

# Targeting mTORC1/2 with OSI-027 inhibits proliferation and migration of keloid keratinocytes

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## ABSTRACT

**Keloid is a dermal proliferative disorder characterized by the excessive keratinocyte proliferation and migration. mTOR over-activation is involved in the process. Here, we show that both mTOR complex 1 (mTORC1) and mTORC2 were hyper-activated in keloid-derived primary keratinocytes. OSI-027, an mTOR kinase inhibitor, potently inhibited proliferation and migration of keloid keratinocytes. OSI-027 disrupted the assembly of mTORC1 (mTOR-Raptor) and mTORC2 (mTOR-Rictor-mLST8). Further, OSI-027 completely blocked the phosphorylations of the mTORC1 substrates (S6K1, S6 and 4EBP1) and the mTORC2 substrate (AKT, at Ser-473). OSI-027 was potent than rapamycin in inhibiting keloid keratinocytes. Moreover, restoring mTORC1 activation by the introduction of the constitutively active S6K1 only partly alleviated OSI-027-induced suppression on keloid keratinocytes. Notably, mTOR2 inhibition by Rictor siRNA also inhibited keloid keratinocyte proliferation and migration, although less efficiently than OSI-027. Together, concurrent targeting of mTORC1/2 by OSI-027 potently inhibits keloid keratinocyte proliferation and migration.**

## INTRODUCTION

Keloid is characterized by the formation of exuberant and autonomous scar tissue that extends the clinical border of the original wound [1–3]. It often forms as a result of dermal injuries, and is considered, to a limited extent, as a benign tumor [1–3]. A set of different chemokines, cytokines and growth factors are over-secreted, which stimulate the proliferation and migration of keratinocytes (and other skin cells) to form keloid skin [1–3]. The hyper-activation of the serine/threonine protein kinase mammalian target of rapamycin (mTOR) plays a pivotal role in the process [4–8].

Signaling by mTOR promotes cell proliferation and migration in a positive manner [9, 10]. The active form of mTOR (phospho-mTOR) is over-expressed in keloid [6, 7]. At least two multiple-protein mTOR complexes exist, including the traditional mTOR complex 1

(mTORC1), and the more recently discovered mTOR complex 2 (mTORC2) [9, 10]. Composed of mTOR, Raptor and PRAS40, mTORC1 is rapamycin sensitive [11, 12] and phosphorylates its target proteins, including p70S6K1 (S6K1) and eIF4E-binding protein 1 (4EBP1), to promote cell proliferation and migration [11–13]. The complex of mTORC2 primarily consists of mTOR, Rictor, Sin1 and mLST8 [14, 15] and functions as an AKT kinase, which phosphorylates AKT at Ser-473 [14, 15]. This mTORC2 complex is also important for cell proliferation and migration [16, 17].

Recent studies have characterized OSI-027 as a novel and potent mTOR kinase inhibitor that displays activity against both mTORC1 and mTORC2 [18–20]. In the current study, we found that targeting mTORC1/2 with OSI-027 inhibited the proliferation and migration of keloid keratinocytes, where mTOR was found to be over-activated.

## RESULTS

### Enhanced cell proliferation and migration in keloid keratinocytes

As described, primary human keratinocytes derived from keloid skin and surrounding normal skin were established and cultured. Viable cell counting assay results demonstrated that the keloid keratinocytes grew faster than the normal keratinocytes (Figure 1A). Meanwhile, an MTT OD assay further showed increased proliferation of keloid keratinocytes compared to normal keratinocytes (Figure 1B). Moreover, in keloid keratinocytes, BrdU (Figure 1C) and [ $H^3$ ] DNA incorporation (Figure 1D) were both increased. These results demonstrate the increased cell proliferation of keloid primary keratinocytes compared to normal keratinocytes (Figure 1A–1D).

Migration of the above-mentioned cells was also tested using phagokinetic track motility [4, 21] and transwell assays. The former measured the migration distance of each single keratinocyte [4, 21], while the latter analyzed the number of migrated cells. As compared to normal keratinocytes, both migration distance (Figure 1E, quantified results are shown in the right panel) and the number of migrated cells (Figure 1F) were increased for keloid keratinocytes. Collectively, these results demonstrate enhanced cell proliferation and migration in keloid keratinocytes.

### OSI-027 potently inhibits proliferation and migration of keloid keratinocytes

Figure 1 demonstrates that keloid keratinocytes showed increased cell proliferation and migration. We next analyzed mTOR signaling in keratinocytes. As shown in Figure 2A, phosphorylation (“p-”) of mTORC1 substrates, including S6K1 and 4EBP1 [9, 10, 13], were significantly increased in keloid keratinocytes (compared to normal keratinocytes). Meanwhile, p-AKT (at Ser-473), an indicator of mTORC2 activation [14, 15], was also higher in keloid keratinocytes (Figure 2A). These results highlighted the over-activation of mTORC1 and mTORC2 in keloid keratinocytes (Figure 2A). Notably, the expression of total mTOR substrates, including S6K1, 4EBP1 and AKT1, were similar in keloid and normal keratinocytes (Figure 2A).

OSI-027 is a recently developed mTOR kinase blocker or dual mTORC1/2 inhibitor [18, 20]. Figure 2B–2D demonstrates that treatment with OSI-027 (100 nM, 72 h) significantly attenuated proliferation of keloid keratinocytes, as determined by MTT (Figure 2B), BrdU ELISA (Figure 2C) and [ $H^3$ ] DNA incorporation assays (Figure 2D). Furthermore, the migration of keloid keratinocytes, tested again by phagokinetic track motility (Figure 2E; data are quantified in the right panel) and transwell assays (Figure 2F), was also suppressed by OSI-027 (100 nM, 48 h). Intriguingly,

OSI-027 treatment (100 nM, 72 h) failed to induce the cell death of keloid keratinocytes as tested by propidium iodide (PI) staining assay (Figure 2G). As a positive control,  $H_2O_2$  (300  $\mu$ M) induced significant cell death (Figure 2G). Collectively, these results confirm that OSI-027 inhibits proliferation and migration of keloid keratinocytes, where mTOR is over-activated.

### OSI-027 blocks mTORC1 and mTORC2 activation in keloid keratinocytes

OSI-027 is a mTOR kinase inhibitor. The activation of mTOR in OSI-027-treated keratinocytes was thus tested. Figure 3A demonstrates that treatment with OSI-027 (100 nM, 2 h) disrupted the assembly of both mTORC1 (mTOR–Raptor complex [22]) and mTORC2 (mTOR–Rictor–mLST8 complex [14, 23]) in keloid keratinocytes as determined by Co-IP. In contrast, the expression of mTOR complex proteins, including mTOR, Raptor, Rictor, and mLST8, was unchanged by OSI-027 (Figure 3A, Inputs). Further studies show that OSI-027 (100 nM, 2 h) treatment almost completely blocked the phosphorylation (“p-”) of mTORC1 substrates, including p70S6K1 (Thr-389), S6 (Ser-235/236) and 4EBP1 (Ser-65) [22] (Figure 3B), as well as that of the mTORC2 substrate, p-AKT (at Ser-473) [14, 23] (Figure 3C). The expression of total p70S6K1, S6, 4EBP1 and AKT1 was unchanged, before and after OSI-027 treatment (Figure 3B and 3C). Notably, ERK activation, detected by anti-p-ERK1/2, was unchanged after OSI-027 treatment (Figure 3D). These results suggest that OSI-027 simultaneously blocks mTORC1 and mTORC2 activation in keloid keratinocytes.

### mTORC1 and mTORC2 are both important in proliferation and migration of keloid keratinocytes

Next, constitutively active S6K1 (T389E, “ca-S6K1-flag-puro”) [24] was introduced to keloid keratinocytes. As shown in Figure 4A, the introduction of ca-S6K1 completely restored S6K1 phosphorylation in OSI-027-treated keratinocytes. S6K1 phosphorylation was even higher than the control level (Figure 4A, quantification). The activation of mTORC2, detected by anti-p-AKT at Ser-473, was still blocked after OSI-027 treatment in ca-S6K1-expressing cells (Figure 4A). Intriguingly, inhibition of the OSI-027-induced proliferation (BrdU ELISA assay, Figure 4B) of keloid keratinocytes was only partly inhibited by ca-S6K1. Similarly, Figure 4C demonstrates that ca-S6K1 only partly restored the migration potential of OSI-027-treated cells as determined by a phagokinetic track migration assay. It should be noted that the mTORC1 blocker, rapamycin (also at 100 nM) was less potent than OSI-027 in inhibiting the proliferation and migration of keloid keratinocytes (Figure 4B and 4C). These results imply that the inhibition of mTORC1 less

potently inhibited keloid keratinocytes than OSI-027, suggesting that activation of both mTORC1 and mTORC2 is required in the process.

To further confirm our hypothesis, an siRNA strategy was applied to silence Rictor, which is the key component of mTORC2 [15]. In keloid keratinocytes, the two non-overlapping Rictor siRNAs (“Rictor siRNA I/II”) both induced dramatic knockdown of Rictor (Figure 4D). Consequently, phosphorylation of the mTORC2 substrate, AKT (at Ser-473), but not the mTORC1 substrate, S6K1, was largely inhibited (Figure 4D). Notably, the silencing of Rictor by targeted siRNAs also inhibited proliferation (Figure 4E) and migration (Figure 4F) of keloid keratinocytes. However, Rictor siRNA-mediated inhibition of keloid keratinocytes was less potent than OSI-027 (Figure 4B–4F). These results further support the notion that mTORC1 and mTORC2 are both important for the proliferation and migration of keloid keratinocytes.

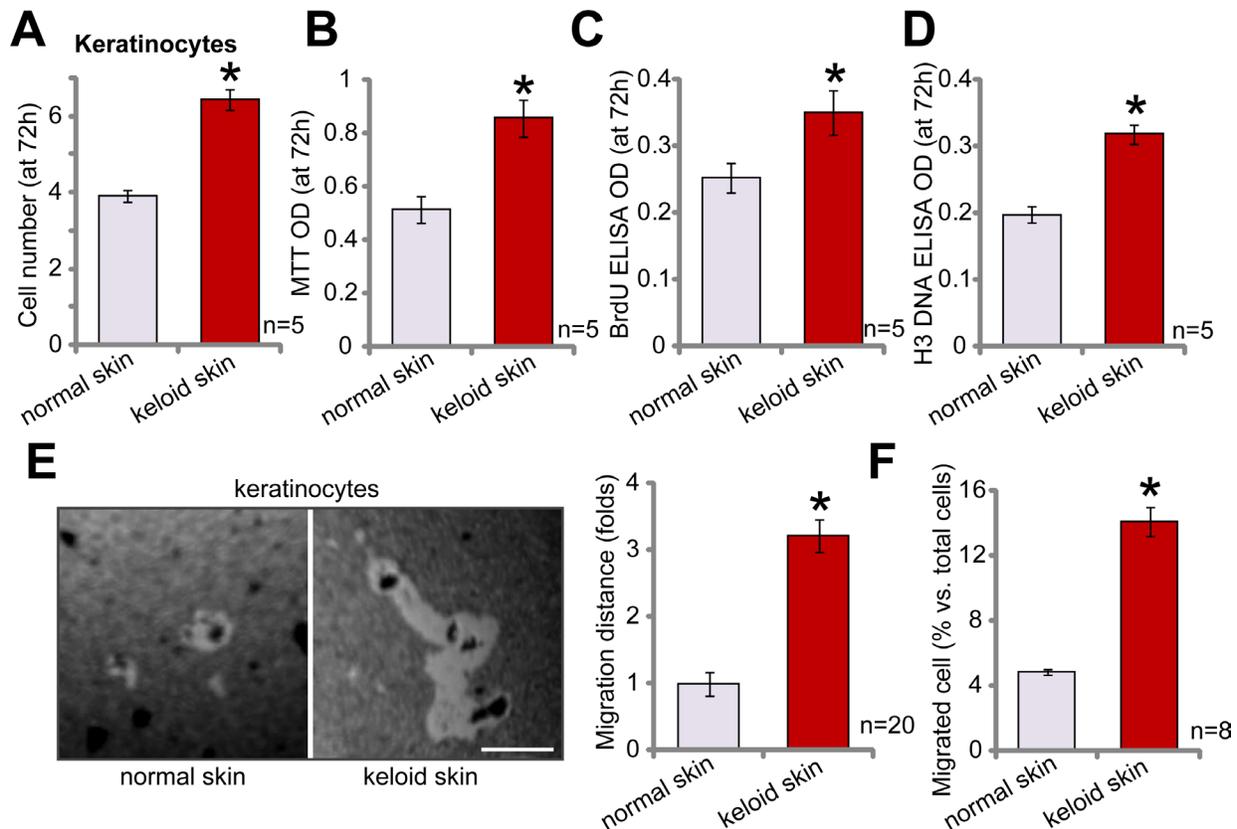
## DISCUSSION

A skin keloid is characterized by the hyper-activity of keratinocytes (and other skin cells) within the wound [1–3].

Recent studies have confirmed mTOR hyperactivation as a potential therapeutic target for keloid [5, 7, 8]. It has been shown that the active form of mTOR (phospho-mTOR) is overexpressed in keloid but not in normal skin [5, 7, 8]. Rapamycin and other mTOR inhibitors were shown to efficiently inhibit keloid keratinocytes [5–7].

Rapamycin binds to FKBP12 binding protein-12 (FKBP12), to form the FKBP12-rapamycin complex that only partially inhibits mTOR kinase activity [25]. There are several limitations when using rapamycin and its analogs (rapalogs) [26–28]: First, rapalogs indirectly inhibit mTOR kinase activity (via binding to FKBP12), causing the inadequate inhibition of mTORC1 [26]. Second, mTORC1 inhibition induces feedback activation of several key mitogenic pathways, including AKT [29, 30] and Erk-MAPK [31]. Third, and most importantly, mTORC1 inhibitors showed no direct effect on mTORC2 activation, the latter promoting cell proliferation and migration [16, 17, 32]. Finally, the bioavailability of rapalogs is often limited [33].

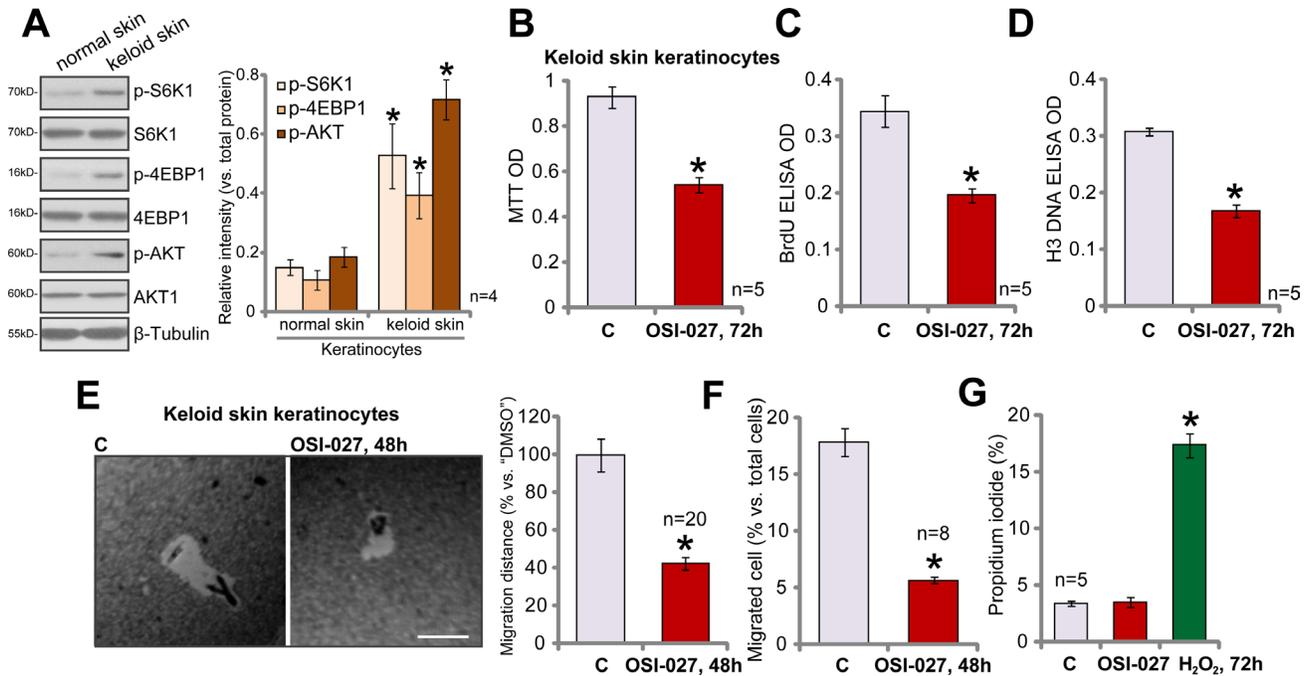
Due to these limitations, direct mTOR kinase inhibitors have recently been developed, termed “second-generation mTOR inhibitors” [26]. OSI-027 is a novel



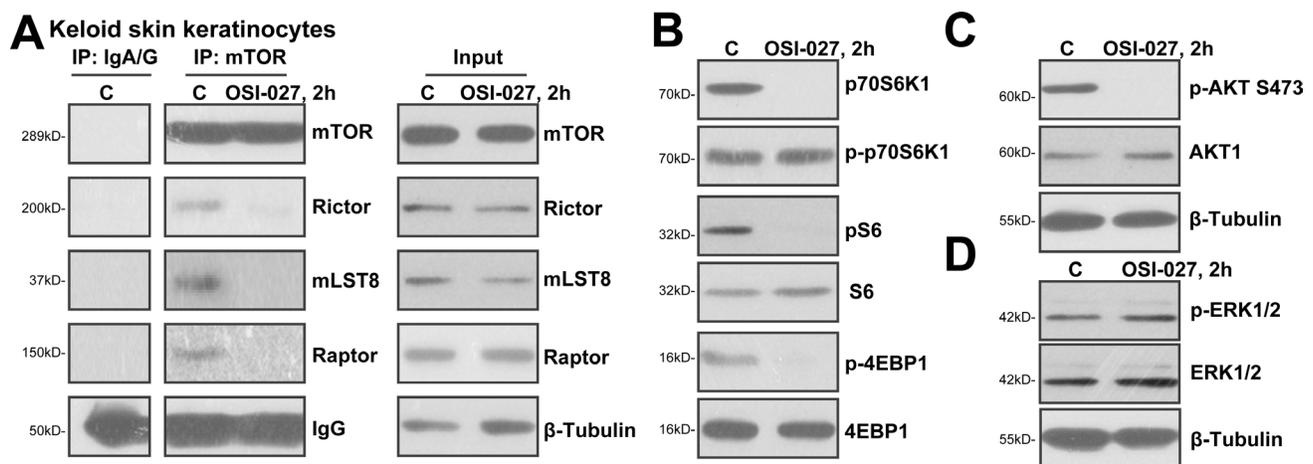
**Figure 1: Enhanced cell proliferation and migration in keloid keratinocytes.** Primary cultured human keratinocytes, derived from keloid and surrounding normal skin, were subjected to cell counting (A), MTT (B), BrdU ELISA (C) and [<sup>3</sup>H] DNA incorporation assays (D) to determine cell proliferation. Cell migration was analyzed using the Phagokinetic track migration assay listed in the text (E and F). For all assays, the same exact number of viable keratinocytes from keloid or surrounding normal skin was initially plated into each well (same for all figures). Data are presented as mean ± standard deviation (SD). \**p* < 0.05 vs. “normal skin” keratinocytes. Bar = 25 μm (E). Experiments were repeated three times, and similar results obtained.

and potent mTOR kinase inhibitor with an extremely low IC-50 (around 10 nM) [18, 20]. Studies demonstrate that OSI-027 does not inhibit PI3K and other protein kinases [18, 20]. In the current study, we showed that OSI-027 disrupted the assembly of mTORC1 (mTOR-Raptor) [14] and mTORC2 (mTOR-Rictor-mLST8) [14, 22] in keloid

keratinocytes. Further, OSI-027 blocked phosphorylation of mTORC1 substrates (S6K1, S6 and 4EBP1) as well as the mTORC2 substrate (AKT at Ser-473) [13]. Unlike rapamycin, OSI-027 treatment did not induce the feedback activation of ERK1/2; It blocked p-AKT at Ser-473. Remarkably, OSI-027 demonstrated significantly more



**Figure 2: OSI-027 potently inhibits proliferation and migration of keloid keratinocytes.** Primary cultured human keratinocytes, derived from keloid and surrounding normal skin, were subjected to a western blotting assay of listed proteins (A, data of four repeated blots were quantified). Primary keloid keratinocytes were treated with OSI-027 (100 nM), vehicle control (0.1% of DMSO, “C”) or H<sub>2</sub>O<sub>2</sub> (300 μM). Cell proliferation (B–D, 72 h), cell migration (E and F, 48 h) and cell death (G, 72 h) were tested by the assays listed in the main text. Data are presented as mean ± standard deviation (SD). \**p* < 0.05 vs. “normal skin” keratinocytes (A) or “C” group (B–G). Bar = 25 μm (E). Experiments were repeated three times, and similar results obtained.



**Figure 3: OSI-027 blocks mTORC1 and mTORC2 activation in keloid keratinocytes.** Cultured primary keloid keratinocytes were treated with vehicle control (0.1% of DMSO, “C”) or OSI-027 (100 nM) for 2 h. Co-immunoprecipitation (Co-IP) was performed to test the association between mTOR and mTOR complex proteins (Rictor, Raptor and mLST8) (A, left panel). The expression of listed proteins was examined by western blot (A, inputs, and B–D). Experiments were repeated three times, and similar results obtained.

effective inhibition of the proliferation and migration of keloid keratinocytes compared with rapamycin.

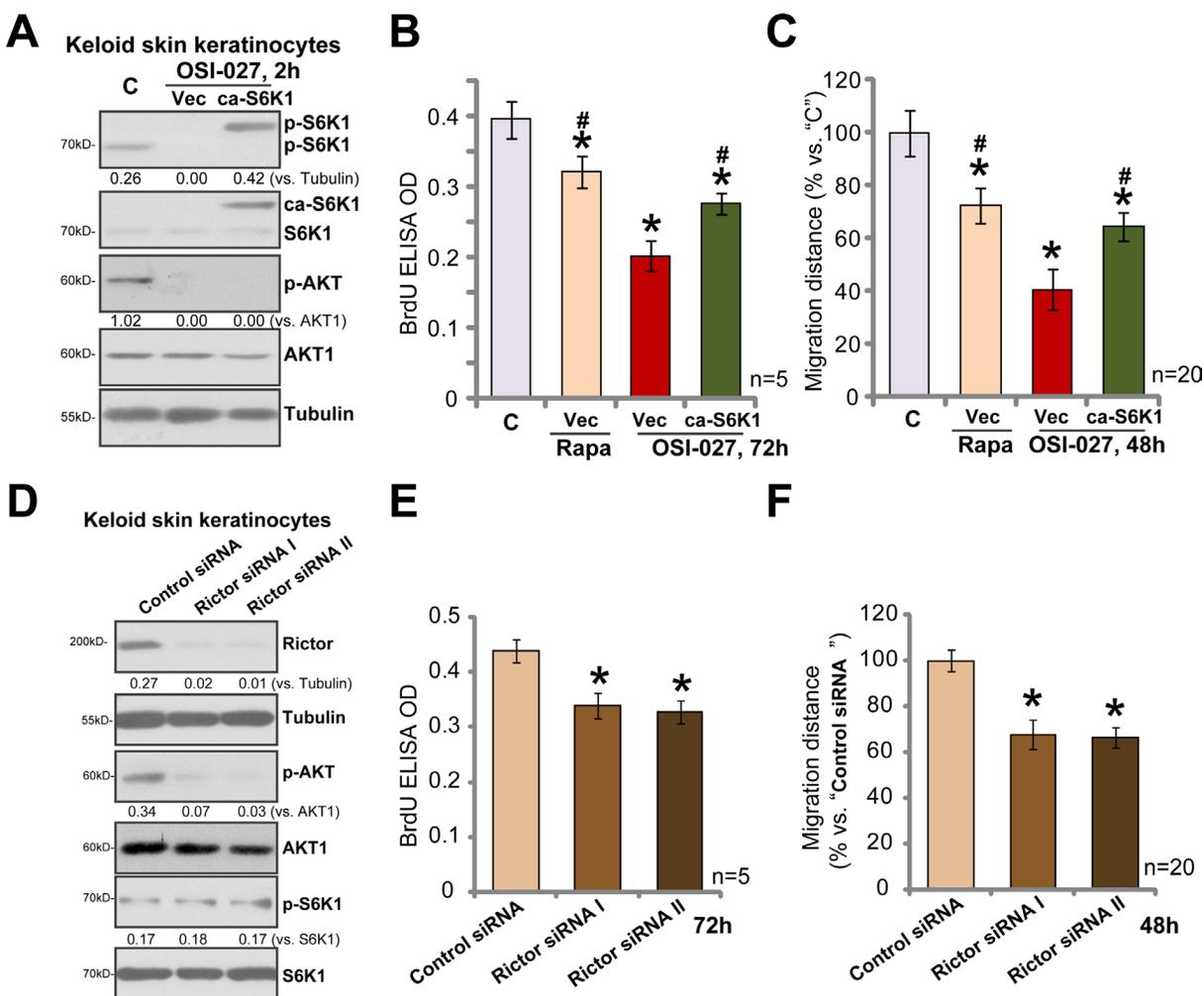
We, here, propose that both mTORC1 and mTORC2 are important in promoting the proliferation and migration of keloid keratinocytes for the following reasons: First, mTORC2, similar to mTORC1, is over-activated in keloid keratinocytes. Second, the mTORC2 substrate AKT (phosphorylation at Ser-473) is known to stimulate cell proliferation and migration [16, 17, 32]. Third, the co-current blockage of mTORC1/2 by OSI-027 was more potent than rapamycin (mTORC1 inhibitor) in inhibiting keloid keratinocytes. Additionally, restoring mTORC1 activation by ca-S6K1 only partly attenuated OSI-027-induced inhibition of keloid keratinocytes. Lastly,

mTOR2 inhibition, by Rictor siRNAs, inhibited keloid keratinocyte proliferation and migration, less efficiently than OSI-027. Therefore, these *in vitro* results imply that targeting both mTORC1 and mTORC2 by OSI-027 may have translational value in treating keloid skin.

## METHODS

### Chemicals, reagents and antibodies

OSI-027 (catalog no. S2624) and rapamycin (catalog no. S1039) were obtained from Selleck LLC (Houston, TX, USA). The  $\beta$ -tubulin antibody (catalog no. T2200) and hydrogen peroxide ( $H_2O_2$ , catalog no. 216763) were



**Figure 4: The importance of mTORC1 and mTORC2 in the proliferation and migration of keloid keratinocytes.** Stable keloid keratinocytes, expressing empty vector ("Vec", pGCL-flag-puro) or the constitutively active S6K1 (T389E, "ca-S6K1"), were treated with 100 nM of OSI-027 or rapamycin ("Rapa") for the times shown. The expression of listed proteins was examined by western blot (A; data were quantified). Cell proliferation (BrdU ELISA assay; B) and migration (phagokinetic track migration assay; data were quantified in C) were examined. Keloid keratinocytes, transfected with 100 nM of Rictor siRNA ("Rictor siRNA I/II") or scrambled control siRNA ("Control siRNA"), were harvested (after 48 h of transfection), and the expression of listed proteins is shown (D; data were quantified). Cells were also subjected to BrdU ELISA proliferation (E) and phagokinetic track migration assays (data were quantified in F). Data were presented as mean  $\pm$  standard deviation (SD). \* $p < 0.05$  vs. vehicle control ("C") (B and C); # $p < 0.05$  vs. OSI-027 treatment in vector cells (B and C). \* $p < 0.05$  vs. "Control siRNA" (E and F). Experiments were repeated three times, and similar results obtained.

purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphorylated ("p")-AKT (Ser 473) antibody (9271), AKT1 antibody (2967), p-p70 S6 kinase 1 (S6K, Thr 398) antibody (9209), S6K antibody (9202), p-4E-BP1 (Ser 65) antibody (9451), 4E-BP1 antibody (9644), p-S6 ribosomal protein (S6, Ser 235/236) antibody (2211), S6 antibody (2317), Rictor antibody (2114), Raptor antibody (2280), mLST8 (GβL) antibody (3274), mTOR antibody (2972), p-p44/42 MAPK (ERK1/2) antibody (9102) and ERK1/2 antibody (9102) were all purchased from Cell Signaling Tech.

### **Culture of primary human keratinocytes**

Epidermal tissues of keloid skin and surrounding normal skin were obtained via biopsy from five informed consent keloid patients (Male, 29/32/35/41/47-year old, administrated at Shanghai Ninth People's Hospital, Shanghai, China). The patients received no prior treatment. The acquired epidermal tissues were minced, washed, and digested by collagenase I (Sigma-Aldrich, catalog no. C0130) [34]. Digestions (2–5) were neutralized, pooled, and filtered. Primary cultures of human keratinocytes were then maintained in Medium 154-CF (Cascade Biologics, M154CF, Portland, OR, USA) supplemented with Human Keratinocyte Growth Supplement plus antibiotics and Ca<sup>2+</sup> (catalog no. S0015) [4]. The medium was provided by Dr. Cao [4]. Skin fibroblasts were abandoned. Protocols using human tissues and cells were conducted according to the principles of the Declaration of Helsinki, and were approved by the Ethics Review Board (ERB) of all authors' institutions. Written, informed consents were obtained from the participants.

### **Cell proliferation assay**

Primary human keratinocytes were seeded into 96-well tissue culture plates (2 × 10<sup>4</sup> cells/well). A routine 3-(4,5-dimethyl-thiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich, catalog no. M2128) dye assay was performed to test cell proliferation. The MTT optical density (OD) value at 490 nm was recorded.

### **BrdU DNA incorporation assay**

Primary human keratinocytes were seeded into 48-well tissue culture plates (5 × 10<sup>4</sup> cells/well) and cultured for 72 h. A 5-bromo-2'-deoxyuridine (BrdU) dye was then added for an additional 16 h. BrdU incorporation into DNA was tested via a BrdU ELISA colorimetric assay (Roche, Shanghai, China, catalog no. 11647229001) according to the manufacturer's protocol. The ELISA OD value at 450 nm was recorded.

### **[H<sup>3</sup>] Thymidine DNA incorporation assay**

Primary human keratinocytes were seeded into 48-well plates (5 × 10<sup>4</sup> cells/well) and cultured for 72 h in

the presence of 1 μCi/mL of tritiated thymidine (Sigma-Aldrich). To determine [H<sup>3</sup>] thymidine incorporation, cells were washed with cold PBS, and DNA precipitated and solubilized as described previously [4]. DNA aliquots were counted and quantified by liquid-scintillation spectrometry, and the [H<sup>3</sup>] thymidine DNA OD recorded.

### **Phagokinetic track migration assay**

A detailed protocol has been previously described [21, 35, 36]. Briefly, 12-well tissue culture plates were coated with fibronectin (10 μg/mL, Sigma-Aldrich, catalog no. 10838039001), and wells then washed with PBS. Afterwards, a microsphere (Sigma-Aldrich, catalog no. NIST1965) suspension was then added to each well. The plates were then centrifuged at 500 × g at 4°C for 20 min. After the supernatant was removed, and freshly trypsinized keratinocytes (1000 cells per well of each treatment) seeded for 48 h, cell migration was observed and photographed under a light microscope.

### **Transwell assay**

The transwell chamber with 12 μm pore filters (Corning, 3403, Shanghai, China) was first pre-coated with 1 mg/mL Matrigel (BD Biosciences, catalog no. 354230, Suzhou, China). Afterwards, keratinocytes (3 × 10<sup>4</sup> cells per chamber) in serum-free medium were plated into the upper chamber. Complete medium (with fetal bovine serum [FBS]) was added to the lower chamber. After 48 h of incubation, cells on the lower surface of the membrane were fixed, stained and then counted in five random fields [37]. Mitomycin (1.5 μg/mL; Sigma-Aldrich) was always added to exclude the influence of cell proliferation.

### **Propidium iodide assay for cell death**

After treatment, keratinocytes were washed with ice-cold PBS, and re-suspended in 70% alcohol, followed by the addition of DNase-free RNase and propidium iodide (PI, Sigma-Aldrich, catalog no. P4170). The suspension was filtered and subjected to analysis by flow cytometer (FACSCalibur; BD Biosciences). The PI percentage was recorded.

### **Western blotting assay**

Keratinocytes were lysed via radioimmunoprecipitation assay (RIPA) lysis buffer (Biyuntian, Wuxi, China). Thirty μg of protein lysate per treatment was separated by a SDS-polyacrylamide gel electrophoresis (PAGE) gel, which was then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, catalog no. IPVH00010, Shanghai, China). After blocking in 5% of milk, the blot was incubated with indicated primary and corresponding secondary antibodies. Enhanced chemiluminescence (ECL) reagents (Amersham Biosciences, Shanghai, China) were applied to

detect antibody–antigen binding. The total gray of each band was quantified via Image J software (NIH, Bethesda, MD). Each lane was loaded with the same amount of quantified protein lysate. The same set of lysate samples were run in sister gels to test different proteins.

### Co-immunoprecipitation

The co-immunoprecipitation (Co-IP) assay protocol has been described previously [38]. Briefly, for each treatment, 800 µg protein lysates were pre-cleared with protein A/G beads (Sigma, catalog no. 11719416001) and then incubated with anti-mTOR antibody (0.2 µg), overnight. Protein A/G beads (30 µL per treatment, Sigma) were added afterwards to the lysates again for another 4 h. The beads were then washed with PBS five times, and one time with cell lysis buffer. The integrity of the mTOR complex was then detected by western blotting assay.

### Constitutively active S6K1 construct and transfection

A constitutively active S6K1 (T389E, “ca-S6K1-flag-puro”) and empty vector (pGCL-flag-puro) were gifts from Dr. Chen [24]. A ca-S6K1 construct was transfected into keratinocytes using Lipofectamine 2000 reagent (Invitrogen, catalog no. 11668027, Shanghai, China). Transfected keratinocytes were then selected by puromycin (2.0 µg/mL, Sigma) for 4 days. The expression of “ca-S6K1” in stable cells was verified by western blotting assay.

### Rictor siRNA

Keloid keratinocytes were seeded into a six-well tissue culture plate at  $1 \times 10^5$  cells per well, grown for 24 h, and transfected with Rictor siRNA I (Cell Signaling Technology, #8649), Rictor siRNA II (Cell Signaling Tech, #8622), or scramble control siRNA (Cell Signaling Technology, #6568), at a final concentration of 100 nM using Lipofectamine 2000 reagent (Invitrogen). To examine siRNA efficiency, cells were harvested 48 h after siRNA transfection, and the silencing of Rictor in transfected cells was confirmed by western blot assay.

### Statistics

Data were expressed as mean  $\pm$  standard deviation (SD). The differential significance was calculated using ANOVA (SPSS 16.0). A *p* value of less than 0.05 was considered statistically significant.

### Author contributions

All authors carried out the experiments, participated in the design of the study and performed the statistical analysis, participated in its design and coordination and helped to draft the manuscript.

### CONFLICTS OF INTEREST

The listed authors have no conflicts of interests.

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