

Isolation of head and neck squamous carcinoma cancer stem-like cells in a syngeneic mouse model and analysis of hypoxia effect

SÓNIA DUARTE¹, AGNÈS LOUBAT³, DAVID MOMIER⁴, MAJLINDA TOPI⁴, HENRIQUE FANECÁ¹, MARIA C. PEDROSO DE LIMA^{1,2}, GEORGES F. CARLE⁴ and VALÉRIE PIERREFITE-CARLE⁴

¹CNC-Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra;

²Department of Life Sciences, University of Coimbra, Coimbra, Portugal; ³IFR50 and ⁴GéPITOS UMR 6235 CNRS, School of Medicine, University of Nice Sophia Antipolis, Nice, France

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Abstract. The incidence of oral tumors is increasing around the world and despite recent advances in early detection and diagnosis, current treatments are still unsatisfactory. Recent data suggest that tumor persistence and recurrence could be due to the presence of a rare cell population called cancer stem cells (CSCs), which are generally spared by traditional treatments. Therefore, identification and characterization of CSCs are extremely important to develop novel and effective treatment strategies for cancer. The aim of this study was to identify and isolate CSCs in an established murine head and neck squamous cell carcinoma (HNSCC) cell line and to investigate the influence of hypoxic conditions on the isolated cell population. Using the expression of the aldehyde dehydrogenase 1 (ALDH1) enzymatic activity, which is now recognized as a CSC marker in various tumors, we isolated a cell population expressing high levels of ALDH1 (ALDH1^{high}) representing 1±0.6% in the murine SCC-VII cell line. These cells were injected subcutaneously in syngeneic animals to evaluate their tumorigenic properties. For the lowest injected cell dose (250 injected cells), tumor occurrence and median tumor size were higher in ALDH1^{high} injected mice than in ALDH1^{low} injected mice. Following an *in vivo* passage and culture in serum-free medium, the percentage of ALDH1^{high} cells increased by 3-fold

in SCC-VII CSCs (oral spheres) compared to the SCC-VII cell line. This percentage was further increased when oral spheres were cultured under hypoxic conditions. In conclusion, this study reports for the first time the isolation of HNSCC CSCs in a syngeneic mouse model and the use of hypoxia as a method to further enrich the ALDH1^{high} cell population.

Introduction

Head and neck squamous cell carcinoma (HNSCC) represents the sixth most common cancer in developed countries and is responsible for approximately 350,000 cancer deaths per year (1). In addition, the incidence of oral tumors is increasing around the world (2). Despite recent advances in early detection and diagnosis, current treatments of oral cancer are unsatisfactory mainly due to the development of distant metastasis and the emergence of treatment-resistant recurrences, resulting in an unchanged 5-year survival rate over the last two decades (3,4). It is now established that tumor persistence and recurrence could be due to the presence of cancer stem cells (CSCs) in some cancers. The cancer stem cell theory of carcinogenesis postulates that normal stem or progenitor cells can eventually become malignant because of the occurrence of genetic and epigenetic alterations (5). Based on their similarity to normal stem cells, CSCs exhibit extensive capability of self-renewal and are likely to be more resistant to therapy (5). Moreover, they have the ability to generate a tumor after injection of a very small number of cells and to reconstitute the cellular heterogeneity typical of the original tumor (6,7). The presence of CSCs has now been evidenced in solid tumors from various origins including head and neck (5,8,9). This rare HNSCC CSC population can be isolated by using cell surface antigens such as CD44 (8) or CD133 (10). The propensity of CSCs to efflux the vital DNA binding Hoechst 33342 dye, due to an increased expression of multiple drug resistance transporter proteins, has also been used as a means to isolate them as a side population (SP) (6). Alternatively, HNSCC CSCs can be isolated on the basis of the expression of the aldehyde dehydrogenase 1 (ALDH1) enzymatic activity (11,12), which is now recognized as a CSC marker in tumors from various origins

Correspondence to: Dr Valérie Pierrefite-Carle, GéPITOS UMR 6235 CNRS UNSA, School of Medicine, Avenue de Valombrose, 06107 Nice cedex 2, France
E-mail: pierrefi@unice.fr

Abbreviations: ALDH, aldehyde dehydrogenase; CSCs, cancer stem cells; DEAB, diethylaminobenzaldehyde; DMEM, Dulbecco's modified Eagle's medium; FACS, fluorescence-activated cell sorting; FCS, fetal calf serum; HNSCC, head and neck squamous cell carcinoma

Key words: head and neck squamous cell carcinoma, cancer stem cells, ALDH1 selection, hypoxia

(13). This last method was described as more specific as the isolated cells appear to be more tumorigenic than the CD44⁺ CSCs (12).

Although primary cancer cells obtained from patients represent the most physiologically relevant material to isolate CSCs, this source presents two main disadvantages. First, it constitutes a very limited resource due to the relatively small size of head and neck tumors and the small percentage of CSCs within the tumor. Second, the human cells need to be administered in immunocompromised animals and, depending on the degree of host immunocompetence, these xenotransplantation assays have been shown to underestimate or overestimate the frequency of cells with tumorigenic potential (14).

In the present study, the objective was to isolate ALDH1^{high} cells in a murine established HNSCC cell line and to determine if these cells would exhibit cancer stem cell-like properties. In addition, we examined the effect of a hypoxic environment on the CSC population *in vitro*.

Materials and methods

Cell culture. The SCC-VII cell line is derived from murine oral squamous cell carcinoma and is able to induce progressive tumors in syngeneic hosts. SCC-VII cells were maintained at 37°C under 5% CO₂, in Dulbecco's modified Eagle's medium (Invitrogen, Cergy Pontoise, France) supplemented with 10% fetal calf serum (FCS) (Dutscher, Brumath, France), penicillin (100 U/ml) and streptomycin (100 µg/ml).

The oralspheres (SCC-VII CSCs) were cultured on ultra-low attachment flasks (Corning, Sigma-Aldrich) in serum-free DMEM/F12 medium (Invitrogen) with penicillin (100 U/ml) and streptomycin (100 µg/ml) supplemented with 10 ng/ml bFGF (Peprotech), 20 ng/ml EGF (Peprotech), 4 µg/ml, heparin (Sigma-Aldrich), 4 mg/ml bovine serum albumin (Sigma-Aldrich), 20 µg/ml insulin (Sigma-Aldrich) and N2 supplement (Invitrogen). Oralspheres were dissociated mechanically and enzymatically, using Accumax reagent (Sigma-Aldrich). This reagent that combines protease, collagenolytic and DNase activities was used to generate single cell suspension for ALDH1 labeling and flow cytometry analysis.

Hypoxia/normoxia. In normoxia, the oralspheres were grown in a humidified 21% O₂ and 5% CO₂ environment. For hypoxia experiments, the oralspheres were grown in a humidified 1% O₂ and 5% CO₂ environment using a MiniGalaxy A incubator (RS Biotech, Ltd). The medium was replaced twice per week after being prewarmed and equilibrated for 4 h in 1% O₂.

ALDH1^{high} cell selection. The ALDEFLUOR kit (StemCell Technologies SARL, Grenoble, France) was used to isolate the cell population with a high aldehyde dehydrogenase 1 (ALDH1) enzymatic activity. Single cell suspensions obtained from freshly trypsinized SCC-VII cells or dissociated oralspheres were suspended in ALDEFLUOR assay buffer. Then, 5 µl of ALDH substrate (BAAA) were added to the cell suspension and the samples were incubated during 40 min at 37°C. As a negative control for each sample of cells, an aliquot was treated with 5 µl of diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor. Fluorescence-activated cell sorting (FACS) was used to analyze the ALDH1^{high} cell population.

The sorting gates were established using the ALDEFLUOR-stained cells treated with DEAB.

Animals and tumor model. Six- to eight-week-old female C3H/HeOuJ mice (Charles River, L'arbresle, France) were used to assess the *in vivo* stem cell properties of the ALDH1^{high} population, compared to the ALDH1^{low} population. All the surgical procedures and the care given to the animals were performed in accordance with institutional guidelines. After anesthetization of the mice, 1,000 or 250 ALDH1^{high} or ALDH1^{low} SCC-VII cells (50 µl) mixed with 50 µl of Matrigel (BD Biosciences, NJ, USA) were injected subcutaneously using a 25-gauge needle. Tumor growth was monitored and after visual detection, the mice were sacrificed and tumor formation was assessed. Tumor volumes were estimated using the formula: length x width² x $\pi/6$ (15).

Tumor dissociation. After excision, the tumors were washed in PBS and DMEM-F12 containing the penicillin/streptomycin (500 U/ml) and amphotericine B (1.25 µg/ml). Then, each tumor was cut into small fragments using sterile scissors and incubated at 37°C with DMEM F-12 containing 1.5 mg/ml collagenase I, 20 µg/ml hyaluronidase and 0.006% of DNase I. After 1 h of digestion, cells were filtered first through a 100-µm nylon sieve, followed by centrifugation at 1,000 rpm for 5 min and then filtered through a 40-µm nylon sieve and centrifuged again. The pellet was suspended in supplemented serum-free DMEM/F12 medium, as described above for culture of oralspheres.

Statistical analysis. The results are expressed either as median [95% confidence intervals (CI)] or as mean \pm standard error. Comparison of tumor volumes between different mice groups was performed using the Mann-Whitney test, which is a non-parametric, two-tailed probability test. P-values were considered to be statistically significant when <0.05 .

Results

Isolation of ALDH1^{high} cells from the murine SCC-VII squamous cell carcinoma cell line. Using the ALDEFLUOR assay and FACS analysis, ALDH1^{high} and ALDH1^{low} cells were isolated from the SCC-VII cell line. Untreated cells exhibited some autofluorescence and the entry of the fluorescent ALDEFLUOR substrate within the cells shifted them to the P1 gate (Fig. 1A). In the presence of the ALDH1 enzyme, substrate cleavage increased its fluorescence, shifting the cells to the P5 gate, which was not observed in the presence of DEAB, the ALDH1 inhibitor. As expected, the majority of SCC-VII cells exhibited low ALDH1 activity, with the percentage of ALDH1^{high} cells being 1 \pm 0.6%. Following sorting, ALDH1^{high} cells were cultured in suspension in serum-free medium with bFGF and EGF. As shown in Fig. 1B, the ALDH1^{high} cells were able to grow as spheres in this medium.

Tumorigenicity of ALDH1^{high} and ALDH1^{low} cells in syngeneic animals. To evaluate the tumorigenicity of ALDH1^{high} and ALDH1^{low} cells, the two cell populations were injected subcutaneously in two different doses (1,000 or 250 cells) in syngeneic C3H mice immediately after sorting. Fig. 2A shows tumor occurrence according to ALDH1 expression and number of

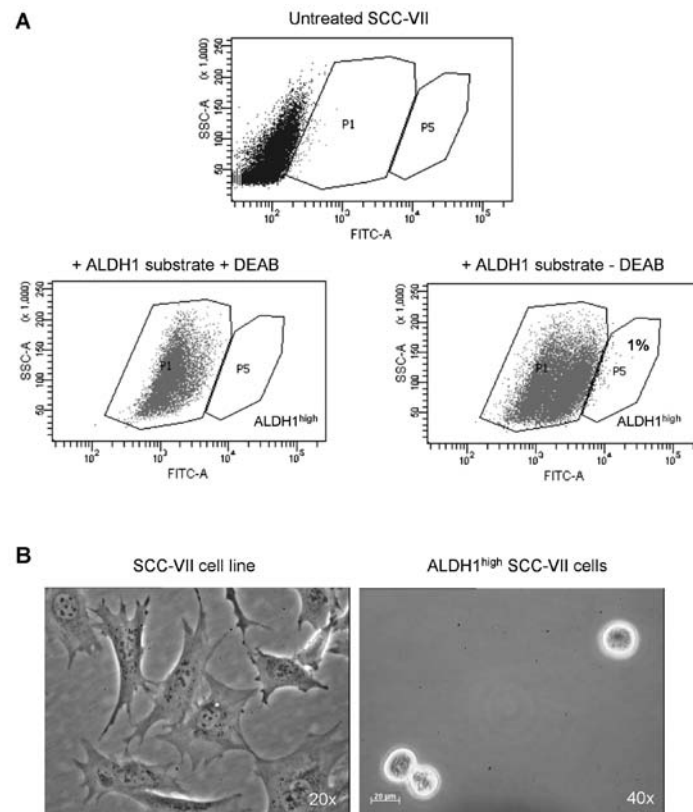


Figure 1. Isolation of squamous cell carcinoma (SCC-VII) cells expressing high levels of ALDH1. (A) Selection of ALDH1^{high} cells from the SCC-VII cell line using the ALDEFLUOR assay. The flow cytometry profiles of untreated cells and cells treated with ALDH1 substrate in the presence or absence of DEAB are presented. The P5 gate contains the ALDH1^{high} cells. (B) Representative photographs of the SCC-VII cell line cultured in DMEM with 10% serum and sorted ALDH1^{high} SCC-VII cells cultured in serum-free medium for 2 days at x20 and x40 magnification, respectively.

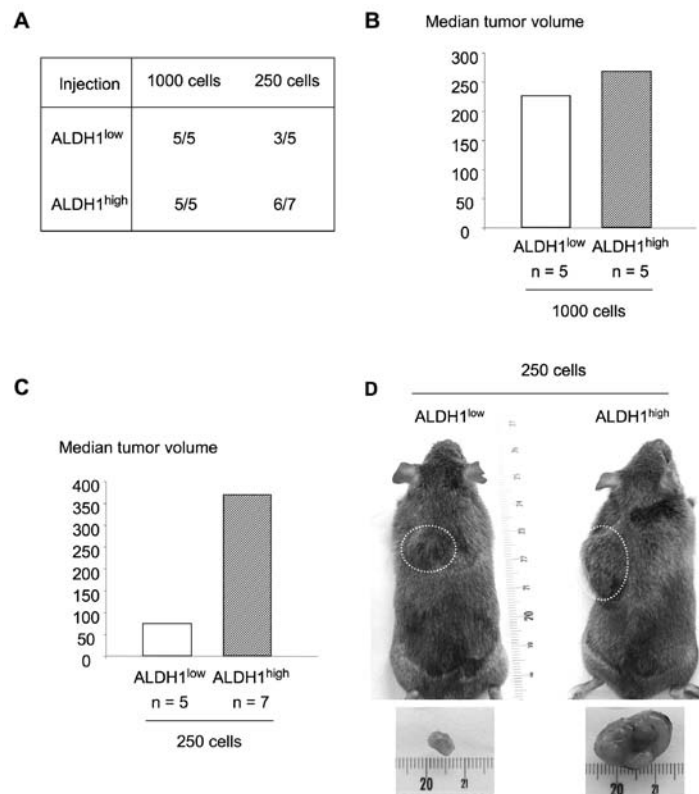


Figure 2. Generation of subcutaneous tumors by injection of sorted cells. (A) Number of tumors resulting from implantations of 250 or 1,000 ALDH1^{high} and ALDH1^{low} cells. (B) Median volume of tumors obtained by injection of 1000 ALDH1^{high} cells or 1,000 ALDH1^{low} cells. (C) Median volume of tumors obtained by injection of 250 ALDH1^{high} cells or 250 ALDH1^{low} cells. (D) Representative tumors in mice injected with 250 ALDH1^{high} cells or 250 ALDH1^{low} cells.

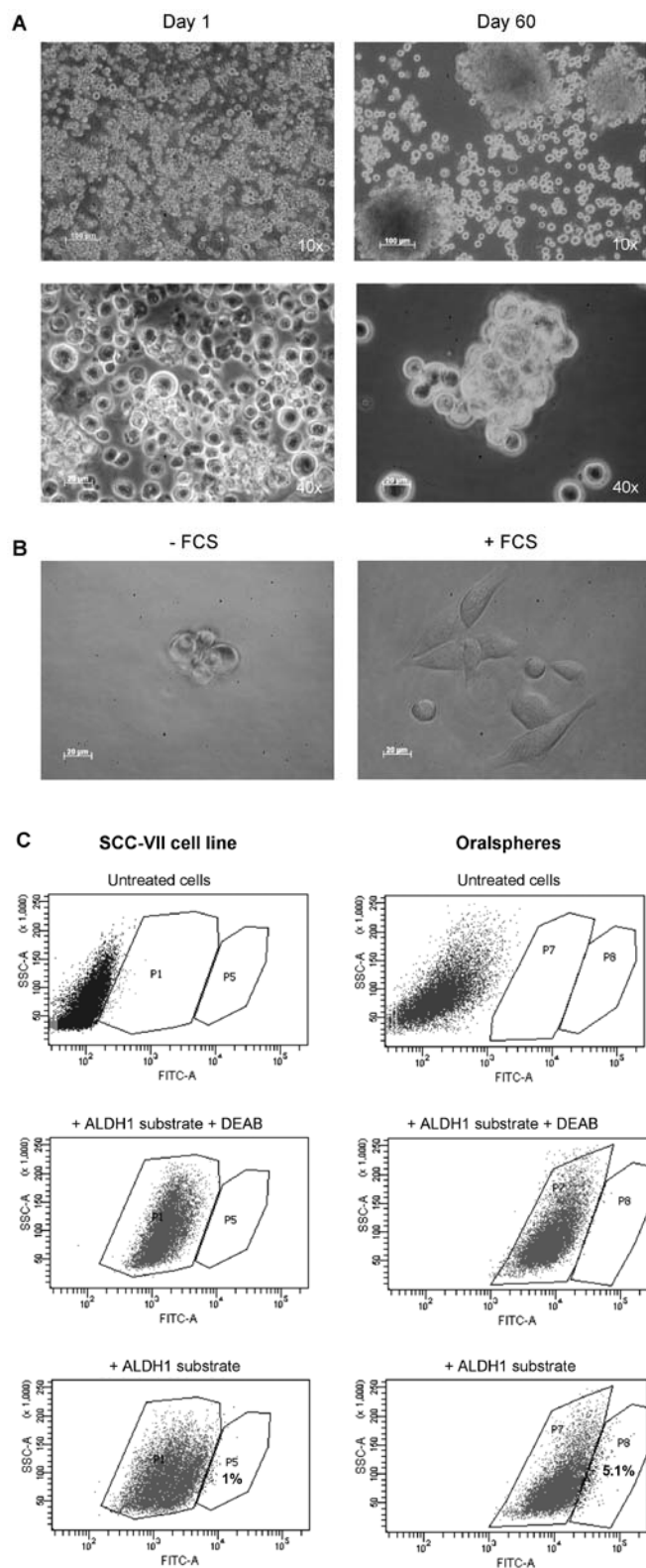


Figure 3. *In vitro* selection of oralspheres from ALDH1^{high} derived-tumors. Tumors generated by injection of 250 ALDH1^{high} SCC-VII cells were dissociated and cultured in serum-free medium containing bFGF and EGF. (A) Representative photographs of oralspheres at days 1 and 60 at x10 and x40 magnification. (B) Differentiation of oralspheres in the presence of serum. After being cultured for >60 days in serum-free medium, the differentiation potential of these cells was evaluated by culturing in DMEM with 10% serum. Representative photographs of oralspheres in the absence of serum (-FCS) and after 24 h of culture in the presence of serum (+FCS) at x40 magnification. (C) Comparison of the percentage of ALDH1^{high} cells in oralspheres and in the SCC-VII cell line by flow cytometry. P5 and P8 represent ALDH1^{high} cells in SCC-VII cell line and oralspheres, respectively.

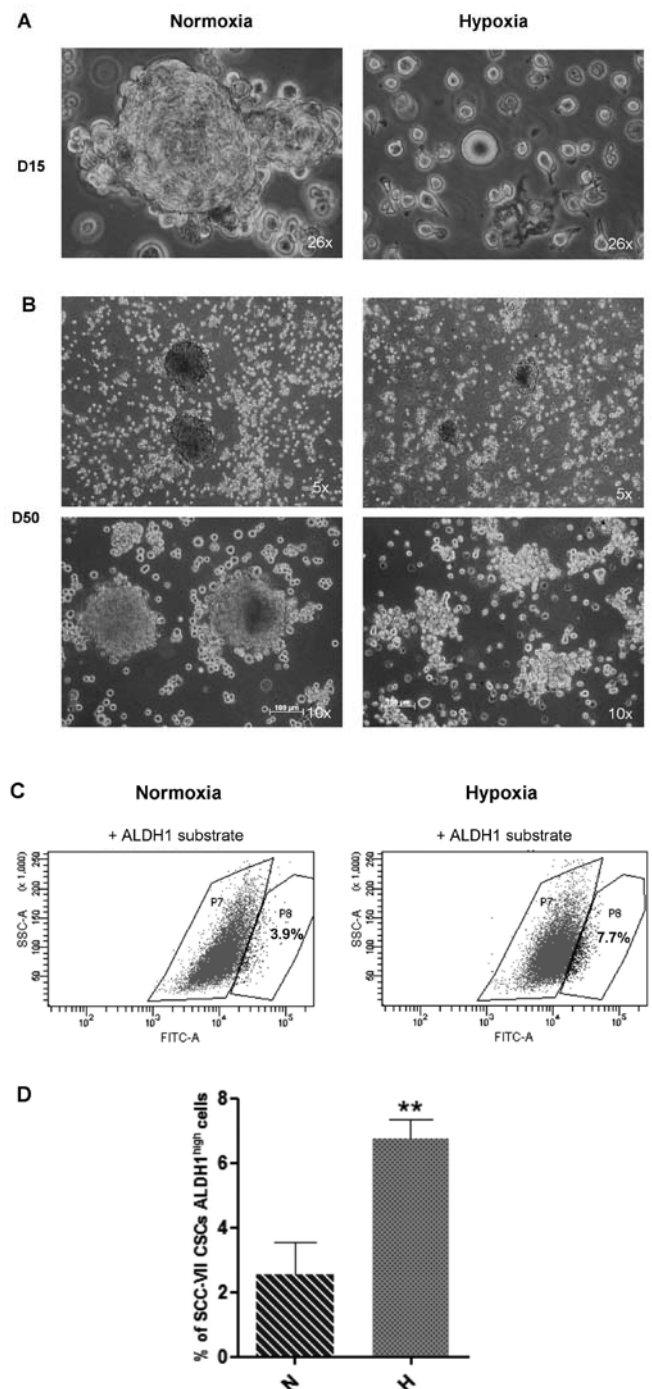


Figure 4. Effect of a hypoxic environment on the growth of oralspheres *in vitro*. (A) Representative photographs of oralspheres cultured under normoxia or hypoxia for 15 days at x26 magnification. (B) Representative photographs of oralspheres cultured under normoxia or hypoxia for 50 days at x5 and x10 magnification. (C) Determination of ALDH1 expression in oralspheres cultured under normal conditions or hypoxia for 50 days. P8 represents ALDH1^{high} cells. (D) Comparison of the ALDH1^{high} percentage in oralspheres cultured under normoxia or hypoxia. **P<0.01 indicate values that differ significantly from those measured under normal conditions.

injected cells. At the highest cell number, both ALDH1^{high} and ALDH1^{low} cells were able to induce tumors in 100% of cases. The median tumor volumes were not statistically different with 226.1 and 267.9 mm³ when generated by ALDH1^{low} and ALDH1^{high} cells, respectively (Fig. 2B). At the lowest number

of injected cells, tumors were developed in 3 out of 5 mice after administration of ALDH1^{low} cells and in 6 out of 7 mice in the case of ALDH1^{high} cells. Although the differences in tumor size were not statistically significant, a 5-fold increase in the median volume of tumors generated by ALDH1^{high} cells (368.4 mm³; 95% CI, 118-784) compared to the ones generated by ALDH1^{low} cells (74.2 mm³; 95% CI, 0-333.4) was observed (Fig. 2C and D). Taken together, these results suggest that ALDH1^{high} cell population is enriched in CSCs.

In vitro selection of oralspheres from ALDH1^{high} derived-tumors. Following tumor dissociation, the cell suspension was cultured in serum-free medium with bFGF and EGF. Spheres, which we called oralspheres, formed by cells aggregates were able to grow exponentially in these conditions (Fig. 3A). To determine the differentiation potential of these cells, oralspheres were then cultured in standard medium in the presence of 10% serum. After 24 h of culture, undifferentiated cells in suspension attached to the plastic, gradually migrating from oralspheres and differentiating into large and adherent cells (Fig. 3B).

The percentage of ALDH1^{high} cells was determined in oralspheres, following an *in vivo* passage, after 3 months of culture in serum-free medium. As shown in Fig. 3C, a slight increase in this percentage was observed with averages of 1% for the SCC-VII cell line and 3% for oralspheres.

Effect of a hypoxic environment on oralspheres in vitro. As CSCs are able to grow under extreme conditions, we then analyzed the effect of hypoxia on the growth of this population. To this aim, oralspheres were cultured under either normoxia (21% O₂) or hypoxia (1% O₂) for 8 weeks. As observed in Fig. 4A, after 15 days oralspheres did not exhibit large spheres under hypoxia as compared to those in normoxia. In addition, under hypoxic conditions, the number of cells decreased and some of them appeared to degenerate and die. The cells finally recovered after 4 weeks under hypoxia, and some spheres could be observed (Fig. 4B). As shown in Fig. 4C and D, comparison of the percentage of ALDH1^{high} cells in oralspheres maintained in normoxia and hypoxia revealed a significant increase of the percentage of ALDH1^{high} cells under the latter conditions with a mean of 6.7% (±1.3) vs. 2.6% (±1.9), under the former conditions (P<0.05).

Discussion

It has been suggested that CSCs could be one of the key determinants of treatment failure in HNSCC like in other cancer types (16,17). So far, most studies regarding HNSCC CSCs have been carried out *in vitro* with only a few studies performed in an *in vivo* environment. Developing *in vivo* models is needed to obtain a comprehensive understanding of the biology of these cells (18). *In vivo* experiments have generally been performed in human cancer cells and immunocompromised animals, and these xenotransplantation assays have been described to result in a great variability in the frequency of cells with tumorigenic potential, depending on the degree of host immunocompetence (14). For these reasons, we decided to isolate CSCs in a syngeneic HNSCC murine model. Although different markers are available for CSC isolation, their detection within the total

tumor bulk remains a challenge (18). ALDH1 was shown to select HNSCC cells exhibiting high self-renewal capacity, chemo- and radioresistance (16), and ALDH1^{high} cells appear to be more tumorigenic than the CD44⁺ CSCs (12). Using this approach in the SCC-VII cell line, we showed that ALDH1^{high} cells represent approximately 1±0.6% of the population, which is in accordance with previously published results (12), while the majority of the cell population presented a low ALDH1 activity. Growth in serum-free conditions has been demonstrated as a suitable method by which HNSCC CSCs can be efficiently cultured *in vitro* in an undifferentiated state (6). This was indeed the case for the SCC-VII ALDH1^{high} cells, in agreement with previously reported results (7,19). Following selection of ALDH1^{high} and ALDH1^{low} cells, the tumorigenic potential of both populations was evaluated in a syngeneic C3H immunocompetent mouse model for oral cancer. For the injection of 1,000 cells, tumor occurrences and volumes were not significantly different between ALDH1^{high} and ALDH1^{low} cells, suggesting that the highest tested cell dose did not discriminate the tumorigenic potential of the two populations. This observation also indicates that cells with a high tumorigenic potential were present in the ALDH1^{low} cell population, strengthening the need for the identification of additional markers of the CSCs. The 250 cell transplantation assay allowed the discrimination between ALDH1^{high} and ALDH1^{low} cells for both tumor volumes and occurrences, suggesting that ALDH1^{high} cells were enriched in CSCs. As expected, these cells were able to grow as spheres in serum-free medium and serum supplementation led to sphere adhesion and differentiation (20).

Stem cell niches are often located in anatomical regions characterized by hypoxic conditions, which are crucial for the maintenance of an undifferentiated state for stem cells from various origins (21). Das *et al* have shown that CSCs localize in hypoxic areas of solid tumors *in vivo* and that they migrate to areas of hypoxia in nude mice (22). These data reinforce the hypothesis that a CSC niche is characterized by a hypoxic environment. According to these findings, we analyzed the effect of hypoxia on the growth of the CSC population. Although SCC-VII CSCs lost their capacity to grow as spheres and some of them even degenerated during the first 15 days, they were then able to recover after an adaptation period. Finally, we compared the ALDH1^{high} cell percentage after an 8-week culture in normoxia or hypoxia. The percentage of ALDH1^{high} cells was significantly higher when the oralspheres were maintained in a hypoxic instead of a normoxic environment, which demonstrated that culturing these cells in hypoxia favoured the enrichment in ALDH1^{high} cells. These results suggest that, as for colorectal cell line-derived CSCs (23), hypoxia is involved in the maintenance of the stem-like phenotype.

Overall, this study reports for the first time the isolation of HNSCC CSCs in a syngeneic mouse model and the use of hypoxia as a method to further enrich the ALDH1^{high} cell population. These cells appear to be a suitable model to develop new therapeutic strategies aiming at eradicating relapses in HNSCC.

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