Stemness in high-grade serous ovarian cancer is a negative prognostic indicator but potentially targetable by EGFR/mTOR-PI3K/aurora kinase inhibitors

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Abstract

Poor chemotherapy response remains a major treatment challenge for high-grade serous ovarian cancer (HGS-OvCa). Cancer stem cells are the major contributors to relapse and treatment failure as they can survive conventional therapy.

Our objectives were to characterise stemness features in primary HGS-OvCa cell lines, correlate stemness markers with clinical outcome and test the response of primary HGS-OvCa cells with stemness features to current and exploratory drugs.

Tissue and ascites samples, treatment-naïve and/or after neoadjuvant chemotherapy, were prospectively collected. Primary cancer cells, cultured under conditions favouring either adherent or spheroid growth, were tested for stemness markers, and the same markers were analysed in tissues for correlation with chemotherapy and survival. Drug sensitivity and resistance testing (DSRT) was performed with 306 oncology compounds.

HGS-OvCa cells in spheroid conditions showed increased expression of stemness markers, including ALDH1A1, as compared to adherent cells, and increased resistance to platinum and taxane. Clustering of treatment-naïve tumours with eight stemness markers identified a distinct subgroup of cancers with enriched stemness features. Expression of ALDH1A1, but not most other stemness markers, was increased after neoadjuvant chemotherapy and its expression in treatment-naïve tumours correlated with chemoresistance and reduced survival. In DSRT, five compounds, including two PI3K-mTOR inhibitors, demonstrated significant activity in both cell culture conditions. Thirteen compounds including EGFR, PI3K-mTOR and aurora kinase inhibitors were more toxic to spheroid cells than adherent cells.

Our results identify stemness markers in HGS-OvCa that associate with decreased response to conventional chemotherapy and reduced survival if expressed at treatment-naïve state. EGFR, mTOR-PI3K and aurora kinase inhibitors are identified as candidates for targeting this cell

population.

Introduction

High grade serous ovarian carcinoma (HGS-OvCa) is an aggressive gynaecological cancer with a 30% survival rate at five years. While most tumours initially respond to treatment, relapse followed by chemoresistance is common [1,2]. The standard treatment for advanced HGS-OvCa is surgery combined with platinum-taxane chemotherapy.

One reason for relapse and treatment failure in ovarian cancer is most likely the non-responsiveness of a subpopulation of cancer cells with stem cell properties [3]. Stem cells represent an important target for novel therapeutic strategies aimed at eradicating ovarian cancer [4]. They are characterised by self-renewal capacity, resistance to apoptosis, and their ability to differentiate into a variety of cells and generate daughter cells [5]. Similarly, cancer cells with stem cell features can be defined by these functional traits [6]. Cancer stem cells were originally characterized in acute myeloid leukemia [7] and later in many different cancers including breast cancer [8], brain cancer [9], colon cancer [5,10] and ovarian cancer [11]. Several stemness marker combinations for HGS-OvCa cancer stem cells have been suggested but a definite marker set remains to be defined [12]. One of the best described stemness marker is aldehyde dehydrogenase isoform I (ALDH1A1), an enzyme that has been used to define cancer stem cells in many cancer types [13], specifically in ovarian cancer [14,15]. In ovarian cancer ALDH1A1 is correlated with stemness properties and poor prognosis [15-17]. The surface marker PROM1 (Prominin-1, CD133) has been associated with ovarian cancer but has more recently been considered controversial [18]. Transcription factors such as OCT4, SOX2, LIN28A, NANOG and c-MYC have been associated with reprogramming human somatic cells to pluripotent stem cells [19] and cancer cells with stemness properties. In ovarian cancer these transcription markers have been associated with poor prognosis [20-22], but only SOX2 has been associated with an ovarian cancer stem cell phenotype [23]. BMI1 overexpression displayed a significant association

with ovarian cancer patients´ low overall survival [24]. Moreover, BMI1 has been linked to cancer cell survival and senescence as well as self-renewing cell divisions of somatic stem cells and it has been found to be overexpressed in, for example, Ewing sarcoma family tumours [25,26] While the markers described above have been associated with many cancers, including ovarian cancer, they have typically been studied as individual markers and not as a marker panel.

Currently, little is known about the drug responses of primary HGS-OvCa cells with stemness features. Drug sensitivity testing has typically been carried out using cells with unknown origin or sometimes lacking genomic features of HGS-OvCa [27-29] and using adherent cell culture conditions, which do not support stemness features [29]. Drug sensitivity testing with conditions favouring stemness features is technically challenging and only few studies have been reported so far [27,30,31].

The aims of this work were to (1) identify markers in primary HGS-OvCa cells that are associated with stemness phenotype, (2) test whether the identified stemness markers are enriched in tumours after treatment with platinum-taxane chemotherapy and (3) whether the same markers are prognostic in treatment naïve HGS-OvCa tumours, and (4) carry out large scale oncology drug testing to study, whether HGS-OvCa cells cultured in conventional adherent methods or stemness promoting conditions display differences in drug response profiles.

Materials and Methods

Patient-derived materials

Tissue and ascites specimens were collected from consented patients at the Department of Obstetrics and Gynecology, Turku University Central Hospital as part of a prospective ovarian cancer study (MUPET/HERCULES study) (ClinicalTrials.gov Id: NCT01276574). The study protocol and use of

all material have been approved by 1) The Ethics Committee of the Hospital District of Southwest Finland (ETMK): ETMK 53/180/2009 § 238 and ETMK 69/180/2010, and 2) Finnish National Supervisory Authority for Welfare and Health in Finland (Valvira): DNRO 6550/05.01.00.06/2010 and STH507A.

Tumour and ascites samples and longitudinal clinical information were collected from 41 patients with stage III or IV HGS-OvCa (Figure S1 and Table S2). Treatment naïve samples were collected during primary debulking surgery or diagnostic laparoscopy. Patients considered primarily inoperable received three cycles of neoadjuvant chemotherapy (NACT) and samples were obtained during interval debulking surgery. Altogether 39 naïve samples and 19 chemotherapy-treated interval samples, including ten paired samples, were available. Primary cell cultures were established from 25 patients of which 19 treatment naïve and seven chemotherapy-treated interval cell lines were established. Three pairs of cell lines were established from the same patients (altogether 6 cell lines). For RNA sequencing, cancer tissue was collected at treatment naïve stage and at interval surgery after 3-4 NACT cycles. Paired material was available from 21 stage III or IV HGS-OvCa patients.

Longitudinal clinical information was collected as described in detail in [32]. The information included stage, detailed recording of tumour dissemination during surgery, treatments and survival information.

To study the co-expression of stemness markers and their association with survival in FIGO stage III-IV HGS-OvCa patients, we used two existing datasets: (1) the microarray expression data of 144 treatment naïve primary tumour samples from patients with clinical data available [33] and (2) the RNA sequencing data of 66 primary HGS-OvCa patients [34]. To make the two datasets comparable, we used eight stemness markers that were included in both platforms.

Cell lines and primary cell culture

Primary cell cultures were established from ascites and tumour tissues. Ascites was centrifuged at 3.0 G for 15 min, followed by gradient centrifugation with Histopaque-1077 to enrich the cancer cell component (Sigma-Aldrich, St. Louis, MO, USA) (Cell lines M019i, M022i and M068i). Cells from primary tumours were isolated by plating approximately 1 mm^3 pieces on 6-well plates. Detached cells were collected weekly within a 4-week period and cultured as adherent or spheroids (see below). Attached cells were also collected when the wells were grown to confluency. The contaminating stromal and immune cells were grown out by passaging the cells for approximately 5 times. During passaging the contaminating cells were either discarded or died. A smear of cells was stained with toluidine blue staining to morphologically confirm the uniformity of the cell population. Cytological samples were also stained with Pax-8 and WT-1 antibodies. The uniform cell cultures were called cell lines.

Conventionally used HGS-OvCa cell lines were Caov-3, (American Type Culture Collection (ATCC) http://www.lgcstandards-atcc.org), Caov-4 (ATCC), OVCAR-4 (National Cancer Institute, NCI, USA), TYK-nu (Health Science Research Resources Bank, (JCRB) Japan), TYK-nuCP-r (JCRB), NIHOVCAR3 (ATCC) and OVCAR-8 (NCI).

The primary and conventional cell lines were investigated in two culture conditions; adherent (2D) and spheroidal (non-adherent, 3D) in regular cell culture flasks. Adherent cells were cultured in DMEM (Euroclone, Milano, Italy) or RPMI medium containing 5-10 % FBS (Lonza, Basel, Switzerland), 100µg/ml penicillin-streptomycin (Gibco Life Technologies, NY, USA) and 2mM Ultraglutamine (Lonza). Spheroidal cell culture medium DMEM-F12 (Lonza) was supplemented with 20 ng/ml EGF (Gibco Life Technologies), 10 ng/ml bFGF (Gibco Life Technologies) and 1x B27 (Gibco Life Technologies). CCD-18Co myofibroblasts (ATCC) were used as feeder cells in the analysis of flow cytometry sorted ALDH positive and negative cells. Cell morphology was analysed

with Primo Vert light microscope and Zen lite software (Carl Zeiss Microscopy Ltd, Goettingen, Germany). Immunofluorescence was detected with LSM780 laser scanning confocal microscope (Carl Zeiss Microscopy Ltd).

Statistical analysis

Differences between the study groups in qRT-PCR and ADLH flow cytometric data were analysed using two-sided t-test. Kaplan-Meier with log-rank test was used for survival analyses. Immunohistochemical as well as RNA sequencing data were analysed with Spearman's correlation analyses. DSRT data was analysed using a web-based pipeline BREEZE (breeze.fimm.fi).

Materials and methods for ATP cell proliferation assay, Quantitative real-time PCR, Aldehyde dehydrogenase activity and ALDH1A1 analysis, RNA sequencing and expression analysis for HGS-OvCa tumours and Drug sensitivity and resistance testing (DSRT) can be found in Supplementary materials and methods.

Results

Cultured primary spheroid HGS-OvCa cells express stemness markers and are more resistant to conventional chemotherapy than adherent cells

We analysed ten selected prominent stem cell markers in primary HGS-OvCa cells grown under spheroid or adherent conditions (Figure 1A). Out of the ten markers, five *(ALDH1A1*, p=0.042; *CIP2A*, p=0.005; *OCT4A*, p=0.021; *SOX2*, p=0.019; *BMI1*, p=0.003) were differentially expressed in the spheroid cells as compared with cells grown in adherent conditions (Figure 1B).

We next compared the response of spheroidal and adherent HGS-OvCa cells to HGS-OvCa standard-of-care chemotherapy agents, platinum and taxane derivatives. Two primary cell lines originating from the same patient, M022 (sample from primary surgery) and M022i (i=sample from an interval

surgery), and three conventional lines (CAOV4, OVCAR4 and OVCAR8) were tested. In all cell lines, cells grown under spheroid conditions were clearly more resistant to both cisplatin (p=0.0141) and paclitaxel (p=0.0188) than adherently grown cells. The results are shown in Figures 1C and D.

Altogether, these results demonstrate that primary HGS-OvCa cells, and especially cells cultured in spheroid conditions, serve as a relevant model to study stemness features in HGS-OvCa. Further, out of ten stemness markers reported in various cancers, five (ALDH1A1, CIP2A, OCT4A, SOX2 and BMI1) were relevant to the HGS-OvCa model.

Stemness markers in treatment naïve HGS-OvCa tumours define a tumour subset with potential survival difference

The ability of the ten stemness markers to classify tumour subsets and predict survival was first studied in the microarray expression dataset consisting of 144 treatment-naïve samples from stage III-IV HGS-OvCa patients. The mRNA expression of eight markers (*ALDH1A1*, *CIP2A*, *c-MYC*, *LIN28A*, *NANOG*, *SOX2*, *PROM1* and *BMI1*) stratified patient samples into two clusters. Of these, cluster 2 (enriched stemness cluster, 32% of all cases) showed significantly higher stemness marker expression than cluster 1 (baseline cluster, 68% of all cases) (Figure 2A). Analysis of each of these markers showed significant enrichment in cluster 2 as compared to cluster 1 (Figure 2B). Similar division in two clusters was shown in an independent RNA sequencing dataset of 66 treatment-naïve samples, with an identical ratio of the two clusters (Figure S3). In Kaplan-Meier survival analysis of the first dataset, patients belonging to cluster 2 had a shorter overall survival than cluster 1 patients (median OS 20 months vs. 33 months, log-rank test p=0.047) (Figure 2C). In the smaller validation set, significant OS differences were not detected.

ALDH positive cells give rise to stem cell –like colonies

As ALDH1A1 is one of the strongest markers of stemness we wanted to further study the association between ALDH and stemness features. We detected the enzyme activity of cultured primary HGS-OvCa cells with Aldefluor, which labels cells with increased ALDH activity. With Aldefluor staining, live cells were separated by flow cytometry to study ALDH high ($ALDH^{bright}$) and low ($ALDH^{dim}$) populations. We consistently observed that only the cells with high ALDH~~1A1~~ activity formed stem-like colonies (Figure 3A). Further analysis of ALDH1A1 expression by western blotting demonstrated that colony derived cells in spheroid conditions expressed more ALDH1A than adherently cultured cells (Figure 3B). Quantitation of the results showed significantly more ALDH1A1 in spheroid cells in three out four cell lines ($p<0.001$) (Figure 3C).

ALDH1A1 expression is increased after platinum-taxane chemotherapy and associated with features of inferior outcome.

We next explored, whether stemness features, and especially expression of ALDH1A1, were induced by chemotherapy. We compared ALDH~~1A1~~ activity of nine HGS-OvCa cell cultures obtained at treatment naïve stage with four HGS-OvCa cell cultures obtained after NACT. The amount of ALDH positive cells, as defined by Aldefluor labelling, was significantly higher in cells obtained after NACT than in cells collected from treatment naïve patients ($p<0.001$) (Figure 4A). This was also seen in ALDH1A immunofluorescence staining of the cells (Figure 4B). To study this finding further, we compared *ALDH1A* qPCR-based mRNA expression in tumour tissue obtained from naïve and interval debulking surgery. The results indicated that *ALDH1A1* expression was increased after platinum-taxane combination therapy ($p<0.05$) (Figure 4C). The increased *ALDH1A* expression could also be visualized by IHC (Figure 4D). When RNA-seq results from a separate set of 21 naïve - interval tissue pairs were compared, we were able to conclude that *ALDH1A1* levels of interval tissues were

significantly higher than *ALDH1A1* levels of naïve tissues ($p<0.0001$) (Figure 4E). In pairwise analysis, 71.4 % (15/21) pairs showed increased ALDH1A1 expression in the interval sample, in 12.8% (3/21) the expression remained stable and in 12.8% (3/21), the expression was reduced in the interval sample (Table S4). Interestingly, in this paired analysis, ALDH1A1 increase in post-treatment samples correlated with c-Myc and BMI1 increase, but showed an inverse correlation with Oct4A, NANOG, SOX2 and Lin28A.

We correlated the RNA-seq-based ALDH1A1 expression in pre-treatment samples with disease dissemination at interval surgery, primary therapy outcome and residual tumour amount. The Spearman's correlation analyses demonstrated that higher levels of ALDH1A1 correlated with more disseminated disease at interval surgery suggesting that patients with higher ALDH1A1 expression do not respond to NACT as well as patients with lower expression levels of ALDH1A1 ($p=0.009$). Furthermore, the results also showed that high expression of ALDH1A1 is indicative of the primary therapy outcome ($p=0.058$) or residual tumour amount ($p=0.082$).

The results demonstrate that HGS-OvCa cells surviving standard chemotherapy were enriched for stemness features. Additionally, the results indicate that ALDH1A1 may be one of the most prominent stemness markers that can be visualized in live cells and in tumour tissue with western blotting, immunofluorescence, RNA expression and IHC.

High-throughput drug sensitivity testing reveals heterogeneity in the drug response of cells cultured in adherent and spheroid conditions

Cancer cells with stemness features are thought to be resistant to chemotherapy, but no comparative high throughput drug tests have been performed with primary HGS-OvCa cultures. Therefore, we carried out drug testing with a panel of 306 oncology compounds using three ascites derived primary HGS-OvCa cell lines after NACT (M019i, M068i and M022i) both at adherent and spheroid

conditions (see Figures 3B and C). The results were calibrated against fresh normal bone marrow cells to compare the general toxicity of the drug with a sensitive healthy tissue (Drug Sensitivity Score, DSS).

The comparison of selective DSS (sDSS) values between cell lines revealed significant differences between individual cell lines and between cell culture conditions. Generally, the M022i cells were more resistant to the tested compounds than the other two primary cell lines M019i and M068i, which showed rather similar response profiles. This is in line with the exceptionally short patient survival of M022i (PFS 3.1, OS 4.0) when compared to the survival of M019i (PFS 2.4, OS 34.3) and M068i (PFS 9.3, OS 11.2) patients. Of all compounds, 31.8% and 31.5% were effective (sDSS >5) against M019i grown in adherent and spheroid conditions, respectively (Figure 5A). For M068i cells the corresponding values were 27.3% and 32.8% (Figure 5B). The proportion of highly effective compounds (sDSS > 15) for M019i was 6.7% and 9.9% and for M068i 5.7% and 8.6%, again tested separately for adherent and spheroid conditions. M022i cells responded to 22.2% of the compounds when grown in adherent conditions and 14.0% when grown as spheroids (Figure 5C). The proportion of highly effective drugs was 3.8% and 1.9%. Interestingly, many classical chemotherapy agents, including cytarabine, decitabine, vinblastine, vincristine and vinorelbine were not cytotoxic against M022i, although they were effective or highly effective towards M019i and M068i cell lines, independent of growth conditions. The efficacy of the drug compounds in each cell line is presented in Table S5.

We identified five compounds that were highly effective against all three cell lines independent of growth conditions. Of these, omipalisib and PF-04691502 are inhibitors of the PI3K-mTOR-pathway, refametinib is a MEK inhibitor, BIIB021 is an HSP90 inhibitor and BMS-754807 is an IGF-1R/InsR inhibitor.

Thirteen compounds appeared to be more effective against cell grown in spheroid conditions than in

adherent conditions. These included three EGFR inhibitors (afatinib, erlotinib and gefitinib), three aurora kinase inhibitors (AZD1152-HQPA, alisertib and AT9283), two PI3K-mTOR pathway inhibitors (apitolisib and AZD2014/vistusertib), two topoisomerase inhibitors (camptothecin and topotecan), a MEK inhibitor (trametinib), a polo-like kinase 1 inhibitor (volasertib) and a nucleoside analogue (gemcitabine). Additionally, the growth inhibitory effect of three EGFR inhibitors (canertinib, dacominitib and neratinib) was more prominent in spheroid conditions in M019i and M068i cell lines.

Altogether, these results suggest that ovarian cancer cells with stemness features can be targeted with compounds already in clinical use or in trials. Of special interest are compounds inhibiting EGFR, PI3K-mTOR and aurora kinase activities.

Discussion

Intertumoural and intratumoural heterogeneity of HGS-OvCa provides a great challenge for effective treatment and for selecting the optimal study design for translational research. One facet of the heterogeneity is the presence of the rare population of cells with stemness features, which may play a pivotal role in tumour initiation, growth, chemoresistance, and recurrence [35,36]. In this study, we have correlated clinical samples with *in vitro* analyses to identify conditions promoting stemness features and to define a stemness marker panel for HGS-OvCa. Using these markers we show that the expression of stemness markers in treatment-naïve tumours indicates reduced survival and that stemness markers are upregulated in cancer cells surviving chemotherapy. We further show that cells grown under conditions favouring stemness demonstrate sensitivity to a limited number of oncology compounds.

In spite of numerous studies, a definitive HGS-OvCa stemness marker panel is still to be defined. Due to the heterogeneity of proposed cell surface markers, we focused mostly on intracellular markers with a key role in controlling the pathways leading to spheroidal, stem cell –like phenotype. All the

included markers, ALDH1A1, PROM1, c-MYC, LIN28A, NANOG, OCT3/4, OCT4A, SOX2, CIP2A and BMI1, have previously been associated with stemness, but they have not been studied to this extent as a marker set. Of the analysed markers, ALDH1A1, CIP2A, SOX2, OCT4A and BMI1 were significantly increased in primary HGS-OvCa cells cultured under conditions favouring stemness. However, when expression was analysed in tumour tissues, c-MYC, LIN28A and NANOG also clustered in these same tumours, in which the other markers were upregulated. These results show that several transcription factors and pathway regulators are concomitantly altered in HGS-OvCa cells with stemness features.

There is evidence that stemness features in treatment-naïve tumours indicate poor response to chemotherapy and aggressive behaviour. The most consistent results have been achieved with ALDH1 IHC, where abundant ALDH1A1 immunoreactivity has been associated with poor platinum free survival (PFS) and overall survival (OS) (reviewed in [37]). Correlations have also been achieved with other putative stemness markers, including CD44 and PROM1, although these results have been more ambiguous. Instead, the correlation of mRNA expression of stem cell marker panels with HGS-OvCa outcome has been tested in only a few studies. In the current study we demonstrate via cluster analysis that in treatment naïve tumours, ALDH1A1 expression coincides with several other stemness markers, and that high expression of the marker panel correlates inversely with overall survival.

The expression of ALDH1A1 is increased not only in HGS-OvCa tumours after chemotherapy, in line with earlier studies [38], but also in primary cell cultures from these tumours. Interestingly, pairwise comparison of samples collected at treatment-naïve stage and after NACT demonstrated an increased ALDH1A1 expression but stable or reduced expression of several other stemness markers. Further studies, including single cell analyses, are needed to explain this finding, which may be indicative of phenotypic plasticity of cancer stem cells challenged by chemotherapy.

HGS-OvCa stem cells are thought to convey chemoresistance, which results in treatment failure with

conventional chemotherapy. Therefore, one of our primary aims was to identify compounds that are effective against HGS-OvCa cells with stemness features. We performed comparative high throughput drug screening with 306 compounds under conventional culture conditions and with conditions favouring the stemness features, using primary cell cultures. We are not aware of any previous studies, in which a similar approach would have been used. A general finding from these experiments was that there is individual variation between patients, but also between culture conditions; the most sensitive donor responded to twice as many compounds as the most resistant donor. Surprisingly, we did not find significant overall differences in response rates between cells grown in adherent and spheroid growth conditions. The results pinpoint candidate compounds with general efficacy independent of donor and compounds that demonstrate some selectivity towards stemness conditions. Altogether, five drugs were highly effective independent of the donor or culture condition and thirteen compounds showed preference towards cells grown in conditions favouring stemness features. A potential weakness of the drug screen results is the fact that adherent and spheroid cells were grown in different culture media, the effect of which cannot be ruled out.

The compounds that demonstrated efficacy independent of culture conditions or donor included kinase inhibitors targeting PI3K and mTOR. These results are consistent with findings that indicate a central role of the PI3K-mTOR pathway both in ovarian cancer biology [39] and with adverse outcome of the disease [40]. Interestingly, IGF-1R receptor, a transmembrane tyrosine kinase receptor, which transmits signal via AKT-PI3K or MAPK-ERK pathways, has been associated with cancer stem cell functionality in ovarian cancer as well as tumorigenicity and maintenance of cancer stem cell phenotype in breast cancer [41,42]. Our discovery of tyrosine kinase inhibitor (BMS-754807) showing effectivity towards stem like -cells is in agreement with this finding. Furthermore, there are recent promising results on the combinatorial therapy of mTOR- PI3K and MEK inhibitors

in ovarian cancer [43]. Furthermore, combined inhibition of MEK and Src was recently shown to deplete ALDH1A1 positive ovarian cancer cells with stem cell characteristics [44].

Altogether six EGFR inhibitors demonstrated preference towards cells with stemness conditions in at least two of the three donors. While clinical trials with afatinib, erlotinib and gefitinib have failed to demonstrate efficacy in ~~HG-SOC~~ HGS-OvCa, at least as single agents, no studies with the irreversible pan-ERBB inhibitors canertinib, dacominitib and neratinimib in HGS-OvCa have been published. There is limited information on the association of chemotherapy resistance and EGFR expression or activation in HGS-OvCa cells. The above-referred MEK/Src inhibition study [44] showed a critical role for EGFR signalling as an alternative pathway to MEK/MAPK in supporting cancer stem cell viability. Another recent study showed that dacomitinib, one of the effective EGFR inhibitors in our panel, prevents growth of chemoresistant cells by inhibiting aurora kinase B activity [45]. This is in line with our results demonstrating that several aurora kinase inhibitors effectively killed cells grown in stemness conditions. Aurora kinases have been reported to play a role in ovarian cancer stem cell biology [46,47] and aurora kinase inhibitors have shown efficacy towards conventional ovarian cancer cell lines. The current study is the first to demonstrate efficacy in primary cell cultures. Recently, clinical trials with aurora kinase inhibitors alisertib and AMG-900 ([48,49]) have demonstrated clinical benefit. Similarly, the first phase I study combining mTOR inhibitor ADZ2014 and weekly paclitaxel showed promising results in HGS-OvCa, warranting further clinical trials ([50]).

In conclusion, we have established an experimental model to study HGS-OvCa cancer stem cell phenotype using primary cell cultures. Expression of *in vitro*-induced stemness markers, including ALDH1A1, defines a subset of treatment-naïve HGS-OvCa tumours with reduced survival probability. High ALDH1A1 expression in treatment-naïve samples is indicative of poor chemotherapy response and its expression is increased during chemotherapy. Most notably

compounds targeting EGFR, PI3K and aurora kinase demonstrate activity towards cancer stem cells. In the future, treatments targeting the signalling pathways vital for cancer stem cells could help to eradicate the cell population that remains resistant to conventional chemotherapy.

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Author contributions statement

JH and AA contributed to material acquisition. KK, PR, KH, LL and PM carried out experiments. KK, PR, PC, KH, TK and SP analysed the data. KK, PR, PC and KH generated figures. KK and OC contributed to study design, data interpretation and literature search. PÖ, SG, KW, SH, KK and OC were involved in writing the paper. All authors had final approval of the submitted version.

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Figure legends

Figure 1. HGS-OvCa cell phenotype depends on culture conditions.

Eleven patient-derived and seven commonly used HGS-OvCa cell lines were grown as adherent cells or under conditions supporting spheroidal growth. **A**. Morphology of two patient-derived cell lines, M022 and M022i (i = interval) grown in spheroid (sph) or adherent (adh) conditions. Scale bar = 100 µm **B**. Expression of selected stemness-related markers in spheroidal and adherent cells. Out of ten stemness related markers, five (*ALDH1A1, CIP2A, OCT4, SOX2 and BMI1*) were significantly upregulated in primary spheroidal cell cultures. Three replicates. * $p< 0.05$, ** $p< 0.01$ (n=16, two-sided t-test). **C**. Platinum (cisplatin) and taxane (paclitaxel) sensitivity of HGS-OvCa cells grown under spheroidal or adherent conditions. Higher activity area (AA) indicates reduced sensitivity. * $p< 0.05$. (paired t-test) 3 replicates. **D**. Platinum (Cisp; blue) and taxane (PTX; red) drug response curves for each cell line. Spheroids = triangles, adherent cells = boxes. * $p< 0.05$. ** $p< 0.01$. *** $p< 0.001$.

Figure 2. Expression of stemness markers in treatment naïve HGS-OvCa tumors is associated with survival.

A. Expression heatmap of eight stemness markers in 144 treatment naïve HGS-OvCa tumours in a microarray dataset [33]. Tumour samples were clustered by k-means clustering (k=2). Cluster 1 = baseline cluster. Cluster 2 = enriched stemness cluster. **B**. Violin plots of stemness marker expression in cluster 1 and cluster 2 samples. Results are deduced from the same dataset. Significant expression differences for each gene between cluster 1 and cluster 2 patients are marked by asterisk (* for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$, **** for $p< 0.0001$ and ***** for $p< 0.00001$). **C**. Kaplan-

Meier survival curves of the different clusters. Cluster 1 patients are associated with shorter overall survival than cluster 2 patients (Median OS 20 mo vs. 33 mo, log-rank test p=0.047).

Figure 3. ALDH as a marker for stemness –like phenotype.

A. Primary HGS-OvCa cells were sorted by flow cytometry for $ALDH^{bright}$ and $ALDH^{dim}$ cells and single cells were plated on myofibroblast feeder cells. Only $ALDH^{bright}$ cells gave rise to colonies (marked with a circle). Scale bar 100 µm. **B**. Primary HGS-OvCa cells grown under spheroid or adherent conditions were analysed for ALDH1A1 protein expression. **C**. Quantitation of western blot results (n = 4). (* for $p< 0.05$, ** for $p< 0.01$, *** for $p< 0.001$, **** for $p< 0.0001$).

Figure 4. ALDH is increased in HGS-OvCa tumors after platinum-taxane chemotherapy.

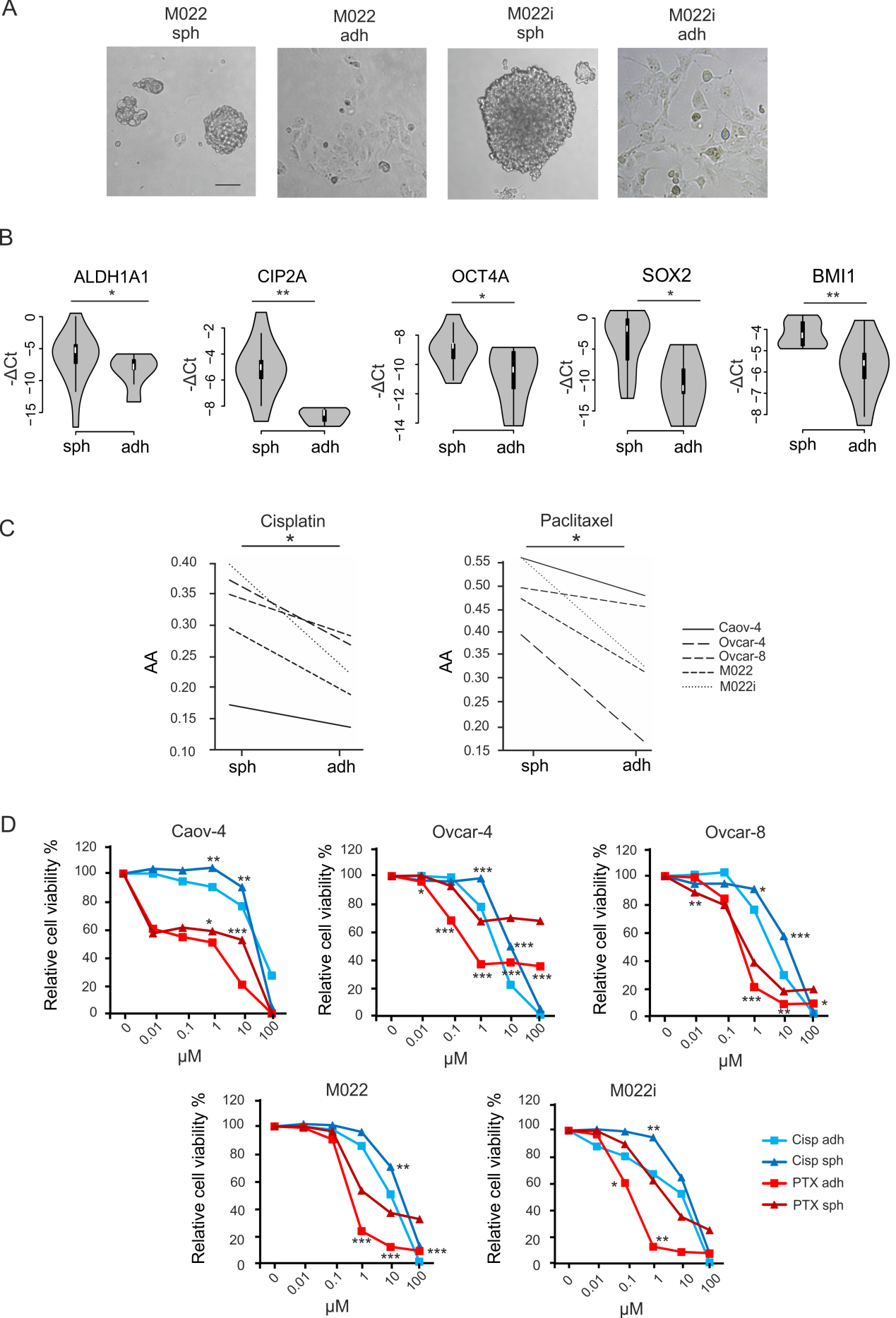
A. Flow cytometry analysis of Aldefluor stained treatment naïve (n=21) and chemotherapy treated (n=6) adherent (2-5 replicates) and spheroidal (2-20 replicates) cells after three platinum-taxane chemotherapy cycles. The percentage of Aldefluor positive cells is significantly higher in the interval HGS-OvCa cells (*** $p< 0.001$). **B**. Immunofluorescence staining of ALDH1A1 shows increased number of ALDH1A positive cells (red) in the interval sample. Blue = DAPI. Scale bar 100 µm. **C**. *ALDH1A* mRNA expression in tumour tissues (n=25) measured by qPCR from treatment naïve and chemotherapy treated interval samples. *ALDH1A* expression is significantly increased in chemotherapy treated samples (* $p<0.05$). **D**. ALDH1A1 IHC of representative treatment naïve and chemotherapy treated tissue specimens. Note strong ALDH1A1 immunoreactivity in cancer cells of chemotherapy treatment. Scale bar 100 µm. **E**. RNA-seq-based *ALDH1A* expression in tumour tissues (n=21) from treatment naïve and chemotherapy treated samples. *ALDH1A1* expression is significantly higher interval tissues (*** $p<0.001$).

Figure 5. Response of spheroid and adherent primary HGS-OvCa cells to oncology compounds.

Comprehensive DSRT was performed with 306 compounds. Three correlation plots are presented on primary ovarian cancer cell lines, M019i (**A.**), M068i (**B.**) and M022i (**C.**). Correlation plots represent drug sensitivity scores (sDSS) of spheroidal (y-axis) and adherent (x-axis) cells. Drugs with sDSS score over 5 are considered effective and sDSS scores over 15 represent highly effective drugs. Dots on the correlation plots represent drugs.

BM = BMS-754807, Om = omipalisib, R e= refametinib, BI = BIIB021, PF = PF-04691502, Cn = canertinib, Da = dacomitininb, Ne = neratinib, Er = erlotinib, Af = afatinib, Gf = gefitinib, Tr = trametinib, 2014 = AZD2014, Ap = apitolisib, Ca = camptothecin, To = topotecan, Ge = gemcitabine, 928 3= AT9283, Al = alisertib, 1152 = AZD1152-HQPA, De = decitabine, Vn = vinorelbine, Vb = vinblastine, Vc = vincristine, Cy = cytarabine, Vo = volasertib

Red = EGFR inhibitor (6), Pink = Aurora-kinase inhibitor (3), Orange = mTOR/PI3K inhibitor/MEK inhibitor (6), Green = IGFR -/HSP90 -/topoisomerase -/polo-like kinase inhibitor (5), Blue = Classic/Nucleoside analogue (6).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M022 sph
M022 adh
M022i sph
M022i adh
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CIP2A
OCT4A
SOX2
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-ΔCt
sph
adh
C
Cisplatin
Paclitaxel
AA
Caov-4
Ovcar-4
Ovcar-8
M022
M022i
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Relative cell viability %
μM
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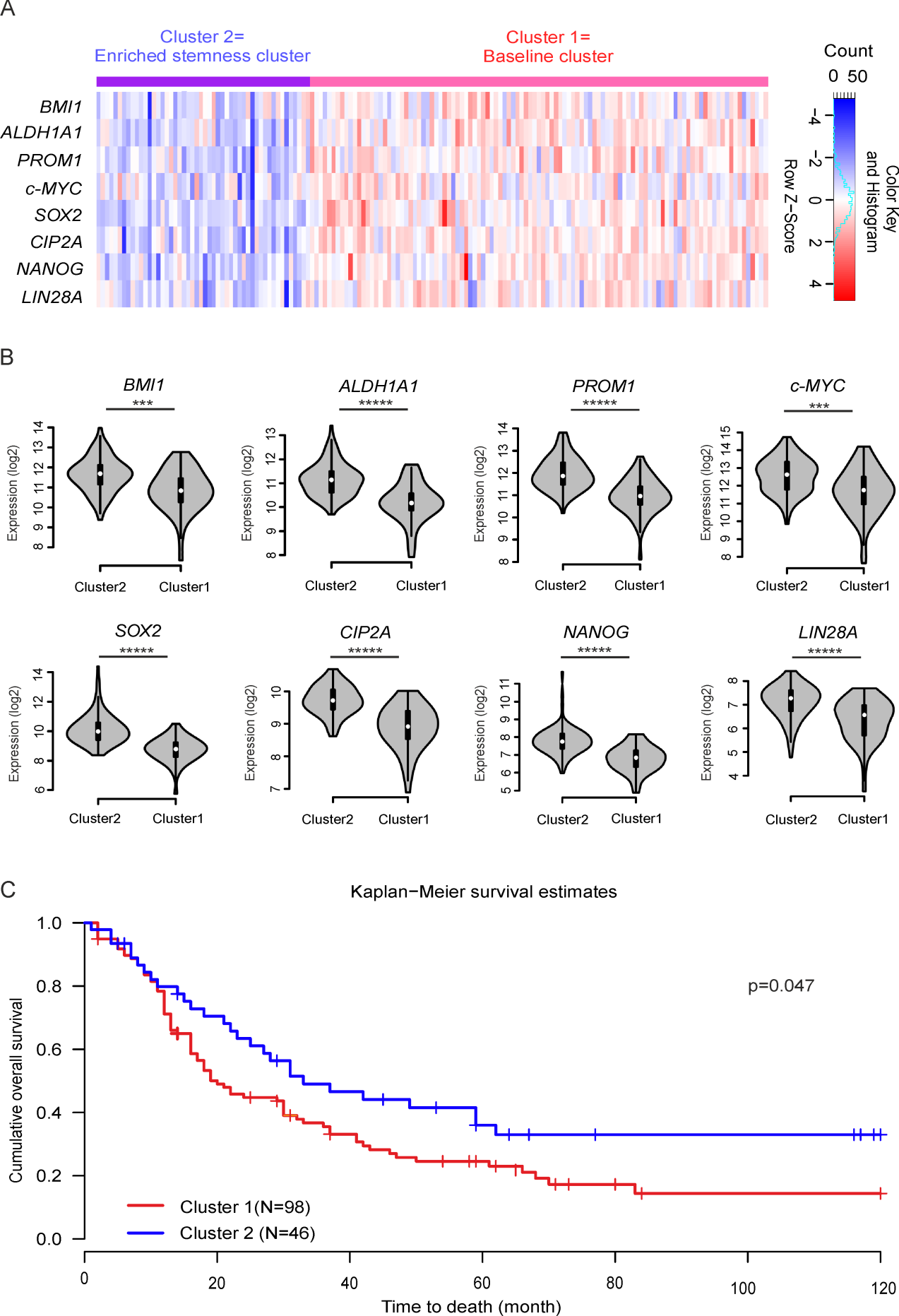
PTX sph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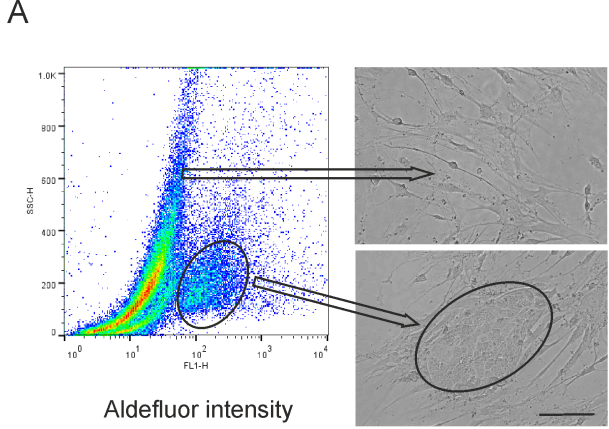
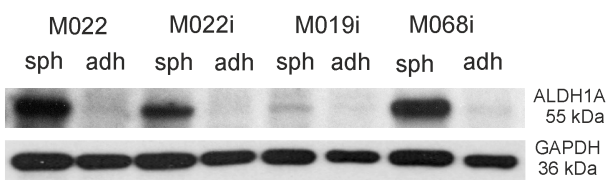
Cluster 2=
Enriched stemness cluster
Cluster 1=
Baseline cluster
Count
0 50
BMI1
ALDH1A1
PROM1
c-MYC
SOX2
CIP2A
NANOG
LIN28A
Row Z-Score
Color Key
and Histogram
B
BMI1
ALDH1A1
PROM1
c-MYC
SOX2
CIP2A
NANOG
LIN28A
Expression (log2)
Cluster2
Cluster1
C
Kaplan−Meier survival estimates
Cumulative overall survival
p=0.047
Cluster 1(N=98)
Cluster 2 (N=46)
Time to death (month)

A


1.0K
800
600
400
200
0
SSC-H
10^0
10^1
10^2
10^3
10^4
FL1-H
Aldefluor intensity

B


M022
M022i
M019i
M068i
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adh
sph
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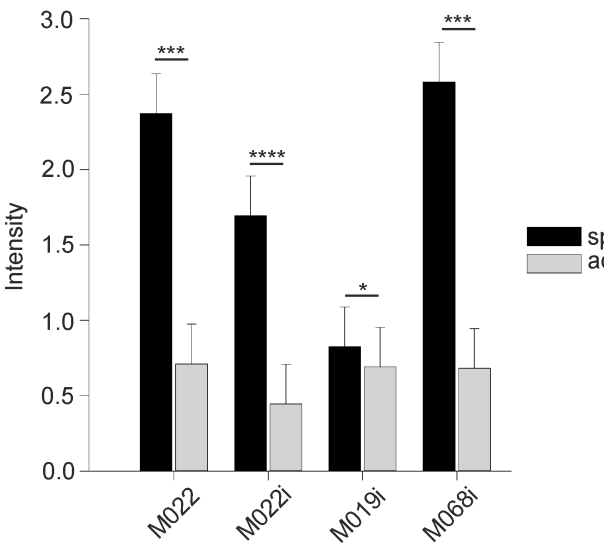
sph
adh
ALDH1A1
55 kDa
GAPDH
36 kDa

C


3.0
2.5
2.0
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Intensity
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sph
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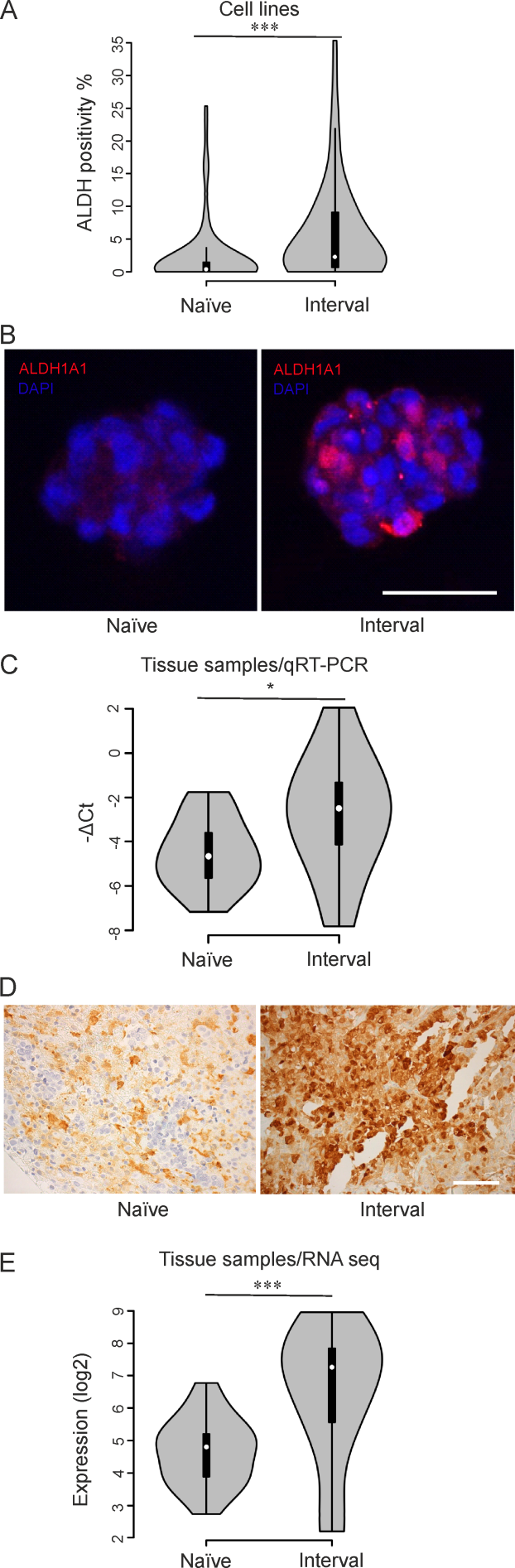
M022
M022i
M019i
M068i


A
Cell lines
***
ALDH positivity %
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5
10
15
20
25
30
35
Naïve
Interval
B
ALDH1A1
DAPI
ALDH1A1
DAPI
Naïve
Interval
C
Tissue samples/qRT-PCR
*
-ΔCt
2
0
-2
-4
-6
-8
Naïve
Interval
D
Naïve
Interval
E
Tissue samples/RNA seq
***
Expression (log2)
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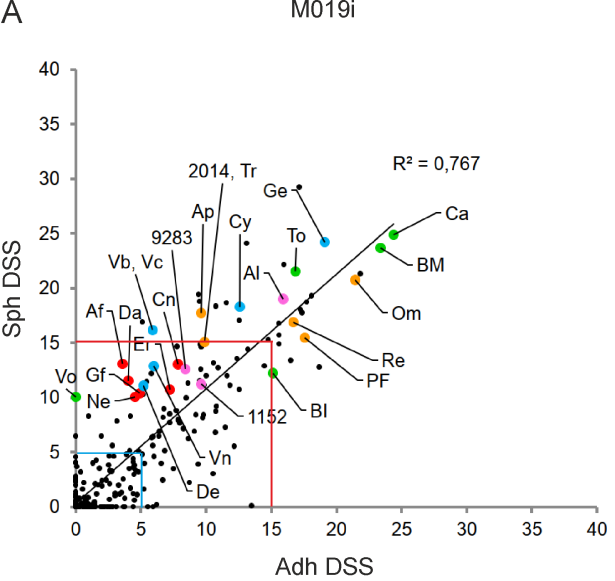
3
4
5
6
7
8
9
Naïve
Interval

A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Adh DSS
2014, Tr
Ge
Ap
Cy
Ca
To
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BM
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Cn
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Vo
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Ne
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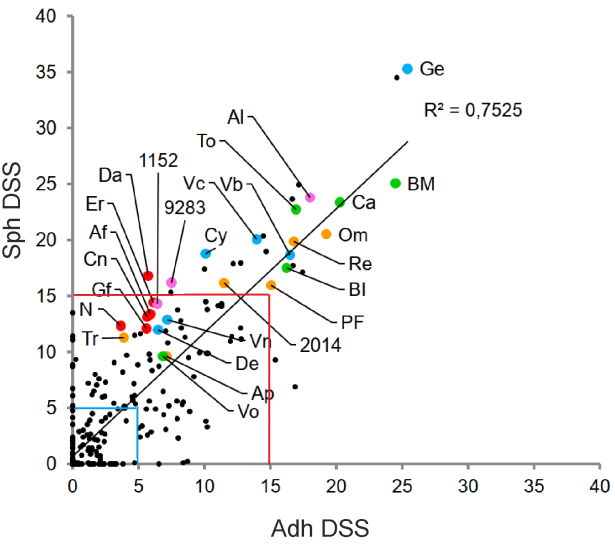
Vn
De

B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Ge
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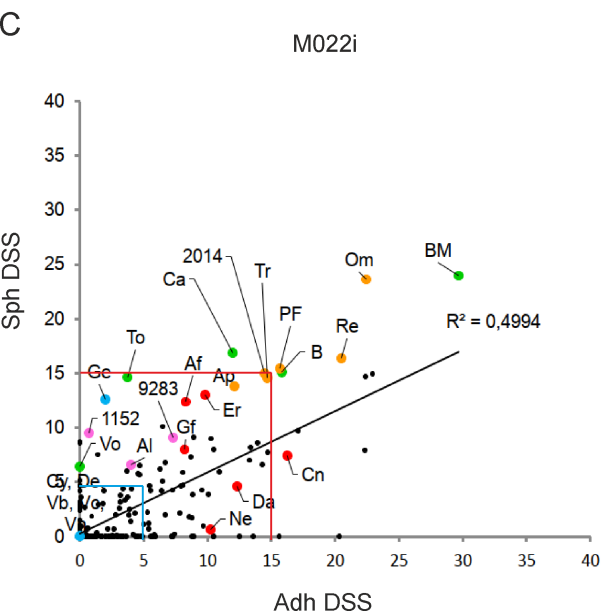
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