Orosphere assay: A method for propagation of head and neck cancer stem cells

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ABSTRACT: *Background.* Recent evidence suggests that head and neck squamous cell carcinomas (HNSCCs) harbor a small subpopulation of highly tumorigenic cells, designated cancer stem cells. A limiting factor in cancer stem cell research is the intrinsic difficulty of expanding cells in an undifferentiated state in vitro.

Methods. Here, we describe the development of the orosphere assay, a method for the study of putative head and neck cancer stem cells. An orosphere is defined as a nonadherent colony of cells sorted from primary HNSCCs or from HNSCC cell lines and cultured in 3-dimensional soft agar or ultralow attachment plates. Aldehyde dehydrogenase activity and CD44 expression were used here as stem cell markers.

Results. This assay allowed for the propagation of head and neck cancer cells that retained stemness and self-renewal.

Conclusion. The orosphere assay is well suited for studies designed to understand the pathobiology of head and neck cancer stem cells. © 2012 Wiley Periodicals, Inc. *Head Neck* **35:** 1015–1021, 2013

KEY WORDS: squamous cell carcinoma, suspension culture, sphere, self-renewal, stemness

INTRODUCTION

The cancer stem cell hypothesis provides a plausible mechanism for tumor recurrence and metastatic spread.[1] According to the cancer stem cell hypothesis, a small subpopulation of cancer cells is highly tumorigenic and is capable of self-renewal and multipotency.[2] Cells with such features may constitute the "drivers'' of the tumorigenic process.[2] If this hypothesis were indeed true for head and neck squamous cell carcinomas (HNSCCs), selective targeting of these cancer stem cells would be essential to improve patient outcomes. Following the discovery of cancer stem cells in HNSCC,[3] investigators throughout the world have begun studies to understand the pathobiology of these cells. The development and optimization of a method for in vitro expansion of head and neck cancer stem cells in an undifferentiated state would be beneficial for the progress of research in this area, and hopefully will accelerate the process of developing improved treatment modalities for HNSCCs.

Two cardinal properties of stem cells allow for their identification and purification: (1) self-renewal, that is, the ability of stem cells to self-perpetuate, and (2) multipotency, that is, the ability of cells to undergo differentiation and generate the complex cellular components observed in a tissue/organ or in cancer.[4–6] It is possible to maintain human head and neck cancer stem cells in an undifferentiated state by serially passaging them in vivo, in immunodeficient mice.[7] However, this strategy is time-consuming and expensive. Furthermore, it is difficult to perform mechanistic studies of signaling pathways involved in the biology of cancer stem cells exclusively in animal models. A third property of stem cells, that is, the ability to form spheres and grow under low attachment conditions,[8,9] inspired the development of in vitro assays for the study of normal and cancer stem cells.

Exploiting the fact that stem cells possess anchorage independence, that is, the ability to survive and proliferate in suspension cultures unlike the nonstem cells,[8,9] adherent-free culture conditions have been proposed as the basis for in vitro assays for propagation of cancer stem cells. Suspension cultures have been used as a method to study stem cell properties in several tumor types, including those of the breast and brain.[10,11] Most of these suspension cultures are done in 3-dimensional (3D) structures, such as soft agar matrices or dishes coated with fibronectin or matrigel.[12–14] These strategies allow for stem cell expansion and proliferation, making them a valuable assay for self-renewal. However, the setup of these cultures is technically challenging, and the intrinsic difficulty associated with the retrieval of the cells from their matrix makes this method not ideal when mechanistic studies involving serial passaging, flow cytometry, or gene-expression analyses are required. In an attempt to address such issues, the culture of cells in low-attachment

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plates has been proposed as an alternative strategy to deprive cells from anchorage, while facilitating their retrieval of cells for further analysis.[15–17]

Fluorescence-activated cell sorting (FACS) and magnetic bead sorting are common approaches for the identification and isolation of putative stem cells.[18,19] Using FACS, we observed that the fraction of putative cancer stem cells in primary HNSCC is small.[7] Here, we describe a method for the propagation of head and neck cancer stem cells: the orosphere assay. The designation reflects the fact that this method was optimized for studies of stem cells sorted from tumors or cell lines derived from the oral cavity and head and neck region. This method enables the expansion of cancer stem cells in an undifferentiated state by culturing them in ultralow attachment plates or in 3D soft agar matrices. The use of ultralow attachment plates allowed for serial passaging of cells (ie, demonstration of self-renewal), and for the retrieval of cells for mechanistic studies.

MATERIALS AND METHODS

Sorting and culture of head and neck cancer stem cells

HNSCC cells (UM-SCC-74A, UM-SCC-74B; gift from Dr. Carey, University of Michigan) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Grand Island, NY), 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. The identity of the tumor cell lines was confirmed by genotyping at the University of Michigan DNA sequencing core facility. Alternatively, putative cancer stem cells were isolated from primary tumors, as described.[7] Briefly, informed consent was obtained from 2 patients prior to surgical removal of HNSCC under a protocol approved by the University of Michigan Institutional Review Board (IRB). The information about the tumor site and patient demographics is described in Supplementary Table S1. The specimens were cut into small pieces, minced until they passed through a 25-mL pipette tip, and suspended in a 9:1 solution of DMEM-F12 (Hyclone, Waltham, MA) containing collagenase and hyaluronidase (Stem Cell Technologies, Vancouver, BC, Canada). The mixture was incubated at 37°C for 1 hour and passed through a 10-mL pipette every 15 minutes for mechanical dissociation. Cells were filtered through a 40-μm nylon mesh (BD Falcon, Franklin Lakes, NJ), washed with low glucose DMEM (Invitrogen) containing 10% FBS, and centrifuged at 800 rpm for 5 minutes. Single-cell suspensions obtained from primary specimens (as well as from HNSCC cell lines) were washed, counted, and resuspended at 10^6 cells/mL PBS. The Aldefluor kit (Stem Cell Technologies) was used to identify cells with high aldehyde dehydrogenase (ALDH) activity. Briefly, cells were suspended in activated Aldefluor substrate (BAA) or in DEAB (specific ALDH inhibitor) for 45 minutes at 37°C. Then, cells were exposed to anti-CD44 antibody (clone G44–26BD; BD Pharmingen, Franklin Lakes, NJ) and lineage (Lin) markers (ie, anti-CD2, CD3, CD10, CD16, CD18; BD Pharmingen). Viable cells are identified with 7-aminoactinomycin (7-AAD; BD Pharmingen). FACS-sorted cells were cultured in low glucose DMEM (Invitrogen), 10% fetal bovine serum, and 100 U/mL penicillin–streptomycin in low-attachment conditions, as described in the following text. Cells were defined as putative head and neck cancer stem cells (ALDH+CD44+Lin−) or control cells (ALDH−CD44−Lin−). To induce cell differentiation, FACS-sorted cells were cultured in regular tissue culture plates (BD Falcon). All studies were done in triplicate wells per condition. Experiments with cell lines were performed at least 3 independent times to verify reproducibility of the data and experiments with cells retrieved from primary HNSCC were performed twice independently.

Orospheres in ultralow attachment plates

FACS-sorted cells (5×10^3 cells/well) were seeded in 6-well ultralow attachment plates (Corning, New York, NY) and cultured in low-glucose DMEM, 10% fetal bovine serum, and 100 U/mL penicillin–streptomycin at 37°C and 5% CO_2. Orospheres were arbitrarily defined as a nonadherent colony of at least 25 cells. Orospheres can be mechanically dissociated into single-cell suspensions and then reseeded in new ultralow attachment plates to generate secondary and tertiary orospheres (indicative of self-renewal).

Orospheres in soft agar

Alternatively, orospheres can be generated using low melting point agarose (Invitrogen). Six-well regular attachment plates (Fisher) were precoated with a layer of 1.2% agarose mixed with an equal volume of 2× DMEM (Invitrogen) to make an inert basal layer. This layer is solidified at room temperature for 45 minutes. Then, 500 FACS-sorted cells/well were resuspended in 2× DMEM (Invitrogen) mixed with equal volumes of 0.6% agarose. After the second agarose layer gelified at room temperature for 30 minutes, 500 μL low-glucose DMEM (Invitrogen) was added onto the surface of the 3D matrix and cells were incubated at 37°C thereafter. Usually the orospheres in soft agar were visualized after 7 days. Quantification of the number of orospheres/well was done under light microscopy.

Immunocytochemistry

For immunocytochemistry, 2×10^3 FACS-sorted cells/well were cultured in LabTek II Chamber Slide (Thermo Scientific, Rochester, NY) or in ultralow attachment plates for up to 7 days. Antigen retrieval was performed using Dako Retrieval solution (S1699; Carpinteria, CA) with gradual warming up from 40 to 98°C within 40 minutes. Slides were incubated in 3% hydrogen peroxidase for 10 minutes. Primary antibodies against Cytokeratin 17 (1:200; Abcam, ab2502; San Francisco, CA) or Involucrin (1:200; Abcam ab27496) were incubated at 4°C overnight. Following a 20-minute incubation with appropriate secondary antibodies, the Romulin AEC Chromogen Kit (Biocare Medical, Concord, CA) was used to visualize the proteins.

Immunofluorescence and confocal imaging

For confocal imaging, 2×10^3 FACS-sorted cells/well were seeded in a 24-well ultralow attachment plate

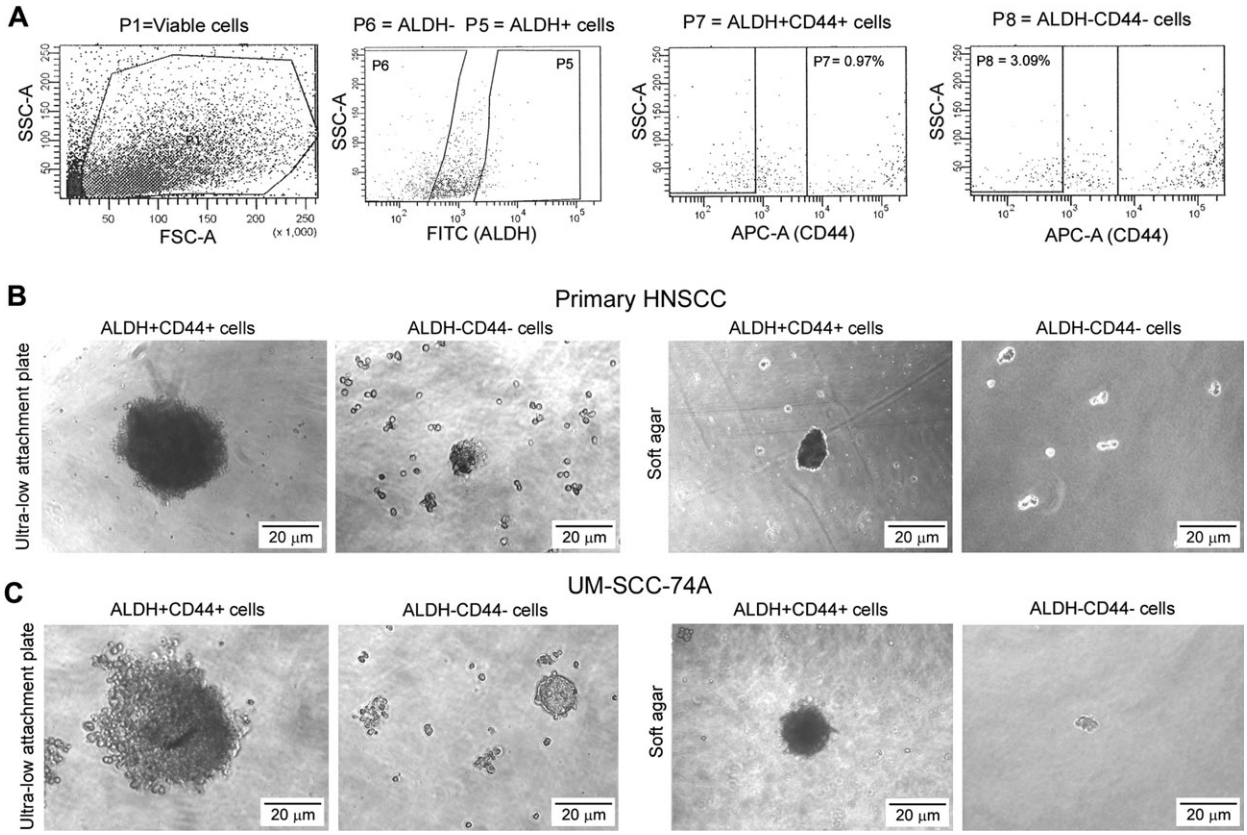

FIGURE 1. In vitro propagation of putative head and neck cancer stem cells in orospheres. (A) Representative flow cytometry sorting of putative cancer stem cells from a primary human head and neck squamous cell carcinoma. Shortly after surgery, single-cell suspensions were prepared by digestion of the tumor specimen with collagenase and hyaluronidase. Viable cells (P1) were isolated using 7AAD and are gated for positivity (after eliminating lineage cells) to ALDH (P5), using DEAB (ALDH inhibitor) as reference. ALDH-negative cells are found in P6. The cells were then gated against CD44 in sequence to select ALDH+CD44+Lin- (P7 = 0.97%) and ALDH-CD44-Lin- (P8 = 3.09%). (B, C) Representative photomicrographs of orospheres arising from ALDH+CD44+Lin– and ALDH–CD44–Lin– cells sorted from a primary HNSCC (B) or from a HNSCC cell line, that is, UM-SCC-74A (C). Cells were cultured either in ultralow attachment plates or in 3D soft agar matrices.

(Corning). Orospheres were fixed in cold 10% buffered formalin (Fisher, Pittsburgh, PA) for 30 minutes. For immunofluorescence, primary antibodies were prelabeled with Alexafluor 488 or 594 using a Zenon labeling kit (Molecular Probes, Z25007, Z25102; Invitrogen). Primary antibodies, that is, anti-ALDH1 (1:50; BD Biosciences, 61195; Franklin Lakes, NJ); CD44 (1:200; Abcam, ab51037) were added directly to the plate and incubated at 4°C overnight. Orospheres were transferred to LabTek II Chambered Coverglass (Thermo Scientific) and mounted with Prolong Gold antifade mounting medium with DAPI (Invitrogen). Confocal imaging was performed using Leica Inverted Confocal SP5X (Leica, Los Angeles, CA). Postprocessing was done with NIH ImageJ software.

Statistical analyses

One-way analysis of variance (ANOVA) followed by appropriate post hoc tests was performed using the SigmaStat 2.0 software (SPSS, Chicago, IL). Statistical significance was determined at $p < .05$.

RESULTS

We have recently shown that ALDH+CD44+Lin– cells sorted from primary HNSCC exhibit self-renewal and are more tumorigenic than control ALDH–CD44–Lin– cells.[7] Such features characterize the ALDH+CD44+Lin– cells as putative head and neck cancer stem cells. Here, we describe the characterization and optimization of a method that was developed to propagate and to evaluate the stem cell properties of cells derived from primary head and neck tumors or from HNSCC cell lines. Single-cell suspensions were prepared from freshly dissected human HNSCC or from HNSCC cell lines. Cells were sorted for high/low ALDH activity (Aldefluor kit) and CD44 expression. A representative flow sorting of the head and neck cancer stem cells from a primary human HNSCC (HN 03) is shown in Figure 1A, wherein the percentage of lineage-negative viable ALDH+CD44+Lin– is 0.97%, whereas the percentage of lineage-negative viable noncancer stem cells (ALDH–CD44–Lin–) is 3.09%. The percentage of ALDH+CD44+Lin– and ALDH–CD44–Lin– cells was calculated using as reference the total number of viable cells in the specimen. After flow sorting, cells were cultured under low-attachment conditions to form nonadherent spheres designated orospheres. To generate these orospheres, we optimized conditions for HNSCC cells cultured either in ultralow attachment plates or in soft agar 3D matrices. Although orospheres can be readily seen within 3 days in ultralow attachment plates, it takes

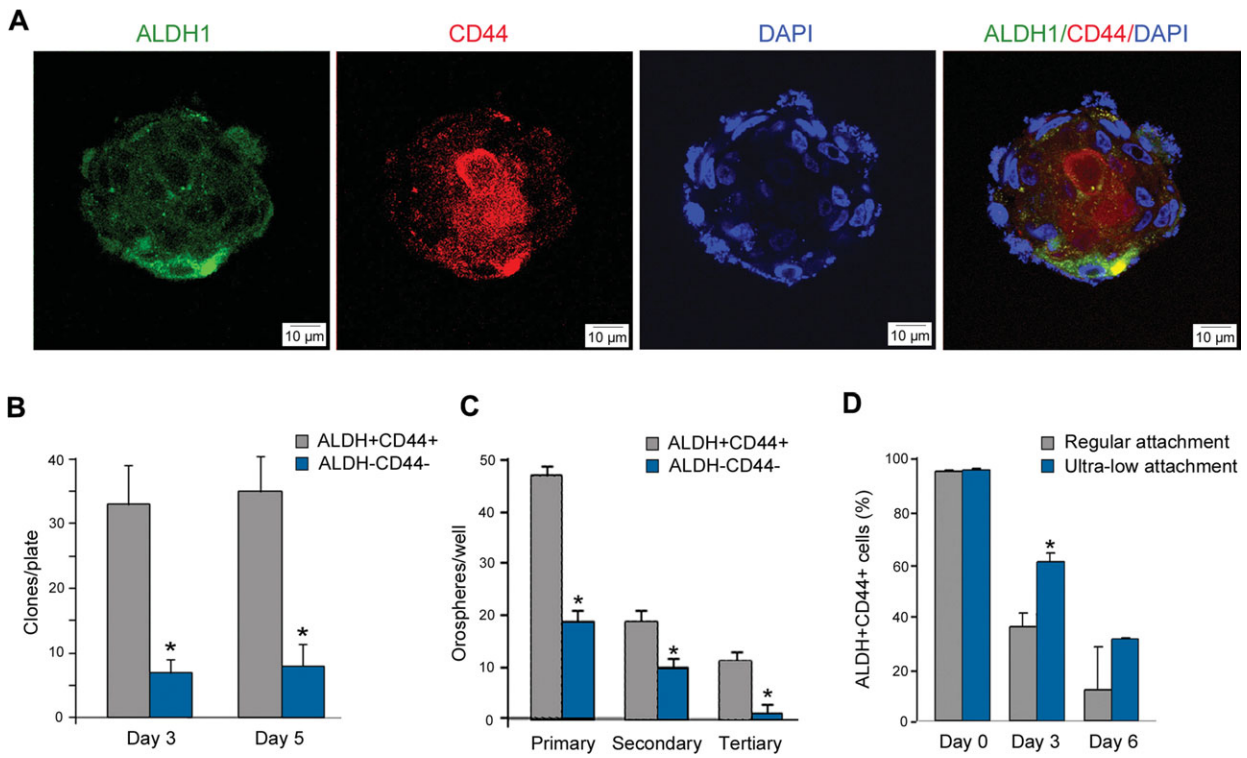

FIGURE 2. Characterization of stem cell properties of orospheres. (A) Confocal microscopy of an orosphere generated from the UM-SCC-74B cell line and stained for the stem cell markers ALDH1 (green) and CD44 (red), along with nuclei staining with DAPI (blue). (B) Graph depicting the number of clones arising from one individual cancer stem cell (ALDH+CD44+) or noncancer stem cell (ALDH–CD44–) per well of a 96-well ultralow attachment plate. (C) Graph depicting the number of orospheres generated from serial passage assays that evaluate self-renewal of putative cancer stem cells (ALDH+CD44+) or control cells (ALDH–CD44–). (D) Graph depicting the percentage of ALDH+CD44+ cells (FACS) over time when cultured in regular attachment or ultralow attachment conditions ($n = 3$). Asterisk depicts $p < .05$, when data are analyzed against controls within same time point.

approximately 7 days to generate orospheres in soft agar (Figures 1B, 1C). Notably, the orospheres shown here were generated either from cells sorted from one primary human HNSCC (Figure 1B) or from a head and neck cancer cell line, that is, UM-SCC-74A (Figure 1C).

To begin to understand the biology of the cells forming the orospheres, we cultured them for 3 days in ultralow attachment plates and visualized the expression of the 2 stem cell markers used to sort the cells initially (ALDH1 and CD44) by confocal microscopy (Figure 2A). To determine if the culture of putative cancer stem cells in low attachment represents a self-renewal method resulting in stem cell expansion, and not just an aggregation of stem-like cells, we seeded a single ALDH+CD44+ cell/well in 96-well ultralow attachment plate and monitored its clonal expansion for 5 days (Figure 2B). We observed that a higher number of individual clones were formed by the putative cancer stem cells (ALDH+CD44+) when compared with control ALDH–CD44– cells ($*p < .05$, $n = 3$). A clone was defined as a colony of at least 10 cells, starting from a single cell.

To evaluate if the orosphere assay is a valid method for testing self-renewal of head and neck cancer stem cells, we cultured orospheres generated from ALDH+CD44+ cells or control cells for 3 days under ultralow attachment conditions. Then, the orospheres were mechanically dissociated and reseeded as single-cell suspension in new ultralow attachment plates. This process was repeated serially to generate secondary and tertiary orospheres (Figure 2C). This experiment revealed 2 general trends: (1) more orospheres were generated from the ALDH+CD44+ than from the control cells over time, demonstrating the self-renewal of the putative cancer stem cells; and (2) a progressive decrease in the overall number of orospheres was observed between the primary and the tertiary passages.

We wanted to ascertain that suspension culture in low-attachment plates was the reason for the continued enrichment of cancer stem cells, and that ALDH+CD44+ cells do retain their stemness over time. We therefore cultured ALDH+CD44+ cells in regular attachment conditions or in ultralow attachment plates. FACS analysis revealed the maintenance of a higher percentage of ALDH+CD44+ cells when culturing in ultralow attachment conditions as compared with regular attachment plates (Figure 2D; Supplementary Figure S1). The reverse experiment was performed to evaluate if the same putative head and neck cancer stem cells (ALDH+CD44+) would lose their stemness and differentiate when cultured in regular attachment plates. This analysis was performed by immunostaining for Cytokeratin 17 (an epithelial stem cell marker) and Involucrin (a differentiated cell marker).[20,21] We observed that on day 0, the ALDH+CD44+ cells were more spherical and expressed high

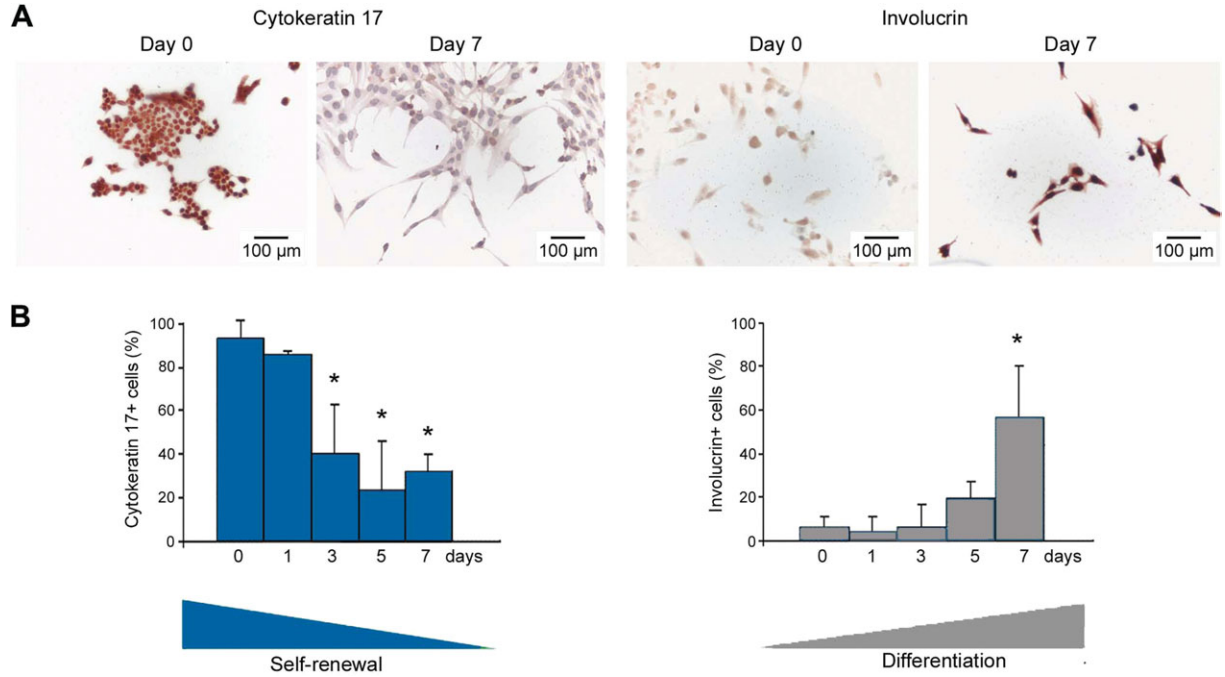

FIGURE 3. Characterization of the differentiation of ALDH+CD44+ cells cultured in regular attachment plates. (A) Representative photomicrographs of Cytokeratin 17 and Involucrin immunostaining of ALDH+CD44+ cells cultured under regular attachment conditions for 1 week. (B) Graph depicting the percentage of cells cultured in regular attachment plates and that stained positive for Cytokeratin 17 or Involucrin over time. Asterisk depicts $p < .05$, when data are analyzed against baseline (day 0).

levels of Cytokeratin 17 and low levels of Involucrin (Figures 3A, 3B). By day 7, the ALDH+CD44+ cells became more elongated and reversed the expression levels of Cytokeratin 17 and Involucrin.

To further evaluate the impact of culture conditions on the stemness of ALDH+CD44+ cells over time, we cultured ALDH+CD44+ cells in regular or ultralow attachment conditions and evaluated ALDH1 expression by Western blots. We observed that ALDH1 was not expressed in cells that were cultured under regular attachment conditions at day 3, and thereafter (Supplementary Figure S2). In marked contrast, expression of ALDH1 was maintained at the same level as baseline at 3 days, and somewhat decreased, but still clearly present, in cells cultured in ultralow attachment conditions after 7 days. Oral keratinocytes, that is, fully differentiated cells, were used as controls for this experiment.

DISCUSSION

The orosphere assay is conceptually derived from suspension cultures developed to study normal or cancer stem cells from tissues such as the brain, breast, or prostate.[9,10,22,23] Pioneer work from Reynolds and Weiss demonstrated that cells dissected from the striatum of the adult mouse brain could be cultured as free-floating spheres and exhibited stem cell properties.[9,22] The Wicha laboratory characterized human mammary stem/progenitor cells from reduction mammoplasties based on their anchorage independence and survival in low-attachment plates.[10] These seminal findings provided the conceptual framework for the development of sphere-based assays as a means to propagate cancer stem cells in an undifferentiated state in vitro. Here, we describe a method in which putative cancer stem cells are sorted from heterogeneous HNSCC primary tumors or from established HNSCC cell lines. These putative cancer stem cells differentiate under regular attachment conditions and generate heterogeneous tumor cell monolayers within a few days. On the other hand, the same cells cultured in low-attachment conditions are capable of retaining stem-like cell properties (see Figure 4). Notably, the method described here is clearly inspired by the existing protocols from other tumor types, but was optimized for use in head and neck tumor models.

One of the critical challenges facing stem cell studies is the definition of markers that discriminate highly tumorigenic cells (cancer stem cells) from cells that possess low tumorigenic potential. Mounting evidence suggests that stem cell markers are tumor-specific, and that CD44, CD133, and ALDH are emerging as useful markers in HNSCC. Seminal work from the Prince laboratory used CD44 expression as a marker for the identification of a subpopulation of highly tumorigenic stem cells in primary HNSCC.[3] More recently, it was shown that CD44+ cells sorted from an HNSCC cell line cultured in uncoated dishes formed tumor spheres that were resistant to chemotherapeutic drugs.[24] CD133, a transmembrane glycoprotein, is considered a putative marker for cancer stem cells in head and neck tumors. CD133-positive cells sorted from HNSCC cell lines or primary tumors showed enhanced clonality and tumorigenicity when compared

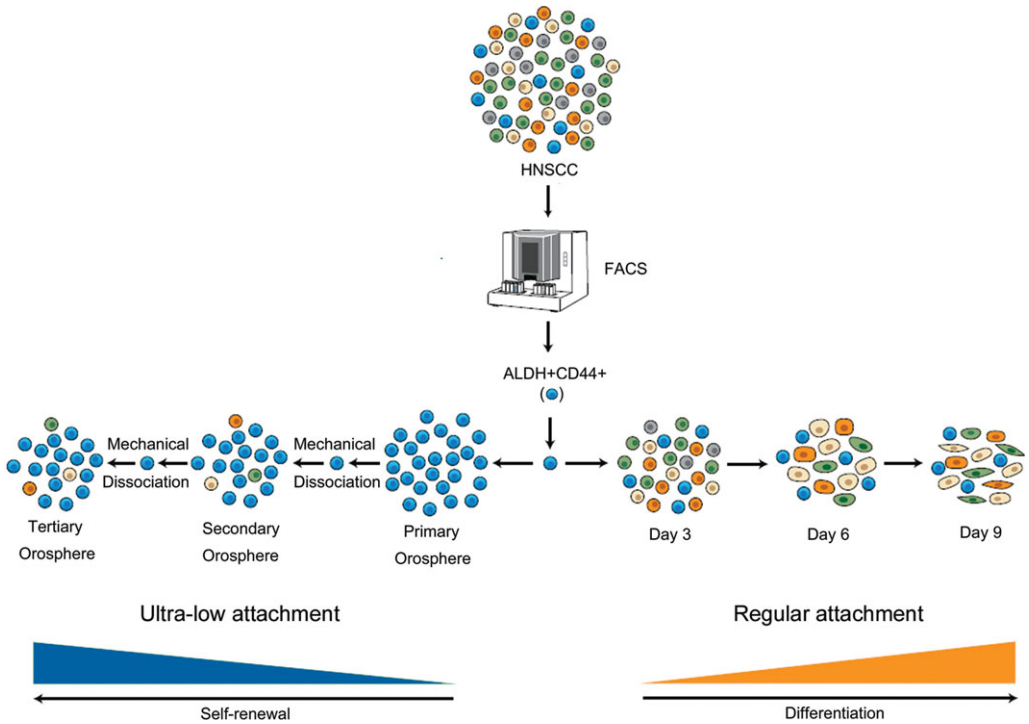

FIGURE 4. Diagram illustrating the in vitro propagation of putative head and neck cancer stem cells using the orosphere assay. Single-cell suspensions are prepared from head and neck squamous cell carcinomas and sorted for stem cell markers, such as ALDH and CD44. The putative cancer stem cells can be serially passaged and expanded in ultralow attachment conditions using the orosphere assay. Alternatively, these cells can be differentiated when cultured in regular attachment conditions generating a heterogeneous cancer cell line.

with control cells.[25–27] Alternatively, ALDH activity, which was initially characterized as a useful stem cell marker in breast cancer,[28] was also validated in head and neck tumor models.[29,30] Of note, since most markers are expressed in both normal and pathologic stem cells, it is plausible that the combination of markers may enhance one's ability to identify cancer stem cells from complex primary tumor tissues. Indeed, it has been recently observed that the combination of ALDH activity and CD44 expression further discriminates a small subpopulation (<3%) of cells in primary HNSCC that exhibit stem-like properties and are highly tumorigenic.[7]

As with most methods, the orosphere assay has its inherent limitations, as follows: (1) the overall number of orospheres decreases upon serial passaging; and (2) the percentage of ALDH+CD44+ cells is higher in ultralow attachment plates than in regular culture plates, but it decreases over time. Collectively, these findings suggest that there might be a certain degree of cell differentiation even in low-attachment conditions in vitro. Although these limitations can be overcome by expanding cancer stem cells in vivo,[3,7] such a strategy makes the process of propagating cells in an undifferentiated state labor and animal intensive, and expensive. Although the "orosphere'' assay has the advantages of being technically simple, reproducible, and relatively inexpensive, one must remain mindful of the limitations of the assay and interpret the data with caution. Therefore, the orosphere assay should be used in combination with appropriate animal models.

We described here the protocols for generating orospheres in either soft agar 3D matrices or in ultralow attachment plates. Careful consideration should be given to the advantages and disadvantages of each method, before selecting the best approach for a specific experimental question. The soft agar method is more time consuming. One has to precoat the plate with a layer of agarose, wait for its gelification, apply a second layer containing both agarose and cells, wait again, and finally cover the 3D gel with culture medium. Along the same lines, it takes about 7 days to generate orospheres in soft agar, whereas it takes only 3 days in ultralow attachment plates. In addition, the soft agar approach does not allow for retrieval of the cells for mechanistic studies (eg, flow cytometry, gene-expression analyses) or for serial passage studies (eg, to evaluate self-renewal properties). As a potential advantage though, the soft agar assay tends to be a more rigorous testing of stem cell properties. We observed that noncancer stem cells do not survive well under these conditions and do not readily form orospheres. On the other hand, the culture of undifferentiated cells in ultralow attachment plates is simpler, since there is no need for coating and gelification steps. This culture condition is highly suitable for the retrieval of cells for serial passage or for mechanistic studies. Knowing the pros and cons of both strategies should direct the decision process toward selecting the soft agar or the ultralow attachment approach.

The field of cancer stem cell biology has attracted much attention in recent years due to the discovery that

these cells may drive the progression of certain tumor types, including HNSCC. As such, the emergence of targeted therapy against cancer stem cells could have a significant impact on the survival of patients with head and neck cancer. The authors believe that the development and characterization of methods to propagate and study the behavior of cancer stem cells in vitro may ultimately contribute to the discovery of mechanism-based therapies for HNSCC.

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