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CHI3L1 results in poor outcome of ovarian cancer by promoting properties of stem-like cells

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Abstract

The role of chitinase-3-like protein 1 (CHI3L1) in ovarian cancer and the possible mechanisms were elucidated. CHI3L1 is a secreted glycoprotein and associated with inflammation, fibrosis, asthma, extracellular tissue remodeling and solid tumors. Our previous study showed CHI3L1 could be a potential prognostic biomarker for epithelial ovarian cancer and could protect cancer cells from apoptosis. Therefore, clinical data and quantitation of CHI3L1 of ovarian cancer patients, tumor spheroid formation, side-population assays, Aldefluor and apoptotic assays, ELISA, RT-PCR, immunoblotting and animal experiments were performed in two ovarian cancer cells lines, OVCAR3 and CA5171, and their CHI3L1-overexpressing and -knockdown transfectants. High expression of CHI3L1 was associated with poor outcome and chemoresistance in ovarian cancer patients. The mRNA expression of CHI3L1 in CA5171 ovarian cancer stem-like cells was 3-fold higher than in CA5171 parental cells. CHI3L1 promoted the properties of ovarian cancer stem-like cells including generating more and larger tumor spheroids and a higher percentage of ALDH⁺ in tumor cells and promoting resistance to cytotoxic drug-induced apoptosis. CHI3L1 could induce both the Akt (essential) and Erk signaling pathways, and then enhance expression of β -catenin followed by SOX2, and finally promote tumor spheroid formation and other properties of ovarian cancer stem-like cells. OVCAR3 CHI3L1-overexpressing transfectants were more tumorigenic *in vivo*, whereas CA5171 CHI3L1-knockdown transfectants were not tumorigenic *in vivo*. CHI3L1 critically enhances the properties of ovarian cancer stem-like cells. CHI3L1 or CHI3L1-regulated signaling pathways and molecules could be potential therapeutic targets in ovarian cancer.

Key Words

- ▶ ovarian cancer
- ▶ cancer stem-like cells
- ▶ chitinase-3-like protein 1
- ▶ SOX2
- ▶ β -catenin (CTNNB1)

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Introduction

Ovarian carcinoma is the leading cause of death among all gynecologic malignancies, with more than 70% of women presenting with advanced-stage disease (Cohen *et al.* 2014). The annual incidence rate of ovarian cancer in the United States was 12.2 per 100,000 and the death rate was 8.2 per 100,000 in 2007 (U.S. Cancer Statistics

Working Group); these rates were respectively 9.12 and 3.09 in Taiwan (Chiang *et al.* 2013). The poor prognosis for these patients is the result of a lack of symptoms, difficulties in early diagnosis, insufficient accurate tumor markers and deficiency of knowledge about ovarian tumor cell biology (Pinke *et al.* 2008). The standard

treatment for ovarian cancer is surgical tumor debulking, followed by platinum-containing chemotherapy (Agarwal & Kaye 2003, Nagourney *et al.* 2003). Conventional prognostic parameters for ovarian carcinoma are tumor stage, residual tumor volume after surgery, histologic type, differentiation grade and response to chemotherapy (van der Burg *et al.* 1988). However, these factors do not present a comprehensive picture of the tumor biology of ovarian cancer and are frequently interrelated. Thus, identifying the molecular pathways of ovarian cancer is extremely important for diagnosis and treatment of this disease.

Increasing evidence supports the hypothesis that a subpopulation of cancer cells with stem cell features lurks within cancers (Crea *et al.* 2009, Clevers 2011, Visvader 2011, Monchamont *et al.* 2012, Pylväs-Eerola *et al.* 2016). The proportion of these cancer stem-like cells or cancer-initiating cells in tumors is generally less than 2% (Tang *et al.* 2007). Compared to other cells, these cancer stem-like cell populations are more tumorigenic, metastatic, invasive, prone to angiogenic switch and resistance to chemotherapy and radiotherapy (Bapat *et al.* 2005, Wu & Alman 2008, Crea *et al.* 2009, Ahmed *et al.* 2010, Visvader 2011, Monchamont *et al.* 2012, Steg *et al.* 2012). The multipotent cancer stem-like cells with these properties drive histologic heterogeneity within the tumors (Park *et al.* 2010). The high incidence of disease recurrence may also contribute to the multidrug resistance and multiple histological phenotypes of the multipotency of stem cell-like properties (Raghavan *et al.* 2017). Chitinase-3-like protein 1 (CHI3L1 or YKL-40) is a secreted glycoprotein with a molecular weight of about 40 kDa. It is a member of the glycosyl hydrolase 18 family, lacks chitinase activity, and is produced by the various types of cells, including inflammatory cells and cancer cells (Libreros & Iragavarapu-Charyulu 2015, Wiley *et al.* 2015). Expression levels of CHI3L1 in serum or tissues positively correlate with various types of cancer, including ovary, lung, breast and brain (Johansen *et al.* 2004, Park *et al.* 2010, Ku *et al.* 2011, Chiang *et al.* 2015a), and with several inflammatory or autoimmune diseases, including coronary artery disease, diabetes mellitus and rheumatoid arthritis (Kazakova *et al.* 2013, Hansen *et al.* 2015, Wiley *et al.* 2015). CHI3L1 is hypothesized to play a role in cancer cell proliferation, migration, invasion and metastasis. Although CHI3L1 can be a marker associated with a poorer clinical outcome of different tumors, its biologic functions and possible mechanisms of action remain under investigation.

Our previous study identified that ovarian cancerous tissues expressed higher CHI3L1 than ovarian benign and

normal tissues (Chiang *et al.* 2015a). Besides, CHI3L1 was associated with poor prognosis of advanced-stage ovarian cancer patients (Chiang *et al.* 2015a). To explore the possible biological functions of CHI3L1 in this cancer, we hypothesized that CHI3L1 could promote its cancer stem-like cell properties. We first found that RNA levels of CHI3L1 in CA5171 ovarian cancer stem-like cells were 3-fold higher than those in CA5171 parental cells. Moreover, CA5171 spheroid cells are more chemoresistant and prone to tumorigenesis than their parental cells. CHI3L1 promotes properties of ovarian cancer stem-like cells, including generating larger and more numbers of tumor spheroids, higher percentages of ALDH⁺ tumor cells, and resistance to cytotoxic drug-induced apoptosis. CHI3L1 could induce both of the Akt (essential) and Erk signaling pathways, and then enhances expression of β -catenin followed by SOX2, and finally promotes tumor spheroid formation and the various properties of ovarian cancer stem-like cells. *In vivo*, OVCAR3 CHI3L1-overexpressing transfectants were more tumorigenic, whereas CA5171 CHI3L1-knockdown transfectants were less tumorigenic. Our findings suggest that CHI3L1 and its related axis (CHI3L1- β -catenin-SOX2) may provide a promising target for the treatment of ovarian cancer.

Materials and methods

Patients and specimens

From 2000 Jan to 2016 Jun, 113 women with advanced-stage (stages III and IV) ovarian carcinoma treated in our institute were enrolled. The Institutional Review Board of the hospital approved the study protocol and the patients provided informed signed consent after Jun, 2007. Tissue specimens were collected during surgery, immediately frozen in liquid nitrogen and stored at -70°C until analysis. The characteristics of disease were defined according to the system of International Federation of Gynecology and Obstetrics (FIGO; Prat 2014). The optimal debulking surgery was defined as when the maximum diameter of a residual tumor was <1 cm. After completion of the primary treatment, history taking, pelvic/rectal examination and regional lymph nodal palpation would be arranged every 3 months for 3 years, and every 6 months thereafter. Recurrence was recorded when tumor marker levels were greater than or equal to twofold the upper limit of normal in two consecutive tests with 2-week intervals, findings of imaging studies and aspiration cytology were abnormal or when there was histological confirmation from a tissue biopsy. The period from completion of the primary

Table 1 Clinico-pathologic characteristics of the ovarian carcinoma patients ($n = 113$).

Characteristic	Patients ($n = 113$)
Age (<50/>50, year-old)	20/93
Disease status	
FIGO stage (III/IV, cases)	97/16
Histology (serous/clear cell/endometrioid/mix, cases)	85/15/11/2
Tumor grade (1/2/3, cases)	7/2/104
Debulking surgery (optimal/suboptimal, cases)	57/56
Biomarker	
CHI3L1 mRNA expression level (median (range))	7.6 (0.00–668.0)
Outcome	
Disease recurrence (yes/no, cases)	86/27
Disease-related death (yes/no, cases)	53/60

FIGO, International Federation of Gynecology and Obstetrics.

treatment until the diagnosis of disease recurrence was defined as disease-free survival (DFS). The time from the diagnosis of the disease until the date of death or last visit was defined as overall survival (OS).

Cancer cell lines

We used two OVCAR3 and CA5171 ovarian cancer cell lines in our studies. OVCAR3 cells purchased from American Type Culture Collection were maintained in RPMI-1640 medium supplemented with 0.01 mg/mL bovine insulin and 20% FBS and grown at 37°C under 5% CO₂ (Chiang *et al.* 2015b). The molecular authentication of OVCAR3 cell line used in this study was confirmed by STRS analyses as showed in Supplementary Figs 1 and 2 (see section on [supplementary data](#) given at the end of this article). The other ovarian cancer cell line, CA5171, established in our lab, was cultured in RPMI-1640 supplemented with 20% FCS, 50 units/mL penicillin/streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate and 2 mM nonessential amino acids and grown at 37°C under 5% CO₂ (Chiang *et al.* 2015b). The passage numbers of the cells used in the following experiments were from P3 to P5.

Tumor spheroid formation

To generate tumor spheroids, we seeded OVCAR3, OVCAR3/CHI3L1-C4, OVCAR3/CHI3L1-C9, CA5171, CA5171/shCHI3L1-S1 and CA5171/shCHI3L1-S2 cells under stem cell conditions as follows: serum-free minimal essential media (RPMI-1640) supplemented with 1% FBS in 6-well (1×10^6 cells/mL), 24-well (5×10^4 cells/mL), or 96-well (1×10^4 cells/mL) Ultra Low Attachment plates (Corning, Corning, NY, USA) and subsequent organization into tumor spheroids for at least 48 h (Sato *et al.* 2016).

Side-population assays

Single CA5171 cell suspensions were stained with 5 µg/mL Hoechst 33342 dye (Sigma-Aldrich) for 45 min at 37°C. Propidium iodide was used to exclude nonviable cells. A total of 100 µM verapamil (Sigma-Aldrich) was then added to inhibit drug efflux pump proteins. The cells were analyzed using a MoFlo XDP cell sorter (Beckman Coulter; Chiba *et al.* 2006).

Aldefluor assay

ALDH is a marker of stemness phenotypes. Aldefluor reagent (StemCell Technologies, Vancouver, Canada), a fluorescent non-toxic substrate for ALDH, was used to detect ALDH activity in spheroids. Spheroids were suspended in Aldefluor assay buffer containing the ALDH substrate and incubated for 45 min at 37°C. As a negative control, suspended spheroids in the same conditions were treated with diethylaminobenzaldehyde, a specific ALDH enzyme inhibitor. Propidium iodide was added for a 15 min before analysis on a BD FACSCalibur flow cytometer (Nakahata *et al.* 2015).

Apoptotic assays

To evaluate the apoptosis of various tumor cell lines, their transfectants and their spheroids treated with cytotoxic drugs, cells were first incubated with 1 µM of paclitaxel or 300 µM of cisplatin for 48 h. Then cells were stained with FITC-conjugated annexin V and 7-AAD (BD Biosciences, San Jose, CA, USA), following the manufacturer's instructions, and finally analyzed by flow cytometry as described earlier. Cells treated with DMSO were used as the negative control.

Xenograft animal model

Six to 8-week-old female SCID mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan) and bred in the animal facility of the School of Medicine of National Taiwan University. All animal procedures were carried out according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals by the animal ethic committee of College of Medicine, National Taiwan University.

In a limit dilution assay, SCID mice (3 per group) were challenged with 1×10^6 , 1×10^5 or 1×10^4 CA5171 parental and CA5171 cancer stem-like cells (cultured for 21 days) subcutaneously. Before being injected, cells were resuspended in a 100 μ L 1:1 RPMI-1640 and PanaceaGel (Menicon Co., Ltd., Kasugai, JP) mixture. Tumor growth was monitored weekly by inspection and palpation, and the size of each tumor was measured and recorded.

To compare tumor growth of cancer stem-like cells with or without expression of CHI3L1, equal numbers of various cells (1×10^6 , 1×10^5 , or 1×10^4) were cultured to form spheroids under stem cell conditions for 48 h. Total spheroids of each group were then harvested and suspended in the single cell suspension. All cancer stem-like cells were resuspended in 100 μ L 1:1 RPMI-1640 mixed with PanaceaGel (Menicon Co., Ltd.), injected subcutaneously into the right leg of SCID mice. Tumor growth was monitored and recorded twice per week.

Reverse-transcription-PCR (RT-PCR)

Total mRNA of various tissue specimens, ovarian cancer cell lines and their transfectants was first isolated by TRIzol RNA isolation kit according to the manufacturer's instructions. The Moloney murine leukemia virus reverse transcriptase kit was then used to generate all the cDNA template. To generate CHI3L1, the sense primer TGTGAAGGCGTCTCAAACAG and anti-sense primer AATTCGGCCTTCATTTCCTT were used in PCR for 30 cycles, as described previously (Chiang *et al.* 2015a). GAPDH was used as the housekeeping gene, generated with the sense primer ACCCAGAAGACTGTGGATGG and anti-sense primer TGCTGTA GCCAAATTCGTTG and used for 30 cycles at 58°C, as described (Chiang *et al.* 2015a). Analysis of PCR products was performed by electrophoresis (1% agarose gel with ethidium bromide) (Sigma) staining in TBE (a buffer mixture of Tris base, boric acid and EDTA) solution.

Quantitative real-time PCR (QRT-PCR)

The cDNA templates were synthesized as previous described (Chiang *et al.* 2015a). The primer Hs00609691_m1 (TaqMan Assays, Life Technologies Corporation) was used to detect the expression of CHI3L1 by a LightCycler Real-Time detection system (Roche Diagnostics) with 50 cycles of 10s at 95°C, 10s at 60°C and 10s at 72°C. Internal control was performed using the LightCycler h-GAPDH housekeeping gene set (Roche Applied Science) with 50 cycles of 10s at 95°C, 15s at 55°C and 15s at 72°C.

The expression of CHI3L1 was calculated by using the comparative $2^{-\Delta\Delta C_t}$ method as described previously (Livak & Schmittgen 2001). The C_t value was counted by the number of cycles needed for amplification-generated fluorescence to reach a specific threshold of detection. The expression level of CHI3L1 in each sample was used to follow equation: Relative expression level of CHI3L1 = $2^{-\Delta\Delta C_t}$, $\Delta C_t = C_{t_{\text{target (CHI3L1)}}} - C_{t_{\text{housekeeping (GAPDH)}}$.

Enzyme-linked immunosorbent assay

ELISA was performed to evaluate CHI3L1 concentrations in various cancer cell media. Cells (2.5×10^5 cells/mL) were seeded in a six-well culture plate and incubated for 48 h. Culture media were then collected and detected using ELISA kits (R&D Systems).

Immunoblotting

Immunoblotting assays were performed to detect various expression levels of CHI3L1, stemness-related proteins and signaling pathway proteins in OVCAR3, CA5171 and their transfectant cells. To measure alterations in stemness gene expression during spheroid formation, cancer cells (1×10^6 cells/mL) were suspended in six-well Ultra Low Attachment plates (Corning) and incubated for 48 h for spheroid formation. Spheroids were harvested and lysed in protein extraction buffer containing proteinase K inhibitor. The BCA Protein Assay Kit (Thermo Fisher Scientific) was used to quantify protein extracts. Equal amounts of protein were resolved on 10% SDS-PAGE gels, transferred to a PVDF/nylon membrane (EMD Millipore) and probed with Abs specific to α -tubulin (Abcam), β -catenin (Abcam), CHI3L1 (R&D Systems), Nanog (OriGene Technologies, Rockville, MD, USA) and SOX2 and Oct-4 (Cell Signaling). The membrane was then incubated with HRP-conjugated rabbit anti-mouse (Hycult Biotechnology, Uden, Netherlands) and goat anti-mouse (R&D Systems) secondary Abs. The ECL western blotting system (Perkin Elmer) was used to visualize specific bands,

and ImageQuant 5.0 software (Molecular Dynamics Inc., Sunnyvale, CA, USA) was used to measure protein levels through densitometric analysis.

For signaling pathways involved in the spheroid formation, OVCAR3 tumor cells were seeded in six-well Ultra Low Attachment plates under stem cell conditions as described earlier and treated with various concentrations of recombinant human CHI3L1 protein (R&D Systems) for 30 min. To evaluate the phosphorylation of various proteins, Phospho-p44/42 MAPK, Phospho-Jnk, p44/42 MAPK, Akt, JNK (all from Cell Signaling) and phospho-Akt (Santa Cruz Biotechnology) were used as primary Abs, and HRP-conjugated rabbit anti-mouse (Hycult Biotechnology) was used as the secondary Ab.

In the signal transduction pathways blockade assays, OVCAR3 cells cultured in Ultra Low Attachment plates under stem cell conditions were first treated with the respective inhibitor (PD98059, 50 μ M; LY294002, 50 μ M; Sigma-Aldrich) for 2 h, followed by exposure to recombinant human CHI3L1 protein for 30 min. Cells were then extracted and immunoblotted to detect expression of the respective molecules in various signaling pathways, as previously described.

Generation of overexpressing and knockdown CHI3L1 in ovarian cancer cell lines

The lentivirus-packaging plasmids, PLKO_AS3w.puro, Δ 8.91 and pMD.G were purchased from the National RNAi Core Facility (Academia Sinica, Taiwan). Full-length CHI3L1 was first generated by PCR using human placenta cDNA as the template and the following set of primers: sense, Hu-CHI3L1_S_NheI GGCTAGCACCATGGGTGTGAAGGCGTCTCAAAC; and anti-sense, (PstI) Hu-CHI3L1_AS_ PstI TAACTGCAGCTCAGCCTGGGACTCAGCA (PCR conditions). The results were cloned into the NheI/PstI sites of PLKO_AS3w.puro vector, designated as pLKO_AS3w CHI3L1.puro. To generate CHI3L1 lentivirus, 293T cells were co-transfected with three plasmids, pLKO_AS3w-CHI3L1.puro, Δ 8.91 and pMD.G, in a total transfection volume of 6 mL of Opti-MEM medium containing Lipofectamine2000 reagent in 10 cm dishes and incubated at 37°C under 5% CO₂ for 48 h (Chiang *et al.* 2015a).

To generate the CHI3L1-overexpressing OVCAR3 transfectants, OVCAR3 cells were infected by CHI3L1 lentivirus in culture medium with polybrene (10 μ g/mL) and incubated at 37°C under 5% CO₂ for 48 h (Chiang *et al.* 2015a). The PLKO_AS3w.puro was used to generate the blank lentivirus as the negative control. To select

stable CHI3L1-overexpressing OVCAR3 transfectants, puromycin (3 μ g/mL) was added to the culture medium and incubated at 37°C under 5% CO₂ for more than 48 h. The puromycin-resistant CHI3L1-overexpressing OVCAR3 transfectants OVCAR3/CHI3L1-C4 and OVCAR3/CHI3L1-C9 were individually picked, expanded and used in the subsequent experiments.

To generate CHI3L1-knockdown CA5171 transfectants, CHI3L1 shRNA plasmid was purchased from the National RNAi Core Facility (Academia Sinica, Taiwan). The CHI3L1 shRNA lentivirus was generated by co-transfection with shRNA plasmid and Δ 8.91 and pMD.G in Opti-MEM medium containing Lipofectamine2000 and incubated at 37°C under 5% CO₂ for 48 h (Chiang *et al.* 2015a). The PLKO_AS3w.puro was used to generate the blank lentivirus as the negative control. CA5171 cancer cells were infected by CHI3L1 shRNA lentivirus in culture medium with polybrene (10 μ g/mL) and incubated at 37°C under 5% CO₂ for 48 h. For selecting stable CHI3L1-knockdown CA5171 transfectants, puromycin (3 μ g/mL) was added to the culture medium and incubated at 37°C under 5% CO₂ for more than 48 h. The puromycin-resistant CHI3L1-knockdown CA5171 transfectants, CA5171/shCHI3L1-S1 and CA5171/shCHI3L1-S2, were individually picked, expanded and used in the subsequent experiments.

Transfection of siRNA (small interfering RNA)

siRNA for SOX2 and CTNNB1 (β -catenin) was designed by and purchased from OriGene Technologies Inc. (Rockville, MD, USA). The duplex sequence of SOX2 was SR304528C-rGrGrUrUrGrArCrArCrGrUrUrGrGrUrArArUrUrAr rUAA, and the duplex sequence of CTNNB1 (β -catenin) was SR301063A-rGGAUCACAAGAUGGAAUUAUCAA. The negative control was the siRNA duplex carrying a 27-mer scrambled sequence from OriGene's siRNA gene-specific products. OVCAR3 and CHI3L1-overexpressing OVCAR3 transfectants were seeded in 6-well culture plates (Corning) overnight and then transfected with siRNA SOX2 or siRNA CTNNB1 (β -catenin) in a final concentration of 100 nM using Lipofectamine 2000 transfection reagent (Invitrogen) and in a total of 2 mL of serum-free RPMI 1640 medium. After incubation at 37°C, 5% CO₂ for 12 h, 2 mL of RPMI 1640 medium containing 20% FBS was added. All cells were harvested and prepared for the subsequent experiments.

Statistical analysis

SPSS version 15.0 (SPSS Inc) was used for data analysis. Statistical analyses were carried out using Kruskal–Wallis

or one-way ANOVA test. Survival curves were generated using the Kaplan–Meier method, and differences in survival curves were calculated using the log-rank test. A *P* value <0.05 was considered to indicate statistical significance.

Ethics approval and consent

Animal handling and procedures were approved by the animal ethic committee of College of Medicine, National Taiwan University (20160416). The Institutional Review Board of the National Taiwan University Hospital approved the study entitled ‘Screening and identification of novel diagnostic and prognostic biomarkers on ovarian cancers’ (200706002R) to collect the human tissues after acquiring the informed consents.

Results

High mRNA expression level of CHI3L1 correlated with chemoresistance and shorter DFS and OS

The clinicopathological characteristics of these cases are shown in Table 1. The mean follow-up duration was 39.0 months. The serous type was 75% (85/113) and tumor grade 3 was 92% (104/113) in all cases. The percentage of optimal debulking in all case was 50%. The median mRNA expression level of the CHI3L1 was 7.6 (range 0.0–668.0).

Chemoresistant patients had the higher median of CHI3L1 mRNA level (18.5 ± 16.3 vs 4.3 ± 2.5 , Kruskal–Wallis test, *P*=0.001, Fig. 1A) than chemosensitive patients. Besides, high CHI3L1 group (≥ 7.6) had both of shorter DFS (Fig. 1B) and OS (Fig. 1C) than low CHI3L1 group (log-rank test, *P*<0.001).

Based on the clinical and outcome results, we hypothesized that the biologic role of CHI3L1 in ovarian cancer is to promote ovarian cancer stem cell formation.

Characteristics of CA5171 cancer stem-like cells in parental and spheroid cells

Representative figures of CA5175 parental cells and CA5171 spheroids are shown in Fig. 2A. Figure 2B shows representative results for side-population assays by flow cytometry. The percentages of CA5171 parental cells pumping out Hoechst33342 decreased from 2.8 to 0.7% under verapamil treatment. The estimated proportion of stem-like cells of the parental CA5171 line was around 2.1% by side-population assays.

CA5171 parental cells and spheroids expressing aldehyde dehydrogenase (ALDH) were further evaluated. As shown in Fig. 2C, 3.8% of CA5171 parental cells expressed ALDH, whereas 55% of CA5171 spheroid cells did so. These results indicated that percentages of cancer CA5171 stem-like cells increased by changing the culture media from regular medium to stem cell culture medium.

Spheroid cells are more chemoresistant to cytotoxic drugs and tumorigenesis than are their parental cells

The chemoresistant capabilities between CA5171 parental cells and spheroids were also evaluated. The IC₅₀ for paclitaxel in CA5171 parental cells was around 0.11 μM (Fig. 2D) whereas for CA5171 spheroid cells, it was >1 μM. In addition, the IC₅₀ values for cisplatin in CA5171 parental cells and their spheroid cells were 172.3 and 604.2 μM, respectively (Fig. 2D).

Figure 1

Correlation between the mRNA expression levels of CHI3L1 and chemoresistance and outcome of 113 ovarian cancer patients. (A) Correlation between the mRNA expression levels of CHI3L1 and chemoresistance. Chemoresistant patients had higher levels of CHI3L1 than chemosensitive patients (18.5 vs 4.3 , *P* = 0.001, Kruskal–Wallis test). (B) Correlation between mRNA expression levels of CHI3L1 and disease-free survival (DFS) of patients. The high CHI3L1 group had shorter DFS than low CHI3L1 group (*P* < 0.001, Log-rank test). (C) Correlation between mRNA expression levels of CHI3L1 and overall survival (OS) of patients. The high CHI3L1 group had shorter OS than low CHI3L1 group (*P* < 0.001, Log-rank test).

Figure 2

Characteristics of CA5171 cancer stem-like cells. (A) Representative images of CA5171 parental cells and spheroids in low- (40×) and high- (200×) power fields photographed using an inverted phase-contrast microscope. CA5171 parental cells could be cultured to CA5171 spheroids under stem cell conditions. (B) Side-population assays of CA5171 parental cells treated with DMSO or verapamil (100 μM), stained with Hoechst 33342 dye, and then analyzed by flow cytometry. The percentages of CA5171 parental cells pumping out Hoechst33342 decreased from 2.8 to 0.7% under verapamil treatment. (C) ALDH⁺ CA5171 parental cells and spheroids stained in Aldefluor assays. 3.8% of CA5171 parental cells expressed ALDH compared to 55% of CA5171 stem-like cells. (D) Cytotoxicities of CA5171 parental cells and spheroids treated with cytotoxic drugs by MTT assays. CA5171 spheroids had significantly higher IC₅₀ concentrations compared to parental cells under either paclitaxel or cisplatin treatment. (E) *In vivo* tumor growth experiments with CA5171 parental cells and its spheroids. Cell numbers for *in vivo* tumorigenesis were fewer in CA5171 spheroid cells than in CA5171 parental cells. A full color version of this figure is available at <https://doi.org/10.1530/ERC-18-0300>.

We also assessed *in vivo* tumorigenic capabilities. The subcutaneous tumors in CA5171 parental and spheroid cell-bearing mice are shown in Fig. 1E. Injection with 1×10^4 CA5171 parental cells yielded no tumorigenesis in the mice; however, injection with 1×10^4 CA5171 spheroid cells resulted in tumorigenesis in two out of six animals (Fig. 2E). These results indicate that CA5171 spheroid cells are more chemoresistant and prone to *in vivo* tumorigenesis than are CA5171 parental cells.

Exogenous CHI3L1 promotes the properties of ovarian cancer stem-like cells

The roles and biologic functions of CHI3L1 in ovarian cancer stem-like cells were further investigated. Representative images of CHI3L1 mRNA expression levels in CA5171 parental cells and its spheroids are shown in Fig. 3A. The CA5171 spheroids expressed 3-folds of CHI3L1 mRNA higher than CA5171 parental cells (Fig. 3B). Figure 3C shows representative results for spheroids from OVCAR3 parental cells treated with recombinant human CHI3L1 protein. The average spheroid sizes (around 150–250 μm) of OVCAR3 parental cells treated with 1000 ng/mL of CHI3L1 were larger than with 100 ng/mL of CHI3L1 or PBS only (100–150 μm) (Fig. 3C).

In addition, the percentages of ALDH⁺ OVCAR3 cells treated with CHI3L1 increased in a dose-dependent

manner (1.2% in PBS, 3.6% in 100 ng/mL and 4.3% in 1000 ng/mL; Fig. 3D). Protein levels of stemness-related genes including SOX2, NANOG, OCT4 and CTNNB1 (β-catenin) were analyzed by immunoblotting. As shown in Fig. 3E, the protein levels of SOX2 and CTNNB1 (β-catenin) of OVCAR3 parental cells increased in a dose-dependent manner when treated with recombinant CHI3L1. In contrast, protein levels of NANOG or OCT4 did not change under treatment with recombinant CHI3L1 (Fig. 3E).

Apoptotic assays of OVCAR3 cells treated with CHI3L1 were further evaluated. With culture of cells under stem cell conditions with or without CHI3L1, OVCAR3 cells treated with 1000 ng/mL of CHI3L1 had the lowest percentages of apoptotic cells ($17.6 \pm 0.1\%$) compared with those treated with 100 ng/mL of CHI3L1 ($12.6 \pm 0.2\%$) or PBS alone ($8.3 \pm 0.1\%$) ($P < 0.001$, one-way ANOVA) (Fig. 3F).

CHI3L1-overexpressing cancer cells generate the properties of cancer stem-like cells

The mRNA expression levels of CHI3L1 in OVCAR3/CHI3L1 transfectants and CA5171/shCHI3L1 transfectants are shown in Fig. 4A. The concentrations of CHI3L1 protein (ng/mL) in the culture media of OVCAR3/CHI3L1 transfectants (C4 (52.8 ± 3.7) and C9 (59.6 ± 2.4))

Figure 3

CHI3L1 expression promotes cancer stem-like cell properties in OVCAR3 tumor cells. (A) CHI3L1 mRNA expression levels in CA5171 parental cells and spheroids by RT-PCR. CHI3L1 RNA expression was higher in CA5171 spheroids than in CA5171 parental cells. (B) Bar graphs of CHI3L1 mRNA expression levels in CA5171 parental cells and spheroids by RT-PCR. The CA5171 spheroids expressed CHI3L1 3 folds higher than CA5171 parental cells. (C) The representative images of spheroids from OVCAR3 parental cells treated with recombinant human CHI3L1 protein. OVCAR3 parental cells treated with CHI3L1 (1000 ng/mL) generated larger spheroid sizes than treated with PBS or 100 ng/mL of CHI3L1. (D) Percentages of ALDH⁺ OVCAR3 tumor cells treated with different concentrations of CHI3L1, as detected by Aldefluor assays and flow cytometric analysis. OVCAR3 parental cells under stem cell conditions treated with CHI3L1 increased the ratio of ALDH⁺ OVCAR3 cancer cells. (E) Immunoblotting analysis of expression of stemness-related molecules in OVCAR3 tumor cells treated with different concentrations of CHI3L1. Cultured OVCAR3 parental cells under stem cell conditions with CHI3L1 regulated expression of β-catenin and SOX2 with no change in Nanog or OCT4. (F) Percentages of apoptotic cells of OVCAR3 tumor cells treated with different concentrations of CHI3L1, as detected in apoptotic assays and by flow cytometric analysis. CHI3L1 enhanced the viability of OVCAR3 parental cells under stem cell conditions.

were higher than those of OVCA3/mock transfectants (5.5 ± 0.3) ($P < 0.001$, one-way ANOVA) (Fig. 4B). However, the concentrations of CHI3L1 protein (ng/mL) in the culture media of CA5171/shCHI3L1 transfectants (S1 (5.6 ± 0.6) and S2 (2.8 ± 0.5)) were lower than those of CA5171/mock transfectants (25.0 ± 1.6) ($P < 0.001$, one-way ANOVA) (Fig. 4B).

The representative images of spheroid formation in different OVCAR3 transfectants and CA5171 transfectants are shown in Fig. 4C. The OVCAR3/CHI3L1-C4 (103.3 ± 10.6) and C9 (103.5 ± 7.4) transfectants had more tumor spheroids (spheroid size $> 100 \mu\text{m}$ was counted) than OVCAR3/mock (29.0 ± 3.5) transfectants ($P < 0.001$, one-way ANOVA; Fig. 4D). In contrast, the tumor spheroids of CA5171/shCHI3L1-S1 (24 ± 3.5) and S2 (28 ± 6.1) transfectants were fewer than CA5171/mock (71 ± 9.3) transfectants ($P = 0.001$, one-way ANOVA) (Fig. 4D).

Representative images of ALDH⁺ cells in various ovarian parental lines and their CHI3L1 transfectants detected by flow cytometric analyses are shown in Fig. 4E. The OVCAR3/CHI3L1 transfectants (C4 ($16.4 \pm 1.2\%$) and C9 ($18.3 \pm 1.0\%$)) had higher percentages of ALDH⁺ cells

than OVCAR3/mock transfectants ($1.2 \pm 0.1\%$) ($P = 0.017$, one-way ANOVA; Fig. 4F). The CA5171/shCHI3L1 transfectants (S1 ($25.6 \pm 2.5\%$) and S2 cells ($28.1 \pm 2.3\%$)), however, had lower percentages of ALDH⁺ cells than the CA5171/mock transfectants ($51.4 \pm 0.7\%$) ($P = 0.002$, one-way ANOVA; Fig. 3F).

Immunoblotting analysis of various CHI3L1 transfectants for stemness-related genes was performed. The protein expression levels of SOX2 and β-catenin (CTNNB1) were higher in both OVCAR3/CHI3L1 transfectants (C4 and C9) than in OVCAR3/mock transfectants. In contrast, protein levels of SOX2 and β-catenin were lower in CA5171/shCHI3L1 transfectants (S1 and S2) than in CA5171/mock transfectants (Fig. 4G).

CHI3L1 stimulates both of Erk and Akt phosphorylation during tumor spheroid formation

We then further evaluated the signaling pathways involved in CHI3L1-related tumor spheroid formation. The representative images of immunoblotting results are

Figure 4

Expression of CHI3L1 in ovarian cancer cells altered the quantity of spheroids and stemness properties and stimulated both phospho-Akt and phospho-Erk pathways under stem cell conditions. (A) CHI3L1 RNA expression levels of OVCAR3 parental cells and their CHI3L1 transfectants and of CA5171 parental cells and their shCHI3L1 transfectants, as detected by RT-PCR. (B) CHI3L1 protein concentrations in culture media of OVCAR3 parental cells and their CHI3L1 transfectants and of CA5171 parental cells and their shCHI3L1 transfectants as detected by ELISA. CHI3L1 mRNA and protein expression levels were both high in OVCAR3 CHI3L1 transfectants, whereas they were low in CA5171 shCHI3L1 transfectants, as detected by RT-PCR and ELISA. (C) Representative images of spheroid formation of OVCAR3 parental cells and their CHI3L1 transfectants, and CA5171 parental cells and their shCHI3L1 transfectants. (D) Bar graphs of the numbers of spheroids in OVCAR3 parental cells and their CHI3L1 transfectants and of CA5171 parental cells and their shCHI3L1 transfectants. Numbers and sizes of spheroids of OVCAR3 CHI3L1 transfectants under stem cell conditions were both increased compared with controls (mock), but those of CA5171 shCHI3L1 transfectants were both decreased compared with controls. (E) Representative images of the percentages of ALDH⁺ OVCAR3 parental cells and their CHI3L1 transfectants and of CA5171 parental cells and their shCHI3L1 transfectants, measured by flow cytometry. (F) Bar graphs of the percentages of ALDH⁺ OVCAR3 parental cells and their CHI3L1 transfectants and of CA5171 parental cells and their shCHI3L1 transfectants (right) by Aldefluor assays. The ratio of ALDH⁺ cells in spheroids of OVCAR3 CHI3L1 transfectants was increased compared with controls (mock), whereas that of ALDH⁺ cells in spheroids of CA5171 shCHI3L1 transfectants was decreased. (G) Expression levels of various stemness-related genes in OVCAR3 parental cells and their CHI3L1 transfectants and of CA5171 parental cells and their shCHI3L1 transfectants, by immunoblotting analysis. Expression of β -catenin and SOX2 was increased in spheroids of OVCAR3 CHI3L1 transfectants under stem cell conditions but decreased in spheroids of CA5171 shCHI3L1 transfectants, and there was no change in Nanog or OCT4. (H) Phosphorylation of various signaling molecules of OVCAR3 tumor cells treated with different concentrations of CHI3L1 detected by immunoblotting. CHI3L1 stimulated phosphorylation of Akt and Erk in spheroids of OVCAR3 parental cells under stem cell conditions. (I) Phosphorylation of various signaling molecules of OVCAR3 parental cells and their CHI3L1 transfectants and of CA5171 parental cells and their shCHI3L1 transfectants, as detected by immunoblotting. Under stem cell conditions, phosphorylation of Akt and Erk increased in spheroids of OVCAR3 CHI3L1 transfectants and decreased in spheroids of CA5171 shCHI3L1 transfectants.

shown in Fig. 4H. The phosphorylation of Erk1/2 and Akt increased in cells treated with recombinant CHI3L1 under stem cell conditions (Fig. 4H). However, the phosphorylation of JNK or cleavage of Notch1 did not change even under CHI3L1 treatment.

The CHI3L1-overexpressing OVCAR3 transfectants (OVCAR3/CHI3L1-C4 and -C9) also revealed increasing phosphorylation of Erk1/2 and Akt compared with the OVCAR3/mock transfectants (Fig. 4I). CHI3L1-downregulated CA5171 transfectants

(CA5171/shCHI3L1-S1 and -S2), on the other hand, had less Erk1/2 or Akt phosphorylation than the CA5171/mock transfectants (Fig. 4I).

CHI3L1 could enhance tumor spheroid formation and characteristics of cancer stem-like cells through the Erk and Akt signaling pathways

To confirm the essential signaling pathway of CHI3L1 in tumor spheroid formation, specific pathway inhibitors such as LY294002 for Akt or PD98059 for Erk were further tested. As shown in Fig. 5A, Erk phosphorylation decreased when cells were cultured with recombinant human CHI3L1 and PD98059 (lane 3) or PD98059 alone (lane 5). In addition, Akt phosphorylation also decreased with cells cultured in recombinant human CHI3L1 and LY294002 (lane 4) or LY294002 alone (lane 6).

We then evaluated if a specific inhibitor of the Akt or Erk signaling pathway could inhibit the CHI3L1-regulated spheroid formation and the generation of stem-like cells. The representative figures of spheroid formation of various OVCAR3 and CA5171 cells and their respective CHI3L1 transfectants are shown in Fig. 5B and C. The spheroid sizes and numbers of OVCAR3 parental cells and OVCAR3/CHI3L1 transfectants decreased under treatment with PD98059 or LY294002 (Fig. 5B). The sizes and numbers of CA5171 parental cells and CA5171/shCHI3L1 transfectants also decreased under these treatments (Fig. 5C).

We then further investigated the percentages of ALDH⁺ cells. As shown in Fig. 5D, the percentages of ALDH⁺ OVCAR3 cells cultured with recombinant human CHI3L1 significantly decreased when pretreated with PD98059 ($4.2 \pm 0.1\%$, $P < 0.001$) or LY294002 ($2.9 \pm 0.1\%$, $P < 0.001$) compared with the control group ($6.4 \pm 0.1\%$) (one-way ANOVA). The percentages of ALDH⁺ cells in OVCAR3 CHI3L1 transfectants were also lower when cells were pretreated with LY294002 (C4: $2.7 \pm 0.3\%$; C9: $2.6 \pm 0.3\%$) or PD98059 (C4: $8.0 \pm 0.8\%$; C9: $8.6 \pm 1.0\%$) compared with controls (C4: $15.7 \pm 0.5\%$, $P < 0.001$; and C9: $16.5 \pm 0.5\%$, $P < 0.001$) (one-way ANOVA) (Fig. 5E). In addition, the OVCAR3/CHI3L1 transfectants treated with LY294002 had lower percentages of ALDH⁺ cells than those treated with PD98059 ($P < 0.001$, one-way ANOVA).

We further evaluated if the inhibition of CHI3L1-regulated signaling pathways could enhance sensitivity to cytotoxic drugs. As shown in Fig. 5F, OVCAR3 parental cells cultured with recombinant human CHI3L1 ($138.4 \pm 3.7\%$) under stem cell conditions had higher percentages of viable cells than those treated with PBS

($100.0 \pm 4.0\%$) when subsequently treated with paclitaxel. However, the percentages of OVCAR3 parental cells treated with PD98059 ($50.3 \pm 0.6\%$) or LY294002 ($8.7 \pm 0.1\%$) were lower than the OVCAR3 parental cells treated with PBS, when subsequently cultured with paclitaxel (one-way ANOVA, $P < 0.001$) (Fig. 5G). The OVCAR3/CHI3L1 transfectants pretreated with LY294002 (C4: $12.5 \pm 0.7\%$; C9: $11.3 \pm 0.7\%$) also had significantly lower percentages of viable cells than those pretreated with PD98059 (C4: $117.9 \pm 4.3\%$; C9: $107.9 \pm 8.7\%$) ($P < 0.001$, one-way ANOVA) when cultured with paclitaxel.

CHI3L1 regulates expression of β -catenin and SOX2 stemness-related molecules through the Akt and Erk signaling pathways

We further evaluated which stemness-related molecules were regulated by CHI3L1 during tumor spheroid formation. As shown in Fig. 5H, the OVCAR3/CHI3L1 transfectants (C4 and C9) expressed higher levels of β -catenin and SOX2 molecules than OVCAR3 parental cells. The expression levels of Nanog or OCT4 did not differ between OVCAR3 parental cells and OVCAR3/CHI3L1 transfectants (Fig. 5H). PD98059 could only inhibit minor expression of SOX2, not β -catenin, in OVCAR3 parental cells and OVCAR3/CHI3L1 transfectants. LY294002, however, suppressed the expression of both β -catenin and SOX2 in OVCAR3 parental cells and OVCAR3/CHI3L1 transfectants. Our results revealed that CHI3L1 could regulate expression of β -catenin and SOX2 during spheroid formation, mainly through the Akt, not Erk, signaling pathway.

β -catenin was upstream of SOX2 among the CHI3L1-regulated tumor spheroid formation and cancer stem-like characteristics

We further assessed if CHI3L1 could regulate the formation and characteristics of cancer stem-like cells through β -catenin and SOX2. The β -catenin siRNA or SOX2 siRNA inhibited expression of their respective molecule in OVCAR3/CHI3L1 transfectants. Inhibition of β -catenin by β -catenin siRNA also suppressed the expression of SOX2 (Fig. 6A), but the inhibition of SOX2 by SOX2 siRNA did not influence the expression of β -catenin. As shown in Fig. 6B, the sizes and numbers of tumor spheroids of OVCAR3/CHI3L1 transfectants significantly decreased under treatment with β -catenin siRNA compared with those treated with scramble siRNA.

Figure 6

Knockdown of SOX2 or β -catenin impaired the formation of spheroids stimulated by CHI3L1. (A) The expression levels of SOX2 and β -catenin in OVCAR3 CHI3L1 transfectants after transfection with siRNA SOX2 or siRNA β -catenin, as detected by immunoblotting. Protein suppression of β -catenin by siRNA in spheroids of OVCAR3 CHI3L1 transfectants under stem cell conditions alone prevented SOX2 protein expression, but protein suppression of SOX2 by siRNA did not affect the protein expression of β -catenin. (B) OVCAR3 parental cells and their CHI3L1 transfectants after transfection with siRNA SOX2 or siRNA β -catenin for 48 h were harvested and cultured to spheroids under stem cell conditions and photographed (40 $\times$) after 48 h. The formation of spheroids of OVCAR3 CHI3L1 transfectants decreased after the suppression of SOX2 or β -catenin protein expression by siRNA. (C) Representative images of the percentages of ALDH⁺ OVCAR3 parental cells and their CHI3L1 transfectants after transfection with siRNA SOX2 or siRNA β -catenin and culture to spheroids, measured by flow cytometry. (D) Bar graphs of the percentages of ALDH⁺ OVCAR3 parental cells and their CHI3L1 transfectants after transfection with siRNA SOX2 or siRNA β -catenin and culture to spheroids, measured by Aldefluor assays after 48 h. The ratio of ALDH⁺ cells in spheroids of OVCAR3 CHI3L1 transfectants under stem cell conditions was also decreased after the suppression of SOX2 or β -catenin protein expression by siRNA. (E) Percentages of apoptotic cells of OVCAR3 CHI3L1 transfectants transfected with siRNA SOX2 or siRNA β -catenin and cultured to spheroids with paclitaxel, as measured by apoptotic assays and flow cytometric analysis after 48 hours. siRNA SOX2 or siRNA β -catenin decreased the viability of OVCAR3/CHI3L1 transfectants under stem cell conditions and paclitaxel treatment. (F) *In vivo* limiting dilution assays for spheroids of OVCAR3 CHI3L1 transfectants. (G) *In vivo* limiting dilution assays for spheroids of CA5171 shCHI3L1 transfectants. The tumorigenicity of spheroids of OVCAR3 CHI3L1 transfectants increased faster than in OVCAR3 parental cells, as did the tumorigenicity of spheroids of CA5171 parental cells compared to CA5171 shCHI3L1 transfectants.

siRNA ($P>0.05$, one-way ANOVA). The β -catenin siRNA (C4: $71.8\pm2.9\%$; C9: $71.1\pm0.9\%$) and SOX2 siRNA (C4: $72.3\pm3.3\%$, C9: $74.8\pm3.4\%$) also decreased the percentages of viable OVCAR3/CHI3L1 transfected cells treated with paclitaxel compared to scramble siRNA (C4: $94.7\pm1.3\%$, $P=0.002$; C9: $92.5\pm3.3\%$, $P=0.002$, one-way ANOVA) (Fig. 6E).

CHI3L1 regulates the formation of cancer stem-like cells to enhance the tumorigenesis of ovarian cancer *in vivo*

We next investigated if OVCAR3/CHI3L1 transfectants could be more tumorigenic than OVCAR3 parental cells *in vivo*. As shown in Fig. 6F, when injected with 1×10^6 of the respective tumor cells, some of the mice

challenged with the OVCAR3/CHI3L1 transfectants (4/8 for C4 and 3/8 for C9) showed tumorigenesis. However, none of the mice challenged with OVCAR3 parental cells did (Fig. 6F). With the injection of 1×10^6 or 1×10^5 CA5171/mock cells, four of eight and one of eight mice respectively showed tumorigenesis (Fig. 6G). As also shown in Fig. 6G, none of the mice showed tumorigenesis following injection with 1×10^6 CA5171/CHI3L1-S1 or CA5171/CHI3L1-S2 transfectants (CA5171/CHI3L1-knockdown transfectants).

Taken together, these results indicate that CHI3L1 can promote *in vivo* tumorigenesis by enhancing the quantity of cancer stem-like cells.

Discussion

We investigated the biological functions of CHI3L1 and if it could be as a potential target in ovarian cancer. Chiang *et al.* reported that high CHI3L1 correlated with the poor outcome of ovarian cancer (Chiang *et al.* 2015a). However, the mechanism of CHI3L1 in cancer still needs to be identified. We first identified that ovarian cancer cells could generate spheroids during low-serum culture media. These spheroid cells are more chemoresistant and tumorigenic than their parental cells. Besides, cancer stem-like cells expressed higher CHI3L1 levels than parental cells. Both exogenous and endogenous CHI3L1 could promote the generation of cancer spheroids and properties of ovarian cancer stem-like cells including the expression of ALDH, stemness-related molecules SOX2 and β -catenin and drug-resistance. CHI3L1 could activate both the Akt (essential) and Erk signaling pathways during the generation of tumor spheroids and cancer stem-like cell properties. The blockade of CHI3L1 and molecules in its related signaling pathway could inhibit tumorigenesis *in vivo*.

Cancer stem cells play important roles in tumor growth and metastasis (Chabner & Murphy 2005, Horst *et al.* 2009, Wakamatsu *et al.* 2012), and metastasis is an intractable problem in cancer therapy. One hypothesis of cancer metastasis is that cancer stem-like cells separate first from the local tumor site and enter into the circulation as seeds that then land at various distant sites (Bapat *et al.* 2005, Clevers 2011). However, cancer cells cannot survive for long periods in single cell suspension (Mehlen & Puisieux 2006, Buchheit *et al.* 2014), whereas tumor spheroids have proved to be one form in which cancer cells survive longer and circulate within the blood vessels. To form a tumor spheroid, tumor cells need to become cancer stem-like cells (Weiswald *et al.* 2015), so the

quantity of spheroids, including number and size, reflects the numbers of cancer stem-like cells. CHI3L1 promoted the formation of tumor spheroids in our study (Figs 2B and 3C), and earlier results have shown that ovarian cancer patients with the advanced-stage disease have higher CHI3L1 than those with the early stage disease (Weiswald *et al.* 2015). We hypothesize that ovarian cancer tumors highly expressing CHI3L1 could generate more tumor spheroids, resulting in disease progression and tumor metastasis.

Cancer stem cells also could underlie chemoresistance to cytotoxic drugs in the treatment of ovarian carcinoma (Weiswald *et al.* 2015). Chemotherapy is always used in metastatic disease, but chemoresistance has been a big challenge, and breaking through it has become a primary focus. Cancer stem-like cells are among the possible explanations for the generation of chemoresistance (Weiswald *et al.* 2015). Somasagara *et al.* reported that chemoresistance could be induced by regulating RAD6 gene, which was also associated with the stem cell signaling. RAD6 could activate TLS (PCNA-Ub) and FA/homologous recombination (FANCD2-Ub) to protect ovarian cancer cells from DNA damage caused by chemotherapy (Somasagara *et al.* 2017).

Patients with chemoresistance are reported to have higher CHI3L1 than those whose cancers are chemosensitive (e.g., in ovarian (Chiang *et al.* 2015a), glioblastoma (Ku *et al.* 2011, Weiswald *et al.* 2015) and lung cancer (Johansen *et al.* 2004)). CHI3L1 increased chemoresistance in ovarian cancer cells in our previous study and generated chemoresistance by boosting numbers of cancer stem-like cells in the current work (Fig. 5F and G; Chiang *et al.* 2015a). Thus, CHI3L1 could be a potential target for reversing chemoresistance by reducing the numbers of cancer stem-like cells.

The major challenges of current ovarian cancer treatments are the lack of good prognostic factors, initiation of tumor cells, chemoresistance and high recurrence rate. Cancer stem-like cell correlate with chemoresistance and recurrence (Crea *et al.* 2009, Ahmed *et al.* 2010, Steg *et al.* 2012, Jung *et al.* 2016, Pylväs-Eerola *et al.* 2016) and CHI3L1 is highly expressed in several human malignancies, including glioblastoma, lung cancer, hepatoma, breast and ovarian cancers (Johansen *et al.* 2004, Chiba *et al.* 2006, Park *et al.* 2010, Ku *et al.* 2011, Chiang *et al.* 2015a). Targeting cancer stem cells is an important and novel strategy for cancer therapy. These cells can be tracked through expression of specific surface markers like CD44, CD133 and ALDH (Monzani *et al.* 2007, Horst *et al.* 2009, Wakamatsu *et al.* 2012)

Figure 7

Schematic of a model of CHI3L1 promotion of tumor spheroid formation and the characteristics of cancer stem-like cells. When ovarian tumors initiate and grow, chronic inflammation arises in the tumor microenvironment. Cancer cells and/or inflammatory cells secrete abundant CHI3L1 in ascites or extracellular matrix. CHI3L1 regulates cancer cells to form tumor spheroids by enhancing expression of stemness-related genes through activation of Akt and Erk signaling pathways. These tumor spheroids then increase chemoresistance and survival of tumor cells, finally migrating to new sites to generate metastasis. A full color version of this figure is available at <https://doi.org/10.1530/ERC-18-0300>.

through stemness genes such as Nanog, OCT-4 and SOX2 (Kitamura *et al.* 2013, Almozyan *et al.* 2017, Lee *et al.* 2017) or by the unique pattern of staining with certain dyes such as Hoechst 33342 (Goodell *et al.* 1996). SOX2 and β -catenin are two intermediate molecules to target in cancer stem-like cells by blocking the function of CHI3L1. Targeting the downstream molecules of CHI3L1 can be another strategy for inhibiting the formation of cancer stem-like cells. SOX2 and β -catenin were intermediate molecules in CHI3L1-regulated cancer stem-like cell formation in our study (Fig. 6B). Our results showed that blocking phosphor-Akt could suppress the expression level of SOX2 or β -catenin and impair spheroid formation in single cancer cell suspension. The major functions of SOX2 and β -catenin in generating spheroids, however, still need further study. The downstream pathway of SOX2 or β -catenin may offer more specific and novel targets for ovarian cancer therapy, as has already been discerned in other cancers (Garros-Regulez *et al.* 2016, Hwang *et al.* 2016). Recently, the CHI3L1 neutralization antibody, mAY, was shown to confer anti-angiogenic properties and promote apoptosis by inhibiting CHI3L1 (Faibish *et al.* 2011). He and colleagues reported that IL13Ra is the receptor for CHI3L1, offering IL13Ra another potential target for inhibiting CHI3L1 function (Faibish *et al.* 2011). Small molecule-based inhibition of the Akt or Erk pathway may be another strategy for impairing the function of CHI3L1. Further studies are needed to investigate these possibilities.

Figure 7 illustrates a hypothesized pathway based on our findings in this and our previous study. When ovarian cancer cells initiate and grow, chronic inflammation arises in the tumor microenvironment. Cancer cells and/or inflammatory

cells secrete abundant CHI3L1 in ascites or extracellular matrix. CHI3L1 then regulates cancer cells to form tumor spheroids by the expression of stemness-related β -catenin and then SOX-2 through activation of the Akt (essential) and Erk signaling pathways. These tumor spheroids then increase chemoresistance and the survival of tumor cells, finally migrating to other sites to establish metastases.

In conclusion, cancer stem-like cells are associated with tumor metastasis, resistance to chemotherapy and radiotherapy and tumor recurrence in many studies (Chabner & Murphy 2005, Chiba *et al.* 2006, Crea *et al.* 2009, Monchamont *et al.* 2012, Raghavan *et al.* 2017). Targeting these cells is thus an important and novel strategy to decrease the possibility of tumor metastasis, chemo- or radioresistance and recurrence and to extend patient survival. Our findings suggest that CHI3L1 and its related axis (CHI3L1- β -catenin-SOX2) may provide a promising target for the treatment of ovarian cancer.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/ERC-18-0300>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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