APPLICATION NOTE

EVOS FL Auto 2 Imaging System

Effect of phosphoinositide 3-kinase (PI3-kinase) inhibition on wound healing and cell proliferation

Introduction

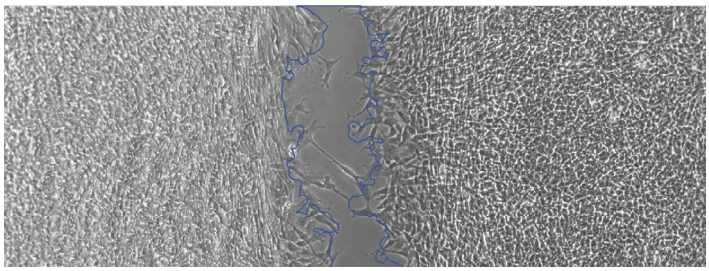
Cell proliferation and migration are two essential parts of normal wound healing. Both of these cellular functions are regulated by PI3-kinases. Cell proliferation is regulated by PI3-kinases through their activation of a G-protein, Rac GTPase, which activates AKT, causing a series of additional interactions. These interactions cause a range of downstream effects, including activation of mTOR, downregulation of apoptosis, and upregulation of the cell cycle, collectively resulting in increased proliferation [1,2].

While the role of PI3-kinases in cell proliferation is well understood, their role in cell motility is not as clearly defined. However, it is known that RAS binds directly to PI3-kinase to increase cell motility. Preventing RAS from binding to PI3-kinase results in upregulation of E-cadherin expression due to an increase in expression of the protein reelin. This increase in focal adhesion protein decreases cell motility and migration [3]. Additional data have shown that PI3-kinase regulates actin through regulation of the protein cofilin [4]. Thus, PI3-kinases are capable of regulating cell motility as well as cell proliferation.

Here we demonstrate the use of cell-tracking reagents, proliferation assays, and live-cell kinetic assays to qualitatively and quantitatively analyze the effects of PI3-kinase inhibition on human dermal fibroblasts and epidermal keratinocytes during wound healing and cell proliferation.

Materials

- Invitrogen™ EVOS™ FL Auto 2 Imaging System (Cat. No. AMAFD2000)
- Invitrogen™ EVOS™ Onstage Incubator (Cat. No. AMC1000)
- Invitrogen™ EVOS™ 20x Objective (Cat. No. AMEP4624)
- Invitrogen™ EVOS™ 10x Objective (Cat. No. AMEP4623)
- Invitrogen™ EVOS™ Cy®5 Filter Cube (Cat. No. AMEP4656)



- Invitrogen™ EVOS™ DAPI Filter Cube (Cat. No. AMEP4650)
- Invitrogen™ EVOS™ GFP Filter Cube (Cat. No. AMEP4651)
- Invitrogen™ Celleste™ Image Analysis Software (Cat. No. AMEP4816)
- Invitrogen™ Click-iT™ Plus EdU Alexa Fluor™ 488 Imaging Kit (Cat. No. C10637)
- Invitrogen™ NucBlue™ Live ReadyProbes™ Reagent (Cat. No. R37605)
- Invitrogen™ CellTracker™ Deep Red Dye (Cat. No. C34565)
- Gibco™ Human Dermal Fibroblasts, neonatal (HDFn) (Cat. No. C-004-5C)
- Gibco™ Medium 106 (Cat. No. M-106-500)
- Gibco™ Low Serum Growth Supplement (LSGS) (Cat. No. S-003-10)
- Gibco™ Human Epidermal Keratinocytes, neonatal (HEKn) (Cat. No. C-001-5C)
- Gibco™ EpiLife™ Medium, with 60 µM calcium (Cat. No. MEPI500CA)
- Gibco™ Human Keratinocyte Growth Supplement (HKGS) (Cat. No. S-001-5)

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Methods

Wound healing time-lapse imaging

Neonatal human dermal fibroblasts were plated in a 35 mm MatTek™ dish and allowed to grow to confluency. The cells were then labeled with 1 µM CellTracker Deep Red Dye for 30 minutes in serum-free medium with 5% CO_2 at 37°C. Cells were washed once with serum-free medium before being placed back in serum-containing medium. Cells were scratch wounded using a pipette tip and placed on the EVOS Onstage Incubator with 80% humidity and 5% CO_2 at 37°C. The EVOS FL Auto 2 Imaging System was then set to automated imaging, acquiring an image every hour for 25 hours using autofocus in the transmitted-light channel to minimize phototoxicity.

Wound healing assay

Neonatal human dermal fibroblasts or neonatal human epidermal keratinocytes were plated in a 12-well plate and allowed to grow to confluency. The cells were then labeled with 1 µM CellTracker Deep Red Dye for 30 minutes in serum-free medium with 5% CO_2 at 37°C. Cells were washed once with serum-free medium before being placed back in serum-containing medium. Each well was scratch wounded using a pipette tip. Four wells of dermal fibroblasts per condition were treated with vehicle (DMSO), 25 nM wortmannin, or 250 nM wortmannin. Four wells of epidermal keratinocytes per condition were treated with vehicle (DMSO), 50 nM LY294002, or 500 nM LY294002. Cells were placed on the EVOS Onstage Incubator with 80% humidity and 5% CO_2 at 37°C. The EVOS FL Auto 2 Imaging System was set to automated imaging, scanning 30% of each well starting from the center of each well, using autofocus to find the focal plane. Images were acquired every 8 hours for either 24 or 32 hours. The scratch wounds were analyzed using the wound healing app in Celleste Image Analysis Software. For each wound, a region of interest was drawn around the entire wound at time 0 and the area was quantified. This same region of interest was then applied to all following time points and the remaining wound area was quantified. The region of interest was then moved to an unwounded area of the scan to establish a baseline for the area in between cells. When the area of the wound reached the baseline, it was considered healed.

Proliferation assay

Neonatal human dermal fibroblasts or neonatal human epidermal keratinocytes were plated in a 96-well plate at a density of 3,500 cells per well. The next day, six wells of dermal fibroblasts per condition were treated with vehicle (DMSO), 25 nM wortmannin, or 250 nM wortmannin. Six wells of epidermal keratinocytes per condition were treated with vehicle (DMSO), 50 nM LY294002, or 500 nM LY294002. Both dermal fibroblasts and epidermal keratinocytes were cultured overnight in normal cell culture conditions. The next day, proliferation of each population was measured using the Click-iT Plus EdU Alexa Fluor 488 Imaging Kit following the procedure listed in the product information sheet. Proliferation rates were quantified using Celleste Image Analysis Software.

Results

EVOS FL Auto 2 Imaging System effectively measures wound healing

To test the ability of the EVOS FL Auto 2 system to image wound healing, human dermal fibroblasts were plated, allowed to grow to confluency, and scratch wounded with a pipette tip. The cells were then imaged every hour for 25 hours using the EVOS FL Auto 2 system. Cells imaged in this manner demonstrated a normal rate of wound healing over a 25-hour period (Figure 1).

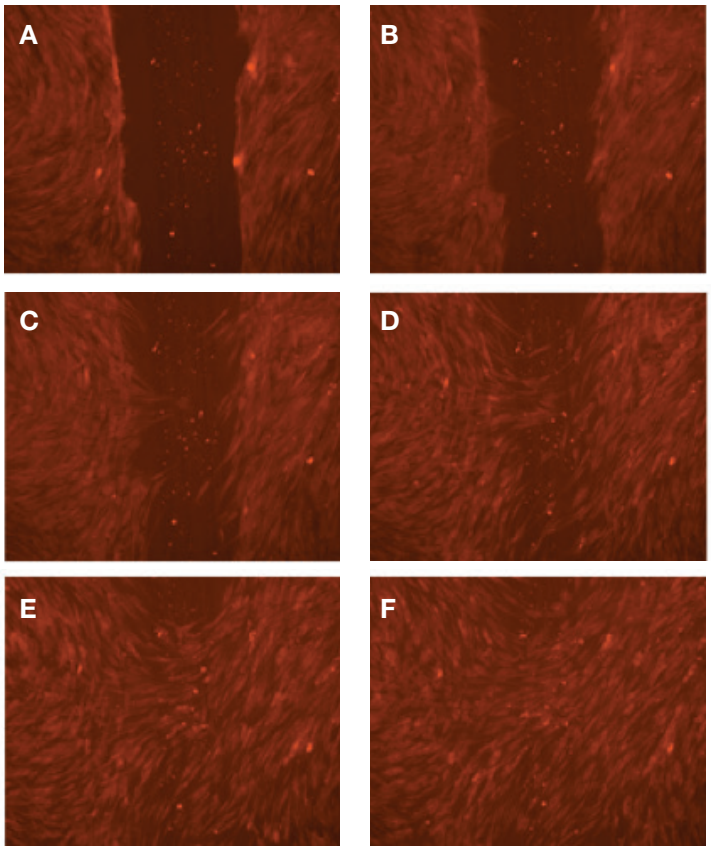

Figure 1. Time-lapse imaging of human dermal fibroblasts—scratch wound healing. Images taken at **(A)** 0 hr, **(B)** 5 hr, **(C)** 10 hr, **(D)** 15 hr, **(E)** 20 hr, and **(F)** 25 hr of dermal fibroblast wound healing. Cells show a normal rate of wound healing over a 25-hour period.

PI3-kinase inhibition results in slower wound healing

The effect of PI3-kinase inhibition on wound healing was tested using both human dermal fibroblasts and epidermal keratinocytes. PI3-kinase activity was inhibited in human dermal cells using the covalent inhibitor wortmannin. Cells treated with wortmannin or a DMSO vehicle control were imaged every 8 hours for either 24 or 32 hours using the EVOS FL Auto 2 system. Imaged cells visually demonstrated a slower rate of wound healing when treated with wortmannin than cells treated with the vehicle alone (Figure 2A–I). The images were then quantified for percent wound area over the 24-or 32-hour time course using the wound healing app included in Celleste Image Analysis Software. Quantification of percent wound area shows a clear, dose-dependent decrease in growth rate when PI3-kinase is inhibited by wortmannin (Figure 2J).

To show that PI3-kinase inhibition resulting in a slower rate of wound healing is not cell line or inhibitor specific, the human epidermal keratinocytes wound healing rate was tested in the presence of the PI3-kinase inhibitor LY94002 or a vehicle control (DMSO). Quantification of human epidermal keratinocytes again showed a dramatic, dose-dependent decrease in growth rate when PI3-kinase was inhibited by LY294002 (Figure 2K).

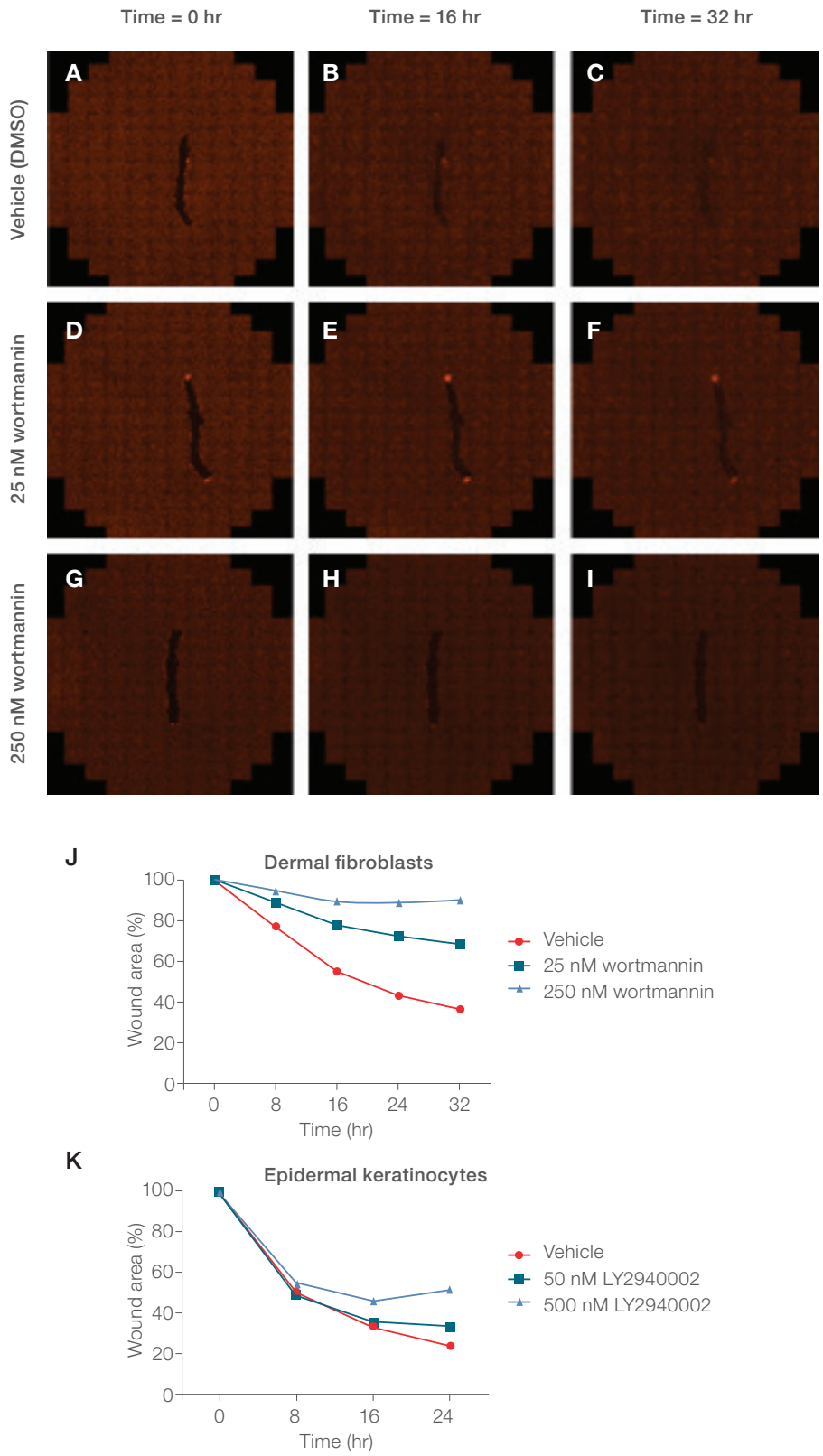

Figure 2. Effects of PI3-kinase inhibition on scratch wound healing. Time-lapse imaging of entire scratch wounds in dermal fibroblasts qualitatively shows that wounds imposed on cultures treated with either **(D–F)** 25 nM or **(G–I)** 250 nM wortmannin heal slower than wounds treated with **(A–C)** vehicle (DMSO) only. Quantitative analysis shows that **(J)** dermal fibroblasts and **(K)** epidermal keratinocytes treated with the PI3-kinase inhibitors wortmannin or LY294002 heal wounds more slowly in a dose-dependent manner, compared to cells with the control treatment.

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PI3-kinase inhibition results in decreased proliferation

The effect of PI3-kinase inhibition on proliferation was determined for both human dermal fibroblasts and epidermal keratinocytes by treating both cell types with a vehicle control (DMSO) or a PI3-kinase inhibitor (wortmannin or LY24900). The cells were then visually analyzed using the Click-iT Plus EdU Alexa Fluor 488 Imaging Kit (Figure 3A, B). Cell images were then quantified using Celleste Image Analysis Software. Both human dermal fibroblasts and epidermal keratinocytes demonstrated a decrease in cell proliferation when PI3-kinase inhibitors were present (Figure 3C, D).

Conclusions

Inhibition of PI3-kinases decreases both cell migration and proliferation in human dermal fibroblast and epidermal keratinocyte scratch wound models. These effects were easily visualized and quantified using the EVOS FL Auto 2 Imaging System and Celleste Image Analysis Software.

References

1. Rafalski VA, Brunet A (2011) Energy metabolism in adult neural stem cell fate. *Prog Neurobiol* 93(2):182–203.
2. Peltier J, O'Neill A, Schaffer DV (2007) PI3K/Akt and CREB Regulate adult neural hippocampal progenitor proliferation and differentiation. *Dev Neurobiol* 67(10):1348–1361
3. Castellano E, Molina-Arcas M, Krygowsaka AA, et al. (2016) RAS signalling through PI3-kinase controls cell migration via modulation of reelin expression. *Nat Commun* 7:1–13
4. Kolsch V, Charest PG, Firtel RA (2008) The regulation of cell motility and chemotaxis by phospholipid signaling. *J Cell Sc* 121:551–559

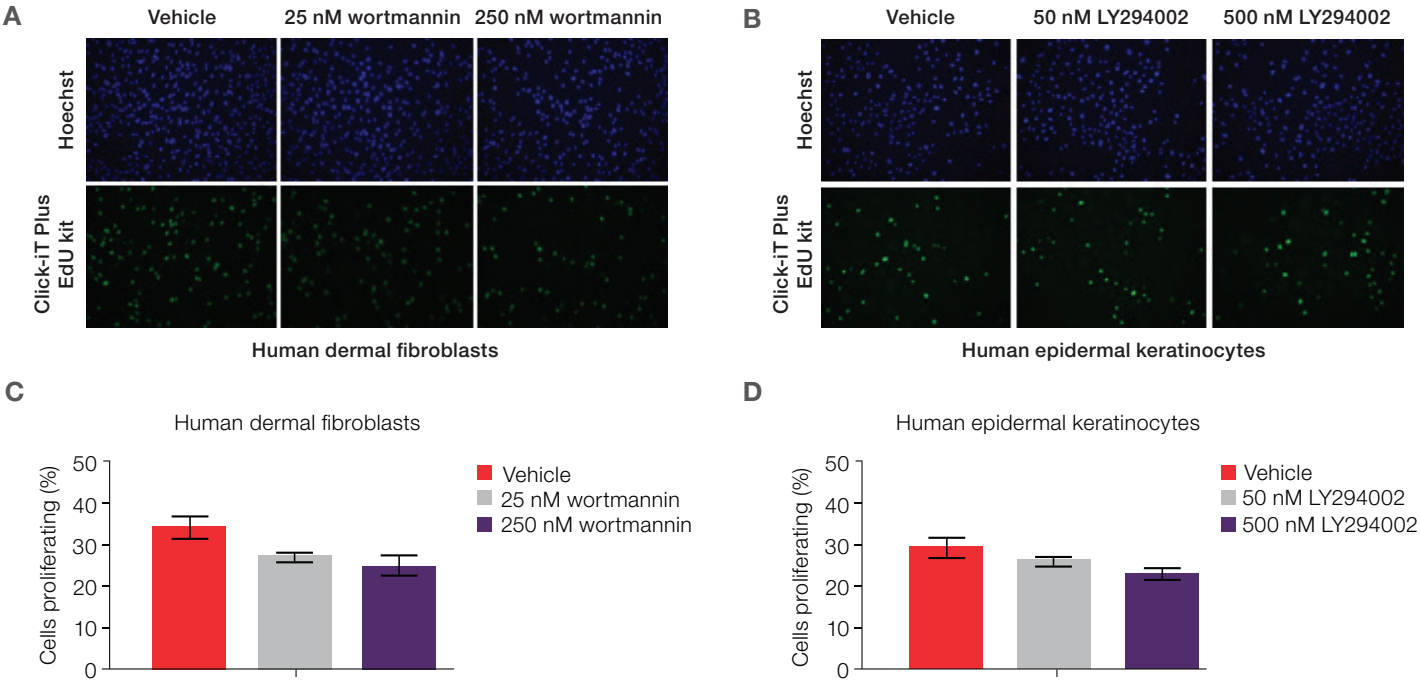

Figure 3. Effects of PI3-kinase inhibition on proliferation. Human dermal fibroblasts were treated with wortmannin, and human epidermal keratinocytes were treated with LY294002. Cell proliferation was **(A, B)** visualized using the Click-iT Plus EdU Alexa Fluor 488 Imaging Kit, and **(C, D)** quantified using Celleste Image Analysis Software. Both cell types showed a decrease in proliferation in the presence of PI3-kinase inhibitors. Significance for dermal fibroblasts: vehicle vs. 25 nM wortmannin, $p<0.05$; vehicle vs. 250 nM wortmannin, $p<0.001$; 25 nM wortmannin vs. 250 nM wortmannin, not significant (NS). Significance for epidermal keratinocytes: vehicle vs. 50 nM LY294002, NS; vehicle vs. 500 nM LY294002, $p<0.001$; 50 nM LY294002 vs. 500 nM LY294002, NS. N = 3 for all conditions.

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