Identification and characterization of stemlike cells in human esophageal adenocarcinoma and normal epithelial cell lines

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Objective: Recent studies have suggested that human solid tumors may contain subpopulations of cancer stem cells with the capacity for self-renewal and the potential to initiate and maintain tumor growth. The aim of this study was to use human esophageal cell lines to identify and characterize putative esophageal cancer stem cell populations.

Methods: To enrich stemlike cells, Het-1A (derived from immortalized normal esophageal epithelium), OE33, and JH-EsoAd1 (each derived from primary esophageal adenocarcinomas) were cultured using serum-free media to form spheres. A comprehensive analysis of parent and spheroid cells was performed by flow cytometry, Western blot analysis, immunohistochemistry and polymerase chain reaction array to study cancer stem cell-related genes, colony formation assays to assess clonogenicity, xenotransplantation to assess tumorigenicity, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays to assess chemosensitivity to 5-fluorouracil and Cisplatin.

Results: For all cell lines, clonogenicity, tumorigenicity, and chemoresistance to 5-fluorouracil and Cisplatin were significantly higher than for spheroid cells compared with parent cells. Spheroids exhibited an increased frequency of cells expressing integrin $\alpha 6^{bri}$/CD71^{dim}, and Achaete-scute complex homolog 2 messenger RNA and protein were also significantly overexpressed in spheroid cells compared with parent cells.

Conclusions: The higher clonogenicity, tumorigenicity, and drug resistance exhibited by spheroids derived from Het-1A, OE33, and JH-EsoAd1 reflects an enrichment of stemlike cell populations within each esophageal cell line. Esophageal cells enriched for integrin $\alpha 6^{bri}$/CD71^{dim} and/or overexpressing Achaete-scute complex homolog 2 would appear to represent at least a subpopulation of stemlike cells in Het-1A, OE33, and JH-EsoAd1. (J Thorac Cardiovasc Surg 2012;144:1192-9)

ET/BS

Biologic models have considered the progression of Barrett esophagus (BE) to esophageal adenocarcinoma (EADC) as a multistep process associated with clonal evolution of tumor cell populations.[1] More recently, increasing evidence suggests that human solid tumors may arise from a small subpopulation of cancer stemlike cells, with capacity for asymmetric cell division, self-renewal, differentiation, and resistance to chemotherapeutic agents.[2,3] Although the establishment of tumors and serial transplantation in immunocompromised mice remains the most widely accepted assay to validate a cancer stem cell (CSC) subpopulation, the identification of cell surface markers and the ability of CSCs to form spheres in vitro has greatly facilitated the identification of cancer stemlike cells. Using such approaches, putative CSCs have now been reported in various malignancies of the gastrointestinal tract,[4,5] but have yet to be identified in human esophageal cancer.[6-8] In the most comprehensive study of human BE and EADC tissues performed to date, a subpopulation identified as tumor-initiating stemlike cells did not express any of the common cell surface antigens (CD24, CD29, CD34, CD44, CD133, CD166, epithelial cell adhesion molecule [EpCAM], and β-catenin) established previously as CSC markers in other human malignancies.[8]

Therefore, in view of the current lack of progress in identifying CSCs in human esophageal tissues, we explored an alternative strategy using human cell lines derived from primary EADC tumors and normal esophageal epithelium. Stem-like cells were enriched by sphere culture, and a comprehensive analysis of parent and spheroid cells was performed by flow cytometry, Western blot analysis, immunohistochemistry, polymerase chain reaction (PCR) -array to identify CSC-related genes; colony formation assays, xenotransplantation, and chemosensitivity assays to identify and characterize putative esophageal CSC subpopulations.

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Abbreviations and Acronyms

Ascl2 = Achaete-scute complex homolog 2
BE = Barrett esophagus
CDDP = cis-diamminedichloroplatinum(II), Cisplatin
CI = confidence interval
CSC = cancer stem cell
DMEM = Dulbecco's Modified Eagle Medium
EADC = esophageal adenocarcinoma
EDTA = ethylenediamine tetraacetic acid
ELDA = extreme limiting dilution assay
EpCAM = epithelial cell adhesion molecule
5-Fu = 5-fluoruracil
FBS = fetal bovine serum
IHC = immunohistochemistry
Lgr5 = leucine-rich repeat containing G protein-coupled receptor
mRNA = messenger ribonucleic acid
MTT = 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
PBS = phosphate-buffered saline
PCR = polymerase chain reaction
RPMI = Roswell Park Memorial Institute
Wnt = wingless type

MATERIALS AND METHODS

Human Esophageal Cell Lines

Two primary EADC cell lines (OE33 and JH-EsoAd1) and 1 immortalized normal esophageal epithelial cell line (Het-1A) were studied. OE33[9] and Het-1A[10] were purchased from the American Type Culture Collection (Manassas, Va) and the European Cell Culture Collection (Porton Down, UK), respectively. JH-EsoAd1[11] was generously provided by Drs Eshleman and Maitra (Johns Hopkins University, Baltimore, Md). Parent cell lines were cultured in monolayers utilizing Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) for Het-1A, Roswell Park Memorial Institute (RPMI) 1640 medium with 10% FBS for OE33, and RPMI with 20% FBS for JH-EsoAd1. Cells were cultured under standard conditions (37°C and 5% CO_2) and passaged using 0.25% trypsin/ethylenediamine tetraacetic acid (EDTA) upon 70% to 80% confluence.

Sphere Culture

From each parent cell line, 5×10^4 cells were seeded into 25-cm^2 Ultra-Low Attachment Cell Culture Flasks (Corning, NY) containing 5 mL serum-free sphere culture medium, comprising DMEM/F-12, basic fibroblast growth factor (20 ng/mL), epidermal growth factor (20 ng/mL), and 1 × B27 supplement.[12] Cells were cultured for 2 to 4 weeks until spheroids (each with 50-100 cells per sphere) formed. Parent cells were detached by incubation with 0.05% trypsin/EDTA, and spheroid cells were isolated using a 50-μm cell strainer and dissociated with 0.25% trypsin/EDTA. Detached parent cells and dissociated spheroid cells were washed with phosphate-buffered saline (PBS), counted, and suspended in fresh culture medium or PBS.

Flow Cytometry and Cell Sorting

Parent cells were stained with primary antibodies (Abcam, Cambridge, Mass) to the following CSC-related markers: CD24, CD34, CD44, CD71, CD133, EpCAM, anti-integrin α6, Musashi 1, and Oct4. For cytoplasmic and nuclear markers, cells were fixed and permeabilized with IntraPrep Permeabilization Reagent (Beckman Coulter, Mississauga, Ontario, Canada) before staining. For unconjugated antibodies, a fluorescein isothiocyanate-conjugated secondary antibody (goat antirabbit immunoglobulin G) was used. Antibody-stained cells were measured with a Coulter Epics XL and XL-MCL Flow Cytometer, and data were analyzed using Flowjo software (FlowJo 8.86, Ashland, Ore) to determine the intensity and percentage of positive-staining cells.

Based on flow cytometry results obtained from parent cells, Musashi 1 and integrin α6/CD71 were evaluated further in spheroid cells. Fluorescence-activated cell sorting was performed using the Epics Elite Esp cell sorting system (Beckman Coulter, Brea, Calif) to isolate 4 cell subsets reflecting integrin α6/CD71 expression—integrin $\alpha6^{bri}$/$CD71^{dim}$, integrin $\alpha6^{bri}$/$CD71^{bri}$, integrin $\alpha6^{dim}$/$CD71^{dim}$, and integrin $\alpha6^{dim}$/$CD71^{bri}$—using PE-conjugated anti-CD71 and fluorescein isothiocyanate-conjugated anti-integrin α6 antibodies (BD Bioscience, San Diego, Calif). Cell subsets were harvested, then washed with PBS for downstream colony and sphere formation assays.

Sphere Formation Assay

Sphere formation assays[13] were performed using 96-well Ultra-Low Attachment Cell Culture Plates. Cells were mixed with serum-free sphere culture media and seeded into each well at 250, 50, 10, and 2 cells per well (12 wells for each concentration). Plates were incubated for 2 to 4 weeks until spheres formed; wells containing spheroid cells were counted as positive. The number of positive wells (per 12 wells at each cell dose) was used to quantitate sphere-forming ability using extreme limiting dilution analysis (ELDA).[14]

Colony Formation Assay

Colony formation assays[13] were performed using 96-well cell culture plates coated with 0.1 mL bottom agar mixture (DMEM, 10% FBS, 0.6% agar). After the bottom layer solidified, cells were mixed with top agar (DMEM, 10% FBS, 0.3% agar), and seeded into each well at 100, 33, 10, and 3 cells per well (12 wells for each concentration). Plates were incubated for 4 weeks until colonies were large enough to be visualized. Colonies were stained with 0.005% Crystal Violet for 1 hour and counted; wells containing clones were counted as positive. The number of positive wells (per 12 wells at each cell dose) was used to quantitate clonogenicity using ELDA.[14]

Tumorigenic Assay

All animal studies were performed in accordance with the *Guide to the Care and Use of Experimental Animals* of the Canadian Council on Animal Care, and were approved by the University of Saskatchewan Committee on Animal Care and Research Ethics Board (protocol no. 20090037). Het-1A, OE33, and JH-EsoAd1 cells were implanted into several species of nude mice, including Hsd:Athymic Nude-Foxn1^{nu}, BALB/c OlaHsd-Foxn1^{nu}, and HsdOla:ICRF-Foxn1^{nu} (Harlan Laboratories Inc, Indianapolis, Ind); and Jax Nu/J and NOD.Cd-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ mice (The Jackson Laboratory, Bar Harbor, Mass). Seven-week-old BALB/c OlaHsd-Foxn1^{nu} mice and NOD.Cd-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ mice were selected for inoculation with OE33 and JH-EsoAd1, respectively. Parent and spheroid cells (1×10^7, 1×10^6, and 1×10^5 cells for OE33; 1×10^6, 2×10^5, and 4×10^4 cells for JH-EsoAd1) were injected subcutaneously into the flank of each mouse. The incidence of tumors (per 5 inoculations at each cell dosage) was recorded weekly and was used to calculate tumorigenicity using ELDA.[14] When each animal was euthanized, tumors were excised, measured (to calculate tumor volume), and bisected. One part was fixed in 10% formalin and embedded in paraffin and the other was snap frozen in liquid nitrogen and stored at –80°C.

Immunohistochemistry

Formalin-fixed xenograft tumor sections (4 μm thick) derived from OE33 and JH-EsoAd1 parent and spheroid cells were first stained with hematoxylin–eosin to determine histopathology. Immunohistochemistry (IHC) was performed on adjacent serial sections to study protein expression and distribution using primary antibodies against CD44 (1:100 dilution), CD71 (1:10 dilution), CD133 (1:50 dilution), and integrin α6 (1:150 dilution). Tissue staining was detected using the EnVision Dual Link Kit (Dako, Markham, Ontario, Canada) following the manufacturer's instructions. Controls were run in parallel with test sections, and included sections that were stained with nonimmune normal goat serum or with preabsorbed antiserum (negative controls). Interpretation of sections was performed by 2 investigators experienced in IHC analysis, and included an assessment of subcellular distribution (cytoplasmic or nuclear) of staining, intensity (0, +, 2+, 3+) and percentage of immunopositive cells (per high-power field).

Chemosensitivity Assay

Chemosensitivity of Het-1A, OE33, and JH-EsoAd1 to 5-fluorouracil (5-Fu) and Cisplatin (cis-diamminedichloroplatinum(II) [CDDP]), the 2 most widely used chemotherapeutic agents for EADC in current clinical practice, was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. Parent and spheroid cells were seeded in 96-well plates at 2×10^4 per well in 100 μL medium and, after 24 hours of incubation, were treated with serially diluted 5-Fu or CDDP (1.00-4.00 μg/mL) for an additional 72 hours. Cell proliferation in each well was determined using MTT assays.[15] Briefly, 10 μL MTT solution was added to each well, and the plate was incubated for 4 hours at 37°C. A total of 100 μL of solubilization solution was added to each well, followed by overnight incubation at 37°C. The plate was read at a wavelength of 570 nm/650 nm using a standard SpectraMax microplate reader. The ratio of absorbance in treated wells versus untreated control wells reflected cell proliferation, which was expressed as a percentage. All experiments were repeated at minimum in triplicate.

PCR Array

Total RNA was extracted from parent and spheroid cells using the All-Prep DNA/RNA Mini Kit (Qiagen, Mississauga, Ontario, Canada). cDNA was synthesized by reverse transcription of 2 μg total RNA by use of random primers and Superscript Reverse Transcriptase III (Invitrogen, Burlington, Ontario, Canada).

Gene expression profiles were determined using PCR arrays (SABiosciences, Frederick, Md) to detect messenger ribonucleic acid (mRNA) expression of 253 stem cell, drug resistance, and wingless-type (Wnt)-related genes. All reagents and materials for each PCR array were purchased from SABiosciences, and experiments were performed on the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, Calif). Fold changes of gene expression in spheroid cells relative to corresponding parent cells were calculated using Web-based software from SABiosciences (http://www.sabiosciences.com/pcr/arrayanalysis.php). Upregulation or downregulation of a gene was defined as a change > or <2-fold, respectively.

Western Blot Analysis

Based on results of the PCR stem cell array, we utilized Western blot analysis to study expression of Achaete-scute complex homolog 2 (Ascl2) protein in cell lines and xenografts (parent and spheroid cells). We also studied expression of the leucine-rich repeat containing G protein-coupled receptor (Lgr5), a related Wnt signaling pathway target gene and potential CSC marker. Whole-cell lysates of parent and spheroid cells were isolated using RIPA buffer with proteinase inhibitor cocktail (Sigma-Aldrich, Oakville, Ontario, Canada). Protein samples were heated at 95°C for 10 minutes, separated on an 8% or 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel, and transferred to a nitrocellulose film by electroblotting. Primary antibodies against Ascl2, Lgr5, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; a loading control) were purchased from Abcam). The Licor 2-color Western blotting kit (Lincoln, Neb) was used to detect specific bands, and all experiments were performed according to standard protocols from Abcam and Licor. The stained bands were recorded and analyzed quantitatively with the Licor Oldssey image system and Quantity One software (Bio-Rad, Mississauga, Ontario, Canada). The relative protein expression of Ascl2 and Lgr5 was expressed as the ratio of the normalized volume of a specific band for each cell type versus the normalized volume of the band for parent Het-1A cells.

Statistical Analysis

Data were expressed as percentages, mean ($\pm$ standard deviation) or median and 95% confidence intervals), and analyzed using the Student t test, Mann-Whitney U test, or Wilcoxon test as appropriate. Kaplan-Meier and log-rank tests were used for comparing tumor frequency in animals. All analyses were conducted using SPSS 15.0 (Chicago, Ill). The estimated frequencies of sphere, clone, and tumor formation for each cell type (parent and spheroid) were quantitated using ELDA incorporating an online Web tool, as reported previously.[14] Statistical significance was set at $P < .05$.

RESULTS

Flow Cytometry of Parent Cells and Spheroid Cells

Flow cytometric analysis of parent cells revealed consistent negativity for CD24, CD34, and CD133, and consistent high positivity for CD44 (Het-1A, 90%; OE33, 84%; JH-EsoAd1, 98%) and Oct4 (Het-1A, 63%; OE33, 69%; JH-EsoAd1, 99%). CD71 was highly positive in Het-1A (73%) and JH-EsoAd1 (89%), but only moderately positive in OE33 (41%). Moderate positivity (Het-1A, 16%; OE33, 34%; JH-EsoAd1, 43%) was found for Musashi 1. EpCAM was negative in Het-1A and OE33, but slightly positive (6%) in JH-EsoAd1. Integrin α6 was slightly positive in Het-1A (5%), low in OE33 (0.2%), and moderately positive in JH-EsoAd1 (44%). Less than 1% of parent cells were positive for integrin $\alpha 6^{bri}/CD71^{dim}$ (Het-1A, 0.11%; OE33, 0.01%; JH-EsoAd1, 0.21%).

Flow cytometry was performed on Het-1A, OE33, and JH-EsoAd1 spheroid cells to evaluate further Musashi 1 and integrin α6/CD71. No differences in Musashi 1 staining were found between parent and spheroid cells of any cell line. However, as illustrated in Figure 1, significant differences in integrin $\alpha 6^{bri}/CD71^{dim}$ staining were seen for parent cells (Het-1A, 0.11%; OE 33, 0.01%; JH-EsoAd1, 0.21%) and spheroid cells (Het-1A, 21.3%; OE33, 5.73%; JH-EsoAd1, 11.3%).

Clonogenicity and Tumorigenicity of Parent Cells and Spheroid Cells

All Het-1A, OE33, and JH-EsoAd1 parent and spheroid cells formed colonies in soft agar. As summarized in Table 1, spheroid cells demonstrated significantly enhanced clonogenicity compared with parent cells for all cell lines.

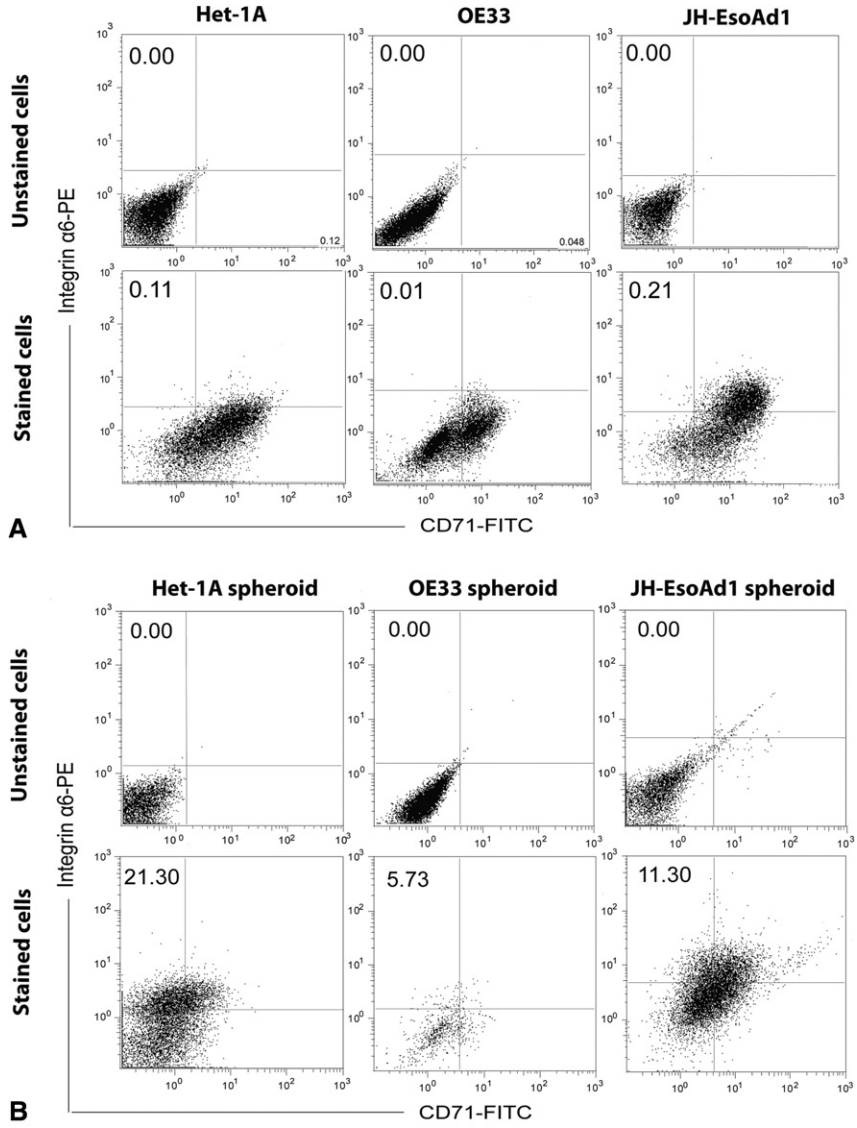

FIGURE 1. Flow cytometry was used to study expression of integrin $\alpha 6$ (vertical axis) and CD71 (horizontal axis) in Het-1A, OE33, and JH-EsoAd1 parent cells (A) and spheroid cells (B). Relative to unstained cells (*top row*, A and B), significant differences in staining were seen for each cell line between parent cells (*lower row*, A) and spheroid cells (*lower row*, B). Significant differences in integrin $\alpha 6^{bri}$/$CD71^{dim}$ staining (*upper left quadrant* of each panel) were seen between parent cells (Het-1A, 0.11%; OE33, 0.01%; JH-EsoAd1, 0.21%) compared with spheroid cells (Het-1A, 21.3%; OE33, 5.73%; JH-EsoAd1, 11.3%). *FITC*, Fluorescein isothiocyanate conjugate.

Neither Het-1A parent cells or spheroid cells, regardless of cell numbers implanted, resulted in any xenograft tumor formation. At 10 weeks, of 15 parent or spheroid cell inoculations, 1 tumor (6.7%) resulted from OE33 parent cells, and 4 tumors (26.7%) resulted from OE33 spheroid cells. For JH-EsoAd1, 1 tumor (6.7%) resulted from inoculation of parent cells and 8 tumors (53.3%) resulted from inoculation of JH-EsoAd1 spheroid cells. At study termination (29 weeks for OE33, 13 weeks for JH-EsoAd1), OE33 parent cell inoculation resulted in 2 tumors (13.3%; median volume, 1.7 mm^3), and OE33 spheroid cell inoculation resulted in 6 tumors (40.0%; median volume, 500 mm^3); JH-EsoAd1 parent cell inoculations resulted in 6 tumors (40.0%; median volume, 275 mm^3), and JH-EsoAd1 spheroid cell inoculation resulted in 13 tumors (86.7%; median volume, 1098 mm^3). Kaplan-Meier analysis confirmed a greater frequency of tumors resulting from spheroid cell inoculation compared with parent cell inoculation at study termination for each cell line (OE33, $P = .12$; JH-EsoAd1, $P < .01$), which was also confirmed by ELDA (OE33, $P = .06$; JH-EsoAd1, $P < .01$; Table 1).

Xenograft Tumor Histology and IHC

Histologic examination of OE33 and JH-EsoAd1 xenografts confirmed that adenocarcinomas resulted from inoculation of parent cells and spheroid cells (OE33 and

TABLE 1. Clonogenicity and tumorigenicity of Het-1A, OE33, and JH-EsoAd1 parent and spheroid cells determined by extreme limiting dilution analysis

Cell line	Parent cells, median frequency (95% CI)	Spheroid cells, median frequency (95% CI)	*P* value
Clonogenicity			
Het-1A	1/51.46 (1/92.80-1/28.50)	1/8.13 (1/15.20-1/4.30)	<.01
OE33	1/49.40 (1/85.8-1/28.5)	1/15.10 (1/27.50-1/8.60)	<.01
JH-EsoAd1	1/196.20 (1/409.00-1/94.20)	1/72.40 (1/124.00-1/42.20)	<.05
Tumorigenicity			
OE33	$1/36.10 \times 10^5$ ($1/145.00 \times 10^5$-$1/8.98 \times 10^5$)	$1/8.69 \times 10^5$ ($1/20.50 \times 10^5$-$1/3.69 \times 10^5$)	.06
JH-EsoAd1	$1/4.58 \times 10^5$ ($1/1080 \times 10^5$-$1/1.94 \times 10^5$)	$1/0.41 \times 10^5$ ($1/1.12 \times 10^5$-$1/0.15 \times 10^5$)	<.05

Median frequencies estimated from extreme limiting dilution analysis.[14] *CI*, Confidence interval.

JH-EsoAd1). Adenocarcinomas derived from spheroid cells were of higher grade compared with tumors resulting from inoculation of parent cells. IHC analysis revealed consistently negative staining in all control sections, and tissues stained for CD133 and CD71. Positive cell membrane staining was seen for CD44 in xenografts derived from parent cells (OE33: 3+, 60%; JH-EsoAd1: 2+, 30%) and spheroid cells (OE33: 2+, 30%; JH-EsoAd1: 3+, 90%) for all cell lines. Cytoplasmic and cell membrane staining was consistently positive (2+, 80%) for integrin α6 in all xenograft tumors arising from parent and spheroid cells of OE33 and JH-EsoAd1.

Characterization of Integrin α6/CD71 Cell Subpopulations

As summarized in Table 2, ELDA showed that integrin $\alpha6^{bri}/CD71^{dim}$ subpopulations of Het-1A, OE33, and JH-EsoAd1 cell lines contained more clonogenic and sphere-forming cells compared with each of the other subpopulations.

Chemosensitivity of Parent Cells and Spheroid Cells

As shown in Figure 2, spheroid cells of all cell lines exhibited lower proliferation rates compared with corresponding parent cells: Het-1A: 0.66 ± 0.08 (sphere) verses 0.13 ± 0.01 (parent), $P < .01$; OE33: 0.47 ± 0.09 (sphere) verses 0.29 ± 0.06 (parent), $P < .05$; JH-EsoAd1: 0.68 ± 0.08 (sphere) verses 0.34 ± 0.03 (parent), $P < .01$.

Compared with parent cells, all spheroid cells were resistant to 5-Fu. Het-1A spheroid cells were also resistant to CDDP, as was OE33 at higher doses (4 μg/mL). JH-EsoAd1 spheroid cells exhibited similar chemosensitivity to CDDP to their parent cells at all doses studied.

mRNA Expression Profile of CSC-Related Genes

PCR array of 253 CSC related genes revealed different gene expression profiles among each cell line. Table 3 summarizes the 3 most frequently upregulated and downregulated genes in spheroid cells (relative to parent cells) for each cell line. Only 1 gene, Ascl2, was found to be consistently upregulated in all cell lines: Het-1A, 2.5-fold; OE33,

TABLE 2. Clonogenicity and sphere formation of Het-1A, OE33, and JH-EsoAd1 parent cell subpopulations expressing different levels of integrin α6 and CD71

Cell line	Clonogenicity		Sphere formation	
Subpopulation	Median frequency (95% CI)	*P* value	Median frequency (95% CI)	*P* value
Het-1A				
Integrin $\alpha6^{bri}/CD71^{dim}$	1/84.1 (1/145-1/48.9)	—	1/7.09 (1/12.80-1/3.94)	—
Integrin $\alpha6^{bri}/CD71^{bri}$	1/331.9 (1/820-1/134.4)	<.01	1/38.34 (1/68.10-1/21.60)	<.01
Integrin $\alpha6^{dim}/CD71^{dim}$	1/170.1 (1/339-1/86)	.09	1/27.77 (1/48.60-1/15.85)	<.01
Integrin $\alpha6^{dim}/CD71^{bri}$	1/186.7 (1/380-1/91.8)	.06	1/18.29 (1/31.90-1/10.48)	.02
OE33				
Integrin $\alpha6^{bri}/CD71^{dim}$	1/112 (1/202-1/61.9)	—	1/7.09 (1/12.80-1/3.94)	—
Integrin $\alpha6^{bri}/CD71^{bri}$	1/395 (1/1049-1/148.6)	.02	1/28.24 (1/49.50-1/16.12)	<.01
Integrin $\alpha6^{dim}/CD71^{dim}$	1/544 (1/1687-1/175.5)	<.01	1/86.67 (1/15.50-1/49.90)	<.01
Integrin $\alpha6^{dim}/CD71^{bri}$	1/312 (1/753-1/129.4)	.04	1/17.47 (1/30.50-1/10.00)	.03
JH-EsoAd1				
Integrin $\alpha6^{bri}/CD71^{dim}$	1/124 (1/219-1/69.9)	—	1/6.62 (1/11.90-1/3.69)	—
Integrin $\alpha6^{bri}/CD71^{bri}$	1/350 (1/1086-1/112.8)	.07	1/8.27 (1/14.90-1/4.59)	.61
Integrin $\alpha6^{dim}/CD71^{dim}$	1/890 (1/2730-1/290.5)	<.01	1/29.17 (1/51.20-1/16.63)	<.01
Integrin $\alpha6^{dim}/CD71^{bri}$	1/1636 (1/6577-1/407.1)	<.01	1/20.03 (1/34.90-1/11.50)	<.01

P values presented with reference to integrin $\alpha6^{bri}/CD71^{dim}$ cells. Median frequencies estimated from extreme limiting dilution analysis.[14] *CI*, Confidence interval.

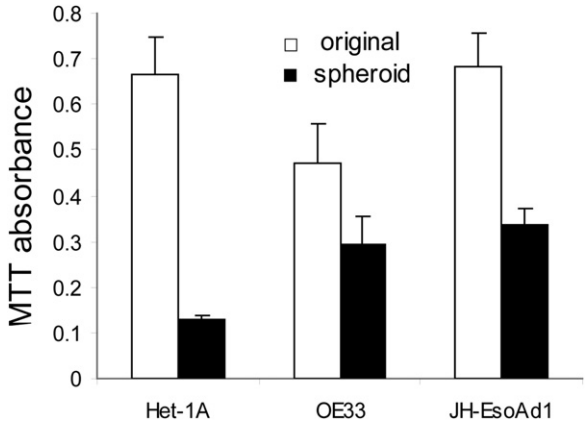

FIGURE 2. Cellular proliferation rates were significantly ($P < .005$) lower for spheroid cells compared with original (parent) cells for each cell line. *Bars* reflect standard error. *MTT*, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

+10.2-fold; and JH-EsoAd1, +4.8-fold upregulation in spheroid cells relative to parent cells.

Western Blot Analysis of Ascl2 and Lgr5

Western blot analysis of Ascl2 protein expression revealed a predicted positive 28-kDa band in each cell line (Figure 3, *A*). An additional higher molecular weight band (estimated, 48 kDa) was also found in each tumor cell line (OE33 and JH-EsoAd1 parent and spheroid cells), but was not detected in Het-1A. Quantitative analysis showed that relative to Ascl2 protein expression in Het-1A parent cells, Ascl2 protein was significantly ($P < .05$) overexpressed in OE33 parent cells and underexpressed in JH-EsoAd1 parent cells (Figure 3, *B*). For each cell line, Ascl2 protein expression was significantly higher in spheroid cells compared with parent cells (Het-1A: 1.71 ± 0.42 vs 1.00 ± 0.00, $P < .05$; OE33: 4.70 ± 1.05 vs 2.74 ± 0.63, $P < .05$; JH-EsoAd1: 1.29 ± 0.25 vs 0.27 ± 0.09, $P < .01$). Similar patterns of Ascl2 protein expression were also found in OE33 and JH-EsoAd1 xenografts derived from parent cells and spheroid cells. Lgr5 expression was consistently negative in all cell lines and xenografts (parent and spheroid cells).

TABLE 3. PCR array of stem cell related genes summarizing the 3 most frequently upregulated and downregulated genes in Het-1A, OE33, and JH-EsoAd1 spheroid cells relative to parent cells

Gene expression	Het-1A	OE33	JH-EsoAd1
Upregulated (-fold*)			
	SFRP 4 (+36.6)	CD 3 (+49.2)	FOS (+39.9)
	BMP 3 (+21.3)	WNT 1 (+32.5)	CYP 3A5 (+32.5)
	FORK A2 (+19.4)	CCN A2 (+30.2)	DKK 1 (+8.8)
Downregulated (-fold*)			
	ABC B1 (–68.4)	WNT 5B (–61.7)	ALD 1 (21.4)
	FZD 4 (–57.8)	FORK A2 (–45.2)	FZD 2 (–17.8)
	MYO 1 (–24.2)	BMP 2 (–13.9)	FGF 1 (–17.1)

SFRP, Secreted frizzled-related protein; *CD*, cluster of differentiation marker; *FOS*, v-fos oncogene; *BMP*, bone morphogenic protein; *WNT*, wingless-type signaling pathway; *CYP*, cytochrome 450 family; *FORK*, forkhead box; *CCN*, cyclin; *DKK*, Dickkopf homolog; *ABC*, adenosine triphosphate-binding cassette family; *ALD*, aldehyde dehydrogenase; *FZD*, frizzled homolog; *MYO*, myogenic differentiation; *FGF*, fibroblast growth factor. *Fold change: upregulation (+) or downregulation (–).

DISCUSSION

In keeping with recent results from a comprehensive evaluation of 17 surgically resected human EADCs in which IHC was used to stain for a panel of CSC markers (CD24, CD29, CD34, CD44, CD133, CD166, EpCAM, and β-catenin),[8] we found that all esophageal cell lines exhibited consistent high positivity for CD44 and Oct4, and negativity for CD24, CD34, and CD133. Although EpCAM was consistently negative in Het-1A and OE33, slight positivity (6%) was seen in JH-EsoAd1. Moderate positivity was also seen for Musashi 1 in each parent cell line (Het-1A, 16%; OE33, 34%; JH-EsoAd1, 43%), comparable with expression levels reported in human esophageal tissues.[16] To date, relatively few studies have evaluated esophageal cancer cell lines specifically, derived predominantly from squamous cell carcinomas.[17,18] Upregulation of several key stem cell-related genes (including Oct4), Wnt and Notch signal pathway-related genes was reported recently in side populations of EC9706 and EC109,[17] and increased expression of β-catenin and β_1 integrin were reported in a radio-resistant cell line derived from TE-2, which also contained a higher percentage of side population cells compared with the parent cell line.[18]

We found that spheroid cells derived from Het-1A, OE33, and JH-EsoAd1 enriched significantly for integrin $\alpha6^{bri}$/$CD71^{dim}$ compared with parent cells (Figure 1). Our finding of enhanced in vitro clonogenicity and spherogenicity (Table 2) for cells expressing integrin $\alpha6^{bri}$/$CD71^{dim}$ further supports the notion that these markers reflect at least a subpopulation of esophageal stemlike cells in each cell line studied. Indeed, integrin $\alpha6^{bri}$/$CD71^{dim}$ has been reported to enrich human corneal epithelial/stem progenitor cells,[19] and keratinocyte stem cell populations.[20,21] A minor subpopulation of undifferentiated cells enriched for integrin $\alpha6^{bri}$/$CD71^{dim}$ has also been reported in the basal layer of the mouse esophagus, a potential murine CSC niche.[22] Although recent evidence had been presented to support a central role for integrins in human embryonic stem cell culture, the precise molecular pathways underlying the contribution of integrins to CSC biology have yet to be defined. By regulating cellular adhesion in the extracellular matrix, it has been suggested that integrins have a central role in maintaining stem cells in their microenvironment, and have been implicated specifically in the bone marrow

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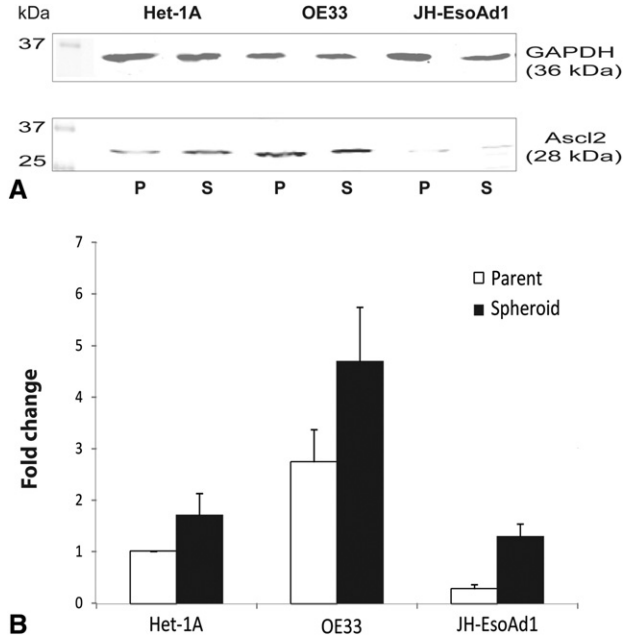

FIGURE 3. Expression of Achaete-scute complex homolog 2 (Ascl2) in parent and spheroid cells of Het-1A, OE33 and JH-EsoAd1. A, Western blot analysis illustrating increased Ascl2 protein expression in spheroid cells relative to parent cells of each cell line. B, Relative to Ascl2 protein expression in Het-1A parent cells, Ascl2 protein expression was significantly ($P < .05$) overexpressed in OE33 parent cells, whereas JH-EsoAd1 parent cells underexpressed Ascl2 protein (the fold change relative to Het-1A parent cells is shown on the *vertical axis*). For each cell line, Ascl2 protein was significantly ($P < .05$) overexpressed in spheroid cells relative to parent cells. *Bars* reflect standard error. *GAPDH*, Glyceraldehyde 3-phosphate dehydrogenase; *P*, parent cells; *S*, spheroid cells.

microenvironment, a potential CSC niche contributing to chemoresistance.

PCR array was also used to study the expression of CSC-related genes in esophageal cell lines and spheres; the 3 most frequently up- and downregulated genes are shown in Table 3. However, relative to parent cells, only Ascl2 mRNA was found to be consistently upregulated in Het-1A, OE33, and JH-EsoAd1 spheroid cells. Corresponding overexpression of Ascl2 protein was confirmed subsequently by Western blot analysis in each cell line (Figure 3), and in OE33 and JH-EsoAd1 xenografts. In addition to the predicted positive 28-kDa band in each cell line, an additional band of higher molecular weight (estimated, 48 kDa) was found only in each of the tumor cell lines (OE 33 and JH-EsoAd1 parent and spheroid cells), but was not detected in Het-1A. A higher molecular weight band, most likely a consequence of overexpression of a long mRNA Ascl2 transcript, has been reported previously to encode an open reading frame for an uncharacterized protein,[23] although further characterization will be required to evaluate its specific role in human malignancy.

To our knowledge, this is the first report of Ascl2 mRNA and protein expression in human esophageal cell lines and xenografts. As a direct transcriptional target of Wnt, Ascl2 has been implicated in the maintenance of human intestinal stem cells, in addition to regulating cell proliferation by modulating progression through the cell cycle G2/M checkpoint.[23] Relative to low levels of expression in normal colonic epithelia, Ascl2 mRNA has been reported to be overexpressed in primary colorectal adenocarcinomas, and in colorectal liver metastases with 11p15.5 gain, in which the induction by Ascl2 of several downstream target genes appeared to confer a unique CSC expression signature.[24] Ascl2 has also recently been implicated in drug resistance to various anticancer agents (including 5-Fu)[25]—an important characteristic of the CSC phenotype limiting the clinical efficacy of many current cytotoxic chemotherapeutic agents.

In summary, we have demonstrated that human esophageal cell lines derived from normal esophageal epithelia and primary EADCs, when grown in serum-free media to form spheroids, each exhibited increased clonogenicity, tumorigenicity, and drug resistance, reflecting an enrichment of stemlike cell populations. Integrin $\alpha 6^{bri}$/CD71^{dim} would appear to define at least a subpopulation of cancer stemlike cells in human esophageal cell lines, with the potential to direct future clinically relevant therapeutic strategies for human esophageal malignancy. Our novel finding of Ascl2 overexpression in esophageal cell lines and xenografts represents a particularly promising area for further investigation of esophageal tumor/CSC biology.

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Discussion

Dr Jules Lin *(Ann Arbor, Mich)*. Congratulations on your very nice presentation. I have several questions that I would like to ask individually. Het-1A is a normal squamous esophageal cell line. Why did you choose to use a normal cell line? In your comparison you looked at 2 tumor cell lines and a normal squamous cell line looking for a commonality between the 3. Would it have been more appropriate to compare the tumor cell lines to find something that was overexpressed when compared to the normal cell line?

Dr Casson. Esophageal cell lines are quite notoriously difficult to get a hold of. I would question whether Het-1A, which has been around for 20-odd years, is truly normal at this point. It was originally derived from normal epithelium but has been immortalized. We put that in—really as, in quotes—the ''negative control.'' But, again, I would caution the interpretation using any of these cell lines.

Dr Lin. One of the key concepts of CSCs is tumorigenicity. Out of the 3 cell lines from your data that you presented, 2 of them formed tumors in the nude mice. The Het-1A did not, for parent or spheroid cells. However, the 2 markers that you mentioned, the Ascl2 and the integrin $\alpha 6^{bri}$/CD71, were both up in Het-1A. In fact, the integrin α6/CD71 ratio was 21% in Het-1A and the Ascl2 was up higher than the Johns Hopkins adenocarcinoma cell line. What conclusion can you draw about those markers?

Dr Casson. Your first comment about not being able to grow Het-1 in animals, that's consistent with the published data. I don't think anybody has been able to reproduce that cell line. So we really were left with the 2 EADC cell lines for these types of xenotransplantation experiments.

In terms of the significance of Ascl2 overexpression, at this point it's very early. I think what we need to do is reproduce these results using serial xenograft transplantations along the fairly classic lines used to determine tumorigenicity using human tissues. The problem we have, of course, is that the particular marker, the integrin α6/CD71, the viability of cells based on cell sorting just does not give you sufficient viability to establish those as xenografts at this point.

Dr Lin. Did you look at any functional studies looking at these 2 genes? Did you try to transfect Ascl2 or integrin α6 to see if it affects spheroid formation, colony formation, or tumorigenicity?

Dr Casson. No, we haven't, but that would be the next logical step.

Dr Lin. My final question: Did you look at any primary tumors to determine whether either of these markers were overexpressed in primary tumors?

Dr Casson. We have not looked at the primary tumors. One of the references I put on from Jan van Lanschot's lab, that was the most comprehensive study using primary tumors so far, and, again, I would say that there were no classic stem cell markers identified in that series of about 17 tumors. That was reported in the *Journal of Pathology* in 2010.

Dr Lin. Thank you. Nice presentation.

Dr Ori Wald *(Jerusalem, Israel)*. I think that another approach to isolate CSCs might be first to select for chemotherapy resistance and then to reexamine the markers. I believe that this represents more what happens in humans. We first treat our patients with chemotherapy and, as a result, selection for chemotherapy-resistant cells occurs. By adopting such an approach, this is another approach you may try to validate the results.

Dr Casson. That's a good point. I would say that CSC research for solid tumors, unlike the leukemias, is really in its infancy and really only has been looked at in the past few years. Good comment.