

Characterization of Melanoma Cells Capable of Propagating Tumors from a Single Cell

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Abstract

Questions persist about the nature and number of cells with tumor-propagating capability in different types of cancer, including melanoma. In part, this is because identification and characterization of purified tumorigenic subsets of cancer cells has not been achieved to date. Here, we report tumor formation after injection of single purified melanoma cells derived from three novel mouse models. Tumor formation occurred after every injection of individual CD34⁺p75⁻ melanoma cells, with intermediate rates using CD34⁻p75⁻ cells, and rarely with CD34⁻p75⁺ cells. These findings suggest that tumorigenic melanoma cells may be more common than previously thought and establish that multiple distinct populations of melanoma-propagating cells (MPC) can exist within a single tumor. Interestingly, individual CD34⁺p75⁻ MPCs could regenerate cellular heterogeneity after tumor formation in mice or multiple passages *in vitro*, whereas CD34⁺p75⁻ MPCs underwent self-renewal only, showing that reestablishment of tumor heterogeneity is not always a characteristic of individual cells capable of forming tumors. Functionally, single purified MPCs were more resistant to chemotherapy than non-MPCs. We anticipate that purification of these MPCs may allow a more comprehensive evaluation of the molecular features that define tumor-forming capability and chemotherapeutic resistance in melanoma.

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Introduction

Cancers are frequently composed of heterogeneous cell populations that can be defined by differential expression of cell surface markers and the ability to propagate tumors in mouse models. It has been hypothesized that only rare cell populations within a heterogeneous tumor are capable of tumor propagation (1). These tumorigenic cell subsets, known as cancer stem cells (CSC), are thought to express cell surface markers traditionally associated with tissue stem cells and are responsible for generating and maintaining tumor heterogeneity (1, 2). This “CSC hypothesis” has been supported by numerous studies showing prospective purification of human cancer cell subsets that have the ability to give rise to tumorigenic and nontumorigenic progeny. Cells capable of reforming heterogeneous tumors were rare in these studies,

comprising only 0.0001% to 1% of cells. This has been shown with pools of sorted CSCs (10^2 to 10^7 cells) in several cases (3–13), but in the absence of purified single-cell injections, it is not clear which individual cells are capable of tumor formation or generating phenotypically diverse offspring.

In contrast, some studies have shown that tumorigenic cells may not be uncommon. Tumors have been formed in mouse models by injection of 20 human prostate cancer cells (14) or individual murine lymphoma or leukemia cells (15). It is not clear why the measured frequency of tumorigenic cells varies widely, but differences have been attributed to specific characteristics of the malignancy/model (16), the relative inability of human tumor cells to grow in a foreign host, and possible residual immune surveillance (17). In addition, injection conditions have a large effect on measured tumorigenic frequency, as coinjection with the Matrigel preparation of mouse EHS sarcoma extracellular matrix decreases the number of human cells required to form a tumor by 100- to 10,000-fold (14, 17). Recently, an average of 27% of uncultured individual human melanoma cells were shown to be capable of tumor formation using a model based on coinjection with Matrigel into immunodeficient nonobese diabetic/severe combined immunodeficient *Il2r γ ^{-/-}* mice (17). This study established that single-cell human xenografts are possible and supports the notion that tumorigenic cells are more common than previously believed, although prospective purification of tumorigenic cells was not achieved. The high efficiency of tumorigenesis in this study coupled with the difficulty in prospective enrichment of tumor-forming cells raised the possibility that differences between individual cancer cells may be minor. However, the idea that a tumor is composed

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of cancer cells with similar phenotypes and tumorigenic potential is at odds with other data showing purification of cancer cell populations with enhanced tumorigenicity (3–13).

Efforts to precisely characterize the biological properties of tumorigenic cell subsets would be greatly accelerated if these cell fractions could be definitively distinguished from non-tumor-forming cells (2). To this end, we isolated cellular subsets based on tissue stem cell surface markers from short-term cultures and uncultured melanomas derived from three novel mouse melanoma models. Examination of the expression of a variety of stem cell markers in these mouse melanomas revealed two potentially useful markers: CD34, which is present on follicular melanocytic stem/progenitor cells (18), and p75/Ngfr, a neural crest stem cell marker (19). The phenotypic properties of individual sorted melanoma cells were evaluated in culture and in single-cell engraftment studies in immunodeficient nude mice (Supplementary Fig. S1). Examination of these functional differences in cell subsets after fluorescent-activated cell sorting (FACS) using the cell surface markers CD34 and p75 revealed two distinct tumorigenic subsets within all individual tumors, which we denote here as melanoma-propagating cells (MPC). Single CD34⁺p75⁻ MPCs formed tumors on every occasion following single-cell injection, whereas individual CD34⁺p75⁻ MPCs formed tumors 57% of the time. Interestingly, CD34⁺p75⁺ cells only rarely formed tumors, showing that this subset predominantly contains non-MPCs. The uniformly tumorigenic CD34⁺p75⁻ MPCs failed to reestablish heterogeneity after single-cell engraftment or following serial passaging in culture. In contrast, individual CD34⁻p75⁻ MPCs frequently self-renewed and reestablished tumor heterogeneity, indicating that this subset has many of the proposed characteristics of CSCs (1). Finally, we found that MPC subsets were more resistant to high-dose chemotherapies than non-MPC subsets. These results support data showing that tumorigenic melanoma cells are not rare (17) and, for the first time, show multiple distinct tumorigenic subsets of cancer cells based on single-cell injections. Further characterization of these purified subsets may enable greater resolution of the molecular pathways responsible for the progression and therapeutic resistance in MPC subsets.

Materials and Methods

Conditional mouse melanomas. *Tyr::CreER^{T2}*, *Cdkn2a^{lox}*, *β-catenin^{lox-ex3}*, *Braf^{CA}*, and *Pten^{lox}* mice were previously described (21–24). Median tumor-free survival (T_m) was calculated by the Mantel-Cox log-rank test. Tumor-free survival in the *Braf^{CA}* and *Pten^{lox/lox}* cohort has been previously reported (23).

Immunostaining for FACS. Short-term tumor cultures were maintained in DMEM/F12 (1:1) with 5% fetal bovine serum and 1% penicillin/streptomycin (complete medium) at 37°C and 5% CO₂. Approximately 5×10^5 cells were stained in a total volume of 100 μL blocking buffer with 1.0 μg anti-CD44, 0.5 μg anti-CD34 (eBioscience), 1:1,000 anti-p75/Ngfr, or 1.0 μg anti-CD133/Prom1 (Chemicon). Alexa Fluor 488/647/750 (Molecular Probes, Invitrogen) secondary antibodies were all used at 1:1,000 dilutions. Propidium iodide (1 μg/mL)

and forward scatter/side scatter signals were used for dead cell and doublet discrimination. Only gates for live single cells were used for analyses/sorts. Each sample was treated independently and gates/quadrants were fixed where secondary antibody-only negative controls gave ≤0.01% positivity. Samples were analyzed with the BD LSRII or FACSria machines with recording of ≥10K events per sample.

Single-cell colony formation and nude mouse engraftments. Single-cell sorting was performed with the BD FACSria sorting system. In brief, 96-well tissue culture-treated plates (BD Falcon) were used for 1 cell per well sorting into 200 μL complete medium per well for colony formation assays. For engraftments, live phase-bright cells were individually picked by 10 μL pipette tip in a 3 μL volume and deposited in a mix of 1 volume Matrigel matrix (BD Biosciences) and 2 volumes of complete medium for a final s.c. injection volume of 150 μL. Tumor volumes were measured by electronic caliper and estimated by the following equation: $a \times b \times c \times 0.5233$.

Fresh tumor dissociation. Induced melanomas were dissociated by finely mincing with sterile scalpel in complete medium followed by collagenase digestion for 3 h and 0.025% trypsin/0.2 mmol/L EDTA for 30 min, triturated, and filtered to yield a single-cell suspension before FACS.

Chemotherapy treatments. Temozolomide (LKT Laboratories) and cisplatin (Sigma-Aldrich) were used at IC₉₀ concentrations of 500 μmol/L for 24 h and 0.5 μg/mL for 4 h, respectively, before wash and complete medium replacement.

Results

Induction and validation of melanoma in novel mouse models. We generated three novel mouse models of melanoma by conditional melanocyte-specific recombination of genes that are thought to play important roles in human melanoma formation and progression (20). These models were based on inactivation of tumor suppressors *Cdkn2a* and *Pten* (21), *Cdkn2a* and *Pten* inactivation with *β-catenin* stabilization (22), and *Braf* activation combined with *Pten* loss (23). All three models used the *Tyr::CreER^{T2}* allele in which loxP-containing sites are recombined in mouse melanocytes following induction of activity by topical or systemic application of 4-hydroxytamoxifen (4-OHT; ref. 24). Melanoma formation was completely penetrant over a period of 9 months in mice following inactivation of *Cdkn2a* and *Pten* in melanocytes. Median tumor-free survival was not statistically different between mice with induction of stabilized *β-catenin* compared with those with wild-type *β-catenin* in the setting of *Cdkn2a* and *Pten* inactivation (Fig. 1A). These findings are in keeping with the recent discovery that *β-catenin* stabilization caused downregulation of *Cdkn2a* in a mouse melanoma model (25). Melanomas had a spindle and epithelioid cell morphology and produced melanin pigment (Fig. 1B). Mice with either *Cdkn2a* or *Pten* inactivation, or *β-catenin* stabilization-only failed to form tumors (Fig. 1A). Representative tumors 1118 and 1111 (*Cdkn2a* and *Pten* inactivated) and 1445 (*Cdkn2a* and *Pten* inactivated with *β-catenin* stabilization) were dissociated and

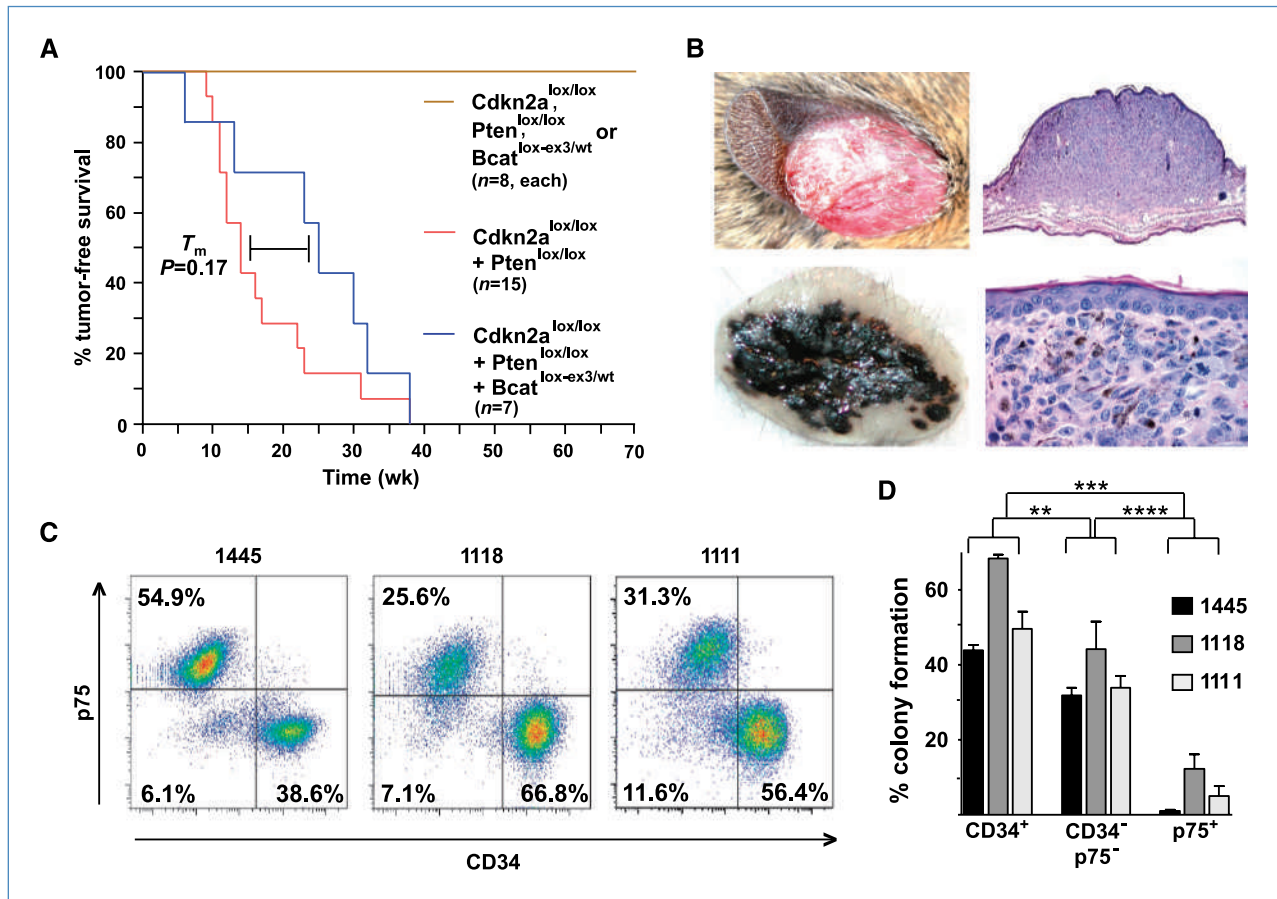


Figure 1. Mouse melanomas contain putative MPCs based on single-cell FACS. **A**, Kaplan-Meier survival graph of 4-OHT–treated mouse cohorts. Median tumor-free survival times (T_m) between the two tumor-bearing cohorts were not significantly different ($P = 0.17$). **B**, *top*, representative pictures of a mouse melanoma (1118) arising on the ear and low-power view of histologic section from ear melanoma; *bottom*, mouse melanoma (3297) from the flank with mixed pigmentation. H&E staining reveals melanin pigment and spindled and epithelioid cells with atypical mitoses. **C**, dual-color flow cytometry with CD34 and p75 showed three major subsets in each cultured melanoma. **D**, CD34⁺ and CD34⁻p75⁻ sorted fractions in all three melanomas exhibited high and moderate colony formation frequencies, respectively, whereas p75⁺ cells only rarely formed colonies. **, $P < 0.001$, ANOVA, for CD34⁺ versus CD34⁻p75⁻ groups; ***, $P < 0.0001$, ANOVA, for CD34⁺ versus p75⁺ groups; ****, $P < 0.0001$, ANOVA, for CD34⁺p75⁻ versus p75⁺ groups.

recombination was validated by PCR on tumor tissue DNA (Supplementary Fig. S2A). Single-cell suspensions were also prepared from these tumors and cultured briefly (three or fewer passages). Cultured cells stained positive for the melanocytic/melanoma markers Tyrp1 and S100 (26, 27), and melanosomes were focally present on electron microscopy of tumor sections, confirming melanocytic differentiation (Supplementary Fig. S2B and C).

Induced melanomas contain distinct cellular subsets.

We sought to prospectively purify tumorigenic cellular subsets that expressed known tissue stem cell or melanoma surface antigens in each cultured melanoma, including CD44, c-Kit/CD117, CD20, Epcam, CD202/Tie2, SSEA-1, Prom1/CD133, CD34, and p75/Ngfr. Flow cytometry revealed that CD44 was universally present in tumor cells and that CD117, CD20, Epcam, CD202, and SSEA-1 were not expressed at appreciable levels (Supplementary Fig. S3A), making these poor candidates for identifying distinct cellular subsets among these tumors. Prom1/CD133-positive populations,

averaging 0.5%, 0.03%, and 0.2% of cells, were detected in melanoma cultures 1445, 1118, and 1111, respectively (Supplementary Fig. S3B) and are consistent with low Prom1⁺ frequencies seen in other studies (4, 6). We sorted single Prom1⁺ and Prom1⁻ cells into 96-well plates using FACS and examined colony formation (defined as $\geq 25\%$ well coverage) for up to 2 months. Greater than 98% average purity in the sorted subsets was confirmed using flow cytometry (Supplementary Fig. S3C). In culture 1445, we observed that colonies formed more frequently from Prom1⁺ single cells than from Prom1⁻ cells ($P = 0.039$); however, in culture 1118, more colonies were formed in the Prom1⁻ than the Prom1⁺ subset ($P = 0.001$) and were not statistically different in culture 1111 ($P = 0.25$; Supplementary Fig. S3C). These conflicting results suggested that Prom1 expression did not reliably distinguish MPCs in these models.

Dual-color flow cytometry using antibodies against CD34 and p75/Ngfr revealed three subpopulations in each culture: CD34⁺p75⁻ (designated CD34⁺), CD34⁻p75⁻, and CD34⁻p75⁺

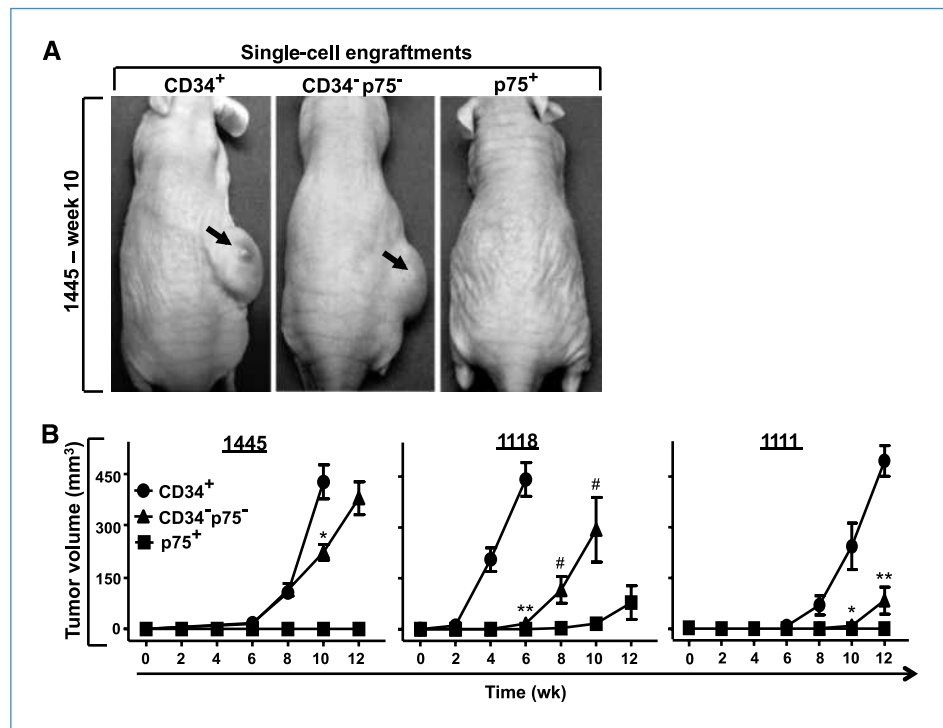
(designated p75⁺; Fig. 1C). Post-sort flow cytometry validation showed >99.0% average purity in these sorted cell fractions (Supplementary Fig. S3D). Rare CD34⁺p75⁺ signals from each culture could not be confirmed cytometrically or microscopically after sorts and therefore were not investigated further (data not shown). Colony formation assays with single-cell FACS showed two putative MPC subsets, CD34⁺ and CD34⁺p75⁻ cells, characterized by high and intermediate rates of colony formation, respectively. In contrast, low colony frequencies were observed with p75⁺ single cells (Fig. 1D). The relative lack of colony formation in p75⁺ cells was not secondary to apoptosis, as purified fractions showed no substantial increase in Annexin V positivity or propidium iodide uptake after FACS when compared with the other subsets (Supplementary Fig. S3E). Single-cell FACS without p75 antibody (i.e., CD34⁺ versus CD34⁻ sorts) showed significantly fewer colonies formed from CD34⁻ fractions, indicating that anti-p75 antibody was not responsible for colony propagation failure (Supplementary Fig. S3E). Cell cycle analysis showed that CD34⁺, CD34⁻p75⁻, and p75⁺ cell subsets were represented at similar proportions in G₁, S, and G₂-M phases of the cell cycle, suggesting that marker expression was stable with respect to phase of cell cycle (Supplementary Fig. S3F). Immunofluorescent analysis of cultured tumors confirmed mutually exclusive expression of CD34 and p75 (Supplementary Fig. S4A). Original tumor sections showed similar subset fractions, showing that short-term culture had not markedly changed cell surface marker expression (Supplementary Fig. S4B).

Two MPC populations identified by single-cell tumor formation assays. The tumorigenicity of the putative MPC

subsets (CD34⁺ and CD34⁻p75⁻) and non-MPC subset (p75⁺) was evaluated following single-cell s.c. injections into nude mice. Single CD34⁺ cells formed tumors at 15 of 15 sites. CD34⁻p75⁻ single-cell injections resulted in tumors at 10 of 15 sites and had slightly slower tumor growth, indicating intermediate rates of tumor propagation. Single-cell injections of p75⁺ cells failed to form tumors at any of 12 engraftment sites from cultures 1445 or 1111 (Fig. 2A and B; Supplementary Table S1A). Increasing the number of p75⁺ cells per injection to either 100 or 1,000 cells also did not result in tumor formation in these cultures (data not shown). Interestingly, tumors formed with two of six p75⁺ cells from melanoma 1118 but grew significantly slower relative to CD34⁺ or CD34⁻p75⁻ tumors, indicating a lower proportion of MPCs in this particular p75⁺ melanoma subset (Fig. 2B). Taken together, these findings show that two major MPC populations exist within these mouse melanomas as shown by their ability to form tumors following single-cell injection. The proportion of total MPCs within cultured tumors was high (45–80% of cells) and overlaps with the range reported for single-cell injections of unpurified human melanoma cells (17).

To determine if short-term culturing altered tumor-forming frequencies, we performed injections of purified single cells derived from uncultured melanomas produced by conditional activation of Braf and loss of Pten (Braf/Pten; refs. 23, 28). Braf/Pten melanomas also contained representative populations of CD34⁺, CD34⁻p75⁻, and p75⁺ cells in primary tumors. Colony formation assays with single-cell FACS following short-term culture of melanoma 2697 showed high rates of colony formation with CD34⁺ cells, intermediate rates with CD34⁻p75⁻ cells, and very low rates with p75⁺ cells, consistent

Figure 2. Purified single-cell s.c. injections into nude mice reveal multiple MPC subsets. A, representative photographs of nude mice bearing tumors of culture 1445 grown from one CD34⁺ cell (left), one CD34⁻p75⁻ cell (middle), and a mouse without tumors from p75⁺ cell injections (right). B, tumor growth over time revealed that CD34⁺ cells propagated tumors significantly faster than CD34⁻p75⁻ tumors in all three melanomas. Two tumors that formed from p75⁺ single-cell inoculations in culture 1118 grew significantly more slowly compared with all other subsets. *, $P < 0.05$, unpaired Student's *t* test, CD34⁺ versus CD34⁻p75⁻; **, $P < 0.005$, unpaired Student's *t* test, CD34⁺ versus CD34⁻p75⁻; #, $P < 0.05$, unpaired Student's *t* test, CD34⁻p75⁻ versus p75⁺.



with observations seen in the other mouse melanoma models (Fig. 3A). Single-cell injections of cells from freshly dissociated, uncultured Braf/Pten melanomas 3297 and 3474 formed tumors at six of six total sites with single CD34⁺ cell injections, two of six sites with CD34⁻p75⁻ cells, and zero of six sites with p75⁺ cells (Fig. 3B). CD34⁺ cell-derived tumors could be distinguished histologically from CD34⁻p75⁻ cell-derived tumors by their uniform epithelioid/rhabdoid cytology and increased pleomorphism (Supplementary Fig. S5C). In contrast, CD34⁻p75⁻ cell-derived tumors had a more heterogeneous appearance with edematous stromal regions that resembled the parental murine melanomas more closely. CD34⁺ cell-derived tumors formed the largest tumors by 10 weeks and were similar to the rates and growth characteristics observed using short-term melanoma cultures (Supplementary Table S1B). PCR analysis of the tumors revealed that they were uniformly composed of cells with recombined Pten (Supplementary Fig. S5B), confirming that tumors were formed from injection of an individual melanoma cell and not a contaminating normal cell, as Pten is not recombined in the latter. Together, these results establish that the CD34⁺ and CD34⁻p75⁻ cells define distinct MPC subsets in all three mouse melanoma models examined in this study.

Reestablishment of tumor heterogeneity is not characteristic of all MPC subsets. We sought to assess the functional characteristics of individual cells within MPC subsets, including evaluation of self-renewal and reestablishment of tumor heterogeneity (CSC traits) as well as the ability to convert from one cell subset to another (plasticity). We chose to test for these properties in single purified cells, as studies performed using pools of sorted cells could be confounded by preexisting heterogeneity and by signaling interactions between adjacent cells. Colonies derived from single-cell FACS experiments were cultured for at least three additional passages *in vitro* and cell subsets were reanalyzed by flow cytometry, as summarized in Supplementary Table S2. All CD34⁺ clones failed to reestablish heterogeneity, producing progeny that were on average >98% CD34⁺ on first passage and >99% CD34⁺ by the third passage (Fig. 4A). CD34⁺ clones continued to uniformly produce CD34⁺ daughter cells even after 10 passages (data not shown). In contrast, CD34⁻p75⁻ single cells were capable of generating phenotypic heterogeneity, giving rise to CD34⁺, CD34⁻p75⁻, and p75⁺ cells that persisted over multiple passages; however, the extent of this heterogeneity was variable between clones and even between passage number for some individual clones (Fig. 4B). In addition, a minority of CD34⁻p75⁻ clones did not reestablish heterogeneity. Single p75⁺ cells from cultures 1445 and 1111 infrequently formed colonies, typically comprising >95% p75⁺ cells. In contrast, colony-forming p75⁺ cells from culture 1118 could produce CD34⁻p75⁻ and p75⁺ progeny (Fig. 4C). The tendency of 1118 p75⁺ cells to produce CD34⁻p75⁻ cells may reflect enhanced plasticity in this primary melanoma culture relative to the other p75⁺ cultures studied and could account for why two of six injection sites formed tumors. These experiments show that distinct MPC populations from the same tumor can have vastly different propensities to reestablish a heterogeneous population of tumor cells.

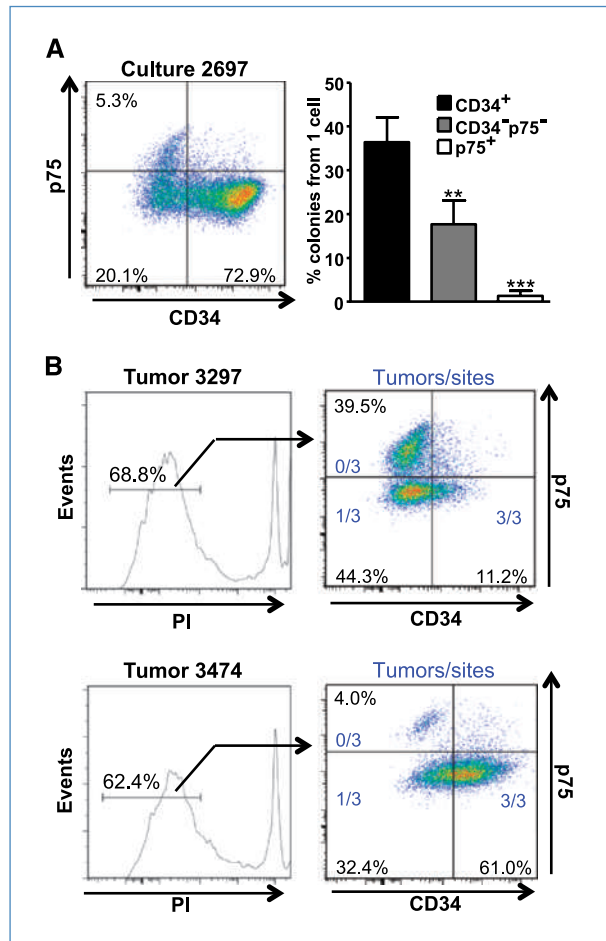


Figure 3. Evaluation of MPC subsets in cultured and uncultured melanomas using the Braf/Pten mouse model. *A, left*, flow cytometry on Braf/Pten cultured tumor 2697; *right*, 2697 single-cell FACS confirmed high, intermediate, and low colony-forming rates with CD34⁺, CD34⁻p75⁻, and p75⁺ single cells, respectively, indicating that MPC subsets are the same across all three mouse melanoma models. Columns, mean ($n = 3$); bars, SE. **, $P < 0.001$, unpaired Student's *t* test; ***, $P < 0.0001$, unpaired Student's *t* test, relative to CD34⁺. *B, left*, fresh Braf/Pten tumors 3297 and 3474 were dissociated and stained with propidium iodide and sorted to deplete dead/apoptotic cells. Live cells were then stained with CD34 and p75 antibodies for subset sorts followed by single-cell inoculations of live cells into nude mice. *Right*, marker profiles of parental tumors 3297 and 3474. Numbers in blue within quadrants represent tumors grown per total sites injected for that subset.

Furthermore, the self-renewing nature of CD34⁺ clones shows that generation of cellular diversity, a CSC trait, does not necessarily correlate with the capacity to propagate colonies or tumors.

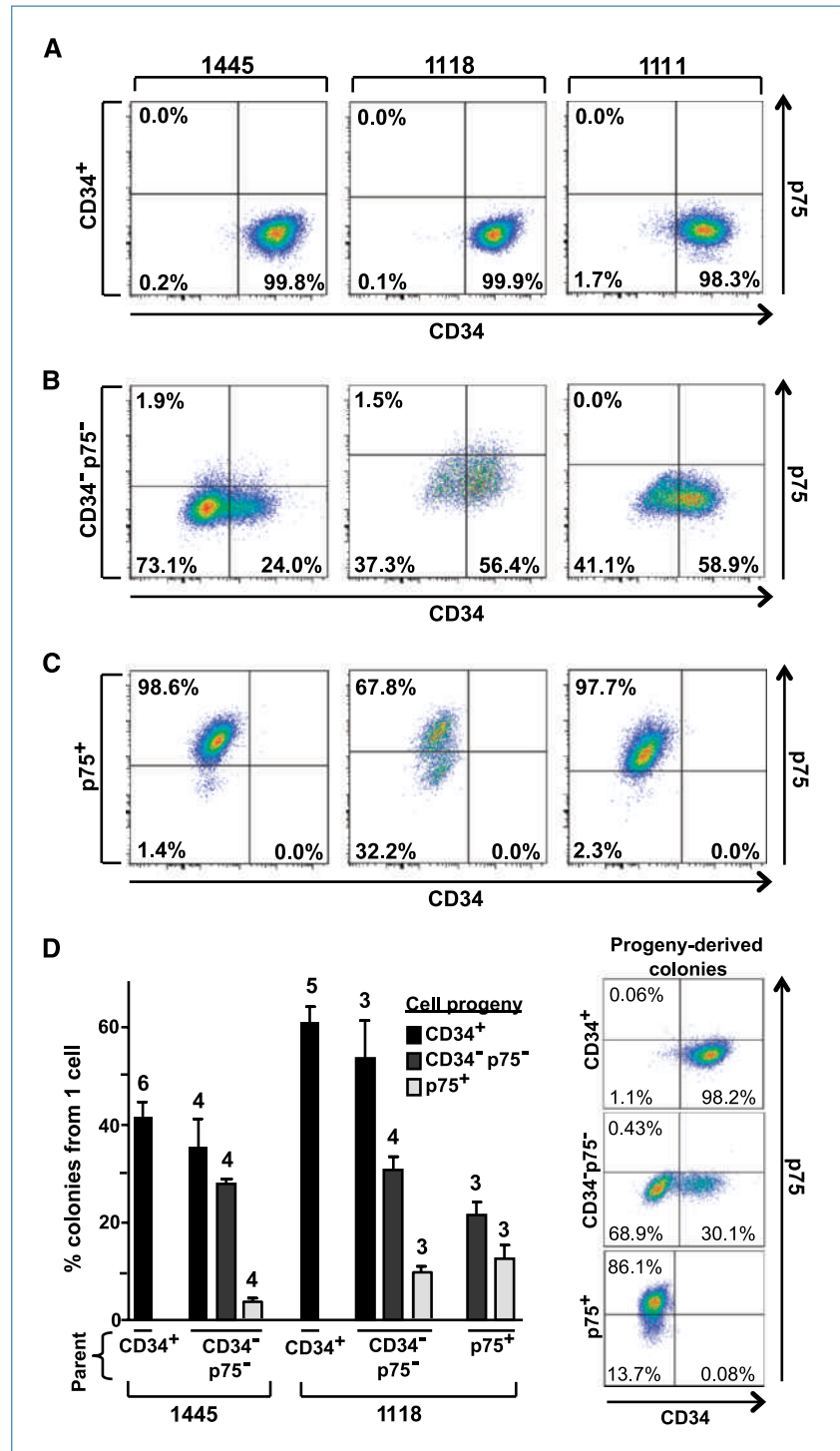
Evaluation of serial single-cell colony formation assays performed on representative clones from passage 3 in melanoma cultures 1445 and 1118 showed that CD34⁺ progeny produced from either single CD34⁺ or CD34⁻p75⁻ parental cells formed colonies at a similar high frequency. CD34⁻p75⁻ progeny exhibited intermediate rates of colony propagation, whereas p75⁺ progeny from heterogeneous colonies derived from either CD34⁻p75⁻ or p75⁺ parental clones had low rates

of colony formation, consistent with parental cell behavior. Additionally, the extent of phenotypic heterogeneity among colonies formed from these individual progeny was similar to that of colonies formed from the parental cells (Fig. 4D). These *in vitro* clonal studies support the idea that individual CD34⁻p75⁻ MPCs have the capacity to reestablish cell hetero-

geneity, including the two distinct populations of MPCs, whereas CD34⁺ MPCs do not exhibit this property.

To determine if cellular heterogeneity was reestablished in the process of tumor formation from single-cell injections, we dissociated tumors produced in this manner and analyzed tumor cells by flow cytometry. Tumors derived

Figure 4. Evaluation of the reestablishment of cellular heterogeneity in the progeny of individual melanoma cell-derived colonies. **A**, colonies grown from CD34⁺ single cells mainly produced CD34⁺ progeny. **B**, CD34⁻p75⁻ clones gave rise to progeny with variable cell surface marker expression. **C**, infrequent colonies from p75⁺ single cells in cultures 1445 and 1111 predominantly gave rise to p75⁺ cells; however, p75⁺ colonies formed in culture 1118 generated both p75⁺ and CD34⁻p75⁻ progeny. **D, left**, serial single-cell FACS on cell progeny from colonies was analyzed for colony-forming ability. Numbers above data columns indicate the number of clones analyzed. **Columns**, mean; **bars**, SE. **Right**, representative flow cytometry on melanoma 1118 serial colonies formed from a CD34⁺ cell (*top*), CD34⁻p75⁻ cell (*middle*), and a p75⁺ cell (*bottom*) each sorted from a CD34⁻p75⁻ cell-derived colony.



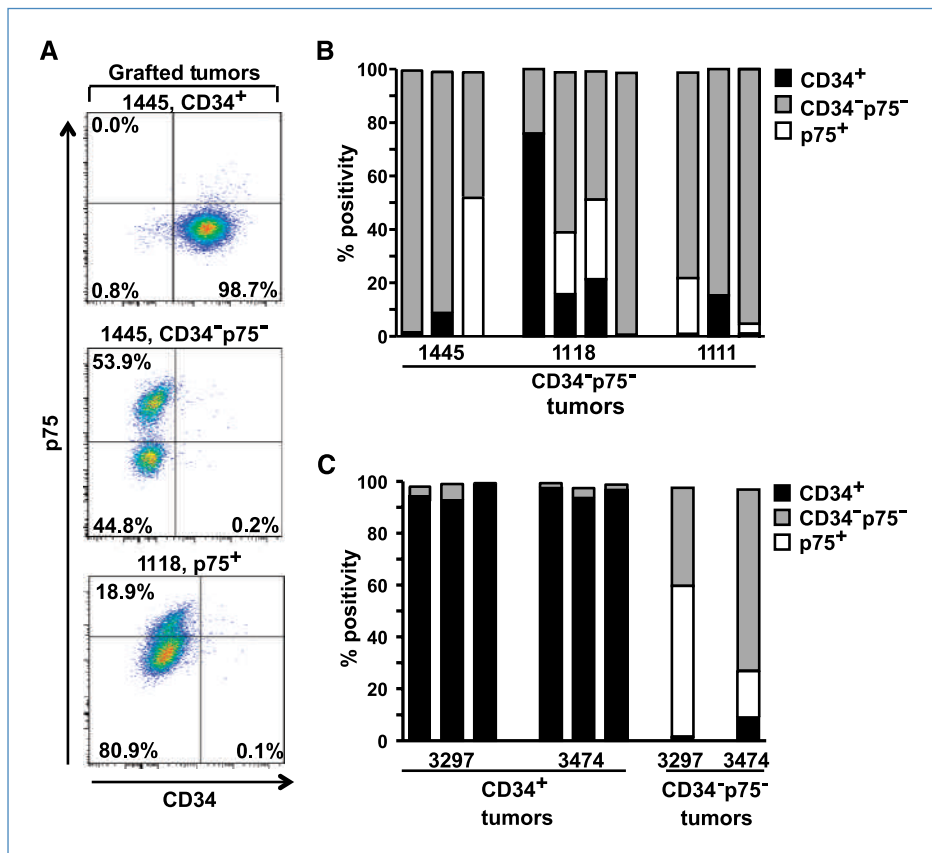


Figure 5. Analysis of the reestablishment of cellular heterogeneity in grafted tumors. A, engrafted tumors were dissociated and analyzed by flow cytometry. Tumors from single cells displayed similar marker phenotypes compared with *in vitro* colonies grown from single cells. Representative flow analyses are displayed for tumors grown from one CD34⁺ cell (top) and one CD34⁻p75⁻ cell (middle) from culture 1445 and one p75⁺ cell (bottom) from culture 1118. CD34⁺ single cells predominantly self-renewed *in vivo* in contrast with CD34⁻p75⁻ cells. B, single-cell CD34⁻p75⁻-derived tumors from short-term melanoma cultures showed variability in their propensity to reestablish heterogeneity, similar to observations seen in culture. C, individual CD34⁺ cells injected directly from freshly dissociated tumors self-renewed to form homogeneous CD34⁺ tumors, whereas those derived from individual CD34⁻p75⁻ reestablished heterogeneity, consistent with short-term culture engraftments.

from CD34⁺ cells were almost exclusively composed of CD34⁺ cells (97.6 ± 0.8%; Fig. 5A and C). Some tumors formed from CD34⁻p75⁻ single-cell engraftments were composed almost entirely of CD34⁻p75⁻ cells (Fig. 5B). However, other tumors, including those grown from injection of CD34⁻p75⁻ uncultured single cells, could produce all cellular subsets (Fig. 5A–C). The only two tumors that grew from injected p75⁺ single cells, both from culture 1118, were composed of both CD34⁻p75⁻ cells and p75⁺ cells, suggesting that some p75⁺ cells from this melanoma are capable of generating a heterogeneous tumorigenic phenotype, although this phenomenon may be uncommon as it occurred in only one of five melanomas characterized (Fig. 5A). These *in vivo* data confirm that the majority of CD34⁻p75⁻ MPCs can both self-renew and reestablish heterogeneity, two traits that conform to the accepted definition of CSCs (1, 2). In contrast, CD34⁺ MPCs are essentially self-renewing and therefore do not rigorously conform to the definition of CSCs despite their uniform tumorigenicity.

Multiple MPC subsets are resistant to chemotherapy.

Resistance to chemotherapy is frequently characteristic of tumorigenic cells (1, 27). To determine if chemotherapeutic resistance correlated with the ability of melanoma cell subsets to form tumors, cultures 1445 and 1118 were incubated with concentrations of cisplatin or temozolomide determined to cause ~90% cell death (IC₉₀). Surviving cells were

allowed to recover and proliferate until 75% confluence before being analyzed for cell subsets. Treatment with IC₉₀ temozolomide or cisplatin resulted in a large decrease in the proportion of p75⁺ cells and an increase in the proportion of CD34⁺ cells. CD34⁻p75⁻ frequencies increased in melanoma 1445 only, potentially indicating chemotherapeutic susceptibility differences between melanoma models (Fig. 6A).

To functionally evaluate chemoresistance of purified subsets on an individual cell basis, single cells were sorted separately into wells containing IC₉₀ concentrations of cisplatin or temozolomide and colony formation rates were determined for up to 8 weeks. After normalization to untreated colony-forming rates, between 4% and 22% of CD34⁺ cells could survive chemotherapy and propagate colonies. In addition, 1% to 6% of CD34⁻p75⁻ cells were capable of forming colonies. However, p75⁺ single cells were incapable of colony formation in the presence of these chemotherapeutic agents (Fig. 6B). These findings show that MPC subsets contain high-dose chemotherapy-resistant cells. Furthermore, the p75⁺ cell subset that rarely formed tumors is also the most sensitive to chemotherapy.

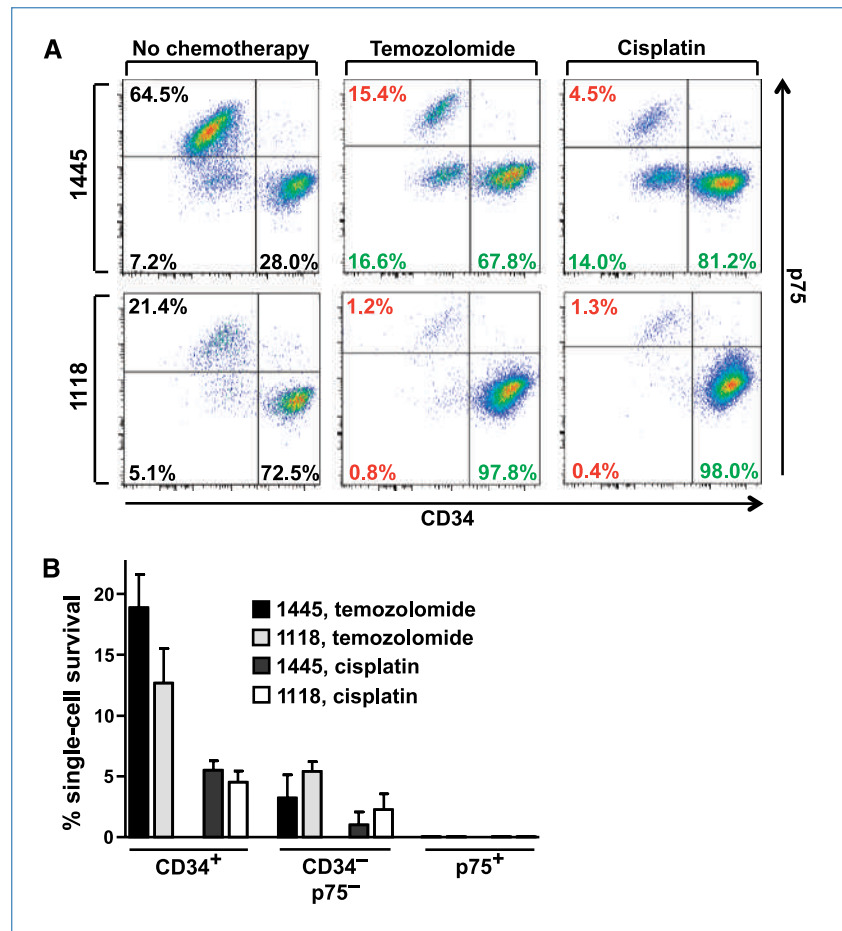
Discussion

Previous studies of cancer-propagating cells have required large numbers of human cells to grow tumors in

immunodeficient mice, suggesting that tumorigenic cells may be rare in human cancers (3–13). However, this idea has recently been challenged through the demonstration of much higher tumorigenic frequencies in experiments involving coinjections of single unpurified human melanoma cells and Matrigel into highly immunodeficient mice (17) or single unpurified murine leukemia/lymphoma cell injections into syngeneic mice (15). These observations suggest that more accurate tumorigenic rates may be obtained using enhanced immunodeficient mouse models as human cancer recipients or transplantation of tumor cells within the same species such as mouse into mouse. Indeed, several recent investigations into mouse models of mammary and brain tumorigenesis have shown higher tumorigenic rates using mouse-in-mouse engraftments (29–31). Likewise, tumorigenic frequencies in our mouse melanoma models were found to be high (average, 53%). In this report, we have prospectively purified multiple MPC subsets and for the first time successfully show a uniformly tumorigenic subset based on single-cell injections. Moreover, the enrichment of these MPC subsets has allowed us to begin characterization of their functional properties at the level of individual cells.

In many cancer types, xenografted tumors resulting from injections of enriched pools of cancer-propagating cells have generally been shown to both self-renew and reestablish the heterogeneity of surface marker expression observed in the parental tumor or derived cell cultures; therefore, these properties have been proposed as characteristics of CSCs (1, 32). A similar analysis of marker expression has not been performed to date in studies showing tumor formation following injection of single cancer cells. In this study, the CD34⁺p75⁻ subset that we identified was enriched for individual cells that could self-renew and reestablish cellular heterogeneity both *in vitro* and *in vivo*, in keeping with established views of CSC behavior. The observation that some CD34⁻p75⁻ MPCs did not reestablish heterogeneity under the same conditions suggests either biological differences among a lower proportion of cells in this subset or, alternatively, that cell decisions to undergo symmetrical or asymmetrical divisions in this subset may be stochastic. CD34⁺ MPCs, on the other hand, underwent self-renewal almost exclusively, a pattern that we observed both in culture and in xenografted tumors showing that tumorigenic rate does not necessarily correlate with CSC behavior in all cases. Finally, p75⁺ subsets were typically incapable of tumor propagation, although one of five

Figure 6. Multiple subsets of melanoma MPCs exhibit chemoresistance. **A**, treatment of parental melanoma cultures 1445 and 1118 with 500 μmol/L temozolomide or 0.5 μg/mL cisplatin (IC₉₀ doses) yields marked reduction of the p75⁺ populations and persistence of the CD34⁺ subsets. Numbers highlighted in red indicate a loss in subset percentage, whereas green indicates percentage gain compared with vehicle controls (*left*). **B**, single-cell FACS of subsets sorted directly into temozolomide- or cisplatin-spiked medium showed that some CD34⁺ and CD34⁻p75⁻ cells were capable of recovering from each chemotherapy treatment, whereas p75⁺ cells were not. Percent survival shown was normalized to untreated controls. Columns, mean (n = 3); bars, SE.



melanomas examined contained a smaller percentage of slowly proliferating p75⁺ MPCs (tumor 1118, Fig. 2B). Although it is possible that an enhanced immunodeficient mouse model or different conditions may yield high rates of p75⁺ tumorigenic cells, we feel this is unlikely as their universal low rate of colony formation *in vitro*, a system devoid of immune modulation and supportive Matrigel microenvironment, corresponds to the observed *in vivo* propagating rates. The long latency and presence of only one tumor per mouse (Fig. 1A and B) imply that the melanomas used to derive cell cultures each arose from a single cell. As such, our observations reveal that single melanoma tumors have the capacity to generate multiple MPC subsets.

Poorly differentiated forms of cancer are often resistant to chemotherapy and radiation therapy. These features are also associated with tumorigenic cells (33–35). However, previous investigations have been unable to evaluate the chemotherapeutic response of individual cancer-propagating cells, as uniformly tumorigenic cells have not been previously identified. In this study, we show that purified MPCs are relatively resistant to high-dose cisplatin and temozolomide, whereas the non-MPC subset is sensitive to these treatment modalities. These findings raise the possibility that some partial responses to chemotherapy, whether observed in the laboratory or in the clinic as a decrease in tumor burden, may in fact reflect preferential killing of a cell subset that is not tumorigenic. In addition, individual CD34⁺ MPCs, which did not conform to the CSC definition, were the most capable of resisting high-dose chemotherapy, indicating that the ability to reestablish tumor heterogeneity does not necessarily correlate with increased chemoresistance. We anticipate that

the purification of uniformly tumorigenic cells will allow for the comprehensive evaluation of phenotypic, genetic, and epigenetic features that define MPCs and the chemoresistant cells associated with those subsets.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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