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Novel monoclonal antibody against integrin α3 shows therapeutic potential for ovarian cancer

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Abstract

Ovarian cancer has a high recurrence rate after platinum-based chemotherapy. To improve the treatment of ovarian cancer and identify ovarian cancer-specific antibodies, we immunized mice with the human ovarian carcinoma cell line, SKOV-3, and generated hybridoma clones. Several rounds of screening yielded 30 monoclonal antibodies (mAbs) with no cross-reactivity to normal cells. Among these mAbs, OV-Ab 30-7 was found to target integrin α3 and upregulate p53 and p21, while stimulating the apoptosis of cancer cells. We further found that binding of integrin α3 by OV-Ab 30-7 impaired laminin-induced focal adhesion kinase phosphorylation. The mAb alone or in combination with carboplatin and paclitaxel inhibited tumor progression and prolonged survival of tumor-bearing mice. Moreover, immunohistochemical staining of ovarian patient specimens revealed higher levels of integrin α3 in cancer cells compared with normal cells. By querying online clinical databases, we found that elevated ITGA3 expression in ovarian cancer is associated with poor prognosis. Taken together, our data suggest that the novel mAb, OV-Ab 30-7, may be considered as a potential therapeutic for ovarian cancer.

KEYWORDS
cell apoptosis, focal adhesion kinase, integrin α3, laminin, monoclonal antibody, ovarian cancer

1 | INTRODUCTION

Ovarian cancer is the most lethal gynecological cancer in the world. According to global cancer statistics, 295 414 new cases of ovarian cancer were diagnosed and 184 799 women died of the disease in 2018.[1] The overall 5-y survival rate for women diagnosed with ovarian cancer is 47%, and for women diagnosed with late-stage disease, the 5-y survival rate is only 29%.[2] About 75% of patients are diagnosed at advanced stages due to a lack of symptoms at early stages and a lack of reliable biomarkers for cancer screening.[3] Currently, 2 main therapeutic strategies are used as first-line treatments for advanced ovarian cancer. One is optimal cytoreductive surgery followed by combination platinum- and taxane-based chemotherapy, and the other is neoadjuvant chemotherapy prior to interval debulking surgery.[4] Approximately 80% of patients respond to first-line chemotherapy, but more than 70% of patients with advanced disease suffer a recurrence within 5 y and develop drug resistance.[5-7]

To improve patient survival and limit the damage to normal cells, targeted drugs, such as bevacizumab and poly (ADP-ribose) polymerase (PARP) inhibitors, have been developed.[8-10] Bevacizumab is a humanized vascular endothelial growth factor A (VEGF-A)-targeting monoclonal antibody (mAb) that can inhibit tumor angiogenesis. The US FDA approved bevacizumab in 2018 for use in combination with carboplatin and paclitaxel, followed by bevacizumab monotherapy, to treat ovarian cancer.[11] Several clinical trials have shown that combined bevacizumab and chemotherapy can prolong progression-free survival from 2 to 4 mo; however no significant improvements have been reported for overall survival.[12-14] Additionally, PARP inhibitors, such as fluzoparib, olaparib, and rucaparib, can inhibit tumor progression by disrupting DNA repair,[15,16] and clinical studies have shown that these drugs have therapeutic potential for ovarian cancers with BRCA gene mutations.[17,18] Although some studies have reported novel targeted therapies for ovarian cancer, there are still many more potentially useful surface molecules to be explored. Moreover, in spite of recent therapeutic advances, patient prognosis for ovarian cancer is still far from satisfactory.

Integrins are $\alpha\beta$ heterodimeric cell adhesion receptors that bind to extracellular matrix proteins and play important roles in cell adhesion, proliferation, and migration.[19] Integrin $\alpha3$ and integrin $\beta1$ form a heterodimer that can bind to laminin[20-22] and is involved in the development of many organs, such as skin, kidney, lung, and brain.[23-27] Several studies have identified integrin $\alpha3$ as a promising target molecule in brain and ovarian cancers, with potential utility in diagnosis and therapy.[28-30] However, the cellular function of integrin $\alpha3$ in ovarian cancer remains largely unknown. One study found that reductions in integrin $\alpha3$ protein induced by WW domain-containing oxidoreductase gene led to diminished cancer cell tumorigenicity.[31] Conversely, 2 other studies indicated that expression of integrin $\alpha3$ is inversely correlated with metastatic potential of human ovarian cancer.[32,33] Therefore, further work will be required to understand the mechanistic role of integrin $\alpha3$ in ovarian cancer.

Because antibodies have high specificity for their target antigens, mAb therapeutics often cause fewer adverse effects in patients compared with traditional chemotherapy.[34] Moreover, antibodies that specifically recognize ovarian cancer cells may be useful in diagnostic and/or therapeutic applications. In this work, we produced a mAb against ovarian cancer cells and investigated its therapeutic potential. Utilizing hybridoma technology, we generated mAbs from BALB/c mice after immunizing the animals with the ovarian cancer cell line, SKOV-3.

2 | MATERIALS AND METHODS

2.1 | Cell lines

The cell lines used in this study include mouse myeloma cell P3/NSI/1-Ag4-1 (NS-1), human ovarian carcinoma SKOV-3 (ATCC® HTB-77™) and ES-2 (ATCC® CRL-1978™), colorectal carcinoma HCT-116 (ATCC® CCL-247™), neuroepithelioma HTB-10 (ATCC® HTB-10™), hepatocellular carcinoma Mahlavu, lung carcinoma H460 (ATCC® HTB-177™), lung adenocarcinoma CL1-5, osteosarcoma U-2 OS (ATCC® HTB-96™), pancreatic adenocarcinoma MIA PaCa-2 (ATCC® CRL-1420™), breast adenocarcinoma MDA-MB-231 (ATCC® HTB-26™), oral carcinoma SAS, kidney carcinoma A-498 (ATCC® HTB-44™), human umbilical vein endothelial cells (HUVECs), foreskin fibroblast CCD-1112Sk (ATCC® CRL-2429™), and normal NNM cells. Primary NNM cultures were grown from biopsies of patients with nasal polyposis.[35] The use of NNM cultures was approved by the Human Subject Research Ethics Committee, Institutional Review Board, Academia Sinica (AS-IRB01-06008). NS-1 cells were purchase from Bioresource Collection and Research Center (Hsinchu, Taiwan). CL1-5 cells were acquired from Prof. Pan-Chyr Yang[36]; Mahlavu cells were obtained courtesy of Dr. M. Hsiao (Genomic Research Center, Academia Sinica); SAS cells were obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan); HUVECs were purchased from Lonza (Walkersville, MD, USA). Other cells were purchased from ATCC (Manassas, VA, USA). HUVEC were cultured in EBM-2 medium (Lonza), H460 in RPMI1640 (Thermo Fisher Scientific) medium, MDA-MB-231 in Leibovitz's L-15 medium and SAS in DMEM and Ham's F12 1:1 mix medium (Thermo Fisher Scientific). Other cell lines were cultured in DMEM. All culture media contained 10% fetal bovine serum (Thermo Fisher Scientific). The cell lines were all maintained at 37°C in humidified air with 5% CO_2.

2.2 | Generation of mAbs against ovarian cancer cells

The procedure to generate mAb against ovarian cancer cells has been described previously.[37,38] In brief, female 6-wk-old BALB/cJ mice (Taiwan National University Animal Center, Taipei, Taiwan) were immunized by intraperitoneal (ip) injection of 1×10^7 live SKOV-3 cells without adjuvant. After the first immunization, 1×10^7 SKOV-3 cells mixed with 0.5 mL completed Freund's adjuvant was administered via ip injection. At 3 wk after the booster injection,

final boosters with 1×10^7 cells in PBS were administered via ip injection. On the fourth day after the final booster, splenocytes were harvested from the spleens of immunized mice and fused with NS-1 myeloma cells using 50% polyethylene glycol (Thermo). Fused cells were suspended in DMEM supplemented with hypoxanthine-aminopterin-thymidine (Sigma-Aldrich) and hybridoma cell growth supplement (MP Biomedicals), after which cells were seeded onto 96-well plates. The hybridomas that recognized SKOV-3, but not human foreskin fibroblast CCD-1112Sk cells, were then clonally selected by limiting dilution, before being preserved in liquid nitrogen. Ascites were produced in pristane-primed BALB/cJ mice, and mAbs were purified using Protein G Sepharose 4 Fast Flow antibody purification resin (GE Healthcare Biosciences). The protocol for mouse experiments was approved by the Committee on the Ethics of Animal Experiments of Academia Sinica (AS IACUC: 15-09-853).

2.3 | Identification of mAb target protein

OV-Ab 30-7 was cross-linked with Protein G Sepharose (GE Healthcare Biosciences) using 60 mmol/L dimethyl pimelimidate. Total SKOV-3 cell lysates were applied to an OV-Ab 30-7-conjugated protein G affinity column. After washing, the proteins bound to OV-Ab 30-7 were eluted with elution buffer (0.2 mol/L glycine, pH 2.5, 150 mmol/L NaCl and 1% NP-40), and the eluates were neutralized with 1 mol/L Tris-HCl, pH 9.1.[39] The eluates were then separated by SDS-PAGE then stained using a SilverQuest™ Silver Staining Kit (Thermo Fisher Scientific). The band of interest was cut from the gel, reduced with 50 mmol/L dithioerythritol in 25 mmol/L ammonium bicarbonate (ABC) at pH 8.5 for 1 h at 37°C, and alkylated with 100 mmol/L iodoacetamide in ABC for 1 h at room temperature. After washing with 50% acetonitrile in ABC, the gel was soaked in 100% acetonitrile and incubated with 0.02 μg trypsin for 16 h at 37°C. The digested peptides were extracted with 50% acetonitrile in 5% TFA and concentrated using a Concentrator (Eppendorf). The sample was analyzed by LC-MS/MS sequencing, performed by the Core Facility for Proteomics and Structural Biology Research at Academia Sinica.

2.4 | Western blot analysis

Total cells were lysed using RIPA buffer (Cell Signaling Technology) containing protease inhibitor cocktail (Roche, Basel, Switzerland). Protein concentration was determined using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). For non-reducing western blotting, protein was mixed with Native Sample Buffer (Bio-Rad) and, for reducing blots, protein was mixed with 4× Laemmli Sample Buffer containing 2-mercaptoethanol (Bio-Rad). Equal amounts of protein were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Merck Millipore). The membrane was blocked with 1% bovine serum albumin (BSA), and incubated with antibody

against PARP (sc-8007, Santa Cruz Biotechnology), FAK (#3285 Cell Signaling Technology), phospho-FAK (#8556, Cell Signaling Technology), integrin α3 (ab131055, Abcam), integrin β1 (#9699, Cell Signaling Technology), p53 (#2524, Cell Signaling Technology) or p21 (#2947, Cell Signaling Technology) at a 1:1000 dilution overnight. The membrane was then incubated with HRP-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature, and signal was detected using an ECL kit (Merck).

2.5 | Flow cytometry

To determine whether the mAbs specifically bind SKOV-3 cells, SKOV-3, and CCD-1112Sk cells were cultured overnight and then suspended using trypsin. Total cells (2×10^5) were transferred to 96-well shrill-base plates and washed with FACS buffer (PBS containing 1% fetal bovine serum). After centrifugation (300 g at 4°C), the FACS buffer was carefully removed and cells were incubated with the hybridoma supernatant, or 1 μg/mL purified mAb or control antibody for 1 h at 4°C. Cells were then washed twice with FACS buffer and incubated with 100 μL phycoerythrin (PE)-labeled goat anti-mouse IgG (1/250 dilution) (Jackson ImmunoResearch) for 30 min at 4°C. Next, cells were washed twice with FACS buffer and suspended in 400 μL FACS buffer. Fluorescence signals were measured on a FACScan instrument (BD FACSCanto™ II, BD Biosciences, San Jose, CA, USA).

2.6 | Cellular ELISA

For hybridoma screening, 1×10^4 SKOV-3 or CCD-1112Sk cells were plated into each well of 96-well polystyrene plates and, after an overnight incubation, cells were fixed with 2% paraformaldehyde for 20 min. The solution was removed and cells were washed with PBS twice, then blocked with 1% BSA in PBS for 4 h at 4°C. The hybridoma supernatants were diluted 2-fold with 1% BSA, and then incubated for 1 h at room temperature before incubation with HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) for 1 h. Plates were washed, and o-phenylenediamine dihydrochloride (Thermo Fisher Scientific) was used as a substrate; reactions were incubated for 7 min and stopped by the addition of 3 N HCl. Absorbance at 490 nm was measured on a microplate reader (Molecular Devices).

2.7 | Immunoprecipitation assay

SKOV-3 cell lysates and recombinant integrin α3 (#TP320975, OriGene, Rockville, MD, USA) or integrin β1 (#H00003688-P01, Abnova, Taipei, Taiwan) were applied to OV-Ab 30-7 or NM IgG-coupled Protein G Sepharose (GE Healthcare Biosciences, Boston) for l h. Then the beads were washed 4 times with PBS containing

1% Tween-20. Proteins were eluted from the Protein G Sepharose with 2-mercaptoethanol and subsequently separated by SDS-PAGE for Western blotting. NM IgG-coupled Protein G Sepharose served as a negative control.

2.8 | Apoptosis assay

For apoptosis screening, cells were seeded and treated with hybridoma supernatant or antibodies for 6 h. Then cells were trypsinized and stained with FITC-annexin-V antibody and PI (BD Biosciences) in 200 μL reaction buffer for 20 min at room temperature. The samples were analyzed by FACScan (BD FACSCanto™ II, BD Biosciences). Cells positive for FITC or both PI and FITC were counted as apoptotic, and those positive for PI only were considered necrotic cells.

2.9 | Sphere-forming assay

In total, 1×10^4 SKOV-3 and 2×10^3 ES-2 suspended single cells were seeded into an ultra-low attachment dish (Corning). Cells were cultured in serum-free DMEM-F12 with 10 ng/mL basic fibroblast growth factor (bFGF), 20 ng/mL epidermal growth factor (EGF) and B-27 Supplement (Thermo Fisher Scientific). After 10 d, spheres were photographed and lysed for western blotting.

2.10 | Immunofluorescence staining

Cells were seeded on cover slips and fixed with 2% paraformaldehyde for 20 min. After washing, cells were blocked with PBS containing 1% BSA for 30 min. Blocked cells were then incubated at room temperature with purified mAbs (2 μg/mL) or NM IgG (2 μg/mL), in 1% BSA. After 1 h incubation, cells were washed and incubated with Alexa Fluor 488 goat-anti-mouse antibodies. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; 1:10 000) (Thermo Fisher Scientific).

2.11 | Active caspase staining

SKOV-3 and SKOV-3 ITGA3-knockdown cells were treated with OV-Ab 30-7 (20, 2, 0.2 μg/mL) or NM IgG (20 μg/mL) for 6 h, and analyzed according to the manufacturer's instructions for the CaspGLOW™ fluorescein active caspase-3, 8, and 9 staining kit (Thermo Fisher Scientific). In brief, cells were harvested and incubated with FITC-conjugated caspase-3, 8, or 9 specific inhibitors for 1 h. After washing, cells were stained with APC-annexin-V (BD Biosciences) and PI. Finally, the cells were analyzed by flow cytometry (BD FACSCanto™ II, BD Biosciences). Cells negative for PI staining were analyzed for APC-annexin-V and active caspase-FITC.

2.12 | Cell adhesion assay

SKOV-3 cells were detached by 10 mmol/L EDTA in serum-free DMEM medium. Detached cells were washed with DMEM containing 1% BSA to remove the EDTA (Merck), and then incubated with OV-Ab 30-7 for 20 min. After incubation, 6×10^3 cells were seeded onto laminin-coated 96-well plates, which had been prepared by incubation with laminin 10 μg/mL (LN332, BioLamina, Sundbyberg, Sweden) overnight at 4°C. After 45 min, the non-adherent cells were removed by washing with PBS, and the adhered cells were quantified by the MTT (Merck) assay.

2.13 | Immunohistochemistry (IHC) staining

In total, 31 human ovarian cancer surgical specimens were obtained from the archives of the Department of Pathology, National Taiwan University Hospital. This study was approved by the National Taiwan University Hospital Research Ethics Committee. For IHC, 4 μm paraffin-embedded sections were stained using the Super Sensitive™ IHC Detection System (BioGenex) according to the manufacturer's directions. Anti-integrin α3 (MAB1952Z, Merck) and anti-laminin antibody (ab17107, Abcam) were diluted to 1:100 and incubated with tissue sections overnight. Samples with less than 1% positive cells were considered as negative staining. The intensity of staining in positive cases was graded as weak, moderate, or strong. Staining was evaluated by a pathologist, Dr. Kuo, K-T.

2.14 | Knockdown of ITGA3

Stable ITGA3-knockdown (clone: TRCN0000057714) or luciferase-knockdown control (clone: TRCN0000231719) SKOV-3 cells were generated by transduction of lentiviruses with short hairpin RNAs cloned into the pLKO.1 vector. RNAi reagents were obtained from the National RNAi Core Facility located at the Institute of Molecular Biology/Genomic Research Center, Academia Sinica, Taipei, Taiwan. The human shRNA library was TRC-Hs 1.0. Stable cells were selected with puromycin for 1 wk.

2.15 | In vitro migration assays

For the migration assay, 5×10^4 cells were suspended in serum-free DMEM and plated onto membranes (8-μm pore size, Merck) that were coated with laminin (LN332, BioLamina). Medium containing 10% FBS was added to the lower chamber as a chemoattractant. Following incubation for 18 h at 37°C, non-invading cells on the upper surface of the filter were removed with a cotton swab. The invading cells on the lower surface of the filter were fixed and stained with hematoxylin and eosin. The cell motility and invasiveness were determined by counting cells in 5 microscopic fields per well, and the extent of invasion was expressed as the average number of cells per field.

2.16 | Antitumor efficacy

The procedure to evaluate antitumor efficacy in vivo was modified from a previous study.[40] In brief, NOD/SCID mice were obtained from the Jackson Laboratory and were bred in the core facility of the Institute of Cellular and Organismic Biology at Academia Sinica. Female NOD.CB17-Prkdcscid/JNarl mice (4-6 wk old), were injected ip with 1×10^7 SKOV-3 cells suspended in 100 μL DMEM (serum free). To investigate the therapeutic efficacy of OV-Ab 30-7, 20 mg/kg OV-Ab 30-7 or NM IgG was simultaneously injected with the tumor cells. Mouse survival was recorded twice a week.

For the xenograft mouse model, 5×10^6 SKOV-3 cells were injected subcutaneously in the dorsal right flank. When the tumor reached 100 mm^3, the mice were randomly divided into 2 treatment groups (each n = 8): NM IgG (20 mg/kg), OV-Ab 30-7 (20 mg/kg). The OV-Ab 30-7 and control IgG were injected twice weekly through the tail vein. Tumor diameters were measured using calipers at 4-d intervals, and tumor volumes were calculated using the formula, width$^2 \times$ length $\times$ 0.52.

For combination therapy, tumor-bearing mice were randomly sorted into 6 groups (each group, n = 6): Group A, Vehicle control; B, PTX (5 mg/kg); C, CBP (30 mg/kg); D, CBP (15 mg/kg) + PTX (2.5 mg/kg); E, OV-Ab 30-7 (20 mg/kg); and F, CBP (15 mg/kg) + PTX (2.5 mg/kg) + OV-Ab 30-7 (20 mg/kg). Treatments were initiated 1 h after inoculation with tumor cells. Drug and antibodies were administered by ip injection twice a week for 4 wk consecutively. Mice survival rates were recorded twice a week.

For the in vivo bioluminescence assay, SKOV-3 and ES-2 cells were infected with Lenti-luc virus (lentivirus containing the luciferase gene). After puromycin selection, 1×10^7 SKOV-3-Luc or 2×10^6 ES2-Luc cells were ip injected into 4- to 6-wk-old female NOD/SCID mice. At 2 d after inoculation of tumor cells, 150 mg/kg D-luciferin (Goldbio) was injected ip, and bioluminescence was quantified by an Xtreme II (Bruker). Mice were then separated into 3 groups: Group A, Vehicle control; B, CBP (15 mg/kg) + PTX (2.5 mg/kg); and C, CBP (15 mg/kg) + PTX (2.5 mg/kg) + OV-Ab 30-7 (20 mg/kg). Drug and antibody were administered by ip injection twice a week for 4 wk consecutively. Tumor progression was monitored by bioluminescence quantification. Mouse body weight and survival rate were recorded. Animal care was carried out in accordance with the guidelines of Academia Sinica, Taiwan. The protocol was approved by the Committee on the Ethics of Animal Experiments at Academia Sinica. PTX was provided by ScinoPharm Taiwan, Ltd. (SPF2039, Tainan, Taiwan) and CBP were purchased from Abcam (ab120828).

2.17 | Statistical analysis

The binding, apoptosis, caspase activities, and migration assays were analyzed using a two-tailed unpaired Student t test. P-values below .05 were considered significant for all analyses. Values were expressed as means ± SD unless otherwise specified. All data were derived from at least 3 independent experiments. Survival analyses were performed using Kaplan-Meier survival curves, and significant differences between groups were tested using a log-rank test. Correlation coefficients were assayed by Spearman analysis. IHC staining for protein co-expression was analyzed by Pearson chi-squared test.

3 | RESULTS

3.1 | Generation and characterization of mAbs against ovarian cancer cells

Compared with other epithelial ovarian cancer subtypes, clear cell ovarian carcinoma is more frequently aggressive and drug resistant.[41] Therefore, we utilized SKOV-3 cells to develop a new therapeutic mAb for clear cell carcinoma. SKOV-3 cells are a well characterized cell line that is frequently used for ovarian cancer antigen studies.[42-44] We injected SKOV-3 cells into the peritoneal cavity of BALB/cJ mice 3 times at 3-wk intervals to serve as an immunogen for antibody production. More than 2800 hybridoma clones against ovarian cancer cells were generated by this procedure. After characterizing the clones by both cellular ELISA and flow cytometry, we selected 30 hybridoma clones with high reactivity and specificity against SKOV-3 cells and no cross-reactivity with CCD-1112Sk cells (Supporting information Figure S1A,B). To identify mAbs from each selected clone, single hybridoma cells were seeded into wells of a 96-well plate by serial dilution. The mAbs were tested by cellular ELISA (Figure S1C) to confirm their binding affinity for SKOV-3 cells. All 30 mAbs showed high binding affinity for SKOV-3 cells without cross-reactivity to CCD-1112Sk cells. We also tested binding of the mAbs to other cancer cell lines, including ES-2, HCT-116, HTB-10, Mahlavu, H460, MDA-MB-231, MIA PaCa-2, U-2OS, CL1-5, SAS, and A-498. Additionally, binding to non-cancerous cells, NNM, CCD-1112Sk, and HUVECs, was also tested. Most of the mAbs showed low affinity to normal cells (Figure S2A).

To identify the target proteins of these mAbs, SKOV-3 whole-cell lysates were used for western blotting under denaturing conditions. OV-Ab 1-21, OV-Ab 2-1, OV-Ab 4-2, OV-Ab 5-3, OV-Ab 8-11, OV-Ab 9-2, OV-Ab 10-6, OV-Ab 11-1, OV-Ab 12-1, and OV-Ab 13-2 all recognized a protein of 38 kDa. Three other mAbs, OV-Ab 15-23, OV-Ab 20-4, and OV-Ab 22-13, recognized 2 proteins with molecular weights of 170 and 120 kDa on the reducing gel. OV-Ab 7-12 recognized a protein with a molecular weight of approximately 170 kDa on the reducing gel. The remaining mAbs did not obviously recognize any particular protein in either reducing or non-reducing western blots (Figure S2B). Additionally, only OV-Ab 15-23 weakly recognized proteins with molecular weights of 170 and 120 kDa in CCD-1112Sk cell lysates. The rest of the mAbs produced either low or undetectable bioluminescence signals when incubated with CCD-1112Sk cell lysates, as compared with the signals from SKOV-3 cells lysates (Figure S2C). Immunofluorescence staining revealed that OV-Ab 3-5, OV-Ab 7-12, OV-Ab 10-6, OV-Ab 13-2,

OV-Ab 25-3, and OV-Ab 30-7 exhibited extremely high cell-surface binding to SKOV-3 cells, without any detectable binding to NNM cells (Figure S2D). We then used the bicinchoninic acid method to standardize the concentrations of these 6 mAbs and determined the relative binding affinity to SKOV-3 cells with cellular ELISA and flow cytometry. We found that among these 6 mAbs, OV-Ab 30-7 had the best affinity for SKOV-3 cells (Figure S3A,B).

To evaluate the ability of mAbs to induce cancer cell apoptosis, we incubated SKOV-3 cells with each mAb for 6 h and then stained the cells with PI and FITC-labeled annexin-V. OV-Ab 6-3, OV-Ab 13-2, OV-Ab 16-13, OV-Ab 19-13, OV-Ab 25-3, and OV-Ab 30-7 all induced apoptosis in more than 20% of the cell population (Figure S1D). Importantly, we found that, in addition to showing the highest affinity to SKOV-3 cells, OV-Ab 30-7 also produced the highest percentage of apoptotic cells (Figure S1D and Table S1).

3.2 | Identification of integrin α3 as the target molecule for OV-Ab 30-7

To identify which surface protein of the SKOV-3 cells was recognized by OV-Ab 30-7, we cross-linked protein G beads with the mAb and immunoprecipitated SKOV-3 total cell lysates. We identified a 120 kDa protein as a target by silver staining the immunoprecipitate on an SDS-PAGE gel (Figure 1A). Subsequently, the bands were excised from the gel and analyzed by mass spectrometry, revealing 31% sequence coverage of integrin α3 (Figure 1B) and 29% coverage of integrin β1 (Figure S5C). As OV-Ab 30-7 could pull down both

integrin α3 and integrin β1 proteins (Figures 1C and S5A), we then used the recombinant proteins for immunoprecipitation to determine which one was the antigen. Only integrin α3 was precipitated by OV-Ab 30-7 (Figure S5B). Moreover, small hairpin RNA (shRNA)-mediated knockdown of integrin α3 mRNA (shITGA3) in SKOV-3 cells drastically reduced OV-Ab 30-7 binding affinity (Figure 1D) and OV-Ab 30-7-induced cell apoptosis (Figure 2A) compared with cells with control knockdown of luciferase (shLuc). Efficient integrin α3 knockdown was validated by western blotting (Figure S5D). Together, these results showed that OV-Ab 30-7 specifically recognizes integrin α3.

3.3 | OV-Ab 30-7 induces SKOV-3 cell apoptosis through integrin α3

No other antibodies have been previously reported to directly induce apoptosis in ovarian cancer cells. Thus, our finding that OV-Ab 30-7 dose-dependently induced apoptosis in SKOV-3 cells (derived from ascites of a patient with ovarian serous cystadenocarcinoma) and ES-2 cells (derived from a poorly differentiated ovarian clear cell carcinoma tumor) with no effect on ITGA3-knockdown SKOV-3 cells or normal epithelial NNM cells (Figure 2A) warranted further exploration.

We treated SKOV-3 cells and ITGA3-knockdown cells with OV-Ab 30-7 for 6 h and then probed caspase 3, caspase 8, or caspase 9 activities by flow cytometry. The activation levels of all 3 caspases were dose-dependently enhanced by OV-Ab 30-7 antibody treatment, but the levels were not significantly affected

FIGURE 1 Identification of integrin α3 as the target antigen of OV-Ab 30-7. A, Silver staining of proteins eluted from OV-Ab 30-7-conjugated protein G affinity column. E1, E2: elution supernatants from protein G-OV-Ab 30-7 affinity column. Ab only: 5 μg/mL OV-Ab 30-7. B, Peptide sequences of the target protein were deduced from MS/MS analysis. Identified peptides are shown in red. C, SKOV-3 cell lysates were immunoprecipitated with normal mouse (NM) IgG, MAB2056 commercial anti-integrin α3 antibody, or OV-Ab 30-7 antibody. Western blotting was performed with rabbit anti-integrin α3 antibody. D, Flow cytometric analysis of OV-Ab 30-7 binding to ITGA3 and luciferase knockdown SKOV-3 cells. Cells (2×10^5 per condition) were incubated with different concentrations of OV-Ab 30-7 and detected with PE-conjugated anti-mouse IgG. $*P < .05$

(A) OV-Ab 30-7

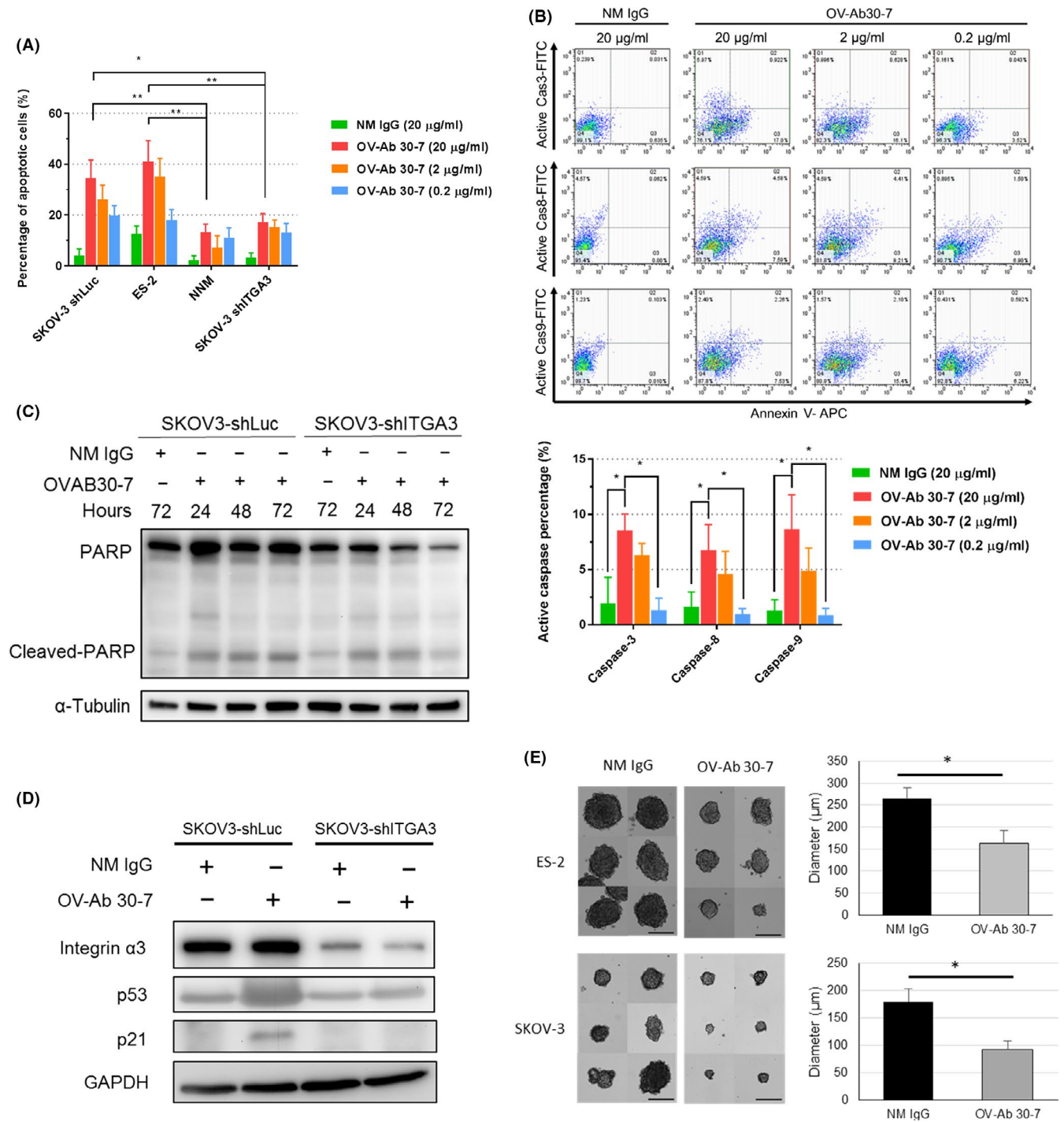(B) Coverage Integrin α3 31%

(C) IP

IB: anti-integrin α3

(D)
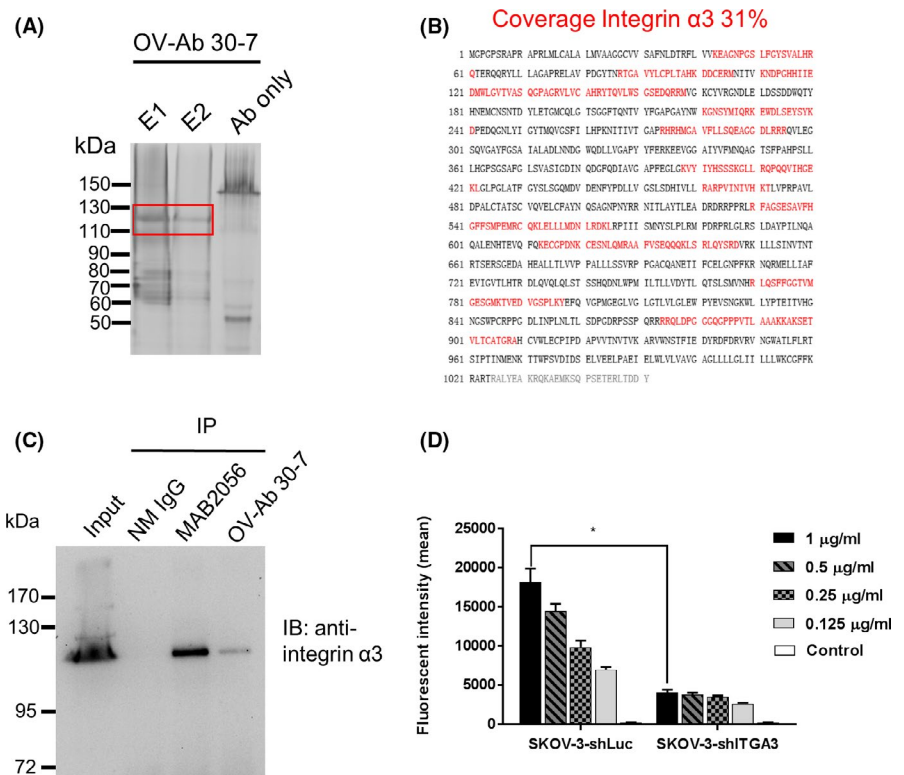
FIGURE 2 OV-Ab 30-7 induces apoptosis, p53 upregulation, caspase activation, and PARP cleavage in SKOV-3 cells. A, Apoptosis of SKOV3-shLuc, ES-2, NNM, and SKOV-3 integrin α3-knockdown cells. FITC-annexin-V and PI staining were used to detect apoptosis after incubation with 0.2, 2, or 20 μg of OV-Ab 30-7 or NM IgG for 6 h. *$P < .05$, **$P < .01$. B, Flow cytometry analysis of caspase-3, 8, and 9 activation in SKOV-3 cells. Cells were treated with NM IgG or OV-Ab 30-7 for 6 h at the indicated concentrations. Representative histograms are shown, with quantitative results below. C, Western blot analysis of cleaved PARP in SKOV-3 cells. Cells were treated with OV-Ab 30-7 or NM IgG (40 μg/mL) for 24, 48, and 72 h. D, Western blot analysis of p53 and p21. Cells were treated with OV-Ab 30-7 or NM IgG for 30 min. E, Representative images of ES-2 (upper) and SKOV-3 (lower) cells treated with 20 μg/mL OV-Ab 30-7 or NM IgG. Quantification of the spheroid diameters is shown to the right. Scale bar, 200 μm

in ITGA3-knockdown cells at any dose (Figures 2B and S6A). Furthermore, we treated SKOV-3 cells with OV-Ab 30-7 for 24, 48, or 72 h and then assessed PARP cleavage by western blot. OV-Ab

30-7 treatment led to an increased level of cleaved PARP protein compared with NM IgG. Moreover, OV-Ab 30-7-induced PARP cleavage was attenuated by knockdown of ITGA3 (Figure 2C). We
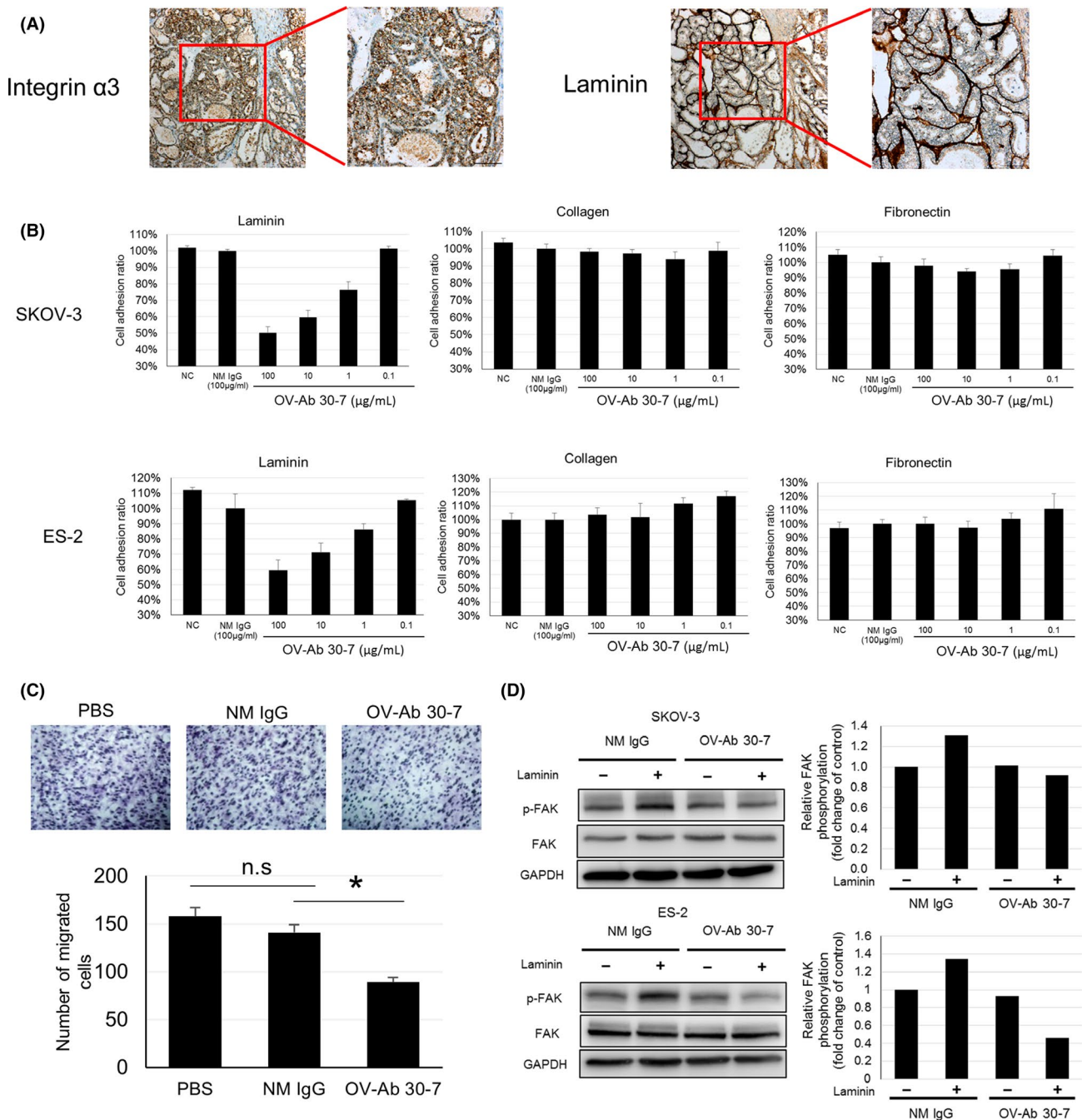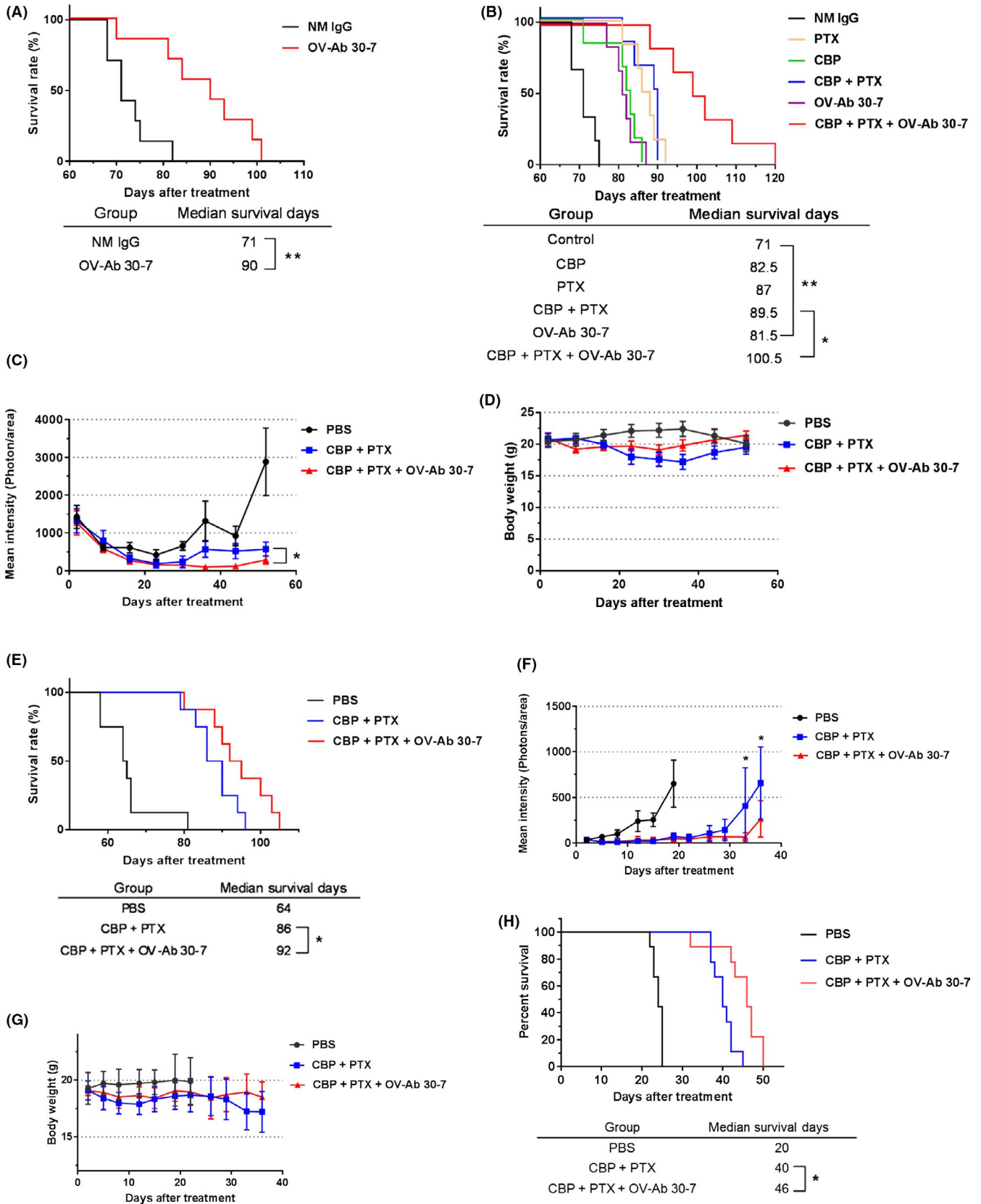
FIGURE 3 Integrin α3 is co-expressed with laminin and OV-Ab 30-7 can block laminin-integrin signaling. A, IHC staining of integrin α3 and laminin-α5 in ovarian cancer tissue reveals co-expression of the proteins. Scale bars, 100 μm (×200). B, Cell adhesion assay. SKOV-3 and ES-2 cells were seeded on laminin, fibronectin or collagen-coated plates for 45 min. After washing, adherent cells were quantified relative to control. C, Transwell migration assay. SKOV-3 cells were treated with PBS, NM IgG, or OV-Ab 30-7 (20 μg/mL), then seeded on laminin-coated wells for 8 h. D, SKOV-3 and ES-2 cells were pre-incubated with OV-Ab 30-7, NM IgG for 15 min at 37°C, then seeded on plates with or without laminin coating for 30 min. Western blot analysis of the indicated proteins was performed. Right panel shows the quantification of phosphorylated FAK signal from western blots

FIGURE 4 OV-Ab 30-7 inhibits tumor growth in vivo. NOD/SCID mice were injected ip with 1×10^7 SKOV-3 cells (A) followed by treatment with 20 mg/kg OV-Ab 30-7 or NM IgG. OV-Ab 30-7-treated mice had prolonged survival compared with those treated with NM IgG (each group n = 7). B, At 1 h after tumor cell injection, mice were treated with NM IgG, PTX, CBP, OV-Ab 30-7, PTX + CBP, or PTX + CBP combined with OV-Ab 30-7 (each group n = 6). Kaplan-Meier survival curves were generated, and the median survival day is shown. C-E, Mice injected ip with 1×10^7 SKOV-3-Luc cells were treated with PBS, CBP + PTX, or CBP + PTX combined with OV-Ab 30-7. C, Tumor bioluminescence, D, body weight, and E, survival were monitored on the indicated days. Each group, n = 8. F-H, NOD/SCID mice injected ip with 2×10^6 ES2-Luc cells were treated with PBS, CBP + PTX, or CBP + PTX combined with OV-Ab 30-7. F, Tumor bioluminescence, G, body weight, and H, survival were monitored. Each group, n = 9 Error bars, SD. *$P < .05$, **$P < .01$

(A)

Survival rate (%) vs Days after treatment

Group	Median survival days	
NM IgG	71	**
OV-Ab 30-7	90	

(B)

Survival rate (%) vs Days after treatment

Group	Median survival days	
Control	71	
CBP	82.5	
PTX	87	**
CBP + PTX	89.5	
OV-Ab 30-7	81.5	*
CBP + PTX + OV-Ab 30-7	100.5	

(C)

Mean intensity (Photon/area) vs Days after treatment

(D)

Body weight (g) vs Days after treatment

(E)

Survival rate (%) vs Days after treatment

Group	Median survival days	
PBS	64	
CBP + PTX	86	*
CBP + PTX + OV-Ab 30-7	92	

(F)

Mean intensity (Photons/area) vs Days after treatment

(G)

Body weight (g) vs Days after treatment

(H)

Percent survival vs Days after treatment

Group	Median survival days	
PBS	20	
CBP + PTX	40	*
CBP + PTX + OV-Ab 30-7	46	

also found that p53 and p21 were upregulated in SKOV-3 cells after OV-Ab 30-7 treatment, an effect that was also attenuated by knockdown of ITGA3 (Figure 2D). Collectively, these results suggested

that OV-Ab 30-7 induced ovarian cancer cell apoptosis by targeting integrin α3. We then sought to investigate whether OV-Ab 30-7 could induce cell apoptosis in breast and pancreatic cancer. We

FIGURE 5 Integrin α3 and laminin expression in ovarian cancer correlate with poor prognosis. A, IHC staining of integrin α3 in ovarian cancer and tumor-adjacent normal ovary tissue. B, Positive and negative integrin α3 IHC staining in the different types of ovarian cancer. C, Overall survival of ovarian cancer patients from the TCGA database stratified by expression of ITGA3, ITGB1, or both. Gene expression was determined by RNA sequencing and reported as median number Fragment Per Kilobase of exon per Million (FPKM) reads. The cut-off value for ITGA3 was 35.34, and for ITGB1, it was 44.09 FPKM. D, In total, 31 clinical ovarian cancer specimens were analyzed by IHC for laminin-α5 and integrin α3. Representative images of weak, moderate, and strong staining are shown. The number of cases with each staining intensity is indicated in the table below ($P < .01$). E, Relationships between ITGA3 and laminin-related gene expression in patients from the TCGA database. Data were visualized by cBioPortal (https://www.cbioportal.org/) and evaluated with Pearson's correlation analysis. F, Kaplan-Meier survival curves of ovarian cancer patients stratified by LAMA3, LAMB3, and LAMC2 expression. G, Kaplan-Meier survival curves for ovarian cancer patients stratified by ITGA3 expression combined with laminin-related gene (LAMA3, LAMB3, LAMC2) expression

found that OV-Ab 30-7 could induce apoptosis in MDA-MB-234 and BxPC3 cells, which have a high binding affinity for the antibody. In contrast, cells with low binding affinity, such as AsPC1, SU.86.86, ZR75 and SKBR3, did not undergo apoptosis after OV-Ab 30-7 treatment (Figure S7A, B).

As integrin expression is known to regulate formation of spheroids by ovarian cancer cells,[45,46] we next examined whether OV-Ab 30-7 could affect spheroid formation in 3D cultures. The results showed that spheroid diameter was significantly smaller when cells were treated with OV-Ab 30-7 compared with control antibody NM IgG (Figure 2E).

3.4 | OV-Ab 30-7 inhibits integrin α3-laminin signaling in SKOV-3 cells

Laminin serves as the ligand for the integrin α3β1 heterodimer.[47,48] Using IHC staining, we found that integrin α3 and laminin were both expressed in ovarian tumor tissue (Figure 3A). We then examined whether OV-Ab 30-7 could block integrin α3 binding to major components of the extracellular matrix. Plates were pre-coated with laminin, collagen, or fibronectin. Then, cells were mixed with OV-Ab 30-7 or control Ab and seeded onto the pre-coated plates. OV-Ab 30-7 inhibited SKOV-3 and ES-2 cell adhesion to the laminin-coated plate in a dose-dependent manner, but cell binding was not inhibited in fibronectin or collagen-coated plates. (Figure 3B). Furthermore, we found that OV-Ab 30-7 inhibited SKOV-3 cell migration on a laminin-coated substrate (Figure 3C). Next, we probed the intracellular signaling pathways activated by integrin. We found that FAK proteins were phosphorylated when cells were seeded on a laminin-coated plate, and this effect was inhibited by OV-Ab 30-7 in both SKOV-3 and ES-2 cells (Figure 3D).
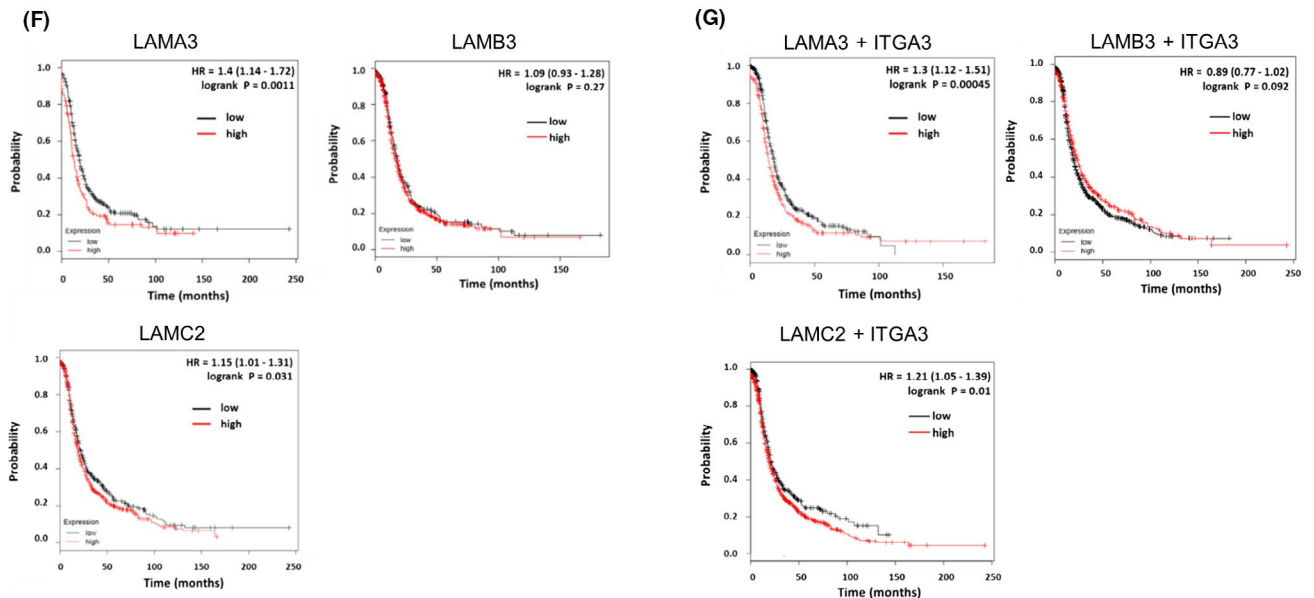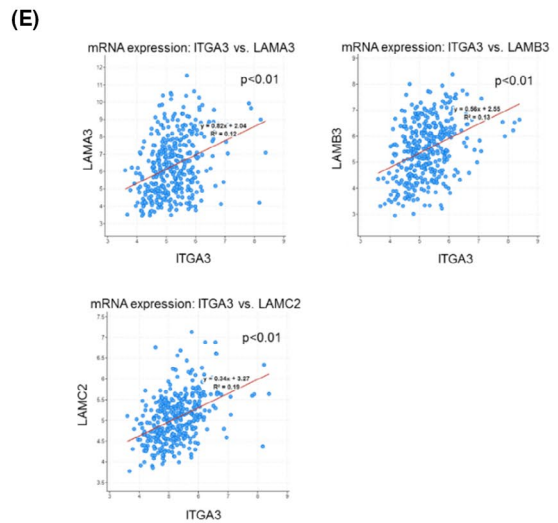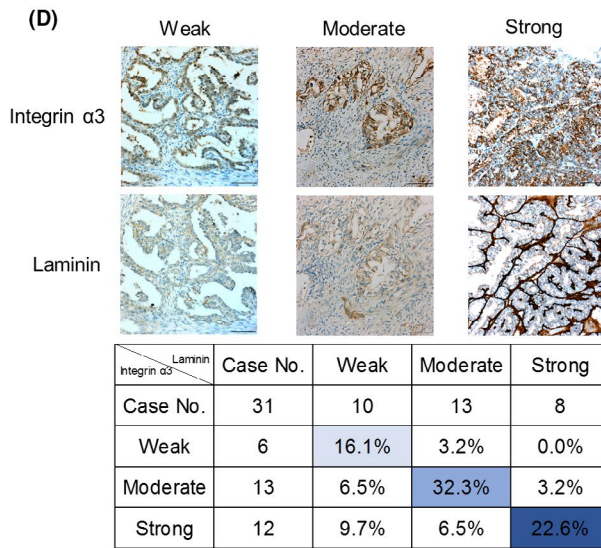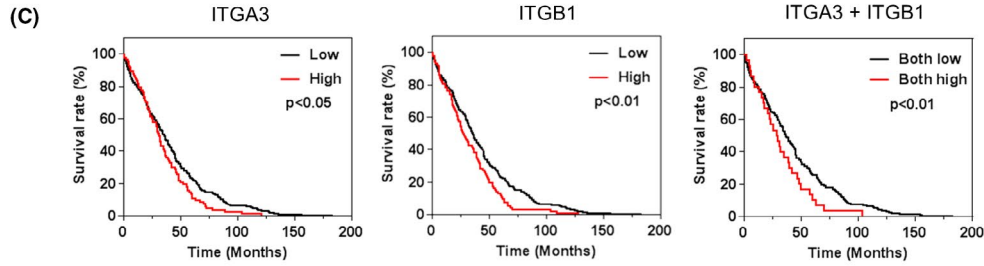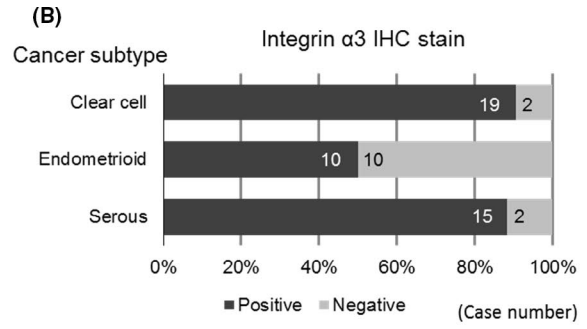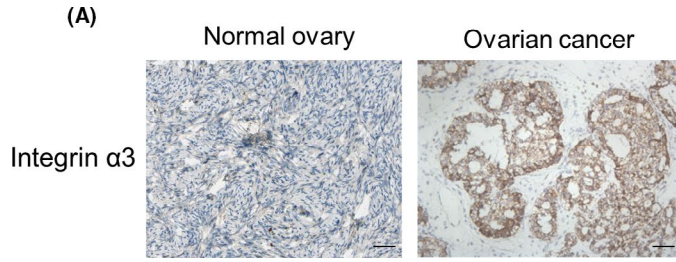
3.5 | OV-Ab 30-7 improves ovarian cancer survival in vivo

To evaluate the therapeutic potential of OV-Ab 30-7 for primary ovarian cancer tumors, we treated a xenograft mouse model with OV-Ab 30-7 or vehicle control. However, OV-Ab 30-7 treatment did not significantly inhibit tumor growth compared with the vehicle control (Figure S8A, B). Adverse effects of OV-Ab 30-7 were then

evaluated in tumor-bearing mice treated with twice-a-week injections of OV-Ab 30-7 for 4 wk consecutively at a dose of 20 mg/kg per injection. The vital organs, including the heart, kidney, and lung, exhibited no significant pathological changes (Figure S8C). To further probe metastatic disease, we co-injected SKOV-3 cells with OV-Ab 30-7 or NM IgG into the peritoneal cavity of SCID mice. When SKOV-3 cells were co-injected with OV-Ab 30-7 (Figure 4A), the median survival of mice was longer than that of the group receiving NM IgG. Next, to examine whether the OV-Ab 30-7 has the ability to improve the currently preferred treatment for ovarian cancer, PTX, CBP, and OV-Ab 30-7 were co-treated as a combination therapy in vivo. At 1 h after SKOV-3 cells were injected into the peritoneal cavity, mice were treated with PTX, CBP, and OV-Ab 30-7 alone or in combination. Again, tumor-bearing mice treated with OV-Ab 30-7 exhibited longer median overall survival compared with NM IgG-treated animals. Moreover, combining OV-Ab 30-7 treatment with PTX and CBP was significantly more effective than combined PTX and CBP treatment (Figure 4B). To examine tumor growth in the mice, we injected SKOV-3-Luc or ES2-Luc cells, which stably express firefly luciferase, into the peritoneal cavity. To mimic clinical treatment for metastatic disease, 2 d after tumor cell injection, mice were treated with PTX and CBP or a combination of OV-Ab 30-7 with both chemotherapeutics (Figure 4C-H). Tumor growth was monitored non-invasively using bioluminescence imaging. PTX and CBP combined with OV-Ab 30-7 showed better tumor growth inhibition and prolonged survival without affecting body weight when compared with the PTX and CBP combination (Figure 4C-H).

3.6 | Integrin α3 and laminin are overexpressed in ovarian tumors and Integrin α3 overexpression correlates with poor prognosis

IHC staining of integrin α3 in ovarian cancer and tumor-adjacent normal ovary tissues are shown in Figure 5A. Integrin α3 was found to be overexpressed in tumors compared with normal tissue. To examine the integrin α3 expression profile in ovarian cancer, we analyzed 3 different histological types of tissue by IHC staining and found that integrin α3 was most often highly expressed in clear cell (19/21, 90.47%) and serous type (15/17, 88.24%) ovarian cancers; only half of endometrioid type tissues (10/10, 50%) were considered to have high expression (Figure 5B). Furthermore, clinical data from

(A)

Normal ovary Ovarian cancer

Integrin α3

(B)

Integrin α3 IHC stain

Cancer subtype

Clear cell	19	2
Endometrioid	10	10
Serous	15	2

■ Positive ■ Negative   (Case number)

(C)

ITGA3 — Low, High, p<0.05

ITGB1 — Low, High, p<0.01

ITGA3 + ITGB1 — Both low, Both high, p<0.01

(D)

Weak Moderate Strong

Integrin α3

Laminin

Integrin α3 \ Laminin	Case No.	Weak	Moderate	Strong
Case No.	31	10	13	8
Weak	6	16.1%	3.2%	0.0%
Moderate	13	6.5%	32.3%	3.2%
Strong	12	9.7%	6.5%	22.6%

(E)

mRNA expression: ITGA3 vs. LAMA3 p<0.01

mRNA expression: ITGA3 vs. LAMB3 p<0.01

mRNA expression: ITGA3 vs. LAMC2 p<0.01

(F)

LAMA3 — HR = 1.4 (1.14 - 1.72), logrank P = 0.0011, low, high

LAMB3 — HR = 1.09 (0.93 - 1.28), logrank P = 0.27, low, high

LAMC2 — HR = 1.15 (1.01 - 1.31), logrank P = 0.031, low, high

(G)

LAMA3 + ITGA3 — HR = 1.3 (1.12 - 1.51), logrank P = 0.00045, low, high

LAMB3 + ITGA3 — HR = 0.89 (0.77 - 1.02), logrank P = 0.092, low, high

LAMC2 + ITGA3 — HR = 1.21 (1.05 - 1.39), logrank P = 0.01, low, high

TCGA databases were analyzed, showing that patients had significantly better survival when tumors expressed low levels of ITGA3, ITGB1, or both ($P < .05$, Figure 5C). As histology type and tumor grade influence survival of ovarian cancer patients,[49] we used the Kaplan-Meier Plotter database (http://kmplot.com/analysis/index.php?p=service&cancer=ovar) to analyze the association of prognosis and ITGA3 expression according to histology type and tumor grade. The results showed that high expression of ITGA3 correlated with poor survival in serous and endometrioid histology types (Figure S9A), and high expression also significantly correlated with poor survival in grade 3 serous type ovarian cancer. (Figure S9B). Thus, higher expression of ITGA3 correlated with poor prognosis in ovarian cancer, especially in the high grade serous type. In addition, integrin α3 expression was positively correlated with laminin expression, according to IHC staining of 31 ovarian cancer patient samples (Figure 5D). By visually analyzing TGCA data with the cBio Cancer Genomics Portal (http://cbioportal.org)[50,51], we found that expression levels of laminin-related genes, including *LAMA3*, *LAMB3*, and *LAMC2*, were positively correlated with *ITGA3* expression in ovarian cancer (Figure 5E), consistent with our IHC results. Therefore, we also analyzed the correlations between patient survival and *LAMA3*, *LAMB3*, and *LAMC2* expression using the Kaplan-Meier Plotter database. Elevated *LAMA3* and *LAMC2* expression alone or in combination with high *ITGA3* expression correlated with poor prognosis. However, *LAMB3* expression was not significantly correlated with survival when analyzed alone or in combination with *ITGA3* expression (Figure 5F,G).

4 | DISCUSSION

Targeted cancer therapies are often more effective and less harmful to normal cells than traditional chemotherapy, as exemplified by PARP inhibitors and anti-angiogenesis drugs currently in clinical trials for ovarian cancer.[52] In this study, we developed novel mAbs that specifically target ovarian cancer. We generated hybridomas that accurately target the ovarian cancer cell line, SKOV-3, and then studied the cellular effects of these mAbs. Subsequently, OV-Ab 30-7 was found to target the surface protein, integrin α3 (but not integrin β1), and directly induced apoptosis in cancer cells, as evidenced by activation of caspase-3, increased cleavage of PARP, and upregulation of p53 and p21 proteins. To the best of our knowledge, this is the first study to describe an anti-integrin α3 mAb that directly induces cancer cell death, rather than stimulating antibody-dependent cellular cytotoxicity or complement-dependent cytotoxicity.

OV-Ab 30-7 can block the laminin-integrin interaction and inhibit downstream FAK protein phosphorylation. It is well known that activation of FAK protein by laminin-integrin α3 binding promotes tumor growth and metastasis.[53,54] Previous studies have found that laminin enhanced cellular migration by binding to the integrin α3β1 heterodimer, and blockade of this interaction by anti-laminin mAb strongly inhibited integrin α3β1-mediated adhesion and migration of cancer cells.[21,55] In ovarian cancer, FAK overexpression was found in 68% of

invasive ovarian cancers and is associated with advanced tumor stage, distant metastasis and poor prognosis.[56] FAK signaling is also associated with ovarian cancer resistance to PTX.[57,58] Treatment of taxane-resistant cell lines with FAK inhibitor can override PTX resistance by decreasing YB-1 transcription factor phosphorylation in an Akt-dependent pathway.[57] Additionally, FAK can promote cell survival by activating the phosphatidylinositol 3-kinase-AKT signaling pathway and suppressing p53-mediated apoptosis.[59] Moreover, deactivation of FAK by antibody,[60,61] antisense oligonucleotide[62] and siRNA[63] were all shown to induce apoptosis in various cancer cells. Therefore, the OV-Ab 30-7 blockade of integrin α3 binding to laminin, which inhibits laminin-induced FAK phosphorylation, is a well supported and likely mechanism to produce therapeutic effects in ovarian cancer.

The role of integrin α3 in ovarian cancer has not been conclusively demonstrated. We found that integrin α3 is overexpressed in ovarian cancer compared with normal ovarian tissue. Furthermore, our analyses of patient survival using online databases showed significant reductions of progression-free survival when *ITGA3* expression level was high. Not surprisingly, laminin expression was correlated with integrin α3 expression, and it was also correlated with poor prognosis in ovarian cancer. Due to the small sample size in our experiment, the expression profiles we found for integrin α3 and laminin might not accurately reflect the entire population of ovarian cancer patients. However, our findings were consistent with several other cancer types, such as breast, colon, lung, oral, pancreatic, and liver cancers.[64-73]

Ovarian cancer is the deadliest gynecological cancer, largely because it is often diagnosed at advanced stages.[74] Combination of PTX and a platinum drug is a standard first-line treatment for advanced and metastatic ovarian cancer. Although most patients respond well to this primary treatment, the majority eventually develop a recurrence.[5] To evaluate the therapeutic potential of OV-Ab 30-7, we investigated the therapeutic effect of the antibody in a model of metastatic ovarian cancer. We treated a human ovarian cancer metastatic animal model with either PTX + CBP alone or PTX + CBP in combination with OV-Ab 30-7. Our results revealed that administration of OV-Ab 30-7 enhanced the antitumor activity of PTX + CBP. Importantly, OV-Ab 30-7 markedly prolonged the median overall survival of metastatic tumor-bearing mice without causing significant changes in body weight. Hence, OV-Ab 30-7 may potentially increase the therapeutic index of the current metastatic ovarian cancer treatment regimens, when used in combination.

Currently, there are 2 integrin-targeting mAbs that have the potential to be used as treatments for ovarian cancer. One is Volociximab, a high-affinity, chimeric antibody against human α5β1-integrin.[75] Volociximab inhibits survival and proliferation of endothelial cells, and it also inhibits ovarian cancer cell metastasis in vivo.[76] However, this antibody failed in a phase II clinical trial due to insufficient efficacy for preventing cancer progression and adverse effects (NCT00516841).[77] The other is Intetumumab (CNTO95), which is a human monoclonal antibody against pan-αv-integrin.[78] Treatment with Intetumumab induced complete response in 1 patient with ovarian cancer in a phase

I clinical study.[79] This drug has not yet progressed to advanced ovarian cancer trials.[80] A previous study also reported a humanized mAb, ONS-M21, which could specifically recognize integrin α3 in gliomas and medulloblastomas without reacting to normal brain tissue.[30] However, to the best of our knowledge, there is currently no integrin α3-targeting therapeutic antibody under development. Targeting integrin α3 might cause adverse effects as the protein is required for basement-membrane structures within the kidney, lung, and skin[81,82] and it is also widely expressed in both fetal and adult epidermis.[23,83] Despite this potential, we found that OV-Ab 30-7 did not induce normal epithelial cell apoptosis, raising the possibility that OV-Ab 30-7 induces cancer cell apoptosis due to oncogenic addiction; this idea is similar to in our previous study on the anti-EpCAM antibody, EpAb2-6.[40,84] Nevertheless, more extensive experiments are needed to address the potential side-effects of OV-Ab 30-7. Treating the antibody by peritoneal injection might minimize off-target effects while retaining anti-cancer activity as ovarian cancer cells usually disseminate through the peritoneal cavity.[85] The epitope of OV-Ab 30-7 has not been determined. Several studies have found that mAbs may bind to a specific protein conformation or glycosylation site critical for cancer-specificity.[86,87] As OV-Ab 30-7 inhibits laminin-induced signaling, we hypothesized that the epitope may be associated with the laminin binding domain on integrin α3. Additionally, we should note that because mouse antibodies are highly immunogenic in humans, OV-Ab 30-7 must be humanized prior to clinical application.

In the present study, we demonstrated that OV-Ab 30-7 recognizes integrin α3 and can inhibit ligand-mediated integrin-FAK signaling, while directly inducing ovarian cancer cell death. Our study introduces integrin α3 as a potential therapeutic target in ovarian cancer and provides a novel biologic drug candidate for this purpose.

DISCLOSURE

All authors have no conflicts of interest to disclose.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.