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LGI3 promotes human keratinocyte migration in high-glucose environments by increasing the expression of β-catenin

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The leucine-rich repeat LGI family member 3 (LGI3) has been reported to regulate various functions in epidermal keratinocytes. In this study, we investigated the effects of LGI3 on keratinocyte migration in environments with different glucose concentrations. Our results showed that cell migration is markedly impaired in high-glucose environments compared to in low-glucose environments (control). Nevertheless, the use of LGI3 in high-glucose environments restores cell migration to the normal level. Therefore, we performed LGI3 knockdown to identify the role of LGI3 in cell migration. It was observed that transfecting LGI3 siRNA into HaCaT cells reduces the expression of LGI3 and inhibits wound closure. These results indicate that LGI3 is deeply involved in wound healing in high-glucose environments. Western blot analysis showed that in high-glucose environments, LGI3 increases the phosphorylation of Akt, forkhead box protein O1, and focal adhesion kinase. However, no change was observed in the levels of glycogen synthase kinase 3β, c-Jun N-terminal kinase, extracellular signal-regulated kinase, or p38 mitogen-activated protein kinase. Further results showed that LY294002, a specific inhibitor of phosphatidylinositol 3-kinase, reduced LGI3-induced cell migration. It is generally known that Akt activation leads to the accumulation of β-catenin, an important mediator of keratinocyte migration. LGI3 greatly increased the expression of β-catenin in high-glucose environments comparison to that in the low-glucose environments. Taken together, these data indicate that LGI3 induces keratinocyte migration in high-glucose environments as a result of β-catenin accumulation via Akt phosphorylation. Therefore, LGI3 can be considered a new treatment option for diabetic wound healing.

1. Introduction

Leucine-rich repeat LGI family member 3 (LGI3) is predominantly found in the brains of mice. Notably, LGI3 is involved in neuronal exocytosis *via* interacting with syntaxin 1 (Park et al. 2008), promotes β-amyloid endocytosis in astrocytes (Okabayashi and Kimura 2008), and induces neuronal differentiation (Park et al. 2010). It is known to be regulated by neuronal restriction silencer elements and AP-2 at the transcript level (Lee et al. 2006). LGI3 inhibits adipogenesis *via* the LGI3 receptor, a disintegrin and metalloproteinase domain-containing protein 23 (Kim et al. 2012), and increases the expression of inflammatory genes, including tumor necrosis factor-α, in macrophages (Kim et al. 2015). It is also largely expressed in various skin cells, including keratinocytes, fibroblasts, and melanocytes (Lee et al. 2012). Notably, increased LGI3 resulting from stimulation with ultraviolet B protects human keratinocytes (Lee et al. 2012). In human keratinocytes, LGI3 promotes differentiation through the Akt pathway (Kim et al. 2018). LGI3 is also known to be a new melanogenic cytokine in melanocytes (Jang et al. 2014). However, its role in skin cells has not yet been fully elucidated.

Skin is considered a strong barrier that protects against pathogenic organisms and external forces (Hameedaldeen et al. 2014). When this barrier breaks down—a phenomenon that can occur in response to various conditions, such as diabetes mellitus (DM)—the body cannot function properly. DM is responsible for a large percentage of morbidity and mortality worldwide; it is characterized by high plasma glucose levels and various other complications, such as enhanced rate of skin infections and chronic ulcerations that might lead to amputation (Danaei et al. 2011; Hu and Lan 2016; Lan et al. 2011). Particularly, it has been reported in many studies that patients with DM have reduced wound healing ability, leading to conditions such as diabetic foot (Hu and Lan 2016; Huang et al. 2017; Lan et al. 2008). Wound healing is a very complex process that involves various cellular phenomenon and biochemical pathways, such as hemostasis, inflammation, angiogenesis, and reepithelialization (Gugerell et al. 2016; Lan et al. 2008). As soon as an injury occurs, keratinocytes migrate to the wound area and induce re-epithelialization, which involves a series of processes, such as cell migration and proliferation (Mi et al. 2018; Raja et al. 2007). Cell migration occurs via a series of steps involving signaling networks and the production of extracellular matrix proteins (Sun et al. 2009).

Notably, the Akt signaling pathway plays a crucial role in the migration and differentiation, and in determining the wound healing ability of keratinocytes. In epidermal cells, forkhead box protein O1 (FOXO1), focal adhesion kinase (FAK), and β-catenin are located downstream of the Akt pathway (Jeong et al. 2013; Zhao and Guan 2011). Inhibitors of the Akt pathway are known to reduce the migration of keratinocytes (Jeong and Kim 2004; Jeong et al. 2013). FOXO1 is a transcription factor that plays an important role in wound healing. In the skin, FOXO1 is involved in epidermal morphogenesis, fibroblast apoptosis, and acne pathogenesis (Tsitsipatis et al. 2017). FAK also plays a major role in wound healing, and cell migration, proliferation, and survival (Singkhorn et al. 2018). In a previous study, we found that LGI3 promotes HaCaT cell migration following the accumulation of β-catenin (Jeong et al. 2013). However, the effects of LGI3 in high-glucose environments on the migration of keratinocytes have not yet been reported. Thus, in this study, we investigated the relationship between LGI3 and wound healing in high-glucose environments.

The aim of this study was to test the hypothesis that LGI3 regulates the migration of human keratinocytes in high-glucose environments. Our results showed that LGI3 has the potential to serve as a therapeutic target for wound healing in high-glucose environments.

2. Investigations and results

2.1. LGI3 promotes HaCaT cell migration in high-glucose environments

To investigate the effect of LGI3 on the migration of HaCaT cells in low- and high-glucose environments, a scratch migration assay was performed. After 24 h of LGI3 treatment, we monitored the closure of the scratch using a phase contrast microscope. In this study, a low glucose concentration (5.5 mM) was defined as a normal environment and a high glucose concentration (25 mM) was defined as a diabetes environment. Notably, HaCaT cells present in the high-glucose environment showed significantly delayed would closure in comparison to cells present in the low-glucose environment. As mentioned, LGI3 promotes keratinocyte migration. However, in the low-glucose environment, LGI3 did not increase the cellular migration rate (Fig. 1). Interestingly, treatment with LGI3 in a high-glucose environment significantly facilitated the migration of keratinocytes. In addition, HaCaT cells were treated with 19.5 mM mannitol as an osmotic pressure control; this environment was found to be similar to the low-glucose environment without mannitol (Fig. 1). We had previously reported that human keratinocytes produce LGI3 (Lee et al. 2006), and LGI3 promotes keratinocyte migration following the accumulation of β-catenin (Jeong et al. 2013). Thus, to further confirm the expression of LGI3 and β-catenin in keratinocytes in low- as well as high-glucose environments, a western blot analysis was performed; this revealed that the endogenous levels of LGI3 and β-catenin in the high-glucose environment were much lower than those in the low-glucose environment (Fig. 2). These findings suggest that exogenous LGI3 may aid the migration of HaCaT cells in high-glucose environments.

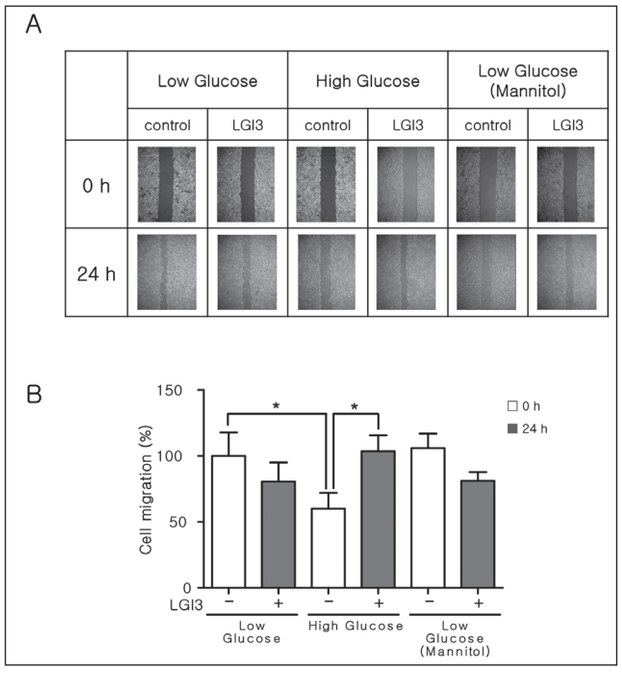

Fig. 1: Effect of leucine-rich repeat LGI family member 3 (LGI3) on the migration of HaCaT cells. (A) Scratch wound healing assays were performed in cultures as described in Section 2. After the HaCaT cells were grown in a six-well plate, they were serum-starved for twenty-four hours. The cells were then treated with LGI3 (10 ng/mL) in both low-glucose (5.5 mM) and high-glucose (25 mM) environments for twenty-four hours. Mannitol (19.5 mM) was used as an osmotic pressure control. A digital video camera was used to create phase contrast photomicrographs of the scratch widths. (B) The graph illustrates the cell migration rate. HaCaT cells remained in the media for 0 h (□) and 24 h (■). The data is presented as the mean ± standard deviation (SD) of wound widths amongst 10 randomly chosen wound fields. It is expressed as a percentage of the control. $^*P < 0.01$.

2.2. Effects of LGI3 knockdown on keratinocyte migration

As shown in Fig. 2, LGI3 is expressed at high levels in low-glucose environments. To further identify the effects of LGI3 on the migration of keratinocytes, we next investigated the effects of LGI3 knockdown on keratinocytes in low-glucose environments. We observed that transfecting LGI3 siRNA into HaCaT cells resulted in reduced expression of LGI3 (Fig. 3A). Furthermore, in the scratch migration assay LGI3 knockdown resulted in reduced wound closure (compared to that observed upon transfecting cells with siRNA control; Fig. 3B). This decreased LGI3 level is similar to that observed in the high-glucose environment. Hence, these results indicate that the migration of keratinocytes may be dependent on the LGI3 level.

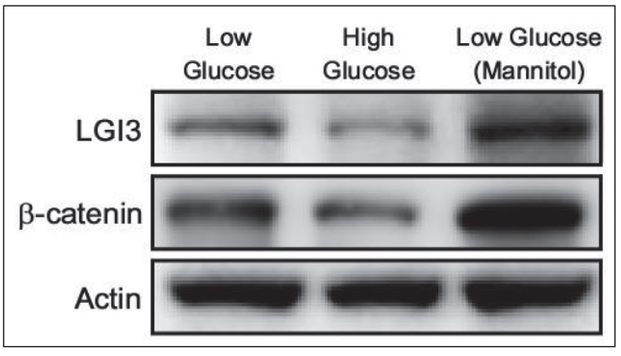

Fig. 2: Expression of endogenous leucine-rich repeat LGI family member 3 (LGI3) and β-catenin in keratinocytes. Low-glucose (5.5 mM) and high-glucose (25 mM) environments were used to culture the HaCaT cells. Mannitol (19.5 mM) was used as an osmotic pressure control. Western blotting with antibodies against LGI3 and β-catenin was utilized to analyze the whole-cell lysates. Actin expression levels confirmed that the proteins were loaded in equal concentrations.

2.3. Effects of LGI3 on cell-migration-related signaling pathways

To examine how LGI3 regulates cell migration, we next investigated cell-migration-related signaling pathways in low- and high-glucose environments. It has previously been reported that Akt activation increases keratinocyte migration (Jeong et al. 2013). Thus, we performed a western blot analysis in the absence or presence of LY294002, a selective PI3K inhibitor. Notably, in the high-glucose environment, LGI3 treatment increased the phosphorylation of Akt, whereas in the low-glucose environment, it had no effect. Furthermore, FOXO1 and FAK—which are the downstream molecules of the Akt pathway—were also found to be phosphorylated after LGI3 treatment in the high-glucose environment, but not in the low-glucose environment. However, our results show that the levels of GSK3β, JNK, ERK, and p38 MAPK were not influenced after LGI3 treatment (Fig. 4). These data indicate that LGI3-activated Akt restored cell migration in the high-glucose environment. Thereafter, we performed a keratinocyte migration assay in the absence or presence of LY294002 (Fig. 5), and we found that cell migration was significantly reduced in both low- and high-glucose environments when LY294002 was used. These results show that the Akt pathway plays a key role in keratinocyte migration.

2.4. Effects of LGI3 on β-catenin expression

It has been reported that the Akt pathway regulates the accumulation of β-catenin, a critical mediator of HaCaT cell migration (Puccinelli et al. 2010; Sun et al. 2009). Hence, we measured the level of β-catenin in LGI3-treated cells in low- and high-glucose environments. As shown in Fig. 6, LGI3 strongly increased the expression of β-catenin in the high-glucose environment in comparison to that in the untreated control. These findings indicate that LGI3 increases the accumulation of β-catenin *via* the Akt

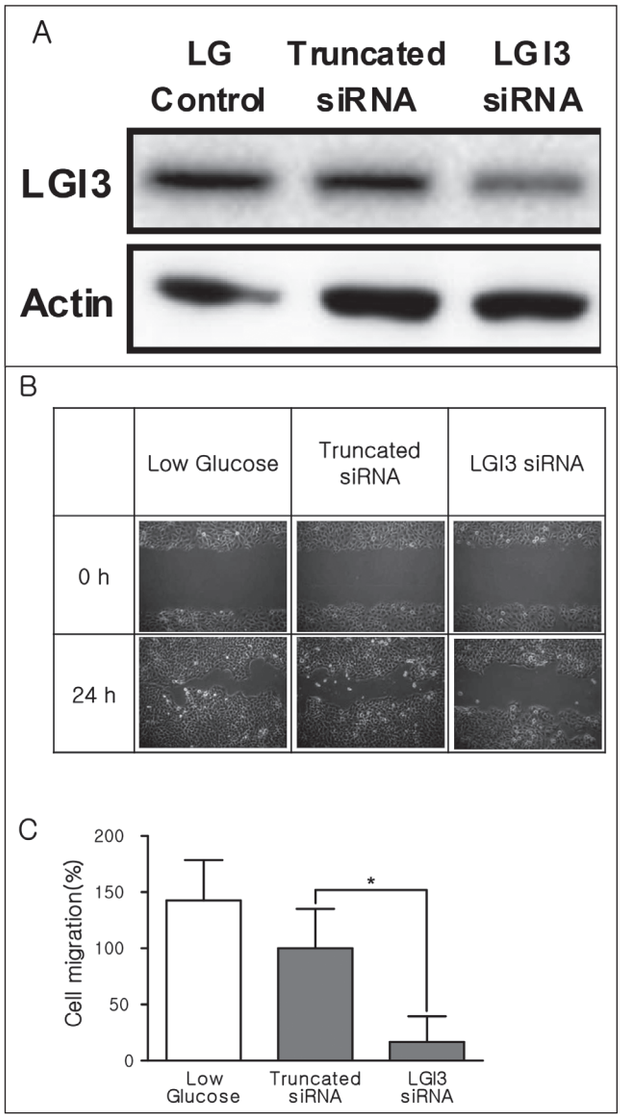

Fig. 3: Effect of leucine-rich repeat LGI family member 3 (LGI3) knockdown on the migration of keratinocytes. HaCaT cells in a low-glucose environment were treated with LGI3 siRNA. Truncated siRNA was used as a control. (A) Western blotting with antibodies against LGI3 was utilized to analyze the whole-cell lysates. Equal protein loading was confirmed based the level of actin expression. (B) Scratch wound healing assays were performed in cultures as described in Section 2. After the HaCaT cells were grown in a six-well plate, they were serum-starved for twenty-four hours. A digital video camera was used to create phase contrast photomicrographs of the scratch widths. (C) The graph illustrates the cell migration rate. The data is presented as the mean ± standard deviation (SD) of wound widths amongst 10 randomly chosen wound fields. It is expressed as a percentage of the control. $^{*}P < 0.01$ compared to the truncated siRNA control.

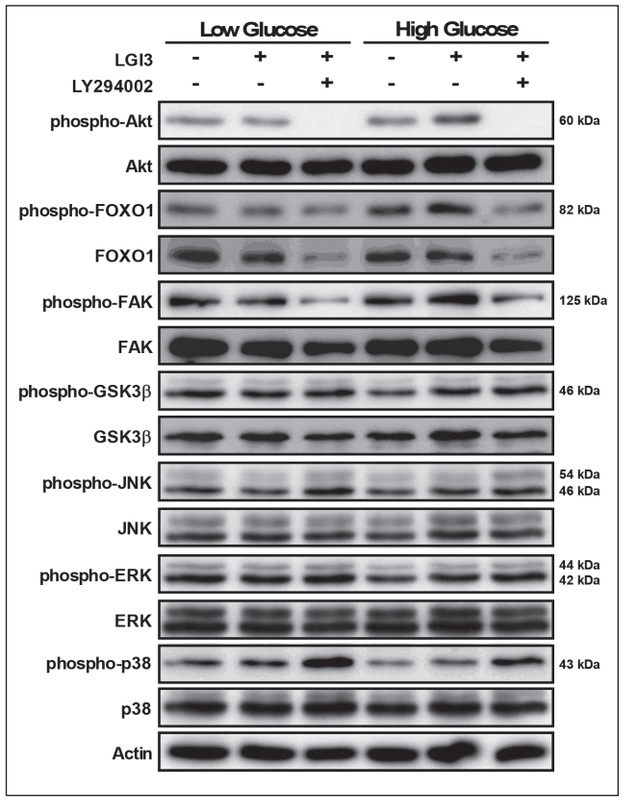

Fig. 4: Effects of leucine-rich repeat LGI family member 3 (LGI3) within signaling pathways related to migration of keratinocytes. Following serum starvation, HaCaT cells were initially treated with LY294002 (20 µM) for 30 min. This was followed by an LGI3 (10 ng/mL) treatment; the cells were then incubated for 1 hour in low- and high-glucose environments. Western blotting was performed to analyze whole-cell lysates, with antibodies against phospho-Akt, phospho-FOXO1, phospho-FAK, phospho-GSK3β, phospho-JNK, phospho-ERK, and phospho-p38 MAPK. Equal concentrations of protein loading were confirmed using the total Akt, forkhead box protein O1 (FOXO1), focal adhesion kinase (FAK), GSK3β, JNK, ERK, p38, and actin expression levels.

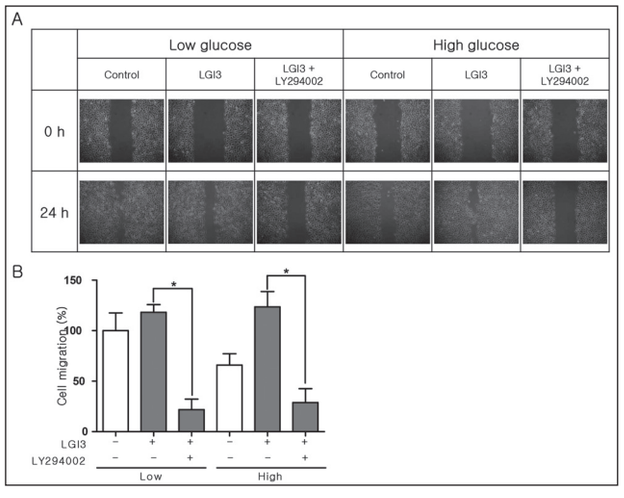

Fig. 5: Effect of leucine-rich repeat LGI family member 3 (LGI3) on the migration of keratinocytes in the presence of LY294002. (A) Scratch wound healing assays were performed in cultures as described in Section 2. Following serum starvation, HaCaT cells were initially treated with LY294002 (20 µM) for 30 min. This was followed by an LGI3 (10 ng/mL) treatment; the cells were then incubated for twenty-four hours in low- and high-glucose environments. A digital video camera was used to create phase contrast photomicrographs of the scratch widths. (B) The graph illustrates the cell migration rate. The data is presented as the mean ± standard deviation (SD) of wound widths amongst 10 randomly chosen wound fields. It is expressed as a percentage of the control. $^{*}P < 0.01$.

pathway in high-glucose environments. As a result, our results show that LGI3 increases wound healing via the accumulation of β-catenin in high-glucose environments.

3. Discussion

Wound healing is one of the most complex biological processes that occur in the human body. When the skin is damaged, it requires rapid and efficient wound healing. During wound repair, a mass of dysfunctional fibrous tissue is formed, which is usually known as a scar. In contrast, fetal tissues that are damaged early during pregnancy can be completely regenerated without fibrosis in a process similar to regeneration (Gurtner et al. 2008; Singh et al. 2017). Keratinocytes in the wounded area temporarily suspend terminal differentiation and prepare for migration. Re-epitheliali-

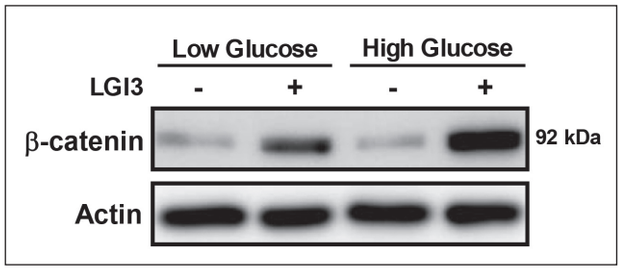

Fig. 6: Effects of leucine-rich repeat LGI family member 3 (LGI3) on the expression of β-catenin. Following serum starvation, LGI3 (10 ng/mL) was used to treat the HaCaT cells. The cells were then incubated for twenty-four hours in low- and high-glucose environments. Western blotting with antibodies against β-catenin was utilized to analyze the whole-cell lysates. Actin expression levels confirmed that the proteins were loaded in equal concentrations.

zation is one of the wound healing processes that occurs *via* keratinocyte migration and proliferation, and is an essential process for rebuilding the skin barrier after an injury (Wang et al. 2018).

Patients with DM have a decreased wound healing ability compared to the normal population, and that this leads to the formation of foot ulcers. However, the mechanisms involved have not yet been accurately identified (Greenhalgh 2003; Gurtner et al. 2008). Our results showed that high-glucose environments result in a significantly reduced wound closure ability (Fig. 1), supporting the fact that patients with DM exhibit delayed wound healing. It has also been demonstrated in various studies that high glucose concentrations suppress keratinocyte migration (Hu and Lan 2016; Huang et al. 2017; Li et al. 2019). In the present study, we demonstrated that LGI3 induces keratinocyte migration in high-glucose environments, suggesting that it could promote wound healing in patients with DM.

We have previously reported that LGI3 promotes keratinocyte migration by Akt activation (Jeong et al. 2013). We hypothesized that LGI3 induces cell migration *via* the Akt pathway in high-glucose environments. Notably, the Akt pathway affects migration as well as numerous cellular functions, such as cell survival, chemotaxis, and motility (Kim et al. 2020). Activation of the Akt pathway by platelet-derived growth factor enhances cell migration by inducing stabilization and translocation of β-catenin (Xiong et al. 2010). Furthermore, the binding of β-catenin to E-cadherin contributes to cell–cell interaction (Park et al. 2008). In addition, β-catenin stabilizes cell adhesion by binding to the actin cytoskeleton that regulates migration (Jeong et al. 2013; Liou et al. 2002). It has been reported in many studies that β-catenin signaling regulates the proliferation, migration, and differentiation of keratinocytes (Mendoza-Reinoso and Beverdam 2018). In addition, both the Akt pathway and β-catenin signaling regulate apoptosis and cell survival as well as cell–cell contact, differentiation, and migration (Andl et al. 2003). When β-catenin accumulates in the cells, it translocates into the nucleus and binds to transcription factors belonging to the TCF-LEF family (Cheon et al. 2006). However, it has also been reported that the stabilization of β-catenin that migrates to the nucleus inhibits keratinocyte migration by preventing the production of epidermal growth factor (Stojadinovic et al. 2005). In our experiments, we found that wound healing increases with the increase in β-catenin under LGI3 treatment in high-glucose environments.

FOXO1 is a transcription factor that is considered one of the downstream molecules of the Akt pathway and a crucial factor in wound healing (Ponugoti et al. 2012). Under normal conditions, FOXO1 induces the migration of keratinocytes by upregulating the expression of transforming growth factor-β (Ponugoti et al. 2013). Further, FOXO1 impairs wound healing *via* the regulation of matrix metalloproteinase-9, especially in conditions with high glucose concentrations (Zhang et al. 2017). However, the role of FOXO1 in normal and diabetic wound healing is controversial. As shown in Fig. 4, we demonstrated that LGI3 treatment in high-glucose environments increases the phosphorylation of Akt and subsequently FOXO1. When the Akt signaling pathway is activated, FOXO1 becomes phosphorylated and the transcriptional activity decreases (Hameedaldeen et al. 2014; Miao et al. 2019). Therefore, we suggest that decreased FOXO1 may help inhibit the impairment of wound healing, i.e., it helps wound healing.

In conclusion, our results showed that LGI3 is involved in the induction of keratinocyte migration in high-glucose environments. LGI3 regulates keratinocyte migration *via* the phosphorylation of FOXO1 and accumulation of β-catenin through the Akt pathway. Therefore, LGI3 can be considered as a treatment option for diabetic wound healing. Further research is still needed to investigate the effects and mechanisms of FOXO1 and β-catenin in epithelial wound healing, particularly in high-glucose environments.

4. Experimental

4.1. Materials

Recombinant LGI3 was prepared as previously reported (Park et al. 2010). LY294002 was purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against phospho-Akt (#9271), Akt (#9272), phospho-extracellular signal-regulated kinase (ERK) (#9101), ERK (#9102), phospho-p38 mitogen-activated protein kinase (MAPK) (#9211), p38 MAPK (#9212), phospho- c-Jun N-terminal kinase (JNK) (#9251), JNK (#9258), phospho-glycogen synthase kinase 3β (GSK3β) (#9336), GSK3b (#9315), phospho-FOXO1 (#9461), FOXO1 (#2880), and β-catenin (#9581) were purchased from Cell Signaling Technology. Antibodies specific for phospho-FAK (#44624) were purchased from Invitrogen (Waltham, MA, USA). Antibodies against LGI3 (T-18), FAK (sc-271126), and actin (sc-47778) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibodies against anti-mouse IgG (PI-2000) and anti-rabbit IgG (PI-1000) were purchased from Vector Laboratories (Burlingame, CA, USA). Mannitol was purchased Sigma-Aldrich Co. (St. Louis, MO, USA).

4.2. Cell culture

HaCaT human keratinocytes were purchased from Cell Lines Service (Eppelheim, Germany). The cells were grown in Dulbecco's modified Eagle's medium supplemented with low-glucose (5.5 mM) and high-glucose (25 mM) in a 5% CO_2 atmosphere at 37 °C; these media also contained 10% fetal bovine serum (PAN-Biotech, Aidenbach, Germany), 50 µg/mL streptomycin, and 50 µg/mL penicillin. The glucose levels used followed those described in previous reports (Lan et al. 2008, 2009). The concentration of glucose in normal plasma is known to be around 105 mg/dL (equivalent to 5.8 mM). As in most previous studies, we regarded 25 mM as a diabetic condition. Therefore, we considered 5.5 mM as low glucose and 25 mM as high glucose in this study.

4.3. Migration assay

A migration assay was performed in a serum-free medium for a period that did not exceed 24 h. For the scratch wound migration assay, HaCaT cells were grown in six-well plates to 100% confluence and serum-starved for 24 h. Perpendicular wounds were made by dragging a sterile yellow micropipette tip across each cell plate. The plates were then washed with phosphate-buffered saline (PBS) and replenished with a fresh medium alone or with a medium containing LGI3 (10 ng/mL). Mannitol (19.5 mM) was used as the osmolarity control. Cell migration into the scratch was monitored under a phase contrast microscope (model: IX50-S8F; Olympus Tokyo, Japan) using an ocular grid at 0 and 24 h, and was photographed using a DCM-800 digital camera equipped with ScopePhoto software (ScopeTek, Inc., Hangzhou, China). The wound widths were measured in 10 randomly chosen fields. Cell migration was calculated using the following formula: Cell migration = (initial wound width − final wound width)/10 fields. The migration rate (%) is expressed as the percent migration, with migration in the untreated control representing 100%. All experiments were repeated at least three times.

4.4. Western blot analysis

HaCaT cells were cultured in low-glucose (5.5 mM) and high-glucose (25 mM) environments for seven days. Protein samples were prepared using a mammalian protein extraction reagent (Pierce, Rockford, IL, USA) supplemented with a complete protease inhibitor mixture (Roche, Mannheim, Germany). The samples were then separated on 10% sodium dodecyl sulfate polyacrylamide gels and transferred onto polyvinylidene fluoride membranes, which were blocked with 4% dried milk prepared in PBS containing 0.1% Tween 20. Blots were incubated with the appropriate primary antibodies at a dilution of 1 : 1000. Membrane-bound primary antibodies were detected using secondary antibodies conjugated with Clarity Western ECL Substrate (Pierce Chemical Co., Rockford, IL, USA). Images of the blotted membranes were obtained using a LAS 4000 Lumino Image Analyzer (Fujifilm, Tokyo, Japan).

4.5. Transfection

Lipofectamine 2000 (Invitrogen, Camarillo, CA, USA) was used to transfect cells with siRNA according to the manufacturer's protocol. The following siRNAs were used: human LGI3 siRNA (sense, CUCUCUAUGUGCGCACCAA[dTdT]) and negative control siRNA with a nontargeting sequence for humans, rats, and mice (sense, UCACAACCUCCUAGAAAGAGUAGA) (Dharmacon, Lafayette, CO, USA). After the cells were treated with siRNA for 24 h, western blot analyses were conducted.

4.6. Statistics

Differences between the results were assessed for significance using Student's *t*-test.

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Conflicts of interest: The authors have no conflicts of interest to declare.

Authors' contributions: S.Y.K. performed the research and wrote the manuscript. Y.Y.K. and I.W.K. performed the research. H.Y.Y. were involved in the study design and data interpretation. D.S.K. contributed to the experimental design; data interpretation; and writing, editing, and submission of this manuscript. All the authors read and approved the final manuscript.

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