IL6/JAK1/STAT3 Signaling Blockade in Endometrial Cancer Affects the ALDH^hi/CD126^ Stem-like Component and Reduces Tumor Burden

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Abstract

Cancer stem-like cells (CSC) may be critical to maintain the malignant behavior of solid and hematopoietic cancers. Recently, patients with endometrial cancer whose tumors expressed high levels of aldehyde dehydrogenase (ALDH), a detoxifying enzyme characteristic of many progenitor and stem cells, exhibited a relative reduction in survival compared with patients with low levels of ALDH. Given evidence of its role as a CSC marker, we hypothesized that high level of ALDH activity (ALDH^hi^) in a tumor might positively correlate with the presence of stem- and progenitor-like tumor cells in this disease setting. In support of this hypothesis, ALDH could be used to enrich for CSC in endometrial cancer cell lines and primary tumors, as illustrated by the increased tumor-initiating capacity of ALDH^hi^ cells in immunodeficient mice. ALDH^hi^ cells also exhibited greater clonogenic and organoid-forming capacity compared with ALDH^lo^ cells. Notably, the number of ALDH^hi^ cells in tumor cell lines and primary tumors inversely correlated with differentiation grade. Expression analysis revealed upregulation of IL6 receptor subunits and signal transducers CD126 and GP130 in ALDH^hi^ endometrial cancer cells. Accordingly, targeted inhibition of the IL6 receptor and its downstream effectors JAK1 and STAT3 dramatically reduced tumor cell growth. Overall, our results provide a preclinical rationale to target IL6 or its effector functions as a novel therapeutic option in endometrial cancer. Cancer Res; 75(17): 3608–22. ©2015 AACR.

Introduction

In general, malignancies are thought to have a hierarchical organization with cancer stem cells (CSC), endowed with self-renewal and differentiation capacity, which continuously fuel tumor growth through unbalanced symmetric (giving rise to more CSCs) and asymmetric (generating more committed progenitors and differentiated cells) cell divisions (1). In view of their central role in underlying malignant behavior and of their intrinsic resistance to cytotoxic treatments, CSCs have received much attention as a unique target for the development of novel tailor-made therapeutic approaches. Resistance of CSCs to conventional therapies has been shown to result from multiple mechanisms (2), including the increased expression of detoxifying enzymes such as aldehyde dehydrogenase (ALDH). Upon chemotherapy and irradiation, ALDH alters aldehydes (oxygen, carbon, and hydrogen) within a cell to prevent DNA damage. Increased ALDH enzyme activity has been found in CSCs in colon, ovarian, prostate, and breast cancers (3–5). In addition, increased mRNA expression of the ALDHI gene in endometrial cancer has been detected, and its expression level negatively correlates with overall survival (6).

The cellular activity and the relative multiplicity of CSCs within a tumor are likely regulated by signals from the tumor microenvironment. In endometrial cancer, high levels of IL6 were found to negatively correlate with overall survival (7). IL6 is secreted upon tissue injury and stress (8). Although innate immune cells represent the major IL6 source within the tumor microenvironment, tumor cells were also shown to express IL6 in autocrine fashion. Upon its secretion, IL6 binds to its receptor (IL6R) with the ubiquitously expressed second transducer GP130. Binding of IL6 to CD126 and their association with GP130 triggers the activation of Janus kinases (JAK), which phosphorylate STAT3, thus modulating the expression of a broad spectrum of downstream target genes (9). Recent studies showed that IL6 and its downstream effector STAT3 are highly expressed in therapy-insensitive endometrial cancers and overall represent poor prognosis indicators for this type of uterine malignancies (7, 10, 11). Here, we took advantage of ALDH enzymatic activity as a marker to enrich for CSCs in human endometrial cancer cell lines and...
primary tumors and to identify novel prognostic markers and therapeutic targets.

Materials and Methods

Animals

NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ (NSG) mice were bred and maintained in the Erasmus MC animal facility (EDC) under standard conditions. All protocols involving animals were approved by the Dutch Animal Experimental Committee and were conform to the Code of Practice for Animal Experiments in Cancer Research established by the Netherlands Inspectorate for Health Protection, Commodities and Veterinary Public Health (The Hague, the Netherlands, 1999).

Primary tumor collection

For the aims of this study, established cell lines and hystereotomy resection specimens from patients were used. Only tumors from patients where no neoadjuvant chemo- and/or radiation therapy was applied were considered for the transplantation experiments. Tumor samples were obtained from resection specimens upon arrival at the Department of Pathology, according to the Code of Conduct for Proper Secondary Use of Human Tissue established by the Dutch Federation of Medical Scientific Societies. The following primary tumor samples were used to generate endometrial cancer patient-derived xenografts (PDX) by transplantation and propagation in NSG immunodeficient mice (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ): HET-01 (grade I, well-differentiated, estrogen receptor (ER)\(^+\), progesterone receptor (PR)\(^+\)); HET-02 (grade II, intermediate differentiation grade, ER\(^+\), PR\(^-\)); HET-04 (grade III, poorly differentiated, ER\(^-\), PR\(^-\)); HET-07 (grade I, well-differentiated, ER\(^+\), PR\(^+\)); and HET-10 (grade I, well-differentiated, ER\(^+\), PR\(^+\)).

Statistical analysis

Statistical analysis was performed using SPSS software (version 16; SPSS). Differences between groups were analyzed using the Kruskal–Wallis statistical test. When \(P < 0.05\), the Dunn multiple comparison was performed and, upon confirmation, \(P < 0.05\) was considered statistically significant. If only two groups were analyzed, a \(t\) test was performed, and a result of \(P < 0.05\) was considered statistically significant.

Cell lines and culture

HEC1B, HECA1, RL952, and Ishihikawa cell lines were obtained from ATCC and ECACC and cultured in DMEM containing 5\% FCS and penicillin/streptomycin, or in serum-free stem cell media (DMEM/F12 supplemented with 1\% v/v B27, 20 ng/mL basic FGF (bFGF), 20 ng/mL EGF, 100 \(\mu\)g/mL gentamycin). The identity of each of the cell lines used was confirmed by DNA fingerprinting with 13 microsatellite markers (Amel, C5F1PO, D2S1338, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D19S433, D21S11, FGA, THO1, TPOX, vWA) and comparison with the analogous data provided by ATCC and ECACC. Notably, although no PR expression was found in the tumors derived from the HEC1A cells, the ATCC datasheet relative to this cell line states its PR\(^+\) status.

Flow cytometry and in vivo transplantation assays

To further purify primary tumor cells from contaminating components, we used antibodies raised against well-characterized endothelial (CD31-APC; BD Biosciences) and hematologic (CD45-APC, TER119-APC; BD Biosciences) lineage (Lin\(^-\)) antigens to deplete Lin\(^-\) cells by gating the CD31\(^-\)/CD45\(^-\) population (12). CD133\(^+\) cells were detected with the CD133/2-APC (293C3; MACS), according to the manufacturer’s instruction. Dead cell discrimination was performed with Hoechst 33258 (1:10,000; Invitrogen). Single-cell suspensions of Lin\(^-\) tumor cells (i.e., enriched for epithelial cells) and cell lines were stained with ALDH reagents (Aldesfluor, STEMCELL Technologies Inc.) alone and in combination with CD126-PE (BD Biosciences). Labeled cells were either used for analysis or sorting. FACS was performed with a FACSAria Cell Sorting System (BD Biosciences). Cells were sorted into DMEM supplemented with 5\% FCS and penicillin/streptomycin. After sorting, cells were centrifuged at 1,300 rpm for 10 minutes, resuspended in Matrigel (BD Biosciences), and subcutaneously injected into <12-week-old NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) female mice in a 100 \(\mu\)L volume. The relative frequency of CSCs in endometrial cancer was estimated by limiting dilution analysis. The size (volume) of the resulting tumors was calculated according to the formula \(V = \frac{a b^2}{2}\), where \(a\) and \(b\) are the longest and shortest tumor diameters, respectively (13). Data are given in mm\(^3\).

The following small-molecule inhibitors and chemotherapeutics were used: nifuroxazine (VETRALAN) was from Sigma-Aldrich; nuxoltinib was from InvivoGen; and cisplatin was from Selleckchem.com. All chemicals were dissolved in DMSO and diluted to keep DMSO at a final concentration of 0.1\% in all the samples.

IHC analysis

Paraffin sections (5 \(\mu\)m) were stained with hematoxylin and eosin (H&E) or antibodies directed against ER\(\alpha\) (clone 1D5, 1:100), PR (clone PgR636, 1:100), and Ki67 (clone TEC-3, 1:200) using the EnVision system Kit (Dako). Counterstained with hematoxylin, and scanned with an NDP slide scanner (Hama-matsu). Quantification of ER and PR staining was done by counting positive cells in three independent sections from each individual tumor (ImageJ software; NIH, Bethesda, MD). The numbers of positive cells were counted per high-power field at a 10\(\times\) magnification, throughout the entire section.

Single-cell suspensions

To obtain single-cell suspensions for FACS analysis, cell lines were trypsinized in 0.05 mmol/L EDTA, whereas tumor tissues were dissociated both mechanically and enzymatically. Briefly, tumor tissue specimens were digested for 20 minutes at 37\(^\circ\)C with DNase I (50 U/mL; Roche) and collagenase A (3 mg/mL; Sigma-AL bocsh). Samples were then passed through a 40-\(\mu\)m cell strainer (BD Biosciences) and the resulting single-cell suspensions used for further analysis.

In vitro Matrigel assay

After sorting, cells were centrifuged at 1,300 rpm for 10 minutes and resuspended in Matrigel (BD Biosciences) in a 50 \(\mu\)L volume and cultured in 24-well dishes in serum-free stem cell media (DMEM/F12 supplemented with 1\% v/v B27, 20 ng/mL bFGF, 20 ng/mL EGF, 100 \(\mu\)g/mL gentamycin; ref. 14).
Clonogenicity assay

Single cells were sorted by FACS in 96-well plates in serum-supplemented growth media. After sorting, each well was inspected by microscopy for the presence of one cell per well. After 1 week of culture (37°C, 5% CO2), clonogenicity was assessed for each cell line and sorted subpopulation by counting the number of positive wells.

Cell proliferation assay

Sorted cells were seeded in 96-well dishes at 500 cells per well and cultured in serum-supplemented medium for 70, 120, and 170 hours. At the indicated time points, cells were fixed with 70% ethanol and stained with 0.1% crystal violet in water. Crystal violet was solubilized with acetic acid followed by optical density (OD) measurement at 570 nm with a microplate reader (BioRad), as readout for cell growth.

Toxicity assays (MTT)

Cells were seeded in 96-well plates at the indicated density, left overnight to allow full recovery and adhesion, and then treated with cisplatin and/or IL6 pathway inhibitors. Viability was assayed at time zero and after 3-day treatment using the MTT viability assay. Briefly, cells were incubated for 3 hours in culture medium supplemented with 0.45 mg/ml MTT (Sigma-Aldrich). The 96-well plates were then centrifuged at 1,000 rpm for 5 minutes and culture medium was removed. MTT formazan precipitates were solubilized with DMSO. OD reading was performed at 490 nm with a microplate reader.

Gene expression analysis

Expression profiling analysis was performed with the RT2 Profiler Cell Surface Markers PCR Array (Qiagen) according to the manufacturer’s instructions. The resulting expression profiles were validated by qPCR, as previously described (15). Expression levels were normalized for the endogenous reference GAPDH gene. Primer sequences are specified in the online Methods section.

Results

The percentage of ALDHlo endometrial cancer cells inversely correlates with their differentiation status

Previously, it was shown that endometrial cancers expressing high ALDH1 mRNA levels are characterized by a reduced survival rate (6). To follow-up on this observation, we further investigated ALDH enzymatic activity in a panel of four endometrial cancer cell lines [HEC1A, HEC1B, RL952, and IK (Ishikawa)] and 5 primary tumors (PDX; HET-01, -02, -04, -07, -10) by Aldefluor reagents followed by flow cytometry analysis. We found that the percentages of ALDHlo cells in our panel of 4 endometrial cancer cell lines ranged from 52% in HEC1B to 1% to 2% in IK cells (Fig. 1A and B). A similarly broad spectrum of ALDH activities was found among the PDX from primary endometrial tumors where the percentages of ALDHhi cells ranged from 3% (HET-04) to 5% (HET-01; Fig. 1A and B).

Next, single-cell suspensions were obtained from the panels of endometrial cancer cell lines and primary tumors sorted by FACS (to deplete Lin- and dead cells) and subcutaneously injected into NSG mice at relatively high multiplicities (103–105) to allow tumors to develop for histologic analysis. When the tumors reached a size of approximately 1 cm3, mice were sacrificed and tumors were analyzed by IHC for the expression of ER and PR (16–18). Tumors derived from the HEC1B and HEC1A cell lines did not show any ER and PR expression, whereas tumors obtained from the RL95-2 and IK cell lines did express both hormone receptors, albeit at different levels (Fig. 1C). Notably, ER/PR expression was absent in the two cell lines (i.e., HEC1B and HEC1A) encompassing high percentages of ALDHhi cells, whereas those with smaller ALDHhi fractions (i.e., RL95-2 and IK) gave rise to tumors with increased ER/PR expression (Fig. 1C). The same trend was observed among primary tumors (Fig. 1C).

Overall, these results indicate that the percentage of ALDH cells in endometrial cell lines and primary tumors inversely correlates with their ER/PR status and most likely with their differentiation grade (16–18).

ALDHhi cells are characterized by increased proliferation, clonogenicity, and organoid-forming capacity

To investigate functional differences between ALDHhi and ALDHlo endometrial tumor cells, we assessed their proliferation rate in conventional culture conditions and their capacity to form self-renewing organoids in serum-free growth conditions.

We first stained our panel of endometrial cancer cell lines and primary tumors with Aldefluor reagents, sorted 500 ALDHhi and ALDHlo cells by FACS into each well of 24-well TC-coated dishes, and cultured them in serum-supplemented medium for 70, 120, and 170 hrs. Cells were then lysed and measured at OD490 as a read-out for cell growth. After 170 hours (and in 3 cases already at 120 hours), ALDHhi cells derived from both endometrial cancer cell lines and primary tumors showed a significantly increased growth rate when compared with ALDHlo cells (Fig. 2A).

Next, we assessed whether, apart from their enhanced growth rate, ALDHhi endometrial cancer cells were also endowed with increased clonogenicity when compared with their ALDHlo counterpart. To this aim, single ALDHhi, ALDHlo, and bulk cells were sorted by FACS into 96-well dishes (a total of 64 well for each subpopulation) and cultured in serum-supplemented medium. As shown in Supplementary Table S1A, ALDHhi cells derived from our panel of endometrial cancer cell lines show increased clonogenicity when compared with ALDHlo and bulk cells.

To investigate their organoid-forming capacity in 3-dimensional (3D) serum-free conditions, a feature of stem and progenitor cells, ALDHhi and ALDHlo cells were sorted by FACS and cultured in Matrigel in stem cell media (i.e., serum-free with specific growth factors; ref. 14). As shown in Fig. 2B, ALDHhi cells derived from...
Figure 2.

A, proliferation analysis of ALDH^{Hi} and ALDH^{Lo} cells from endometrial cancer cell lines and PDX cultured under conventional (serum-supplemented) conditions, as measured by crystal violet detection at 570 nm. ALDH^{Hi} cells, black-filled circles; ALDH^{Lo} cells, white-filled circles. B, spheroid formation assay of ALDH^{Hi} and ALDH^{Lo} cells from endometrial cancer cell lines and PDX. ALDH^{Hi} cells, black-filled circles; ALDH^{Lo} cells, white-filled circles. C, examples of organoids derived from ALDH^{Hi} (left) and ALDH^{Lo} (right) cells. Bar, 100 μm. D, relative size of organoids derived from ALDH^{Hi} cells and their ALDH^{Lo}-derived counterparts. The bars indicate the fold change between the diameters of ALDH^{Hi}- and ALDH^{Lo}-derived organoids, based on triplicate experiments, as a measure of the overall increased size of the former compared with the latter.
both cell lines and primary tumors gave rise to a significantly increased number of organoids when compared with ALDH^lo^ cells. Also, ALDH^hi^-derived organoids were of larger size than their ALDH^lo^ derived counterparts (Fig. 2C and D). In addition, we also observed differences in the time needed to initiate organoid formation among cell lines: whereas HEC1A and HEC1B cells gave rise to organoids within 8 days of 3D culture, RL95-2 and IK cells were significantly slower (14–21 days; data not shown).

Overall, these results indicate that ALDH^hi^ endometrial cancer cells from both established cell lines and primary tumors are characterized by increased proliferative rates, clonogenicity, and organoid-forming capacity when compared with ALDH^lo^ cells.

**Endometrial ALDH^hi^ cancer cells have increased tumor-initiating capacity**

The above features of ALDH^hi^ endometrial cancer cells are indicative of a possible enrichment of CSCs within this subpopulation. To assess the tumor-initiating capacity of these cells, ALDH^hi^, ALDH^lo^, and bulk cells were isolated by FACS from the panels of endometrial cancer cell lines and primary tumors and subcutaneously injected into NSG mice at different limiting dilutions, ranging from 10 to 10^5^. As shown in Table 1, among the endometrial cancer cell lines, ALDH^hi^ cells clearly showed increased tumor-forming capacity when compared with bulk and ALDH^lo^ cells. However, whereas this was evident even at extremely low multiplicities (i.e., 10 and 100 transplanted cells) in the cell lines characterized by the highest percentages of ALDH-positive cells (i.e., HEC1B, HEC1A, and RL95-2, giving rise to the least differentiated tumors), in the case of the IK cell line (i.e., encompassing a small minority of ALDH-positive cells and giving rise to the most differentiated tumors), ALDH^hi^ cells revealed an increased tumor-initiating capacity only when higher multiplicities were used (at least 10^3^ injected cells). Lower IK cell multiplicities (10 and 100) did not give rise to any tumor formation upon transplantation in NSG mice (Table 1, endometrial cancer cell lines). The same effect was observed among endometrial cancer cells derived from primary tumors: whereas in the case of those tumors encompassing more than 10% ALDH-positive cells (and with a low degree of differentiation), an increased tumor-initiating capacity could be demonstrated at extreme limiting dilutions (10 and 100 for HET-04, HET-02, and HET-10), the most differentiated ones with the least ALDH-positive component (~5%; HET-07 and HET-01) revealed increased tumor-forming activity only when at least 10^3^ cells were injected (Table 1, primary patient-derived tumors).

Apart from their increased tumor-forming capacity, ALDH^hi^ cells also initiated subcutaneous tumor growth with a significantly shorter latency than observed with ALDH^lo^ cells (Fig. 3A). Moreover, ALDH^hi^-derived tumors were considerably larger than those obtained with ALDH^lo^ cells (Fig. 3B). These results were further confirmed by Ki67 IHC analysis of the tumors obtained by subcutaneous injection of ALDH^hi^ and ALDH^lo^ cells from both cell lines and primary tumors (Fig. 3C). A clear increase in Ki67-positive cells is evident in the tumors obtained by injection of ALDH^hi^ tumor cells when compared with their ALDH^lo^ counterpart.

**Table 1. Summary of limiting dilution transplantation assays of sorted ALDH^hi^, ALDH^lo^, and bulk cells derived from endometrial cancer cell lines and primary, patient-derived tumors.**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Sorted population</th>
<th>10^1^</th>
<th>10^2^</th>
<th>10^3^</th>
<th>100</th>
<th>10</th>
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<tr>
<td>HEC1B ALDH^hi^</td>
<td>8/8 (100%)</td>
<td>8/8 (100%)</td>
<td>12/12 (100%)</td>
<td>4/4 (100%)</td>
<td>3/4 (75%)</td>
<td></td>
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<tr>
<td>HEC1B ALDH^lo^</td>
<td>8/8 (100%)</td>
<td>8/8 (100%)</td>
<td>3/4 (75%)</td>
<td>1/4 (25%)</td>
<td></td>
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<tr>
<td>HEC1B Bulk</td>
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<td>8/8 (100%)</td>
<td>7/8 (88%)</td>
<td>3/4 (75%)</td>
<td>0/4 (0%)</td>
<td></td>
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<tr>
<td>HEC1A ALDH^hi^</td>
<td>8/8 (100%)</td>
<td>8/8 (100%)</td>
<td>3/4 (75%)</td>
<td>1/4 (25%)</td>
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<tr>
<td>HEC1A ALDH^lo^</td>
<td>8/8 (100%)</td>
<td>8/8 (100%)</td>
<td>7/8 (88%)</td>
<td>3/4 (75%)</td>
<td>0/4 (0%)</td>
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<tr>
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<td>8/8 (100%)</td>
<td>7/8 (88%)</td>
<td>3/4 (75%)</td>
<td>0/4 (0%)</td>
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<tr>
<td>RL95-2 ALDH^hi^</td>
<td>8/8 (100%)</td>
<td>8/8 (100%)</td>
<td>2/8 (25%)</td>
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<td>RL95-2 ALDH^lo^</td>
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<td>7/8 (88%)</td>
<td>3/4 (75%)</td>
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<td>7/8 (88%)</td>
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<tr>
<td>IK ALDH^hi^</td>
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<td>5/8 (62%)</td>
<td>1/4 (25%)</td>
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<td>HET-04 ALDH^hi^</td>
<td>n.d</td>
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<td>1/4 (25%)</td>
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<tr>
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<td>7/8 (88%)</td>
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<tr>
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<tr>
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<td>n.d</td>
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</table>

**NOTE:** ALDH^hi^, ALDH^lo^, and bulk (control) cells were sorted by FACS from cell lines and primary tumor cells and injected subcutaneously in NSG mice at different multiplicities (10^1^, 10^2^, 10^3^, and 10^4^ cells for each transplantation). The number of tumors grown out of the total number of transplantation (%) is indicated. Abbreviation: n.d., not detected.
Figure 3.
A, limiting dilution transplantation analysis of bulk (red line), ALDH<sup>hi</sup> (black solid line), and ALDH<sup>lo</sup> (black stippled line) cells from endometrial cancer cell lines and PDX. Numbers of tumor growths out of the total transplantations are specified next to each line in the graphs. B, tumor size analysis from the limiting dilution transplantations. ALDH<sup>hi</sup> cells, black-filled circles; ALDH<sup>lo</sup> cells, white-filled circles; bulk cells, gray-filled circles. * statistically significant differences. C, Ki67 IHC analysis of tumors obtained by subcutaneous injections of FACS-sorted HECIA ALDH<sup>lo</sup> and ALDH<sup>hi</sup> cells into NSG mice. Similar results were obtained with other cell lines and primary tumors (not shown).
To establish the self-renewal capacity of the endometrial CSCs, tumors obtained by subcutaneous transplantation of both ALDH<sup>hi</sup> and ALDH<sup>lo</sup> cells from the HET-02 and HET-07 primary tumors were again reduced to single-cell suspension, sorted again by FACS into ALDH<sup>hi</sup> and ALDH<sup>lo</sup> fractions, and again transplanted (10<sup>3</sup> cells) into recipient NSG mice. Supplementary Table S2 summarizes the results relative to a total of three rounds of serial transplantations. Clearly, whereas ALDH<sup>lo</sup> rapidly lose their tumor-initiating capacity already after two rounds, ALDH<sup>hi</sup> cells demonstrate self-renewal by retaining their ability to form subcutaneous tumors throughout the serial transplantation passages.

Next, in line with previous reports on breast CSCs showing that epithelial-to-mesenchymal transition (EMT) is functionally linked to cancer stemness (19), we analyzed by RT-qPCR the expression of several EMT genes in the ALDH<sup>hi</sup> and ALDH<sup>lo</sup> populations of the endometrial cancer cell lines HEC1A and RL95. However, in most cases, no significant differences were observed between the two subpopulations with the only
exception of TWIST1 and TWIST2, upregulated in ALDHhi cells from the HEC1A cell line (Supplementary Fig. S1).

Overall, our cumulative in vivo data strongly suggest that endometrial ALDHhi cancer cells from both immortalized cell lines and primary, patient-derived tumors are enriched in CSCs with enhanced tumor-propagating and self-renewing capabilities.

Expression of the CD126 gene, encoding for the IL6 receptor, is increased in ALDHhi endometrial cancer cells

To identify cell surface antigens that may serve as markers of endometrial CSCs to allow both their further enrichment by FACS and their specific targeting, ALDHhi and ALDHlo cells from the HEC1A cell line were sorted and total RNA extracted for profiling analysis. This was conducted by a commercially available RT-qPCR array encompassing 84 genes whose functions are relevant to the cell surface (see Materials and Methods).

The analysis revealed that the CD72, CD79b, IL1R2 (interleukin 1 receptor 2), KLRC1 (Killer ligand receptor 1), TNFRSF8 (TNF receptor soluble factor 8), and IL6R (interleukin 6 receptor; also known as CD126) genes were upregulated in ALDHhi cells when compared with their expression in ALDHlo cells. Vice versa, genes upregulated in ALDHlo cells when compared with their expression in ALDHhi included CD2, CD3g, CD4, IL2R, S100A8, and TNFRSF4 (TNF receptor soluble factor 4; Fig. 4A).

Previously, it was shown that increased IL6 levels are associated with poor prognosis among patients with endometrial cancer (7, 10, 11). From this perspective, the observed increased expression of the gene encoding the IL6R (IL6R or CD126) in ALDHhi cells is of interest as CD126 may represent both a candidate cell surface marker for the enrichment of endometrial CSCs and a therapeutic target in view of the readily available antibodies and small-molecule inhibitors. We therefore set to validate the above results by RT-qPCR analysis of the IL6R/CD126 gene and extended our expression analysis to the IL6 gene, encoding for the IL6 ligand, and to the IL6ST (also known as GP130) gene. The latter encodes for a signal transducer, the activation of which is dependent upon the binding of IL6 to its receptor IL6R (20, 21).

As depicted in Fig. 4B, we confirmed a significant increase in the expression of the CD126 gene in ALDHhi cells from both endometrial cancer cell lines (HEC1A, P < 0.05; RL95-2, P < 0.05) and primary tumors (HET-04, P < 0.05; HET-07, P < 0.05). Moreover, expression of the IL6ST/GP130 gene was also significantly increased in ALDHhi cells in the HEC1A and RL95-2 cell lines (P < 0.05) and in the primary tumors HET-04, HET-07, and HET-10 (P < 0.05). Of interest, expression of the gene encoding for the IL6 ligand was significantly increased in ALDHhi endometrial cancer cells both from cell lines (HEC1A and IK; P < 0.05) and primary cancers (HET-04, HET-07, and HET-10; P < 0.05; Fig. 4B).

Overall, the expression profiling data indicate that ALDHhi endometrial cancer (stem) cells preferentially express the main IL6R CD126 and signal transducer GP130, whereas the IL6 ligand is predominantly expressed in ALDHhi cells. Hence, membrane-bound members of the IL6 signaling pathway, such as CD126, offer opportunities for both the further enrichment of endometrial CSCs and their specific therapeutic targeting.

ALDHhi CD126hi endometrial cancer cells have increased spheroid- and tumor-forming capacity

To assess whether CD126 indeed represents a candidate cell surface marker for the enrichment of endometrial CSCs, we stained the panels of cell lines and primary tumors with Alde conceptual reagents in combination with an antibody directed against membrane-bound CD126. Double positive (ALDHhi/CD126hi) cells were always detected in secondary tumor cell lines and primary endometrial tumors at percentages roughly reflecting the fraction of ALDHhi cells (see Fig. 5A and relative legend).

Next, the four cell subpopulations (ALDHhi/CD126hi, ALDHhi/CD126lo, ALDHlo/CD126hi, and ALDHlo/CD126lo) arising from three cell lines and from four primary tumors were sorted by FACS and analyzed for their proliferation rate in conventional culture conditions and for their capacity to form self-renewing organoids in serum-free growth conditions, as described above. In all cases, ALDHhi/CD126hi cells had an increased growth rate even when compared with ALDHhi cells (Fig. 5B). The same was true for the spheroid-forming capacity of ALDHhi/CD126hi cells that was invariably the highest, thus suggesting a further enrichment for CSCs by combining Alde conceptual reagents. With the antibody directed against CD126 (Fig. 5C). Furthermore, ALDHlo/CD126hi endometrial cancer cells were also shown to have increased clonogenicity when compared with ALDHhi/CD126hi, ALDHlo/CD126lo, and ALDHhi/CD126lo cells (Table I, primary patient-derived tumors).

Last, the four cell subpopulations (ALDHhi/CD126hi, ALDHhi/CD126lo, ALDHlo/CD126hi, and ALDHlo/CD126lo) arising from the HEC1A cancer cell line were again sorted by FACS and transplanted subcutaneously at limiting dilution (100 cells) into NSG mice. Also in this in vivo assay, the double-positive ALDHhi/CD126hi endometrial cancer cells invariably gave rise to a tumor (4 of 4) with early onset when compared with ALDHhi/CD126lo (2 of 4), ALDHlo/CD126lo (1 of 4), and bulk (1 of 4) cells (Fig. 5D).

Hence, CD126 represents, next to ALDH, a valid marker for the enrichment of endometrial CSCs both from immortalized cell lines and primary tumors. This is of note as similar attempts to further enrich the ALDHhi CSC population by using a well-known CSC marker such as CD133, previously shown to be expressed in endometrial cancer stem/progenitor cells (22), did not result in any significant result: as shown in Supplementary Fig. S2, 3 of the 4 endometrial cancer cell lines here used were CD133-negative.

Figure 5.
A, representative ALDHhi/CD126 flow cytometric analysis of endometrial cancer cell lines (HEC1A, IK, and 1B) and primary tumors (HET-07 and -10). The relative size (percentage of total cells) of each ALDHhi/CD126 subpopulation is specified in the corresponding quadrant. B, proliferation analysis of endometrial cancer cell lines and PDX. Cells were sorted for ALDH and CD126 and cultured with serum-supplemented medium in TC dishes. Proliferation was measured by crystal violet dye at 570 nm. C, spheroid formation assay of endometrial cancer cell lines and PDX sorted for ALDH and CD126. Sorted cells were cultured in Matrigel with serum-free (stem cell) medium. The bars indicate the average number of organoids observed from triplicate experiments. *: statistically significant differences between individual subpopulations. D, limiting dilution transplantation analysis of the HEC1A endometrial cancer cell line sorted for ALDH and CD126. ALDHhi/CD126hi cells, black solid line; ALDHhi/CD126lo, black thin line; ALDHlo/CD126hi, black stippled line; bulk cells, red line. Numbers of tumor growths out of the total transplantations are specified next to each line in the graph.
Targeting IL6 signaling inhibits growth of endometrial cancer (stem) cells both in vitro and in vivo

As mentioned above, the observation according to which both the main receptor (CD126) and signal transducer (GP130) of IL6 are expressed in endometrial CSCs is of interest in view of the opportunities they provide to develop targeted therapies.

We explored the possibility of targeting the IL6 signaling pathway both by an antibody directed against the CD126 receptor and by small-molecule inhibitors directed against its downstream effectors STAT3 (nifuroxazide) and JAK1 (ruxolitinib). To this aim, HEC1A cells were stained with Aldefluor reagents, sorted by FACS, and cultured in the presence of IL6 (positive control), anti-CD126 antibody, nifuroxazide (STAT3i), and ruxolitinib (JAK1i) for 8 days. As depicted in Fig. 6A, the presence of the IL6 ligand in the culture medium significantly stimulated proliferation of HEC1A ALDH<sup>hi</sup> cells but not of ALDH<sup>lo</sup> cells. In contrast, in the presence of either the CD126 antibody or the STAT3 and JAK1 inhibitors, both ALDH<sup>hi</sup> and ALDH<sup>lo</sup> cell numbers significantly declined (P < 0.05).

Next, we extended and validated the observed HEC1A growth-inhibiting effects of the CD126 antibody and of the STAT3 and JAK1 inhibitors on the panel of 4 endometrial cancer cell lines. Notably, whereas the inhibitory effects of both the antibody and the small-molecule inhibitors were confirmed for the ALDH<sup>hi</sup> fractions of the HEC1B, RL95-2, and IK cell lines, ALDH<sup>lo</sup> cell numbers were affected only in the HEC1A and B cell lines (Fig. 6A and B). This apparent inconsistency of the results is likely due to the very large percentage of ALDH-positive cells in the HEC1 cell lines and to the possible coexisting presence of ALDH-expressing cells in the ALDH<sup>lo</sup> fraction.

Similar results were obtained when the ALDH<sup>hi</sup> and ALDH<sup>lo</sup> subpopulations of the endometrial cancer cell lines were cultured in Matrigel in stem cell (serum-free) medium: a significant reduction in the organoid-forming capacity was observed in the ALDH<sup>hi</sup> fraction and in the bulk parental cell lines (Fig. 6C).

To confirm that targeting of IL6/JAK1/STAT3 signaling affects endometrial CSCs not only in cultured cells but also in an in vivo therapeutic setting, HEC1A-bulk, -ALDH<sup>hi</sup>, and -ALDH<sup>lo</sup> cells were sorted by FACS and subcutaneously transplanted into NSG mice. Tumors were then allowed to reach an average size of about 80 to 100 mm<sup>3</sup>. From this time point on (~24 days after transplantation), tumor-bearing animals were administered intraperitoneally either with the STAT3 or JAK1 inhibitor three times a week for a total of 3 weeks. As shown in Fig. 6D and E, the size of the tumors derived from ALDH<sup>hi</sup> and bulk cells was significantly reduced by both small-molecule inhibitors. In contrast, tumors derived from ALDH<sup>lo</sup> cells were not affected by the 2 specific STAT3 and JAK1 inhibitors. In the latter case, ALDH<sup>lo</sup>-derived tumors even appeared to be increased in size, although the differences with the control (PBS) group never reached statistical significance.

ALDH<sup>hi</sup> endometrial cells are resistant to cisplatin and sensitive to combination of cisplatin and IL6 signaling inhibitors

Finally, we tested the chemoresistance of endometrial ALDH<sup>hi</sup> cells and assayed the therapeutic potential of a combinatorial treatment comprising a conventional cytotoxic drug as cisplatin together with the above antibody or small-molecule inhibitors targeting the IL6 pathway.

To this aim, we chose the HEC1A line because of its well-known resistance to cisplatin (23), sorted it into ALDH<sup>hi</sup> and ALDH<sup>lo</sup> cells by FACS, and cultured them in the presence of control medium and of cisplatin (50 and 100 μmol/L). Notably, ALDH<sup>lo</sup> cells showed a striking resistance against cisplatin, whereas ALDH<sup>hi</sup> cells demonstrated high sensitivity to the same drug (Supplementary Fig. S3A). Next, we tested bulk HEC1A cells for their capacity to grow in the presence of the various the JAK1/STAT3 inhibitors and CD126 antibody as single agents and in comparison with cisplatin (Supplementary Fig. S3B). This allowed us to establish a baseline to evaluate the therapeutic efficacy of the combinatorial treatments. As shown in Supplementary Fig. S3C, HEC1A cells grown in the presence of combinations of cisplatin with either the CD126 antibody or the STAT3 and JAK1 inhibitors for 3.5 days and then cultured in control medium for 3 weeks were severely affected and were unable to regrow. A very different result was obtained with cisplatin alone (50 and 100 μmol/L) where, in agreement with the observations shown in Supplementary Fig. S3A, substantial regrowth was observed.

Discussion

Endometrial cancer is the most common gynecologic malignancy in the industrialized world and is generally characterized by a favorable outcome due to the early presentation of disease-related symptoms such as abnormal vaginal bleeding. At that time point, endometrial cancer is often confined to the endometrium without myometrial invasion and lymph node metastasis and can be treated by hysterectomy and bilateral salpingo-oophorectomy with or without adjuvant treatment with a 5-year survival rate of 86% (24). However, survival is poor in patients with recurrent or metastatic endometrial cancer (5-year survival rate, 15%; ref. 12). These patients additionally receive platinum-based chemotherapies (e.g., cisplatin and doxorubicin or carboplatin and paclitaxel).

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As for the majority of solid malignancies, endometrial cancer encompasses CSCs capable of self-renewal and differentiation (25). Endometrial CSCs have been prospectively enriched both as side population cells (26) and by the use of markers known to be associated with CSCs in other tumor types (25). Additional and more general methods, for example, the increased activity of detoxifying enzymes such as ALDH in stem cells, have been successfully applied to enrich CSCs in a broad spectrum of malignancies, including colon, ovarian, prostate, and breast cancer (3–5, 27). Our results indicate that ALDHhi/CD126+ cells from endometrial cancer cell lines and PDX are enriched in CSCs. Notably, we could not find any correlation between expression of CD133 and the ALDHhi subpopulation enriched in CSCs (Supplementary Fig. S2). The CD133-positive nature of IK cells was in agreement with previous observations (28).

We show that the ALDHhi CSCs are characterized by enhanced proliferation, which opens the possibility that other, possibly more quiescent CSC species are present within endometrial malignancies as previously observed for other tumor types (29).

To sort and quantify ALDHhi and ALDHlo cells from endometrial cancer cell lines and PDX, we have here preferred the enzymatic Aldefluor assay to ALDH1 IHC. The main advantage of Aldefluor resides in its functional nature able to detect ALDH activity from several aldehyde dehydrogenases whether IHC is mainly based on the protein encoded by the main gene ALDH1.

The CSC-ALDHhi connection is likely to be of clinical relevance, as increased ALDH1 gene expression was found to negatively correlate with overall survival in endometrial cancer (6). This was here confirmed by the inverse correlation found between ALDH levels and tumor differentiation with low-grade, well-differentiated tumors characterized by lower ALDH activity when compared with high-grade lesions (Fig. 1). Although the use of specific PR isoforms (rather than pan-antibodies) as differentiation markers in endometrial cancer has been debated in the literature (17, 18), it is generally accepted that an overall decreased PR/ER expression characterizes most poorly differentiated lesions.

The cellular activity and the relative size of the CSC subpopulation within a tumor are regulated, among others, by inflammatory signals from the microenvironment (30). In endometrial cancer, high expression levels of the inflammatory cytokine IL6 and of its downstream effector STAT3 are often found in therapy-inexperienced endometrial cancers where they represent poor prognosis indicators (11, 20). Notably, several studies implicate both IL6 and STAT3 as important regulators of self-renewal of CSCs in a broad spectrum of tumor types (31–36). IL6 represents the link between inflammation and malignant transformation, as it activates the NF-kB pathway that, on its turn, promotes IL6 expression in a positive feedback loop (37). Finally, activation of the IL6 signaling cascade results in the de novo generation of CSCs through induction of EMT (38). The latter is also in agreement with our previous study, where increased expression levels of IL6 and STAT3 were detected in endometrial tumors displaying EMT features (39). Our RT-qPCR analysis of several EMT-related genes in endometrial cancer cell lines identified differential expression between the ALDHhi and ALDHlo subpopulations of the HEC1A cell line only in the case of TWIST1 and TWIST2 (Supplementary Fig. S1). Notably, a previous study failed to find any correlation between the expression of EMT and CSC markers on a large cohort of primary endometrial cancers (40).

Hence, targeting the IL6 pathway in endometrial cancer holds therapeutic potential especially in view of the availability of IL6 blocking antibodies (e.g., tocilizumab; refs. 41, 42) and STAT3 inhibitors (e.g., ruxolitinib) that interfere with the upstream JAK2 (43). Our data reinforce and extend the therapeutic potentials of small-molecule inhibitors and antibodies targeting the IL6 pathway in endometrial cancer: we show that expression levels of the genes encoding for the IL6R CD126 and for its signaling transducer GP130 are significantly elevated in endometrial CSCs when compared with their non-CSC counterpart. Notably, the latter appears to have elevated IL6 gene expression that, in combination with the more CSC-specific expression of its receptor and signaling transducer, is suggestive of a dynamic equilibrium between CSCs and their more committed equivalents in endometrial malignancies based on IL6 secretion. Previously, it was shown that IL6 secretion mediates the dynamic equilibrium between CSCs and NSCCs (non–stem cancer cells) in a cellular model of breast cancer (44). IL6 is thought to maintain the relative size of these cellular subpopulations over many generations by converting NSCCs into CSCs, although it is unclear how the expression pattern of IL6 and its receptor CD126 and signaling transducer GP130 is distributed between the two subpopulations of breast cancer cells. Here, we show that expression of the IL6 gene is enhanced in more committed endometrial cancer cells, whereas CD126 and GP130 are mainly expressed in CSCs. Hence, paracrine IL6 secretion from endometrial NSCCs is likely to stimulate self-renewal of the CSCs endowed with the IL6R and signaling transducer. Vice versa, autocrine IL6 stimulation among endometrial NSCCs will be effective only in the minority of cells expressing CD126 and GP130, which are likely to function as a reservoir for de novo CSC formation.

We also show that the previously reported resistance of the HEC1A cell line to cisplatin (23) is mainly due to the CSC-enriched ALDHhi fraction (Supplementary Fig. S3). However, this CSC subpopulation can be efficiently targeted when cytostatic drugs such as cisplatin are combined with small molecules and antibodies directed against members of the IL6 pathway expressed by CSCs. Improved therapeutic efficacy of the combinatorial treatments was observed with a significant increase in cell death, thus compromising the ability of the cancer cell to regrow upon withdrawal of the selective pressure (Supplementary Fig. S3). Notably, similar results were obtained with RL95-2 using cisplatin in combination with either the STAT3 inhibitor nifurtumoxide or with the anti-CD126 antibody (data not shown).

CSCs coexist within primary malignancies together with other more committed subpopulations in a dynamic equilibrium, the mechanisms of which are to date poorly understood (45). Our results on the expression patterns of the IL6 ligand in NSCCs and of its signaling receptor and transducer in endometrial CSCs represent the first step toward the elucidation of the cellular and molecular mechanisms underlying the dynamic equilibrium among functionally distinct subpopulations of cancer cells. Moreover, our data offer novel therapeutic targets for patients with endometrial cancer with recurrent or metastatic endometrial cancer, often showing resistance to conventional chemotherapy. In these cases, combinatorial treatments where platinum-based cytotoxic therapies directed at the bulk of the tumor cells (e.g., cisplatin and doxorubicin or carboplatin and paclitaxel) are administered together with IL6 signaling inhibitors (e.g., tocilizumab) and/or STAT3 inhibitors (e.g., ruxolitinib) targeting the CSC component of the tumor and are likely to have improved therapeutic efficacy.
Disclosure of Potential Conflicts of Interest

C. Burger is a consultant/advisory board member for Roche Medical Company. No potential conflicts of interest were disclosed by the other authors.

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