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Targeting LRRC15 inhibits metastatic dissemination of ovarian cancer

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Abstract

Dissemination of ovarian cancer (OC) cells can lead to inoperable metastatic lesions in the bowel and omentum that cause patient death. Here we show that LRRC15, a type-I 15-leucine-rich repeat-containing membrane protein, highly overexpressed in OC bowel metastases compared to matched primary tumors and acts as a potent promoter of omental metastasis. Complementary models of OC demonstrated that LRRC15 expression leads to inhibition of anoikis-induced cell death and promotes adhesion and invasion through matrices that mimic omentum. Mechanistically, LRRC15 interacted with β1-integrin to stimulate activation of focal adhesion kinase (FAK) signaling. As a therapeutic proof of concept, targeting LRRC15 with the specific antibody-drug conjugate ABBV-085 in both early and late metastatic OC cell line xenograft models prevented metastatic dissemination, and these results were corroborated in metastatic patient-derived OC

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Conflict of Interest

The authors declare no potential conflicts of interest

xenograft models. Furthermore, treatment of 3D-spheroid cultures of LRRC15-positive patient-derived ascites with ABBV-085 reduced cell viability. Overall, these data uncover a role for LRRC15 in promoting OC metastasis and suggest a novel and promising therapy to target OC metastases.

Keywords

LRRC15; adhesion; ovarian cancer; metastasis; ABBV-085

Introduction

Widespread peritoneal dissemination at the time of diagnosis is a hallmark of ovarian cancer (OC) [1]. Peritoneal dissemination and attachment of OC to the mesothelium [2] culminates predominantly in omental and bowel metastasis resulting in malignant bowel obstruction (MBO) [3], which is the major cause of mortality [4] and a common problem in recurrent disease. Despite advances in treatment, the molecular drivers of OC dissemination remains poorly defined thereby impeding development of therapeutic approaches to target bowel metastasis in patients with OC [5].

A major challenge is the lack of faithful models that replicate OC progression and dissemination to dissect and therapeutically exploit molecular vulnerabilities. The recent report of the first comprehensive molecular characterization of primary OC and matching bowel mets, identified genes that are differentially expressed. The causal role of any of these candidate genes in OC dissemination and bowel metastasis is unclear and needs to be clarified in order to pave the way for future studies investigating the potential clinical benefit to be gained from targeting these mechanisms, if evidences emerge causally linking them with malignant spread.

Of these genes, a type-I 15-leucine-rich repeat containing membrane protein encoding LRRC15 gene was found to be highly represented in bowel mets and our preliminary investigation had revealed its role in peritoneal metastasis and growth [6]. LRRC15 located on chromosome 3 at 3q29 is a 581 amino acid containing membrane protein that belong to the LRR superfamily that lack an intracellular signaling domain [7–8] and was reported to be highly expressed on cancer-associated fibroblasts within the active stroma in numerous solid tumors and directly in the epithelial cancer cell compartment of mesenchymal tumors like glioblastoma, sarcomas and melanoma [9]. Previously LRRC15 has been functionally linked to regulating cell-cell and cell-extracellular matrix (ECM) interactions by likely partnering with a range of ECM proteins including fibronectin, laminin and collagen IV through its extracellular leucine-rich repeats [10,11]. Increased stromal expression of LRRC15 is reported in multiple solid tumor types including breast, ovarian and cervical cancers [6, 12–14]. Therefore, the objective of our current study was to further investigate the role of and characterize the mechanism(s) through which LRRC15 promote OC dissemination and bowel and/or omental metastasis. In this study using multiple OC cell lines and xenotransplantation models and a range of functional and molecular assays we have determined a mechanistic link between LRRC15 expression and promotion of OC

metastasis. We also provide the first preclinical proof-of-concept evidence that LRRC15 expression creates a therapeutic vulnerability which could be targeted using the humanized anti-LRRC15 antibody drug conjugate ABBV-085, to blockade OC dissemination and bowel and/or omental metastasis.

Materials and methods

Reagents:

ABBV-085 (anti-LRRC15 humanized IgG1 antibody, consisting of hydrophobic interaction chromatography-enriched E2 in which approximately two anti-mitotic monomethyl auristatin E (MMAE) payloads are conjugated per antibody through a protease cleavable valine–citrulline (vc) linker 29), Isotype mAb and Isotype-vc-MMAE-E2 drug control were received from AbbVie, CA, USA [9]. Other reagents and antibodies used were listed in Supplementary Table S1.

Cell Culture:

Human OC cell lines, LP9/TERT-1 mesothelial cells [15], immortalized normal ovarian fibroblast NOF151hTERT and TRS3 stromal cells, immortalized normal ovarian surface epithelial cells VOSE and IOSE523 [16] were maintained as shown in Supplementary Table S2. Patient-derived ascites were obtained with the approval and written informed consent (IRB-1288-03 to Dr. Shridhar) through the Mayo Clinic Ovarian SPORE program and in collaboration with the University of Minnesota Cancer Center Tissue Procurement Facility with IRB approval and cultured as mentioned [17,18]. The studies were conducted in accordance with recognized ethical guidelines (e.g., Declaration of Helsinki, CIOMS, Belmont Report, U.S. Common Rule).

Generation of knock down (KD) and overexpressed (OE) stable clones:

LRRC15 KD was performed in the OVCAR5 cells with shLRRC15 (Sigma-Aldrich) targeting 3’UTR [Sequence: sh1-GCTATGAAAGAGAGAAGGAAA and sh2-CCAGGTTTCTTCTTCTCTTAA] and stable overexpression (OE) of HA-LRRC15 (Sino Biologicals) in OVCAR7 cell line using standard transfection guidelines and reagents.

ITGB1 knock out (KO) OVCAR5 cells were generated using Crispr/cas9 method using sgRNA clone [target site: TTTGTGCACCACCCACAATT, Genecopoeia] using manufacturer protocol.

Spheroid formation assay:

OVCAR7 EV (empty vector control) and OVCAR7 LRRC15 OE (LRRC15 overexpression) and OVCAR5 NTC (non-targeted control) and OVCAR5 sh1 (LRRC15 stable KD) cells were seeded in ultra-low attachment 24-well plates (2,500 cells/well) for spheroid formation assay. Spheroids were allowed to form for 6 days and imaged using EVOS FL Auto Imaging System (Life Technologies). At least 3 wells were imaged for each biological replicate. Spheroids were manually quantified.

Anoikis assay:

1×10^6 OVCAR5 NTC and sh1 cells and OVCAR7 EV-transfected and LRRCl5 overexpressed cells were grown in suspension for 0-6 days. Cell viability was assessed by MTT assay from the re-plated cultures grown for 6 days [19] and cell survival ability was analyzed by clonogenic assay [20].

Adhesion to mesothelial cells:

LP9/TERT-1 mesothelial cells were grown on 96-well plates. OVCAR5 NTC, sh1/sh2 cells and OVCAR7 EV, LRRC15 cells were pre-labeled with 10mM celltracker™green (CMFDA) for 45 min at 37°C. The labeled cells were washed and 2×10^4 cells/well was added to the mesothelial cells in 4 replicates. After incubation for indicated times, the non-adherent cells washed and fluorescence was quantified using Scion Image Software (Scion Corp, MD, USA).

Adhesion to fibronectin and other ECM proteins:

Each of 2μg/ml fibronectin, 10mg/ml poly-L-lysine, 0.1mg/ml collagen, 1mg/ml laminin, 0.5mg/ml vitronectin was added to 96-well plates in 4 replicates and allowed to air dry. 2×10^4 CMFDA-labeled cells/well were seeded on coated plates and after incubation for indicated times, the non-adherent cells were washed, and fluorescence quantified using Scion Image Software.

Scratch wound-healing assay:

The OVCAR5 NTC and sh1 KD cells and OVCAR7 EV and LRRC15 OE cells were scratched with 10μl pipette tips and replaced with fresh media. 0hr/24hr images were captured using an EVOS inverted microscope (ThermoFischer Scientific).

Immunoblot analysis: Whole cell lysates were subjected to immunoblot analysis [21] against antibodies: LRRC15, cleaved PARP1, cleaved Caspase3, ITGB1, phospho-FAK^{y397}, FAK, MMP2, PCNA, GAPDH and β-actin, visualized using fluorophore-conjugated secondary antibodies (LICOR) by LI-COR OdysseyFc Imaging System (Nebraska, USA) as described [19]. Details of the antibodies used were listed in Supplementary Table S1.

3D invasion assay:

The 3D culture model mimicking the surface layers of the omentum was assembled in fluoroblock transwell inserts with 8μm pores using normal omental fibroblasts (NOF) and human peritoneal mesothelial cells (HPMC), isolated from human omentum, as described previously [22]. Briefly, NOFs ($4x10^3$) mixed with 1.82μg of collagen Type 1 in 50μl DMEM were seeded in transwell fluroblock inserts (8μm pores, $0.6cm^2$) and incubated at 37°C for 5 hours. Thereafter, $5x10^4$ HPMCs in 50μl DMEM were seeded on top of this basement membrane like layer to form a confluent monolayer resembling the mesothelium. CMFDA labeled OVCAR5 NTC or sh1 cells and OVCAR7 EV or LRRC15 OE ($5x10^4$/500μl) cells were seeded in serum free DMEM on the 3D culture and 700 μl of DMEM with 10% FBS was added in the lower chamber to serve as a chemoattractant. Invasion is stopped after 8h by fixing with 4% paraformaldehyde. The invaded fluorescent

ovarian cancer cells on the lower surface of the inserts were imaged (5 fields/insert) using the EVOS FL auto microscope (Life Technologies) and counted.

3D adhesion assay:

The 3D culture model mimicking the surface layers of the omentum was assembled in 96-well Black/clear-bottom tissue culture plates as described in the 3D Invasion assay. Briefly, $2x10^3$ NOFs along with 0.91μg of collagen Type 1 in each well and overlayed with $2.5x10^4$ HPMCs. CMFDA labeled NTC/sh1 and EV/LRRC15 OE ($2.5x10^4$/50 μl) cells were seeded on the 3D culture. Cells were allowed to attach for 30 minutes, and the total fluorescence was quantified using a SynergyH1 plate reader (BioTek). The wells were washed with PBS to remove the nonadherent cells and the residual fluorescence was measured to quantify the percentage of adherent cells.

In vivo adhesion assay:

6-week old female NSG mice (3 mice/group) were injected with the CMFDA fluorescent labelled LRRC15 shRNA or NTC control OVCAR5 cells or LRRC15 overexpressing or EV control OVCAR7 cells intraperitoneally (1 million cells in 0.5 ml PBS). Mice were euthanized 3 hrs post injection. Omentum and peritoneum were dissected and washed with PBS to remove nonadherent cells. The attached cancer cells were dissociated by incubating in trypsin at 37°C with gentle oscillation. The dissociated cells were collected, and their fluorescence was quantified using a SynergyH1 plate reader.

Immunoprecipitation assay:

Equal amount of cell lysates were incubated with anti-ITGB1 or anti-LRRC15 separately for 24hr at 4°C followed by protein A/G-agarose beads addition for 24hr, processed following the the manufacturers protocol and was probed for LRRC15 or ITGβ1 respectively.

Immunofluorescence imaging:

NTC, sh1 OVCAR5 and EV, LRRC15 OVCAR7 cells were seeded on fibronectin-coated cover slips (10μg/ml) and allowed to attach overnight [21], probed for anti-ITGβ1, anti-LRRC15 and anti-vinculin antibodies and captured using Zeiss-LSM 510 confocal microscope.

Flow-cytometric extracellular antigen staining:

NTC and sh1/sh2 cells were fixed, permeabilized and probed for primary-tagged anti-ITGβ1 and anti-CD44 as discussed [23]. The samples were analyzed using a LSR II FACS analyzer (BD Biosciences).

Proximity ligation assay (PLA):

NTC, sh1 OVCAR5 and EV, LRRC15 OVCAR7 cells were seeded on fibronectin (10μg/ml)-coated chamber slides and permitted to attach overnight. The cells were processed using the manufacturers protocol of the Duolink® In Situ Red Starter Kit where the cells are probed for either rabbit anti-LRRC15 or mouse anti-ITGB1 or both followed by ligation

and amplification process and the signal was captured using Zeiss-LSM 510 confocal microscope.

Mouse xenograft model of OVCAR5 cells:

$5X10^6$ cells were injected intraperitoneally (i.p.) into 4-5weeks old, female athymic nude mice. In the post-treatment study model the treatment was initiated one week after tumor cell injection and mice were randomized into 3 groups of 5 each and treatment was continued for 4 weeks. Conversely, in the pre-treatment model the mice were randomized into 3 groups of 7 mice each and the treatment was started 3 days prior to tumor cell injection and continued for 2 weeks. Treatment includes ABBV-085, a monomethyl auristatin E (MMAE)-containing antibody-drug conjugate (ADC, 6mg/kg) and the controls Isotype mAb (6mg/kg) and Isotype-vc-MMAE-E2 drug control (6mg/kg) were injected i.p.; eventually all mice were euthanized at day36. All experimental use of animals will comply with the guidelines of the Institutional Animal Care and Use Committee (IACUC) at the Mayo Foundation following approved protocols.

PDX model of OC:

Fresh tissues were collected from OC patients after consent in accordance with the Mayo Clinic Institutional Review Board through the Mayo Clinic Ovarian Tumor Repository. Tumor grafts were developed as previously described [24,25] under the guidelines of Institutional Animal Care and Use Committee. Briefly, ~0.3ml of minced fresh patient tumor was mixed 1:1 with media and i.p. injected into 6-8weeks old female SCID mice. Mice were monitored by ultrasound and treatment was started when tumors reached 0.5–$1cm^2$. For post-treatment model of PH081, treatment started 2weeks after tumor injection. Mice were randomized in 3 groups each of 10 and therapy started with i.p. administration of Isotype mAb (6mg/kg), the Isotype-vc-MMAE-E2 (6mg/kg) and ABBV-085 (6mg/kg) for a total of 6 treatments at an interval of 4 days. For the pre-treatment model of both PH127 and PH081, similar treatment was administered 3 days prior to the tumor injection followed by another 5 dosage at an interval of 4 days. The tumor mass was assessed weekly by ultrasound; mean of three measurements per session for each animal were provided. All experimental use of animals follow approved protocols under the guidelines of the Institutional Animal Care and Use Committee (IACUC) at the Mayo Foundation.

Immunohistochemistry (IHC):

IHC studies were performed on formalin fixed de-paraffinized sections as previously described [19]. Expression analysis in the xenograft tumor tissue was probed against Ki-67. IHC study for LRRC15 expression was performed in the primary tumor and their autologous omental mets from OC patients and on patient tissue microarray (TMA) containing 27 clear cell, 28 endometrioid and 8 high grade serous tumors as discussed in [6]. TMA slide was scanned on the Aperio ScanScope AT Turbo brightfield instrument (Leica Biosystems) at ×40 magnification and a resolution of 0.25μm/pixel. LRRC15 expression level was scored for intensity and the data was plotted.

Statistical analysis:

All investigation was performed in triplicates for 3 independent experiments unless mentioned otherwise. The results were expressed as mean±standard deviation. Significant changes (*p<0.05, **p<0.01) were determined by two-sided unpaired t-tests, unless otherwise noted.

Results

LRRC15 expression is associated with poor OC patient outcome

RNA sequence of bowel met biopsies compared to matching primary tumors in patients with OC (n=21) identified LRRC15 gene among few others to be significantly represented at high levels in bowel mets (metastatic lesions) [6]. Analysis of LRRC15 expression in OC patients (n=1436) using Kaplan-Meier analysis (Figure 1A) showed higher expression of LRRC15 was significantly associated with adverse outcome. TCGA analysis of high-grade serous subtype showed 16% cases with LRRC15 amplification (Figure 1B). Similar to bowel mets [6], LRRC15 was highly expressed in the stroma of omental mets (7/15) compared to autologous primary tumors (Figure S1A); with no significant changes in 6/15 samples (Figure S1B). Additionally LRRC15 expression was determined by immunohistochemistry on a patient TMA containing 27 clear cell, 28 endometrioid and 8 high grade serous tumors. Patient characteristics were described in supplementary table S3. Levels of LRRC15 expression were categorized as no (0), low (1+), moderate (2+) and high (3+) staining and plotted (Figure S1C). Representative triplet sections showing 2+ intensity in the different histologies is shown in figure S1C. 7 of 27 (26%) clear cell tumors, 16 of 28 (57%) endometrioid tumors and 5 of 8 (62.5 %) high grade serous tumors expressed high levels of LRRC15. Expression analysis of LRRC15 in the tumor and stromal compartments in the TMA revealed stromal LRRC15 positive staining in 1 of 7 LRRC15+ (14.2%) clear cell tumors, 3 of 16 LRRC15+ (18.7%) endometrioid tumors and 2 of 5 LRRC15+ (40%) high grade serous tumors (Figure S1D).

LRRC15 expression promotes multiple cellular properties necessary for OC dissemination and omental metastasis

Peritoneal metastases is regulated by the ability of OC cells to shed from the primary tumor, survive during transit as free-floating single cells or cell aggregates, and subsequently attach to and invade through mesothelial lining of the abdominal cavity to colonize various metastatic sites [26,27]. We, therefore, investigated the role of LRRC15 in this metastatic cascade. Western blot analysis showed that OVCAR5 and 8 cells robustly expressed LRRC15 with Kuramochi, DOV13, Hey1 and TRS3 cells with lower levels of LRRC15 (Figure 1C–D). In contrast, normal ovarian surface epithelial VOSE and IOSE523 cell lines, the normal ovarian fibroblast cell line NOF151hTERT and the LP9/TERT-1 mesothelial cells were deficient in LRRC15 (Figure 1C–D). Additionally, immunoblot analysis of OC patient-derived tumorografts (PDX) showed high expression of LRRC15 in PH127 and PH081 tumors (Figure 1E).

Next, we investigated the role of LRRC15 in OC metastasis as described schematically in the Figure 2A. We generated knock down (KD) LRRC15 expression clones in OVCAR5

cells using two different shRNAs (sh1 and sh2) and the NTC as control (expressing non-targeting control) (Figure 2B), and overexpression clones of LRRC15 in OVCAR7 cell line with no detectable levels of LRRC15 and generated OVCAR7 Cl2/Cl3 (LRRC15 overexpression, OE) or EV (empty-vector control) (Figure 2C). To mimic the *in vivo* microenvironment, cells were initially cultured on 3D ultra-low attachment plates as spheroids for 6 days to assess whether sphere formation depends on LRRC15 expression. LRRC15 expressing OVCAR5 and OVCAR7 OE cells showed significantly more larger spheroids compared to the LRRC15 KD and vector transfected cells respectively (Figure 2D and F). Cells were replated under 2D conditions to confirm that LRRC15 overexpressing cells were viable and survived in an environment not attached to ECM using MTT and CFA assays respectively. Interestingly, KD of LRRC15 in OVCAR5 cells led to loss of survival and colony forming ability. The surviving fraction of dissociated single cells replated in 2D-tissue culture dishes at clonal densities further suggested that LRRC15 depletion promoted anoikis (Figure 2E and H). In contrast OVCAR7/LRRC15 cells showed improved cell viability and colony forming ability compared to controls (Figures 2G and J), suggesting LRRC15 overexpression promotes anoikis resistance. Consistent with these results, levels of cleaved PARP1 as determined by immunoblot analysis showed increased expression in OVCAR5 KD cells and decreased expression in OVCAR7/LRRC15 cells compared to their respective controls (Figures 2I and K).

To understand the role of LRRC15 in cell-to-cell adhesion and how the altered expression of LRRC15 affects the ability of cancer cells to adhere to mesothelial cells, OVCAR5 and OVCAR7 derivative cell lines were pre-labeled with CMFDA and added on top of the LP9/TERT-1 mesothelial cells monolayer. Fluorescence intensity was measured at the indicated time points which reflect the degree of cancer cell adhesion to mesothelial cells. We found a significant reduction in the adhesion property of the LRRC15 deficient cells to the mesothelial layer, however the LRRC15 overexpressed cells showed an increased adherence phenotype compared to EV cells (Figure 3A–B). We also assessed the ability of the fluorescently labeled cells to adhere to the omentum surface using the 3D culture model mimicking the surface layers of the omentum [22] (Figure 3C). A significant decrease in the adherence percentage (17%) of OVCAR5 KD cells was observed compared to NTC cells (41.5%, Figure 3D,E). In contrast a significant increase in the adhesion phenomenon under similar assay condition was obtained in the LRRC15/OVCAR7 cells compared to control (Figure 3F). However, no alteration was obtained in the proliferative ability of the either the LRRC15 KD or overexpressed cells compared to their respective controls (Figure S2A–B).

Given that the LRRC15 deficient cells loses their adherence property to the omental layer known to express both fibronectin and vitronectin [28], fluorescently labeled OVCAR5 NTC and sh1 KD cells were seeded on the top of the poly-l-lysine, collagen1A1, fibronectin (FN), laminin and vitronectin coated plates for 1hr and the percent adhered cells were counted. Results indicate that KD cells showed a significant reduction in adhesion to fibronectin, collagen1A1 and vitronectin (Figure S2C, 3G), with no significant change in adherence to the poly-l-lysine and laminin coated plates. To further validate, we performed an *in vivo* adhesion assay by injecting CMFDA-labeled OVCAR5 KD and NTC cells intraperitoneally into NSG female mice. After 3hrs, mice were sacrificed, omentum and peritonium were dissected, washed with PBS, and adherent cancer cells were dissocated with trypsin. The

fluorescence intensity of the dissociated cancer cells was measured to quantify the extent of adhesion. A significant decrease in fluorescence intensity indicative of reduced adherence rate was observed in the KD group of mice compared to the control cohort (Figure 3H,I). To confirm we also performed the *in vivo* adhesion assay injecting CMFDA-labeled OVCAR7 LRRC15 overexpressed (OE) and EV control cells intraperitoneally in similar manner as mentioned above and the data revealed significant upregulation in fluorescence intensity suggestive of increased adherence rate observed in the LRRC15 OE group of mice compared to the control cohort (Figure 3J). These results highlight the importance of LRRC15 in OC metastatic cascade.

LRRC15 regulates focal adhesion (FA) formation in OC cells

Activation and clustering of integrins upon binding to ECMs lead to FA formation and the complex formation was analyzed in the OVCAR5 and OVCAR7 derivatives when grown on FN-coated plates and immunostained for vinculin and F-actin. Earlier reports establish vinculin as a principal regulator of the FA formations [29–31] and have shown that vinculin downregulation results in reduced adhesion and increased migration compared to vinculin expressing wild-type cells. In contrast, vinculin overexpression increases the number/masses of FAs and diminished cell motility [32]. Confocal imaging against fluorescently tagged vinculin (red) showed larger and numerous FA complexes mostly in the interior of the OVCAR5 sh1 KD cells compared to the smaller and fewer ones towards the periphery of the control cells (Figure 4A). Moreover, the OVCAR7/LRRC15 cells showed redistribution of vinculin co-localizing with F-actin (green) in the periphery of cancer cells compared to EV cells (Figure 4B). Percent vinculin positive cells were calculated in a total of 50 cells per group is shown Figure 4C. Together, the results are in agreement with the increased migratory phenotype observed in cells with LRRC15.

Having shown that LRRC15 promotes adherence to the omentum and alteration in FA formation, we determined if LRRC15 had a role in invading through the omentum surface in a 3D-culture model mimicking the surface layers of the omentum. The 3D-culture system was assembled by a confluent monolayer of human primary mesothelial cells seeded over a layer of Type-I collagen and normal omental fibroblasts (Figure 4D). Fluorescently labeled OVCAR5 NTC and KD cells and OVCAR7/LRRC15 and EV control cells were seeded on the 3D-culture matrix as previously described [33] and allowed to invade for 12hr. A significant increase in invasion was obtained in the LRRC15/OVCAR7 cells compared to the vector control cells (Figures 4E and G). In contrast, sh1 KD cells showed reduced invasion compared to control cells under similar assay conditions (Figures 4F and H). Furthermore, LRRC15 OE OVCAR7 cells showed increased migration potential compared to EV control cells whereas the sh1 KD cells showed the reverse effect (Figures 4I) in the wound-healing migration assay.

LRRC15 regulates multiple ECM gene expression and directly binds to β1-integrin

Among the well-defined cell surface receptors that binds to various ECM proteins are integrins, particularly α5β1-integrins in promoting metastasis in OC is well recognized [34,35]. LRRC15 was necessary for binding to fibronectin, laminin and collagen (Figure 3G) and β1-integrin can heterodimerize with several α-subunits to form receptors for these

three matrix [36]. Moreover, FA formation involves integrin signaling mediated, at least in part, through engagement of β1-integrin. CD44, is another adhesion molecule that can bind to both fibronectin and collagen and associated with poor prognosis in OC patients [37,38]. Therefore, we studied the expression of β1-integrin and CD44 in OVCAR5 KD and NTC cells grown on FN-coated plates by flow cytometry. LRRC15-deficient OVCAR5 cells showed 80% downregulation of ITGB1 and 40% reduction in CD44 expression respectively compared to LRRC15 proficient control cells (Figures 5A–C, S2D and E).

Having found that there is a reduction in ITGB1 expression in the KD cells, we surmised that LRRC15 binding to FN in the ECM, may enhance ligand activation of β1-integrin by interacting with each other. Confocal microscopy revealed a reduction in the co-localization between LRRC15 with ITGB1 in KD cells compared to control cells, however a strong interaction was observed in the OVCAR7/LRRC15 cells (Figure 5D–E). The interaction was validated when LRRC15 co-precipitated with β1-integrin in the OVCAR5 NTC-control but not in KD cells (Figure 5F). Similar interaction was also obtained in the OVCAR7/LRRC15 cells compared to EV control (Figure 5G). Consistent with these findings, the reverse IP against LRRC15 also immunoprecipitated β1-integrin in the OVCAR5/NTC and OVCAR7/LRRC15 cells (Figures 5F–G, panel3–4 respectively). To further validate the interaction, we performed the proximity-ligation assay to quantify the interaction between LRRC15 and ITGB1 in the above mentioned cell types (Figure S3A–B). A significant attenuation in the red fluorescent signal measuring positive interaction was obtained in the LRRC15 sh1 KD cells compared to the NTC cells (Figure S3A,C). In contrast the LRRC15 OE OVCAR7 cells showed more positive interaction compared to EV controls (Figure S3B,C).

LRRC15-β1 integrin interaction activates FA signaling

Given that LRRC15 associates with β1-integrin and KD cells have more vinculin positive FA, we hypothesized that formation and de-assembly of FAs that are in part regulated by focal adhesion kinase (FAK) activity may be altered and reflective of the LRRC15 levels. OVCAR5 KD and NTC cells grown on FN did not show significant auto-phosphorylation of FAK at pY397 at 6hr, whereas activation was observed in OVCAR7/LRRC15 cells (Figures 5H–I respectively). The functional significance of the β1-integrin interaction with LRRC15 was investigated by assessing the effect of ITGB1 knock out (KO) which shows a reduction in migratory phenotype of the OVCAR5 cells (Figure S3D). LRRC15 KD and ITGB1 KO cells alone and in combination results in significant decrease in migration rate in OC cells while the reverse is true in LRRC15 overexpressed cells and in the cells rescued for LRRC15 and ITGB1 in their respective KD cells (Figure S3E–F). To further validate, we performed adhesion assays on the scrambled (scr) control OVCAR5 cells expressing LRRC15 and ITGB1 KO OVCAR5 cells that still retain LRRC15 expression on FN coated plates before and after pre-blocking the FN binding sites with recombinant LRRC15 protein and monoclonal antibody (mAb) from AbbVie that is a strong binder of LRRC15. The data shows that pre-blocking LRRC15 binding to FN inhibits the adhesion of both LRRC15 expressing OVCAR5 NTC cells and ITGB1 KO cells that retain LRRC15 expression (Figure S3G–H).

Therapeutic targeting of LRRC15 suppresses omental/bowel metastasis in OVCAR5 xenograft model

We demonstrated that LRRC15 depletion led to suppression of both tumorigenesis and metastatic spread in xenograft model, implicating LRRC15 as a novel therapeutic target for OC [6]. Recently ABBV-085, an ADC-directed against LRRC15 was found to be safe and efficacious against LRRC15 cancer-positive and cancer-negative/stromal-positive preclinical models both as a monotherapy and/or in combination with standard therapies [9]. To address the efficacy of ABBV-085 in OC, we tested the effect of ABBV-085 *in vitro* in OVCAR5 cells. As shown in figure S4A–B, ABBV-085 dose-dependent reduction in cell viability was observed for LRRC15-expressing OVCAR5 NTC cells but not in KD cells.

To evaluate *in vivo* efficacy of ABBV-085 in suppressing early and late OC metastasis, two different OVCAR5 xenograft models were used. In the early metastatic model (Figure 6A) we evaluated the efficacy of ABBV-085 in preventing the adhesion of OC cells to the omentum/peritoneum, where the therapy was initiated 3days before OC cell injection. As referred [9], after 3days tumor cells were i.p. injected into female athymic nu/nu mice followed by 5 doses of i.p. injections of ABBV-085 and the control drugs at an interval of every 3days. Treatment with ABBV-085 showed almost no tumor burden in the mice compared to the isotype-mAb and isotype-vc-MMAE drug-treated controls (Figures 6B–C). Comparative analysis of tumor weight and mean abdominal circumference across the cohort also revealed the efficacy of ABBV-085 in preventing the adhesion of OC cells compared to control groups (Figures 6D–E). Reduced Ki67 staining was observed in the ABBV-085 treated group compared to controls (Figure 6F–G).

Since majority of the OC patients have widespread peritoneal dissemination and ascites at time of diagnosis, the efficacy of ABBV-085 was assessed after 1 week of tumor cell injection when they have had enough time to adhere to the peritoneal cavity. To determine if ABBV-085 can still be effective in inhibiting the micro-metastasis, in the late metastatic models, 6 doses each of ABBV-085 and the respective control drugs were administered at an interval of 3days and the mice euthanized on day36 when the tumor burden exceeded 10% of the body weight in the control isotype-mAb cohort (Figure S5A). A significant regression of the metastatic growth was obtained in the ABBV-085 treated mice cohort compared to the controls (Figures S5B–C). However, isotype-vc-MMAE-E2 drug-control presented modest inhibitory side-effect mainly due to the highly cytotoxic nature of the drug in this model. Similar comparative analysis of tumor weight and mean abdominal circumference showed significant reduction in the metastatic dissemination and tumor growth in ABBV-085 treated mice (Figures S5D–E) and reduction in Ki67 levels (Figure S5F–G). Liver histology as assessed by H&E staining were normal in control and treated groups in both the models (Figure S6A). Both the early and late metastatic model of OC xenograft suggests that the targeted therapy of LRRC15-positive cancer cells with ABBV-085 prevents tumor growth.

ABBV-085 reduces cell viability in LRRC15 expressing human patient-derived ascites

To understand the clinical relevance, we screened the differential expression of LRRC15 in 7 patient-derived ascites cultures [17,18] and the details of the clinical characteristics of the patients were given in the supplementary table S4. Confocal imaging of ascitic

cultures using human epithelial specific antigen marker (EpCAM) and fibroblast activated protein marker (FAP) showed that ascitic cells are predominantly epithelial in nature (Figure S6B) and immunoblot analysis revealed that 4 of 7 samples A4832, A3626, JM067 and DC378 showed LRRC15 expression (Figure 7A). To determine the efficacy of ABBV-085, the ascitic cells were cultured as 3D spheroids and treated in triplicates with indicated concentrations of ABBV-085 and the drug controls and cell viability was measured analyzing ATP levels using CellTiter-Glo. We observed a dose-dependent reduction in cell viability in the 4 LRRC15-expressing ascitic models upon ABBV-085 treatment compared to control drugs (Figure 7B–E). However, no significant changes in the ATP levels were observed in the LRRC15 non-expressing A7683 and AM812 cells (Figures 7F–G). Furthermore, CMFDA-labeled A4832 and AM812 ascites cells were seeded on top of fibronectin-coated plates for 2hr and percent adhered cells were counted upon treatment with ABBV-085 and drug controls. While LRRC15-positive A4832 cells showed a significant reduction in adhesion to fibronectin, however no such significant change was observed in adherence of LRRC15 non-expressing AM812 cells (Figures S6C–D). Additionally, western blot analysis on LRRC15 expressing ascites A4832 and JM067 showed no change in the expression of LRRC15 following treatment with M25 and MMAE-E2 controls and ABBV-085 (Figures S6E–F). Together it suggests that ABBV-085 treatment targets LRRC15 expressing OC ascites.

Efficacy of ABBV-085 in the PDX model of OC

Given the importance of targeting LRRC15 and the need for preclinical models to evaluate the efficacy of ABBV-085 in OC, we selected the two PDX PH081 and PH127 models expressing LRRC15 (Figure 1D) for our further studies. Since our *in vivo* adhesion model establishes that a time of 3hr is enough for the cells to adhere to peritoneum/omentum (Figure 3H–I), we evaluated the efficacy of ABBV-085 in the pre-treatment metastatic PH127-PDX model. For the early metastatic model of PH127 xenograft, we found that only 3 mice out of 11 formed tumors in the ABBV-085 group (Figure 8A–B) signifying the effect of ABBV-085 in inhibiting the adhesion of LRRC15 expressing cells to the peritoneum compared to 10 out of 11 and 9 out of 13 in isotype-vc-MMAE and mAb controls respectively. A detailed analysis at the initial time of detectable tumor engraftment by ultrasound also showed similar effect in the PH127 xenograft and Ki67 staining also showed reduced expression in the ABBV-085 treated group (Figure 8C, S7A–B). Decreased p-FAK^{y397} levels and a reduction in ECM infiltration was found in ABBV-085 treated cohort compared to controls (Figures S7C–D). Additionally, western analysis of LRRC15 showed that there is no change in the expression of LRRC15 itself within the ABBV-085 treated and drug control treated groups (Figures S7E). No toxicity was also observed in the ABBV-085 treated group compared to the drug control groups by analyzing the liver histology (Figure S7F).

We also determined the efficacy of ABBV-085 in both early and late metastatic model for PH081 PDX. For the early metastatic model of PH081 xenograft, treatment was initiated 3days prior to tumor cell injection followed by 6 doses of treatment at an interval of 4days (Figure 8D). The tumor volume was measured for 15weeks by ultrasound. A significant regression in tumor volume was observed in ABBV-085 treated animal cohort by week

8 compared to two control groups (Figure 8E). For the late metastatic model, 6 doses of treatment at an interval of 4days were started after 2weeks of tumor cell injection when the tumors reached 0.5-1cm^2 and mice were monitored for reduction in tumor volume for 6weeks. To our surprise, there was no difference in tumor growth in ABBV-085 treated cohort compared to controls (Figure 8F). ABBV-085 treated cohort showed reduced Ki67 levels in the PH081 early metastatic model (Figure S8A–B). However, no adverse effects like weight loss or poor health due to treatments was noted in all the PDX models. Similar decreased levels of p-FAK^{y397} and reduced ECM infiltration was found in ABBV-085 treated cohort compared to controls (Figures S8C–D). Furthermore, immunoblot analysis of LRRC15 showed no change in the expression between the ABBV-085 treated and drug control treated groups (Figure S8E). Additionally the H&E staining of the tumor tissue on the omentum from PH081 PDX model show that tumor cells were able to adhere and form tumors (Figure S8F). Together, the result strongly supports that ABBV-085 effectively prevents adhesion and *de novo* tumor growth but may not suppress the previously established tumors.

Discussion

Metastasis is the most significant step in cancer progression as it limits the curative surgical treatment for OC. Due to the essential nature of cell-to-cell adhesion, cell-to-matrix adhesion, and resistance to anoikis in successful metastatic progression, critical regulators that control these steps may play an important role in OC dissemination. A better understanding of the regulators of various steps in omental/bowel metastasis in OC is essential to clinically facilitate therapeutic approaches that will treat this deadly complication and improve patient prognosis.

Our recent interest in identifying regulators that promotes metastasis to the bowel/omentum in OC revealed a specific metastatic gene signature (n=21 genes) using RNASeq analysis. Among the most significantly overexpressed genes in bowel metastases, we found that 13 of the 21 ECM-related genes expressed only in the stroma with an exception of LRRC15 which was found to be present both in stroma and epithelial compartments [6]. Increasing evidence showed that most of these genes were associated with regulation of EMT, fibrosis and metastasis. For example, reports revealed that MFAP5 coordinates binding to α5β3 integrin and activates collagen genes through ERK signaling in CAFs [39,40]. Additionally, stromal expression of fibroblast activating protein, FAP [41], COL11A1 and POSTN1 emphasizes the significance of the stromal component in the metastatic development [42]. Affymetrix GeneChip of breast tumors showed high differential LRRC15 expression in cells metastasizing to the bone compared to the brain [43]. Bignotti et al. reported LRRC15 as one of upregulated gene in omental mets amongst 120 genes compared to unmatched OC [44] and studies by Reynolds et al. [45], showed that LRRC15 binds to ECM proteins including collagen and fibronectin. However, whether LRRC15 has a crucial role to play in the metastatic cascade of OC was never explored. In this study, we identified LRRC15 as playing a significant role in OC metastasis by promoting anoikis resistance, increased adhesion and colonization to omentum/peritoneum and invasion using different approaches. Mechanistically we report for the first time that in OC, LRRC15-expressing cells adhere

to fibronectin and promote the clustering of β1-integrin followed by the activation of FAK signaling and promote metastasis.

Among the stromal markers, Yeung et al. showed that targeting using MFAP5 ADC reduces fibrosis and increases chemosensitivity in both the ovarian and pancreatic cancers [39]. Additional report by Fabre et al. showed that targeting FAP with OMTX705 ADC prevents tumor growth supporting its clinical development [46]. However, clinical development of these ADCs is still warranted. In contrast, LRRC15 targeting ADC, ABBV-085 was evaluated in phase 1 clinical trial in sarcomas and other advanced solid tumors (NCT02565758) [47]. Promising antitumor efficacy was obtained when ABBV-085 is used as monotherapy or in combination therapy in xenograft models of several solid tumors including sarcoma, pancreatic, head and neck, breast, glioblastoma and lung [9]. Additionally, ABBV-085 monotherapy attenuates tumor growth in LRRC15 positive soft-tissue sarcomas (STS) PDX models [48] including the highly chemo-refractive undifferentiated pleomorphic sarcomas (UPS) with poor survival rate and treatment options [49]. Our *in vivo* analysis for the first time showed the efficacy of targeting LRRC15 by ABBV-085 in preventing the adhesion and inhibiting metastasis of LRRC15-expressing cancer cells using both the OVCAR5-based early and late metastatic xenograft models. Additionally, ABBV-085 treatment inhibits tumor burden and dissemination in the early metastatic model of both the LRRC15 positive PH127 and PH081 PDX xenograft and validates that ABBV-085 prevents early adhesion phenomenon of the cancer cells required for metastatic dissemination.

However, to our surprise, we found no difference in tumor growth in ABBV-085 treated cohort compared to the two control groups in the late metastatic model of the PH081 xenograft. The reason for this unexpected finding is, we believe is the propensity of cancer cells to seed in the peritoneal/omental/bowel may occur within a day or 2 allowing these micro-metastases to start growing at the rate to reach a tumor volume after 2weeks when the drug treatment was initiated. The seeding of the micro-metastatic tumors may have counteracted the effect of ABBV-085 to prevent adhesion of LRRC15-expressing cells to the preferred “soil”. Treatment with ABBV-085 may have targeted LRRC15-expressing clones, however due to the long latent period and tumor heterogeneity the LRRC15 non-expressing clones may have survived in this favorable environment and overgrew once the LRRC15-expressing cells were eliminated. Taken together, the result suggest that ABBV-085 is able to prevent adhesion of tumor cells when the mice were pre-treated with the drug.

Our additional studies showed promising antitumor efficacy with ABBV-085 in the LRRC15-positive OC ascites cells while no effect in LRRC15 non-expressing cells. One of the arguments against targeting proteins overexpressed in metastatic sites of OC is that, the majority of tumors on the omentum and the ovary are optimally debulked and therefore targeting them may have negligible clinical significance. However, it does not take into account the presence of minimal residual disease (MRD) that potentially results in recurrent disease. Due to its unique tumor biology, which is initially dependent on attachment to a fibronectin-rich mesothelial surface, OC may be especially suited to a LRRC15–targeting approach.

Tumor-stromal interaction that contributes to inherent and acquired multidrug resistance is the major reason for chemotherapy failure in most cancers [49,50]. Thus, improvement of anti-fibrotic/anti-stromal therapies was in development and LRRC15 as a mesenchymal marker should be potentially exploitable in solid tumor therapy. Our data demonstrates that ABBV-085 is highly effective in treating micro metastases and in killing tumor cells in ascites. Since most ovarian cancer patients suffer from relapse and development of ascites, following the standard of care cytoreductive surgery and chemotherapy, ABBV-085 can be effective in prevention of such conditions. Till date analysis on the expression of LRRC15 in relation to recurrent and minimal residual disease (MRD) is missing and is associated with poor survival. Identification of MRD biomarkers is critical specifically if they are drugable targets such as LRRC15 and may lead to effective treatment. As we continue to develop an improved understanding of the complex interactions in the microenvironment, we will be able to advance stroma-targeting strategies for more-effective anticancer treatments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Statement of Significance

This study identifies that LRRC15 activates β1-integrin/FAK signaling to promote ovarian cancer metastasis and shows that the LRRC15-targeted antibody-drug conjugate ABBV-085 suppresses ovarian cancer metastasis in preclinical models.

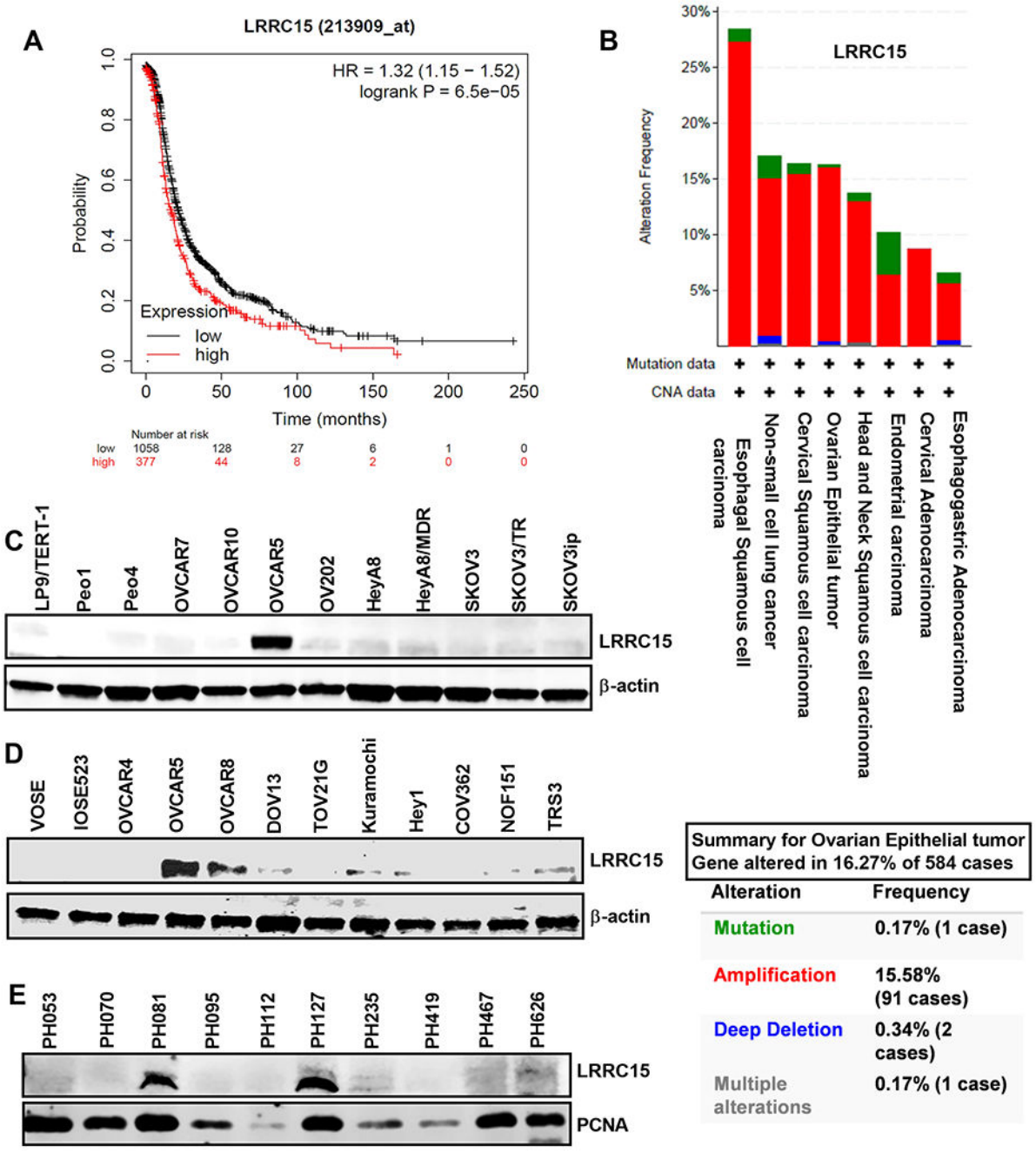

Figure 1: LRRC15 is associated with ovarian cancer.

(A) Kaplan-Meier progression free survival (PFS) analysis shows high LRRC15 expression is associated with worse PFS in a cohort of 1436 OC patients. (B) TCGA analysis of LRRC15 expression in several cancer types along with the percent gene altered as evaluated in the ovarian epithelial tumor. (C-D) Immunoblot analysis of LRRC15 in OC cell lines and in the OC fibroblast cell lines NOF15hTERT and TRS3 and in the normal ovarian surface epithelial cells VOSE and IOSE523. β-actin was used as loading control. (E) Western blot analysis of LRRC15 and PCNA expression as control in 10 OC PDX tumors.

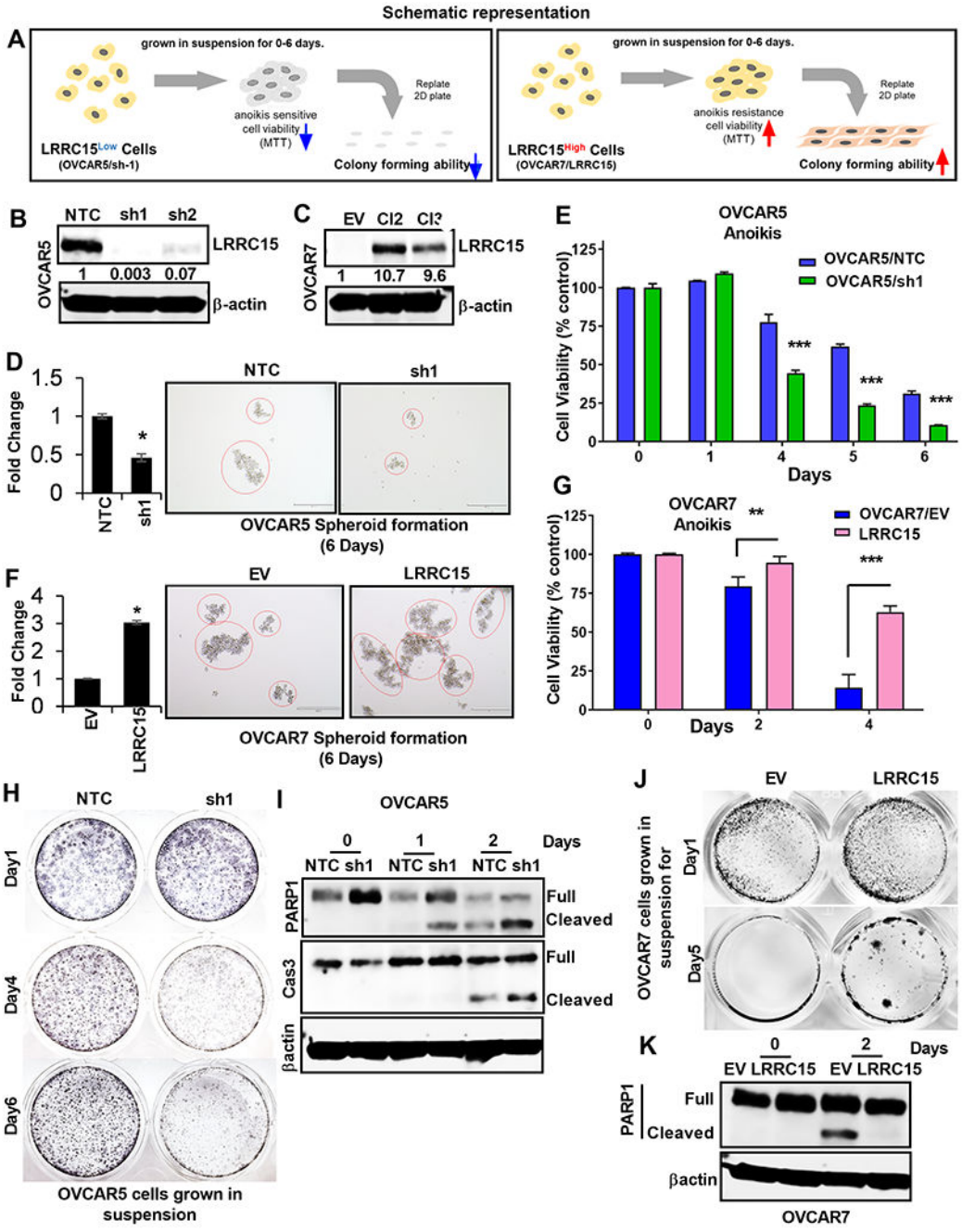

Figure 2: LRRC15 renders cells resistant to anoikis.

(A) Schematic representation of the experimental protocol . (B) Immunoblot analysis of LRRC15 expression in OVCAR5 NTC and sh1/sh2 KD cells and (C) in the OVCAR7 empty vector (EV) transfected control cells and Cl2 and Cl3 LRRC15 OE cells. β-actin was used as a loading control. Fold change was calculated using the Image J software, normalized to endogenous control, and provided beneath the panel. (D) 3D-spheroid formation assay was performed for 6 days in OVCAR5 NTC control and LRRC15 sh1 KD cells. Quantification as fold change was provided. (E) OVCAR5 NTC and sh1 cells spheroids were subsequently

transferred into adhesive plates for the indicated time points followed by MTT assay. The percent cell viability was scored and plotted. Results show the mean±SEM (***p < 0.001). (F) Spheroid formation assay was performed in OVCAR7 EV control and LRRC15 OE cells and represented as fold change. (G) Cell viability assay was performed in the mentioned cells for the indicated time points in similar manner. The percent cell viability was plotted with the mean±SEM (**p<0.01, ***p<0.001). (H) Colony forming assay was performed with OVCAR5 NTC and sh1 spheroid culture transferred in 6-well adhesive plates and imaged upon staining with Coomassie blue for the mentioned time points. (I) Immunoblot analysis of cleaved PARP1 and cleaved caspase-3 levels was performed under similar conditions for days 0 to 2. (J-K) Colony forming assay and western blot analysis for cleaved PARP1 was performed in the OVCAR7 EV and LRRC15 overexpressed cells in a similar manner. β-actin used as loading control.

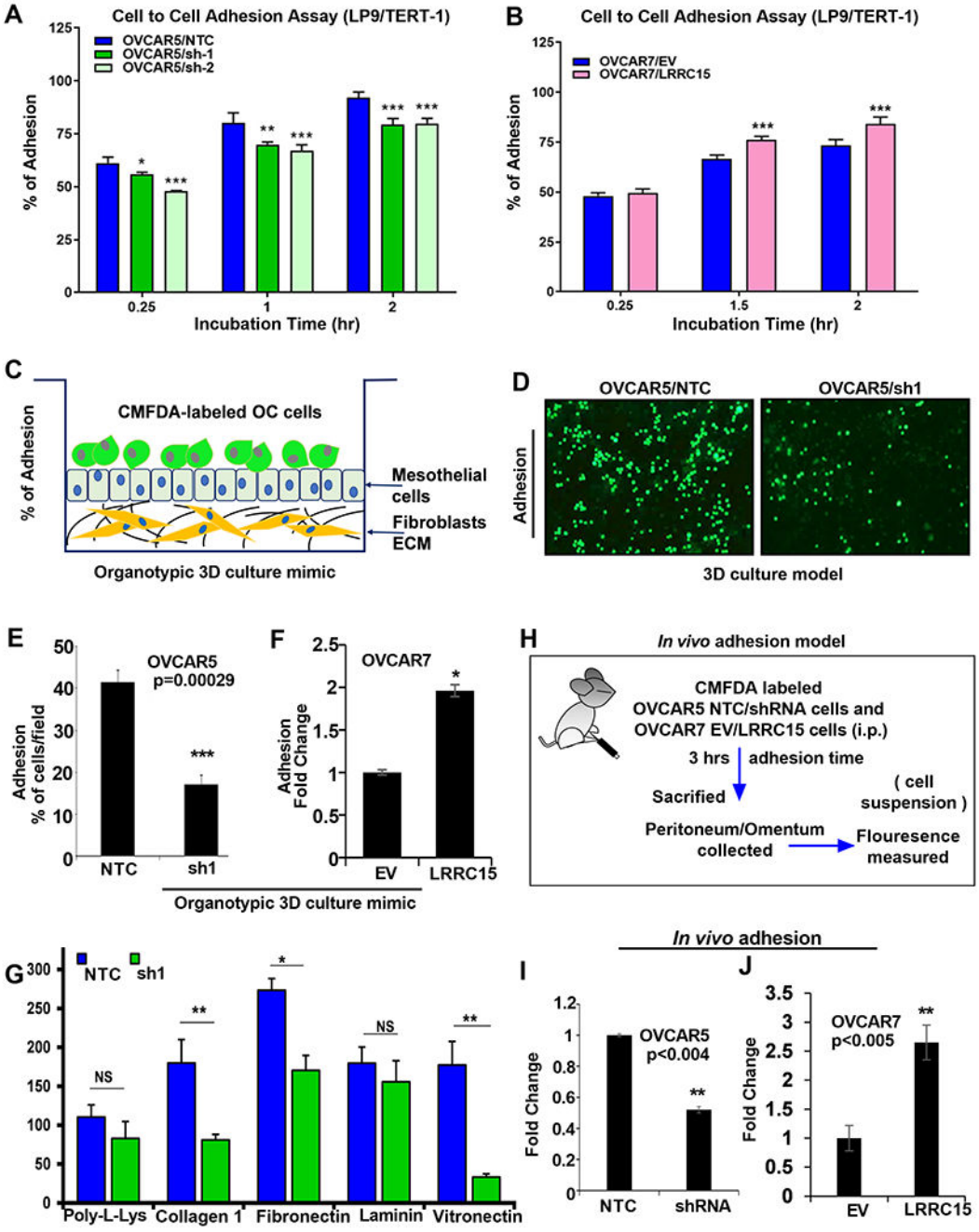

Figure 3: LRRC15 knock down abrogates the adhesion phenomenon in the OC cells.

(A) OVCAR5 NTC, sh1/sh2 and (B) OVCAR7 EV and LRRC15 overexpressed cells were pre-labeled with fluorescent CMFDA and seeded onto the top of the LP9/TERT-1 mesothelial monolayer culture. Fluorescent intensity was measured at the mentioned time points which reflects the percent of cancer cells that gets adhered to the mesothelial layer and the percent cell adherence after normalization was plotted as mean ± SEM (*p<0.05, **p < 0.01, ***p < 0.001). (C) Schematic representation of the organotypic 3D culture model of the surface layers of the omentum. (D) Representative images of

fluorescently labeled NTC and sh1 OVCAR5 cells adhering to the 3D culture model . (E) The percent adherent NTC and sh1 OVCAR5 cells were measured and plotted as mean ± SEM (***p < 0.001). (F) The percent of empty vector (EV) and LRRC15 overexpressing (LRRC15) OVCAR7 cells adhering to the 3D culture were measured and plotted as mean ± SEM (*p < 0.05). (G) OVCAR5 NTC and sh1 cells were pre-labeled with CMFDA and then seeded onto the top of the culture dishes that were pre-coated with poly-l-lysine, collagen1A1, fibronectin, laminin and vitronectin respectively. Percent adhered cells were represented as mean ± SEM (*p<0.05, **p<0.01). (H) Schematic representation of the *in vivo* adhesion assay in NSG mice model of OC. *In vivo* adhesion was performed by intraperitoneal injection of CMFDA labelled NTC and LRRC15 shRNA OVCAR5 cells or EV and LRRC15 OVCAR7 cells in female NSG mice (n=3/group). After 3hrs the mice were sacrificed and the peritoneum/omentum tissues were collected, cancer cells dissociated, and fluorescence intensity was measured in the cell suspension. The fold change in fluorescent intensity was plotted as a meadure of adhesion for (I) NTC vs. LRRC15 shRNA cells and (J) EV vs. LRRC15 overexpressing cells (**p<0.01).

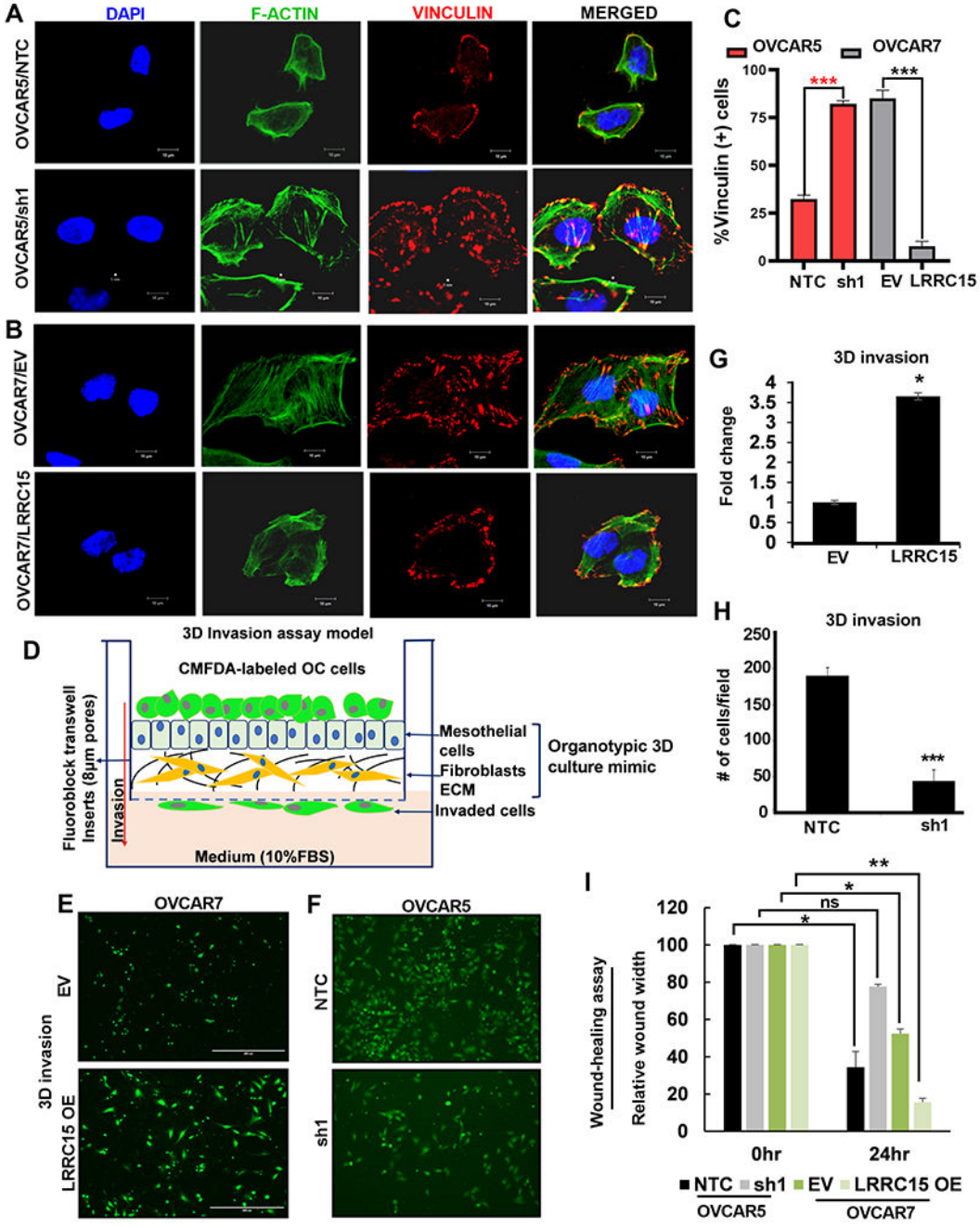

Figure 4: Altered LRRC15 expression reveals distinct pattern of FA complex formation in OC cells.

(A) OVCAR5 NTC and sh1 cells were grown on fibronectin coated coverslips for 24hrs followed by IF study against F-actin (green) and vinculin (red) using the confocal microscopy. DAPI is used to stain the nucleus and the merged images are represented. (B) Similar IF assay was performed in the OVCAR7 EV and LRRCl5 OE cells and the images are provided. Scale bar 10µm. (C) Percentage of vinculin positive cells in a total of 50 cells were counted and represented as bar graph (***p < 0.001). (D) Schematic representation of the invasion assay through the organotypic 3D culture model of the omentum surface . (E)

CMFDA labeled EV control and LRRC15 OVCAR7 cells were allowed to invade the 3D culture matrix for 12hrs and the representative images were provided. (F) Similar invasion assay was performed in the OVCAR5 NTC and sh1 cells. (G-H) Percent invaded cells were scored for the 3D invasion assay and presented as mean ± SEM respectively (*$p<0.05$, ***$p<0.001$). (I) Wound healing assay was performed in the OVCAR5 NTC and sh1 cells and in the EV control and LRRC15 OE OVCAR7 cells the relative wound width was calculated using the ImageJ software and represented (*$p<0.05$,**$p < 0.01$).

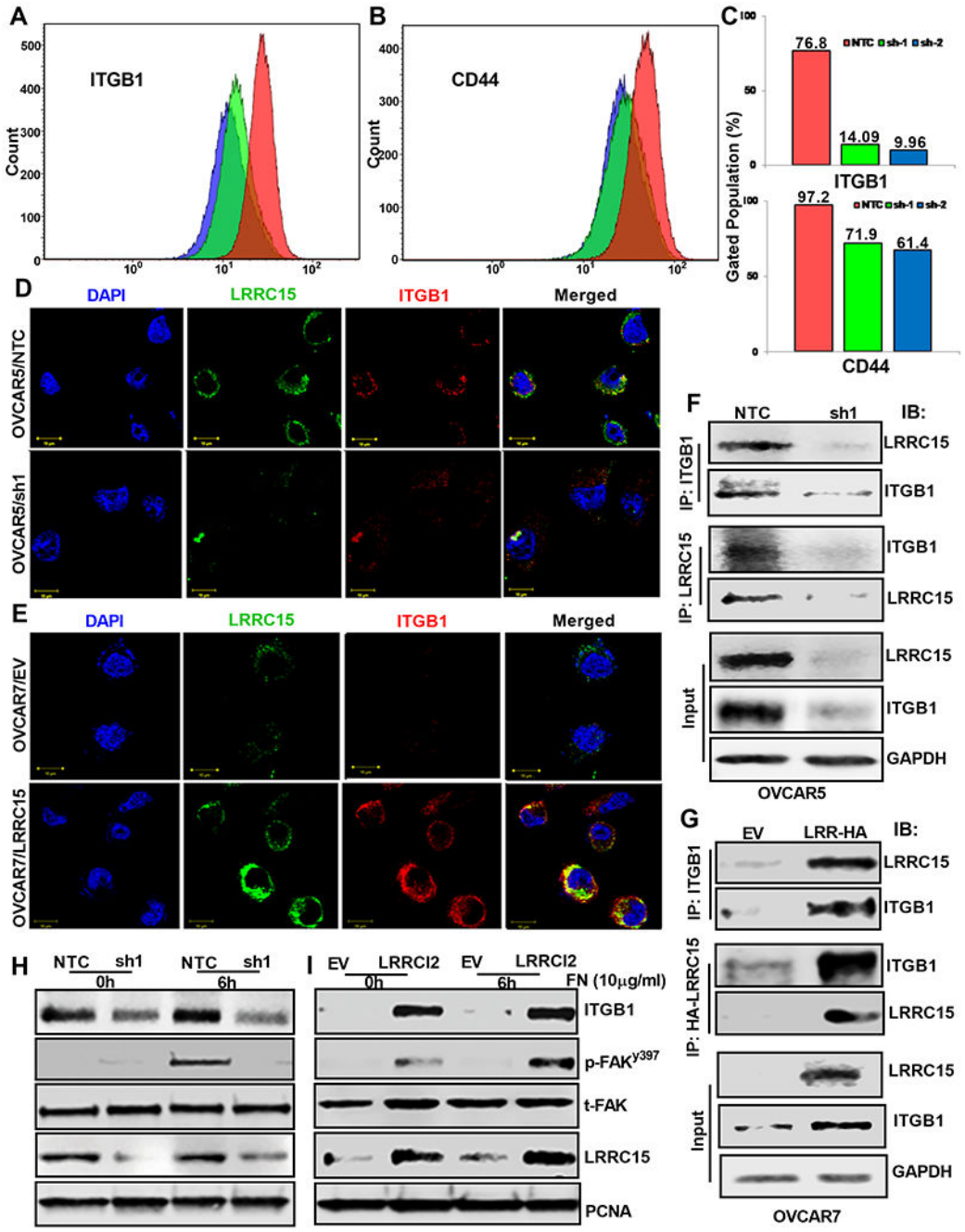

Figure 5: β1 integrin-LRRC15 interaction activates the FAK signaling.

(A-B) OVCAR5 NTC, sh1 and sh2 cells were grown in fibronectin coated plates for 24hrs followed by flow cytometry analysis against fluorescently tagged ITGB1 and CD44. (C) Percent of cells with positive signal were plotted. (D) Co-localization studies between LRRC15 (green) and ITGB1 (red) in the OVCAR5 NTC and sh1 cells and (E) in the OVCAR7 EV and LRRCl5 cells was evaluated using the confocal imaging. DAPI was used to stain the nucleus and the merged images were represented in both the cases. Scale bar 10μm. (F) OVCAR5 NTC and sh1 cell extracts were immunoprecipitated with anti-ITGB1

and the co-precipitated LRRC15 was detected by western analysis and vice versa. (G) Similar immunoprecipitation studies were performed in the OVCAR7 EV and LRRC15 overexpressing cells. GAPDH was used as a loading control in both the cases. (H) NTC and sh1 OVCAR5 cells were grown on FN coated plates for 6hrs followed by western blot analysis. FAK pathway activation was performed by analyzing the p-FAK^{y397} and total FAK levels. (I) Similar immunoblot analysis of OVCAR7 EV and LRRCl5 cells. PCNA was used for loading control. LRRC15 KD and OE was confirmed by probing against LRRC15 in the cell lysates respectively.

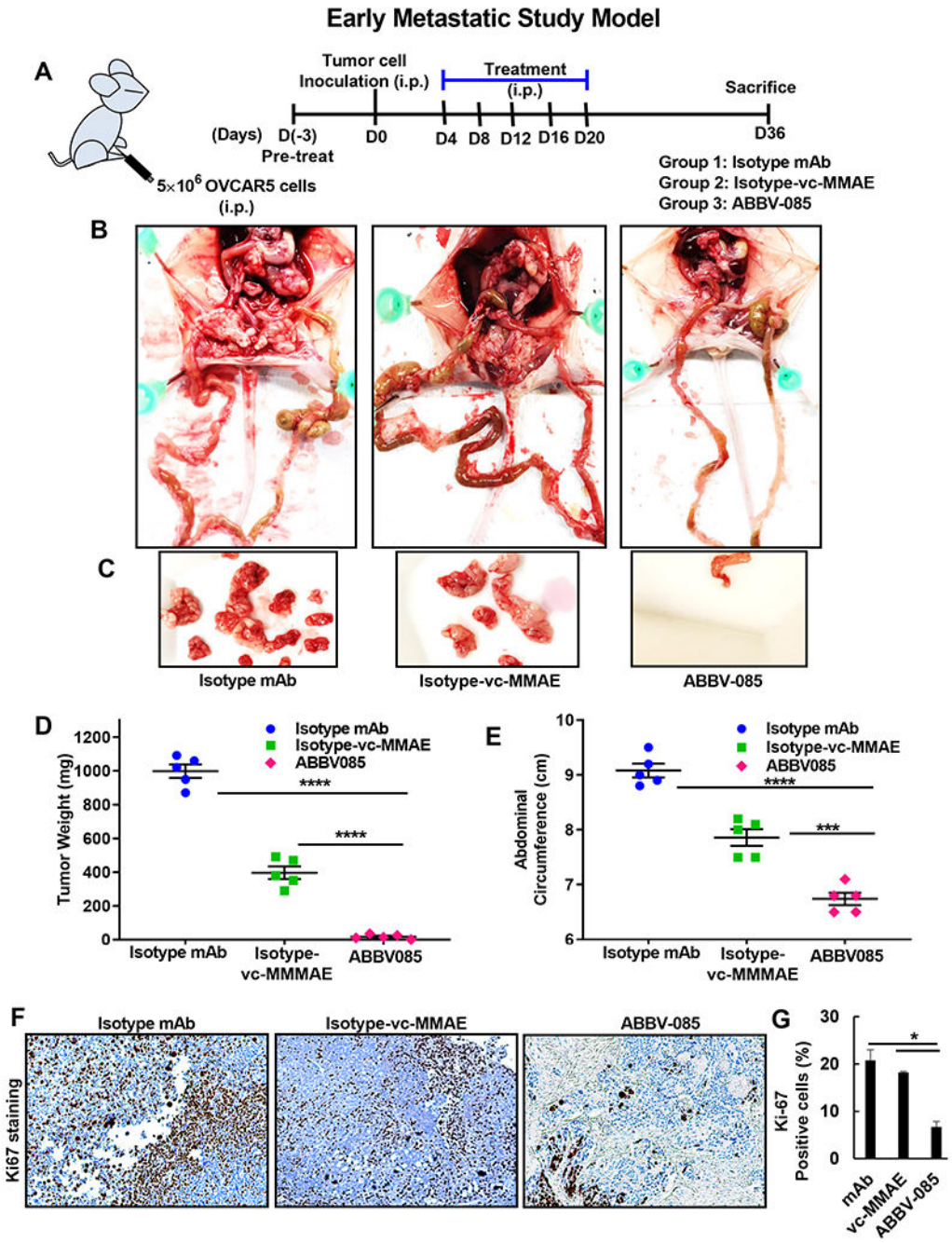

Figure 6: ABBV-085 prevents peritoneal adhesion and inhibits tumor growth in early metastatic model of OVCAR5 xenografts.

(A) Schematic representation of the early metastatic study model in mice OC xenograft. (B) Pre-treatment with control Isotype-mAb antibody (6mg/kg), the Isotype-vc-MMAE-E2 drug control (6mg/kg) and ABBV-085 (6mg/kg) was initiated 3 days prior followed by intraperitoneal OVCAR5 inoculation. Treatment was continued for 2 weeks as described previously and the animals were euthanized at day 36. Representative images of the mice with the tumor burden and metastatic nodes were shown. (C) Representative images for the tumor burden per mice for the 3 treatment groups were provided (****p<0.0001).

(D) Graphical representation of the excised tumor weights in the 3 treatment cohorts (****p<0.0001, ***p<0.001). (E) Abdominal circumference of each animal measured on the day 36 across the treatment groups. (F) Ki67 staining in each of the treated group was performed. (G) Percent Ki67 positive cells were quantified and represented.

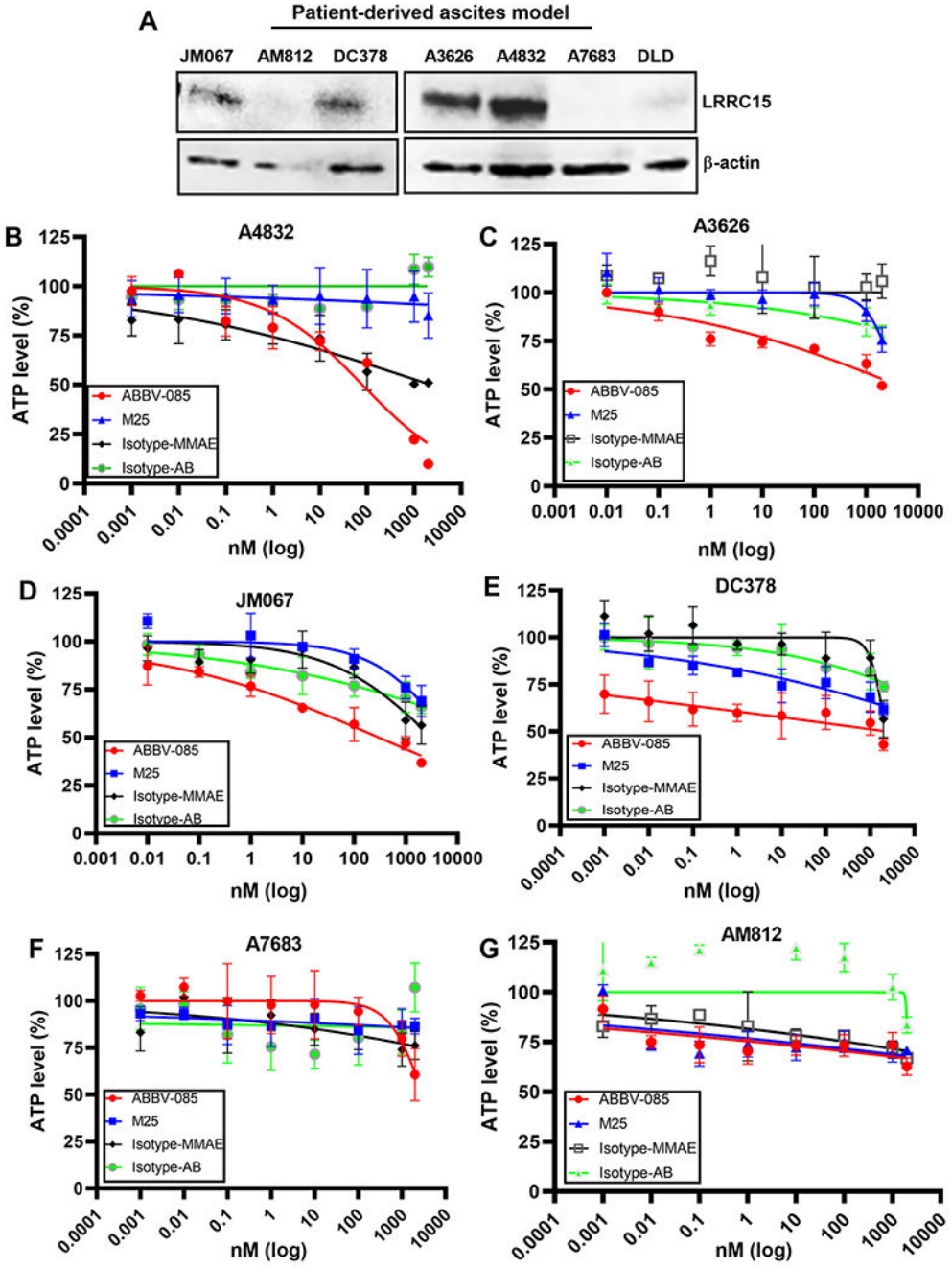

Figure 7: Treatment with ABBV-085 reduces cell viability in LRRC15-expressing human patient-derived ascites.

(A) Analysis of LRRC15 expression in 7 human patient-derived ascites. β-actin was shown as a loading control. (B) Percent ATP level was analyzed upon treatment with ABBV-085 and drug controls in LRRC15 expressing A4832, (C) A3626, (D) JM067, (E) DC378 and LRRC15 non-expressing (F) A7683 and (G) AM812 ascites cells.

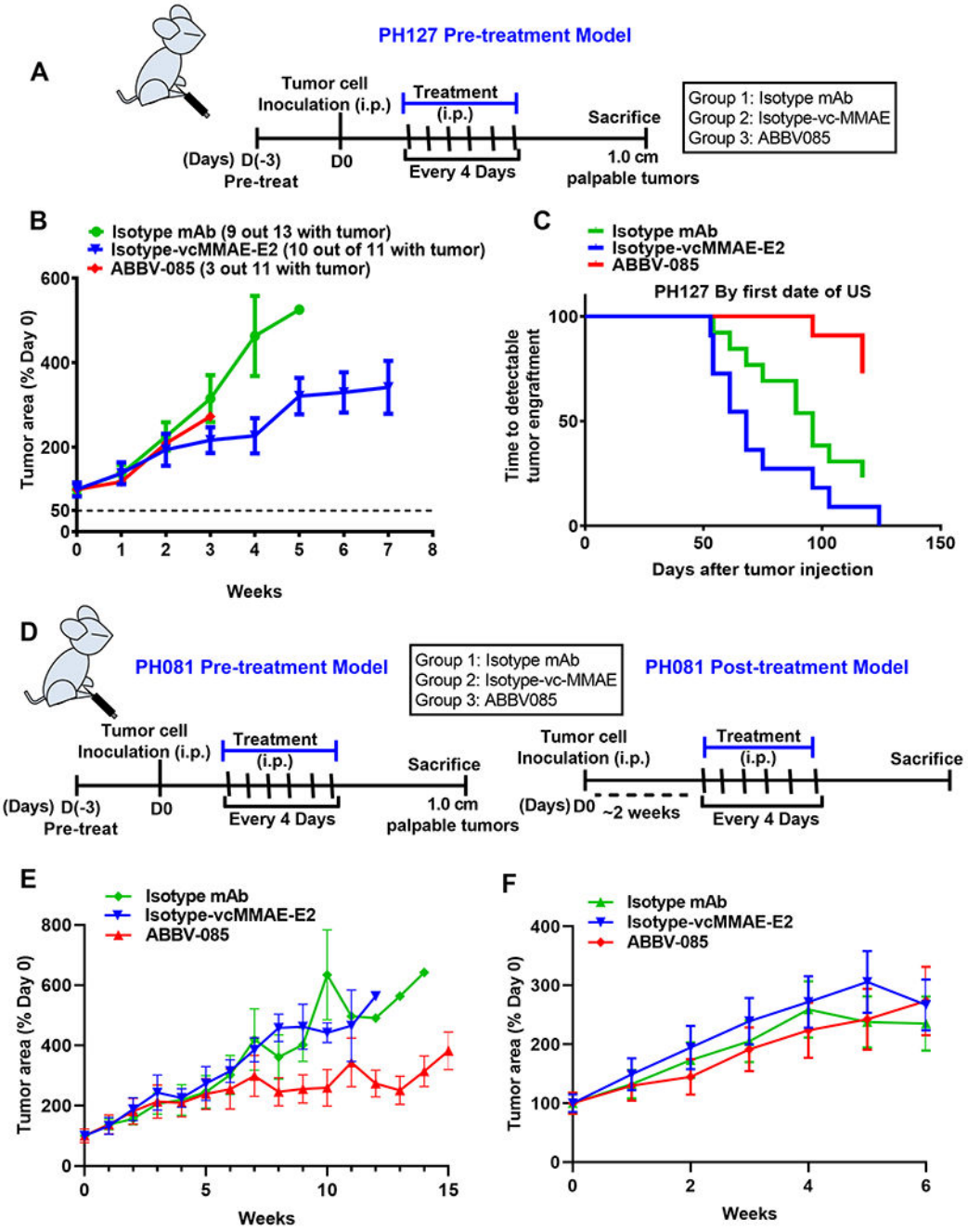

Figure 8: Therapeutic efficacy of ABBV-085 in the PH127 and PH081 OC PDX xenograft model.

(A) Schematic representation of the early metastatic model of PH127 PDX xenograft. (B) Percent change in tumor area in PH127 PDX early metastatic model following treatments with ABB-085 and control drugs as determined by ultrasound (US) weekly. (C) Graphical analysis of the time required for the detectable tumor engraftment in each mouse from the three treatment cohorts. (D) Schematic representation of PH081 PDX in the pre- and post-treatment models. (E) Percent change in tumor area in the PH081 PDX early

metastatic model and in the late metastatic model (F) following treatments with the drugs as determined by ultrasound weekly.