Cancer Research

CD271 Expression on Patient Melanoma Cells Is Unstable and Unlinked to Tumorigenicity

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Abstract

The stability of markers that identify cancer cells that propagate disease is important to the outcomes of targeted therapy strategies. In human melanoma, conflicting data exist as to whether hierarchical expression of CD271/p75/NGFR (nerve growth factor receptor) marks cells with enriched tumorigenicity, which would compel their specific targeting in therapy. To test whether these discrepancies relate to differences among groups in assay approaches, we undertook side-by-side testing of published methods of patient-derived melanoma xenografting (PDX), including comparisons of tissue digestion procedures or coinjected Matrigel formulations. We found that $CD271^-$ and $CD271^+$ melanoma cells from each of seven patients were similarly tumorigenic, regardless of assay variations. Surprisingly variable CD271 expression patterns were observed in the analyses of sibling PDX tumors ($n = 68$) grown in the same experiments from either $CD271^-$ or $CD271^+$ cells obtained from patients. This indicates unstable intratumoral lineage relationships between $CD271^-$ and $CD271^+$ melanoma cells that are inconsistent with classical, epigenetically based theories of disease progression, such as the cancer stem cell and plasticity models. SNP genotyping of pairs of sibling PDX tumors grown from phenotypically identical $CD271^-$ or $CD271^+$ cells showed large pairwise differences in copy number (28%–48%). Differences were also apparent in the copy number profiles of $CD271^-$ and $CD271^+$ cells purified directly from each of the four melanomas (1.4%–23%). Thus, CD271 expression in patient melanomas is unstable, not consistently linked to increased tumorigenicity and associated with genetic heterogeneity, undermining its use as a marker in clinical studies. *Cancer Res; 76(13); 3965–77. ©2016 AACR.*

Introduction

Extensive effort has been made across a wide range of cancers to identify markers of cells that propagate malignant disease. Indeed, the effectiveness of targeted cancer therapies depends on identifying markers in or on cancer cells that are reproducibly associated with malignant behaviors. Some of the most useful markers are those generated by genetic mutation. In some melanomas, for example, mutated BRAF is a stable, ubiquitous marker (1) that dominantly drives disease progression (2). This necessitates the continued targeting of BRAF even in treatment approaches that seek to overcome mechanisms of BRAF inhibitor resistance (e.g., ClinicalTrials.gov identifier: NCT02159066).

Markers related to epigenetically driven malignant states are also potential therapy targets. For example, propagation of melanoma cell lines is abrogated by targeting the histone H3 lysine 4 demethylase JARID1B, which otherwise reversibly drives slow-cycling, tumorigenic cell subpopulations (3). Markers expressed specifically on cancer stem cells (CSC) are particularly appealing targets in cancers that follow a CSC model (4). However, the usefulness of markers that define epigenetically driven mechanisms of disease progression depends on their stability, which is seldom tested in uncultured cancer cells. The targeting in patients of cancer markers that are not consistently expressed and/or that do not consistently define malignant states in cancer cells will not offer substantial clinical benefit.

In melanoma, cells distinguished by differences in expression of the neural crest stem cell marker CD271/p75/NGFR were shown by multiple groups (5, 6) to have differing abilities for tumor formation in patient-derived xenograft (PDX) assays. In these studies, $CD271^+$ cells were more tumorigenic than $CD271^-$ cells, and analyses of secondary tumors suggested hierarchical relationships between $CD271^-$ and $CD271^+$ cells, consistent with a CSC model. Studies of cell lines also found enriched tumorigenicity among slow-cycling fractions of $CD271^+$ melanoma cells (7) and a functional role for CD271 in disease propagation (8).

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However, these data have not been universally reproducible. Using highly efficient PDX assays (9), none of 16 cell surface markers, including CD271, was found to identify melanoma cells with enriched tumorigenic potential (10). Moreover, regardless of the phenotypes of injected cells, expression of numerous markers in secondary PDX tumors largely recapitulated expression patterns in parental melanomas, consistent with plastic rather than hierarchical relationships between marker-defined melanoma cell subpopulations (11, 12). Understanding why these data differ from previous studies is essential to determine whether CD271 is consistently linked to melanoma cell tumorigenicity in a manner that would compel its testing, and/or the testing of $CD271^+$ melanoma cells, as therapeutic targets.

Proposed (6, 13, 14) reasons for these disparate data include differences in the PDX tumorigenesis assays used in each study (e.g., use of trypsin by Quintana and colleagues; ref. 9), as the frequencies of tumorigenic cells in melanomas varied across studies by 2 to 3 orders of magnitude (13). However, other explanations are also plausible. For example, the melanomas tested might have varied biologically, with some following a CSC model and others not. In addition, in testing cancer cell hierarchies by evaluating marker reexpression in transplanted tumors, stochastic tumor-to-tumor variation might confound comparisons of studies that evaluated only small numbers of tumors.

To test CD271 stability and to examine variations in PDX melanoma assays, we evaluated tumorigenic potential and CD271 expression in melanoma cells prepared and transplanted side-by-side under different assay conditions. Regardless of the assay, we were unable to find evidence that melanoma follows a CSC model associated with CD271 expression. Moreover, substantial variation was seen among CD271 reexpression patterns and in the genotypes of sibling PDX tumors, and genetic differences were apparent between $CD271^-$ and $CD271^+$ cells in the same melanomas. These data indicate that the specific targeting of CD271-expressing cells is unlikely to improve melanoma therapy, and that *in vivo* marker expression in uncultured human cancer cells may be far less predictable than is typically assumed.

Materials and Methods

Tumor cell preparation

Patient melanomas were obtained with consent under Peter MacCallum Cancer Centre Human Research Ethics Committee protocol 10/02. Tumors were mechanically dissociated with a McIlwain tissue chopper (Mickle Laboratory Engineering). Enzymatic tumor dissociations were performed according to published methods (5, 6, 9; see Supplementary Methods). Digestion of PDX melanomas and patient melanomas was identical.

Cell labeling and flow cytometry

Antibody labeling was performed for 40 minutes on ice. Cells from patient samples were stained with directly conjugated antibodies to human HLA-A, B, C (1:5, G46-2.6-FITC, BD Pharmingen), human CD45 (1:5, HI30-APC, BD Pharmingen), human CD31 (1:800, WM59-APC, eBioscience) and CD235a [glycophorin A; 1:2000, GA-R2 (HIR2)-APC, BD Pharmingen] to enable selection of $HLA^+CD45^-CD31^-CD235a^-$ (Lin^-) cells (Supplementary Fig. S3). Cells from PDX tumors were stained with directly conjugated antibodies to human HLA-A, B, C (as above), mouse CD45 (1:200, 30-F11-APC, BD Pharmingen), mouse Ter119 (1:100, TER119-APC, BD Pharmingen), and mouse CD31 (1:100 APC, 390-APC, eBioscience). For detecting CD271, anti-human CD271 (1:33, NGFR-PE, Miltenyi Biotec) was added. The Lin^-CD271^- gate was defined on the basis of control cells labeled with Lin markers but not with anti-CD271. Labeled cells were resuspended in 10 μg/mL DAPI (Roche) and analyzed and/or sorted (Supplementary Fig. S3) on a FACSAria Cell Sorter (Becton Dickinson) with a 70-μm nozzle. Sorted cells were routinely reanalyzed for purity, which was typically >95%.

Tumorigenesis assays

After sorting, cells were counted and resuspended in staining media with 25% high concentration (HC) Matrigel (BD Biosciences) unless otherwise indicated. Animal experiments were performed under Peter MacCallum Cancer Centre Animal Ethics and Experimentation Committee protocols E421 and E526. Subcutaneous injections were performed in each flank and in the interscapular region of NOD/CB17Prkdcscid Il2rgtm1 Wjl/SzJ (NOD/SCID $Il2rg^{-/-}$, NSG) mice (Jackson Laboratories). Tumors were evaluated weekly by palpation and caliper measurement.

IHC

Primary antibodies were monoclonal mouse anti-CD133 (gift from Andreas Behren, Olivia Newton-John Cancer Research Institute, Australia), polyclonal rabbit anti-JARID1B (Novus Biologicals, NB100-97821), polyclonal rabbit anti-ABCB5 (Sigma, HPA026975), and monoclonal rabbit anti-ALDH1A1 (Abcam, ab52492), or isotype controls. Sections were heated at 60°C for 30 minutes and dewaxed using a Leica Jung autostainer XL. Antigen retrieval was performed in trisodium citrate buffer at 125°C for 3 minutes, followed by a wash in Tris-buffered saline and 0.1% Tween-20 (TBST). Sections were incubated in 3% peroxidase block for 20 minutes, washed in TBST, and then blocked with 1%–2% horse serum. Primary antibodies were incubated at room temperature for 60 minutes or at 4°C overnight. Sections were washed in TBST, incubated for 45 minutes in ImmPRESS HRP anti-mouse or anti-rabbit Ig (peroxidase) polymer detection (Vector Laboratories, MP-7402), and washed again. Signal detection was performed with 1–2 drops of AEC substrate-chromogen (Dako, K3464), followed by rinsing in water and counter staining with hematoxylin. Sections were cover slipped with Aquatek. Quantification of marker expression was performed by counting cells in random 20× fields of 800–1,500 cells per field using the cell counter plugin of ImageJ (http://rsb.info.nih.gov/ij/).

DNA/RNA isolation and cDNA synthesis

DNA was extracted from pellets of cells (40,000–1,000,000 cells) purified by flow cytometry (Supplementary Fig. S3). DNA was extracted using DNeasy Blood and Tissue kits (Qiagen). Samples were eluted twice with 20–50 μL of AE buffer. DNA concentration and purity were measured on a NanoDrop2000 UV-Vis spectrophotometer (Thermo Scientific). RNA was extracted from cells using PureLink RNA Mini kits (Ambion). cDNA was synthesized using a single step protocol SuperScript VILO cDNA Synthesis Kit (Invitrogen), with input RNAs ranging from 6–8 ng per reaction.

qRT-PCR

qRT-PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems) on a StepOne Plus Real Time PCR System (Applied Biosystems), using commercial primer kits (Hs_GAPDH_2_SG QuantiTect Primer Assay, Hs_NGFR_1_SG QuantiTect Primer Assay (Qiagen). Data were analyzed using

StepOne Plus Real Time LinRegPCR software and normalized to GAPDH expression.

Statistical analysis

Tumor growth rates were determined by maximum tumor diameter (in mm) divided by time elapsed (in weeks) from the date tumors first became palpable. Differences between mean growth rates were compared using unpaired *t* tests. Non-normally distributed datasets were log-transformed prior to performance of *t* tests. Tumorigenic cell frequencies were calculated using ELDA (extreme limiting dilution analysis; ref. 15). To account for false negative tumor engraftments due to premature death of mice, mice were excluded that died without any tumors before the mean time from injection to palpability for all tumors in the same experiment.

SNP Genotyping

Genomic DNA (200 ng) was diluted to 50 ng/μL and genotyped on Illumina Beadchip HumanOmni2.5-8 (∼2.5M SNP loci) or HumanOmniExpress (∼700k SNP loci) arrays in the Australian Genome Research Facility. Data were analyzed using a novel method, the *t* statistic (see Supplementary Methods).

Results

Detection of CD271 expression in melanoma cells is not affected by trypsin-based tumor dissociation

To investigate effects of tumor dissociation, we dissociated the same melanomas according to three different protocols [Q (9), C (6) and B (5)] used previously to test the CD271-based CSC model (Fig. 1A, Supplementary Fig. S1, and Supplementary Methods). Differences included use in the Q protocol of a brief 0.05% trypsin–EGTA incubation, which was proposed to explain differences between studies (6, 14). Patient or PDX melanomas (Supplementary Fig. S2) were minced and evenly divided into three tubes prior to side-by-side dissociation (Fig. 1A). No consistent differences in cell yields were observed (Fig. 1B and C).

If use of trypsin in melanoma dissociation impairs binding to CD271 by anti-CD271 antibodies, as proposed (6, 14), then the trypsin-containing Q protocol should yield fewer flow cytometry–detected $CD271^+$ cells than the C and B protocols (6). We thus labeled cells with antibodies to allow specific gating of human melanoma cells (Supplementary Fig. S3; ref. 10) and CD271-expressing cells. Unlike previous studies (6), trypsin-based dissociation did not consistently reduce proportions of $CD271^+$ cells (Fig. 2A). Consistent with this, no differences in *CD271* mRNA levels were detectable in $CD271^-$ cells purified after dissociation via the Q, B, or C protocols (Fig. 2B). These data indicate that melanoma dissociation with low concentration trypsin does not affect the flow cytometry–based detection of CD271 on human melanoma cells.

Robust tumor formation from $CD271^-$ and $CD271^+$ melanoma cells regardless of tumor dissociation method

We next asked whether the method of melanoma dissociation could affect tumorigenic potential in unselected $CD271^-$ or $CD271^+$ melanoma cells. Unfractionated Lin^- cells, $CD271^-$ cells, and $CD271^+$ cells were isolated by flow cytometry from seven patient melanomas that had been dissociated side-by-side in the Q, C, or B protocols. Sorted cells were mixed in 25% high concentration Matrigel and transplanted subcutaneously into NOD/SCID $Il2rg^{-/-}$ (NSG) mice (Fig. 3A). The frequencies of cells with tumorigenic potential in each isolated cell fraction were calculated using limit dilution analysis (15) (Fig. 3B and Supplementary Fig. S4).

In all experiments, tumor formation was routinely observed from injections of as few as ten Lin^-, $CD271^-$, or $CD271^+$ cells, regardless of the method of tumor dissociation. Examples of this are shown in Fig. 3A. Across all experiments, the empirically determined frequency of tumorigenic Lin^- cells was 1/63 when evaluated following dissociation in the Q protocol, compared with 1/113 and 1/127 for the C and B protocols, respectively (Fig. 3B; $\chi^2P > 0.05$ for all comparisons). For both $CD271^-$ and $CD271^+$ cells, tumors formed robustly and with similar regularity for all numbers of all phenotypes of injected cells. In some experiments, $CD271^+$ cells were more likely to form tumors than $CD271^-$ cells (e.g., in melanoma 12-1036 dissociated via B method, tumorigenic cell frequencies were 1/273 in $CD271^-$ cells and 1/149 in $CD271^+$ cells; $\chi^2P = 0.5$; Supplementary Fig. S4). However, the opposite was observed in other experiments (e.g., in melanoma 12-1036 dissociated via C method, tumorigenic cell frequencies were 1/67 in $CD271^-$ cells and 1/121 in $CD271^+$ cells; $\chi^2P = 0.2$, Supplementary Fig. S4). Across all transplants, tumor formation was more efficient from cells dissociated by the Q and C methods (tumorigenic cell frequencies 1/71 and 1/119, respectively) than by the B method (tumorigenic cell frequency 1/170; $\chi^2P < 0.001$). Consistent with previous data (10), no differences were observed at necropsy between rates of metastasis in NSG mice with tumors generated from $CD271^-$ or $CD271^+$ cells (Fig. 3C).

Effects of Matrigel formulation on PDX tumor formation from purified melanoma cells

Matrigel is an extracellular matrix compound that enhances the tumorigenicity of human melanoma cells in NSG mice (9). It contains numerous structural proteins and growth factors that can impact cell biology assays (16–18). As published assays of CD271-associated melanoma tumorigenesis used different commercially available preparations of Matrigel [standard (5, 6) or high concentration (HC; ref. 10)], we next tested the effects of Matrigel formulation on melanoma cell tumorigenicity in PDX assays.

Cells were dissociated from each of three melanomas according to the Q protocol. Flow cytometrically purified Lin^- cells were evenly divided prior to side-by-side resuspension into 25% final concentrations of one of four different Matrigel formulations (HC, standard, HC growth factor–reduced, and HC phenol red–free) and injection into NSG mice. Although no differences in tumor formation were noted among cells transplanted with HC, standard, and HC phenol red–free Matrigel, HC growth factor–reduced Matrigel revealed tumorigenic potential in 2- to 3-fold more melanoma cells than the other formulations (Fig. 4A).

To test whether published differences in Matrigel formulation affect tumorigenicity associated with CD271 expression, additional aliquots of the same Lin^-, $CD271^-$, and $CD271^+$ cells from two of the experiments in Fig. 3B (Q dissociation protocol) were injected after side-by-side resuspension in 30% standard Matrigel (5, 6) or 25% high concentration Matrigel (9, 10). Tumor formation and the growth rates of tumors were similar regardless of the type of Matrigel used or of the phenotype of injected cells (Fig. 4B; $P > 0.2$ for all comparisons).

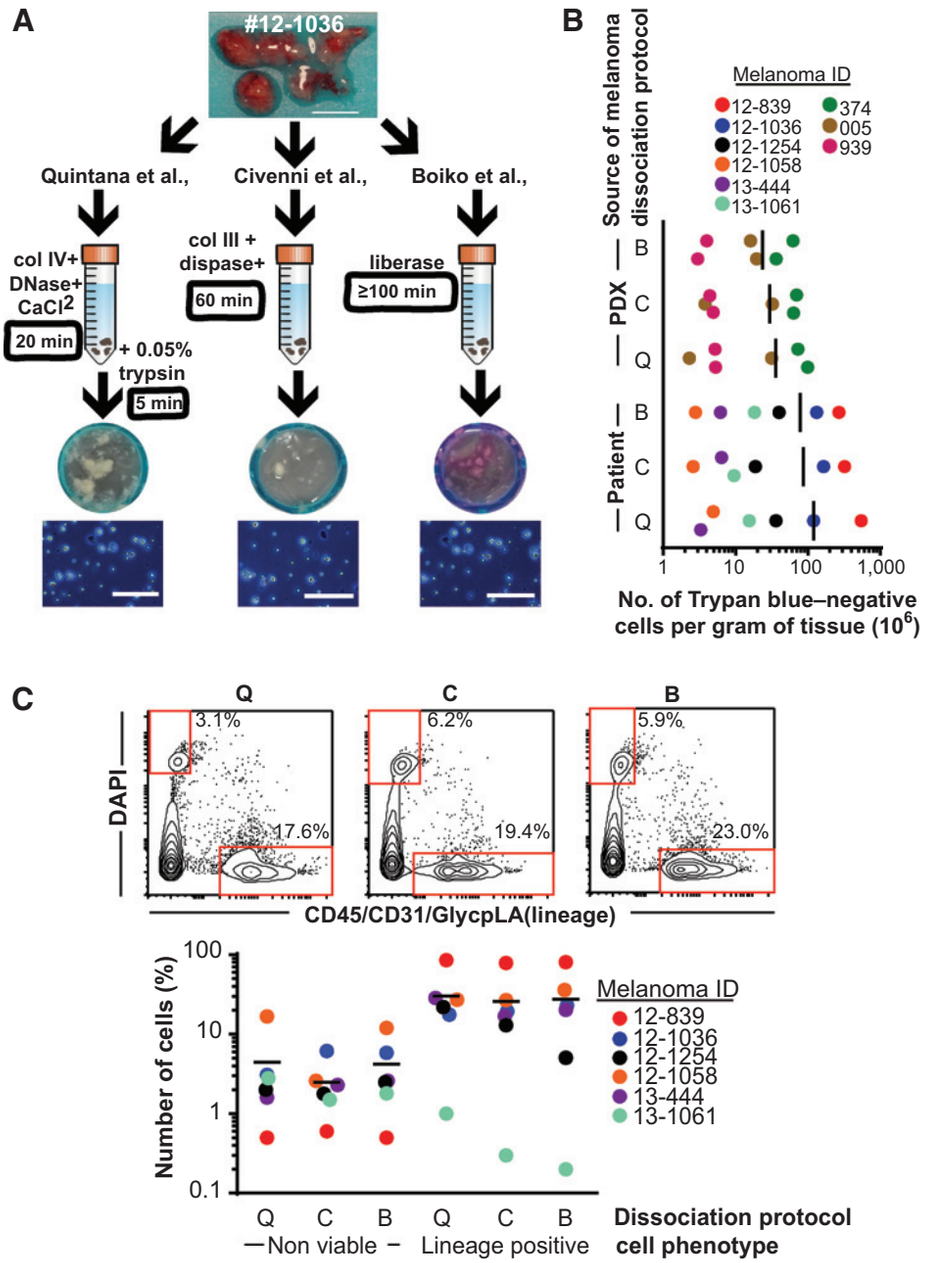


Figure 1.
Comparable yields of cells across different methods of melanoma dissociation. A, side-by-side dissociation according to different enzymatic methods [Quintana and colleagues (Q; ref. 9), Civenni and colleagues (C; ref. 6), Boiko and colleagues (B; ref. 5)] of a lymph node metastasis (top; bar, 1 cm) from patient 12-1036. The tumor was chopped, mixed, separated equally, and dissociated. Dissociated cells were filtered (bottom circles show filter membranes) and stained with Trypan blue (bottom; bars, 50 μm). B, yields of viable cells from patient ($n = 6$) and PDX ($n = 6$) melanomas dissociated according to different published methods (Q, C, and B). Dots, individual values, color-coded according to patient ID; lines, average values ($P > 0.05$, t test). C, yields of nonviable and nonmelanoma cells from six patient melanomas dissociated according to different published methods (Q, C, and B). Top, flow cytometry analyses of cells from the same melanomas dissociated via methods Q, C, and B and labeled with "lineage markers" (CD45, CD31, and CD235a) and DAPI. Red boxes, percentages of nonviable cells (DAPI$^+$) and nonmelanoma cells (lineage$^+$), respectively. Bottom, proportions of nonviable (left) and nonmelanoma (right) cells in the gated populations (see Supplementary Fig. S3) of six patient melanomas dissociated side-by-side according to Q, C, or B methods. Dots, individual values, color-coded according to patient ID; lines, average values ($P > 0.05$, t test). No consistent differences were observed according to dissociation method.

Serial transplantability of CD271$^-$ and CD271$^+$ cells from PDX melanomas

We also tested whether CD271 expression might be linked to the ability of melanoma cells to renew their malignant potential through serial transplantation. Secondary PDX tumors grown from CD271$^-$ or CD271$^+$ patient melanoma cells were dissociated (Q method) and cells labeled with anti-CD271 antibody prior to analysis by flow cytometry. Notably and consistent with previous studies (7, 10), CD271 was heterogeneously expressed in secondary tumors regardless of the CD271 phenotype of cells that give rise to them (Fig. 5).

CD271$^-$ and CD271$^+$ cells were purified from these secondary tumors and transplanted into tertiary recipients. For melanoma 12-1036, tertiary transplantation was unusually inefficient from a secondary tumor derived from CD271$^-$ cells, whereas both CD271$^-$ and CD271$^+$ cells from a CD271$^+$ cell–derived secondary tumor were readily passaged (Fig. 5B). However, in another melanoma (13-061), cells from secondary tumors were similarly and efficiently transplantable regardless of their CD271 content and regardless of the CD271 phenotype of cells that produced them. Thus, the serial transplantability of uncultured melanoma cells in these experiments was not consistently linked to CD271 expression.

CD271 reexpression is highly variable in secondary PDX melanomas

Evaluating marker reexpression in secondary tumors is essential for testing the CSC model. In the CD271-based CSC model of melanoma progression (5, 6), CD271$^+$ cells sit atop the hierarchy,

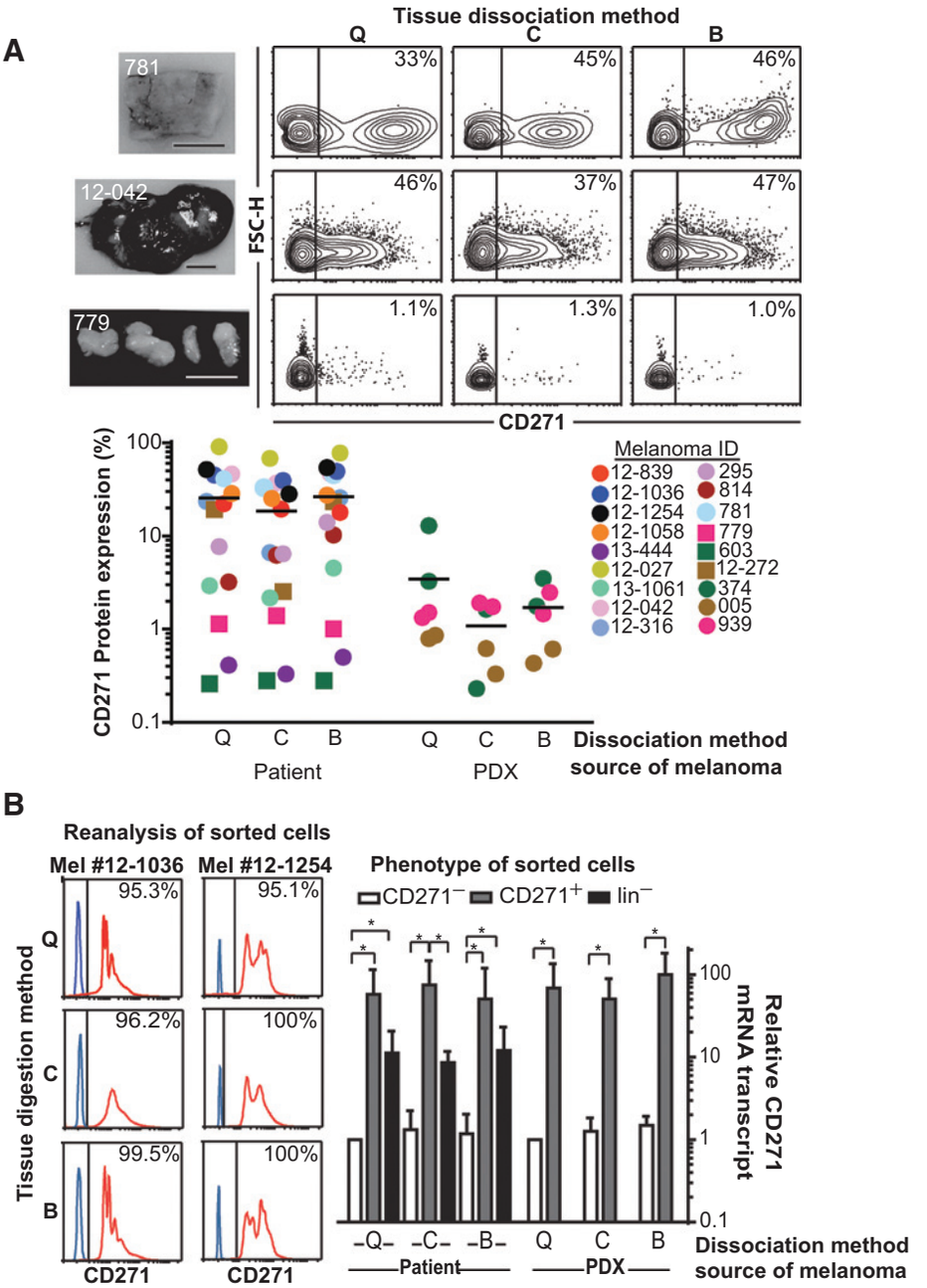
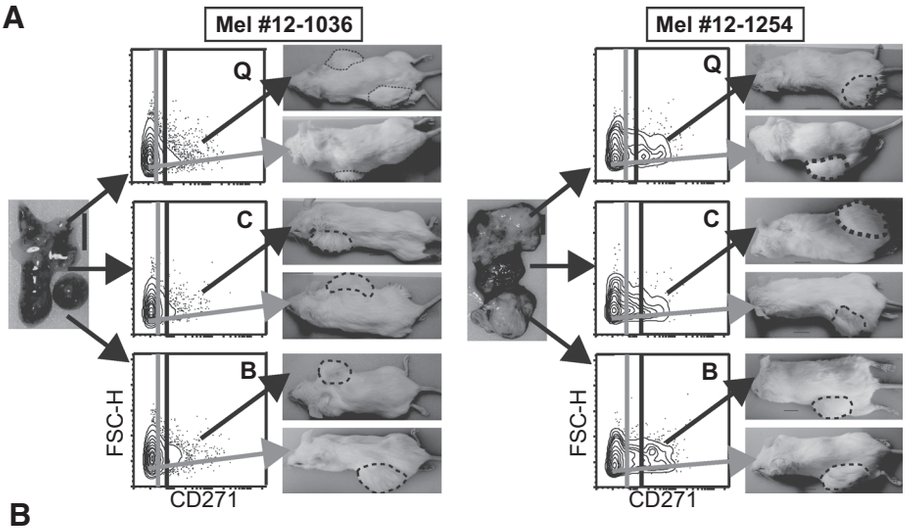


Figure 2.
Detection of CD271 in dissociated melanomas cells does not vary according to trypsin exposure. A, top, flow cytometry evaluation of CD271 expression in three patient melanomas (781, 12-042 and 779; bars, 1 cm) after side-by-side dissociation according to different published methods (Q, C, and B). Percentages of CD271$^+$ cells are shown, defined by unstained controls. Bottom, CD271 detection by flow cytometry in patient ($n = 12$) and PDX ($n = 6$) melanomas following side-by-side dissociation according to published methods (Q, C, and B). Dots, individual values, color-coded according to patient ID; lines, average values. A consistent effect was not observed of the method of cell dissociation on CD271 protein detection by flow cytometry ($P > 0.05$, t test). B, CD271 mRNA expression in CD271$^-$ and CD271$^+$ cell fractions purified by flow cytometry. Left, histograms showing purities of sorted CD271$^-$ and CD271$^+$ cells derived from two patient melanomas (12-1036 and 12-1254) following side-by-side dissociation according to published methods (Q, C, and B). Purities of sorted cells in these experiments were usually >95%. Right, fold-change differences in CD271 mRNA expression according to cell phenotype and method of tumor dissociation in patient ($n = 4$) and PDX melanomas ($n = 5$). In each experiment, CD271 mRNA expression was referenced to expression in Q method CD271$^-$ cells. CD271 mRNA levels are shown as means ± SD; *, $P < 0.02$, t test. No differences were apparent in CD271 mRNA expression in CD271$^-$ cells purified from the same melanomas via Q, C, or B dissociation methods. Quintana and colleagues (Q; ref. 9), Civenni and colleagues (C; ref. 6), Boiko and colleagues (B; ref. 5).

such that CD271$^+$ cells generate progeny that recapitulate the CD271 heterogeneity of the parental tumor, whereas CD271$^-$ cells produce no or very few CD271$^+$ cells. As published studies (6, 10) only evaluated CD271 reexpression in small numbers of PDX tumors derived from CD271$^-$ and CD271$^+$ melanoma cells, with contrasting results, we wondered whether studying more tumors would reveal unexpected variation in CD271 reexpression patterns.

We thus evaluated CD271 expression in most ($n = 68$) of the PDX tumors shown in Figs. 3 and 4 that were derived from transplantations of Lin$^-$, CD271$^-$, and CD271$^+$ cells isolated from two patient melanomas (12-1036 and 12-1254). Although a small proportion of tumors derived from CD271$^-$ and CD271$^+$ cells had CD271 expression patterns consistent with hierarchical relationships between these cells in the parental melanoma (e.g., tumors ST11 and ST14 in Supplementary Fig. S5), most did not. One tumor derived from CD271$^+$ cells and one from Lin$^-$ cells contained no CD271$^+$ cells. All other tumors displayed heterogeneity of CD271 expression, regardless of the phenotype of injected cells that generated them (Fig. 6B).

However, in contrast to studies that found stable, plastic equilibration of markers in melanoma (3, 10), the proportions of



B

Dissociation protocol	Cell phenotype	Number of tumors/ number of injections: Cells per injection 1,000	50–100	10	Melanoma-initiating cell frequency (95% confidence interval)	
Q	CD271$^-$	5/5	18/30	6/30	1/88	(1/58–1/135)
	CD271$^+$	6/6	18/25	7/20	1/61	(1/39–1/96)
	Lin$^-$	6/6	14/21	7/13	1/63	(1/38–1/103)
	Total	17/17	50/76	20/63	1/71	(1/55–1/92)
C	CD271$^-$	5/5	14/24	3/15	1/98	(1/59–1/162)
	CD271$^+$	5/5	10/22	1/23	1/158	(1/88–1/285)
	Lin$^-$	5/5	12/24	4/12	1/113	(1/67–1/190)
	Total	15/15	36/70	8/50	1/119	(1/88–1/162)
B	CD271$^-$	4/4	11/29	2/12	1/173	(1/100–1/298)
	CD271$^+$	4/4	7/21	1/17	1/216	(1/111–1/418)
	Lin$^-$	4/4	7/15	3/18	1/127	(1/67–1/242)
	Total	12/12	25/65	6/47	1/170	(1/119–1/242)

C

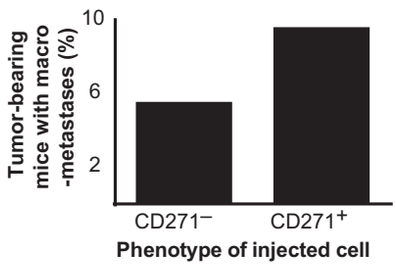


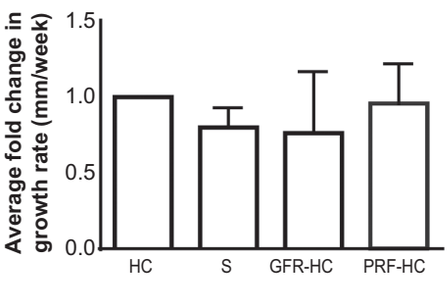
Figure 3.
Lack of effect of tumor dissociation method on CD271-associated tumorigenicity in human melanoma cells. A, tumor formation in NSG mice from 10–1000 flow cytometrically purified CD271$^-$ or CD271$^+$ cells following side-by-side dissociation of lymph node metastases from patients 12-1036 (left) and 12-1254 (right; bars, 1 cm) according to different methods (Q, C, and B; see Fig. 1), followed by antibody labeling. Cells were sorted according to CD271 expression (dot plots show gating strategies) and injected subcutaneously into NSG mice. Tumors (within dotted lines) formed robustly from CD271$^-$ and CD271$^+$ cells regardless of the method used to dissociate cells. B, summary of tumorigenesis data from the subcutaneous transplantation into NSG mice of the indicated number of CD271$^-$, CD271$^+$, and Lin$^-$ cells dissociated side-by-side, according to published methods (Q, C, and B), from seven patient melanomas (12-1036, 12-1254, 13-1061, 12-316, 13-444, 12-042, 12-272). Tumorigenic cell frequencies were calculated using limit dilution analysis (15). C, percentage of mice ($n = 97$) with distant macrometastasis from subcutaneous tumors grown from CD271$^-$ or CD271$^+$ cells ($P = 0.46$, Fisher exact test). Quintana and colleagues (Q; ref. 9), Civenni and colleagues (C; ref. 6), Boiko and colleagues (B; ref. 5).

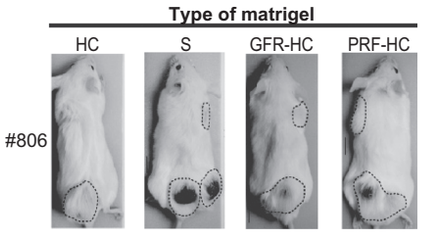
CD271$^+$ cells varied widely even among sibling secondary tumors (Fig. 6B). For example, separate injections of 100 CD271$^-$ cells from melanoma 12-1036 formed tumors that contained 54.6% and 6.7% CD271$^+$ cells, respectively (Fig. 6A, tumors ST3 and ST4). Similarly, separate injections of 1,000 CD271$^-$ cells from melanoma 12-1254 formed tumors that contained 24% and 11% CD271$^+$ cells, respectively (Supplementary Fig. S5, tumors ST12 and ST13). CD271 expression patterns were clearly different among tumors in each pair, despite tumors being grown from inoculates of the same numbers of cells taken from the same purified cell pools. By immunohistochemical staining, variation (median 6.5-fold, range 1.5–23.0) among sibling PDX tumors was also apparent in the expression of other markers previously linked to the CSC model in melanoma [ALDH (19, 20), CD133 (21), and ABCB5 (22)]. In contrast, the expression of JARID1B, which plastically regulates the propagation of melanoma cell lines (3), was stable (median difference 1.2-fold, range 1.1–1.4; Fig. 6C and Supplementary Fig. S6).

These data confirm that intratumoral relationships between CD271$^-$ and CD271$^+$ melanoma cell subpopulations are not hierarchical in a manner predicted by the CSC model. However, the striking variability of CD271 expression is not consistent with

A

Melanoma	Matrigel	Number of tumors/Number of injections: Cells per injection 1,000	100	10	Melanoma-initiating cell frequency (95% confidence interval)	
All	HC	3/3	6/9	3/9	1/67	(1/32-1/140)
	S	3/3	4/9	6/9	1/71	(1/34-1/150)
	GFR-HC	3/3	8/9	5/6	1/24*	(1/10-1/61)
	PRF-HC	3/3	5/9	4/6	1/70	(1/33-1/151)





B

Matrigel	Cell phenotype	Number of tumors/Number of injections: Cells per injection 1,000	100	10	Melanoma initiating cell frequency (95% confidence interval)	
HC	CD271$^-$	3/3	3/9	2/9	1/162	(1/66-1/396)
	CD271$^+$	4/4	6/12	3/9	1/103	(1/51-1/208)
	Lin$^-$	4/4	5/12	2/6	1/138	(1/102-1/640)
	Total	11/11	14/33	7/24	1/129	(1/82-1/202)
S	CD271$^-$	3/3	2/6	1/9	1/187	(1/64-1/550)
	CD271$^+$	3/3	4/12	3/12	1/154	(1/71-1/333)
	Lin$^-$	2/2	3/12	1/3	1/256	(1/102-1/640)
	Total	8/8	9/30	5/24	1/192	(1/114-1/322)

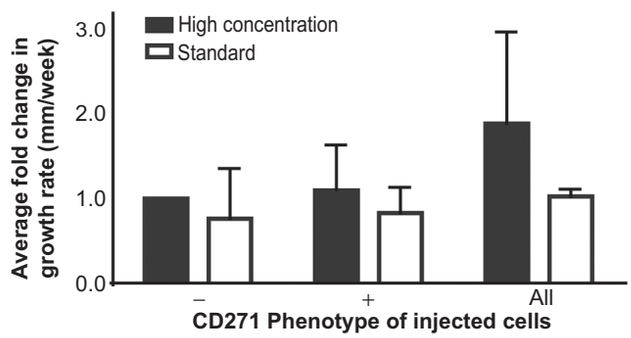


Figure 4.

Lack of effect of Matrigel formulation on CD271-associated tumorigenicity in human melanoma cells. A, tumor formation in NSG mice following subcutaneous injection of unselected (Lin$^-$; Supplementary Fig. S3) tumor cells from dissociated PDX (176, 374) and patient (806) melanomas. Cells were mixed prior to injection in 25% final concentrations of different formulations of Matrigel [high concentration (HC), standard (S), growth factor reduced (GFR)-HC or phenol red–free (PRF)-HC]. Top, summary tumorigenesis data for all injections. Mixture of cells in GFR-HC Matrigel resulted in 2- to 3-fold higher efficiencies of tumor formation (*, $P < 0.05$, χ^2). Bottom left, average fold-change (relative to HC tumors) in growth rates of PDX melanomas in the top table, grouped according to the type of Matrigel use to establish them. Bars, means ± SD (all P values > 0.05, Student t test). Bottom right, tumor formation (within dotted lines) in NSG mice following transplantations of 10 Lin$^-$ melanoma cells from patient melanoma 806 after mixing in different types of Matrigel. B, top, summary tumorigenesis data for all injections of CD271$^-$, CD271$^+$, and Lin$^-$ cells at the indicated numbers, following dissociation of patient melanomas (12-1036, 12-1254) by the Q method and mixture in either 25% HC Matrigel or 30% S Matrigel, as per published studies (5, 6, 9). Frequencies of tumorigenic cells were not significantly different for any parameter ($P > 0.2$, χ^2). Bottom, average fold-change (relative to HC CD271$^-$ tumors) in growth rates of PDX melanomas in the top table (all = Lin$^-$). Bars, means ± SD (all P values > 0.05, Student t test).

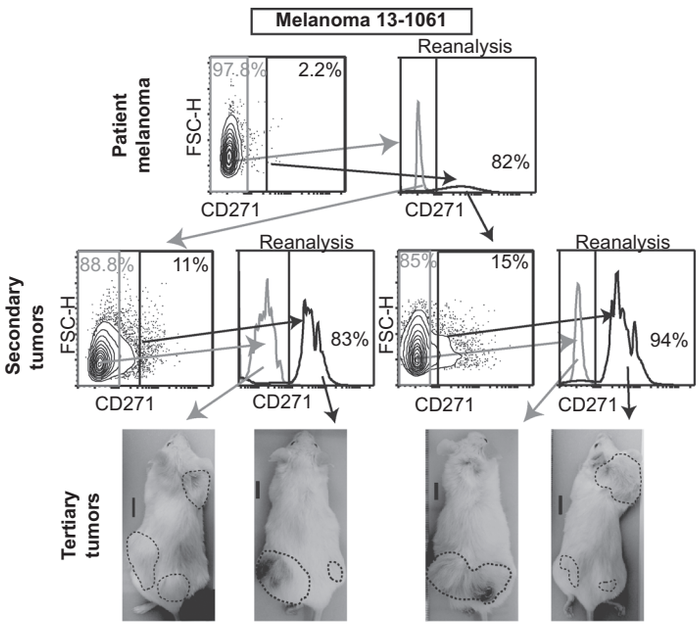
classical models of cancer cell plasticity that predict reproducible distributions of marker expression. We thus wondered whether highly variable CD271 expression among sibling PDX melanomas could be associated with genetic variation (11, 23).

Genetic differences among sibling PDX tumors grown from purified melanoma cells

To test genetic differences linked to CD271 expression in PDX melanomas, we genotyped Lin$^-$ cells sorted by flow cytometry from four pairs of sibling tumors (ST3/4, ST6/7, ST10/11, and ST12/13 in Fig. 6A; Supplementary Fig. S5). Tumors in each pair clearly differed in CD271 expression pattern despite being grown from cells obtained from the same pools of CD271-purified cells. Genomic DNA was extracted from Lin$^-$ cells from each tumor and subjected to SNP genotyping using Illumina Human OmniExpress 715K arrays.

To evaluate differences in genomic copy number alterations (CNA) between tumors in each pair, we developed a novel approach for comparative SNP array analysis (see Supplementary Methods) to address limitations in published algorithms (24), which we found inaccurately assigned copy number changes in tumor genomes with complex ploidy and heterogeneity characteristics. To compare copy number profile between tumor pairs, we calculated t statistics in 100 SNP

A



B

Melanoma	Phenotype of cells that produced secondary tumors	Phenotype of cells used to generate tertiary tumors	Number of tertiary tumors/number of injections		Melanoma-initiating cell frequency (95% confidence interval)	
			Cells per injection			
			100	10		
12-1036	CD271−	CD271−	0/6	0/6		(<1/220)
		CD271+	1/6	0/6	1/609	(1/86–1/4300)
	Total		1/12	0/12	1/1269	(1/179–1/8987)
	CD271+	CD271−	5/6	1/6	1/56	(1/23–1/136)
		CD271+	2/6	3/6	1/106	(1/39–1/248)
	Total		7/12	4/12	1/80	(1/42–1/154)
13-1061	CD271−	CD271−	6/6	4/6	1/9	(1/3–1/25)
		CD271+	5/6	1/6	1/56	(1/23–1/136)
	Total		11/12	5/12	1/30	(1/15–1/60)
	CD271+	CD271−	6/6	5/6	1/6	(1/2–1/15)
		CD271+	4/6	4/6	1/47	(1/19–1/114)
	Total		10/12	9/12	1/27	(1/14–1/55)

Figure 5.
Serial transplantability of PDX tumors grown from CD271$^-$ and CD271$^+$ melanoma cells. A, PDX tumor formation in NSG mice from 100 CD271$^-$ or CD271$^+$ cells first purified from a lymph node melanoma metastasis from patient 13-1061 and then serially from secondary PDX melanomas. Dot plots show gating strategies for CD271 sorting. Tertiary tumors (within dotted lines; bars, 1 cm) formed robustly and regardless of CD271 phenotype from cells purified from both patient and secondary PDX melanomas. B, summary PDX tumor formation data from serial transplantations in NSG mice of CD271$^-$ and CD271$^+$ cells first purified from two patient melanomas (12-1036, 13-1061) and then again from secondary tumors. The proportions of tumorigenic cells in secondary tumors were estimated by limiting dilution analysis of tertiary tumor formation data.

windows across the genome, independently for both log R ratio (LRR) and for a transformed B allele frequency (BAF). Regions of copy number difference were found by applying a min-run-max-gap algorithm (25). Genetic differences among tumors within a pair were summarized as the proportions of tumor genome affected by divergent CNAs. In three control technical replicate assays of DNAs extracted from PDX melanomas, the *t*-statistic algorithm detected copy number differences of 0%–0.01% (data not shown).

Tumors in each sibling PDX melanoma pair contained clear genetic differences (Fig. 7A and B). For example, tumors ST3/4 differed genetically by 28% and tumors ST12/13 differed genetically by 48% (Fig. 7B). Genetic differences were either CNAs present in one tumor but not detectable in the other [e.g., heterozygous loss of chromosome (chr) 11 in tumor ST3 but not ST4; Fig. 7A, double-headed arrows], or differences in the proportions of genetically distinct subclones present within heterogeneous tumors (e.g., chr 8 in PDX tumor ST11 was ~70% diploid and ~30% monoploid, whereas in tumor ST10 chr 8 was ~85% diploid and ~15% monoploid; Supplementary Fig. S7, middle plots, double-headed arrows). Genetic differences among sibling PDX melanomas were not only due to release of evolutionary bottlenecks in parental tumors by transplantation of small numbers of cells, as they did not correlate with whether tumors were grown from the same or different numbers of inoculated cells (Fig. 7B, bottom). These data reveal a surprising degree of genetic divergence among PDX tumors grown from phenotypically identical patient melanoma cells.

Intratumoral genetic heterogeneity is linked to differences in CD271 expression in melanoma

We next tested whether CD271$^-$ and CD271$^+$ cells within the same melanomas might be genetically distinct subclones. Genomic DNA from flow cytometrically purified CD271$^-$ and CD271$^+$

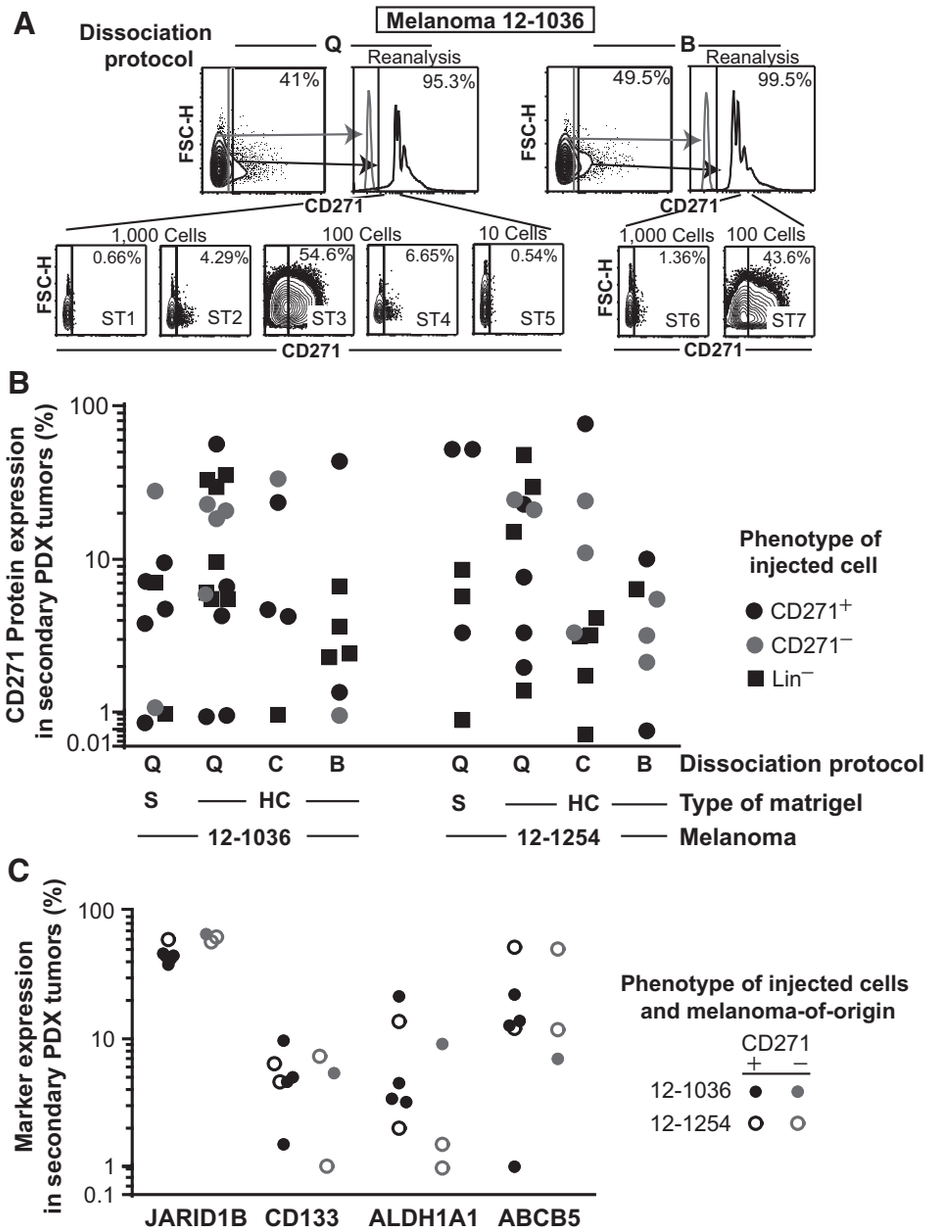


Figure 6.

Highly variable marker reexpression patterns in PDX tumors grown from CD271$^+$ and CD271$^-$ cells. A, flow cytometrically purified CD271$^-$ and CD271$^+$ cells from patient melanomas (12-1036 dissociated according to Q or B methods) were injected into NSG mice to form secondary PDX tumors, which were then analyzed for CD271 expression. Top plots, CD271 expression in patient tumors at left and reanalyses of sorted cells at right. Bottom plots, CD271 expression in secondary PDX tumors (ST1, ST2, etc.) derived from injections of the indicated numbers of cells taken from the same pools of purified CD271$^+$ (black plots) or CD271$^-$ (red plots) cells. B, percentages of CD271$^+$ cells in all (n = 68) tumors grown in NSG mice from CD271$^-$ (gray circle), CD271$^+$ (black circle), and Lin$^-$ (black square) cells obtained from melanomas 12-1036 and 12-1254. Highly varied percentages of CD271$^+$ cells were observed in sibling PDX melanomas regardless of the PDX assay method. C, quantification of expression of JARID1B, CD133, ALDH1A1 and ABCB5 in sibling PDX melanomas grown from CD271$^+$ (black) and CD271$^-$ (gray) cells from melanomas 12-1036 and 12-1254. Quintana and colleagues (Q; ref. 9), Civenni and colleagues (C; ref. 6), Boiko and colleagues (B; ref. 5).

cells from patient ($n = 1$) and PDX ($n = 4$) melanomas underwent SNP genotyping to identify copy number differences using the t statistic method (see above). In 4 of 5 (80%) melanomas, genetic differences (range 1.4%–23%) were apparent between CD271$^-$ and CD271$^+$ cells in the same melanomas (Fig. 7C–G and Supplementary Fig. S8). For example, in patient melanoma 13-609, a subclone characterized by loss of heterozygosity (LOH) and focal deletions in chr 9 and a chr 19q amplification was identified in CD271$^-$ cells, whereas the complementary CD271$^+$ population contained a mix of this subclone plus another subclone with LOH and no focal deletions on chr 9, LOH in chr 14, and chr 19 diploidy (Fig. 7C–F). In other melanomas, differences in proportions of subclones were evident between CD271$^-$ and CD271$^+$ cells. For example, chr 12 in mel 1254 was approximately 8% tetraploid and approximately 92% triploid in CD271$^+$ cells and approximately 25% tetraploid and approximately 75% triploid in CD271$^-$ cells (Supplementary Fig. S8, bottom right plots, blue arrows).

As the human *CD271/p75/NGFR* gene locus is on chromosome 17, we looked carefully in this region for copy number differences between CD271$^-$ and CD271$^+$ cells, finding none. We also looked for divergent copy number changes affecting genes whose products were found to regulate expression of CD271, such as *IFNG* (26), *IFNB1* (27), *TNF* (28), *BMP2* (29), *BMP7* (30), *EGR2* (31), *RUNX2* (32), *VDR* (33), and *FOXO3A* (34). None of these CD271-regulatory genes was directly affected by differential copy number between CD271$^-$ and CD271$^+$ cells. Nonetheless, genetic differences were associated with differences in CD271

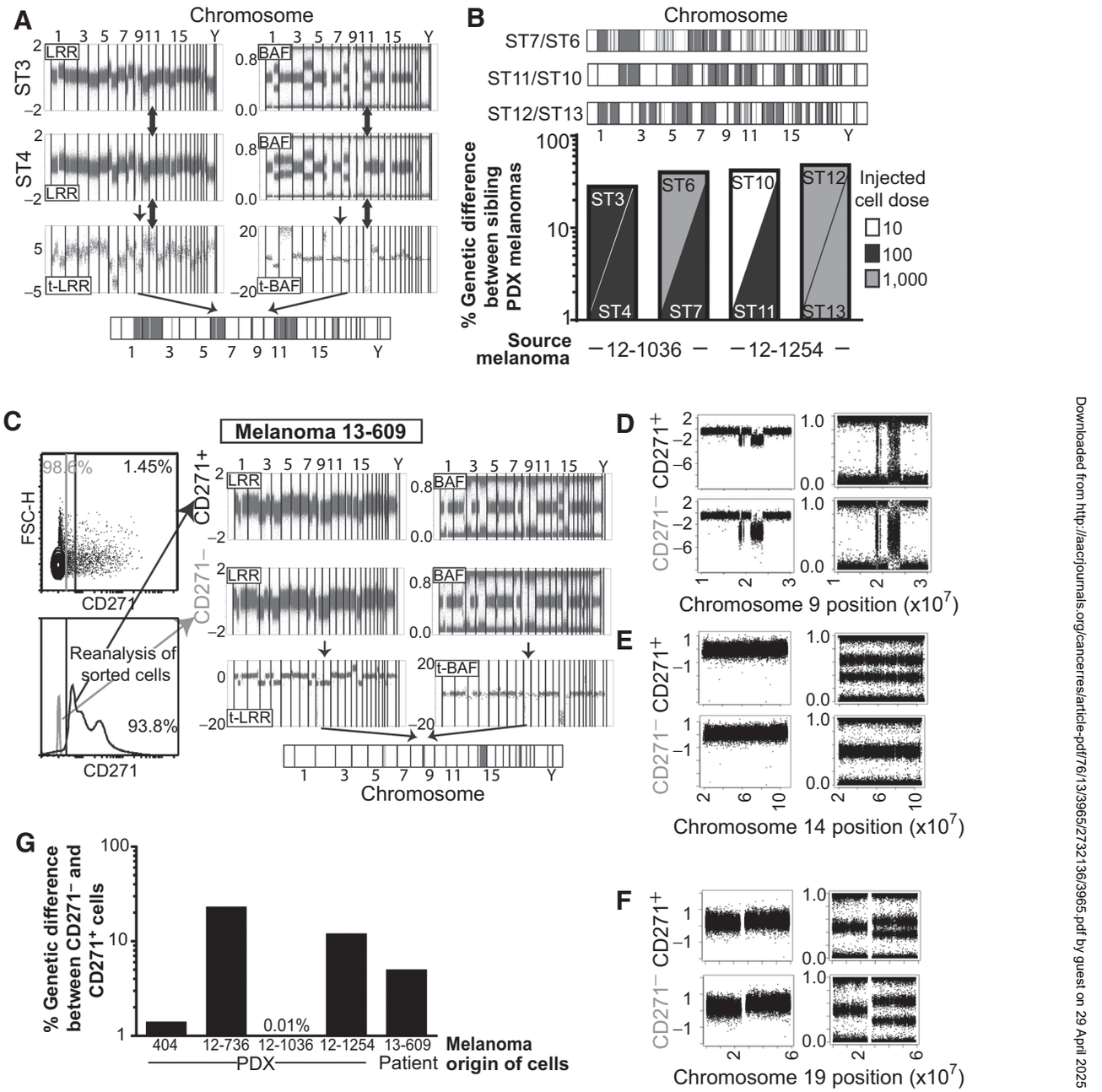


Figure 7.

Genetic heterogeneity linked to CD271 expression among and within melanomas. A, genomic copy number differences among sibling PDX tumors. Copy number alterations in sibling PDX tumors (ST3 and ST4) grown from separate 100-cell injections of CD271$^+$ cells from the same patient melanoma (see Fig. 6A). LogR ratio (LRR) data shown to left and B allele frequencies (BAF) to right. t-LRR and t-BAF values indicate differences between the raw LRR and BAF signals. Double-headed arrows, chr 11. Heatmap indicates genomic regions (gray) that differ in copy number between tumors. B, top, heatmaps indicating regions of copy number difference (gray) between three other sibling PDX tumor pairs (ST6 vs. ST7, ST10 vs. ST11, ST12 vs. ST13; see Fig. 6 and Supplementary Fig. 5A). Bottom, quantitation of genomic differences within each sibling PDX tumor pair. Histograms are colored to indicate the cell dose used to establish the tumors in each pair. Values indicate proportional genomic differences in copy number among tumors in a pair (range 28%–48%). C, genomic copy number differences among CD271$^-$ and CD271$^+$ cells in a patient melanoma. Left, flow cytometry analyses of CD271 expression in patient melanoma 13-609 (top, sorting gates are shown) and in sorted (bottom histograms) CD271$^-$ (gray) and CD271$^+$ (black) cells submitted for SNP genotyping (right). Right, LRR and BAF plots derived from SNP genotyping of sorted CD271$^-$ and CD271$^+$ cells from patient melanoma 13-609. t-LRR and t-BAF values indicate differences between raw LRR and BAF signals from each cell population. D–F, LRR (left) and BAF (right) plots indicating copy number differences on chromosomes 9 (D), 14 (E), and 19 (F) between CD271$^-$ and CD271$^+$ cells from patient melanoma 13-609. G, quantitation of genomic differences among CD271$^-$ and CD271$^+$ cells in four PDX (404, 12-736, 12-1036, 12-1254) and one patient (13-609) melanoma. Values indicate proportional genomic differences in copy number among the two CD271-defined subpopulations in each melanoma [range (0% to 23%)].

expression among uncultured cells or their progeny in most of the patient and PDX melanomas we studied.

Discussion

We found that human melanoma does not follow a CD271-based CSC model, regardless of variations in PDX assays. In side-by-side comparisons of published methods for testing the CSC model in melanoma (5, 6, 9), we found no consistent effect of assay variation on tumorigenesis, which was routinely observed from low numbers of $CD271^-$ and $CD271^+$ patient melanoma cells (Figs. 3 and 4). The contrasting conclusions by different groups regarding the CD271-based CSC model in melanoma are thus not explained by differences in the processing of cells prior to xenotransplantation, as has been proposed (6, 10, 14). Regardless of the method of cell preparation, uncultured human $CD271^+$ melanoma cells do not consistently have more tumorigenic potential than $CD271^-$ melanoma cells.

A definitive explanation for differences among published studies remains obscure. The use of different strains of recipient mice is one potential reason, as $CD271^-$ melanoma cells may be more suppressed or more efficiently cleared, compared with $CD271^+$ cells, in mice that are less immunocompromised than NSG mice (6). However, this is not relevant to the CSC model, which describes mechanisms for differences in cell-intrinsic tumorigenic potential among cancer cells, rather than differences in cell-extrinsic regulation of cancer cell fate (11), including via xenogeneic immune mechanisms.

It is possible that some differences among studies relate to genomic instability in melanoma cells (Fig. 7; ref. 35), as the random acquisition of favorable or deleterious genotypes could promote or impede tumorigenicity in different experiments. Increased tumorigenicity might be linked to increased CD271 expression if a particular set of genetic changes caused both these features. In some experiments we observed, as others have (5, 6), that $CD271^+$ melanoma cells were more tumorigenic than $CD271^-$ cells (Fig. 4B). However, in other experiments the opposite was seen (Fig. 3B). This suggests that genetic changes that increase tumorigenic potential in melanoma can be associated with reduced CD271 expression. Genetic divergence linked to phenotypic and functional differences among malignant subclones has been recognized in other cancers (36–38).

Although variations in marker expression among sibling PDX tumors could relate to microenvironmental differences that drive varied epigenetic determinants of expression, we identified clear copy number differences (28%–48%) among sibling tumors with distinct CD271 expression profiles. $CD271^-$ cells were genetically distinguishable from $CD271^+$ cells in 4 of 5 melanomas, containing different and complex mixtures of shared and, in some cases, distinct clones (Fig. 7C–G and Supplementary Fig. S8). Although these intratumoral copy number differences could not simply explain differences in CD271 protein expression as a consequence of varying doses of genes encoding CD271 or its known regulatory proteins, the expression of CD271 and its regulators are likely to be modulated by other factors affected by the dynamic genetic changes we observed to promote or inhibit CD271.

These data are consistent with studies of phenotypically distinct breast cancer cells (39, 40), in which only small genetic differences were concluded to exist among putative breast CSC populations. As far as we are aware, our study is the first to identify extensive intratumoral genetic differences among uncultured human cancer cells that are phenotypically distinct in a manner previously assumed to be driven by stable epigenetic mechanisms. This raises the likelihood that CD271 expression is modulated by competing, genetically unstable subclones, perhaps via cross-talk (41, 42), resulting in widely variable expression patterns. The instability of CD271 expression in melanoma renders it an unattractive therapeutic target.

A key concept underpinning the CSC model is the hierarchical organization in tumors of cells that are phenotypically distinct (43). Consistent with some previous studies (10), but in contrast to others (6), our analyses of CD271 expression in secondary tumors revealed no evidence of hierarchical relationships between $CD271^-$ and $CD271^+$ melanoma cells. However, in evaluating a large number of secondary PDX tumors, we also observed striking variation in CD271 reexpression patterns (Fig. 6B), even among tumors grown in the same experiments from the same numbers of phenotypically identical cells (Fig. 6A and Supplementary Fig. S5). Indeed, most secondary PDX melanomas had CD271 expression levels more than 50% different from parental tumors (Fig. 6B). Not only are these data inconsistent with a CSC model, they are also not consistent with a classical plasticity model of marker expression, in which reequilibration of marker-defined cell populations occurs in a predictable manner (11, 44, 45). *In vivo* marker expression patterns in tumors composed of uncultured cancer cells may be far more variable than they are in highly passaged cell lines or than they appear when only small numbers of PDX tumors are evaluated.

The CSC and plasticity models of cancer progression are most useful if marker expression states are stably linked to malignant potential. If such links are only transiently and variably present during disease progression, then targeting these markers will not substantially benefit patients. Indeed, although epigenetic changes drive a variety of phenotypes and functional states in cancer, if they do so on a profoundly unstable genomic background, their importance will likely be reduced over the course of a patient's disease as genetically distinct subclones, modulated by new epigenetic states, are selected. The presence of substantial intratumoral genomic variation, such as we observed in this study, decreases the usefulness of the CSC and plasticity models for understanding mechanisms of progression in genetically unstable cancers.

Including data from this study and from other studies (5, 6, 10), CD271 expression has been found to be independent of tumorigenicity in most patient melanomas evaluated. Although others found evidence for hierarchical cellular organization in melanoma with other markers (20, 22), the genomic instability and variability in marker expression we identified indicate that extended testing of these markers is warranted before they, and the cells they mark, can be considered promising therapeutic targets. To identify reliable and useful markers of cancer cells that might be worth targeting in patients, comprehensive *in vivo* evaluation is required of the stability and functional associations of marker expression in uncultured cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Development of methodology: S.E. Boyle, C.G. Fedele, V. Corbin, E. Wybacz, R. Young, A.T. Papenfuss, M. Shackleton

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.E. Boyle, C.G. Fedele, E. Wybacz, P. Szeto, J. Lewin, A. Wong, J. Spillane, D. Speakman, S. Donahoe, D. Gyorki, M.A. Henderson
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.E. Boyle, V. Corbin, E. Wybacz, R. Young, R. Fuller, A.T. Papenfuss, M. Shackleton
Writing, review, and/or revision of the manuscript: S.E. Boyle, C.G. Fedele, V. Corbin, R. Young, J. Lewin, J. Spillane, D. Speakman, D. Gyorki, M.A. Henderson, R.W. Johnstone, M. Shackleton
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.E. Boyle, V. Corbin, E. Wybacz, M. Pohl, M.A. Henderson
Study supervision: R.W. Johnstone, M. Shackleton

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