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DISSERTATION

Enrichment of cancer stem cells from head and neck cancer by anchorage independent culture and related research

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Abbreviations

7-AAD	7-Amino-Actinomycin D
ALDH1	Aldehyde dehydrogenase class 1
α-SMA	alpha-smooth muscle actin
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
CSC	cancer stem cell
DMEM	Dulbecco's Modified Eagled Medium
E-cadherin	Epithelial cadherin
EMT	Epithelial-to-mesenchymal transition
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
HNSCC	Head and neck squamous cell carcinoma
MET	Mesenchymal-to-epithelial transition
MMP	Matrix metalloprotease
PBS	Phosphate buffered saline
PE	Phycoerythrin
SDC	Spheroid-derived cell
SC	Stem cell
SOX2	SRY (sex determining region Y)-box 2
TGFβ	Transforming growth factor-beta
TF	transcription factor

Abstract

Accumulating evidence suggests that self-renewal and differentiation capabilities reside only in a subpopulation of tumor cells, termed cancer stem cells (CSCs) that can be identified by their aldehyde-dehydrogenase-isoform-1 (ALDH1) activity. We quantified and enriched ALDH1+ cells within HNSCC cell lines by an anchorage independent culture method and subsequently characterized their phenotypic and functional properties and epithelial-to-mesenchymal transition (EMT). On patients' tumor samples, we investigated the relationship of ALDH1 and α-smooth muscle actin (SMA) expression to metastasis of HNSCC. All cell lines formed spheroids that could self-renew and be serially re-passaged. ALDH1 expression was significantly higher in spheroid-derived cells (SDCs). ALDH1+ cells possessed self-renewal capability. SDCs had significantly higher invading activity. mRNA of the stemness-related transcription factor (TF) genes Sox2, Nanog, and Oct3/4 was significantly increased in SDCs of all cell lines. SDCs had a higher G0 phase proportion, showed high-level expression of α-SMA and Vimentin, but significantly decreased E-Cadherin expression. In 60 tumor samples from HNSCC patients, ALDH1 and α-SMA expression level was proven to closely correlate to lymph node metastasis. HNSCC-lines harbor potential CSCs, characterized by ALDH1 and stemness marker TF expression as well as properties like invasiveness, quiescence, and EMT. CSCs can be enriched by anchorage-independent culture techniques, which may be important for the investigation of their contribution to therapy resistance, tumor recurrence and metastasis.

ZUSAMMENFASSUNG

Neue Daten zeigen, dass die Selbsterneuerungs- und Differenzierungskapazität nur in einer kleinen Subpopulation von Tumorzellen, genannt Krebsstammzellen (cancer stem cells, CSC) vorkommt, die durch ihre Aldehyddehydrogenase-Isoform-1 (ALDH1) Aktivität identifiziert werden kann. Wir haben ADLH1+ Zellen aus HNSCC Zelllinien quantifiziert, durch adhäsionsunabhängige Kulturmethoden angereichert und anschliessend ihre phänotypischen und funktionellen Eigenschafen sowie deren Epithel-zu-Mesenchym Übergang (EMT) charakterisiert. An Patientenproben haben wir die Beziehung zwischen ALDH1 und α-smooth muscle actin (SMA) Expression mit der Metastasierung von HNSCC untersucht.

Alle Zelllinien bildeten Spheroide, die selbsterneuernd und seriell passagierbar waren. ALDH1 Expression war signifikant höher in als Spheroide gewachsenen Zellen (spheroid-derived cells, SDCs). ALDH1+ Zellen besassen die Fähigkeit zur Selbsterneuerung. SDCs hatten signifikant höhere Invasionsaktivität. mRNA der „stemness"-vermittelnden Transkriptionsfaktorgene (TF) Sox2, Nanog und Oct3/4 war signifikant erhöht in SDCs aller Zelllinien. SDCs hatten einen höheren Anteil an Zellen in Go Phase, hatten eine höhere Expression an α-SMA und Vimentin, aber signifikant reduzierte E-Cadherin Expression. In Tumorproben von 60 HNSCC Patienten konnte eine enge Korrelation zwischen ALDH1 und α-SMA Expressionshöhe zum Lymphknotenmetastasierungsstatus gezeigt werden. HNSCC Zelllinien beinhalten potentielle CSCs, die durch ALDH1 und Stammzelltranskriptionsfaktorexpression sowie Eigenschaften wie Invasivität, proliferative Quieszenz und EMT charakterisiert werden. CSC können durch Adhäsions-unabhängige Zellkulturtechniken angereichert werden, was für die Erforschung ihres Beitrags zu Therapieresistenz, Tumorrezidivierung und Metastasierung wichtig sein kann.

Introduction

1.1. Squamous cell carcinoma of head-and-neck

Cancers that are known collectively as head-and-neck cancers usually begin in the squamous cells that line the moist, mucosal surfaces inside the head and neck. These squamous cell cancers are often referred to as head-and-neck squamous cell carcinomas (HNSCC). Cancers of the head and neck are further categorized by the area of the head or neck in which they begin (for example: nasal cavity, sinuses, lips, mouth, throat, or larynx). Alcohol and tobacco abuse are the most common risk factors for head-and-neck cancer. HNSCC are the sixth most prevalent type of malignancy worldwide[1]. Despite advances in therapy, which have improved quality of life, survival rates have remained static over the past decades. Mortality from this disease remains high due to the development of distant metastases and the emergence of eventually inoperable local and regional recurrences that have low responsiveness to radiation- or chemotherapy. Therefore, despite significant improvements in surgery, radiation- and chemotherapy, long-term survival rates in patients with advanced stage HNSCC have not significantly increased in the past 30 years [1]. Even in the case of stage I disease, where 90% of patients can be cured, 10% relapse with fatal outcome. In more advanced stages, the subset of patients who fail to respond to therapy or suffer from recurrences increases, for so far unknown reasons. It is, therefore, desirable to develop a deeper understanding of the biology of this disease to adapt current therapeutic strategies and to develop therapies that are more effective.

1.2. Cancer stem cells in HNSCC

Cancer stem cells (CSCs) are defined by their ability to (i) fully recapitulate the tumor of origin when transplanted into immune deficient mouse hosts, and (ii) self-renew, demonstrated by their ability to be serially transplanted. These properties suggest that CSCs are required for tumor maintenance and metastasis. Thus, it has been predicted that CSC elimination is required for cure. This prediction has profoundly altered paradigms for cancer research, compelling investigators to prospectively isolate CSCs to characterize the molecular pathways regulating their behavior.

In head-and-neck cancer, Prince et al. were the first to demonstrate that the population of HNSCC cells possess the properties of cancer stem cells [4], but a relatively high number of CD44+ cancer cells (>5000 cells) were needed to initiate new tumors in immune deficient mice. Moreover, in the head and neck CD44 and CD44v6 expression does not distinguish normal from benign or malignant epithelia. CD44s and CD44v6 were abundantly present in

the great majority of cells in head-and-neck tissues, including carcinomas [5]. Thus, identification of more specific CSC markers for HNSCC is necessary. Recently, in breast cancer, lung cancer, hepatoma, head-and-neck, and colon cancer high aldehyde dehydrogenase 1 (ALDH1, also known as ALDH1A1) activity was shown to identify the CSC [6; 7; 8; 9; 10]. However, in breast cancer the ALDH1+ population shows a surprisingly small overlap with the previously described CD44+/CD24−/low phenotype (0.1–1.2%). The cells bearing both phenotypes appeared to have highly enhanced tumorigenicity, being able to generate tumors from as few as 20 cells [6]. CD133 was also proven to be a candidate CSC maker for HNSCC [11]. It remains to be determined if there is also a small overlap with stem cell markers in HNSCC.

1.3. Epithelial-to-mesenchymal transition (EMT)

EMT is an important process during normal embryonic development and wound healing [12; 13; 14; 15] and allows epithelial cells to undergo morphological changes. These changes lead to the breakdown of cell–cell and cell–extracellular matrix connections, allowing cell migration to other locations in the body during tissue reconstruction and repair [16]. EMT is also recognized to be important in cancer cell migration/metastasis and has been linked to progression in several types of cancer [13; 14; 17; 18; 19]. In HNSCC, overexpression of EMT-associated transcription factors (e.g. Twist and/or Snail) have been observed [20; 21]. Nijkamp et al. showed a putative association of loss of E-cadherin and gain of vimentin with enhanced migration of tumor cells and increased metastatic risk in HNSCC patients using immunohistochemistry (IHC) staining [22]. DNA microarray analysis comparing 75 genes predictive of disease in high-risk and low-risk groups of HNSCC found that genes involved in EMT and nuclear factor-kB (NF-kB) signaling were the most prominent molecular characteristics of high-risk tumors [23].

1.4. EMT and cancer stem cells

Mani et al. first showed the connection between EMT and CSC by demonstrating an up-regulation of stem cell markers through the induction of EMT in mammary epithelial cells and breast cancer cells [24]. In the past years, additional groups have demonstrated that the induction of EMT not only promotes tumor cell invasion and metastasis, but also contributes to drug resistance and enriches for cells with the phenotype and properties of CSCs [17; 18; 24; 25; 26; 27; 28]. Biddle et al. reported CSCs in HNSCC that can switch between two phenotypes: migratory CD44+/EpCAM^{low} EMT CSCs expressing EMT markers and a

mesenchymal phenotype, and proliferative CD44+/EpCAM^{high} non-EMT CSCs with epithelial characteristics [29]. To metastasize successfully, EMT CSCs required an ALDH+ phenotype. The group proposed a model where HNSCC CSCs are able to migrate to other locations in the body using the EMT phenotype, where the ALDH+ cell fraction is capable of reverting back into the non-EMT phenotype by the reverse process of MET (mesenchymal-to-epithelial transition); these MET cells can then form the metastasis through proliferative activity. This model may also apply to our HNSCC cultures. Another mediator of EMT is the pro-inflammatory factor S100A4 which is involved in the regulation of a variety of biological effects including cell motility, metastasis, angiogenesis, differentiation and survival [30]. In HNSCC cell lines, overexpression of S100A4 resulted in an EMT phenotype, and enhanced stem cell-like properties, migration and invasion. Conversely, knockdown of S100A4 reduced their self-renewing and tumorigenic properties in vitro and in vivo [31]. Overexpression of the putative CSC marker CD133 was also reported to enhance tumor stemness and the tumorigenic potential of HNSCC cell lines [32]. Moreover, up-regulation of CD133 increased phosphorylation of Src and induced EMT transformation, suggesting CD133/Src signaling to be a regulatory switch for EMT and cancer stem-like cell properties in HNSCC [32].

1.5. Anchorage independent culture

The ability of spheroid formation under anchorage independent culture is increasingly being used to evaluate stem cell activity in normal tissue and putative CSC. They rely on the ability of most stem cells of epithelial origin to grow anchorage independently in suspension forming clusters of cells on non-adherent surfaces. The neurosphere is the best-studied sphere assay. The central nervous system cells grown on non-adherent surfaces give rise to neurospheres that have the capacity for self-renewal and can generate all of the principal cell types of the brain [33; 34]. The capacity for repeated generation of neurospheres from single cells is generally viewed as evidence of self-renewal [35]. Recently, spheroids isolated from gliosarcoma rat cell lines were also shown to possess cancer stem-like cells [36]. There is also emerging evidence that spheroids grown from HNSCC cell lines or even primary tumor are enriched for CSC. To generate spheres, single cells were seeded in ultra-low attachment plates at a low density. After five to seven days, representative spheroids formed and could be collected for following experiments. However, it has to be noted that spheres still consist of heterogeneous populations, which are enriched in CSC but are not entirely pure and can not be produced from any cell line.

2. Objectives

Heterogeneity of tumor tissue has been accounted for in recent years by a hierarchy-based model in which CSCs have the ability both to self-renew and to give rise to differentiated tumor cells and are responsible for the overall organization of a tumor. Research into CSCs has progressed rapidly and concomitantly with recent advances in the biology of normal tissue stem cells, resulting in the identification of CSCs in a wide range of human tumors. Many efforts have been made to isolate or enrich CSCs based on functional and phenotypic properties of target cells. Recently, EMT has been firstly reported to play an important role in initiating CSCs in breast cancer, but the correlation between EMT and CSCs in head-and-neck cancer remained unclear. In the present work, we tried to prove if anchorage-independent cell culture techniques allow the generation of spheroid-cultures and if these cultures were enriched for cells with functional and phenotypic properties characterizing CSC of HNSCC. Here, we also provide evidence that myofibroblasts can be derived from HNSCC using a spheroid cell culture model that enriches for CSC-like cells as characterized by a high proportion of ALDH1 positivity, proliferative quiescence, and invasive capacity.

Material and Methods

3.1. Cell lines

The HNSCC cell line panel was composed of UD (University of Düsseldorf, Dr. Henning Bier) -SCC -1, -2, UM (University of Michigan, Tom Carey) -SCC -11B and UT (University of Turku, Dr. Reidar Grenman) -SCC -9, -22, -24A. The suffixes A, B, and C indicate cell lines derived from the primary tumor (A), metastatic (B) or recurrent (C) disease. These immortal HNSCC lines have been in culture from primary tumor for approximately 40 to 60 generations.

3.2. Suspension culture for spheroid formation

Adherent monolayer cells were grown in normal 25 cm^2 culture flasks in DMEM supplemented with GlutaMAX^{TM}-I (Gibco, Paisley, UK), 1x containing 10% heat inactivated FBS and 1% Penicillin/Streptomycin, until confluency. Cells were washed with PBS twice and detached using Trypsin/EDTA solution (Biochrom AG, Berlin, Germany). The reaction was stopped by adding complete culture medium. After 5 minutes, cells were resuspended in serum-free Quantum 263 (PAA, Cölbe, Germany) medium, supplemented with 10 ng/ml EGF and 10 ng/ml bFGF (Biochrom AG, Berlin, Germany). To generate spheroids, single cells were plated in Ultra-Low Attachment plates at a density of $2x10^4$ cells/ml. Cells were kept in the incubator at 37°C in a humidified atmosphere with 5% CO_2 content. At day three, half of the medium was replaced. After 5 to 7 days, representative pictures were taken and spheroids were collected by filtration through a 40 μm mesh (BD Biosciences, Heidelberg, Germany) for the following experiments.

3.3. Multicolor flow-cytometric (FACS) analysis and sorting

The ALDH activity of spheroid- and monolayer-derived cells was determined by using the Aldefluor assay kit (StemCell Technologies, Durham, NC, USA) and all the manipulation was followed by manufacturer's instruction. Next, for cell surface antigen phenotyping, cells were stained with 20 μl anti-CD24-PE, 20 μl anti-CD44-APC and 5 μl 7-AAD (BD bioscience, San Jose, CA, USA) or 5 μl anti-E-Cadherin (Biolegend, San Diego, CA, USA) on spheroid-derived cells (SDC) and monolayer-derived cells (MDC) and analyzed by flow cytometry, respectively. For ALDH1+ cells FACS sorting, cells were separated on an Aria cell sorter (BD Bioscience) and cells treated with DEAB were used as negative control.

3.4. Phenotyping stemness of SDC in vitro

3.4.1 Clone formation assay

After FACS sorting, the $ALDH1^+$ and $ALDH1^-$ cells were inoculated to 6-well plates at a density of 1000 cells/ml in DMEM medium supplemented with 10% FBS. Cells were cultivated under standard culture conditions for 2 weeks and then the number of colonies was counted microscopically.

3.4.2 Semi-quantification of stemness-related gene expression in SDC

Total RNA was extracted by using a Qiagen RNeasy kit, then converted to cDNA with the Omniscript First-Strand synthesis system using random primers. qRT-PCRs were carried out using ABI Power SYBR Green mix and run on a BioRad Chromo4 (BioRad, Hercules, CA, USA). Reactions were carried out in triplicate with RT controls, the gene of Ribosomal protein HL32 was used as a reference gene, and data were analyzed using the modified delta delta Ct method. Primer sequences are listed in Tab.1.

Table 1: Primer sequences used in Realtime-PCR

Transcript name	Forward primer sequence	Reverse primer sequence
SNAIL1	GGCGCACCTGCTCGGGGAGTG	GCCGATTCGCGCAGCA
Nanog	AATACCTCAGCCTCCAGCAGATG	TGCGTCACACCATTGCTATTCTTC
SNAIL2	GGGGAGAAGCCTTTTTCTTG	TCCTCATGTTTGTGCAGGAG
Twist1	GGAGTCCGCAGTCTTACGAG	TCTGGAGGACCTGGTAGAGG
Oct3/4	GACAGGGGGAGGGGAGGAGCTAGG	CTTCCCTCCAACCAGTTGCCCCAAAC
Sox2	GGGAAATGGGAGGGGTGCAAAAGAGG	TTGCGTGAGTGTGGATGGGATTGGTG
hL32 (reference)	AGCTCCCAAAAATAGACGCAC	TTCATAGCAGTAGGCACAAAGG

3.4.3 Invasion assay

Single cell suspensions of SDC and MDC were resuspended in 1% (w/v) BSA-DMEM culture medium and seeded into the upper compartments of BD BioCoat^{TM} Matrigel Invasion Chambers (BD Bioscience)), and DMEM supplemented with 10% FCS was added to the lower compartment according to manufacturer's instructions. The invasion chamber was kept for 24 h under standard culture conditions. After incubation, the non-invading cells were removed from the upper surface of the membrane by gentle scrubbing, and the cells on the lower surface of the membrane were stained with crystal violet. Cell counting was facilitated by photographing the membrane through the microscope and 3 fields per membrane of triplicate membranes were counted at 200x magnification.

3.4.4 Cell cycle analysis and Ki-67 staining

The parental cells and spheroids were harvested, dissociated into single cells and resuspended in 70% ethanol by adding ethanol dropwise at −20°C while vortexing. The cells were stored at −20°C overnight then washed with PBS, centrifuged and 1 μg/ml FITC-conjugated mouse anti-human Ki-67 (DAKO, Glostrup, Denmark) was added to the cell pellet and incubated for 20 min at room temperature. Finally, cells were resuspended in 1 ml of a cocktail containing propidium iodide (2 μg/ml) and RNAse (100 μg/ml) (Fermentas Life Science, St. Leon-Rot, Germany) and stained for 30 min followed by FACS analysis.

3.4.5 Immunofluorescence

Indirect immunofluorescence staining was performed on spheroids and on parental adherent cultures of cancer cells for α-SMA and Vimentin. Spheroids were collected by filtering the suspension culture medium through a 40 μm mesh (BD Bioscience), and then whole spheroids were sedimented by cytospin (1500 rpm, 5 min) onto slides. Cells were fixed in 4% (w/v) paraformaldehyde then permeabilized with 0.1% Triton X-100 in PBS solution for 10 min. First, mouse anti-human Vimentin (1:50 from stock; Santa Cruz Biotechnology, Heidelberg, Germany) and mouse anti-human α-SMA diluted (1:50 from stock; Dako) antibodies were added and incubated overnight at 4^{o}C. Then the secondary antibody, Alexa488-conjugated goat anti-mouse IgG (Invitrogen, Karlsruhe, Germany) or Alexa568-conjugated donkey anti-mouse IgG (1:200 from stock) (Invitrogen), was added and incubated for 60 min at room temperature. Counterstaining was performed by using DAPI (1 μg/ml Roche, Mannheim, Germany). For quantification, the average percentages of positive cells from three experiments were estimated. Due to superposition in the case of SDC, cell proportions are less reliable. Therefore, no significance was calculated for Vimentin and α-SMA.

3.4.6 Immunohistochemistry Double Staining

ALDH1 and α-SMA double staining was performed on paraffin sections of HNSCC and the diagnoses were confirmed by pathologists. All the sections were deparaffinized in xylene then hydrated with an ethanol gradient. The anti-human α-SMA (1:50 from stock; Dako) and anti-human ADH1 (1:100 from stock; BD Bioscience) antibodies were incubated with sections for 1 h at room temperature, respectively, and the staining process was performed by using Dako EnVision™ doublestain system, all the manipulations were followed by manufacturer's instructions. The score was given by counting the positively staining cells' percentage by microscopy (x400), the positive cells ≤10% counted as negative (-), 11%—50% as weak

positive（+, 51%—75% as positive (++) and >75% as strong positive (+++). Staining ≥ (++) was evaluated as high expression.

4. Results

4.1. Anchorage independent culture can enrich cells with stem cell characteristics from HNSCC cell lines

All five HNSCC-derived cell lines (UD-SCC1, UT-SCC22, UMSCC11B, UT-SCC9, UT-SCC24A) were proven to be able to form spheroids ranging from 50 to 500 cells per spheroid in serum-free medium under non-adherent conditions. The SDCs could form new spheroids even at a clonal density of 1,000 cells per ml. In contrast, the parental monolayer cell cultures grown under the same conditions did not form spheroids. When the spheroids were transferred back to a regular tissue culture flask, they could regrow to a confluent monolayer which was identical to the parental cell lines (Fig. 1). By using Matrigel invasion chambers, we found that the SDCs showed increased (2.1 to 8.6 fold higher) invading activity compared to their parental cells. It was reported that Oct4, Sox2, and Nanog, which form a self-organized core of transcription factors (TF), maintain pluripotency and self-renewal of human embryonic stem cells. Real-time PCR testing proved that mRNA levels of Oct3/4, Sox2, and Nanog were all significantly increased in SDCs (Fig. 2). Furthermore, we analyzed the cell cycle by FACS and the data showed that in all 5 cell lines, the proportion of cells in the G0 phase showed a highly significant increase in SDCs, indicating that SDCs contain a higher proportion of quiescent cells than parental MDCs.

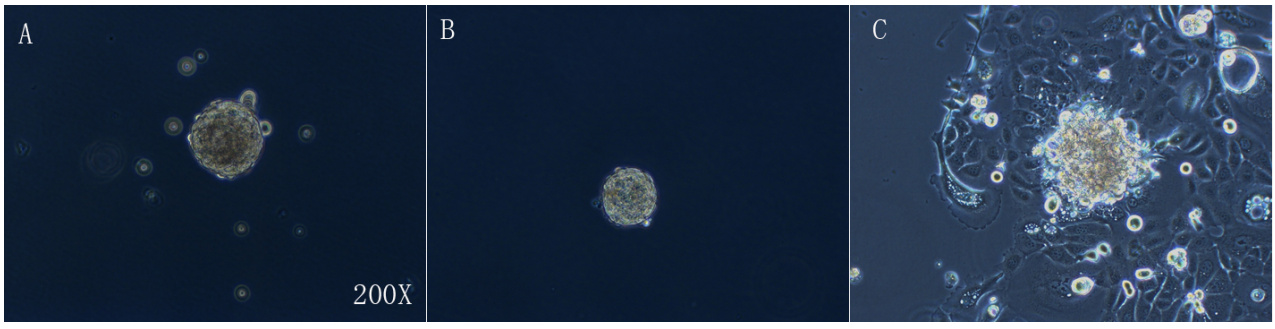

Figure 1: Spheroids generated from HNSCC lines in serum-free medium have self-renewing capacity. Representative pictures of the cell line UD-SCC1 are shown. (A) The first generation of spheroids. (B) A subspheroid formed after seeding at a concentration of 1,000 cells/ml. (C) Spheroid adhering and growing to confluence after replating into flasks coated for tissue culture [37].

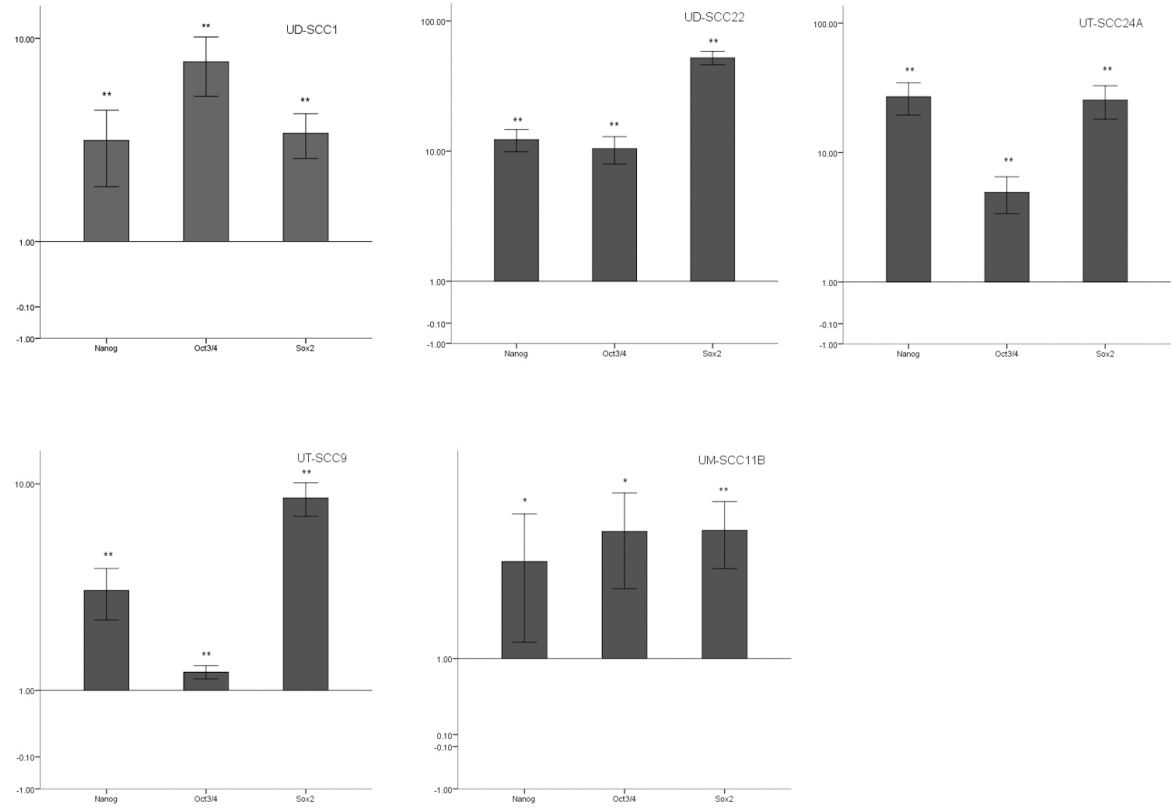

Figure 2: Quantitative PCR analysis of mRNA expression of stemness-related transcription factors. Messenger RNA isolated from spheroids and monolayer cultures was quantified for expression of the indicated TF. The ratio of expression in spheroid to monolayer cells is given. Significant differences were * $p<0.05$ and ** $p<0.01$. The mRNA level of stemness-related TF Nanog, Oct3/4, and Sox2 were generally increased in spheroids[37].

4.2. Phenotypic characterization of SDC

The expression of the putative stem cell marker ALDH1 and CD44/CD24 were analyzed by multicolor FACS-analysis. Interestingly, all SDC had an increased number of ALDH1 positive cells as compared to parental cell lines. The highest proportion of ALDH1 cells was found in spheroids generated from the UD-SSC1 cell line (46.4±8.1%), which was 5-fold higher than the corresponding parental cell line (9.4±5.3%) (Fig. 3). We also investigated the CD44 and CD24 expression in HNSCC cell lines and found that the proportion of CD44+/CD24- cells is highly variable (0.5–97%). The overlap with ALDH1+ cells was small (0–2.2%) except for the two cell lines UM-SCC11B and UT-SCC22 which had an overlap of about 33% and were composed to over 95% of CD44+/CD24- cells. Interestingly, the proportion of CD44+/CD24- cells was not consistently enriched by the spheroid culture method.

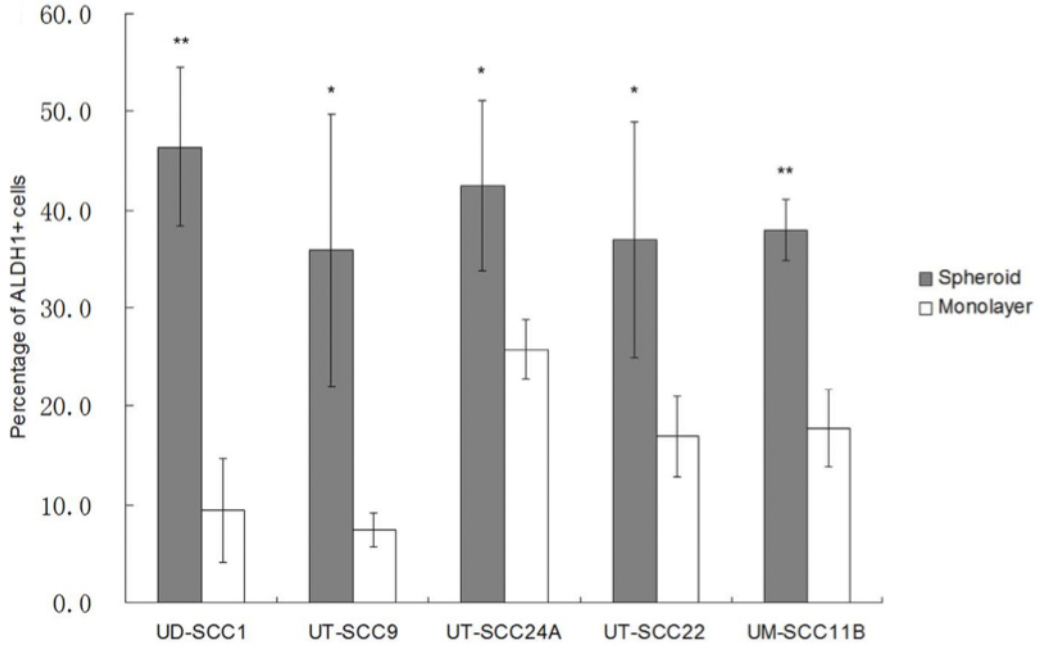

Figure 3: ALDH1 expression in parental cells and spheroids. The results represent the expression of ALDH1 in cells derived from spheroid cultures (hedged columns) compared to monolayer cells (open columns). ALDH1 expression of SDC is generally significantly enhanced (** $p<0.01$, * $p<0.05$)[37].

4.3. A subpopulation of SDC have acquired features of myofibroblasts and may have undergone EMT

We investigated the expression of myofibroblast markers Vimentin and α-SMA in SDC of 3 cell lines which have different ALDH1 expression levels in monolayer culture (low: UD-SCC1 with 9.4±5.3%, medium: UT-SCC22 with 16.9±4.1%, high: UT -SCC24A with 25.8±3.0%). We found that the different cell lines and SDC generated from these cell lines varied in the proportion of cells expressing Vimentin and α-SMA. UD-SCC1 showed no α-SMA expressing cells and weak (12.5±2.5%) Vimentin expression of the cells in the monolayer culture. In contrast, UT-SCC24A and UT-SCC22 monolayer-derived cells had a relatively high frequency of Vimentin and α-SMA expressing cells which indicates that these cell lines have both epithelial and mesenchymal characteristics. Interestingly, these two cell lines have a relatively high percentage of cells in the monolayer cultures that are ALDH1 positive. Nevertheless, as spheroid cultures, all cell lines contained more α-SMA and Vimentin positive cells than the corresponding parental monolayer cell lines. The epithelial marker E-Cadherin was also investigated. The number of E-Cadherin expressing cells in SDCs and MDCs was quantified by FACS. The SDCs showed significantly increased proportions of E-Cadherin negative to low expressing cells compared to their monolayer-derived counterparts, indicating loss of cellular adherence in this subpopulation. These findings were further supported by real-time PCR which showed that the expression level of Snail1 the key transcriptional factor involved in EMT were significantly increased in all 5

SDCs. Interestingly, when the spheroids regrew out as a monolayer culture, the α-SMA and Vimentin level decreased, and most of the cells that grew out from the spheroids stained negative for these two markers.

4.4. ALDH1 and α-SMA expression may have an impact on lymph node metastasis of HNSCC

Paraffin-embedded tumor samples from 60 HNSCC patients were investigated, 36 of which had lymph node metastasis. We found that there was a varying expression status of ALDH1 in HNSCC, and the higher expression level of ALDH1 was more common in patients with lymph node metastasis. α-SMA positive cells were mainly located at the tumor's invading border, and its expression level was also increased in patients with lymph node metastasis. ALDH1 and α-SMA also showed high correlation in tumors with positive lymph nodes (Fig. 4).

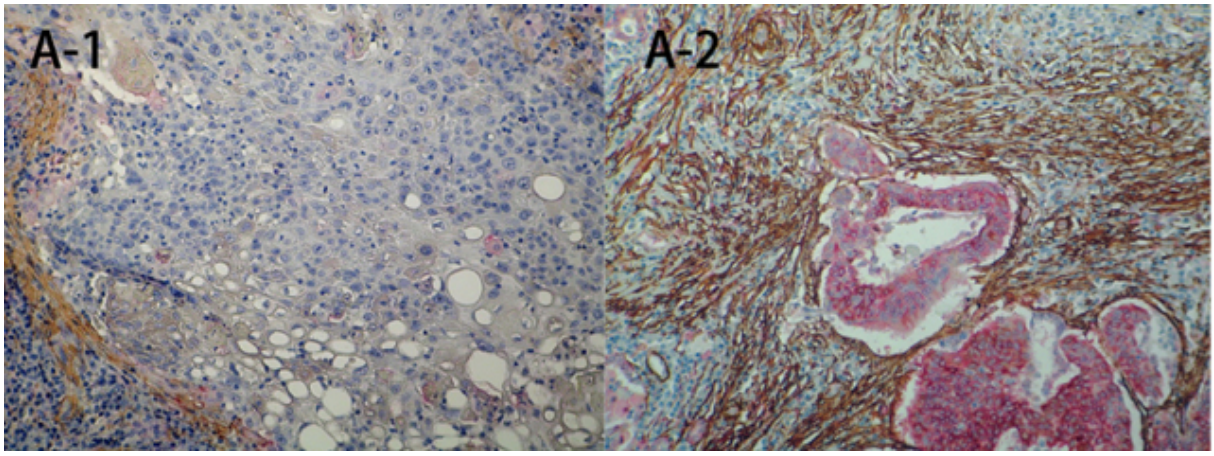

Figure 4: ALDH1 and α-SMA expression in HNSCC patients. The expression of ALDH1 (red) and α-SMA (brown) increased significantly in metastasized lymph node (A-2) compared with primary lesion (A-1) of the same patient. (representative pictures).

Table 2: ALDH1 and α-SMA expression in HNSCC patients

	α-SMA		ALDH1		α-SMA&ALDH1	
LN Metastasis[1]	High	Low	High	Low	High/High	Low/Low
Yes	29*	7	27**	9	26**	6
No	11	13	5	19	3	11

The number of patients with highly expressed ALDH1 and /or α-SMA was significantly increased when lymph node metastasis existed. (**p<0.01 *p<0.05)

Discussion

The cancer stem cell (CSC) model of tumor development and progression states that tumors, like normal adult tissues, contain a subset of cells that both self-renew and give rise to differentiated progeny. Like in other tissues, these stem cells are a minority of the whole tissue, and are the only cells that can maintain tumor growth indefinitely. The remaining cells, though actively proliferating and making up the majority of the cells in the tumor, are also undergoing differentiation-like changes and are destined to die. The self-renewal properties of the CSCs are thus the real driving force behind tumor growth. Prince et al. demonstrated that a minor population of CD44+ HNSCC cells possess CSC characteristics, which could give rise to new tumors in vivo (<5,000 cells injected). Mack et al. questioned the use of CD44 as a specific CSC marker in HNSCC since CD44 was abundantly expressed in most tumor cells within HNSCC (60%-100%) [10]. ALDH1 was recently shown to be a more suitable marker to identify putative CSC of HNSCC and other epithelial cancers. In HNSCC, Chen et al. showed that only 3000 ALDH1+ cells from five HNSCC patients sufficed in xeno-transplanted mice in all cases to generate visible tumors 6 weeks after injection, while 10^4 ALDH1- cells failed to generate tumors. Here, we first proved that through anchorage-independent cell culture methods, we could enrich ALDH1+ cell from HNSCC cell lines. The stem cell-like characteristics of these cells were further analyzed by comparing cell cycle and the expression of embryonal TFs as well as functional characteristics with the parental monolayer cell lines. The phenotypic characterization of SDCs revealed that spheroids do not consist of a homogeneous cell population and a subpopulation had characteristics of mesenchymal cells with expression of EMT-markers. This may indicate that these cells may exert an EMT-program and demonstrates a potentially close relationship between CSC and cells with an activated EMT program which may have derived from the CSC.

Spheroids are three-dimensional (spherical) clusters of tumor cells grown from one or several cell clones. As compared to cell doubling times measured in monolayer culture, the rate and pattern of spheroid growth in vitro better matches that observed in tumors in vivo. Anchorage independent growth has been shown to be a property shared by normal tissue cells that exhibit stem cell properties. In our study, we also identified the expression of widely used CSC marker CD44+/CD24- in HNSCC cell lines and showed that the frequency of CD44+/CD24- was highly variable (from 0.5% to 97%) and could not be enriched by spheroid culture, which is in contrast to the finding in breast cancer [38; 39]. On the contrary, ALDH1 positive cells were found to be highly enriched in spheroid cultures from all five HNSCC cell lines. If seeded at a very low density, FACS-sorted ALDH1+ cells formed

significantly more colonies then the ALDH- population, indicating a higher proliferative capacity of this subpopulation. Cancer and normal stem cells (SC) share proliferative properties of self-renewal, quiescence and expression of key transcription factors. Chickarmane et al. set up a computer-model to describe the key transcription factor combinations in SC and defined high levels of Oct3/4, Sox2, and Nanog [40]. In our study, the expression of Oct3/4, Sox2, and Nanog was up-regulated in SDC in all five HNSCC-derived cell lines. This implies that SDCs may have enhanced stem cell properties. We also succeeded in enriching human skeletal muscle progenitor cells using the same culture method. In SDCs derived of skeletal muscle cells, called myospheres, expression of skeletal progenitor cell markers Pax7, ALDH1, Myod, and Desmin, and of the stem cell markers Nanog, Sox2, and Oct3/4 is significantly elevated over adherently grown cells or the initial tissue biopsy [41]. Adult stem cells are maintained in a quiescent state but are able to exit quiescence and rapidly expand and differentiate in response to stress. The quiescent state appears to be necessary for preserving the self-renewal of stem cells and is a critical factor in the resistance of cancer stem cells (CSCs) to chemotherapy and targeted therapies. We demonstrated that CSC populations derived from HNSCC and cervical cancer cell lines were less sensitive to MHC class I-restricted alloantigen-specific CD8+ CTL lysis as compared to matched MDC, but IFN-γ pretreatment could enhance CD8+ CTL lysis of SDC [42]. In our study, we found that SDC had a higher proportion of Ki-67 negative cells compared to the corresponding monolayer cultures. This indicates that SDCs are more quiescent than cells derived from monolayer. We also found that SDCs have an increased invasion capacity, which implies that these cells may have a higher ability to metastasize.

Accumulating evidence showed that in cancer, tissue invasion is driven by the stroma [43; 44]. Myofibroblasts and cancer-associated fibroblasts are important components of the tumor stroma. The origin of myofibroblasts remains controversial. In this study, we demonstrated that myofibroblast-like cells are a subpopulation of SDC. These spheroids derived exclusively from cloned tumor cells. It can therefore be concluded that the myofibroblasts could only emerge from epithelial cancer cells by altering the epithelial to a mesenchymal phenotype through EMT. More importantly, we found that over 90% of SDC stained positive for α-SMA and Vimentin while the percentage of ALDH1+ cells ranged from 37% to 46.4%. A significant decrease of E-Cadherin expression and increase of snail1 expression was also found in SDCs, indicating a loss of epithelial trait. These marker expressions were reversible under culture conditions, where spheroids could adhere and cells grew radially out to form a monolayer. Taken together, we showed that cells derived from spheroids, which had

undergone a transition to a mesenchymal phenotype, maintained CSC characteristics such as expressing a high level of the CSC marker ALDH1, an increased proportion of G0 phase cells, increased invading capacity, and elevated mRNA levels of stem cell-related transcription factors such as Nanog, Sox2, and Oct3/4. To further investigate the role of myofibroblasts in promoting metastasis in HNSCC and its connection to ALDH1, we compared the expression of these two markers in HNSCC tissue samples. Data showed that α-SMA positive cells were mainly located at a tumor's invading border, and enhanced expression of α-SMA and ALDH1 was closely connected to lymph node metastasis. In summary, in this study we provide an anchorage-independent culture method of HNSCC cell lines suitable for the enrichment of cells with cancer stem cell characteristics and of cells undergoing EMT. Whether this method would select the tumorigenic cells from non-manipulated human HNSCC samples, remains to be demonstrated. Functionally, SDCs showed higher invading capacity than monolayer cells. FACS sorted ALDH1+ cells showed higher colony forming ability than ALDH1- cells. Our findings may prove useful to investigate the phenomenon of EMT in vitro and thus support in vivo studies investigating the role of CSC and EMT in the spread of HNSCC.

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Selected Publications

1. **Chen C**, Wei Y, Hummel M, Hoffmann TK, Gross M, Kaufmann AM, Albers AE. Evidence for epithelial-mesenchymal transition in cancer stem cells of head and neck squamous cell carcinoma. PloS One, 2011，6(1):e16466. IF=3.73(2012)
2. Wei Y, Li Y, **Chen C**, Stoelzel K, Kaufmann AM, Albers AE. Human skeletal muscle-derived stem cells retain stem cell properties after expansion in myosphere culture. Exp Cell Res, 2011，317(7):1016-27. IF=3.557(2012)
3. Liao T, Kaufmann AM, Qian X, Sangvatanakul V, **Chen C**, Kube T, Zhang G, Albers AE. Susceptibility to cytotoxic T cell lysis of cancer stem cells derived from cervical and head and neck tumor cell lines. J Cancer Res Clin Oncol. 2013,139(1):159-70. IF=2.914(2012)

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Evidence for Epithelial-Mesenchymal Transition in Cancer Stem Cells of Head and Neck Squamous Cell Carcinoma

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Abstract

Initiation, growth, recurrence, and metastasis of head and neck squamous cell carcinomas (HNSCC) have been related to the behavior of cancer stem cells (CSC) that can be identified by their aldehyde-dehydrogenase-isoform-1 (ALDH1) activity. We quantified and enriched ALDH1$^+$ cells within HNSCC cell lines and subsequently characterized their phenotypical and functional properties like invasion capacity and epithelial-mesenchymal transition (EMT). Spheroid culture enriched CSC from five HNSCC cell lines by up to 5-fold. In spheroid-derived cells (SDC) and the parental monolayer-derived cell line ALDH1, CD44, CD24, E-Cadherin, α-SMA, and Vimentin expression was compared by flow-cytometry and immunofluorescence together with proliferation and cell cycle analysis. Invasion activity was evaluated by Matrigel assay and expression of stemness-related transcription factors (TF) Nanog, Oct3/4, Sox2 and EMT-related genes Snail1 and 2, and Twist by real-time PCR. All cell lines formed spheroids that could self-renew and be serially re-passaged. ALDH1 expression was significantly higher in SDC. ALDH1$^+$ cells showed increased colony-formation. The proportion of cells with a putative CSC marker constellation of CD44$^+$/CD24$^-$ was highly variable (0.5% to 96%) in monolayer and spheroid cultures and overlapped in 0%–33% with the CD44$^+$/CD24$^-$/ALDH1$^+$ cell subset. SDC had significantly higher invading activity. mRNA of the stemness-related genes Sox2, Nanog, and Oct3/4 was significantly increased in SDC of all cell lines. Twist was significantly increased in two while Snail2 showed a significant increase in one and a significant decrease in SDC of two cell lines. SDC had a higher G0 phase proportion, showed high-level expression of α-SMA and Vimentin, but significantly decreased E-Cadherin expression. HNSCC-lines harbor potential CSC, characterized by ALDH1 and stemness marker TF expression as well as properties like invasiveness, quiescence, and EMT. CSC can be enriched by anchorage-independent culture techniques, which may be important for the investigation of their contribution to therapy resistance, tumor recurrence and metastasis.

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Introduction

HNSCC accounts for approximately 6% of all cancer cases and for about 650,000 new cases and 350,000 deaths worldwide each year [1,2,3]. Advances in therapy have improved quality of life, but survival rates have remained unchanged over the past decades. Mortality from this disease remains high because of the development of distant metastases and the emergence of local and systemic recurrences resistant to chemo- and radiotherapy. It is therefore essential to develop a deeper understanding of the biology of this disease in order to develop more effective therapeutic approaches.

Evidence has recently been accumulating to support the hypothesis that tumors contain a small subpopulation of cells called cancer stem cells (CSC), which exhibit self-renewing capacities and are responsible for tumor maintenance and metastasis [4]. CD44$^+$/CD24$^-$cells have been firstly proposed to exhibit CSC properties in breast cancer [5].

Subsequently, CD133 was found to identify CSC in brain tumors [6], colorectal carcinoma [7], and pancreatic carcinoma [8]. In HNSCC, Prince et al. first demonstrated that a CD44$^+$ population of cells possesses the properties of CSC [9], but relatively high numbers of these cells (>5,000 cells) were needed to generate new tumors in immunodeficient mice indicating either a low frequency of CSC or a low specificity of CD44 as CSC-marker in HNSCC. The latter hypothesis is supported by the observation that CD44s and CD44v6 expression does not distinguish normal from benign or malignant epithelia of the head and neck. CD44s and CD44v6 were abundantly present in the great majority of cells in head and neck tissues, including carcinomas [10]. Thus, the identification of more specific CSC markers for HNSCC is desirable. Recently, high aldehyde dehydrogenase 1 (ALDH1, also known as ALDH1A1) activity was shown to identify the CSC in HNSCC and other epithelial cancers [11,12,13,14,15]. However, in breast cancer the ALDH1$^+$ population shows a surprisingly

small overlap with the previously described $CD44^+/CD24^-$ phenotype of only 0.1–1.2%. Interestingly, in breast cancer the cells bearing both phenotypes appeared to be highly tumorigenic, being able to generate tumors from as few as 20 cells [15]. It remains to be determined if the same phenotypic pattern of stem cells in HNSCC is associated with a similar tumorigenic potential.

Non-adherent sphere assays are increasingly being used to evaluate stem cell activity in normal tissue and putative CSC. The neurosphere is the best-studied sphere assay. Central nervous system cells grown on nonadherent surfaces give rise to neurospheres that have the capacity for self-renewal and can in principal generate all the cell types of the brain [16,17]. The capacity for repeated generation of neurospheres from single cells is generally viewed as evidence of self-renewal [18]. Recently, it was described that spheroid-derived cells (SDC) from gliosarcoma rat cell lines possess CSC capacity [19]. So far, it is unknown whether spheroid cultures of HNSCC can enrich for CSC.

Normal hematopoietic stem cells are able to self-renew and give rise to all blood cell types. They are non- or very slow-dividing cells and reside within bone marrow niches in a dormant condition. The ability to maintain this non-dividing condition, or in other terms, stay in the G0 phase of cell cycle, is called quiescence [20]. It has been proposed that CSC share many qualities with normal stem cells since the vast majority of them also should stay quiescent and be able to self-renew. Because a majority of anti-cancer drugs target actively dividing (cycling) cells, quiescent CSC remain alive and may be the cause for relapses and progression of cancer. Ki-67 antigen is the prototypic cell cycle related nuclear protein, expressed by proliferating cells in all phases of the active cell cycle (G1, S, G2 and M phase), but it is absent in resting (G0) cells.

Another capability of CSC is to perform epithelial–mesenchymal transition (EMT), a key step during embryogenesis [21,22,23] and wound healing [24]. Recent evidence suggests that genetic programs relevant for EMT are also transiently activated in epithelial cancers playing a role in cancer progression, through which epithelial cancers invade tissues and metastasize. Although the EMT program is necessary for normal development, the aberrant activation of EMT contributes to various pathologic conditions, including fibrosis and carcinoma progression [25,26]. During EMT epithelial cells break down cell-cell and cell-extracellular matrix contacts and migrate to other locations in the body [27]. During cancer progression, EMT seems to provide cancer cells with the capacity to infiltrate the surrounding tissue and ultimately metastasize to distant sites [28]. Recently, it has been reported that the induction of EMT in differentiated

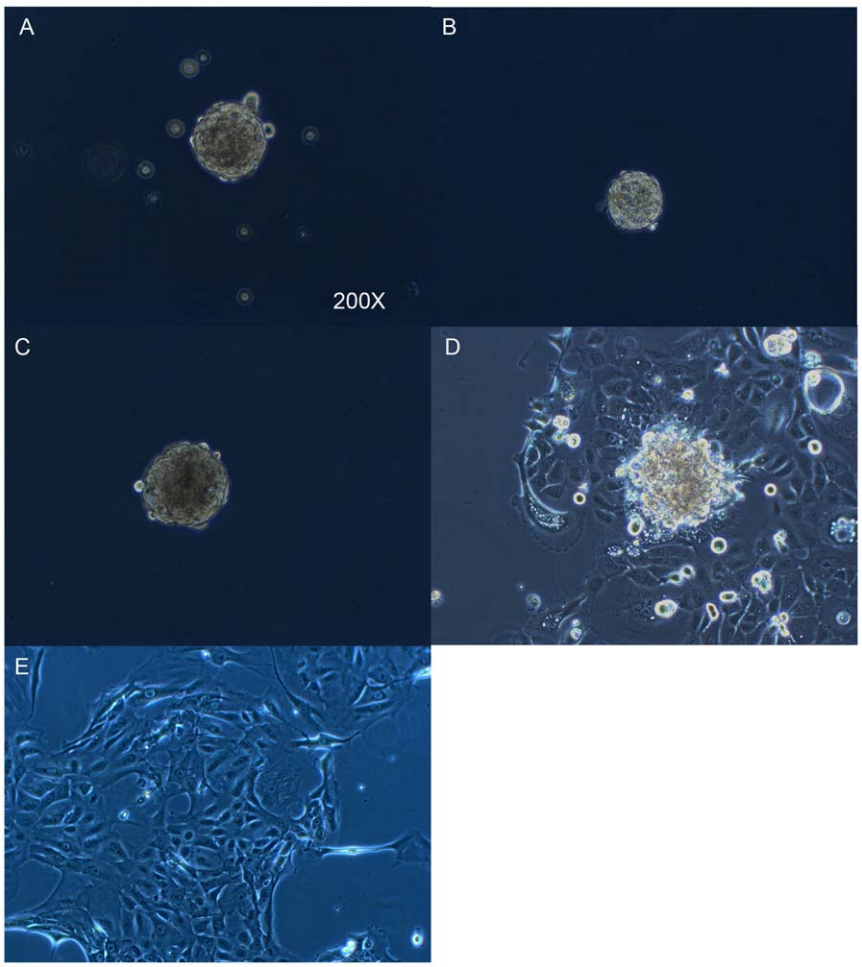

Figure 1. Spheroids generated from HNSCC lines in serum-free medium have self-renewing capacity. Representative pictures of the cell line UD-SCC1 are shown. (**A**) The first generation of spheroids. (**B**) A subspheroid formed after seeding at a concentration of 1,000 cells/ml. (**C**) A spheroid formed in the 21st generation. (**D**) Spheroid adhering and growing to confluence after replating into flasks coated for tissue culture. (**E**) Parental cell line grown permanently as a monolayer culture.
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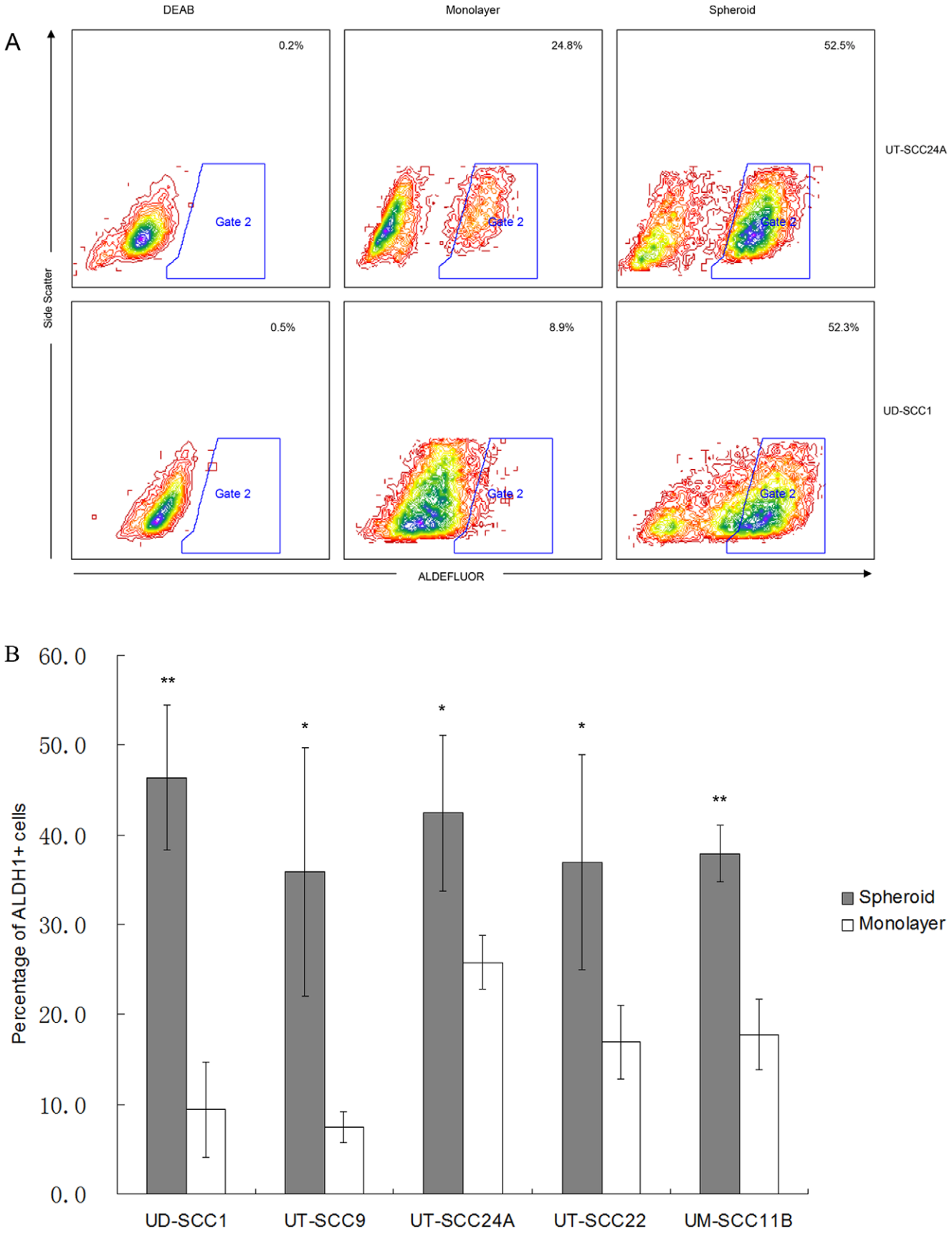

Figure 2. ALDH1 expression in parental cells and spheroids. (**A**) Representative FACS-analysis of two cell lines after Aldefluor staining. Comparison of frequencies of ALDH1 positive cells in monolayer to SDC and DEAB control. DEAB is a specific inhibitor of ALDH1. (**B**) The results

represent the expression of ALDH1 in cells derived from spheroid cultures (hedged columns) compared to monolayer cells (open columns). ALDH1 expression of SDC is generally significantly enhanced (A, B). UD-SCC22, UT-SCC24A, and UM-SCC11B displaying a partial mesenchymal phenotype have already relatively high ALDH1 expression in parental cell lines. (** $p<0.01$, * $p<0.05$).
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immortalized human mammary epithelial cells led to the acquisition of the $CD44^+/CD24^-$ stem cell phenotype [29]. Moreover, it was shown that these putative $CD44^+/CD24^-$ CSC isolated from neoplastic human breast tissues expressed high mRNA levels encoding the EMT-associated markers Snail1 (SNA), Snail2, and Twist. Malignant tumors consist of cancer cells and tumor-associated host cells, with the latter attracting more interest recently because of their participation in tumor invasion and metastasis, and therapeutic response [30,31]. Myofibroblasts are the most important components of the tumor stroma. Nevertheless, the origin of these cells remains controversial so far. The expression of alpha-smooth muscle actin (α-SMA) is considered the marker of the fully differentiated myofibroblasts. Emerging evidence shows that myofibroblasts can be derived from the epithelial (tumor) cells via EMT [32,33]. This notion is supported by the observation that compact spheroids formed by ovarian cancer cells in ascites display contractile behavior, possess high invading capacity in vitro and the expression of α-SMA, which is also associated with high contractile capacity [34].

In the present work, we tested if anchorage-independent cell culture techniques allow the generation of spheroid-cultures and if

Table 1. $CD44^+/CD24^-$ phenotype is highly variable in HNSCC and does not consistently overlap with the $ALDH1^+$ subpopulation.

Frequency (%)	UD-SCC1	UT-SCC9	UT-SCC24A	UT-SCC22	UM-SCC11B
$CD44^+/CD24^-$ in monolayer cultures	0.44±0.13	0.11±0.07	0.98±0.2	95.27±0.8	95.4±1.25
$CD44^+/CD24^-$ in spheroid cultures	0.96±0.71	3.28±3.54	0.88±0.19	95.17±1.46	96.47±1.62
$ALDH1+/CD44^+/CD24^-$ in monolayer cultures	0.11±0.1	0.08±0.13	0.36±0.33	19.07±1.17	17.37±3.33
$ALDH1+/CD44^+/CD24^-$ in spheroid cultures	0.66±0.52	2.22±2.61	0.53±0.41	33±10.66*	33.4±3.04**

(Data were stated as: mean±standard deviation, %=proportion of positive cells, *$p<0.05$, **$p<0.01$).
doi:10.1371/journal.pone.0016466.t001

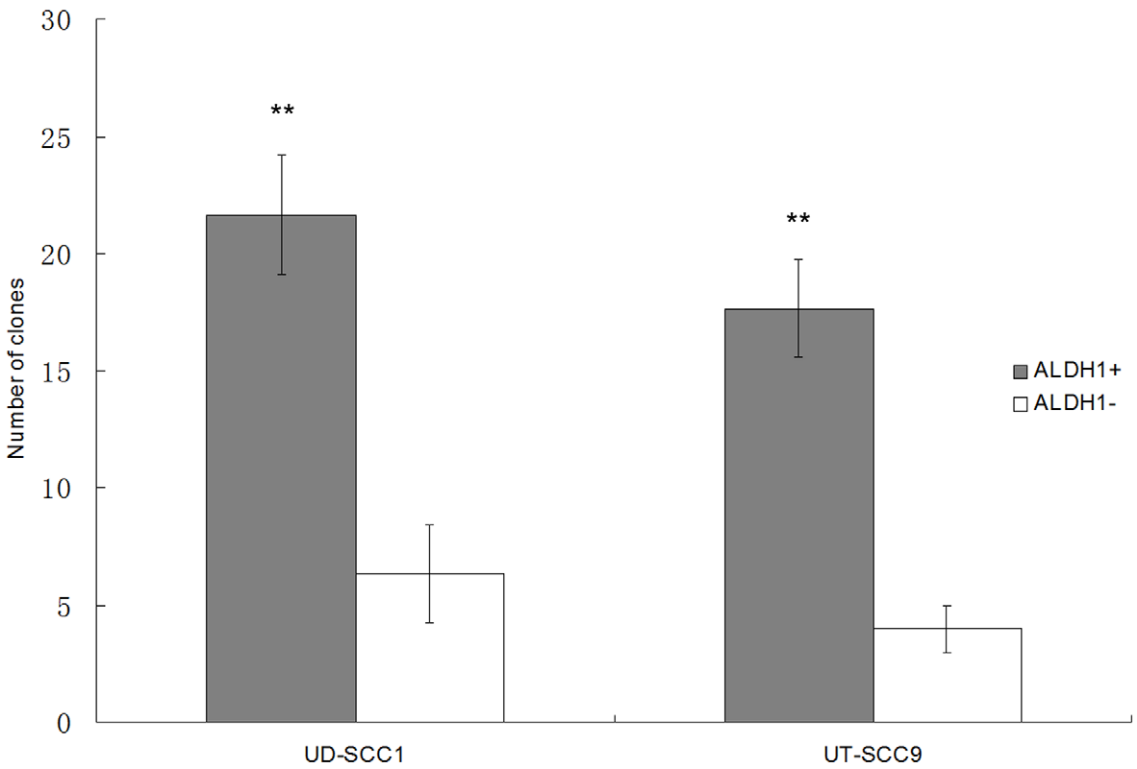


Figure 3. Clone formation assay with FACS-sorted and cloned cells. Two thousand ALDH1-sorted cells were seeded and after 14 days, the colonies that formed were quantified. The $ALDH1^+$ subpopulation in UD-SCC1 and UT-SCC9 cell lines have a higher clone formation efficiency as compared to the $ALDH1^-$ subpopulation (** $p<0.01$).
doi:10.1371/journal.pone.0016466.g003

Table 2. Single ALDH1$^+$ cells show higher clone formation activity than ALDH1$^-$ cells.

Spheroid-formation	Name of cell line			
	UT-SCC9 ALDH1-	UT-SCC9 ALDH1+	UD-SCC1 ALDH1-	UD- SCC1 ALDH+
Yes	1	19	0	23
No	135	92	104	96
Sum	136	111	104	119
Formation Rate	0.7%	17.10%*	0%	19.30%**

Analyzed by chi-square test,
*$p<0.05$,
**$p<0.01$.
doi:10.1371/journal.pone.0016466.t002

these cultures were enriched for cells with functional and phenotypic properties characterizing CSC of HNSCC. Here, we provide evidence that myofibroblasts can be derived from HNSCC using a spheroid cell culture model that enriches for CSC-like cells as characterized by a high proportion of ALDH1 positivity, proliferative quiescence, and invasive capacity.

Results

HNSCC cell lines contain cells with self-renewing capacities that form tumor spheroids

Several different approaches have been used to identify CSC. The strategy used for our experiments was the in vitro spheroid

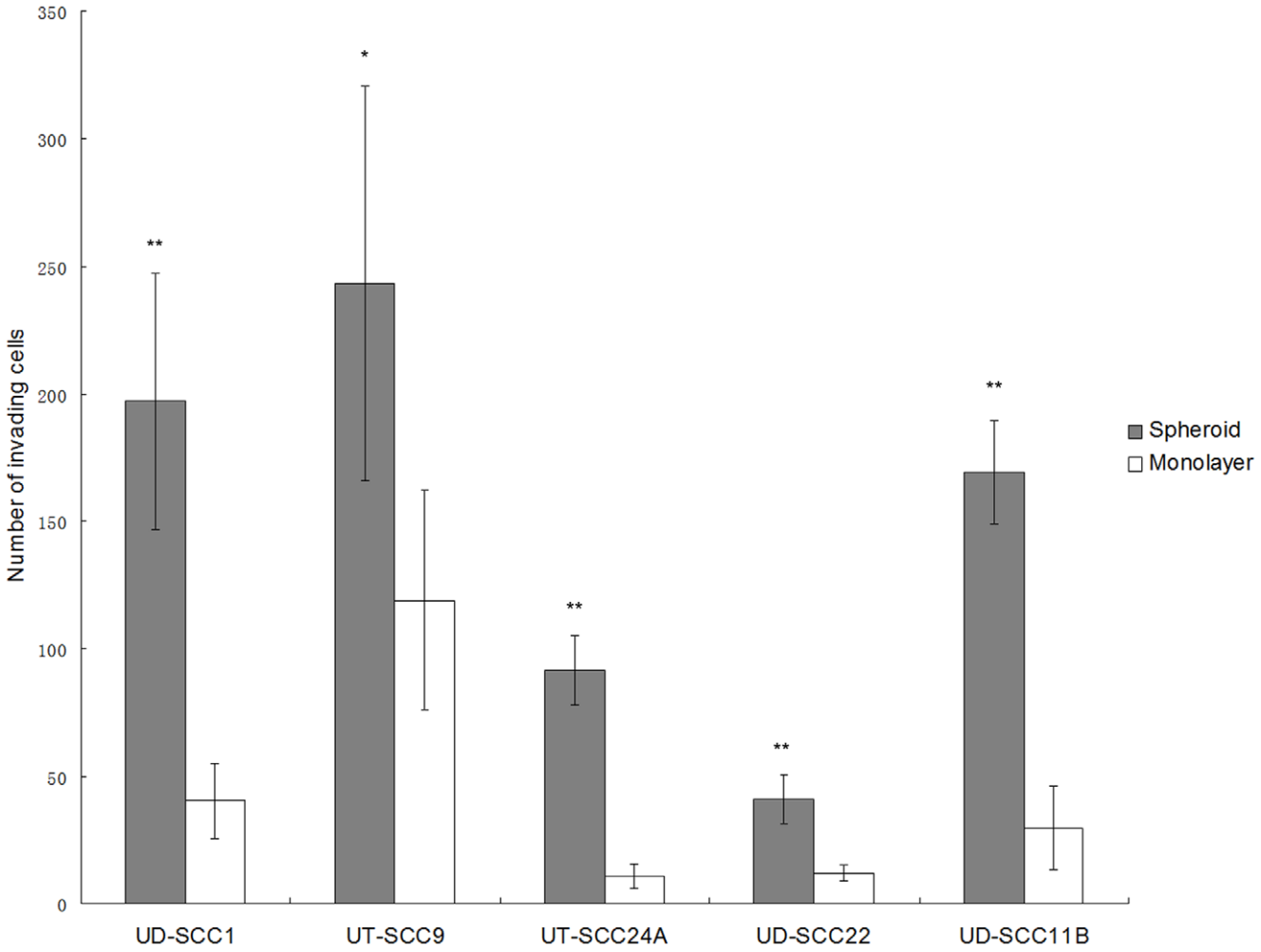
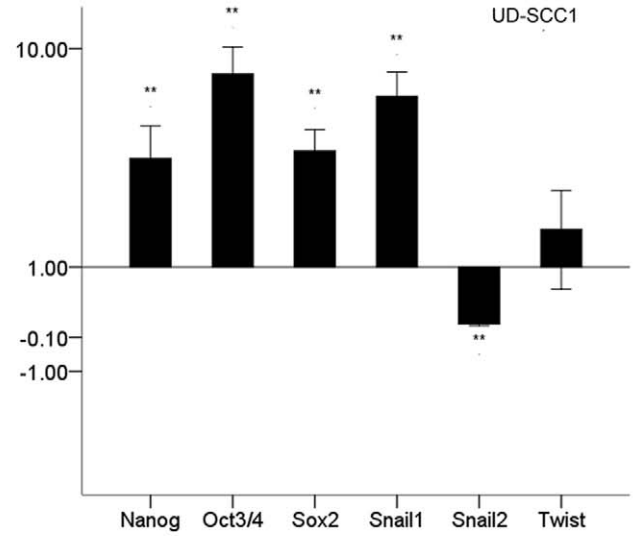


Figure 4. ECM invasion assay. Matrigel invasion chambers were used to compare the invading capacity between cells derived from spheroids (hatched columns) or monolayer (open columns). SDC from all cell lines investigated show higher invading activity in vitro than monolayer-derived cells. (** $p<0.01$, * $p<0.05$).
doi:10.1371/journal.pone.0016466.g004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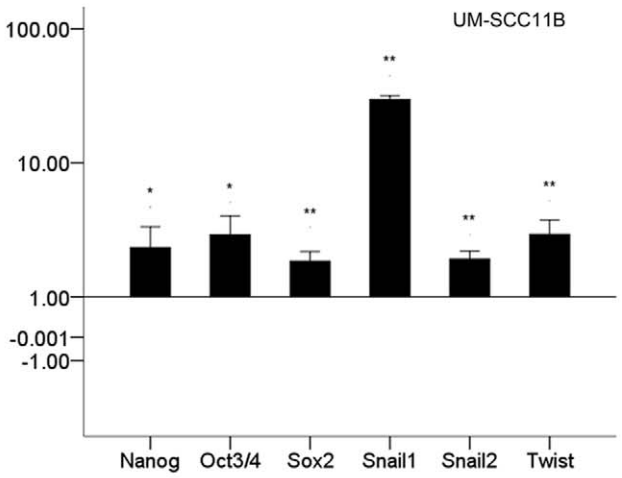
-1.00
Nanog
Oct3/4
Sox2
Snail1
Snail2
Twist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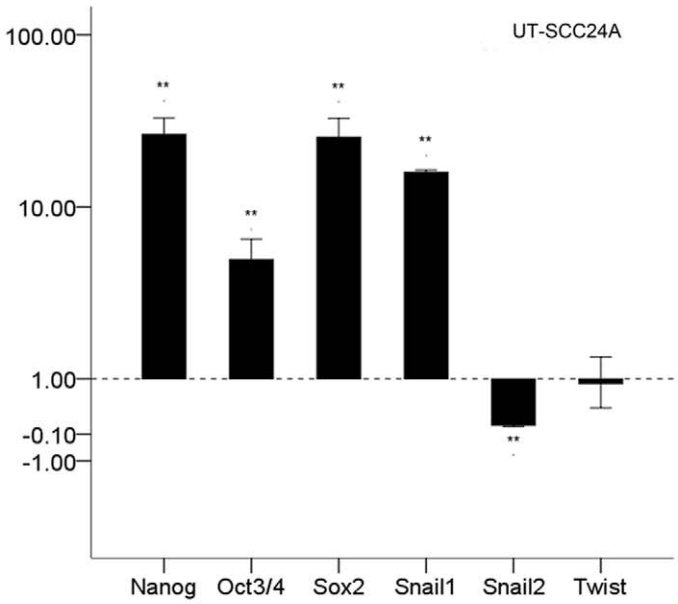
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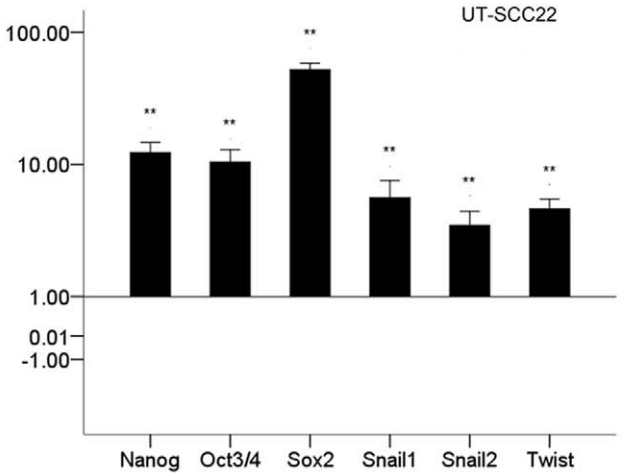
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Oct3/4
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Snail2
Twist

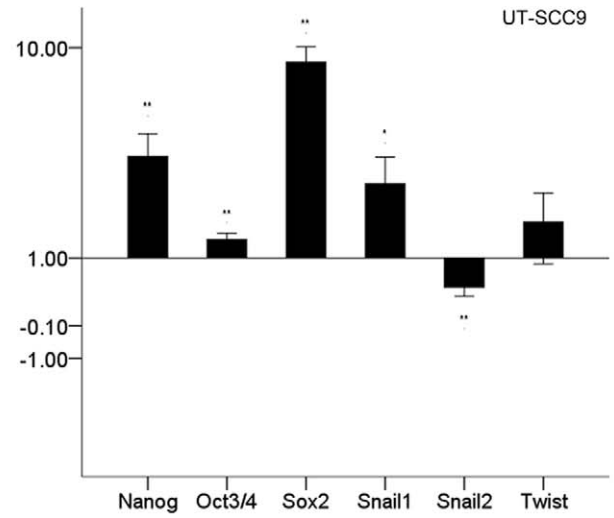

UT-SCC9
10.00
1.00
-0.10
-1.00
Nanog
Oct3/4
Sox2
Snail1
Snail2
Twist

Figure 5. Quantitative PCR analysis of mRNA expression of stemness-related transcription factors. Messenger RNA isolated from spheroids and monolayer cultures was quantified for expression of the indicated TF. The ratio of expression in spheroid to monolayer cells is given. Significant differences were * $p<0.05$; ** $p<0.01$. The mRNA level of stemness-related TF Nanog, Oct3/4, and Sox2 were increased remarkably in spheroids. The key TF in EMT Snail1 was also increased in all spheroids but another two TF involved in EMT, Snail2 and Twist, showed an expression pattern depending on the CD44/CD24 status.
doi:10.1371/journal.pone.0016466.g005

colony formation method. We investigated the ability of five HNSCC-derived cell lines (UD-SCC1, UT-SCC22, UM-SCC11B, UT-SCC9, UT-SCC24A) to grow anchorage independently as spheroid-cultures. Cells were plated at a density of 20,000/ml. After in vitro culture for 5–10 days in serum-free medium under nonadherent conditions, all investigated cell lines formed spheroids, ranging from 50 to 500 cells per spheroid (Figure 1A). The self-renewing capacity of these tumor spheroids was assessed using the method published by Ghods et al [19]. Briefly, the spheroids were collected and dissociated into a single cell suspension that was subsequently plated at a clonal density of 1,000 cells per ml. Tumor subspheroids ranging from 20 to 40 cells were evident after 10 to14 days (Figure 1B, C). In contrast, the parental monolayer cell cultures grown under the same conditions did not form spheroids if plated at this low concentration even after 21 days. When the spheroids were transferred back to a regular tissue culture flask coated for monolayer cell culture, the spheroids adhered to the flask and cells grew out from the spheroid and formed a confluent monolayer. The phenotype of these cells was identical to the parental cell lines (Figure 1D).

Phenotypic characterization of SDC

The expression of the putative stem cell marker ALDH1 in SDC and the respective parental monolayer cell line used as control was compared to the $CD44^+/CD24^-$ expressing population by multicolor FACS-analysis.

Interestingly, all SDC had an increased number of ALDH1 positive cells as compared to parental cell lines (Figure 2). The highest proportion of $ALDH1^+$ cells was found in spheroids generated from the UD-SSC1 cell line ($46.4\pm8.1\%$) which was 5-fold higher than the corresponding parental cell line ($9.4\pm5.3\%$).

Tumor cells with a $CD44^+/CD24^-$ phenotype were shown to have CSC properties in a variety of solid tumors [4]. We therefore also investigated comparatively the CD44 and CD24 expression in HNSCC cell lines and found that the proportion of $CD44^+/CD24^-$ cells is highly variable (0.5–97%). The overlap with $ALDH1^+$ cells was small (0–2.2%) except for the two cell lines UM-SCC11B and UT-SCC22 which had an overlap of about 33% and were composed to over 95% of $CD44^+/CD24^-$ cells. Interestingly, the proportion of $CD44^+/CD24^-$ cells was not consistently enriched by the spheroid culture method (Table 1). This result raised the question how well ALDH1 is suited for the identification of cells with CSC properties in HNSCC. To approach this question, ALDH1 positive and negative cells from the spheroids generated from the cell lines UT-SCC9 and UD-SCC1 were separated by FACS sorting. Subsequently, their capacity for colony formation was assessed. UT-SCC9 and UD-SCC1 were chosen because as compared to the other cell lines they generated more spheroids with higher enrichment of $ALDH1^+$ cells. The data show that $ALDH1^+$ cells can form 3 to 4 times more clones than $ALDH1^-$ cells (Figure 3). Light microscopic observation after 2 weeks showed that the clones formed by $ALDH1^+$ cells on average contained 197 (197 ± 47) cells compared with 33 (33 ± 16) cells in clones generated from $ALDH1^-$ cells ($p<0.01$). Our data also show that single $ALDH1^+$ cells can significantly better regenerate a spheroid in an anchorage-independent culture with serum-free medium complemented with bFGF and EGF (UT-SCC9: 17.1%, UD-SCC1: 19.3%), whereas under the same conditions single $ALDH1^-$ cells regenerated only in one case a spheroid. (Table 2, Figure S1).

In summary, SDC generated from HNSCC lines express high levels of the putative CSC marker ALDH1, form significantly more clones, which also significantly regenerate more often into spheroids then $ALDH1^-$ cells and have a varying overlap with the $CD44^+/CD24^-$ population.

SDC show increased invading capacity in vitro

Central to the definition of CSC is the capability to initiate and drive the growth of the primary tumor and of invasion and metastasis. A close relationship between the percentage of cells with CSC phenotype ($CD44^+/CD24^-$) in the primary tumor and the development of metastasis in breast-cancer was recently reported [35]. Also, indication for the metastatic potential of cells with CSC-phenotype in breast cancer comes from the observation that breast cancer cells detected in the bone marrow predominantly showed this phenotype [36].

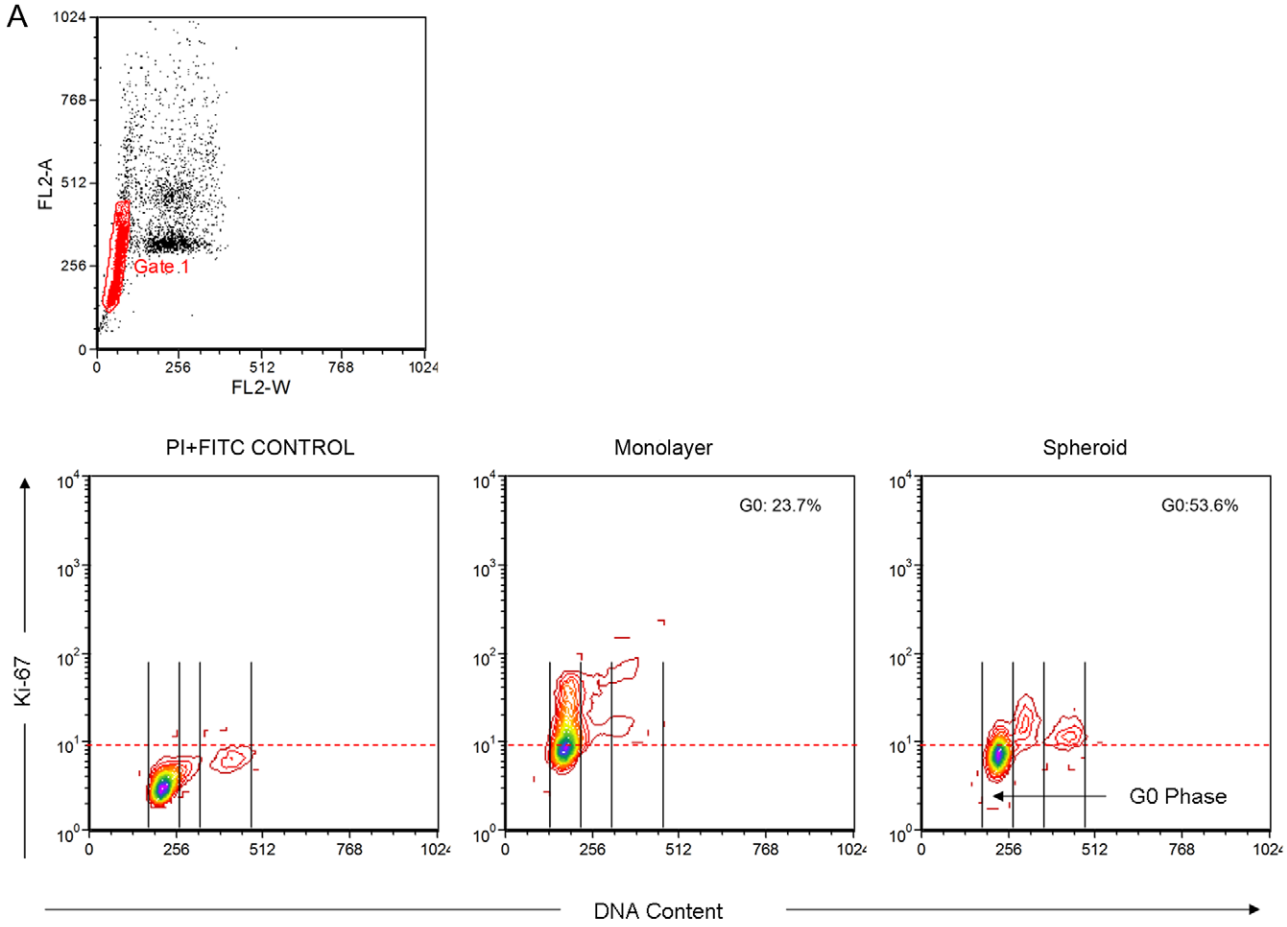
In analogy to this observation, we asked whether CSC derived of spheroid cultures of HNSCC display a similar invasive potential. By using a Matrigel invasion chamber, we compared the invading capacity of cells either raised in spheroid or monolayer culture. SDC from all five tested cell lines showed a significantly increased invading capacity of 2.1 to 8.6 fold over the parental control (Figure 4).

Overexpression of stemness-related genes in SDC

It was reported that Oct4, Sox2, and Nanog, which form a self-organized core of transcription factors (TF), maintain pluripotency and self-renewal of human embryonic stem cells [37,38]. We wanted to know if CSC also share this feature of TF expression with embryonal stem cells (ES). For this purpose, we quantitatively compared the mRNA expression of these TF between SDC and parental monolayer-derived cells. We found that the mRNA levels of Oct3/4, Sox2, and Nanog were all significantly increased in the SDC. The highest increase was observed in UT-SCC22, where a 52-fold increase in Sox2 expression was found in spheroids. The smallest change was found in UT-SCC9, where a 1.23-fold increase in Oct3/4 expression was found in spheroids. The key TF involved in EMT, Snail1 was also significantly increased in all SDC generated from the five different HNSCC cell lines. Interestingly, two other TF involved in EMT, Snail2 and Twist, showed an expression pattern in accordance with the CD24 status. In UD-SCC1, UT-SCC9, and UT-SCC24A where most of the cells are $CD44^+/CD24^+$, the Snail2 level decreased in spheroids while Twist showed no significant change. However, in UT-SSC22 and UM-SCC11B, which were composed to over 95% of $CD44^+/CD24^-$ cells Snail2 and Twist, showed a significant increase (Figure 5). These findings implied that Snail2 and Twist expression level were correlated to the CD24 phenotype.

SDC are more quiescent than their parental monolayer-derived counterparts

For cell cycle analysis by FACS, the cell lines UT-SCC24A, UT-SCC9, UD-SCC1, UT-SCC22, and UM-SCC11B grown in spheroid or monolayer culture were stained for Ki-67-FITC and DNA counterstained by propidium iodide. Cells residing in the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FL2-A
FL2-W
Gate.1
PI+FITC CONTROL
Monolayer
Spheroid
G0: 23.7%
G0:53.6%
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Ki-67
DNA Content

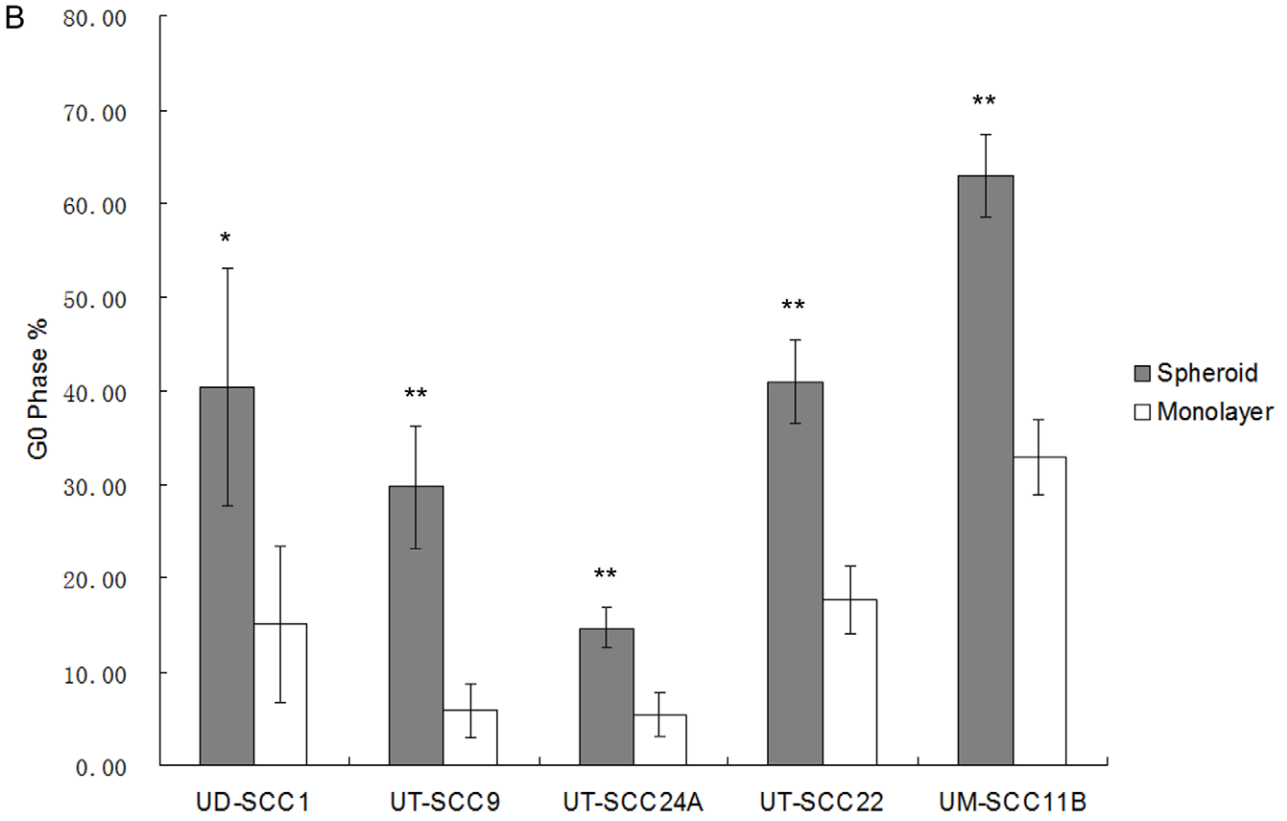

B
G0 Phase %
Spheroid
Monolayer
UD-SCC1
UT-SCC9
UT-SCC24A
UT-SCC22
UM-SCC11B

Figure 6. Cell cycle analysis of SDC and monolayer-derived cells by FACS analysis. (**A**) Double staining with Ki-67 and propidium iodide for the measurement of proliferation and cellular DNA content. Single cells were gated on FL2-W and FL2-A. DNA content and Ki-67 expression was evaluated by flow cytometry analysis. (**B**) Frequencies of quiescent cells in SDC as comparison to monolayer-derived cells. In all cases, the cells derived from spheroids contain a highly significant larger percentage of quiescent (G0) cells. (** $p<0.01$).
doi:10.1371/journal.pone.0016466.g006

G1/G0 peak that were negative for Ki-67 staining were regarded to be the quiescent fraction (G0 phase). In all cell lines, the proportion of cells in the G0 phase showed a highly significant increase in SDC, indicating that SDC contain a higher proportion of quiescent cells than parental monolayer-derived cells (Figure 6).

A subpopulation of cells in spheroids have acquired features of myofibroblasts and may have undergone EMT

Myofibroblasts are components of the tumor stroma and may have a role in constructing the CSC-niche, which may help to preserve the stemness of CSC. Nevertheless, the origin of these cells remains unknown. Myofibroblasts produce several factors, which may stimulate proliferation of cancer cells and facilitate infiltration of tissue. In the literature, non-adherent spheroids generated from cancer cell lines have been shown to contain putative CSC. These spheroids could be maintained in culture for long periods, which implies that the spheroids may provide a niche suitable for the maintenance and growth of CSC. Therefore, we asked if i) myofibroblasts exist in the niche created by spheroids, ii) are these myofibroblasts derived from epithelial tumor cells through EMT, and iii) are there indications that this process is reversible?

To address this matter, we investigated the expression of myofibroblast markers Vimentin and α-SMA in SDC of 3 cell lines which have different ALDH1 expression levels in monolayer culture (low: UD-SCC1: 9.4±5.3%, medium: UT-SCC22: 16.9±4.1%, high: UT-SCC24A: 25.8±3.0%). We found that the different cell lines and SDC generated from these cell lines varied in the proportion of cells expressing Vimentin and α-SMA (Table 3). UD-SCC1 showed no α-SMA expressing cells and weak (12.5±2.5%) Vimentin expression of the cells in the monolayer culture. In contrast, UT-SCC24A (Figure 7) and UT-SCC22 monolayer-derived cells had a relatively high frequency of Vimentin and α-SMA expressing cells which indicates that these cell lines have both epithelial and mesenchymal characteristics. Interestingly, these two cell lines have a relatively high percentage of cells in the monolayer cultures that are ALDH1 positive (Table 3). Nevertheless, as spheroid cultures, all cell lines contained more α-SMA and Vimentin positive cells than the corresponding parental monolayer cell lines.

EMT is also accompanied by loss of cell-cell contacts to epithelial cells, characterized by a down-regulation of E-Cadherin expression. The number of E-Cadherin expressing cells in SDC and monolayers was quantified by FACS (Figure 7, Table 3). The SDC showed significantly increased proportions of E-Cadherin negative to low expressing cells compared to their monolayer-derived counterparts, indicating loss of cellular adherence in this subpopulation.

Finally, we also tested the re-adhesion of spheroids to culture plastic. Interestingly, when the spheroids attached to the flask and began to grow out as a monolayer culture, the α-SMA and Vimentin level decreased, and most of the cells that grew out from the spheroids stained negative for these two markers (Figure 8).

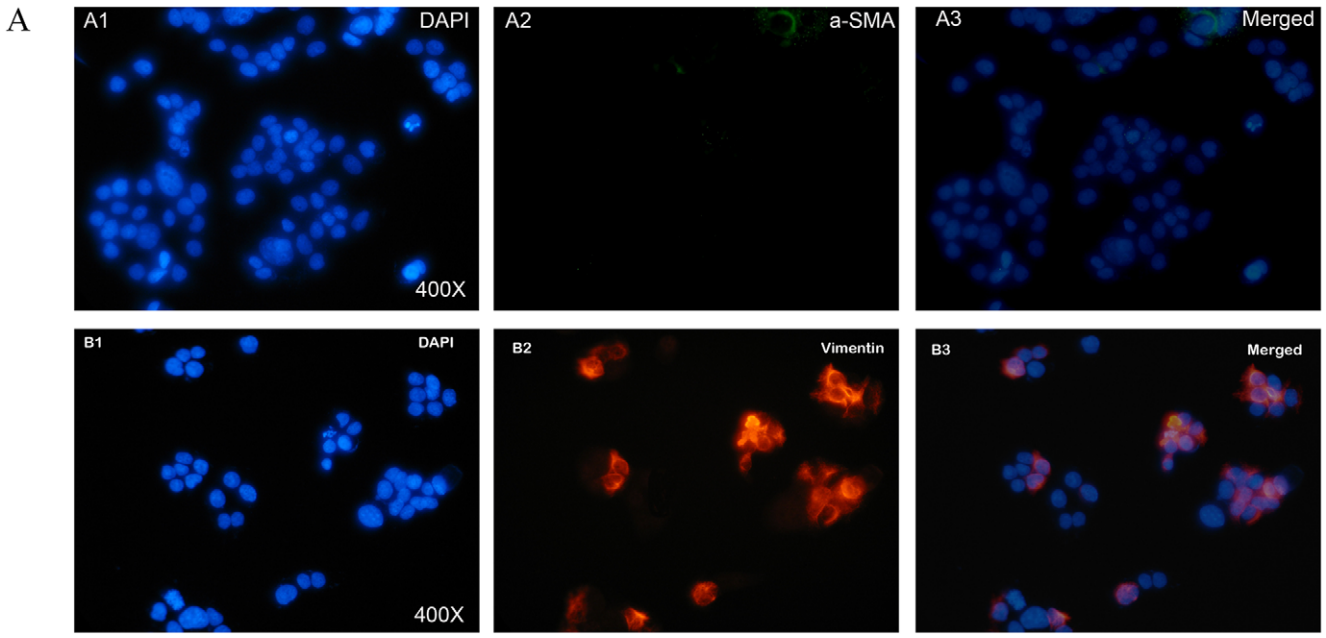
Discussion

In tumor biology many efforts have been made to explain the embryonal-like features of transformed cancer cells. The ability of CSC to rebuild the tumor from a single cell could explain many of the differences that discriminate tumor cells from differentiated somatic cells like immortality, quiescence, invasion leading to metastasis, and recurrence after treatment. Prince and colleagues [9], demonstrated that a minor population of $CD44^+$ HNSCC-cells possess CSC characteristics, which could give rise to new tumors in vivo (≈5,000 cells injected). Mack and colleagues questioned the use of CD44 as a specific CSC marker in HNSCC since CD44 was abundantly expressed in most tumor cells within HNSCC (60%-100%) and therefore could not be used to distinguish normal from benign or malignant epithelia of the head and neck [10]. ALDH1 was recently shown to be a more suitable marker to identify putative CSC of HNSCC and other epithelial cancers [11,12,13,14,15]. In HNSCC, Chen et al showed that 3000 $ALDH1^+$ HNSCC cells from five patients in xenotransplanted mice resulted in all cases in the generation of visible tumors 6 weeks after injection, while 10^4 $ALDH1^-$ cells failed to generate tumors. [12,39]. We describe a method for the propagation and enrichment of $ALDH1^+$ cell cultures of HNSCC cell lines. The stem cell-like characteristics of these cells were analyzed by comparing surface antigen expression and the expression of embryonal TF as well as functional characteristics of the parental monolayer cell lines. The observation that spheroid-cultures do not consist of a homogeneous cell population triggered further subanalyses that revealed a cell population with expression of EMT-markers. This may indicate that these cells have an activated EMT-program and demonstrates a potentially close relationship between CSC and cells with an activated EMT program which may have derived from the CSC.

Spheroids are three-dimensional (spherical) clusters of tumor cells grown from one or several cell clones. As compared to cell doubling times measured in monolayer culture, the rate and pattern of spheroid growth in vitro better matches that observed in tumors in vivo [40]. Anchorage independent growth has been shown to be a property shared by normal tissue cells that exhibit stem cell properties [41]. Also CSC derived from melanoma [42], breast cancer [43], and gliosarcoma [19] were able to be propagated anchorage independently and display the phenotype of nonadherent spheroids.

As exemplified in breast cancer for example, putative CSC ($CD44^+/CD24^-$) can be enriched in-vitro by isolating mammospheres from suspension cultures [43,44]. In our study, we found the frequency of $CD44^+/CD24^-$ cells in HNSCC lines to be highly variable (from 0.5% to 97%). Moreover, they could not be enriched by spheroid culture. In contrast, ALDH1 positive cells were found to be highly enriched in spheroid cultures from all five HNSCC cell lines. If seeded at a very low densitiy, FACS sorted $ALDH1^+$ cells formed significantly more colonies then the $ALDH^-$ population, indicating a higher proliferative capacity of this subpopulation. Others have shown, that $ALDH1^+$ or $ALDH1^+/CD44^+/CD24^-$ cell populations isolated from HNSCC-patients have a higher tumorigenic potential then $ALDH1^-$ cells even if they were $CD44^+24^-$ if xenotransplanted in mice [12].

Cancer and normal stem cells (SC) share proliferative properties of self-renewal, quiescence and expression of key transcription factors (TF). Chickarmane et al. [45] set up a computer-model to describe the key transcription factor combinations in SC and defined high levels of Oct3/4, Sox2, and Nanog. On the other hand, Ji et al.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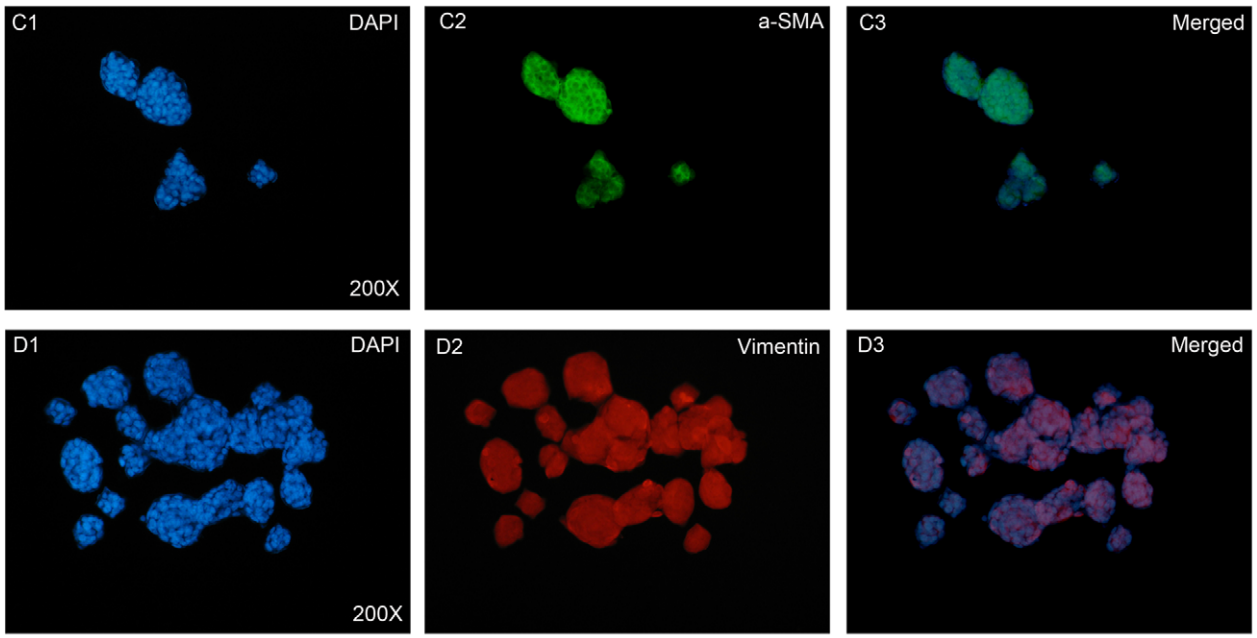
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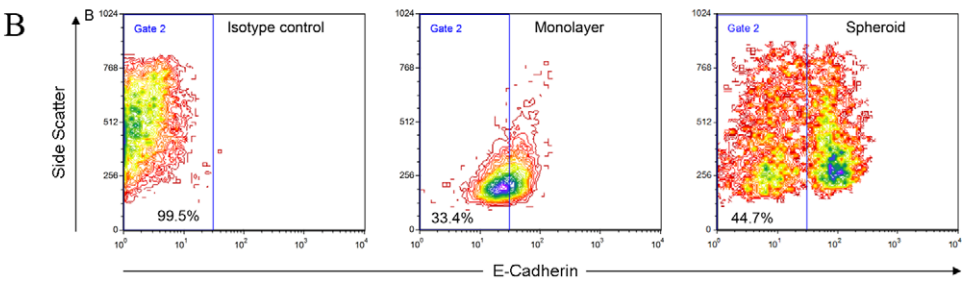
B
Side Scatter
Gate 2
Isotype control
99.5%
Gate 2
Monolayer
33.4%
Gate 2
Spheroid
44.7%
E-Cadherin

Figure 7. Analysis of the expression of α-SMA and Vimentin expression by immunofluorescence and E-Cadherin by FACS of monolayer versus SDC. (**A**) Immunofluorescence staining of monolayer (A1-3 and B1-3) and spheroid (C1-3 and D1-3) cultures from UT-SCC24A with antibodies directed against α-SMA (green) and Vimentin (red). All of the SDC (panel C and D) show high levels of the mesenchymal markers α-SMA and Vimentin compared to their monolayer counterparts. (**B**) E-Cadherin expression analysis by FACS. Compared to their monolayer counterparts, spheroids showed decreased E-Cadherin expression; two populations of cells derived from UT-SSC24A can be separated according to the E-Cadherin expression level, which indicates that the SDC have acquired myofibroblast characteristics by EMT.
doi:10.1371/journal.pone.0016466.g007

[46] compared the role of Oct3/4 and Nanog in transformed (t-hPSCs), and normal human pluripotent stem cells (hPSCs). Unlike normal SC, self-renewal and survival of t-hPSCs was found to be independent of Oct3/4 expression. In contrast, genetic knockdown of Nanog caused complete loss of self-renewal concomitantly with apoptosis in t-hPSCs. In our study, the expression of Oct3/4, Sox2, and Nanog was up-regulated in SDC in the five HNSCC derived cell lines. This implies that SDC may have stem cell properties.

Studies dealing with gene expression profiling using in vivo invasive cells or cells undergoing EMT in vitro, revealed a switch from a proliferative to an invasive phenotype. In agreement to this observation, the cell proliferation marker Ki-67 has been shown to label only a small minority of cells at the invasive front as compared to the more differentiated central part of the tumor, suggesting that non-proliferating tumor cells that have escaped the tumor mass could have metastatic potential [47]. In our study, we found that SDC had a higher proportion of Ki-67 negative cells compared to the corresponding monolayer cultures. This indicates that SDC are more quiescent than cells derived from monolayer. We also found that SDC have an increased invasion capacity, which implies that these cells may have a higher ability to metastasize.

Myofibroblasts are a kind of mesenchymal cell playing a key role in tumor invasion and metastasis and therapeutic response. They can produce several factors which may stimulate proliferation of cancer cells and facilitate their infiltration [31]. However, the origin of these myofibroblasts is not clear. EMT has been reported to play an important role in initiating CSC [29]. In this study, we demonstrated that the myofibroblast-like cells are a subpopulation of SDC. These spheroids derived exclusively from cloned tumor cells. It can therefore be concluded that the myofibroblasts could only emerge from epithelial cancer cells by altering the epithelial to a mesenchymal phenotype through EMT. More importantly, as we found, that over 90% of SDC stained positive for α-SMA and Vimentin while the percentage of $ALDH1^+$ cells ranged from 37% to 46.4%. A significant decrease of E-Cadherin expression could be demonstrated in SDC, indicating a reduction of cell-cell contact in spheroid cultures. These marker expressions were reversible under culture conditions, where spheroids could adhere and cells grew radially out to form a monolayer. Taken together, these data indicate that spheroid cultures contain at least two populations that at their extremes may have either CSC- or mesenchymal characteristics, while many cells are in transition between these phenotypes. We believe that cells derived from spheroids, which had undergone a transition to a mesenchymal phenotype, maintained CSC-characteristics such as expressing a high level of the CSC marker ALDH1, an increased proportion of G0 phase cells, increased invading capacity, and elevated mRNA levels of stem cell-related transcription factors such as Nanog, Sox2, and Oct3/4.

In summary, in this study we provide an anchorage independent culturing method of HNSCC cell lines suitable for the enrichment of cells with cancer stem cell characteristics and of cells undergoing EMT. Whether this method would select, starting from non-manipulated human HNSCC samples, the tumorigenic cells remains to be demonstrated. Functionally, SDC showed higher invading capacity than monolayer cells. FACS sorted $ALDH1^+$ cells showed higher colony forming ability than $ALDH1^-$ cells. Our findings may prove useful to investigate the phenomenon of EMT in vitro and thus support in vivo studies investigating the role of CSC and EMT in the spread of HNSCC.

Materials and Methods

Cell lines

The HNSCC cell line panel was composed of UD (University of Düsseldorf, Henning Bier) -SCC 1, UM (University of Michigan, Tom Carey) -SCC -11B and UT (University of Turku, Reidar Grenman) -SCC -9, -22, -24A [48,49]. The suffixes A, B, and C indicate cell lines derived from the primary tumor (A), metastatic (B) or recurrent (C) disease. These immortal HNSCC lines have been in culture from primary tumor for approximately 40 to 60 generations.

Suspension culture for spheroid formation

Adherent monolayer cells were grown in normal 25 cm^2 culture flasks (TPP, Switzerland) in DMEM (Gibco, Paisley, UK) supplemented with GlutaMAXTM-I, 1x containing 10% heat inactivated FCS and 1% Penicillin/Streptomycin, until confluency. Cells were washed with PBS twice and detached using Trypsin/EDTA solution. The reaction was stopped by adding complete culture medium. After 5 minutes, cells were resuspended in serum-free Quantum 263 medium (Biochrom AG, Berlin, Germany), supplemented with 10 ng/ml EGF and 10 ng/ml bFGF (Biochrom).

Table 3. Relative expression of EMT markers by monolayer and SDC-derived cells.

	α-SMA	Vimentin	E-Cadherin	ALDH1
UD-SCC1 Monolayer SDC[1]	no expression SDC: >90%	12.5±2.5% SDC: >90%	95.4±2.8% 83.5±3.1%**	9.4±5.3% 46.4±8.1%**
UT-SCC24A Monolayer SDC	6.0±1.4% SDC: >90%	36.3±5.3% SDC: >90%	62.4±4.5% 49.0±5.6%*	25.8±3.0% 42.5±8.7%*
UT-SCC22 Monolayer SDC	23.7±3.0% SDC: >90%	42.7±5.3% SDC: >90%	84.3±4.2% 63.1±5.0%**	16.9±4.1% 37.0±11.9%*

[1)]due to superposition cell proportions were estimated.
**P<0.01,
*P<0.05.
doi:10.1371/journal.pone.0016466.t003

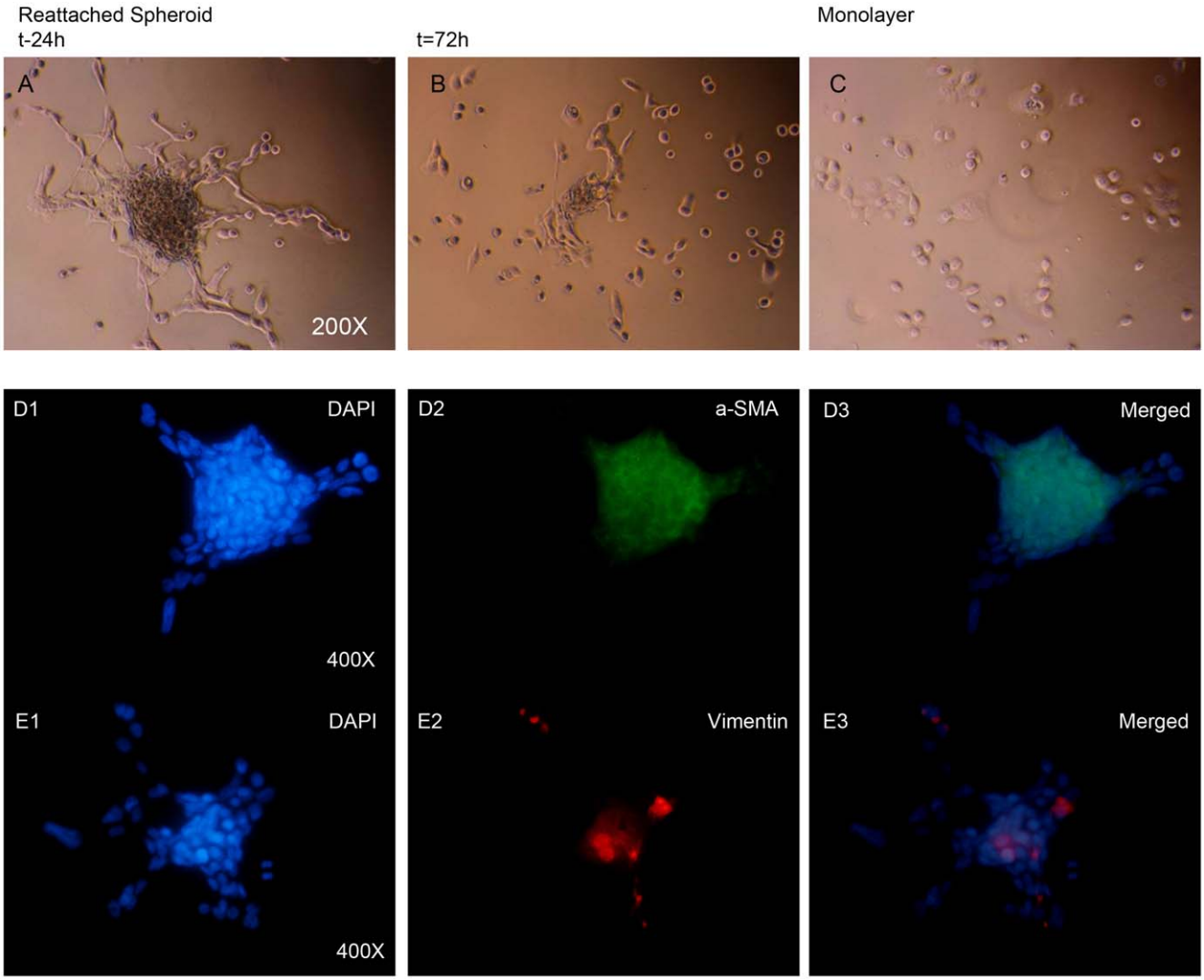

Figure 8. Changes in putative EMT marker expression upon reattachment of spheroids to an adhesive support. (**A–C**) Light-microscopic images with phase contrast form UD-SCC1 spheroids reattaching to cover slides during the process of re-growth to a monolayer phenotype. (A) 24 h after reattachment, some spindle-like cells grow out from adhered spheroids. (B) 72 h after reattachment, most of the attached cells' shape looks like their parental monolayer counterpart (C). (**D–E**) Indirect immunofluorescence staining of reattached spheroids after 24 h stained with DAPI (D1, E1), α-SMA (D2; green), and Vimentin (E2; red). Pictures D3 and E3 show the overlay with nuclear staining. Arrows depict outgrowing cells radiating from the spheroid. The expression of α-SMA and Vimentin decreased in outgrowing cells (arrows) after reattachment and most of the staining was confined to spheroids while most of the cells grown from spheroids were unstained.
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To generate spheroids, single cells were plated in Corning* Ultra-Low Attachment plates (Fisher Scientific, Loughborough, UK) at a specific density of 2×10^4 cells/ml. Cells were kept in the incubator at 37°C in humidified atmosphere with 5% CO_2 content. At day three, half of the medium was replaced. After 5 to 7 days, representative pictures were taken and spheroids were collected by filtration through a 40 μm mesh for the following experiments.

Multicolor flow-cytometric (FACS) analysis and sorting

The identification of aldehyde dehydrogenase 1 (ALDH1) activity from spheroid-derived and HNSCC monolayer cells was conducted by using the ALDEFLUOR assay (StemCell Technologies, Durham, NC, USA). Spheroids were collected using a 40 μm mesh and disaggregated into single cells by Trypsin/EDTA digestion for 10 min followed by 20 times up- and down pipetting using a 1000 μm pipette tip. The single cell suspension was washed twice in PBS buffer then suspended in ALDEFLUOR assay buffer containing ALDH substrate (BAAA, 1 mol/l per 1×10^6 cells) and incubated for 40 min at 37°C. As a negative control, for each sample of cells an aliquot was treated with 50 mmol/l diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor.

Next, for cell surface antigen phenotyping, cells were resuspended in 100 μl Aldefluor incubation buffer then stained with 20 μl anti-CD24-PE (BD bioscience, San Jose, CA, USA), 20 μl anti-CD44-APC (BD bioscience) and 5 μl 7-AAD (BD bioscience) or 5 μl anti-E-Cadherin (Biolegend, San Diego,CA, USA) per 10^6 cells. The cells were then incubated at 4°C for 15 min in the dark. Following incubation, cells were washed once with cold FACS buffer.

For FACS sorting, cells were resuspended in PBS buffer at 1×10^7 cells per ml and separated on an Aria cell sorter (BD Biosciences). The sorted cells could not be assessed for purity by reanalysis due to the low numbers of cells obtained. The sorting

Table 4. Primer sequences used for RT-PCR in TF expression analysis.

Transcript name	Forward primer sequence	Reverse primer sequence
SNAIL1	GGCGCACCTGCTCGGGGAGTG	GCCGATTCGCGCAGCA [51]
Nanog	AATACCTCAGCCTCCAGCAGATG	TGCGTCACACCATTGCTATTCTTC
SNAIL2	GGGGAGAAGCCTTTTTCTTG	TCCTCATGTTTGTGCAGGAG
Twist1	GGAGTCCGCAGTCTTACGAG	TCTGGAGGACCTGGTAGAGG
Oct3/4	GACAGGGGGAGGGGAGGAGCTAGG	CTTCCCTCCAACCAGTTGCCCCAAAC
Sox2	GGGAAATGGGAGGGGTGCAAAAGAGG	TTGCGTGAGTGTGGATGGGATTGGTG
hL32 (reference)	AGCTCCCAAAAATAGACGCAC	TTCATAGCAGTAGGCACAAAGG [50]

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gates were established, using as negative controls the cells treated with DEAB.

Clone formation assay

After FACS sorting, the ALDH1$^+$ and ALDH1$^-$ cells were inoculated to 6-well plates at a density of 1000 cells/ml in DMEM medium supplemented with 10% FBS. Cells were cultivated under standard culture conditions for 2 weeks and then the clone numbers were counted microscopically.

Spheroid culture is one of the three-dimensional culture systems that have been reported. After FACS sorting, ALDH1$^+$ and ALDH1$^-$ cells were inoculated into Ultra-low attachment 96-well plates (Fisher Scientific, Loughborough, UK) by using the limiting dilution method. All wells were examined carefully under microscopical vision and the wells with more than one cell or no cells were excluded from further analysis. Cells were resuspended in serum-free Quantum 263 medium (Biochrom AG, Berlin, Germany), supplemented with 10 ng/ml EGF and 10 ng/ml bFGF (Biochrom). Fresh medium was added every week. After 3 weeks, the cells contained in each well were counted and photographed.

Quantitative real-time PCR

Total RNA was extracted by using a Qiagen RNeasy kit (Qiagen, Hilden Germany), then converted to cDNA with the Omniscript First-Strand synthesis system (Qiagen) using random primers (Qiagen). qRT-PCRs were carried out using ABI Power SYBR Green mix (ABI, Applied Biosystems Inc, Foster City, CA, USA) and run on a BioRad Chromo 4 (BioRad, München, Germany). Reactions were carried out in Triplicate with RT controls, the gene of Ribosomal protein HL32 was used as a reference gene [50], and data were analyzed using the modified delta delta Ct method. Primer sequences are listed in table 4.

Invasion assay

Cells cultivated in a monolayer or in a spheroid-culture were separated into single cells by careful trypsin digestion and resuspended in 1% BSA-DMEM culture medium. Cells (5×10^4) were seeded into the upper compartments of BD BioCoat^{TM} Matrigel Invasion Chambers (BD Bioscience), and DMEM supplemented with 10% FBS was added to the lower compartment according to manufacturer's instructions. The invasion chamber was kept for 24 h under standard culture conditions. After incubation, the non-invading cells were removed from the upper surface of the membrane by gentle scrubbing, and the cells on the lower surface of the membrane were stained with crystal violet. Cell counting was facilitated by photographing the membrane through the microscope and 3 fields per membrane of triplicate membranes were counted under 200× magnification (Axiovert, Axiovision, Zeiss, Germany).

Cell cycle analysis and Ki-67 staining

The parental cells and spheroids were harvested, dissociated into single cells and resuspended in 70% ethanol by adding ethanol at −20°C dropwise while vortexing. The cells were stored at −20°C overnight then washed with PBS, centrifuged and 1 µg FITC-conjugated mouse anti-human Ki-67 (DAKO, Glostrup, Denmark) was added to the cell pellet and incubated for 20 min at room temperature. Finally, cells were resuspended in 1 ml of a cocktail containing propidium iodide (2 µg/ml) and RNAse (100 µg/ml) (Fermentas Life Science, St. Leon-Rot, Germany) and stained for 30 min followed by FACS analysis.

Immunofluorescence

Indirect immunofluorescence staining was performed on spheroids and on parental adherent cultures of cancer cells for α-SMA and Vimentin. Spheroids were collected by filtering the suspension culture medium through a 40 µm mesh (BD bioscience), and then whole spheroids were cytospined (1500 rpm, 5 min) onto slides. Cells were fixed in 4% paraformaldehyde then permeabilized with 0.1% Triton in PBS solution for 10 min. First, mouse anti-human Vimentin (1:50 from stock; Santa Cruz Biotechnology, Heidelberg, Germany) and mouse anti-human α-SMA diluted (1:50 from stock; Dako) antibodies were added and incubated overnight at 4°C. Then the secondary antibody, Alexa488-conjugated goat anti-mouse IgG (Invitrogen, Karlsruhe, Germany) or Alexa568-conjugated donkey anti-mouse IgG (1:200 from stock) (Invitrogen), was added and incubated for 60 min at room temperature. Counterstaining was performed by using DAPI (1 µg/ml Roche, Mannheim, Germany). For quantification, the average percentages of positive cells from three experiments were estimated. Due to superposition in the case of SDC, cell proportions are less reliable. Therefore, no significance was calculated for Vimentin and α-SMA.

Supporting Information

Figure S1 Single ALDH1$^+$ cells proliferated and formed a spheroid significantly more often then ALDH1$^-$ cells. (**A**) FACS-sorting strategy for SDC of UD-SCC1. Representative pictures of the cell line UD-SCC 1 are shown. (**B**) ALDH1$^-$ or ALDH1$^+$ single cells were cultured in 96-well ultra-low attachment plates. Three weeks after inoculation, 19.3% of ALDH1$^+$ SDCs formed spheroids while ALDH1$^-$ cells failed to form spheroids except for one case ($p<0.01$).
(TIF)

Author Contributions

Conceived and designed the experiments: AEA CC AMK. Performed the experiments: CC YW. Analyzed the data: AEA AMK MH TKH. Contributed reagents/materials/analysis tools: AEA AMK. Wrote the paper: AEA CC AMK YW MH TKH. Obtainted permission for use of cell line: TKN. Contributed to the revision of the paper: MG.

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Human skeletal muscle-derived stem cells retain stem cell properties after expansion in myosphere culture

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Susceptibility to cytotoxic T cell lysis of cancer stem cells derived from cervical and head and neck tumor cell lines

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Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Publications

1. **Chen C**, Wei Y, Hummel M, Hoffmann TK, Gross M, Kaufmann AM, Albers AE. Evidence for epithelial-mesenchymal transition in cancer stem cells of head and neck squamous cell carcinoma. PloS One, 2011，6(1):e16466.
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[Component of the dissertation]

Affidavit

I, Chao, Chen certify under penalty of perjury by my own signature that I have submitted the thesis on the topic Enrichment of cancer stem cells from head and neck cancer by anchorage independent culture and related research，I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations indicated. The sections on methodology and results correspond to the URM (s.o) and are answered by me. My contributions in the selected publications for this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author of correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date _____ Signature

Declaration of any eventual publications

[Chao, Chen] had the following share in the following publications:

Publication 1: **Chao Chen**, Yan Wei, Michael Hummel, Thomas K. Hoffmann, Manfred Gross, Andreas M. Kaufmann, Andreas E. Albers, Evidence for Epithelial-Mesenchymal Transition inCancer Stem Cells of Head and Neck Squamous Cell Carcinoma, PLoS One, 2011

Contribution in detail (please briefly explain): Independently completed all the experiments and statistical analysis involved the article.

Publication 2: Yan Wei, Yuan Li, **Chao Chen**, Katharina Stoelzel, Andreas M. Kaufmann, Andreas E. Albers, Human skeletal muscle-derived stem cells retain stem cell properties after expansion in myosphere culture, Experimental cell research, 2011

Contribution in detail (please briefly explain): Applied the anchorage independent culture method into myosphere culture, and analyzed the cell surface marker expression by flow cytometry.

Publication 3: Tian Liao, Andreas M. Kaufmann, Xu Qian, Voramon Sangvatanakul, **Chao Chen**, Tina Kube, Guoyou Zhang, Andreas E. Albers, Susceptibility to cytotoxic T cell lysis of cancer stem cells derived from cervical and head and neck tumor cell lines, J Cancer Res Clin Oncol, 2012

Contribution in detail (please briefly explain): Established the reliable method for enriching CSCs from different cell lines and optimized the ALDH1 assay by using flow cytometry

Signature, date and stamp of the supervising University teacher

_____

Signature of the doctoral candidate

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