# The impact of Icariside II on human prostate cancer cell proliferation, mobility, and autophagy via PI3K-AKT-mTOR signaling pathway

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**Research**

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## Abstract

# Background

The flavonol glycoside icariside Ⅱ (ICA II) has been shown to exhibit a range of anti-tumor properties. Herein we evaluated the impact of ICA II on the proliferation, motility, and autophagy activity of human prostate cancer cells, and we further evaluated the molecular mechanisms underlying these effects.

# Methods

Herein, we treated DU145 human prostate cancer cells with a range of ICA II doses. We then evaluated the proliferative abilities of these cells via CCK-8 assay, whereas apoptosis and cell cycle status were assessed via flow cytometry. We further utilized wound healing and transwell assays to probe the impact of ICA II on migratory and invasive activities, while autophagy was assessed via laser confocal fluorescence microscopy. Western blotting was further utilized to measure LC3-II/I, Beclin-1, P70S6K, PI3K, AKT, mTOR, phospho-AKT, phospho-mTOR, and phospho-P70S6K levels, with RT-PCR being used to evaluate the expression of these same genes at the mRNA level.

# Results

We found that ICA II was capable of mediating a dose- and time-dependent suppression of prostate cancer cell proliferative activity, while also causing these cells to enter a state of cell cycle arrest and apoptosis. We further determined that ICA II treatment was associated with significant impairment of prostate cancer cell migratory and invasive abilities, whereas autophagy was enhanced in treated cells relative to untreated controls. Levels of p-P70S6K, p-mTOR, p-AKT, and PI3K were all also decreased by ICA II.

# Conclusion

Our results indicate that ICA II treatment is capable of suppressing human prostate tumor cell proliferation and disrupting migratory activity while enhancing autophagy through PI3K-AKT-mTOR signaling. As such, ICA II may be an ideal candidate drug for the treatment of prostate cancer.


## Background

Prostate cancer (PC) remains one of the leading causes of cancer and death among men. [1] PC treatments are typically treated via chemotherapy, radical prostatectomy, and hormone therapy,[2] with individuals with advanced disease often undergoing androgen deprivation therapy (ADT).[3] While initially efficacious in those with androgen-sensitive PC, most patients eventually exhibit ADT resistance such

that their disease is reclassified as castration-resistant PC (CRPC) and has a poor prognosis.[2,3] As such, it is vital that novel treatments for CRPC be identified.

The flavanol glycoside icariside II (ICA II) is a primary compound isolated from the traditional Chinese medicinal compound *Herba epimedii*.[4,5] ICA II has been found to exhibit a diverse array of biological and pharmacological activities, functioning to combat osteoporosis, sexual dysfunction, and cancer as shown in multiple studies.[4,5] Autophagy is a key catabolic process that is active in eukaryotic cells,[6] and it has recently been highlighted as a potentially viable therapeutic target for the treatments of CRPC.[3,7] The phosphatidylinositol 3-kinase-protein kinase B-mammalian target of rapamycin (PI3K-AKT-mTOR) signaling pathway is essential within cells[8], with mTOR in particular serving to regulate activities such as cellular motility, proliferation, and autophagy.[9] ICA II has been shown to inhibit the COX-2/PGE 2 pathway and to induce mitochondria-dependent apoptosis in PC-3 PC cells. [10] The present study was therefore designed with the goal of evaluating the impact of ICA II on the proliferative, migratory, and autophagy activity of human PC cells, and to explore the mechanisms underlying such activity.

## Materials And Methods

# 1.1 Materials

DMEM, fetal bovine serum (FBS), and penicillin/streptomycin were from Gibco (Life Technologies, NY, USA). PBS, protease and phosphatase inhibitor cocktails, bovine serum albumin (BSA), RIPA lysis buffer, stripping buffer, propidium iodide (PI), and thioglycollate were from Sigma Aldrich (MO, USA). An annexin V-FITC-base apoptosis detection kit, a CCK-8 assay kit, and transwell chambers (with or without Matrigel pre-coating) were from BD Biosciences (CA, USA). Antibodies specific for microtubule-associated protein 1A/1B-light chain 3 (LC3), Beclin1, P70S6K, PI3K, AKT, mTOR, phospho-AKT, phospho-mTOR, and phospho-P70S6K were from Cell Signaling (MA, USA).

# 1.2 Cell Culture

DU145 PC cells from Peking University First Hospital were cultured in DMEM containing 10% FBS and penicillin/streptomycin at 37 °C in a 5% CO2 incubator.

# 1.3 Cell Proliferation Assay

A CCK-8 assay was used to assess the impact of ICA II on DU145 cell proliferative activity. Briefly, DU145 cells were added to a 96-well plate and were treated for 24 or 48 h using 0, 10, 20, 40, or80 μM ICA II. A CCK-8 kit was then used based on provided directions, with absorbance (OD) at 450 nm being evaluated via Multiclan EX plate reader (Thermo Fisher Scientific, MA, USA). Proliferation (%) = (OD of treated cells-OD of blank sample)/OD of control sample - OD of blank sample) × 100.

# 1.4 Assessment Of Cell Cycle Progression

The impact of ICA II on cell cycle progression in DU145 cells was assessed using PI. Briefly, cells were treated for 48 h using 0, 20, or 40 μM ICA II in 6-well plates, after which they were fixed overnight using 70% at − 20 °C. Cells were then washed two times using PBS prior to being stained using PI. A flow cytometer was then used to evaluate these cells, with FlowJo being used for data analysis.

# 1.5 Assessment Of Cellular Apoptosis

Apoptosis was assessed using an Annexin V-FITC/PI kit. Briefly, we treated DU145 cells for 48 h using 0, 20, and 40 μM ICA II for 48 h in 6-well plates, after which the cells were washed two times with chilled PBS before being resuspended in a 100 uL volume of binding buffer. Next, 5 uL each of PI and Annexin V-FITC was added to each sample, and cells were stained for 10 minutes protected from light. An additional 400 uL of binding buffer was then added to each sample prior to analysis with a flow cytometer within 1 hour. FlowJo was used for data analysis.

# 1.6 Wound Healing Assay

DU145 cell migration was assessed via wound healing assay. Briefly, we treated Du145 cells in 6-well plates for 48 h with 0, 20, or 40 μM ICA II. A micropipette tip was then used to generate a wound in the cell monolayer, with PBS being used to remove detached cells. Serum-free media was then added, and wound closure was assessed via light microscopy at appropriate time points.

# 1.7 Invasion Assay

DU145 cell invasion was assessed with a Transwell assay system. Briefly, DU145 cells were treated for 48 h using 0, 20, or 40 μM ICA II in 6-well plates. Cells were resuspended in serum-free DMEM and added to the upper chamber of a matrigel-coated Transwell insert (8um pore size), with DMEM containing 15% FBS being added to the lower well. After a 24 h incubation, invasive cells were fixed using 4% paraformaldehyde, stained with crystal violet, and imaged via microscopy.

# 1.8 Morphological Assessment

Following a 48 h incubation with 0, 20, or 40 μM ICA II in 6-well plates, DU145 cells were added to 6-well plates for 24 h and were transfected transiently with the GFP-LC3 plasmid using Lipofectamine 2000 (Invitrogen, Shanghai, China) based on provided directions. Following an additional 24 h, cells were used to assess autophagy by evaluating these GFP-LC3 overexpressing cells via confocal laser scanning microscope (Carl Zeiss, Shanghai, China).

# 1.9 Western Blotting

Following a 48 h incubation with 0, 20, or 40 μM ICA II in 6-well plates, DU145 cells were collected, rinsed with cold PBS, and lysed with RIPA buffer that contained protease and phosphatase inhibitors. Cellular proteins were then separated via SDS-PAGE, transferred to PVDF membranes, and these blots were blocked using 5% BSA for 1 hour. These blots were next probed overnight at 4 ℃ using primary anti-LC3, Beclin1, P70S6K, PI3K, AKT, mTOR, p-AKT, p-mTOR, and p-P70S6K. Next, blots were rinsed using TBST, and were then probed for 1 h using HRP-linked secondary antibodies, after which an ECL Advance Detection System was utilized for protein detection.

# 1.10 RT-PCR

Gene expression in DU145 cells was assessed via RT-PCR after a 48 h incubation with 0, 20, or 40 μM ICA II in 6-well plates. Primers specific for LC3-II/I, Beclin-1, phospho-PI3K, phospho-AKT, phospho-mTOR, PI3K, AKT, mTOR, and GAPDH cDNA sequences from the NCBI database[11]. were designed using the Oligo 6.71 software and synthesized by Shanghai Bioengineering Co., Ltd. An Ultra-Pure RNA kit was used to extract cellular RNA, which was then treated with DNase prior to being used to prepare cDNA with the HiFiScript cDNA Synthesis kit (Cwbiotech).

GAPDH-F: TCAAGAAGGTGGTGAAGCAGG, GAPDH-R: GCGTCAAAGGTGGAGGAGTG; Becliin-1-F: GGTTGCGGTTTTTCTGGGAC, Beclin-1-R: ACGTGTCTCGCCTTTCTCAA; mTOR-F: CCTCCATCCACCTCATCA, mTOR-R: GACGCCAAGACACAGTAG LC3-I/II-F: TTCTTCCTGCTGGTGAAC, LC3-I/II-R: GAGGCGTAGACCATATAGAG; P70S6K-F: CCATGAAGGTGCTTAAAAAGGCA, P70S6K-R: TTCCACCAGTCTGAAAGGCA.

# 1.11 Statistical analysis

Statistical testing was conducted using SPSS 22.0 (SPSS Inc., IL, USA) by researchers not involved in conducting this study. Data were compared via one-way ANOVAs, with $P < 0.05$ as the significance threshold.

## Results

# ICA II disrupts the proliferation of DU145 cells in a dose- and time-dependent fashion

We began by assessing the proliferative activity of DU145 cells following treatment with a range of ICA II concentrations (0, 10, 20, 40, and 80 μM) for 24 or 48 h. We found that ICA II treatment was associated with significant reductions in the proliferation of these PC cells ($P < 0.01$), with the magnitude of this suppression being both time- and dose-dependent (Fig. 1).

### ICA II alters the cell cycle progression of DU145 cells

We next evaluated DU145 cell cycle progression after treatment for 48 h with a range of ICA II doses (0, 20, and 40 μM). ICA II-treated cells exhibited a higher frequency of cells in the G1 phase relative to untreated control cells ($P < 0.01$), whereas ICA II treatment was associated with a significant reduction in the frequency of cells in the S phase of the cell cycle ($P < 0.01$). These results thus suggested that ICA II can induce G1 phase arrest in DU145 cells (Fig. 2).

### ICA II induces the apoptotic death of DU145 cells

We next used Annexin V/PI staining to assess rates of apoptosis in DU145 cells following a 48 h treatment with a range of ICA □ doses (0, 20, and 40 μM). We found that ICA II treatment was associated with significantly higher rates of apoptosis in treated cells relative to untreated cells ($P<0.01$), suggesting that this compound is capable of inducing the apoptotic death of these PC cells (Fig. 3).

### ICA II impacts the migratory activity of DU145 cells

Next, a wound healing assay was utilized as a means of assessing DU145 cell migration following a 48 h treatment with a range of ICA II concentrations (0, 20, and 40 μM). We observed significantly reduced wound healing in the ICA II-treated cell samples relative to control untreated wells ($P<0.01$), thus suggesting that ICA II can inhibit DU145 cell migration (Fig. 4).

### ICA II suppresses the invasive activity of DU145 cells

DU145 cell invasive activity was next assessed via Transwell assay following treatment with a range of ICA II doses (0, 20, and 40 μM) for 48 h. We observed significant reductions in the number of invasive cells in ICA II-treated wells relative to control untreated wells ($P<0.01$), suggesting that ICA II is an inhibitor of DU145 cell invasive activity (Fig. 5).

### ICA II induces autophagic activity in DU145 cells

We next employed a laser confocal fluorescence microscopy approach to evaluate the impact of ICA II treatment (0, 20, and 40 μM) for 48 h on autophagic activity in DU145 cells. We observed enhanced autophagosome formation in ICA II-treated DU145 cells relative to control untreated cells ($P<0.01$), suggesting that ICA II is capable of promoting autophagy within DU145 cells (Fig. 6).

### Evaluation of autophagy, PI3K, mTOR, and Akt-related protein expression

Western blotting was next employed to monitor protein levels in DU145 cells following a 48 h treatment with ICA □ (0, 20, and 40 μM). We found that ICA II treatment was linked to a significant increase in the protein-level expression of LC3-II/I and Beclin-1 relative to control samples ($P<0.01$), whereas ICA II treatment was linked to reductions in the expression of PI3K, p-AKT ($P<0.05$), p-mTOR, and p-P70S6K levels relative to those levels observed in control samples ($P<0.01$). In contrast, total mTOR, AKT, and P70S6K expression levels were comparable between ICA II-treated and control group samples ($P>0.05$). Figure 7,8.

### Evaluation of autophagy, PI3K, mTOR, and Akt-related gene expression

Finally, we assessed the mRNA level expression of genes related to autophagy and PI3K/Akt/mTOR activity via RT-PCR in DU145 cells after a 48 h treatment with ICA  (0, 20, and 40 μM). We observed significant increases in mRNA level expression of LC3-II/I and Beclin-1 in ICA II-treated cells relative to controls ($P<0.01$), whereas ICA II reduced the expression of PI3K, AKT, and mTOR relative to expression levels in control cells ($P<0.01$). We also found that ICA II treatment was associated with a reduction in P70S6K expression relative to control samples ($P<0.05$). Figure 9,10.

# Discussion

Cancer remains the second most prevalent cause of death globally, with PC being the most common cancer to affect men.[1] Between 10% and 20% of men with metastatic PC develop CRPC within a 5-year period, and following the onset of such castration resistance the median survival time for these patients is just 14 months.[1,3] Chemotherapy remains the primary treatment for those with CRPC,[3] and entails the administration of an array of natural or synthetic pharmacological compounds that can help to disrupt tumor cell growth or division. Docetaxel is currently employed as a first-line treatment for CRPC, as it has been shown to exhibit survival advantages for treated patients, but it is associated with negative side effects such as diarrhea, hair loss, and myelosuppression.[3,7,12] DUl45 cells are androgen-independent human PC cells and are commonly used as a model for studies of CRPC. Novel therapeutic agents capable of treating CRPC are urgently needed.

Tumor growth and development is associated with increased invasiveness and dysregulated apoptotic cell death.[13,14] Several different anti-tumor drugs seek to modulate these activities via either disrupting tumor cell proliferation and/or via inducing apoptotic tumor cell death.[10,15] Traditional herbal medicines represent a potentially ideal source of compounds for use in chemotherapeutic regimens,[16] with drugs such as camptothecin, paclitaxel, and emodin all having been developed from natural plant sources.[17,18] Previous work has demonstrated that ICA II is capable of mediating anti-tumor activity against hepatic carcinoma, breast cancer, and gastric cancer via a range of mechanisms.[10,16] The P13K-Akt-mTOR signaling axis is a key regulator of protein synthesis within cells stimulated using an array of growth factors, influencing both autophagic activity and survival in a context-dependent fashion. ICA II treatment of osteosarcoma cells has recently been shown to be associated with reduced PI3k-AKT pathway activation.[16] Herein, we therefore explored the anti-cancer properties of ICA II by using it to treat DU145 PC cells. We ultimately determined that ICA II was able to reduce the proliferative, migratory, and invasive activity of these cells while inducing excessive mitophagy therein via PI3K-AKT-mTOR signaling mechanisms.

Cancer cells are largely defined by the fact that they can undergo unrestrained proliferation while resisting apoptotic death.[18] Chemotherapeutic drugs function by disrupting these activities, leading cells to be less proliferative and/or more sensitive to apoptotic signaling. [19] There is previous evidence that ICA II can

induce lung cancer cell apoptosis.[16] The cell cycle defines the basic steps whereby cells are able to replicate and divide, and it is traditionally divided into the G0, G1, S, and G2/M phases[7,13] Cell cycle arrest is a primary checkpoint that can help to restrain tumor cell growth,[16] and as such many chemotherapeutic compounds function by inducing such cell cycle arrest within cancerous cells.[16,20] Herein, we determined that ICA II was capable of significantly reducing DU145 cell proliferation in a dose- and time-dependent fashion, while also inducing G1 phase arrest and apoptosis within these PC cells. As such, the anti-proliferative activity of ICA II may be attributable to its ability to induce cell cycle arrest.

Metastatic progression is a complex, multi-step process wherein tumor cells undergo changes in their migratory, invasive, proliferative, phenotypic, and angiogenic properties that enable them to expand and spread to distant metastatic sites within affected individuals.[21–23] Both invasion and migration are key components of the metastatic process.[12] A number of studies have explored the ability of ICA II to modulate the invasivity and migratory capabilities of lung, gastric, and esophageal cancer cells.[10,16] Herein, we determined that ICA II was able to significantly inhibit DU145 PC cell invasion and migration.

Autophagy is a key regulator of cell cycle progression, apoptosis, and cellular migration.[6,24] Autophagy serves as a catabolic process in eukaryotic cells,[25] and is a vital means of maintaining intracellular homeostasis in physiological and pathological contexts.[6] While it can promote cell survival in some cases, in other settings autophagy can trigger apoptotic cell death depending on the intracellular signaling pathways that are engaged in a given cell.[6] Autophagic cell death is an alternative form of programmed cell death that is distinct from apoptosis and that has been observed in the context of PC.[6,26] Autophagy is associated with a disruption of apoptotic induction, whereas caspase activity that is induced during apoptosis can, in turn, disrupt autophagic processes. In some contexts, however, autophagy and related signaling processes can trigger tumor regression and cell death.[26] The specific mechanisms governing autophagy are highly complex and tightly regulated.

Beclin-1 and LC3 are key proteins that serve to regulate the formation of autophagosomes, thereby controlling autophagy initiation.[12] Beclin-1 interacts with Bcl-2, and was the first mammalian protein shown to be involved in the process of autophagy owing to its key role in inducing autophagosome formation.[22,27] Beclin-1 overexpression has been reported to inhibit HeLa cellular growth *in vivo* and *in vitro*, as it induces enhanced autophagic activity and apoptotic death in these tumor cells. The protein LC3 can exist in both solid and lapidated forms known respectively as LC3-I and LC3-II.[28] LC3-II is commonly monitored as a membrane marker for autophagic vacuole formation, and as such it can be used to gauge autophagic activity within individual cells.[29] Indeed, GFP-LC3 is a commonly utilized tool for the assessment of autophagy within cultured cells.[30] Numerous natural compounds have demonstrated the ability to induce mitophagy via the regulation of key autophagic pathways.[15] For example, 3-methyladenine (3-MA) can disrupt the formation of autophagosomes via modulating the Beclin-1 pathway.[19,30] Herein, we determined that ICA II is capable of enhancing autophagic activity within DU145 cells, as evidenced by the increased expression of Beclin-1 and LC3-II in these cells.

The PI3K-Akt-mTOR signaling pathway is associated with autophagic activity, and controls the differentiation, survival, proliferation, and migration of cells.[26] Akt functions as the primary mediator of PI3K signaling, with mTOR being a downstream target of this PI3K/Akt signaling pathway.[7,9] mTOR functionality is primarily governed by the activity of two different complexes (mTOR1 and mTORC2), with mTORC1 kinase activity serving to suppress autophagy and to maintain homeostatic cell growth, proliferation, and survival. PI3K-Akt-mTOR activity has been shown to suppress autophagy, whereas inhibiting this activity in contrast results in enhanced autophagic activity.[6,9,31] Herein, we found that ICA II was able to induce mitochondrial autophagy within DU145 PC cells, as evidenced by the upregulation of Beclin-1 and downregulation of PI3K, AKT, mTOR, and P70S6K levels within these cells.

PI3k-Akt-mTOR signaling is a key regulator of CRPC cell survival and proliferation, regulating disease progression through crosstalk with other important intracellular signaling processes associated with survival, differentiation, and autophagic activity. A number of different drugs that induce autophagy have been shown to mediate specific PI3K-AKT-mTOR pathway downregulation, suggesting that the modulation of this pathway may be a viable approach to inducing targeted cell death in tumor cells. Herein, we found that ICA II was able to inhibit PC cell proliferation, invasion, and migration while simultaneously promoting apoptosis and excessive mitochondrial autophagy. The molecular mechanism underlying this phenotype may be associated with disrupted PI3K-AKT-mTOR pathway signaling that ultimately results in autophagic induction. This study is, however, limited by the fact that ICA II is a multi-functional anti-tumor compound that can modulate the activity of a range of intracellular targets. As such, our results may only offer insights into one of several mechanisms whereby ICA II modulates PC cell proliferation and survival. Together, our findings suggest that ICA II may be a viable therapeutic agent for the treatment of CRPC patients.

# Conclusions

Herein, we determine that ICA II was able to exert a number of antitumor activities via reducing cellular proliferation, impairing migratory/invasive activity, and inducing excessive mitophagy through PI3K-AKT-mTOR signaling in human PC cells. As such, ICA II may be a viable therapeutic candidate for the treatment of this deadly form of cancer.

# Abbreviations

ICA II: Icariside Ⅱ; PC:Prostate cancer; ADT: androgen deprivation therapy; CRPC: castration-resistant PC; PI3K-AKT-mTOR: phosphatidylinositol 3-kinase-protein kinase B-mammalian target of rapamycin; FBS: fetal bovine serum; BSA: bovine serum albumin; PI: propidium iodide; LC3: microtubule-associated protein 1A/1B-light chain 3; OD: absorbance; 3-MA:3-methyladenine.

# Declarations

# Availability of data and materials

The datasets used in this study are available from the corresponding author upon reasonable request.

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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# Authors' contributions

Yuexin Liu designed the research. Shuang Li, Yunlu Zhan, and Yingwei Xie conducted the experiments. Yonghui Wang and Yuexin Liu wrote the manuscript. Shuang Li, Yunlu Zhan, Yingwei Xie, Yonghui Wang, and Yuexin Liu revised the manuscript. All authors read and approved the fnal manuscript.

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# Figures

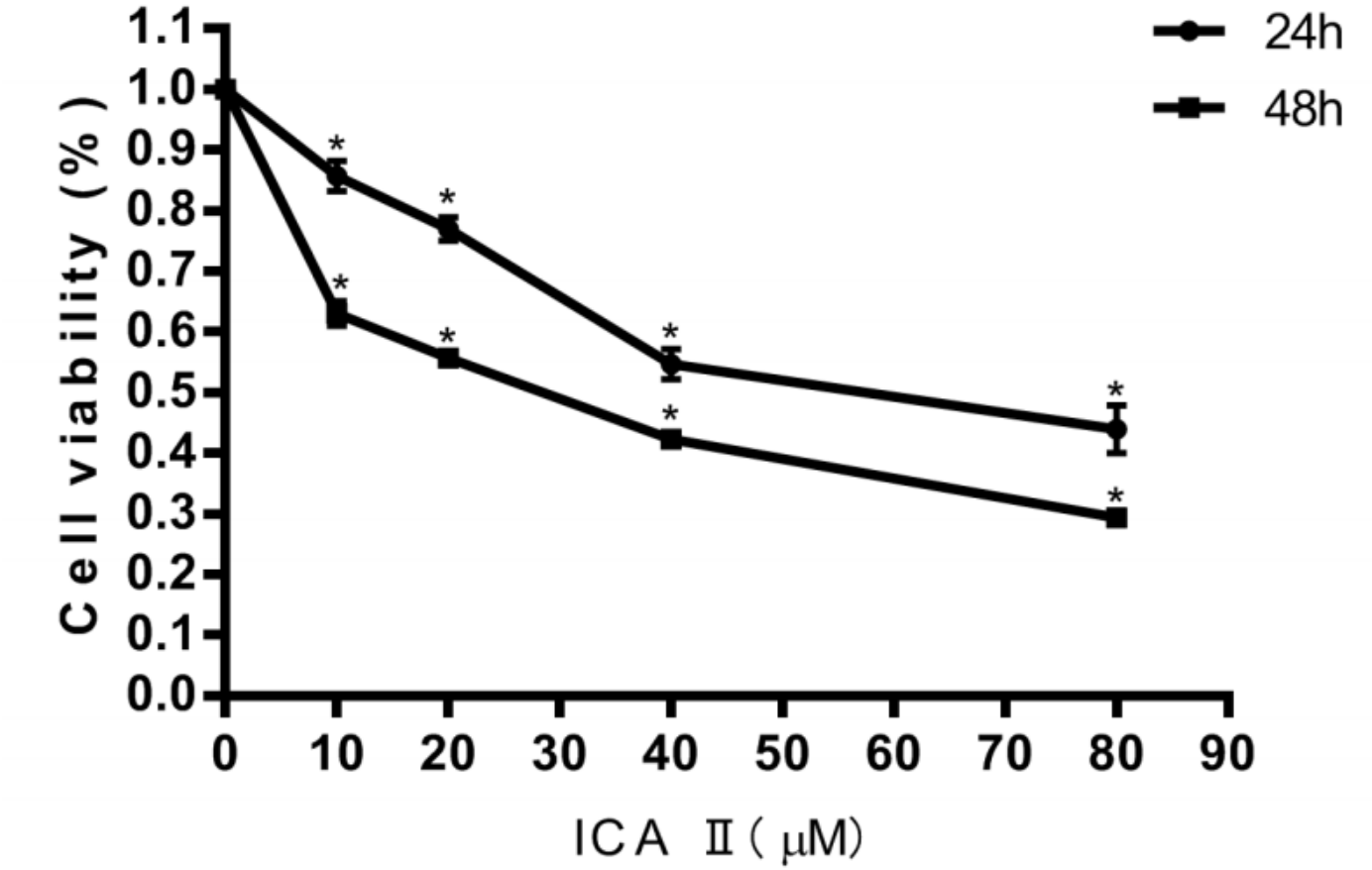
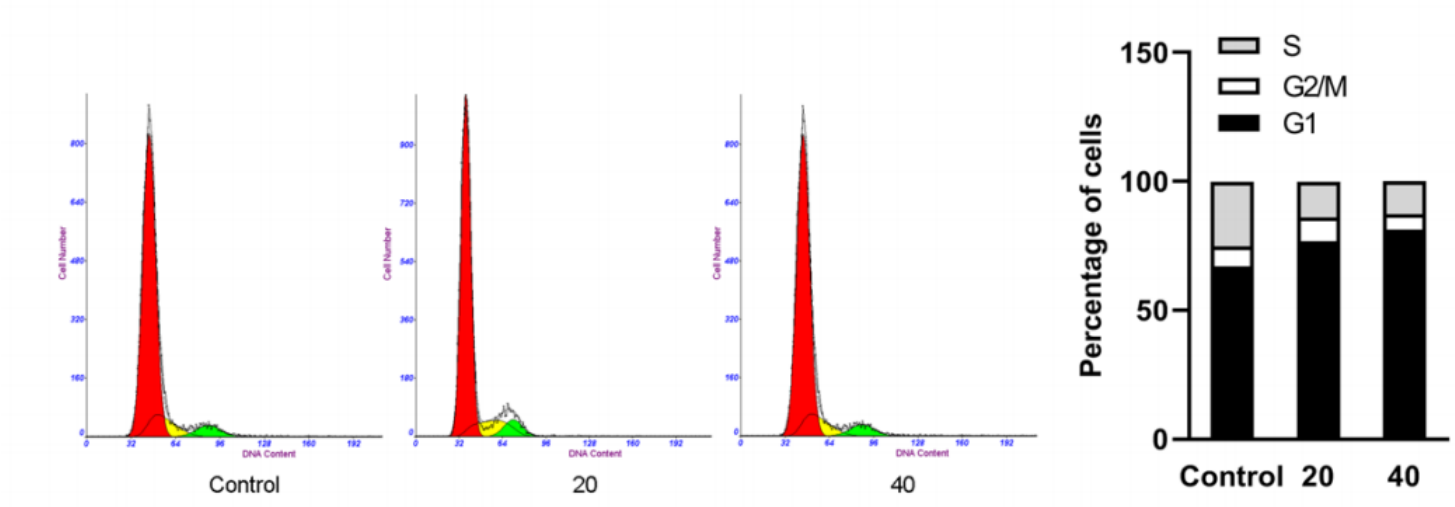


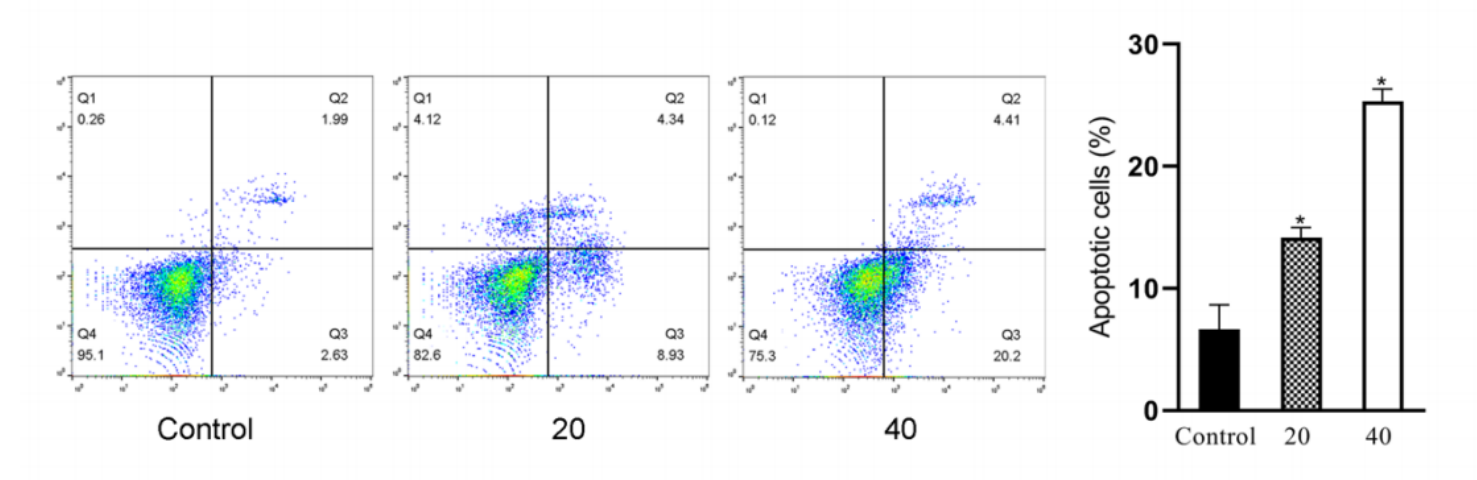

**Figure 1**

The impact of ICA II treatment on DU145 cells viability *: (P <0.01), vs control.



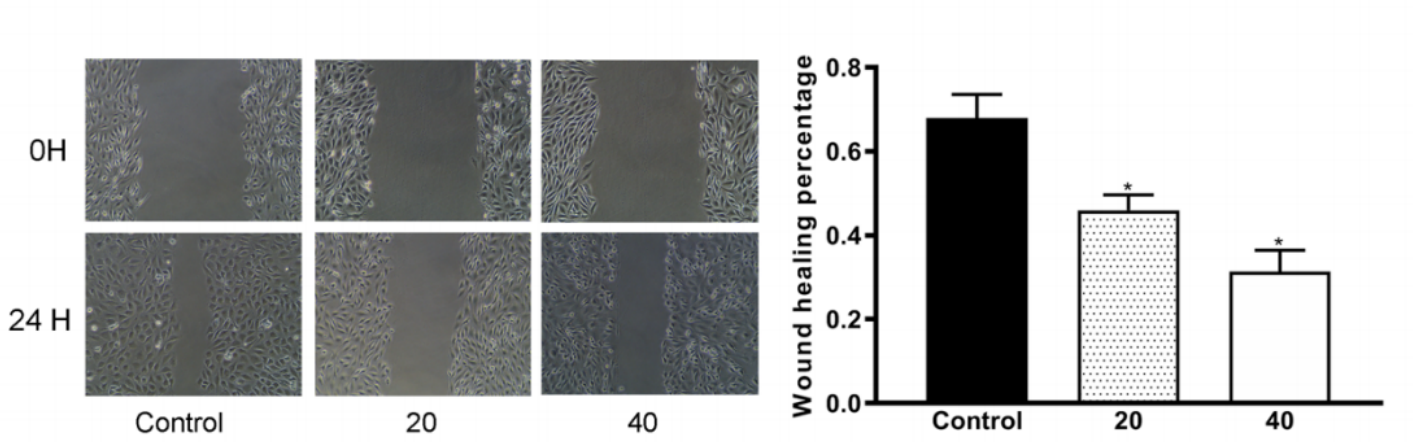
## Figure 2

The impact of ICAII on DU145 cell cycle distribution. Following a 48 h treatment with ICA II, DU145 cells were assessed via flow cytometry (left). Percentages of cells in different cell cycle phases are also shown (right)



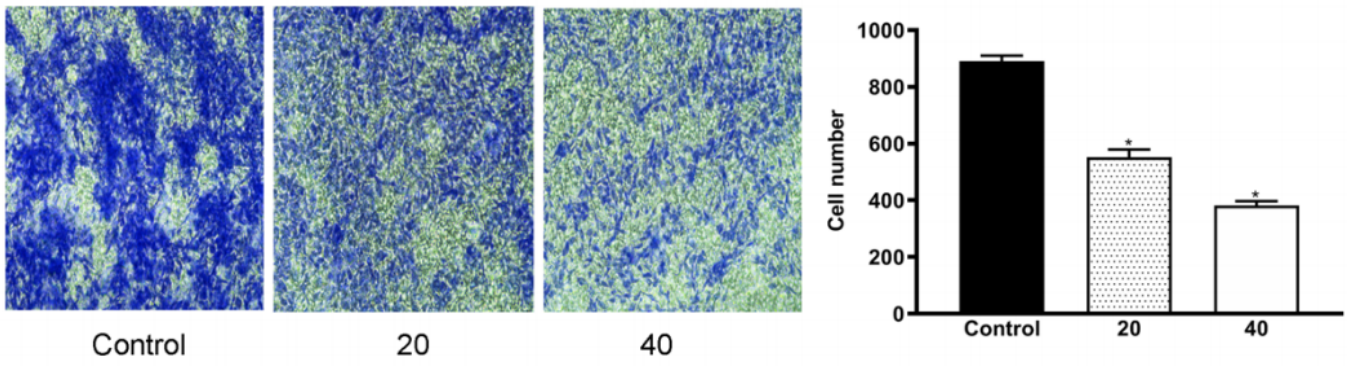

## Figure 3

The impact of ICAII on DU145 cellular apoptosis. Cells were treated for 48 h with ICA Ⅱ (0, 20, or 40 μM), after which flow cytometry was used to assess apoptosis (left). Percentages of apoptotic cells are shown on the right. *: (P <0.01), vs control.



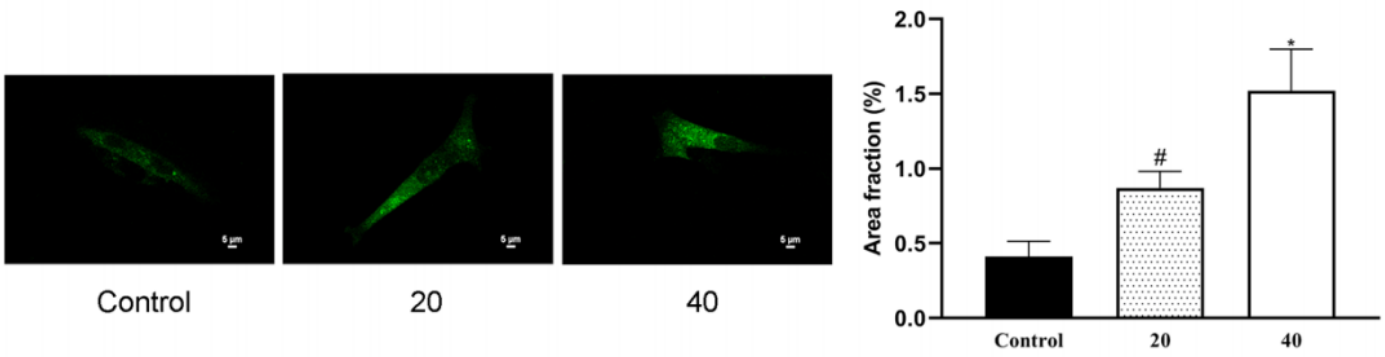

## Figure 4

The impact of ICAII treatment on DU145 cell migratory activity. Following a 48 h ICA Ⅱ treatment (0, 20, and 40 μM), DU145 monolayers were wounded using a pipette tip (left), with wound healing percentages shown on the right. *: (P <0.01), vs control.



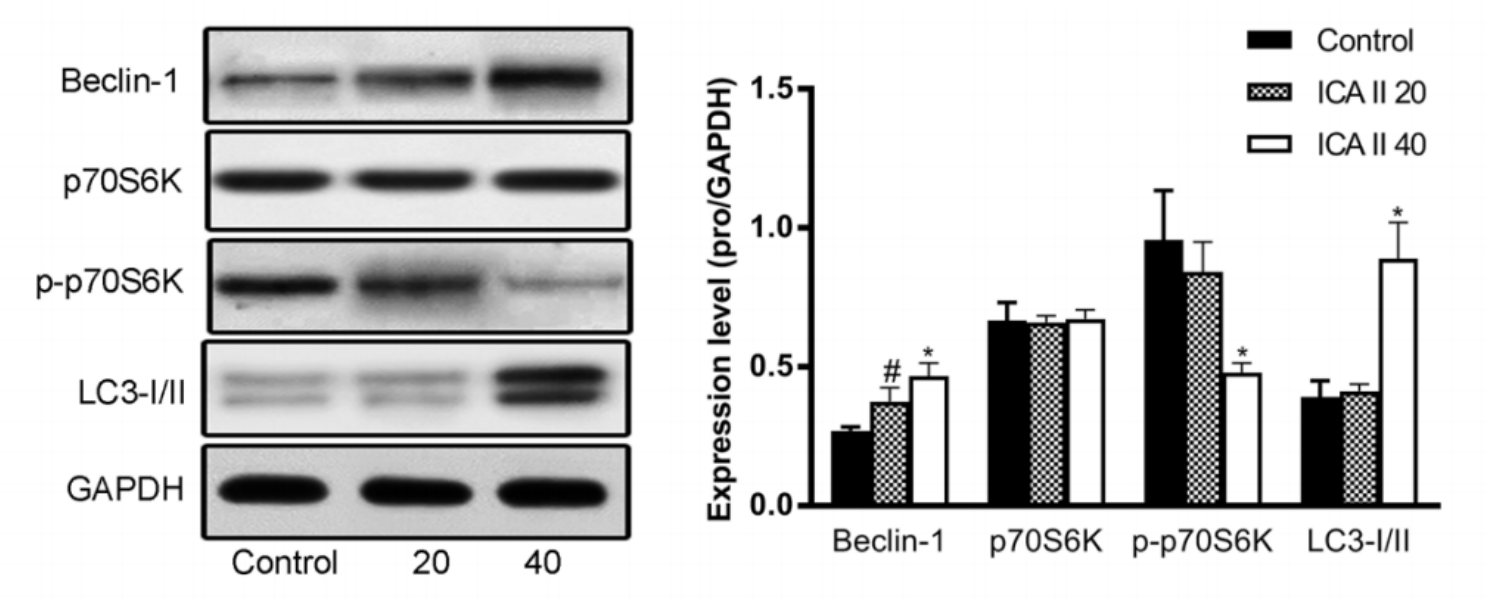

## Figure 5

The impact of ICAII on DU145 cell invasivity. DU145 cells were treated for 48 h using ICA Ⅱ (0, 20, and 40 μM), after which their invasivity was assessed via Transwell assay (right). *: (P <0.01), vs control.



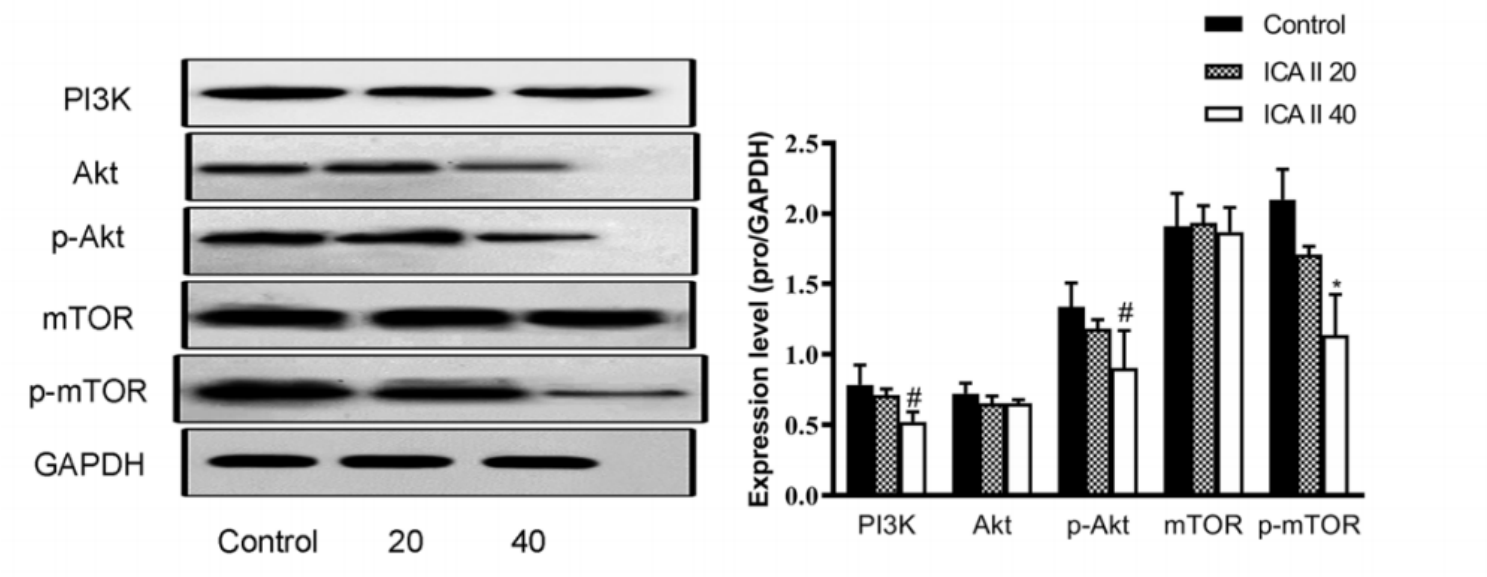

## Figure 6

The impact of ICAII on DU145 cell mitophagy. DU145 cells were treated for 48 h using ICA Ⅱ (0, 20, and 40 μM), and were transfected using the GFP-LC3 plasmid. The percentage of area fraction was determined (right). *: (P <0.01), vs control; #: (P <0.05), vs control.



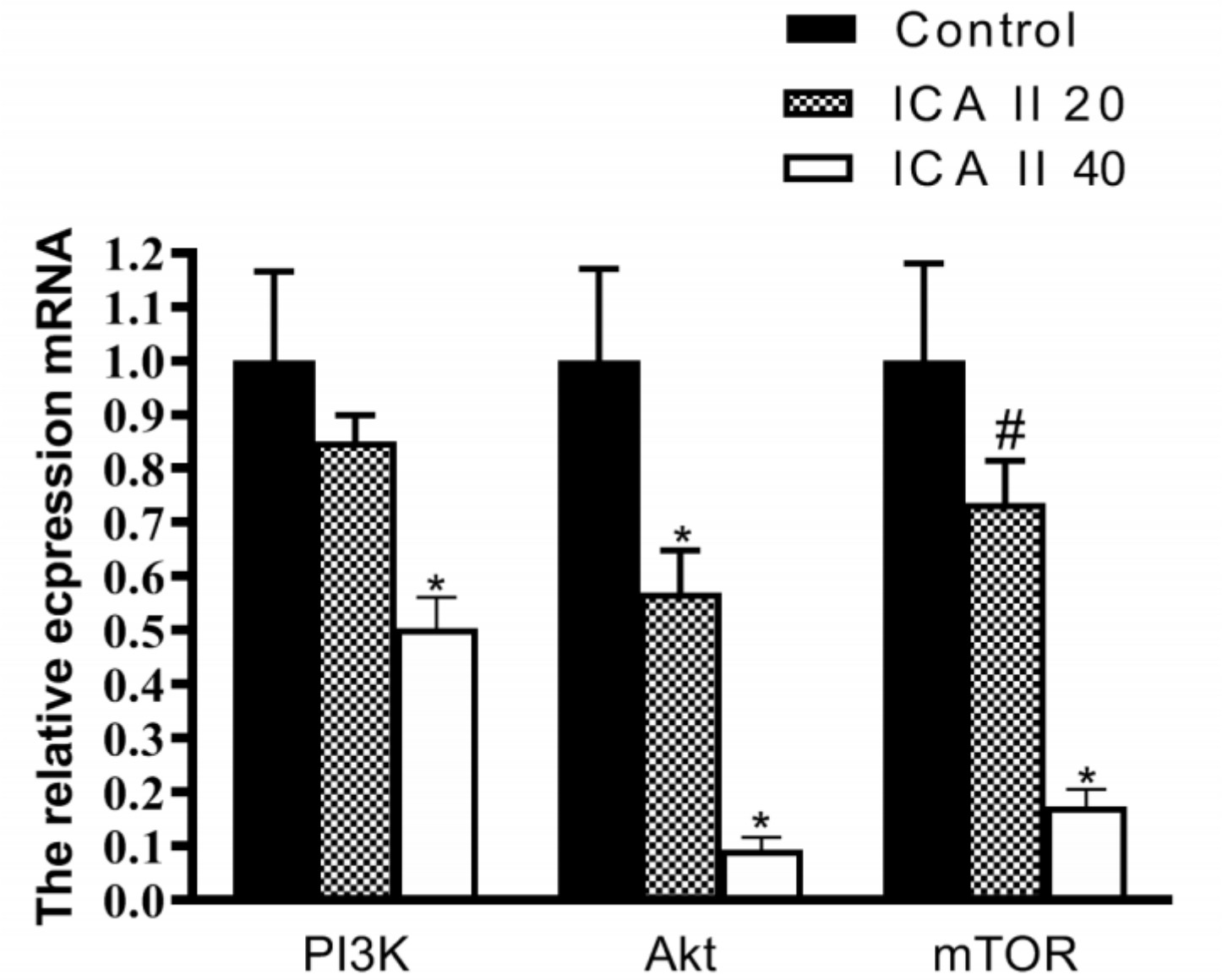

## Figure 7

The impact of ICAII on the expression of autophagy-related proteins in DU145 cells. DU145 cells were treated for 48 h using ICA Ⅱ (0, 20, and 40 μM), after which protein levels were assessed via Western blotting, with GAPDH as a loading control. Mean protein levels are shown in the histogram. *: (P <0.01), vs control; #: (P <0.05), vs control.




## Figure 8

The impact of ICAII on the expression of proteins in the PI3K-AKT-mTOR pathway in DU145 cells. *: (P <0.01), vs control; #: (P <0.05), vs control.




## Figure 9

The impact of ICAII on the expression of autophagy-associated genes in DU145 cells. DU145 cells were treated for 48 h using ICA II (0, 20, and 40 μM), after which real-time PCR was used to assess gene expression. *: (P <0.01), vs control; #: (P <0.05), vs control.

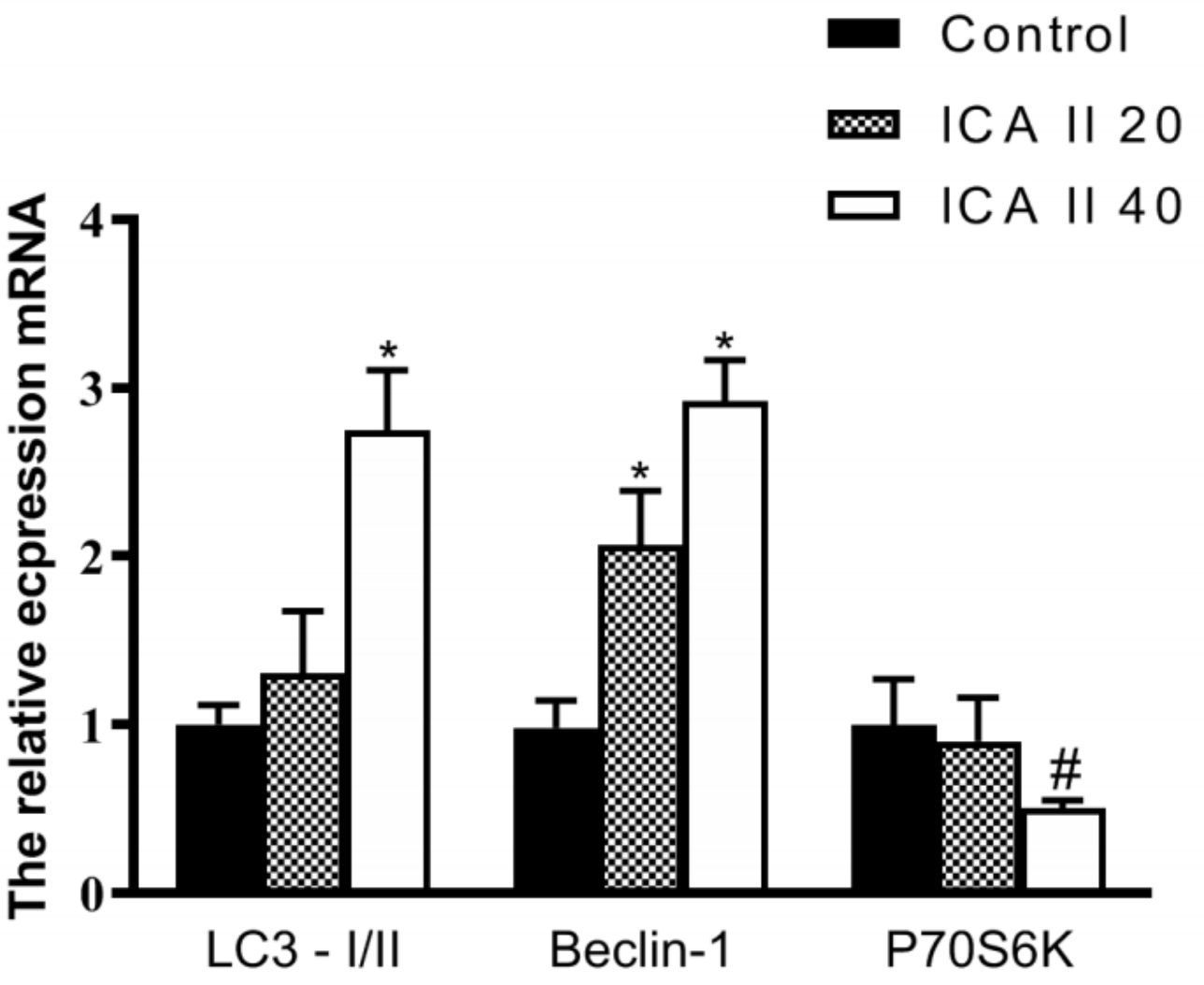



**Figure 10**

The impact of ICAII on the expression of genes related to PI3K-AKT-mTOR signaling in DU145 cells. *: (P <0.01), vs control; #: (P <0.05), vs control.