

The stem cell markers Oct4A, Nanog and c-Myc are expressed in ascites cells and tumor tissue of ovarian cancer patients

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Abstract

Purpose The aim of this study was to examine the expression of established stem cell markers in ascites and tumor tissue obtained from ovarian cancer patients.

Methods Mononuclear cells present in ascites were collected by density gradient centrifugation. Intracellular flowcytometry was used to assess the putative presence of stem cell markers. RT-PCR was used to detect full length Oct4A, a splice variant Oct4B, implicated in glioma and breast cancer, Oct4 pseudogenes and c-Myc. Genes were cloned and sequenced to determine putative mutations. Confocal laser scanning microscopy was performed to localize the markers in ascites cells as well as in tumor tissue. Material from carcinomas other than epithelial ovarian carcinoma served as control.

Results A small quantity of cells in ascites and in tumor tissue of ovarian cancer patients was detected that expresses c-Myc, Oct4A and Nanog. Besides Oct4A, present in the nucleus, also the cytoplasmic resident Oct4B splice variant was detected. Remarkably, c-Myc was found partially in the cytoplasm. Since no mutations in c-Myc were found that could explain the cytoplasmic localization, we hypothesize that this is due an IL-6 induced c-Myc shuttle factor.

Conclusions The expression of stem cell genes was detected in a small proportion of tumor cells present in ascites as well as in tumor tissue. IL-6 plays an important role in the induction of c-Myc.

Keywords Cancer stem cells · Oct4 · c-Myc · Nanog · Ovarian cancer

1 Introduction

Ovarian cancer is a devastating disease with a high mortality and relapse rate. Due to late patient presentation, 70 % of the patients are diagnosed at an advanced stage with a five-year survival rate less than 30 %. Most patients respond well to primary treatment, either cytoreductive surgery followed by chemotherapy or chemotherapy followed by cytoreductive surgery. Notwithstanding this treatment, 80 % of the patients experience recurrent disease. Moreover, the development of a chemotherapy resistant tumor aggravates the situation. Evidence has been presented that cells enriched for tumor-initiating cells are less vulnerable to chemotherapeutic drugs. Increasing experimental evidence suggests that those tumor-initiating cells (TICs) play a decisive role in the initiation and progression of tumors. Consequently, targeting the TIC has been put forward as a new treatment modality [4, 30, 32, 33, 37]. However, TICs with distinct tumorigenic abilities have been identified [21, 48], as well as a large variation in their frequency [25, 45]. Several markers for TICs have been described, but those appeared to exhibit a wide variety of expression in tumors [18] and also to display opposite clinical outcomes even in the same tumor setting [9, 27, 46]. Moreover, TICs do not appear to be a stable entity but, instead, show plasticity [1, 10, 15, 29, 62]. The TIC compartment has been subdivided into long-term TICs, tumor transient amplifying cells and delayed contributing TICs [19]. Only the long-term TICs maintain tumor formation in serial xenografts, and these cells are considered as cancer stem cells (CSCs). The CSC is a rare cancer cell and is able to self-renew, giving rise to another malignant stem cell and a cell that undergoes

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massive proliferation and differentiation leading to the phenotypically and functionally more mature cancer cells [2, 3]. CSCs were originally described in leukemia [7], and later on in extended to solid tumors [3, 11, 21, 53]. All these studies demonstrated that isolated CSCs were capable of initiating primary tumors and metastases in immunodeficient mice.

However, for ovarian cancer the CSC concept has been challenged, albeit that ovarian CSC-like tumor initiating cells with a distinct tumorigenic capability have been identified [6, 54, 55]. Initially, ovarian CSCs were identified and isolated by using several cell surface markers from different ovarian tumor cell lines as well as primary tumor tissues [22, 57, 66]. Previously, we examined tumor cells from a large array of patients with different histological subtypes. The expression of the putative ovarian CSC markers CD24, CD44, CD117, CD133, ABCG2 and ALDH was tested. The expression of those markers was found to be diverse among patients, and none of them could be used as a general marker for CSC [18]. Consistently, Stewart and coworkers established a quantitative assay that enables the characterization of TICs from serous ovarian cancer, and they also found that the tumor initiating cell phenotype is heterogeneous among patients [54]. This suggests that CSC is an instable entity, a phenomenon called plasticity [24], just like a chameleon that changes its color depending on the circumstances [10, 15].

Our goal is to assess whether ovarian CSCs express established stem cell markers. As the tumor-initiating cells are composed of heterogeneous cells and currently characterized putative ovarian TICs may represent populations of tumor cells at different differentiation stages, we need to search for real stem cell signatures [19, 41]. A comprehensive approach for finding cancer stem cells is to focus on genes involved in pluripotency. These genes are known to maintain the pluripotent state of embryonic stem cells and are able to dedifferentiate somatic cells into induced pluripotent stem cells (iPS). Those genes may aid in the establishment of tumors and may be re-expressed by the malignant stem cells [40]. Since CSCs may derive from transformed normal stem cells or from differentiated progenitor cells that have acquired self-renewal, we asked whether the genes essential for the production of iPS induced pluripotent stem (iPS) cells are involved in tumor initiation. The first gene we tested, Oct4, is exclusively expressed in pluripotent stem cells and no iPS cell has been produced without using Oct4. Most importantly, it has been reported that 80 % of healthy people have natural immunity against Oct4, which makes it a suitable target for immunotherapy [14, 16]. This treatment potential requires additional research [31]. Subsequently, we tested c-Myc, which is also implicated in iPS cell induction [59] and Nanog needed for stem cell maintenance [39]. We found that Oct4, c-Myc and Nanog are expressed by a rare tumor cell population. And interestingly, we found that IL-6 is correlated with induction of abnormal c-Myc expression in the tumor cells.

2 Material and methods

2.1 Collection of the tumor cells and primary tumor sphere culture

Patients were informed and agreed to use their ascites and tumor tissue according to the local institutional board. Material is collected and assigned a number to protect the privacy of the patient according to local rules. Isolated cells are frozen and stored in liquid nitrogen. Samples are chosen from this collection when the diagnosis is high-grade primary ovarian cancer and the ascites is obtained before treatment. To get single cell suspensions, ascites was filtered through a 100 μ m cell strainer (BD Falcon™); tumor tissue was washed, minced with sterile blades to small pieces and dissociated by incubating 15 min at 37 °C in HBSS (Invitrogen) containing collagenase type IA (50 μ g/ml), DNase (10 μ g/ml) and trypsin inhibitor (1 μ g/ml). Mononuclear cells were then isolated from single cell suspension by centrifugation over Lymphoprep™ (Axis-Shield). Cells were plated in low adhesion plates and were maintained in KnockOut™ DMEM/F-12 medium (Invitrogen) supplemented with recombinant human EGF and FGF (10 ng/ml; R&D systems) at 37 °C with 5 % CO₂. Because mature cancer stem cells will not survive in this condition, enrichment for cancer stem cells is expected.

2.2 Reverse transcription PCR analysis

For reverse transcription PCR analysis of c-Myc, Oct4 and hydroxymethylbilane synthase (HMBS), total RNA was extracted from frozen ascites-derived mononuclear cells with the Quick-RNA™ MiniPrep (ZYMO RESEARCH) according to the manufacturer's instructions. Then RNA was treated with DNase I (Invitrogen) and reverse-transcribed into cDNA by using random hexamers and M-MLV reverse transcriptase (Invitrogen). Control samples were obtained without reverse transcriptase. c-Myc PCR was performed with Taq DNA polymerase (Bioke); while Oct4 and HMBS PCRs were performed with AmpliTaq Gold® DNA Polymerase (Applied Biosystems). The primer sequences are shown in Tables 1 and 2. The reaction mixtures recommended by the manufacturers were used. The initial denaturation step with Taq DNA polymerase was 3 min at 94 °C; with AmpliTaq Gold DNA Polymerase 10 min at 94 °C. The PCR thermal profiles are listed in Tables 1 and 2 (35 cycles). The final elongation for each reaction was 5 min at 72 °C. HMBS was used as control for cDNA quality. The positive controls were c-Myc full-length cDNA plasmid (BC000141, ImaGenes) and Oct4 full-length cDNA plasmid (BC117435, ImaGenes). The PCR products were separated on 1.5 % agarose gels, stained with 10000 times diluted Nancy 520 (Invitrogen) and visualized under UV light. Control RNA was obtained from dendritic cells

Table 1 Primer sequences used for PCR

Name	Primer sequence	Expected product (base pairs)		
c-Myc	Full-length	1FW 5'-gcagagggagcgcggg-3'	829	
		1RV 5'-ccagcttctctgagacgacg-3'		
		2FW 5'-accagagtttcatctcgcac-3'	881	
		2RV 5'-gacaggatgatgctgtgg-3'		
		3FW 5'-aacacacaacgtctggagc-3'	717	
		3RV 5'-ggctcaatgataattgcc-3'		
	Threonine 58 site	FW 5'-gcagagggagcgcggg-3'	829	
		RV 5'-ccagcttctctgagacgacg-3'		
	Nuclear Localization Signal	FW 5'-accagagtttcatctcgcac-3'	881	
		RV 5'-gacaggatgatgctgtgg-3'		
	Oct4	Full-length	FW 5'-catggcgggacacctggct-3'	1086
			RV 5'-cctcagtttgatgcatgggag-3'	
Isoform A		FW 5'-cttctgccccctccaggt-3'	496	
		RV 5'-aaatagaacccccagggtgagc-3'		
Isoform B		FW 5'-gtttatgttctacaagtcttc-3'	711	
		RV 5'-5' -cctcagtttgatgcatgggag-3'		
Nuclear Localization signal		FW 5'-accctgtgcccgtgaa-3'	526	
		RV 5' cctcgtgtgcatagtcg-3'		
HMBS		FW 5'-ggcaatgcgctgcaa-3'	64	
		RV 5'-gggtaccacgcgaatcac-3'		

that were generated in vitro from monocytes that were cultured with IL-4 and GM-CSF for 6 days.

2.3 Cloning and sequence analysis

PCR products were extracted from the gel with Nucleospin Extract II (MACHEREY-NAGEL) and inserted into a pGEM-T-easy Vector (Promega) according to the manufacturers' instructions. The constructs were transformed into DH5 α -competent bacteria by heat shock. Ampicillin-resistant and β -gal-negative clones were selected and amplified. Plasmids were isolated using the Nucleospin Plasmid QuickPure (MACHEREY-NAGEL) according to the protocol. The plasmids with an insert were sequenced with T7 and M13 reverse primers from two directions. The obtained DNA sequences were analyzed with the NCBI nucleotide blast program.

2.4 Flow cytometric analysis

Cells were fixed with 4 % paraformaldehyde for 4 min on ice, washed with PBA (PBS with 0.5 % bovine serum albumin and 0.01 % NaN₃) and then permeabilized with PBA containing 0.5 % saponin and 2 % human serum and incubated with antibodies for 30 min on ice: c-Myc AlexaFluor488 (clone 9E10, AbD Serotec), c-Myc AlexaFluor647 (clone 9E10, AbD Serotec), goat anti-human Nanog-NL493 (R&D Systems), goat anti-human/mouse Oct3/4-NL493 (clone 240408, R&D Systems). Antibodies were diluted in the permeabilization buffer. Before flow cytometric analysis the cells

were washed and resuspended in PBA solution. Cells were then examined using a CyAn™ ADP flow cytometer (BeckmanCoulter) and data was analyzed with FlowJo.

2.5 Immunofluorescence staining and microscopy

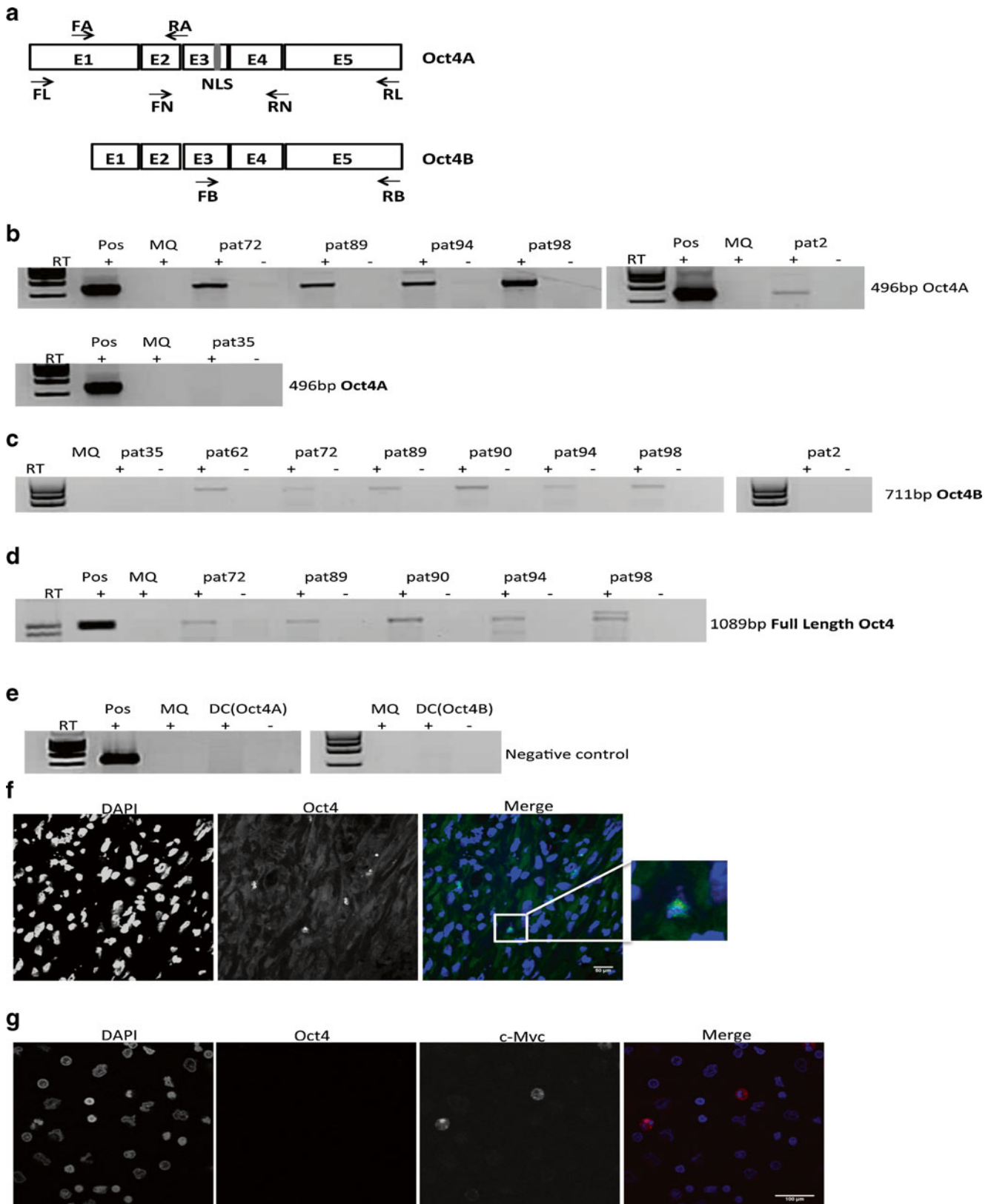
Ascites-derived tumor cells were rinsed in PBS, fixed with 2 % paraformaldehyde for 15 min, permeabilized with 0.1 % Triton X-100 for 5 min, and blocked with CLSM buffer (PBS containing 3 % BSA and 10 mM glycine) with 1 % human serum for 30 min at room temperature. Then cells were labeled with intracellular antibodies c-Myc Alexa Fluor 647

Table 2 PCR thermal profiles

Primer set	Denaturation (Temp/Time)	Annealing (Temp/Time)	Elongation (Temp/Time)
c-Myc full-length 1FW/RV	94 °C/1 min	62 °C/1 min	72 °C/1 min
c-Myc full-length 2FW/RV	94 °C/1 min	58 °C/1 min	72 °C/1 min
c-Myc full-length 3FW/RV	94 °C/1 min	58 °C/1 min	72 °C/1 min
c-Myc Thr58 site	94 °C/1 min	62 °C/1 min	72 °C/1 min
c-Myc NLS	94 °C/1 min	58 °C/1 min	72 °C/1 min
Full-length Oct4	94 °C/1 min	62 °C/1 min	72 °C/1 min
Oct4 A	94 °C/1 min	60 °C/1 min	72 °C/1 min
Oct4 B	94 °C/1 min	60 °C/1 min	72 °C/1 min
Oct4 NLS	94 °C/1 min	56 °C/1 min	72 °C/1 min
HMBS	94 °C/1 min	60 °C/1 min	72 °C/1 min

and Oct3/4-NL493 for 30 min. Nuclei were counterstained with DAPI. Appropriate isotype controls were stained in parallel for each primary antibody. After antibody labeling,

cells were adhered to poly-L-lysine-coated cover slips and fixated again with 2 % paraformaldehyde. Cover slips were mounted onto glass slides with Mowiol. Subsequently, cells



were examined by a laser scanning confocal microscope equipped with 60×/1.35NA oil UPLSAPO lens (Olympus FV1000), and the images were analyzed with OLYMPUS FLUOVIEW Version 1.6a Viewer.

2.6 Immunofluorescence staining and microscopy

Frozen tumor tissue embedded in embedding matrix (CellPath) was cut into sections of 4 μm, air dried, and fixed with 4 % paraformaldehyde. After rinsing with PBST (PBS containing 0.05 % TritonX-100), tissue sections were blocked in PBS containing 10 % normal donkey serum, 0.1 % PBST and 1 % bovine serum albumin for 20 min. Tissue sections were then stained 3 h at room temperature with c-Myc and Oct4 antibodies 1:5 diluted in blocking buffer followed by DAPI staining for 10 min. Cover slips were mounted onto glass slides with tissue sections using Mowiol and tissues were also examined using confocal scanning fluorescent microscopy (Olympus).

3 Results

3.1 Full length Oct4A is expressed in tumor and ascites-derived cells

Oct4 is an embryonic stem cell specific transcription factor. It is an important marker for undifferentiated embryonic stem cells and is expressed at high levels in undifferentiated embryonic stem cells and down regulated upon differentiation [36]. We first tested Oct4 expression at the mRNA level. Several studies showed that the different isoforms can lead to false positive signals during RT-PCR analysis [35, 67]. So, a dedicated primer set to distinguish the Oct4A, Oct4B and Oct4 pseudogenes was used [67]. The primer sets and exon usage are depicted in Fig. 1a. Oct4A expression was detected in ascites-derived tumor cells from eight out of eight patients tested, regardless of histological subtypes. Five representative samples are shown (Fig. 1b). One negative sample derived from a sarcoma patient (p35) is also

shown. We designed primers that specifically amplify a fragment of Oct4B, and found that seven out of seven tested patients do also express Oct4B. Six representative samples are shown (Fig. 1c). Two patients were found to be negative for Oct4B, one with a sarcoma (p35) and one with an endometrioid adenocarcinoma (p2) (Fig. 1c). The latter patient (p2) expressed Oct4A mRNA only (Fig. 1b and c). In addition to the main isoforms, the highly homologous pseudogenes of Oct4 may also cause artifacts in RT-PCR experiments [35, 67]. To test the presence of full-length Oct4A, we used a published primer set to amplify the entire coding sequence [67]. Indeed, a PCR product of full-length Oct4A was detected in all the patients tested (Fig. 1d). In some patients more bands were visible, indicating the expression of pseudogenes. Collectively, these results demonstrate the expression of the full-length Oct4A gene in ovarian tumor cells.

In order to exclude false positive results caused by genomic DNA contamination or aspecific binding of the primers, we took along cDNA from human dendritic cells (DCs) of a healthy donor as negative control. Indeed, we did not detect either Oct4A or Oct4B mRNA expression in DCs, excluding genomic DNA contamination in our procedure (Fig. 1e).

Using immunofluorescent microscopy for Oct4, we confirmed Oct4 protein expression in ovarian tumor tissue from all the patients tested ($n=4$). Remarkably, Oct4 is expressed in the nucleus as well as the cytoplasm (Fig. 1f). As a negative control we also performed immunofluorescent staining of the patient (p35) that did not express Oct4 mRNA, and consistently, Oct4 protein was undetectable (Fig. 1g). However, this patient does express c-Myc.

It has been shown that point mutations within the nuclear localization signal (NLS) of Oct4 yielded a mutant that localizes randomly throughout the cell and is defective in transactivating target genes [43]. To exclude that the presence of Oct4A in the cytoplasm is due to mutation of the NLS site, we designed primer sets that specifically amplify the Oct4 NLS coding sequence and performed RT-PCR analysis with RNA isolated from tumor cells. The PCR products were inserted into pGEM-T-easy vector and sequenced. Because no mutation was found (data not shown) this indicates that only Oct4B is present in the cytoplasm. Collectively, our results suggest that full-length Oct4A is expressed in rare ovarian tumor cells.

3.2 c-Myc is expressed in tumor and ascites-derived cells

We reasoned that other pluripotency genes may also be expressed in ovarian tumor cells. So, c-Myc was tested, a proto-oncogene that regulates functional gene and protein networks involved in tumorigenesis. We did RT-PCR analysis with three primer sets covering the entire coding sequence of c-Myc (Fig. 2a). We tested 12 patients of different histological subtypes, and 11 of them expressed the full-length c-Myc mRNA transcript. Two representative patients are

◀ **Fig. 1** Detection of Oct4 in ovarian tumor tissue and ascites-derived tumor cells. Total RNA is isolated from frozen tumor cells and reverse transcribed into cDNA. Oct4A-, Oct4B- and full length Oct4-specific primer sets were used to specifically amplify a fragment of Oct4A, full length Oct4B and full length Oct4A, respectively. **a** schematic drawing of exon organization of the Oct4 isoforms. *Arrows* show PCR primer positions. NLS indicates the nuclear localization signal. **b** Oct4A-specific PCR product. **c** Oct4B PCR product. **d** full length Oct4A PCR product. **e** RT-PCR analysis of Oct4 expression in human dendritic cells. Positive control was Oct4 plasmid, negative controls were obtained without reverse transcriptase during cDNA synthesis or using milliQ water as template during PCR. **f** expression of Oct4 protein in ovarian tumor tissue sections. Oct4 expression and its subcellular localization were determined by immunocytochemistry (*green*). Cell nuclei were counterstained with DAPI (*blue*). Scale bar is 50 μm. **g** Oct4 protein expression was not detected in patient 35 that did not express Oct4 mRNA. Scale bar is 100 μm

depicted in Fig. 2b. The c-Myc positivity was not due to genomic DNA contamination or aspecific binding of the

primers because the same RT-PCR reaction using cDNA from DCs of a healthy donor was negative (Fig. 2c).

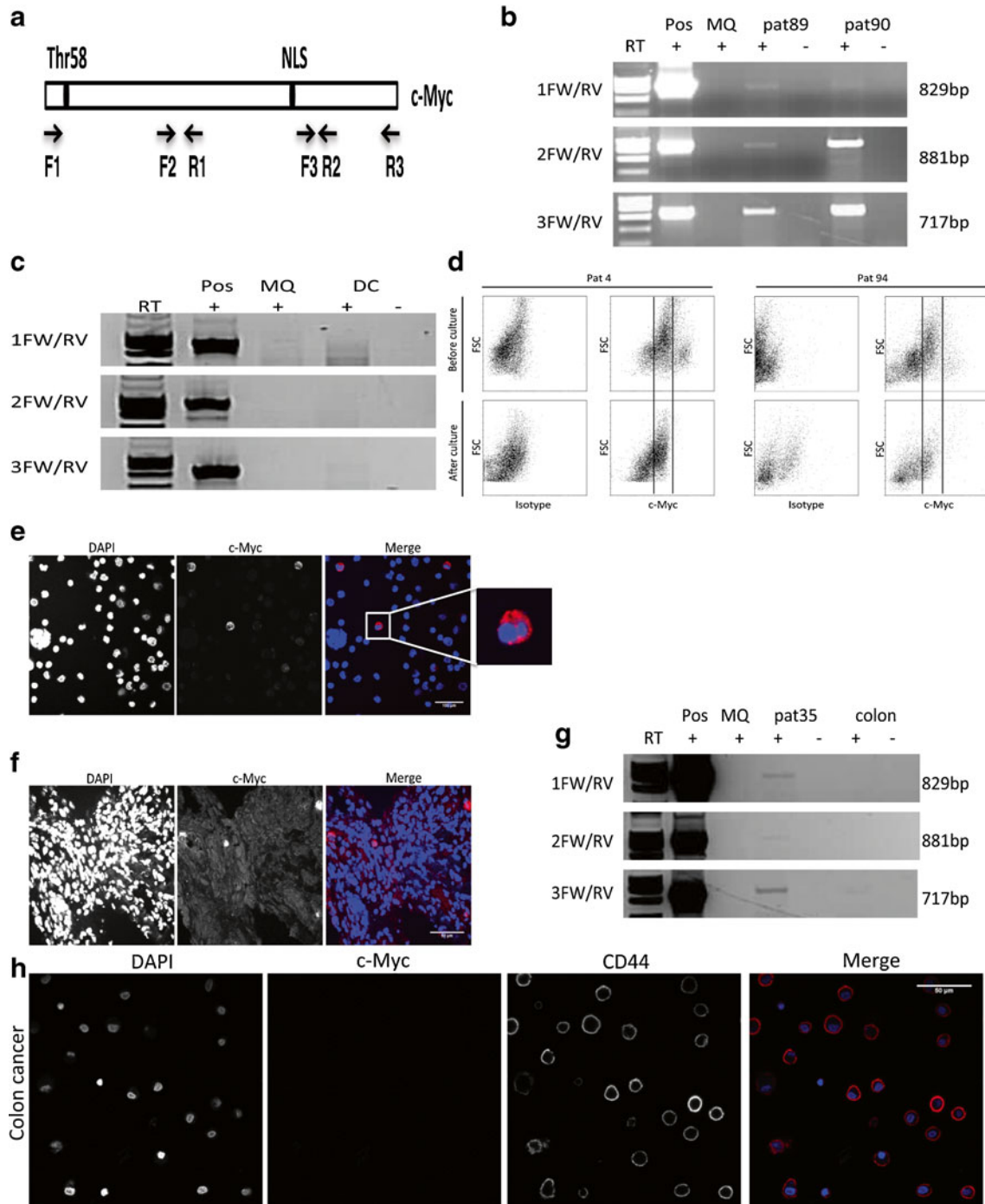


Fig. 2 Detection of c-Myc in ovarian tumor tissue and ascites-derived tumor cells. **a** three primer sets were designed to amplify the entire c-Myc coding sequence. NLS represents nuclear localization signal. **b** RT-PCR analysis of c-Myc expression in ascites cells. **c** RT-PCR analysis of c-Myc expression in dendritic cells from a healthy donor. Positive control was a c-Myc plasmid, negative controls were obtained without reverse transcriptase during cDNA synthesis or using milliQ water as template during PCR. **d** c-Myc expression in ascites derived cells at the onset and after in vitro culture using stem cell conditions measured by flow cytometry. **e**

c-Myc protein was detected in ascites cells using confocal microscopy, c-Myc was expressed in both nuclei and cytoplasm (red). DAPI was used to stain the nuclei (blue). Scale bar is 100 μ m. **f** c-Myc expression was confirmed by immunocytochemistry in tumor tissue sections. Scale bar is 50 μ m. **g** c-Myc expression as determined with PCR in ascites cells from a sarcoma and a colon cancer patient. **h** c-Myc protein was not detected in colon tumor cells using confocal microscopy. DAPI was used to stain the nuclei (blue). CD44-APC was used to stain the tumor cell surface. Scale bar represents 50 μ m

In addition to c-Myc mRNA, we also detected c-Myc protein by FACS in ascites-derived tumor cells as well as tumor tissue in almost all 50 patients tested. Interestingly, as depicted in Fig. 2d, intracellular staining showed that a small percentage of cells expresses very high levels of c-Myc while a major proportion expresses intermediate levels of c-Myc. The small population highly expressing c-Myc was always detected in freshly isolated tumor cells as well as cultured ones; an intermediate population was detected in fresh tumor cells but not always in cultured cells. Consistently, c-Myc expression in ovarian ascites cells as well as tumor tissue was detected by immunofluorescent microscopy ($n=6$, Fig. 2e and f). Furthermore, besides nuclear localization, c-Myc was also detectable in the cytoplasmic compartment of rare tumor cells. As shown in Fig. 2g and h, c-Myc was undetectable in a colon tumor specimen.

Next, we wanted to understand why c-Myc is abnormally expressed in the tumor cell cytoplasm, since c-Myc protein is normally located in the nucleus and is virtually undetectable in quiescent cells. Several possibilities for this aberrant cellular localization exist. Firstly, mutations could be present at the NLS site. It has been shown that complete removal of the c-Myc NLS resulted in mutant proteins that are distributed in both the nucleus and cytoplasm, but retained rat embryo cell cotransforming activity [12]. To test this, we designed primer sets that specifically amplify the NLS coding sequence and performed RT-PCR analysis with RNA isolated from tumor cells. The PCR products were inserted into pGEM-T-easy vector and sequenced. Five clones were sequenced from two patients but no mutation was found in the amplified sequences (data not shown).

Secondly, a mutation at the c-Myc Thr58 site leads to higher c-Myc levels, which are due to slower turnover of c-Myc protein. Phosphorylation of Thr58 induces c-Myc degradation. Normal turnover of c-Myc happens within 15–30 min, and mutations at the Thr58 site result in higher levels of c-Myc protein with enhanced transforming capability as shown in lymphoma [5]. However, after sequencing six clones containing the Thr58 PCR products from three patients, no mutation at this position was found (data not shown).

Finally, we investigated the effects of interleukin-6 (IL-6) on c-Myc expression in ovarian tumor cells. Yigit et al. showed previously that the IL-6 level is high in ascites of patients with malignant EOC, and that a positive correlation does exist between IL-6 concentration in ascites and tumorload after cytoreductive surgery [65]. Guided by those data, we cultured ovarian tumor cells with or without IL-6 in stem cell medium, and c-Myc and Oct4 expressions were measured after 2 weeks of culturing. As shown in Fig. 3, FACS analysis confirmed that c-Myc expression was upregulated in cells treated with a high concentration of IL-6 (150 ng/ml). In contrast, Oct4 expression was independent

of IL-6. From these results we can conclude that IL-6 sustains c-Myc expression in ovarian tumor cells in vitro.

3.3 Tumor cells express of Oct4, c-Myc and Nanog

Since ectopic expression of Oct4 and c-Myc are both needed in reprogramming somatic cells to induced-pluripotent stem cells [52], we hypothesized that they are co-expressed by the same cell population. We found rare cells expressing both Oct4 and c-Myc by FACS analysis (Figs. 3 and 4a). The c-Myc + Oct4+ cells account for a small percentage (~0.05–3.5 %) of the total cell population in ascites. After culture for 1–2 weeks in stem cell medium, we dissociated the tumor spheres using trypsin and performed the same analysis. Comparable results were obtained. Moreover, we also analyzed the expression of these two markers by immunofluorescent staining on sections of tumor tissue ($n=4$). Indeed, such rare double positive cells were found (Fig. 4b).

Next, we tested Nanog. Nanog is another key gene in the maintenance of pluripotency in mammalian cells. It cooperates with Oct4 and Sox2 to establish embryonic cell identity and its absence leads to cell differentiation and loss of pluripotency [38, 63]. Nanog expression was determined by FACS analysis and found to be expressed by the c-Myc expressing cell population (Fig. 4c). As cancer stem cells can originate from reprogrammed progenitor cells by regaining the self-renewal machinery, we considered the rare cell population in tumor tissue and ascites expressing Oct4, c-Myc or Nanog to be putative ovarian cancer stem cells.

3.4 Primary ovarian tumors form tumor spheres

To enrich for cells expressing stem cell markers, we established cultures from primary solid ovarian tumors and ascites. Culture conditions that favored stem cell growth were used. Regardless of histological subtypes, within 1–2 weeks of primary culture some of the tumors and ascites yielded a minority of cells that demonstrated growth into clusters, termed tumor spheres (Fig. 4d). During culture of the spheres only slight differences occurred in the percentage of c-Myc and Oct4 double positive cells (Fig. 4a). We never saw sphere cultures where Oct4, c-Myc and Nanog positive cells became the most prominent cells present, in agreement with a recently developed model to explain sphere culture [44].

3.5 Expression of CD24, CD44 and CD133 by Oct4/c-Myc positive cells

Triple staining was performed to elucidate the CD24, CD44 and CD133 expression by Oct4/c-Myc positive cells. Results from a patient having 0.2 % and one with only 0.02 % Oct4/c-Myc positive cells are depicted in Fig. 5. Only partial overlap of the markers is observed.

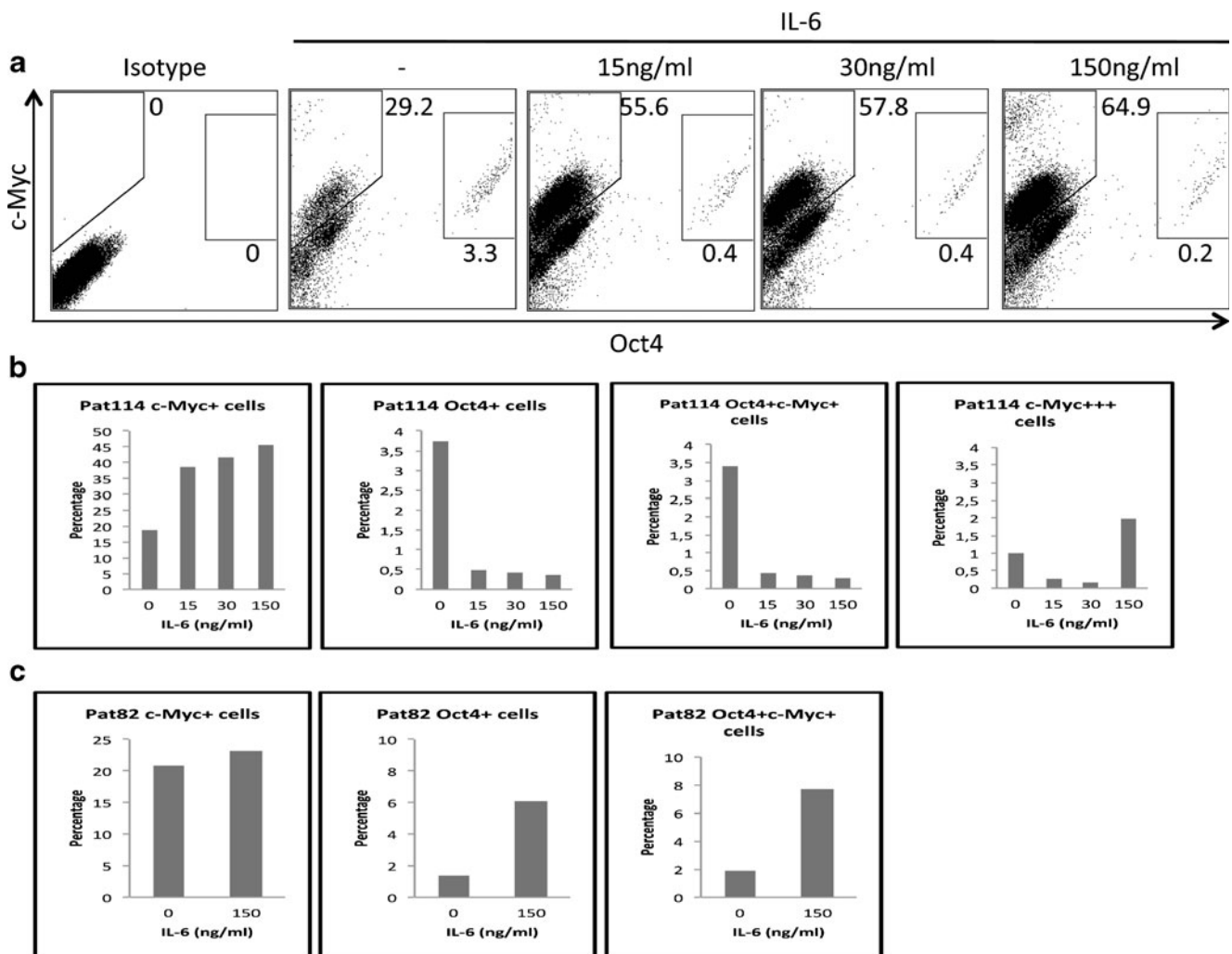


Fig. 3 c-Myc expression in IL-6 culture. Ascites-derived tumor cells were cultured with or without IL-6 in stem cell medium. After 2 weeks the cells were tested. **a** FACS analysis of Oct4 and c-Myc expression. **b**

and **c** two representative patients showing percentage of highly c-Myc expressing cells and c-Myc and Oct4 double positive cells after 2 week culture in different IL-6 concentrations

4 Discussion

The recent identification of growth and differentiation pathways responsible for cancer stem cell proliferation and survival will help in the discovery of novel therapeutic targets for immune elimination of CSCs. However, the existence of ovarian CSC is still heavily debated because no consistent expression of stem cell marker proteins in cancer stem cells has been found, leading to opposite conclusions [9, 13, 18, 23, 26, 27, 46, 57]. In our study, we identified a rare cell population that is positive for Oct4, c-Myc and Nanog present in primary ovarian carcinoma, and considered those cells as putative ovarian cancer stem cells.

Expression of Oct4 in cancer has been described before [58], and several studies have shown the Oct4 pseudogenes are transcribed in human cancers as well as tumor cell lines [56, 67]. However, due to the presence of different isoforms and expressed pseudogenes it is not clear which isoform is

actually expressed in older papers [67]. We show that full length Oct4 is expressed in ovarian cancer. Accordingly, Oct4 protein was detected in tumor tissue and ascites-derived tumor cells. Lee et al. showed that the subcellular localization of Oct4 isoforms differs. Oct4A is localized in the nucleus, whereas Oct4B is mainly expressed in the cytoplasm [34]. Furthermore, they also showed that Oct4A and Oct4B differ in their ability to confer self-renewal, i.e., only Oct4A can sustain stem cell properties [8, 34]. Indeed, we detected Oct4 visualized by a monoclonal antibody in both nuclei as well as cytoplasm in rare tumor cells. This indicates that the Oct4 signal detected in nuclei was Oct4A and in cytoplasm was Oct4B. The expression of Oct4A indicates that the cells are undifferentiated. Recently, Oct4 and Nanog were described to be higher expressed in side population cells obtained from ovarian cancer cell lines than the bulk of the cells, confirming the expression of stem cell markers as described here [61].

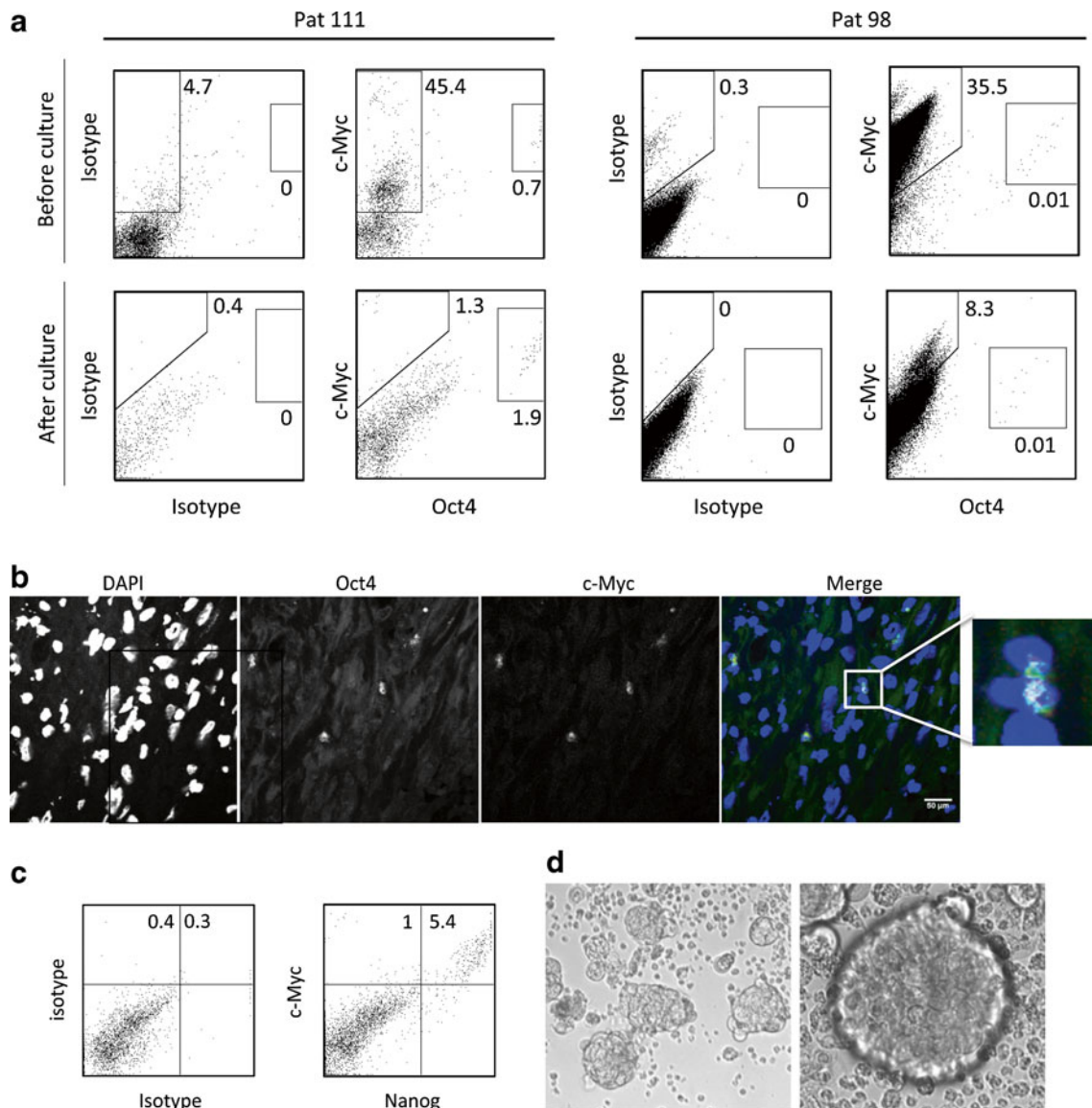


Fig. 4 Oct4, c-Myc and Nanog are expressed by the same population of tumor cells. **a** FACS analysis of c-Myc and Oct4 double positive cells at the onset and after 2 weeks culture under stem cell conditions. **b** Confocal imaging of Oct4 (green) and c-Myc (white) expression in ovarian tumor tissue. DAPI was used to stain the nuclei (blue). Scale bar

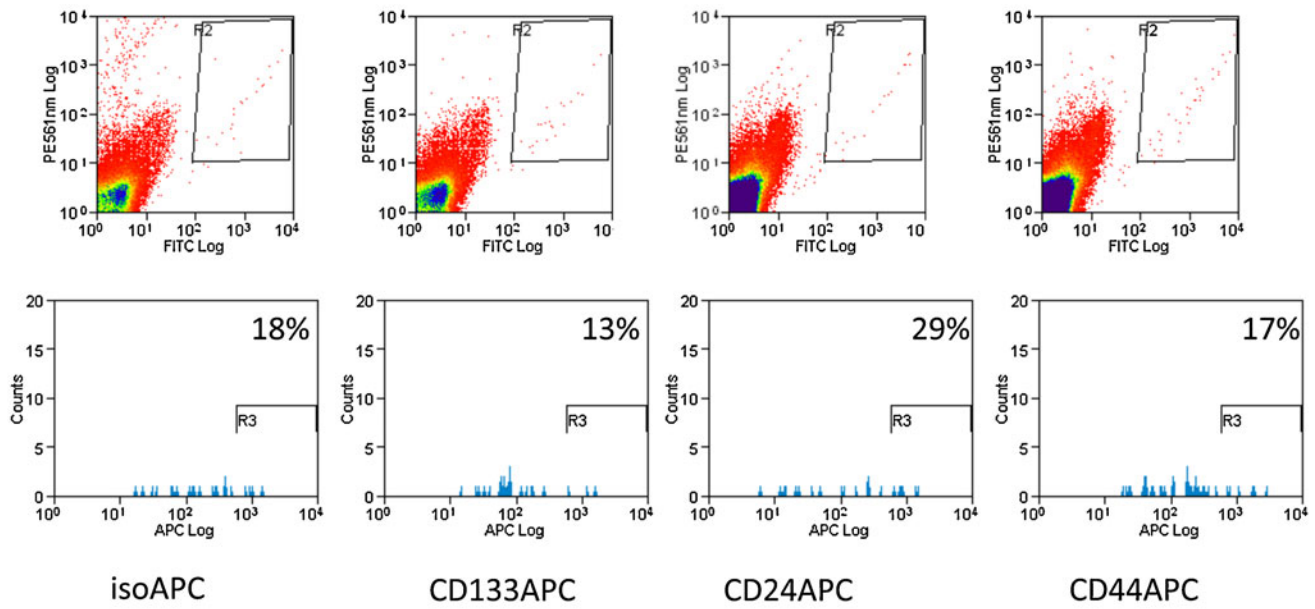
is 50 μ m. **c** FACS analysis of c-Myc and Nanog protein expression in tumor cells. Isotypes were used as negative controls. **d** primary tumor cells were cultured in serum-free DMEM/F-12 medium containing growth factors, EGF and FGF. After approximately 2 weeks tumor spheres were observed

Additionally, we also detected c-Myc and Nanog expression in the same cell. Also c-Myc and Nanog are important in maintaining the pluripotency of stem cells [20, 38, 60, 63]. Recently, the level of activation of Nanog was correlated with a CSC-like phenotype [42]. c-Myc protein is normally expressed in the nucleus and is virtually undetectable in quiescent cells. However, over-expression of c-Myc is detected in many human tumors, and is always associated with an aggressive and poorly differentiated state. c-Myc was also detected in the cytoplasm. We attribute this abnormal c-Myc expression to IL-6. Shi et al. showed that IL-6 can induce c-Myc translation in multiple myeloma cells and that c-Myc is shuttled to cytoplasm by the RNA-binding protein hnRNP A1

[50]. Regulation of stem cell genes or even tumor development by cytokine indicates a strong correlation between the tumor and its microenvironment [64].

Besides the cell population that is highly positive for c-Myc and Oct4, we also found an intermediate c-Myc + population in freshly isolated as well as thawed frozen tumor cells from almost all the patients tested. In contrast, after culturing in stem cell medium, the c-Myc intermediate population was undetectable anymore in some cases. We believe this is due to the sphere forming culture system. Serum-free medium allows for the maintenance of an undifferentiated stem cell state, and the addition of EGF and FGF induces the proliferation of multipotent, self-renewing and expandable stem cells

Pat172 OCT-4FITC, c-MYCPE

0.02% OCT4⁺ c-MYC⁺

Pat174 OCT-4FITC, c-MYC

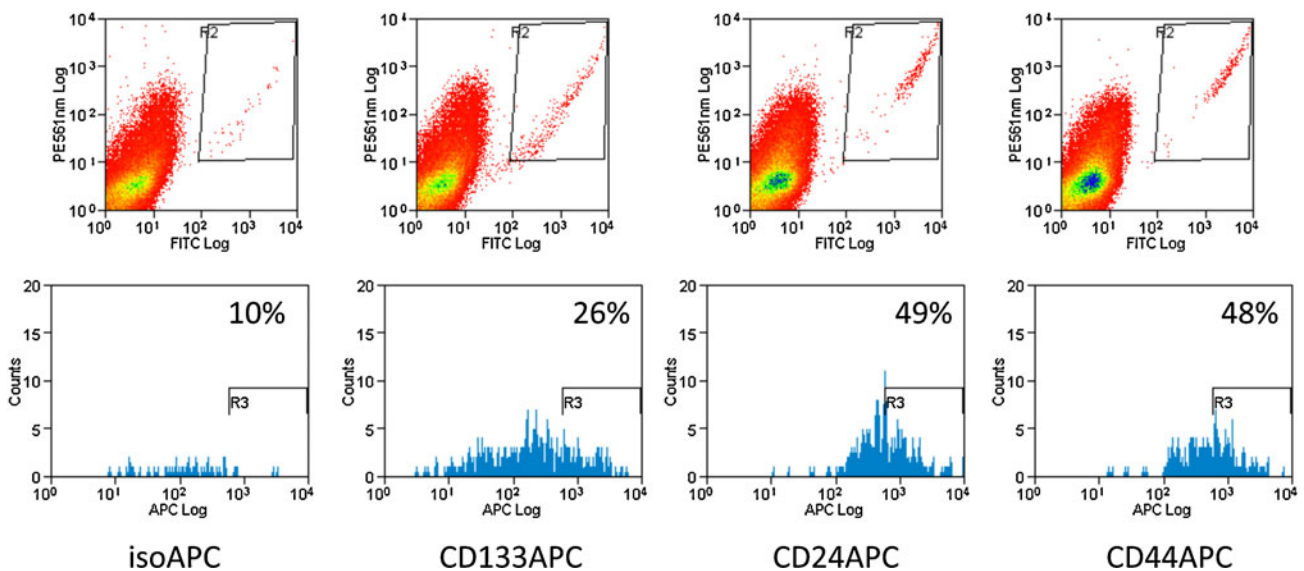
0.2% OCT4⁺ c-MYC⁺

Fig. 5 Triple staining of ascites derived tumor cells. Oct4/c-Myc cells were gated and their expression of CD24:CD44 and CD133 is shown in the second and fourth row. The results of two patients are depicted, one with a relatively high percentage Oct4/c-Myc positive cells and one with a very low percentage

[47]. We argue that the c-Myc⁺ cells represent more differentiated cells than c-Myc⁺ Oct4⁺ cells that are not able to survive in serum-free medium. Moreover, the percentage of c-Myc and Oct4 double positive cells remains stable before and after culture. In contrast, others using surface markers to identify ovarian CSCs showed a wide range of variation in the percentage of ovarian CSC bearing the surface marker CD44, or even phenotypic deterioration of CSC positive for CD133 [41, 54]. Moreover, in human colon cancer, tumor initiating cell populations with distinct tumorigenic capabilities were found [19]. We assume that ovarian cancer harbors a heterogeneous

population of tumor initiating cells, but that only the ones that express genes involved in maintaining pluripotency can drive tumor formation. On the other hand, in a few patients the c-Myc and Oct4 double positive cell population was very low before culture. After culturing in stem cell media a rare cell population (~1%) of tumor cells highly expressing c-Myc, Nanog and Oct4 was observed. This indicates that the tumor spheres are heterogeneous, consisting of both undifferentiated as well as differentiated cells. Similarly, others also confirmed the existence of a more differentiated population in spheroid cultures of tumor cells [41]. Due to the plasticity of cancer stem

cells [29], the c-Myc + Oct4+ cells obtained after culturing are probably derived from the intermediate c-Myc + cells. Only a partial overlap of Oct4/c-Myc was found with CD24, CD44 and CD133, extending previous results [17, 26, 27, 51].

In conclusion, we argue that the c-Myc, Oct4 and Nanog positive cells are putative ovarian cancer stem cells for several reasons: 1) Oct4A is only expressed in undifferentiated pluripotent cells and is down regulated upon differentiation [36]. Its expression by tumor cells may indicate that those malignant cells regain stem cell properties and remain undifferentiated; 2) Oct4A is the most important factor in producing iPS cells. Since CSCs may originate from reprogrammed progenitor cells, expression of Oct4A is a crucial step to become a functionally active stem cell; 3) c-Myc and Nanog are expressed alongside Oct4, all genes needed for reprogramming to induced pluripotent stem cells. c-Myc, Nanog and Oct4 upregulation may be due to regaining the self-renewal machinery in the tumor microenvironment; 4) the Oct4 + c-Myc + cells are rare and remain stable during culture in vitro. Due to lack of cell surface markers, we could not prove the c-Myc + Oct4 + Nanog + cells are tumorigenic in vivo by xenografting them into immunodeficient mice. However, our study provides a generally functional approach for the characterization and identification of CSCs from tumors. A putative solution could be to label the cells with a molecular beacon specific for Oct4A. It has been shown that cells can survive such a labeling procedure, but the labeling has to be fast to minimize breakdown of the beacon. Fast labeling was performed using a beacon coupled to a cell penetrating peptide, however no data are available to prove that those labeled cells still have retained their oncogenic properties [28, 49].

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