

Tumorigenic Cells Are Common in Mouse MPNSTs but Their Frequency Depends upon Tumor Genotype and Assay Conditions

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SUMMARY

Tumor-initiating cells have been suggested to be rare in many cancers. We tested this in mouse malignant peripheral nerve sheath tumors (MPNSTs) and found that 18% of primary and 49% of passaged MPNST cells from *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} mice formed tumors upon transplantation, whereas only 1.8% to 2.6% of MPNST cells from *Nf1*^{+/-}; *p53*^{+/-} mice did. MPNST cells of both genotypes require laminin binding to β 1-integrin for clonogenic growth. Most MPNST cells from *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} mice expressed laminin, whereas most MPNST cells from *Nf1*^{+/-}; *p53*^{+/-} mice did not. Exogenous laminin increased the percentage of MPNST cells from *Nf1*^{+/-}; *p53*^{+/-} but not *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} mice that formed tumorigenic colonies. Tumor-forming potential is common among MPNST cells, but the assay conditions required to detect it vary with tumor genotype.

INTRODUCTION

The question of which cells contribute to cancer growth and progression has fundamental implications for therapy. If tumor growth and metastasis are driven primarily by rare, or at least infrequent, cancer stem cells then cancer might be more effectively treated by specifically targeting the cancer stem cells (Reya et al., 2001; Pardal et al., 2003; Dick, 2008). On the other hand, if many cancer cells are capable of driving tumor growth and metastasis it will be necessary to eliminate most or all cancer cells.

Evidence suggests that small populations of cancer stem cells drive the growth and progression of a number of cancers, including some germ lineage cancers (Kleinsmith and Pierce, 1964; Illmensee and Mintz, 1976), myeloid leukemias (Lapidot et al., 1994; Bonnet and Dick, 1997; Yilmaz et al., 2006; Oravec-Wilson et al., 2009), breast cancers (Al-Hajj et al., 2003), gliomas (Singh et al., 2004), and colon cancers (Dalerba

et al., 2007; O'Brien et al., 2007; Ricci-Vitiani et al., 2007; Merlos-Suárez et al., 2011). In each case, transplantation of cancer cells into highly immunocompromised mice revealed a small, phenotypically distinct subpopulation of cells that was uniquely capable of transferring disease and forming phenotypically diverse nontumorigenic progeny. This suggests that some cancers are hierarchically organized, with small numbers of cancer stem cells that sustain tumor growth by forming large numbers of cancer cells with limited proliferative potential (Reya et al., 2001; Pardal et al., 2003; Dick, 2008).

In contrast to this model, human melanomas (Quintana et al., 2008, 2010), mouse melanomas (Held et al., 2010), and some mouse leukemias (Kelly et al., 2007; Williams et al., 2007) have many cells with tumorigenic potential. For example, 30% of single cells obtained directly from melanomas in patients and over 50% of cells obtained from primary mouse melanomas can form tumors in highly immunocompromised mice (Quintana

Significance

Our results demonstrate that mouse MPNSTs contain high frequencies of tumorigenic cells, which were similar upon transplantation into immunocompetent or immunocompromised mice. However, tumor genotype influenced the laminin expression pattern, and therefore the dependence of MPNST cells on exogenous laminin and the frequency of tumorigenic cells that could be detected in some assays. Although laminin binding to β 1-integrin was important for clonogenic growth, the laminin could either be intrinsic or extrinsic to MPNST cells. Tumors of different genotypes, even with the same malignant phenotype, can therefore require different assay conditions to detect the full range of cells with tumorigenic potential.

et al., 2008; Held et al., 2010; Quintana et al., 2010). These results raise three critical questions.

- (1) Is melanoma unique among solid cancers in having common tumorigenic cells?
- (2) Can high frequencies of tumorigenic cells be detected in immunocompetent recipients for some cancers or does the immune system play such a central role in the control of tumor-initiating cells that common tumorigenic cells can only be detected in immunocompromised recipients? To address this question it is necessary to examine mouse models of cancer, which can be transplanted into syngeneic recipients without a confounding xenogeneic immune barrier that rejects human cells by mechanisms that are very different from the autologous immune responses that can occur within patients.
- (3) Finally, what other factors influence the frequency of cancer cells that can form tumors?

Malignant peripheral nerve sheath tumors (MPNSTs) are aggressive soft tissue sarcomas that arise within peripheral nerves. Most MPNSTs arise in the context of Neurofibromatosis type 1, a hereditary tumor syndrome caused by mutations in *NF1* that encodes Neurofibromin, a GTPase-activating protein that negatively regulates Ras signaling (Rubin and Gutmann, 2005). About 30% of NF1 patients develop benign plexiform neurofibromas, which can transform into MPNSTs. Plexiform neurofibromas and MPNSTs arise from Schwann cells in peripheral nerves (Zhu et al., 2002; Joseph et al., 2008; Zheng et al., 2008). Beyond the loss of NF1 function, *CDKN2A* (*p16^{Ink4a}*), *CDKN2D* (*p14^{Arf}*, the human ortholog of mouse *p19^{Arf}*), and *TP53* are often inactivated in MPNSTs by deletion or promoter hypermethylation (Menon et al., 1990; Perrone et al., 2003; Agensen et al., 2005). Mutations of these genes in mice also lead to the development of MPNSTs. Thus, mice that are heterozygous for a deletion of both *Nf1* and *p53* (*Nf1^{+/-}; p53^{+/-}*; Cichowski et al., 1999; Vogel et al., 1999) and mice heterozygous for *Nf1* and deficient for *Ink4a/Arf* (*Nf1^{+/-}; Ink4a/Arf^{-/-}*; Joseph et al., 2008) develop MPNSTs.

We developed in vitro and in vivo assays to determine the frequency of MPNST cells with tumorigenic potential in these mouse models of MPNST.

RESULTS

More Tumorigenic Cells Were Detected among Primary MPNST Cells from *Nf1^{+/-}; Ink4a/Arf^{-/-}* Mice

Nf1^{+/-}; Ink4a/Arf^{-/-} and *Nf1^{+/-}; p53^{+/-}* mice were backcrossed onto a C57BL/Ka background (at least six generations) and then aged to monitor the development of MPNSTs. Approximately 25% to 50% of *Nf1^{+/-}; Ink4a/Arf^{-/-}* mice and *Nf1^{+/-}; p53^{+/-}* mice developed MPNSTs on the legs and abdomens, typically between 4 and 7 months of age, as previously reported (Cichowski et al., 1999; Joseph et al., 2008). Tumors of both genotypes grew rapidly and at comparable rates (data not shown). Three of four primary and four of four secondary MPNSTs from *Nf1^{+/-}; p53^{+/-}* mice exhibited *p16^{Ink4a}* and *p19^{Arf}* expression, whereas *p16^{Ink4a}* and *p19^{Arf}* were not detected in MPNSTs from *Nf1^{+/-}; Ink4a/Arf^{-/-}* mice, as expected

(Figure S1 available online). *p53* was not detected in most MPNSTs from *Nf1^{+/-}; p53^{+/-}* mice, as expected (Vogel et al., 1999), but was detected in most MPNSTs from *Nf1^{+/-}; Ink4a/Arf^{-/-}* mice (data not shown).

We sacrificed *Nf1^{+/-}; Ink4a/Arf^{-/-}* and *Nf1^{+/-}; p53^{+/-}* mice at 6.1 ± 0.9 or 5.3 ± 1.4 (mean \pm SD) months of age, respectively, for analysis. Tumors of both genotypes contained tightly packed spindle cells with tapered nuclei arranged in a fascicular pattern (Figures 1A–1F). Of 25 tumors analyzed, 24 were focally positive for the Schwann cell marker S100 β (Figures 1A and 1D). In tumors of both genotypes, we observed frequent mitotic figures (an average of 1.1 ± 0.7 in *Nf1^{+/-}; Ink4a/Arf^{-/-}* MPNSTs and 1.6 ± 0.6 in *Nf1^{+/-}; p53^{+/-}* MPNSTs per high power field, objective \times ocular magnification = $400\times$) and a high frequency of Ki-67 positive cells ($39 \pm 16\%$ for *Nf1^{+/-}; Ink4a/Arf^{-/-}* and $52 \pm 9\%$ for *Nf1^{+/-}; p53^{+/-}* MPNSTs; Figures 1C, 1F, and 1G).

We also administered bromo-deoxyuridine (BrdU) to tumor-bearing mice. After 6 hr of administration, 10% and 14% of cells were BrdU+ in primary MPNSTs from *Nf1^{+/-}; Ink4a/Arf^{-/-}* and *Nf1^{+/-}; p53^{+/-}* mice, respectively. Similar results were obtained when secondary MPNSTs were analyzed: 9% and $10 \pm 6\%$ of cells from MPNSTs obtained from *Nf1^{+/-}; Ink4a/Arf^{-/-}* and *Nf1^{+/-}; p53^{+/-}* mice incorporated BrdU, respectively. After 5 or 10 days of administration, over 80% of cells were BrdU+ in MPNSTs of both genotypes (Figure 1H). MPNSTs from both *Nf1^{+/-}; Ink4a/Arf^{-/-}* and *Nf1^{+/-}; p53^{+/-}* mice therefore contained similarly high frequencies of rapidly dividing cells.

To obtain MPNST cells for experiments, primary tumors of comparable size from mice of both genotypes were enzymatically dissociated and stained with antibodies. Only tumors that were confirmed to be MPNSTs by histopathology were included in our analysis. The cells were sorted by flow cytometry to exclude debris (based on light scatter), dead cells (4',6-diamidino-2-phenylindole+ (DAPI+)), hematopoietic cells (Ter119+ or CD45+), and endothelial cells (CD31+; Figure 1I). Viable cells that were negative for hematopoietic/endothelial markers were used in all experiments. These cells comprised an average of $48 \pm 17\%$ and $44 \pm 14\%$ (mean \pm SD) of nucleated cells in MPNSTs from *Nf1^{+/-}; Ink4a/Arf^{-/-}* and *Nf1^{+/-}; p53^{+/-}* mice, respectively.

We injected MPNST cells subcutaneously, into sciatic nerves, or under the kidney capsules of wild-type C57BL/Ka mice. The kidney capsule is a common site for heterologous cell grafting, where many cell types are able to efficiently engraft. We rarely observed the formation of tumors after subcutaneous injection, even when we injected large numbers of cells, but transplanted cells readily formed tumors in sciatic nerve and under the kidney capsule at higher cell doses (Table S1). At lower cell doses the cells engrafted more efficiently under the kidney capsule, where the cells were retained after injection (Table S1). Injection of cells into the endoneurial space increased pressure within nerves and led to the ejection of the injected material through the needle track, rendering the assay nonquantitative in peripheral nerve. For this reason, subsequent experiments to quantify the frequency of cells with tumorigenic potential were conducted by transplanting cells under the kidney capsule.

We transplanted aliquots of 100 cells, ten cells, or single cells from ten primary MPNSTs obtained from *Nf1^{+/-}; Ink4a/Arf^{-/-}* mice and eight primary MPNSTs from *Nf1^{+/-}; p53^{+/-}* mice under

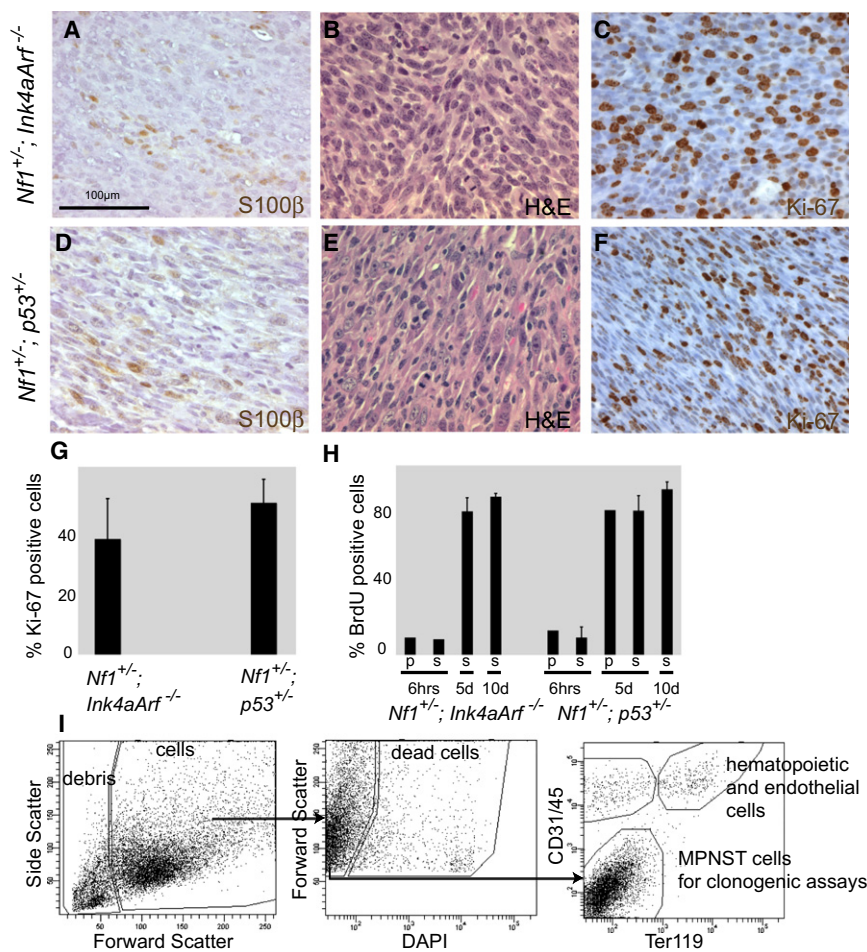


Figure 1. *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} and *Nf1*^{+/-}; *p53*^{+/-} Mice Develop MPNSTs that Contain a High Frequency of Proliferating Cells

(A–F) Paraffin sections of *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} and *Nf1*^{+/-}; *p53*^{+/-} MPNSTs were stained with antibodies against S100β (A and D), hematoxylin and eosin (B and E), and antibodies against Ki-67 (C and F). Sections were counterstained with hematoxylin (blue) in (A), (D), (C), and (F). Each panel reflects a different section.

(G) The percentage of MPNST cells that were Ki-67-positive was determined for four tumors of each genotype on at least 20 high-power fields (400× magnification, ocular x objective) for each tumor.

(H) The percentage of MPNST cells (that stained negatively for blood or endothelial markers) in mice bearing primary (p) or secondary (transplanted into wild-type mice; s) MPNSTs treated with BrdU for either 6 hr or 5 or 10 days that were BrdU-positive. The data reflect one primary and eight secondary tumors from *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} mice and three primary and nine secondary tumors from *Nf1*^{+/-}; *p53*^{+/-} mice. All statistics represent mean ± SD.

(I) Isolation of MPNST cells from primary tumors using flow cytometry.

See also Figure S1 and Tables S1 and S2.

the kidney capsule of C57BL/Ka mice. We monitored the mice for at least 15 weeks after transplantation. When tumors became evident by palpation or at the end of the experiment, the mice were sacrificed for gross and histological examination. Sorted events from the debris fraction (Figure 1I) did not form tumors upon transplantation and cells from the fractions that stained positively for hematopoietic or endothelial markers rarely formed tumors (Table S2). When viable MPNST cells from the nonhematopoietic/nonendothelial fraction of *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} mice were injected, 28 of 29 mice (97%) injected with 100 cells, 28 of 39 mice (72%) injected with 10 cells, and 16 of 76 mice (21%) injected with single cells formed MPNSTs (Table 1). Almost all of these tumors were observed within ten weeks after transplantation and appeared histologically similar to the primary tumors from which they derived (Figure S2A). Limit dilution analysis indicated that 18 ± 14% (mean ± SD; range = 6% to 53%) of cells from primary MPNST formed tumors after transplantation. Tumorigenic cells were therefore common within primary MPNSTs obtained from *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} mice.

When nonhematopoietic/nonendothelial cells from primary tumors obtained from 8 *Nf1*^{+/-}; *p53*^{+/-} mice were injected, 24 of 37 mice (65%) injected with 100 cells, 6 of 43 mice (14%) injected with 10 cells, and 2 of 38 mice (5%) injected with single cells formed MPNSTs (Table 1). All of these tumors were observed within ten weeks after transplantation and appeared

histologically similar to the primary tumors from which they derived (Figure S2B). Limit dilution analysis indicated that an average of 1.8 ± 1.6% (mean ± SD; range = 0.6% to 5.0%) of cells from these MPNSTs formed tumors after

transplantation. The frequency of tumorigenic cells in MPNSTs from *Nf1*^{+/-}; *p53*^{+/-} mice was therefore significantly lower (10-fold, *p* = 0.008) in this assay than in MPNSTs from *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} mice. This demonstrates that MPNST genetic background can affect tumorigenic cell frequency.

MPNSTs from *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} Mice Retain Higher Frequencies of Tumorigenic Cells upon Secondary Transplantation

We wondered whether the reduced frequency of tumorigenic cells within primary MPNSTs from *Nf1*^{+/-}; *p53*^{+/-} mice reflected increased infiltration of normal cells into these tumors as compared to MPNSTs from *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} mice. To test this, we performed secondary transplants of dissociated cells from tumors that arose under the kidney capsule of primary recipient mice. Transplanted MPNST cells form focal masses that are distinct from normal kidney tissue. We reasoned that only cancer cells would proliferate extensively in primary recipient mice, reducing or eliminating normal cells from the passaged tumors. Therefore, if normal cells within primary MPNSTs from *Nf1*^{+/-}; *p53*^{+/-} mice diluted the cancer cells, then secondary transplantation of these MPNSTs should eliminate the difference in tumorigenic cell frequency among MPNSTs of different genotypes. When MPNST cells from eleven first-generation grafts from five different primary MPNSTs from

Table 1. Tumor Formation by Primary MPNST Cells

| Tumor | Injected Cell Dose (No. of Tumors/ No. of Injections) | | | Cells that Formed Tumors (%) | 95% CI (%) |
|--------------------------------------------------------|-------------------------------------------------------------|----------------|----------------|------------------------------------|---------------|
| | 100 Cells | 10 Cells | 1 Cell | | |
| <i>Nf1^{+/-}; Ink4a/Arf^{-/-}</i> mice | | | | | |
| 10 | 3/3 | nd | 1/8 | 13 | 1.9–95 |
| 11 | 1/2 | 1/2 | 2/6 | 16 | 4.3–61 |
| 12 | 5/5 | 4/6 | 6/8 | 27 | 11–65 |
| 14 | 3/3 | 4/4 | 0/8 | 18 | 6.6–48 |
| 15 | 3/3 | 3/6 | 0/7 | 6.0 | 2.1–18 |
| 16 | 4/4 | 6/6 | 4/10 | 53 | 22–100 |
| 17 | 4/4 | 4/5 | 1/9 | 15 | 5.9–37 |
| 23 | nd | nd | 1/10 | 10 | nd |
| 29 | nd | 2/5 | 1/10 | 6.2 | 1.9-20 |
| 35 | 5/5 | 4/5 | nd | 16 | 5.4–48 |
| Total | 28/29 (97%) | 28/39 (72%) | 16/76 (21%) | 18 | 6.8–55 |
| <i>Nf1^{+/-}; p53^{+/-}</i> mice | | | | | |
| 1 | 1/3 | 0/4 | 1/10 | 0.7 | 0.1–3.0 |
| 3 | 4/5 | 0/5 | nd | 1.3 | 0.5–3.6 |
| 4 | 4/5 | 2/5 | 1/10 | 2.6 | 1.0–6.8 |
| 5 | 4/5 | 1/5 | 0/10 | 1.7 | 0.6–4.3 |
| 6 | 2/4 | 0/5 | 0/8 | 0.6 | 0.1–2.2 |
| 7 | 5/5 | 3/8 | nd | 5.0 | 1.8–13 |
| 8 | 4/6 | 0/5 | nd | 1.0 | 0.3–2.6 |
| 9 | 0/4 | 0/6 | nd | nd | nd |
| Total | 24/37 (65%) | 6/43 (14%) | 2/38 (5%) | 1.8** | 0.5–4.0 |

Tumorigenic cell frequency significantly differed in this assay between MPNSTs from *Nf1^{+/-}; Ink4a/Arf^{-/-}* and *Nf1^{+/-}; p53^{+/-}* mice (** $p = 0.008$). nd, not determined; CI, confidence interval.

For tumor histology, see Figure S2.

Nf1^{+/-}; Ink4a/Arf^{-/-} mice were injected into secondary recipients, 14 of 15 (93%) ten-cell injections and 44 of 99 (44%) single-cell injections gave rise to tumors (Table 2). Virtually all tumors became palpable within ten weeks of transplantation. Limit dilution analysis indicated that $49 \pm 8.6\%$ (mean \pm SD; range = 40% to 58%) of cells from each of these serially transplanted MPNST formed tumors after transplantation. Thus, approximately half of the cells within MPNSTs are capable of forming tumors by the time the MPNSTs have been passaged once.

When MPNST cells from six first-generation grafts from four primary MPNSTs from *Nf1^{+/-}; p53^{+/-}* mice were injected into secondary recipients, 6 of 32 (19%) injections of 10 cells and 3 of 30 (10%) injections of single cells gave rise to tumors (Table 2). All tumors became palpable within ten weeks of transplantation. Limit dilution analysis indicated that an average of $2.6 \pm 2.3\%$ (mean \pm SD; range = 0.3% to 5.7%) of cells from each of these serially transplanted MPNSTs formed tumors after transplantation. Thus, MPNSTs from *Nf1^{+/-}; Ink4a/Arf^{-/-}* mice retained a significantly (19-fold; $p = 2 \times 10^{-5}$) higher frequency of tumorigenic cells than MPNSTs from *Nf1^{+/-}; p53^{+/-}* mice,

Table 2. Tumor Formation after Secondary Transplantation of MPNST Cells

| | Injected Cell Dose | | | Cells that Formed | 95% |
|--------------------------------------------------------|--------------------|----------------|----------------|----------------------|---------|
| Tumor | 100 Cells | 10 Cells | 1 Cell | Tumors (%) | CI (%) |
| <i>Nf1^{+/-}; Ink4a/Arf^{-/-}</i> mice | | | | | |
| 9 (N = 1) | nd | nd | 4/8 | 50 | nd |
| 10 (N = 1) | nd | nd | 5/9 | 56 | nd |
| 11 (N = 3) | nd | 3/3 | 8/25 | 40 | 21–76 |
| 12 (N = 4) | nd | 11/12 | 15/38 | 40 | 24–65 |
| 17 (N = 2) | nd | nd | 12/19 | 58 | nd |
| Total | nd | 14/15 (93%) | 44/99 (44%) | 49 | 22–71 |
| <i>Nf1^{+/-}; p53^{+/-}</i> mice | | | | | |
| 3 (N = 2) | 7/9 | 2/11 | 1/10 | 1.8 | 0.9–3.6 |
| 4 (N = 2) | 3/10 | 0/10 | 0/10 | 0.3 | 0.1–1.0 |
| 5 (N = 1) | 4/4 | 1/5 | 2/10 | 5.7 | 1.9–17 |
| 8 (N = 1) | 3/4 | 3/6 | nd | 2.5 | 0.9–7.1 |
| Total | 17/27 (63%) | 6/32 (19%) | 3/30 (10%) | 2.6** | 0.9–7.3 |

Tumorigenic cell frequency significantly differed in this assay between MPNSTs from *Nf1^{+/-}; Ink4a/Arf^{-/-}* and *Nf1^{+/-}; p53^{+/-}* mice (**, $p = 2 \times 10^{-5}$).

nd, not determined; CI, confidence interval.

even upon secondary transplantation. This suggests that the reduced frequency of tumorigenic cells within primary MPNSTs from *Nf1^{+/-}; p53^{+/-}* mice did not reflect the presence of normal cells in these tumors.

MPNSTs from *Nf1^{+/-}; Ink4a/Arf^{-/-}* Mice Retain Higher Frequencies of Tumorigenic Cells upon Transplantation into NOD/SCID ILR2^γnull Mice

To test whether MPNST cells from *Nf1^{+/-}; p53^{+/-}* mice were more immunogenic than MPNST cells from *Nf1^{+/-}; Ink4a/Arf^{-/-}* mice we transplanted dissociated cells from primary MPNSTs into immunocompetent C57BL/Ka recipients or into highly immunocompromised NOD/SCID ILR2^γnull mice, which lack B, T, and natural killer cells (Shultz et al., 2005). We found that 16%–20% of cells from two primary MPNSTs from *Nf1^{+/-}; Ink4a/Arf^{-/-}* mice formed tumors irrespective of whether they were transplanted into C57BL/Ka or NOD/SCID ILR2^γnull mice. Similarly, 1%–5% of MPNST cells from *Nf1^{+/-}; p53^{+/-}* mice formed tumors in C57BL/Ka recipients and 0.6%–2.5% formed tumors in NOD/SCID ILR2^γnull recipients (Table 3). These results indicate that the recipient immune system has little effect on the frequency of MPNST cells that form tumors after transplantation, irrespective of genotype, and the reduced frequency of tumorigenic MPNST cells from *Nf1^{+/-}; p53^{+/-}* mice cannot be explained by increased immunogenicity.

Higher Percentage of MPNST Cells from *Nf1^{+/-}; Ink4a/Arf^{-/-}* Mice Formed Colonies in Nonadherent Culture as Compared to *Nf1^{+/-}; p53^{+/-}* Mice

We cultured primary mouse MPNST cells, 1 cell/well in 96 well plates for 10–14 days in nonadherent conditions (Molofsky et al., 2003). We found that $35 \pm 20\%$ (mean \pm SD) of MPNST

Table 3. Tumor Formation by Primary MPNST Cells in Immunocompetent C57BL/Ka Mice and Immunocompromised NOD/SCID IL2R γ ^{null} Mice

| Tumor | Recipient Mice | Injected Cell Dose | | | Tumorigenic Cells (%) | 95% CI |
|----------------------------------------------------|----------------------------------------|--------------------|----------|---------|-----------------------|---------|
| | | 100 Cells | 10 Cells | 5 Cells | | |
| Nf1 ^{+/-} ; Ink4a/Arf ^{-/-} (35) | C57BL/Ka | 5/5 | 4/5 | | 16 | 5.4–48 |
| Nf1 ^{+/-} ; Ink4a/Arf ^{-/-} (35) | NOD/SCID IL2R γ ^{null} | 5/5 | 4/5 | | 16 | 5.4–48 |
| Nf1 ^{+/-} ; Ink4a/Arf ^{-/-} (40) | C57BL/Ka | | | 4/7 | 17 | 6.2–46 |
| Nf1 ^{+/-} ; Ink4a/Arf ^{-/-} (40) | NOD/SCID IL2R γ ^{null} | | | 5/8 | 20 | 7.9–49 |
| Nf1 ^{+/-} ; p53 ^{+/-} (7) | C57BL/Ka | 5/5 | 3/8 | | 5.0 | 1.8–13 |
| Nf1 ^{+/-} ; p53 ^{+/-} (7) | NOD/SCID IL2R γ ^{null} | 4/5 | 3/7 | | 2.5 | 1.0–6.5 |
| Nf1 ^{+/-} ; p53 ^{+/-} (28) | C57BL/Ka | 3/5 | 1/8 | | 1.0 | 0.4–2.8 |
| Nf1 ^{+/-} ; p53 ^{+/-} (28) | NOD/SCID IL2R γ ^{null} | 2/5 | 1/8 | | 0.6 | 0.2–2.1 |

The frequency of tumorigenic cells and the 95% confidence interval (CI) around this frequency were determined by extreme limiting dilution (ELDA) software (see [Experimental Procedures](#) for software URL).

cells from 12 different Nf1^{+/-}; Ink4a/Arf^{-/-} mice formed colonies, and 62% of these colonies (62 of 100) formed tumors after transplantation under the kidney capsule of C57BL/Ka mice. In contrast, only 4.1 ± 3.4% of MPNST cells from 12 Nf1^{+/-}; p53^{+/-} mice formed colonies, and 32% of these colonies (13 of 40) formed tumors after transplantation into C57BL/Ka mice (Table 4). Averaging across tumors, 23 ± 21% of single MPNST cells from Nf1^{+/-}; Ink4a/Arf^{-/-} mice and 2.0 ± 2.9% of MPNST cells from Nf1^{+/-}; p53^{+/-} mice formed tumorigenic colonies. This indicates that tumorigenic cells were also significantly less common (11-fold, $p = 0.02$) in MPNSTs from Nf1^{+/-}; p53^{+/-} mice as compared to Nf1^{+/-}; Ink4a/Arf^{-/-} mice, even when these cells were initially propagated in culture instead of transplanting uncultured cells directly in vivo (as in Tables 1, 2, 3).

The reduced colony formation by MPNST cells from Nf1^{+/-}; p53^{+/-} mice was largely attributable to increased cell death as compared to MPNST cells from Nf1^{+/-}; Ink4a/Arf^{-/-} mice. Ten to 14 days after adding MPNST cells to culture, we carefully examined the wells. We found that 43 ± 2.4% of wells in which we had plated single MPNST cells from Nf1^{+/-}; Ink4a/Arf^{-/-} mice did not contain any cells, indicating that these cells must have undergone cell death. In contrast, 69 ± 13% of wells in which we had plated single MPNST cells from Nf1^{+/-}; p53^{+/-} mice did not contain any cells. This suggests that a significantly ($p = 0.02$) higher percentage of single MPNST cells from Nf1^{+/-}; p53^{+/-} mice underwent cell death after being added to culture. The remaining wells that did not contain colonies but were not empty contained small numbers of cells (<10), indicating that these cells underwent a limited number of divisions.

We examined the number of apoptotic cells in MPNSTs in vivo and in MPNST cells cultured under nonadherent conditions (Figure S3). MPNST tumors in Nf1^{+/-}; Ink4a/Arf^{-/-} and Nf1^{+/-}; p53^{+/-} mice in vivo contained similar frequencies of cleaved caspase-3 positive cells. In culture, we found that a significantly higher percentage of cells from Nf1^{+/-}; p53^{+/-} mice compared to Nf1^{+/-}; Ink4a/Arf^{-/-} mice expressed cleaved caspase-3 after 12 days of culture (5.8 ± 3.4% versus 2.2 ± 2.4%; mean ± SD; $p = 0.03$). MPNST cells from Nf1^{+/-}; Ink4a/Arf^{-/-} mice are therefore more likely to survive nonadherent culture than MPNST cells from Nf1^{+/-}; p53^{+/-} mice, even though no difference in the frequency of apoptotic cells was observed in tumors in vivo. This suggests that differences in the rate of cell death between

MPNSTs from different genetic backgrounds are context-dependent. We therefore decided to explore whether changes in assay conditions could increase the frequency of clonogenic MPNST cells.

Laminin Is Expressed by More MPNST Cells in Nf1^{+/-}; Ink4a/Arf^{-/-} Mice than in Nf1^{+/-}; p53^{+/-} Mice

One common way of improving the growth and survival of cells in culture is by growing cells adherently on extracellular matrix, rather than nonadherently, as described above. Adherent culture on laminin, in particular, enhances the growth and survival of neural stem/progenitor cells and glioma cells (Sun et al., 2008; Pollard et al., 2009; Lathia et al., 2010). Because MPNST cells from Nf1^{+/-}; p53^{+/-} mice did not exhibit increased cell death in vivo, in contrast to what was observed in culture, we wondered whether MPNST cells were exposed to laminin in the tumor environment in vivo. We examined the expression of laminin-111/-211 (laminin α 1/ β 1/ γ 1 and α 2/ β 1/ γ 1) on sections through MPNSTs from Nf1^{+/-}; Ink4a/Arf^{-/-} and Nf1^{+/-}; p53^{+/-} mice (Figures 2A–2D). In MPNSTs from Nf1^{+/-}; Ink4a/Arf^{-/-} mice laminin-111/-211 was widely expressed by most tumor cells, including cancer cells and vascular/perivascular stromal cells (Figures 2B and 2D). In contrast, in MPNSTs from Nf1^{+/-}; p53^{+/-} mice laminin appeared to be expressed by many fewer cells and to be mainly vascular/perivascular (Figures 2A and 2C).

We found that dissociated MPNST cells from Nf1^{+/-}; Ink4a/Arf^{-/-} mice exhibited much stronger laminin-111/211 staining than MPNST cells from Nf1^{+/-}; p53^{+/-} mice (Figure 2E). Indeed, 70 ± 11% of MPNST cells from Nf1^{+/-}; Ink4a/Arf^{-/-} mice and 28 ± 22% of MPNST cells from Nf1^{+/-}; p53^{+/-} mice stained with laminin by flow cytometry. This demonstrates that most MPNST cells from Nf1^{+/-}; Ink4a/Arf^{-/-} mice carry laminin with them after dissociation, whereas only a minority of MPNST cells from Nf1^{+/-}; p53^{+/-} mice do so. If laminin promotes the survival or proliferation of MPNST cells, then MPNST cells from Nf1^{+/-}; p53^{+/-} mice would be expected to depend more upon exogenous laminin, as compared to MPNST cells from Nf1^{+/-}; Ink4a/Arf^{-/-} mice.

We administered a 6 hr pulse of BrdU to mice and then stained sections through their MPNSTs with antibodies against BrdU and laminin-111/211 to assess the localization of dividing cells relative to laminin-expressing cells. BrdU+ cells tended to

Table 4. Colony Formation by Single Primary MPNST Cells Cultured under Nonadherent Conditions and Tumor Formation by These Colonies after Transplantation In Vivo

| Tumor | Single Cells that Formed Colonies (%) | Tumors/Colonies Injected (%) | Single Cells that Formed Tumorigenic Colonies (%) |
|------------------------------------------------------------------|---------------------------------------|------------------------------|---------------------------------------------------|
| <i>Nf1</i> ^{+/-} ; <i>Ink4a/Arf</i> ^{-/-} mice | | | |
| 6 | 26 | 2/9 (22) | 5.8 |
| 7 | 47 | 4/5 (80) | 38 |
| 8 | 39 | 2/7 (29) | 11 |
| 9 | 14 | 5/11 (45) | 6.2 |
| 10 | 22 | 5/10 (50) | 11 |
| 11 | 34 | 8/13 (62) | 21 |
| 12 | 66 | 12/13 (92) | 60 |
| 14 | 11 | 4/5 (80) | 8.7 |
| 15 | 7.6 | 2/7 (29) | 2.2 |
| 16 | 38 | 9/11 (82) | 31 |
| 17 | 66 | 9/10 (90) | 59 |
| 23 | 55 | nd | nd |
| Mean ± SD | 35 ± 20 | 62/100 (62) | 23 ± 21 |
| <i>Nf1</i> ^{+/-} ; <i>p53</i> ^{+/-} mice | | | |
| 1 | 10 | nd | nd |
| 2 | 3.1 | nd | nd |
| 3 | 6.1 | 0/10 (0) | 0.0 |
| 4 | 2.4 | 1/5 (20) | 0.5 |
| 5 | 0.5 | nd | nd |
| 6 | 8.3 | 4/4 (100) | 8.3 |
| 7 | 0.5 | nd | nd |
| 8 | 5.6 | 4/10 (40) | 2.2 |
| 9 | 1.6 | nd | nd |
| 12 | 0.5 | 1/1 (100) | 0.5 |
| 13 | 8.1 | 1/6 (17) | 1.4 |
| 14 | 2.9 | 2/4 (50) | 1.5 |
| Mean ± SD | 4.1 ± 3.4** | 13/40 (32) | 2.0 ± 2.9* |

For each tumor, we determined the percentage of cells that formed colonies in culture and the percentage of these colonies that formed tumors upon transplantation into C57BL/Ka mice. The last column represents the product of these statistics, that is, the percentage of MPNST cells that formed tumorigenic colonies.

**p = 3 × 10⁻⁵; *p = 0.02. All statistics represent mean ± SD.

nd, not determined.

For data on cell death in culture, see Figure S3.

localize adjacent to laminin-expressing cells and regions of the tumor that lacked laminin staining also had fewer dividing cells. MPNSTs from *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} and *Nf1*^{+/-}; *p53*^{+/-} mice included 9 ± 5 and 8 ± 2% (mean ± SD) BrdU+ cells, respectively (Figures 2F–2H). In MPNSTs from *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} mice, laminin was widely expressed such that almost all cells (88 ± 18%), including nearly all BrdU+ cells (97 ± 1%), were adjacent to laminin-stained cells (Figures 2F and 2H). However, in MPNSTs from *Nf1*^{+/-}; *p53*^{+/-} mice, laminin was more restricted in its expression such that only 39 ± 6% of MPNST cells were adjacent to laminin-stained cells. Yet, most BrdU+ cells (83 ± 7%) remained adjacent to laminin-stained cells in these tumors (Figures 2G and

2H). Therefore, BrdU+ cells were more than twice as likely as all MPNST cells to localize adjacent to laminin-expressing cells in MPNSTs from *Nf1*^{+/-}; *p53*^{+/-} mice. This is consistent with the possibility that laminin may promote the proliferation of MPNST cells, though laminin staining may also be associated with vasculature that promotes proliferation through other mechanisms.

MPNSTs from *Nf1*^{+/-}; *p53*^{+/-} but Not *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} Mice Form More Colonies on Laminin

If laminin promotes clonogenic growth by MPNST cells and MPNST cells from *Nf1*^{+/-}; *p53*^{+/-} mice are less likely to carry laminin after dissociation (Figure 2E), then exogenous laminin might stimulate clonogenic growth by MPNST cells from *Nf1*^{+/-}; *p53*^{+/-} mice to a greater extent than MPNST cells from *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} mice. To test this, we compared colony formation by single MPNST cells from both genetic backgrounds in nonadherent cultures and in adherent cultures on laminin-111 (Figure 3A). Adherent culture on laminin did not affect the percentage of MPNST cells from *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} mice that formed colonies compared to nonadherent cultures (Figure 3A); however, it significantly increased the percentage of MPNST cells from *Nf1*^{+/-}; *p53*^{+/-} mice that formed colonies from 6.7 ± 9.8% to 25 ± 18% (p = 0.003; Figure 3A). Although the percentage of cells that formed colonies varied between tumors, the rate of colony formation on laminin was always at least as high as in nonadherent cultures, and the effect was statistically significant in a paired t test (p = 0.003). Adherent culture on fibronectin or collagen may have enhanced the rate of tumor formation to some extent, but the effects were variable among tumors and not statistically significant (Table S3). Exposure to laminin thus increased colony-formation by MPNST cells from *Nf1*^{+/-}; *p53*^{+/-} mice but not *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} mice, consistent with the difference in endogenous laminin expression between these genetic backgrounds.

Adherent colonies grown on laminin by MPNST cells from *Nf1*^{+/-}; *p53*^{+/-} mice, but not *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} mice, also tended to be larger than those that grew in nonadherent cultures (data not shown). Consistent with this, a significantly higher frequency of cells within colonies from *Nf1*^{+/-}; *p53*^{+/-} mice incorporated a pulse of BrdU when cultured on laminin-111 compared to nonadherent culture conditions, whereas adherent culture on laminin did not significantly affect the rate of BrdU incorporation into colonies cultured from *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} mice (Figure 3B).

To assess the proliferative potential of MPNST cells that formed colonies on laminin, we dissociated the colonies that arose from single cells and replated them into secondary cultures. Twenty-three of 24 (96%) colonies from three primary MPNSTs obtained from *Nf1*^{+/-}; *p53*^{+/-} mice gave rise to secondary colonies, yielding an average of 2573 ± 626 secondary colonies per primary colony. An average of 30 ± 10% of cells within the primary colonies formed secondary colonies upon replating. These results suggest that many MPNST cells from *Nf1*^{+/-}; *p53*^{+/-} mice have the potential to proliferate extensively if assayed under permissive conditions.

To assess whether colony-forming MPNST cells from *Nf1*^{+/-}; *p53*^{+/-} mice had the potential to form tumors in vivo, we dissociated individual colonies and injected the cells under the kidney capsule of C57BL/Ka mice. In these experiments,

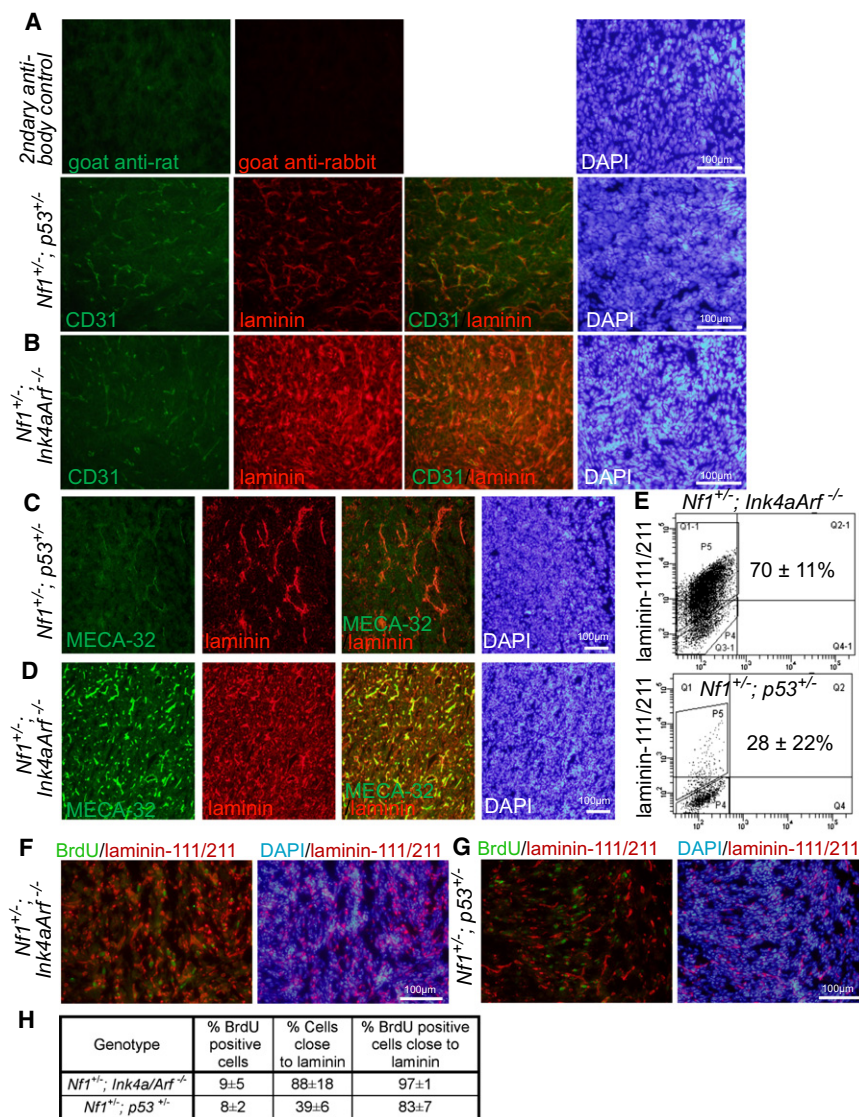


Figure 2. Laminin Is Expressed by Most MPNST Cells from *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} Mice but Only by a Subset of MPNST Cells from *Nf1*^{+/-}; *p53*^{+/-} Mice

(A–D) MPNSTs from *Nf1*^{+/-}; *p53*^{+/-} (A and C) and *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} (B and D) mice were stained with antibodies against laminin-111/211, CD31, and MECA-32 (to label endothelial cells), as well as DAPI to identify nuclei.

(E) Dissociated MPNST cells, after exclusion of blood and endothelial cells, were stained with antibodies against laminin-111/211 and analyzed by flow cytometry. Numbers reflect the percentage of cells that stained more strongly than isotype control (3–5 MPNSTs/genotype, $p = 0.02$ between the two genotypes).

(F–H) Mice bearing MPNSTs were treated with BrdU for 6 hr and cryosections were stained with DAPI, as well as antibodies against laminin-111/211 and BrdU. In MPNSTs from *Nf1*^{+/-}; *p53*^{+/-} mice, BrdU+ cells were 2.1-fold more likely ($p = 0.04$) than the average MPNST cell to localize adjacent to laminin (the data reflect three tumors/genotype and 2,200–3,200 cells/tumor). All statistics are mean ± SD.

MPNST cells varies with tumor genotype and assay conditions and that many primary MPNST cells are capable of forming tumors in immunocompetent mice.

To test whether endogenous laminin expression distinguishes intrinsically tumorigenic from intrinsically nontumorigenic subpopulations of MPNST cells, we sorted laminin-positive and laminin-negative MPNST cells from both genetic backgrounds and transplanted them under the kidney capsule of C57BL/Ka mice. Both laminin-positive and laminin-

negative MPNST cells formed tumors, though the rate of tumor formation by the laminin-positive cells was significantly higher in both genetic backgrounds (2.2-fold difference, $p = 0.02$ for cells from *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} mice; 2.5-fold difference, $p = 0.049$ for *Nf1*^{+/-}; *p53*^{+/-} mice; Figures 3F and 3G). Because 6.6% and 1.3% of laminin negative MPNST cells from *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} mice and *Nf1*^{+/-}; *p53*^{+/-} mice, respectively, formed tumors in vivo, our data do not support the idea that laminin negative cells intrinsically lack tumor-forming capacity. Rather, the data suggest that laminin increases the clonogenic potential of MPNST cells but that it does not matter whether the laminin is intrinsic or extrinsic to the cells. It remains possible there is a hierarchy of tumorigenic and nontumorigenic MPNST cells that can be identified with other markers. Thus, our data demonstrate that many MPNST cells are capable of forming tumors, depending on assay conditions and genetic background, but the formal question of whether MPNSTs exhibit a hierarchical organization consistent with the cancer stem cell model is not addressed by our study.

25 ± 18% of the MPNST cells formed adherent colonies on laminin-111 and 36 of 54 colonies formed tumors in vivo (Figure 3C). Thus, 19 ± 13% of single MPNST cells from *Nf1*^{+/-}; *p53*^{+/-} mice formed tumorigenic colonies in the presence of laminin-111. The histological appearance of the tumors that arose from transplanted colonies was similar to primary MPNSTs (Figure S4).

Given the increased colony formation by MPNST cells on laminin, we tested whether the tumorigenicity of uncultured primary MPNST cells in vivo was increased by mixing the cells with Matrigel (of which laminin is the main component; Kleinman and Martin, 2005). Suspension of primary MPNST cells in Matrigel prior to injection approximately doubled the frequency MPNST cells from both genetic backgrounds that formed tumors (Figures 3D and 3E). Limit dilution analysis indicated that 4.7% of primary MPNST cells from *Nf1*^{+/-}; *p53*^{+/-} mice and 30% of primary MPNST cells from *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} mice formed tumors when injected under the kidney capsule with Matrigel. These results further emphasize that the frequency of tumorigenic

negative MPNST cells formed tumors, though the rate of tumor formation by the laminin-positive cells was significantly higher in both genetic backgrounds (2.2-fold difference, $p = 0.02$ for cells from *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} mice; 2.5-fold difference, $p = 0.049$ for *Nf1*^{+/-}; *p53*^{+/-} mice; Figures 3F and 3G). Because 6.6% and 1.3% of laminin negative MPNST cells from *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} mice and *Nf1*^{+/-}; *p53*^{+/-} mice, respectively, formed tumors in vivo, our data do not support the idea that laminin negative cells intrinsically lack tumor-forming capacity. Rather, the data suggest that laminin increases the clonogenic potential of MPNST cells but that it does not matter whether the laminin is intrinsic or extrinsic to the cells. It remains possible there is a hierarchy of tumorigenic and nontumorigenic MPNST cells that can be identified with other markers. Thus, our data demonstrate that many MPNST cells are capable of forming tumors, depending on assay conditions and genetic background, but the formal question of whether MPNSTs exhibit a hierarchical organization consistent with the cancer stem cell model is not addressed by our study.

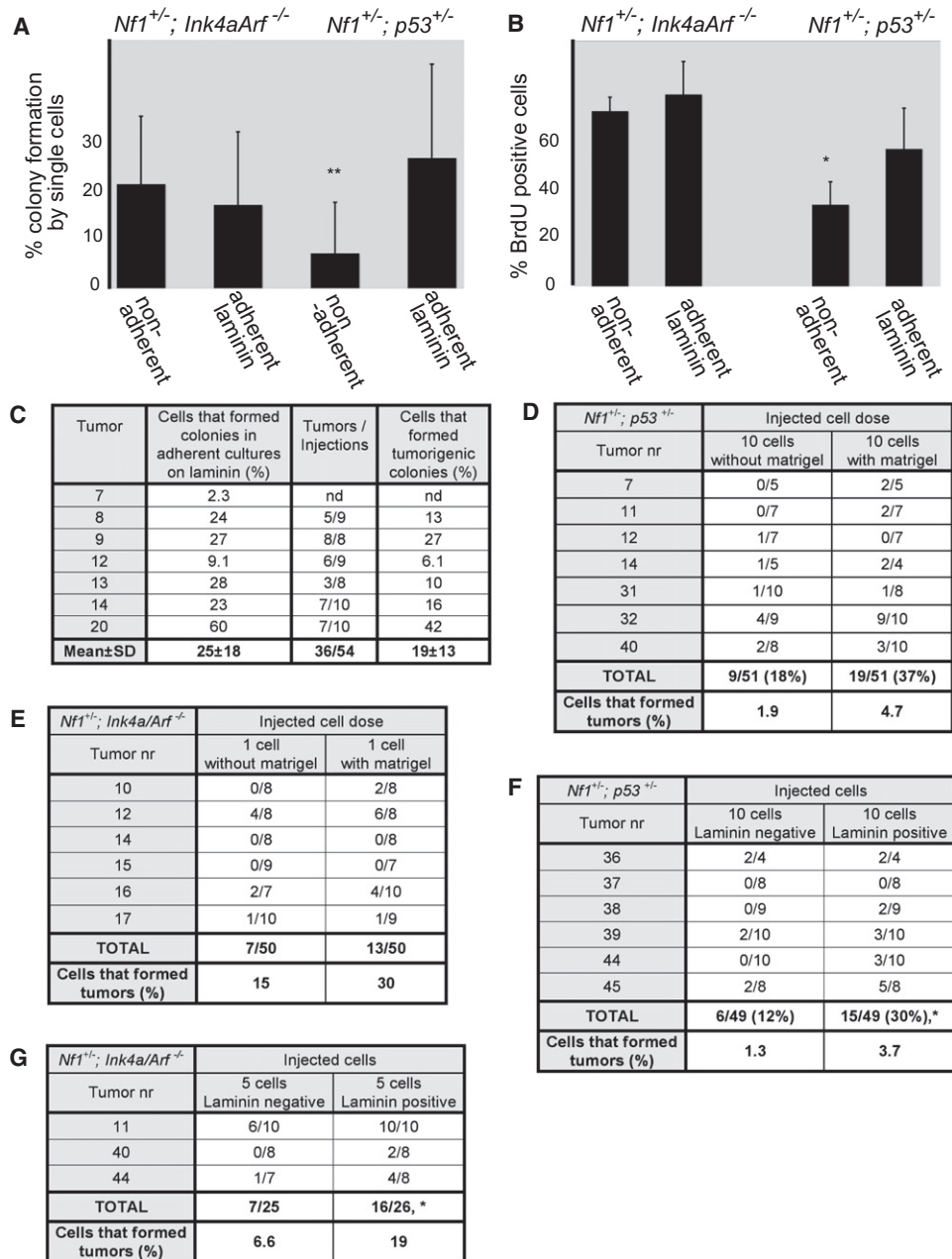


Figure 3. MPNST Cells from *Nf1*^{+/-}; *p53*^{+/-} Mice Formed More Colonies When Plated Adherently on Laminin

(A) Single MPNST cells from *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} (N = 6) and *Nf1*^{+/-}; *p53*^{+/-} mice (N = 7) were sorted into individual wells of 96-well plates and cultured either nonadherently or adherently on laminin-111. **, p = 0.003 by paired t test between adherent and nonadherent cultures of cells from *Nf1*^{+/-}; *p53*^{+/-} mice.

(B) 10,000 MPNST cells from *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} or *Nf1*^{+/-}; *p53*^{+/-} mice (three tumors each) were cultured nonadherently or adherently on laminin-111 for 1–3 days. Then a 3 hr pulse of BrdU was administered (*p < 0.05). Error bars in (A) and (B) represent SD.

(C) Adherent colonies from single MPNST cells from *Nf1*^{+/-}; *p53*^{+/-} mice grown on laminin-111 were trypsinized, injected under the kidney capsules of C57BL/Ka mice, and then monitored for the ability to form tumors for 15 weeks. The last column represents the percentage of MPNST cells that formed adherent colonies and that went on to form tumors upon transplantation of the colonies in vivo (the product of columns 1 and 2). nd, not determined.

(D and E) Freshly isolated MPNST cells from *Nf1*^{+/-}; *p53*^{+/-} (D) or *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} (E) mice were transplanted under the kidney capsule of C57BL/Ka mice, with or without Matrigel, and then monitored for tumor formation for at least 15 weeks.

(F and G) Freshly dissociated laminin-positive or laminin-negative MPNST cells from *Nf1*^{+/-}; *p53*^{+/-} (F) or *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} (G) mice were transplanted under the kidney capsule of C57BL/Ka mice. Cells that stained positively for laminin were more likely to form tumors in both genetic backgrounds (p = 0.02 for *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} mice and p = 0.049 for *Nf1*^{+/-}; *p53*^{+/-} mice, paired t test).

See also Table S3 and Figure S4.

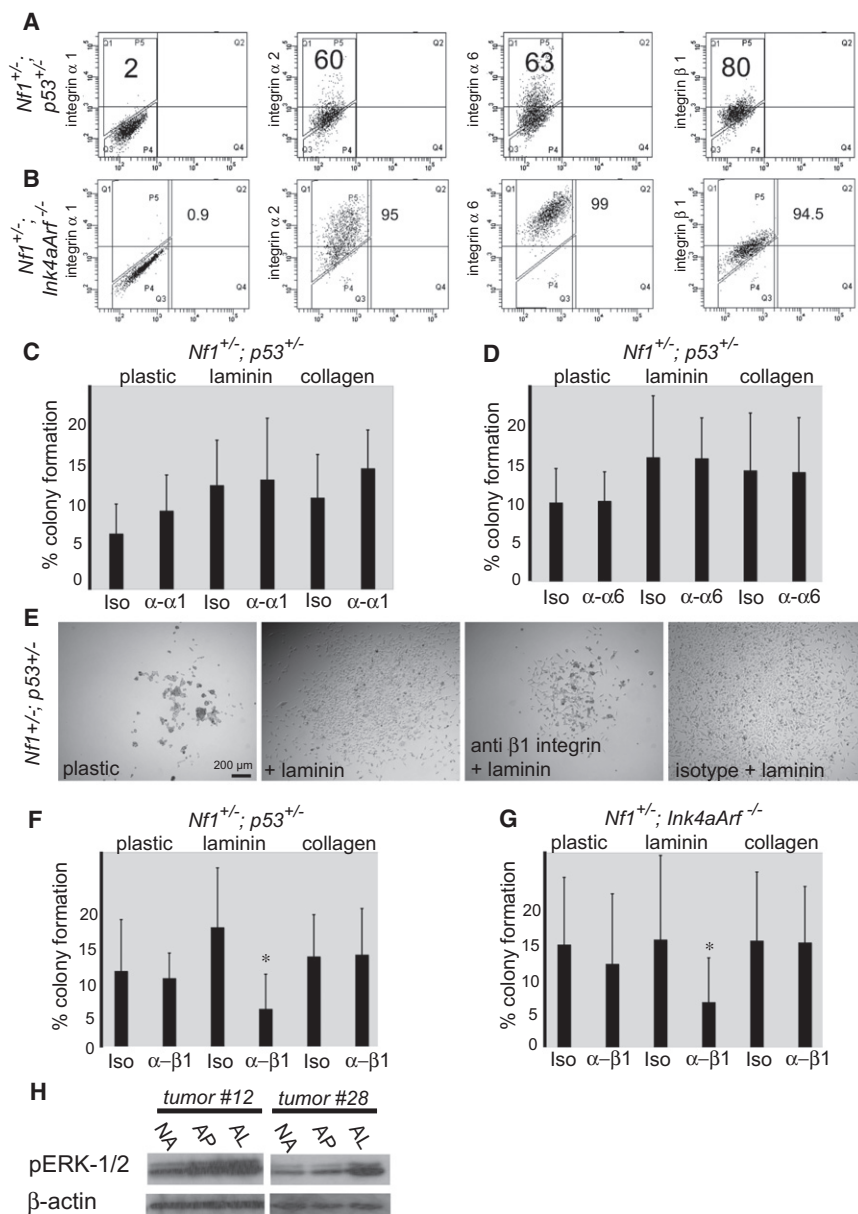


Figure 4. MPNST Cells Expressed Laminin Receptors and Blocking Antibodies against $\beta 1$ Integrin Decreased Colony Formation on Laminin

(A and B) Expression of indicated integrins by MPNST cells from $Nf1^{+/-}; p53^{+/-}$ (A) and $Nf1^{+/-}; Ink4a/Arf^{-/-}$ (B) mice. Numbers reflect the percentages of cells that stained more strongly than isotype control.

(C and D) Colony formation by MPNST cells from $Nf1^{+/-}; p53^{+/-}$ mice on the indicated substrate treated with function-blocking antibodies against $\alpha 1$ (C) or $\alpha 6$ (D) integrin. (Iso means isotype control antibody.)

(E) Representative pictures of colonies formed by MPNST cells from $Nf1^{+/-}; p53^{+/-}$ mice in the presence of function blocking antibodies against $\beta 1$ integrin or isotype control.

(F and G) The effect of blocking antibody against $\beta 1$ integrin on the percentage of cells that formed colonies. *, $p = 0.02$ for $Nf1^{+/-}; p53^{+/-}$ mice (F) and $p = 0.05$ for $Nf1^{+/-}; Ink4a/Arf^{-/-}$ (G) on laminin. No significant difference on plastic or collagen.

(H) Western blots of extracts from MPNSTs cells obtained from $Nf1^{+/-}; p53^{+/-}$ mice using antibodies against phospho-ERK1/2. Cells were cultured for three days under nonadherent conditions (NA), adherently on plastic (AP), or adherently on laminin-111 (AL). Error bars represent SD.

See also Figure S5.

Laminin Promotes the Survival and Proliferation of MPNST Cells via $\beta 1$ Integrin

$\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 7\beta 1$, and $\alpha 6\beta 4$ integrins are laminin receptors (Miner and Yurchenco, 2004). By flow cytometry, we found that integrins $\alpha 2$, $\alpha 6$, and $\beta 1$ were widely expressed on the surface of MPNST cells from $Nf1^{+/-}; Ink4a/Arf^{-/-}$ and $Nf1^{+/-}; p53^{+/-}$ mice (Figure 4A). Integrins $\alpha 1$ (Figure 4A) and $\beta 4$ (data not shown) were rarely expressed at detectable levels. Thus, MPNST cells appear to express multiple laminin receptors, including $\alpha 2\beta 1$ and $\alpha 6\beta 1$. Laminin-111/211 is also strongly expressed by basement membranes under the kidney capsule (Figure S5), just as in MPNSTs (Figure 2), potentially explaining why the kidney capsule creates a permissive environment for tumor formation by MPNST cells.

We examined whether antibodies that block the binding of laminin-111 to integrin receptors would impede colony forma-

tion. We were unable to find a preservative-free antibody against $\alpha 2$ -integrin suitable for use in these experiments. Addition of blocking antibodies against $\alpha 1$ -integrin (Figure 4C) or $\alpha 6$ -integrin (Figure 4D) to culture did not affect the percentage of MPNST cells from $Nf1^{+/-}; p53^{+/-}$ mice that formed colonies or the size of colonies. In contrast, addition of blocking antibodies against $\beta 1$ -integrin (Milner and Campbell, 2002) but not isotype control antibody significantly decreased the percentage of MPNST cells from $Nf1^{+/-}; p53^{+/-}$ mice (Figure 4F)

and $Nf1^{+/-}; Ink4a/Arf^{-/-}$ mice (Figure 4G) that formed colonies on laminin-111, as well as the size of these colonies (Figure 4E), but did not affect the percentage of cells that formed adherent colonies on uncoated plastic or collagen 1 (Figures 4F and 4G). These data demonstrate that laminin binding to $\beta 1$ -integrin-containing receptors increases the clonogenicity of MPNST cells from both genetic backgrounds.

Laminin promotes the survival of Schwann cells (Yu et al., 2005), and MPNSTs arise from Schwann cells (Zhu et al., 2002; Joseph et al., 2008; Zheng et al., 2008). Integrin receptors signal through the ERK pathway (Guo and Giancotti, 2004), and ERK provides a survival and proliferation signal to Schwann lineage cells (Newbern et al., 2011) and MPNST cells (Ambrosini et al., 2008). We found that adherent culture on laminin-111 increased the levels of phosphorylated ERK1/2 in MPNST cells from some $Nf1^{+/-}; p53^{+/-}$ mice compared to nonadherent culture or

adherent culture on plastic (Figure 4H). Laminin binding to $\beta 1$ -integrin may therefore promote the survival and proliferation of MPNST cells by increasing ERK signaling.

DISCUSSION

Our data demonstrate that cells with tumorigenic potential are common in MPNSTs of both genotypes but that tumors with different genotypes sometimes require different assay conditions to read-out the full spectrum of cells with tumorigenic potential. If tumor genotype commonly influences the assay conditions that must be used to detect tumorigenic cells then assay conditions that detect the full spectrum of tumorigenic cells in one cancer patient may not detect the full spectrum of tumorigenic cells in other patients with the same cancer type. Studies of mouse lung cancers (Curtis et al., 2010), mouse mammary cancers (Vaillant et al., 2008), and mouse myeloid leukemias (Somerville and Cleary, 2006; Kennedy et al., 2007) have shown that cancers from different genetic backgrounds have different frequencies of tumorigenic cells, differences in the extent to which they follow a cancer stem cell model, or tumorigenic cells that express different markers. Human acute lymphoblastic leukemia samples that differ with respect to *CDKN2A/B* genotype also display differences in leukemogenic cell frequencies in immunocompromised mice (Notta et al., 2011). We demonstrate here that tumors of different genotypes can have similarly high frequencies of cells with tumorigenic potential but can require different assay conditions to detect these tumorigenic cells. Furthermore, we show that sarcomas sometimes contain common tumorigenic cells.

Our data suggest that the binding of laminin to $\beta 1$ -integrin-containing receptors promotes the survival and proliferation of MPNST cells. MPNST cells from *Nf1*^{+/-}; *p53*^{+/-} and *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} mice depended upon the binding of laminin to $\beta 1$ -integrin for proliferation and survival (Figure 4), but MPNST cells from *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} mice did not require the addition of exogenous laminin to culture (Figures 3A and 3B), presumably because these cells were much more likely to carry endogenous laminin as compared to MPNST cells from *Nf1*^{+/-}; *p53*^{+/-} mice. Suspension of MPNST cells from either genetic background in Matrigel doubled the frequency of cells that formed tumors in vivo (Figures 3D and 3E), and dividing cells in primary MPNSTs in vivo almost always had contact with laminin-expressing cells (Figures 2F–2H). Thus, laminin appears to be one important determinant of clonogenicity in MPNST cells, but it does not appear to matter whether the laminin is intrinsic or extrinsic to the MPNST cells.

Our results raise the possibility that the failure to restore critical extracellular matrix cues after tumor dissociation may lead to underestimates of the frequency of tumor-initiating cells. The common use of sphere-formation assays in nonadherent cultures to identify tumor-initiating cells may exacerbate this problem by depriving dissociated cancer cells of extracellular matrix.

Our data demonstrate that melanoma is not unique among solid tumors in containing common cells with tumorigenic potential. We have demonstrated that at least 30% of human melanoma cells obtained directly from patients have the potential to form tumors upon transplantation into NOD/SCID IL2 γ ^{null}

mice (Quintana et al., 2008, 2010). An even higher percentage of single cells obtained from primary mouse melanomas can form tumors upon transplantation (Held et al., 2010). Whereas some cancers are likely to have only rare cells with the potential to proliferate extensively, other cancers are likely to contain common tumorigenic cells, and it remains uncertain what fraction of cancers fall in each category. Some mouse leukemias contain common leukemogenic cells (Somerville and Cleary, 2006; Kelly et al., 2007; Williams et al., 2007). However, other hematopoietic malignancies contain only rare leukemogenic cells (Lapidot et al., 1994; Kennedy et al., 2007), even when mouse leukemias are transplanted into histocompatible mice (Yilmaz et al., 2006; Oravecz-Wilson et al., 2009). A recent study discovered that human hematopoietic stem cells engraft more efficiently in female as compared to male mice (Notta et al., 2010), a variable that has rarely been taken into account in xenotransplantation experiments. Thus, whereas many solid cancers have appeared to contain only rare tumorigenic cells (Ishizawa et al., 2010), an open question concerns the extent to which existing assays underestimate the frequency of cells with tumorigenic potential.

Our observation that approximately 20% of MPNST cells from *Nf1*^{+/-}; *p53*^{+/-} and *Nf1*^{+/-}; *Ink4a/Arf*^{-/-} mice have the potential to form tumors is likely an underestimate, because there are likely to be important features of the tumor environment that are not adequately modeled in the tumorigenesis assays we used. For example, it is possible that tumor-associated fibroblasts or tumor-infiltrating inflammatory cells promote the survival or proliferation of MPNST cells (Le et al., 2009; Staser et al., 2010) in a way that is not initially replicated when we place small numbers of dissociated MPNST cells in culture or under the kidney capsule in vivo. More sophisticated models of this tumor microenvironment could reveal even higher frequencies of MPNST cells that have the potential to form tumors.

Although we found that cells with tumorigenic potential were common in mouse MPNSTs, a separate question concerns the spectrum of cells that are actually fated to contribute to disease progression in vivo. Our data demonstrating that many cells divide rapidly within MPNSTs (Figure 1H) suggests that many cells contribute to tumor growth. However, it is also possible that many of these cells divide only a limited number of times and that only a small fraction of the cells with the potential to proliferate extensively actually does so in vivo. Fate-mapping studies will be required to assess whether many or few MPNST cells actually contribute extensively to tumor growth or disease progression in vivo.

The question of whether MPNSTs follow a cancer stem cell model, in which cancer cells are hierarchically organized into intrinsically different subpopulations of tumorigenic and nontumorigenic cells, also remains an open question. One possibility is that MPNSTs contain common tumorigenic cells with no hierarchical organization. Another possibility is that MPNSTs contain a shallow hierarchy in which common tumorigenic cells differentiate into nontumorigenic MPNST cells. Even if the latter is the case, MPNST would be quite different from other cancers suggested to follow the cancer stem cell model.

Our data demonstrate that cells with tumorigenic potential are common in MPNSTs and that tumors from different genetic backgrounds can require different assay conditions to read-out

the full spectrum of cells with tumorigenic potential. This suggests that efforts to quantify the frequency of tumorigenic cells in human cancers may be complicated by confounding effects of tumor genotype on assay conditions. As assays improve it is likely that estimates of tumorigenic cell frequencies will continue to increase in many cancers. It remains to be determined what fraction of cancers have only rare tumorigenic cells and what fraction of cancers have common tumorigenic cells. Determining which cancers fall in each category has fundamental implications for therapy as attempts to target small subpopulations of cancer cells are not likely to improve the treatment of cancers in which many cells can drive disease progression.

EXPERIMENTAL PROCEDURES

Mice

Nf1^{+/-} (Jacks et al., 1994) mice were bred with *Ink4aArf*^{-/-} mice (Serrano et al., 1996) to yield compound mutant *Nf1*^{+/-}; *Ink4aArf*^{-/-} mice (Joseph et al., 2008). *Nf1*^{+/-}; *Ink4aArf*^{-/-} mice and *Nf1*^{+/-}; *p53*^{+/-} mice (Cichowski et al., 1999) were backcrossed on a C57BL/Ka background for at least six generations. Genotyping was performed as described by Joseph et al. (2008). All animal experiments were performed in accordance with protocols approved by the University of Michigan's Committee on the Use and Care of Animals.

Dissociation of MPNSTs and Flow Cytometry

Tumors were harvested from mice and minced using a scalpel. Minced tissue was washed in Ca²⁺ and Mg²⁺-free Hank's buffered salt solution (HBSS; Invitrogen, Grand Island, NY, USA), centrifuged, and treated for 20 min at 37°C with a mixture of 25% Ca²⁺ and Mg²⁺-free HBSS, 25% Collagenase IV (Worthington [Lakewood, NJ, USA]; resuspended to 2,000 U/ml in HBSS containing Ca²⁺ and Mg²⁺, Invitrogen), and 50% Trypsin-EDTA solution (0.05%, Invitrogen) with constant agitation. The reaction was quenched with staining medium consisting of L15 medium (Invitrogen), 1 mg/ml BSA (Sigma-Aldrich, St. Louis, MO, USA), 10 mM HEPES (BioWhittaker # 17-738E), 1% penicillin/streptomycin (Invitrogen), supplemented with DNase 1 (100 U/ml; Sigma), and filtered through 70 µm nylon filter (Fisher Scientific, Ottawa, Ontario). The cells were centrifuged, resuspended in staining medium, and then counted on a hemocytometer. Cells were stained with antibodies against CD31 (BioLegend, San Diego, CA, USA), CD45 (eBioscience, San Diego, CA, USA), and Ter119 (BioLegend) in 200 µl of staining medium for 20 min at 4°C. The cells were then washed and resuspended in staining medium supplemented with DAPI (10 µg/ml; Sigma). To evaluate the expression of laminin receptors, cells were stained with antibodies against integrin α 1 (BD Biosciences, San Diego, CA, USA), α 2 (BD Biosciences), α 6 (eBioscience), and integrin β 1 (BD Biosciences), as well as appropriate secondary antibodies prior to staining with blood lineage markers. Flow cytometry was performed with a FACSVantage SE-dual laser, three-line flow cytometer, or with a FACSAria II (Becton Dickinson, Franklin Lakes, NJ, USA).

Cell Culture

Cells were typically cultured in 96-well tissue culture plates (Corning, Lowell, MA, USA) at a density of 1 cell/well. For nonadherent cultures, ultra-low binding plates (Corning) were used. For adherent cultures, plates were coated with fibronectin (Biomedical Technologies, Stoughton, MA, USA), laminin-111 (Invitrogen), or collagen-1 (BD Bioscience), all at 50 µg/ml. The culture medium for both adherent and nonadherent cultures was based on a 5:3 mixture of Dulbecco's modified eagle medium -low:Neurobasal medium (Invitrogen). The medium was supplemented with 15% chick embryo extract (prepared as described; Stemple and Anderson, 1992), 20 ng/ml recombinant human bFGF (R&D Systems, Minneapolis, MN), 20 ng/ml IGF1 (R&D Systems), 1% N2 supplement (Invitrogen), 2% B27 supplement (Invitrogen), 50 mM 2-mercaptoethanol (Acros Organics), 35 ng/ml retinoic acid (Sigma), and 1% penicillin/streptomycin (Invitrogen). In some cases, the medium was supplemented with laminin-111 or fibronectin, both at 50 µg/ml. All cultures were maintained at 37°C in 6% CO₂/balance air. For experiments using blocking antibodies,

cells were cultured at a density of 50–100 cells/well on 24-well plates on plastic or laminin-111 (50 µg/ml) in the presence or absence of antibodies against integrin α 1 (BD Bioscience), α 6 (clone GoH3; R&D Systems), or β 1 (BD Bioscience) or corresponding isotype controls (all at 10 µg/ml). Colony formation was assessed after 5–8 days.

Kidney Capsule Injections

MPNST cells were resuspended in 30 µl of sterile staining medium. Mice were anesthetized by intraperitoneal injection of ketamine/xylazine. After shaving the skin and prepping with betadine (Fisher Scientific), a 1 cm longitudinal incision was made in the skin of the abdomen and in the body wall above the kidney. Cell suspensions were injected under the capsule of the exposed kidney using an insulin syringe with a 31G needle (BD Bioscience). In some cases, 50% Matrigel (BD Bioscience) was added to the cell suspension. The incisions were closed using absorbable suture (Tevdek, Genzyme Surgical #7-734). Animals were given antibiotic water (1.1 g/l neomycin sulfate and 10⁶ U/l polymixin B sulfate) and monitored closely. Tumor formation was assessed by weekly palpation of the abdomen or by magnetic resonance imaging for at least 15 weeks. The frequency of tumorigenic cells and the 95% confidence interval were calculated using extreme limiting dilution software (<http://bioinf.wehi.edu.au/software/elda/index.html>).

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.ccr.2011.12.027.

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