Intravital Imaging Reveals Transient Changes in Pigment Production and Brn2 Expression during Metastatic Melanoma Dissemination

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Abstract

How melanoma acquire a metastatic phenotype is a key issue. One possible mechanism is that metastasis is driven by microenvironment-induced switching between noninvasive and invasive states. However, whether switching is a reversible or hierarchical process is not known and is difficult to assess by comparison of primary and metastatic tumors. We address this issue in a model of melanoma metastasis using a novel intravital imaging method for melanosomes combined with a reporter construct in which the Brn-2 promoter drives green fluorescent protein (GFP) expression. A subpopulation of cells containing little or no pigment and high levels of Brn2::GFP expression are motile in the primary tumor and enter the vasculature. Significantly, the less differentiated state of motile and intravasated cells is not maintained at secondary sites, implying switching between states as melanoma cells metastasize. We show that melanoma cells can switch in both directions between high- and low-pigment states. However, switching from Brn2::GFP high to low was greatly favored over the reverse direction. Microarray analysis of high- and low-pigment populations revealed that transforming growth factor (TGF) β2 was up-regulated in the poorly pigmented cells. Furthermore, TGFβ signaling induced hypopigmentation and increased cell motility. Thus, a subset of less differentiated cells exits the primary tumor but subsequently give rise to metastases that include a range of more differentiated and pigment-producing cells. These data show reversible phenotype switching during melanoma metastasis. [Cancer Res 2009;69(20):7969–77]

Introduction

Melanoma is a type of skin cancer arising from the aberrant proliferation of melanocytes. Once melanoma begins to spread the prognosis deteriorates (1). Melanocytes originate from a subpopulation of neural crest cells called melanoblasts (2). During development, melanoblasts migrate through the embryo. Melanoblasts stop migrating when they reach the skin and reside in the lower levels of the epidermis or in the dermis, depending on species, and differentiate to melanocytes (3). The differentiation of melanoblasts to mature melanocytes is associated with down-regulation of the transcription factor Brn-2/POU3F, which represses the transcription factor MITF (4). MITF is a key regulator of melanocyte function, and high levels of MITF activity promote melanogenesis (5–7). Melanogenesis occurs in organelles called melanosomes. Pigment is produced in stage 3 and 4 melanosomes before being exported. However, the level of pigment produced is not the same for all melanocytes and is controlled by a range of genetic and environmental factors (3). Although MITF promotes pigment production, it suppresses invasion (8, 9). Conversely, Brn-2, which represses MITF, can promote invasion in vitro (4). Brn-2 expressing subpopulations of cells can be found within melanoma (4). However, the behavior and stability of Brn-2–expressing cells in vivo is not known.

Two models to account for melanoma metastasis can be envisaged. First, acquisition of prometastatic genetic changes, such as NEDD9 amplification (10), could lead to a more invasive phenotype. This model predicts that metastases would have a genetic signature different from the majority of cells in the primary tumor (1, 11). Alternatively, metastasis may be driven by epigenetic events that could be reversible. This is supported by the observations of Hoek and colleagues (12). These changes may be initiated by signals originating in the tumor microenvironment that need not necessarily be maintained at secondary sites. Definitive proof that reversible phenotype switching occurs during metastasis is difficult to obtain by comparison of primary and metastatic tumors. Therefore, we used live imaging of a melanoma model to directly analyze motile cells escaping from the primary tumor and those that have entered the blood. The results reveal that disseminating cells are poorly pigmented and express high levels of a Brn2-GFP reporter. However, this phenotype can be reversed at metastatic sites.

Materials and Methods

Cell Culture

B16F2 and B-RafV600E melanoma cells (called 4599; ref. 13) were cultured in DMEM with 10% fetal calf serum.

Pigmentation Experiments

B16F2 or 4599 cells were stimulated or inhibited with 2 μg/mL αMSH (Calbiochem 05-23-0751), 10 μmol/L H89 (Sigma), 1 ng/mL transforming growth factor (TGF) β1 (Peprotech 100-21C), or 1 ng/mL TGF β2 (R&D Biosystems 302-B2) for 48 h.

Imaging Techniques

In vitro. A ZeissLSM510META confocal microscope with a femtosecond-pulsed Ti-Sapphire laser was used. Pigment was imaged using transmitted-light without phase-contrast or NISREVE signal was captured by exciting the cells with NRA wavelengths between 700 to 850 nm and collecting visible light emission. To determine the emission spectrum between 385 to 680 nm, a ZeissMETA detector was used. Analysis of cell migration speed was carried out by tracking phase-contrast movies of B16F2 cells plated on
collagen/Matrigel gels and treated with α-MSH, TGFβ1 or TGFβ2 for 24 h before imaging. Tracks were calculated using Metamorph (Molecular Devices) and Mathematica (Wolfram) software.

**In vivo.** Mouse procedures were carried out in accordance with PPL70/6164. Depending on availability, either C57/BL6 or Nude mice were injected s.c. with 10^6 B16-GFP, B16-GFP-CAAX, or B16-mRFP-CAAX Brn2::GFP cells. Intravital imaging was carried out on anaesthetized mice with tumors using a Zeiss LSM510 confocal microscope. An incision was made in the skin to expose the tumor. The animal was placed on a heated stage. Tumor areas were imaged for 20 min at 30- to 60-s intervals. Collagen fibers were visualized by second harmonic signals (410–450 nm), pigment was imaged at 565 to 615 nm and 635 to 680 nm following excitation at 850 nm (–20 mW power at objective). GFP-CAAX was imaged at 500 to 550 nm following excitation with either a 488 nm laser or 850 nm; excitation using the later wavelength also resulted in signal being detected from NIRVE. To obtain cells from the circulation, vessels draining from the tumor were cut and were allowed to drain onto a coverslip for 1 min. PBS was then added to dilute the sample before analysis for green fluorescent protein (GFP)–positive melanoma cells. Details of quantification are given in Supplementary Data. For analysis of sorted Brn2::GFP–high and Brn2::GFP–low and pigment–high and pigment–low populations 10^6 cells were injected into the tail vein of C57/BL6 mice. Lung colonies were imaged after 11 to 12 d.

**Flow Cytometry**

Analysis was carried out on an FACS Aria cell sorter (Becton Dickinson). Details of fluorescence-activated cell sorting (FACS) settings are provided in Supplementary Data.

**Microarray**

RNA was extracted from B16F2 cells sorted from tumors by FACS using RNeasy mini kit (Qiagen). Microarray analysis was carried out by the Paterson Institute using Affymetrix chips (MOE430 2.0).

**Immune Fluorescence**

Frozen sections of B16 tumors were fixed in 4% PFA, followed by 0.2% TritonX-100 and blocking with 5% bovine serum albumin (BSA). Anti-F4/80 (Abcam) was used to detect macrophages. Pigment was imaged as described above.

Further details of imaging methods are available in Supplementary Data.

**Results**

**Intravital imaging of a melanoma model.** B16F2 melanoma cells can enter the vasculature and spread to the lymphatic system and lungs. To learn more about this process, we performed intravital imaging of these tumors. B16F2 cells were engineered to express GFP before s.c. injection into mice. Tumor imaging was performed using single photon excitation at several locations with high resolution for 20-minute periods. Only a fraction of cells were motile in the primary tumor (Fig. 1A1 and Supplementary Movie S1). Figure 1Aiii shows overlaid images of three time points in red, green, and blue. Nonmotile cells appear white—the composite color generated from red, green, and blue. Motile cells are seen by the separation of the colors. Motile cells moved with speeds ranging from 0.2 to 5 μm/minute and had irregular, rounded, and rapidly changing morphology (Fig. 1B and Supplementary Movie S1). Nonmotile cells had a diverse array of shapes ranging from amorphous to some with extended “dendritic” morphologies that were never observed in motile cells. We speculated that the motile subpopulation of cells observed in the melanoma model might have an altered differentiation status compared with the nonmotile population.

**Visualization of pigment in melanoma cells and melanocytes following near infra red excitation.** Pigment containing melanosomes can be identified by imaging cells with transmitted light (Fig. 2A). However, this method cannot be applied in live tumors. During our tumor imaging, we noticed a vesicular pattern of light emission from pigment producing B16 tumors following illumination with NIR wavelengths. We speculated that this could originate from pigment containing vesicles. Figure 2A shows a remarkable correlation between these signals and transmitted light imaging of melanosomes. Visible light was emitted across a broad spectrum of visible wavelengths following NIR illumination between 700 to 900 nm (Fig. 2A, right). We will use the abbreviation NIRVE (Near IR Visible Emission) to describe this unexpected phenomenon. Correlative analysis of NIRVE signal with transmission electron microscopy of the same cells revealed that NIRVE signal originated from stage 3 and 4 melanosomes (orange and red asterisks), which contain melanin, but not stage 2 melanosomes, which do not contain pigment (Fig. 2B). Treating B16F2 cells with either α9i or α-MSH, to inhibit PKA-dependent melanogenesis or stimulate melanin production, respectively, caused corresponding changes in NIRVE signal (Fig. 2C). NIRVE signal could also be detected in another melanoma culture, 4599, but not in breast cancer cells (Supplementary Fig. S1A). Imaging of mouse skin showed that NIRVE signal is also detected in hair and around the base of some hair follicles, which is where melanocytes are located (Fig. 2D and Supplementary Movie S2 for animation through z-sections; refs. 14, 15). As further demonstration of the utility of this method, we found that NIRVE signal is also detected in formalin-fixed paraffin-embedded human melanoma samples (Supplementary Fig. S1B). Together, these data show that NIRVE emission is a novel and specific means of imaging melanin pigment that can be widely applied to image tissues in situ.

**Motile cells in vivo lack pigment.** Fig. 3A shows that pigment could also be imaged in B16F2 tumors in vivo and was usually in puncta near the cell periphery, which was defined by GFP-CAAX expression. Timelapse analysis revealed that the NIRVE signal was dynamic (Supplementary Movie S3), possibly as a result of melanosome movement. Immunohistochemistry revealed that some NIRVE signal could also be found within melanoma associated macrophages, which are known to take up melanin (13, 14), but not breast tumor-associated macrophages (Supplementary Fig. S1C). B16F2 melanoma cells can metastasize. This process begins with cell motility in the primary tumor (11). Figure 3B and Supplementary Movie S4 show examples of the motile behavior of B16F2-GFP cells in vivo. Strikingly, motile cells contained very little pigment (Fig. 3B). The amount of pigment contained in motile and nonmotile cells was quantified. The majority of the cells in the primary tumors are nonmotile; therefore, the distribution of pigment in the nonmotile cells was similar to the distribution for all the cells. However, the motile cells contained lower levels of pigment than the nonmotile cells (Fig. 3C, compare second and third panels). Cells were also collected from the vasculature and imaged: these cells also had significantly less pigment than nonmotile cells in the primary tumor.

Despite finding that the motile cells in the primary tumor contained a low level of pigment, spontaneous secondary metastatic tumors were highly pigmented (for example, in the draining lymph nodes; Fig. 3Di). Furthermore, experimental lung metastases were also pigmented. This was confirmed by analysis of NIRVE signal at metastatic locations (Fig. 3C and Di), although some less pigmented cells were still present in metastases. These results suggested that the hypopigmentation of invasive melanoma cells could be a transient phenotype.
Brn2 promoter activity is up-regulated in motile cells. A failure to produce pigment does not necessarily indicate a change in differentiation status. We therefore sought another method to investigate the differentiation status of the motile melanoma cells. Brn-2/POU3F2 expression is high in migratory melanoblasts in culture and decreases as melanocytes differentiate (16, 17). To investigate the relationship between Brn-2 expression and melanoma dissemination, we engineered B16 cells to contain the Brn-2 promoter driving the expression of GFP; these cells were also engineered to express mRFP-CAAX constitutively. Supplementary Fig. S2 confirms that these cells down-regulate GFP expression in response to the prodifferentiation stimulus αMSH. Brn2::GFP was heterogeneously expressed in vivo (Fig. 4A). Furthermore, GFP levels were high in motile cells in vivo, indicating elevated activity of the Brn-2 promoter (Fig. 5A, yellow arrows). However, high levels of GFP expression were not sufficient to confer motility as not all GFP-positive cells were motile (red arrows). We quantified the fluorescence intensity of GFP in numerous cells that were nonmotile, motile, circulating, or growing in the lungs. Figure 4B shows a clear shift toward elevated Brn-2 promoter activity in the motile and circulating cells that is largely downregulated in cells growing in the lungs. The data presented above suggest that Brn-2 promoter activity and pigment production should be inversely related. We investigated this by simultaneous imaging of Brn-2 promoter–driven GFP expression and NIRVE signal. Figures 4C and D show that cells with high levels of pigment have low levels of Brn-2::GFP expression. Many cells had low levels of both indicating that there is not a simple inverse correlation between the two variables. Cells expressing high levels of Brn2::GFP generally had low levels of pigment although some cells expressed above average levels (Fig. 4D, top right quadrant).

Switching between high- and low-pigment production and Brn2 expression states. The data outlined above indicate that melanoma cells in transit from primary to secondary sites have less differentiated characteristics that are only found in a subset of cells in primary tumors and metastases. This could be explained by reversible switching between states. Melanoma cells could reduce pigment production and elevate Brn2 expression as they exit primary tumors and reverse these changes when they have arrived at secondary locations. Another possibility is the switching between states is unidirectional. In this model, high-Brn2 low-pigment cells can produce low-Brn2 and high-pigment progeny but not vice versa. The heterogeneity at primary and metastatic sites would arise from the differentiation of the Brn2 high/pigment low cells. To test these contrasting possibilities, we isolated either Brn2::GFP-high and Brn2::GFP-low or pigment-high and pigment-low populations from primary tumors and reintroduced them into mice.

We developed a FACS method for sorting cells based on their level of pigment. We found a robust difference in light emitted around 450 nm following excitation at 405 nm between more pigmented α-MSH–treated cells and less pigmented control cells or H89-treated cells, which contain almost no pigment (Fig. 5A; see also Fig. 1C). The most highly and least pigmented B16F2 cells were isolated from xenograft tumors based on 450 to 40 emission (Fig. 5Bi). These cells were then expanded in culture before injection into the tail vein of mice. After 11 to 12 days, the lungs were analyzed. Figure 5Bi shows that a similar spectrum of pigment levels were observed in melanoma colonies derived from both pigment-high and pigment-low populations. These data show that switching can occur in both directions between high- and low-pigment production states.

We performed a similar series of experiments with cells sorted for levels of Brn2::GFP expression. Figure 5C shows that Brn2::GFP-high cells produced colonies with both GFP-positive and GFP-negative cells. This result indicates that Brn2-high cells can produce Brn2-low progeny. In contrast, Brn2::GFP-low cells produced colonies with predominantly Brn2::GFP-low cells and only very few Brn2::GFP-high cells (Fig. 5Ci). These data indicate that switching from a Brn2-high state to a Brn2-low state is favored over low to.
high switching. The number of melanoma colonies obtained with high- and low-pigment and Brn2::GFP-high and Brn2::GFP-low populations were equivalent (data not shown).

**TGFβ2 is up-regulated in cells lacking pigment.** We next wished to investigate molecular differences between more and less pigmented cells that might explain the switching between high- and low-pigment states. We sorted pigment-high and pigment-low cells as described above and performed microarray analysis. Supplementary Table S1 lists the genes differentially regulated between the two cell populations. The green text indicates genes more highly expressed in the cells lacking pigment (this population contains the motile population observed in vivo). From the genes up-regulated in cells lacking pigment, we chose to investigate the function of TGFβ2 further. TGFβ2 is extensively

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**Figure 2.** Pigment containing melanosomes can be identified by visible light emission following near IR illumination. **A**, B16F2 cells stimulated with α-MSH were imaged by transmitted light (i) and laser scanning microscopy following illumination at 800 nm (ii) merged image of emission at 410 to 450 nm (cyan), 500 to 550 nm (green), and 565 to 615 nm (red). Scale bar, 25 μm. Emission and excitation spectra of near IR–stimulated visible emission are shown in iii and iv, respectively. A.u., arbitrary unit. **B**, transmitted light and NIRVE images (dark red) of B16 cells are shown overlaid (left). A cell with a long process and high NIRVE signal is visible surrounded by cells with low NIRVE signal. Transmission electron microscopy images of the same area are shown. **Middle left**, a low magnification image of the NIRVE-positive cell and surrounding cells. Inset panels 1 to 3, high-magnification images of the areas indicated on the low-magnification image. White, orange, and red asterisks are placed adjacent to stage 2, 3, and 4 melanosomes, respectively. **C**, top, changes in pigment following α-MSH or H89 treatment. **Bottom,** NIRVE signal in green following α-MSH or H89 treatment; red, filamentous actin (F-actin). Scale bar, 50 μm. **D**, ear skin of mouse showing collagen (blue, collected between 420–490 nm following illumination at 800 nm) and NIRVE signal (red, collected between 575-630 nm following illumination at 800 nm). See also Supplementary Movie S2. Arrows, pigment-producing melanocytes. *, some NIRVE signal is also collected.
implicated in cancer progression and is also a target gene of TGFβ signaling (19). Therefore, high TGFβ2 expression may indicate a positive feedback loop of TGFβ signaling in less pigmented melanoma cells. Consistent with this, we observed an inverse pattern of pigmentation and activation of TGFβ signaling as judged by pSmad3 staining (data not shown).

**TGFβ signaling induces hypopigmentation and cell motility.**

We next tested whether TGFβ signaling could affect pigment levels.
We found that both TGFβ1 and TGFβ2 induced hypopigmentation of melanoma cells at relatively low doses (Fig. 6A). This was confirmed by reduction of NIRVE signal following stimulation with TGFβ in both α-MSH–treated B16 and 4599 melanoma cells (Fig. 6B). We also investigated whether there was a connection between TGFβ signaling and melanoma motility. When plated on deformable collagen/Matrigel matrix in vitro B16F2 appeared elongated with some dendritic protrusions (Fig. 6C). Treatment of cells with α-MSH enhanced the dendritic morphology that is characteristic of a differentiated melanocyte. However, treatment of cells with either TGFβ1 or TGFβ2 inhibited dendrite formation and cells appeared more rounded (Fig. 6Ci). Furthermore, treatment with either TGFβ1 or TGFβ2, but not α-MSH, significantly increased cell motility (Fig. 6Ci). These data show that TGFβ signaling can reverse characteristics found in differentiated melanocytes including pigment production and dendritic morphology, and increase cell motility.

**Discussion**

Heterogeneity between cancer cells in the same tumor is a feature of many cancers. In the case of melanoma, distinct Brn2- and MITF-expressing populations can be found in the same tumor (4). It has been suggested that reversible changes in between cell states underlie metastatic behavior. Melanoma cells can switch reversibly between more and less pigmented states (18), and interconversion between proliferative and invasive states has been reported in primary tumors (12). However, it is unclear how changes in these states may relate to the various stages of melanoma dissemination, if changes in cell state are truly reversible and what factors may drive these changes. In this study, we use intravital imaging to show transient changes in pigment production and differentiation status during the metastatic process.

Both primary tumors and metastases contain Brn2-high and Brn2-low cells and pigment-high and pigment-low cells. Combined analysis of Brn2::GFP levels and NIRVE signal showed that Brn2-high cells generally had lower levels of pigment than Brn2-low cells. Nonetheless, some Brn2-high cells had above average levels of pigment and many cells had low levels of both pigment and Brn2 expression (Fig. 4D). Our intravital imaging revealed that only Brn2-high/pigment-low cells were motile. Therefore, it is the coincidence of elevated Brn2 with very low pigment levels that most closely correlates with motility. These traits are characteristics...
Figure 5. Flow cytometry analysis of pigment content. A, FACS profile of B16F2 cells with or without stable GFP expression (left). Effect of H89 or α-MSH on violet laser stimulated 450 nm emission is shown by contour plots. i, B16F2-GFP CAAX tumor cells isolated from xenograft tumors in nude mice. Contour plots show GFP (515–45 nm) against Violet (450–70 nm) emission. Blue line, gate used to sort GFP-expressing cells; magenta lines, the gates used to sort the top and bottom 10% pigmented cells. ii, pigment-high and pigment-low populations were reintroduced into the tail vein of mice and the resulting lung colonies were imaged. Representative images are shown with NIRVE signal (black) and GFP (green). iii, quantification of pigment levels assessed on a cell by cell basis in colonies generated from pigment-high and pigment-low populations. Relative frequency of pigment levels is shown (data from 12 mice in three independent experiments).

B, i, B16F2-GFP CAAX tumor cells isolated from xenograft tumors in nude mice. Contour plots show GFP (515–45 nm) against Violet (430–70 nm) emission. Blue line, gate used to sort GFP-expressing cells; magenta lines, the gates used to sort the top and bottom 10% pigmented cells. ii, pigment-high and pigment-low populations were reintroduced into the tail vein of mice and the resulting lung colonies were imaged. Representative images are shown with NIRVE signal (black) and GFP (green). iii, quantification of pigment levels assessed on a cell by cell basis in colonies generated from pigment-high and pigment-low populations. Relative frequency of pigment levels is shown (data from 12 mice in three independent experiments).

C, i, B16F2 Brn2::GFP/mRFP-CAAX tumor cells isolated from xenograft tumors in nude mice. Contour plots show red (600–40 nm) against GFP (515–45 nm) emission. Blue line, gate used to sort mRFP-expressing cells; green lines, the gates used to sort the top and bottom 10% Brn2::GFP cells. ii, Brn2::GFP-high and Brn2::GFP-low populations were reintroduced into the tail vein of mice and the resulting lung colonies were imaged. Two representative images are shown with mRFP-CAAX signal (red) and Brn2::GFP (green). *, highlights three cells that have converted back to high levels of Brn2::GFP expression. iii, quantification of Brn2::GFP levels assessed on a cell by cell basis in colonies generated from Brn2::GFP-high and Brn2::GFP-low populations. Relative frequency of Brn2::GFP levels is shown (data from 10 mice in three independent experiments). *, P < 0.01 Mann-Whitney test.
of less differentiated more melanoblast-like cells. The less differentiated nature of the disseminating cells could result from cell extrinsic factors transiently promoting dedifferentiation. Alternatively, there may be unidirectional switching between states and only cells that are intrinsically less differentiated would be competent to metastasize. We find that switching of pigment and Brn2 expression can occur in both directions. Although, Brn2-low cells rarely converted to melanoblast-like Brn2-high cells (Fig. 5C). The conversion to high Brn2 expression may result from a rare combination of cell extrinsic factors or may simply be stochastic. These data support a transient and reversible switch to a less differentiated phenotype during melanoma dissemination (Supplementary Fig. S3).

For melanoma cells to become motile and begin disseminating, a combination of conditions need to occur. First, the cell must be in a Brn2-high state. However, Brn2 expression is not sufficient to completely suppress pigment production or induce motility in vivo. Therefore, additional cues are required to further suppress levels of pigment and induce motility. Our microarray data and subsequent analysis show that TGFβ signaling is a prime candidate for this additional signal. Elevated levels of TGFβ ligands in the primary tumor could suppress pigment production and promote motility. However, these changes may not be maintained if TGFβ levels are low at secondary sites. TGFβ2 has previously been reported to be increased in metastatic melanoma (19) and is associated with a more invasive phenotype (9). The inverse correlation between pigment and TGFβ signaling that we observe in tumors is consistent with previous reports that TGFβ antagonizes MITF function and melanosome maturation (20, 21). TGFβ signaling in the less differentiated disseminating melanoma cells may also promote mesenchymal characteristics and even stem cell traits (22). Our microarray analysis shows that less pigmented cells have elevated levels of a mesoderm-specific transcript. However, we did not find evidence for stem cell behaviors in our system. All the populations we describe have similar clonogenic potential both in vitro and in vivo (data not shown). This is consistent with a recent report that melanoma lacks a stem cell compartment (23). TGFβ did not promote Brn-2::GFP expression (data not shown). The factors that drive Brn2 expression in a subset of cells are unclear, but they could include B-Raf or β-catenin signaling (24, 25).

Figure 6. TGFβ induces hypopigmentation, inhibits dendrite formation, and increases cell motility. A, cell pellets of B16F2 cells treated with H89, αMSH, or TGFβ for 48 h are shown. Numbers indicate digital analysis of pellet darkness. B, NIRVE (white, collected between 410–530 nm following illumination at 790 nm) and F-actin (red) merged images of B16F2 and 4599 mouse melanoma cells treated with αMSH for 24 h and where indicated TGFβ1 or TGFβ2 for 24 h before α-MSH treatment are shown. Scale bar, 50 μm. C, i, B16F2 cells plated on deformable collagen/Matrigel matrix (cyan) and stained for F-actin (green) and DNA (red). Scale bar, 50 μm. ii, box plots of cell speeds in micrometers per hour taken from phase contrast movies of B16F2 cells plated on deformable collagen/Matrigel matrix over 16-h period. *, P < 0.01 Mann-Whitney test.
Several features of this model have parallels in the normal physiology of melanoblasts and melanocytes. First, less differentiated melanoblasts that are migratory during development. Differentiation is essentially an irreversible process and this may explain why we observe Brn2-low cells converting to Brn2-high cells with low frequency. Second, reversible transitions between high and low levels of pigment production frequently occur in melanocytes; either to coordinate with the hair growth or to respond to UV exposure of the skin. In both cases, TGFβ signaling is implicated in reducing pigment levels (21, 26). However, in these cases, TGFβ signaling does not cause cell motility, probably because the cells are well differentiated.

To conclude, both primary and metastatic melanoma contain a heterogeneous mix of more and less differentiated cells. Strikingly, actively disseminating cells are more uniform with low levels of pigment and high levels of Brn2 expression. However, neither of these traits is stable. Changes in pigment production are reversible and may be transiently induced by TGFβ signaling in the primary tumor. Although, Brn2-high cells give rise to cells expressing low levels of Brn2 and high levels of pigment. Thus, although only a subset of less differentiated cells leave the primary tumor, they redifferentiate at secondary sites.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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