Human Cathelicidin Antimicrobial Peptide LL-37 Promotes Lymphangiogenesis in Lymphatic Endothelial Cells through the ERK and Akt Signaling Pathways

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Abstract

LL-37, the only member of the cathelicidin family of cationic antimicrobial peptides in humans has been shown to exhibit a wide variety of biological actions in addition to its antimicrobial activity. However, the lymphangiogenic effect of LL-37 has not been elucidated yet. In this study, we examined the effects of LL-37 on lymphangiogenesis and evaluated the underlying molecular mechanisms. LL-37 treatment significantly increased the migration and tube-like formation of human dermal lymphatic microvascular endothelial cells (HDLECs) and promoted the expression of lymphangiogenic factor in HDLECs. Treatment with LL-37 increased phosphorylation of ERK and Akt proteins in HDLECs, and pretreatment with ERK and Akt inhibitors significantly blocked the LL-37-induced HDLEC migration and tube-like formation. Furthermore, to investigate the involvement of formyl peptide receptor-like 1 (FPRL-1) signaling in LL-37-induced lymphangiogenesis, HDLECs were treated with an FPRL-1 antagonist. Pretreatment with the FPRL-1 antagonist inhibited LL-37-induced phosphorylation of ERK and Akt proteins and attenuated LL-37-induced HDLEC migration and tube-like formation. These data indicated that LL-37-induces lymphangiogenesis in lymphatic endothelial cells via FPRL1, and the activation of the ERK and Akt-dependent signaling pathways.

Keywords: LL-37, Lymphangiogenesis, ERK, Akt, FPRL1

1. Introduction

The extraction of a tooth initiates a series of repair processes involving both soft and hard tissues. Delayed wound healing of the socket after tooth extraction is likely to cause postoperative infection. Wound healing is a complex physiological process involving hemostasis, inflammation, proliferation, and maturation [1]. Recently, the importance of lymphatic vessel formation in wound healing has also been reported [2]. The lymphatic system is crucial for the immune barrier function and tissue fluid balance [3]. During inflammation, lymphangiogenesis takes place to enhance the transport of filtered fluid, proteins, and immune cells [3]. A previous study indicated that parathyroid hormone (PTH) increased bone formation and promoted blood and lymphatic vessel formation in wound healing after tooth extraction [4]. Therefore, the regulation of lymphangiogenesis may also be important for improving wound healing after tooth extraction.

Lymphangiogenesis, a complex process of sprouting of new lymphatic vessels is regulated by various growth factors. Vascular endothelial growth factor- (VEGF-A, VEGF-C, VEGF-D) and angiopoietin- families (Ang1, Ang2) are known to bind to specific receptors and activate several downstream signaling pathways [5]. Previous studies indicated that extracellular signal-regulated kinase (ERK1/2) and

phosphatidylinositol 3-kinase (PI3K/Akt) signaling pathways are important for lymphatic endothelial cell survival, migration, and proliferation [6].

Antimicrobial peptides are effector molecules of innate immunity known to play a major role in host defense against various pathogenic microbes; they are present in almost all vertebrates, invertebrates, and plants' species. [7]. In mammals, antimicrobial peptides are mainly categorized into two families: cathelicidins and defensins [8]. LL-37 is a 37 amino-acid cationic peptide generated by the cleavage of the human cationic antimicrobial protein 18 (hCAP18) C-terminal end; of note, it is the only member of the cathelicidins' family in humans [8]. LL-37 is expressed in a variety of cells such as keratinocytes, intestine cells, colon enterocytes, skin epithelial cells, neutrophils, T cells, monocytes, NK cells, and mast cells [9]. In the oral cavity, LL-37 has been detected in saliva, gingival crevicular fluid, and salivary glands [10-13], and has been reported to help prevent infection and maintain a healthy oral environment [14].

LL-37 is known to exhibit a wide variety of biological actions in addition to its antimicrobial activity through the activation of several different types of receptors [15]. Previous studies have demonstrated that LL-37 participates in inflammation, wound healing, and angiogenesis [16-18]. Additionally, several reports have demonstrated that LL-37 activates G-protein coupled receptors (GPCRs) such as the formyl peptide

receptor-like 1 (FPRL1), tyrosine kinase receptors such as the epidermal growth factor receptor (EGFR), toll-like receptors (TLR), and the P2X7 purinergic channel receptor (P2X7R) [15]. Consequently, various intracellular signaling pathways including ERK1/2, c-Jun N-terminus kinase (JNK), and PI3K/Akt are activated by the action of LL-37 on these receptors [18-21]. A recent study has indicated that LL-37 promotes angiogenesis via FPRL1 following activation of the PLC-γ/PKC/NF-κB, ERK1/2, and PI3K/Akt pathways [18]. However, the lymphangiogenic effects of LL-37 have not been elucidated. In this study, we examined the effects of LL-37 on lymphangiogenesis and evaluated the underlying molecular mechanisms.

2. Materials and Methods

2.1. Materials

LL-37 was obtained from PEPTIDE INSTITUTE, INC. (Osaka, Japan). The LL-37 scrambled peptide was purchased from Eurogenetec (Fremont, CA, USA). The ERK (SCH772984) and Akt (MK-2206) inhibitors were obtained from Cayman (Ann Arbor, MI, USA). The FPRL1 antagonist (WRW4) was purchased from Abcam (Cambridge, UK).

2.2. Cell culture

Human dermal lymphatic microvascular endothelial cells (HDLECs) were purchased from Lonza (Walkersville, MD, USA). HDLECs were cultured in endothelial cell basal medium-2 (EBM-2) (Lonza), supplemented with EGM-2 Microvascular Endothelial Cell Growth Medium (MV) at 37 °C, 5% CO_2. Cells between passages 3 and 5 were used for the experiments.

2.3. Cell proliferation assay

Cell proliferation was analyzed using the Premix WST-1 Cell Proliferation Assay System (Takara, Tokyo, Japan) according to the manufacturer's instructions. Briefly, 1×10^3 HDLECs were cultured in 96-well plates for 4 h and treated with LL-37 (1–1000 nM) for 24 – 72 h. Cells were incubated with Premix WST-1 for 40 min, and the absorbance was measured at 450 nm using a microplate reader (Multiscan FC, ThermoFisher Scientific, Rockford, IL, USA).

2.4. Migration assay

The migration assay was performed with transwell 24-well tissue culture plates composed of a polycarbonate membrane with 8 μm pores (Corning Inc., Corning, NY,

USA). HDLECs were seeded on the inner chamber of the transwell plate at a concentration of $1x10^5$ cells/100 μl. The inner chamber was placed into the outer chamber, filled with 600 μl EBM-2, which contained LL-37 (1–1000 nM), LL-37 scrambled peptide (100 nM), or saline, and incubated for 6 h at 37 ˚C. In some experiments, HDLECs were pretreated with SCH772984 (10 μM), MK-2206 (10 μM), or WRW4 (10 μM) for 60 min and then, the migration assay was performed. Cells that migrated onto the outer surface of the membrane were fixed with cold methanol and 4% paraformaldehyde and stained using the May-Giemsa method. The number of migrated cells was counted in five randomly-chosen fields (in triplicates) at a magnification of x10 for each sample.

2.5. Tube formation assay

The formation of tube-like structures by HDLECs on growth factor-reduced Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was assessed according to the manufacturer's instructions. Before the experiment, HDLECs were placed in EBM-2 for 15 h for serum starvation. Then, cells were seeded onto Matrigel-coated plates at 5 x 10^4 cells/cm^2 in EBM-2 medium and incubated with LL-37 (1–1000 nM), LL-37 scrambled peptide (100 nM), or saline at 37 °C for 24 h. In some experiments, HDLECs were pretreated with

SCH772984 (10 μM), MK-2206 (10 μM), or WRW4 (10 μM) for 60 min before the tube formation assay was performed. Network formation was assessed using an inverted phase-contrast microscope (Olympus, Tokyo, Japan), and photomicrographs were taken at ×10 magnification. The degree of tube formation was quantified by measuring the length of tubes in 5 randomly chosen fields from each well using the Image-J program. Each experiment was repeated 3 times.

2.6 siRNA transfection

HDLECs were transfected with siRNAs targeting human VEGF-A, VEGF-C, Ang1 (Thermo Fisher Scientific, Waltham, MA, USA), and FPRL1 (Horizon Discovery Ltd., Cambridge, UK), or scrambled control siRNA (Thermo Fisher Scientific) using the lipofectamine RNAiMAX reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, each siRNA was diluted with Opti-MEM I reduced serum medium (Life Technologies, Carlsbad, CA, USA), and then, mixed with the lipofectamine RNAiMAX reagent for 5 min at room temperature. The mixture was then added to HDLECs in 6-well culture plates. After 3 days of incubation at 37°C and 5% CO_2, cells were used for the subsequent experiments. Quantitative reverse transcription (RT)-PCR was performed to evaluate the expression levels of each target gene.

2.7. Quantitative RT-PCR analysis

Total RNA was isolated with ISOGEN (NIPPON GENE, Tokyo, Japan) from HDLECs cultured in EGM-2 MV growth medium with or without LL-37 (100 nM) for 48 h. Reverse transcription was performed with 1 μg of RNA, random primers, and the MMLV reverse transcriptase (ReverTra Ace-α, TOYOBO, Osaka, Japan). Quantitative real-time PCR was performed using the CFX Connect Real-Time System (Bio-Rad, Hercules, CA, USA) and the THUNDERBIRD SYBR Green PCR kit (Toyobo, Osaka, Japan). The sequences of all of the primers used are shown in Table 1. mRNA expression was calculated in relation to the expression of GAPDH mRNA and all data are presented as fold change, compared to the respective controls.

2.8. Immunoblotting analysis

HDLECs were treated with LL-37 (100 nM) at the indicated time-points and were lysed in RIPA lysis buffer (Thermo Fisher Scientific). Cell lysates were subjected to immunoblotting. In some experiments, HDLECs were pretreated with SCH772984 (10 μM), MK-2206 (10 μM), or WRW4 (10 μM) for 60 min. The following primary antibodies were used: anti-LYVE-1 (Acris Antibodies, Inc. San Diego, CA, USA), anti-

VEGF Receptor 3 and anti-FPRL1 (Abcam, Cambridge, UK), anti-phospho-ERK (Thr-202/Tyr-204), anti-ERK, anti-phospho-JNK (Thr-183/Tyr-185), anti-JNK, anti-phospho-p38 (Thr-180/Tyr-182), anti-p38, anti-phospho-Akt (Ser-473), anti-Akt, and anti-β-actin (Cell Signaling Technology, Danvers, MA, USA). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and an enhanced chemiluminescence (ECL) system were obtained from GE Healthcare (Buckinghamshire, UK).

2.9. Statistical analysis

The data are presented as mean ± standard deviation (S.D.) values. Comparisons between 2 groups were performed using the Student's *t*-test. Multiple comparisons (among three or more groups) were performed using one-way ANOVA. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of LL-37 on HDLECs proliferation

To examine the effect of LL-37 on HDLECs proliferation, cells were incubated with different concentrations of LL-37 (1–1000 nM) for 24–72 h. Overall, treatment with LL-37 did not promote the proliferation of HDLECs (Fig. 1a). However, the HDLECs growth

was suppressed by the addition of a high LL-37 concentration (1000 nM) after 72 h of incubation (Fig. 1a).

3.2. Effect of LL-37 on the migration and tube-like formation of HDLECs

To evaluate whether LL-37 induces HDLECs migration, HDLECs were treated with LL-37 (1–1000 nM) or saline as the control. Treatment with LL-37 promoted HDLECs migration (Fig. 1b). In fact, the quantitative analysis demonstrated that LL-37 significantly promoted HDLECs migration, peaking at about 100 nM (Fig. 1b). Next, we investigated whether LL-37 induces the formation of lymphatic endothelial cell tube structures. Treatment with LL-37 enhanced HDLECs tube-like structures; of note, the quantitative analysis indicated that LL-37 treatment significantly promoted the formation of tube-like structures even at low concentrations (1 nM; Fig. 1c). To further confirm the observed promotion of HDLECs migration and tube-like formation was dependent on the LL-37 peptide function, we repeated the assays using an LL-37 scrambled peptide. Importantly, treatment with the LL-37 scrambled peptide had no effect on both HDLECs migration and tube-like formation (Fig. 1d, 1e). These results demonstrate that, indeed, the LL-37-induced migration and tube-like formation are dependent on its bioactive properties.

3.3. LL-37 promotes lymphangiogenic factor expression in HDLECs

Recently, various lymphangiogenic factors such as the VEGF and the angiopoietin families have been identified [5]. Here, we investigated whether the treatment with LL-37 induces lymphangiogenic factor expression in HDLECs. VEGF-A, VEGF-C, and Ang1 mRNA levels were significantly increased in the LL-37-treated group, compared with the non-treated group (Fig. 2a). To further analyze the involvement of lymphangiogenic factors in the LL-37-induced HDLECs migration and tube-like formation, knockdown experiments were performed using siRNAs against VEGF-A, VEGF-C, and Ang1. Transfection with siRNAs targeting VEGF-A, VEGF-C, and Ang1 resulted in 43%, 45%, and 53% reduction of each gene expression (Fig. 2b). Importantly, the knockdown of VEGF-A and VEGF-C decreased LL-37-induced HDLECs migration and tube-like formation (Fig. 2c, 2d). On the other hand, the knockdown of Ang1 did not impact the effect of LL-37 on HDLECs migration and tube-like formation (Fig. 2c, 2d).

3.4. LL-37 activates ERK and Akt signaling in HDLECs

It is well established that various intracellular signals are involved in the migration and tube-like formation of HDLECs [6]. We, therefore, examined the potential signaling

pathways involved in LL-37-induced HDLECs migration and tube-like formation. Treatment with LL-37 (100 nM) increased phosphorylation of ERK and Akt proteins in HDLECs (Fig 3a), while there was no effect regarding JNK and p38 protein activation. Of note, treatment with LL-37 had no effect on total ERK, JNK, p38, and Akt levels (Fig. 3a).

3.5. Involvement of ERK and Akt signaling in LL-37-induced migration and tube-like formation in HDLECs

Next, we investigated the participation of ERK and Akt signaling in LL-37-induced migration and tube-like formation. We confirmed that pretreatment with ERK (SCH772984) and Akt (MK-2206) inhibitors significantly suppressed the LL-37-induced phosphorylation of ERK and Akt proteins (Fig. 3b). Further, we investigated whether inhibition of ERK and Akt signaling attenuated LL-37-induced migration and tube-like formation in HDLECs. Pretreatment with SCH772984 and MK-2206 significantly inhibited LL-37-induced HDLECs migration (Fig. 3c). Furthermore, inhibition of ERK and Akt signaling also suppressed LL-37-induced tube-like formation of HDLECs (Fig. 3d).

3.6. LL-37 induces HDLECs migration and tube-like formation in an FPRL1 signaling-depending manner

LL-37 has been reported to induce a variety of biological actions via multiple receptors, such as FPRL1, epidermal growth factor receptor, ERBb2, or P2X7 [15]. Interestingly, a previous study showed that LL-37 induces an angiogenic potential in vascular endothelial cells via FPRL1 signaling [18]. Therefore, we examined whether FPRL1 signaling is involved in LL-37-induced HDLECs migration and tube-like formation. We first examined the expression of FPRL-1 in cultured HDLECs. Western blot analysis showed that cultured HDLECs expressed the lymphatic endothelial hyaluronic acid receptor-1 (LYVE-1) and the vascular endothelial growth factor receptor 3 (VEGFR3), which are specific lymphatic endothelial cell markers; moreover, we confirmed that FPRL-1 protein was expressed in cultured HDLECs (Fig. 4a). To further investigate the involvement of FPRL-1 signaling in the LL-37-induced phosphorylation of ERK and Akt proteins, HDLECs were treated with an FPRL-1 antagonist (WRW4), and the kinases relative phosphorylation levels were assessed. Importantly, treatment with WRW4 suppressed LL-37-induced phosphorylation of ERK and Akt proteins (Fig. 4b), and had no effect on total ERK and Akt protein levels. Next, we investigated whether the inhibition of FPRL-1 signaling attenuated LL-37-induced migration and tube-like

formation in HDLECs. Treatment with WRW4 significantly suppressed LL-37-induced migration (Fig. 4c) and tube-like formation (Fig. 4d). Furthermore, to corroborate these data, knockdown experiments were performed using a siRNA against FPRL-1. Transfection with siRNA targeting FPRL1 resulted in a 64% reduction of FPRL-1 expression (Fig. 5a). Importantly, in line with the above results, FPRL-1 knockdown significantly decreased LL-37-induced HDLECs migration and tube-like formation (Fig. 5b, 5c). Overall, these results indicate that that LL-37-induced lymphangiogenesis is dependent on FPRL1 signaling.

4. Discussion

In mammals, at least two distinct groups of antimicrobial peptides are known: cathelicidins and defensins are the most representative ones [8]. It is well known that antimicrobial peptides show various biological actions besides antimicrobial activity [16-18, 22]. However, there are few reports on their effects on lymphangiogenesis. Although a previous study has demonstrated that β-defensins promote lymphangiogenesis by inducing lymphatic endothelial cell migration [23], as far as we are concerned there are no investigations on the effect of cathelicidins on lymphangiogenesis. In the present study, we show for the first time that the human cathelicidin LL-37 promotes lymphangiogenesis

in lymphatic endothelial cells. Furthermore, we also elucidated the molecular mechanism involved.

Lymphangiogenesis is regulated via a process involving proliferation, migration, and sprouting of lymphatic endothelial cells. It is well known that lymphangiogenesis is regulated by various growth factors such as those from the VEGF and angiopoietin families [5]. Previous studies have reported that LL-37 promotes VEGF expression in several cell types [24, 25], but, whether LL-37 induces the expression of lymphangiogenic factors in lymphatic endothelial cells has not been elucidated. In the present study, we have shown that treatment with LL-37 promoted VEGF-A, VEGF-C, and Ang1 expression in HDLECs, and that the knockdown of VEGF-A and VEGF-C decreased LL-37-induced migration and tube-like formation. These results indicate that VEGF-A and VEGF-C may, at least in part, affect LL-37-induced lymphangiogenesis in an autocrine manner.

LL-37 is associated to diverse biological actions through various intracellular signaling pathways [19-21]. We have shown that treatment with LL-37 increased phosphorylation of ERK and Akt in HDLECs. Mitogen-activated protein kinase (MAPK) and PI3K/Akt signals have been reported to play an important role in promoting cell proliferation, migration, and lumen formation [6]. Of note, the expression of

lymphangiogenic factors such as VEGF-A and VEGF-C is regulated by ERK and Akt signaling [26]. We hypothesize that the expression of LL-37-mediated lymphangiogenic factors may be induced via the ERK and Akt signaling pathways. Still, our observation that the LL-37 treatment did not impact HDLECs proliferation, goes against the activation of ERK and Akt signaling pathways. Previous studies reported that the duration of ERK and Akt signaling activation is important to control cellular functions, such as proliferation and differentiation [27, 28]. Of note, in our study, the LL-37-induced activation of ERK and Akt signals peaked at 20 minutes. It is possible that the duration of LL-37-mediated signaling was not enough to induce the proliferation of HDLECs; of note, in our context, it is unclear whether the activation pattern of ERK and Akt by LL-37 is transient or continuous. Thus, further studies are required to understand the impact of LL-37-mediated ERK and Akt activation, in a dynamic way, and extrapolate to the context of cell proliferation.

The treatment with ERK and Akt inhibitors effectively suppressed LL-37-induced migration and tube-like formation in HDLECs. These data demonstrate that LL-37-mediated lymphangiogenesis in lymphatic endothelial cells is induced via both ERK and Akt-dependent signaling. Curiously, in the present study, treatment with the Akt inhibitor MK2206 also suppressed ERK phosphorylation in HDLECs. Consistent with our data, a

previous study has shown that MK2206 attenuated phospho-ERK signals in human bladder carcinoma [29]. Furthermore, LY294002 a PI3K inhibitor that also affects Akt signaling, inhibited ERK phosphorylation in various cell types [30]. Although Akt and ERK pathways are activated independently, a cross-talk between them has been reported [31]. However, and particularly in lymphatic endothelial cells, it is unclear what molecules are involved in such cross-talk between the Akt and ERK pathways. Therefore, further studies are required to fill this gap in knowledge.

The various biological functions of LL-37 are induced via various cell surface receptors such as FPRL1, EGFR, TLR, and P2X7R [15]. FPRL1 signaling is known for its important role in endothelial cells, particularly in the regulation of various intracellular signals. A previous study demonstrated that LL-37 induced proliferation and tube formation in umbilical vein endothelial cells via FPRL1 signaling [32]. Another study also reported that LL-37 induced angiogenesis via FPRL1 *in vitro* and *in vivo* [18]. In the present study, we have shown that FPRL1 is also expressed in HDLECs. Importantly, we have also shown that, in HDLECs, the pharmacological inhibition of FPRL1 (with WRW4) effectively inhibited LL-37-induced phosphorylation of ERK and Akt proteins. Moreover, both the treatment with WRW4 and the knockdown of FPRL1 significantly

suppressed LL-37-induced migration and tube-like formation in HDLECs, indicating that LL-37-induced lymphangiogenesis is dependent on the FPRL1 signaling pathway.

Functional lymphangiogenesis is important for the wound healing process; in fact, delayed or failed lymphatic vessel regeneration impairs wound healing [2]. Lymphangiogenesis can be induced using different approaches. For instance, previous reports showed that administration of adenoviral and recombinant VEGF-C induced lymphangiogenesis [33, 34]. However, repeated administration of high doses of VEGF-C could cause edema and hyperplasia of blood vessels due to their abnormal hyperpermeability [35]. The mechanisms by which LL-37 potentially induces wound healing likely involve several wound repair components involved in re-epithelialization, granulation tissue formation, and angiogenesis [17, 18]. Our present data suggest that lymphangiogenesis may also be involved in the LL-37-induced wound healing process. A previous study indicated that skin injury rapidly induced LL-37 expression in granulation tissue, as early as 24 hours after injury; of note increased expression of LL-37 has also been observed on the 5^{th} day following injury [36]. Interestingly, another report indicated that lymphangiogenesis was induced during wound healing in granulation tissue from day 5 onward; however, by day 9, very few lymphatic vessels

remained [37]. Interestingly, these observations suggest that the expression of LL-37 and induction of lymphangiogenesis occur almost simultaneously during wound healing.

LL-37 plays an important role in maintaining a healthy oral environment [14]. However, the precise roles of LL-37 in oral wound healing remain unclear. In the oral cavity, LL-37 is found in the saliva, gingival crevicular fluid, and salivary glands [10-13]. In fact, previous studies reported that the salivary LL-37 concentration in healthy subjects was 0.14 μg/ml, while that in periodontitis patients was 0.225 – 0.33 μg/ml [10, 11]. Furthermore, another study revealed that the concentration of LL37 in the gingival crevicular fluid was 1.4 μg/ml in healthy subjects, 3 μg/ml in periodontal patients, and 4.6 μg/ml in gingivitis patients [12]. Together, these studies suggest that the LL-37 concentration in the oral cavity is increased in the context of inflammation. It is expected that the external administration of LL-37 to the wound site would promote lymphangiogenesis and enhance the wound healing effect. Therefore, LL-37 is emerging as a potential therapeutic. However, some reports indicate that LL-37 did not promote wound healing [38, 39]. Fibrillin is the major structural protein of connective tissue microfibrils [40]. Fibrillin is involved in the regulation of transforming growth factor-β (TGF-β), a family of profibrotic cytokines that has been linked to many fibrotic diseases [40]. Lymphatic endothelial cells produce fibrillin and participate in fibrillin deposition

in human skin [41]. Therefore, excessive lymphatic vessel formation after LL-37 administration may cause fibrosis after wound healing. In the present study, LL-37 induced a lymphangiogenic effect at a concentration of 100 nM (0.458 μg/ml) in lymphatic endothelial cells *in vitro*. Of note, this concentration is about 3 times higher than that in the saliva of healthy subject saliva. However, this concentration is also about one-third of that in the gingival crevicular fluid of healthy subjects. Since in the present study, we only examined the LL-37 lymphangiogenic effect *in vitro*, a detailed study of the onset time and duration of treatment, and the LL-37 dose and route of administration *in vivo*, e.g. in the context of periodontal disease is required to ultimately understand the potential of this antimicrobial peptide as a therapeutic agent for oral disease.

In conclusion, our data demonstrate that the LL-37-induced lymphangiogenesis in lymphatic endothelial cells is mediated by FPRL1, following the activation of the ERK and Akt-dependent signaling pathways. LL-37 may be a potential therapeutic for oral wound healing.

Declarations:

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Figure legends

Fig. 1 Effects of LL-37 on the proliferation, migration, and tube-like formation ability of HDLECs. (a) HDLECs were treated with LL-37 (1–1000 nM) or saline for 24 – 72 h, and then, a WST-1 assay was performed. Results are presented as mean ± S.D. (n=3). *$p < 0.05$. (b) Representative photomicrographs of migrating HDLECs treated with LL-37 (1–1000 nM) or saline for 6 h. Migrating cells were counted in five fields randomly chosen from the triplicated chambers. *$p < 0.05$ *vs*. vehicle. (c) Representative photomicrographs of tube-like formation in HDLECs treated with LL-37 (1–1000 nM) or saline for 24 h. Quantitative analysis of the tube length is shown (n=3). *$p < 0.05$ *vs*. vehicle. (d) Representative photomicrographs of migrating HDLECs treated with the LL-

37 scrambled peptide (100 nM) or saline for 6 h. Migrating cells were counted in five fields randomly chosen from the triplicated chambers. *$p < 0.05$ *vs*. vehicle, $^{\#}p < 0.05$ *vs*. LL-37. (e) Representative photomicrographs of tube-like formation in HDLECs treated with LL-37 scrambled peptide (100 nM) or saline for 24 h. Quantitative analysis of the tube length is shown (n=3). *$p < 0.05$ *vs*. vehicle. $^{\#}p < 0.05$ *vs*. LL-37. *Scale bar*, 50 μm.

Fig. 2 Effects of LL-37 on lymphangiogenic factors' expression in HDLECs. (a) Expression of genes encoding lymphangiogenic factors in HDLECs cultured in growth medium with or without LL-37 (100 nM) for 48 h. mRNA expression was calculated in relation to the expression of GAPDH mRNA and all data are presented as fold change, compared to the respective control conditions (LL-37–). Results are presented as mean ± S.D. (n=3). *$p < 0.05$. (b) Quantitative RT-PCR was performed to evaluate the expression levels of each gene. *$p < 0.05$ *vs*. vehicle. (c) Representative photomicrographs of migrating HDLECs transfected with each siRNA, and treated with LL-37 (100 nM) or saline for 6 h. Migrating cells were counted in five fields randomly chosen from the triplicated chambers. *$p < 0.05$. (d) Representative photomicrographs of tube-like formation in HDLECs transfected with each siRNA, and treated with LL-37 (100 nM) or

saline for 24 h. Quantitative analysis of the tube length is shown (n=3). *$p < 0.05$. *Scale bar*, 50 μm.

Fig. 3 Involvement of ERK and Akt signaling in LL-37-induced migration and tube-like formation in HDLECs (a) Immunoblotting with the indicated antibodies was performed on HDLECs treated with LL-37 (100 nM) at the indicated times. Representative blots are shown. (b) Effects of ERK and Akt inhibitors on LL-37-induced phosphorylation of ERK and Akt proteins. HDLECs were pretreated with SCH772984 (10 μM) or MK-2206 (10 μM) for 1 h, and then treated with LL-37 (100 nM) for 15 min. Relative phosphorylation levels were normalized to the total protein signals. Results are presented as mean ± S.D. (n=3). *$p < 0.05$ *vs*. LL-37(–)/SCH772984 (–)/MK-2206 (–), $^{\#}p < 0.05$ *vs*. LL-37 (+)/SCH772984 (–)/MK-2206 (–). (c) Involvement of ERK and Akt signaling in LL-37-induced HDLECs migration. HDLECs were pretreated with SCH772984 (10 μM) or MK-2206 (10 μM) for 1 h, and then with LL-37 (100 nM) for 6 h. Migrating cells were counted in five fields randomly chosen from the triplicated chambers. Results are presented as mean ± S.D. *$p < 0.05$ *vs*. LL-37(–)/SCH772984 (–)/MK-2206(–), $^{\#}p < 0.05$ *vs*. LL-37(+)/SCH772984(–)/MK-2206(–). (d) Involvement of ERK and Akt signaling in LL-37-induced tube-like formation in HDLECs. HDLECs

were pretreated with SCH772984 (10 μM) or MK-2206 (10 μM) for 1 h, and then treated with LL-37 (100 nM) for 24 h. Quantitative analysis of tube length is shown (n=3). *p < 0.05 *vs*. LL-37(–)/SCH772984 (–)/MK-2206 (–), $^{\#}p$ < 0.05 *vs*. LL-37(+)/SCH772984 (–)/MK-2206 (–). *Scale bar*, 50 μm.

Fig. 4 FPRL1 signaling is involved in LL-37-induced migration and tube-like formation in HDLECs. (a) Immunoblotting with the indicated antibodies was performed on HDLECs. Representative blots are shown. (b) Effects of the FPRL-1 antagonist on LL-37-induced phosphorylation of ERK and Akt proteins. HDLECs were pretreated with WRW4 (10 μM) for 1 h, and then treated with LL-37 (100 nM) for 15 min. Relative phosphorylation levels were normalized to the total protein signals. Results are presented as mean ± S.D. (n=3); *p < 0.05 *vs*. LL-37(–)/WRW4 (–), $^{\#}p$ < 0.05 *vs*. LL-37(+)/WRW4 (–). (c) Involvement of FPRL1 signaling in LL-37-induced HDLECs migration. HDLECs were pretreated with WRW4 (10 μM) for 1 h, and then treated with LL-37 (100 nM) for 6 h. Migrating cells were counted in five fields randomly chosen from the triplicated chambers. Results are presented as mean ± S.D. (n=3); *p < 0.05 *vs*. LL-37(–)/WRW4 (–), $^{\#}p$ < 0.05 *vs*. LL-37(+)/WRW4(–). (d) Involvement of FPRL1 signaling in LL-37-induced tube-like formation in HDLECs. HDLECs were pretreated with WRW4 (10 μM)

for 1 h, and then treated with LL-37 (100 nM) for 24 h. Quantitative analysis of the tube length is shown (n=3). *p < 0.05 *vs*. LL-37(–)/WRW4(–), $^{\#}p$ < 0.05 *vs*. LL-37(+)/WRW4(–). *Scale bar*, 50 μm.

Fig. 5 Knockdown of FPRL1 suppresses LL-37-induced migration and tube-like formation of HDLECs. (a) Quantitative RT-PCR was performed to evaluate the expression levels of FPRL1. *p < 0.05 *vs*. Control. (b) Representative photomicrographs of migrating HDLECs transfected FPRL1 siRNA and treated with LL-37 (100 nM) or saline for 6 h. Migrating cells were counted in five fields randomly chosen from the triplicated chambers. *p < 0.05. (c) Representative photomicrographs of tube-like formation in HDLECs transfected FPRL1 siRNA and then treated with LL-37 (100 nM) or saline for 24 h. Quantitative analysis of the tube length is shown (n=3). *p < 0.05. *Scale bar*, 50 μm.

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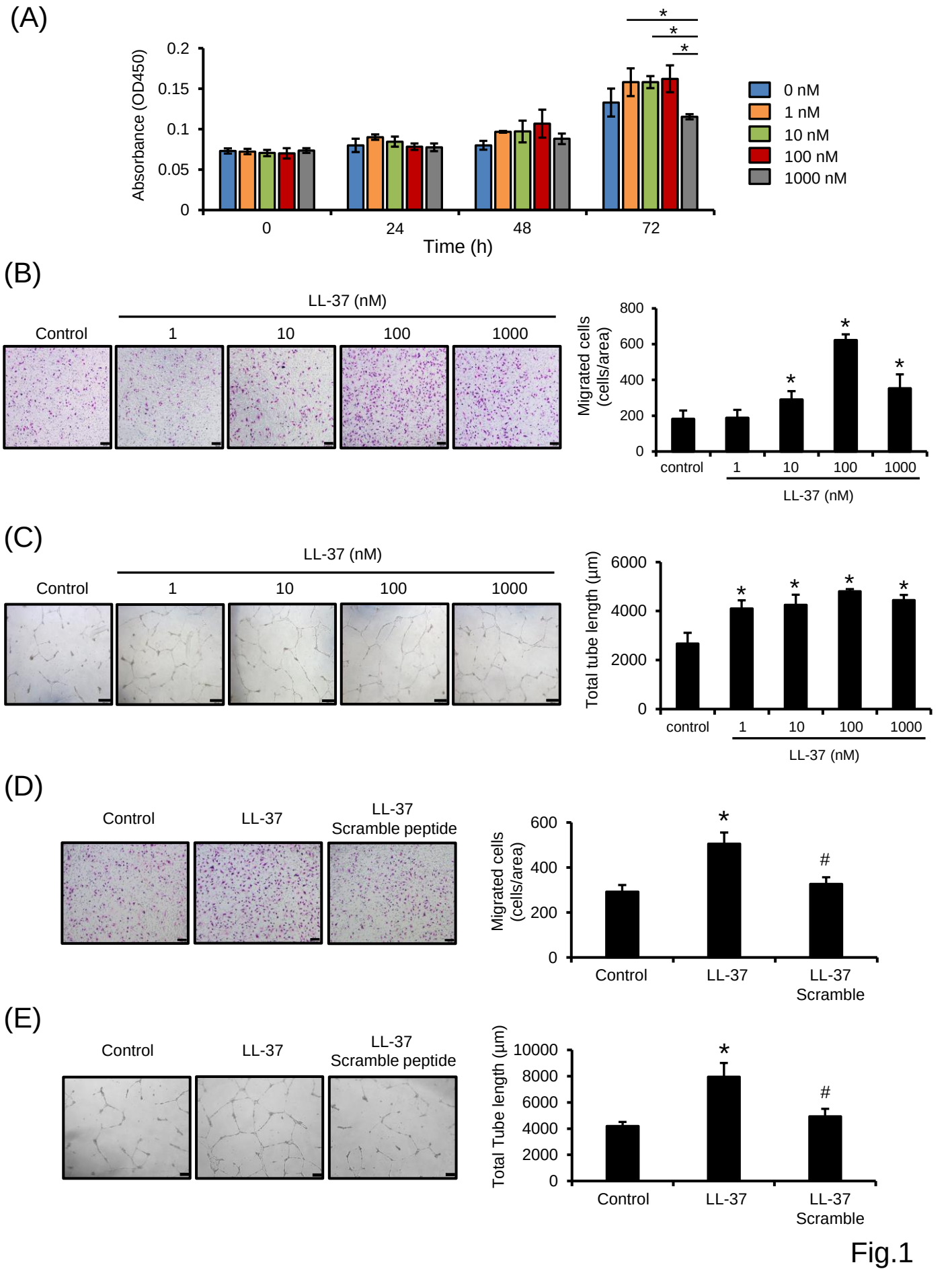

(A)
Absorbance (OD450)
0.2
0.15
0.1
0.05
0
0
24
48
72
Time (h)
*
*
*
0 nM
1 nM
10 nM
100 nM
1000 nM
(B)
LL-37 (nM)
Control
1
10
100
1000
Migrated cells (cells/area)
800
600
400
200
0
control
1
10
100
1000
LL-37 (nM)
(C)
LL-37 (nM)
Control
1
10
100
1000
Total tube length (μm)
6000
4000
2000
0
control
1
10
100
1000
LL-37 (nM)
(D)
Control
LL-37
LL-37 Scramble peptide
Migrated cells (cells/area)
600
400
200
0
Control
LL-37
LL-37 Scramble
(E)
Control
LL-37
LL-37 Scramble peptide
Total Tube length (μm)
10000
8000
6000
4000
2000
0
Control
LL-37
LL-37 Scramble

Fig.1

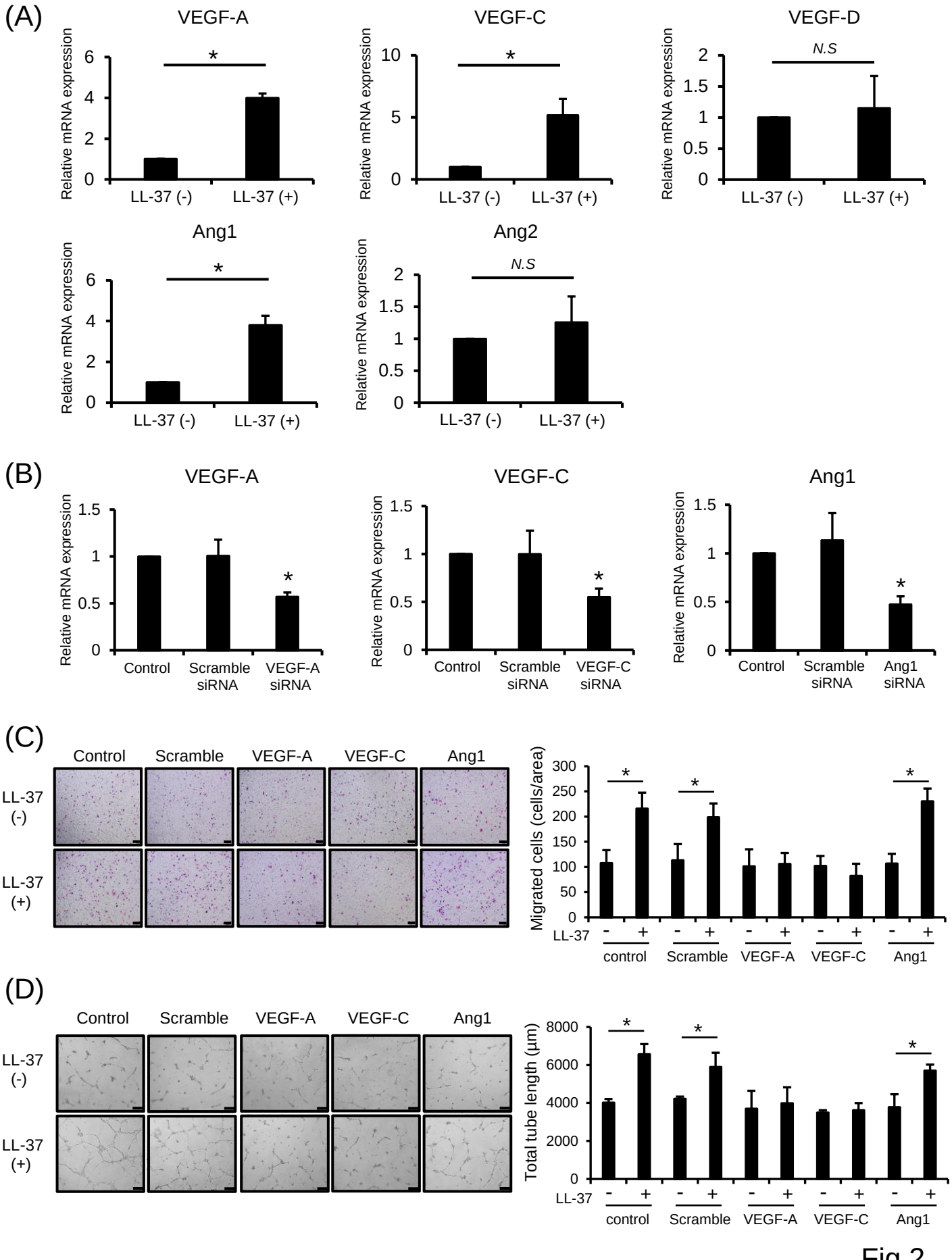

(A)
VEGF-A
VEGF-C
VEGF-D
Ang1
Ang2
Relative mRNA expression
LL-37 (-)
LL-37 (+)
*
N.S
(B)
Control
Scramble siRNA
VEGF-A siRNA
VEGF-C siRNA
Ang1 siRNA
(C)
Control
Scramble
VEGF-A
VEGF-C
Ang1
LL-37 (-)
LL-37 (+)
Migrated cells (cells/area)
control
(D)
Total tube length (μm)

Fig.2

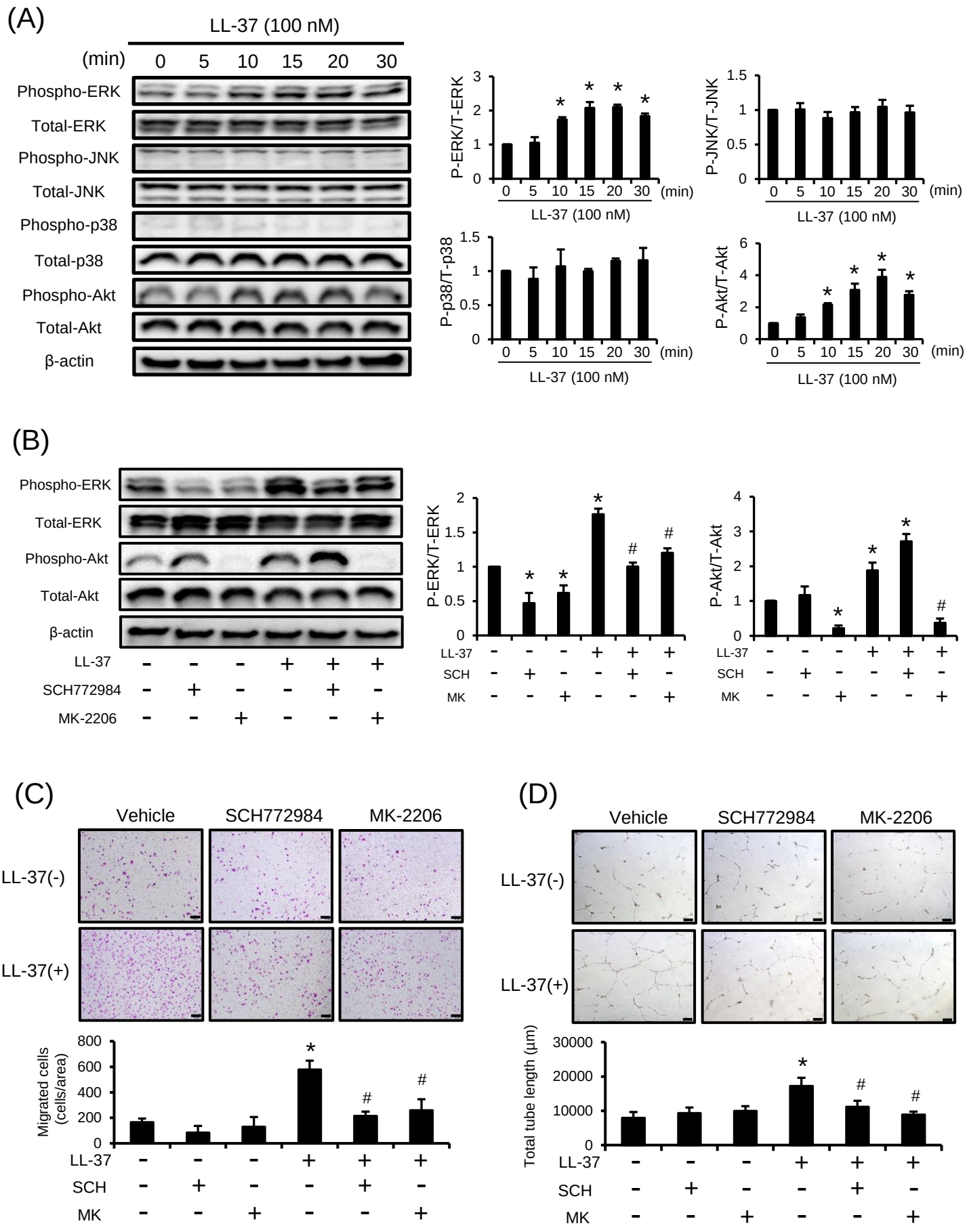

(A)
LL-37 (100 nM)
(min) 0 5 10 15 20 30
Phospho-ERK
Total-ERK
Phospho-JNK
Total-JNK
Phospho-p38
Total-p38
Phospho-Akt
Total-Akt
β-actin
P-ERK/T-ERK
P-JNK/T-JNK
P-p38/T-p38
P-Akt/T-Akt
(min)
LL-37 (100 nM)
(B)
Phospho-ERK
Total-ERK
Phospho-Akt
Total-Akt
β-actin
LL-37
SCH772984
MK-2206
P-ERK/T-ERK
P-Akt/T-Akt
LL-37
SCH
MK
(C)
Vehicle
SCH772984
MK-2206
LL-37(-)
LL-37(+)
Migrated cells (cells/area)
LL-37
SCH
MK
(D)
Vehicle
SCH772984
MK-2206
LL-37(-)
LL-37(+)
Total tube length (μm)
LL-37
SCH
MK

Fig. 3

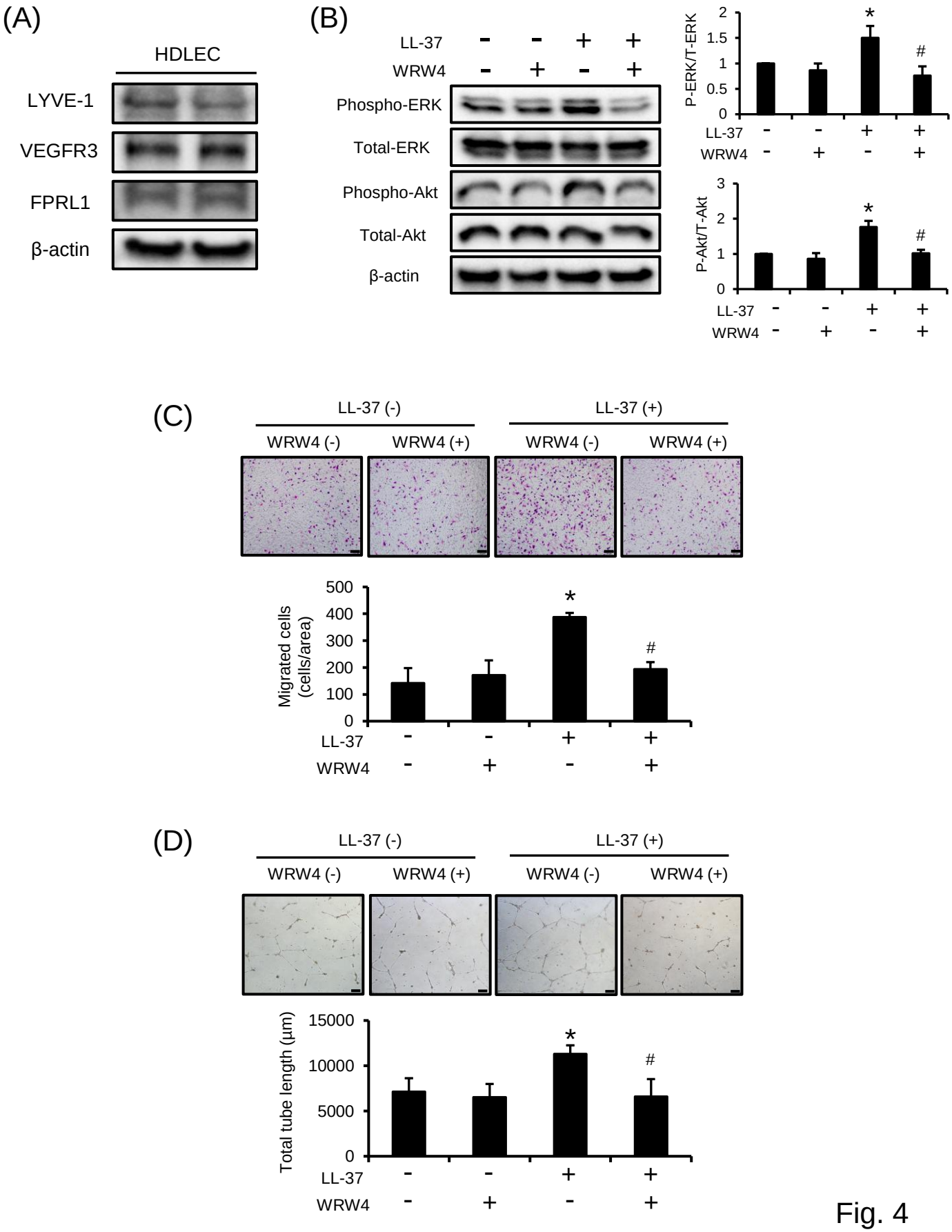
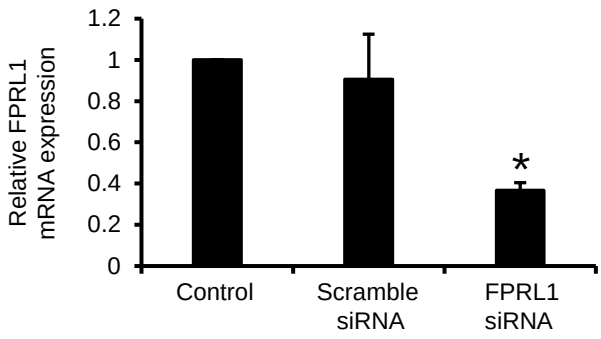
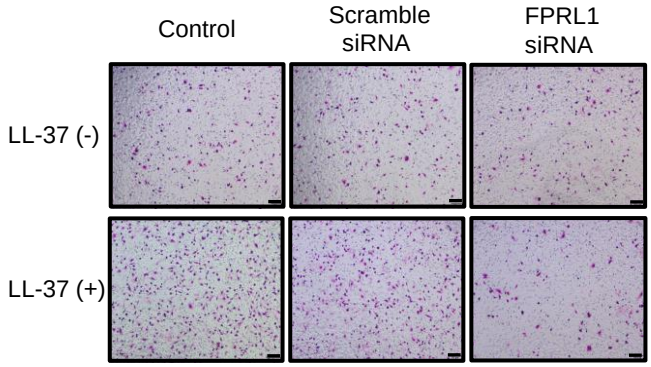

(A)
HDLEC
LYVE-1
VEGFR3
FPRL1
β-actin
(B)
LL-37
WRW4
Phospho-ERK
Total-ERK
Phospho-Akt
Total-Akt
β-actin
P-ERK/T-ERK
P-Akt/T-Akt
(C)
LL-37 (-)
LL-37 (+)
WRW4 (-)
WRW4 (+)
Migrated cells (cells/area)
(D)
Total tube length (μm)

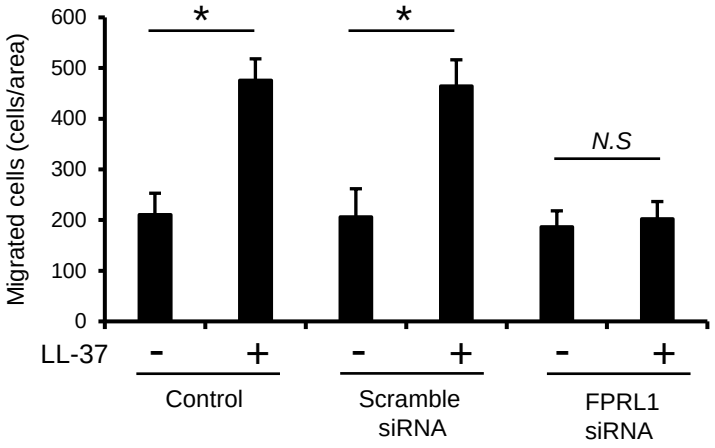
Fig. 4

(A)

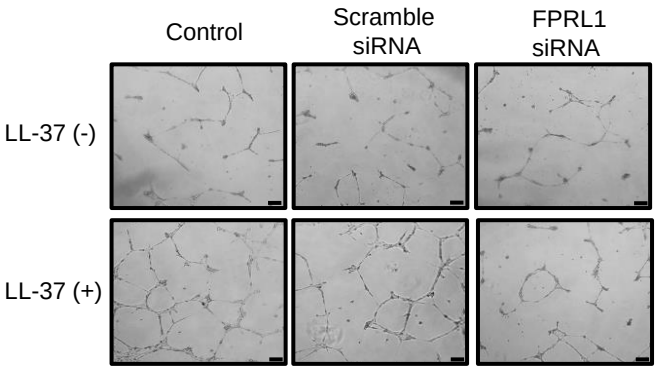

Relative FPRL1 mRNA expression
1.2
1
0.8
0.6
0.4
0.2
0
*
Control
Scramble siRNA
FPRL1 siRNA

(B)


Control
Scramble siRNA
FPRL1 siRNA
LL-37 (-)
LL-37 (+)


Migrated cells (cells/area)
600
500
400
300
200
100
0
*
*
N.S
LL-37
-
+
-
+
-
+
Control
Scramble siRNA
FPRL1 siRNA

(C)


Control
Scramble siRNA
FPRL1 siRNA
LL-37 (-)
LL-37 (+)

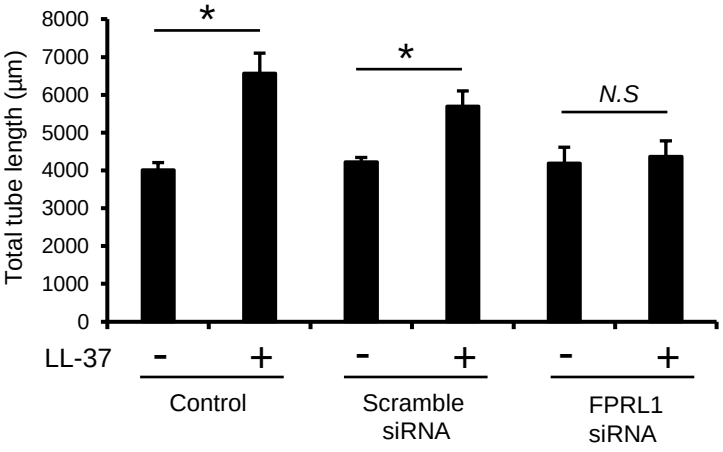

Total tube length (μm)
8000
7000
6000
5000
4000
3000
2000
1000
0
*
*
N.S
LL-37
-
+
-
+
-
+
Control
Scramble siRNA
FPRL1 siRNA

Fig.5

Table 1

Nucleotide sequences of primers used for PCR

Gene		Sequence (5'—3')
VEGF-A	Forward	TTCTGGGCTGTTCTCGCTTC
	Reverse	CTAGCACTTCTCGCGGCTC
VEGF-C	Forward	CCAAAAAGCTACACCGACGC
	Reverse	CCTCACAGGAAACCGGACAT
VEGF-D	Forward	ATCCCATCGGTCCACTAGGT
	Reverse	TGGTACTCTTCCCCAGCTCA
angiopoietin 1 (Ang1)	Forward	TGGCTTGGATGTGCAACCTT
	Reverse	GTGTGACCGTTCAGCATGGA
angiopoietin 2 (Ang2)	Forward	GAACCAGACGGCTGTGATGA
	Reverse	AGGGAGTGTTCCAAGAGCTG
FPRL1	Forward	ACATTTATCATCTCATGGCACAGG
	Reverse	CTCTTCTTCCCATTGGATCGCT
GAPDH	Forward	CGACCACTTTGTCAAGCTCA
	Reverse	AGGGGAGATTCAGTGTGGTG