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Lung Cancer in Mice Induced by the Jaagsiekte Sheep Retrovirus Envelope Protein Is Not Maintained by Rare Cancer Stem Cells, but Tumorigenicity Does Correlate with Wnt Pathway Activation

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Abstract

JSRV, a simple beta-retrovirus, is the etiologic agent of ovine pulmonary adenocarcinoma, a form of non–small cell lung cancer in sheep and goats. It has been shown that the envelope protein alone is sufficient to induce tumorigenesis in the lungs of mice when delivered via an adeno-associated viral vector. Here, we tested the hypothesis that JSRV envelope–induced tumors are maintained by a small population of tumor-initiating cells, termed cancer stem cells. To test this hypothesis, dissociated cancer cells were sorted from envelope-induced tumors in mouse lung based on the putative stem cell markers Sca-1, CD34, and CD133, the pluripotency-associated transcription factor Oct4, and the level of Wnt signaling. No association with increased tumor-initiating capacity was found with any of the cell-surface markers. In addition, we were unable to detect any evidence of Oct4 expression in tumor-bearing mouse lung. However, tumor cells possessing an active Wnt signaling pathway did show a significant correlation with increased tumor formation upon transplantation. Limiting dilution transplant analysis suggests the existence of a large fraction of cells with the ability to propagate tumor growth, with increasing tumor initiation potential correlating with activated Wnt signaling. *Mol Cancer Res; 10(1); 86–95. ©2011 AACR.*

Introduction

Lung cancer is the most common cause of cancer-related deaths worldwide; moreover, it is the second most common form of cancer for both men and women, after prostate and breast cancer, respectively (1). The overall 5-year survival rate is lower than 15% (2). These statistics indicate, in part, a poor understanding of the etiology and nature of the disease. Importantly, at least 15% of lung cancer cases cannot be attributed to smoking (1). Lung cancer is thus by no means a completely preventable disease. A more representative model of lung cancer is needed before significant therapeutic advances can be made.

An evolving paradigm in cancer biology is that tumor growth is dependent on a minor population of cancer stem cells (CSC; ref. 3). CSC have been identified in cancers of the hematopoietic system, breast, pancreas, prostate, and other tissues (4). Such cells are generally defined on the basis of expression of specific cell-surface markers, with CD133 in particular known to be a conserved stem marker in several cancers (5). Identification of CSC predicts the need to target therapy not at the bulk of the tumor, but rather, the small number of CSC responsible for cancer maintenance (6).

Jaagsiekte sheep retrovirus (JSRV) causes lung cancer in sheep (7), and our laboratory has shown that adeno-associated virus (AAV) vectors expressing the JSRV envelope (Jenv) protein alone can induce lung cancer in mice (8). Histologically, tumors in mice are similar to those occurring in natural infections of sheep (9). In mice, transduction of lung epithelial cells initially leads to widespread Jenv expression by 1 week posttransduction, but the bulk of this expression is transient and disappears within weeks (S. K. Wootton and A. D. Miller, unpublished data). Tumors arise only in the lower airway, suggesting that a limited number of cells are susceptible to Jenv-induced transformation and tumorigenesis. We hypothesized that the subset of cells permissive to Jenv-induced transformation were the bronchioalveolar stem cells (BASC) of the distal lung. Initial evidence suggested that BASC expansion occurs early during lung tumorigenesis, supporting this hypothesis (10). Similarly, it has been shown that cutaneous stem cell expansion occurs during chemical promotion of skin carcinogenesis, with the CSC fraction of skin carcinomas sharing many properties (including definitive cell-surface markers) with the normal stem cells; lineage tracing showed these tumors arose preferentially from the cutaneous stem cells (11).

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Although a stem cell origin of cancer does not necessarily imply a CSC subpopulation within developed tumors, we wondered whether markers used to enrich for BASC might also identify the tumor-initiating fraction of the resulting tumor. This question is especially relevant in light of recent studies that were unable to identify any SPC^{pos} CC10^{pos} BASC in the ovine lung, leaving open the possibility that sheep lung progenitors exhibit a different marker phenotype or that the ovine lung epithelial hierarchy is fundamentally different from that of mice (12, 13). Furthermore, several reports have provided evidence that repudiates the definition, if not existence, of BASC (14–16). The existence of CSC has not been investigated in virally induced tumors, an important question given that 20% of human cancer burden worldwide is associated with infectious agents (17). Moreover, development of a mouse model of lung CSC would allow for assessment of therapies targeted toward the CSC population.

We have developed a novel method to transplant lung tumor cells orthotopically and noninvasively via intranasal inhalation. This is in contrast to the more common technique in which putative CSC are assayed for tumor-initiating capacity via subcutaneous injection, an approach that has raised many objections involving immune tolerance and niche incompatibility. Moreover, our use of syngeneic tumors and transplant recipients circumvents concerns over xenograft immunogenicity issues. Our system avoids these issues and allows us to show tumor-initiating capacity in a physiologically relevant environment. Nonetheless, orthotopic transplantation ultimately proved to be a less sensitive method for assaying tumorigenic potential of very small numbers of cells, so subcutaneous transplant experiments were also conducted.

To address the existence of CSC within Jenv-induced tumors, we tested a number of cell-surface markers identified on BASC and CSC of other tissues. In addition, we examined expression of the pluripotency-associated transcription factor Oct4 in these tumors as well as the presence of Wnt signaling. These results, combined with limiting dilution transplant analysis, suggest that Jenv-induced lung cancer in mice is not maintained by a discrete population of CSC, although tumor cells do possess a range of tumor initiation potentials that correlate with active Wnt signaling.

Materials and Methods

Experimental procedures in mice

Animal studies were conducted following approval by the Institutional Animal Care and Use Committee of the Fred Hutchinson Cancer Research Center. Initial transplant experiments testing BASC markers were conducted using 129S6/SvEvTac-*Rag2^{tm1Fwa}* mice. Nonobese diabetic severe-combined immunodeficient (NOD/SCID) mice were used in subsequent transplants, unless as otherwise noted for experiments employing NOD/SCID gamma (NOD.Cg-*Prkdc^{scid} Il2rg^{tm1Wjl}*/SzJ) mice. *B6:129S4-Pou5f1^{tm2(EGFP)Jae}*/J mice were used as tumor donors for experiments analyzing Oct4

expression. All mouse strains were obtained from The Jackson Laboratory. JSRV envelope tumors were induced in mice by intranasal administration of AJJenv or AEJenv (which produce apparently identical tumors) as previously described (18). Briefly, the Jenv cDNA under the control of either the JSRV (AJJenv) or ENTV (AEJenv) *LTR* promoter was cloned into identical AAV2 vector backbones, and virus was produced using the AAV6 capsid, which has a very high transduction efficiency in the lung (19). Mice were sacrificed upon signs of distress, generally 2 to 3 months postinfection.

Lung injury was induced by intranasal administration of 0.5 mU/g body weight bleomycin (Novaplus) in PBS 2 weeks prior to cell transplant, when alveolar cell depletion is most evident (10), or by intraperitoneal injection of 0.2 mg/g body weight naphthalene in corn oil (final concentration 20 mg/mL) 3 days prior to transplant. Male mice were used for all experiments described here because of increased experimental variation observed when using females.

For immunocompetent mouse strains used as tumor cell recipients, a drug-based immunosuppression protocol was developed. 30 μg/g body weight cyclosporine A (PLIVA) and 0.3 μg/g body weight FTY720 (ChemieTek) in PBS were administered daily via intraperitoneal injection starting 3 days prior to Jenv vector administration.

Cell culture

The Rag2/Jenv clone 1 (RJenv c1) lung tumor cell line was obtained by growing disaggregated, Jenv tumor–bearing mouse lung in a 1:1 mixture of conditioned medium from NIH 3T3 cells and keratinocyte serum-free medium (KSFM) plus 10% FBS at 37°C in a 5% CO$_2$ atmosphere. Confluent NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) + 5% FBS for 72 hours to produce conditioned medium. Epithelial colonies were subcloned and tested for Jenv expression by immunohistochemistry as previously described (8). RJenv c1 was the only Jenv-expressing lung epithelial line that we were able to successfully isolate. A549 human lung carcinoma cells were purchased from American Type Culture Collection, grown for a short period, and were stored under liquid nitrogen until use. A549 cells were grown in DMEM plus 10% FBS at 37°C in a 5% CO$_2$ atmosphere.

For "tumor sphere" culture conditions, RJenv c1 and A549 cells were grown in DMEM/F12 (1:1) plus B-27 supplement (Invitrogen), 100 ng/mL EGF, and 50 ng/mL bFGF (PeproTech) in Costar Ultra Low Attachment plates for at least 2 weeks prior to fluorescence-activated cell sorting (FACS) analysis.

FACS analysis and sorting

Single-cell suspensions from whole lung were prepared by mechanical and enzymatic digestion. After animal sacrifice, lungs were perfused with 3 mL of 50 U/mL dispase (Worthington). Dissected lungs were then placed in DMEM + 10% FBS, diced into small chunks, and passed twice through a series of 16 G to 18 G syringe needles. Collagenase (400 U/mL; Worthington) and 50 U/mL DNase I (Invitrogen) were added to the medium, and the

tissue was incubated at 37°C. Lungs were sequentially passed through smaller gauge needles every 30 minutes for a total of 2 hours and a final 27 G needle. The entire suspension was then filtered through a 40-μm cell strainer to eliminate any nondisaggregated tissue.

Tumor cell suspensions, as well as cell line suspensions [obtained by treatment of cell monolayers with PBS (without Ca^{++} or Mg^{++}) plus 1 mmol/L EDTA to retain surface markers that might be lost by trypsinization] were stained with α-Jenv polysera obtained from immunocompetent mice infected intranasally with AJJenv (9) as well as with α-Sca-1–FITC (fluorescein isothiocyanate) and α-CD34-PE (both from BD Pharmingen) or α-CD133–FITC (eBioscience). α-Mouse IgG-Alexa Fluor 647 (Invitrogen) was used as a secondary antibody for Jenv staining. Cells were sorted on BD FACS Vantage or Aria flow sorters.

Tumor cell transplants

Dissociated and sorted tumor cells were transplanted either orthotopically or subcutaneously as noted. For orthotopic transplants, lung-injured mice were lightly anesthetized and cell suspensions were administered intranasally as previously described (8) in four 50 μL doses separated by 20 minutes each. For subcutaneous transplants, sorted cells were resuspended in 100 μL DMEM plus 25% Matrigel (BD) and injected into the flank. Mice for each experiment were all sacrificed simultaneously at 2 to 3 months posttransplantation.

Histology and tumor burden analysis

Lungs and subcutaneous tumors were perfused with PBS and fixed in 2% paraformaldehyde in PBS immediately after sacrifice. Each lobe or subcutaneous tumor was paraffin embedded and sections were laid out on slides, sampling 5 cross-sections (corresponding to each lobe) of all sacrificed mouse lungs. Jenv staining was done as previously described (8). Tumors were counted, and tumor area as a fraction of the total lung area was determined using ImageJ software. Subcutaneous tumors were easily dissected away from the surrounding tissue and were weighed for analysis.

Wnt reporter vectors

BARVS, BARLS, and control fuBARVS and fuBARLS (found unresponsive) vectors were generous gifts from Randy Moon (20). These reporters express luciferase or the fluorescent yellow marker Venus (VS) under the control of multimerized TCF/LEF DNA-binding sites. RJenv c1 cells were transfected with plasmid DNA by the standard $CaCl_2$ method and selected with 2 μg/mL puromycin until all control cells were dead. After selection, cells were plated at low density so that individual colonies could be selected. For RJenv c1/BARVS transplant experiments, clone 2 was selected due to its ability to show the most inducible response to Wnt agonist 6-bromoindirubin-3′-oxime (BIO). Cells were always treated with 100 nmol/L BIO overnight prior to transplant to ensure that all cells capable of Wnt signaling were Venus positive.

Statistical analysis

All statistics were calculated in Microsoft Excel, using the Data Analysis pack as necessary. The χ^2 test was used to determine the significance of tumor formation associated with Venus-positive or Venus-negative cell transplantation. The 1,000- and 100-cell transplants into NOD/SCID recipients were omitted as these were below the threshold of tumor formation. All other P values were calculated using the Student's t test.

Results

Lung injury improves engraftment of orthotopically transplanted lung cancer cells

Utilizing a novel orthotopic transplantation protocol, we tested the ability of Jenv-expressing tumor cells to engraft and form new tumors in naive NOD/SCID mice. Tumor-bearing mice were generated by intranasal administration of a Jenv-expressing AAV vector and lungs were harvested approximately 2 months later, after significant weight loss and labored breathing was apparent. Initially, an unsorted preparation of cells from tumor-bearing whole lung was used as the transplant source. Given the physiology of the lung, especially with regard to lung fluid movement mediated by ciliated cells that is designed to expel foreign material introduced into the lung, as well as the need to generate physical space for cell engraftment, we reasoned that lung injury and promotion of a wound repair environment might increase the efficiency of tumor formation after
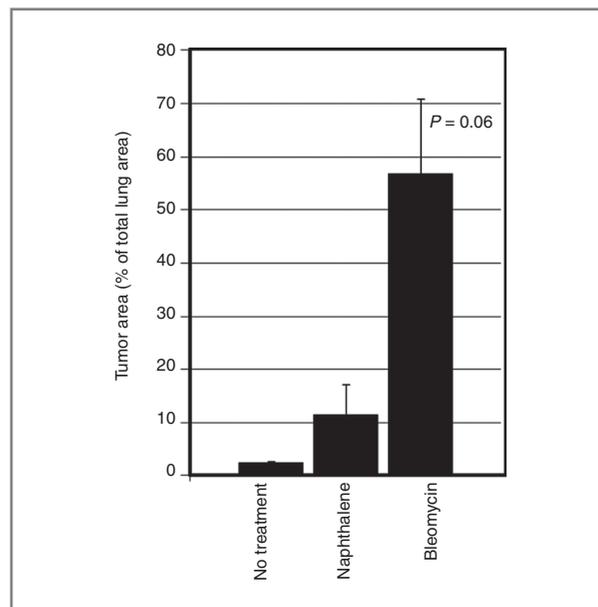
Figure 1. Treatment with lung epithelium-ablating drugs results in greater tumor formation after orthotopic transplantation. Naphthalene or bleomycin was administered to NOD/SCID mice prior to orthotopic tumor cell transplantation (see Materials and methods). Pretreatment with naphthalene or, more significantly, bleomycin resulted in a greater tumor burden after orthotopic transplantation as measured by the fraction of lung cross-sections covered by tumors at approximately 2 months posttransplantation.

administration of Jenv^{pos} cancer cells. Two animals for each group were treated with either naphthalene, which selectively ablates Clara (bronchiolar) cells, or bleomycin, which exhibits a broader toxicity to multiple lung epithelial cell types (15). On the basis of the quantitative analysis of representative lung sections from each recipient mouse, naphthalene treatment slightly increased tumor burden over untreated animals, whereas bleomycin treatment increased tumor burden dramatically (Fig. 1). Bleomycin itself has not been reported to be tumorigenic (21) and did not induce tumors in mice over the typical 3-month period we used to measure tumor induction (data not shown). Unless otherwise indicated, all subsequent orthotopic transplants utilized recipient animals pretreated with bleomycin.

Lung tumor cells expressing Sca-1 and CD34 are not enriched for CSC

Lung epithelial cells that express Sca-1 and CD34 have been reported to be enriched for stem/progenitor cells (10). In particular, Sca-1^{pos} CD34^{pos} cells in normal mouse lung also express SP-C and CC10, markers that define the BASC population (10). We hypothesized that these markers might similarly identify tumor-initiating cells. To test this hypothesis, we sorted Jenv^{pos} tumor cells (Fig. 2A) for expression of Sca-1 and CD34 (Fig. 2B). Unlike the previous study (10), cells were not sorted for lack of the vascular and hematopoietic lineage–specific markers PECAM and CD45, because sorting for Jenv expression excludes cells that display these markers. The number of cells transplanted was determined on the basis of the availability after sorting for each experiment (Supplementary Table S1). Somewhat surprisingly, all transplanted cell populations were able to form tumors in recipient mice regardless of expression of either Sca-1 or CD34 (Table 1). Moreover, all resulting tumors maintained similar mixed adenoma/adenocarcinoma histology (Fig. 2C–F). Although variability in tumor formation from animal to animal was high, cells bearing every combination of marker were always capable of tumor formation with no apparent trends. These results indicate that markers used to enrich for BASC do not define a tumor-initiating compartment in fully developed Jenv-induced tumors.

The proposed marker for human lung adenocarcinoma tumor-initiating cells, CD133, does not define Jenv-induced CSC

We next tested the utility of CD133 to identify CSC in Jenv-induced tumors. Initial transplants were done using a tumor cell line derived from a virally induced tumor-bearing mouse, called RJenv c1. A small percentage (∼1%) of RJenv c1 cells expressed CD133 by FACS analysis (Fig. 3A). The ability of the antibody to detect expression of mouse CD133 was confirmed by costaining of CD34^{pos} mouse bone marrow cells (Supplementary Fig. S1). As with Sca-1 and CD34, CD133-expressing and nonexpressing cells showed a similar ability to form tumors upon transplantation (Fig. 3C and D, Table 1). Regardless of marker expression, cells derived from the RJenv c1 cell line, as opposed to primary tumors, showed an increased ability to form tumors after
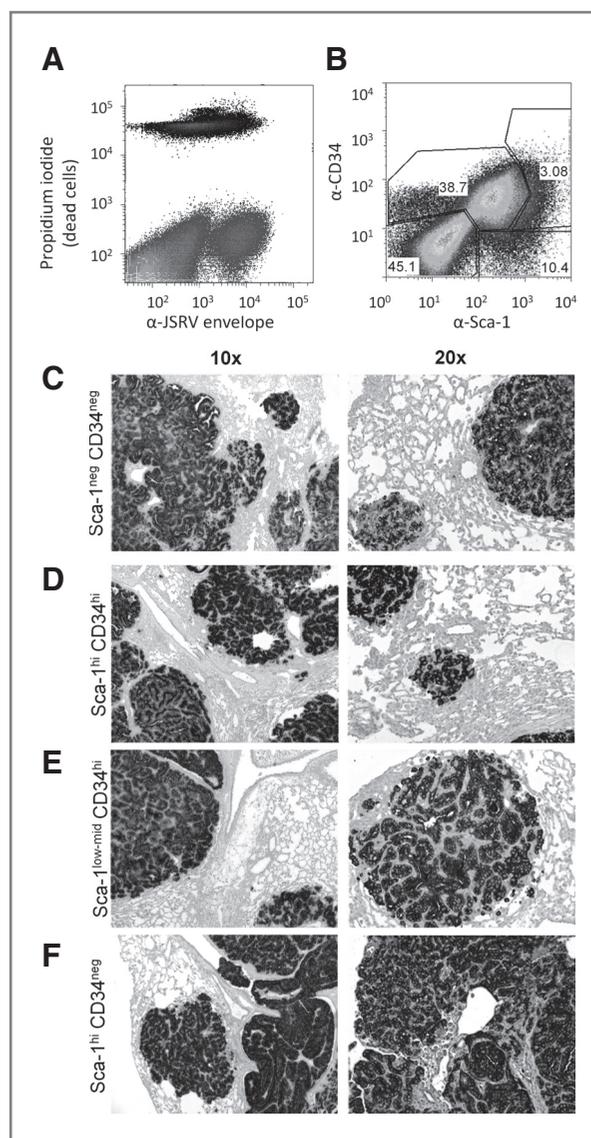
Figure 2. Jenv^{pos} cells are capable of tumor formation upon orthotopic transplantation regardless of BASC marker expression. Single-cell suspensions were obtained from tumor-bearing mice and stained for Sca-1 and CD34 expression. A, representative plot showing the ability to isolate live, JSRV envelope (Jenv) positive cells from a total lung cell preparation. B, representative plot showing CD34 and Sca-1 expression in the Jenv-positive cell fraction. Gray outlines represent gates used to sort these cells prior to transplantation, and the associated number is the percentage of total cells that gate contains. C–F, representative lung cross-sections of recipient animals immunohistochemically stained with Jenv monoclonal antibody after transplantations of Jenv^{pos} cells expressing (C) neither Sca-1 or CD34, (D) high levels of Sca-1 and CD34, (E) intermediate levels of Sca-1 and CD34, or (F) high levels of Sca-1 and little or no CD34 expression. Black staining labels all Jenv^{pos} tumor cells within that cross-section. A mixed adenoma/adenocarcinoma morphology is apparent.

transplant, reflected by the higher mean percentage of tumor-bearing lung area (Table 1). Although not thoroughly investigated, this could be due to genetic and/or epigenetic alterations that occurred during establishment of the cell line

Table 1. Tumor characteristics following orthotopic transplantation of Jenv[pos] lung tumor cells with specific cell-surface marker profiles

Marker profile	Tumor cell source	Number of tumors	Mean tumor area (%)	Number of mice
Sca-1^{hi} CD34^{hi}	AEJenv-infected mouse	18.2 ± 14.2	10 ± 10	5
Sca-1^{hi} CD34^{low}	AEJenv-infected mouse	36.2 ± 5.9	15.2 ± 2.5	3
Sca-1[low-mid] CD34^{hi}	AEJenv-infected mouse	18.4 ± 7.1	8.3 ± 3.5	5
Sca-1^{low} CD34^{low}	AEJenv-infected mouse	35.5 ± 23.4	8.1 ± 7.6	4
CD133^{pos}	RJenv c1 cells	330 ± 230	63 ± 38	3/4[a]
CD133^{neg}	RJenv c1 cells	210 ± 45	57 ± 87	2/7[a]
CD133^{pos}	AJJenv-infected mouse	6.3 ± 2.4	4.7 ± 8.4	6
CD133^{neg}	AJJenv-infected mouse	6.8 ± 2.4	6.4 ± 9.5	6

NOTE: Jenv^{pos} tumor cells, sorted based on cell-surface marker expression, were transplanted into lungs of Rag2 (for Sca-1 and CD34 transplants) or NOD/SCID (for CD133 transplants) mice pretreated with bleomycin. Values for tumor area and tumor quantity are determined per 100,000 cells transplanted to normalize the results. In the case of CD133^{pos} cells from AJJenv-infected mice, the cells were so rare that they were mixed with CD133neg cells to give the same total number of cells transplanted into recipient mice.
[a]The number of evaluable mice over the total tested is shown. Some mice could not be evaluated because of the very high number and convergent growth of individual tumors in these animals.

or the relatively harsh treatment of primary tumor cells during disaggregation.

To confirm that our results using the CD133 marker were not simply an artifact of using a cell line as opposed to primary tumors, we conducted another experiment using primary Jenv tumors as the cell source. Because of the very small number of CD133^{pos} cells present (Fig. 3B), CD133^{pos} cells were added to sorted CD133^{neg} cells to control for cell loss that might result from transplantation of too few cells. Mice receiving CD133^{pos} cells with CD133^{neg} cells did not show an increase in tumor burden compared with mice receiving CD133^{neg} cells alone (Table 1).

These results are in contrast to reports by Eramo and colleagues (4) and Bertolini and colleagues (22) using human tumor samples and cell lines, including the human lung carcinoma cell line A549. To address this difference, a control experiment was done using the human lung cancer line A549 and a human CD133 antibody. CD133 expression could not be detected on A549 cells grown as a monolayer or in sphere culture (Supplementary Fig. S2), despite our ability to stain human bone marrow samples for CD133 as a positive control (Supplementary Fig. S3). These results suggested that reports of CD133 expression and correlation with CSC in human non–small-cell lung carcinoma (NSCLC) cell lines should be reevaluated.

Lack of expression of the pluripotency marker Oct4 in mouse lung

The transcription factor Oct4 plays an important role in maintenance of multipotency and in the generation of induced pluripotent stem cells (iPSC). Several reports have also described expression of Oct4 in mouse lung and lung cancer (23, 24). Accordingly, we tested whether Oct4 expression might discriminate between populations of differentiated and stem-like lung cancer cells. To examine this, an Oct4–GFP reporter mouse (*B6:129S4-Pou5f1[tm2(EGFP)Jae]*/J) was

obtained from the Jackson Laboratory. This mouse contains an internal ribosomal entry site and an eGFP cDNA flanking the 3′ end of the endogenous Oct4 locus. iPSCs generated from these mice show green fluorescent protein (GFP) expression, although the vast majority of adult cells do not (25). Tumors were generated in this mouse by intranasal administration of the Jenv-expressing AAV vector along with daily administration of immunosuppressant drugs (see Materials and methods). Drug-based immunosuppression was necessary because an immunodeficient Oct4 reporter strain was not readily available, and immunocompetent mice are refractory to Jenv-induced tumor formation (8). The mouse was sacrificed after weight loss and labored breathing was apparent.

After digestion of the tumor-bearing lungs, cells were examined for GFP fluorescence by flow cytometry. No GFP-expressing cells could be detected in Jenv^{pos} tumor cells, nor in any other cells in the entire lung digest (Fig. 4). To confirm the identity of the Oct4–GFP reporter mouse strain, mice were genotyped by PCR and were shown to be homozygous for the Oct4–GFP transgene (data not shown). These results strongly argue against any involvement of Oct4 in Jenv-induced lung cancer.

Jenv^{pos} cancer cells showing more active Wnt signaling have a higher tumor-initiation potential

Given our inability to discriminate a CSC population via expression of a cell-surface marker or a transcription factor reporter, we next explored whether activation of a particular signaling pathway might be restricted to CSC. Wnt signaling has been implicated in BASC maintenance (26) and NSCLC metastasis (27), so we reasoned it might serve to help identify tumor-initiating cells.

The presence of an inducible Wnt signaling pathway in lung tumor cells was shown using a luciferase reporter construct containing a β-catenin–responsive promoter
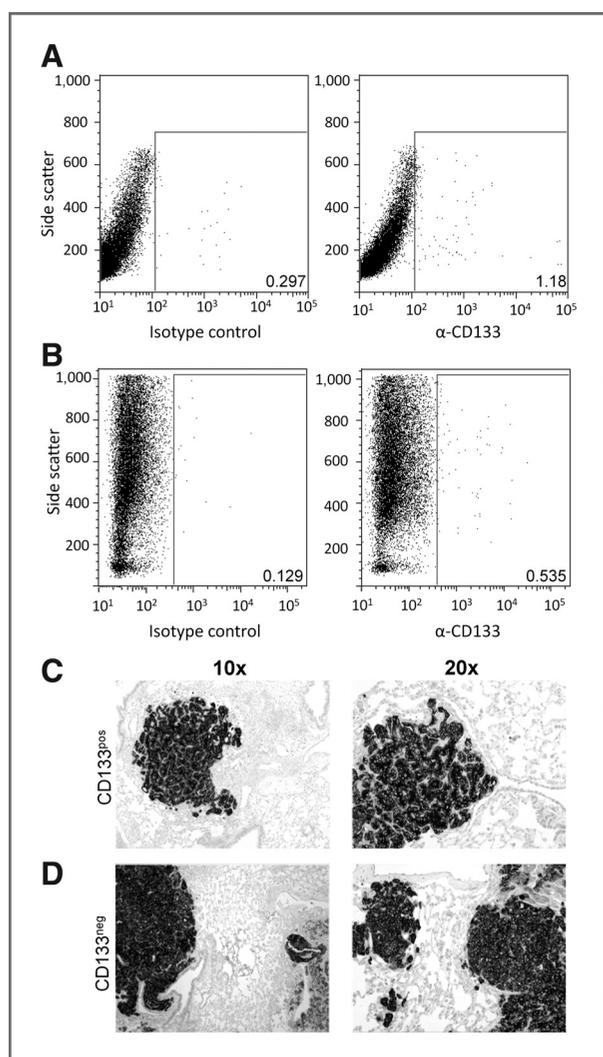
Figure 3. RJenv c1 cells are capable of tumor formation upon orthotopic transplantation regardless of CD133 expression. Single-cell suspensions of primary Jenv tumor cells and RJenv c1 cells were sorted for CD133 expression prior to orthotopic transplantation. A and B, representative plots showing CD133 expression in the Jenv tumor–derived cell line, RJenv c1 (A) and primary Jenv tumors (B). The black outline on the right hand side indicates the cutoff at which a cell was deemed CD133 positive. The percentage of CD133-positive cells is denoted by the number in the lower right hand corner of the plots. C and D, representative lung cross-sections of recipient animals after transplantations of Rjenv c1 possessing (C) or lacking (D) CD133 expression. Tissue sections were stained with a Jenv-specific mAb. A mixed adenoma/adenocarcinoma morphology is apparent.

region, termed *BARLS*. In RJenv c1 lung tumor cells transduced with this reporter, incubation with the Wnt activator BIO resulted in a more than 3-fold increase in luciferase expression (Fig. 5A). Compared with a negative control reporter with a mutated promoter unable to bind β-catenin (fuBARLS), even untreated RJenv c1/BARLS cells showed some baseline level of Wnt signaling, perhaps via autocrine signaling.

Because luciferase cannot be measured on a single cell level, a Venus fluorescent marker–expressing version of the BAR construct was transfected into RJenv c1 to enable sorting for Wnt-activated cells. Analysis of these cells showed a gradient of Venus expression contained within 2 overlapping populations (Fig. 5B). Cells from the periphery of each population were sorted and transplanted into mice subcutaneously (see next section for rationale). In contrast to results with all other tested markers, Venus^{hi} cells did show a substantial increase in tumor-forming ability (Fig. 5C and D). By assigning each transplant either a "1" (developed tumor) or "0" (no tumor), a student's t test was done to compare the probability of a tumor arising in Venus[hi] versus Venus[low] cell transplant recipients. The P value obtained was <0.04, showing the association of Venus expression (and thus Wnt pathway activation) and tumor formation to be highly significant. A χ^2 test was also done, resulting in a highly significant P value, less than 0.02, for the association between Venus[hi] cell transplant recipients and tumor formation. It is important to note, however, that Venus^{low} cells did retain the capacity, albeit reduced, to form tumors upon transplantation. This suggests that a high level of Wnt signaling is favorable for tumor growth but not entirely necessary.

A small number of unsorted cells can consistently reconstitute tumors

The cancer stem cell hypothesis suggests that only a very small number of cells within a tumor are capable of formation of new tumors upon transplantation. To address whether this is the case for Jenv-induced tumors, we carried out a limiting dilution analysis of subcutaneously transplanted RJenv c1 cells. Prior experiments had shown that we could only rarely detect tumor formation after orthotopic transplantation of less than 10,000 cells, so we sought to increase our sensitivity of detection by subcutaneous transplantation.

We determined that 100 RJenv c1 cells could consistently form tumors when subcutaneously transplanted into either NOD/SCID or NOD/SCID *gamma* mice, a more highly immunocompromised strain (Table 2). Furthermore, in 1 of 3 of cases, as few as 10 cells formed detectable tumors. These results suggest that the fraction of cells capable of tumor formation is at least 1/30. In tissue culture, only $23 \pm 9.7\%$ of individual RJenv c1 cells are able to form colonies. Assuming this value represents the maximum number of cells capable of continued proliferation under ideal conditions, the fraction of tumor-initiating cells is at least 1/7. Taking into account a probable reduced seeding efficiency in animals, it is likely that the fraction is significantly higher and is thus at odds with the traditional cancer stem cell hypothesis.

Discussion

Our results indicate that JSRV envelope (Jenv)-induced lung tumors in mice are not maintained by a small or discrete
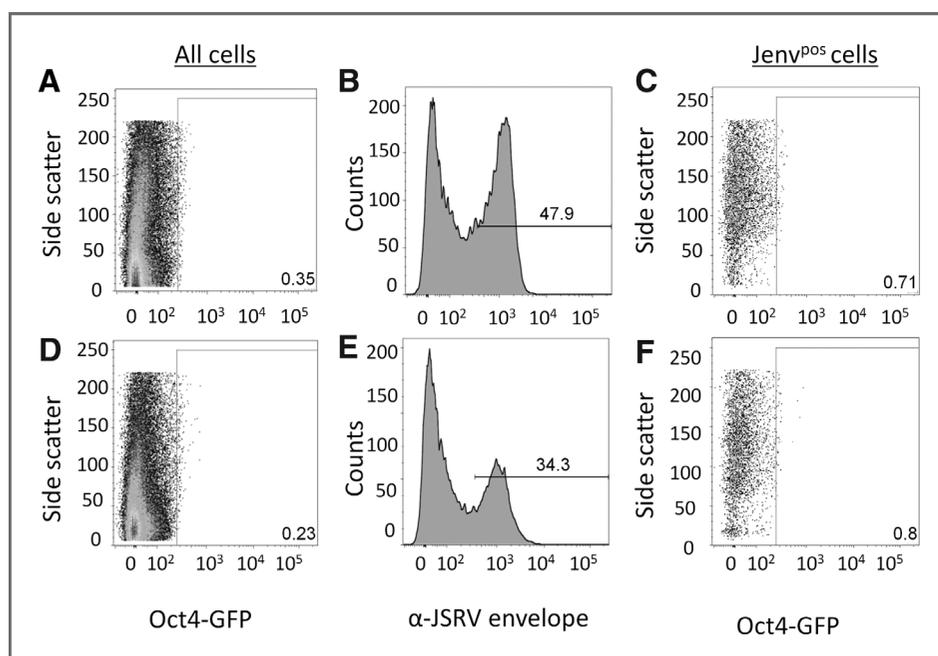
Figure 4. Neither tumor-bearing nor normal mouse lung expresses Oct4. Disaggregated cells from a tumor-bearing Oct4 reporter mouse and a background strain control were examined for GFP expression by FACS. A–C, background strain-negative control animal *B6: 129SF1/J*. D–F, Oct4-GFP reporter strain *B6: 129S4-Pou5f1$^{tm2(EGFP)}$^{Jae}/J* mouse. GFP-positive cells could not be detected in either the total lung digest (A and D) nor the Jenv-expressing fraction (C and F). Percentages of cells within the Oct4 positive gate (denoted by line) are in the bottom right hand corner of each plot. B and E, note the percentage of Jenv-expressing tumor cells in each lung digest.

population of tumor-initiating "cancer stem cells." This is in contrast to reports suggesting CD133 and other markers define a distinct CSC population in human NSCLC. None of the markers described by other groups displayed any ability to discriminate a tumor-initiating cell population in our hands. Furthermore, evidence that as few as 10 and consistently 100 unsorted cells can form tumors upon transplantation calls into question the CSC fraction as a specific drug target. In this case, evaluation of overall efficacy of chemotherapeutic drugs on all tumor cells would be more appropriate. Interestingly, however, this study provides evidence that within the tumor cell population, cells exhibiting more active Wnt signaling are more able to form tumors.

Our initial attempts to define a CSC population utilized a novel orthotopic transplantation method. Given the consistency of the tissue environment into which cells were transplanted, as well as the syngeneic nature of the allograft, any appreciable difference in tumor initiation capacity should have been readily apparent. The hospitality of the recipient lung environment to foreign cell engraftment was further increased by bleomycin-induced lung injury. Nonetheless, significant differences in tumor initiation were never observed. Although variability in the extent of tumor formation was quite high from mouse to mouse and makes it difficult to measure small differences in tumor formation, it is unlikely that such small differences would have biological or clinical relevance.

Sca-1 and CD34 expression on BASC informed our choice to test them as candidate CSC markers. However, although the previous study showed that 100% of lineage^{neg} Sca-1^{pos} CD34^{pos} cells in normal lung also coexpress the BASC markers SPC and CC10 (10), our studies found that Jenv-induced tumors are largely SPC^{pos} but do not contain detectable CC10^{pos} cells (8). This expression pattern is similar to typical NSCLC in mice and humans. Given the high percentage of tumor cells coexpressing Sca-1 and CD34, it is clear that not all of those cells are SPC and CC10 dual positive.

Recent reports suggest that one of the BASC markers, Sca-1, does enrich for tumor-propagating activity in mouse NSCLC, but this result is dependent on the nature of the primary oncogenic lesion (28). Interestingly, Sca-1 expression identified CSC in K-ras^{G12D}/p53^{-} mice, but not in K-ras^{G12D} alone or EGFR$^{T790M-L858R}$-activated oncogene mouse models. It is therefore conceivable that Jenv-induced cancer is analogous to tumors formed in the latter animal models. However, p53 does not seem to be a major barrier to tumor initiation by Jenv, in that p53 knockout mice are no more or less susceptible to Jenv-induced tumorigenesis than background strain controls (unpublished data). How Jenv-induced tumors might compare with the various genetic models of mouse lung cancer, and how this might affect CSC marker expression, remains to be determined. Our results agree with recent reports refuting an important role for BASC in JSRV-induced lung tumorigenesis in sheep (12, 13)

It is of particular relevance that not only did CD133 lack significance as a CSC marker in our model, we also failed to detect its expression in the human lung cancer cell line A549, even though we validated the utility of our anti-human CD133 antibody to detect CD133 on human hematopoietic progenitors. Moreover, even in tumor sphere culture conditions, which reportedly select for CSC growth and CD133 positivity, we were never able to show CD133 expression. Many experiments showing CD133 as a lung CSC marker used A549 and similar human NSCLC cell lines as their tumor source. Our data suggest these claims should be reevaluated.

Several groups have shown Oct4 expression both in normal stem/progenitor cells of the lung (24) and in lung
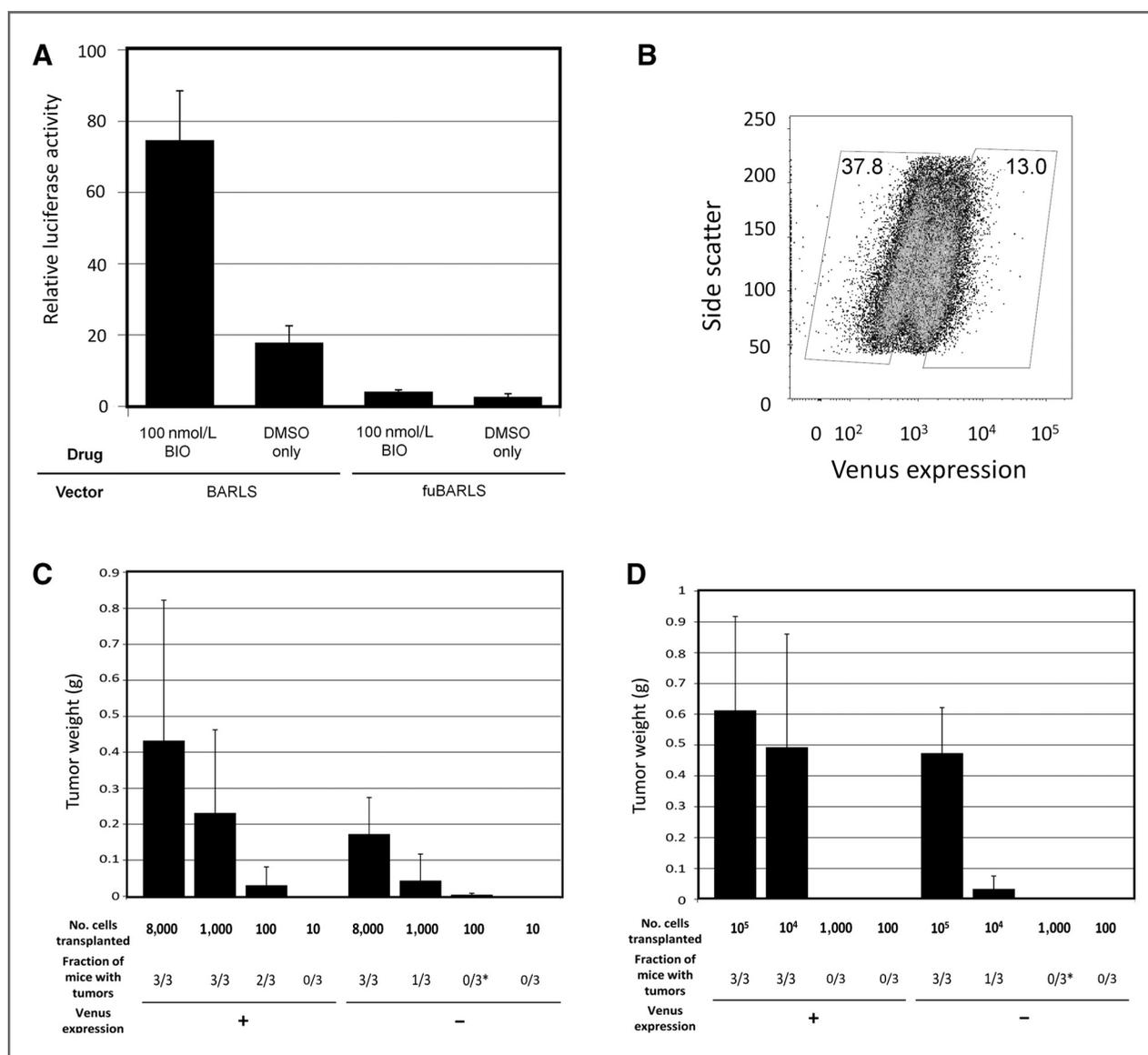
Figure 5. Wnt pathway–responsive reporter expression correlates with an increased ability to form tumors upon subcutaneous transplantation. A, RJenv c1 cells containing the BARLS Wnt reporter vector were treated with the Wnt agonist BIO overnight and assayed for luciferase expression. Treatment with dimethyl sulfoxide (DMSO) alone still resulted in some luciferase expression in comparison with the nonresponsive control promoter in fuBARLS, suggesting some degree of autocrine Wnt signaling in these cells. B, cells were harvested by PBS/EDTA treatment and analyzed for Venus expression by FACS. Cells show a relatively wide range of expression. Gates on the periphery of each population were used to sort Venus[hi] and Venus^{low} cells for transplant. Numbers represent percentage of total cells in gated populations. C and D, tumor formation via subcutaneous transplant of BARVS-sorted RJenv c1 cells in NOD/SCID gamma (C) and NOD/SCID (D) mice. Tumors formed from Venus^{hi} tumor cells were, on average, larger than those formed from Venus^{low} cells. There was also an increased likelihood of tumor formation from smaller numbers of transplanted Venus^{hi} cells versus Venus^{low} cells. Each bar represents the average weight of tumors obtained. The bottom row of the associated table displays what fraction of mice receiving that group of transplanted cells showed tumor formation. *, An extremely small nodule was present, but it did not stain positive for Jenv.

cancer (23). However, Oct4 expression in adult mouse lung has been disputed (29). The aforementioned report relied on examining Oct4 expression in the lung using antibodies to eGFP, which was driven by the endogenous Oct4 locus. We hypothesized that Oct4 expression in the lung could have been overlooked if it were restricted only to BASC. However, our data indicates that this report was correct in showing a complete lack of

Oct4 expression in the mouse lung. BASC are purported to compose approximately 0.1% of a total lung preparation (10). Given that we analyzed nearly 10^6 cells by flow cytometry, the likelihood of missing Oct4–GFP expressing cells is extremely low, calling into question earlier reports of Oct4 expression in the mouse lung. It should be noted that those reports utilized antibodies to Oct4 rather than a transgenic reporter, and given the abundance of

Table 2. Tumor formation after subcutaneous transplantation of RJenv c1 cells

Mouse strain	Cell number	Mice with tumors	Mean tumor weight (g)
NSG[a]	10,000	3/3	ND
NSG	1,000	3/3	ND
NSG	100	3/3	0.64 ± 0.44
NSG	10	1/3	0.0016 ± 0.0029
NOD/SCID	10,000	3/3	ND
NOD/SCID	1,000	3/3	ND
NOD/SCID	100	3/3	0.42 ± 0.38
NOD/SCID	10	1/3	0.0016 ± 0.0027

Abbreviation: ND, not done.
[a]NOD/SCID *gamma* (NSG) mice.

protein-coding Oct4 pseudogenes (30), false-positives seem likely.

There is accumulating evidence that Wnt signaling plays a critical role in lung cancer. Stabilization of β-catenin in a transgenic mouse model results in greatly expanded numbers of BASC (31). Perhaps most striking is the finding that NSCLC metastasis in primary tumors is reliant upon a distinct WNT/TCF signaling program acting through LEF1 and HOXB9 (27). Our data support this finding, in that cells showing active Wnt signaling were more likely to form tumors upon transplantation. Subcutaneous transplantation was employed in this case because we found that we could assay for tumor initiation from a smaller number of transplanted cells, thus increasing the sensitivity of the assay. Wnt-active tumors were, on average, larger than those formed by cells with less active Wnt signaling. The presence of this signaling pathway was not, however, restricted to a well-defined subset of cells. Flow analysis suggests that there exists a continuum of signaling within the total tumor cell population. In future experiments, it will be important to determine whether Wnt signaling is, in fact, restricted to a static fraction of cells or whether cells can turn on and off Wnt signaling in a temporal fashion, for example, as a function of the cell cycle.

A strong argument against a low-level CSC population in our model is the ability of 10 to 100 cells to form tumors when transplanted subcutaneously. This is in contrast to recent claims that, in K-Ras and epidermal growth factor receptor (EGFR) models, 1 in 10,000 cells are tumor initiating (28). Although it is possible that Jenv tumors are fundamentally different than those driven by other oncogenes, it seems likely that tumor-initiating cell frequency is vastly underestimated in the aforementioned models. One major problem with the previous estimate is the inability to determine what fraction of digested lung cells are truly tumor cells versus surrounding tissue and infiltrating cells. Our model avoids this issue because we can easily sort tumor cells based on cell-surface expression

of the Jenv oncoprotein. Moreover, in our experience, enzymatic and mechanical dissociation significantly reduces tumor initiation capacity of individual cells, even though these cells seem viable by propidium iodide and trypan blue staining. Finally, the lung is an organ designed to rid itself of exogenous material by mucus production and fluid flow induced by ciliated cells, which likely eliminates many transplanted cells before they can engraft.

The apparent abundance of CSC within Jenv-induced tumors is not without precedence. In melanomas, it was found that up to 41% of total tumor cells were tumor initiating (32). Admittedly, our experiment to determine the CSC fraction was only done using the RJenv c1 cell line, which could have been selected for greater tumor initiation as a result of adaptation to tissue culture. Nonetheless, most other studies of CSC use cell lines as the tumor source and still find only a small CSC fraction.

Overall our results show that JSRV Env-induced lung cancer in mice is not driven by a minor CSC population. We cannot claim that NSCLC in general does not involve CSC, but our mouse model does recapitulate the features of lung adenocarcinoma seen in humans and in JSRV-infected sheep. In addition, in spite of claims of other groups to the contrary, we were unable to find evidence of CD133 expression in human NSCLC cells lines, nor expression of Oct4 in the mouse lung. Together, these results suggest those findings should be reevaluated and independently replicated to assess their validity. Despite our interpretation that Jenv-induced lung cancer is not CSC driven, our data do indicate that tumor cells exhibiting active Wnt signaling are more capable of driving tumor growth. Although we believe it inappropriate to call these cells CSC, because of their abundance and lack of clear distinction from the total cancer cell population, our data agrees with other reports that Wnt signaling is of fundamental importance in lung cancer. Further studies should be aimed at elucidating the exact role and requirement of the Wnt pathway in the growth and metastasis of NSCLC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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