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Phenotypic Heterogeneity among Tumorigenic Melanoma Cells from Patients that Is Reversible and Not Hierarchically Organized

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SUMMARY

We investigated whether melanoma is hierarchically organized into phenotypically distinct subpopulations of tumorigenic and nontumorigenic cells or whether most melanoma cells retain tumorigenic capacity, irrespective of their phenotype. We found 28% of single melanoma cells obtained directly from patients formed tumors in NOD/SCID IL2Rγ^{null} mice. All stage II, III, and IV melanomas obtained directly from patients had common tumorigenic cells. All tumorigenic cells appeared to have unlimited tumorigenic capacity on serial transplantation. We were unable to find any large subpopulation of melanoma cells that lacked tumorigenic potential. None of 22 heterogeneously expressed markers, including CD271 and ABCB5, enriched tumorigenic cells. Some melanomas metastasized in mice, irrespective of whether they arose from CD271$^-$ or CD271$^+$ cells. Many markers appeared to be reversibly expressed by tumorigenic melanoma cells.

INTRODUCTION

Cancer is a heterogeneous disease, involving differences between tumors as well as between cancer cells within the same tumor. Clonal evolution contributes to this heterogeneity as cancer cells undergo irreversible genetic changes over time, leading to functional and phenotypic differences (Nowell, 1976). Another explanation for heterogeneity within tumors comes from the cancer stem cell model, which posits that tumors are hierarchically organized, with a small subpopulation of tumorigenic cells that generates phenotypically diverse nontumorigenic progeny in a manner similar to normal stem cell differentiation (Kleinsmith and Pierce, 1964; Lapidot et al., 1994; Reya et al., 2001). These models are not mutually exclusive in that cancers that follow the stem cell model would be expected to undergo clonal evolution.

Evidence supports the cancer stem cell model in some acute myeloid leukemias (Bonnet and Dick, 1997; Lapidot et al., 1994), chronic myeloid leukemias (Eisterer et al., 2005; Neering et al., 2007; Oravecz-Wilson et al., 2009), teratocarcinomas (Kleinsmith and Pierce, 1964), breast cancers (Al-Hajj et al., 2003), brain tumors (Read et al., 2009; Singh et al., 2004), and colon cancers (O'Brien et al., 2007; Ricci-Vitiani et al., 2007). In each cancer, markers have been identified that distinguish small,

Significance

In cancers that follow a stem cell model, phenotypically distinct tumorigenic cells form abundant and phenotypically diverse nontumorigenic progeny in a hierarchical manner that resembles normal stem cell differentiation. In contrast to this model, our results indicate that primary cutaneous or metastatic melanomas from patients have common and phenotypically diverse tumorigenic cells that undergo reversible phenotypic changes in vivo. Most of the phenotypic heterogeneity in melanoma is therefore not associated with a loss of tumorigenic potential or organized in stable hierarchies. These data suggest a phenotypic plasticity model in which phenotypic heterogeneity is driven largely by reversible changes within lineages of tumorigenic cells rather than by irreversible epigenetic or genetic changes.

often rare, subpopulations of cancer cells that are greatly enriched for tumorigenic/leukemogenic activity as compared to unfractionated cancer cells. The same markers were concluded to distinguish tumorigenic from nontumorigenic cells in multiple patients, suggesting these cancers adopt reproducible cellular hierarchies. Nonetheless, the robustness of some cancer stem cell markers has been questioned (Joo et al., 2008; Ogden et al., 2008; Wang et al., 2008) and it remains to be determined how generalizable the model is.

Cancer stem cell studies have consistently found that cells from nontumorigenic/nonleukemogenic cancer cell populations are rarely able to form tumors/leukemias, even when assayed under conditions permissive for tumorigenesis by small numbers of cancer stem cells (Al-Hajj et al., 2003; Bonnet and Dick, 1997; Lapidot et al., 1994; O'Brien et al., 2007; Oravecz-Wilson et al., 2009; Read et al., 2009; Ricci-Vitiani et al., 2007; Singh et al., 2004). In cancers that follow this model, nontumorigenic cells have therefore irreversibly lost tumorigenic capacity or only regain this capacity under rare circumstances. The cancer stem cell and clonal evolution models have thus emphasized the role of irreversible epigenetic and genetic changes in determining heterogeneity among cancer cells.

On the other hand, recent studies carried out in cancer cell lines have suggested that some phenotypic and functional attributes of tumorigenic cells can reversibly turn on and off (Mani et al., 2008; Pinner et al., 2009; Roesch et al., 2010; Sharma et al., 2010). This raises the question of whether reversible changes are observed in primary cancers from patients and whether many or few cells in these cancers can undergo such changes. If most cells in a cancer can reversibly gain and lose competence to form a tumor, then this is a transient state rather than a hierarchically determined attribute possessed only by rare cancer stem cells. These studies also raise the separate question of whether phenotypic heterogeneity in patient tumors is driven mainly by irreversible or reversible phenotypic changes.

The growth and metastasis of melanomas has been proposed to be driven by a small subpopulation of melanoma stem cells that can be distinguished from nontumorigenic melanoma cells based on the expression of ABCB5 (Schatton et al., 2008) or CD271 (Boiko et al., 2010). However, the xenotransplantation experiments that were the basis for these conclusions were carried out with assays in which an average of only 1 in 50,000 (see Table S3 in Boiko et al., [2010]) to 1 in 1,090,000 (Schatton et al., 2008) unfractionated melanoma cells formed tumors. We have found that the frequency of tumorigenic melanoma cells that can be detected after xenotransplantation is highly assay-dependent (Quintana et al., 2008). By making a series of changes in assay conditions, we increased the detectable frequency of tumorigenic melanoma cells by several orders of magnitude. We found that one in four cells obtained directly from patients with primary cutaneous or metastatic melanomas are able to form tumors after xenotransplantation into NOD/SCID IL2Rγ^{null} (NSG) mice (see Figure 3c in Quintana et al., [2008]). The conclusion that many melanoma cells are capable of forming tumors has been independently confirmed in primary mouse melanomas (Held et al., 2010) and in human melanoma cell lines (Roesch et al., 2010).

Although many melanoma cells are able to form tumors, it is critical to assess whether melanoma is hierarchically organized into phenotypically distinct subpopulations of tumorigenic and nontumorigenic cells. We have found that both CD133^{+} and CD133^{-} melanoma cells form tumors that exhibit similar heterogeneity in CD133 expression, suggesting that CD133 is reversibly expressed by tumorigenic melanoma cells rather than distinguishing cells at different levels of a hierarchy (Shackleton et al., 2009). The JARID1B histone demethylase regulates the tumorigenic activity of melanoma cell lines and can also reversibly turn on and off, raising the possibility that competence to form a tumor is reversible (Roesch et al., 2010). Brn2 and pigment are also reversibly expressed by cells from melanoma cell lines as they metastasize in vivo (Pinner et al., 2009). These studies raise the question of whether reversible phenotypic plasticity is observed among many, or few, markers in melanomas obtained from patients. We addressed this by carrying out extensive functional and phenotypic analyses of melanoma cells obtained from patients with stage II, III, and IV disease.

RESULTS

We previously reported that 69 of 254 (27%) single, unselected melanoma cells, isolated from four patient melanomas that had been passaged as xenografts in mice, formed tumors after injection into NSG mice (Quintana et al., 2008). We have now extended these studies to evaluate an additional 210 single, unselected cells from melanomas that were either obtained from other xenografts (n = 3 patient tumors, up to two passages in mice) or directly from patients with stage III disease (n = 5 patient tumors). In each case, 15%–50% of single cells formed subcutaneous tumors in NSG mice. Overall, 62 of 210 (30%) single cells formed tumors, including 28% of single cells obtained directly from patients (Table 1). These are minimum estimates of the frequency of tumorigenic melanoma cells as additional assay improvements could further increase the frequency of tumorigenic cells that can be detected.

We also carried out limit dilution assays on cells obtained directly from 11 additional patients with primary cutaneous (stage II or III) or metastatic (stage III or IV) melanomas (Table 1). The stage II melanoma (Breslow depth 3.3 mm, no ulceration, 2 mitoses/mm^2), obtained directly from a patient, contained a high frequency of tumorigenic cells, with five of six injections of ten unselected cells forming tumors. Primary cutaneous melanomas obtained directly from early stage patients with a good prognosis can therefore contain a high frequency of tumorigenic cells, though additional studies will be required to determine whether some stage I or II melanomas contain less frequent tumorigenic cells. The melanomas obtained directly from stage III (n = 9) and IV (n = 1) patients also contained high frequencies of tumorigenic cells. We have thus examined the frequency of cells with tumorigenic potential in melanomas obtained from 18 different patients (see Figure S1 available online for patient details). We observed high frequencies of tumorigenic cells in every case, irrespective of whether the melanomas were obtained directly from patients (n = 16 cases) or xenografted (n = 3), and irrespective of whether they were primary cutaneous melanomas (n = 3), cutaneous or subcutaneous metastases (n = 4), or regional lymph node metastases (n = 12).

Table 1. Summary of Tumor Formation by Single Cells or by Limit Dilution Analyses of Melanoma Cells from Sixteen Stage II, III, and IV Patients

Tumor Origin	Patient	AJCC Clinical Stage (Tumor Site)	Tumors/Injections: Cells Per Injection 1000	100	10	1	Engraftment Rate (%) or Tumorigenic Cell Frequency (95% CI)
Single cell injections							
Directly from patients	526	III (regional LN metastasis)				10/32	31%
	528	III (regional LN metastasis)				4/27	15%
	530	III (regional LN metastasis)				6/36	17%
	534	III (regional LN metastasis)				15/30	50%
	600	III (regional subcutaneous metastasis)				9/30	30%
Xenograft (up to 2 passages)	405	III (regional LN metastasis)				6/15	40%
	501	III (regional LN metastasis)				7/27	26%
	491	III (regional subcutaneous metastasis)				5/13	38%
ALL	n = 8					62/210	30%
Limit dilution analysis							
Directly from patients	610	II (cutaneous primary)		6/6	5/6		1/6 (1/2–1/15)
	486	III (cutaneous primary)	6/6	6/6	2/3		1/9 (1/2–1/39)
	597	III (cutaneous primary)	6/6	6/6	2/6		1/22 (1/8–1/62)
	495	III (cutaneous metastasis)	6/6	6/6	1/3		1/20 (1/5–1/76)
	510	III (regional LN metastasis)		6/6	3/3		>1/21
	514	III (regional LN metastasis)	6/6	6/6	6/6		>1/11
	631	III (regional LN metastasis)		6/6	3/3		>1/21
	632	III (regional LN metastasis)		6/6	6/6		>1/11
	633	III (regional LN metastasis)		6/6	6/6		>1/11
	641	III (regional LN metastasis)		6/6	4/6		1/9 (1/3–1/25)
	608	IV (distant subcutaneous metastasis)		6/6	6/6		>1/11
ALL	n = 11		24/24	66/66	44/54		1/6 (1/4–1/8)

AJCC: American Joint Committee on Cancer, CI: confidence interval, LN: lymph node. Melanoma cells were mixed with Matrigel and injected into NSG mice. Twenty-eight percent (44 of 155) of single cells obtained directly from patients formed tumors. AJCC is the clinical stage of the patient at the time of melanoma removal. See also Figure S1.

No Correlation between Tumorigenic Cell Frequency and Tumor Growth Rate

A prediction that is commonly made based on the cancer stem cell model is that aggressively growing tumors contain a higher frequency of tumorigenic cells. Nonetheless, this prediction has rarely, if ever, been tested. To evaluate this, we transplanted single cells from melanomas obtained from 12 patients (see Figure S1) into NSG mice then compared the growth rates of the resulting tumors with the percentage of single cells that were tumorigenic. Tumors that arose from some patients grew quickly (patients 205, 214, and 491), whereas tumors from other patients grew slowly (patients 405, 481, 487, 501, 526, 528, 530, 534, and 600) (Figure 1A). Although fast-growing tumors grew an average of 3 times faster than slow growing tumors in NSG mice (1.52 ± 0.64 versus 0.50 ± 0.30 mm/week; $p < 0.0001$), there was no correlation with the frequency of tumorigenic cells in NSG mice (Figure 1B). The melanoma that gave rise to the fastest growing tumors (205) also had the lowest frequency (13%) of tumorigenic cells, whereas a melanoma that gave rise to slow-growing tumors (481) had a remarkably high frequency (70%) of tumorigenic cells. Tumors derived directly from patients tended to grow more slowly than tumors derived from xenografts, but these tumors did not tend to have lower frequencies of tumorigenic cells (Figure 1A). Differences in the growth rates of melanomas from different patients in NSG mice are not determined by differences in tumorigenic cell frequency.

All Tumorigenic Melanoma Cells Appear Capable of Indefinite Proliferation

To test whether the proliferative capacity of some tumorigenic melanoma cells may be limited, as observed in some human leukemia-initiating cells (Hope et al., 2004), we formed melanomas in NSG mice from single cells derived from six patients (32 tumors arose from the injection of 58 single cells). We then serially transplanted 100 cell aliquots from each tumor into secondary and tertiary NSG mice. All 32 single-cell derived tumors gave rise to secondary tumors, and all 31 of the tumors from which tertiary transplants were attempted gave rise to tertiary tumors (Figures 2A and 2B; one secondary tumor could not be retransplanted due to the unexpected death of the

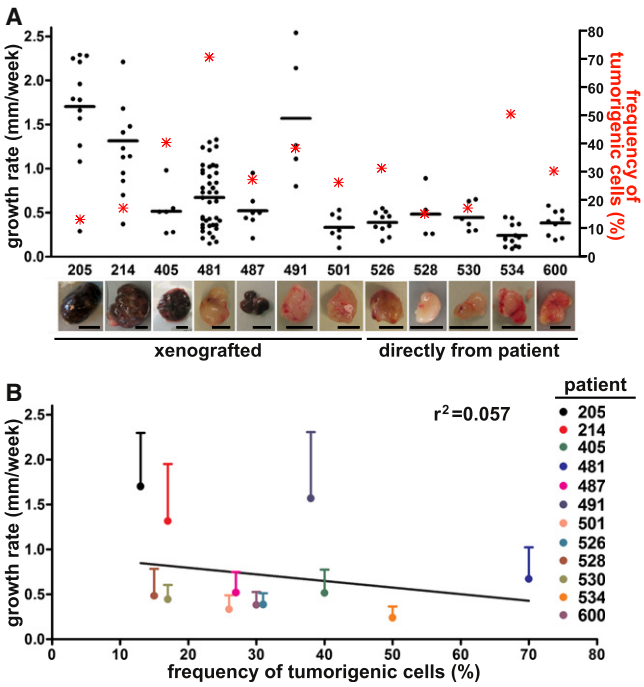


Figure 1. Lack of Correlation between Growth Rate and Tumorigenic Cell Frequency in Human Melanomas

(A) Tumor development after subcutaneous injection into NSG mice of single melanoma cells derived from 12 patients with metastatic disease. Dots represent individual tumors and are plotted against tumor growth rate (left axis). Lines represent the mean growth rate of all tumors derived from each patient. Red stars represent the proportion of single cells derived from each patient melanoma that formed tumors (right axis). Scale bars represent 1 cm.

(B) Linear regression analysis of the mean (±standard deviation [SD]) growth rate of clonal tumors derived from the same 12 patients plotted against the empirically determined frequency of tumorigenic cells in their parent tumors. r^2 value shows the Pearson correlation coefficient, indicating no significant correlation between tumor growth rate in NSG mice and tumorigenic cell frequency in NSG mice.

mouse). In all cases, the single melanoma cells appeared to be capable of unlimited growth, necessitating euthanasia of the mice. Tumor growth rate significantly increased in a few cases between secondary and tertiary transplants (see asterisk in Figures 2E–2H, $p < 0.05$; primary tumor growth rates could not be compared to secondary tumors because primary tumors were initiated with single cells while secondary and tertiary tumors were initiated with 100 cells). In no case did the rate of tumor growth decrease during serial passaging (Figures 2C–2H). We have thus been unable to detect any tumorigenic melanoma cells that have limited tumorigenic potential in vivo.

Both ABCB5$^-$ and ABCB5$^+$ Melanoma Cells Can Form Tumors

We previously published (Quintana et al., 2008) that four markers (CD133, CD166, L1-CAM, and CD49f) were not able to distinguish tumorigenic from nontumorigenic melanoma cells. We have now tested 18 additional markers (ABCB5, CD271, MCAM, E-Cadherin, N-Cadherin, c-kit, CD29, CD44, CD49d, CD49b, A2B5, HNK1, CD54, CD9, CD151, CD10, L6, and CD49e) that we found to be heterogeneously expressed among human melanoma cells out of 85 markers that we examined (for a summary of all tumors and markers see Figure S1 and Table S2). We present data in greatest detail for ABCB5 (Figure 3) and CD271 (Figure 4).

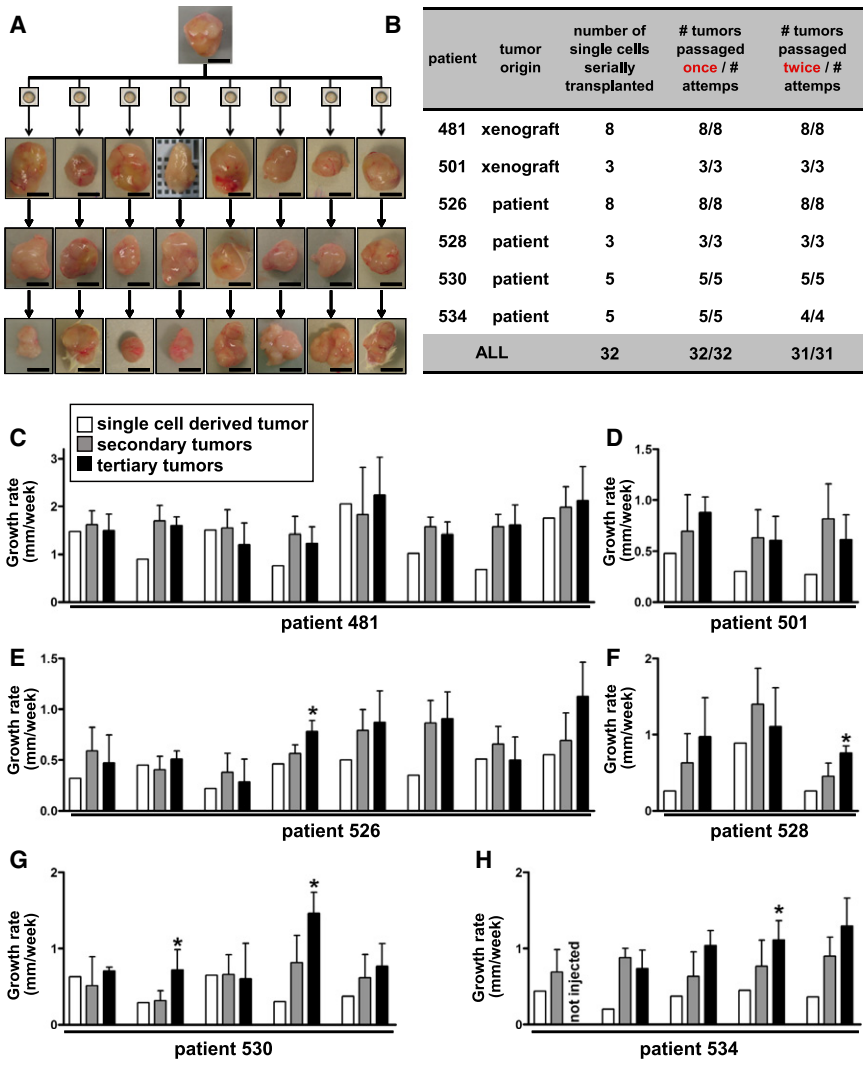
We evaluated ABCB5 expression by flow-cytometry in melanomas from nine patients using dissociation conditions and antibody provided by Schatton et al. (2008). We did not independently test the specificity of this antibody but rather relied on published results (Frank et al., 2003). We detected staining with this antibody in a minority of cells from four of these tumors, ranging from 2.9% to 5.3% of cells (Figure 3A). Because the detectable frequency of ABCB5$^+$ cells (0%–5.3%) was much lower than the frequency of tumorigenic cells (11%–70%) in all tumors, cells with tumorigenic potential could not reside exclusively within the ABCB5$^+$ subpopulation (Figure 3A). We observed no significant difference in the frequency of tumorigenic cells between melanomas with ABCB5$^+$ cells and melanomas without ABCB5$^+$ cells (27 ± 13% and 27 ± 25% respectively; mean ± standard deviation [SD], $p = 0.97$).

To compare the tumorigenic potential of ABCB5$^-$ and ABCB5$^+$ melanoma cells, we separated these cells by flow cytometry from tumors derived from three different patients (Figures 3B and 3C) and transplanted them into NSG mice with Matrigel. All injections of 50 cells, and most injections of 10 cells, formed tumors, irrespective of whether unfractionated, ABCB5$^-$ cells, or ABCB5$^+$ cells were injected (Figure 3D). We were therefore unable to detect any difference in the tumorigenic capacity of ABCB5$^-$ and ABCB5$^+$ melanoma cells.

Both CD271$^-$ and CD271$^+$ Melanoma Cells Can Form Tumors

We also examined CD271 expression in primary cutaneous and metastatic melanomas from 20 patients (Figure 4A). CD271 expression was analyzed in some tumors immediately after removal from the patient (n = 13), and in other tumors after ≤2 passages as a xenograft (n = 9; tumors from some patients were examined both ways). CD271 was heterogeneously expressed in every tumor tested (Figures 4A and 4C), but the range of expression was large: 1%–91% of cells were CD271$^+$. Indeed, CD271$^+$ cells accounted for >50% of the cells in 4 of 13 tumors obtained directly from patients (Figure 4A). Overall, we observed no correlation between the frequency of CD271$^+$ cells and the frequency of tumorigenic cells ($r^2 = 0.004$, Figure 4B). In some cases, the frequency of CD271$^+$ cells was much lower than the frequency of tumorigenic cells (see xenografts from patients 481, 487, 526, 534, and tumor directly from patient 610), whereas in other cases CD271$^+$ cell frequency was much higher than the frequency of tumorigenic cells (see xenografts from patients 491 and 530). This suggests that neither the CD271$^+$ nor the CD271$^-$ cell fraction can consistently contain all tumorigenic melanoma cells.

To directly compare the tumorigenic potential of CD271$^-$ and CD271$^+$ melanoma cells, we isolated these cells from xenografted tumors derived from three patients (491, 526, and 534; ≤2 passages; Figures 4C and 4D), and injected into NSG mice with Matrigel. In each case, tumors arose from only 10 CD271$^-$ or 10 CD271$^+$ cells and were at least as likely to arise from CD271$^-$ as from CD271$^+$ cells (Figure 4D). We also compared the tumorigenicity of CD271$^-$ and CD271$^+$ cells from xenografts from three other patients in NSG mice, but this time without



patient	tumor origin	number of single cells serially transplanted	# tumors passaged once / # attemps	# tumors passaged twice / # attemps
481	xenograft	8	8/8	8/8
501	xenograft	3	3/3	3/3
526	patient	8	8/8	8/8
528	patient	3	3/3	3/3
530	patient	5	5/5	5/5
534	patient	5	5/5	4/4
ALL		32	32/32	31/31

Figure 2. Tumorigenic Human Melanoma Cells Exhibit Indefinite Proliferative Potential on Serial Transplantation

(A) Eight clonal tumors were established from single cells obtained from patient 481. One hundred cell aliquots from each of these tumors were serially transplanted into secondary and tertiary NSG mice. All clonal tumors were successfully passaged twice after being established from single cells, suggesting that all tumorigenic cells had unlimited tumorigenic potential. Scale bar represents 1 cm.

(B) Clonal tumors derived from melanomas from five other patients were passaged similarly. Every attempt was successful.

(C–H) Tumor growth rates for patients 481 (C), 501 (D), 526 (E), 528 (F), 530 (G), and 534 (H). Each group of three bars indicates the growth rates of a clonal tumor (white) and its descendent first (gray) and second (black) generation tumors. During each passage, two to six secondary or tertiary injections were carried out for each tumor line and nearly all such injections gave rise to tumors. The growth rates of these tumors are shown as mean ± SD (*$p < 0.05$ by t test indicates significantly different growth rates in secondary and tertiary tumors).

Matrigel. Again, both CD271$^-$ and CD271$^+$ cells readily formed tumors and neither fraction was enriched for tumorigenic activity (Table S1).

We carried out similar experiments using CD271$^-$ and CD271$^+$ cells from six melanomas obtained directly from patients, including two primary cutaneous tumors, one from a patient with good prognosis stage II disease (Figures 4A, 4C, and 4D). Similar to xenografted tumors, tumors arose readily from CD271$^-$ and CD271$^+$ cells from the four metastatic melanomas (patients 600, 608, 631, and 641), (Figure 4D). In the two primary cutaneous melanomas (patients 597 and 610), tumors were more likely to arise from CD271$^-$ cells (Figure 4D). This is reminiscent of observations from primary mouse melanomas, which also exhibited tumorigenic activity from both the CD271$^-$ and CD271$^+$ fractions, but more tumorigenic activity from CD271$^-$ cells (Held et al., 2010). In our experiments, the less tumorigenic CD271$^+$ cells were a minor subpopulation (2%–12% of cells) in primary cutaneous melanomas, rather than the bulk of tumor cells. It remains unclear whether the reduced tumorigenic capacity of CD271$^+$ cells in these experiments was determined by genetic, epigenetic, or environmental differences.

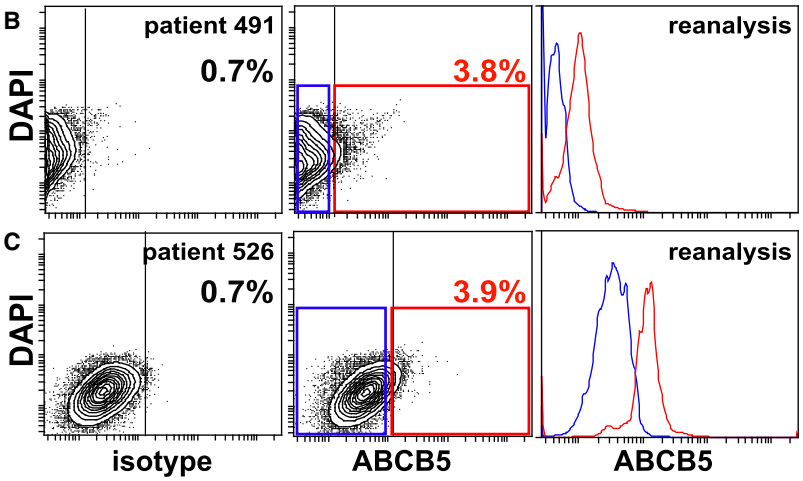
Both CD271$^+$ and CD271$^-$ Cells Form Tumors that Metastasize

We extended our studies to evaluate spontaneous metastasis by melanoma cells. Transplanted melanoma cells could disseminate as a result of inadvertent intravascular injection rather than from spontaneous metastasis; therefore, we first tested whether metastases arose in mice injected subcutaneously with single melanoma cells. NSG mice with subcutaneous tumors that arose from single cells from melanoma 481 were euthanized and examined as tumors approached 20 mm diameter. Macroscopic metastases, involving lymph nodes and/or visceral organs, were visible in most mice (see Figures 5A–5H). This demonstrates that metastases can develop spontaneously in NSG mice with xenografted human melanomas.

To test whether CD271$^-$ and CD271$^+$ melanoma cells form tumors with different metastasis potentials, we established subcutaneous tumors in NSG mice from CD271$^-$ or CD271$^+$ melanoma cells isolated directly from patient 608. Tumors were allowed to grow until they approached 20 mm in diameter or until the mice became ill. When the mice were euthanized, the tumors derived from CD271$^-$ and CD271$^+$ cells had mean ± SD diameters of 16 ± 8 mm and 17 ± 9 mm ($p = 0.66$), respectively, and mean times since injection of cells of 26 ± 6 weeks and 26 ± 5 weeks ($p = 0.85$). Metastases were similarly evident in the kidneys and lungs of all mice that developed subcutaneous tumors, irrespective of whether the tumors derived from CD271$^-$ or CD271$^+$ cells (Figures 5I–5Q). CD271$^-$ and CD271$^+$ melanoma cells from a xenograft derived from patient 205 were also injected in NSG mice, in this case without Matrigel.

A

patient	AJCC clinical stage, tumor site	tumor origin	frequency of ABCB5+	frequency of tumorigenic cells
308	III, Lymph node regional metastasis	xenograft	2.9%	13%
481	III, Subcutaneous regional metastasis	xenograft	0%	70%
487	IV, Cutaneous distant metastasis	xenograft	0%	27%
491	III, Subcutaneous regional metastasis	xenograft	3.8%	38%
492	III, Lymph node regional metastasis	xenograft	0%	>9%
495	III, Cutaneous regional metastasis	xenograft	0%	11%
498	III, Cutaneous primary tumor	xenograft	0%	17%
514	III, Lymph node regional metastasis	xenograft	5.3%	ND
526	III, Lymph node regional metastasis	xenograft	3.9%	31%



D

sorted fraction	#tumors/#injections (cells/injection) 50	10
unfractionated	6/6	16/24
ABCB5-	6/6	23/24
ABCB5+	6/6	23/24

Figure 3. Staining with an Antibody against ABCB5 Is Variable among Melanomas and Does Not Distinguish Tumorigenic from Nontumorigenic Cells in NSG Mice

(A) Frequency of ABCB5$^+$ cells versus frequency of tumorigenic cells in melanomas from nine patients. AJCC stage is the American Joint Committee on Cancer clinical stage of the patient at the time of melanoma removal. Frequency of ABCB5$^+$ cells indicates the percentage of cells that stained above isotype control background by flow cytometry. The frequency of tumorigenic cells in each tumor was determined by either single cell or limiting dilution injections into NSG mice.

(B and C) Separation by flow cytometry of ABCB5$^-$ (blue) and ABCB5$^+$ (red) melanoma cells from patients 491 (B) and 526 (C). Percentages indicate the frequency of cells that stained more strongly than isotype control (left). Reanalyses of sorted cells is shown to the right. Each plot shows viable, human HLA$^+$ cells and excludes mouse hematopoietic (CD45, TER119) and endothelial (CD31) cells as described previously (Quintana et al., 2008).

(D) Tumor formation after injection into NSG mice of unfractionated, ABCB5$^-$, and ABCB5$^+$ cells isolated as in (B) and (C) from three different patients (308: n = 2 experiments, 491: n = 1, 526: n = 1). Almost every injection of 10 ABCB5$^-$ cells or 10 ABCB5$^+$ cells from these three melanomas formed a tumor.

In this experiment, distant metastases were evident in abdominal structures (mesentery, ovaries, fallopian tubes, pararenal tissues) of three of six mice with tumors arising from CD271$^-$ cells and in two of five mice with tumors arising from CD271$^+$ cells (Figure 5R). Some human melanomas spontaneously metastasize in NSG mice, irrespective of whether they arise from CD271$^-$ or CD271$^+$ cells.

Other Markers Fail to Identify Nontumorigenic Melanoma Cells

To identify markers that are heterogeneously expressed by melanoma cells, we screened antibodies against 85 cell surface markers in melanomas from 3 to 19 patients per marker (Table S2). We were unable to detect the expression of 32 antigens, including CD34 and SSEA-1 (CD15). Other markers appeared to be nearly uniformly expressed by melanoma cells, including CD63, which was only heterogeneously expressed in 4 of 13 tumors. Beyond ABCB5 (Figure 3), CD271 (Figure 4), and the four heterogeneously-expressed markers we tested previously (CD133, CD166, L1-CAM, and CD49f; [Quintana et al., 2008]), we also compared the tumorigenic potential of melanoma cells that differed in the expression of MCAM, E-Cadherin, N-Cadherin, c-kit, CD29, CD44, CD49d, CD49b, A2B5, HNK-1, CD54, CD9, CD151, CD10, L6, and CD49e (Figure 6). In each case, marker-negative/low and marker-positive/high cells were separated by flow cytometry from tumors derived from two to three different patients (Figure 6A), and 10 cell aliquots were injected subcutaneously into NSG mice. In no case did we observe clear enrichment of tumorigenic activity in any fraction of cells (Figure 6B). In two of three experiments evaluating L6, tumors were more likely to arise from the L6$^-$ fraction of cells; however, the less tumorigenic L6$^+$ fraction accounted for only 9% of cells. We have therefore been unable to identify any large subpopulation of melanoma cells that lacks tumorigenic activity or any small subpopulation that is enriched for tumorigenic activity.

We also compared the growth rates of tumors to test whether some markers distinguished tumorigenic fractions with intrinsically different growth rates. We compared the rate at which tumors grew from 10 unfractionated cells, 10 marker-negative/low cells, or 10 marker-positive/high cells for 22 different markers: MCAM, E-Cadherin, N-Cadherin, ckit, CD29, CD44, CD49d, CD49b, A2B5, HNK-1, CD54, CD133, CD166, L1-CAM, CD49f, ABCB5, CD271, CD49e, CD9, CD10, CD151, and L6 (Figure S2). For each marker, a total of 6 to 28 tumors per cell fraction were generated from cells isolated from two to four different patients per marker. In no case did we observe a statistically significant difference in the rate at which tumors grew from marker-negative/low versus marker-positive/high fractions of cells. These data indicate there is extensive phenotypic heterogeneity among melanoma cells that does not correlate with differences in tumorigenic capacity or tumor growth rate.

A

tumor origin	patient	AJCC clinical stage, tumor site	frequency of CD271+ cells	frequency of tumorigenic cells
directly from patient	465	IV, Subcutaneous distant metastasis	59%	ND
	481	III, Subcutaneous regional metastasis	18%	ND
	597	III, Cutaneous primary tumor	12%	5%
	600	III, Subcutaneus regional metastasis	33%	30%
	608	IV, Subcutaneous distant metastasis	15%	>9%
	610	II, Cutaneous primary tumor	2%	17%
	625	II, Cutaneous primary tumor	1%	ND
	631	III, Lymph node regional metastasis	62%	>5%
	632	III, Lymph node unknown primary	91%	>9%
	633	III, Lymph node regional metastasis	58%	>9%
	634	III, Cutaneous regional metastasis	11%	ND
	640	II, Cutaneous primary tumor	4%	ND
	641	III, Lymph node regional metastasis	16%	17%
xenograft (up to 2 passages)	308	III, Lymph node regional metastasis	7%	ND
	465	IV, Subcutaneous distant metastasis	19%	ND
	481	III, Subcutaneous regional metastasis	28%	65%
	487	IV, Cutaneous distant metastasis	2%	20%
	491	III, Subcutaneous regional metastasis	30%	11%
	492	III, Lymph node regional metastasis	23%	20%
	526	III, Lymph node regional metastasis	8%	31%
	530	III, Lymph node regional metastasis	82%	17%
	534	III, Lymph node regional metastasis	7%	50%

D

tumor origin	patient	sorted fraction	injection of melanoma cells: #tumors/#injections, cells per injection: 100	10
directly from patient	597	unfractionated	6/6	2/6
		CD271-	6/6	3/6
		CD271+	5/6	0/6
	600	unfractionated	6/6	1/3
		CD271-	12/12	5/12
		CD271+	12/12	7/12
	608	unfractionated	6/6	6/6
		CD271-	5/6	5/6
		CD271+	6/6	6/6
	610	unfractionated	6/6	5/6
		CD271-		6/6
		CD271+		0/6
	631	unfractionated	6/6	3/3
		CD271-		6/6
		CD271+		6/6
	641	unfractionated	6/6	5/6
		CD271-	6/6	6/6
		CD271+	6/6	2/6
	ALL (n=6)	unfractionated	36/36	22/30
		CD271-	29/30	31/42
		CD271+	29/30	21/42
xenograft (up to 2 passages)	491	unfractionated		4/6
		CD271-		2/6
		CD271+		1/3
	526	unfractionated		6/6
		CD271-		4/6
		CD271+		3/6
	534	unfractionated		6/6
		CD271-		12/12
		CD271+		10/12
	ALL (n=3)	unfractionated		16/18
		CD271-		18/24
		CD271+		14/24

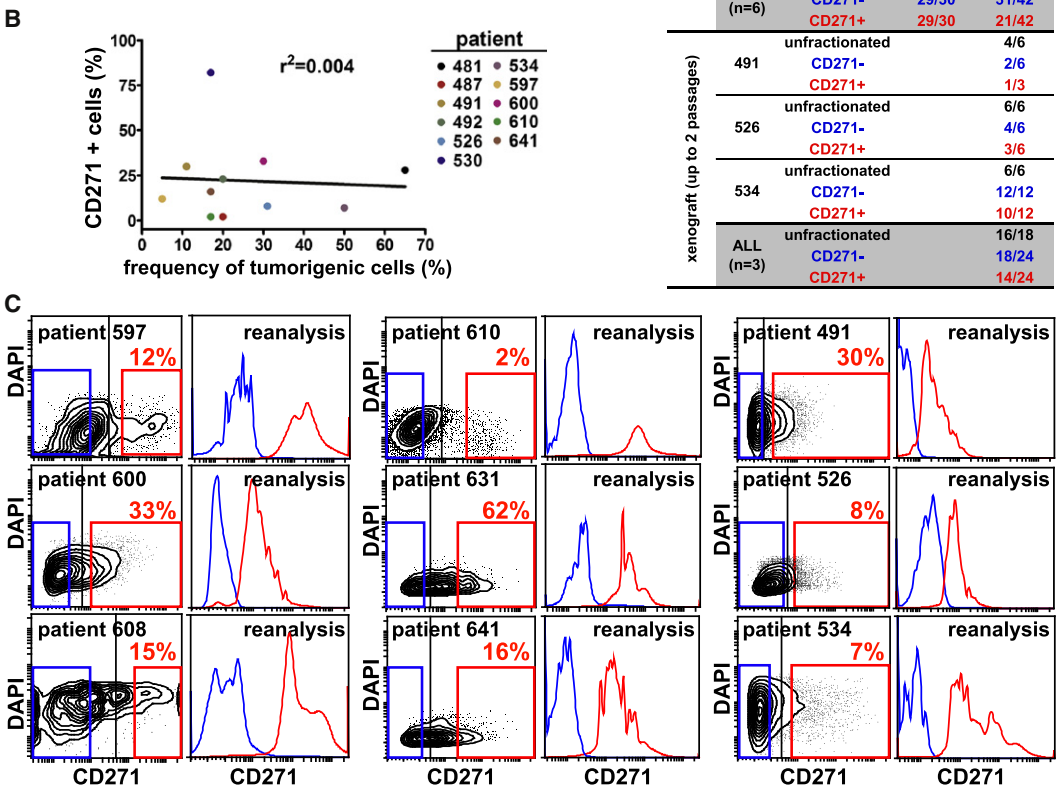


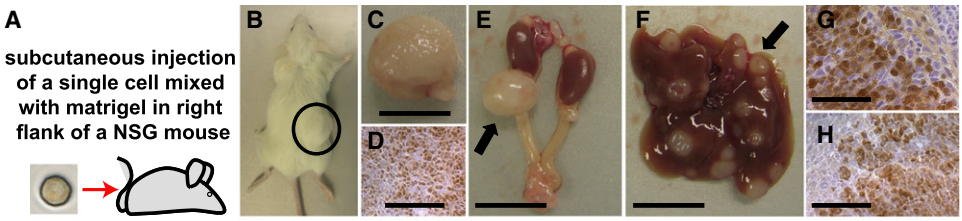
Figure 4. CD271 Expression Does Not Correlate with the Frequency of Tumorigenic Cells and Does Not Enrich Tumorigenic Melanoma Cells

(A) Frequency of CD271$^+$ cells versus frequency of tumorigenic cells in melanomas obtained directly from 13 patients and from nine xenografted tumors ($\leq$2 passages). NGFR5 and C40-1457 anti-human CD271 antibodies were compared side-by-side and gave similar results (data not shown).

(B) Linear regression analysis of the percentage of cells expressing CD271 in tumors from 15 patients, plotted against the frequency of tumorigenic cells in the same tumors. r^2 value (the Pearson correlation coefficient) indicates no correlation.

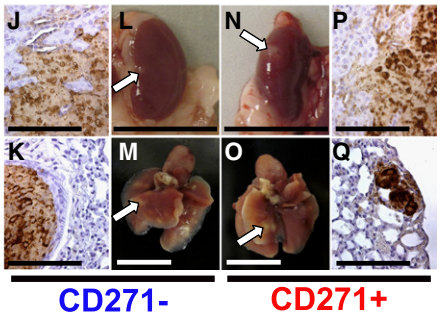
(C) Separation by flow cytometry of CD271$^-$ (blue) and CD271$^+$ (red) melanoma cells from patients 597, 600, 608, 610, 631, 641, 491, 526, and 534. Reanalysis of sorted cells is shown to the right. Each plot shows viable, human HLA$^+$ cells and excludes human or mouse hematopoietic (CD45$^+$, Glycophorin A or TER119$^+$) and endothelial (CD31$^+$) cells.

(D) Tumor formation after injection into NSG mice of unfractionated, CD271$^-$ and CD271$^+$ cells purified as in (C) directly from six patients or from three xenografted ($\leq$2 passages) tumors. Both CD271$^-$ and CD271$^+$ cells readily formed tumors with similar efficiency when isolated from stage III or IV metastatic tumors (600, 608, 631, and 641). CD271$^-$ cells were more tumorigenic in primary cutaneous tumors (597, p = 0.001; 610, p = 0.005), although the less tumorigenic CD271$^+$ cells accounted for only 2%–12% of cells in these tumors. See also Table S1.



I cells injected in NSG mice with matrigel

patient	sorted fraction	#mice with metastases/ # tumor bearing mice cells/injection 100	10
608	unfractionated	2/2	2/2
	CD271-	2/2	1/1
	CD271+	2/2	2/2



R cells injected in NSG mice without matrigel

patient	sorted fraction	# mice with metastases/# tumor bearing mice cells/injection 1,600	400	ALL 1,600-400 cells/injection
205	unfractionated	3/4	1/2	4/6 (67%)
	CD271-	1/3	2/3	3/6 (50%)
	CD271+	2/3	0/2	2/5 (40%)

Figure 5. Spontaneous Metastasis of Human Melanomas in NSG Mice Irrespective of Whether They Derived from CD271$^-$ or CD271$^+$ Melanoma Cells

(A–H) Spontaneous metastasis from a subcutaneous melanoma that arose from the injection of a single melanoma cell derived from a xenograft obtained from patient 481. Fifteen weeks after injection, a subcutaneous tumor was observed at the site of injection (B–D) that metastasized to lymph nodes (not shown), ovaries (E), pancreas (not shown), and liver (F). Immunostaining of the subcutaneous tumor (D), ovary (G), and liver (H) confirmed the presence of S100+ melanoma cells (in brown).

(I–Q) Melanoma metastasis from subcutaneous tumors that arose from the transplantation of CD271$^-$ or CD271$^+$ cells obtained directly from patient 608 (I). Metastases developed in the kidneys (J, L, N, P) and lungs (K, M, O, Q) of NSG mice 23–32 weeks after transplantation, irrespective of whether CD271$^-$ (J–M) or CD271$^+$ (N–Q) cells were transplanted. Metastasis developed with similar efficiency from tumors derived from CD271$^-$ and CD271$^+$ cells (I). Sections of kidney (J, P) and lungs (K, Q) show infiltrated S100+ melanoma cells (in brown). Similar results were obtained when injecting unfractionated, CD271$^-$ or CD271$^+$ cells derived from xenografted tumors from patient 205, without Matrigel (R). Scale bars represent 1 cm (C, E, F, L–O) or 100 μm (D, G, H, J, K, P, Q).

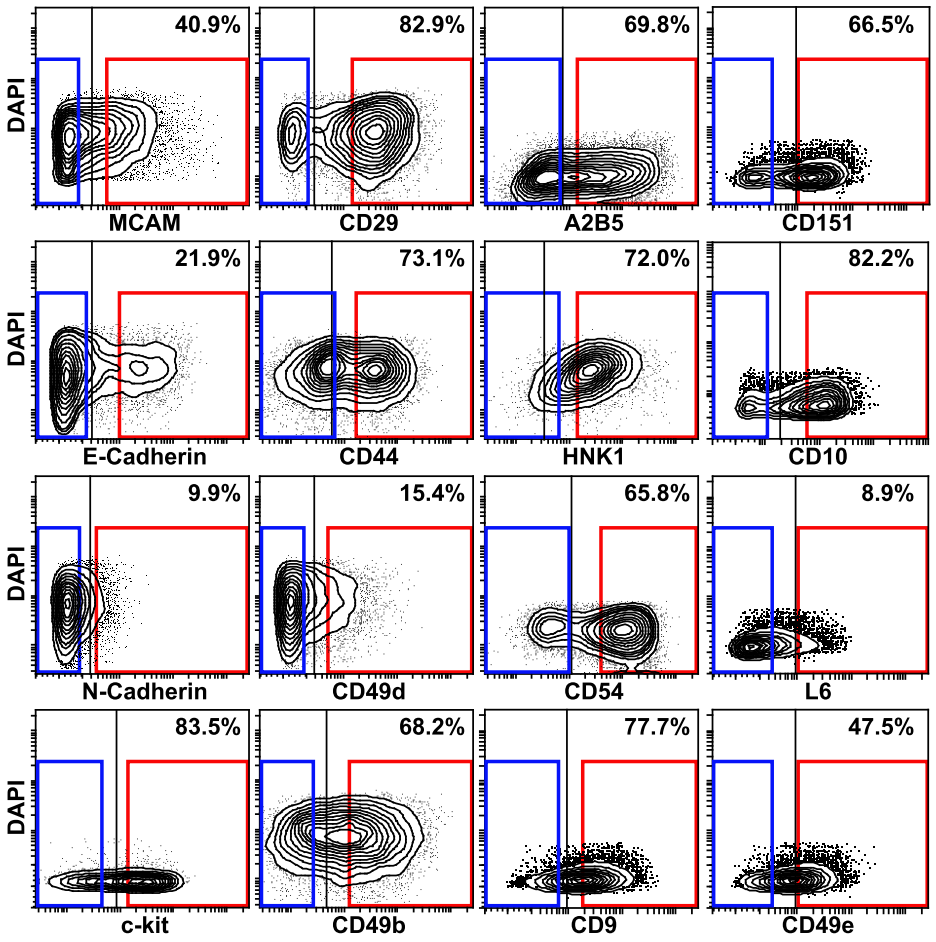
Many Phenotypically Diverse Melanoma Cells Can Recapitulate Tumor Heterogeneity

The capacity of tumorigenic cells to recapitulate the phenotypic heterogeneity of a parental tumor after transplantation is considered a defining characteristic of cancer stem cells. In contrast, phenotypic heterogeneity that arises from clonal evolution might lead to cells that form phenotypically distinct tumors that do not recapitulate the parental tumor. Given that melanoma cells with many different phenotypes were capable of forming tumors (Figure 3, Figure 4, and Figure 6), we tested whether these cells recapitulated the heterogeneity of parental tumors.

We evaluated the phenotypes of tumors that arose from marker-defined fractions of cells in the experiments described in Figures 3, 4, and Figure 6. For every marker tested, we found that secondary tumors were phenotypically similar to the parent tumor, irrespective of whether they arose from marker-negative/low cells or marker-positive/high cells. For example, melanoma cells were isolated based on ABCB5 expression from a tumor in which 3.8% of cells were ABCB5$^+$ (Figure 7A). Both ABCB5$^+$ and ABCB5$^-$ cells formed phenotypically similar tumors in NSG mice that recapitulated the heterogeneity of the parent tumor, with 4.2% to 5.4% of cells that were ABCB5$^+$ (Figure 7A). Similar results were observed in experiments that involved CD166 (Figure 7B), A2B5 (Figure 7C), CD151 (Figure 7D), CD54 (Figure 7E), CD44 (Figure 7F), CD9 (Figure 7G), CD29 (Figure 7H), N-Cadherin (Figure 7I), and CD271 (Figure 7J). This indicates that many melanoma cells are capable of recapitulating tumor heterogeneity after transplantation, irrespective of their phenotype at the time of transplantation. The capacity to recapitulate melanoma heterogeneity is therefore widely shared by many phenotypically diverse melanoma cells.

In some experiments, the marker-negative/low fraction and the marker-positive/high fraction both gave rise to heterogeneous tumors, but the tumors that arose from the positive

A



B

sorted fraction	#tumors/ #injections	sorted fraction	#tumors/ #injections	sorted fraction	#tumors/ #injections	sorted fraction	#tumors/ #injections
unfractionated	17/18	unfractionated	18/24	unfractionated	19/24	unfractionated	16/18
MCAM-	11/18	CD29$^{-/low}$	21/24	A2B5-	21/24	CD151-	13/18
MCAM+	12/18	CD29^{high}	21/24	A2B5+	21/24	CD151+	13/18
unfractionated	17/18	unfractionated	24/30	unfractionated	13/18	unfractionated	12/12
E-Cadherin-	7/12	CD44$^{-/low}$	29/30	HNK1$^{-/low}$	12/18	CD10-	9/12
E-Cadherin+	14/18	CD44^{high}	26/30	HNK1^{high}	9/18	CD10+	11/12
unfractionated	19/24	unfractionated	14/18	unfractionated	13/18	unfractionated	16/18
N-Cadherin-	19/24	CD49d-	15/18	CD54-	15/18	L6-	18/18
N-Cadherin+	18/24	CD49d+	10/15	CD54+	13/18	L6+	8/18
unfractionated	15/18	unfractionated	14/18	unfractionated	17/18	unfractionated	12/12
c-kit-	15/18	CD49b-	9/12	CD9-	17/18	CD49e-	11/12
c-kit+	14/18	CD49b+	14/18	CD9+	16/18	CD49e+	12/12

Figure 6. None of Sixteen Heterogeneously Expressed Markers Distinguished Tumorigenic from Nontumorigenic Melanoma Cells

(A) Analysis by flow cytometry of the heterogeneous expression of MCAM, CD29, A2B5, CD151, E-Cadherin, CD44, HNK1, CD10, N-Cadherin, CD49d, CD54, L6, c-kit, CD49b, CD9, and CD49e. The expression of each marker was analyzed in xenografted tumors derived from at least nine different patients (see Table S2 for details). Blue and red gates show the selection of marker$^{-/low}$ and marker$^{+/high}$ cells for transplantation studies (B), based on isotype labeling (indicated with a vertical line in each plot).

(B) Tumor formation after injection into NSG mice of 10 unfractionated, 10 marker$^{-/low}$, or 10 marker$^{+/high}$ cells isolated by flow cytometry from xenografted tumors obtained from three to five different patients (except for CD10 and CD49e, which were tested in cells from two different patients). Tumors readily formed from every fraction of cells such that no marker distinguished tumorigenic from nontumorigenic melanoma cells. See also Figure S2 and Table S2.

fraction of cells contained a higher percentage of marker positive cells. This was observed in some experiments performed with CD271 (data not shown), CD49e (Figure 7K), CD49f (Figure 7L), L1CAM (Figure 7M), E-cadherin (Figure 7N), and c-kit (Figure 7O). This suggests that there are sometimes genetic or epigenetic differences among phenotypically distinct human melanoma cells that bias the phenotype of the progeny they generate without preventing tumorigenesis or the re-establishment of

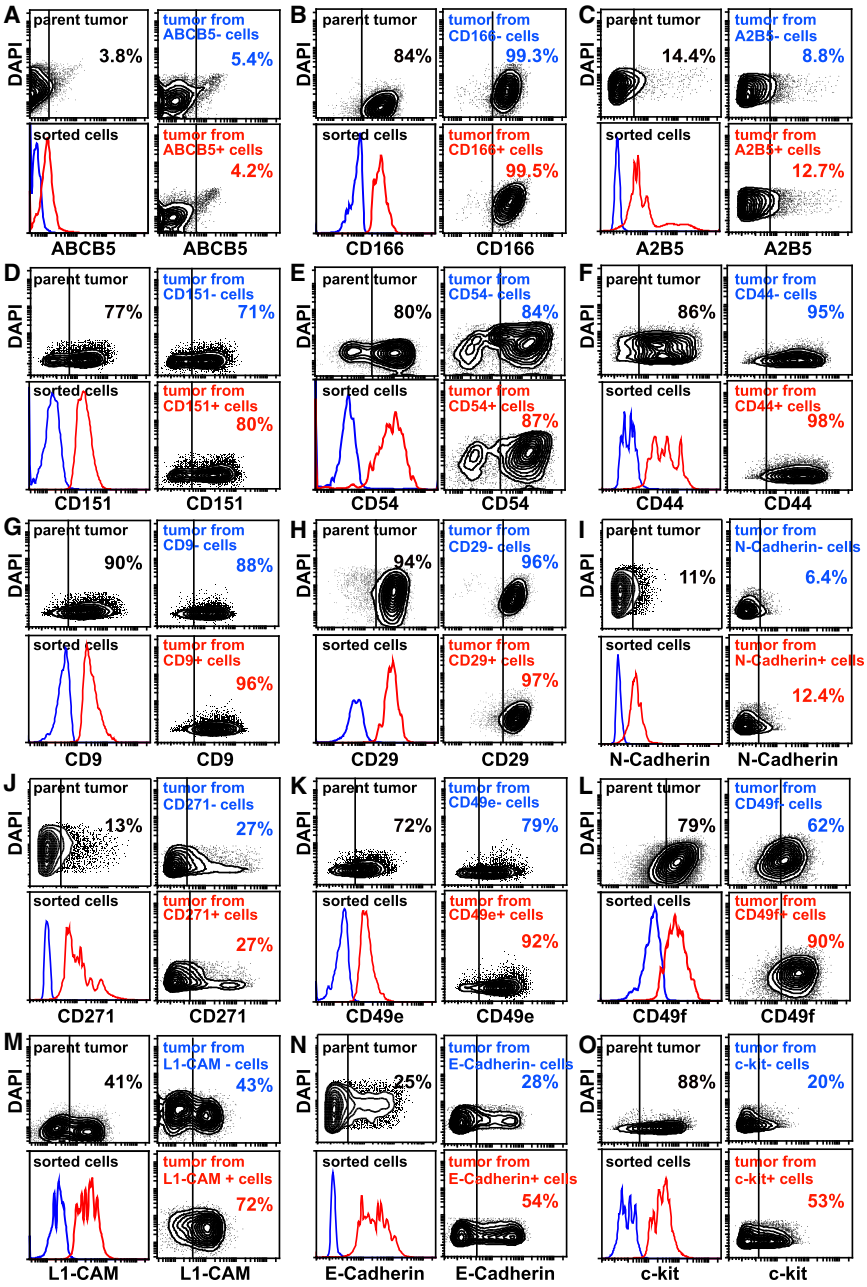


Figure 7. Many Phenotypically Distinct Fractions of Melanoma Cells Can Recapitulate the Heterogeneity of the Tumors from which They Derive

Expression of ABCB5 (A), CD166 (B), A2B5 (C), CD151 (D), CD54 (E), CD44 (F), CD9 (G), CD29 (H), N-Cadherin (I), CD271 (J), CD49e (K), CD49f (L), L1-CAM (M), E-Cadherin (N), and c-kit (O) in parent tumors (upper left) compared with expression in secondary tumors derived from marker$^{-/low}$ and marker$^{+/high}$ fractions (top right and bottom right, respectively). Bottom left panels show reanalyses of the sorted cell fractions used to generate secondary tumors. See also Figure S3. Every marker was tested in two to four separate melanomas, except for CD44, CD49f, E-Cadherin, and c-kit, which were tested in one.

heterogeneity. However, this was only observed in only a minority of experiments, suggesting that such biases are not a major driver of phenotypic heterogeneity.

The capacity of tumorigenic cells to recapitulate the heterogeneity of parental tumors has generally been tested with only a few (one to three) surface markers. We wondered whether analyses of larger numbers of surface markers might reveal phenotypic differences among tumors formed by phenotypically distinct pairs of cells from the same parental tumor. To test this, we analyzed some of the pairs of daughter tumors shown in Figure 7 with panels of 13–16 heterogeneously expressed surface markers. ABCB5$^-$ and ABCB5$^+$ cells not only formed secondary tumors that were indistinguishable with respect to ABCB5 expression, but these tumors were also similar with respect to CD166, CD54, L1-CAM, CD49b, CD49d, CD49f, A2B5, CD271, HNK1, MCAM, E-Cadherin, c-kit, CD44, CD133, and N-Cadherin expression (Figure S3A). Similar results were obtained with pairs of tumors formed by CD271$^-$ and CD271$^+$ cells (Figure S3B), CD54$^-$ and CD54$^+$ cells (Figure S3C), c-kit$^-$ and c-kit$^+$ cells (Figure S3D), L1-CAM$^-$ and L1-CAM$^+$ cells (Figure S3E), and CD44$^-$ and CD44$^+$ cells (Figure S3F). In a few cases, we observed differences in the percentage of cells that stained with individual markers. However, most markers exhibited similar staining patterns in secondary tumors, despite being derived from the transplantation of phenotypically distinct cells. These results suggest many markers are reversibly turned on and off within lineages of tumorigenic melanoma cells.

DISCUSSION

Our experiments suggest that melanoma does not adopt a hierarchy consisting of a minor subpopulation of tumorigenic cells and a majority population of nontumorigenic cells. Melanomas consistently contained high frequencies of tumorigenic cells, irrespective of whether they were primary cutaneous or metastatic melanomas, whether they were from stage II, III, or IV disease, and whether they were obtained directly from patients or after xenografting (Table 1 and Figure 1A, Figure 3A, and 4A). All tumorigenic cells appeared to have the capacity to proliferate indefinitely on serial transplantation (Figure 2).

We have not been able to identify any marker that robustly distinguishes tumorigenic from nontumorigenic melanoma cells despite examining 85 markers and carefully studying the tumorigenic potential of cells that differ in their expression of 22 heterogeneously expressed markers, including ABCB5

(Figure 3), CD271 (Figure 4; Table S1) and CD133 (Shackleton et al., 2009). For all of the markers we studied, tumors with similar growth rates readily arose from all fractions of cells (Figure 6; Figure S4). Despite subdividing melanoma cells using many markers we have been unable to identify any large subpopulation of melanoma cells that lacks tumorigenic potential. Thus, tumorigenic capacity is not restricted to a small subpopulation of melanoma cells but is widely shared among phenotypically diverse cells.

Many phenotypically distinct melanoma cells had the capacity to form tumors that recapitulated the phenotypic diversity of the tumors from which they derived (Figure 7; Figures S5–S10). This suggests that tumorigenic cells appeared to undergo reversible changes in the expression of many markers in vivo. This contrasts with models that attribute phenotypic heterogeneity to the hierarchical differentiation of cancer stem cells into nontumorigenic progeny or to irreversible genetic changes that arise through clonal evolution.

Our results are compatible with the idea that tumorigenic competence might reflect a reversible state in melanoma. Studies of breast cancer cell lines have suggested that tumorigenic activity correlates with the capacity to undergo an epithelial to mesenchymal transition and that cells might reversibly undergo such transitions (Mani et al., 2008). Studies of other cell lines have suggested that therapy resistance can also reflect a reversible state (Sharma et al., 2010). Recent studies of melanoma cell lines have indicated that the JARID1B histone demethylase, Brn2, and pigment are reversibly turned on and off within lineages of melanoma cells in a manner related to cell function (Pinner et al., 2009; Roesch et al., 2010). Transient exposure of glioblastoma cells to perivascular nitric oxide confers tumorigenic competence and stem cell properties, raising the possibility that these attributes reflect a reversible state in brain tumor cells (Charles et al., 2010). These studies make the prediction that in some cancers many cells will be capable of forming phenotypically diverse tumors, without robust hierarchical organization. Our study comprehensively tests this prediction in tumors from patients in vivo, finding that many phenotypic differences among melanoma cells reversibly change within lineages of tumorigenic cells rather than being hierarchically organized.

Although no marker robustly distinguished tumorigenic from nontumorigenic melanoma cells, we observed little tumorigenic activity among $CD271^+$ cells from two primary cutaneous melanomas. In this regard, our data are similar to results from primary mouse melanomas (Held et al., 2010) in that both studies found tumorigenic activity in a high percentage of single cells, but $CD271^-$ cells were more likely to form tumors. However, neither our results nor the results from Held et al. (2010) were consistent with the cancer stem cell model because Held et al. (2010) found that the tumorigenic cells they studied often did not recapitulate the heterogeneity of parental tumors, and the $CD271^+$ cells with limited tumorigenic activity in two tumors in our study represented only 2%–12% of tumor cells, a minor subpopulation of cancer cells.

Our results with CD271 are different from the results reported by Boiko et al. (2010) even though both studies used the same anti-CD271 antibody, both studied a similar spectrum of melanoma stages (mainly stage III), and both studied a combination of xenografted tumors and tumors obtained directly from patients. The most obvious potential explanation for the difference in results lies in the different assays used in the two studies: different enzymatic dissociation conditions (25 min in our study versus up to 3 hr in their study), different injection sites (subcutaneous versus intradermal), and different recipient mice (NSG versus $Rag^{-/-}IL2R\gamma^{-/-}$). An average of 1 in 50,000 unfractionated melanoma cells formed tumors in the study carried out by Boiko et al. (2010) (using four stage III, one stage IV, and one stage II melanoma, see Table S3 in Boiko et al., 2010). In contrast, when we transplanted unfractionated melanoma cells directly from six stage III patients in our prior study, an average of one in four cells formed tumors (Quintana et al., 2008). In our current study, an average of 28% of single, unfractionated melanoma cells obtained directly from five stage III melanoma patients (Table 1) and 17% (one in six) of melanoma cells obtained directly from a stage II melanoma patient (Table 1) formed tumors. Thus, the assay we used appears to be ~10,000-fold more sensitive than the assay used by Boiko et al. (2010). Using this more sensitive assay, we find that $CD271^-$ cells have at least as much ability to form tumors as $CD271^+$ cells. It will now be critical for other labs to independently assess whether they also observe tumor formation by $CD271^-$ melanoma cells.

Although our results argue against the cancer stem cell model in melanoma, they do not mean that other cancers do not follow a stem cell model. We and others have found that most chronic myeloid leukemias (Eisterer et al., 2005; Jamieson et al., 2004; Neering et al., 2007; Oravecz-Wilson et al., 2009) and acute myeloid leukemias (Bonnet and Dick, 1997; Lapidot et al., 1994; Yilmaz et al., 2006) do follow a cancer stem cell model in which leukemogenic cells are rare, phenotypically distinct from the vast majority of other leukemia cells, and robustly hierarchically organized. It will be critical to determine which cancers follow a stem cell model and which do not, so therapies designed to target rare subpopulations of cells are not inappropriately tested in patients whose disease is driven by many diverse cancer cells.

If a marker is identified in future that robustly distinguishes tumorigenic from nontumorigenic melanoma cells, melanoma would still be quite different from cancers, such as myeloid leukemia, that follow a stem cell model. Our observation that many markers are reversibly expressed by tumorigenic melanoma cells contrasts with the obvious morphologic and phenotypic differences between leukemogenic and non-leukemogenic cells. Thus, even if some melanomas do contain a hierarchy, it would be a shallow hierarchy that includes abundant and diverse tumorigenic cells rather than a steep hierarchy driven by rare tumorigenic cells, as described so far in cancers found to follow a stem cell model.

Some have suggested that cancer stem cells might be distinguished from nontumorigenic cancer cells by reduced immunogenicity, allowing them to proliferate more extensively by escaping immune detection (Schatton and Frank, 2009). However, this hypothesis is not testable in human cancers because they cannot be transplanted autologously into patients or into immunocompetent mice. Immunocompetent mice mount a powerful xenogeneic immune response against human cells, making it impossible to assess whether a failure to engraft reflects immune rejection or an intrinsic lack of tumorigenic

capacity. Furthermore, no xenotransplantation model recapitulates the autologous anticancer immune response that occurs in some patients against their own cancers because the xenogeneic immune response is far more powerful and driven by very different mechanisms than autologous immune responses. For these reasons, 30 years of research has found that the preferred system for studying the potential of normal human hematopoietic stem cells (Ito et al., 2002; Shultz et al., 2005) and human leukemic stem cells (Agliano et al., 2008; Sanchez et al., 2009) is NSG mice that are not only highly immunocompromised, but also irradiated to further promote the engraftment of human cells. The failure of human hematopoietic stem cells and leukemic stem cells to engraft in immunocompetent mice does not mean these cells are normally regulated by autologous immune responses in patients because the xenogeneic immune response that rejects these cells from mice involves very different mechanisms.

To identify the spectrum of human cancer cells that have the potential to contribute to disease, these cells must be studied in highly immunocompromised mice. Once the spectrum of cells capable of contributing to disease is identified, a separate and context-dependent question concerns which of these cells are actually fated to contribute to disease in a patient. This question can only be addressed in mouse cancers because no xenograft model reflects the anticancer immune response, or certain other aspects of the environment, in patients.

Our results demonstrate that phenotypic heterogeneity in melanomas obtained from patients is largely driven by reversible changes in a broad range of markers that turn on and off within lineages of tumorigenic cells. This phenotypic plasticity contrasts with both the cancer stem cell and clonal evolution models, which largely attribute heterogeneity to irreversible epigenetic and genetic changes. Although clonal evolution occurs in many cancers, including melanoma, and some cancers follow a stem cell model, our results raise the possibility that pervasive phenotypic plasticity is an independent source of heterogeneity in some cancers.

EXPERIMENTAL PROCEDURES

Tumor Cell Preparation

Melanoma specimens were obtained with informed consent from all patients according to protocols approved by the Institutional Review Board of the University of Michigan Medical School (IRBMED approvals 2004-1058 and 2000-0713). Tumors were mechanically dissociated with a McIlwain tissue chopper (Mickle Laboratory Engineering, Guilford, UK) before sequential enzymatic digestion in 200 U/ml collagenase IV (Worthington, Lakewood, NJ) for 20 min followed by 0.05% trypsin-EGTA for 5 min, both at 37°C. DNase (50–100 U/mL) was added to reduce clumping of cells during digestion. Cells were filtered (40 μm cell strainer) to obtain a single cell suspension. Dead cells and debris were reduced by density centrifugation (1.1 g/ml Optiprep; Sigma, St. Louis, MO) when necessary. To test ABCB5 expression, single cell suspensions were derived after incubation of mechanically dissociated tumor tissue in 10 ml sterile PBS containing 0.1 g/L calcium chloride and 5 ug/ml Collagenase Serva NB6 (SERVA Electrophoresis GmbH) for 3 hr at 37°C (Schatton et al., 2008).

Cell Labeling and Flow Cytometry

All antibody staining was carried out for 20 min on ice, followed by washing and centrifugation. A list of primary antibodies is in Table S2. Anti-ABCB5 antibody (clone 3C2-1D12) was a gift from Markus Frank. Secondary antibodies were conjugated to phycoerythrin (goat anti-mouse IgG or IgM, goat anti-rat IgG, or goat anti-rabbit IgG; Jackson ImmunoResearch, West Grove, PA). Primary isotype controls followed by the same secondary antibodies were used to set background. Cells were subsequently stained with directly conjugated antibodies to human CD45 (HI30-APC; BD Biosciences, San Jose, CA), human CD31 (WM59-APC; eBiosciences, San Diego, CA), and Glycophorin A (HIR2-APC; Biolegend, San Diego, CA) (for tumors obtained directly from patients) or mouse CD45 (30-F11-APC; eBiosciences), mouse CD31 (390-APC; Biolegend), mouse Ter119 (TER-119-APC; eBiosciences) and human HLA-A,B,C (G46-2.6-FITC; BD Biosciences) (for xenograft tumors) to select live human melanoma cells and to exclude endothelial and hematopoietic cells. Cells were resuspended in 10 μg/ml DAPI (Sigma) and analyzed and/or sorted on a FACSAria Cell Sorter (Becton Dickinson, San Jose, CA). After sorting, an aliquot of sorted cells was always reanalyzed to check for purity, which was usually >95%.

Transplanting Melanoma Cells

After sorting, cells were counted and resuspended in staining medium (L15 medium containing 1 mg/ml BSA, 1% penicillin/streptomycin and 10 mM HEPES [pH7.4]) with 25% high protein Matrigel (product 354248; BD Biosciences). Subcutaneous injections of human melanoma cells were performed in each flank and the interscapular region of NOD.CB17-Prkdcscid Il2rgtm1Wjl/SzJ (NOD/SCID IL2Rγ^{null}, NSG) mice (Jackson Laboratories) according to protocols approved by the Committee on the Use and Care of Animals at the University of Michigan (protocol 9055). Tumor formation was evaluated regularly after injection by palpation of injection sites, and tumor diameters were measured with calipers. The presence of human melanomas was confirmed at necropsy by gross appearance, histology, and immunohistochemistry.

Injection of Single Melanoma Cells

Single cells were isolated and identified using methods described previously (Quintana et al., 2008). Briefly, sorted melanoma cells were diluted and aliquoted into 10- μl microwells (Thermo Fisher Scientific, Roskilde, Denmark). Plates were centrifuged at 450 × g for 30 s, and wells containing single cells were identified by phase microscopy. Cell doublets could be identified easily, were rare, and were discarded. Each single cell was drawn into a syringe containing high protein Matrigel (product 354248, BD Biosciences), the well was visually confirmed to no longer contain the cell, and the cell was injected into a NSG mouse as described above.

Histopathology and Immunostaining

Portions of melanoma tumors and mouse organs used in experiments were fixed in 10% neutral buffered formalin, paraffin embedded, sectioned, and stained with hematoxylin and eosin for histopathology analysis. Paraffin-embedded tumors were confirmed as melanomas by staining for S100 expression after quenching endogenous peroxidase activity. Binding of anti-S100 antibody (DAKO) was carried out for 30 min at room temperature, detected by anti-rabbit secondary (30 min at room temperature) and revealed using DAB Chromagen. S100-stained slides were counterstained with hematoxylin.

Statistics

Tumor growth rates were determined by maximum tumor diameter (in mm) at euthanasia divided by elapsed time (in weeks) from injection. Differences between mean growth rates were compared using unpaired Student's t tests. Correlations between tumor growth rates (Figure 1B) or CD271$^+$ cell frequencies (Figure 4B) and tumorigenic cell frequencies were carried out by linear regression analysis using GraphPad Prism 3.0 software. Limiting dilution analyses were carried out using ELDA: Extreme Limiting Dilution Analysis (Hu and Smyth, 2009). Melanoma-initiating cell frequencies were compared using likelihood ratio tests. Statistical significance was defined as $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and two tables and can be found with this article online at doi:10.1016/j.ccr.2010.10.012.

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