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REAL-TIME FLUORESCENCE IMAGING OF SIRT1 CYTOSOLIC TRANSLOCATION UNDER THE TREATMENT OF GROWTH FACTOR DEPRIVATION

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Sirtuins comprise a family of enzymes implicated in the determination of organismal lifespan in yeast and the nematode. Human sirtuin SIRT1 has been shown to deacetylate several proteins in a NAD^+-dependent manner. It is reported that SIRT1 regulates physiological processes including senescence, fat metabolism, glucose homeostasis, apoptosis, and neurodegeneration. In general, SIRT1 has initially been thought to represent an exclusive nuclear protein. However, depending on the cell lines and organisms examined, a partial or temporary cytoplasmic localization was observed in murine pancreatic beta cells and neonatal rat cardiomyocytes. Since SIRT1 deacetylates both histone and nonhistone-proteins, such as a number of transcription factors, changes in subcellular localization probably play a role in the regulation of its function. In the present studies, we investigated the subcellular localization of SIRT1 in response to growth factor deprivation in African green monkey SV40-transformed kidney fibroblast cells (COS-7). Using SIRT1-EGFP fluorescence reporter, we found that SIRT1 localized to nucleus in physiological conditions. We devised a model enabling cell senescence via growth factor deprivation and found that SIRT1 partially translocated to cytosol under the treatment, suggesting a reduced level of SIRT1 activity. We found PI3K/Akt pathway was involved in the inhibition of SIRT1's cytosolic translocation, because inhibition of these kinases significantly decreased the amount of SIRT1 maintained in nucleus. Taken together, we demonstrate that growth factor deprivation induces cytosolic translocation of SIRT1, which suggests a possible connection between cytoplasm-localized SIRT1 and the aging process and provides a new application of single molecule fluorescence imaging of the molecule events in living cells.

Keywords: Senescence; growth factor starvation; SIRT1.

1. Introduction

The silent information regulator 2 (Sir2) proteins are a family of class III histone deacetylases found in organisms from bacteria to humans.[1] Unlike class I and II histone deacetylases, the catalytic activity of Sir2 family depends on the cofactor NAD^+ and is not sensitive to the broad deacetylase inhibitor trichostatin (TSA).[2] Sir2 extends the lifespan of yeast by suppressing recombination in the rDNA region and consequently reducing the formation of extrachromosomal rDNA circles,[3] which is a cause of senescence.[4] There are seven members of the Sir2 family in mammals, of which the protein SIRT1 has the highest sequence similarity to Sir2.[5] SIRT1

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deacetylates a large number of substrates, including p53, Ku70, NF-κB, and forkhead proteins to affect stress resistance in cells[6–9] conferred by CR. SIRT1 also regulates the activities of the nuclear receptor PPAR-γ and PGC-α to influence differentiation of muscle cells, adipogenesis, fat storage in white adipose tissue, and metabolism in the liver.[10–12] SIRT1 is mostly considered to a nuclear protein. Previous studies have reported that it is localized exclusively to the nucleus in male germ cells and in COS-7 cells.[13,14] On the other hand, the cytoplasmic localization of SIRT1 has also been reported in murine pancreatic β cells and neonatal rat cardiomyocytes.[15,16] Dynamic changes in the subcellular localization of *Drosophila* Sir2 during embryonic development have also been reported[17]; hence, it may be a nucleocytoplasmic shuttling protein.[18] However, conclusive evidence about the relationship between the localization of SIRT1 and aging is still unknown.

Because SIRT1 deacetylates histones and various transcription factors in the nucleus, its subcellular localization must affect its function. In this study, we found that SIRT1 translocated to cytoplasm in COS-7 cells treated with growth factor deprivation, a model enabling cell senescence,[19] and inhibition of PI3K activity also significantly decreased the amount of SIRT1 maintained in nuclei. Thus, we speculated that SIRT1 is maintained in nuclei by PI3K/Akt pathway. In the present study, we monitored in real-time the process of cytosolic translocation of SIRT1 induced by growth factor deprivation using confocal microscopy.

2. Methodology

2.1. *Cell culture and treatment*

COS-7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum, 50 units/mL penicillin, and 50 units/mL streptomycin on 22-mm culture glasses in 5% CO_2, at 37°C in a humidified incubator. In all experiments, 85% confluent cultures were used. After transfected with SIRT1-EGFP plasmid for 48 h, COS-7 cells were supplemented with 0.5% serum to imitate growth factor deprivation condition.

2.2. *Chemicals and plasmids*

The SIRT1-EGFP was provided by Prof. Yoshiyuki Horio.[18] To observe the distribution of SIRT1 in real time, COS-7 cells growing at 50~60% confluences were transfected with the SIRT1-EGFP plasmid. Transient transfection was performed with Lipofectamine™ 2000 Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Cells transfected with SIRT1-EGFP were utilized to show SIRT1 protein expression. Transfection efficiency was approximately 60% as determined using fluorescent protein, and maximal levels of protein expression were observed between 24 and 48 h.

2.3. *Laser scanning microscope (LSM510 META)*

Live cell imaging experiments were performed on laser scanning microscopes (LSM510 META), combination system (Zeiss, Jena, Germany) equipped with a Zeiss Plan-Neofluar 40×/1.3 NA Oil Dic objective. The stage of LSM was equipped with a temperature-controlled and CO_2-controlled small incubator (CTI-controller 3700 digital and Tempcontrol 37−2 digital, Zeiss), which maintains the cells at 37°C and 5% CO_2 during the whole experiment. GFP was excited with 488 nm Ar-Ion laser, and fluorescence emission was detected through a 500−550 nm band-pass filter, respectively. During the experiments, the exciting power of 488-nm laser was reduced to the minimal level (0.5−1%) to reduce the possible effects of exciting light. After background subtraction, the average fluorescence intensity per pixel was calculated.

2.4. *SIRT1-EGFP shuttling analysis*

The COS-7 cells transfected with SIRT1-EGFP were incubated for 24 h, in DMEM supplemented with 10% fetal bovine serum, and then treated with as follows: (1) growth factor deprivation for 24 h; (2) 10 μM wortmannin for 24 h. At the selected time points, the images were obtained by confocal microscopy.

2.5. *Quantification of apoptosis by nuclear staining*

Cells under different treatments were stained with 1 μg/ml Hoechst 33258 (blue) to visualize nuclear morphology and distinguish apoptotic cells from normal ones. Uniformly blue-stained nuclei were

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scored as healthy, viable, normal cells, whereas condensed or fragmented nuclei that stained blue were counted as apoptotic. Fluorescence images of the normal and apoptotic cells were examined with a modified commercial microscope system equipped with a mercury lamp (band-pass filter: 352−461 nm), a 395-nm dichroic mirror and a long-pass 397-nm emission filter (LSM510/ConfoCor2, Zeiss, Jena, Germany). The fluorescence images were collected via a Zeiss C-Apochromat objective (40 ×, NA = 1.3).

2.6. *Assessment of apoptosis by flow cytometric analysis based on Annexin V/PI*

For assessment of apoptosis, both floating and adherent cells under different treatments were collected and analyzed. Cells grown on 32-mm culture plastic dish were treated with growth factor deprivation and then incubated for about 7 or 12 h. At the end of the incubation, cells were harvested and washed twice with phosphate-buffered saline (PBS). After centrifugation at 1,500 g for 5 min at room temperature, the cells were stained with Annexin V and propidium iodide (PI, 1 μg/mL). The cell apoptosis distributions were determined on a BD FACSCantoTM II flow cytometer.

2.7. *Statistical analysis*

All data represent at least three independent experiments and are expressed as the mean ±SEM of the mean.

3. Results

3.1. *SIRT1 predominantly locates to nuclear under physiological conditions in COS-7 cells*

To know the subcellular location of SIRT1, we transfected plasmid SIRT1-EGFP, a fluorescence reporter, into cells and analyzed the subcellular distribution of fluorescent-labeled SIRT1 by confocal microscopy. As shown in Fig. 1, we found that SIRT1 predominately maintained in the nuclei in COS-7 cells under physiological conditions, which was consistent with previous study.[13]

3.2. *SIRT1 translocates to cytoplasm under the treatment of growth factor deprivation*

It is well known that overexpression of Sir2 and its orthologs can extend organismal lifespan in a wide range of lower eukaryotes.[3] It is also reported that SIRT1 protein is partially cleaved in apoptotic cells by caspase-1, -3, -8 and its level decreased in aging cells.[19] These studies suggest that SIRT1 may translocate to cytoplasm and then be cleaved or degraded in aging cells. Therefore, we devised a model enabling cell senescence via growth factor deprivation[20] and exclude the possibility of changes of cell nucleus under the growth factor deprivation via nuclear staining and flow cytometric analysis. As indicated in Fig. 2, we found that SIRT1 partially translocated to the cytosol after growth factor deprivation for 7 h. This may be due to a reduction

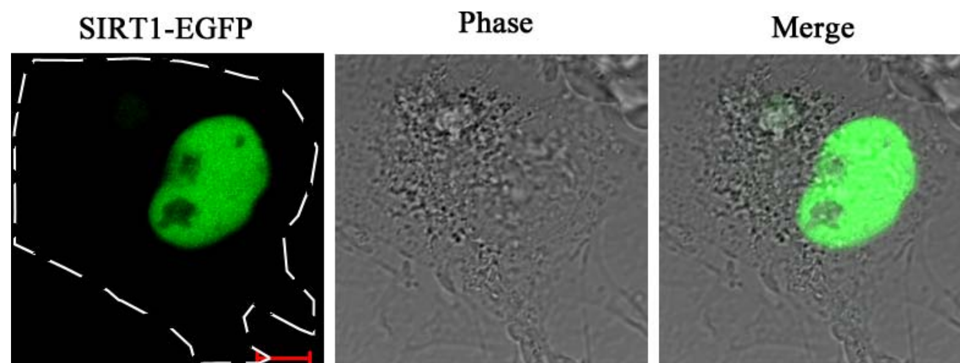

Fig. 1. Subcellular localization of SIRT1-EGFP in COS-7 cells. COS-7 cells were transiently transfected with a SIRT1-EGFP plasmid. After transfected for 48 h, the subcellular localization of the SIRT1-EGFP (green) was monitored by confocal microscopy. The following specific settings were used for light excitation and emissions: excited at 488 nm with an argon ion laser and emission was recorded through a 500−550 nm band-pass filter. With controlled experiments, it was confirmed that the bleaching of the probe was negligible for protocols. Bar for 10 μm.

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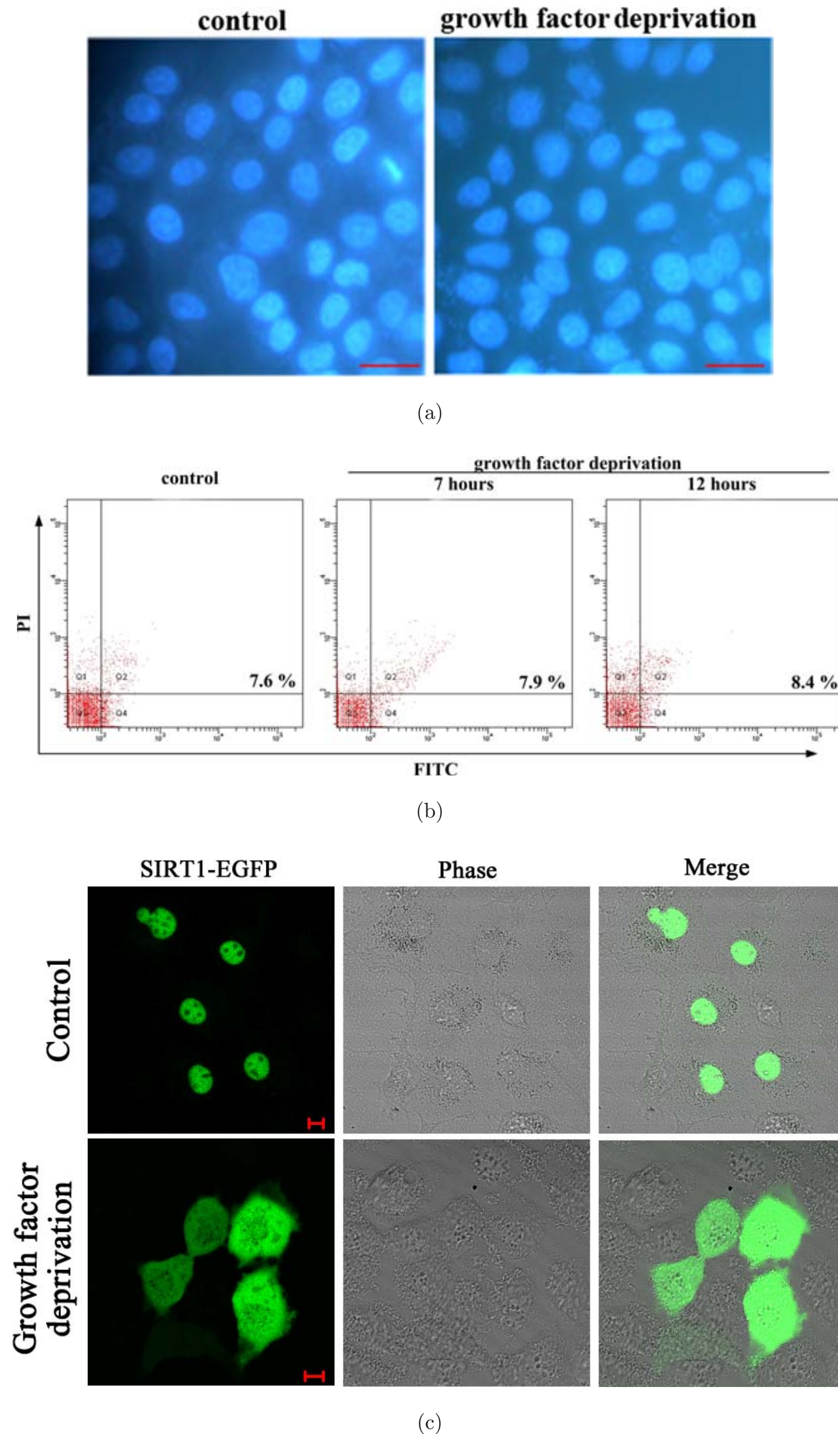

Fig. 2. Real-time fluorescence imaging SIRT1 cytoplasm translocation under the treatment of growth factor deprivation for 7 and 24 h in COS-7 cells. COS-7 cells were stained with Hoechst 33258 to visualize the apoptotic nuclear morphology (a). Bar for 50 μm. The cells were analyzed by a BD FACSCantoTM II flow cytometer after staining with fluorescein-conjugated Annexin V and propidum iodide (PI) (b). COS-7 cells transfected with SIRT1-EGFP were treated 48 h later with or without growth factor deprivation. SIRT1 localization was monitored by LCSM 510 Meta microscopy (c). The percentage of cells on the diagram (d) corresponds to the number of cells distributed between the two types of SIRT1 cellular localization (200−300 counted cells) detected by confocal microscopy; nucleus (gray), nucleus + cytoplasm (white). Cells transfected with SIRT1-EGFP were treated 48 h later with growth factor deprivation for 7 h and then real-time monitoring the dynamic changes of SIRT1 (e). Quantitative analysis of SIRT1-EGFP translocation corresponding to images (f). Data represent the mean ±SD of independent experiments.

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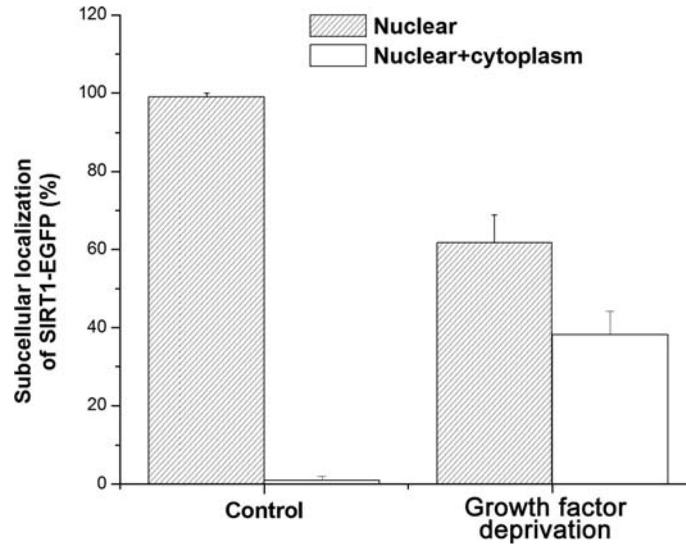
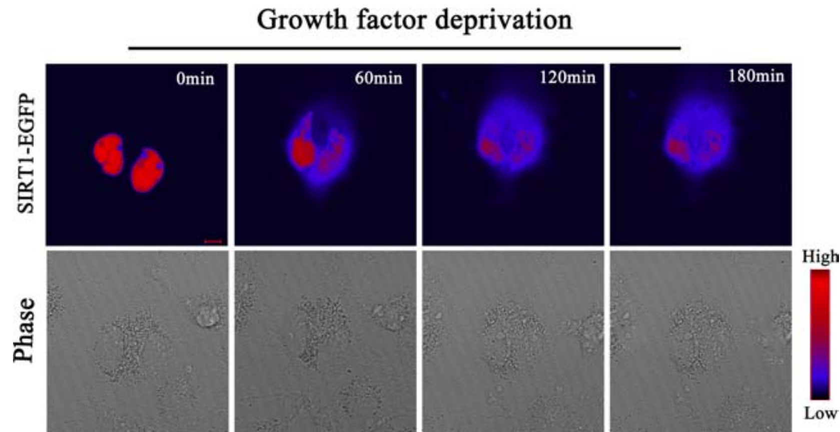
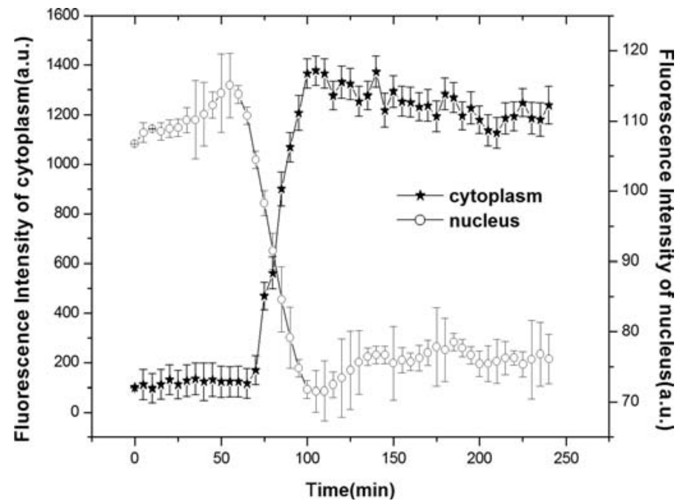


(d)



(e)



(f)

Fig. 2. (*Continued*)

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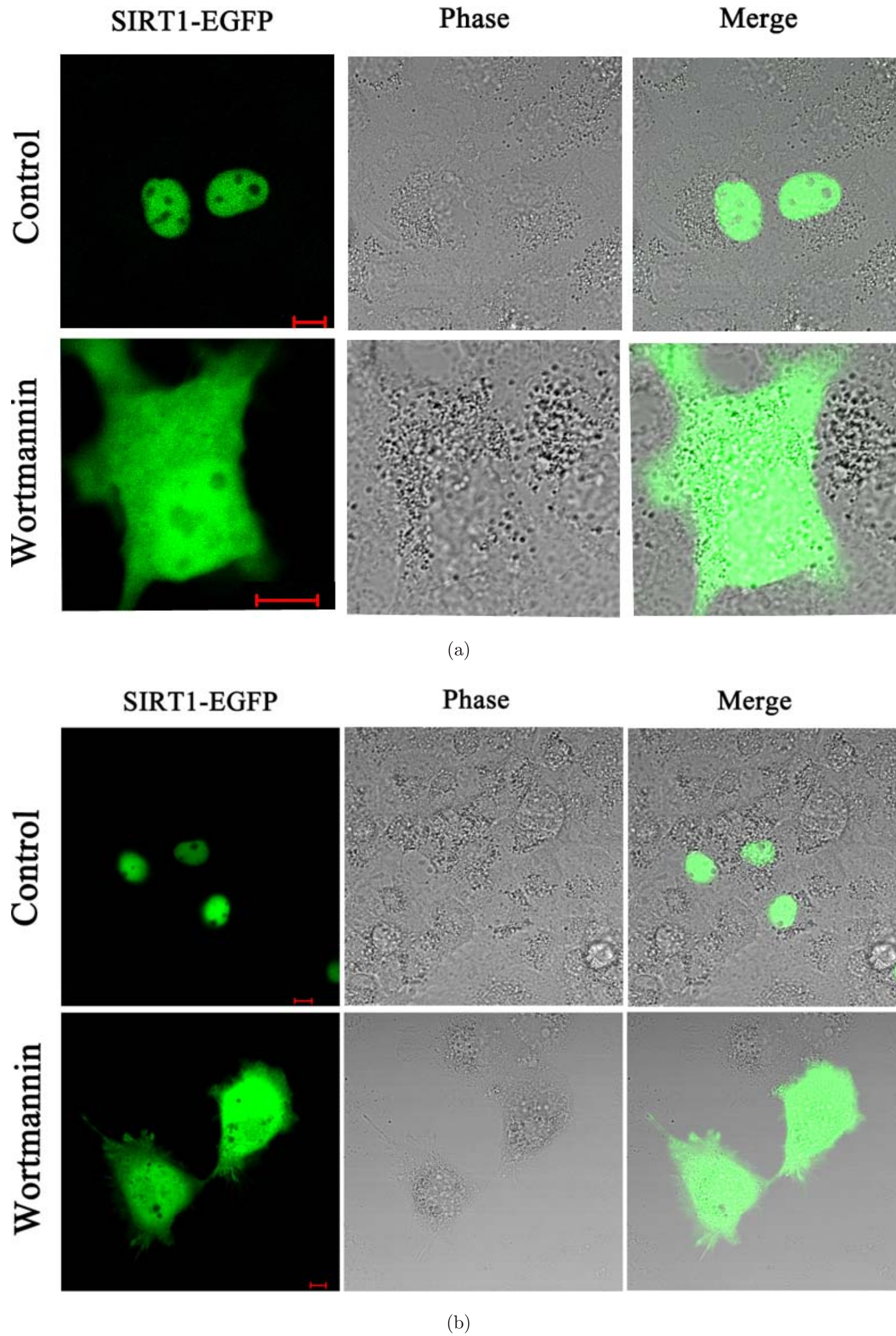

Fig. 3. The PI3K/Akt signaling pathway prevents cytoplasm translocation of SIRT1. COS-7 cells transfected with SIRT1-EGFP were treated 48 h later with or without wortmannin (10 μM) for 24 h. After incubated with wortmannin for 24 h, SIRT1 translocated to cytoplasm (a). (b) SIRT1 cytoplasm translocation in multiple cells under the treatment as (a). (c) Quantification of cells showing SIRT1 translocation was determined in cells by counting under an inverted fluorescence microscope. At least 200 cells were scored for each experimental point. Columns represent the percentage of cells showing SIRT1 localization.

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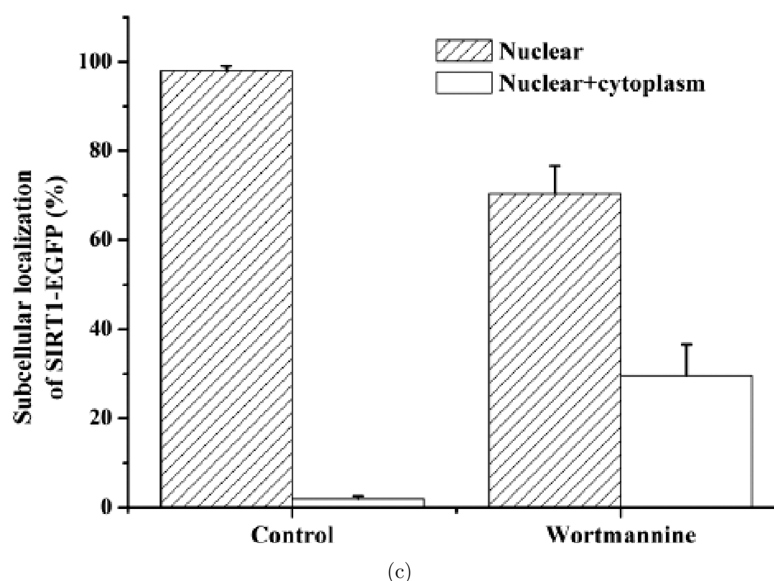

(c)

Fig. 3. (*Continued*)

in kinases activity elicited by growth factor deprivation, further leading to SIRT1 translocation from the nuclei to the cytoplasm similar to other nucleocytoplasmic shuttling proteins such as p53 and FOXO proteins.

3.3. *SIRT1 shuttles to cytoplasm by inactivating PI3K/Akt signaling pathway*

Previous studies have defined that SIRT1 protein is not only present in the nucleus,[13] but also distributed in the cytoplasm in murine pancreatic β cells.[15] These data suggest that SIRT1 might be a nucleocytoplasmic shuttling protein. Like other shuttling proteins, the subcellular distribution of SIRT1 might also be controlled by kinases. Given these probabilities, we examined the effect of PI3K kinases inhibitor wortmannin on the localization of SIRT1-EGFP in COS-7 cells. As shown in Fig. 3, we found that GFP were observed in the cytoplasm after the indicated treatment, suggesting that the inhibitor induced an exclusion of SIRT1-EGFP from the nucleus, resulting in SIRT1 subcellular redistribution. This data suggest a reduced level of SIRT1 activity.

4. Conclusions

The subcellular localization of SIRT1 differs from cell-to-cell *in vivo*; most of cells show nuclear expression of SIRT1 but others express it in the cytoplasm alone or in both the nucleus and cytoplasm, suggesting that the SIRT1 localization may influence its function, because only nuclear SIRT1 is functional in deacetylating histone and transcriptional factors. In this study, we found that SIRT1 was predominantly located at the nuclei in COS-7 cells under physiological conditions, and SIRT1 translocated to cytoplasm from nuclei when the cells were subjected to growth factor deprivation for at least 24 h. We also demonstrated that PI3K/Akt pathway was involved in the maintenance SIRT1 at the nucleus, because inhibition of these kinases significantly decreased the amount of SIRT1 in nuclei.

Some studies have reported that laser irradiation diverse signaling modulates pathways and ultimately affects the cell physiological processes.[21] Consistent with increased levels of Ca^{2+},[22] Gao *et al.* showed that PKC kinases are activated in human lung adenocarcinoma (ASTC-a-1) cells[23] and rat pheochromocytoma (PC12) cells[24] in response to low-power laser irradiation (LPLI). It is also reported that LPLI triggers a significant activation of ROS/Src pathway[25] and promotes formation of circular dorsal ruffles via PI3K/Ras pathway.[26] Meanwhile, elevated levels of Akt phosphorylation is observed in African green monkey SV40-transformed kidney fibroblast cells (COS-7) when stimulated by LPLI ($<50\,J/cm^2$).[27,28] Thus, we speculate that SIRT1 is maintained in nuclei by

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LPLI via PI3K/Akt pathway and needs further investigation. In summary, our data demonstrate that SIRT1 translocates from nuclear to cytoplasm in aging cells and provides a new application of single-molecule fluorescence imaging of the molecule events in living cells.

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