Supporting Information

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SI Text

Methods

Plasmids. The DNA fragments encoding tdTomato, wild-type human FAK-related nonkinase (FRNK WT), an S1034 mutant of human FRNK (FRNK S1034), and a chimeric protein consisting of human CD2 and human FAK (CD2-FAK) were obtained from pRSET-B-tdTomato (a gift from R. Y. Tsien), pcDNA3.1-FRNK, pcDNA3.1-FRNK S1034 (gifts from D. D. Schlaepfer), and pCDM8-CD2-FAK (a gift from S. M. Frisch and K. Vuori), respectively. All of the DNA fragments described above were subcloned into pLV-IRES-neo (a gift from I. Ben-Porath), which was generated by replacing the dsRed2-encoding sequence in pLV-tTRKRAB-Red (a gift from D. Trono) with the DNA sequence of the neomycin-resistance gene and subsequently removing the sequence of tTR-KRAB. In subcloning the tdTomato DNA fragment, the sequence of the internal ribosomal entry site (IRES) and neomycin-resistance gene in pLV-IRES-neo were replaced with the sequence of tdTomato.

To generate lentiviral vectors expressing short hairpin RNAs (shRNAs), hairpin oligomers with the target sequences shown in Table S1 were cloned into either pLKO-puro or pLKO-hygro just as described in ref. 1.

Some of the target sequences described in Table S1 were taken from the Website of the RNAi consortium at Broad Institute (http://www.broad.mit.edu/rnai/trc). None of these sequences match any mouse cDNA sequence registered in BLAST other than that of their own target. The target sequence of scrambled shRNA (sh scrambled) does not match any mouse cDNA sequence registered in BLAST.

Cell Culture. All cells were cultured in a 5% CO_2 humidified incubator at 37°C. Mouse mammary carcinoma cell lines D2.0R, D2.1, and D2A1 were gifts from F. R. Miller and maintained as described in ref. 2. Mouse melanoma cell lines B16F1 and B16F10 were obtained from ATCC and maintained according to the provider's protocol. Lentivirus-mediated gene transfer was performed essentially as described in ref. 1. The transfection of 293T cells with a viral vector and helper constructs was performed in 15-cm culture dishes, and 20 mL of virus-containing medium was filtered and centrifuged at 17,500 × *g*, for 12 h at 4°C. The resulting viral pellet was resuspended in 2 mL of fresh medium, supplemented with 10 μg/mL polybrene, and used for the infection. The selections of cells successfully infected with pLV-IRES-neo-based lentivirus were performed with medium containing 750 μg/mL G418, whereas the selections of cells infected with pLKO-puro- or pLKO-hygro-based lentivirus were performed with medium containing 5 μg/mL puromycin or 300 μg/mL hygromycin, respectively.

For the 3-dimensional, embedded (3D embedded) culture of D2 cells (Fig. S1 *B* and *C*), 12-well culture plates were coated with 100 μL/well of gel consisting of the indicated ECM components and incubated at 37°C for 30 min to allow the gels to solidify. Subsequently, 2,000 cells were resuspended in 500 μL of gel to form a single-cell suspension and layered on top of the previously formed gel, and the plate was incubated at 37°C for another 30 min. After the cell-containing gel had solidified, 1 mL of 2D/3D culture medium (DME/F12 medium supplemented with 2% horse serum, 0.5 μg/mL hydrocortisone, 50 ng/mL cholera toxin, 10 μg/mL insulin, 100 units/mL penicillin, and 100 μg/mL streptomycin) was added on top of the gel. Medium was changed every 4 days. The 2D and 3D culture of B16F1 and B16F10 cells were performed essentially as described in the *Cell Culture* subsection in *Methods* of the main article, except that DME medium supplemented with 2% FCS, 100 units/mL penicillin, and 100 μg/mL streptomycin was used instead of 2D/3D culture medium. For imaging 2D- or 3D-cultured cells (Fig. 3*A*, Fig. S1*B*, Fig. S3, Fig. S4*A*, and Fig. S6*A*), cells were cultured for 10 days, fixed with 2% (wt/vol) paraformaldehyde in PBS at RT for 20 min, and permeabilized with 0.5% Triton-X in PBS on ice for 10 min. Subsequently, cell nuclei were stained with 1 μg/mL DAPI (Sigma). Images were acquired using a Nikon TE300 inverted microscope (Nikon) equipped with a Hamamatsu Orca camera (Hamamatsu Photonics).

Animal Procedures. All of the animal experiments were performed in accordance with the regulations of the Massachusetts Institute of Technology Committee on Animal Care protocol (1008–110-11). After the tail-vein injection of D2 cells, lungs were harvested at the indicated time point, and the whole-lobe images were taken using a Leica MZ12 fluorescence dissection microscope (Leica) equipped with a SPOT-RTKE digital camera (Diagnostic Instruments). Colonies visible at 8× magnification under fluorescence microscopy were counted as macroscopic metastases (macrometastases). For frozen sectioning, 2.5 mL of O.C.T. compound were injected through the trachea immediately before harvesting the lungs. Lungs were snap frozen in liquid nitrogen and sections of 50 μm thickness were placed onto Superfrost Plus slides (Fisher Scientific). Sections were subsequently fixed with 4% (wt/vol) paraformaldehyde in PBS at RT for 10 min, and the nuclei were stained by soaking in 1 μg/mL Hoechst 33342 in PBS, containing 0.1% Triton-X, for 5 min on ice. The quantification of perfusion-resistant cells in the lungs (Fig. 1*B* and Fig. S8) was performed by directly counting the numbers of GFP-positive cells and tdTomato-positive cells on the frozen section slides under fluorescence microscopy. Approximately 500 cells in total were counted per mouse.

Immunofluorescence. For 2D culture, 400 cells were resuspended in 200 μL of 2D/3D culture medium to form a single-cell suspension and plated in 8-well chamber slides (NUNC). For 3D culture, 8-well chamber slides were coated with 40 μL/well of Matrigel and incubated at 37°C for 30 min to allow the gel to solidify. Subsequently, 400 cells were resuspended in 200 μL of 2D/3D culture medium, containing 2% Matrigel, to form a single-cell suspension and added to the Matrigel-coated well. Cells were cultured for 5 days with the medium changed on the fourth day. Fixation and staining were performed essentially as described in ref. 3. M.O.M. blocking reagent (Vector Laboratories) was used instead of the goat-anti mouse $F(ab')_2$ fragment to block endogenous mouse antibodies contained in Matrigel. The types and dilutions of primary antibodies used were the following: anti-FAK (4.47; Upstate, 1:200), anti-phospho-FAK [pY^{397}] (44–624G; Biosource, 1:200), anti-fibronectin (10; BD Transduction, 1:100), anti-integrin α_5 (5H10–27; BD PharMingen, 1:100), anti-integrin α_v (AB1930; Chemicon, 1:500), anti-primed integrin β_1 (9EG7; BD PharMingen, 1:30), anti-paxillin (5H11; Upstate, 1:100), anti-talin (8D4; Sigma, 1:200) and anti-vinculin (VIN-11–5; Sigma, 1:200). The types of secondary antibodies used were Alexa Fluor-546/647 goat anti-mouse/rabbit/rat IgG (Molecular Probes), and they were used at a 1:200 dilution. Filamentous actin was stained using Alexa Fluor 488 phalloidin (Molecular Probes) at a 1:40 dilution. Nuclei were

stained with either 1 μg/mL DAPI or 100 nM YOYO-1 (Molecular Probes) mixed with 200 μg/mL RNaseA (Sigma). Cover glasses were mounted on the slides with ProLong Gold Antifade Reagent (Molecular Probes).

Deconvolution microscopy was performed using a DeltaVision RT restoration microscopy system equipped with an Olympus IX70 inverted microscope (Olympus), a CoolSNAP HQ CCD-camera (Roper Scientific) and a 60× objective lens. After acquisition of the *Z*-series images, they were deconvolved using the software attached in the system (Softworx; Applied Precision). Confocal microscopy was performed using a Zeiss LSM 510 META laser scanning confocal microscope system with a 100× objective lens. Images were processed on Photoshop (Adobe) for the contrast and brightness adjustment.

Immunoblotting. Immunoblotting was performed essentially as described in ref. 4. Briefly, cells were harvested and lysed in RIPA buffer [25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 1× complete protease inhibitor mixture (Roche)]. Then, 40 μg of protein lysate was loaded into each lane of a NuPAGE Novex 4%–12% Bis-Tris Gel (Invitrogen). The types and dilutions of primary antibodies used were the following: anti-(total) FAK (06–543; Upstate, 1:500), anti-phospho-Fak [pY^{397}] (44–624G; Biosource, 1:500), anti-phospho-Fak [pY^{861}] (44–626G; Biosource, 1:500), anti-integrin α_5 (AB1928; Chemicon, 1:4000), anti-integrin α_v (21; BD Transduction, 1:250), anti-integrin β_1 (AB1952P; Chemicon, 1:1000) and anti-α-tubulin (DM1A; Abcam, 1:4000).

To detect FAK phosphorylation (Fig. 3*C*and Fig. 5*C*), cells were cultured on 6-cm culture dishes. For 2D culture, 6×10^4 cells were resuspended in 5 mL of 2D/3D culture medium to form a single-cell suspension and plated on a 6-cm culture dish. For 3D culture, 6-cm culture dishes were coated with 650 μL/dish of Matrigel and incubated at 37°C for 30 min to allow the gel to solidify. Subsequently, 6×10^4 cells were resuspended in 5 mL of 2D/3D culture medium, containing 2% Matrigel, to form a single-cell suspension and added to the Matrigel-coated dish. Cells were cultured for 5 days with the medium changed on the fourth day. The number of cells in each sample after 5 days of culture was determined using cells cultured in an extra dish. For protein lysis, the cells together with Matrigel were collected by scraping. Cells were lysed together with Matrigel in RIPA buffer supplemented with Halt Phosphatase Inhibitor Cocktail (Pierce) for 2 h at 4°C. After being precleared by the incubation with Streptavidin Agarose Resin (Pierce) for 30 min at 4°C, lysate corresponding to 3×10^4 cells at the time of harvesting was incubated with 1 μg of anti-FAK antibody (4.47; Upstate), biotinylated using EZ-Link Micro Sulfo-NHS-LC-Biotinylation kit (Pierce), overnight at 4°C. Lysate was subsequently incubated with streptavidin agarose resin for 2 h at 4°C, and the biotinylated anti-FAK antibody and the FAK protein bound by the antibody were collected by centrifugation. Collected protein was recovered from the streptavidin agarose resin by incubating it in 1× LDS sample buffer (Invitrogen) boiling for 10 min. The sample was then loaded into the lane of a Bis-Tris gel. Before determining the relative band intensity, the intensity of the band for phospho-FAK was normalized against that of the band for total FAK in the same sample.

Quantitative RT-PCR. To obtain total RNA samples of 2D- or 3D-cultured cells for quantitative RT-PCR, cells were cultured in 6-cm culture dishes as described above in the *Immunoblotting* section. Two-dimensionally-cultured cells were harvested by treatment with 0.05% trypsin/0.02% EDTA for 20 min at 37°C. Three-dimensionally-cultured cells were harvested by treatment with Dispase (BD Biosciences) for 30 min at 37°C, followed by treatment with 0.05% trypsin/0.02% EDTA for 30 min at 37°C. RNA preparation and cDNA synthesis were performed using RNeasy Mini kit (QIAGEN) and SuperScript III First-Strand synthesis system (Invitrogen), respectively, both according to the manufacturer's protocol. Quantitative real-time PCR was performed essentially as described in ref. 4. A cDNA sample prepared from 20 ng total RNA was used for each PCR. The PCR, data collection, and data analysis were performed on the MyiQ Single-Color Real-Time PCR detection system (Bio-Rad). The thermal cycling parameters for the PCR were as follows: 95°C for 7 min, followed by 43 cycles of 95°C for 15 sec and 60°C for 90 sec. The standard curve was drawn on the basis of the results from serially diluted samples and used to determine the relative quantity of mRNA. Before determining the relative expression units, the relative mRNA quantity calculated as above was normalized against the relative quantity of β-actin mRNA (*Actb*) in the same sample. The primer set used for each of the mRNA targets is shown in Table S2.

Flow Cytometric Analysis. Flow cytometric analyses were carried out on a FACSCalibur system (BD Biosciences). To determine the number of cells after 2D or 3D culture (Figs. 2 *A* and *B*, 4 *A* and *B*, 5*A*, Fig. S1*C*, Fig. S4*B*, and Fig. S6*B*), cells were harvested by dispase and trypsin/EDTA treatment as described above in the *Quantitative RT-PCR* section, except for the 3D embedded cultures using gels consisting of collagen I or collagen I + Matrigel, in which 1.5 mg/mL collagenase A (Roche) or the 1:1 mixture of 1.5 mg/mL collagenase A and dispase, respectively, were used instead of dispase. Cells were subsequently prepared as a single-cell suspension by pipetting and passing them through a cell strainer (BD Falcon, BD Biosciences). Along with this, D2A1 cells expressing GFP (D2A1-GFP cells) were also prepared as a single-cell suspension, whose concentration was determined using a hemocytometer. Suspension samples of 2D- or 3D-cultured cells were mixed with a specific number of D2A1-GFP cells, and the percentage of GFP-positive cells in the mixed cell populations and in the D2A1-GFP cell population was analyzed by flow cytometry. The number of cells in the original suspension samples was calculated by the following formula: $n = n_A g_A(1 - g_m/100)/g_m - n_A(1 - g_A/100)$, wherein n, n_A, g_A, and g_m stand for the number of cells in the original suspension sample, the number of added D2A1-GFP cells, the percentage of GFP-positive cells within the D2A1-GFP cell population, and the percentage of GFP-positive cells within the mixed cell population, respectively.

To determine the abundance of fluorescent-labeled cells present in the lungs after the tail-vein injection (Fig. 1*A*), lungs were harvested, minced, and treated with 1.5 mg/mL collagenase A, containing 0.1 units/mL DNase I, for 1 h at 37°C. After passing through a cell strainer, dissociated lung cells were analyzed by flow cytometry. To determine whether the cells that extravasated into the lungs were BrdU positive, 1×10^6 of tdTomato-labeled cells were injected into the tail vein. One milligram of BrdU, dissolved in PBS, was injected intraperitoneally twice, at 6 and 3 h before harvesting the lungs, which was performed 7 days after the injection of the cells. Lungs were harvested after the perfusion of the vasculature and dissociated as described above. After dissociation, the cells were fixed and stained for BrdU using FITC BrdU flow kit (BD Biosciences) according to the manufacturer's protocol. The percentage of BrdU-positive cells within the population of tdTomato-positive cells was determined using flow cytometry.

Proliferation and Apoptosis Assay. To determine the proliferation and apoptosis rates in 2D- or 3D-cultured cells, cells were cultured for 5 days in 8-well chamber slides as described above in the *Immunofluorescence* section. Ki67 staining was also performed as described above in the *Immunofluorescence* section. Anti-Ki67 antibody (TEC-3; DakoCytomation) was used at a

dilution of 1:25. TUNEL staining was performed using an In Situ Cell Death Detection kit, TMR red, (Roche) according to the manufacturer's protocol. Images of Ki67- or TUNEL-stained cells were acquired using a Zeiss LSM 510 META laser scanning confocal microscope system with a 40× objective lens. Approximately 200 cells were scored for Ki67 or TUNEL positivity per sample.

1. Stewart SA, et al. (2003) Lentivirus-delivered stable gene silencing by RNAi in primary cells. *RNA* 9(4):493–501.
2. Rak JW, McEachern D, Miller FR (1992) Sequential alteration of peanut agglutinin binding-glycoprotein expression during progression of murine mammary neoplasia. *Br J Cancer* 65(5):641–648.
3. Debnath J, Muthuswamy SK, Brugge JS (2003) Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. *Methods* 30(3):256–268.
4. McAllister SS, et al. (2008) Systemic endocrine instigation of indolent tumor growth requires osteopontin. *Cell* 133(6):994–1005.

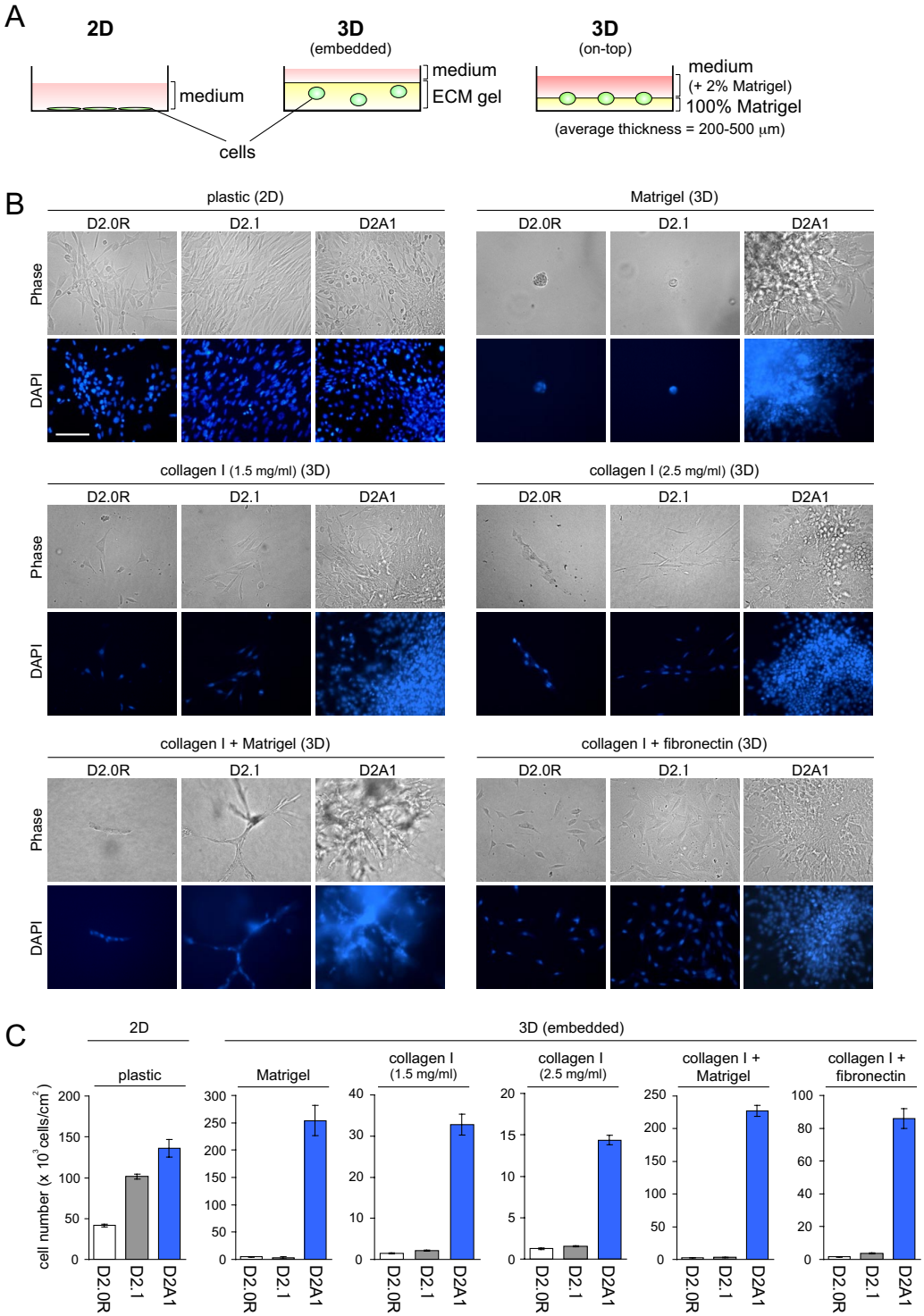

Fig. S1. Three-dimensional culture of D2 cells in various ECM gels. (*A*) A schematic description of culture methods. Five different types of ECM gels (100% Matrigel, 50% Matrigel + 1.5 mg/mL collagen I, 1.5 mg/mL collagen I, 2.5 mg/mL collagen I and 1.5 mg/mL collagen I + 100 μg/mL fibronectin) were tested for 3D-embedded culture method (Fig. S1*B* and Fig. S1*C*), whereas only Matrigel was tested for 3D on-top method (Fig. 2 and Fig. S3). (*B*) D2 cells were cultured under 2D- or 3D-embedded conditions, and the typical morphologies of cells after 10 days of culture are presented. For 3D-embedded culture, D2 cells were mixed within the gel of ECM components (ECM gels), and 5 different types of ECM gels were tested (see Fig. S1*A* and *SI Methods* for the details). Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; light blue). (Scale bar, 0.1 mm.) (*C*) Cell numbers were determined after 10 days of 2D- or 3D-embedded culture. Values are means ± SD ($n = 3$).

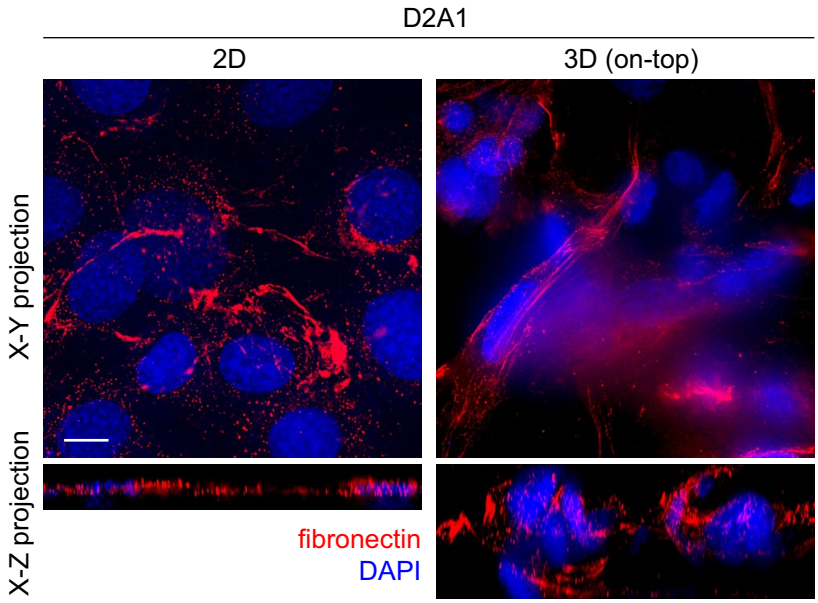

Fig. S2. Arrangement of 2D- and 3D-cultured D2A1 cells. D2A1 cells were cultured under 2D or 3D on-top conditions for 5 days and stained for fibronectin (red). Cell nuclei were stained with DAPI (blue). Shown are maximum intensity projections of 5 (X–Y projection, 2D), 45 (X–Y projection, 3D) or 100 (X–Z projection, 2D and 3D) consecutive focal planes with a step size of 0.5 μm. (Scale bar, 10 μm.) Notably, under 3D on-top conditions, D2A1 cells invaded the Matrigel and reached various depths within the Matrigel layer during the 5 days of culture, whereas these cells exhibited a monolayer arrangement under 2D culture conditions.

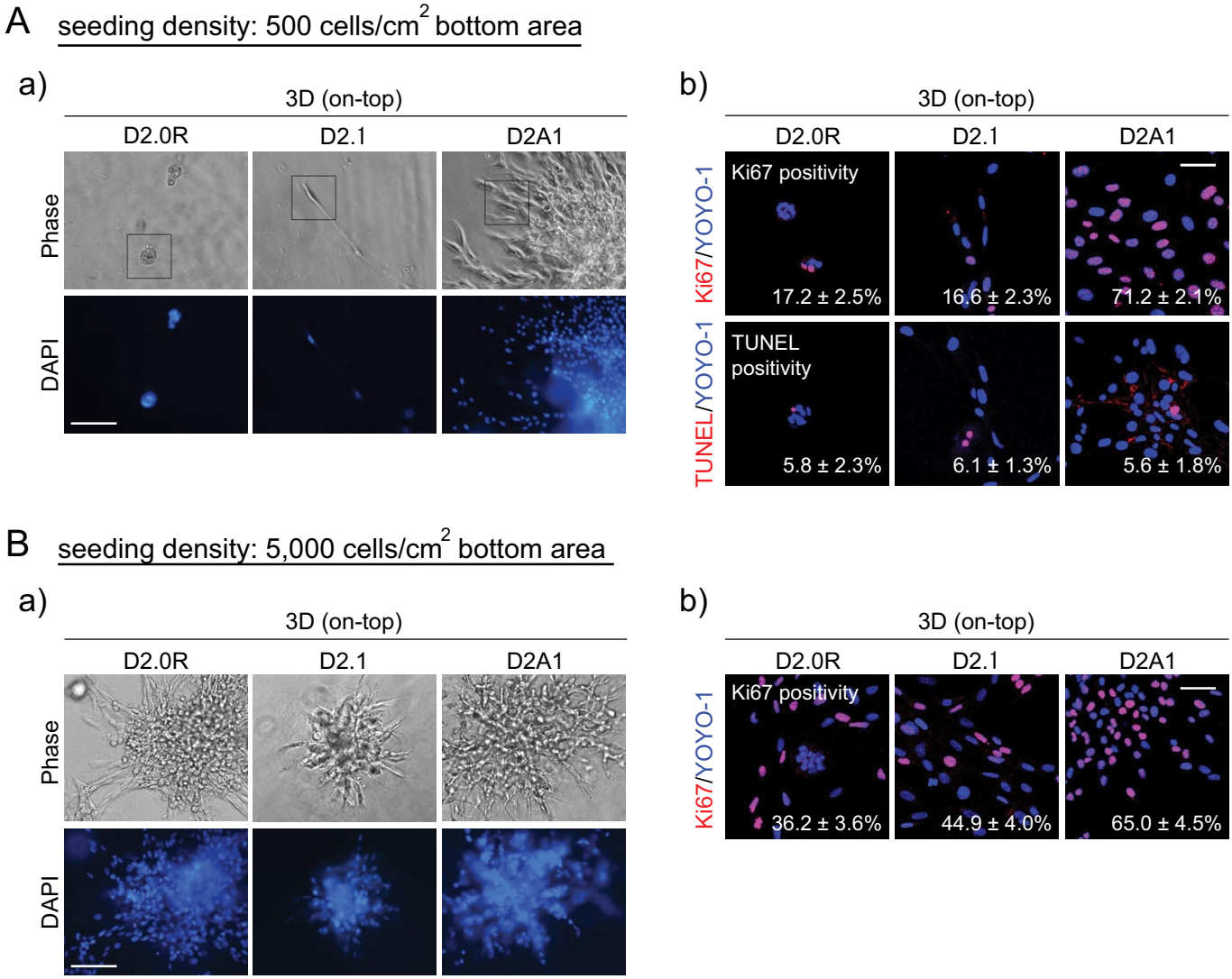

Fig. S3. Three-dimensional culture of D2 cells with a low and a high seeding density. D2 cells were cultured under 3D on-top conditions with a seeding density of 500 cells/cm^2 bottom area (*A*) or 5,000 cells/cm^2 bottom area (*B*). (*Aa* and *Ba*) The typical morphologies of cells after 10 days of culture are presented, and cell nuclei were stained with DAPI (light blue). Enlarged images of the *Boxed* region within the phase-contrast images in *Aa* are also presented in Fig. 3*A*. (Scale bar, 0.1 mm.) (*Ab* and *Bb*) Cells cultured for 5 days were stained for proliferation (Ki67) or apoptosis (TUNEL) marker (both are represented by the red color), and the nuclei were stained with YOYO-1 (blue). The positivity of Ki67 or TUNEL staining within each cell population is indicated. (Scale bar, 50 μm.) Increased initial seeding density facilitated the proliferation of the nonmetastatic D2.0R and D2.1 cells under 3D on-top culture conditions. As discussed in the main text, alterations in cell–cell interaction or cell–Matrigel interaction may support the proliferation of nonmetastatic D2.0R and D2.1 cells when they were seeded at a density of 5,000 cells/cm^2 bottom area. Moreover, high seeding densities enable cells to exert more proteolytic activity to remodel the Matrigel substrate, which may also contribute to the increased proliferation rates of these nonmetastatic cells.

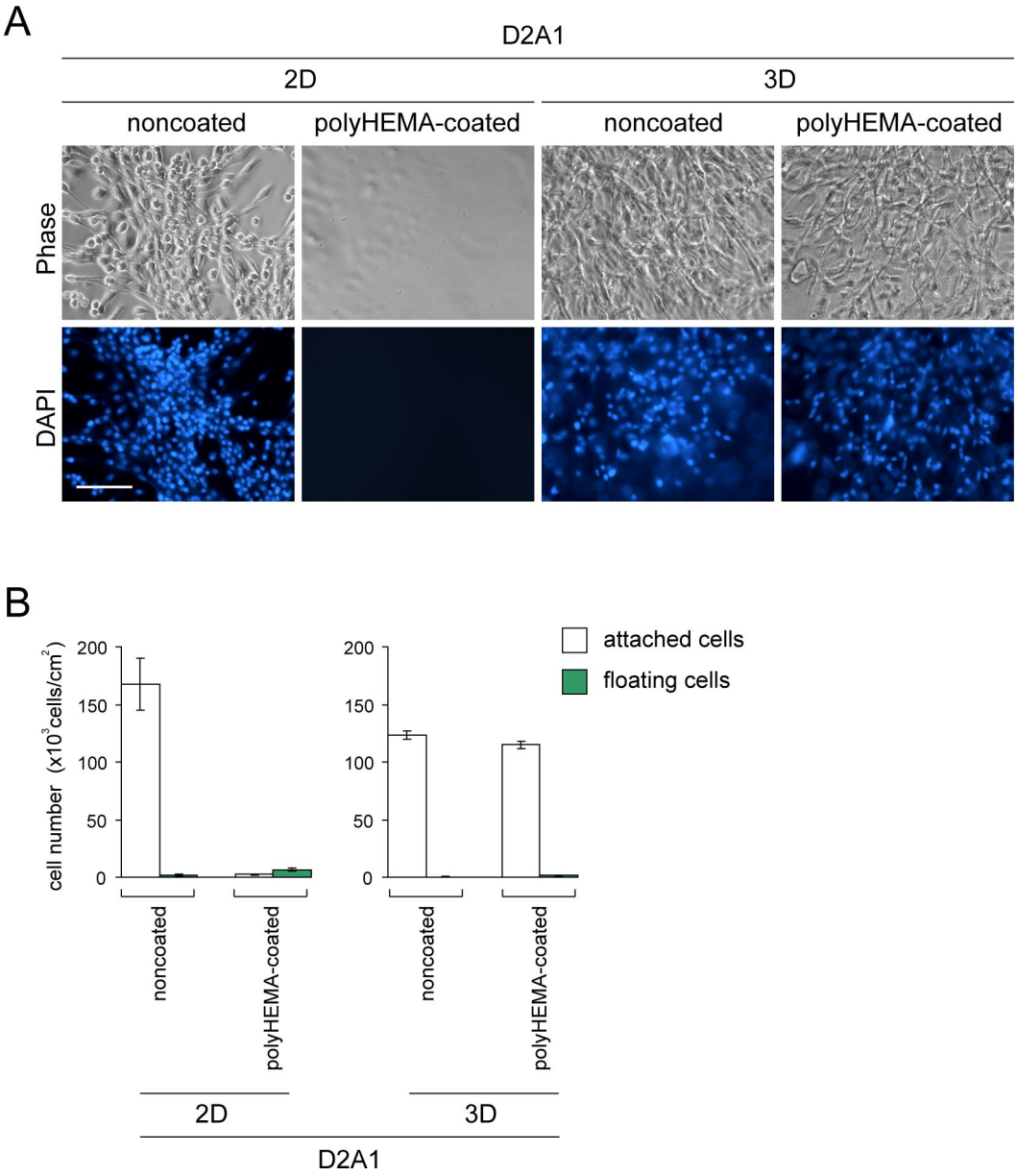

Fig. S4. Two-dimensional and three-dimensional culture on a polyHEMA-coated culture dish. (*A*) D2A1 cells were cultured under 2D or 3D on-top conditions on polyHEMA-coated or noncoated dishes, and the typical morphologies of cells after 10 days of culture are presented. Adherent cells were hardly detectable on the polyHEMA-coated dish under 2D conditions, indicating that polyHEMA-coating effectively blocked the direct attachment of D2A1 cells to the bottom of the dish. (Scale bar, 0.1 mm.) (*B*) Numbers of attached and floating cells were determined after 10 days of 2D or 3D culture on polyHEMA-coated or noncoated dishes. Under 2D conditions, the sum of attached and floating cell numbers were substantially decreased by polyHEMA coating of the culture dish, indicating that cell proliferation is facilitated by the attachment to the bottom of the culture dish under these conditions. In contrast, polyHEMA coating did not clearly affect the resulting cell number under 3D conditions of culture. Values are means ± SD ($n = 3$).

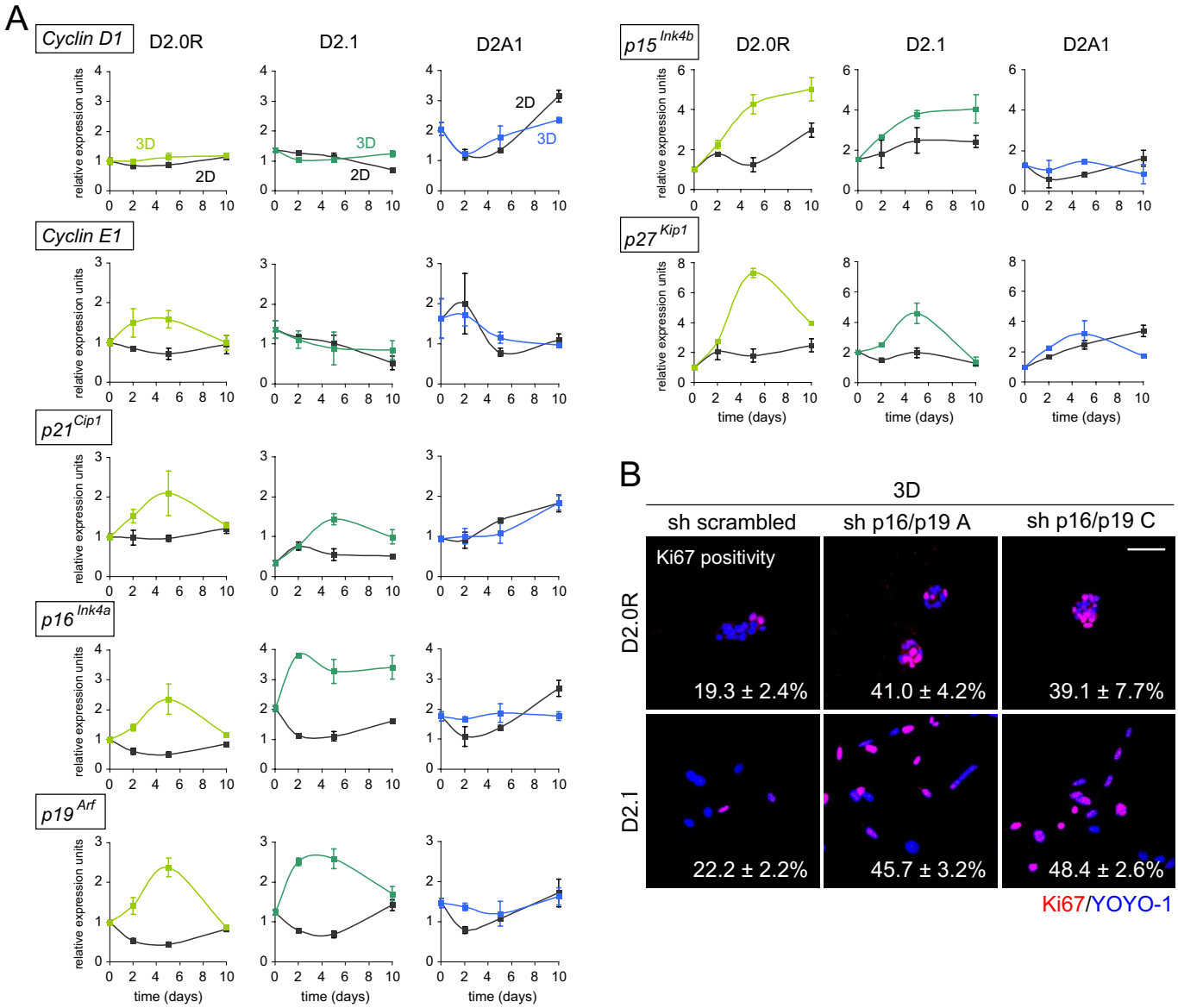

Fig. S5. Role of p16^{Ink4a} and p19^{Arf} in the proliferation arrest of 3D-cultured nonmetastatic cells. (*A*) mRNA samples were extracted from the D2 cells cultured under 2D or 3D conditions for 0, 2, 5, or 10 days. Expression units of each mRNA determined by real-time RT-PCR, relative to that in the sample from D2.0R cells cultured for 0 day, were plotted. The black line in each pair of lines represents the change in the expression under 2D culture conditions, whereas the colored line represents the change in the expression under 3D culture conditions. The *p16^{Ink4a}*, *p19^{Arf}*, *p21^{Cip1}*, and *p27^{Kip1}* mRNAs were up-regulated in D2.0R and D2.1 cells under 3D but not under 2D conditions; significantly, none of these mRNAs was up-regulated in D2A1 cells under either 2D or 3D conditions of culture. (*B*) The effect of p16^{Ink4a} and p19^{Arf} knockdown on the proliferation of 3D-cultured D2A1 cells was analyzed by Ki67 staining (red). The effects of 2 different shRNA constructs against p16^{Ink4a}/p19^{Arf} (sh p16/p19A and C), both of which were designed to simultaneously knock down the expression of both p16^{Ink4a} and p19^{Arf}, and the effect of an shRNA control (sh scrambled) are presented. Cell nuclei were stained with YOYO-1 (blue). The positivity of Ki67 staining within each cell population is indicated. (Scale bar, 50 μm.) Values are means ± SD (n = 3) (*A* and *B*).

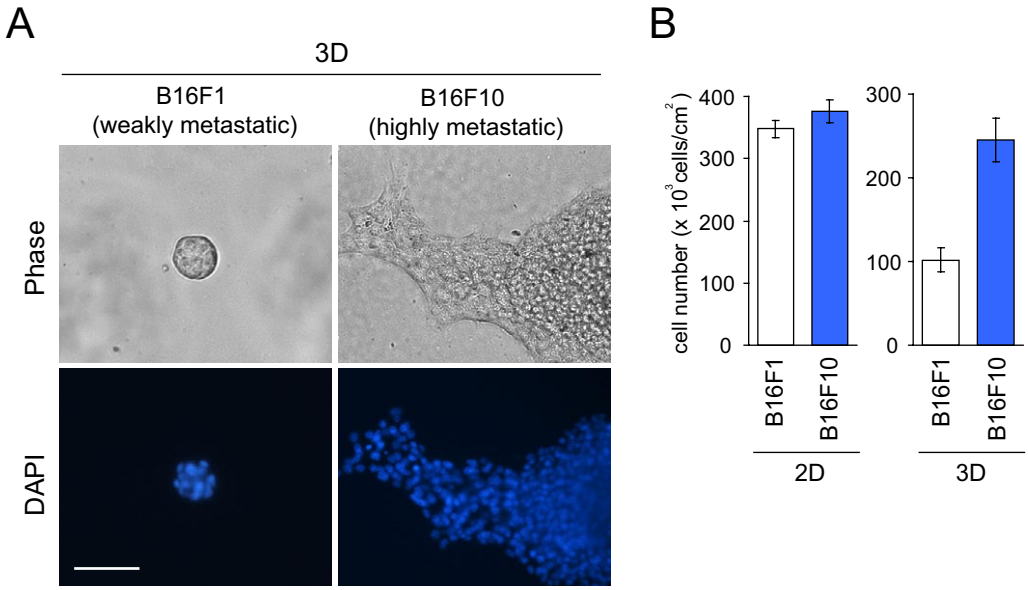

Fig. S6. Two-dimensional and three-dimensional culture of B16 melanoma cells. (*A*) Weakly metastatic B16F1 cells and highly metastatic B16F10 cells were cultured under 2D or 3D on-top conditions, and the typical morphologies of cells after 10 days of culture are presented. Cell nuclei were stained with DAPI (light blue). (Scale bar, 0.1 mm.) (*B*) Cell numbers were determined after 10 days of 2D or 3D culture. Values are means ± SD ($n = 3$).

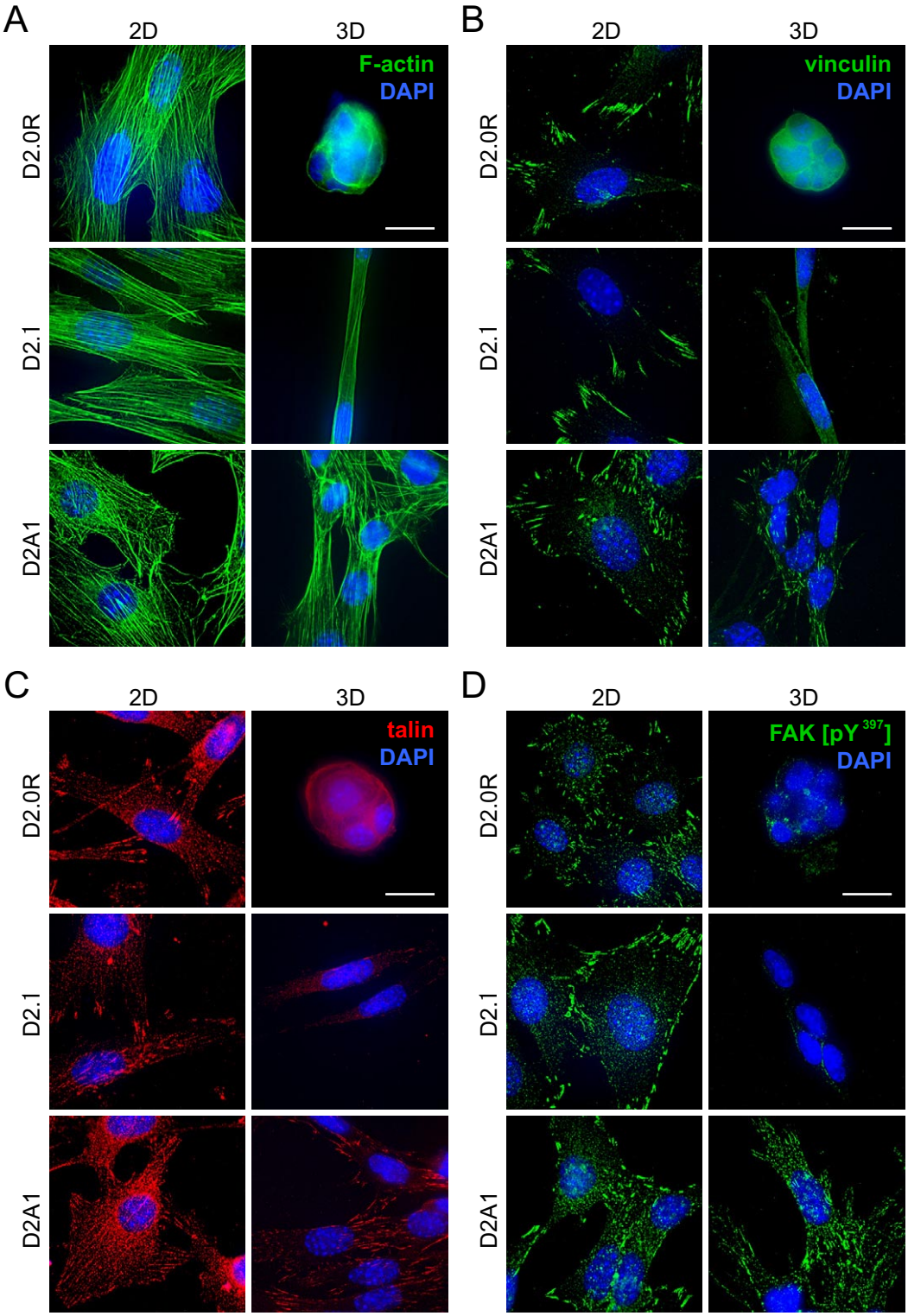

Fig. S7. Cytoskeleton and cell-matrix adhesions under 2D and 3D culture. D2 cells cultured under 2D or 3D on-top conditions for 5 days were stained for filamentous-actin (with phalloidin, *A*; green), vinculin (*B*; green), talin (*C*; red) and phospho-FAK (tyrosine-397 [pY^{397}]) (*D*; green). Cell nuclei were stained with DAPI (blue). (Scale bar, 20 μm.) Notably, under 3D on-top culture conditions, adhesion plaque proteins including paxillin (Fig. 3*B*), vinculin (Fig. S7*B*), and talin (Fig. S7*C*) formed abundant plaques of elongated morphology only in the metastatic D2A1 cells; these adhesion plaque proteins in the 3D-cultured D2.0R cells did not form plaques of oval-like or elongated morphology, and the 3D-cultured D2.1 cells showed far fewer elongated adhesion plaques per cell than exhibited by the 3D-cultured D2A1 cells.

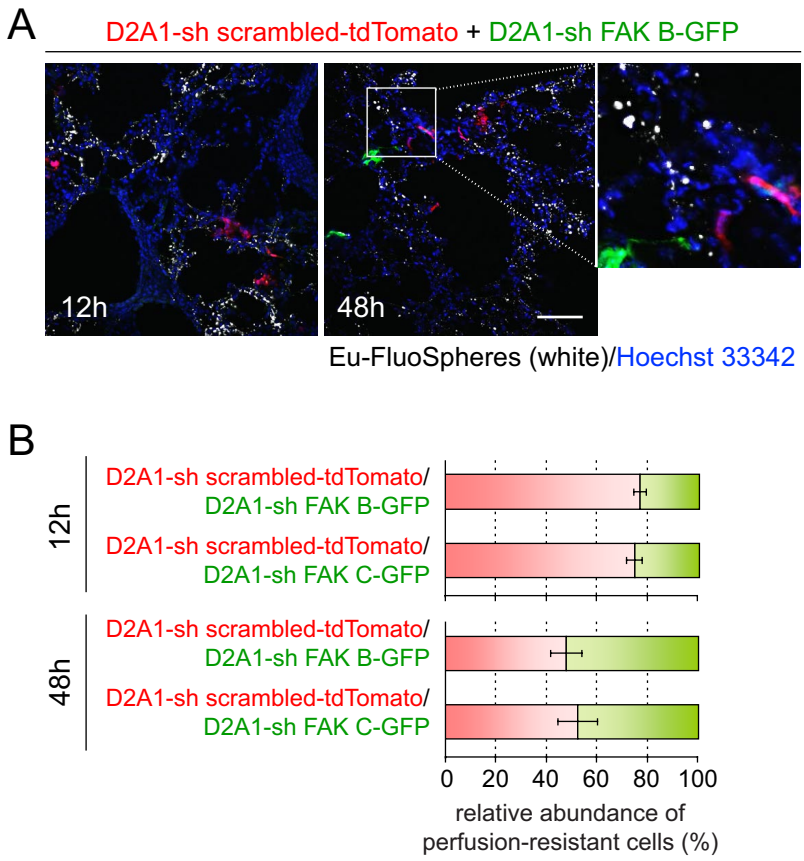

Fig. S8. Effect of FAK knockdown on the extravasation of D2A1 cells in the lungs. (*A*) Lungs were harvested at 12 or 48 h after the coinjection of equal numbers of tdTomato-labeled D2A1 cells expressing an shRNA control (D2A1-sh scrambled-tdTomato) and GFP-labeled FAK-knockdown D2A1 cells (D2A1-sh FAK B-GFP or -sh FAK C-GFP). Shown are the representative images of lung sections at 12 (*Top Left*) and 48 (*Top Middle*) h after the coinjection of D2A1-sh scrambled-tdTomato and D2A1-sh FAK B-GFP cells. An enlarged image of the *Boxed* region within the *Top Middle* image is also presented (*Top Right*). The lung vasculature was perfused by Eu-FluoSpheres just before harvesting the lungs, which subsequently filled the lumen of microvessels (white), and the nuclei were stained with Hoechst 33342 (blue). (Scale bar, 0.2 mm.) (*B*) The relative abundance of perfusion-resistant D2A1-sh scrambled-tdTomato cells and GFP-labeled FAK-knockdown D2A1 cells (D2A1-sh FAK B-GFP or -sh FAK C-GFP) in the lungs at 12 or 48 h after injection were determined as in Fig. 1*B*. Values are means ± SEM ($n = 4$)

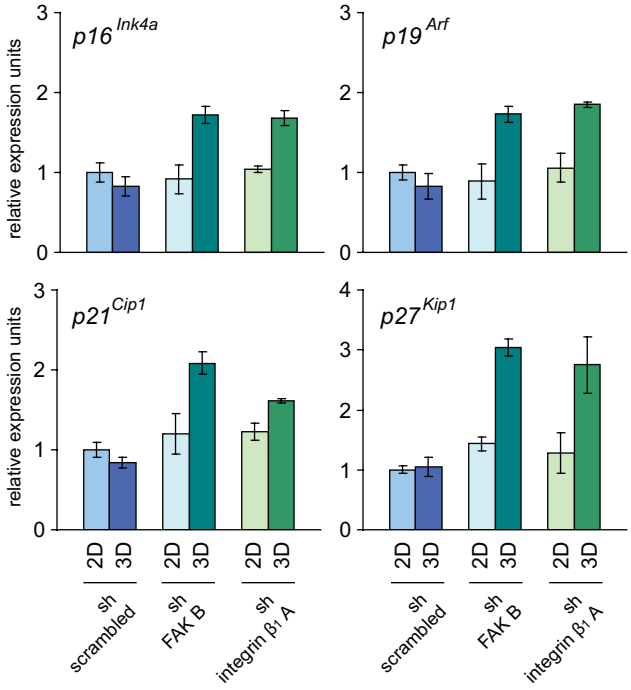

Fig. S9. Role of FAK and integrin β_1 on the transcriptional regulation of cell cycle inhibitors. mRNA samples were extracted from D2A1 cells in which the expression of FAK (sh FAK B) or integrin β_1 (sh integrin β_1 A) was knocked down, and from D2A1 cells expressing an shRNA control (sh scrambled). Cells were cultured under 2D or 3D conditions for 5 days. Plotted are the expression units of each mRNA determined by real-time RT-PCR, relative to that in the sample from 2D-cultured D2A1 cells expressing an shRNA control. The lighter of each pair of bars represents the expression under 2D culture conditions, whereas the darker represents the expression under 3D conditions. Values are means ± SD ($n = 3$).Under 3D conditions of culture, knocking down the expression of either integrin β_1 or FAK in D2A1 cells resulted in the up-regulation of the *p16*^{Ink4a}, *p19*^{Arf}, *p21*^{Cip1}, and *p27*^{Kip1} mRNAs, indicating the role of integrin β_1-FAK signaling axis in the transcriptional regulation of these cell cycle regulators. Notably, these effects were specific for 3D conditions of culture, because under 2D conditions, knocking down the expression of either integrin β_1 or FAK in D2A1 cells did not lead to up-regulation of the mRNAs for these cell-cycle regulators.

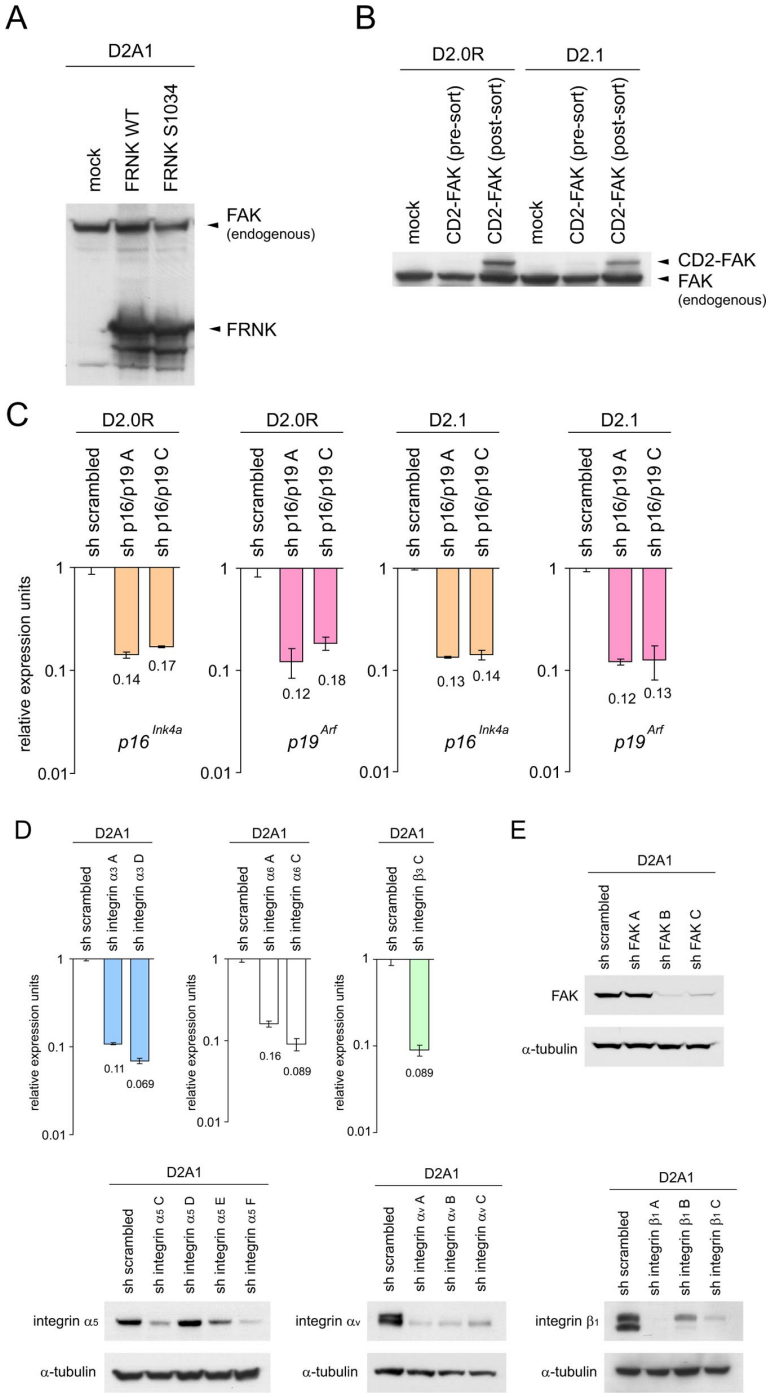

Fig. S10. Overexpressions and knock-downs. (*A*) The expression of ectopically expressed wild-type FRNK (FRNK WT) and S1034 mutant (FRNK S1034) protein in D2A1 cells was analyzed by immunoblotting. (*B*) The expression of ecotopically expressed CD2-FAK protein in D2.0R and D2.1 cells was analyzed by immunoblotting. Infection of these two cells with CD2-FAK-expressing lentivirus and subsequent drug selection yielded only weak expression of CD2-FAK (pre-sort). Therefore, high-CD2-FAK expressing fraction was subsequently enriched by the two rounds of cell sorting, using FITC-labeled anti-CD2 antibody (BD Pharmingen) (post-sort). (*C*) The efficiencies of $p16^{Ink4a}$ and $p19^{Arf}$ knock-down in D2.0R and D2.1 cells were analyzed. The levels of mRNA expression were assessed by real-time PCR. Two different shRNA constructs (sh p16/p19 A and C), each simultaneously targeting the expression of both $p16^{Ink4a}$ and $p19^{Arf}$, were tested. The expression units relative to that of the sample from D2.0R or D2.1 cells expressing an shRNA control (sh scrambled) were plotted. (*D*) The efficiencies of integrin subunits knock-down in D2A1 cells were analyzed. For integrins α_5, α_v, and β_1, the levels of protein expression were assessed by immunoblotting. For integrins α_3, α_6, and β_3, the levels of mRNA expression were assessed by real-time PCR, and the expression units relative to that of the sample from D2A1 cells expressing an shRNA control (sh scrambled) were plotted. The capital letter at the end of each shRNA label is added for distinguishing the different shRNA constructs for the same target, and multiple different constructs were tested for targeting each of integrin α_3, α_5, α_6, α_v, and β_1 expression, whereas a single shRNA construct was tested for targeting the expression of integrin β_3 (sh integrin β_3 C).(*E*) The efficiency of FAK knock-down in D2A1 cells was analyzed. Three different shRNA constructs targeting FAK expression (sh FaK A-C), as well as an shRNA control (sh scrambled) were tested. The levels of protein expression were assessed by immunoblotting.

Table S1. Target sequences used in short-hairpin RNA (shRNA) constructs

Construct	Target sequence
sh integrin α_3 A	CGGATGGACATTTCAGAGAAA
sh integrin α_3 D	GAAACAGCTACATGATTCAGC
sh integrin α_5 C	CCTCAGCAAGAACCTGAACAA
sh integrin α_5 F	CCAGAGAACCAAAGGAAGTCT
sh integrin α_6 A	CCAGGGACTTACAACTGGAAA
sh integrin α_6 C	CGGCACAGCAACCTTGAATAT
sh integrin α_v A	CGAGGGAAGTTACTTCGGATT
sh integrin α_v B	GGCCAGCCCATTGAGTTTGAT
sh integrin α_v C	CGTGTGTTCTTAGGGACTTAA
sh integrin β_1 A	GCACGATGTGATGATTTAGAA
sh integrin β_1 C	CCCGACATCATCCCAATTGTA
sh integrin β_3 C	CATTATGTTTACAGAGGACAA
sh FAK B	CCTGGCATCTTTGATATTATA
sh FAK C	CGGTCCAATGACAAGGTATAT
sh p16/p19 A	TGATGATGGGCAACGTTCACG
sh p16/p19 C	TCAAGACATCGTGCGATATTT
sh scrambled	CAACAAGATGAAGAGCACCAA

Table S2. Primer sequences used in RT-PCR experiments

Target	Primer sequence
Cyclin D1	Forward: GCCGAGAAGTTGTGCATCTA Reverse: GTTCACCAGAAGCAGTTCCA
Cyclin E1	Forward: GTGGCTCCGACCTTTCAGTC Reverse: CACAGTCTTGTCAATCTTGGCA
$p21^{Cip1}$	Forward: CGAGAACGGTGGAACTTTGAC Reverse: CAGGGCTCAGGTAGACCTTG
$p16^{Ink4a}$	Forward: CGCAGGTTCTTGGTCACTGT Reverse: TGTTCACGAAAGCCAGAGCG
$p19^{Arf}$	Forward: GGTCACTGTGAGGATTCAGC Reverse: ACATGTTCACGAAAGCCAGA
$p15^{Ink4b}$	Forward: ACCCTGCCACCCTTACCAGA Reverse: CAGGCGTCACACACATCCA
$p27^{Kip1}$	Forward: TTAATTGGGTCTCAGGCAAACTC Reverse: GCTTCCTCATCCCTGGACAC
Integrin α_3	Forward: CCCAACTACAGGCGGAACA Reverse: ATGGAGAAGAAGCCGTGGAA
Integrin α_6	Forward: GGTTTGGAGCAGATTGTTTGTG Reverse: AGTTCCCGTTTCTTTCTTGAGTTG
Integrin β_3	Forward: CCTTGCTACTCTGCTCATCTGG Reverse: GCGGGTTGTTTGCTGTGT
β-Actin	Forward: ACCGTGAAAAGATGACCCAGA Reverse: AGGCATACAGGGACAGCACA