ORIGINAL ARTICLE

Novel *LAMC2* fusion protein has tumor-promoting properties in ovarian carcinoma

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Abstract

Laminins are heterotrimeric ECM proteins composed of α, β, and γ chains. The γ2 chain (Lm-γ2) is a frequently expressed monomer and its expression is closely associated with cancer progression. Laminin-γ2 contains an epidermal growth factor (EGF)-like domain in its domain III (DIII or LEb). Matrix metalloproteinases can cleave off the DIII region of Lm-γ2 that retains the ligand activity for EGF receptor (EGFR). Herein, we show that a novel short form of Lm-γ2 (Lm-γ2F) containing DIII is generated without requiring MMPs and chromosomal translocation between LAMC2 on chromosome 1 and NR6A1 gene locus on chromosome 9 in human ovarian cancer SKOV3 cells. Laminin-γ2F is expressed as a truncated form lacking domains I and II, which are essential for its association with Lm-α3 and -β3 chains of Lm-332. Secreted Lm-γ2F can act as an EGFR ligand activating the EGFR/AKT pathways more effectively than does the Lm-γ2 chain, which in turn promotes proliferation, survival, and motility of ovarian cancer cells. LAMC2-NR6A1 translocation was detected using in situ hybridization, and fusion transcripts were expressed in ovarian cancer cell tissues. Overexpression and suppression of fusion transcripts significantly increased and decreased the tumorigenic growth of cells in mouse models, respectively.

To the best of our knowledge, this is the first report regarding a fusion gene of ECM showing that translocation of LAMC2 plays a crucial role in the malignant growth and progression of ovarian cancer cells and that the consequent product is a promising therapeutic target against ovarian cancers.

KEYWORDS

extracellular matrix, fusion gene, laminin-γ2, ovarian carcinoma

1 | INTRODUCTION

Alteration of genes, such as cell signaling molecules, receptors, transcription factors, and intracellular signal mediators, can cause life-threatening tumors; however, genes encoding ECMs are not usually included in these cancer-causing genes.

Laminins are major macromolecular components of BMs and are cross-shaped heterotrimeric molecules composed of α, β, and γ chains, which are connected to each other by disulfide bonds.[1,2] Laminins play crucial roles in epithelial cell adhesion to the BM and in tissue architecture. Laminin chains, which include five α, three β, and three γ chains, are encoded by independent genes.[3,4] Among the laminins, Lm-332 is composed of α3, β3, and γ2 chains and is deposited in BMs as a major component.[5-7] The γ chain, Lm-γ2, has unique features.[8] Domains I/II (or coiled-coil domain) of the C-terminal portion are used to form heterotrimeric helixes with other chains and form the stem-like structure of the cross-shape. However, DIII (or LEb) is in the short arm of the cross-shape and has an EGF-like repeat motif. Domain III indeed retains the ability to bind to the EGFR when it is released from the main body as a result of MMP-dependent processing, although it cannot activate EGFR as long as it remains in Lm-332[9] (Figure 1A). Therefore, the DI/II of Lm-γ2 appear to prevent DIII from accessing EGFR freely.

It is well known that EGFR signaling is frequently activated in many types of cancer cells, but the specific ligands are not clearly known.[10-12] Tissue-derived EGF and/or HB-EGF is believed to be a candidate ligand.[13] Although it is possible that the DIII of the Lm-γ2 chain is used as a ligand, it has not been easy to show that DIII is available in cancer tissue as a ligand of EGFR. However, we and others have reported that the Lm-γ2 chain is frequently expressed abundantly in invasive cancer cells, particularly as a monomeric form.[14-16] It is also well known that invasive cancer cells express multiple MMPs, and MT1-MMP (MMP14) and MMP2 are the most powerful combination for cancer cell invasion into the surrounding ECM.[17,18] Interestingly, these MMPs are able to cleave DIII-containing fragment from the Lm-322 or Lm-γ2m chain, and the released DIII fragment can activate EGFR (Figure 1A). However, as most MMP inhibitors have not been effective in clinical trials,[19] MMP-dependent DIII might not be a major EGFR ligand, at least in cancer patients.

The Lm-γ2m chain is produced in many types of cancers, such as esophageal, stomach, colon, and cervical carcinomas, but not in normal tissue.[5,6] We developed a mAb that specifically recognizes the Lm-γ2m chain but not Lm-332. By using an Ab, we developed an easy detection system for the Lm-γ2m chain and showed that the Lm-γ2m chain expressed in tumor tissue is efficiently released into body fluids,[20] with the level in urine and blood functioning as an effective biomarker for bladder carcinoma[21] and hepatocellular carcinoma,[22] respectively. The strong correlation between the Lm-γ2m chain in body fluid and the presence of cancer could have crucial roles for the Lm-γ2m chain in cancer development and progression, although the production of the Lm-γ2m chain might just be a consequence of malignant transformation. Imbalance between laminin chain expression could occur in malignant tumor cells; for example, amplification of the *LAMC2* gene is reported in hepatocellular

carcinoma, nasopharyngeal carcinoma, and squamous cell lung cancer,[23-26] and such amplification could cause overexpression of Lm-γ2 compared to the Lm-α3 and Lm-β3 chains.

Since we reported the close correlation between the production of the Lm-γ2m chain and the invasiveness of cancer cells,[14,20] it has been our particular interest to know how the EGFR ligand activity of Lm-γ2 is turned on and to determine whether Lm-γ2 acts as an oncogene.

In this study, we found, to the best of our knowledge, for the first time that chromosomal translocation produces a novel Lm-γ2 fusion gene, which acts as a driver for cancer malignant progression in ovarian cancer. Whole genome analysis revealed chromosomal rearrangement of the *LAMC2* gene on chromosome 1 and the *NR6A1* gene locus on chromosome 9, although *NR6A1* was in the reverse direction. As a result of translocation, a short form of the Lm-γ2 chain was generated and it consequently activated the EGFR signal pathway. This is presumably the first report that a gene encoding an ECM protein changes to an oncoprotein by chromosomal translocation.

2 | MATERIALS AND METHODS

2.1 | Cell lines and cell culture

Human ovarian cancer cell lines SKOV3, OVCAR8, and IGROV were obtained from the ATCC, and KURAMOCHI and RMGI were from the Japanese Collection of Research Bioresources Cell Bank (National Institute of Biomedical Innovation, Health and Nutrition). The human melanoma cell line MUM2B was a kind gift from Professor Vito Quaranta (Vanderbilt University). The cells were cultured in RPMI-1640 medium with 10% FBS and 1% penicillin-streptomycin, and were maintained in 5% CO_2 at 37°C. Expi293 human kidney embryonic cells (Thermo Fisher Scientific) used for recombinant protein expression were cultured in Expi293 expression medium and maintained in 8% CO_2 at 37°C in a shaker set at a fixed speed of 90 rpm.

2.2 | Ovarian cancer and normal tissue specimens

Formalin-fixed paraffin-embedded tissues were obtained from the archives of the Department of Pathology, Kanagawa Cancer Center. Frozen cancer tissue specimens surgically removed from ovarian carcinoma patients (Tables 1 and 2) were deposited in Kanagawa Cancer Center Biospecimen Center. The study was approved by the ethical committee of the Kanagawa Cancer Center (approval no. 2015-26). Commercially available human normal tissue cDNAs were purchased from Filgen Inc.

2.3 | Antibodies and inhibitors

Anti-Lm-γ2 mouse mAb (D4B5) was purchased from Merck-Millipore. Anti-Akt1/2 rabbit mAb (C67E7), anti-phospho Akt1/2 Ser[473] rabbit mAb (D9E), anti-ERK mouse mAb (3A7), anti-pERK

FIGURE 1 *LAMC2-NR6A1* gene fusion is a result of genomic rearrangement. A, Laminin-γ (Lm-γ2) domain map. Lm-γ2 chain contains four domain structures namely domains I/II (or coiled-coil domain), III (or LEb), IV (or L4), and V (or Lea; DI-V). Lm-γ2 chain cleaved by proteases before and/or after DIII, which contains epidermal growth factor (EGF) ligand-like activity. D4B5 mAb recognizes DIII. BMP1, bone morphogenetic protein 1; mTLDs, mammalian tolloid-like proteases. B, SKOV3 and Mum2B cells were serum-cultured for 24 h with or without MMP inhibitor MMI-270 (MMI), and conditioned media (CM) were collected. Concentrated CM were subjected to western blot analysis using D4B4 mAb. Cont, control. C, Schematic of *LAMC2* (yellow box)-*NR6A1* (blue box) fusion gene. Region in the red box denotes the break point. D, Long and short mRNA of the Lm-γ2F transcript. Supporting reads (red, *LAMC2*; blue, *NR6A1*) and sequencing chromatogram of the *LAMC2-NR6A1* fusion gene from SKOV3 cells are shown. Black bar shows the break point
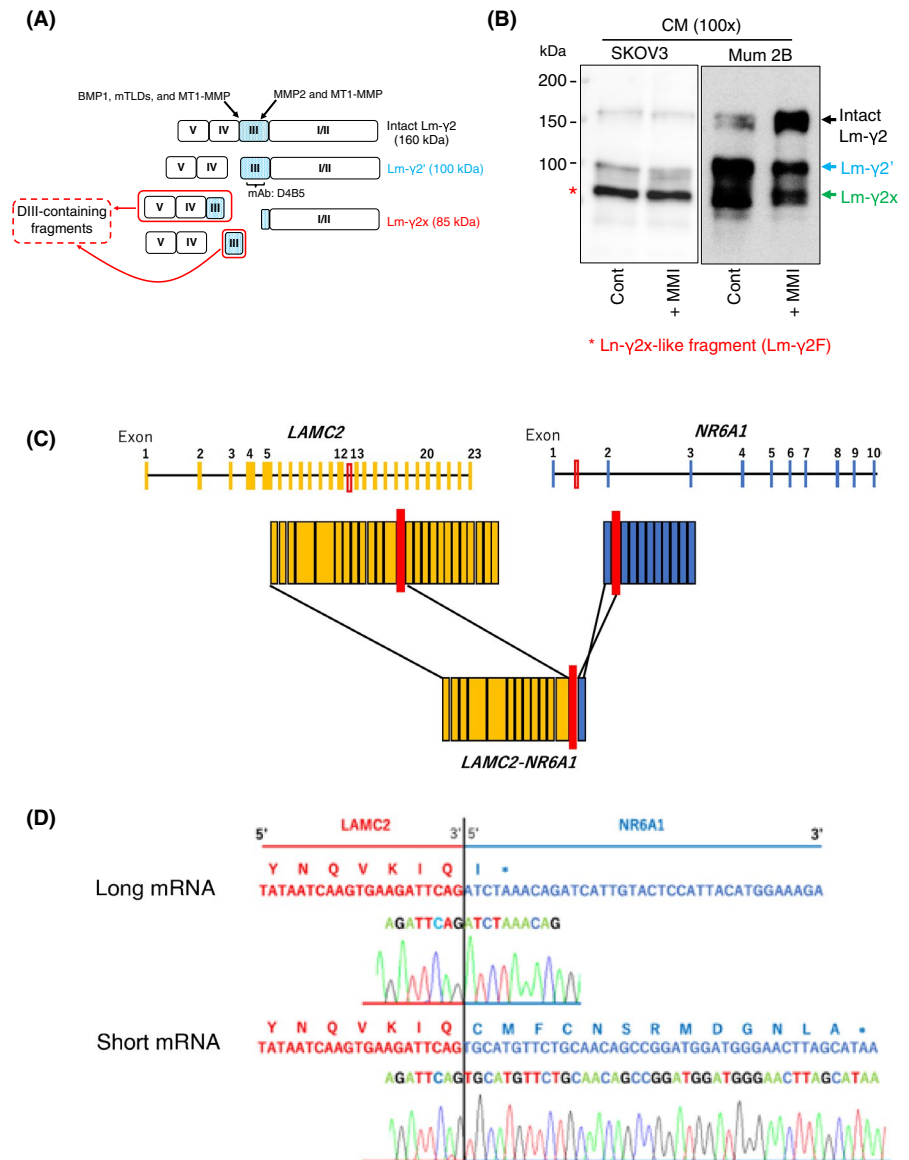
rabbit mAb (D13.14.4E), anti-EGFR rabbit mAb (D38B1), and anti-phospho EGFR Tyr[1068] rabbit mAb (D7A5) were purchased from Cell Signaling Technology. Anti-luciferase mouse mAb (M095-3) was purchased from MBL.

A synthetic hydroxamic MMP inhibitor, MMI-270, was a kind gift from Novartis Pharma. Gefitinib, an EGFR tyrosine kinase inhibitor, was purchased from Sigma.

2.4 | Polymerase chain reaction

We used the following primer sets for reverse transcription and real-time PCR experiments: Lm-γ2 forward, 5′-gctacttcggggacccattg-3′ and reverse, 5′-caagctggacagctgaatgc-3′; Lm-γ2-NR6A1 gene fusion forward, 5′-accagtgcaaagcaggctac-3′ and reverse, 5′-tcagggttgctctttgaga-3′; and GAPDH forward, 5′-aaggctgagaacgggaagcttgtcatcaat-3′ and reverse, 5′-ttcccgtctagctcagggatgaccttgccc-3′. Real-time PCR was carried out using Universal SYBR Green Supermix (Bio-Rad).

2.5 | Expression of recombinant Lm-γ2m and Lm-γ2F proteins

Expi293 cells stably expressing WT Lm-γ2m or Lm-γ2F were generated using a lentivirus protein expression system according to the manufacturer's instructions (Thermo Fisher Scientific). Serum-free CM were collected from transfected Expi293 cells expressing Lm-γ2m- or Lm-γ2F proteins and purified using the anti-Lm-γ2 Ab conjugated column, as described previously.[20]

2.6 | Knockdown of LAMC2 gene using shRNA

The Lm-γ2F shRNA vectors were purchased from Sigma. The ViraPower Lentiviral Expression System (Invitrogen) was used for stable knockdown of the *LAMC2* gene by shRNA in SKOV3 cells, and the cells were maintained in 10 µg/mL blasticidin (Thermo Fisher Scientific). Two shRNA

TABLE 1 Histological information of ovarian cancer tissues

Lane no.	Histological types of ovarian cancer
1	Serous adenocarcinoma, high grade
2	Serous adenocarcinoma, high grade
3	Serous adenocarcinoma, high grade
4	Serous adenocarcinoma, high grade
5	Serous adenocarcinoma, high grade
6	Serous adenocarcinoma, high grade
7	Serous adenocarcinoma, high grade
8	Clear cell carcinoma
9	Mucinous adenocarcinoma
10	Clear cell carcinoma
11	Clear cell carcinoma
12	Clear cell carcinoma
13	Clear cell carcinoma
14	Serous adenocarcinoma, high grade
15	Endometrioid adenocarcinoma
16	Endometrioid adenocarcinoma

TABLE 2 Pathological information of ovarian cancer tissues

Sample ID	Diagnosis	pTNM
OVCa-24	Mucinous carcinoma	pT1aN0M0
OVCa-36	Clear cell adenocarcinoma	pT1aN0M0
OVCa-44	Clear cell adenocarcinoma	pT1aN0M0
OVCa-25	Serous adenocarcinoma, high grade	pT3aN0M0
OVCa-27	Serous adenocarcinoma, high grade	pT3aN0M0
OVCa-23	Yolk sac tumor	pT1aN0M0

Note: Cancer stage categorized according to the TNM Classification of Malignant Tumours (8th edition).

sequences were used: 5′-ctgccaaatttcttgggaatc-3′ (shKD1) and 5′-gccctgtcaatgcaacaacaa-3′ (shKD2).

2.7 | Cell growth assay

Cells were suspended in RPMI-1640 medium containing 0.1% FBS, seeded onto a 6-well culture plate (1×10^3 cells/well; TPP Techno Plastic Products), and incubated for 3 days at 37°C in 5% CO_2. OVCAR8 cells treated with DMSO or gefitinib (Sigma) at indicated concentrations and incubated for 3 days at 37°C in 5% CO_2. The number of living cells was counted with either a hemocytometer or Coulter Counter (Beckman Coulter).

2.8 | Cell migration assays

The Transwell migration assays were undertaken as described previously.[27] Briefly, Transwell inserts with 8-μm pore size filters (Falcon) were inserted into 24-well plates. RPMI-1640 containing 10% FBS was added to the lower chamber and a cell suspension (1×10^5 cells) was placed in the upper chamber. The plates were incubated at 37°C with 5% CO_2 for 20 hours. After incubation, the cells that had migrated to the lower side were stained with 0.5% crystal violet solution and counted using a light microscope at ×10 magnification. The values represent averages from five fields.

2.9 | Soft agar colony formation assay

Soft agar colony assay was carried out using the CytoSelect kit according to the manufacturer's instructions (Cell Biolabs). Briefly, 1×10^4 cells were added to 2× RPMI-1640/20% FBS medium on CytoSelect Matrix and mixed well. Thereafter, 10× CytoSelect Agar Matrix Solution was added, mixed well, and incubated at room temperature for 5 minutes. The plate was then incubated at 4°C for 20 minutes and at room temperature for 30 minutes. Culture medium (50 μL) was added to the mixture and incubated for 7 days at 37°C in 5% CO_2. For quantitation of anchorage-independent growth, matrix solubilization buffer was added to each well. After solubilization, MTT solution was added to the mixture, followed by incubation in the dark for 3 hours at room temperature. The absorbance was measured at 570 nm with a microtiter plate reader.

2.10 | Xenografts

The tumorigenicity of transfected cells was examined in 6-week-old female BALB/c nude mice. Briefly, 1×10^7 cells of each transfectant were suspended in 1 mL growth medium, and 200 μL of the suspension was injected into the intraperitoneal cavity of nude mice after 30 days. Anesthesia was induced through intraperitoneal injection of 2% isoflurane and D-luciferin (150 mg/kg), followed by bioluminescence imaging using the IVIS 200 imaging system (Xenogen). The animals were treated according to the guidelines of Kanagawa Cancer Center.

3 | RESULTS

3.1 | Identification of novel *LAMC2-NR6A1* fusion gene

We previously reported that proteolytic cleavage of the human Lm-γ2 chain by MMPs results in an 85-kDa C-terminal fragment (Lm-γ2x), which promotes motility of epithelial and tumor cells.[28] The N-terminal fragments containing DIII (or LEb) act as an EGFR ligand and promote cell motility through EGFR activation (Figure 1A).[9] Therefore, we explored the expression of Lm-γ2 protein (160 kDa) and its proteolytic fragments (~85–100 kDa) in serum-free CM of ovarian cancer cells by western blotting using D4B5 mAb (anti-Lm-γ2 DIII mAb). In addition to the intact Lm-γ2

chain and its potential proteolytic fragment (Lm-γ2′, 100 kDa; Figure 1B, blue arrow), a Lm-γ2x-like fragment (85 kDa; Figure 1B, red asterisk) was detected in SKOV3 CM in the presence/absence of MMP inhibitor (MMI-270; Figure 1B, left panel). Moreover, the cleavage of the Lm-γ2 chain into two proteolytic fragments was suppressed following treatment with MMI-270 in Mum2B CM (Figure 1B, right panel).

To clarify how the proteinase, independent of Lm-γ2x-like fragment, was generated, we undertook whole genome sequencing of DNA isolated from SKOV3 cells using next-generation sequencing. The whole genome sequencing analysis revealed that Lm-γ2-related fragments are generated from a fusion transcript. The fusion transcript is produced by a chromosome translocation, which joins intron 12 of the *LAMC2* gene on chromosome 1 with intron 1 of the *NR6A1* gene on chromosome 9 (Figure 1C). The translocation resulted in a fusion transcript consisting of a part of the *NR6A1* intron 1 sequence in the opposite direction of the *NR6A1* gene following the *LAMC2* intron 12 sequence. Therefore, we concluded that the Lm-γ2x-like fragment, now termed "Lm-γ2F", was transcribed from this fusion gene. The Lm-γ2F transcript was speculated to be alternatively spliced to generate two isoforms of different lengths, termed long and short mRNA forms,

using two artificially appeared alternative splice acceptor sites in the *NR6A1* intron sequence (Figures 1D and S1). As a stop codon appeared in each deduced mRNA in the ORF following the *LAMC2* exon 12 sequence (Figures 1D and S1), Lm-γ2F seemed to be composed of amino acids 1-619 generated from *LAMC2* exons 1-12 and an additional one or 13 de novo amino acids encoded by the *NR6A1* intron sequence. Laminin-γ2F was found to be devoid of DI/II, which are required for its assembly with Lm-α3 and -β3 chains, but contained a laminin EGF-like domain, DIII. Although the *LAMC2-NR6A1* fusion gene does not generate a full-length novel protein, it produces a short Lm-γ2 peptide chain, which is functionally important in ECM organization as well as a growth factor ligand without a proteolytic event on the cell surface.

To confirm the presence of fusion genes in human normal tissues, we undertook RT-PCR using primers set on exon 12 of *LAMC2* in the forward direction and on intron 1 of *NR6A1* in the reverse direction (Figure S1). The results showed amplification of long and short spliced fragments in SKOV3 cells, but not in human normal tissues. Furthermore, normal tissues showed expression of only WT *LAMC2* gene (Figure 2A). We next examined the expression of the *LAMC2-NR6A1* fusion gene in various ovarian cancer cell lines and tissues using RT-PCR. As shown in Figure 2B, the
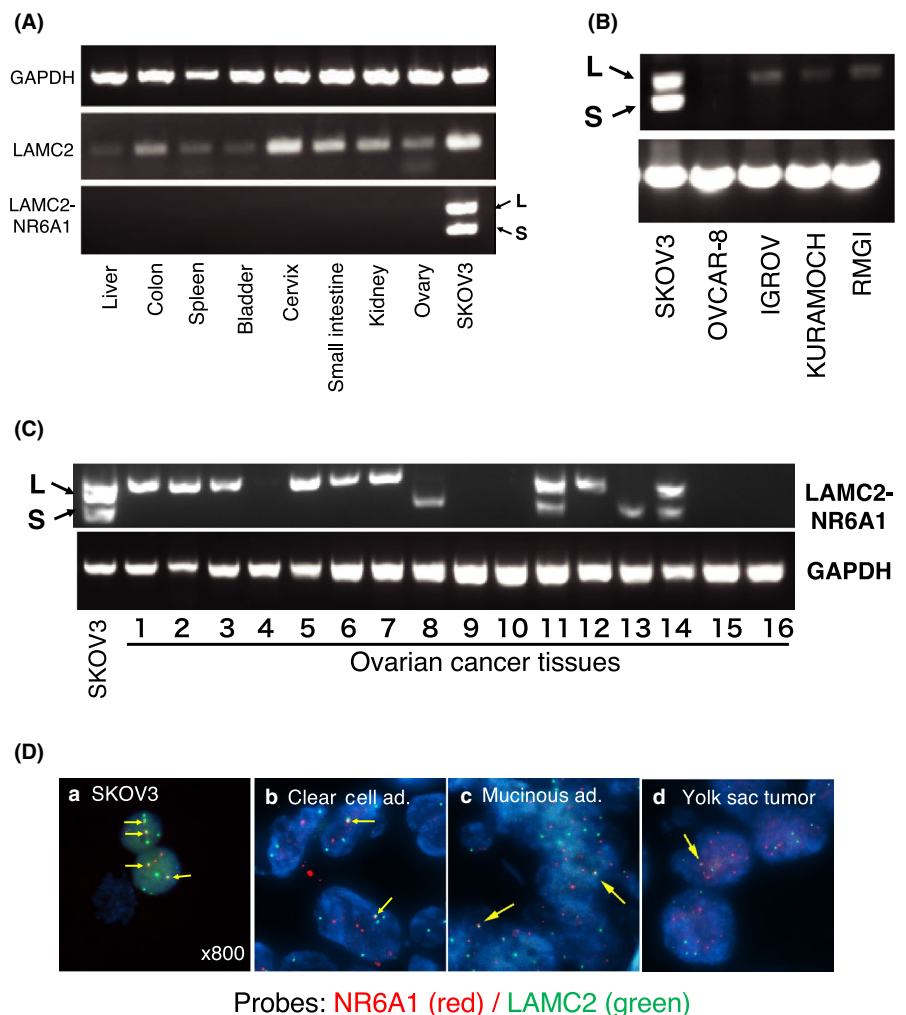

FIGURE 2 Expression of *LAMC2-NR6A1* fusion transcripts in human ovarian cancer tissues, cells, and normal tissues. A-C, Agarose gel images of the PCR products generated by amplifying the *LAMC2-NR6A1* fusion junction from (A) normal human tissues, (B) ovarian cancer cell lines, and (C) ovarian cancer tissues. SKOV3 cells were used as a positive control. Two different lengths of transcripts were amplified. L, long form. S, short form. D, FISH showing *LAMC2-NR6A1* fusion gene in (a) ovarian cancer SKOV3 cells, (b) clear cell adenocarcinoma (ad.), (c) mucinous adenocarcinoma, and (d) yolk sac tumor tissues under cytogenetics imaging solution (Leica CW4000; magnification, ×800). Red signals indicate *NR6A1*; green signals indicate *LAMC2*. Yellow arrows indicate gene fusion
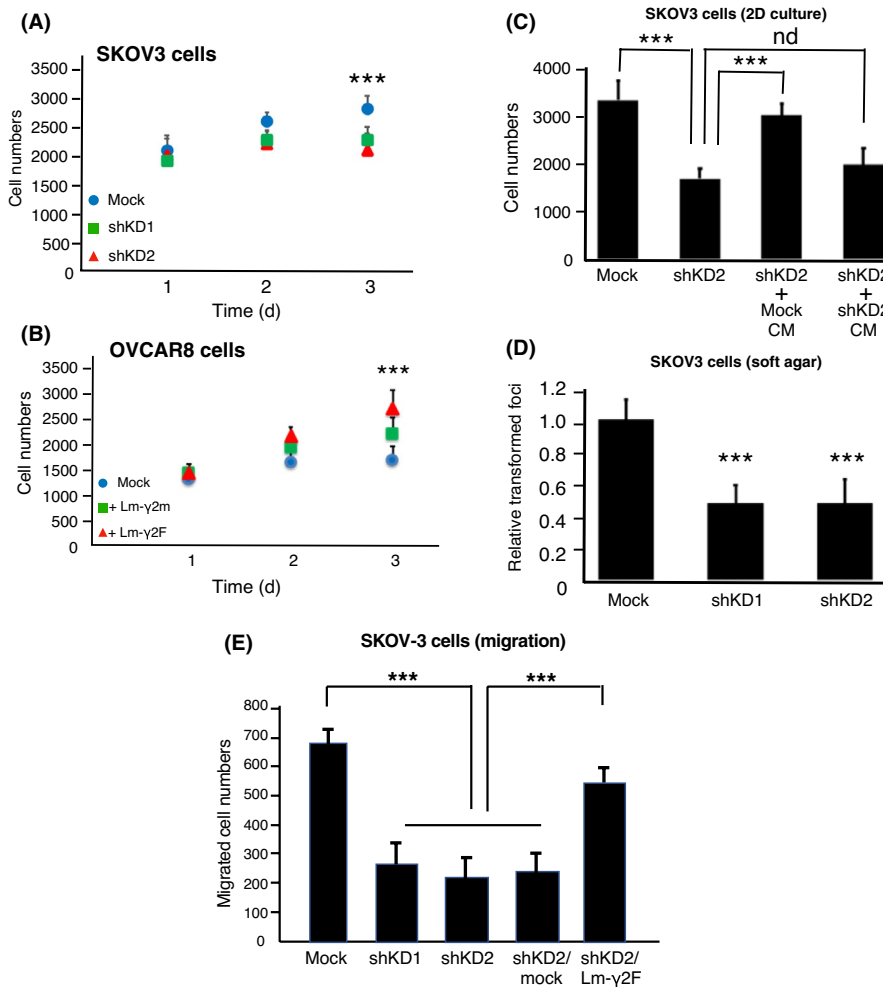
FIGURE 3 Laminin-γ2F (Lm-γ2F) induces ovarian cancer cell growth and motility in vitro. OVCAR8 and SKOV3 cells were used as Lm-γ2F-positive and Lm-γ2F-negative controls, respectively, in subsequent experiments. A, Comparison of cell growth rates between SKOV3 cells transfected with shRNA against Lm-γ2F (shKD1, green square; shKD2, red triangle) and mock (shLacZ, blue circle). B, Comparison of cell growth rates between OVCAR8 cells transfected with intact Lm-γ2 (green square) or Lm-γ2F (red triangle) and mock (blue circle). C, Rescue experiment for shKD2-related growth defect using mock or shKD2-derived conditioned media (CM). D, Soft agar assay using SKOV3 cells transfected with shRNA against Lm-γ2F (shKD1 or shKD2) or mock (shLacZ). E, Cell migration was analyzed using the indicated SKOV3 transfectants. The number of cells that had migrated through the membrane was enumerated. Error bars, ±SD of triplicate experiments. ***$P < .05$, unpaired t test with Welch's correction

LAMC2-NR6A1 fusion gene was detected in all ovarian cancer cell lines, except OVCAR8. Hence, we used SKOV3 as *LAMC2-NR6A1* fusion-positive cells and OVCAR8 as *LAMC-NR6A1* fusion-negative cells for further analysis. Next, we analyzed the expression of the fusion gene in multiple ovarian cancer tissues collected from patients (Table 1). As shown in Figure 2C, 11 of 16 ovarian cancer tissues were positive for the *LAMC2-NR6A1* fusion gene, indicating a frequency of 68% (Figure 2C). Sequencing of the PCR products further confirmed the presence of *LAMC2-NR6A1* fusion gene in these tissue samples. Furthermore, FISH analysis with *LAMC2* and *NR6A1* probes confirmed the presence of gene fusion in SKOV3 cells and ovarian cancer tissues (Table 2 and Figure 2D). Moreover, three of six ovarian cancer tissues, clear cell adenocarcinoma, mucinous adenocarcinoma, and yolk sac tumor (Table 2), showed *LAMC2-NR6A1* gene fusion according to FISH analysis (yellow arrows), indicating a frequency of 50% among the patients.

3.2 | Laminin-γ2F induces ovarian cancer cell growth in vitro

To examine the effects of Lm-γ2F on cancer cell properties, we generated stable SKOV3 transfectants with suppressed expression

of Lm-γ2F using shRNAs (shKD1 and shKD2), and stable OVCAR8 transfectants with ectopic expression of Lm-γ2F (Figure 3). We further confirmed the expression of the corresponding proteins (Figure 4B, bottom panel and Figure S2, respectively). Knockdown of Lm-γ2F reduced the growth of SKOV3 cells (Figure 3A), whereas overexpression of Lm-γ2F increased the growth of OVCAR8 cells in serum-free culture condition (Figure 3B). To examine whether secreted Lm-γ2F directly increased cell growth activity, we collected CM from mock or shKD2 transfected cell cultures and incubated the shKD2-transfected cells in CM for 3 days. The CM obtained from mock transfectant culture could rescue the defective shKD2-transfectant cell growth, whereas CM obtained from shKD2 could not (Figure 3C).

In addition, to explore the role of Lm-γ2F in inducing anchorage-independent growth activity, SKOV3 cells were cultured on soft agar, and the cell number was determined by MTT assay. The cells transfected with shKD1 or shKD2 showed a significant decrease in anchorage-independent growth activity (Figure 3D). We further observed the involvement of Lm-γ2F in regulation of cell motility. Specifically, Lm-γ2F knockdown also reduced the migration of SKOV3 cells, and Lm-γ2F-stable revertant that showed re-expression after introduction of the shRNA-resistant expression plasmid (shKD2/rLm-γ2F) could rescue the defective
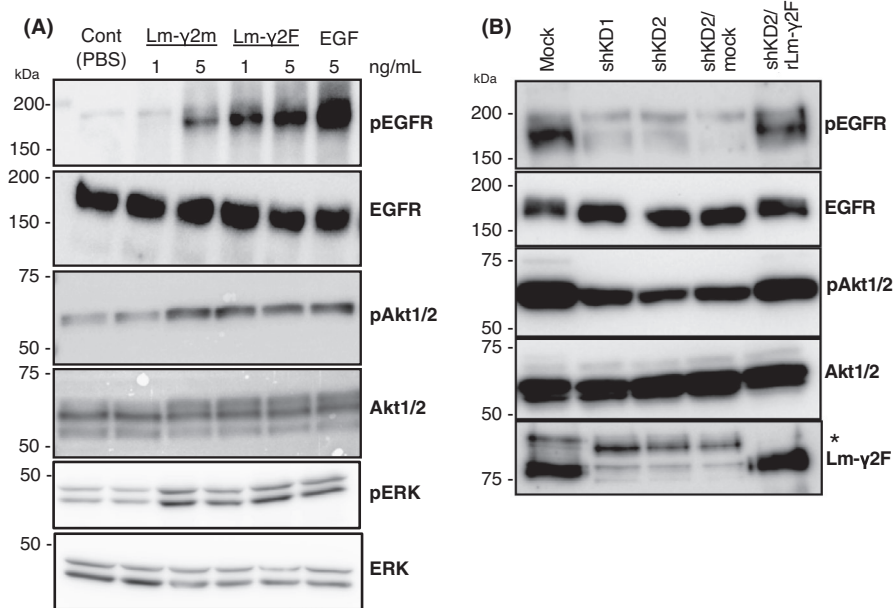
FIGURE 4 Effects of laminin-γ2F (Lm-γ2F) on the activation of epidermal growth factor receptor (EGFR), Akt, and Erk signaling pathways. A, Western blot analysis of the phosphorylation of the EGFR, Akt, and Erk signaling pathways in SKOV3 cells treated with Lm-γ2m, Lm-γ2F, or EGF for 30 min at indicated different concentrations. Cont, control. B, Western blot analysis of the phosphorylation of the EGFR and Akt signaling pathways in SKOV3 cells transfected with shRNA against Lm-γ2F (shKD1 or shKD2) or shKD2 and shLacz (shKD2/mock) or shKD2 and its resistant *LAMC2-NR6A1* fusion gene (shKD2/rLm-γ2F) or mock (shLacZ) after serum starvation for 4 h. Western blot analyses of the phosphoproteins were carried out under different exposure durations (ie, 1 and 5 min). *Nonspecific band

shKD2-transfectant cell motility (Figure 3E). These results indicate that secreted Lm-γ2F is important in promoting oncogenic phenotypes.

3.3 | Laminin-γ2F activates the EGFR pathway

As Lm-γ2F contains multiple EGF-like domains (Figure 1A), we explored its role in EGFR activation. We purified the proteins produced by intact Lm-γ2m (Figure S3, Lm-γ2m, red arrow) or Lm-γ2F (Figure S3, blue arrow) from the CM of the transfected Expi293 cell system using an anti-Lm-γ2m mAb affinity column (Figure S3, elution fraction 2). Activation of EGFR, Akt, and Erk by phosphorylation was confirmed in SKOV3 cells treated with the indicated concentrations of purified proteins for 30 minutes, as indicated by western blotting results using their specific phospho-Abs (Figure 4A). Although an increase in EGFR, Akt, and Erk phosphorylation was observed in the cells treated with both Lm-γ2 proteins in a dose-dependent manner, treatment with Lm-γ2F showed a much higher phosphorylation than Lm-γ2m. Moreover, suppression of Lm-γ2F in SKOV3 cells using shRNAs against Lm-γ2F (shKD1 and 2) clearly decreased EGFR phosphorylation, and re-expression of shKD2-resistant Lm-γ2F (Lm-γ2F) in shKD2-transfected cells recovered the EGFR phosphorylation without serum condition (Figure 4B, right lane). Moreover, the recombinant Lm-γ2F effectively activated the EGFR and Akt signaling pathways in SKOV3 cells, and the PI3K inhibitor (LY294002) decreased *LAMC2* and *LAMC2-NR6A1* gene expression (Figure S4).

These data showed that Lm-γ2F effectively activates EGFR and downstream AKT signaling, and that its gene expression could be regulated by a positive feedback loop between the EGFR-Akt signaling pathway and *LAMC2* and *LAMC2-NR6A1* gene regulation in ovarian cancer cells. Indeed, we confirmed that Lm-γ2F-related growth promotion in OVCAR8 cells could be blocked by using an EGFR tyrosine kinase inhibitor, such as gefitinib (Figure S5).

3.4 | Laminin-γ2F regulates tumor growth in vivo

To examine the effects of Lm-γ2F on tumor growth in vivo, we injected nude mice with ovarian cancer cells transfected with shRNAs against Lm-γ2F (SKOV3) or the fusion gene (OVCAR8). SKOV3 cells transfected with shRNAs significantly reduced tumor formation compared to the cells transfected with mock shLacZ. Re-expression of the fusion gene rescued this and increased tumor formation, as measured by bioluminescence (Figure 5A,B). Furthermore, OVCAR8 cells overexpressing the fusion gene resulted in tumor formation and promoted Akt and Erk activation in vivo (Figures 5C,D and S6).

4 | DISCUSSION

Although chromosomal translocations induce driver gene mutations that promote carcinogenesis of blood tumors and solid tumors, there are no reports about ECM molecule mutations by chromosomal
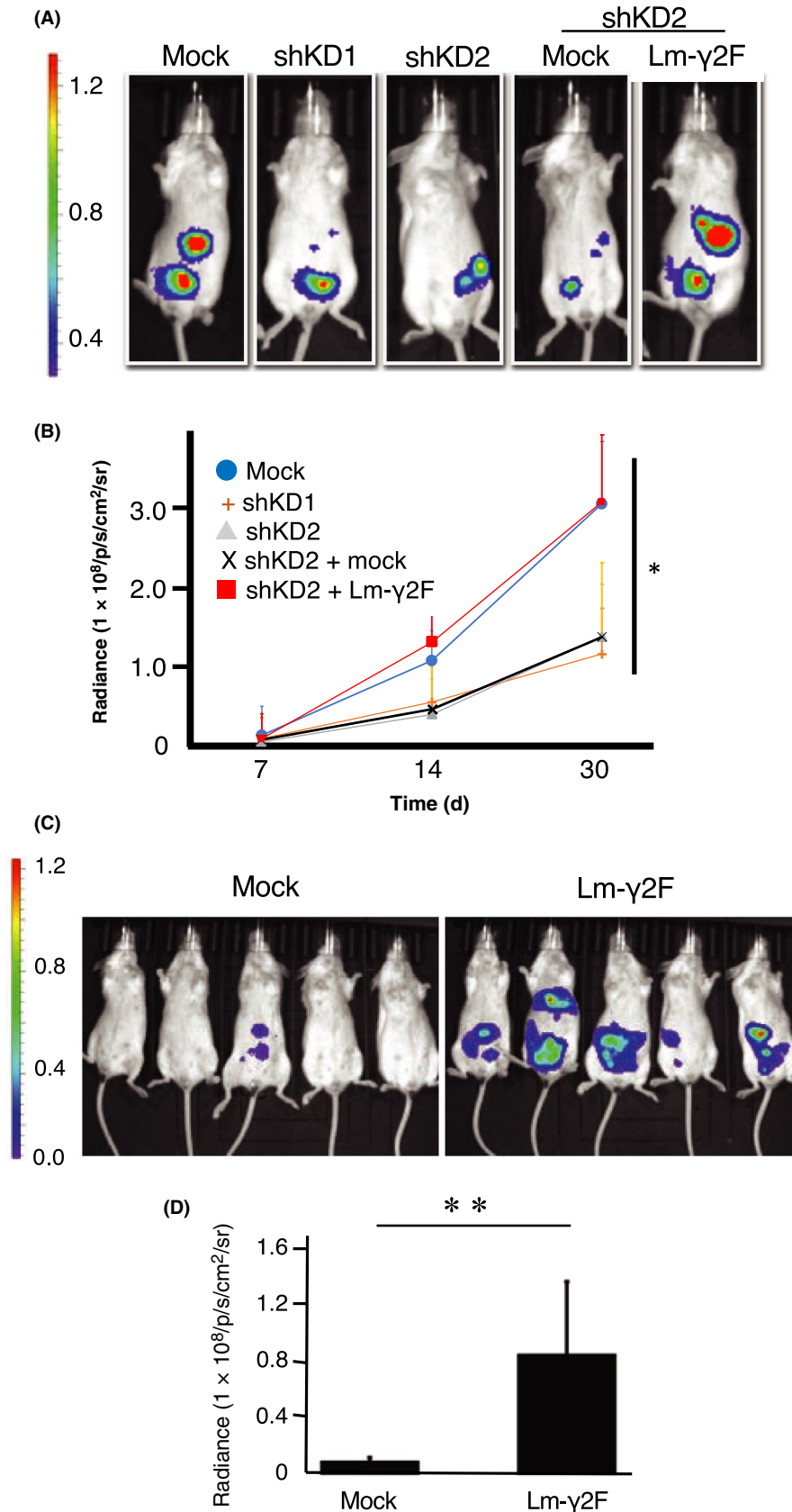
FIGURE 5 Laminin-γ2F (Lm-γ2F) promotes ovarian cancer cell growth in vivo. A, B, Representative bioluminescent (BL) images (A) and quantitative bar plots (B) of mice after i.p. injection of SKOV3 control cells (mock), SKOV3 with Lm-γ2F knockdown (shKD1 and shKD2), or shKD2 and its resistant *LAMC2-NR6A1* fusion gene (shKD2/Lm-γ2F). Error bars, ±SEM, n = 6. *P < .05. C, D, Representative BL image (C) and quantitative bar plots (D) of mice at 30 d after i.p. injection of OVCAR8 control cells (mock) or OVCAR8 cells transfected with *LAMC2-NR6A1* fusion gene (Lm-γ2F). Error bars, ±SEM n = 5. **P < .05

translocation.[12] This is the first report to identify that chromosomal translocation turns an ECM component into an oncoprotein in ovarian carcinomas.

We showed that the translocation between chromosomes 1 and 9 generates the *LAMC2-NR6A1* fusion gene, which further undergoes alternative splicing to generate a C-terminally truncated Lm-γ2,
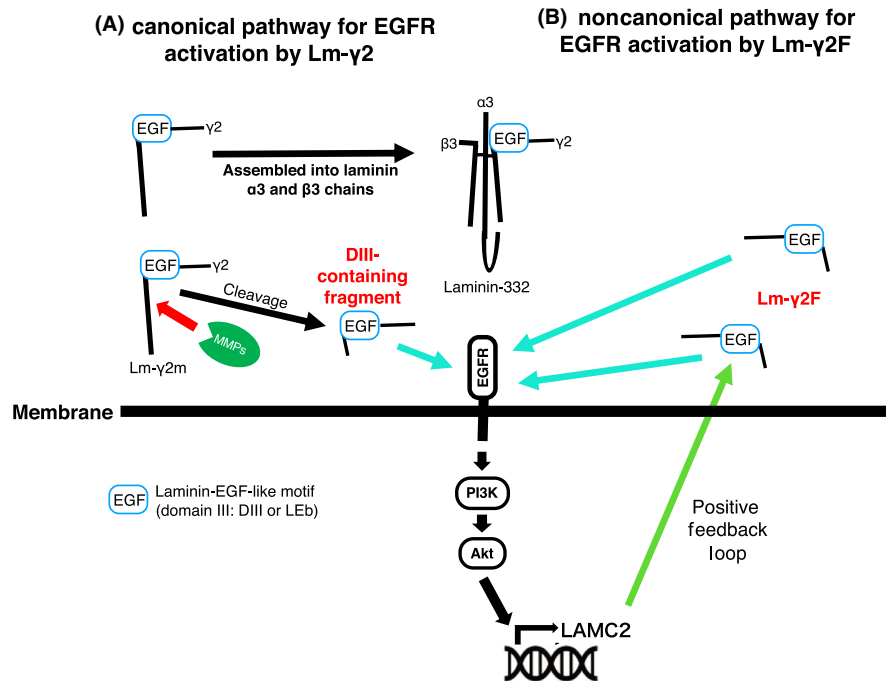
FIGURE 6 Schematic model of activation of the epidermal growth factor receptor (EGFR) signaling pathway by laminin-γ2 (Lm-γ2) chains. A, Canonical pathway of EGFR activation by Lm-γ2 chain. Cleavage of Lm-γ2 chain by MMPs releases the N-terminal domains that contain a laminin-EGF-like motif, which promotes tumor progression by activation of the EGFR pathway. Noncleaved Lm-γ2 chain assembles into other laminin subunits, and forms Lm-332. Cleavage of γ2 chain in Lm-332 by MMPs is more resistant than in monomeric Lm-γ2m (Lm-γ2m).[33] B, Noncanonical pathway of activation of the EGFR pathway by C-terminally truncated Lm-γ2 (Lm-γ2F). Lm-γ2F generated by chromosomal translocation between *LAMC2* and *NR6A1* genes results in the fusion transcript that contains the Lm-EGF-like motif and lacks domain I/II (DI/II) required for its assembly with other Lm subunits (α and β). Lm-γ2F can directly bind to EGFR and activate the EGFR and AKT pathways, in turn increasing Lm-γ2 expression as a result of the positive feedback loop

termed Lm-γ2F. Interestingly, Lm-γ2F cannot assemble with other laminin chains due to the absence of DI/II (or coiled-coil domain) of the Lm-γ2 chain. Moreover, Lm-γ2F is composed of a short arm containing DIII (or LEb), IV (or L4), and V (or Lea) that is comparable to the migratory fragment produced from the Lm-γ2 chain following processing by MMPs (Figure 6). Furthermore, we confirmed that Lm-γ2F can act as an EGFR ligand, promoting tumor growth, motility, and metastasis in vitro and in vivo.

Ovarian cancer frequently recurs after chemotherapy and acquires resistance to anticancer drugs through the upregulation and/or activation of the PI3K/Akt/mTOR and MAPK/Erk pathways.[29, 30] Previous studies have reported that a ligand of EGFR, HB-EGF, plays a critical role in the progression and prognosis of ovarian cancer.[31]

In this study we found that Lm-γ2F efficiently upregulated Erk and Akt phosphorylation and their gene expression by the feedback loop of the EGFR pathway (Figures 4 and S6). Indeed, fusion gene expression was observed in advanced ovarian cancer tissues compared to that of ovarian cancer cell line (Figure 2 and Tables 1 and 2). Presumably, various stimuli from the tumor microenvironment could induce the expression of the fusion gene, resulting in malignant growth activity in ovarian cancers.

In addition to an EGFR ligand-like function, Lm-γ2F could have further functions to regulate tumor differentiation. In general, BMs for cancer cells act as a barrier to invasion, and poorly differentiated

cancer cells lacking BMs frequently show aggressive invasive phenotypes.[19] Therefore, the present result shows that translocation might facilitate the invasive phenotype in ovarian cancer cells expressing Lm-γ2F, due to defect of BM formation, providing new insights into ECM functions involved in ovarian cancer differentiation and malignant phenotypes.

Overexpression of Lm-γ2m is associated with cancer progression and metastasis in vivo,[8, 14] and expression of the *LAMC2-NR6A1* fusion gene was also observed in the advanced stage of ovarian carcinomas (Figures 1D, 2). Thus, similar to Lm-γ2m, Lm-γ2F seems to play a role in not only tumor growth but also in acquisition of malignant progression potentials, such as invasion, metastasis, and chemoresistance, in ovarian cancer.

However, unlike the *LAMC2* gene, *LAMC2-NR6A1* fusion gene expression was not observed in the normal tissues we tested. The *LAMC2-NR6A1* fusion gene and its product could be a cancer-specific biomarker and could be a potent indicator for cancer existence in vivo.

Indeed, a novel 13 amino acid-long polypeptide as a cancer-specific tag generated from the shorter transcript isoform of the fusion gene was identified (Figure 1D). In particular, the shorter transcript isoform was frequently detected in three of five (60%) clear cell carcinoma tissues. Interestingly, the polypeptide was not present in any of the protein databases, when searched. Although CA125 is used as a tumor marker for ovarian carcinoma diagnosis,

the diagnostic accuracy is insufficient to diagnose clear cell carcinoma.[32] Therefore, the novel polypeptide might have the potential to be used for differential diagnosis, although further clinical studies are needed.

In conclusion, we identified a novel gene fusion, *LAMC2-NR6A1*, in ovarian cancer and characterized its role in cancer growth and progression. The fusion gene product, Lm-γ2F, possessed a similar bioactivity for EGFR phosphorylation to that of the EGF ligand at the molar level. Thus, ovarian cancer cells presumably make use of a noncanonical pathway for EGFR activation by Lm-γ2F, which is easier than the canonical pathway of EGFR activation through Lm-γ2m in the tumor microenvironment (Figure 6). Thus, Lm-γ2F could play a crucial role in malignant transformation, including tumor growth, survival, and motility, and could be a novel molecular target for ovarian cancer therapy and diagnostics.

CONFLICT OF INTEREST

The authors have no conflict of interest.

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