Research Article

Molecular Cancer Therapeutics

Role of the 18:1 Lysophosphatidic Acid–Ovarian Cancer Immunoreactive Antigen Domain Containing 1 (OCIAD1)–Integrin Axis in Generating Late-Stage Ovarian Cancer

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Abstract

Chemotherapy resistance in ovarian cancer remains an unsolved problem in caring for women with this disease. We now show that ovarian cancer immunoreactive antigen domain containing 1 (OCIAD1) has higher expression in chemoresistant compared with chemosensitive ovarian cancer cell lines. We have designed a novel secondary cell homing assay (SCHA) to test the ability of cells to withstand chemotherapy and form secondary colonies that could form recurrent disease. OCIAD1 upregulated cells had significantly higher secondary colony-forming ability than had OCIAD1 downregulated cells following treatment with paclitaxel. Additionally, 18:1 lysophosphatidic acid (LPA) increases OCIAD1 expression in a time- and dose-dependent manner. LPA stimulates OCIAD1 serine phosphorylation within two hours of stimulation. Transfection of MKK6 increases OCIAD1 expression but nuclear translocation is inhibited. Inhibition of p38 mitogen-activated protein kinase blocks LPA-induced OCIAD1 expression. Cycloheximide treatment of MKK6-transfected cells does not inhibit OCIAD1 expression, suggesting that MKK6 upregulation is not translationally controlled. OCIAD1 downregulation knocks down LPA-induced cell adhesion to collagen I and laminin 10/11 and specifically inhibits cell attachment to α2, α5, αV, and β1 integrins. Proteomic studies indicate that OCIAD1 is physically attached to α actin 4 and β actin. Thus, OCIAD1 may play a role in cytoskeletal function which can alter sensitivity to paclitaxel. This is the first study to indicate that OCIAD1 is a key player in generating ovarian cancer recurrence; it is functionally controlled by LPA and MKK6 signaling, and inhibition of OCIAD1 could be an important strategy in the management of recurrent ovarian cancer. *Mol Cancer Ther; 9(6); 1709–18. ©2010 AACR.*

Introduction

Ovarian cancer remains the most lethal gynecologic malignancy (1). Despite the introduction of taxanes in the 1980s, the recurrence rates and overall survival for ovarian cancer have remained poor, with less than one third of advanced-stage cases surviving beyond five years (2). Information regarding the physiology and biology of these tumors and *in vivo* responses to standard chemotherapeutics is limited by current clinical management guidelines, consisting of surgical cytoreduction followed by chemotherapy (3). Ovarian cancer patients initially respond to surgery and chemotherapy, but the disease often recurs between six months and two years (4). Recurrent disease frequently becomes unresponsive to chemotherapy and eventually leads to death (5). Thus, it remains unclear if the biology of early- and late-stage ovarian cancer is the same. To better understand the biology of late-stage ovarian cancer, we collected paired primary and metastatic ovarian tumors from eight patients with stage IIIC disease. Using proteomic approaches on these paired tumor sets, we identified the overexpression of ovarian cancer immunoreactive antigen domain containing 1 (OCIAD1) on metastatic tumors compared with paired primary tumors (6). OCIAD1 was first identified by immunoscreening of an ovarian carcinoma cDNA library with ascites fluid from ovarian cancer patients (7). Although this protein was identified in 2001, only one report from our laboratory has been published to date to establish its role in ovarian cancer.

We have already shown that OCIAD1 promotes chemotherapeutic (paclitaxel) resistance against cell detachment in the presence of 18:1 lysophosphatidic acid (LPA; ref. 6). The role of LPA is important as it is omnipresent and upregulated in the blood and ascites of patients suffering from ovarian cancer (8–10). LPA affects all different cellular behaviors in ovarian cancer in terms of cell adhesion (11), cell migration (11), invasion (12), metastasis (12, 13), and cell survival (14). As OCIAD1 is involved in ovarian cancer cell detachment it is imperative that its role in cell adhesion be studied.

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doi: 10.1158/1535-7163.MCT-09-1024

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Cell adhesion is an extremely important phenomenon over the course of ovarian cancer. Initially, cells need to adhere and home to secondary sites to form metastatic foci. Later, when the disease has been reduced to microscopic levels after surgery and chemotherapy, the cell adhesion function helps cells to survive against chemotherapeutic insults and form recurrent disease. The work of other authors has shown that paclitaxel reduces cell adhesion to different extracellular matrix proteins, by downregulating integrin expression (15), whereas LPA increases cell adhesion (11). We have earlier shown that OCIAD1 can be important for cell adhesion to extracellular matrix proteins like collagen I and laminin 10/11 (6). We have also shown that ovarian cancer cell adhesion to laminin 10/11 leads to cleavage of intracellular phospho-lipase A2 (iPLA2) that ultimately helps in the production of LPA (16). The present study determined the mechanism of LPA action on OCIAD1 expression and function, and in an attempt to understand how OCIAD1 is functionally involved in preventing cell detachment in the presence of paclitaxel, our results indicated that OCIAD1 is upregulated by LPA and increases cellular ability to form secondary recurrent colonies after first-line chemotherapy involving paclitaxel.

Materials and Methods

Cell cultures

All cells were cultured in 10% fetal bovine serum containing RPMI1640 medium plus 2 mmol/L L-glutamine at 37°C with 5% CO_2.

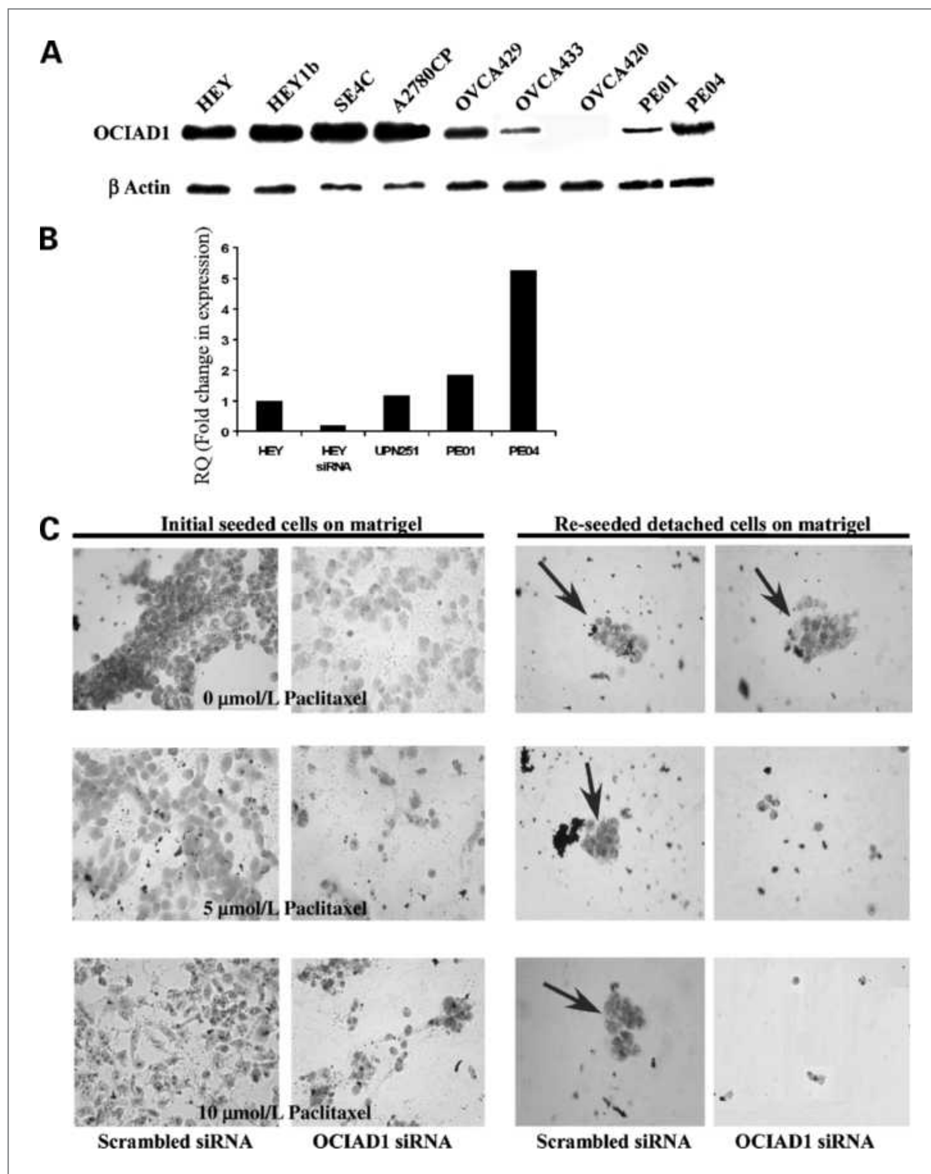

Figure 1. Role of OCIAD1 in late-stage ovarian cancer. A, Western blot of ovarian cancer cell lines, both chemosensitive and chemoresistant expressing OCIAD1. OCIAD1 antibody was used for the Western blot. β Actin was used as the loading control. B, quantitative PCR of OCIAD1 gene was done on several ovarian cancer cell lines, and the results were compared with HEY cell OCIAD1 expression. β2 microglobulin and GAPDH were used as loading controls. C, secondary cell homing assay to test for the ability of cells to form colonies after being challenged with paclitaxel.

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All cell transfections were done using Lipofectamine2000 (Invitrogen) according to the manufacturer's protocol.

Stealth siRNA against OCIAD1 (5′-AAUUGGU-CUUGGAACCUCUGCAUUC-3′and 5′-GAAUGCAGA-GGUUCCAAGACCAAUU-3′) and scrambled siRNA were obtained from Invitrogen.

Western blotting was done using polyvinylidene difluoride membranes. The blots were developed using phospho-p38, total p38, phospho-serine, and phospho-tyrosine (Cell Signaling Technology), and OCIAD1 antibodies and antirabbit IgG as the secondary antibody (Cell Signaling Technology).

OCIAD1 antibody was made and purified in our laboratory (6). Anti-integrin antibodies were obtained from Milipore.

RNA purifications were done by using RNAeasy kit (Qiagen). cDNA generation from RNA was done using kits from Invitrogen. Quantitative PCRs were done using Cyber-Green kit (Invitrogen) according to the manufacturer's recommendation. The primers used for the OCIAD1 gene *QPCR* were 5′-AGA CCA ATT CCC CAC ATA GG-3′ and 5′-TAC TTG TTG CAG CCA AAG GC-3′. The internal control gene β microglobulin was tested using 5′-CTT GTC TTT CAG CAA GGA CTG G-3′ and 5′-CAT GAT GCT GCT TAC ATG TCT C-3′.

Nucleus and cytosol preparation

Cells were fractionated into nucleus and cytosol using the NE-PER kit from Therma Scientific, according to the manufacturer's recommendation.

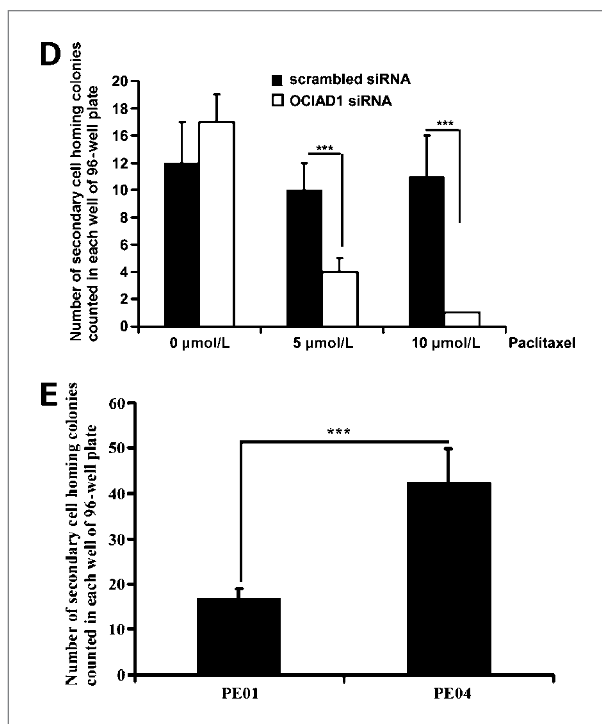

Figure 1. *Continued*. D, count of colonies formed in the experiment shown in C. E, secondary cell homing assay on PE01 and PE04 cells after treatment of cells with 5 µmol/L paclitaxel (Materials and Methods).

Cell adhesion kits

Cell adhesion kits to different integrins were obtained from Milipore.

Secondary cell homing assay

A secondary cell homing assay (SCHA) system was developed in our laboratory to simulate tumor recurrence in a cell culture model. Briefly, cells were starved overnight in serum-free RPMI1640, and 1×10^5 cells were allowed to attach on Matrigel-coated (25 µL; BD Biosciences) 24-well plates on serum-free RPMI1640. After the cells had attached to Matrigel (generally within 2 hours), we treated the cells with 20 µmol/L LPA for 2 hours, and then followed it with different doses of paclitaxel for standardization purpose. The medium was unchanged, but LPA and paclitaxel were added every 24 hours for 48 hours. The cells were kept at 37°C and in 5% CO_2. Seventy-two hours after the initiation of paclitaxel treatment, we collected the medium from the culture plates. The plates were washed thrice with sterile PBS. The washings and the removed medium were spun down at 500 rpm for 10 minutes for collecting the floating, unattached, and detached cells, and were resuspended in 1 mL RPMI1640 with 10% fetal bovine serum. The plate was stained with Cell Stain solution (Milipore) according to the manufacturer's instructions. Trypan blue dye exclusion method was done to check the viability of the floating cells that were collected from the plate. Almost 94% of the cells remained viable. Cells were counted in a hemocytometer, and similar numbers of cells from different experiments were then reseeded on second Matrigel containing 96-well plates in triplicate. This time RPMI1640 with 10% fetal bovine serum was used. The second plate with floating cells from the first plate was allowed to grow for 48 hours at 37°C and in 5% CO_2. The plates were then washed with PBS and the cells were stained with Cell Stain solution (Milipore). The colonies for a particular cell line showed similar size under a 10× microscope. The number of colonies was counted under the microscope and the experiments were done in triplicate. Cells that formed successful and more colonies on the second plates had higher secondary colony-forming efficiency. The second plate colony count was used as a measure for SCHA. After doing a dose curve we have used 5 µmol/L paclitaxel for subsequent experiments of SCHA on ovarian cancer cells.

Phosphorylation enrichment was done using a phosphoprotein enrichment kit (Therma Scientific).

Affinity column purification of OCIAD1

HEY cells were starved overnight in serum-free medium, then treated with LPA for 2 hours lysed in high-salt radioimmunoprecipitation assay (RIPA) buffer [50 mmol/L 0.5 mol/L Tris-HCl (pH8.0), 450 mmol/L sodium chloride, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 1.0% nonyl phenoxylpolyethoxylethanol (NP40), and halt protease and phosphatase inhibitors (Therma Scientific)] on ice for 1 hour. The

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lysates were then centrifuged at 1,000 × *g* at 4°C to remove debris. Protein concentrations of the extracts were measured and equal protein concentrations were used to equilibrate with the OCIAD1 antibody–sepharose 4B column that was prepared in our laboratory for 2 hours at 4°C. The flow through was removed, and the column was washed with 10 times the bed volume. The column was eluted out with 0.1 mol/L Glycine (pH 3.0), and was immediately neutralized with 1.5 mol/L Tris-HCl (pH 8.8).

Mass spectroscopy identification of proteins

Protein bands were cut from gels and were analyzed by mass spectroscopy as has been described before (6).

Statistical analysis was done by measuring the $P < 0.001$ and using Student's *t* test and Bonferroni's corrections.

Results

OCIAD1 plays a role in late-stage ovarian cancer

We tested several ovarian cancer cell lines originating from different stages of the disease. Cell lines originating from stage IVC disease like SE4C (12) and platinum-resistant cell line A2780cp expressed much higher levels of OCIAD1 when compared with cell lines like Ovca420, Ovca433, and Ovca429 (Fig. 1A). We also carried out quantitative PCR assays on different chemoresistant and chemosensitive cell lines (Fig. 1B). One cell line, PE01 (obtained from a patient before chemotherapy and which was chemosensitive), had much less OCIAD1 when compared with the PE04 cell line (this cell line was obtained from the same patient after the disease had recurred and become chemoresistant; ref. 17). The results indicate that chemoresistant cell lines have a much higher expression level of OCIAD1 when compared with cell lines that were sensitive to paclitaxel (Fig. 1B).

Cell adhesion to extracellular matrix proteins is one of the properties for forming secondary recurrent colonies. These secondary nodules are observed in patients after they have undergone first-line surgery and chemotherapy. However, there is no established assay system to test this function of secondary nodule formation. To address this problem, we designed a secondary cell homing assay (SCHA) to test for cellular ability to form recurrence (described in Materials and Methods). In this work we tried to replicate recurrence *in vivo*, a clinical condition most prevalent in ovarian cancers. After the *in vitro* metastatic site was created by SCHA, we tested the efficiency of OCIAD1-overexpressing (HEY-scrambled siRNA) and OCIAD1-low-expression cells (HEY-OCIAD1-siRNA) to detach from the site in the presence of paclitaxel in different doses given over a period of 24 to 72 hours. The OCIAD1-overexpressing cells had a significantly higher ability to form secondary colonies than the OCIAD1-low-expressing cells even after 10 μmol/L paclitaxel treatment (Fig. 1C and D). This experiment showed that OCIAD1 downregulation can compromise cellular ability to defend against paclitaxel and form secondary colonies, thus highlighting the importance of OCIAD1 in the formation of late-stage ovarian cancer. PE01 and PE04 had different levels of OCIAD1 expression as shown by quantitative PCR (Fig. 1B) and Western blotting (data not shown). SCHA was done on these cell lines, and results indicate that PE01 had the ability to form secondary colonies but significantly less than PE04 cells (Fig. 1E). This experiment also shows that the OCIAD1 expression level is a determinant of the formation of secondary colonies after treatment of the cells with paclitaxel.

Mechanism of LPA-dependent OCIAD1 expression

Previous results help us to realize that OCIAD1 has a potential role in the formation of recurrent ovarian cancer. Earlier experiments done in our laboratory showed that LPA stimulates cell adhesion on collagen I (6). We have also previously shown that overexpression of OCIAD1 leads to an increase in LPA-induced cell adhesion (6, 11). Therefore, we wanted to test if LPA modulates OCIAD1 expression in ovarian cancer cells. LPA had the ability to stimulate OCIAD1 in a dose-dependent manner (Fig. 2A). LPA stimulated OCIAD1 expression in a time-dependent manner as well (Fig. 2B). To determine the subcellular location of OCIAD1 on LPA stimulation, we prepared nuclear and cytosolic extracts 2 hours after LPA (20 μmol/L) stimulation and found that the nuclear content of OCIAD1 increased, whereas after 24 hours of LPA stimulation OCIAD1 levels decreased in the nucleus and increased in the cytosol (Fig. 2B). Other ovarian cancer–stimulating molecules like epidermal growth factor and insulin-like growth factor did not stimulate OCIAD1 expression at 2 hours or 24 hours (data not shown). To determine the role of OCIAD1 in the presence of paclitaxel, we tested in HEY cells the ability of paclitaxel to modulate OCIAD1 expression. Low-dose paclitaxel did not inhibit

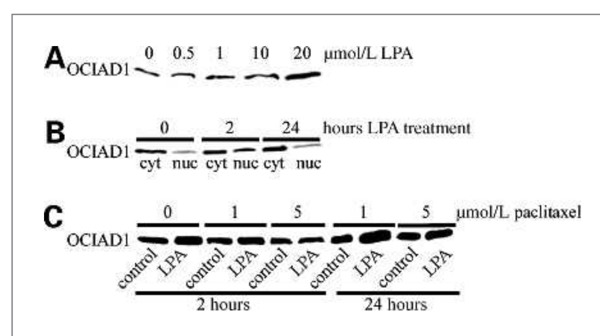

Figure 2. Western blot using OCIAD1 antibody. A, to test the dose response of HEY cells to LPA after 2 hours in stimulating OCIAD1 expression, protein concentrations in the samples were measured and equal concentrations of protein were loaded into all the wells. B, test expression of OCIAD1 in the cytoplasm and nucleus when treated with 20 μmol/L LPA. Protein concentrations in the samples were measured, and equal concentrations of protein were loaded into all the wells. C, test expression of OCIAD1 in cell cytoplasm when HEY cells were treated with LPA and paclitaxel. Protein concentrations in the samples were measured, and equal concentrations of protein were loaded into all the wells.

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basal or LPA-induced OCIAD1 expression, but at higher doses, paclitaxel blocked LPA-induced OCIAD1 expression but not the basal level of OCIAD1 expression (Fig. 2C).

To understand the mechanism of LPA-induced OCIAD1 expression, we tested several signaling molecule inhibitors for their ability to block LPA-dependent OCIAD1 stimulation (Fig. 3Ai). It has already been shown that LPA signals via the LPA receptors and is followed by the activation of Gi/o-PI3K-Erk-p38-Akt (18). Stimulation of the p38 mitogen-activated protein (MAP) kinase leads to cytosolic phospholipase A2 (cPLA2) activation (12). Signal inhibitor assays show that the p38 MAP kinase inhibitor SB 203580 inhibits LPA-induced increase in OCIAD1 expression (Fig. 3Ai). Furthermore, transfection of cells with MKK6 (an upstream activator of p38) and p38AGF (a dominant negative mutant of p38) has shown that MKK6 is involved in cytosolic accumulation of OCIAD1 (Fig. 3Aii). Western blot analysis shows that p38 phosphorylation is upregulated after transfection with MKK6 and is downregulated after p38AGF transfection (Fig. 3Aii). To confirm the role of MKK6 and p38 in the process of LPA-induced OCIAD1 overexpression, we transfected MKK6 in cells and inhibited p38 stimulation of MKK6 by SB 203580 (Fig. 3B). Here also we see that whereas MKK6 stimulates OCIAD1 expression, this MKK6-induced expression is blocked by SB 203580, suggesting that p38 is involved in stimulating OCIAD1 expression. MKK6 inhibited nuclear expression of OCIAD1, suggesting that it could block in some way nuclear transport of the protein and allow it to function in the cytosol.

To determine if this MKK6-p38 pathway–induced OCIAD1 expression is transcriptionally or translationally regulated, HEY cells were transfected with MKK6 to trigger OCIAD1 upregulation, then treated with 50 ng/mL cycloheximide to block protein translation. HEY cells were also transfected with OCIAD1-specific siRNA and scrambled siRNA as a negative control and comparison for the quantitative PCR experiment (Fig. 3C). We observe that cycloheximide treatment of cells does not inhibit MKK6-induced upregulation of OCIAD1 mRNA. OCIAD1-specific siRNA-transfected cells had much lower OCIAD1 mRNA when compared with scrambled siRNA, showing that the results obtained by OCIAD1 primers are specific and not an artifact. Thus, we infer a MKK6-p38 pathway stimulation of OCIAD1 upregulation.

Mechanism of OCIAD1 function

LPA stimulates OCIAD1 phosphorylation. Phosphorylation and dephosphorylation is a common mechanism of different proteins to execute their function in the cell. From an analysis of the protein sequence in silico with different algorithms it was apparent that OCIAD1 can have several putative phosphorylation sites in three serines at 108, 123, and 191 amino acid residues. It was observed that at 2 hours of LPA treatment, the concentration of OCIAD1 phosphoprotein increased when compared with 0 hours of LPA treatment, and at 24 hours the increased phosphorylation of OCIAD1 was reduced to 0-hour levels (Fig. 4A). The phospho-protein-enriched samples were probed with OCIAD1, phospho-serine, and phospho-tyrosine antibodies (Fig. 4B). This shows that OCIAD1 is phosphorylated at a serine residue. Thus, it was understood that LPA stimulates serine phosphorylation of OCIAD1. We are currently working to determine the exact amino acid moieties in the protein that get phosphorylated by LPA stimulation.

OCIAD1 expression levels modulate integrin function. Based on the secondary cell homing assays, we inferred that if OCIAD1 expression levels can alter cell attachment, the integrins should be involved in the process. Integrins are known to interact with extracellular matrix proteins and generate both outside-in and inside-out signaling pathways. We transfected HEY cells with OCIAD1-specific siRNA and scrambled siRNA and tested for their ability to bind to different extracellular proteins (Fig. 5A). OCIAD1 inhibition led to a decrease in LPA-induced cell adhesion to collagen I and laminin 10/11, very little but still statistically significantly to vitronectin, but not on fibronectin, and collagen IV (Fig. 5A). Combinations of α and β integrin subunits have shown that β1 integrin is common to cell adhesion to collagen I and laminin 10/11 (16). We therefore tested for the ability of OCIAD1-negative cells to express β1 integrins. Western blotting results show that β1 integrin expression is not different between scrambled and OCIAD1 siRNA–transfected cells (Fig. 5B). To test what integrins might be involved in the process, we did several cell adhesion assays on α integrin and β integrin arrays using cell adhesion kits (Chemicon; Fig. 5C). LPA stimulation of OCIAD1-positive cells increases adhesion to β1 integrin significantly. However, cell adhesion to β1 integrin is actually downregulated in OCIAD1-inhibited cells. LPA stimulation does not help to recover this decrease in cell adhesion to β1 integrins. This result, along with the expression result in Fig. 5B, indicates that although OCIAD1 does not interfere with β1 expression, OCIAD1 does play a role in β1 integrin function. There was no significant difference in the cell adhesion efficiency to β2 integrins among OCIAD1-positive and -inhibited cells both in the presence and the absence of LPA. Cells did not attach to β3 and β4 integrins. This β1-specific effect shows the functional specificity of OCIAD1 towards β1 integrin. LPA did not increase cell adhesion to any α integrins. However, OCIAD1 inhibition led to reduced cell adhesion to α2, α5, and αV, but not to α3. Reduction of cell binding to α5 and αV is not recovered on LPA treatment. α5 β1 is generally recognized as the integrin for fibronectin, and αV β3 and αV β5 are generally recognized as integrin complexes responsible for binding to vitronectin. We have also seen that LPA can recover the reduced cell affinity to vitronectin and fibronectin in OCIAD1-inhibited cells. Therefore, the inability of LPA to increase cell adhesion to α5 and αV in OCIAD1-inhibited cells raises the possibility of other mechanisms involved in cell attachment to vitronectin and fibronectin.

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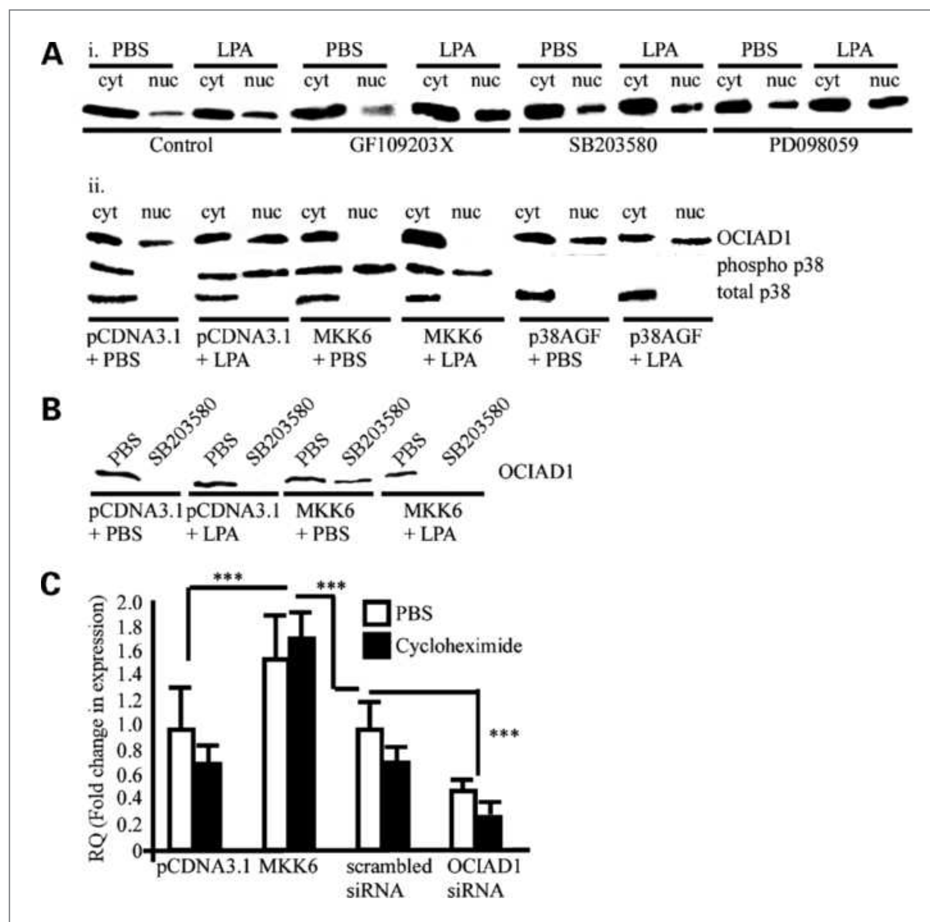

Figure 3. A, i, Western blot to test OCIAD1 expression in the cytoplasm and nucleus of HEY cells when treated with different cell signaling inhibitors. Protein concentrations in the samples were measured and equal concentrations of protein were loaded into all the wells. ii, Western blot to test OCIAD1, phospho p38 when HEY cells were transfected with MKK6 and p38AGF (dominant negative mutant) in the presence and the absence of LPA. Protein concentrations in the samples were measured and equal concentrations of protein were loaded into all the wells. B, Western blot to test OCIAD1 expression when HEY cells were transfected with pCDNA3.1 vector and MKK6 and treated with PBS, LPA, and p38 inhibitor SB 203580. Protein concentrations in the samples were measured, and equal concentrations of protein were loaded into all the wells. C, quantitative PCR results of OCIAD1 gene in HEY cells transfected with pCDNA3.1 vector, MKK6, scrambled siRNA, and OCIAD1 siRNA in the presence and the absence of cycloheximide (50 μg/mL). *Y*-axis shows RQ or fold change in expression (compared with pCDNA3.1-transfected HEY cells treated with PBS). Student's *t* test and Bonferroni's corrections were used for statistical analysis.

OCIAD1 interacts with several cellular proteins. To understand what proteins might be involved in the OCIAD1-integrin pathway, we designed coprecipitation experiments with proteomic techniques. The samples were then resolved on 10% SDS-PAGE. One gel with the samples was Western blotted to test for OCIAD1 expression (Fig. 6A) and another gel was stained with silver stain. The gel was destained to get best visibility of the bands (Fig. 6A). Three bands from the nucleus and three bands from the cytosol were analyzed using liquid chromatography/mass spectroscopy (LC/MS) and identified (Fig. 6B). We consistently see the presence of β actin and α actinin 4 as a coprecipitated molecule with OCIAD1. α Actinin 4 can act as a binding factor of β actin and can also lead to modification of integrin function. We therefore hypothesize that α actinin 4 is the intermediary between OCIAD1 action and integrin function. Bands indicate the presence of eukaryotic translation elongation factor 1 α 2 (EF2α), which is a transcription factor. This raises the possibility that OCIAD1 could be under EF2α transcriptional control.

Discussion

OCIAD1 is important for recurrence of ovarian cancer

Initial studies on OCIAD1 had suggested that it is expressed in metastatic ovarian tumors when compared with paired primaries, and that modulating its levels has an effect on cell adhesion (6). Cell adhesion is an important phenomenon in generating recurrent nodules. This raised the possibility of the role of OCIAD1 in recurrent ovarian

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cancer. We have reported that the OCIAD1 effect was dependent on LPA action (6). Therefore, we sought to further dissect the role of LPA-induced OCIAD1 expression at a molecular level. When OCIAD1 expression was tested in different cell lines, it became apparent that OCIAD1 may play a role in recurrent ovarian cancer. Cell lines that had high chemoresistance expressed more OCIAD1 when compared with the chemosensitive cell lines (Fig. 1A and B). Of particular importance were the results from cell lines PE01 and PE04 that were obtained from the same patient suffering from primary ovarian cancer (PE01) and recurrent and chemoresistant ovarian cancer (PE04). This result highlights the potential importance of OCIAD1 in recurrent and chemoresistant disease. This also raises the possibility that OCIAD1 levels could determine the potential for recurrence in primary tumors, thus opening a possibility for early therapeutic strategy intervention. Recurrence can conceivably occur from cells that were not removed during surgery at microscopic levels, and which had the ability to resist chemotherapeutic insults and grow to form secondary nodules. Using this concept we designed an *in vitro* SCHA. We allowed cells to grow on Matrigel (to replicate basement membrane of the peritoneum where ovarian cancer recurs) and stimulated with LPA (which is present in the peritoneum) then challenged them with paclitaxel (as one would do clinically). Although many cells were detached from the Matrigel following paclitaxel treatment (paclitaxel is known to reduce integrin function; ref. 15), only few cells had the capacity to actually attach on a second Matrigel matrix and grow into colonies. This colony-forming ability of cells after being challenged with paclitaxel may be of significant importance for forming disease recurrence. We observed that cells where OCIAD1 is downregulated lacked the ability to form secondary colonies after being challenged with paclitaxel, whereas cells with high OCIAD1 levels retain the ability to form secondary colonies after paclitaxel challenge (Fig. 1C-E). These experiments indicate that OCIAD1 function is of potential importance in forming recurrent ovarian cancer. These experiments also for the first time showed a strategy to test for cellular ability to form secondary colonies, a test which can be used to determine the potential of cancer cells to develop recurrent disease.

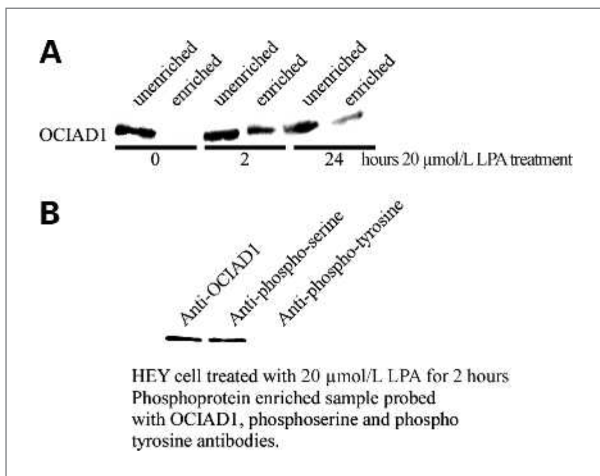

Figure 4. A, Western blot using OCIAD1 antibody in HEY cells treated with LPA. The cell lysates were enriched for phospho-specific proteins. Protein concentrations for unenriched and enriched samples were measured, and equal concentrations of proteins were loaded into the wells. B, HEY cells treated with 20 µmol/L LPA were lysed with RIPA and enriched for phospho-specific proteins. Equal concentrations of protein were loaded on the wells and the different lanes and the Western blots were probed with OCIAD1, phospho-serine and phospho-tyrosine antibodies.

LPA induces OCIAD1 expression and function

We reported earlier that the OCIAD1 function to increase cell adhesion is dependent on LPA stimulation (6). This raises the possibility that stimulation of LPA receptors on cells could lead to signaling changes leading to OCIAD1 expression and function. The possibility was proved right when experiments showed that LPA stimulates OCIAD1 expression within 2 hours in the nucleus of cells and in 24 hours is redistributed into the cytoplasm (Fig. 2B). The signaling pathways originating from the LPA receptors leading to cell migration (11, 16), cell survival (18), and cell invasion (12, 13) had earlier been described in HEY cells. Generally LPA receptor–Gi/o–phosphoinositol-3-kinase–extracellular signal-regulated kinase (Erk)–MKK6–p38 has been described (18) before the signals branch of stimulating cell migration by stimulating cPLA2 for migration (12) or Akt phosphorylation for cell survival (18). To test if OCIAD1 overexpression by LPA stimulation follows the same described pathways, we started treating HEY cells with small molecule inhibitors for phospho kinase C (PKC), Erk, and p38. The results indicate that Erk inhibition does not block LPA-stimulated OCIAD1 (Fig. 3A). However, p38 function inhibition by SB 208530 does lead to inhibition of OCIAD1 expression after LPA stimulation (Fig. 3A). This result indicates that LPA stimulation of OCIAD1 can use other pathways besides Erk to stimulate via p38. PKC inhibition by GF 109203X also did not inhibit LPA-induced OCIAD1 expression. When MKK6 (an upstream mediator of p38) was transfected into cells, OCIAD1 expression went up and the protein was very quickly redistributed into the cytoplasm as no band was observed in the nucleus (Fig. 3B). p38AGF (dominant negative p38) transfection experiments also indicated that LPA-induced upregulation of OCIAD1 expression was inhibited (Fig. 3B). These results indicate that the p38 MAP kinase is involved in OCIAD1 upregulation by LPA stimulation. It is well known that p38 can stimulate many transcription factors to induce protein synthesis in cells. We used cycloheximide to inhibit translation of protein and found out that cycloheximide failed to inhibit OCIAD1 synthesis in cells when cells were transfected with MKK6. This indicates that MKK6-induced p38 stimulation can increase OCIAD1 levels by modulating certain transcription factors in the cells (Fig. 3C). Moreover, Western blots indicate that after MKK6 transfection OCIAD1 is not in the nucleus. MKK6 function can in some

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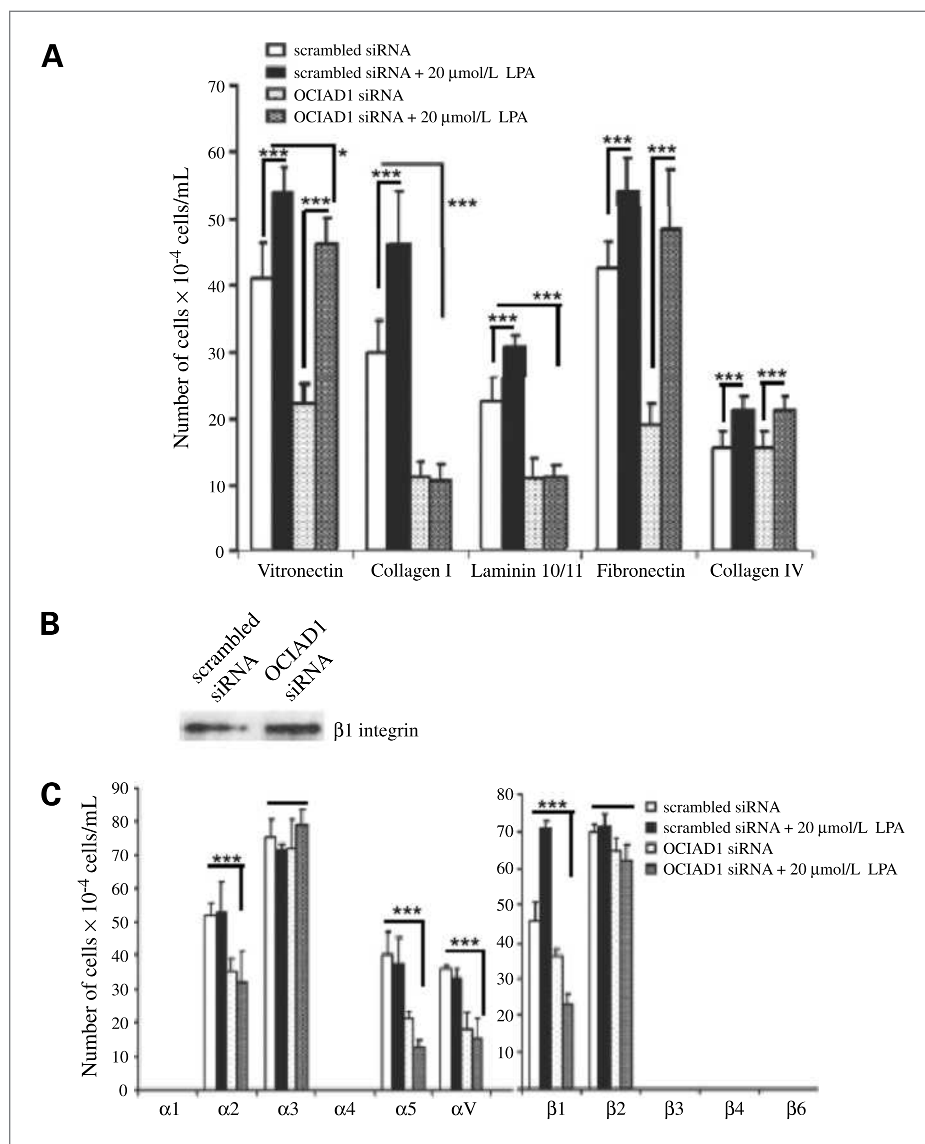

Figure 5. A, cell adhesion assay of HEY cells transfected with scrambled and OCIAD1 siRNA in the presence and the absence of LPA on different extracellular matrix proteins. Statistical significance was measured using Student's *t* test and Bonferroni's corrections. ***, $P < 0.001$. B, Western blot of β1 integrin antibody in HEY cells transfected with scrambled and OCIAD1 siRNA. Protein concentrations in the samples were measured, and equal concentrations of protein were loaded into all the wells. C, cell adhesion assay of HEY cells transfected with scrambled and OCIAD1 siRNA on different integrin receptors. Statistical significance was measured using Student's *t* test and Bonferroni's corrections. ***, $P < 0.001$.

way block the distribution of OCIAD1 to the nucleus or can quickly redistribute OCIAD1 and keep the protein in the cytosol. Allowing the protein to remain in the cytoplasm could be to allow OCIAD1 function.

Phosphorylation and dephosphorylation is one important mechanism of protein function in the cell. Therefore, to understand OCIAD1 function we tested for the ability of OCIAD1 to get phosphorylated. OCIAD1 does get phosphorylated by LPA stimulation (Fig. 4A). Results show that OCIAD1 can be phosphorylated at a serine residue (Fig. 4B). Experimental analysis reveals the presence of three specific phosphorylation sites on serine residue 108, 123, and 191 amino acids. However, a detailed understanding of OCIAD1 phosphorylation and its relevant kinases is still going on in our laboratory. This result indicates that OCIAD1 function could be dependent on OCIAD1 phosphorylation. The initial observation that OCIAD1 expression levels modulate cells efficiency to defend against paclitaxel and subsequent homing to extracellular matrix leads to the hypothesis that OCIAD1 levels can modulate integrin function. As Matrigel is a combination of different extracellular matrix proteins, we tested for the ability of OCIAD1-expressing and -inhibited cells to bind to different extracellular matrix proteins. The increase in cell attachment on LPA stimulation in the presence and the absence of OCIAD1 was not statistically significant on vitronectin, fibronectin, or collagen I (Fig. 5A), but was significantly reduced on collagen I and laminin 10/11. We have already published the importance of ovarian cancer cell adhesion to laminin 10/11 (11, 14) as this adhesion leads to increased LPA production from the cells. As binding to both laminin

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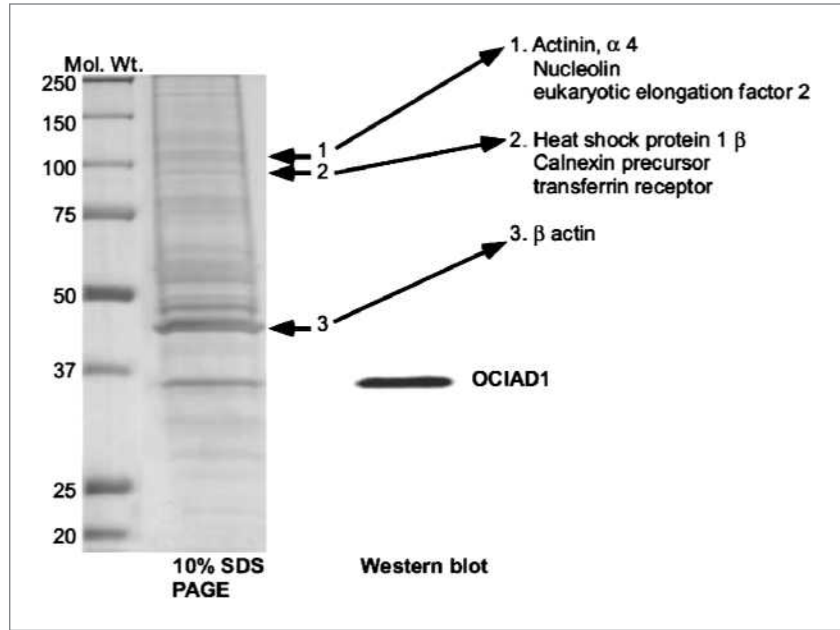

Figure 6. HEY cell lysates were passed through an OCIAD1 antibody-sepharose column. The column elute was run on 10% SDS-PAGE and run on two lanes. One lane was silver stained and the other lane was Western blotted and developed with OCIAD1 antibody. Arrows, bands cut and analyzed by LC/MS. The numbers given to the cut bands were the sample numbers. Results obtained from the LC/MS. The numbers correspond to the sample numbers in cut bands.

10/11 and collagen I involves β1 integrins, we tested for the ability of OCIAD1-inhibited cells to express β1. The results indicated that expression level of β1 did not change in OCIAD1-inhibited cells (Fig. 5B). Therefore, we concluded that OCIAD1 expression levels modulated integrin function. We used cell adhesion assays to test cell adhesion capacity to different integrins. LPA stimulated the ability of OCIAD1 to stimulate β1 integrin binding to cells, which probably explains the ability of cells to stick and home to secondary locations even after being challenged with paclitaxel.

To probe deeper into OCIAD1 and integrin interaction, we stimulated cells with LPA and then prepared cell lysates in RIPA buffer. OCIAD1 was purified from these fractions by passing them through OCIAD1 antibody column. Proteins that were physically interacting with OCIAD1 were identified by SDS-PAGE and then by LC/MS identification techniques. OCIAD1 consistently

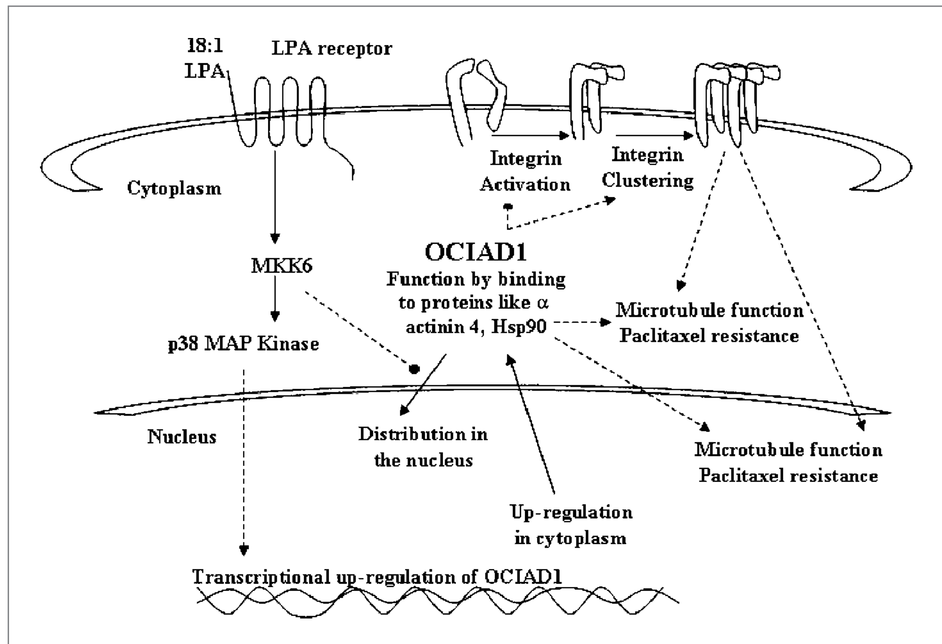

Figure 7. Schematic of hypothetical mechanism of OCIAD1 in modulating integrin function leading to secondary cell homing/paclitaxel resistance. (broken lines, unknown mechanism).

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precipitated α actinin 4 (Fig. 6A and B), which is known to be an adapter molecule leading to modulation of integrin function and maintaining β actin architecture (19). This β actin organization is important for cellular attachment to extracellular matrix leading to increased cell survival and chemoresistance. β Actin can also play a role in inside-out signaling to integrin receptors leading to increased integrin function.

Based on the obtained results, we have developed a working hypothesis for the role of OCIAD1, as shown in the cartoon in Fig. 7.

This is the first report showing that OCIAD1 is an important player in the formation of recurrent ovarian cancer and raises the probability of using OCIAD1 inhibition strategies as a therapeutic measure. Current efforts in our laboratory are directed at solving the intracellular function of OCIAD1 and testing if OCIAD1 inhibition can be used as a strategy to treat metastasis and recurrence *in vivo*.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

Outrun Ovarian Cancer (www.oroc.org).

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Received 11/16/2009; revised 03/25/2010; accepted 04/12/2010; published OnlineFirst 06/01/2010.

References

1. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2008. CA Cancer J Clin 2008;58:71–96.
2. Ozols RF. Treatment goals in ovarian cancer. Int J Gynecol Cancer 2005;15 Suppl 1:3–11.
3. Blagden S, Gabra H. Future directions in the management of epithelial ovarian cancer. Future Oncol 2008;4:403–11.
4. Bukowski RM, Ozols RF, Markman M. The management of recurrent ovarian cancer. Semin Oncol 2007;34:S1–15.
5. Michener CM, Belinson JL. Modern management of recurrent ovarian carcinoma. A systematic approach to a chronic disease. Oncology (Williston Park) 2005;19:1277–85; discussion 85, 88, 93.
6. Sengupta S, Michener CM, Escobar P, Belinson J, Ganapathi R. Ovarian cancer immuno-reactive antigen domain containing 1 (OCIAD1), a key player in ovarian cancer cell adhesion. Gynecol Oncol 2008;109:226–33.
7. Luo LY, Soosaipillai A, Diamandis EP. Molecular cloning of a novel human gene on chromosome 4p11 by immunoscreening of an ovarian carcinoma cDNA library. Biochem Biophys Res Commun 2001; 280:401–6.
8. Xu Y, Fang XJ, Casey G, Mills GB. Lysophospholipids activate ovarian and breast cancer cells. Biochem J 1995;309:933–40.
9. Xu Y, Gaudette DC, Boynton JD, et al. Characterization of an ovarian cancer activating factor in ascites from ovarian cancer patients. Clin Cancer Res 1995;1:1223–32.
10. Sutphen R, Xu Y, Wilbanks GD, et al. Lysophospholipids are potential biomarkers of ovarian cancer. Cancer Epidemiol Biomarkers Prev 2004;13:1185–91.
11. Sengupta S, Xiao YJ, Xu Y. A novel laminin-induced LPA autocrine loop in the migration of ovarian cancer cells. FASEB J 2003;17:1570–2.
12. Sengupta S, Kim KS, Berk MP, et al. Lysophosphatidic acid downregulates tissue inhibitor of metalloproteinases, which are negatively involved in lysophosphatidic acid-induced cell invasion. Oncogene 2007;26:2894–901.
13. Kim KS, Sengupta S, Berk M, et al. Hypoxia enhances lysophosphatidic acid responsiveness in ovarian cancer cells and lysophosphatidic acid induces ovarian tumor metastasis *in vivo*. Cancer Res 2006; 66:7983–90.
14. Sengupta S, Wang Z, Tipps R, Xu Y. Biology of LPA in health and disease. Semin Cell Dev Biol 2004;15:503–12.
15. Menendez JA, Vellon L, Mehmi I, Teng PK, Griggs DW, Lupu R. A novel CYR61-triggered 'CYR61–3 integrin loop' regulates breast cancer cell survival and chemosensitivity through activation of ERK1/ERK2 MAPK signaling pathway. Oncogene 2005;24:761–79.
16. Zhao X, Wang D, Zhao Z, et al. Caspase-3-dependent activation of calcium-independent phospholipase A2 enhances cell migration in non-apoptotic ovarian cancer cells. J Biol Chem 2006;281: 29357–68.
17. Lewis AD, Hayes JD, Wolf CR. Glutathione and glutathione-dependent enzymes in ovarian adenocarcinoma cell lines derived from a patient before and after the onset of drug resistance: intrinsic differences and cell cycle effects. Carcinogenesis 1988;9: 1283–7.
18. Baudhuin LM, Cristina KL, Lu J, Xu Y. Akt activation induced by lysophosphatidic acid and sphingosine-1-phosphate requires both mitogen-activated protein kinase kinase and p38 mitogen-activated protein kinase and is cell-line specific. Mol Pharmacol 2002;62: 660–71.
19. Mizejewski GJ. Role of integrins in cancer: survey of expression patterns. Proc Soc Exp Biol Med 1999;222:124–38.

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