Novel method of cell line establishment utilizing fluorescence-activated cell sorting resulting in 6 new head and neck squamous cell carcinoma lines

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ABSTRACT: *Background.* The purpose of this study was to present the establishment of new cell lines, which is important to cancer research.

Methods. Six new head and neck squamous cell carcinoma cell lines were established using a novel fluorescence-activated cell sorting (FACS) method in order to overcome the barrier of fibroblast overgrowth and the susceptibility of primary tumors to fail in vitro.

Results. Antibodies chosen for specific targeting of epithelial cells and fibroblasts successfully separated cells for line establishment in 6 of 12 attempts, providing an alternative method of establishing head and neck squamous cell carcinoma cell lines. Each attempt at cell line establishment resulted in an epithelial carcinoma population, which was genotyped and catalogued as a unique cell line, and a corresponding fibroblast population.

Conclusion. The selection of antibody markers could be optimized to aid in the establishment of any cancer cell line derived from any tumor tissue; this method is not limited to head and neck cancer. © 2015 Wiley Periodicals, Inc. *Head Neck* 38: E459–E467, 2016

KEY WORDS: cell line, flow cytometry, fibroblasts, squamous cell carcinoma

INTRODUCTION

Cancer cell lines provide an invaluable research tool for the study of this diverse and deadly disease. Cell culture techniques were developed in the early 20th century involving animal cells,[1–3] with immortalized mouse cells being established in 1943.[4] The first human continuous cancer line, HeLa cells, was cultured in 1951 at Johns Hopkins Hospital in Baltimore, Maryland.[5,6] Under laboratory conditions that are quite different than those of the modern era, the establishment of this cervical cancer cell line allowed institutions from all over the world to study the disease in the laboratory without limitations because of specimen availability. After the initial breakthrough of HeLa establishment, human cells were cultured with greater frequency and efficacy.[7–9] Culturing techniques have improved as years have passed, including the introduction of antibiotics, sterile conditions, and laminar flow hoods, as well as the optimization of tissue culture medium formulas.[10–13] As a result of improved cell line establishment methods and culture conditions, cell lines of a variety of cancers have been made available for research, with the most recent cancer cell line encyclopedia containing information on 947 different cell lines from 36 tumor types.[14]

The availability of a library of cancer cell lines is especially important in the study of head and neck cancer, which includes a diverse group of biologically similar cancers from multiple sites. Ninety percent of head and neck cancers are squamous cell carcinomas, mostly occurring in the oral cavity, larynx, and pharynx, with roughly 40,000 new diagnoses each year in the United States.[15,16] Worldwide collections of head and neck cell lines are now being assembled as valuable repositories to reflect the different varieties of the disease.[17–20] Recent interest in the role of human papillomavirus (HPV) in the pathogenesis of head and neck cancer has driven research to compare HPV-positive and HPV-negative tumor types and has increased the need for newly established HPV-positive cell lines.[21,22]

Current methods for establishing cell lines from primary tissue of the head and neck include tumor explant in tissue culture, or mechanical or enzymatic digestion of the tissue and then in vitro growth of attached epithelial cells from single-cell suspensions or partially digested tumor tissue.[17,23] A key concern that arises in these methods is fibroblast overgrowth of the culture. Fibroblasts accompany the primary tumor tissue and usually divide faster than the epithelial population of cancer cells, while also competing for media nutrients and area for expansion. Fibroblasts are typically removed from culture

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through a series of partial trypsinizations, as they will detach from culture flasks or plates before the epithelial population, but other methods of fibroblast elimination have been described.[24,25] Partial trypsinizations are performed multiple times until the fibroblasts are eliminated or become senescent, after an average of 50 population doublings known as the Hayflick limit.[26] This method can take several months before fibroblast growth is arrested, and also risks loss of cancer cells during each successive trypsinization. From 1978 to 1994, our laboratory established over 112 unique University of Michigan-squamous cell carcinoma (UM-SCC) cell lines from 95 different patients, including 17 cell lines from 8 patients who provided more than 1 tumor from either different sites or from different times in the course of their disease. Nearly all of these were established using the partial trypsinization method.[27–29] The overall success rate during this time varied from 30% to 35% of attempts. The average time to successful passage of the tumor cells from mixed epithelial and fibroblast cultures ranged from 195 days for recurrent and metastatic tumors to more than 250 days for previously untreated primary tumors. Using similar methodology, from 1992 until 1997, the University of Pittsburgh established 52 new head and neck cell lines out of 199 tumor samples utilizing the partial trypsinization culture method, successfully establishing cell lines in 26% of the attempts.[19] One hundred one of the tumor samples were deemed unsuccessful because of fibroblast overgrowth or growth of only fibroblasts when culture was attempted. The remaining attempts failed because of contamination of the culture flasks. Although good, these rates of success fall below a level that would allow study of most patients' tumors in a timely manner for identifying targets for therapy. Thus, improvements in cultivation techniques would be of great value.

Herein, we describe a novel method of cell line establishment that removes the need for a series of partial trypsinizations to eliminate fibroblast overgrowth. Careful selection of antibodies specific to surface markers exclusive to either squamous cell carcinomas or to fibroblasts allows for complete separation of the 2 populations by fluorescence-activated cell sorting (FACS). We chose epithelial cell adhesion molecule (EpCAM) and fibroblast surface protein (FSP). EpCAM is a pan-epithelial carcinoma-associated antigen that is expressed in a majority of carcinomas.[30–32] FSP is a surface protein on human fibroblasts that is absent on human epithelial cells.[33–35] This technique allows for isolation of a carcinoma population within several hours of tissue acquisition, as well as conserving the fibroblasts as a separate population, if desired. There exists a potential benefit in being able to expand the cancer cell population without the time and effort involved in having to first eliminate the fibroblasts. This antibody-based sorting method may help to improve upon the frequency that lines are established, as many potential cell lines were lost because of fibroblast overgrowth in culture using traditional partial trypsinization methods.

MATERIALS AND METHODS

Patients

Patients were recruited from the Head and Neck Oncology Division of the Department of Otolaryngology at the University of Michigan and asked to sign an Institutional Review Board approved informed consent to study their tissue, including permission to establish a permanent cell line.

Preparation and digestion of tissue

Primary tumor tissue was transported from the operating room to the laboratory and was washed extensively in Hanks balanced salt solution (HBSS) containing penicillin, streptomycin, and amphotericin B. The tissue was then minced by scalpel blade and digested in Dulbecco's Modified Eagle Medium (DMEM)/F12 (GIBCO, Grand Island, NY) with 1X collagenase/hyaluronidase (Stem Cell Technologies, Vancouver, Canada). After 2 hours of digestion at 37°C, the mixture was strained through a 70 um sieve and the cells were counted before being prepared for flow cytometry. When tumor was not collected on the same day as the cell sort, digested cells were placed in a culture flask and remaining tumor pieces were placed in a separate flask for a further digestion on the day of the sort. All primary samples were sorted by flow cytometry within 72 hours of collection.

Flow cytometry

EpCAM and FSP expression were detected using primary antibodies (CAT#MS-181-P; Neomarkers Marseille, France; CAT#ab11333; Abcam, Cambridge, MA) and fluorophore-conjugated secondary antibodies (BD Pharmingen; eBioscience). Cells were suspended in HBSS (GIBCO) with 2% heat inactivated calf serum added to a concentration of 1 million cells per mL. Five μl of primary antibody was added per mL of cell suspension, and left to incubate on ice for 20 minutes. Cells were then pelleted down and resuspended in HBSS to a concentration of 1 million cells per mL. Five μl of secondary antibody was added per mL of cell suspension for 20 minutes on ice. For EpCAM expression, cell-sorting gates were established using an unstained control population in the allophycocyanin channel with excitation and emission wavelengths of approximately 650 to 660 nm. For FSP expression, cell-sorting gates were established using an unstained control population in the phycoerythrin channel with excitation and emission wavelengths of approximately 565 to 578 nm.

Cell line establishment

Cells collected from flow cytometry were cultured in DMEM containing 20% fetal bovine serum (GIBCO) containing penicillin, streptomycin, nonessential amino acids, and amphotericin B. Once growth without contamination was confirmed, cells were grown in 10% fetal bovine serum media without amphotericin B. Fibroblasts were frozen down before 5 passages, and cell lines were frozen down as often as possible until establishment was confirmed at 20 passages, as described previously.[27] Lines were then further passaged until at least 50 passages and 100 doublings were achieved.

Genomic DNA purification for genotyping

Cells were harvested and washed in phosphate-buffered saline, then frozen at −80°C. The thawed cell

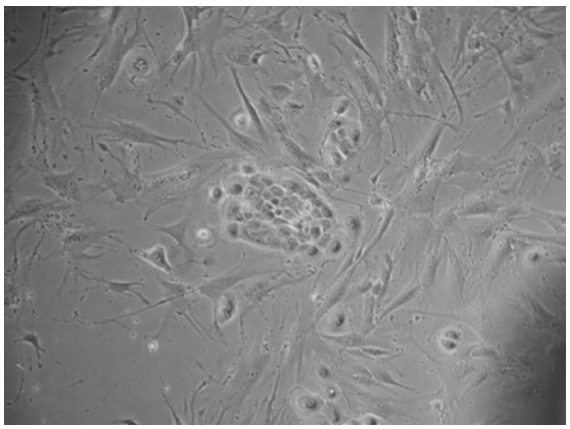

FIGURE 1. An early attempt at cell line establishment from a primary tumor using traditional partial-trypsinization methods. An island of epithelial cells is seen surrounded by fibroblast overgrowth.

pellets were resuspended in 600 μL of Promega nuclei lysis solution (Promega, San Luis Obispo, CA) for 1 hour at 55°C, then allowed to cool to room temperature. Two hundred μl of Promega protein precipitation solution (Promega) was added to each sample on ice for 5 minutes before being centrifuged at 13,000 revolutions per minute for 2 minutes. Supernatant was transferred to a tube containing 600 μL of isopropanol and centrifuged at 13,000 revolutions per minute for 1 minute. Supernatant was aspirated and the DNA pellet washed in 200 μL of 70% ethanol and resuspended in 50 μL of nuclease-free water.

TABLE 1. Six cell lines were established from primary tumors out of 12 attempts.

Sample	Site	TNM classification	Sex	Age, y	Cell line
HN162	Lateral tongue	T4N2bM0	Male	57	
HN165	Larynx	T4N0M0	Male	51	UM-SCC-105
HN166	Oral tongue	T4N2bM0	Male	36	UM-SCC-106
HN171	Floor of mouth	T4N0M0	Male	60	
HN172	Ventral tongue	T4N2cM0	Female	47	
HN173	Lateral tongue	T4N0M0	Female	30	UM-SCC-108
HN174	Lateral tongue	T4N2bM0	Male	25	
HN175	Floor of mouth	T4N1M0	Female	69	
HN176	Base of tongue	T4N1M0	Male	49	UM-SCC-109
HN177	Anterior tongue	T3N0M0	Male	39	UM-SCC-110
HN178	Floor of mouth	T3N0M0	Male	51	
HN181	Lateral tongue	T4N2cM0	Male	60	UM-SCC-111

Abbreviation: UM-SCC, University of Michigan-squamous cell carcinoma.

Analysis of genetic loci

DNA samples were diluted to 0.10 ng/L and were analyzed at the University of Michigan DNA Sequencing Core using the Profiler Plus PCR Amplification Kit (Invitrogen, Carlsbad, CA) in accord with the manufacturer's protocol. The 10 loci D3S1358, D5S818, D7S820, D8S1179, D13S317, D18S51, D21S11, FGA, vWA, and AMEL were analyzed and compared with ladder control samples, as described previously.[18]

Luciferase transduction

UM-SCC-105, UM-SCC-106, and UM-SCC-110 were transduced with human immunodeficiency virus with a luciferase reporter, a lentiviral vector containing a

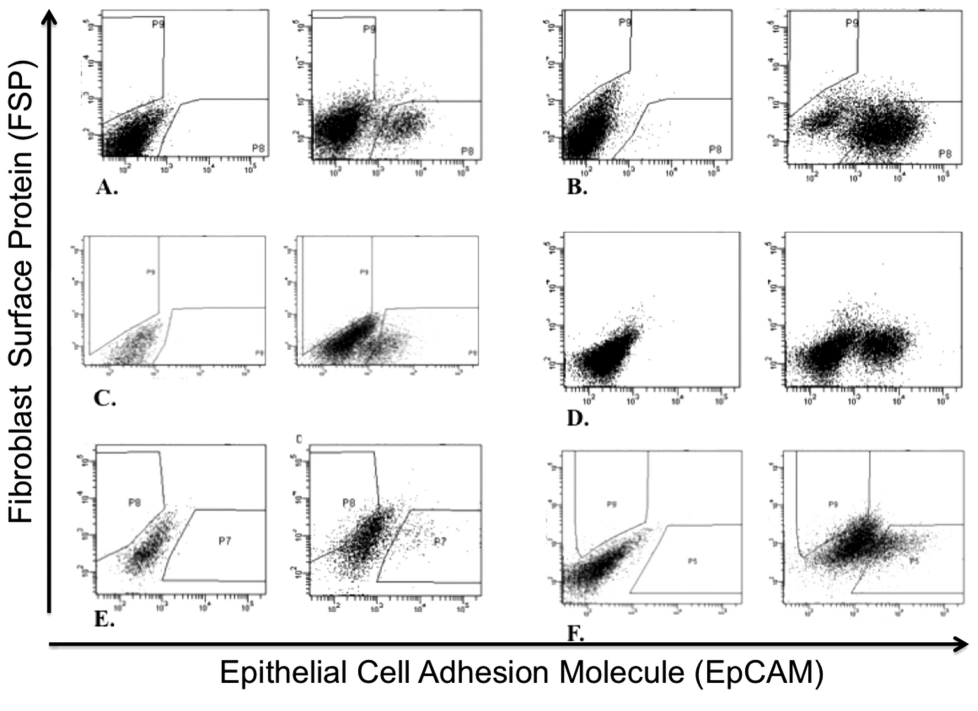

FIGURE 2. Fluorescence activated cell sorter (FACS) plots of each primary tumor that became a successful cancer cell line with the unstained plot on the left and the stained plot on the right. Cells expressing the epithelial cell adhesion molecule (EpCAM) marker shift to the right along the X-axis, whereas cells expressing the fibroblast surface protein (FSP) marker shift up along the Y-axis. Debris and other cell types do not stain for either antibody. Primary tumors shown are (A) HN165; (B) HN166; (C) HN173; (D) HN176; (E) HN177; and (F) HN181.

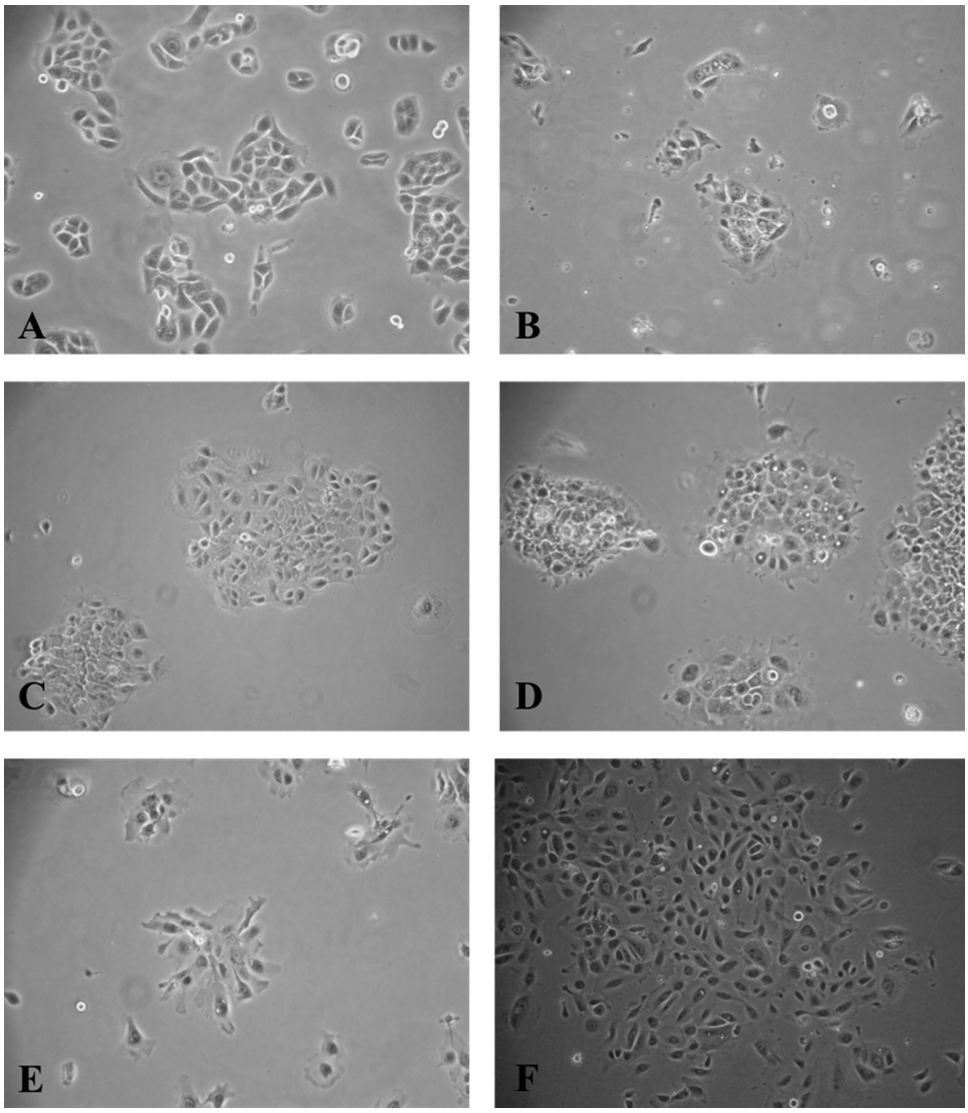

FIGURE 3. Cells from the epithelial cell adhesion molecule (EpCAM)+/fibroblast surface protein (FSP)- population were collected and grown in culture. These cells were established as new carcinoma lines (A) University of Michigan-squamous cell carcinoma (UM-SCC)-105; (B) UM-SCC-106; (C) UM-SCC-108; (D) UM-SCC-109; (E) UM-SCC-110; and (F) UM-SCC-111.

p Lentilox backbone and a cytomegalovirus promoter. Polybrene was added to increase efficiency of the transduction. Successful gene delivery was confirmed via green fluorescent protein visualization in a side-by-side transduction of the human immunodeficiency virus-green fluorescent protein vector under identical conditions.

Flank injections

One hundred thousand cells of luciferase-transduced cells were suspended in a mixture of 100 μL of DMEM and 100 μL of Matrigel extracellular matrix (BD Biosciences, San Jose, CA). The resulting 200 μL volumes were injected subcutaneously into the left flanks of NOD/SCID mice (Jackson Laboratories, Bar Harbor, ME) to examine xenograft potential. One hundred thousand cells of luciferase-transduced cells were mixed with 100,000 tumor-associated fibroblasts (TAFs) derived from the same tumor and injected subcutaneously into the right flanks of the same mouse. Tumor growth was allowed to persist for 12 weeks until harvested for sectioning and digestion. The xenografts were confirmed to match the initial cell lines.

Bioluminescence imaging

All animals injected with luciferase-transduced cells were imaged with the Xenogen IVIS-200 imaging system. Treated mice were given intraperitoneal injections of 100 uL luciferin at a concentration of 40 mg/mL and allowed to sit for 10 minutes before being anesthetized with isoflurane and imaged.

RESULTS

Primary tissue

Previous attempts at cell line establishment using traditional partial trypsinization methods for fibroblast removal resulted in limited success (see Figure 1). Six cell lines were established out of 12 attempts from

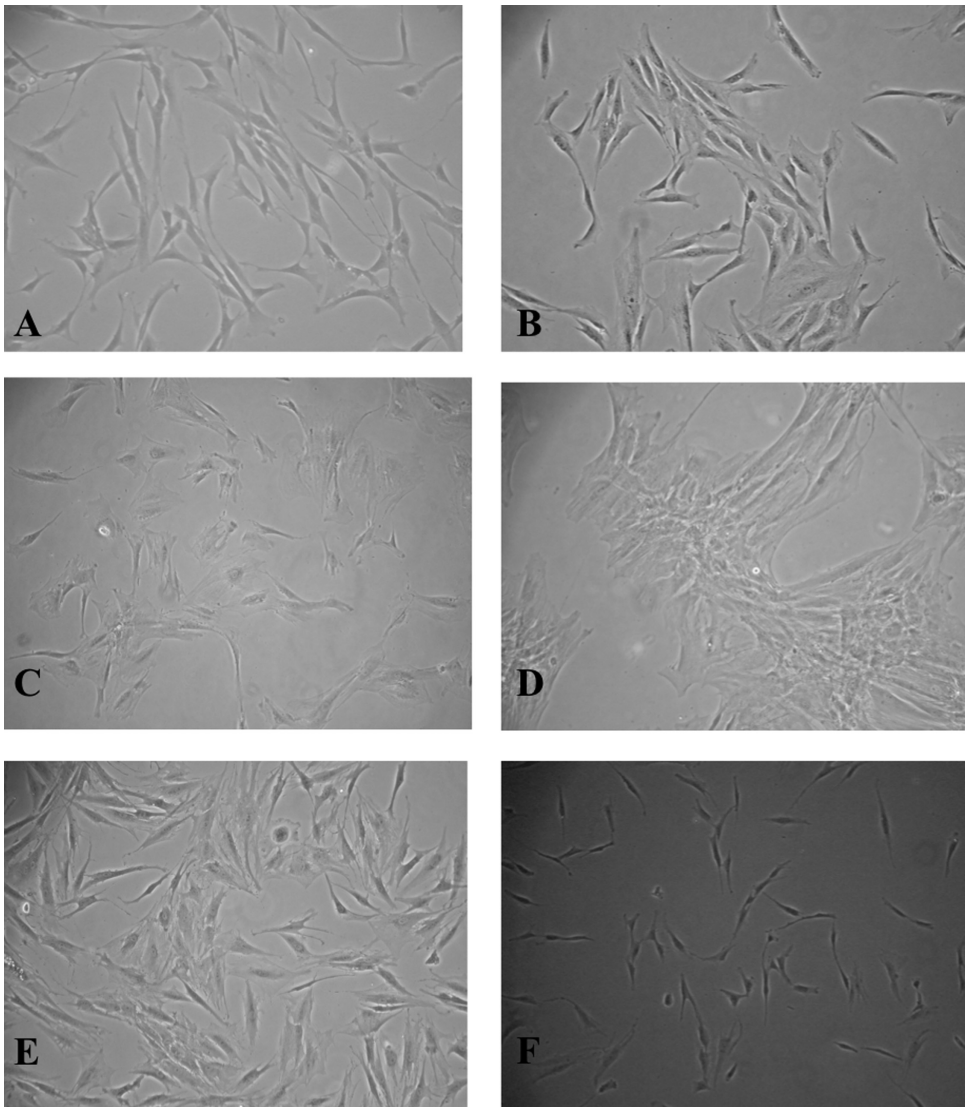

FIGURE 4. Cells from the epithelial cell adhesion molecule (EpCAM)-/fibroblast surface protein (FSP)+ population were collected and grown in culture. These cells were stored as tumor-associated fibroblasts (TAFs) in conjunction with the established cell lines (A) University of Michigan-squamous cell carcinoma (UM-SCC)-105; (B) UM-SCC-106; (C) UM-SCC-108; (D) UM-SCC-109; (E) UM-SCC-110; and (F) UM-SCC-111.

digested tumor tissue using flow cytometry. The site and staging of each case at the time the tissue was obtained is noted (Table 1). Five of the established lines were derived from T4 tumors, and 1 line was established from a T3 tumor.

Flow cytometry

Cells were collected within 72 hours of surgery as epithelial populations (EpCAM+/FSP−) and fibroblast populations (EpCAM-/FSP+). Gates were created in the flow cytometry software from cell suspensions that lacked antibody staining (see Figure 2). Cells stained for markers that fell into these gated regions were collected and grown in culture.

Cell culture

Sorted populations were cultured separately as EpCAM+ and FSP+ populations. The cells cultured as EpCAM+ showed epithelial phenotypes after 24 hours of culture (see Figure 3). The cells cultured as FSP+ showed fibroblast phenotypes after 24 hours of culture (see Figure 4). After 20 passages, the EpCAM+ populations were genotyped as unique new cell lines. Immortalization has been confirmed past 50 passages and at least 100 population doublings for each cell line. The FSP+ populations were frozen at early passages as TAFs paired with each new cell line, as these cells are not expected to be immortalized.

Population resorting

To confirm the validity of this new approach to cell line establishment, 100,000 cells from each line were mixed with an equal number of TAFs derived from the same primary tumor. Every mixed population was easily separated into 2 distinct populations, each composed of roughly 50% of the sample (see Figure 5).

Genotyping

Each pure population of EpCAM+ cells was genotyped as a unique cell line. Each paired population of FSP+

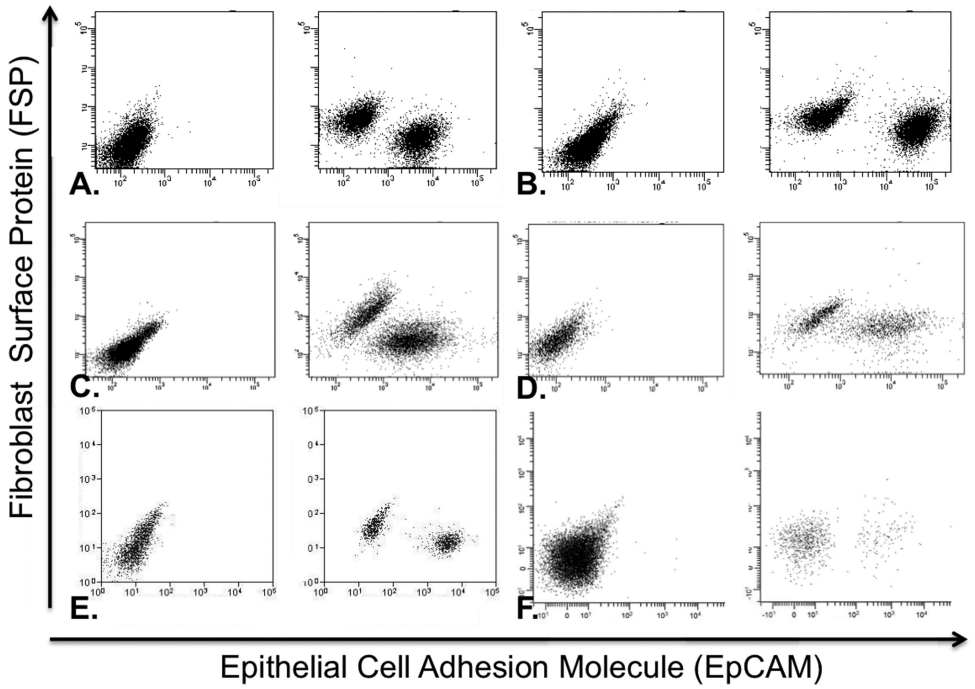

FIGURE 5. Fluorescence activated cell sorter (FACS) plots of each established cell line when mixed with an equal number of tumor-associated fibroblasts (TAFs) and separated. The unstained plot is shown on the left and the stained plot is shown on the right. Cells expressing the epithelial cell adhesion molecule (EpCAM) marker shift to the right along the X-axis, whereas cells expressing the fibroblast surface protein (FSP) marker shift up along the Y-axis. Cell lines with their corresponding TAFs are (A) University of Michigan-squamous cell carcinoma (UM-SCC)-105; (B) UM-SCC-106; (C) UM-SCC-108; (D) UM-SCC-109; (E) UM-SCC-110; and (F) UM-SCC-111.

cells was genotyped to demonstrate the cell line is consistent with the genotype of the donor (Table 2). Interestingly, there is frequent loss of heterozygosity in the tumor cells, for example, loss of the allele 16 in UM-SCC-106 at the D35.1358 locus.

Xenografts

Three cell lines (UM-SCC-105, UM-SCC-106, and UM-SCC-110) were transduced with luciferase and injected into the flanks of NOD/SCID mice to test for tumorigenicity. One hundred thousand cells were injected

TABLE 2. Each epithelial cell adhesion molecule+ cell population was genotyped as a unique cell line.

Cell line	AMEL	D3S1358	vWA	FGA	D8S1179	D21S11	D18S51	D5S818	D13S317	D7S820	Results
UM-SCC-105	X, Y	15, 18	15, 17	18, 21	12, 15	29, 30	12, 16	11	11, 12	9, 11	Unique cell line
UM-SCC-105 fibroblasts	X, Y	15, 18	15, 17	18, 21	12, 15	29, 30	12, 16	11	11, 12	9, 11	Consistent with UM-SCC-105
UM-SCC-106	X	14	17	20, 21	14	30, 31	12	11	10, 11	9, 12	Unique cell line
UM-SCC-106 fibroblasts	X, Y	14, 16	17	20, 21	12, 14	30, 31	12, 14	11, 13	10, 11	9, 12	Consistent with UM-SCC-106
UM-SCC-108	X	17	14, 18	21	12, 13	29, 30.2	15	11	9, 11	8, 12	Unique cell line
UM-SCC-108 fibroblasts	X	17	14, 18	21, 24	12, 13	29, 30.2	15	11	9, 11	8, 12	Consistent with UM-SCC-108
UM-SCC-109	X	16	14, 17	22	8, 13	30, 32.2	12	12, 13	11	8, 11	Unique cell line
UM-SCC-109 fibroblasts	X	14, 16	14, 17	21, 22	8, 13	30, 32.2	12, 15	12, 13	11, 13	8, 11	Consistent with UM-SCC-109
UM-SCC-110	X, Y	15	17, 20	21	8, 9	29	15	9	13	9, 11	Unique cell line
UM-SCC-110 fibroblasts	X, Y	15, 18	17, 19	21, 23	8, 9	29, 31.2	14, 15	9, 11	11, 13	9, 11	Consistent with UM-SCC-110
UM-SCC-111	X	17	17, 18	20, 24	11, 14	31	13, 19	11	8, 14	11	Unique cell line
UM-SCC-111 fibroblasts	X, Y	16, 17	17, 18	20, 24	11, 14	30, 31	13, 19	9, 11	8, 13	11	Consistent with UM-SCC-111

Abbreviation: UM-SCC, University of Michigan-squamous cell carcinoma.
Each fibroblast surface protein (FSP)+ cell population was genotyped consistently with its paired cell line as tumor-associated fibroblasts (TAFs).

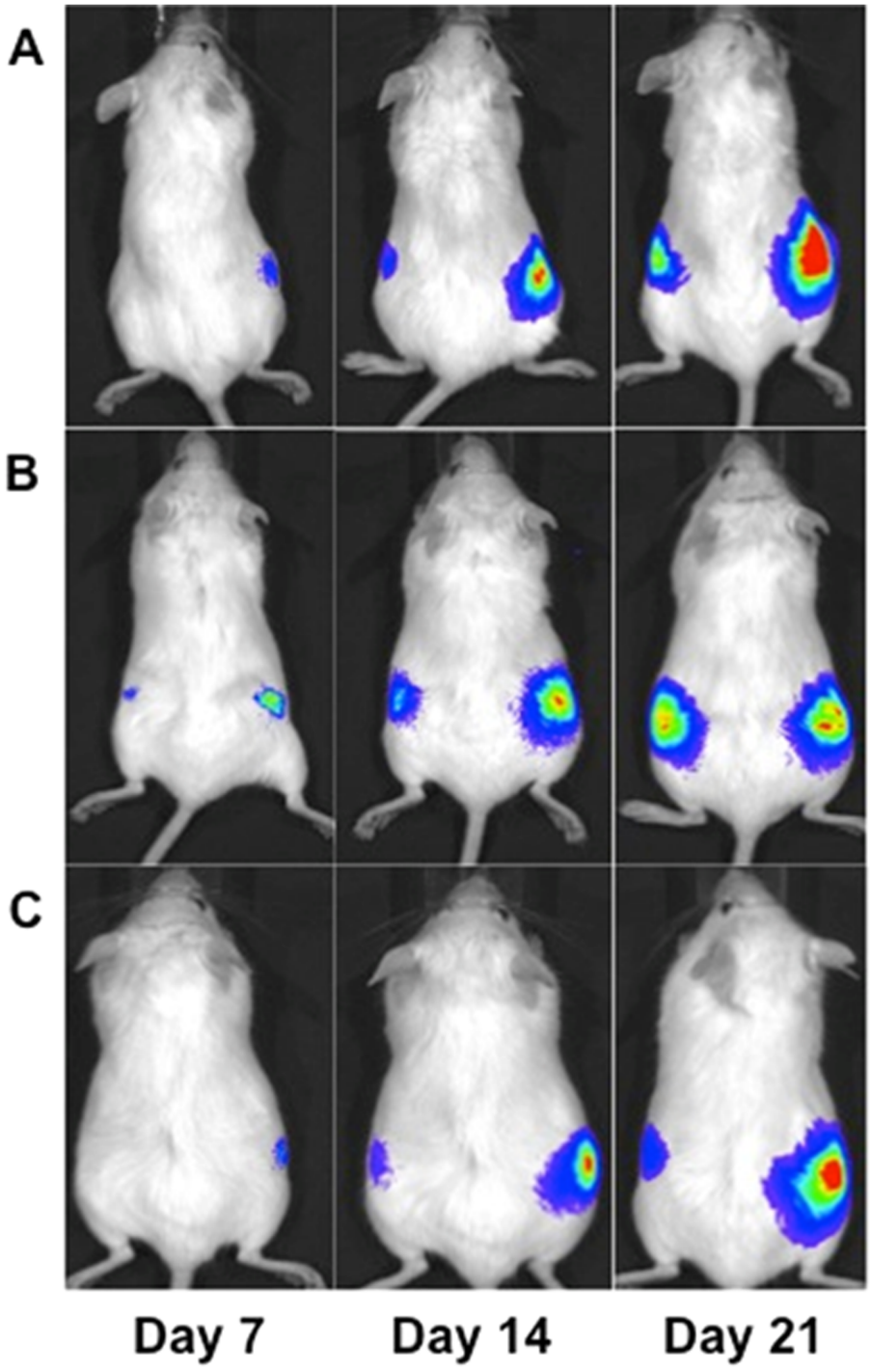

FIGURE 6. Flank xenografts of the newly established cell lines after transduction with luciferase. One hundred thousand transduced cells were injected into the left flank, while 100,000 transduced cells along with 100,000 non-transduced tumor-associated fibroblasts (TAFs) were injected into the right flank of NOD/SCID mice. Tumors initiated earlier when co-injected with fibroblasts and grew larger than tumors that did not have fibroblasts co-injected. Cell lines used are (A) University of Michigan-squamous cell carcinoma (UM-SCC)-105; (B) UM-SCC-106; and (C) UM-SCC-110.

into the left flanks, whereas another 100,000 cells mixed with an equal number of TAFs were injected into the right flank in the same volume (see Figure 6). The fibroblasts were not transduced, so measured bioluminescence was solely from the cancer cells. All injections were able to form flank tumors. The injections with fibroblasts initiated tumor growth earlier and promoted tumor growth when compared to the injections without fibroblasts, as measured by the bioluminescence of the xenografts.

DISCUSSION

Initial attempts to separate the fibroblasts by flow cytometry utilized only the FSP antibody, which did not provide staining that was strong enough to properly separate 2 different cell populations. After addition of the EpCAM antibody, the 2 cell populations were easily separated along 2 axes on a dot plot. The fibroblasts shifted upward along the Y-axis, while the epithelial cells shifted

to the right along the X-axis. Each cell line was unique in its staining pattern. Most cell lines had a strong separation of EpCAM+ cells from the remaining population, but UM-SCC-110 and UM-SCC-111 displayed an EpCAM+ population that only slightly disassociated itself from the other cell types. This suggests that certain carcinomas may express EpCAM at higher levels, and may even express EpCAM as a gradient in a heterogeneous population. The literature suggests that cell lines that express high levels of EpCAM may be more aggressive and capable of metastasis.[30]

Of particular note is the successful establishment of UM-SCC-110. The primary tumor tissue for this cell line was grown in the laboratory for 72 hours before sorting was attempted, and each culture flask seemed to be completely overgrown with fibroblasts. No cells with an epithelial phenotype could be seen under the microscope, but FACS analysis did detect a very small EpCAM+ population that was sorted and cultured. The successful establishment of this cell line would most likely not have been possible using traditional partial trypsinization methods.

Separation of the 2 cell populations was immediate and thorough. Within 24 hours, the sorted cells were successfully growing in culture and displayed either an epithelial phenotype or a fibroblast phenotype. Once enough cells were grown in culture, 100,000 EpCAM+ and 100,000 FSP+ cells from the previous sort were mixed together before being restained and reanalyzed by flow cytometry. In each of the 6 cell lines, the 2 cell types were easily reseparated. The staining patterns differed from the original primary tumor tissue, and we hypothesize that the large population of cells that do not stain for either FSP or EpCAM in the primary tissue are cells that are neither mesenchymal nor epithelial, and constitute other cell types, such as blood cells and lymphocytes. These cells were not collected, but blood draws were conducted for each patient in order to acquire lymphocytes.

There exists much potential from the immediate isolation of a carcinoma population of cells from primary tumor tissue. These cells can be immediately expanded for future applications, rather than having to wait for a pure population devoid of fibroblasts. Our own research involving cells acquired from primary tumors was limited to $<1.0 \times 10^6$ viable tumor cells from each sample, or about the same number of cells in a confluent T-25 culture flask.[36–38] Immediate expansion of sorted cancer cells provides us with the opportunity to produce a much larger number of cells within a matter of weeks. Our future research will rely on this method of cell line establishment in order to grow and harvest large numbers of cancer stem cells within a short period of time for specific applications. This includes the priming of dendritic cells with cancer stem cell lysate in a time frame that allows appropriate treatment for the patient from which the cell line was established, which has been shown to confer antitumor immunity in an animal model.[39,40]

Another benefit from this method of cell line establishment is the acquisition of a pure fibroblast population at the time that the primary tissue is available. As previously mentioned, fibroblasts are not immortal and only go through a limited number of cell divisions before entering senescence. This novel method allows for immediate isolation of a population of TAFs, stromal cells that help contribute to tumor growth.[41,42] Research into the effects of TAFs on the cancer environment has been gaining momentum in the past few years, and a consistent collection of cancer cell lines paired with TAFs from the same source is a useful resource on which to draw.[43] Our initial experiments in a mouse model suggest that the TAFs not only help to initiate tumor growth when grown in conjunction with their patient-specific cancer cell lines, but also help to fuel growth over time. This is an interesting area to explore in the future, as more information about the tumor niche and crosstalk with other cell types is learned.

Genotyping results from these 6 new head and neck cell lines were also made more quickly and more efficiently through this cell line establishment method. Carcinoma cells and fibroblasts from each patient were genotyped as pure populations. Each cell line showed significant locus deviation from the fibroblast population, with the exception of UM-SCC-105, which matched the fibroblast companion on every locus. UM-SCC-105 was the only cell line of the 6 that tested positive for HPV-18. This cell line was established from a never smoker, consistent with fewer chromosome aberrations in HPV-induced tumors than in tumors derived from patients with tobacco smoke carcinogenesis. These results infer that UM-SCC-105 is a viral-driven cancer that does not rely on cell-cycle mutation but rather E6 and E7 protein inhibition of cell-cycle checkpoints.[21] UM-SCC-110 indicated genetic instability leading to DNA polymerase slippage from 19 to 20 at the D3S1358 locus, as well as frequent loss of heterozygosity at 6 of the loci. UM-SCC-111 also indicated genetic instability because of DNA polymerase slippage or absence of mismatch repair from 13 to 14 at the D13S317 and the loss of heterozygosity at 3 of the loci.

CONCLUSIONS

FACS is a useful tool for the continued accumulation of new cancer cell lines, and provides an alternative antibody-based method to the traditional partial trypsinization technique used previously. In a group of 12 primary tumors of the head and neck, 6 were successfully established as cancer cell lines. Identifying and isolating the cancer cells from the accompanying fibroblasts allows for immediate growth of pure cell populations. This method significantly decreases the length of time required for cell line establishment, as well as providing an isolated population of TAFs unique to the established cell line.

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