

## Research Article

## LPA Regulates SOX9 in Ovarian Cancer Cells

Qipeng Fan\*, Qingchun Cai\*, and Yan Xu

Department of Obstetrics and Gynecology, Indiana University School of Medicine, USA

\*These authors contributed equally to this work.

**Corresponding author:** Yan Xu, Department of Obstetrics and Gynecology, Indiana University School of Medicine, 1044 W. Walnut St, Indianapolis, USA, Tel: +(317) 274-3972; E-mail: xu2@iu.edu

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**Abstract**

**Objective:** SOX9 is a master transcription factor that regulates development and stem cell programs. This work is to determine SOX9's potential oncogenic activity and regulatory mechanisms controlling SOX9 protein expression in Epithelial Ovarian Cancer (EOC).

**Methods:** An oncolipid, Lysophosphatidic Acid (LPA) has been tested for its regulatory effect on SOX9 in mouse and human EOC cells. The CRISPR/Cas9 technique was used to knockout (KO) SOX9. The functional assays of SOX9 in EOC include proliferation, anoikis, CD44 expression, and spheroid-formation.

**Results:** LPA dose- and time-dependently up-regulated SOX9 in EOC cells. This up-regulation was likely mediated by the nuclear receptor peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ). SOX9 was involved in cellular activities related to Cancer Stem Cells (CSC), including anoikis-resistance, regulation CSC marker CD44, and spheroid-formation.

**Conclusion:** Our data revealed that LPA is a regulator of SOX9, that is involved in stem cell related activities in EOC. Hence, SOX9, along with its regulatory and signaling pathways, warrants further investigation to critically evaluate their therapeutic significance in EOC.

**Key words:** Cancer stem cells (CSC); Epithelial ovarian cancer (EOC), Gynecological cancers, High grade serous ovarian cancer (HGSOC); Lysophosphatidic acid (LPA), Sex determining region Y-box9 (SOX9); Peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ).

**Introduction**

Epithelial Ovarian Cancer (EOC) is the most deadly gynecological cancer. Specifically targeting cancer stem cells (CSC) represents a major challenge in EOC treatment. Novel and more specific and effective treatments are urgently needed. Identification of critical regulators in EOC CSC properties is pivotally important.

Sex-determining region Y (SRY)-box 9 (SOX9) is a member of the SOX transcription factor family. It plays an important role in sex determination and bone development [1]. In recent years; deregulation of SOX9 has been implicated in various diseases, including fibrosis and cancer. SOX9 plays a tumor-promoting role and is associated with CSC in lung, pancreatic, breast, oral, liver,

colon, and other cancers [2-8]. Regarding to ovary, the role of SOX9 has mainly been studied in Sertoli-Leydig cell tumors and granulosa cell tumors [9-11]. Recently, SOX9 has been shown to allow the survival of EOC cells upon hypoxic condition and its aberrant activation and high expression in human EOC tissues are prominent in patients with aggressive EOC [12]. However, the potential involvement of SOX9 in EOC CSC is totally unknown.

As a critical gene in the development of bones and testes, SOX9 expression is regulated by related factors [13]. However, the regulation of aberrant expression of SOX9 in cancer is much less known and the regulatory factors of SOX9 in EOC cells are essentially unknown [14]. We tested the potential regulatory effects on SOX9 expression exerted by LPA. LPA is a proven and validated oncolipid and target for EOC [15-20]. LPA regulates many known oncogenes [16-18, 21]. However, whether it can regulate SOX9 is unknown in any cells. We tested the regulatory roles of LPA in SOX9 expression and the role of SOX9 pertinent to CSC related cellular properties in mouse and human EOC. Genetic, biochemi-

cal, and cell biological approaches are utilized in the investigation.

## Materials and Methods

### Reagents, Cell Lines and Culture

Oleoyl-LPA was from Avanti Polar Lipids (Birmingham, AL). The following reagents were used: BrP-LPA (EBI, Salt Lake City, UT); Y27632 (Biovision, Milpitas, CA); GW9662 (EMD Corp; Billerica, MA); pertussis toxin (PTX; Invitrogen, Grand Island, NY); H89 and actinomycin D (ActD; Sigma-Aldrich, St. Louis, MO). Anti-SOX9 antibody (Cat. Log # AB5535) was from EMD Millipore (Billerica, MA). The pair of PE01/PE04 cell lines were from Dr. Daniela Matei (Northwestern University); the OVCAR3 cells were obtained from ATCC (Manassas, VA). The ID8, T29, and OVCA433 cell lines were kind gifts from Dr. R. Bast (M.D Anderson), Dr. Jinsong Liu (M.D Anderson), and Dr. Paul F Terranova (University of Kansas Medical Center), respectively. These cell lines were authenticated by ATCC. All cell lines were maintained in a humidified atmosphere at 37°C with 5% CO<sub>2</sub>. ID8 cells (mouse epithelial ovarian cancer cells) were maintained in high glucose DMEM containing 5% FBS (ATCC, Manassas, VA) and 100 µg/mL Penicillin/Streptomycin/ Amphotericin B (PSA). OVCA433 cells and PE01/PE04 cells were cultured in RPMI 1640 with glutamine, 10% FBS (ATCC, Manassas, VA), and 100 µg/mL Penicillin/Streptomycin/Amphotericin B (PSA). OVCAR3 cells were maintained in RPMI-1640 supplemented with 20% FBS, 0.01 mg/mL insulin and 100 µg/mL PSA. PE01/PE04 cells were cultured in RPMI 1640 with glutamine, 10% FBS, and 100 µg/mL PSA. For serum starvation, cells were incubated in the basal medium without FBS or antibiotics. LPA treatment was performed in cells starved from serum for 16-24 hr.

### Stable Cell lines

SOX9 CRISPR lentiVirus HCP217635-LvSG03 and Cas9 pCRISPR-LvSG03 vectors (GeneCopoeia, Rockville, MD) were co-transfected with the delta 8.9 packaging plasmid and the pCMV-VSVG plasmid into 293T cells for virus packaging, using Fugene6 (Promega, Madison, WI). Cell medium was changed to DMEM supplemented with 30% FBS following overnight incubation. After 48 hrs, cell media were harvested and filtered using 0.45 µm filter syringes. PE04 and OVCAR3 cells were infected by packaged viruses in the presence of Polybrene (8 µg/mL) for 48 hrs, followed by selection with puromycin (0.5 µg/mL) for at least 7 days.

### Western Blot Analysis

Western blot analyses were conducted using standard procedures and proteins were detected using primary antibodies and fluorescent secondary antibodies (IRDye 800CW-conjugated or IRDye 680-conjugated anti-species IgG, Li-Cor Biosciences, Lincoln,

NE) as we described previously [22]. The fluorescent signals were captured on an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE) with both 700- and 800-nm channels. Boxes were manually placed around each band of interest, and the software returned near-infrared fluorescent values of raw intensity with background subtraction (Odyssey 3.0 analytical software, Li-Cor Biosciences, Lincoln, NE). The protein MW marker used was the Pre-stained SDS-PAGE Standards, broad range (BIO\_RAD, Cat. Log # 161-0318).

### Cell Proliferation, Anoikis-Resistance, Colony- and Spheroid-Formation Assays

Cell proliferation was analyzed based on MTT hydrolysis using Cell Counting Kit-8 (Dojindo Molecular Technologies, Rockville, MA). Anoikis-resistance and soft agar colony assays were described in detail previously [22]. For spheroid formation, cells were re-suspended at  $1 \times 10^3$  to  $1 \times 10^4$  cells/mL in serum-free DMEM/F12 supplemented with 5 µg/mL insulin (Sigma), 20 ng/mL human recombinant epidermal growth factor (EGF; Invitrogen), 10 ng/mL basic fibroblast growth factor (bFGF; Invitrogen), and 0.4% bovine serum albumin (BSA; Sigma), followed by culturing in 24-or 96-well Ultra Low Attachment plates (Corning, NY). Spheroids were photographed after seven days in culture.

### Immunofluorescence Assay

To assess the expression level of CD44 in EOC cells, immune fluorescence was performed using antibody against CD44 (Ab6124; Abcam, Cambridge, MA). Cells were fixed with 4% Para formaldehyde and permeabilized using a blocking solution consisting of 5% Normal Goat Serum and 0.1% Triton X-100 in PBS. The primary antibodies against CD44 were diluted 1:200 in the same blocking solution.

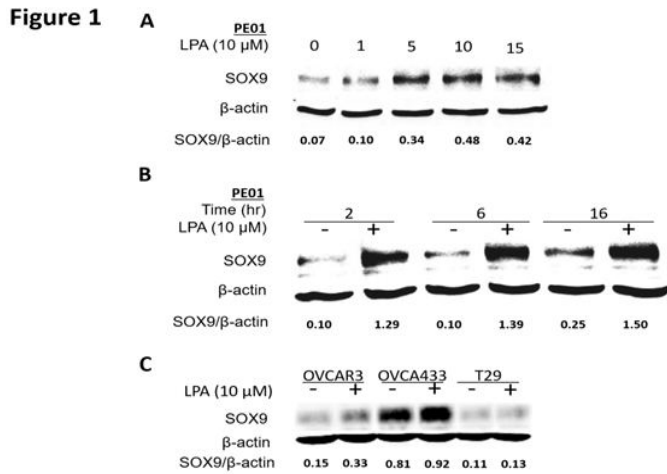
### Statistical Analyses

The Student's t-test was utilized to assess the statistical significance of the difference between two treatments. The asterisk rating system as well as quoting the P value in this study was \* P < 0.05; \*\* P < 0.01; and \*\*\* P < 0.001. A P value of less than 0.05 was considered significant.

## Results

### LPA-dose and -time dependently up-regulated SOX9 in mouse and human EOC cells

We tested the potential effect of LPA on SOX9 expression and found that LPA up-regulated SOX9 in PE01 cells in a dose- and time-dependent manner, with the optimal dose and time being 5-10 µM and 6 hrs, respectively (Figures. 1A and 1B). LPA also up-regulated SOX9 in OVCAR3, another HGSOE cell line, and in OVCA433 EOC cell line, but not in a human ovarian surface epithelial cell (HOSE) line T29 (Figure. 1C).



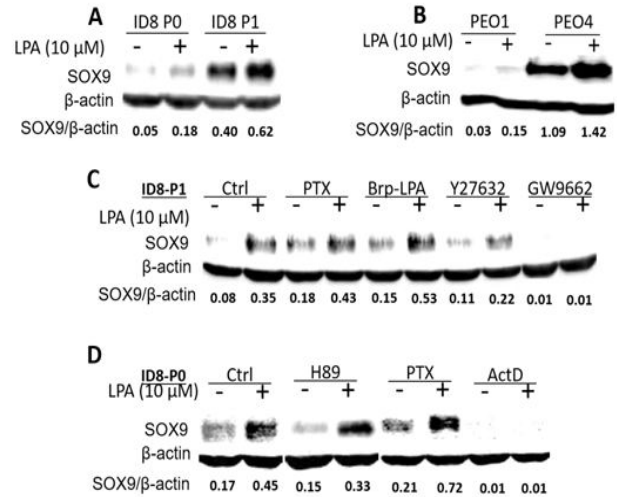
**Figure 1:** LPA induced SOX9 up-regulation in human HGSOC and T29 cells

A. PE01 cells were serum starved for 16 hrs prior to LPA treatment (6 hrs) with concentrations indicated. B. PE01 cells were serum starved for 16 hrs prior to LPA (10 μM) treatment for different times as indicated. C. LPA-induced SOX9 up-regulation in OVCAR3 and OVCA433, but not in the T29 cells. Reproducible results from independent experiments were shown.

### SOX9 expressed at higher levels in more aggressive EOC cells and LPA-induced SOX9 expression was PPARγ-dependent

We have developed a highly aggressive EOC cell line ID8-P1 through *in vivo* passage of ID8-P0 cells in C57BL6 mice [22]. The tumor/ascites formation time is reduced from ~90 days for ID8-P0 cells to ~30 days in different P1 cell lines isolated from tumors in different organs or from ascites [22]. We found that SOX9 was expressed at higher levels in the more aggressive ID8-P1 cells than in ID8-P0 cells. In addition, LPA induced further increases in SOX9 expression in these cells (Figure. 2A). Similarly, in the paired human HGSOC. A. Mouse ID8 P0/P1 cells were serum starved for 16 hrs prior to LPA treatment (10 μM, 6 hrs). ID8-P1 cells expressed higher level of SOX9 than ID8-P0 cells. LPA induced further increases in SOX9 expression in this cells. B. Human PE01/PE04 cells were serum starved for 16 hrs prior to LPA treatment (10 μM, 6 hr); PE04 cells expressed higher level of SOX9 than PE01. LPA induced further increases in SOX9 expression. C. Serum-starved ID8-P1 cells were treated with PTX (100 ng/mL) for 16 hr; BrP-LPA (10 μM), Y27632 (10 μM), and GW9662 (10 μM) for 1 hr, prior to LPA treatment (10 μM, 6 hrs). D. Serum starved ID8-P0 cells were treated with H89 (10 μM) for 1 hr; PTX (100 ng/mL) for 16 hrs or the transcriptional inhibitor ActD (1 μg/mL) for 1 hr, prior to LPA treatment (10 μM, 6 hrs). Reproducible results from independent experiments were shown. PE01/PE04 cell lines, SOX9 was expressed at much higher levels in the drug-resistant PE04 cells than in PE01 cells [23]. (Figure. 2B)

**Figure 2**



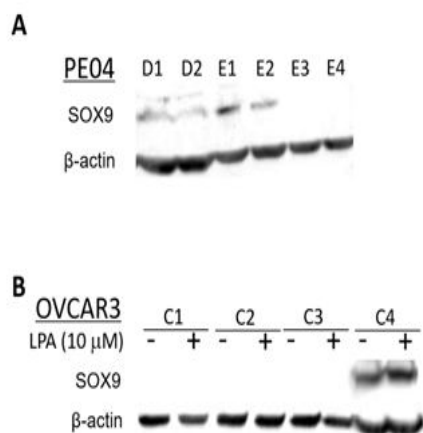
**Figure 2:** Endogenous and LPA-induced SOX9 expression in EOC cells and PPARγ-dependent LPA induction.

The majority of known cellular effects of LPA are mediated by membrane G protein-coupled receptors (GPCRs; LPAR<sub>1-6</sub>) [24, 25, 21, 26]. To determine which LPA receptors are involved in LPA-SOX9 up-regulation, we used BrP-LPA, a pan-LPA receptor [27] Surprisingly, this inhibitor did not significantly block the effect (Figure. 2C). We then employed several selective inhibitors mediated by LPA GPCRs in EOC cells as we and others shown previously [28-32], including pertussis toxin (PTX), a G<sub>i</sub> inhibitor; Y27632, a G<sub>12/23</sub>/Rho-Rock kinase pathway inhibitor; and H89, a G<sub>s</sub>-protein kinase A inhibitor. Consistent with the receptor inhibitor BrP-LPA, these inhibitors had insignificant or only weak effects on LPA-induced SOX9 expression (Figures. 2B, 2C). On the other hand, the PPARγ selective inhibitor GW9662 completely blocked the effect; strongly suggest that LPA-induced SOX9 was mainly mediated by PPARγ. LPA-induced SOX9 expression was sensitive to Actinomycin D (ActD), a transcription inhibitor, suggesting that transcription is involved (Figure. 2D).

### SOX9 was Functionally Involved In CSC Related Activities in EOC Cells

To investigate the role of SOX9 in EOC, we generated SOX9-knockout (KO) clones using the CRISPR/Cas9 system in PE04 and OVCAR3 cells (Figure. 3). We found that SOX9-KO did not affect cell proliferation when cells were cultured in 2D dishes, but significantly reduced anoikis-resistance when cells were cultured in suspension in both PE04 and OVCAR3 cells (Figure. 4). This is very similar to what we have observed in ID8-P1 and -P0 cell [22]. Even though time to tumor/ascites formation is reduced from 90 days to 22-45 days in ID8-P1 vs. -P0 cells, the P1 cells do not gain a proliferation advantage when cultured in 2D dishes, but have greatly enhanced anoikis-resistance.<sup>22</sup> This anchorage-independent growth is related to transformation and CSC properties.

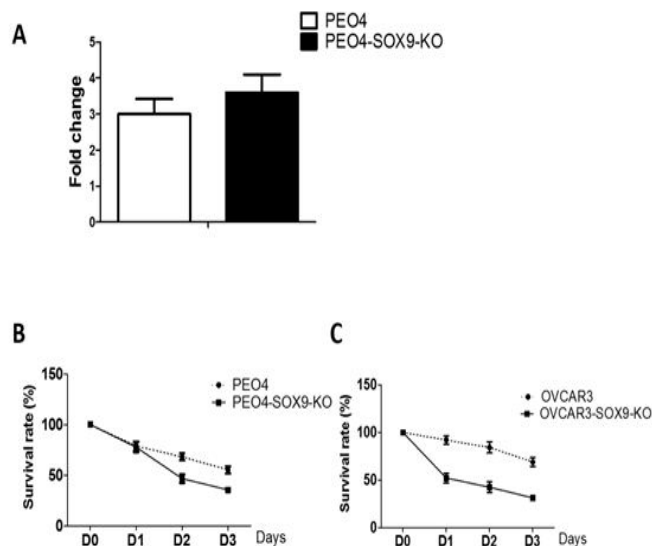
**Figure 3**



**Figure 3:** SOX9-KO clones were generated in PE04 and OVCAR3 cells.

A. Human PE04-SOX9-KO clones were detected by Western blot analyses. The E3 and E4 clones were used in functional studies. B. Human OVCAR3-SOX9-KO clones were detected by Western blot analyses. The C1 and C2 clones were used in functional studies.

**Figure 4**

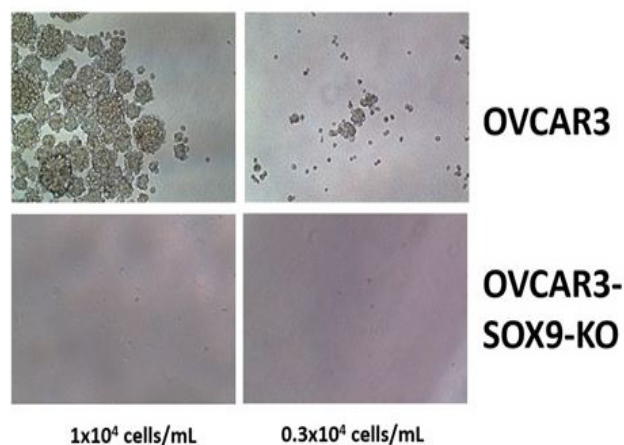


**Figure 4:** SOX 9 did not affect cell proliferation in EOC cells.

A. Cells were cultured in 2D tissue culture dishes and MTT was used to analyze cell proliferation of PE04 and PE04-SOX9-KO cells over 3 days with in the presence of FBS (5%). B and C. Anoikis-resistance in PE04, PE04-SOX9-KO, OVCAR3, and OVCAR3-SOX9-KO cells.

Spheroid formation is a consistent marker for EOC CSC [33]. Spheroids are present in the malignant ascites of essentially all EOC patients and represent a significant impediment to efficacious treatment due to their roles in progression, metastasis, and drug-resistance [34,35]. Spheroids, in general, have high SP, drug-resistance, and CSC activity [36-38]. LPA has been shown recently to be a potent spheroid inducer in EOC cells [39]. We tested whether SOX9 KO affect spheroid formation in EOC cells. As shown in Figure. 5, the spheroid-formation was dependent on the cell density used and under the same conditions, KO of SOX9 essentially diminished spheroid-formation in HGSOC cells.

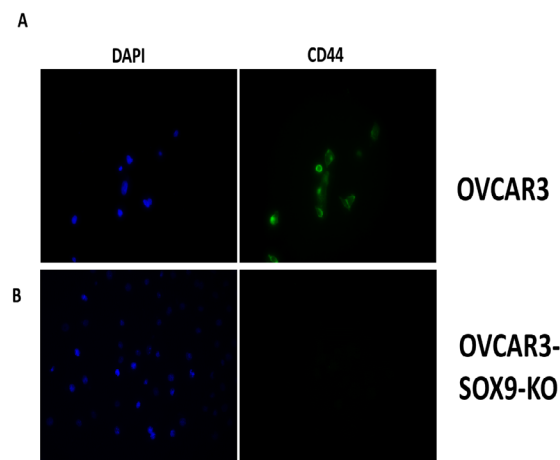
**Figure 5**



**Figure 5:** SOX9-KO blocked spheroid-formation in EOC cells.

Spheroids formed in OVCAR3 and OVCAR3-SOX9-KO cells at different cell concentration as indicated.

**Figure 6**



**Figure 6:** SOX9-KO inhibited CD44 expression spheroid-formation in EOC cells.



Immunostaining of CD44 in OVCAR3 and OVCAR3-SOX9-KO cells with or without LPA (10  $\mu$ M, 24 hrs) treatment.

## Discussion

Compelling evidence has been accumulated in recent years to support the concept that stem cell populations within each individual tumor are key contributors of therapy failure. Thus, it is becoming increasingly important to develop effective CSC targeting strategies. One of the major obstacles in development of therapeutic strategies targeting CSCs is the inherited high diversity and plasticity of CSC cells [40]. Hence, a much better understanding of these features and identification of multiple targets for co-targeting are critical in making progression in this field.

The presented data in this work support this notion. While LPA is a confirmed oncolipid and target in EOC [15- 20], and at least three compounds blocking LPA GPCR receptors have passed phase I and phase II clinical trials for different diseases [25], our study suggests that certain important LPA tumor promoting actions are mediated by PPAR $\gamma$ . Therefore, co-targeting LPA PCR receptors and PPAR $\gamma$  becomes very important in future considerations.

This study provides the first line of evidence that LPA is able to regulate SOX9 and that SOX9 plays crucial roles in CSC-related activities, such as anoikis-resistance, spheroid-formation, and regulation CD44 expression in EOC cells. However, where SOX9 promotes proliferation in gliomas, lung, prostate, endometrial, thyroid and other cancer cells [41- 45], our data suggest that SOX9 is not involved in cell proliferation, at least when analyzed in 2D culture. It is possible that there are cancer type- and/or cell line-dependent distinct effects, since SOX9 has also been shown to have an inhibitory effect on growth/proliferation in certain cells, including breast cancer cells, and melanoma cells [46,47].

Our findings have additional important implications. SOX9 is over-expressed in more aggressive or more drug-resistant EOC cells {comparison of more aggressive ID8-P1 vs. less aggressive ID8-P0; the two HGSOE cell lines derived from the same patient before (PE01) and after (PE04) the onset of drug resistance to cis-platinum, chlorambucil and 5-fluorouracil [23]} in our study. However, SOX9 is not involved in cell proliferation, but rather is involved in certain CSC-related activities. Although un-controlled cell proliferation is one of the most important hallmarks of cancer cells [48], during certain stages of tumor development, and especially when cancer cells undergo stress challenges, such as hypoxia, cell detachment, nutritional starvation, and chemotherapeutic drug treatment, their survival becomes the top priority. For EOC, epithelia cell detachment is a highly pathological relevant stress, since most late stage HGSOE patients develop ascites and large numbers of tumors cells are present in ascites in suspension condition [49]. Long-term survival and low level of proliferation are characteristics of stem cells, making them highly resistant to drug treatment. Cancer cells have many different signaling circuits

as mentioned by Hanahan and Weinberg in their next generation of cancer hallmarks paper [48], and they have the ability to reprogram these circuits in cancer cells under different conditions [40, 50]. Our data suggest that SOX9 is more specifically involved in cell survival and CSC property maintenance programs, which is likely to be a highly interesting target in EOC.

Taken together, we have revealed an innovative LPA-PPAR $\gamma$ -SOX9 signaling pathway and provide strong data to support SOX9's tumor promoting activities in EOC, and in human HGSOE cells in particular. Importantly, SOX9 plays pivotal roles in EOC CSC, which are the critical target for EOC treatment.

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