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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 (HNSCC) harbor a small sub-population of highly tumorigenic cells, named 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 non-adherent colony of cells sorted from primary HNSCC or from HNSCC cell lines and cultured in 3-D soft agar or ultra-low attachment plates. Aldehyde dehydrogenase (ALDH) 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.

Keywords

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 sub-population of cancer cells is highly tumorigenic, 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 (HNSCC), 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

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Competing interests' statement

The authors declare no competing interests.

would be beneficial for the progress of research in this area, and hopefully will accelerate the process of developing improved treatment modalities for HNSCC.

Two cardinal properties of stem cells allow for their identification and purification: A) Self-renewal, *i.e.* the ability of stem cells to self-perpetuate; and B) Multipotency, *i.e.* 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, *i.e.* the ability to form spheres and grow under low attachment conditions,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, *i.e.* the ability to survive and proliferate in suspension cultures unlike the non-stem cells^{8,9}, adherent-free culture conditions have been proposed as basis for *in vitro* assays for propagation of cancer stem cells. Suspension cultures have been utilized 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 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 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 named the orosphere assay. The name 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 ultra-low attachment plates or in 3-D soft agar matrices. The use of ultra-low attachment plates allowed for serial passaging of cells (*i.e.* 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

Head and neck squamous cell carcinoma cells (UM-SCC-74A, UM-SCC-74B; gift from Dr. Carey, University of Michigan) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen; Grand Island, NY, USA), 10% fetal bovine serum, 100 U/ml penicillin, 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 two 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 Figure 1. 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, USA) containing collagenase and hyaluronidase (Stem Cell Technologies; Vancouver, BC, Canada). The mixture was incubated at 37°C for one 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, USA), 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 re-suspended at 10⁶ cells/ml PBS. The Aldefluor kit (Stem Cell Technologies) was used to identify cells with high 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, USA) and lineage (Lin) markers (*i.e.* anti-CD2, CD3, CD10, CD16, CD18; BD Pharmingen). Viable cells are identified with 7-Aminoactinomycin (7-AAD, BD Pharmingen). FACS (Fluorescence Activated Cell Sorting) 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 below. 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 specimens per condition. Experiments were performed at least three independent times to verify reproducibility of the data for all the cell lines and twice for primary HNSCC.

Orospheres in ultra-low attachment plates

FACS-sorted cells (5×10³ cells/well) were seeded in 6-well ultra-low attachment plates (Corning; New York, NY, USA) and cultured in low glucose DMEM, 10% fetal bovine serum and 100 U/ml Penicillin-streptomycin at 37°C and 5% CO₂. Orospheres were arbitrarily defined as a non-adherent colony of at least 25 cells. Orospheres can be mechanically dissociated into single cell suspensions and then re-seeded in new ultra-low 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). 6-well regular attachment plates (Fisher) were pre-coated with a layer of 1.2% agarose mixed with equal volume of 2x 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 2x DMEM (Invitrogen) mixed with equal volumes of 0.6% agarose. After the second agarose layer gelifies at room temperature for 30 minutes, 500 µl low glucose DMEM (Invitrogen) is added onto the surface of the 3-dimensional matrix and cells are incubated at 37°C thereafter. Usually the orospheres in soft agar are visualized after 7 days. Quantification of the number of orospheres/well is done under light microscopy.

Immunocytochemistry

For immunocytochemistry, 2×10³ FACS-sorted cells/well were cultured in LabTek II Chamber Slide (Thermo Scientific; Rochester, NY, USA) or in ultra-low attachment plates for up to 7 days. Antigen retrieval was performed using Dako Retrieval solution (S1699; Carpinteria, CA, USA) with gradual warming up from 40°C 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, USA) 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, USA) 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 ultra-low attachment plate (Corning). Orospheres were fixed in cold 10% buffered formalin (Fisher; Pittsburgh, PA, USA) for 30 minutes. For immunofluorescence, primary antibodies were pre-labeled with Alexafluor 488 or 594 using Zenon labeling kit (Molecular Probes, Z25007, Z25102; Invitrogen). Primary antibodies, *i.e.* 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 anti-fade mounting medium with DAPI (Invitrogen). Confocal imaging was performed using Leica Inverted Confocal SP5X (Leica; Los Angeles, CA, USA). Post-processing was done with NIH Image J software.

Statistical analyses

One-way ANOVA followed by appropriate post-hoc tests was performed using the SigmaStat 2.0 software (SPSS, Chicago, IL). Statistical significance was determined at $P < 0.05$.

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%, while the percentage of lineage-negative viable non-cancer 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 non-adherent spheres named orospheres. To generate these orospheres, we optimized conditions for HNSCC cells cultured either in ultra-low attachment plates or in soft agar 3-D matrices. While orospheres can be readily seen within 3 days in ultra-low attachment plates, it takes approximately 7 days to generate orospheres in soft agar (Figure 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, *i.e.* UM-SCC-74A (Figure 1C).

To begin to understand the biology of the cells forming the orospheres, we cultured them for 3 days in ultra-low attachment plates and visualized the expression of the two 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 ultra-low 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 to control ALDH-CD44- cells ($*P < 0.05$, $n=3$). 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 ultra-low attachment conditions. Then, the orospheres were mechanically dissociated and re-seeded as single cell suspension in new ultra-low attachment plates. This process was repeated serially to generate secondary and tertiary orospheres (Figure 2C). This experiment revealed two general trends: A) 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, B) A progressive decrease in the overall number of orospheres was observed between the primary and the tertiary passage.

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 ultra-low attachment plates. FACS analysis revealed the maintenance of a higher percentage of ALDH+CD44+ cells when culturing in ultra-low attachment conditions as compared to regular attachment plates (Figure 2D; Supplementary Figure 2). 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 levels of Cytokeratin 17 and low levels of Involucrin (Figure 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 ultra-low 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 3). 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 ultra-low attachment conditions after 7 days. Oral keratinocytes, i.e. 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 mamoplasties 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 (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 sub-population of highly tumorigenic stem cells in primary HNSCC.³ More recently, it was shown that CD44+ cells sorted from a 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 to 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 sub-population (<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: A) The overall number of orospheres decreases upon serial passaging; and B) The percentage of ALDH+CD44+ cells is higher in ultra-low 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 strategy makes the process of propagating cells in an undifferentiated state labor and animal intensive, and expensive. While the “orsphere” 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. And 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 3-D matrices or in ultra-low 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 pre-coat 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 3-D gel with culture medium. Along the same lines, it takes about 7 days to generate orospheres in soft agar, while it takes only 3 days in ultra-low attachment plates. In addition, the soft agar approach does not allow for retrieval of the cells for mechanistic studies (*e.g.* flow cytometry, gene expression analyses) or for serial passage studies (*e.g.* 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 non-cancer 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 ultra-low 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 towards selecting the soft agar or the ultra-low 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 head and neck cancer patients. 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 head and neck squamous cell carcinoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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, *i.e.* UM-SCC-74A **(C)**. Cells were cultured either in ultra-low attachment plates or in 3-D soft agar matrices.

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 non-cancer stem cell (ALDH-CD44-) per well of a 96-well ultra-low 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 ultra-low attachment conditions (n=3). Asterisk depicts p<0.05, when data are analyzed against controls within same time point.

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 one 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 $p < 0.05$, when data are analyzed against baseline (day 0).

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 ultra-low attachment conditions using the orosphere assay. Alternatively, these cells can be differentiated when cultured in regular attachment conditions generating a heterogeneous cancer cell line.