocyte monolayers were scratched, suggesting that the effect of FICZ on wound healing might require the molecular machinery stimulated by scratch wounding. Recently, it has been shown that ERK activity is elevated near the edge of the wounds, which depends on transforming growth factor-β (TGF-β) stimulation (Chapnick and Liu, 2014). Another report suggests that TGF-β is activated early after keratinocyte scratching (Fitsialos et al., 2007). Although further studies are necessary to identify the specific target molecules for FICZ, ERK might be activated by FICZ through the TGF-β signaling. As noted above, wound healing is a systematic process, including hemostasis and inflammation. During the hemostasis process, activated platelets release several cytokines, such as TGF-β, which promotes the migration of neutrophils, macrophages, and fibroblasts. Therefore, our future investigation will focus on the relationship between FICZ and TGF-β/ERK signaling in inflammatory cell migration.

We also found that FICZ expedited wound healing in db/db mice, which exhibited wound healing impairments owing to type 2 diabetes (Figure 1i). Diabetic ulcers are still one of the most intractable types of chronic ulcer and seriously affect the vital prognosis. Whereas acute wounds follow wellorchestrated sequential phases (hemostasis, inflammation, proliferation, and remodeling), in chronic wounds, this orderly set of events does not progress and ulcers stall in the inflammatory phase (Loots et al., 1998). Diabetic ulcers are characterized by reduced growth factor production, impaired angiogenic responses, and delayed keratinocyte migration due to the aberrant localization of EGFR (Falanga, 2005; Galiano et al., 2004). Although several growth factor drugs, such as basic fibroblast growth factor in Japan and platelet-derived growth factor in the USA and EU, are applicable to treat diabetic wounds and show significant efficacy by promoting neovascularization and granulation tissue formation (Steed, 2006; Uchi et al., 2009), impaired diabetic wound healing remains a problem. Our findings indicated that FICZ has potential as an activator of keratinocyte migration, and might be beneficial for the treatment of chronic skin wounds.

**MATERIALS AND METHODS**

**Reagents and antibodies**

FICZ was obtained from Enzo Life Sciences (Plymouth Meeting, PA), dissolved in DMSO (Sigma-Aldrich, St. Louis, MO) at a concentration of 100 μM, and further diluted in medium. Tryptophan, benz[a]pyrene, β-naphthoflavone, mitomycin C, cytchalasin D, and PD153035 were obtained from Sigma-Aldrich; U0126 and PD98059 were purchased from Cell Signaling Technology (Danvers, MA); and CH-223191 was obtained from Merck Millipore (Darmstadt, Germany). The antibodies used in this study were rabbit anti-β-actin, rabbit antiphosphorylated ERK1/2 (Thr202/Tyr204), rabbit anti-ERK1/2, rabbit antiphosphorylated Akt (Ser473), rabbit anti-Akt, rabbit antiphosphorylated JNK (Thr183/Tyr185), rabbit anti-JNK, rabbit antiphosphorylated EGFR (Tyr1068), rabbit anti-EGFR, rabbit antiphosphorylated c-Raf (Ser338), rabbit anti-c-Raf, rabbit antiphosphorylated MEK1/2 (Ser217/221), and rabbit anti-MEK1/2 from Cell Signaling Technology (Danvers, MA), as well as rabbit anti-AhR (H-211) from Santa Cruz Biotechnology (Santa Cruz, CA). The horseradish peroxidase-conjugated secondary antibody was purchased from Cell Signaling Technology.

**Wound healing in vivo**

Female C57BL/6, DBA/2, balb/c, db/db (+/Leprdb/db), and db/db (+/Leprdb/db) mice (all from Charles River Laboratories, Kanagawa, Japan) were housed in a vivarium in accordance with the guidelines of the animal facility center of Kyushu University. The mice were maintained on food and water ad libitum. They were anesthetized with sevoflurane, after which full-thickness wounds were made in the dorsal skin using a biopsy punch with a diameter of 6 mm (Kai Industries, Gifu, Japan). On the day of wound creation and every 2 days thereafter, ointment (vaseline containing FICZ: [FICZ: 284.3 ng/g vaseline, 1% DMSO] or vehicle [1% DMSO]) was occlusively applied to each wound (FICZ: 11.01 ± 0.44 ng/mouse) and digital photographs were taken under sevoflurane anesthesia until healing. The wound area was calculated from the photographs using the ImageJ software (NIH, Bethesda, MD).

**Cell culture**

HaCaT cells, a human keratinocyte cell line, were maintained in DMEM containing 10% fetal bovine serum and antibiotics. Normal human epidermal keratinocytes, obtained from Clonetics-BioWhittaker (San Diego, CA), were maintained in serum-free keratinocyte growth medium (Lonza, Walkersville, MD) supplemented with bovine pituitary extract, recombinant epidermal growth factor, insulin, hydrocortisone, gentamicin-amphotericin, transferrin, and epinephrine (Lonza).

**Scratch assay (IncuCyte system)**

HaCaT cells or normal human epidermal keratinocytes were seeded in a 96-well ImageLock tissue culture plate (Essen BioSciences) coated with type I collagen, and at full confluence, cell monolayers were scratched with the wound-maker (Essen BioSciences) to make acellular areas. Then, cells were incubated with FICZ, benz[a]pyrene, β-naphthoflavone, or DMSO supplemented with various inhibitors in DMEM containing 10% fetal bovine serum. Each well was automatically imaged every 2 hours within a CO2 incubator and the relative wound area was measured using the IncuCyte software (Essen BioSciences).

**Cell proliferation assay**

The BrdU incorporation assay and the 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay were performed following the manufacturers’ instructions (Roche, Mannheim, Germany, and Trevigen, Gaithersburg, MD, respectively). Briefly, HaCaT cells were seeded in 96-well plates, and were treated with FICZ (1, 10, or 100 nM) or DMSO for 24 hours. For the BrdU incorporation assay, BrdU was added for the final 2 hours of FICZ treatment. The reaction products were quantified by measuring the absorbance using a plate reader at 450 nm with a reference wavelength of 655 nm (BrdU incorporation assay), or at 570 nm
(3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay). All of the assays were performed in medium containing 10% fetal bovine serum.

Real-time quantitative reverse transcriptase-PCR
Total RNA was isolated from HaCaT cells using the RNeasy Mini kit (Qiagen) or mouse skin samples using the RNeasy fibrous tissue mini kit (Qiagen). Quantitative real-time reverse transcriptase-PCR was performed with PrimeScript RT reagent and SYBR Premix Ex Taq II (Takara Bio, Ohtsu, Japan) in accordance with the manufacturer’s instructions. PCR amplifications were performed with the following cycling conditions: 95 °C for 30 seconds initially, followed by 40 cycles of 95 °C for 5 seconds (denaturation step) and 60 °C for 20 seconds (annealing/extension steps). The cycle threshold for each amplification was normalized using β-actin (internal control). Normalized gene expression is shown as the quantity of gene-specific mRNA relative to that of control mRNA (fold induction). Oligonucleotide primers used in this study are listed below.

Sequences of nucleotides used as primer for PCR amplification:

**human CYP1A1:** sense, 5'-TAGACACTGATGCTGGTGACAG-3'
antisense, 5'-GGGAAGGCTCCATACGACAT-3'

**human β-actin:** sense, 5'-ATGCCGACAGGATGCAAGA-3'
antisense, 5'-GAGTACCTTTGCTAGGAGGA-3'

**mouse CYP1A1:** sense, 5'-CAATGAGTTCAGATGATGTC-3' (fold induction).
antisense, 5'-ATTGCCGACAGGATGCAGA-3'

**mouse CYP1B1:** sense, 5'-CCACAGCCTTAGTGCAGAC-3'
antisense, 5'-GGCAGGAGGGAGAGATG-3'

**mouse G6PD:** sense, 5'-CTACAGGTCCAGATGTC-3'
antisense, 5'-CAGCTTCTCTTCTCCATG-3'

AhR siRNA transfection
AhR siRNA (s1200) and control siRNA (Negative Control #1) were purchased from Ambion (Austin, TX) and transfected as required into HaCaT cells using the HiPerFect Transfection kit (Qiagen) in accordance with the manufacturer’s instructions.

Immunoblotting
HaCaT cells were seeded in six-well plates, and at full confluence, cell monolayers were scratched with a blue pipette tip. Scratch cells were then treated with FICZ (100 nM) or DMSO supplemented with various inhibitors in DMEM for 6 hours and protein lysates from cells were isolated with lysis buffer (25 mM HEPES, 10 mM Na3PO4·12H2O, 1% Triton X-100) and analyzed by SDS-PAGE on a 10% polyacrylamide gel. Proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and probed with specific antibodies. Immunological bands were identified with a horseradish peroxidase-conjugated secondary antibody followed by visualization with a SuperSignal west pico chemiluminescence substrate (Pierce, Rockford, IL).

Statistics
Data are presented as mean ± standard error. The significance of differences between groups was assessed using Student’s unpaired two-tailed t test (when two groups were analyzed) or one-way analysis of variance for three or more groups. A P-value of <0.05 was considered statistically significant.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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