Application Note

Establishing a time course for translocation of FOXO4 in live cells using a novel microfluidic culture platform assay

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Introduction

Forkhead box O (FOXO) belongs to the large Forkhead transcription factor family that were first found to be altered in several types of cancers such as alveolar rhabdomyosarcomas and mixed-lineage leukemias[1,2]. These transcription factors are insulin-sensitive, and can interact with an array of downstream targets and partners that are involved in the regulation of the PI3K-Akt pathway[3-5]. In brief, FOXOs can be regulated in two ways[6]. Activation of the PI3K-Akt pathway results in FOXO translocation from the nucleus to cytosol, where the transcription factor is removed from its targeted genes, such as GADD45 and p27, which are regulators that promote survival and direct the cells to proliferate[5,6]. Upon deactivation of the PI3K-Akt pathway on removal of the growth factor or by chemical inhibition with the fungal steroid metabolite wortmannin, FOXOs return to the nucleus and bind to their target genes, leading to cell cycle arrest and cell death (Figure 1)[5,7]. To date, there are four different FOXO members identified in mammals: FOXO1, FOXO3, FOXO4 and FOXO6[8,9]. Although the post-transcriptional regulation and the shuttling mechanism of FOXOs may be well understood (see review[6,10]), little is known about the dynamics of the translocation machinery. Here, we exploited FOXO4-GFP U2OS reporter cells as a model to extract time course information for FOXO4 protein translocation in the cells upon wortmannin treatment.

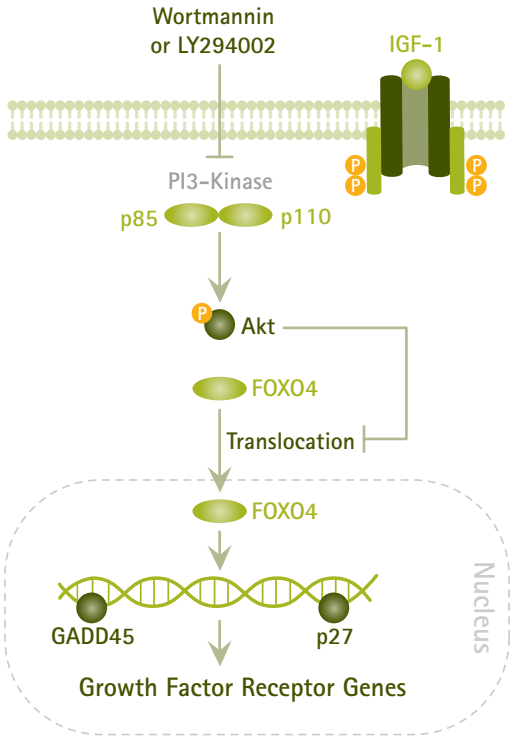

Figure 1.

The fungal metabolite wortmannin is a potent and cell-permeable inhibitor of the FOXO forkhead box transcription factor proteins. Upon treatment with wortmannin, FOXO4 translocates to the nucleus, where it binds target growth factor receptor genes such as GADD45 and p27, which leads to cell cycle arrest and cell death.

In addition to employing FOXO4-GFP reporter cells, we used a microfluidic live cell imaging platform, the CellASIC® ONIX system with M04S switching plates for mammalian cultures, to develop a dynamic cell-based assay for monitoring the translocation process in its entirety. This platform offers temperature and gas control as well as media perfusion for precise environmental control within the associated culture chamber. Using this system, FOXO4-GFP U2OS cells were subjected to wortmannin treatment in the assay buffer for a predetermined time period followed by cell recovery induced by re-introduction of the wash buffer, and FOXO4 protein translocation was visualized at regular intervals by fluorescent microscope. Using this technique, we observed that FOXO4-GFP fusion protein relocated to the nucleus within one hour of administration of wortmannin. In addition, upon removal of the wortmannin treatment, FOXO4-GFP quickly translocated from nucleus to cytosol within one hour.

Combining live cell analysis, fluorescently-tagged reporter cells, and the unique microenvironmental control capabilities of the CellASIC® ONIX system, we were able to establish the first time course studies for translocation of FOXO proteins following deactivation of the PI3K-Akt pathway. The assay method described here provides quantitative information on both FOXO4 nuclear import and export, and thus provides a platform for the discovery of new targets and therapeutic compounds in cancer as well as other diseases.

Materials and Methods

Cell culture, reagents, and live cell analysis systems

Recombinant U2OS cells that are stably expressing human FOXO4 fused to the N-terminus of enhanced green fluorescent protein were purchased from Thermo Scientific, and were used according to the manual. In brief, U2OS cells were maintained in DMEM containing 2 mM L-glutamine, 10% heat-inactivated FBS (Cat. No. ES-009-B), 100 Units/mL penicillin, 100 µg/mL streptomycin (Cat. No. TMS-AB2-C), and 500 µg/mL Geneticin (G418, Life Technologies), under 5% CO_2 at 37°C for up to 10 passages after cells thawed from the cryopreserved vial. The translocation assay was carried out using the assay buffer (DMEM supplemented with 2 mM L-glutamine, 5 mM HEPES, 100 Units/mL penicillin, and 100 µg/mL streptomycin) and the wash buffer (DMEM supplemented with 2 mM L-glutamine, 5 mM HEPES, 100 Units/mL penicillin, 100 µg/mL streptomycin, and 1.4% FBS). The wortmannin used to induce the FOXO4 translocation was purchased from Merck Millipore (Cat. No. 681676). Live cell analysis was performed using the CellASIC® ONIX system equipped with an Merck Millipore Tri-gas Mixer (Cat. No. GM230), and Merck Millipore Microincubator Controller (Cat. No. MIC230).

To seed the cells, the cell suspension was prepared according to the manual at the density of 2 x 10^6/mL, allowing close cell-cell contact. Before introducing the cells into the microfluidic device, the liquid in wells 1, 6, 7, and 8 was first aspirated. The inner ring in wells 6 and 7 was then carefully aspirated. 10 µL of cell suspension was added into the inner ring of well 6; the plate was placed in a laminar flow cell culture hood for 30 minutes to allow cells to load into the microchambers by capillary action. The plate was subsequently placed inside a standard CO_2 incubator; to promote cell attachment, gravity-driven perfusion was used to slowly deliver 300 µL of culture medium to the microchamber. For prolonged culture in the incubator, medium was aspirated in well 1 and 7 every 48 hours and 300 µL of the culture medium was added into well 1 to re-establish gravity-driven perfusion. The medium was replenished every 2 days.

Live cell analysis for dynamic starvation-induced autophagy assay

PBS was pipetted into Wells 1, 2, 3, 6, 7, and 8, but the liquid in the inner ports was not disturbed. 300 µL of the wash buffer were added to wells 2 and 5, while 300 µL of the desired treatments (150 nM of wortmannin or the vehicle in the assay buffer) were added into wells 3 and 4. The plate was affixed to the heater manifold and microincubator MIC230 as described in the CellASIC® manual. The flow program was set using the CellASIC® ONIX FG software for the following parameters for the single-time stimulation experiment:
V2 and V5, 1 Psi, 7 minutes;
V3 and V4, 1 Psi, 60 minutes;
V2 and V5, 1 Psi, 90 minutes

Live cell imaging and image analysis

An Olympus IX-71 inverted microscope was used for live cell imaging. All images were taken with the 40X objective. The fluorescence intensity of the nucleus for each image was determined using a custom developed image processing sequence for manual object identification in CellProfiler software (version 2.1.0 Broad Institute). The analysis module was programmed to perform the following steps: Identify objects manually, measure object intensity, track objects, export to spreadsheet, save images, and lastly export to database. In brief, we identified the nuclear areas for each image manually, and total of eight nuclei per treatment group were measured by applying a fixed threshold. Lastly, all fluorescence intensity numbers were normalized to the intensity of nuclei at time zero.

Results and Discussion

To investigate the FOXO4 translocation process, we first treated the cells with 150 nM wortmannin in the assay buffer for one hour, followed by the administration of the recovery buffer, for another hour. By fluorescence microscopy on live cells in real time, we closely monitored the dynamic changes of the fluorescence intensity in the nucleus during the treatment and recovery phase that were later quantified using CellProfiler software. As expected, FOXO4-GFP signal relocated to the nuclei within one hour of the wortmannin treatment, and was exported out of the nuclei within an hour after the removal of the inhibitor (Figure 2). The changes in fluorescence intensity in the nuclei were also quantified and plotted in Figure 3.

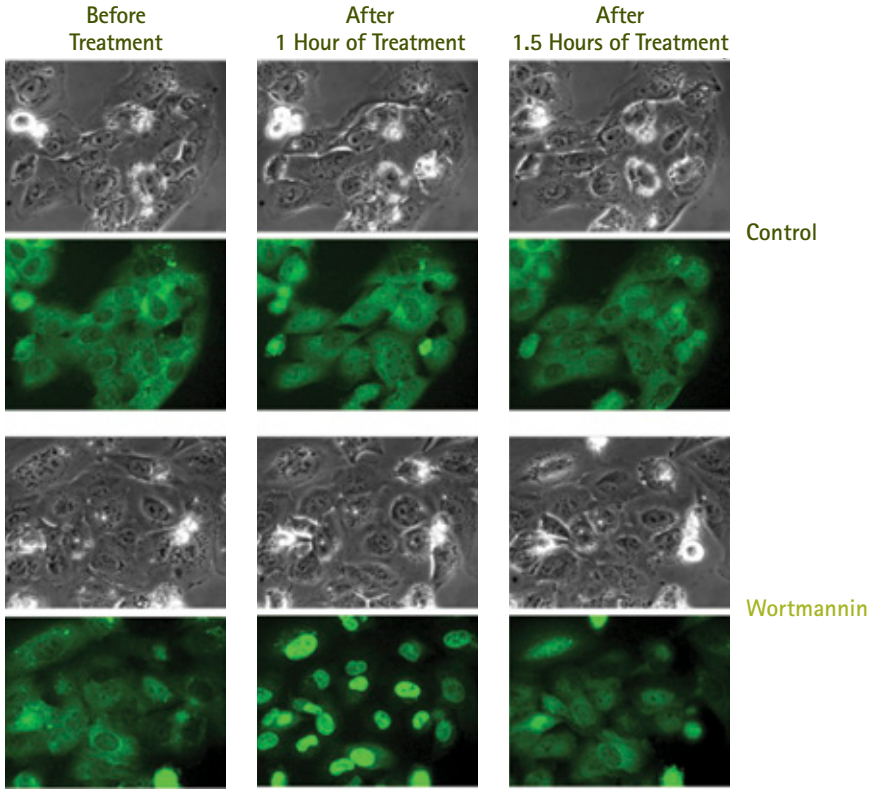

Figure 2.

Live cell images of FOXO4 translocation in FOXO4-GFP U2OS reporter cells. FOXO4-GFP U2OS reporter cells were cultured with a programmable device capable of exchanging reagents automatically. Wash buffer was perfused to establish the fluorescence baseline. Vehicle control (0.5% DMSO in assay buffer) and 150 nM wortmannin were then introduced to inhibit the PI3K pathway, leading to FOXO4 protein translocation to the nucleus. The wortmannin treatment was then withdrawn and cells were allowed to recover in wash buffer. during which FOXO4 was observed to relocate to the cytoplasm.

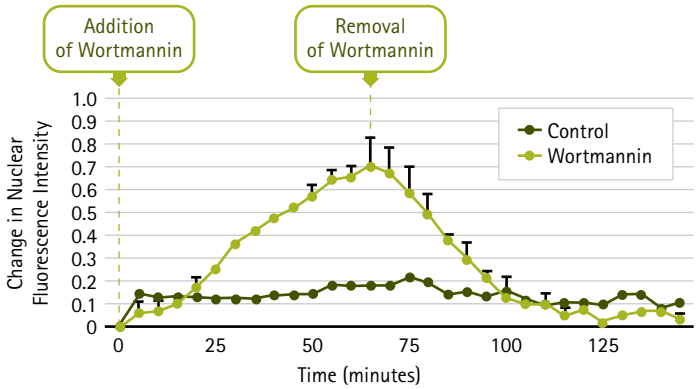

Figure 3.

Changes of the fluorescence intensity in the tracked nuclei upon treatments. The fluorescence intensity of GFP in the nuclei in Figure 2 was determined using a custom developed image processing sequence for object identification (Cell Profiler, version 2.1.0 Broad Institute). All fluorescence intensity numbers were normalized to the numbers at time zero (T0). Error bars represent S.D. of the fluorescence intensity in a total of 8 cells per time point.

Conclusions

With use of microfluidic live cell analysis, we were able to instantaneously monitor the intracellular relocation of FOXO4 in reporter cells in response to 150 nM wortmannin treatment. The CellASIC® ONIX system not only enabled observation of the temporal features of translocation in real time, but also dissected the dynamics of the FOXO translocation process at the single cell level. By performing live cell analysis using the CellASIC® ONIX microfluidic platform, we created a dynamic assay that not only has the potential to simultaneously monitor multiple intracellular components throughout the entire protein translocation process without disruption, but also allows the researcher to precisely manipulate culture parameters (media flow, inducer/inhibitor concentration, gas content) and discover translocation mechanisms that the end-point assays cannot offer. Consequently, this platform may be capable of simulating conditions of pulse exposure to drug compounds, and could potentially provide novel and vital information for compound profiling by enabling quantification of the rate of protein translocation.

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