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## **PALMD supports spheroid integrity and anoikis resistance in ovarian cancer cells**

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## Abstract

Peritoneal metastasis of ovarian cancer is mediated by multicellular spheroids that survive under anchorage-independent conditions. However, the molecular mechanisms underlying spheroid survival remain unclear. This study aimed to investigate the role of palmdelphin (PALMD), a membrane-associated protein of the paralemmin family, in ovarian cancer spheroid adaptation. A human ovarian cancer cell culture (OVCSC) derived from malignant ascites was established, and its spheroid-forming and cancer stem-like features, including CD44, EpCAM, and ALDH expression, were confirmed. Transcriptomic analyses of MESOV cells cultured under two-dimensional and three-dimensional conditions revealed extensive gene expression remodeling, with 3,791 differentially expressed genes. *PALMD* was significantly upregulated in three-dimensional MESOV cell culture compared with two-dimensional culture. Functional suppression of *PALMD* in OVCSC cells was associated with increased p53 expression, disrupted spheroid integrity, enhanced vimentin expression, and elevated apoptosis, as shown by Annexin V, terminal deoxynucleotidyl transferase dUTP nick end labeling, and caspase 3/7 assays. Endothelial co-culture further increased *PALMD* expression and was associated with reduced p53 and increased VEGF expression levels. These findings suggest that PALMD contributes to spheroid integrity and anoikis resistance in ovarian cancer cells and may represent a context-dependent regulator of peritoneal metastasis.

**Keywords:** ovarian cancer; PALMD; anoikis; spheroid; peritoneal metastasis; three-dimensional culture

## Introduction

Ovarian cancer is characterized by a distinctive metastatic route in which tumor cells disseminate within the peritoneal cavity. Rather than depending primarily on hematogenous spread, ovarian cancer cells detach into ascitic fluid, form multicellular spheroids, and subsequently attach to mesothelial and stromal surfaces. The clinical burden associated with this intraperitoneal, spheroid-forming route is reflected by the fact that relapse occurs in more than 85% of patients with advanced ovarian cancer, and the peritoneum is the predominant site of recurrence; among patients with advanced disease, 75% of recurrences involve the peritoneum and 40% are confined to the peritoneum [1,2]. Spheroids also contribute to residual microscopic disease, chemoresistance, and postoperative recurrence [3–7]. Peritoneal metastasis requires tumor cell survival after detachment from the extracellular matrix. Detachment normally induces anoikis, a form of programmed cell death that prevents inappropriate survival of epithelial cells outside their native tissue [8,9]. Ovarian cancer spheroids acquire anoikis resistance through coordinated changes in cell–cell adhesion, cytoskeletal organization, epithelial–mesenchymal plasticity, metabolic adaptation, and interactions with the local microenvironment [10–14]. Despite the clinical importance of spheroid-mediated recurrence, the molecular mechanisms that enable spheroid adaptation and survival remain incompletely understood. Palmdelphin (PALMD), a member of the palemmin protein family, has been implicated in membrane dynamics and cell shape control [15]. Studies in endothelial cells have further shown that PALMD regulates nuclear resilience to mechanical stress and that PALMD deficiency activates NF- $\kappa$ B signaling in valvular endothelial cells [16,17]. PALMD is also a p53-associated molecule that participates in cell death responses following DNA damage [18]. Although PALMD has recently been reported to inhibit ovarian cancer stem specification via downregulation of ring finger protein 145 (RNF145) [19], whether PALMD regulates multicellular spheroid integrity and anoikis resistance in ascites-adapted ovarian cancer cells remains unknown. This unresolved question is relevant because ovarian cancer spheroid cells with stem-like properties contribute to tumor generation, metastasis, and chemotherapy resistance [20]. This study aimed to investigate whether PALMD contributes to ovarian cancer spheroid adaptation and survival and to evaluate its potential relevance to spheroid-mediated peritoneal metastasis. Transcriptomic analyses of MESOV cells (an in vitro and in vivo model of ovarian cancer) under two-dimensional (2D) and three-dimensional (3D) culture conditions were integrated with functional experiments using a patient-

derived ovarian cancer spheroid-forming cell culture (OVCSC) established from malignant ascites. A strength of this design was the combination of an established ovarian cancer model for transcriptomic screening with a clinically relevant, ascites-derived spheroid model for functional validation. The findings showed that PALMD was induced during 3D adaptation, that PALMD depletion disrupted spheroid integrity and enhanced apoptosis, and that endothelial niche-related conditions increased PALMD expression. These findings suggest that PALMD is a context-dependent regulator of spheroid survival that may be relevant to peritoneal metastasis and could inform strategies aimed at disrupting ovarian cancer spheroid persistence.

## **Results**

### **OVCSC cells show spheroid-forming and cancer stem-like features**

To establish a clinically relevant model of ovarian cancer cells that survive in ascitic fluid, OVCSC cells derived from malignant ascites were characterized under spheroid-forming conditions. OVCSC cells formed multicellular spheroids under ultra-low attachment conditions, supporting their suitability for studying anchorage-independent survival (Fig. 1a). Flow cytometry analysis showed that OVCSC cells expressed cancer stem-like surface markers, with CD44-positive and EpCAM-positive fractions of 38.1% and 52.8%, respectively, whereas the CD45-positive fraction was low (0.3%) (Fig. 1b). OVCSC cells also demonstrated high aldehyde dehydrogenase (ALDH) activity, with an ALDH-positive fraction of 51.5% (Fig. 1c). Together, these findings indicate that OVCSC cells retain spheroid-forming and cancer stem-like features and provide a suitable patient-derived model for the functional analysis of anoikis resistance.

### **Transcriptomic analysis identifies *PALMD* as a 3D-associated gene in MESOV cells**

To identify genes associated with spheroid adaptation, transcriptomic analyses were performed using MESOV cells cultured under 2D adherent and 3D spheroid-forming conditions. Heatmap analysis revealed distinct gene expression patterns between MESOV-2D and MESOV-3D cultures, indicating broad transcriptional remodeling during 3D adaptation (Fig. 2a). Volcano plot analysis further demonstrated substantial differential gene expression between the two culture conditions (Fig. 2b). Using thresholds of  $Q < 0.05$  and  $|\log_2 \text{fold change}| \geq 1$ , 3,791 differentially expressed genes (DEGs) were identified, including 1,810 upregulated and 1,981 downregulated genes in MESOV-3D cells compared with MESOV-2D cells. Among these

DEGs, *PALMD* was significantly upregulated in MESOV-3D cells compared with MESOV-2D cells (fold change = 2.76, log2 fold change = 1.47,  $Q = 1.30 \times 10^{-7}$ ) (Fig. 2c). These findings suggest that *PALMD* is associated with transcriptional programming during 3D spheroid adaptation in ovarian cancer cells. To further characterize the biological features associated with 3D adaptation, representative DEGs upregulated or downregulated in MESOV-3D cells relative to MESOV-2D cells were summarized (Table 1). Genes associated with extracellular matrix organization, cell adhesion, proteolysis, and stemness, including *PODXL*, *CDH6*, *MMP2*, *COL1A1*, *CTSD*, and *SOX2*, were markedly upregulated in 3D culture. These findings indicate that MESOV cells undergo coordinated remodeling of adhesion-, matrix-, proteolysis-, and stemness-related programs during spheroid formation. A complete list of DEGs is provided in Supplementary Table S1.

### ***PALMD* knockdown increases p53 expression in OVCSC cells**

To examine the functional role of *PALMD* in OVCSC cells, *PALMD*-knockdown cells, hereafter referred to as sh*PALMD* cells, were generated using shRNA targeting *PALMD*. Western blotting confirmed that *PALMD* protein expression was efficiently reduced in sh*PALMD* cells under both 2D and 3D culture conditions (Fig. 3a, b). Notably, *PALMD* knockdown was associated with increased p53 protein expression, particularly under 3D culture conditions (Fig. 3a, c). These findings indicate that *PALMD* was successfully suppressed in OVCSC cells and suggest an inverse association between *PALMD* expression and p53-related stress signaling during spheroid adaptation.

### ***PALMD* knockdown disrupts spheroid integrity and induces partial epithelial–mesenchymal transition (EMT)-like changes**

Considering that *PALMD* has been implicated in membrane and cytoskeletal regulation, the impact of *PALMD* depletion on spheroid morphology and epithelial–mesenchymal plasticity was evaluated. Under adhesion conditions, shControl OVCSC spheroids maintained a relatively compact morphology, whereas sh*PALMD* spheroids showed increased spreading and partial dissociation at the adhesion surface, suggesting reduced spheroid integrity (Fig. 4a). Next, the expression of EMT-related markers was assessed. Western blotting showed that *PALMD* knockdown increased vimentin expression, particularly under 3D culture conditions. E-cadherin and N-cadherin levels showed modest changes (Fig. 4b); however, these changes did not indicate

a complete cadherin switch. These findings suggest that PALMD depletion disrupts spheroid integrity and induces partial EMT-like changes characterized mainly by vimentin upregulation.

### ***PALMD* knockdown enhances apoptosis in OVCSC spheroids**

The association of spheroid integrity disruption caused by PALMD depletion with apoptosis under anchorage-independent conditions was explored. Annexin V/propidium iodide flow cytometry showed that *PALMD*-knockdown OVCSC spheroids contained a higher proportion of early apoptotic cells than did shControl spheroids (Fig. 5a, b). Consistently, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining demonstrated increased DNA fragmentation in *PALMD*-knockdown spheroids compared to shControl spheroids (Fig. 5c). Caspase 3/7 activity was also significantly increased in *PALMD*-knockdown cells (Fig. 5d). These findings indicate that PALMD depletion enhances apoptosis in OVCSC spheroids and that PALMD contributes to anoikis resistance under detachment conditions.

### **Endothelial co-culture enhances PALMD expression and reduces p53 expression in OVCSC cells**

Given that ovarian cancer spheroids interact with stromal and endothelial components during peritoneal metastasis, the influence of endothelial co-culture on PALMD expression in OVCSC cells was examined. OVCSC cells were cultured under 3D conditions, exposed to endothelial cell-conditioned medium, or co-cultured with HUEhT-1 endothelial cells using a horizontal co-culture system (Fig. 6a). Western blotting showed that PALMD expression increased in both endothelial cell-conditioned medium and HUEhT-1 co-culture conditions compared to 3D culture alone (Fig. 6b, c). In contrast, p53 expression decreased under co-culture conditions (Fig. 6b, c). Vascular endothelial growth factor (VEGF) concentrations in the culture supernatant increased over time during OVCSC–HUEhT-1 co-culture, suggesting that endothelial interactions generated a niche-like microenvironment associated with pro-survival signaling (Fig. 6d). Immunofluorescence analysis further supported the increased PALMD and reduced p53 signal intensities observed under co-culture conditions (Supplementary Fig. S1). These findings suggest that endothelial niche-related signaling is associated with increased PALMD and reduced p53 expression in OVCSC cells.

## Discussion

This study aimed to determine whether PALMD contributes to spheroid adaptation and anoikis resistance in ovarian cancer cells. The principal findings were as follows: first, patient-derived OVCSC cells established from malignant ascites retained spheroid-forming and cancer stem-like features; second, PALMD was induced during 3D spheroid adaptation in MESOV cells; third, PALMD depletion disrupted spheroid morphology, increased vimentin expression, and enhanced apoptosis; and fourth, endothelial co-culture enhanced PALMD expression while reducing p53 expression. These findings identify PALMD as a context-dependent regulator of spheroid integrity and anoikis resistance in ovarian cancer cells.

The clinical relevance of this study lies in the improved understanding of the biology of peritoneal metastasis. For successful peritoneal metastasis, ovarian cancer cells must survive after detachment, maintain multicellular spheroids in ascitic fluid, and subsequently attach to peritoneal surfaces. Therefore, anoikis resistance is a prerequisite for metastatic competence [8–10]. Anoikis resistance in cancer is mediated through multiple mechanisms, including altered cell adhesion, cytoskeletal remodeling, epithelial–mesenchymal plasticity, activation of survival signaling, suppression of apoptotic pathways, and metabolic adaptation [21]. The present findings suggest that PALMD supports several of these anoikis-resistance mechanisms. PALMD depletion disrupted spheroid integrity, increased vimentin expression, enhanced p53 expression, and increased apoptosis, as shown by Annexin V, TUNEL, and caspase 3/7 assays. These results indicate that PALMD may help ovarian cancer spheroids survive anchorage-independent stress by maintaining spheroid architecture, supporting cytoskeletal and partial EMT-like plasticity, and limiting p53-associated apoptotic responses.

The transcriptomic analyses of MESOV cells strengthen this interpretation. MESOV cells showed broad gene expression remodeling during the transition from 2D to 3D culture, and PALMD was significantly induced in 3D culture. Genes associated with extracellular matrix organization, cell adhesion, proteolysis, and stemness, including *PODXL*, *CDH6*, *MMP2*, *COL1A1*, *CTSD*, and *SOX2*, were also markedly upregulated. Considering that MESOV is an established ovarian cancer model and that OVCSC cells were derived from clinical ascites, the

combination of these systems suggests that PALMD induction is not merely a culture-specific artifact but rather part of an adaptive program associated with spheroid biology.

PALMD knockdown increased vimentin expression and disrupted spheroid architecture, whereas changes in E-cadherin and N-cadherin levels were more modest. These findings do not support a complete cadherin switch but are more consistent with partial epithelial–mesenchymal transition (EMT)-like plasticity. Hybrid epithelial–mesenchymal states have been proposed to support collective invasion, stemness, and stress tolerance [22–25]. In ovarian cancer spheroids, maintenance of this intermediate state may allow cells to balance cohesion with plasticity. PALMD may contribute to this balance by regulating membrane and cytoskeletal adaptations required for spheroid integrity under detachment conditions.

The relationship between PALMD and p53 identified in the current study was also notable. PALMD is a p53-associated molecule involved in DNA damage responses [18]. In our experiments, PALMD depletion increased p53 expression, whereas endothelial co-culture increased PALMD and reduced p53 expression. These results suggest that the PALMD–p53 relationship may be context-dependent in ovarian cancer spheroids. Rather than acting solely as a pro-apoptotic p53 target, PALMD may be repurposed in detached or niche-supported cancer cells to promote adaptation and survival. Further studies are required to determine whether this regulation depends on TP53 mutational status, transcriptional feedback, protein stability, or endothelial-derived signals.

The endothelial co-culture experiments suggest that microenvironmental conditions may reinforce PALMD-associated survival signaling. During peritoneal metastasis, ovarian cancer spheroids encounter mesothelial, endothelial, and stromal cells, which provide survival cues [26–30]. Increased VEGF expression during co-culture is consistent with the development of a pro-survival niche. VEGF has been implicated in ovarian cancer growth, angiogenesis, ascites formation, and peritoneal dissemination [31]. Therefore, the observed increase in VEGF during OVCSC–HUEhT-1 co-culture supports the biological relevance of endothelial niche-related signaling in this model. The concomitant increase in PALMD expression suggests that PALMD may participate in niche-mediated protection during early peritoneal attachment.

This study has several strengths. First, the use of OVCSC cells derived from malignant ascites allowed functional analysis in a clinically relevant spheroid-forming model. Second, the

combination of transcriptomic screening in MESOV cells with functional validation in patient-derived OVCSC cells reduced the likelihood that PALMD induction was a model-specific artifact. Third, multiple complementary assays, including western blotting, spheroid morphology assessment, Annexin V staining, TUNEL staining, caspase 3/7 activity measurement, endothelial co-culture, VEGF measurement, and immunofluorescence, supported the conclusion that PALMD is associated with spheroid survival. This study also has limitations. First, the main functional experiments were performed using a single patient-derived OVCSC culture; therefore, validation in additional patient-derived cultures and established ovarian cancer cell lines is needed. Second, although the data support a role for PALMD in spheroid survival relevant to peritoneal metastasis, direct in vivo metastasis experiments were not performed. Third, the molecular mechanism by which PALMD regulates apoptosis, vimentin expression, p53 signaling, and endothelial niche-related responses remains to be clarified. Finally, the present data indicate that PALMD is associated with anoikis resistance but do not establish whether PALMD alone is sufficient to drive spheroid survival.

In conclusion, PALMD is induced during 3D spheroid adaptation and supports spheroid integrity and anoikis resistance in ovarian cancer cells. PALMD depletion increased p53 expression, disrupted spheroid architecture, induced partial EMT-like changes, and enhanced apoptosis, whereas endothelial niche-related conditions increased PALMD expression and reduced p53 expression. These findings suggest that PALMD is a context-dependent regulator of ovarian cancer spheroid survival and may represent a potential target for strategies aimed at disrupting peritoneal metastasis. Future studies should validate these findings in additional patient-derived models and in vivo systems and should clarify the molecular mechanisms linking PALMD to p53 signaling, cytoskeletal adaptation, and endothelial niche-mediated survival.

## **Methods**

### **Ethics statement**

The Institutional Review Board of Teikyo University School of Medicine approved the use of human ascites-derived ovarian cancer cells (approval number: 13-003-7). All procedures were performed in accordance with relevant guidelines and regulations. The patients provided written

informed consent for the use of their clinical samples for research purposes. All personally identifiable information was removed before analysis.

### **Patient-derived ovarian cancer culture**

OVCSC cells were established from the malignant ascites of patients with advanced ovarian cancer. Floating tumor-derived cells were collected after abdominal puncture, washed, and cultured under spheroid-forming conditions. The OVCSC was used as a patient-derived ovarian cancer spheroid-forming culture.

### **Cell lines and culture conditions**

MESOV cells were obtained from ATCC (CRL-3272) and cultured according to the manufacturer's instructions. For the 2D culture, cells were maintained under adherent conditions. For the 3D culture, cells were seeded onto ultra-low-attachment culture plates and maintained under spheroid-forming conditions. HUEhT-1 endothelial cells were obtained from the JCRB Cell Bank and cultured on collagen I-coated dishes in endothelial cell growth medium. Cells were maintained at 37 °C in a humidified incubator with 5% CO<sub>2</sub>. MESOV cells were authenticated using short tandem repeat profiling and routinely tested negative for mycoplasma contamination.

### **Flow cytometry and ALDH assay**

To characterize the cancer stem-like and lineage marker profiles of OVCSC cells, flow cytometry and ALDH assays were performed. OVCSC cells were stained with antibodies against CD44, EpCAM, CD45, and CD133, with appropriate unstained and secondary antibody controls. ALDH activity was assessed using the ALDEFLUOR Kit (STEMCELL Technologies, Vancouver, BC, Canada) according to the manufacturer's protocol. The proportions of marker-positive and ALDH-positive cells and relative mean fluorescence intensities were analyzed.

### **Immunofluorescence staining**

Cells or spheroids were fixed with 4% paraformaldehyde, permeabilized, blocked, and incubated with primary antibodies against PALMD, p53, and relevant stemness-associated markers. Alexa Fluor-conjugated secondary antibodies were used for detection, and the nuclei were counterstained with DAPI. Images were acquired using fluorescence or confocal microscopy. Scale bars are indicated in the corresponding figures.

### **RNA sequencing and differential expression analysis**

Bulk RNA sequencing was performed using MESOV cells cultured under 2D and 3D conditions, with three biological replicates for each condition. Read counts were normalized using the trimmed mean of M values. Differential expression analysis was performed by comparing the 3D and 2D cultures. Genes with  $Q < 0.05$  and absolute  $\log_2$  fold change  $\geq 1$  were considered differentially expressed. Heatmaps and volcano plots were generated from the processed differential expression table.

### **Microarray analysis of co-cultured OVCSC cells**

To explore transcriptional changes induced by endothelial niche-related conditions, OVCSC cells were cultured under control or endothelial co-culture-related conditions. Total RNA was extracted, quality controlled, and hybridized using Affymetrix Clariom S arrays (Thermo Fisher Scientific, Waltham, MA, USA). Raw CEL files were processed using robust multichip average normalization implemented in Transcriptome Viewer software (Kurabo Industries Ltd., Osaka, Japan). The microarray findings were used as supportive exploratory data.

### ***PALMD* knockdown**

*PALMD* expression was suppressed using shRNA targeting *PALMD*. The control cells were transfected with a non-targeting control vector. Knockdown efficiency was confirmed by western blotting. When transient knockdown was performed, *PALMD*-specific and control siRNA were introduced according to the manufacturer's instructions.

### **Western blotting**

Cells were lysed in protein extraction buffer, and protein concentrations were determined. Equal amounts of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The membranes were blocked and incubated with primary antibodies against *PALMD*, p53, E-cadherin, N-cadherin, vimentin, and beta-actin, followed by appropriate secondary antibodies. Signal intensities were detected using a chemiluminescence imaging system and quantified relative to beta-actin levels.

### **Spheroid morphology assay**

Control and *PALMD*-knockdown OVCSC spheroids were cultured under ultra-low attachment conditions and observed microscopically during adhesion. Spheroid compactness, spreading, and

dissociation were compared between the groups using representative images and, where applicable, quantitative image analysis.

### **Apoptosis and anoikis assays**

To determine whether PALMD depletion enhanced apoptosis and anoikis under anchorage-independent conditions, apoptosis was assessed using Annexin V/propidium iodide staining followed by flow cytometry. Early apoptotic and late apoptotic or necrotic populations were quantified using standard gating. DNA fragmentation was evaluated using the DeadEnd™ Fluorometric TUNEL System (Promega, Madison, WI, USA) according to the manufacturer's protocol. Caspase 3/7 activity was measured using the Caspase-Glo 3/7 Assay System (Promega, Madison, WI, USA) according to the manufacturer's protocol. Anoikis sensitivity was evaluated under ultra-low attachment or spheroid-forming conditions.

### **Endothelial co-culture and VEGF measurement**

To examine whether endothelial niche-related conditions modulate PALMD and p53 expression in OVCSC cells, OVCSC cells were co-cultured with HUEhT-1 endothelial cells using a horizontal co-culture system or exposed to endothelial-conditioned medium. PALMD and p53 expression levels were evaluated using western blotting and immunofluorescence. VEGF concentrations in the culture supernatants were measured using a Human VEGF Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol.

### **Statistical analyses**

Statistical analyses Data are presented as means  $\pm$  standard deviations unless otherwise stated. Each experiment was performed with at least three independent biological replicates unless otherwise indicated. Data distribution was assessed using the Shapiro–Wilk test and visual inspection of Q–Q plots. For normally distributed data, differences between two groups were analyzed using two-sided unpaired Student's t-tests. For comparisons among more than two groups, one-way analysis of variance followed by Tukey's post hoc test was performed.

### **Use of artificial intelligence tools**

During manuscript preparation, a large language model tool was used to assist with language editing, structural organization, and formatting. The authors reviewed and edited all content and take full responsibility for the accuracy, integrity, and validity of the final manuscript.

## Data Availability

The RNA-seq data generated in this study have been deposited in the DDBJ Sequence Read Archive under BioProject accession number PRJDB42459. The corresponding Run accession numbers are DRR1061129–DRR1061134. Processed differential expression results are provided as Supplementary Table S1.

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**Author contributions**

M.K. and K.N. conceived and designed the study. M. K., Y. M., K. T., R. W., K. S., and T. K. performed the experiments. S.W., M.N., Y.T., H.N., M.H. and H.H. contributed to data acquisition, cell culture, molecular analyses, and data interpretation. K.N. provided clinical and scientific insights. K.N., Y.O. supervised the project. M.K. and K.N. drafted the manuscript. All authors have reviewed and approved the final manuscript.

**Competing interests**

The authors declare no competing interests.

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## Figure legends

### Figure 1. Characterization of OVCSC patient-derived ovarian cancer spheroids.

(a) Representative phase-contrast image of OVCSC cells cultured under ultra-low attachment conditions, showing formation of multicellular spheroids. (b) Flow cytometry analysis of OVCSC surface marker expression. OVCSC cells showed CD44 and EpCAM positivity, whereas CD45 expression was minimal, supporting their tumor-derived and cancer stem-like characteristics. Appropriate unstained and secondary antibody controls were included. (c) ALDEFLUOR assay showing high aldehyde dehydrogenase activity in OVCSC cells. Percentages indicate positive cell fractions. ALDH, aldehyde dehydrogenase; EpCAM, epithelial cell adhesion molecule; OVCSC, ovarian cancer spheroid-forming cell culture.

### Figure 2. Transcriptomic remodeling of MESOV cells during 3D culture.

(a) Heatmap showing differentially expressed genes between MESOV cells cultured under 2D and 3D conditions. (b) Volcano plot showing upregulated and downregulated genes in 3D culture compared with 2D culture. Genes were considered differentially expressed at  $Q < 0.05$  and  $|\log_2 \text{fold change}| \geq 1$ . (c) PALMD expression in individual MESOV 2D and 3D culture samples. Each dot represents an independent biological replicate, and bars indicate mean  $\pm$  SD. TMM, trimmed mean of M values.

### Figure 3. *PALMD* knockdown increases p53 expression in OVCSC cells.

(a) Representative western blot analysis of *PALMD* and p53 expression in control and *PALMD*-knockdown OVCSC cells cultured under 3D and 2D conditions. Actin was used as a loading control. (b) Densitometric quantification of *PALMD* expression normalized to actin. (c) Densitometric quantification of p53 expression normalized to actin. Data are presented as means  $\pm$  SDs from at least three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ .

**Figure 4. *PALMD* knockdown disrupts spheroid integrity and induces partial EMT-like changes.**

(a) Representative phase-contrast images of shControl and *PALMD*-knockdown OVCSC spheroids under adhesion conditions. *PALMD*-knockdown spheroids show increased spreading and partial dissociation compared with shControl spheroids. Scale bars, 200  $\mu$ m. (b) Western blot analysis and densitometric quantification of EMT-related markers, including vimentin, E-cadherin, and N-cadherin, in shControl and *PALMD*-knockdown OVCSC cells cultured under 3D and 2D conditions. Actin was used as a loading control. Data are presented as means  $\pm$  SDs from three independent experiments. \*P < 0.05.

**Figure 5. *PALMD* knockdown enhances apoptosis in OVCSC spheroids.**

(a) Representative Annexin V/propidium iodide flow cytometry plots of shControl and sh*PALMD* OVCSC spheroids. Q4 indicates early apoptotic cells, and Q2 indicates late apoptotic or necrotic cells. (b) Quantification of early apoptotic cells. (c) Representative TUNEL staining images of shControl and sh*PALMD* OVCSC spheroids. DAPI was used for nuclear staining, and FITC indicates TUNEL-positive apoptotic cells. (d) Caspase 3/7 activity in shControl and sh*PALMD* OVCSC cells. Data are presented as means  $\pm$  SDs. Annexin V assays were performed with four independent experiments, and caspase 3/7 assays were performed with three independent experiments. \*P < 0.05, \*\*P < 0.01.

**Figure 6. Endothelial co-culture enhances *PALMD* expression and reduces p53 expression in OVCSC cells.**

(a) Schematic illustration and representative phase-contrast images of the OVCSC–HUEhT-1 endothelial co-culture system. OVCSC cells and HUEhT-1 cells were cultured in connected chambers, allowing exchange of soluble factors between the two cell populations. (b) Representative western blots of *PALMD* and p53 expression in OVCSC cells cultured under 3D culture, endothelial cell-conditioned medium, and HUEhT-1 co-culture conditions. Actin was used as a loading control. (c) Densitometric quantification of *PALMD* and p53 expression

normalized to actin. Data are presented as means  $\pm$  SDs from three independent experiments. \*P < 0.05, \*\*P < 0.01. **(d)** VEGF concentration in culture supernatants during OVCSC–HUEhT-1 co-culture, measured by enzyme-linked immunosorbent assay.

**Table****Table 1. Representative differentially expressed genes in MESOV cells cultured under 3D conditions compared with 2D conditions.**

| Gene            | Direction in 3D | Fold change | log2FC | Q value                | Functional relevance                                       |
|-----------------|-----------------|-------------|--------|------------------------|------------------------------------------------------------|
| <i>PALMD</i>    | Up              | 2.76        | 1.47   | $1.30 \times 10^{-7}$  | Membrane dynamics; candidate spheroid survival factor      |
| <i>PODXL</i>    | Up              | 32.58       | 5.03   | $2.09 \times 10^{-20}$ | Cell surface glycoprotein; adhesion and cancer progression |
| <i>CDH6</i>     | Up              | 56.99       | 5.83   | $8.88 \times 10^{-20}$ | Cadherin family; cell-cell adhesion                        |
| <i>MMP2</i>     | Up              | 11.76       | 3.56   | $1.93 \times 10^{-18}$ | Extracellular matrix remodeling and invasion               |
| <i>COL1A1</i>   | Up              | 11.72       | 3.55   | $1.05 \times 10^{-18}$ | Extracellular matrix component                             |
| <i>CTSD</i>     | Up              | 12.56       | 3.65   | $1.11 \times 10^{-19}$ | Protease; survival and invasion-associated pathways        |
| <i>SOX2</i>     | Up              | 13.74       | 3.78   | $1.43 \times 10^{-18}$ | Stemness-associated transcription factor                   |
| <i>SPON2</i>    | Up              | 28.95       | 4.86   | $2.78 \times 10^{-19}$ | Extracellular matrix-associated protein                    |
| <i>MFAP2</i>    | Up              | 49.36       | 5.63   | $9.23 \times 10^{-19}$ | Matrix-associated protein                                  |
| <i>AKR1C3</i>   | Up              | 29.13       | 4.86   | $1.11 \times 10^{-19}$ | Metabolic adaptation/steroid metabolism                    |
| <i>CCN1</i>     | Down            | 0.045       | -4.47  | $1.16 \times 10^{-20}$ | Matricellular signaling                                    |
| <i>SLC7A5</i>   | Down            | 0.044       | -4.50  | $2.09 \times 10^{-20}$ | Amino acid transporter                                     |
| <i>FOSL1</i>    | Down            | 0.073       | -3.78  | $5.07 \times 10^{-20}$ | AP-1 transcription factor                                  |
| <i>SERPINE1</i> | Down            | 0.093       | -3.43  | $4.49 \times 10^{-18}$ | ECM remodeling; plasminogen activator inhibitor            |
| <i>PHGDH</i>    | Down            | 0.149       | -2.75  | $6.96 \times 10^{-18}$ | Serine biosynthesis/metabolic pathway                      |

Differential expression analyses were performed using three biological replicates for each condition. Genes were selected based on their statistical significance and biological relevance to spheroid formation, cell adhesion, extracellular matrix remodeling, epithelial–mesenchymal plasticity, and anoikis resistance. Fold change indicates the expression in 3D culture relative to that in 2D culture. A complete list of the DEGs is provided in Supplementary Table S1. 2D, two-dimensional; 3D, three-dimensional; DEG, differentially expressed gene; FC, fold change

Fig. 1

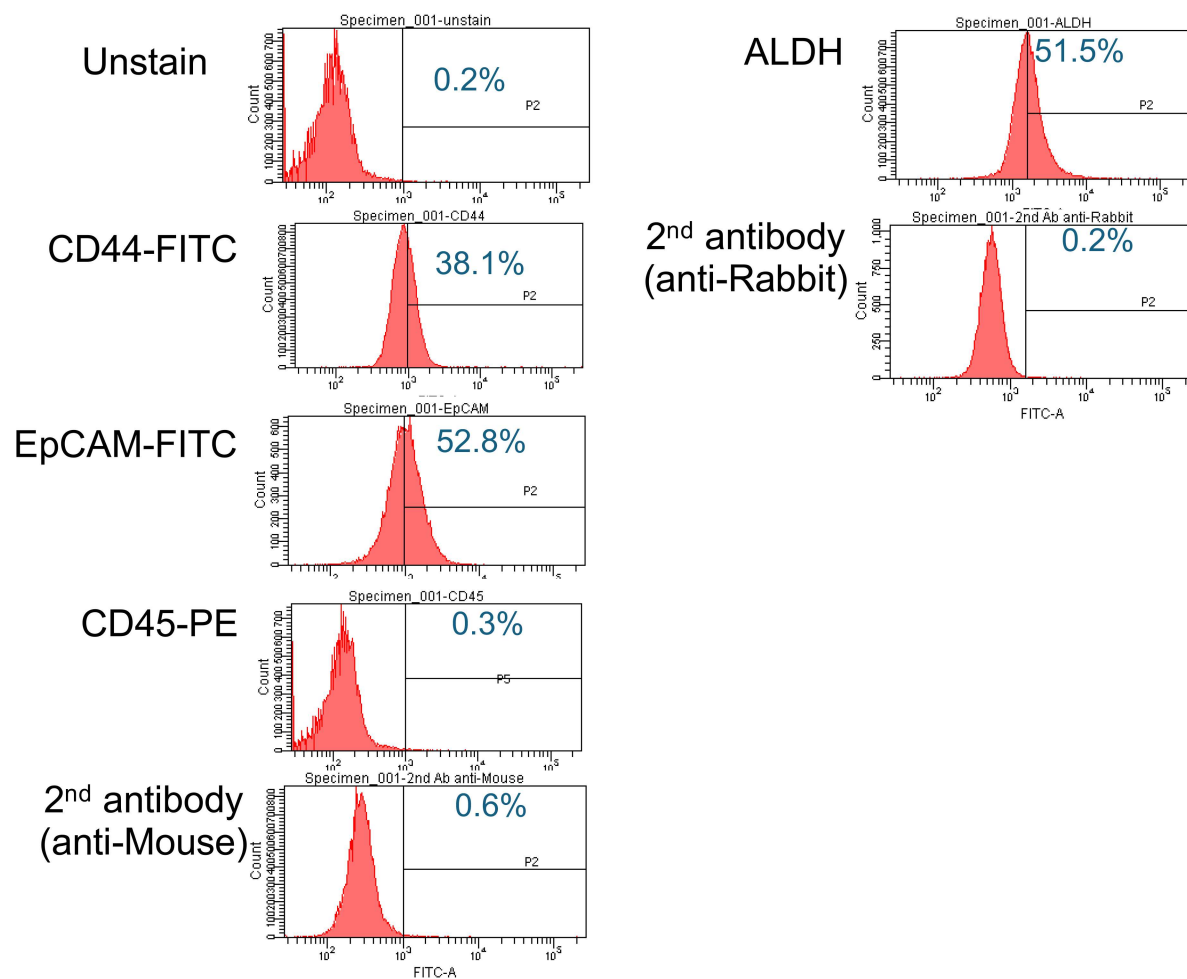
a

Representative OVCSC spheroid image



Fig. 1

b



c

ALDEFLUOR assay

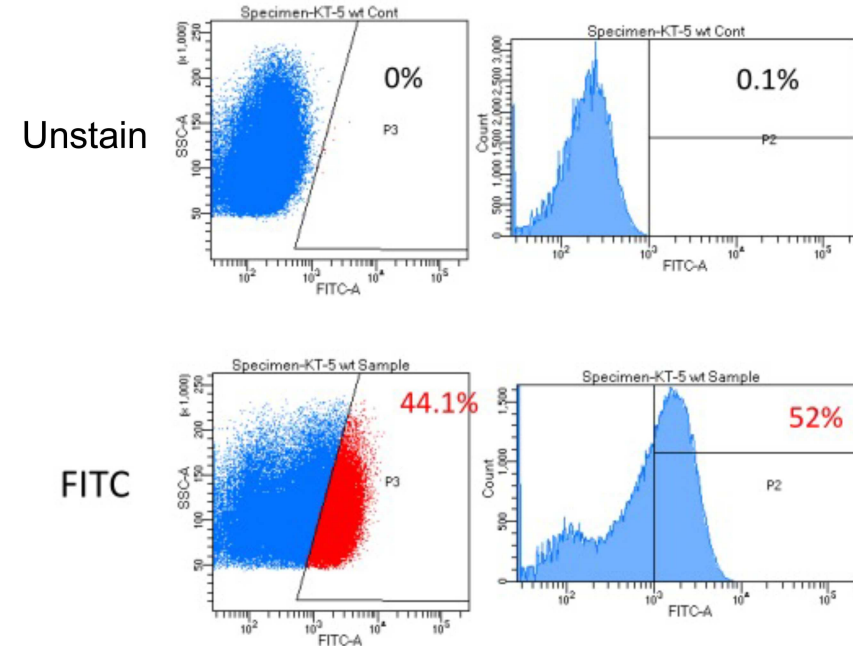
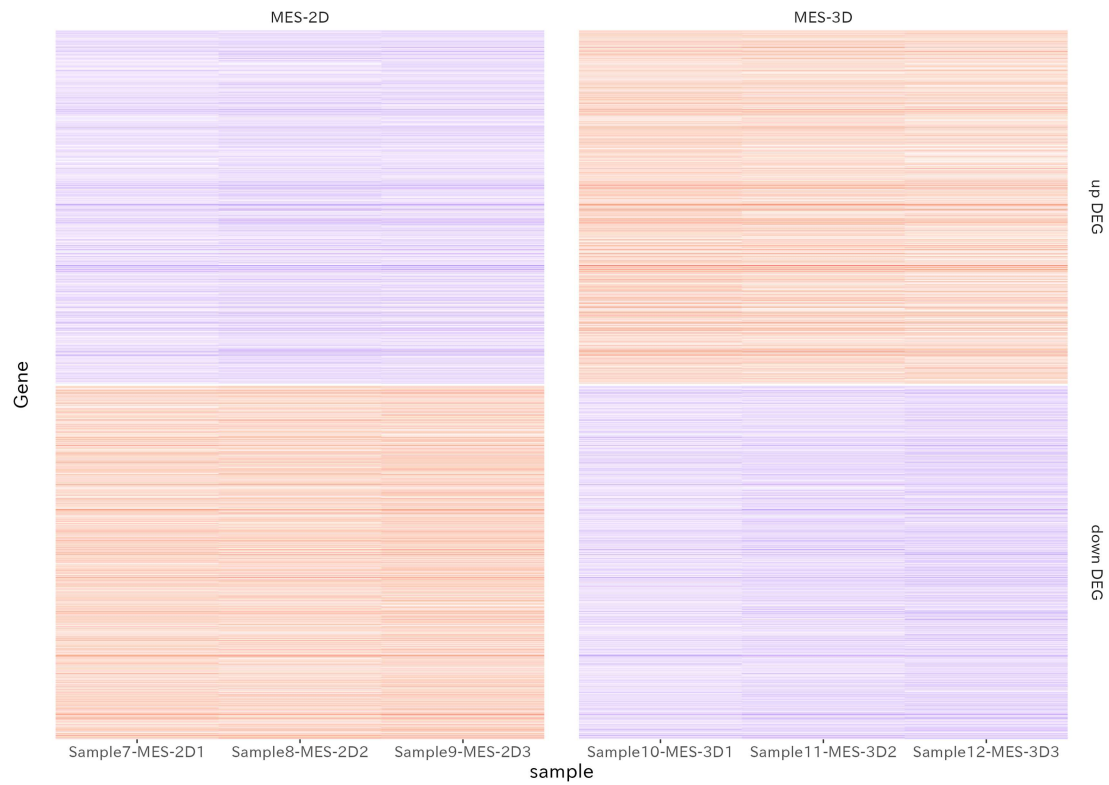


Fig. 2 **PALMD is upregulated during three-dimensional adaptation of MESOV cells**

a



b

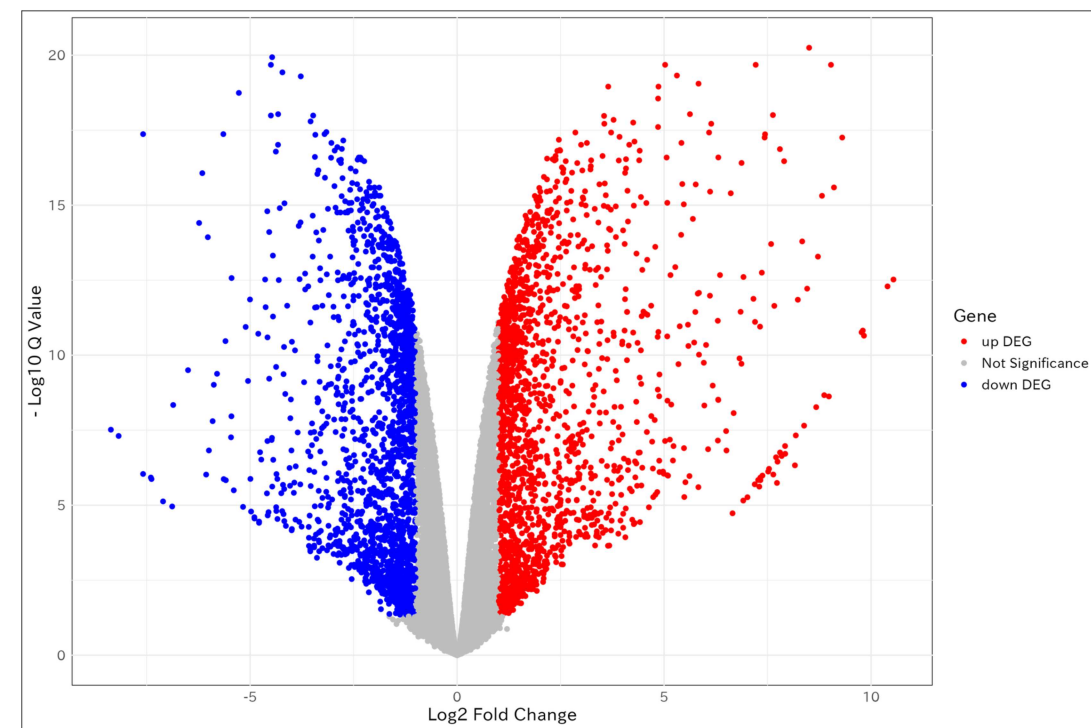
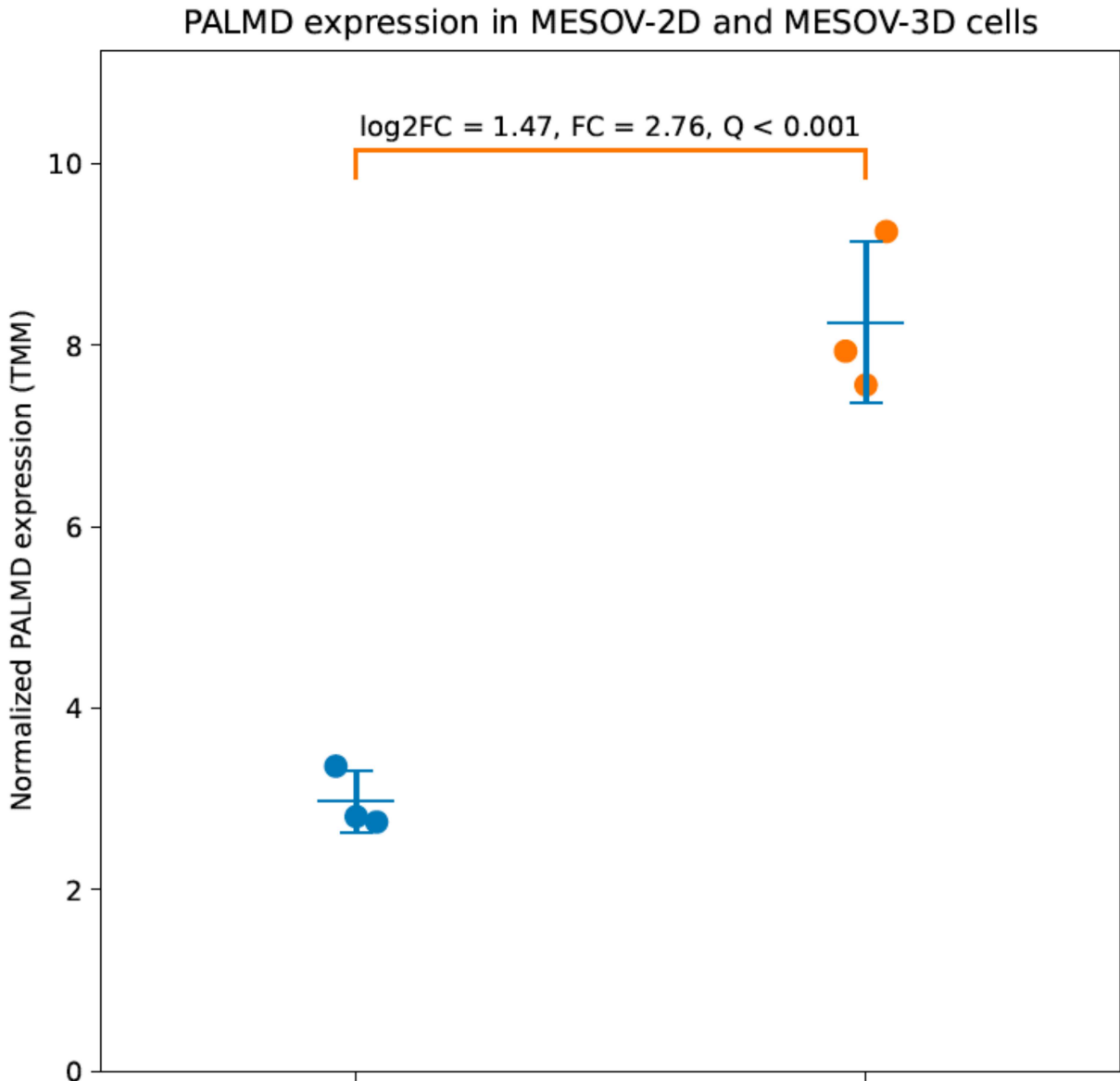


Fig. 2 **PALMD is upregulated during three-dimensional adaptation of MESOV cells**

C



| Sample           | Group    | PALMD normalized expression |
|------------------|----------|-----------------------------|
| Sample7-MES-2D1  | MESOV-2D | 3.36                        |
| Sample8-MES-2D2  | MESOV-2D | 2.81                        |
| Sample9-MES-2D3  | MESOV-2D | 2.74                        |
| Sample10-MES-3D1 | MESOV-3D | 7.93                        |
| Sample11-MES-3D2 | MESOV-3D | 7.56                        |
| Sample12-MES-3D3 | MESOV-3D | 9.25                        |

Fig. 3 PALMD knockdown increases p53 expression in OVCSC cells

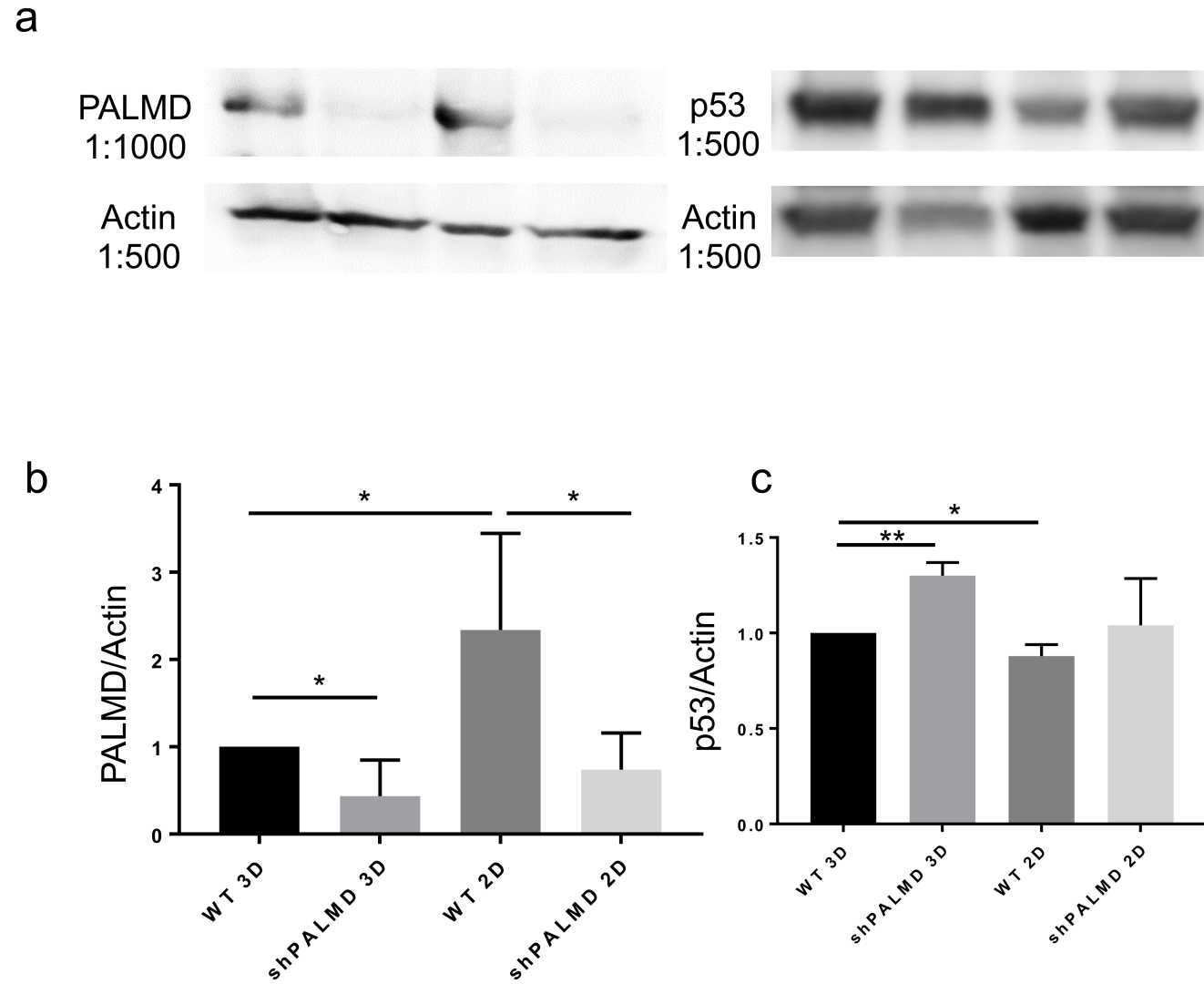


Fig. 4 PALMD knockdown disrupts spheroid integrity and induces partial EMT-like changes

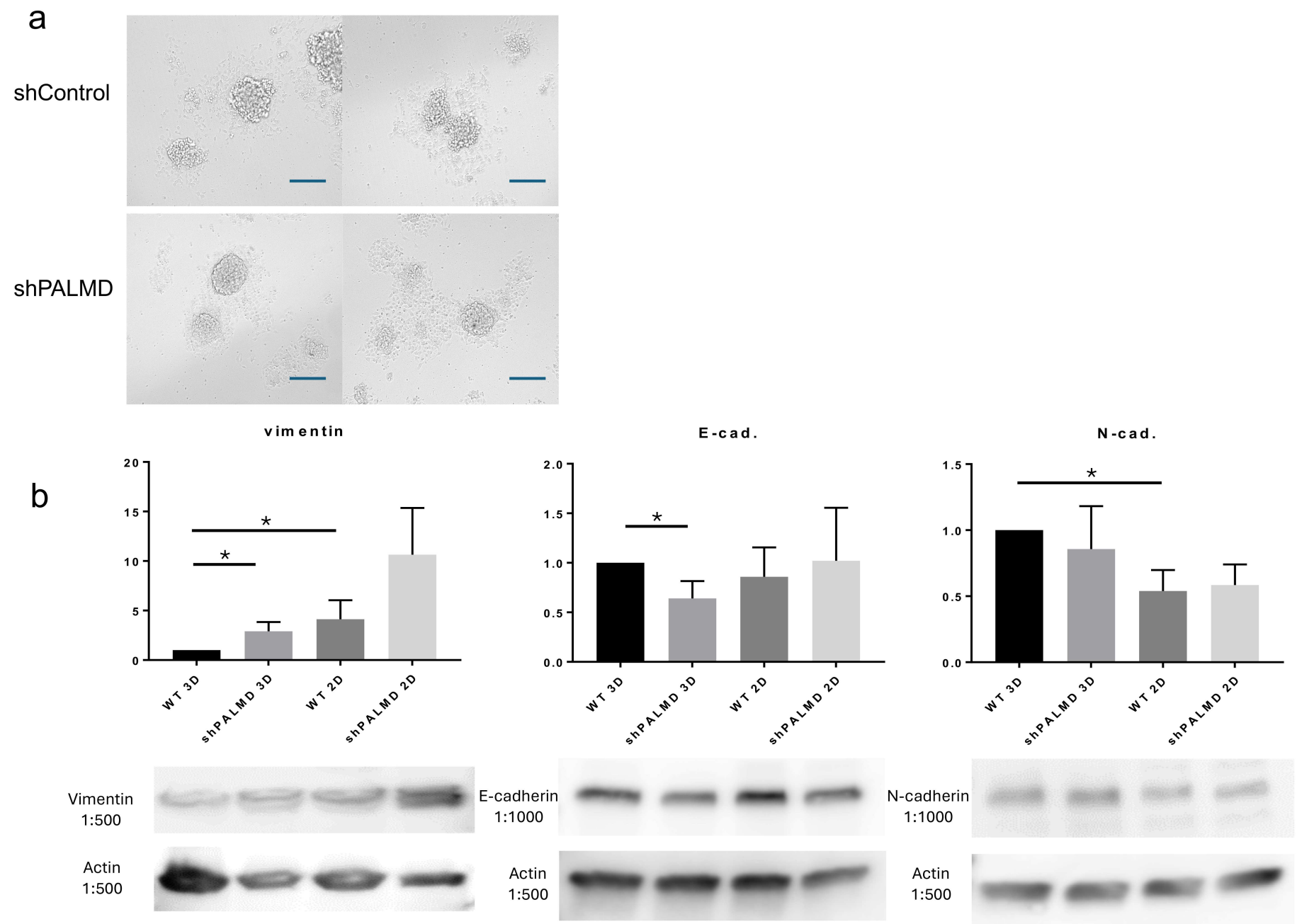


Fig. 5 PALMD knockdown enhances apoptosis in OVCSC spheroids

a

Annexin V/PI flow cytometry

b

Quantification of early apoptotic cells

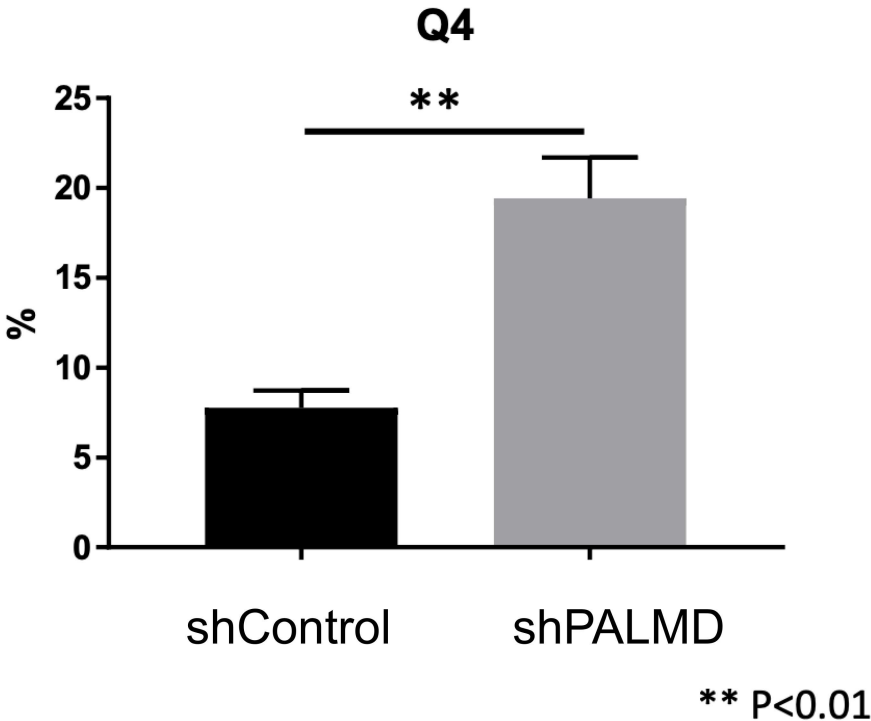
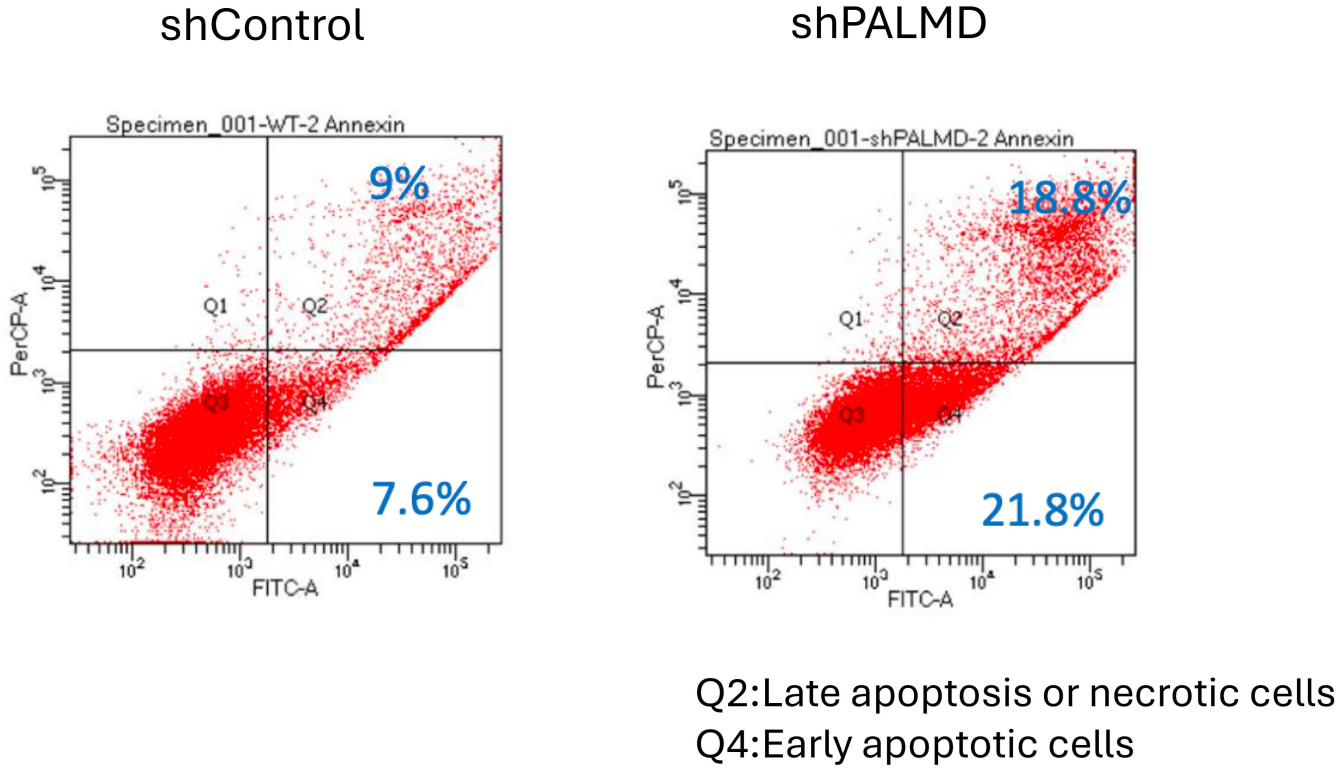


Fig. 5 PALMD knockdown enhances apoptosis in OVCSC spheroids

c

TUNEL assay

Positive control

shControl

shPALMD

DAPI

FITC(Apoptosis)

Merge

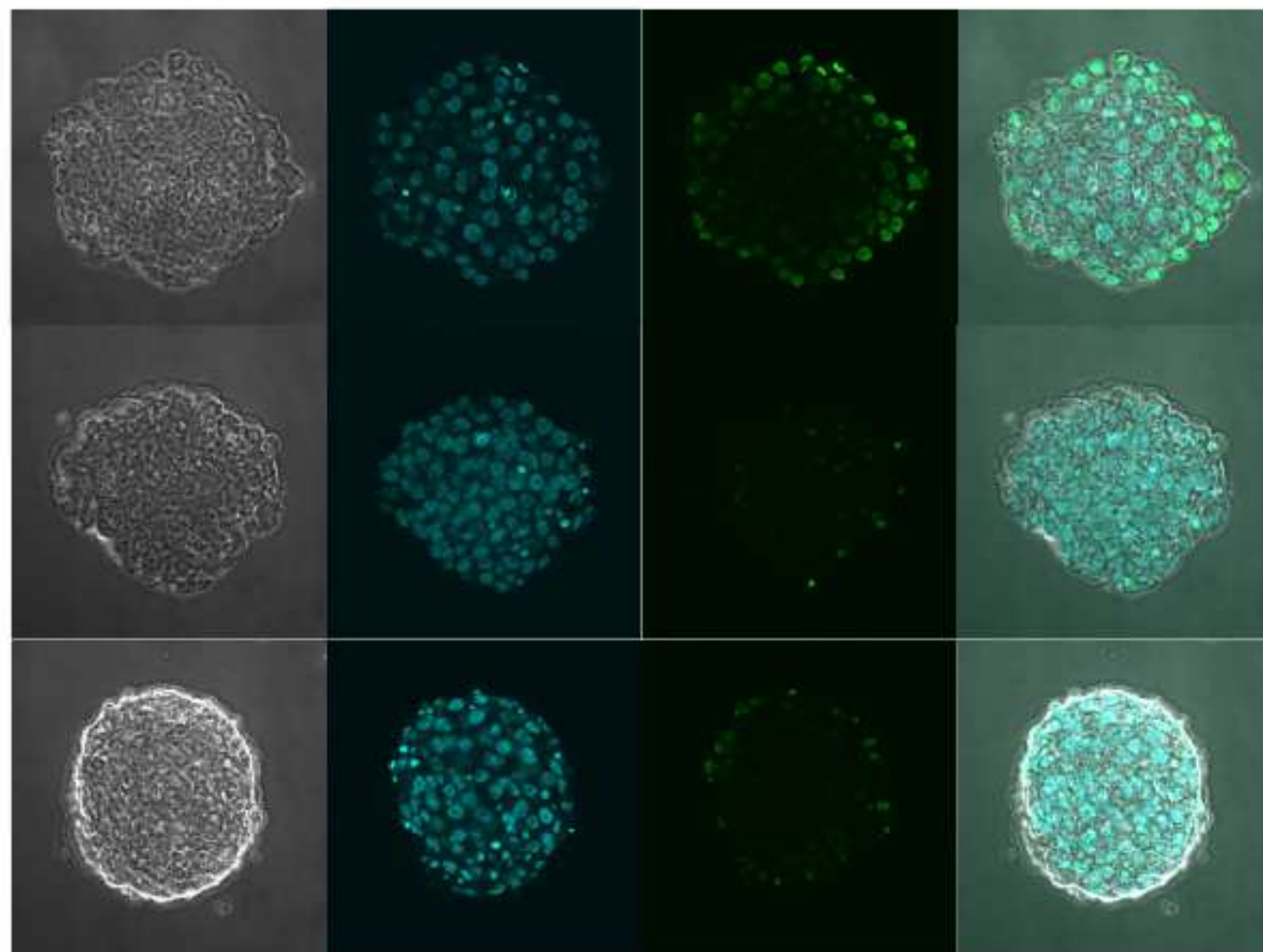


Fig. 5 PALMD knockdown enhances apoptosis in OVCSC spheroids

d

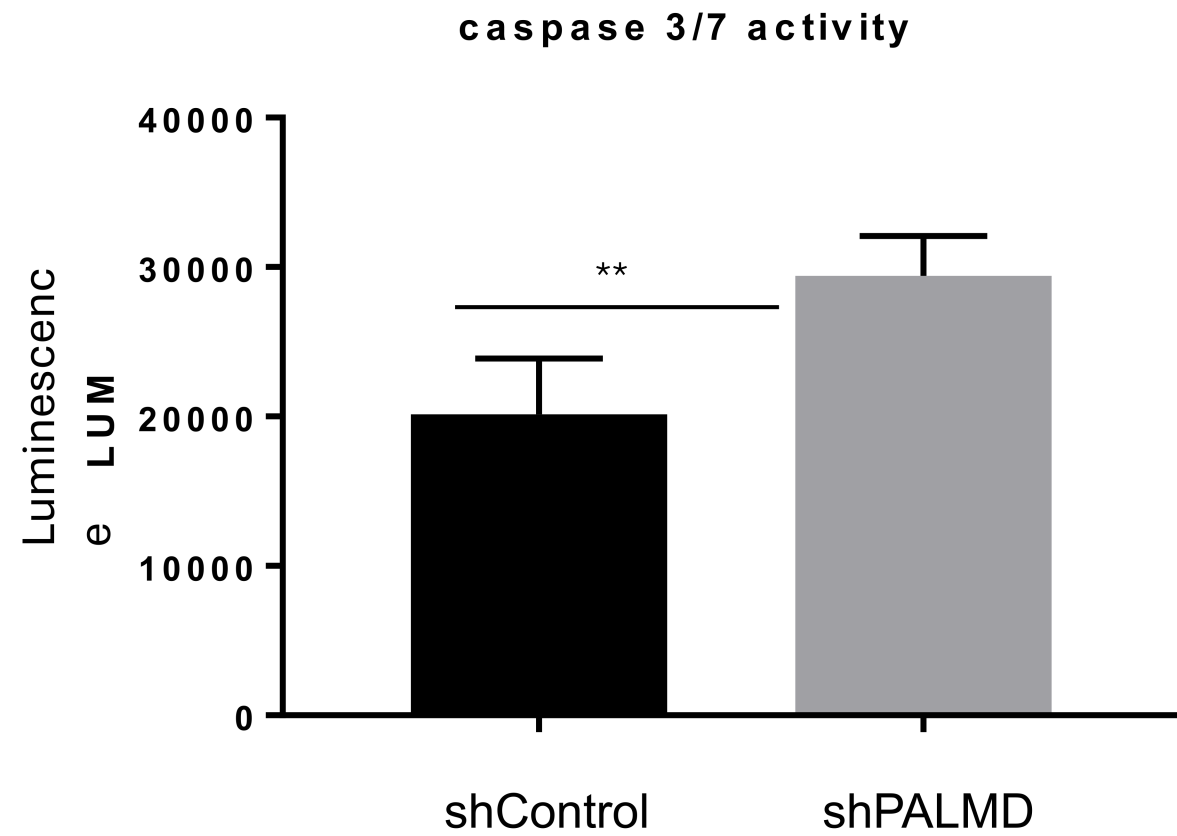
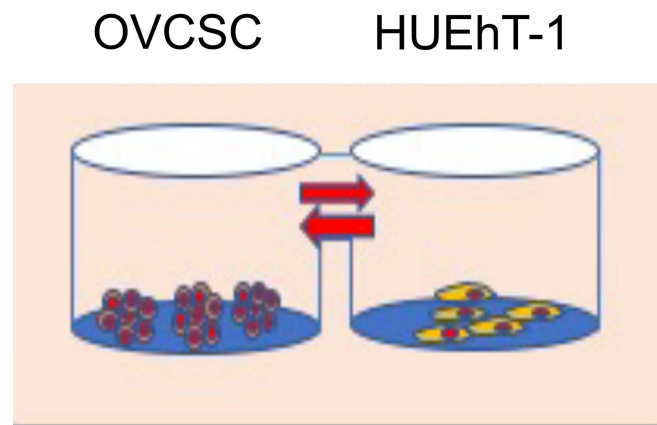


Fig. 6 **Endothelial co-culture enhances PALMD expression and reduces p53 expression in OVCSC cells**

a

OVCSC–HUEhT-1 co-culture system



1hr

OVCSC



HUEhT-1



3hr



6hr

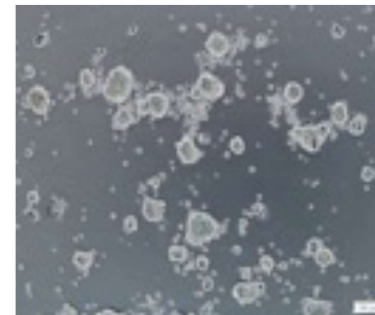
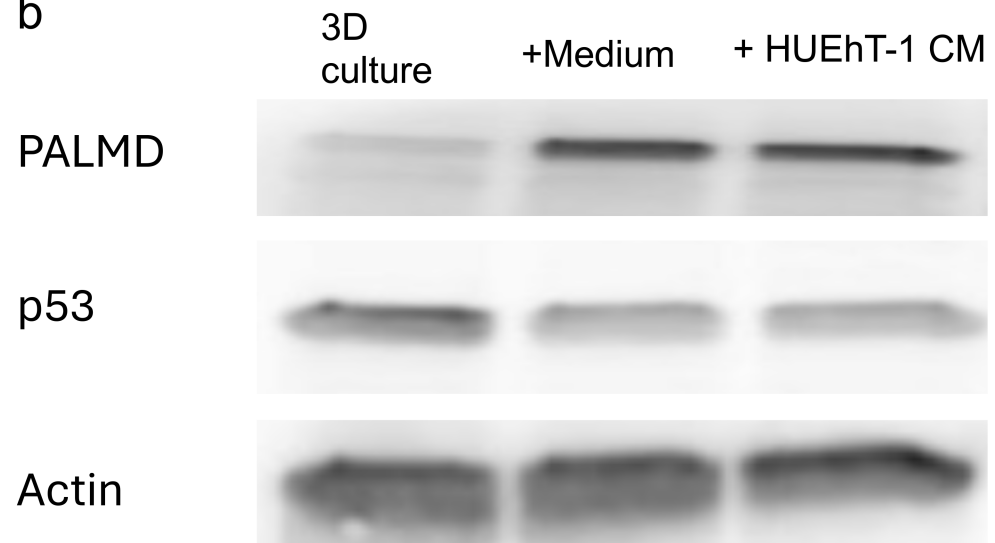
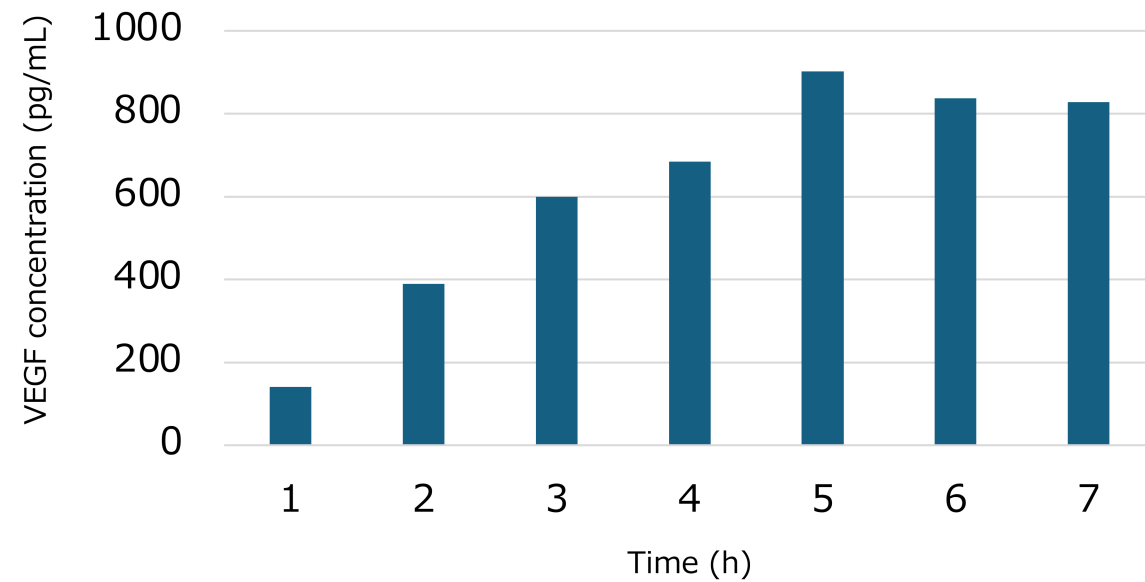


Fig. 6

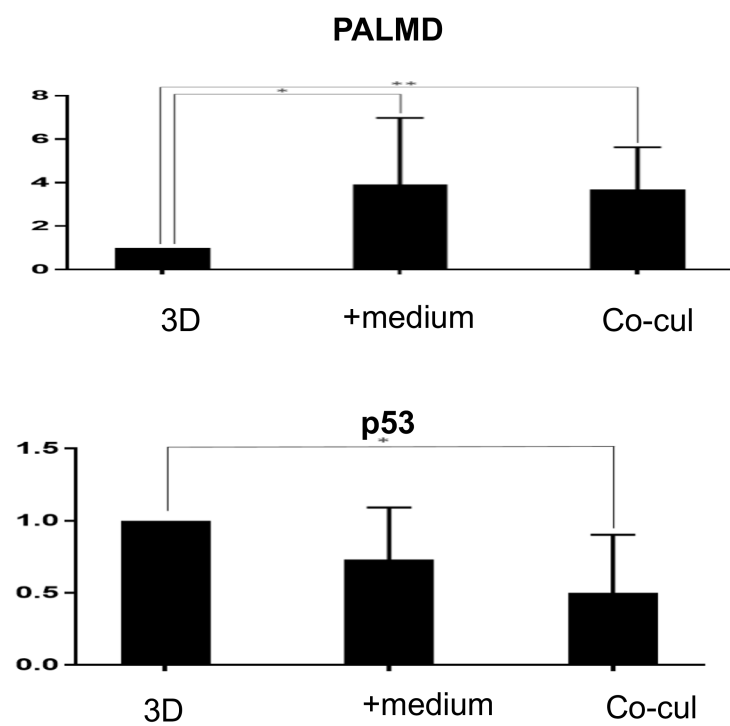
b



d



c



## Supplementary Files

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- [SupplementaryFig.S1.tif](#)