α-Actinin-4 Is Required for Amoeboid-type Invasiveness of Melanoma Cells

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Background: Melanoma cells invasion through the dermis directly correlates with death, defining migration as critical.

Results: ACTN4 down-regulation limits aggressive melanoma cells to a mesenchymal phenotype that retards collagen I matrix invasiveness.

Conclusion: Amoeboidal down-regulation limits aggressive melanoma cells to a mesenchymal phenotype that retards collagen I matrix invasiveness.

Significance: This finding provides for a role of ACTN4 in melanoma invasion and implicates a linkage between actin cytoskeleton and melanoma progression.

α-Actinin-4 (ACTN4), a key regulator of the actin cytoskeleton, is up-regulated in melanoma, though its role in melanoma remains speculative. We have discovered that in WM1158, a highly aggressive melanoma cell line, down-regulation of ACTN4 by shRNA induces a collagen I-dependent amoeboidal-to-mesenchymal transition. Re-expression of low levels of WT ACTN4 but not similar expression levels of ACTN1 successfully restores the amoeboidal morphology and limits collagen I gel compaction. A truncated ACTN4 mutant 1–890, which lacks the C-terminal tail, fails to rescue the amoeboidal morphology and to compact collagen I gel. Interestingly, in three-dimensional collagen I gels, ACTN4 KD cells are more polarized compared with cells in which scrambled shRNA is expressed. Surprisingly, ACTN4 KD cells migrate faster than the ones expressing the scrambled shRNA on a collagen I gel (two-dimensional) although these two cell lines migrate similarly on tissue culture. Most importantly, down-regulation of ACTN4 significantly reduced invasion of WM1158 cells into the three-dimensional collagen I gel, a representative of the dermis. Taken together, these findings suggest that ACTN4 plays an important role in maintaining the amoeboidal morphology of invasive melanoma and thus promoting dissemination through collagen-rich matrices.

Melanoma, a potentially deadly skin cancer, originates in the pigment-producing melanocytes of the basal layer of the epidermis. Patients with melanoma can be cured by surgical resection if they are diagnosed at a very early stage when the transformed melanocytes migrate radially. However, the rate of 5-year survival of melanoma patients dramatically declines once the tumor cells invade vertically through the dermal matrix, and then metastasize to distant organs. These disseminated tumors respond poorly if at all to current chemotherapies (1). Thus, understanding how melanomas transit the dermis would be key to limiting death from this cancer.

To invade the dermal matrix, melanoma cells need to invade into the dermis by changing their morphology, altering contacts with the extracellular matrix (2), communications with their surrounding stromal cells (2, 3), and finally actively migrating (4). We have found that melanoma invasiveness through a matrix occurs by amoeboidal movement (5). The change to an amoeboidal morphology by invading carcinomas is associated with activation of the small GTPase Rho (6). This was also noted with the melanomas moving into collagen-rich matrix (5). Ho et al. also have found that knockdown of RhoJ inhibits melanoma invasion through a change in actin cytoskeletal dynamics (7). These findings argue for alterations of the cytoskeleton and linkage to the membrane as operative in melanoma invasion.

Recent studies revealed that α-actinin-4 (ACTN4) is up-regulated in both biopsies and in cultured artificial skin suggesting that ACTN4 may play roles in the metastasis of melanoma cells (8). ACTN4, a member of a family of actin crosslinking proteins, has been implicated in cancer metastasis and is considered as a biomarker of cancer diagnosis (9). Increased expression of ACTN4 has been reported in melanoma cell lines WM983a and WM1158, compared with normal melanocytes in which only trace amounts are detected (8). In fact, ACTN4 is one of the most up-regulated proteins in the dermis when comparing melanoma-invaded with non-involved regions of skin. Of interest, when melanoma cells are activated by low dose proton beam irradiation, ACTN4 expression is increased (10). These findings implicate ACTN4 as being involved in the invasion and metastasis of melanoma. However, the precise biological function of ACTN4 in melanoma cells remains elusive. ACTN4 is comprised of three dominant domains: N-terminal actin binding domain, central spectrin repeats, and C-terminal calcium binding motifs. These

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‡ The abbreviations used are: ACTN4, α-actinin-4; TIRF, total internal reflection fluorescence; TIR-FM, total internal reflection fluorescence microscopy; KD, knockdown; RSF, radial stress fiber; TA, transverse arcs.
domains drive its biochemical functions of cross-linking actin filaments, bridging the cytoskeleton to cell membrane and regulating transcription. This suggests a critical role of ACTN4 in transcellular contractility and cell shape (11–13). In this study, we report that expression of ACTN4 controls the amoeboidal and mesenchymal phenotype and thus invasion of melanoma cell lines through collagen matrices.

MATERIALS AND METHODS

Melanocytes were cultured in DermaLife M medium supplemented with growth factors and other chemical components (Lifeline Cell Technology, Frederick, MD). Melanoma cell line WM35, a gift from the Coriell Institute for Medical Research (Camden, NJ), was cultured in MCDB 153:L15 medium mixture at a ratio of 4:1 in volume with addition of 10% fetal bovine serum, 5 μg ml−1 insulin, 2 mm CaCl2, and 1× pen/strip antibiotics. Melanoma cell line IgR3 was cultured in RPMI medium with addition of 10% fetal bovine serum and 1× pen/strip antibiotics. Melanoma cell lines WM983a, WM983b, and WM1158 were cultured in DMEM (1 g liter−1 glucose):L15 medium mixture at a ratio of 3:1 in volume with addition of 10% fetal bovine serum and 1× pen/strip antibiotics. Collagen I was purchased from BD Biosciences (San Jose, CA). Small hairpin RNAs were synthesized at Integrated DNA Technologies (Coralville, IA). Lipofectamine 2000 was purchased from Invitrogen (Grand Island, NY). Polyclonal actin antibody was purchased from Sigma Aldrich. ACTN1 and ACTN4 antibodies were purchased from Santa Cruz (Dallas, TX).

Small Hairpin RNA (shRNA) and Transfection—Sense and antisense oligonucleotides of human ACTN4 shRNA were denatured, annealed and then cloned into vector pSilencer 2.0-U6 (Life Technology) (Ambion, Grand Island, NY). Scrambled shRNA vector was purchased from Origene (Rockville, MD). Both scrambled (WM1158) and ACTN4 (ACTN4 KD) shRNAs were transfected into melanoma cells using Lipofectamine according to the manufacturer’s manual. Stable colonies were selected and cultured in complete growth medium in the presence of 1.5 μg/ml puromycin. To construct a shRNA-resistant ACTN4, DNA mutagenesis was performed by PCR. PCR fragments were then cloned into expression vector pEGFP-N1. Positive colony was further confirmed by DNA sequencing.

Immunoblotting—Melanoma cells were cultured in 6-well tissue culture plates until they were confluent. Cells were washed briefly with phosphate-buffered saline (PBS) in the absence of calcium and magnesium and then lysed in RIPA buffer in the presence of 1× protease inhibitors cocktails set V (Billerica, MA). The lysate was left on ice for 5 min prior to brief sonicating. After centrifugation at 13,000 × g for 10 min at 4 °C, the supernatant was transferred to a new microcentrifugation tube. The concentration of total protein was determined using Thermo Scientific” Pierce” BCA” Protein Assay (Rockland, IL). 10 μg of total proteins mixed with 5× SDS sample buffer in the presence of β-mercaptoethanol was boiled for 3 min prior to loading on SDS-PAGE. Proteins were then transferred to polyvinylidene difluoride (PVDF) membrane and immunoblotted with appropriate primary antibodies according to standard immunoblotting protocol.

Immunofluorescence—Cells grown on coverslips were rinsed briefly with PBS and then fixed with 4% formaldehyde for 30 min at room temperature, followed by permeabilizing with 0.2% Triton X-100 on ice for 5 min. After three washes with PBS, cells were incubated with 1% bovine serum albumin (BSA) for 30 min at room temperature. Cells were incubated with various primary antibodies diluted in 1% BSA for 1 h at room temperature and then washed three times with PBS (10 min each wash). Subsequently, fluorescent secondary antibody diluted in 1% BSA was added and incubated with cells for 45 min at room temperature followed with three washes of PBS. For staining of actin filaments, rhodamine phalloidin diluted in 1% BSA was added and incubated with permeabilized cells for 15 min at room temperature. After complete washing, coverslips were then transferred inversely to a glass slide carrying a drop of Mowiol 4-88 (Sigma) and stored in the refrigerator overnight. Images were taken with Olympus microscopy at the Center for Biological Imaging, University of Pittsburgh.

Total Internal Reflection Fluorescence (TIRF) Microscopy—Cells transiently express vinculin-eGFP were imaged using the objective-type total internal reflection fluorescence microscopy (TIR-FM) as described previously (14). A CFI Apochromat series 1.49 NA TIR-FM objective was mounted on a Nikon Eclipse Ti inverted microscope equipped with a Perfect Focus System device, Agilent 4 color launch and Elements AR software. Images were collected using an Andor Zyla 5.5 camera, while all colors were collected with a single quad pass (405, 488, 561, 633 nm) cube to ensure images were sorted with a high...
speed (20 ms) FLI (Fingerlakes) filter wheel. Exposure times were 100 ms, and images were collected every 2 min.

Cell Compaction Assay—Cellular compaction of melanoma cells was assessed using a previously described method (15). In brief, parental and ACTN4 KD cells were harvested from monolayer culture using 0.25% trypsin/EDTA treatment and resuspended in growth medium. Neutralized collagen I solution (1 mg/ml) containing 10⁶ cells/ml cells was dispensed into 24-well tissue culture plates (0.5 ml/well). Collagen solutions were left to polymerize for 60 min at 37 °C in a humidified incubator with 5% CO₂ followed by overlaying 1 ml of growth medium per well on the matrix. The matrices were then gently released from the edge of each gel using a long and thin forceps and further incubated for 24 h. Compaction was determined by

**FIGURE 2. ACTN4 controls morphology of melanoma cell line WM1158 in a collagen I-dependent manner.** A, WM1158 cells were transfected with scrambled (WM1158) or ACTN4 shRNA (ACTN4 KD) and stable cell lines were selected. Expression levels of indicated proteins were detected using immunoblotting to demonstrate specific down-regulation. A representative of three independent experiments is shown. B, cells grown on coverslips with or without collagen I coating for 18 h were stained with rhodamine phalloidin demonstrating the mesenchymal morphology of the knockdown cells. Shown are representative images of three independent experiments. Scale bar, 30 μm. C, ratio of length to width of cells was calculated using Image J software. Fifty cells from different areas were randomly chosen. ***, p < 0.01 with respect to WM1158 and KD + ACTN4 on collagen. D, shown are representative images of WM1158 and ACTN4 KD cell cultured on collagen I-coated coverslips for 1 h prior to staining with rhodamine phalloidin. On the left, the square areas of the right panels are enlarged to highlight the filaments. Quantitative results represent the average number of RSF per cell. Twenty cells were analyzed for each sample. Scale bar, 20 μm. E, both WT ACTN4-eGFP and ACTN4 shRNA-resistant ACTN4-eGFP were transfected into WM1158 and ACTN4 KD cells. Images are representative of transfected cells randomly chosen from different area. This demonstrates that the re-expression construct is not down-regulated by the shRNA against the endogenous ACTN4. Scale bar, 60 μm. F, WM1158 ACTN4 KD cells expressing WT ACTN4-eGFP were cultured on collagen I-coated coverslips for 18 h and then were stained with rhodamine phalloidin to demonstrate restoration of a rounded morphology. Shown is the representative image of three independent experiments. Scale bar, 60 μm.
measuring the area of the top surface of collagen I gel using PhotoShop software.

**Cell Motility Assay**—Cell migration was performed according to a motility assay described previously (16). Briefly, cells were plated on 6-well tissue culture plate and grown to confluence in complete growth medium. Then, cells were scraped using a rubber policeman to create a wound area. A further culture for 24 h was performed to allow cells to migrate into this denuded area. Photographs were taken at 0 and 24 h, respectively. The relative distance migrated by the cells was analyzed using Photoshop software.

**Live Cell Migration Assay on Collagen I Gel**—Neutralized collagen I solution (2.5 mg/ml) was dispensed into 12-well tissue culture plates (0.5 ml/well) and left to polymerize for 60 min. Photographs were taken at 0 and 24 h, respectively. The relative distance migrated by the cells was analyzed using Photoshop software.

**FIGURE 3.** ACTN1 only partially restores the morphology of ACTN4 KD WM1158 cells. A, immunoblots of indicated proteins. Shown is a representative of three independent experiments. B, representative images of ACTN4 KD WM1158 cells stably expressing wild type ACTN4-eGFP or ACTN1-eGFP. Scale bar, 50 μm. C, ratio of length to width of green fluorescent cells. At least fifty cells for each sample were randomly chosen. *, p < 0.05 with respect to WM1158 and KD + ACTN4-eGFP. D, integrated fluorescent intensity of individual cell was measured using Photoshop software, and the ratio of length to width was calculated using ImageJ software. Numbers below the x axis stand for the cells numbered in B, E, ratio of length to width of ACTN4 KD cells that express low level of ACTN4 (ACTN4-eGFP(L)) or ACTN1 (ACTN1-eGFP(L)). At least fifty cells for each sample were randomly chosen. **, p < 0.01 with respect to WM1158 and KD + ACTN4-eGFP(L). E, relative levels of ACTN1 and ACTN4 protein (by immunoblotting and the calculation is based and normalized to the intensity of GFP immunoblotting band in A and mRNA (by quantitative RT-PCR) in WM1158 cells, with ACTN1 levels arbitrarily set at one. Note: the ACTN1 antibody also recognizes ACTN4. G, cells grown on coverslips coated with collagen I for 18 h were fixed and stained with rhodamine phalloidin. Shown are representative images of three independent experiments. Scale bar, 20 μm.
at 37 °C in a humidified incubator with 5% CO₂. To create a wound area, a sterile 1.5 × 8 mm coverslip was gently laid on the center of the surface of the polymerized collagen I matrix. Cells were then seeded (5 × 10⁵ cells/well) and left to attach for 2h at 37 °C in a humidified incubator with 5% CO₂. The coverslip was then carefully removed using an extra fine forceps without disturbing the matrix surface and cells. Unattached cells were completely washed away with growth medium. Cells migrating into the acellular area were imaged under Nikon live microscopy, and migration speed was calculated using Meta-morph software.

In Vitro Invasion Assay—Cells were seeded in 12-well tissue culture plate (5 × 10⁵ cells/well) coated with collagen I until most of cells attached and partially spread. Unattached cells were completely washed away by PBS followed by addition of 1 ml of 2.5 mg/ml neutralized collagen I solution containing 1× EMEM medium. Collagen solutions were left to polymerize for 60 min at 37 °C in a humidified incubator with 5% CO₂ followed by overlaying 2 ml of complete growth medium per well onto the matrix for further culture. After 3 days, the collagen I matrix was fixed with 4% formaldehyde diluted in PBS for 30 min at room temperature followed by three washes with PBS. Cells were then permeabilized with 0.2% Triton X-100 on ice for 10 min prior to staining with 1 g/ml DAPI for 16 h at 4 °C. After throughout washing, cells invaded into the collagen I matrix to

FIGURE 4. ACTN4 controls morphology of melanoma cell line IgR3. A, IgR3 cells were transfected with scrambled or ACTN4 shRNA and stable cell lines were selected. Expressions of indicated proteins were detected using immunoblotting. A representative of three independent experiments is shown. B, cells grown on coverslips coated with collagen I for 18 h were fixed and stained with rhodamine phalloidin. Shown are representative images of three independent experiments. Scale bar, 20 μm.

FIGURE 5. Integrin β1 antibody blocks mesenchymal morphology of WM1158 ACTN4 KD cells. A, cells were incubated with indicated concentration of monoclonal integrin β1 antibody at room temperature for 1 h and then were plated on collagen I-coated coverslips for a 1-h incubation. Cells were fixed and stained with rhodamine phalloidin. Shown are representative images of three independent experiments. Scale bar, 20 μm. B, ratio of length to width of cells was calculated using Image J software. Fifty cells from different areas were randomly chosen.
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a distance of 100 μm above the bottom of the matrix gel were imaged under confocal microscopy and then counted.

Statistical Analysis—To access statistical significance, we used Student’s t test for pair-wise comparisons and one-way ANOVA with Tukey post test (for multiple comparisons) using GraphPad Prism (GraphPad Software, San Diego, CA). Graphs show mean ± S.E.*, p < 0.05; **, p < 0.01.

RESULTS

Expression of ACTN4 but Not ACTN1 Differs in Different Melanoma Lines—ACTN4 has been suggested to be a biomarker in the diagnosis of human cancer (17, 18) and one that we have reported as up-regulated in invasive melanoma (8). As ACTN4 has been linked to cell motility (11, 17, 19, 20), we sought to determine whether this molecule drives invasion of melanomas. Expanding on earlier work, we find that ACTN4 levels are higher in melanoma cells compared with melanocytes, though absolute levels vary between cell lines (Fig. 1A). Interestingly, ACTN1, which bundles parallel actin filaments and is ubiquitous in non-muscle cells (21), is similar among all detected cell lines. Examining the role of ACTN4 in melanoma migration, we find that levels do not directly correlate with the rate of closure of an in vitro wound healing (cell scrape) assay performed in two dimensions on tissue culture plastic plates (Fig. 1B).

Down-regulation of ACTN4 Limits the Amoeboidal Morphological Transition—We earlier reported that knock-out of ACTN4 significantly inhibits the rate of migration in fibroblasts (11). Herein we queried whether this was similar in melanoma cell invasiveness by assessing this in the fastest melanoma line, WM1158. We stably transfected WM1158 with specific ACTN4 shRNA to accomplish >90% decrease in ACTN4 protein expression when compared with scrambled shRNA-transfected cells, whereas the protein expression of ACTN1 is not affected by ACTN4 shRNA (Fig. 2A), suggesting ACTN4 is specifically down-regulated by shRNA.

We had earlier found that melanoma migration in two dimensions is distinct from that during migration through three-dimensional matrices, such as the skin dermis, similar to other tumors (22, 23). Invasive melanomas switch between a mesenchymal and an amoeboidal morphology to penetrate such collagen I-rich matrices (4). As ACTN4 has been suggested to link the cytoskeleton to cell membrane (11, 24–26), we determined whether this morphologic change is affected by varying ACTN4 levels. Both wild type (scrambled shRNA) and ACTN4 knockdown (KD) cells were rounded when plated on tissue culture plastic, but upon plating on the top of a collagen I gel, only the ACTN4 KD cells assumed an elongated mesenchymal shape (Fig. 2, B and C). Interestingly, both radial stress fiber (RSF) and transverse arcs (TA) are noted in WM1158 cells while only TAs are visible in ACTN4 KD cells (Fig. 2D), suggesting the ACTN4 is required for RSF assembly. This finding is consistent with previous studies in which ACTN1 is required for the assembly of RSF in human osteosarcoma (U2OS) cells (27, 28). To exclude the possibility that this morphologic switch in WM1158 is due to a down-regulation of unknown protein by ACTN4 shRNA, we re-expressed full-length C-terminal eGFP-tagged ACTN4 in which 6 nucleotides (wobble bases) within its shRNA region are replaced with shRNA-resistant nucleotides (Fig. 2E). (All ACTN4-eGFP and ACTN4-eGFP-(1–890) expressed in ACTN4 KD cells refer to ACTN4 shRNA resistant). Re-expression of ACTN4 in ACTN4 KD cells restored the amoeboidal morphology (Fig. 2F), which suggests that the mesenchymal morphology of ACTN4 KD cells is specifically caused by the loss of ACTN4.

As ACTN4 and ACTN1, two non-muscle isoforms of α-actinin protein family are highly homologous and exhibit similar actin binding activity, we queried whether expression of ACTN1 could also rescue the morphology of ACTN4 KD WM1158 cells. This was tested even though the two non-muscle isoforms have been shown to present distinct function and localization in different cells (29, 30). As shown in Fig. 3B, the expression levels of exogenous ACTN1 and ACTN4 in ACTN4 KD WM1158 varied between cells though on average they were similar (Fig. 3A as determined by immunoblotting). Therefore, we quantified the ratio of length to width of all imaged cells in terms of the amount of either ACTN4 or ACTN1 expressed. Any detectable levels of ACTN4 rescued the amoeboidal morphology (Fig. 3, C, D, and E). Only higher levels of ACTN1 could revert the ACTN4 KD WM1158 cells back toward the amoeboidal morphology, whereas lower levels (ACTN1-eGFP(L)) remained mesenchymal (Fig. 3, B, D, and E). Surprisingly, we found that WM1158 cells do not express equal levels of ACTN1 and ACTN4. As shown in Fig. 3F, WM1158 cells express about twice the ACTN1 as ACTN4 determined at both protein and mRNA levels. That lower, physiological levels of ACTN4 but not low levels of ACTN1 restored the ACTN4 KD WM1158 morphology suggests that ACTN4 is the preferential effector in maintaining cell morphology of melanoma cells.

To ensure that these changes are not unique to the WM1158 line, a distinct invasive melanoma line, IgR3, was manipulated
FIGURE 7. ACTN4 knockdown reduces the number of focal adhesions. A, WM1158 Cells grown on collagen I-coated coverslips for 2 h were fixed, permeabilized, and stained for vinculin (all three cell variants) and rhodamine phalloidin (WM1158 and ACTN4 KD) or ACTN4-GFP being re-expressed. Images are representative of three independent experiments. Quantitation of the number of focal adhesions per cell and the relative length of focal adhesion are shown. Fifty cells were randomly chosen. **, p < 0.01 with respect to WM1158 and KD + WT ACTN4. Scale bar, 20 μm. B, images are taken using TIRF at indicated time points. C, number of focal adhesion plaques larger than 1 μm after 10- and 20-min observations were quantified. The percentage is calculated by dividing the FA number at time 0 by FA number at time 10 and 20, respectively. Ten cells for each cell line were chosen. Scale bar, 20 μm.
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and found to similarly present a polarized mesenchymal morphology in the absence of ACTN4 (Fig. 4, A and B). Consistent with this dependence on collagen-dependent morphology, the morphologic switch in ACTN4 KD WM1158 cells was blocked by antibodies to integrin-β1 (Fig. 5). These findings show that melanoma cell morphology in the presence of collagen fibrils is dependent on ACTN4.

**ACTN4 Knockdown Results in Greater Transcellular Contractility and Morphology in Three-dimensional Collagen Gels**—The elongated mesenchymal morphology presents transcellular stress fibers as noted upon actin-phalloidin staining (Fig. 2). This would suggest that the stresses are transmitted to the matrix, rather than in squeezing cells through small openings in complex matrices (5). To determine whether mesenchymal ACTN4 KD WM1158 cells have increased cell contractility, we performed a collagen I gel compaction assay. As shown in Fig. 6, ACTN4 knockdown significantly increases cell compaction of WM1158 cells compared with scrambled shRNA-expressing cells. Re-expression of ACTN4-eGFP in ACTN4 KD cells limits the gel compaction to control levels. In line with the effect of exogenous ACTN1 on the rescue of morphology of ACTN4 KD WM1158 cells (Fig. 3E), ACTN1-eGFP(L) expressing KD cells fail to restore the gel compaction although KD cells in which both high and low level of ACTN1 are expressed partially limits the gel compaction. In contrast, similarly low levels of ACTN4 (ACTN4-eGFP(L)) fully restored the compaction to the level of WM1158 cells suggesting that ACTN4 plays a crucial role in maintaining normal cell contractility. ACTN4 has been suggested to be involved in the formation of focal adhesion by bridging to membrane from the actin filaments. Thus, we investigated if the morphological changes of ACTN4 KD cells are related to altered focal adhesions using immunofluorescence of vinculin. As shown in Fig. 7A, WM1158 cells present focal adhesions across the entire ventral contact surface. In contrast, ACTN4 KD cells only form few and larger focal adhesions behind the leading cell edge and along the cell periphery with these contacts generally absent in the cell body. This finding concurs with previous reports in fibroblasts and in osteosarcoma cells (11, 28). The pan-cellular adhesions were fully restored by re-expression of ACTN4-eGFP. To further determine if knockdown of ACTN4 affects the turnover of focal adhesion, total internal reflection fluorescence microscopy was performed on WM1158 and ACTN4 KD cells in which vinculin tagged with GFP at its C terminus was transiently expressed. As shown in Fig. 7B, the lifetime of focal adhesion plaque in ACTN4 KD cells is significant longer than in WM1158. These data suggest that ACTN4 is required in maintaining normal cell focal adhesion dynamics in melanoma cells.

The above data show that ACTN4 knockdown affects cell morphology as noted in two dimensions, but its effect on morphology in three dimensions is to be determined. Within a three-dimensional artificial matrix we also note more amoeboid morphology with intact ACTN4 (Fig. 8). Again, the ACTN4 KD cells appear elongated and polarized comparing to scrambled shRNA-expressing cells. Re-expression of ACTN4-eGFP fully rescues morphology of ACTN4 KD cells suggesting that ACTN4 plays an important role in maintaining cell morphology in the three-dimensional environment.

**Figure 8.** ACTN4 knockdown elongates cells in a three-dimensional collagen I matrix. Cells grown in a three-dimensional collagen I matrix for 24 h were fixed and imaged under Olympus fluorescent microscopy. The ratio of cell length to width was calculated using ImageJ software. At least 50 cells that were in focus were randomly chosen for each cell line. The maximum length and width in the plane of the imaging were measured. Images are representative of three independent experiments. Cells marked with stars represent cells assessed. The box-and-whisker plots are mean and 25–75% for the box, 10–90% for the whiskers. Scale bar, 50 μm. *, p < 0.05 with respect to WM1158 + eGFP and KD + WT ACTN4-eGFP.

The C Tail of ACTN4 Is Essential for Its Function in Cell Morphology and Contractility—Our recent studies highlighted that the C tail of ACTN4 is required for interacting with the N terminus of the antiparallel ACTN4 molecule during the formation of the homodimer (32) that is essential for its actin binding activity (13). To determine if this tail is also important for the function of ACTN4 in the radial filaments in melanoma cells, we expressed ACTN4-eGFP-(1–890), a truncated ACTN4 mutant that lacks the C tail in ACTN4 KD WM1158 cells to determine whether the compaction and the amoeboidal morphology on a two-dimensional collagen I gel as well as in the three-dimensional environment is restored. Expression of ACTN4-eGFP-(1–890) fails to restore the amoeboidal morphology (Fig. 9A), limit the compaction (Fig. 9B), or reset the ratio of length to width in the three-dimensional collagen I gel back to levels noted with full-length ACTN4 (Fig. 9C). As ACTN4-(1–890) has been shown to be more sensitive to the cleavage by m-calpain (13), we questioned whether the failure of ACTN4-(1–890) to restore the morphology of ACTN4 KD cells was due to its degradation in cells. However, by immunoblotting we found that the expression level of ACTN4-(1–890) in ACTN4 KD cells was similar to WT ACTN4 (Fig. 9D). Together, these data suggest that the C tail of ACTN4, possibly via actin binding capacity, is essential for its normal function in melanoma cells.
ACNT4 Knockdown Enhances the Migration of WM1158 on the Two-dimensional Collagen I Gel but Impairs Invasiveness in the Three-dimensional Collagen I Gel—Previous studies from ourselves and others suggested that ACTN4 plays an important role in cell migration of fibroblasts and several tumor cells including colorectal cancer, breast cancer, ovarian cancer, and lung carcinoma cells (9, 11, 33–35). To investigate whether knockdown of ACTN4 also affects the migration of melanoma cells, we performed live cell migration assay on WM1158 and ACTN4 KD cells cultured on a collagen I gel for 12 h. To our surprise, we found that ACTN4 KD WM1158 cells migrated faster than scrambled shRNA-transfected cells (Fig. 10A) although cells with or without ACTN4 migrated at similar speeds on tissue culture plate without collagen I coating (Fig. 10B). This phenomenon is the opposite to our previous finding in which knock-out of ACTN4 significantly inhibited the migration of fibroblasts and is probably due to the enhanced cellular contractility and mesenchymal morphology. This result also suggests that the enhanced migration of ACTN4 KD cells is collagen I-dependent.

During invasion and dissemination, tumor cells need to alter their shape within their microenvironment so as to fit through
pores in the matrix (22). As the above data showed that knockdown of ACTN4 causes an amoeboidal-mesenchymal transition, we sought to determine if this transition affects the invasiveness of WM1158 cells in a three-dimensional collagen I gel that mimics an in vivo cellular microenvironment. As shown in Fig. 11, the invasiveness of ACTN4 KD cells is significantly impaired compared to control cells suggesting that the ACTN4-dependent amoeboidal morphology is essential for maintaining the invasion of WM1158 cells.

DISCUSSION

ACTN4, a member of the family of actin-crosslinking proteins, has been shown to play an important role in the regulation of the cytoskeleton and has been linked to tumor invasion and metastasis (9, 33−35). ACTN4 expression levels have been reported to increase in several human cancers including breast carcinoma, colorectal cancer, non-small cell lung cancer, pancreatic cancer, and ovarian cancer (9, 17, 33, 36, 37). Consistent with these earlier works, we have reported that ACTN4 is increased in melanoma (8) and in this study that melanoma cell lines including RGP, VGP, and MGP all express high levels of ACTN4 (but not ACTN1) when compared with melanocytes.

We interpreted this as indicating that ACTN4 may contribute to the dissemination of melanoma cells. Melanocytes-cum-melanoma cells need to acquire the ability to move among the epidermal cells and then invade through a dense collagen I-rich matrix as the transformed cells progress through radial to vertical growth phases. For metastatic dissemination, invasive melanoma cells also need to alter their contacts with ECM and surrounding stromal cells in a microenvironment and change their cytoskeletal organization (3) so as to squeeze through small apertures in the dermis by amoeboid migration (5, 6). Therefore, deciphering how melanocytes accomplish this morphological transition and the mechanisms of invasion are the key to understanding why malignant melanoma is such a life-threatening disease.

Our findings revealed that ACTN4 was required to maintain a compacted and amoeboidal shape in two-dimensional and three-dimensional collagen I-rich environments. The invasive ability of the mesenchymal ACTN4 KD WM1158 cells through the three-dimensional collagen I matrix was significantly inhibited. The amoeboidal to mesenchymal transition coincided with the loss of RSF and would be consistent with the putative role of ACTN4 in linking the cytoskeleton to the membrane (25, 26). This may be why down-regulation of ACTN4 but not ACTN1 impairs the migration of astrocytoma cells (20).
The reason why ACTN4 KD cells expressing low level of ACTN1 present mesenchymal morphology could be due to the co-localization of ACTN1 with cortical actin (Fig. 3G), which may restrict the reorganization or alignment of actin stress fiber and thus the formation of cell polarity. Recently, ACTN1-ACTN4 heterodimers have been suggested to be the most abundant form in many cell lines and may have different properties and interacting proteins compared with homodimerized ACTN1-ACTN1 and ACTN4-ACTN4 (31). Thus, the unavailability of heterodimerized ACTN1-ACTN4 in ACTN4 KD cells may also limit the function of ACTN1 in the maintenance of melanoma cell morphology.

Dimerization of ACTN4 involves the C terminus of ACTN4 via the interaction between the N terminus of one molecule and the C terminus of its partner (32) with this sequence also being required for actin binding activity of ACTN4 (13). The C terminus of ACTN4 has also been shown to be required for the formation of a protein complex consisting of actin, ACTN4, and JRAB/MICAL-L2 that exists at the membrane in MTD-1A cells with tight junctions (25). In A431 cells, ACTN4 colocalizes with NK-ρ8 along actin stress fibers and in membrane lamellae (26). All the above evidence suggests that ACTN4 may directly link cytoskeleton to the cell membrane probably via an integrin-dependent manner (39). Indeed, in this study, our findings suggest that the mesenchymal morphology of ACTN4 KD cells is mediated by β1-integrin due to the collagen I dependence of the elongated morphology. Loss of ACTN4 appears to limit actin cytoskeleton interaction with the cell membrane resulting in an extended cell. Although ACTN1 is abundant in ACTN4-eGFP-(1–890)-expressing ACTN4 KD cells, and ACTN4 and ACTN1 are high homology, ACTN1 lacks the flexible N-terminal domain that exists in ACTN4 and has been suggested to be required for the homodimerization.3 In short, the findings suggest different, if overlapping functions of ACTN4 and ACTN1 in regulating cell shape.

Of interest is that these changes in morphology appear to be dependent on the presence of the main matrix component of the dermis, collagen I. As a consequence of the amoeboidal to mesenchymal transition, ACTN4 KD WM1158 cells migrate faster on the two-dimensional collagen I matrix than WM1158 cells (expressing the control scrambled shRNA). The enhanced motility of ACTN4 KD cells on collagen I matrix is probably due to: 1) the elongated/mesenchymal morphology, which results in an enhanced cell contractility. Our live cell spreading data (not shown) reveal that WM1158 cells spread symmetrically with many highly dynamic protrusions at the edges of cells. In contrast, ACTN4 KD cells produce stable and elongated protrusion, which results in a mesenchymal shape. 2) Impaired rate of turnover of focal adhesion in ACTN4 KD cells, which is probably required for persistent migration. On the other hand, the decreased number of focal adhesion plaques may also be essential for the rapid morphology change, which enhances the cell movement. However, this ability to move rapidly across a surface may not translate into invasiveness through a tight matrix that may not have cleavage planes or pores sufficiently large to allow for cells to penetrate without significant deformation (40, 41). The impaired invasive ability of ACTN4 KD cells in the three-dimensional collagen I matrix implies that the amoeboidal morphology is required for efficient invasiveness of WM1158. Because of the loss of ACTN4, ACTN4 KD WM1158 cells are not capable of restoring their morphology to amoeboid when they are embedded within the collagen I matrix (Fig. 8).

In summary, these findings place ACTN4 as a key element for melanoma cell invasion through the dermis by controlling the cell shape required to penetrate this dense matrix. This also strongly supports the contention that ACTN4 differs from ACTN1 in integrating the membrane with the actin cytoskeleton possibly by bridging between actin and phosphoinositides on the membrane (13, 17, 38) rather than simply bundling actin filaments. Further studies are needed to decipher the molecular mechanisms by which the expression of ACTN4 but not ACTN1 is elevated in melanoma and the increase of ACTN4 expression regulates the metastasis and invasiveness. An understanding of the mechanisms may provide insights in considering ACTN4 as a therapeutic target for melanoma patients.

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