Human CD271-Positive Melanoma Stem Cells Associated with Metastasis Establish Tumor Heterogeneity and Long-term Growth

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Abstract

Human melanoma is composed of distinct cell types reminiscent of neural crest derivatives and contains multipotent cells that express the neural crest stem cell markers CD271(p75NTR) and Sox10. When isolated from solid tumors by using a method that leaves intact cell surface epitopes, CD271-positive, but not CD271-negative, cells formed tumors on transplantation into nude or nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice. These tumors fully mirrored the heterogeneity of the parental melanoma and could be passaged more than 5 times. In contrast, in more immunocompromised NOD/SCID/IL2rnull mice, or in natural killer cell-depleted nude or NOD/SCID mice, both CD271-positive and CD271-negative tumor cell fractions established tumors. However, tumors resulting from either fraction did not phenocopy the parental tumors, and tumors derived from the CD271-negative cell fraction could not be passaged multiple times. Together, our findings identify CD271-positive cells as melanoma stem cells. Our observation that a relatively high frequency of CD271/Sox10-positive cells correlates with higher metastatic potential and worse prognosis further supports that CD271-positive cells within human melanoma represent genuine cancer stem cells. Cancer Res; 71(8); 3098–109. ©2011 AACR.
the definition of melanoma stem cells. Intriguingly, the incidence of such cells in patient biopsies is associated with poor prognosis.

Materials and Methods

Tissue microarray analysis

All analyses involving human melanoma tissue were carried out in accordance with the ethical committee in canton Zurich. The tissue microarray used here comprised 54 primary melanomas, 141 melanoma metastases, and 53 melanoma cell lines and was generated as described (12, 13). Coexpression of CD271 and Sox10 was identified by double immunostaining (Sox10, 1:800, R&D, MAB2864; CD271, 1:50, Miltenyi Biotec, 130-091-883). The staining procedure was done as previously described (12). The frequency of cells that express CD271 plus Sox10 is presented as mean ± SD and median with interquartile range (IQR). Values were compared by using the Mann–Whitney U test with exact P values. Tumor-specific survival was analyzed by using Kaplan–Meier curves and compared between groups by log-rank test. SPSS 15 software (SPSS Inc.) was used for statistical analyses. Two-tailed P values of 0.05 or less were considered statistically significant.

Mice

BALB/c-Swiss nude (CAnNCg-Foxn1nu/Crl. Nude) and NSG (NOD.Cg-Prkdcsclcid Il2rgtm1Wjl/S. NSG) mice were obtained from Charles River. NOD/SCID (NOD.CB17/JHliHsd- Foxn1nu/Crl, Nude) and NSG (1:800; R&D, MAB2864), CD271 (1:200; Alomone Labs, ANT-007), MelanA (1:50; Abcam, Ab785), S100P (1:400; DAKO, Z0611), HMB-45 (1:100; DAKO, M0634), microphthalmia-associated transcription factor (MITF; 1:100; Acris, DM4625), neuron-specific class III beta-tubulin (TuJ1; 1:200; Sigma, T8660), neurofilament 160 (NF; 1:200; Sigma, N5264), neuron-specific enolase (NSE; 1:100; Abcam, Ab53025), Peripherin (1:100; Abcam, ab4666), smooth muscle actin (SMA; 1:400; Sigma, A2547), and green fluorescent protein (GFP; 1:500; Abcam, Ab290). Sections were subsequently incubated with following secondary antibodies for 1 hour at room temperature: Cy3-conjugated goat antimouse (1:500; Jackson Immunoresearch Laboratories), Cy3-conjugated goat antirabbit (1:500; Jackson Immunoresearch Laboratories), Alexa 488-conjugated goat antimaouse (1:500; Invitrogen). All slides were counterstained with Hoechst 33342 (Invitrogen). In addition, sections were stained with hematoxylin and eosin (H&E). Stainings on cultured cells were done as described (14).

Quantification of positive cells was done by ImageJ software (NIH). Three images from each biopsy were taken randomly. Images were imported into ImageJ, converted to 8-bit formats, and subjected to automatic threshold. The “Analyze Particles” function was used to count positive cells.

Flow cytometry and FACS

All steps for flow cytometry were carried out in RPMI-1640 supplemented with 10% fetal calf serum (Invitrogen), 5% Pen/Strep (Invitrogen), and 2 mmol/L EDTA. For sorting or analysis, single cell tumor samples were stained with antihuman CD271 (Miltenyi Biotec; FITC-conjugated: 130-091-917, or APC-conjugated: 130-091-884), antihuman CD4 (1:100; BD Pharmingen, 554477), or anti-ABCB5 antibody (1:200; clone 3C2-1D12, a gift from Markus Frank). Samples were measured with FACSscan (BD Biosciences) and analyzed with Diva software (BD Biosciences). Sorting was done with FACSaria (BD Biosciences), and the purity of sorted fractions was 95% to 99% of CD271-positive cells in the CD271-enriched fraction and less than 0.5% of CD271-positive cells in the CD271-depleted fraction.

Natural killer cell depletion

Mice were injected i.p. with 50 µL polyclonal rabbit anti-asialo GM1 antibodies (Wako Chemicals, catalogue no. 986-10001) in 150 µL PBS every 5 days starting at the day of melanoma cell engraftment, which resulted in a greater than 95% depletion of CD3− DX5− natural killer (NK) cells for the duration of the experiment as measured by flow cytometry (15).

Sphere culture

Sphere cultures were established as previously described for neural stem cells (16). Briefly, single cells were plated in flasks
(Nunc) coated with Poly(2-hydroxyethylmethacrylate) (Poly-Hema; Sigma) at a density of 20,000 viable cells/mL in DMEM-F12 1:1 media (Gibco) containing 1 × B-27 supplement (Invitrogen), 20 ng/mL FGF2 (PeproTech), 10 ng/mL EGF (PeproTech), and 5% Pen/Strep (Invitrogen). The resulting spheres were collected after 7 to 10 days by gentle centrifugation (800 rpm), dissociated by using PBS containing 2 mmol/L EDTA, and replated into Poly-Hema–coated culture flasks. To ensure plating of single, viable cells, the dissociated sphere cells were passed through a 40-μm nylon mesh followed by Trypan blue examination. To assess the relative sphere numbers over passage, spheres were grown for 7 days, counted, dissociated, and replated under the same conditions.

Melanoma cell lines

Cell lines M990115 and M010817 were previously described (17). The cell line M070302 was established from surplus material from a xenograft (patient 2481) generated in nude mice. Cell lines were grown in sphere culture condition as describe earlier in the text.

Transduction of melanoma with GFP-expressing lentivirus

The GFP-expressing lentivirus was produced as described (18). Lentivirus-containing supernatants were collected 48 hours after transfection, filtered through a 0.22-μm membrane, and transferred to target cells (see the following text). We xenografted a fresh human melanoma into nude mice, processed the xenograft into single cells and cultured them under sphere conditions. Cells were transduced with GFP-lentivirus at the eighth passage. After 1 week, resulting GFP-tagged cells were checked by FACS and injected s.c. into nude, NOD/SCID, and NSG mice, and sections of the resulting xenografts were stained for the expression of 11 markers. The transplanted bulk melanoma cells gave rise to tumors in all cases. Importantly, 100% of all xenografts in nude and NOD/SCID mice were exact phenocopies of the parental tumors with respect to the expression of all markers tested (Fig. 1; Supplementary Fig. S2; Table 1). In contrast, all xenografts in NSG mice (n = 7) differed from the parental tumors and lacked cells expressing one or more of the following markers: CD271, MITF, S100P, and neuronal markers (Fig. 1; Supplementary Fig. S2; Table 1). Quantification of cells expressing a given marker confirmed the consistency of CD271/Sox10, S100P, and MITF expression between nude and NOD/SCID xenografts and the respective parental tumors, and the discrepancies in marker expression between NSG xenografts and original patient tumors (Fig. 1C).

Because the major difference between NSG mice and nude or NOD/SCID mice is the absence of NK cells in NSG mice, we addressed whether NK cells influence the phenotype of xenografted tumors by depleting NK cells from nude and NOD/SCID mice (15). Intriguingly, xenotransplanted melanoma cells did not phenocopy the parental tumor in NK-depleted NOD/SCID mice (Fig. 1A–C; Supplementary Fig. S3; Table 1). Thus, the level of immunocompetence of the xenotransplant recipient crucially affects the capacity of human melanoma cells to form tumors resembling the respective patient melanoma.

Results

Xenotransplantation into highly immunocompromised mouse models fails to phenocopy the cellular heterogeneity of parental human melanoma samples

Because melanoma derives from the neural crest cell lineage, we expect putative melanoma stem cells to exhibit features of neural crest stem cells (NCSC) and individual tumors to comprise cells expressing the NCSC markers CD271 and Sox10 (19, 20) as well as cells with features of different neural crest derivatives. To examine the cellular heterogeneity of a given patient’s tumor, we analyzed 19 independent melanoma metastases (Supplementary Table S1) for the expression of 11 NCSC, melanocytic, neural, and mesenchymal markers and found that the majority of the samples were positive for most or all of these markers (Fig. 1A and B; Supplementary Fig. S1).

To investigate mechanisms of human melanoma formation and propagation, it is imperative to use a model that faithfully reproduces the parental phenotype, which we will call phenocopy hereafter. Melanoma cell xenotransplantation into different models of immunodeficient mice, including highly immunocompromised NSG mice, previously led to conflicting results (3, 7). To address which mouse model(s) for melanoma formation might best recapitulate the cellular composition of parental tumors, 1,000 cells from the bulk of various patient samples were injected s.c. into nude, NOD/SCID, and NSG mice, and sections of the resulting xenografts were stained for the expression of 11 markers. The transplanted bulk melanoma cells gave rise to tumors in all cases. Importantly, 100% of all xenografts in nude and NOD/SCID mice were exact phenocopies of the parental tumors with respect to the presence of all markers tested (Fig. 1; Supplementary Fig. S2; Table 1). In contrast, all xenografts in NSG mice (n = 7) differed from the parental tumors and lacked cells expressing one or more of the following markers: CD271, MITF, S100P, and neuronal markers (Fig. 1; Supplementary Fig. S2; Table 1). Quantification of cells expressing a given marker confirmed the consistency of CD271/Sox10, S100P, and MITF expression between nude and NOD/SCID xenografts and the respective parental tumors, and the discrepancies in marker expression between NSG xenografts and original patient tumors (Fig. 1C).
dissociated followed by enzymatic digestion with collagenase (8), a mix of collagenase and dispase (9), or collagenase and trypsin (10, 11, 21). Therefore, we addressed whether these different tumor digestion protocols might affect detectability of surface markers used to directly isolate putative tumorigenic melanoma cell populations. Strikingly, the percentage of retrievable CD271- and ABCB5-positive cells was consistently reduced in conditions that included a trypsin incubation step (11), whereas CD44 was not affected (Supplementary Fig. S4). These results show that a strong proteolytic activity can selectively damage surface protein epitopes and thus lead to reduced detectability by the corresponding antibodies. This unavoidably results in underestimation of surface marker expression in human melanoma cells and in negative fractions being contaminated by cells actually expressing the selected marker. For this reason, in this study human biopsies and xenografts were dissociated in the absence of trypsin activity.

Using this gentle protocol for tumor dissociation and cell isolation, FACS-sorted CD271-positive melanoma cells from primary xenografts consistently promoted tumor formation in nude and NOD/SCID mice, whereas the CD271-negative cell fraction never gave rise to tumors (Fig. 2A; 32 transplantations representing 7 distinct patient tumors). In contrast, we did not detect differences in tumor initiation in NSG mice between CD271-positive and CD271-negative cells from primary xenografts (12 transplantations each, representing 3 distinct...
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Table 1. Phenocopy of the parental tumors by grafted bulk melanoma cells

<table>
<thead>
<tr>
<th>Marker</th>
<th>Nude</th>
<th>N/S</th>
<th>NSG</th>
<th>N/S_GM1</th>
</tr>
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<tbody>
<tr>
<td>CD271/Sox10</td>
<td>100</td>
<td>100</td>
<td>71</td>
<td>100</td>
</tr>
<tr>
<td>S100P</td>
<td>100</td>
<td>100</td>
<td>71</td>
<td>80</td>
</tr>
<tr>
<td>HMB45</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>20</td>
</tr>
<tr>
<td>MITF</td>
<td>100</td>
<td>100</td>
<td>57</td>
<td>20</td>
</tr>
<tr>
<td>Tuj1</td>
<td>100</td>
<td>100</td>
<td>86</td>
<td>60</td>
</tr>
<tr>
<td>NF</td>
<td>100</td>
<td>100</td>
<td>86</td>
<td>60</td>
</tr>
<tr>
<td>NSE</td>
<td>100</td>
<td>100</td>
<td>71</td>
<td>80</td>
</tr>
<tr>
<td>Peripherin</td>
<td>100</td>
<td>100</td>
<td>57</td>
<td>40</td>
</tr>
<tr>
<td>All markers</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
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</table>

NOTE: Capacity of unsorted melanoma cells to generate phenocopies of parental tumors in different mouse hosts. Xenografts were generated in nude, N/S, NSG, and N/S mice treated with anti-asialo GM1 antibodies. Values represent the percentage of xenografts that phenocopy the parental tumor with respect to expression of the indicated marker.

To specifically address this issue, we infected melanoma cells from a xenograft of patient sample 2481 with a GFP-expressing lentivirus, followed by FACS of GFP/CD271 double-positive cells and plating of those cells at clonal density. Although 19 ± 4% of the CD271-positive cells gave rise to clones consisting of more than 10 cells after 2 weeks in culture, such clones were not observed in the CD271-negative cell fraction (Fig. 2C). Three clones derived from GFP/CD271 double-positive founder cells were selected and subjected to differentiation assays in cell culture, revealing the emergence of multiple cell types, such as cells expressing CD271/Sox10, neuronal and melanocytic markers, and SMA (Fig. 2D). Thus, the CD271-positive melanoma cell fraction contains a population that is clonogenic and multipotent in cell culture, similar to NCSCs.

To address the multipotency of CD271-positive melanoma cells in vivo, cells from the selected clones were grafted s.c. into nude and NSG mice, and the xenografts were analyzed for expression of the 11 markers as described before. In nude mice, the 3 clones were able to establish a cellular hierarchy with all 11 markers present in the xenografts. In particular, 10.2 ± 2.0% of the xenografts derived from a CD271-positive cell expressed CD271. Moreover, although most smooth muscle cells present in the xenografts were host derived, all xenografts comprised cells double positive for SMA and GFP, revealing that some smooth muscle cells in the tumor originated from CD271-positive melanoma cells. In contrast, neither the NCSC markers CD271/Sox10 nor S100P, MITF, and neuronal traits were consistently expressed in xenografts derived from clones transplanted into NSG mice. Moreover, we did not find any GFP/SMA double-positive cells in the NSG grafts, suggesting that the NSG host environment either suppresses the in vivo multipotency of CD271-positive cells or supports the growth of melanoma cells with restricted developmental capacities (Fig. 2D; Supplementary Fig. S6).

CD271-positive melanoma cells have self-renewal capacity and sustain long-term tumor growth in vivo

To address whether CD271-positive cells have self-renewal capacity, in addition to being multipotent (Fig. 2B–D), we...
carried out sphere assays under conditions that are known to support sphere formation and self-renewal of normal skin-derived NCSCs (16). CD271-positive cells isolated from fresh melanoma tissue or from melanoma cell lines readily generated spheres that contained CD271/Sox10-positive cells and that could be propagated by serial passaging, whereas the few spheroid aggregates generated by the CD271-depleted melanoma cell fraction displayed a reduced passaging capacity.
in nude mice, even after multiple passages melanoma stem cells faithfully phenocopy the parental tumor/C6 cells (Fig. 3C; percentage of CD271-positive cells in primary time period. To test this, we serially passaged tumors generated from unsegregated patients such tumors could not be maintained NK-depleted nude mice (Fig. 2; refs. 9, 10), we expected that cells can generate tumors in NSG, NK-depleted NOD/SCID, or propation. Therefore, although CD271-negative melanoma melanoma stem cells is a prerequisite for long-term tumor generated from unsegregated patients CD271-positive cells was maintained in xenografts directly (Fig. 3D; Supplementary Fig. S7) but also the frequency of with respect to neural crest-specific differentiation markers we analyzed first and second passage xenografts by immuno-

Table 2. Phenocopy of the parental tumors by CD271-positive melanoma cells

<table>
<thead>
<tr>
<th>Marker</th>
<th>% of xenografts phenocopying patient tumors (n)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Nude CD271⁺</td>
</tr>
<tr>
<td>CD271/Sox10</td>
<td>100 (4)</td>
</tr>
<tr>
<td>S100P</td>
<td>100 (4)</td>
</tr>
<tr>
<td>HMB45</td>
<td>100 (4)</td>
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<tr>
<td>MITF</td>
<td>100 (4)</td>
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<td>Tuj1</td>
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<td>NF</td>
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<tr>
<td>NSE</td>
<td>100 (4)</td>
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<tr>
<td>Peripherin</td>
<td>100 (4)</td>
</tr>
<tr>
<td>All markers</td>
<td>100 (4)</td>
</tr>
</tbody>
</table>

NOTE: Capacity of CD271-positive versus CD271-negative melanoma cell fractions to generate phenocopies of parental tumors in nude or NSG Mice. Values represent the percentage of xenografts generated by CD271-positive (CD271⁺) or CD271-negative (CD271⁻) cells that phenocopy the parental tumor with respect to expression of the indicated marker.

(Fig. 3A and B). Determining the ratio between numbers of secondary over primary and quinternary over quaternary spheres showed that CD271-positive, but not CD271-negative, cells have an extensive self-renewal potential and expand their stem cell activity over time (Fig. 3B).

To confirm the self-renewal potential of CD271-positive melanoma cells in vivo, we isolated CD271-positive and CD271-negative cells from primary xenografts generated by bulk tumor cells in nude mice and retransplanted these cell fractions. We found that only CD271-positive cells were able to generate secondary tumors in nude mice (Fig. 3C and D; Supplementary Fig. S7, "first passage": n = 4 for each biopsy). Likewise, CD271-positive cells reisolated from secondary xenografts consistently generated tertiary tumors, unlike CD271-negative cells (Fig. 3C and D; Supplementary Fig. S7, "second passage"). To test whether the cellular heterogeneity originating from CD271-positive cells is a stable trait in vivo, we analyzed first and second passage xenografts by immuno-fluorescence. Intriguingly, not only the cellular heterogeneity with respect to neural crest-specific differentiation markers (Fig. 3D; Supplementary Fig. S7) but also the frequency of CD271-positive cells was maintained in xenografts directly generated from unsegregated patients’ melanoma cells and in tumors obtained by serial in vivo passaging of CD271-positive cells (Fig. 3C; percentage of CD271-positive cells in primary xenograft, first pass, and second pass, respectively: 10 ± 1%, 11 ± 3%, 8 ± 2%; n = 4). Hence, CD271-positive melanoma stem cells faithfully phenocopy the parental tumor in nude mice, even after multiple passages in vivo.

Our data suggest that the presence of CD271-expressing melanoma stem cells is a prerequisite for long-term tumor propagation. Therefore, although CD271-negative melanoma cells can generate tumors in NSG, NK-depleted NOD/SCID, or NK-depleted nude mice (Fig. 2; refs. 9, 10), we expected that such tumors could not be maintained in vivo over a prolonged time period. To test this, we serially passaged tumors generated in NSG mice from CD271-positive or CD271-negative cells, using tumors produced by CD271-positive melanoma cells in nude mice as positive control. In both nude and NSG mice, tumors originating from CD271-positive cells allowed propagation in vivo over 5 passages, in accordance with the earlier-described in vivo self-renewal capacity of CD271-positive cells in nude mice (Fig. 4). In striking contrast, tumors derived from CD271-negative cells in NSG mice exhausted with time and could only be propagated in vivo for 3 to 4 passages (Fig. 4). Quantification of CD271-expressing cells in consecutively produced NSG xenografts revealed that the capacity for tumor propagation over several passages was associated with the presence of CD271-positive melanoma stem cells in each xenograft (Fig. 4A–C). CD271-negative cells, however, were unable to produce CD271-positive cells, even after xenograft passaging (Fig. 4A and D). It should be noted, however, that although CD271-positive cells generate tumors in NSG mice that can be serially passaged, those tumors did not phenocopy the parental tumor (Fig. 2).

Thus, NSG mice, NK-depleted NOD/SCID mice, or NK-depleted nude mice provided a host environment permissive for tumor growth by CD271-negative cells, whereas such cells were unable to initiate tumorigenesis in NOD/SCID or nude mice. In contrast, CD271-positive melanoma stem cells were tumorigenic in all mouse models tested. In any case, only in nude or NOD/SCID mice CD271-positive melanoma stem cells produced tumors that completely phenocopied the parental melanoma even on serial xenotransplantation, whereas they failed to do so in NSG mice.

Expression of the NCSC markers CD271/Sox10 correlates with metastatic potential and poor prognosis of melanoma

To verify our findings with a large number of patient samples, we stained tissue microarrays containing more than 200 different melanoma biopsies of primary melanomas,
melanoma metastases, and melanoma cell lines for cells coexpressing the NCSC transcription factor Sox10 (20) and CD271 that is found both in NCSCs (16, 19) and in melanoma-initiating cells (9). Consistent with its expression in NCSCs and melanocytes, nuclear Sox10 was found in the majority of cells in melanoma cell lines, primary melanomas, and

Figure 3. CD271-positive melanoma cells have the capacity of self-renewal in vitro and in vivo, in contrast to CD271-negative melanoma cells. A, light microscopy (left) of a representative human melanoma sphere and immunofluorescent staining (right) for CD271 (red) and Sox10 (green; white arrow, inset). Scale bar, 50 μm. B, propagation of CD271−derived (white bars) and CD271+/−derived (black bars) spheres by serial passaging. Spheres were obtained from melanoma cell lines (M90115 and M010817; ref. 17), and metastatic melanoma lesions (824 and 4286). The data (mean ± SD, n = 4) are given as ratios of second/first passage and fifth/fourth passage, respectively. C, number of CD271-positive cells (mean ± SD) in primary xenografts obtained by direct transplantation of patients’ tumor material (n = 4), in secondary tumors derived from CD271-positive cells selected from primary xenografts (n = 4; first passage), and in tertiary tumors derived from CD271-positive cells selected from secondary tumors (n = 4; second passage). D, human melanoma (patient 4286) and corresponding primary and secondary xenografts derived in nude mice from CD271-positive cell fractions were stained for the NCSC markers CD271 (red) and Sox10 (green; white arrows, insets) and the differentiation markers MelanA, S100P, HMB45, MITF, TuJ1, NF, NSE, Peripherin, and SMA (in green). Tissues were counterstained with Hoechst 33342. Expression or absence of a given marker is labeled by + or −, respectively. Bottom, tissues were stained with H&E. Scale bar, 100 μm.
metastases (Fig. 5A). In contrast, the number of cells with detectable CD271 expression was highly variable. Among primary melanoma biopsies, we found 31 biopsies without detectable CD271 expression (57%), 14 biopsies with less than 5% CD271-positive cells (26%), and 9 biopsies with more than 5% of CD271-positive cells (17%). Intriguingly, however, the proportion of CD271/Sox10 double-positive cells in primary tumors without evidence of metastasis was significantly less than that in primary tumors of patients, who developed metastases during 5-year follow-up (P = 0.01; Fig. 5B). In addition, there was a higher proportion of CD271/Sox10-positive cells in metastases as compared with primary tumors without evidence of metastasis (P = 0.04, Fig. 5C). Similarly, the proportion of CD271/Sox10-positive cells was significantly increased in cell lines derived from metastases as compared with cell lines derived from primary tumors (P = 0.01; Fig. 5C). Thus, both in primary tumors with evidence of metastasis and in metastatic lesions, the number of CD271/Sox10-positive cells was relatively increased, suggesting that their frequency is associated with the metastatic potential in human melanoma.

To specifically address this issue, we focused our analysis on 54 primary malignant melanomas of a sentinel lymph node study for which tumor-specific survival data were available (12). Within this cohort, a frequency of CD271/Sox10-positive cells greater than 5% was associated with poor tumor-specific survival (P = 0.03; Fig. 5D). Together, these findings support the hypothesis that an elevated frequency of melanoma cells expressing NCSC markers is a prognostic factor for the development of metastasis.

Discussion

In this study we show that human melanoma contains cells that fulfill the definition of cancer stem cells, including the capacity for extensive in vivo self-renewal, maintaining long-term tumor growth, and faithfully recapitulating the cellular composition of the patient tumor, from which the cells have been derived. These melanoma stem cells share properties with normal NCSCs, the precursors of melanocytes in the skin. Indeed, human melanoma stem cells express the NCSC markers CD271 and Sox10, and, similar to NCSCs, have the capacity to self-renew and to generate multiple cell types in vitro and in vivo. CD271-expressing melanoma cells have most recently been shown to exhibit an increased tumor-initiating capacity as compared with CD271-negative cells in fully immunocompromised mice (9). In addition, as we show here, CD271-positive melanoma cells are not only able to initiate tumorigenesis, but invariably regenerate heterogeneous tumors analogous to the parental tumors in patients, even...
Importantly, CD271-positive cells were required for continuous melanoma growth, as long-term passaging and expansion of tumors was dependent on the presence of a CD271-positive cell fraction in the tumor.

The recent debate on whether particular surface markers can be used to distinguish tumorigenic from nontumorigenic melanoma cells in human biopsies (8–11) has pointed out the importance of establishing appropriate methods for cancer cell isolation from solid tumors and for the study of tumorigenic properties in vivo (3, 11, 22). Our work shows that an excess of proteolytic trypsin activity during tumor digestion and cell fraction preparation can significantly reduce the percentage of cells positive for a given surface marker in immunolabeling assays. In particular, trypsin treatment substantially lowered the detectability of CD271- and ABCB5-positive cells by specific antibodies as compared with more gentle protocols of tumor digestion, presumably because of proteolytic cleavage of surface epitopes. Our findings conceivably explain the discrepancies between conflicting reports on the nature of melanoma subpopulations with tumor-initiation potential. Specific markers defining tumorigenic cells have been identified in studies avoiding trypsin during melanoma cell fraction preparation (refs. 8, 9; this study), whereas no such markers were identified when trypsin has been included in the tumor digestion protocol (10, 11). Thus, we propose that trypsin treatment of melanoma cells can yield cell fractions false-negative for a given cell surface marker. The resulting contamination of supposedly marker-negative cell fractions by cells actually expressing the marker might explain why in some experimental setups cells positive for CD271 or ABCB5 as well as cells seemingly negative for these markers seemed to be equally tumorigenic and to give rise to tumors reexpressing the markers (10, 11). Moreover, loss of surface epitopes might generally affect the capacity of a tumor cell to associate with the surrounding tissue on transplantation and to initiate tumor formation.

Strikingly, complete phenocopies of parental tumors were never obtained in NSG mice, irrespective of whether bulk tumor cells or isolated cell fractions were used for xenograft experiments. Furthermore, only fully immunocompromised NSG mice provided a host environment permissive for tumor growth by CD271-negative cells, whereas these cells were unable to initiate tumorigenesis in more immunocompetent models. In contrast, CD271-expressing melanoma stem cells were tumorigenic in all mouse models tested, indicating a specific capacity for immune evasion by the stem cell population. The apparent immunoselection of melanoma stem cells in xenografts involves the innate immune system, as NK cell depletion in nude or NOD/SCID mice restored the capacity of CD271-negative cells to form tumors. In agreement with these results, NK and NKT cells have been shown to play a central part during immune surveillance of chemically induced skin tumor in mice (23–25). In addition, there are many lines of evidence supporting the concept of immunoediting also in human tumors, including melanoma (26, 27).

There are a number of mechanisms by which tumor cells may escape or suppress an immune response (28). Melanoma cells might achieve immunogenic tolerance by promoting apoptotic cell death or inactivation of antigen-reactive cells (29) or by inducing an immunosuppressive environment as provided, for instance, by increased levels of immunosuppressive macrophages and neutrophils (30). Interestingly, it has recently been shown that ABCB5-positive melanoma cells have the capacity to inhibit interleukin-2-dependent T-cell proliferation.
activation and to induce tolerization by regulatory T cells (31). Reduced expression levels of specific tumor antigens, such as Melan-A/MART-1, tyrosinase, and gp-100, might present another mechanism for immune evasion by melanoma cells. Notably, CD271- and ABCB5-positive melanoma cells express low levels of melanoma-associated antigens such as MART-1, supporting the idea that melanoma cells expressing melanoma stem cell markers escape the immune system attack by the host (refs. 9, 29, 31; G. Civenni and L. Sommer, unpublished data). Of note, we identified a subpopulation of CD271-positive melanoma cells that also express ABCB5 (G. Civenni and L. Sommer, unpublished data), although the functional implication of this finding remains to be addressed. All together, these results suggest that in patients, melanoma stem cells might be able to evade or modulate the immune response, allowing these cells to promote tumorigenic growth and to provide resistance to immunotherapy. The hypothesis that these processes are relevant in patients is further supported by our data that only in mice with a certain level of immunocompetence, CD271-positive melanoma cells generated tumors fully phenocopying the original patient melanoma. However, because xenotransplantation models cannot accurately recapitulate the immune response induced by cancer in human patients, the interaction between cancer cell populations and the immune system should be addressed by using syngeneic melanoma mouse models, in which components of the anticancer immune system can be manipulated during tumor initiation and progression.

Our data are consistent with the idea that CD271-positive melanoma cells play a crucial role in tumor formation in human patients. This is further supported by our clinical data obtained with an extensive tissue array of melanoma samples. These show an association between the proportion of CD271/Sox10-positive cells in primary melanoma and metastatic disease as well as poor tumor-specific survival. Hence, high numbers of melanoma stem cells expressing NCSC markers might influence aggressiveness and the metastatic behavior of malignant melanoma. This conceivably reflects intrinsic, NCSC-like features of melanoma stem cells, given that normal NCSCs have the capacity to extensively migrate through embryonic tissue before differentiation into melanocytes and other cell types. In support of this, increased levels of CD271 expression in melanoma have been associated with enhanced invasive potential in culture (32). Interestingly, CD271/Sox10 expression in metastatic primary melanoma was higher than that in metastatic lesions, which could be explained by different patient groups. Whereas all primary melanomas (metastatic versus nonmetastatic) were primary tumors without chemotherapy or vaccination therapy, tissue of the metastases was obtained from patients after adjuvant therapies. Possibly, this therapy might result in a lower prevalence of cells with CD271/Sox10 expression in the metastases. Alternatively, the site of primary metastatic tumor formation might offer a microenvironment more favorable for stem cells than provided by distant metastases. In any case, however, the proportion of CD271/Sox10-positive cells can potentially be used as a predictor of metastases and consequently poor tumor-specific survival. This in turn suggests that monitoring melanoma cells with NCSC-like features in primary melanoma might be of great prognostic relevance.

Several biopsies of primary, mostly nonmetastatic melanoma did not contain CD271/Sox10-positive cells according to our TMA analysis. CD271/Sox10-positive cells might therefore be involved in the formation of metastatic melanoma, whereas less aggressive tumors might originate from other types of melanoma-initiating cells. By extrapolation one could argue that tumor aggressiveness depends on whether the key oncogenic mutation occurred in a normal stem cell or in a more restricted progenitor cell (33). However, although the melanoma stem cells identified in this study display similar marker expression and potential as normal NCSCs in the adult skin (16, 34), melanoma-initiating cells could also arise by dedifferentiation of more mature melanocytic cells (35). Similarly, we cannot exclude that depending on the context or the oncogenotype, CD271/Sox10-negative melanoma cells emerge from other tumor cells, for instance on a process reminiscent of an epithelial-to-mesenchymal transition during metastasis or by epigenetic modification (36, 37). Intriguingly, however, at least in the conditions chosen for this study, CD271 expression was a stable trait of human melanoma-initiating cells, and we did not find any evidence for CD271-positive cells originating from the CD271-negative tumor cell fraction even after prolonged incubation in vivo. Moreover, CD271-negative cells did not acquire properties of actual melanoma stem cells, in that they could neither reproduce the cellular heterogeneity of parental tumors nor sustain long-term tumor formation.

In conclusion, the identification of human melanoma stem cells required for continuous tumor growth points to potential culprits of tumor formation in patients. In the future, it might be possible to establish specific treatments that reduce tumorigenesis by elimination of these cells or by targeting "stemness" in melanoma (1). Thus, efforts should be made to develop drugs able to promote differentiation of melanoma stem cells, to selectively kill these cells, or to specifically block their self-renewal and expansion.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

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