

Claudin-3 is Frequently Overexpressed in Ovarian Cancer and Promotes Migration and Invasion

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Research

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Abstract

Objective

Claudins are integral membrane proteins associated with tight junctions. Previous studies have shown that claudin-3 (CLDN3) is frequently upregulated in epithelial ovarian cancer (EOC). The relevance of CLDN 3 responsible for the invasion and metastasis of ovarian carcinoma is unclear. The aim of the study was to investigate the suppressive effects of CLDN3 in ovarian cancer cells and normal ovarian cells.

Materials and methods

Ovarian cancer tissues and their adjacent nontumorous tissues were investigated in this study. The expressions of CLDN3 in these cells were detected by Western blot and Immunohistochemistry (IHC). The cellular viability was determined by MTT assay. Cell Invasive capabilities in vitro were determined by a modified Boyden chamber invasion assay. Cellular Migration capabilities were determined Using Wound-healing/Scratch Assay.

Results

The expression of CLDN3 was 90% (18/20) in the ovarian cancer which was significantly higher than that in normal tissues (50%, 6/12) ($p < 0.05$). The CLDN3 protein expression was significantly suppressed in SKOV3 cells with siRNA silencing Claudin-3 gene. CLDN3 expression is associated with cellular viability. The cellular migration and invasion abilities were significantly decreased in CLDN3-knockdown SKOV3 cells compared with control cells. Upregulation of CLDN3 showed an increased metastasis ability in normal ovarian cell IOSE80.

Conclusion

Claudin-3 expression has been shown to promote cellular viability, migration and invasion of ovarian cells. Elucidation of the roles of CLDN3 and treatment targeting on CLDN3 in ovarian cancers might provide a new opportunity for therapy.

Introduction

Ovarian carcinoma remains the most lethal of gynecologic malignancies [1]. Although many patients with ovarian cancer have an initial favorable response to the standard combination of surgery and chemotherapy, nearly 90% later develop recurrent chemotherapy-resistant cancer and inevitably succumb to their disease [2]. New effective therapies are urgently needed for those patients with advanced-stage ovarian cancer who either do not respond to initial therapy or develop recurrent disease.

Claudins are integral membrane proteins associated with tight junctions. Two members of the claudin protein family, claudin-3 (CLDN3) and CLDN4, are overexpressed in epithelial ovarian tumors relative to normal ovarian tissue [3–6]. Meanwhile, Claudin-3 and -4 have also been identified as the epithelial receptors for Clostridium perfringens enterotoxin (CPE), a potent cytolytic toxin [7–9]. CPE triggers lysis of mammalian epithelial cells through interactions with claudin-3 and -4 receptors while cells that do not express CPE receptors are protected from the lethal effects of CPE because they fail to bind the toxin [10]. Given their high expression in ovarian tumor cells and the possibility that their overexpression may disrupt tight junction barrier function and contribute to tumorigenesis, targeting CLDN3 using siRNA is an attractive option as a potential therapy for ovarian cancer.

In this study, we have compared the expression of CLDN3 between human ovarian tumor tissues and healthy tissues. We further treated ovarian cell lines with siRNA silencing Claudin-3 gene, and the results showed that the cellular viability and metastasis was significantly reduced. These promising results suggest that silencing of CLDN3 warrants further development as a new therapeutic option for ovarian cancer.

Materials And Methods

Cell lines and cell culture

ovarian cancer cell line SKOV3 and normal ovarian cell line IOSE80 were used in this study. Cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin.

Cells were cultured in a humidified atmosphere at 37 °C containing 5% CO². All experiments were performed at a [confluency](#) of around 70%.

Patients and tissue samples

Ovarian cancer tissues and their adjacent nontumorous tissues were investigated in this study. The tissues were collected immediately after surgical resection prior to any other therapeutic intervention at the Zhujiang Hospital of Southern Medical University (Guangzhou, China). All samples were confirmed by histology. Informed consent was given by all of the patients. The study protocol was approved by the Clinical Research Ethics Committee of the Zhujiang Hospital of Southern Medical University of Medical Sciences.

Immunohistochemistry

IHC staining was performed using the standard streptavidin-biotin-peroxidase complex method. Briefly, paraffin sections of the tissues were deparaffinized, blocked with 10% normal goat serum for 10min, and

incubated with anti-CLDN3 polyclonal antibody (Invitrogen, 1:800 dilution) overnight at 4°C. The tissue section was then incubated with biotinylated goat anti-rabbit immunoglobulin at a concentration of 1:75 at 37°C for 30min. The status of CLDN3 expression was assessed by two independent investigators without prior knowledge of clinicopathologic data.

Western Blot

Total protein was extracted and the concentrations were determined by Bradford Protein Assay. 30µg of protein from each sample was separated on 8~12% SDS-PAGE and transferred onto polyvinylidene fluoride membrane. Blots were detected by incubation with antibodies to CLDN3 (Invitrogen), active β-catenin and GAPDH (Cell Signaling, Danvers, MA).

MTT assay

Cells were seeded in 96-well plates at a density of 3000 cells/well in 100 µl medium and allowed to attach overnight. The MTT assay was performed after 72 h incubation to determine [cellular proliferation and viability](#). Absorbance was measured at wavelength of 590 nm using an [enzyme-linked immunosorbent assay](#) plate reader (BioRad, München, Germany).

In vitro cell invasion assay

The invasive capabilities of the cells were determined using a modified Boyden chamber invasion assay. Cells were cultured to about 80% confluence and serum starved overnight. Transwell inserts (BD Biosciences, San Jose, CA) of 8-µm pore size were coated with 150 µl of 1:60 diluted Matrigel (BD Biosciences). Three hundred thousand cells after overnight starvation were plated onto the top of each of the coated filters in 150 µl of serum-free medium. Three hundred microliters of the same medium containing 20% fetal calf serum was placed in the lower chamber to act as a chemoattractant. After 24 hours of culture, cells that did not invade through the pores of the transwell inserts will be manually removed with cotton swabs and the inserts were fixed in cold methanol for 10 minutes and then stained with 0.01% crystal violet in 20% ethanol. After washing thoroughly, colorimetric readings were taken at 595 nm. The experiment was performed twice with each sample in triplicate. To assess cell migration, assays were carried out as above except that cells were plated on top of uncoated (Matrigel-free) inserts.

Measurement of Migration Using Wound-healing/Scratch Assay

Cells were grown to confluence on 60-mm cell culture dishes and a scratch was made through the cell monolayer using a pipette tip. After washing with Hank's balanced salt solution, fresh culture medium

was added and the cells were incubated at 37°C in a humid environment with 5% CO₂. Wound closure was observed and photographed at 0, 12, and 24 hours after making the scratch to monitor the invasion of cells into the wounded area.

Statistics

Statistical analysis was performed with the SPSS standard version 16.0 (Chicago, IL) or GraphPad Prism 5 (GraphPad Software, La Jolla, CA). Results expressed as mean ± SD were analyzed using the Student t test. P value less than 0.05 was considered statistically significant.

Results

Immunohistochemical staining Shows CLDN3 expression Elevated in ovarian cancer tissues

The CLDN3 expression in ovarian cancer tissues (n=20) and their adjacent normal ovarian tissues (n=12) was determined by using immunohistochemistry. The criteria for grading of CLDN3 expression were as follows: negative (-): ≤5% positive cells; low to moderate (+): 5~50% positive cells; strong (++) : 50~100% positive cells. Normal ovarian tissues showed negative or low expression of CLDN3. In contrast, the expression of CLDN3 was elevated in ovarian cancer tissues. 30% cases showed moderate expression and 60% case showed strong expression in ovarian cancer patients, while the moderate and strong expression cases were 33% and 17%, separately. Together, the expression of CLDN3 was 90% (18/20) in the ovarian cancer which was significantly higher than that in normal tissues (50%, 6/12) (p=0.05) (Fig 1).

CLDN is elevated in ovarian cancer cell lines and can be reduced by CLDN3-specific siRNA

In order to evaluate CLDN3 expression in ovarian cell lines, ovarian cancer cell line SKOV3 and normal ovarian cell line IOSE80 were evaluated by Western Blot. As shown in Fig 2A, CLDN3 protein was not detected in normal ovarian cell line IOSE80, but was present in ovarian cancer cell line SKOV3. Next, we constitutively knocked down their expression in ovarian cancer cell line SKOV3 using siRNA vectors. The results revealed that CLDN3 protein expression was significantly suppressed in SKOV3 cells, with the relative grey value lower than mock-control and non-specific-control cells (NC control cells) (P<0.001) (Fig 2B, C). The normal ovarian cell line IOSE80, which showed lower level of CLDN3 protein expression, was also transfected with lentiviral vectors expressing CLDN3 and results showed the CLDN3 expression significantly increased after transfection compared with control cells, with the relative grey value higher than mock-control and non-specific-control cells (P<0.001). These results showed high expression of

CLDN3 in ovarian cancer cell lines, and confirm that CLDN3 can be reduced by CLDN3-specific siRNA (Fig 2D, E).

CLDN3 expression is associated with cellular viability

In order to determine the effect of CLDN3 on the growth of ovarian cancer cells and normal ovarian cells, MTT assay was conducted to determine the cellular proliferation. We first performed transient siRNA-mediated knockdown of CLDN3 expression in ovarian cancer cell line SKOV3 and tested the cellular viability. The results showed that OD values decreased at every time point tested. The significant changes started from 1.5 days of cell culture (Fig 3A). To further confirm role of CLDN3 in cellular survival, CLDN3 was upregulated in IOSE80 cells. Consistent with what was observed with SKOV3 cells, we found that stable overexpression of this protein led to increased cellular viability compared to mock-transfected cells or parental IOSE80 cell (Fig 3B).

Migration suppressing ability of CLDN3 in ovarian cancer cells

Since CLDN3 is a tight junction (TJ) protein and disruption of TJ barrier could facilitate dissociated cancer cells to metastasize [11], we thus determine the migrative role of CLDN3 in SKOV3 and IOSE80 cells by wound-healing assays. The migration of SKOV3 cells was significantly suppressed in CLDN3-knockdown SKOV3 cells compared with control cells after 12-hour transfection ($P < 0.05$). Twenty-four hours post siCLDN3 transfection, the wound-healing assays showed a significant larger migration distance, indicating a decreased cellular migration ability in CLDN3-knockdown SKOV3 cells compared with control cells ($P < 0.05$) (Fig 4A, B). Similarly, upregulation of CLDN3 in normal ovarian cell IOSE80 showed a smaller migration distance, indicating an increased migrative ability in IOSE80-CLDN3 cells compared with the control cells (Fig 4C, D).

Invasion suppressing ability of CLDN3 in ovarian cancer cells

Invasion suppressing ability of CLDN3 was further investigated in SKOV3 and IOSE80 cells by Boyden chamber matrigel invasion assays. As shown in Fig 5A, less cells were shown in SKOV3-siRNA/CLDN3 cells, indicating that the invasive ability was suppressed in CLDN3-knockdown SKOV3 cells compared with control cells (Fig 5B). Similarly, upregulation of CLDN3 in normal ovarian cell IOSE80 showed that more cells could invaded through the pores of the transwell inserts, indicating an increased invasive ability in IOSE80-CLDN3 cells compared with the control cells (Fig 5C, D).

Discussion

Different members of the claudin gene family are abnormally regulated in several human cancers [9]. CLDN3 is reported overexpressed in breast [12], prostate [13], and endometrial cancer of serous papillary or clear-cell histology [14]. There is evidence that it is overexpressed in other tumor types as well, including lung and kidney [15]. In this study, consistent with the previous studies, we found CLDN3 proteins highly expressed in ovarian cancer tissues. Highly expression of these proteins was rare in normal ovarian tissues, clearly associating the presence of these proteins with malignancy. Our finding is in agreement with C.D. Hough et al. research who identified two claudin genes CLDN3 and CLDN4 highly up-regulated in ovarian cancer [3]. Work from several groups has now confirmed the overexpression of these proteins in ovarian cancer, and these findings may have significant implications for detection, prognosis and therapy of ovarian cancer [16].

Actually, Claudin-3 expression was found associated with poor outcome in ovarian cancer [17], and another study also found that claudin-3 expression was associated with higher grade ovarian tumors [18]. Thus, there is a possibility that claudin3 overexpression plays an important role in the process of ovarian cancer development. Currently, studies concerning the correlation of CLDN3 expression with ovarian cancer invasion and metastasis have rarely been reported. In this study, we treated ovarian cell lines with siRNA silencing Claudin-3 gene and upregulated the CLDN3 expression in normal ovarian cancers, its expression has been shown to promote cellular viability, migration and invasion of ovarian cells. In this case, the role of claudin overexpression in ovarian cancer would be limited to two possibilities. First, claudin-3, not normally present in ovarian tissues, may interfere with normal TJ formation which located in the junctional complex of epithelial cells and endothelial cells [7]. This has been previously observed that overexpressing CLDN2 in MDCK cells reduces the tightness of TJ in these cells [8]. Another possibility is that claudin-3 may be required for signaling through important survival or proliferative pathways in ovarian cancer, regardless of their role in TJ permeability. Further experiments to prove a functional role in ovarian tumorigenesis are currently under way.

Elucidation of the roles of CLDN3 in ovarian cancers might provide new opportunity for therapy. Mechanism based targeting of the claudin3 may inhibit cell proliferation and metastasis. Although the mechanism relevant to claudin3 overexpression in ovarian cancer is still being investigated, this report unequivocally identifies claudin-3 related to the cellular viability, migration and invasion of ovarian cells. Considering the difficulties associated with ovarian cancer therapy, it is important to aggressively pursue alternative treatment strategies for this disease. Our investigations of this approach have suggested that targeting the CLDN3 might be extremely promising and clinical trials will be crucial in determining the feasibility of this strategy.

Conclusion

In summary, we found CLDN3 immunohistochemical expression was elevated in ovarian cancer tissues compared with healthy ovarian tissues. This study reveals that CLDN3 immunoexpression may be a potential marker for ovarian cancer as CLDN3 expression has been shown to promote cellular viability,

migration and invasion of ovarian cells. Elucidation of the roles of CLDN3 and treatment targeting on CLDN3 in ovarian cancers might provide a new opportunity for therapy.

Abbreviations

CLDN3: claudin-3; CPE: Clostridium perfringens enterotoxin ; FBS: fetal bovine serum ; IHC: Immunohistochemistry ; TJ: tight junction

Declarations

Acknowledgements

Not applicable.

Authors' contributions

Chenyuan Hao, Yifeng Wang: Study design and manuscript editing; Chenyuan Hao: Data analysis and manuscript preparation; Xiaomei Liang: parts of the experiments. All authors read and approved the final manuscript.

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No fund was contributed to the current study.

Availability of data and materials

All data generated or analyzed during this study are included in this published article. The data that support the findings of this study are available.

Ethics approval and consent to participate

This study was approved by the Ethical Committee of Zhujiang Hospital of Southern Medical University.

Consent for publication

Not applicable.

Acknowledgements

Not applicable.

Competing interests

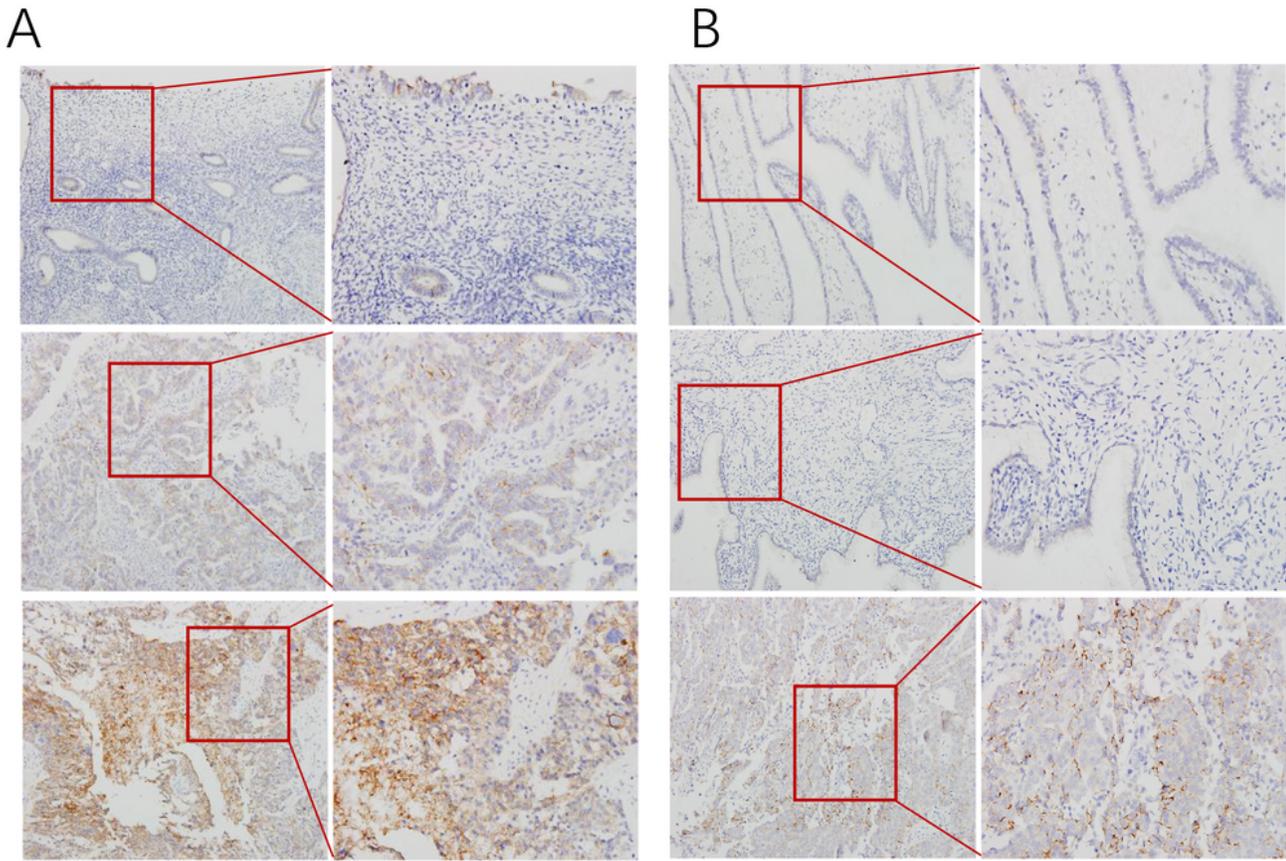
The authors declare that they have no competing interests.

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Figures



C CLDN3 expression

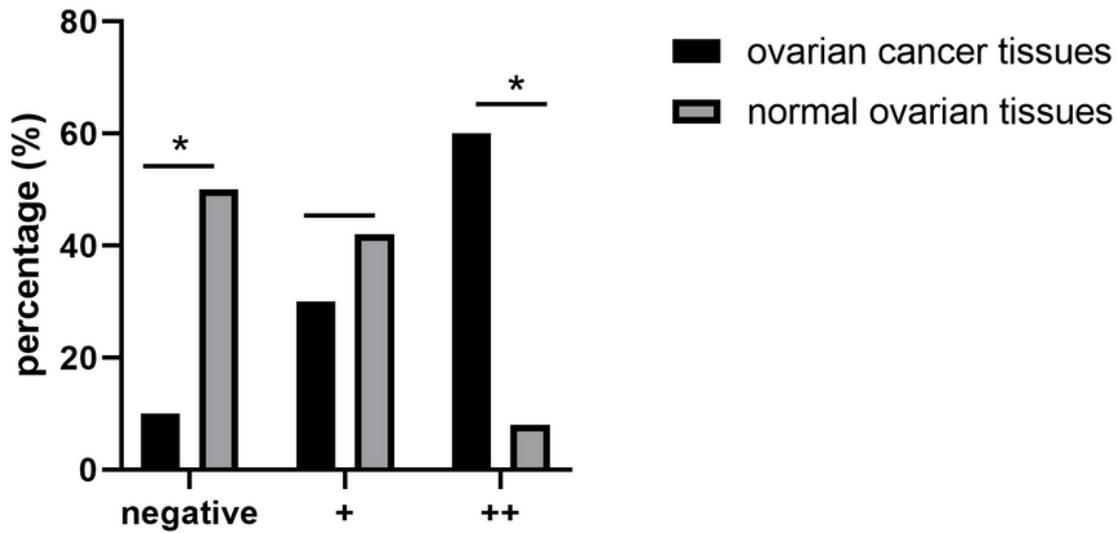
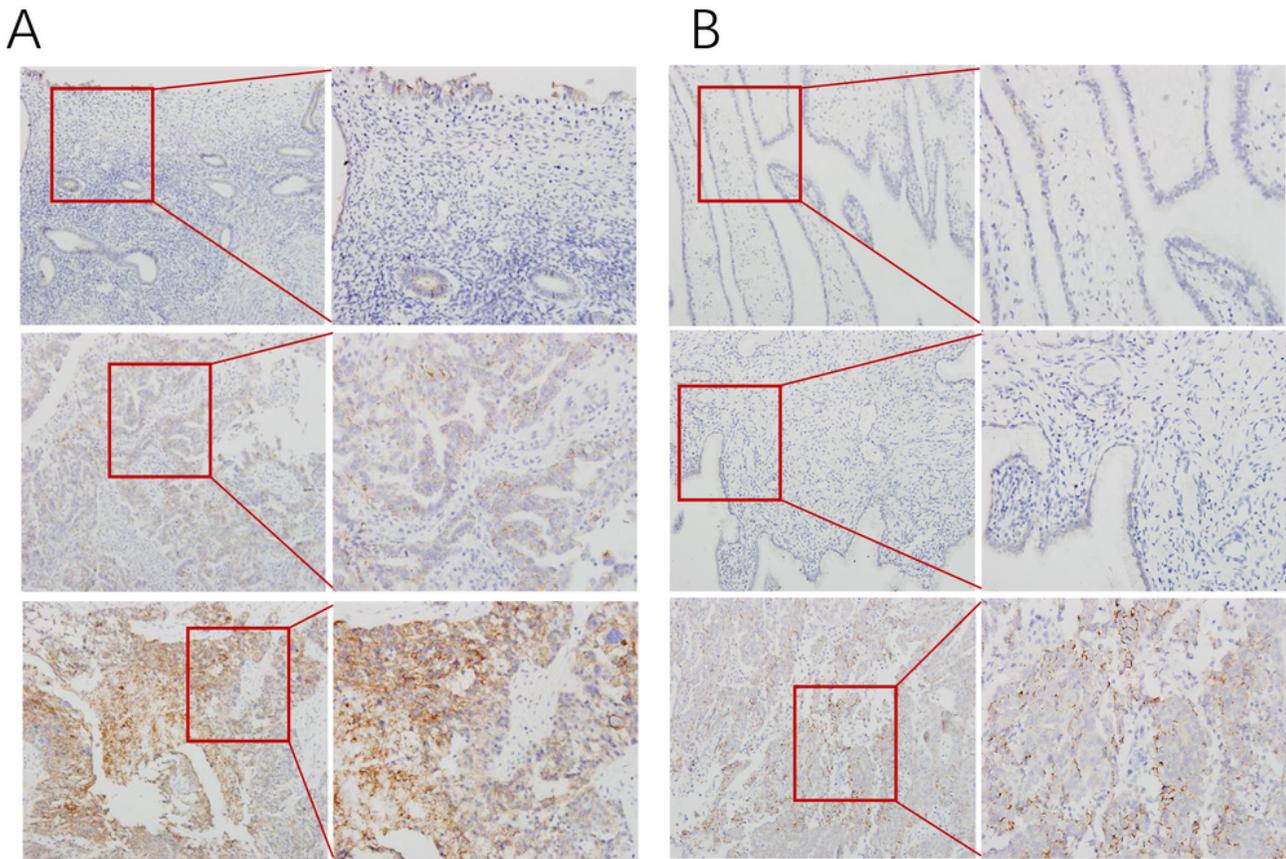


Figure 1

The CLDN3 expression in ovarian cancer tissues (n=20) and their adjacent normal ovarian tissues (n=12) was determined by using immunohistochemistry. The criteria for grading of CLDN3 expression were as follows: negative (-): $\leq 5\%$ positive cells; low to moderate (+): 5~50% positive cells; strong (++) : 50~100% positive cells. Normal ovarian tissues showed negative or low expression of CLDN3. In contrast, the expression of CLDN3 was elevated in ovarian cancer tissues. 30% cases showed moderate

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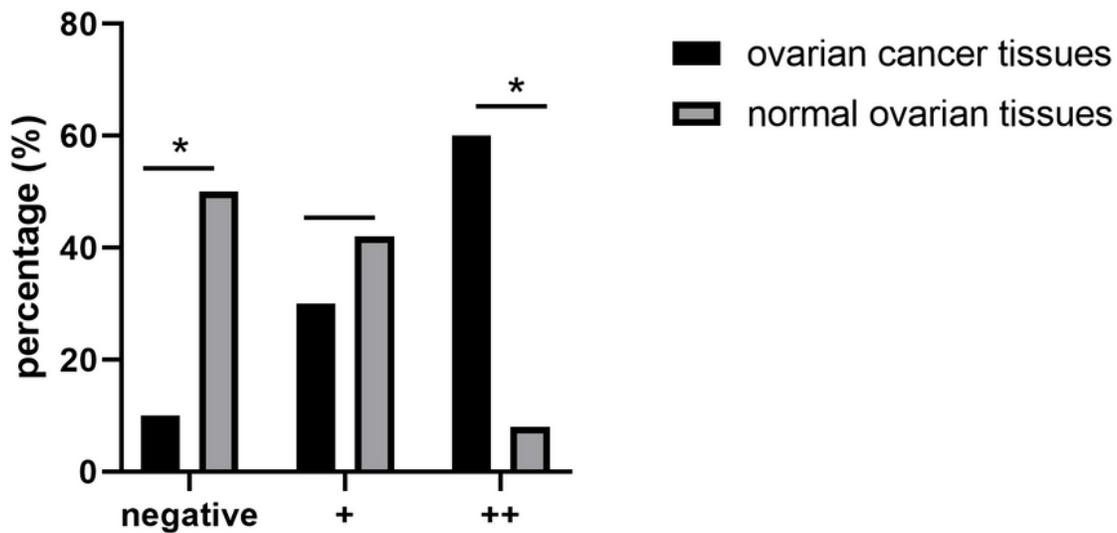
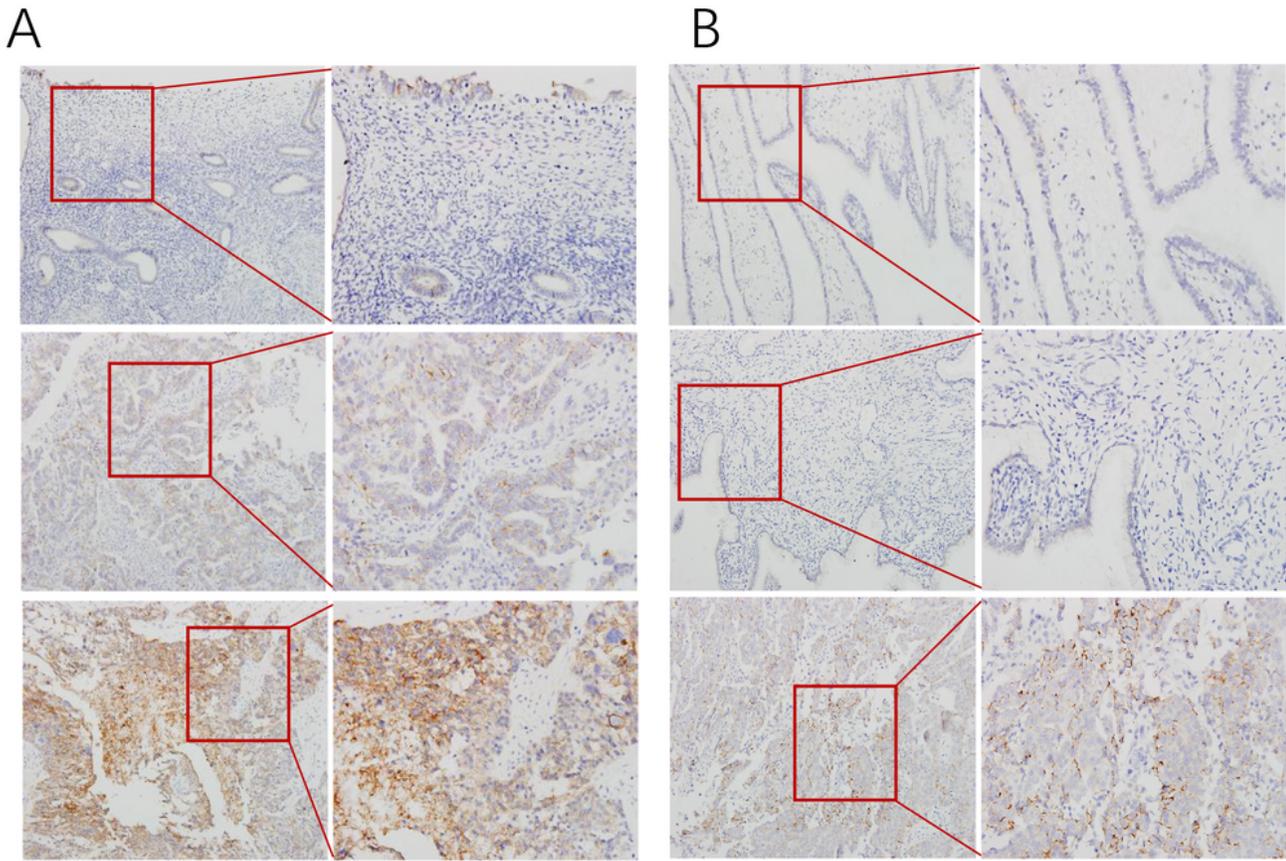


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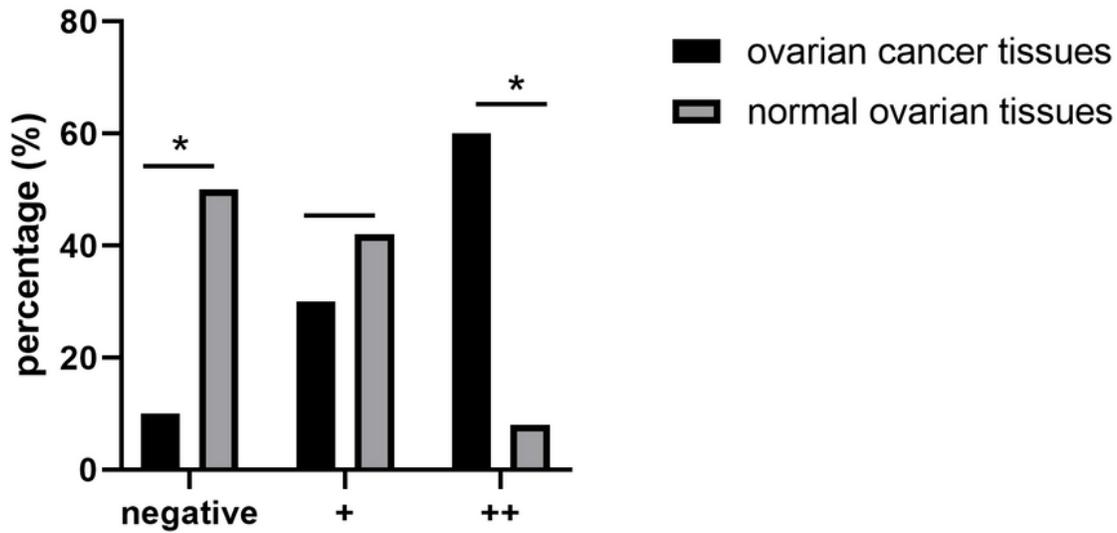
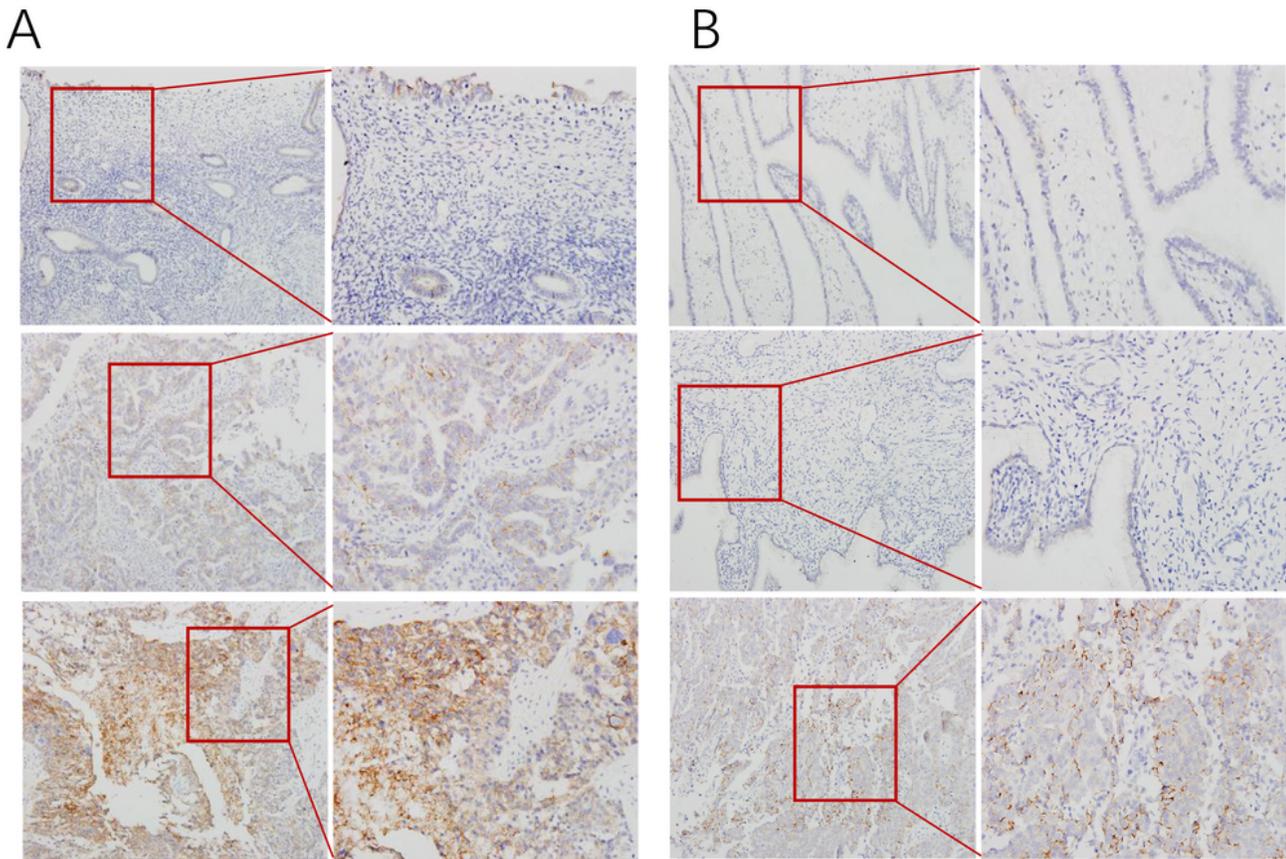


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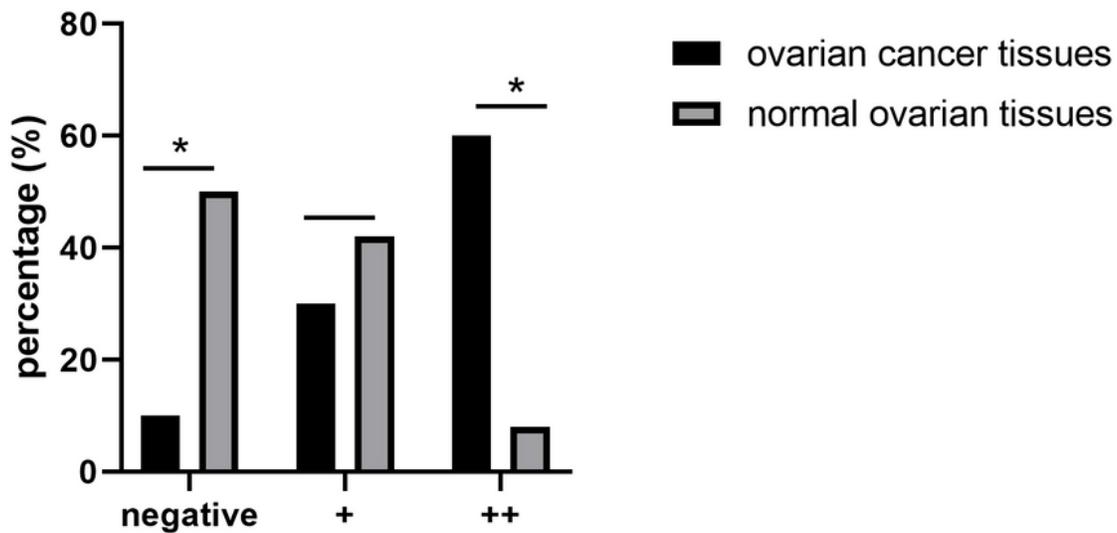
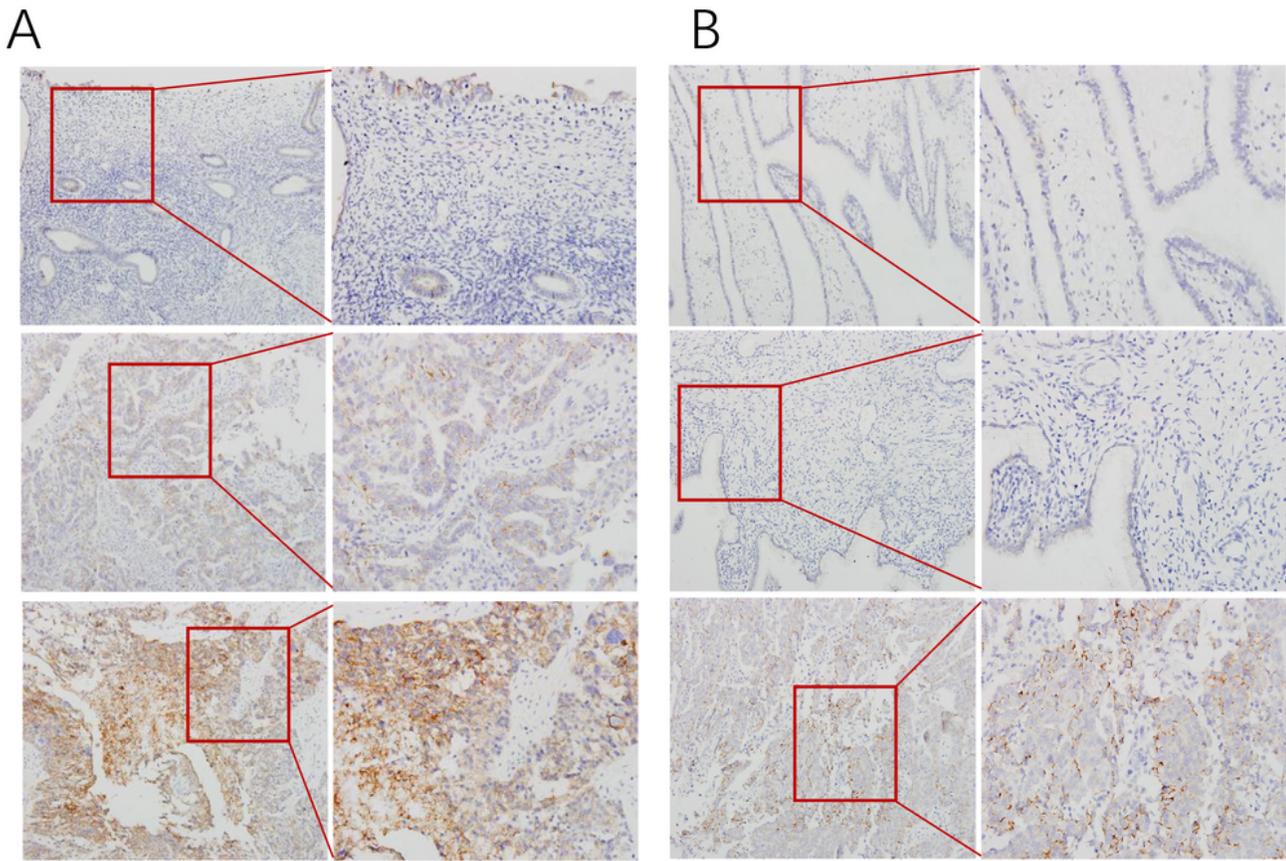


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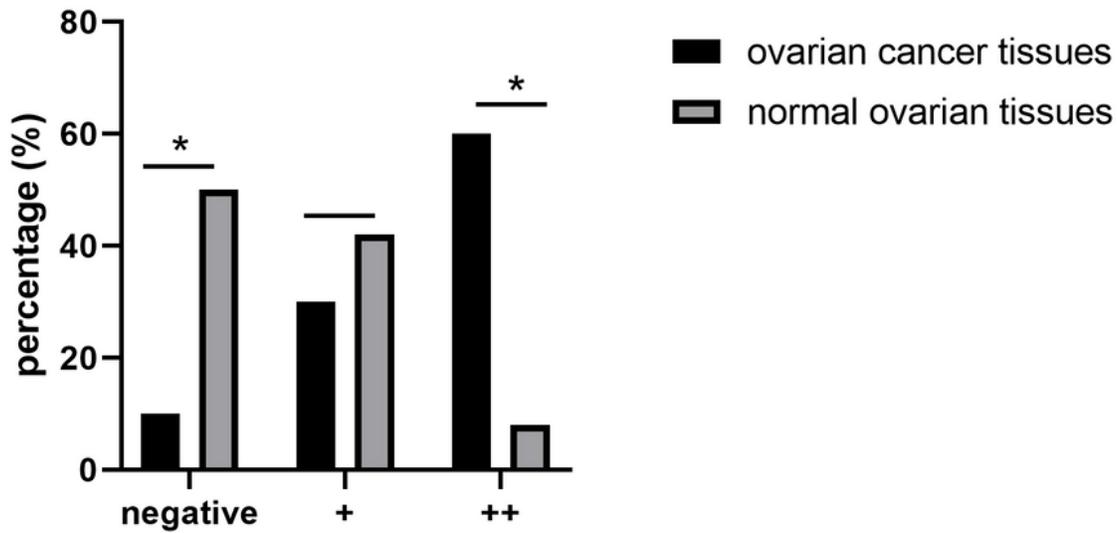


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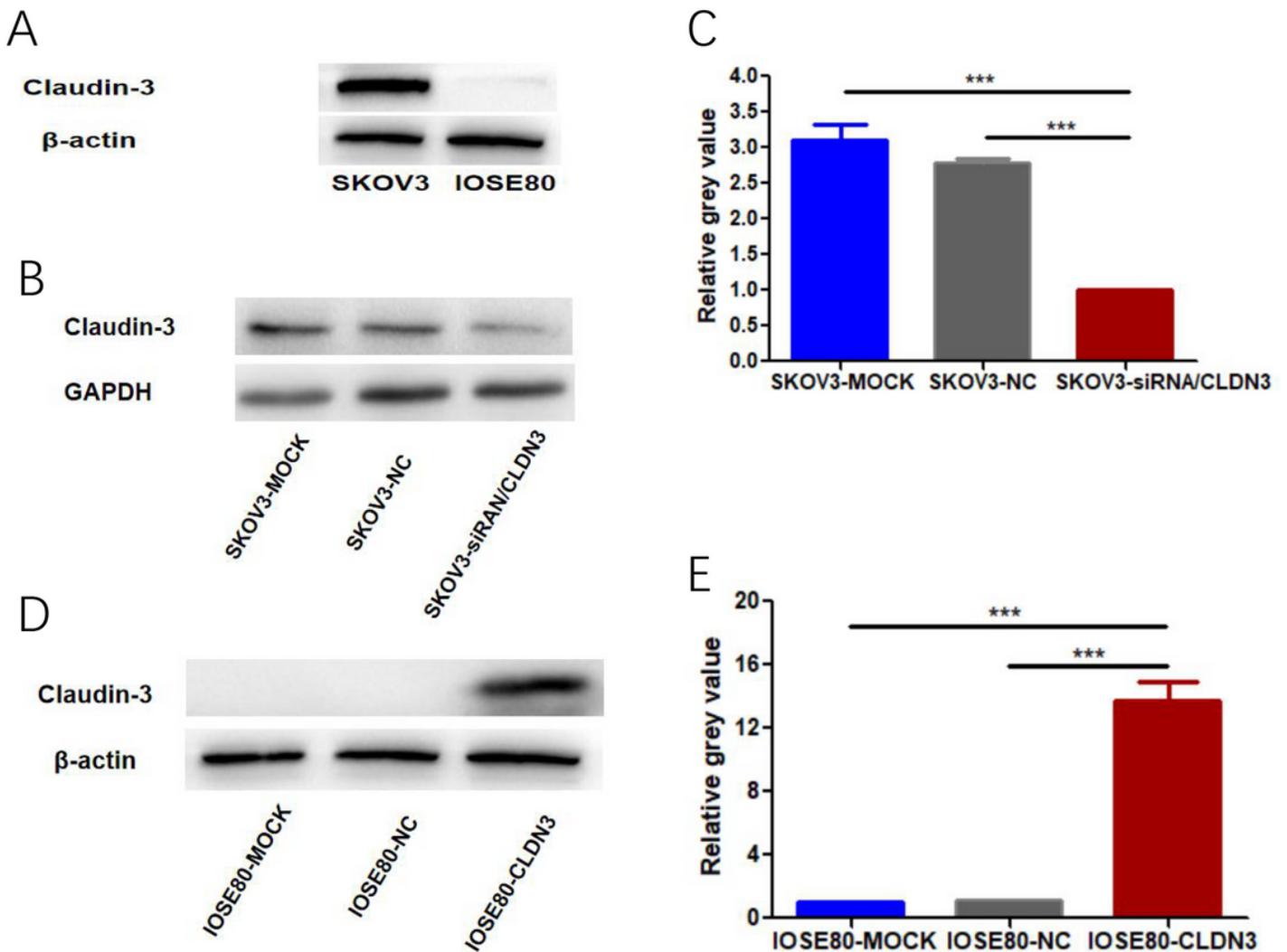


Figure 2

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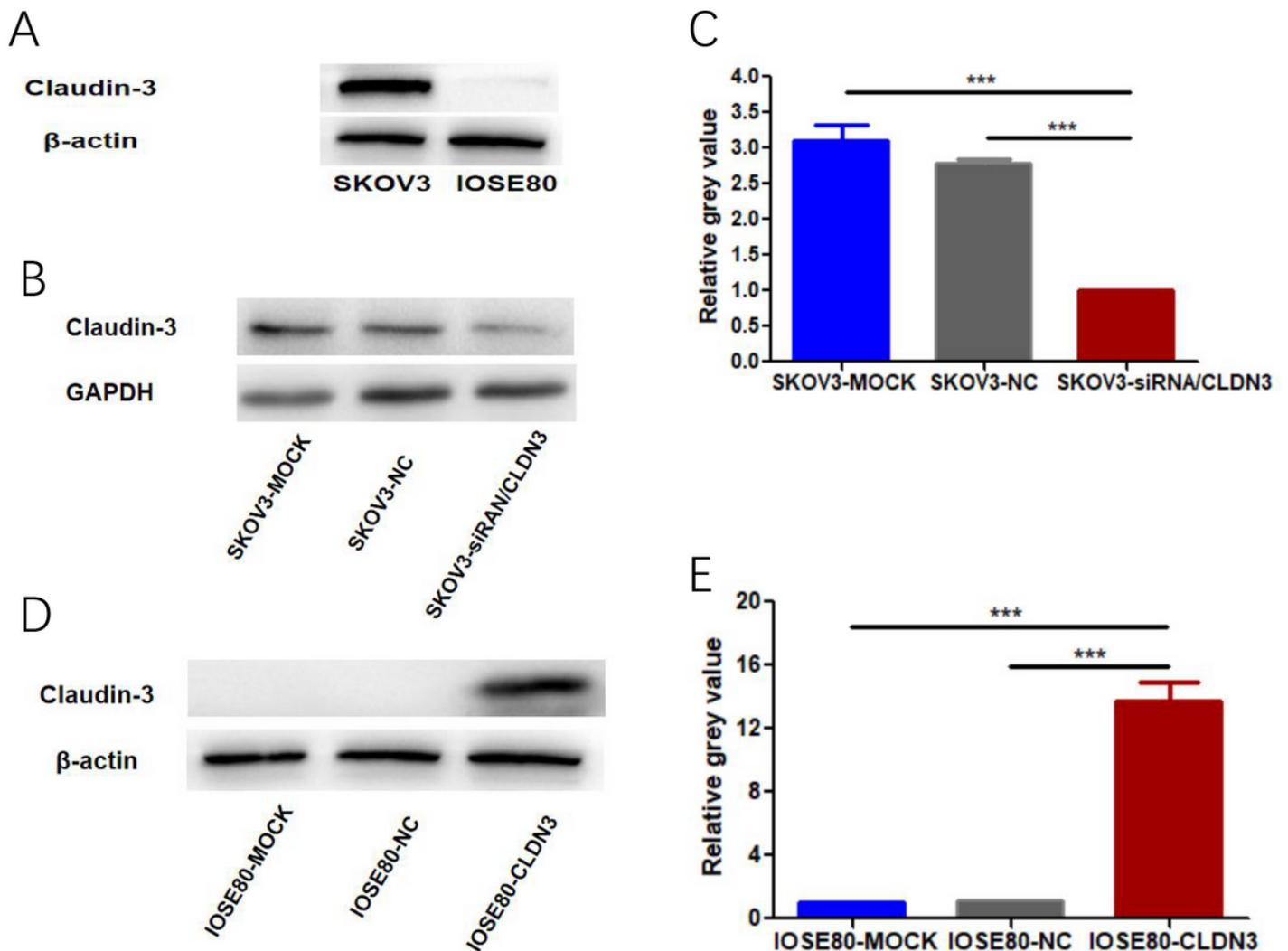


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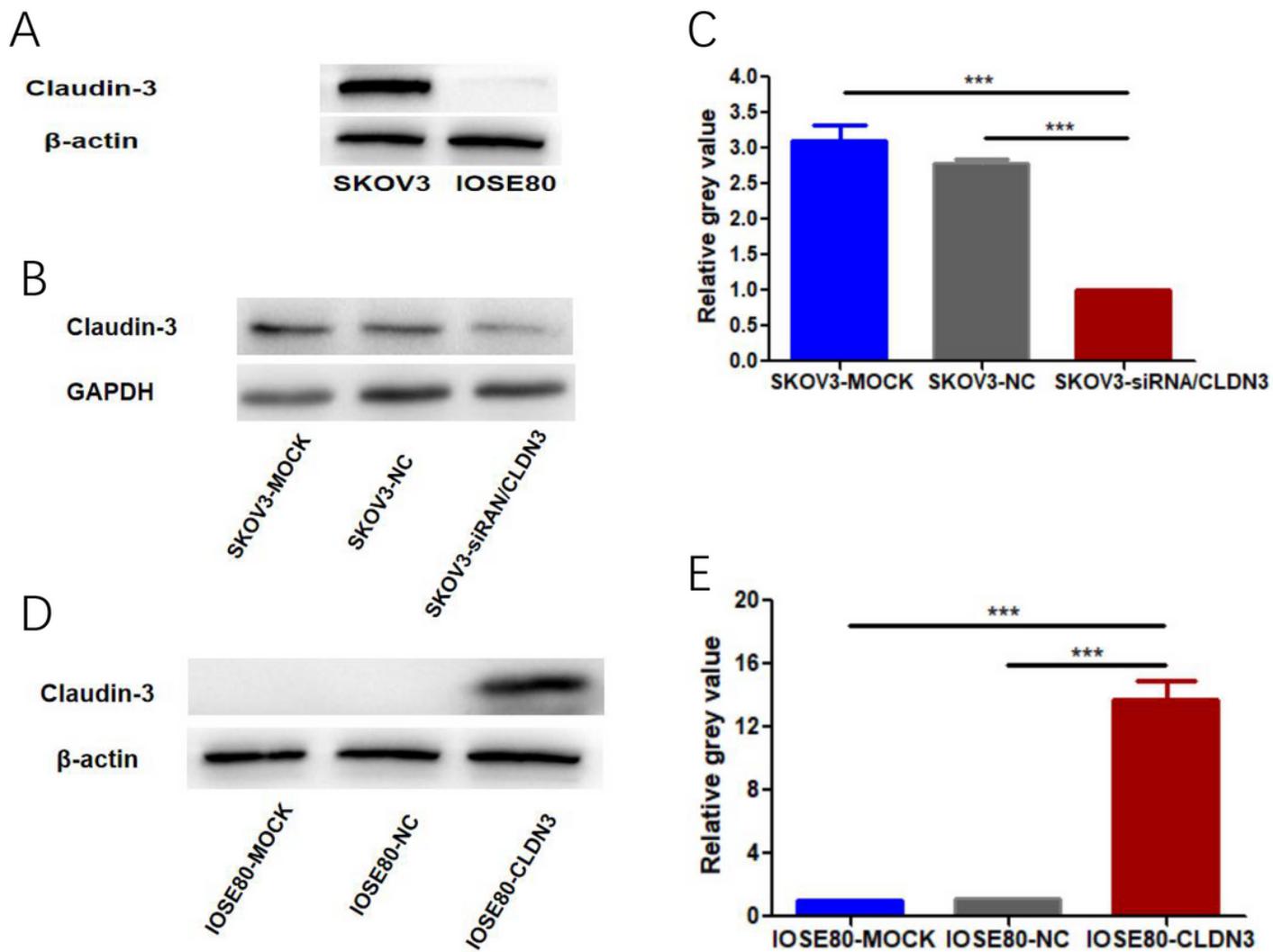


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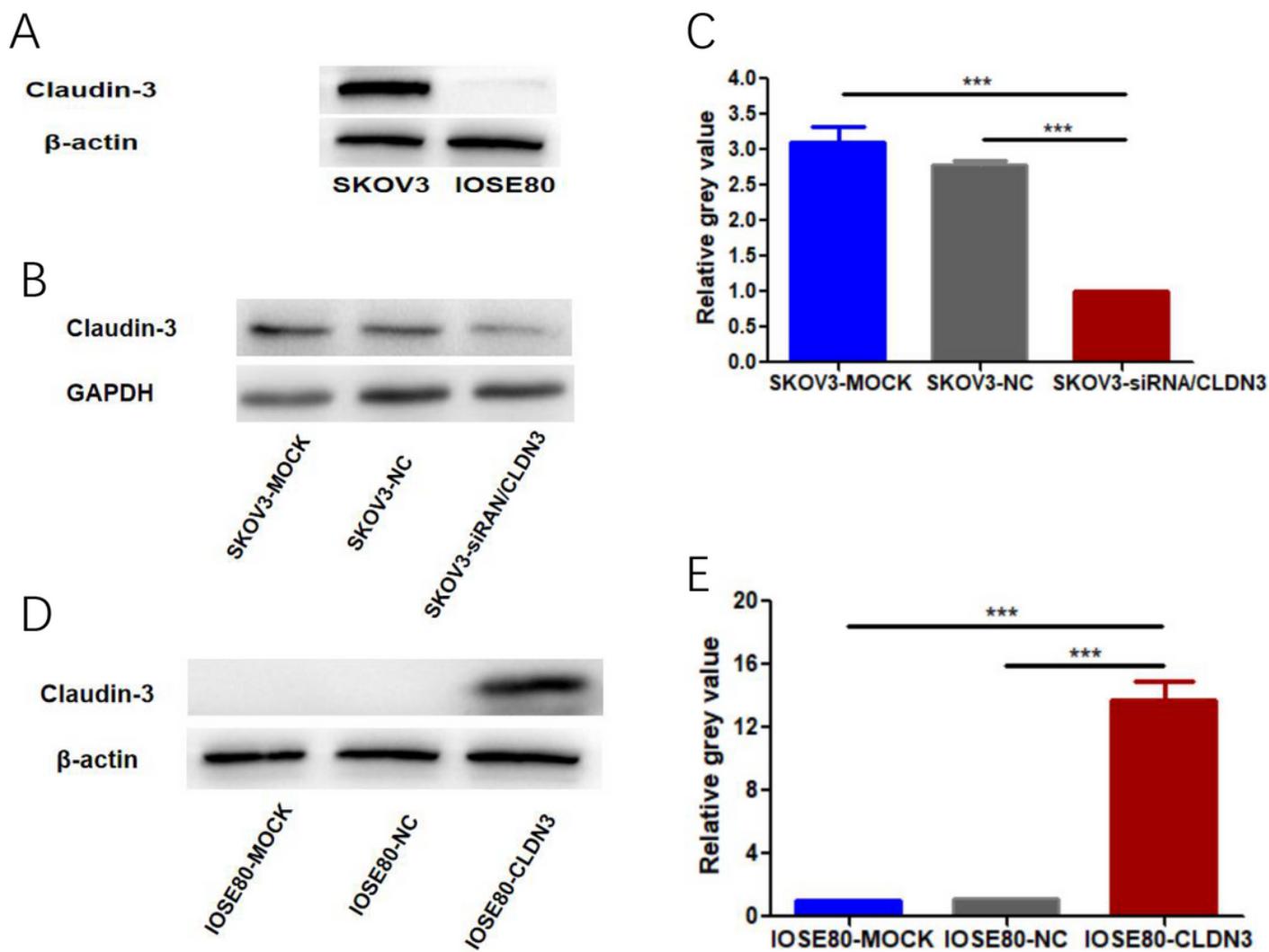


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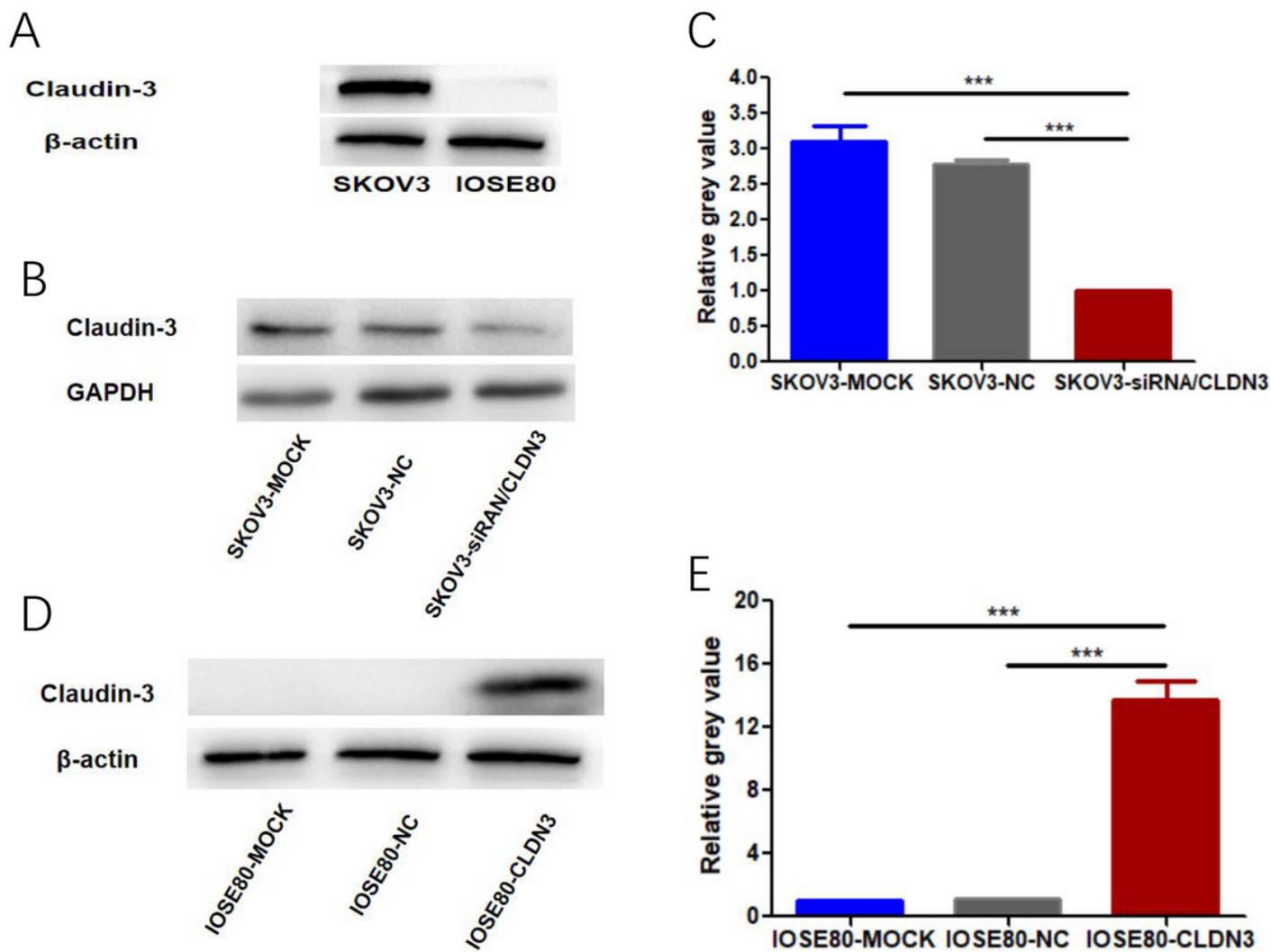


Figure 2

In order to evaluate CLDN3 expression in ovarian cell lines, ovarian cancer cell line SKOV3 and normal ovarian cell line IOSE80 were evaluated by Western Blot. As shown in Fig 2A, CLDN3 protein was not detected in normal ovarian cell line IOSE80, but was present in ovarian cancer cell line SKOV3. Next, we constitutively knocked down their expression in ovarian cancer cell line SKOV3 using siRNA vectors. The results revealed that CLDN3 protein expression was significantly suppressed in SKOV3 cells, with the relative grey value lower than mock-control and non-specific-control cells (NC control cells) ($P < 0.001$) (Fig 2B, C). The normal ovarian cell line IOSE80, which showed lower level of CLDN3 protein expression, was also transfected with lentiviral vectors expressing CLDN3 and results showed the CLDN3 expression significantly increased after transfection compared with control cells, with the relative grey value higher than mock-control and non-specific-control cells ($P < 0.001$). These results showed high expression of CLDN3 in ovarian cancer cell lines, and confirm that CLDN3 can be reduced by CLDN3-specific siRNA (Fig 2D, E).

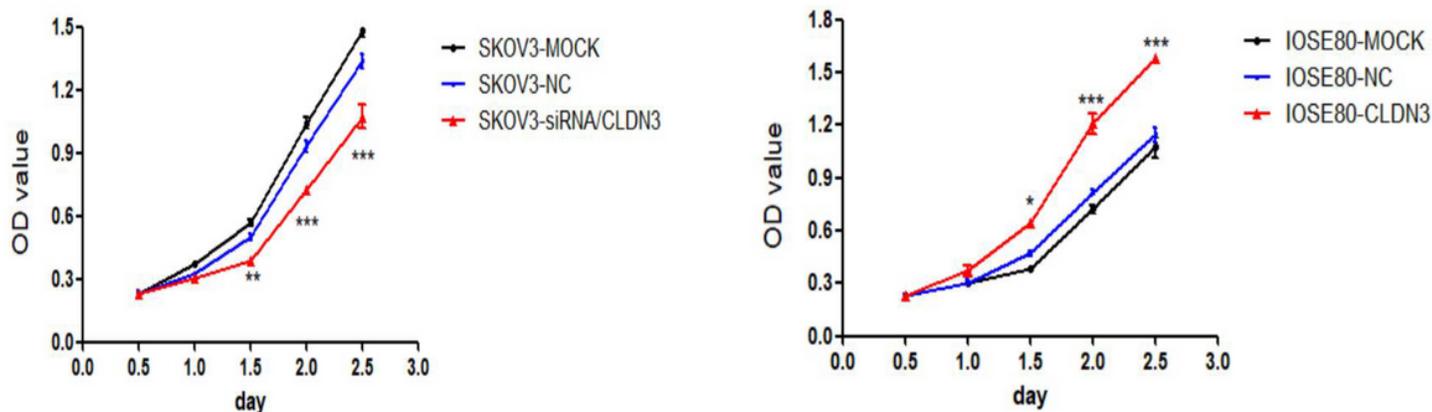


Figure 3

In order to determine the effect of CLDN3 on the growth of ovarian cancer cells and normal ovarian cells, MTT assay was conducted to determine the cellular proliferation. We first performed transient siRNA-mediated knockdown of CLDN3 expression in ovarian cancer cell line SKOV3 and tested the cellular viability. The results showed that OD values decreased at every time point tested. The significant changes started from 1.5 days of cell culture (Fig 3A). To further confirm role of CLDN3 in cellular survival, CLDN3 was upregulated in IOSE80 cells. Consistent with what was observed with SKOV3 cells, we found that stable overexpression of this protein led to increased cellular viability compared to mock-transfected cells or parental IOSE80 cell (Fig 3B).

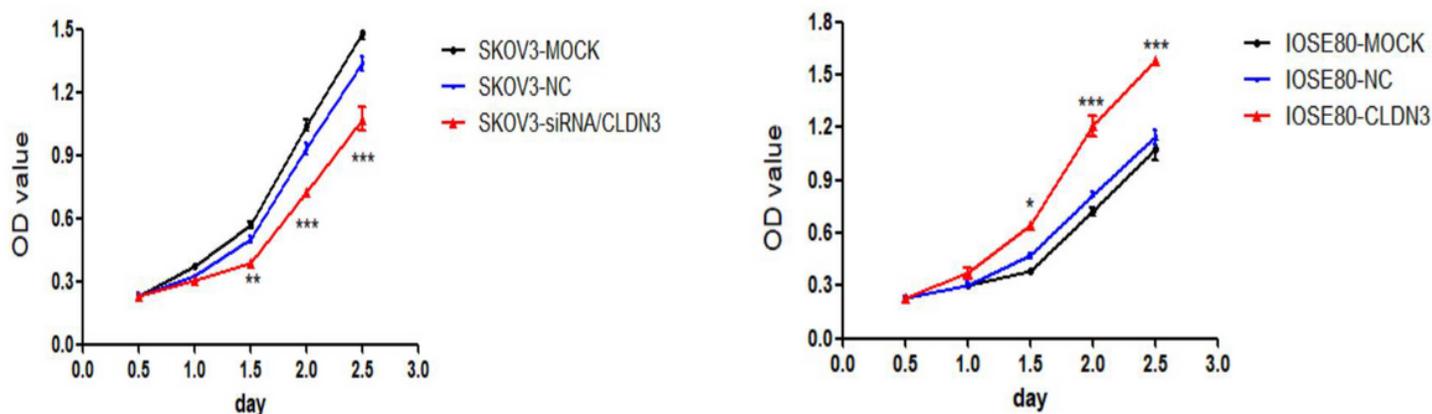


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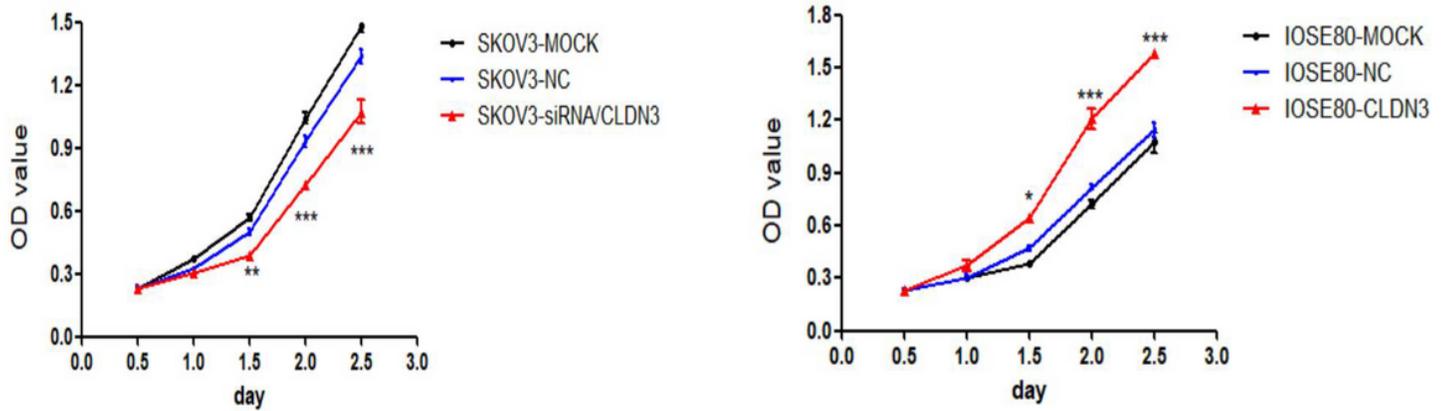


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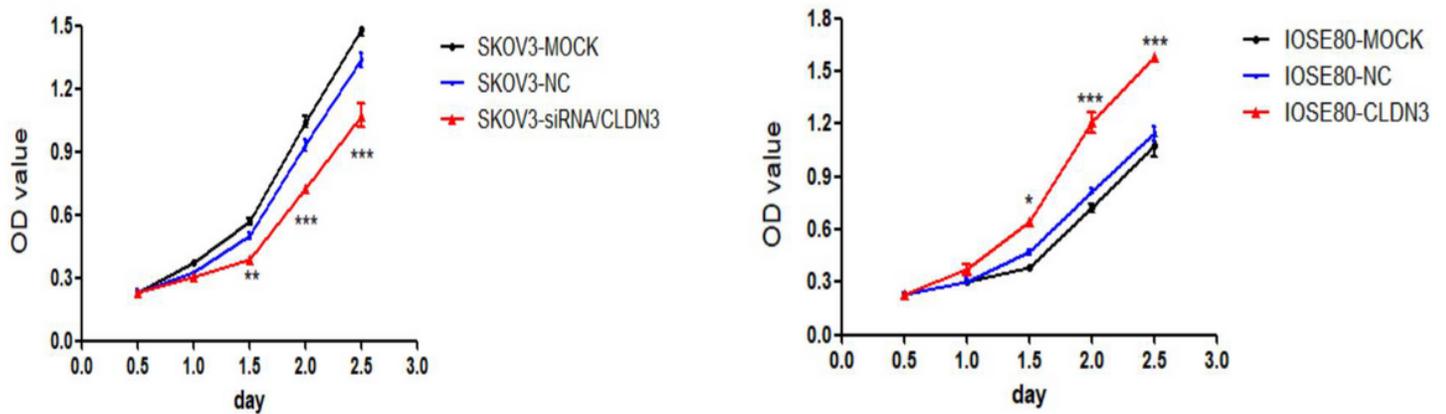


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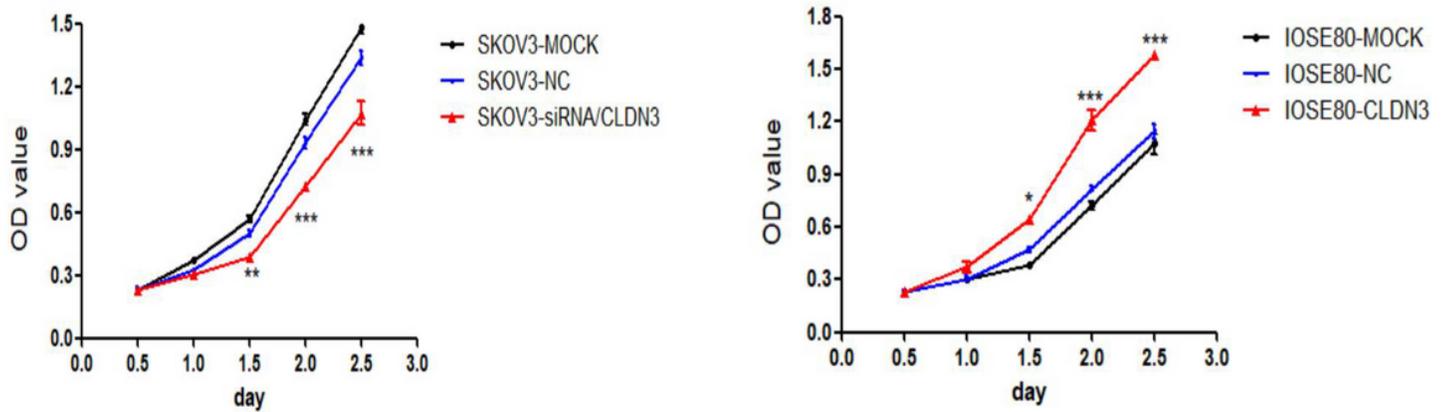


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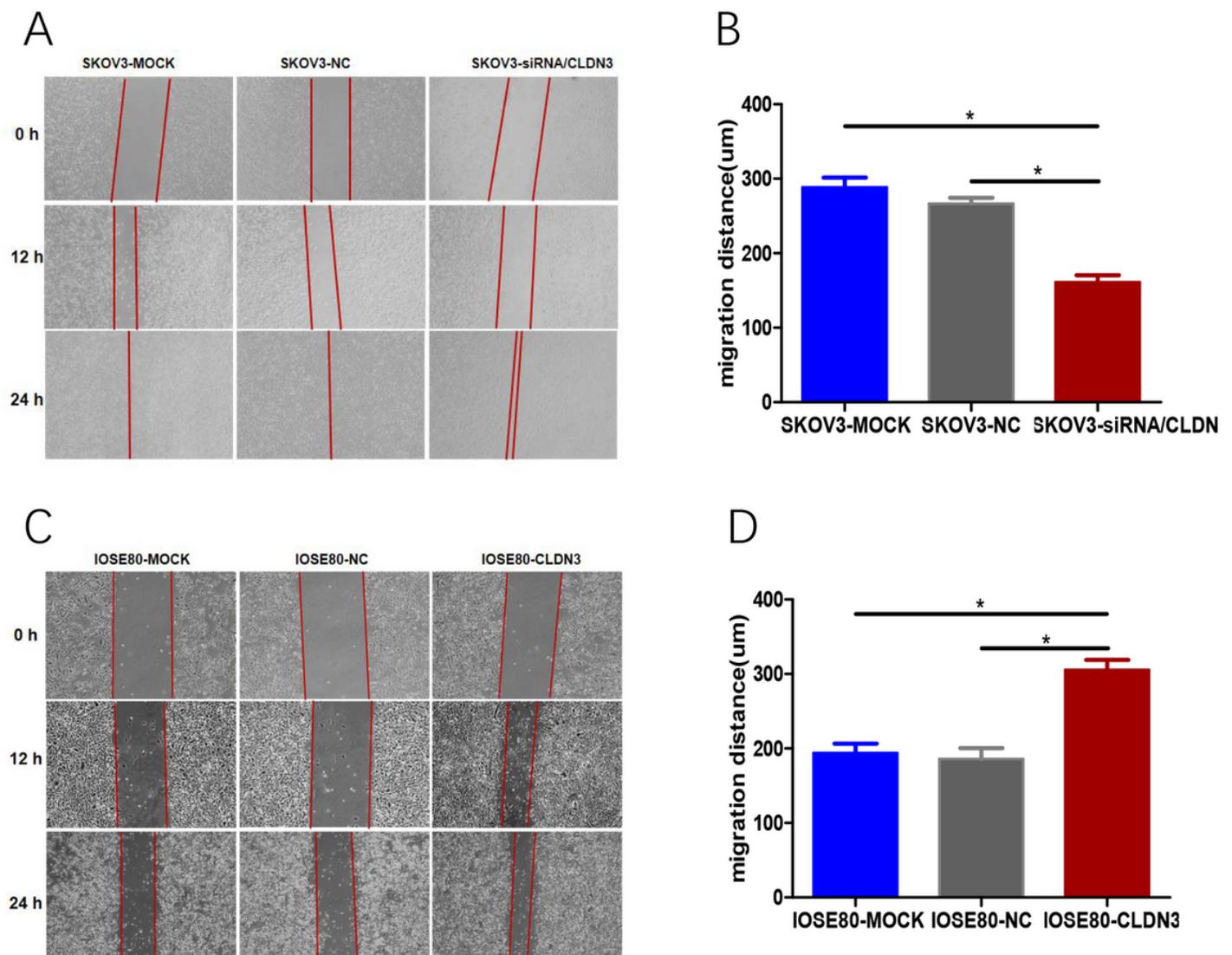


Figure 4

Since CLDN3 is a tight junction (TJ) protein and disruption of TJ barrier could facilitate dissociated cancer cells to metastasize [11], we thus determine the migrative role of CLDN3 in SKOV3 and IOSE80 cells by wound-healing assays. The migration of SKOV3 cells was significantly suppressed in CLDN3-knockdown SKOV3 cells compared with control cells after 12-hour transfection ($P < 0.05$). Twenty-four hours post siCLDN3 transfection, the wound-healing assays showed a significant larger migration distance, indicating a decreased cellular migration ability in CLDN3-knockdown SKOV3 cells compared with control cells ($P < 0.05$) (Fig 4A, B). Similarly, upregulation of CLDN3 in normal ovarian cell IOSE80 showed a smaller migration distance, indicating an increased migrative ability in IOSE80-CLDN3 cells compared with the control cells (Fig 4C, D).

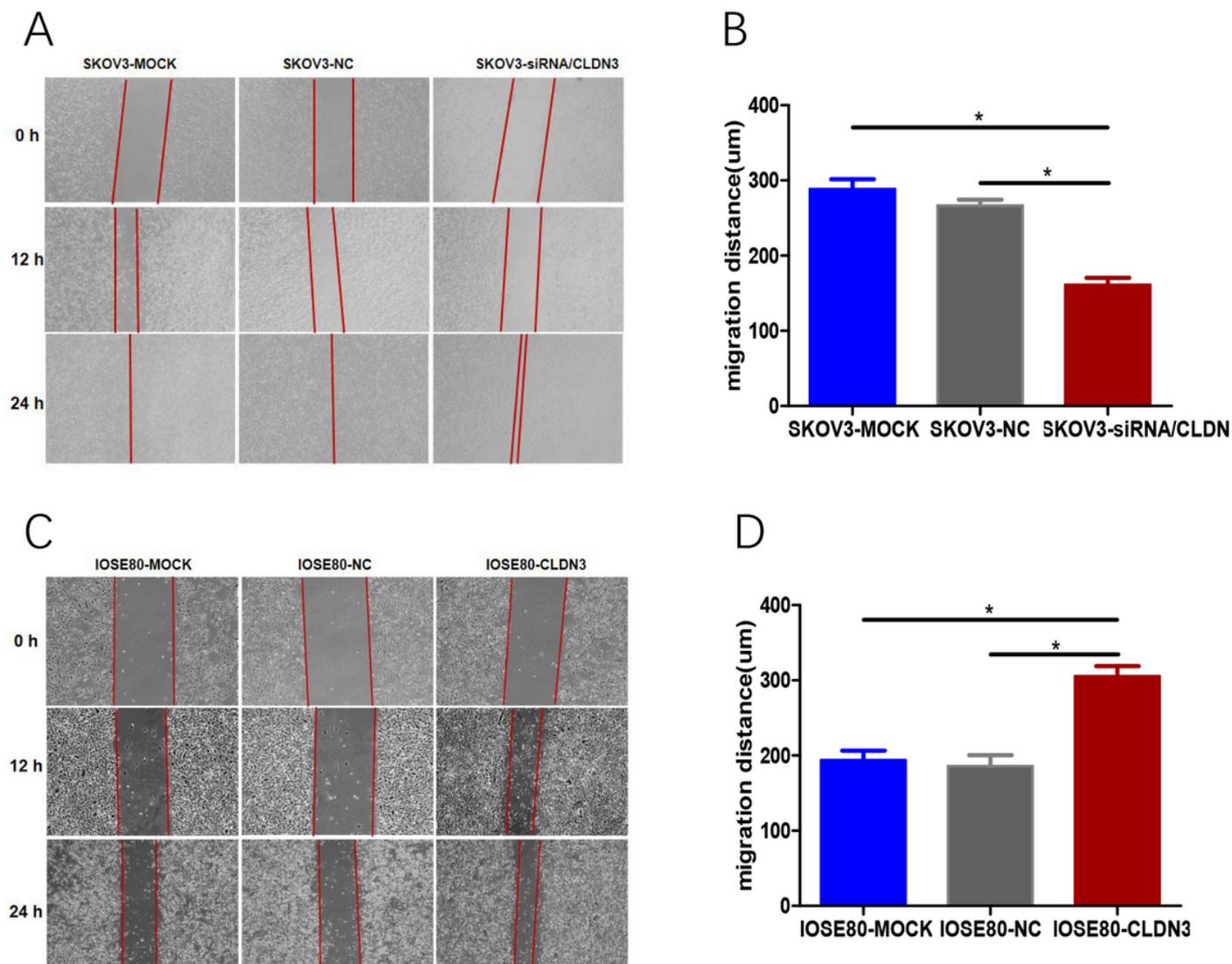


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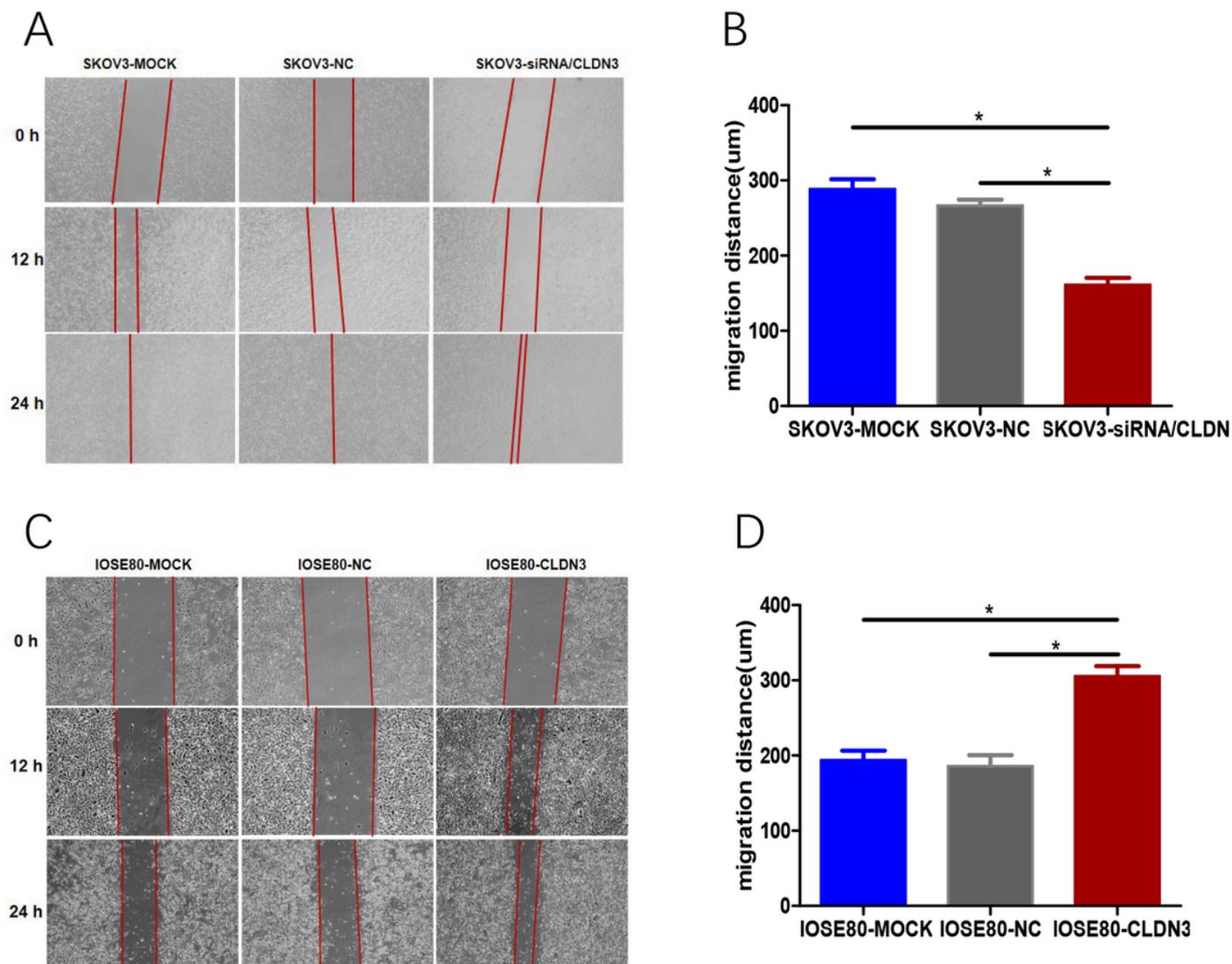


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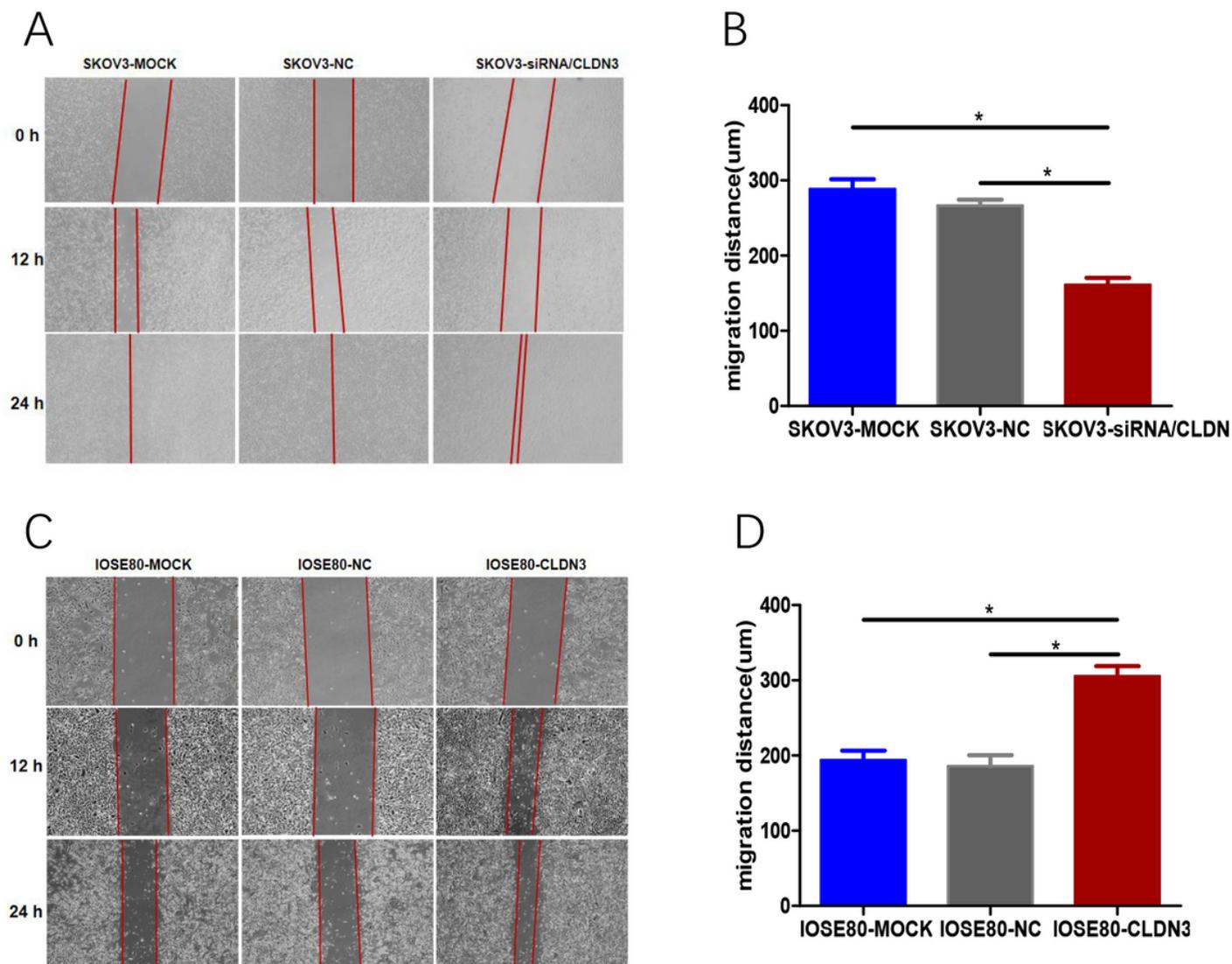


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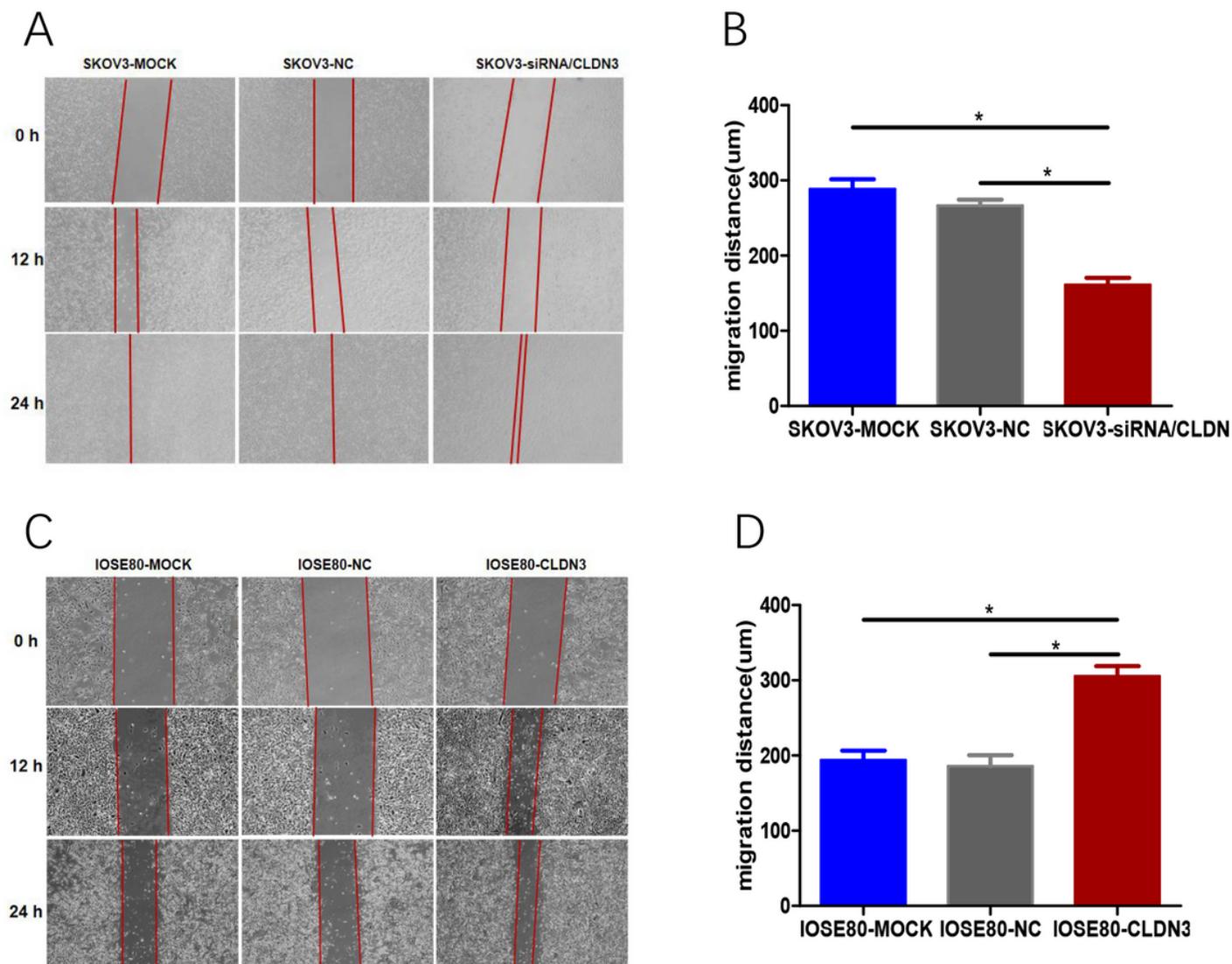


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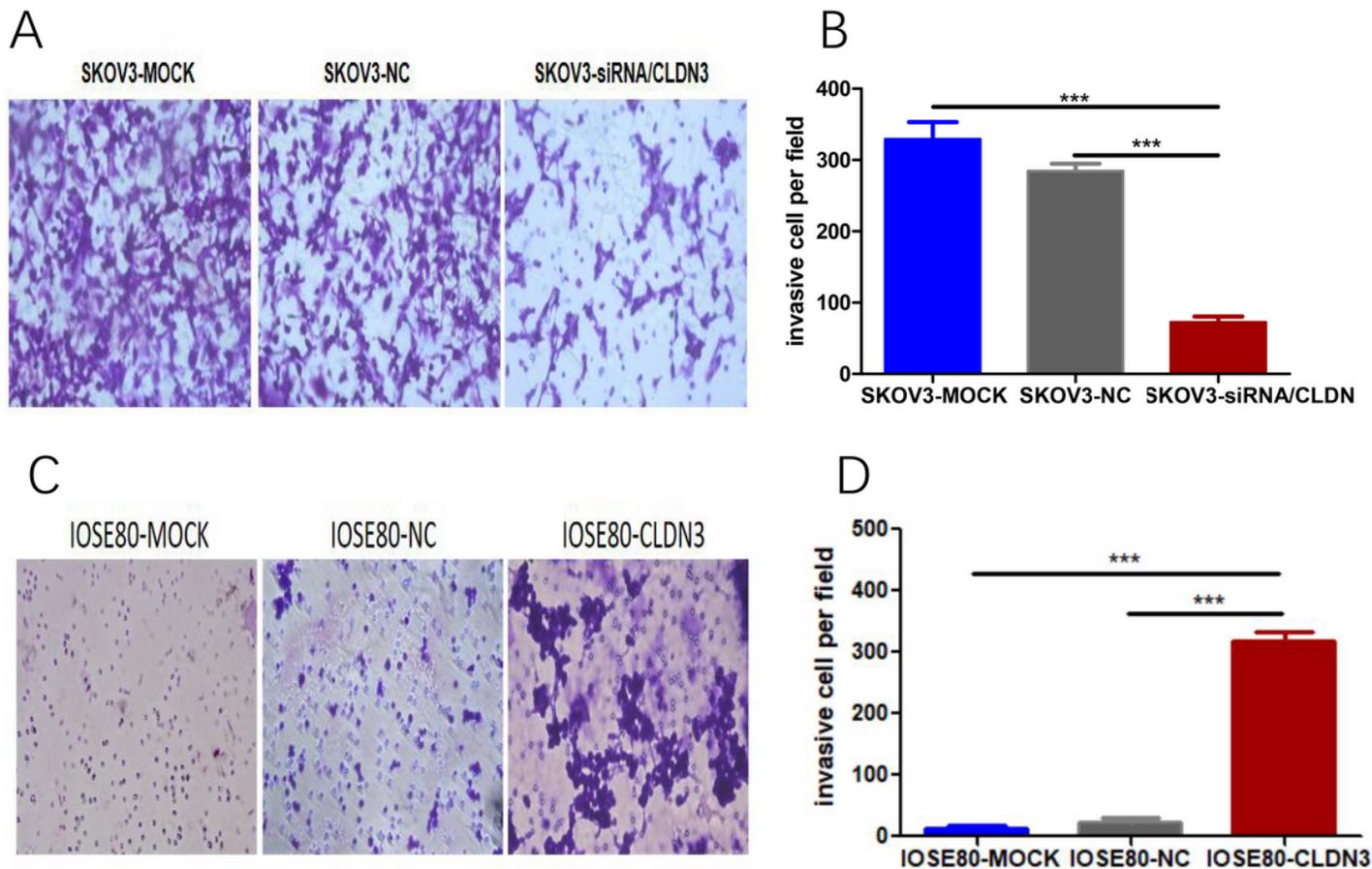


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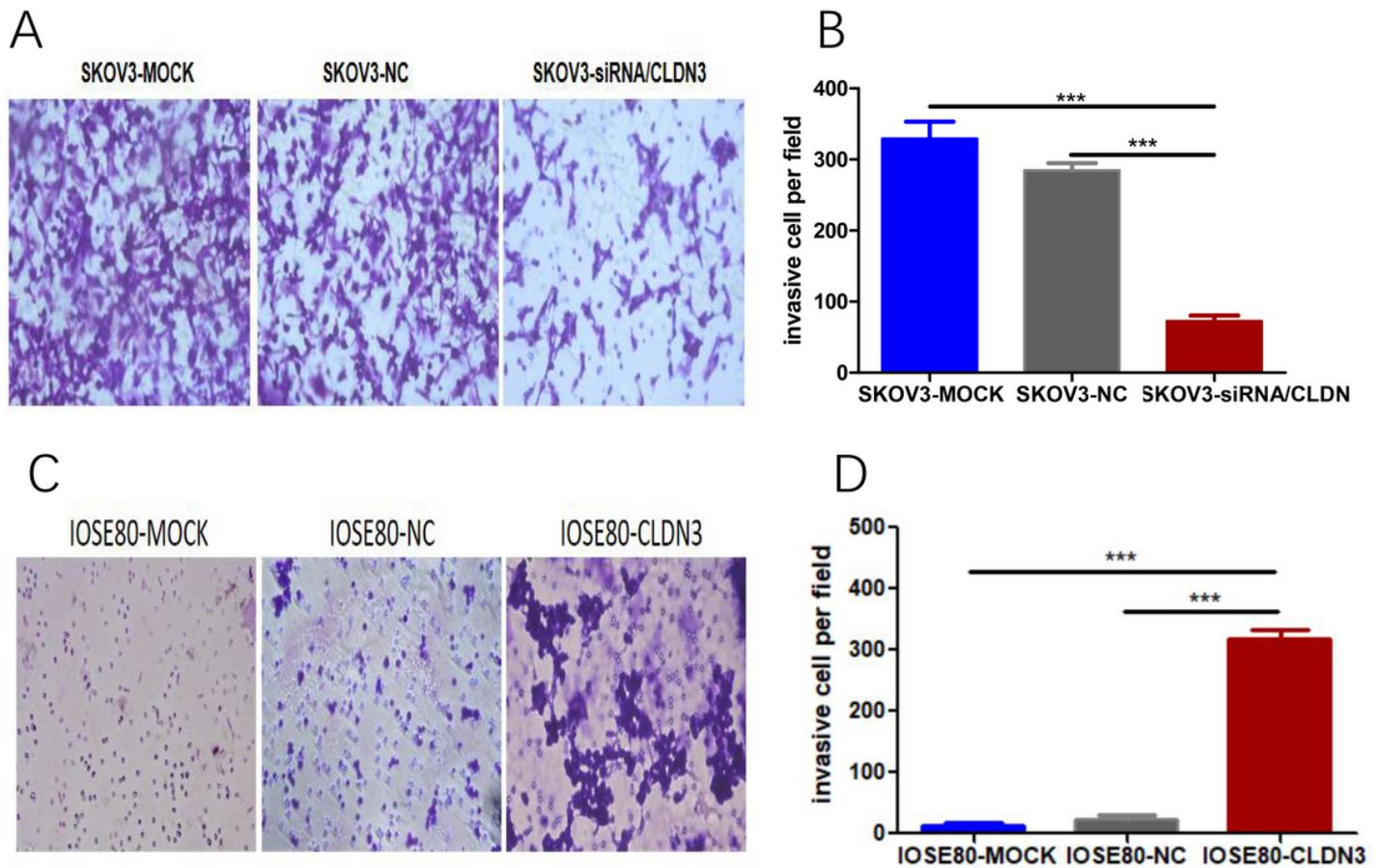


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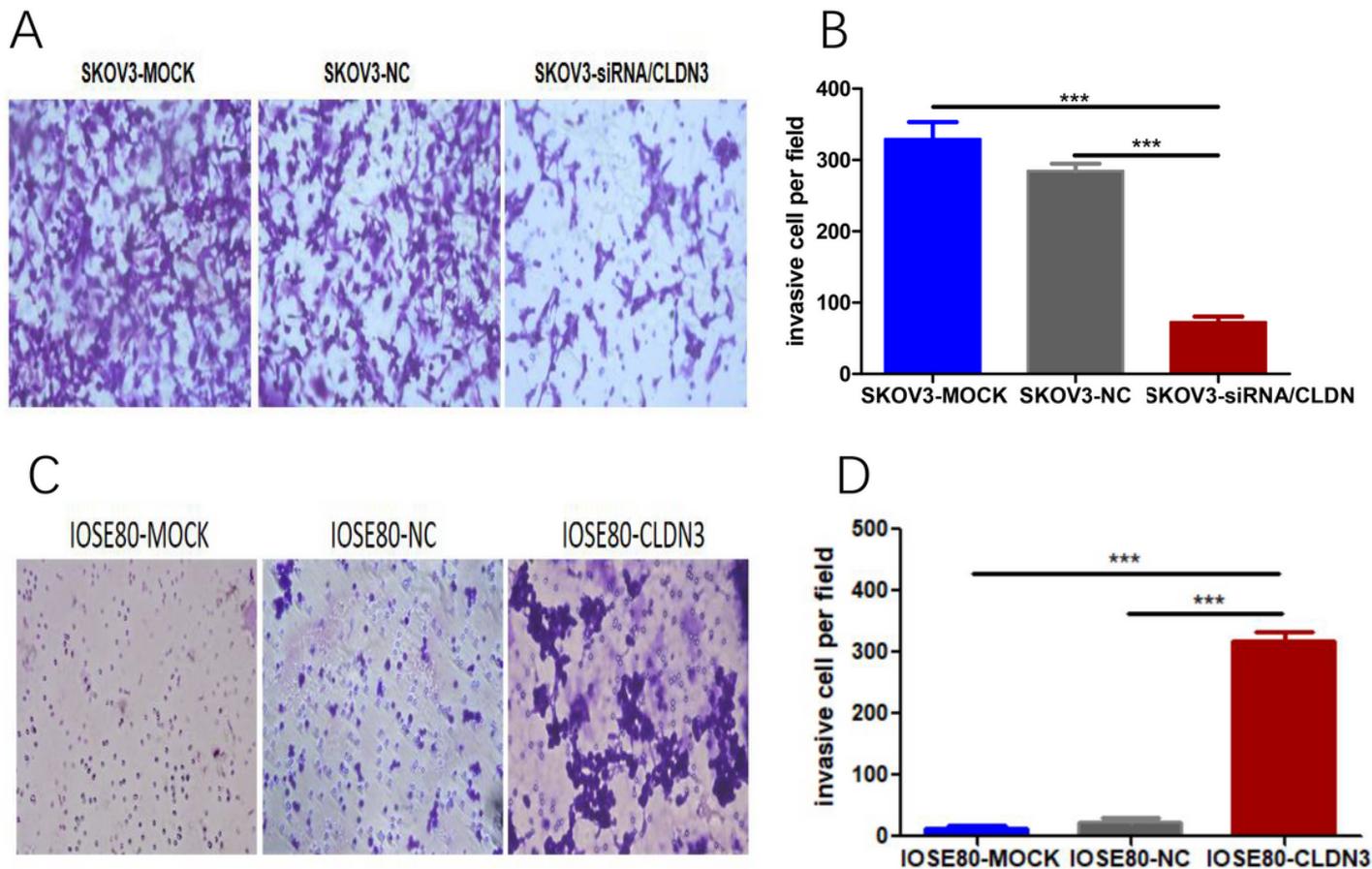


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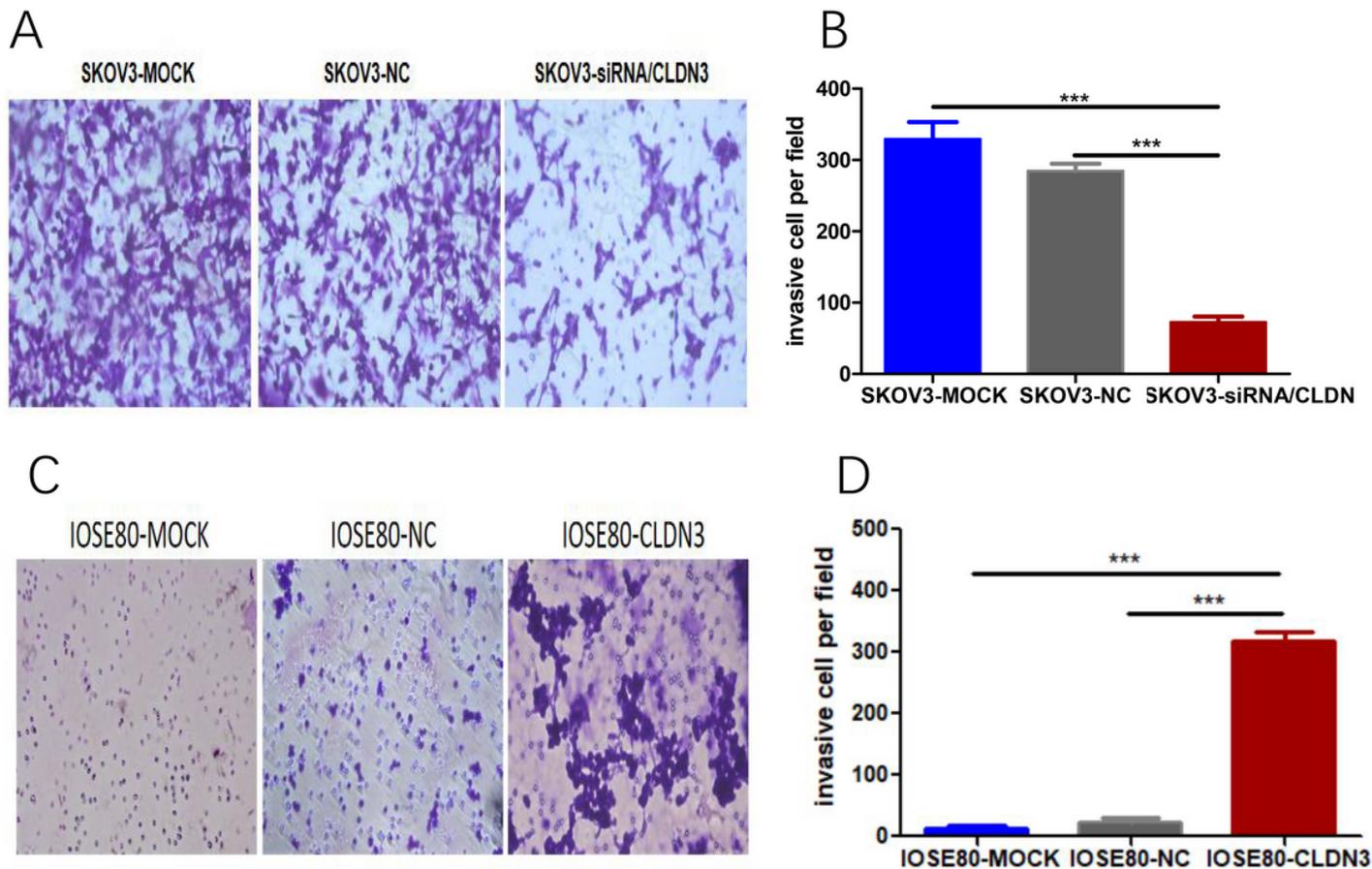


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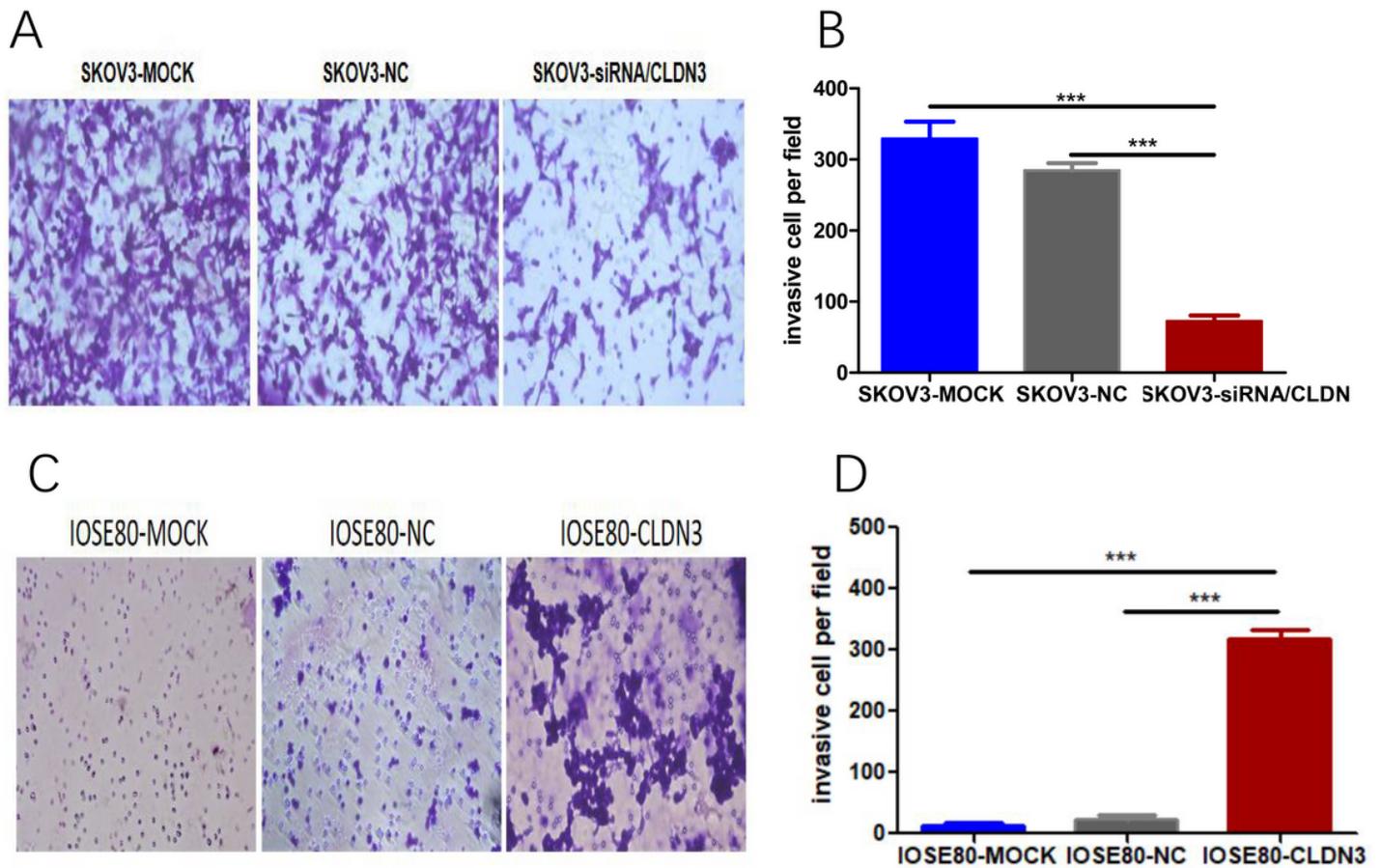


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