Original Article
Fascin-1 knock-down of human glioma cells reduces their microvilli/filopodia while improving their susceptibility to lymphocyte-mediated cytotoxicity

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Abstract: Cancer cells derived from Glioblastoma multiforme possess membranous protrusions allowing these cells to infiltrate surrounding tissue, while resisting lymphocyte cytotoxicity. Microvilli and filopodia are supported by actin filaments cross-linked by fascin. Fascin-1 was genetically silenced within human U251 glioma cells; these knock-down glioma cells lost their microvilli/filopodia. The doubling time of these fascin-1 knock-down cells was doubled that of shRNA control U251 cells. Fascin-1 knock-down cells lost their transmigratory ability responding to interleukin-6 or insulin-like growth factor-1. Fascin-1 silenced U251 cells were more easily killed by cytolytic lymphocytes. Fascin-1 knock-down provides unique opportunities to augment glioma immunotherapy by simultaneously targeting several key glioma functions: like cell transmigration, cell division and resisting immune responses.

Keywords: Fascin-1, shRNA, glioblastoma, microvilli, filopodia, CTL

Introduction

Glioblastoma multiforme (GBM) are aggressive, lethal brain tumors. This cancer is usually fatal within five years (2014 Central Brain Tumor Registry; http://www.cbtrus.org/) due to its very invasive nature. Even with the use of temozolomide, an alkylating agent that targets the DNA of these brain cancers, GBM patient survival is still limited. Immunotherapy is an attractive approach for the treatment of GBM, for it allows the host's activated lymphocytes a chance to destroy these cancerous cells [1, 2]. These activated immune cells should produce more durable responses by preventing tumor recurrence. Dendritic cell (DC)-based tumor vaccines improved the survival of patients with the mesenchymal sub-type of GBM [3]. Myeloid-derived suppressor cells, T regulatory cells, immunosuppressive cytokines/factors, and im-

muno-modulatory cell surface molecules all limit the host's immune response via inhibited antigen presentation or diminished lymphocyte effector function [4-16].

We previously reported that glioma cells resisted lymphocyte-mediated cytotoxicity *in vitro* via a complex surface topography [17]. The glioma cell's surface possesses numerous microvilli and microspikes that physically prevent cytolytic lymphocytes from killing glioma cells, just as a sea urchin avoids predators by using its spines as a physical defense. Clinical GBM specimens also display microvilli and filopodia and can contain mitochondria, suggesting these structures actively search for weak spots between normal brain tissue that make it easy for the tumor migration once fertile areas for invasion are detected [18, 19]. Filopodia are the long cylindrical protrusions coming from the

cell membrane that extended outward from the cell body. These extended protrusions express integrin and growth factor receptors, which allow the glioma to search for weak spots and initiate the invasion process [19-25]. Micro-projections also display various matrix proteases (MT1-MMP/MMP14, MMP2 and MMP9) which help digest the surrounding matrix and allow macrophages, myoblasts and breast cancers to transmigrate through enlarged openings between cells or into an extracellular matrix [26-30]. Glioma cells also express these same matrix metalloproteases, including the membrane-bound MT-MMP/MMP14 [31-34]. Thus, these structures are actively involved in very dynamic and complex processes.

Filopodia and microvilli are internally supported by cross-linked polymerized actin (filamentous actin). Upon a brief five-minute treatment with cytochalasin B, the microvilli rapidly regressed [17]. Likewise, when adherent glioma cells detach from their substrates, these rounded-up non-adherent cells became optimal targets for various human effector lymphocytes since these target cells lost their defensive microvilli. Consequently, the current cytolytic assays may over-estimate the amount of cytolytic effector function that occurs within the *in situ* environment.

Fascin was initially discovered and cloned from sea urchin oocytes [35]. Fascin is an important scaffolding protein that strengthens this actin-based cytoskeleton by cross linking the parallel actin filaments into tightly compacted rope-like strands [36-38]. Two actin binding regions reside within the third and fourth domains of the globular fascin-1 molecule allow two different actin filaments to be cross-linked into stronger bundles. These interlocked strands increase the tensile strength and stiffness of these membrane protrusions. Filopodia exerts tension upon the substrate and can elicit movement of the cell in the direction of chemo-attractants that the receptors on the filopodia detect [23, 25, 27-30].

There are three members of the fascin family (FSCN-1, 2 and 3); each protein has a restricted tissue expression within normal tissue [23, 27, 31]. Fascin-1 is primarily expressed within the mesenchymal and nervous tissue, like neurons, glial cells and vascular endothelial cells. Fascin-2 is expressed within retinal photoreceptor/sensitive cells; while Fascin-3 is found

within the testes. Most work has studied fascin-1. Fascin-1 is highly expressed with various human cancers, including astrocytic-derived tumors [20, 30, 39-41], and its expression increases with the cancer's grade status and correlates with a poorer prognosis in other cancer types, too [39-50].

Using either transient siRNA or stable shRNA constructs, fascin-1 was genetically silenced within human U251 glioma cells. The siRNA achieved a better knock-down efficacy with a 90% knock-down, while the stable transduced shRNA-fascin-1 cells were inhibited by 50-70%. Our best selected fascin-1 knock-down clone possessed a 70% inhibition. In both silencing systems, the U251 cells lost the majority of their microvilli/filopodia and assumed a more rounded squamous appearance. The shRNA driven fascin-1 knock-down cloned U251 cells had a slower *in vitro* growth rate and became less invasive as demonstrated by its inhibited ability to penetrate through 8 micron pores in response to interleukin-6 (IL-6) or insulin-like growth factor-1 (IGF-1), known chemo-attractants for human glioma cells. The expression of HLA-A2 and the tumor associated antigen, the glioma Big Potassium (gBK) ion channel was unaltered by stable fascin-1 knock-down. Consequently, the loss of these membrane protrusions allowed these glioma cells to be more susceptible to lymphocyte-mediated killing within 6 hour cytotoxicity assays. Therefore, fascin-1 knock-down improves lymphocyte cytolytic activity, while simultaneously slowing glioma growth and inhibiting their ability to migrate *in vitro.* Thus, strategies which inhibit fascin-1 expression may enhance immunotherapy against various GBM by three different mechanisms: lowered growth rates, less invasiveness and improved cytolytic potential of effector lymphocytes.

Materials and methods

Cell lines and cell culture

The human U251 glioma cell line has been previously described [51]. These cells were grown in DMEM media supplemented with 5% fetal bovine serum and standard antibiotics.

Fascin-1 knock-down

The human glioma cells were genetically knocked-down for fascin-1 by using either short

interfering RNA (siRNA) (sc-35359) or short hairpin (shRNA) (sc-35359-sh or control shRNA sc-108060) constructs from Santa Cruz Biotechnology (Dallas, TX) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) or the Santa Cruz transfection reagent (sc-29528). The transfections were performed according to the manufacturer's protocols. The cells were seeded in 6-well plate and grown to 50-70% confluence. The fascin-1 or control constructs were mixed with the transfection reagent. After 30-minute incubations at room temperature, the cells were washed with the complete medium and incubated.

After 1-3 days after siRNA transfection, the cells were examined. For the shRNA transfected cells, the cells were incubated at 37 C in the CO_2 incubator; the cells were cultured in medium containing puromycin. After puromycin selection, single cells were isolated by limiting dilution to screen the cells with the lowest fascin-1 expression. The best known-down clone was then used, which had a 70% knock down as detected by both molecular and protein detection analyses.

Quantitative real time-reverse transcriptase polymerase chain reaction (qRT-PCR)

The following primers were synthesized by Operon Biotech (Germantown, MD): fascin-1 forward: 5'-ACGGCAACGTGACCTGCGAG-3'; fascin-1 reverse: 5'-GACTGCAGCGACCAG CGACC-3'. 18S RNA Forward and 18S reverse were 5'-CAGGATTGACAGATTGATAGC TCT-3' and Reverse-5'-GAGTCTCGTTCGT TATCGGAATTA-3', respectively.

Total RNA was isolated from the cells using the Qiagen' s RNeasy plus mini kit and one microgram RNA were subjected to cDNA synthesis using the cDNA synthesis kit from Bio-Rad. The cDNA was then used for real-time PCR analysis using C1000 Thermal Cycler (Bio-Rad, Hercules, CA). The thermal profile was 95°C for 15 minutes, followed by 40 cycles of 95°C for 15 seconds and 58°C for 30 seconds, finally holding at 4°C.

Samples were run in triplicate, and a reaction without cDNA was used to establish baseline fluorescence levels with 18S RNA. The fluorescent signal was plotted versus cycle number, and the threshold cycle (C_T) was determined at the cycle number where an increase above background fluorescence could be reliably detected. Each PCR run also included non-template controls containing all reagents except cDNA. After cycling, a melting curve was produced by slow denaturation of the PCR end products to validate the specificity of amplification. The relative quantification of expression of the gene was determined by $2^{-\Delta CT}$ as described earlier [51-53].

Intracellular flow cytometry

Exponentially growing glioma cells (1×10^7 cells) at 50-70% confluence were first trypsinized and then fixed in FCM™ Fixation Buffer (Santa Cruz Biotechnology) on ice for 30 minutes. The cells were washed twice in ice-cold PBS. The cells were permeabilized with Santa Cruz Biotech's Permeabilization buffer for 15 minutes on ice. The cells were washed again with FCM Wash buffer twice. The re-suspended cells were divided into 10^6 cell aliquots and then incubated with the primary monoclonal IgG1 antibody anti-fascin-1 antibody (Santa Cruz Biotechnology, sc-21743) for 1 hr. The cells were washed twice and the secondary antibody-conjugated with fluorescein isothiocyanate (FITC) (Vector Labs, Burlingame, CA) was incubated on ice for another hour. After washing the cells twice, ten thousand cells were analyzed with a Becton Dickinson FACS Calibur flow cytometer (Mountain View, CA). We used the Kolmogorov-Smirnov statistic's test, to show significant differences in flow cytometry expressions (P<0.05) between the various cell lines [51-53].

Immunofluorescent microscopy

Glioma cells were incubated upon coverslips that were collagen coated. The cells were fixed with 2% paraformaldehyde and permeabilized with ice-cold methanol/acetone. Tissues containing the glioma were stained with a 1:50 dilution of the anti-fascin-1 antibody. The tissue was washed and then incubated one hour with a 1:200 dilution of the secondary antibody-FITC conjugated (Vector Labs, Burlingame, CA). Tissue samples were washed and mounted with Prolong Gold anti-fade reagent (Molecular Probes, Eugene, OR). Samples were imaged and analyzed using the Eclipse E800 Microscope.

For some experiments intact non-fixed cells were stained with fluorescent wheat germ agglutinin which stains extracellular glycoproteins to better outline the cell-surface topography [17].

Atomic force microscopy

Five thousand tumor cells were plated onto sterile gelatin coated 12 mm coverslips cultured within 24-well plates. The cells were allowed to adhere to the matrix for 16-18 hrs. Cells were fixed in 1% glutaldehyde here for 30 minutes at 37°C. The fixative was replaced by PBS and transported to the AFM facility at room temperature. At the AFM facility, the samples were mounted onto a J-piezoscanner of an atomic force microscope (Nanoscope III, Digital Instruments, Santa Barbara, CA) equipped with a fluid cell. The cantilevers with oxide sharpened silicon nitride tips were 100 μm long. The images were collected in tapping height mode at frequencies of 9.2 kHz with a scanning frequency of 1 Hz [36]. We used this technique in our previous paper [17].

Doubling times

Five thousand glioma cells were placed in each well of the 24 well plate in triplicate replicates. The doubling time of each cell was calculated by the equation: Number of divisions = (log 10 N1 - log 10 N0)/log 2 [54]. N0 is the number of cells found at Day 0 and N1 is the number obtained on a later day. After days 1, 2 and 3, the cells were trypsinized and counted. Population-doubling time hour is calculated by dividing 24, 48 or 72 hours by the number of divisions that were calculated. The doubling times of cells from the 3 days were then averaged, and the standard deviation was calculated.

Transmigration studies

The Chemo-Tx disposable chemotaxis system with 8 μm pores from NeuroProbe (Gaithersburg, MD) was used according to the manufacturer's directions. Twenty thousand U251 cells in PBS were loaded in the top chamber with either non-transfected U251 or with fascin-1 knock-down U251 cells. The bottom chamber contained either 20% fetal bovine serum, or 100 ng/ml of either recombinant interleukin-6 (IL-6) or insulin-like growth factor (IGF-1) to induce glioma transmigration [55, 56]. The human cytokines were purchased from Peprotech (Rocky Hill, NJ). The number of cells transmigrating from the upper to lower chambers after an overnight incubation (five hours) was determined in sextuplicate replicates. Fluorescent cells at the bottom of the well in the 96 well

plates were determined by the NOVOStar fluorescence plate reader BMG Labtech Inc. (Cary, NC). A student's t-test was then used to verify significant differences (P<0.05) occurred between the experimental groupings from the control [51-53].

Cytolytic lymphocytes

Lymphokine Activated Killer Cells (LAK): After informed consent, forms were signed, human peripheral blood mononuclear cells were collected at Hoag Hospital, and the cells were isolated after a centrifugation using Ficoll-Hypaque. Aliquots were frozen until needed. Frozen PBMCs were thawed in a 37°C water bath, resuspended in warm AIM-V (Gibco, Grand Island, NY) with antibiotics and centrifuged at 2000 rpm for 5 minutes at room temperature. The cell pellet was re-suspended at 3×10^6 cells per ml in AIM-V with antibiotics and supplemented in 3,000 units/ml of recombinant human interleukin-2 (rhIL-2) (Chiron, Emeryville, CA). The cells were incubated in the humidified air at 37°C in 5% CO_2 for 7 days. On day 5, the cells were counted by Trypan-blue dye exclusion assay and re-suspended in 80% fresh AIM-V at 3×10^6 cells per ml plus 3,000 units/ml rhIL-2. The cell cultures exhibited typical clustering and phenotype of IL-2 activated lymphocytes as well as cytolytic activity against natural killer sensitive cell lines like K562 [17].

Peripheral-blood mononuclear cells were collected from an HLA-A2+ donors after informed consent forms were signed at UCLA. The DC was pulsed with $gBK_{704-712}$: gBK1-SLWRLESKG or $gBK_{722-730}$: gBK2- GQQTFSVKV peptides as previously described [51-53]. The mononuclear cells were ficolled and then incubated in human GM-CSF (1,000 units/ml) and IL-4 (1,000 units/ml) in AimV (Gibco/Invitrogen, San Diego, CA) at 37°C. The recombinant cytokines were obtained from CellGenix USA (Antioch, Illinois). The autologous T lymphocytes were then incubated with the antigen pulsed DC and then expanded with 10 ng/ml IL-2 for 1 week and then re-stimulated with fresh antigen pulsed DC and then fed every 3-4 days with IL-2, IL7 and IL-15 for the next couple weeks.

Cytotoxicity assay

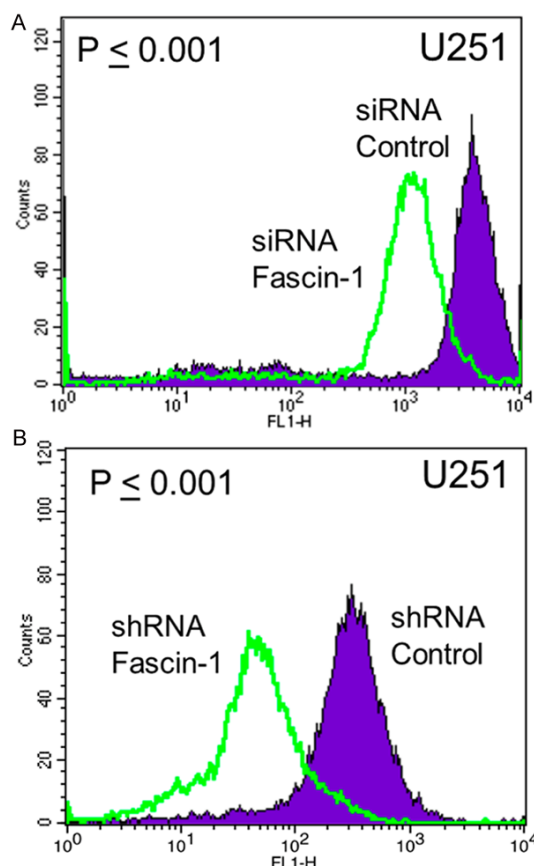The U251 target cells were plated (10,000 cells/well) into the wells of 96 well flat bottom

Figure 1. Intracellular flow cytometry of fascin-1 protein level knocked down by siRNA and shRNA. (A) The U251 cells, siRNA control (shaded area) or siRNA fascin-1 knockdown (light line) and (B), shRNA control (shaded area) or initial non-cloned shRNA fascin-1 knockdown U251 cell (light line) were examined by intracellular flow cytometry using the monoclonal antibody towards fascin-1. Ten thousand cells were analyzed. By the Kolmogorov-Smirnov statistics' test, the fluorescent readings were determined to be statistically different (P<0.001) from each other.

plates over night to allow them to adhere. A separate aliquot of U251 cells was detached by incubating the cells in a versene buffer for 20 minutes. The detached cells and the attached U251 cells were labeled with Cr^{51} (MP Bio-medicals, Indianapolis, IN) as we have previously done [51]. After 3 washes, the cells were incubated with the effector cells at either 50:1, 25:1 or 12:1 effector: target cell ratios in quadruplicate cultures. Triton X-100 (1%) treated cells served as the maximum release. U251 cells incubated without any effector cells served as the spontaneous release values. After 6 hours, the supernatants was collected and analyzed on the scintillation co-

unter. Percent specific release was calculated using theequation: % Specific Release =

$$\frac{CPM\left(experiment\right) - CPM\left(spontaneous\ release\right)}{CPM\left(maxmum\right) - CPM\left(spontaneous\ release\right)} \times 100$$

Data from the cytotoxicity assays were analyzed using a student's t-test. Values were considered significantly different at the P<0.05 levels.

Results

Fascin-1 knock-down alters the glioma morphology and loses membranous projections

U251 and other glioma cells display a complex cell surface topography with numerous microvilli, microridges, microspikes and filopodia both *in vitro* and *in vivo* [17-19]. When U251 glioma cells were treated with cytochalasin B to disrupt the actin cytoskeleton or these adherent cells were physically detached from their substrates, the microvilli disappeared within 5 minutes. These membrane projections are composed of polymerized actin filaments held together by a cross-linking protein called fascin-1. Previous studies associated the presence of fascin-1 with glioma morphology and function [20, 39, 40].

Our preliminary work was done by using siRNA fascin-1 knock-down techniques. With the siRNA knock-down of the fascin-1 gene in U251 cells, we obtained about 90% knock-down as determined by qRT-PCR. This knock-down was then verified at the protein level by using a monoclonal antibody directed towards fascin-1 and assessed by doing intracellular flow cytometry within three days (**Figure 1**). The morphology of glioma cells changed in response to this genetic silencing.

Representative picture of the morphology of the U251 cells after two days after siRNA transfection is displayed in **Figure 2**. Non-transfected U251 cells changed from a stretched fibroblastic-like morphology (Top Row of panels) to a more rounded-squamous looking one, (Second Row of panels). The non-transfected or siRNA control (not shown) U251 cells displayed a nice well-organized cytoskeleton with many strands of polymerized F-actin (green) associated with fascin-1 proteins (red). The yellow shows where the co-localization of the F-actin and fascin-1 occurs. The long filopodia and the leading edges (lammelipodia) are both observed to be
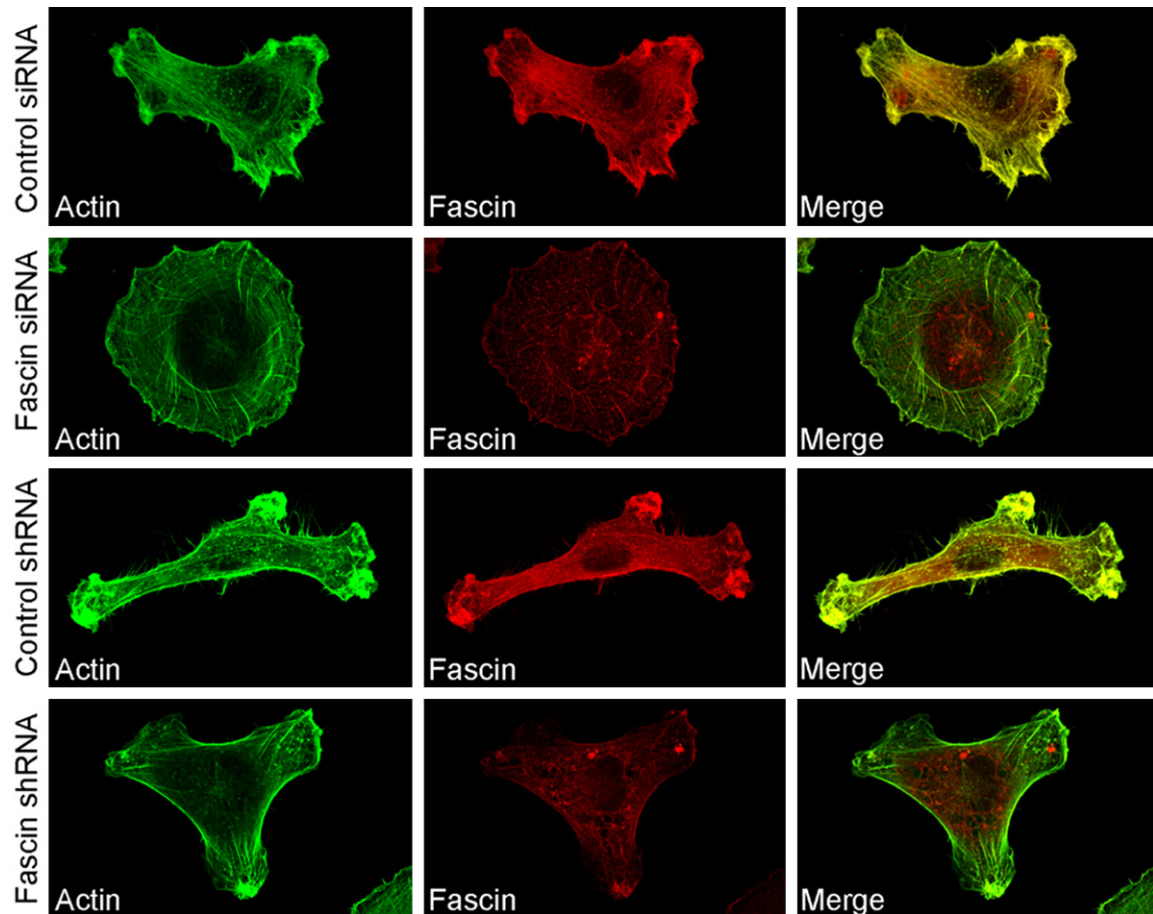
Figure 2. The morphology of U251 cells before and two days after fascin-1 siRNA knock-down. U251 cells (untreated Top Row) and siRNA-fascin-1 knock-down (2 days after transfection, Second Row), shRNA control (third row) or shRNA fascin-1 (bottom row) were allowed to adhere overnight onto a slide. The cells were fixed, permeabilized and then stained with the anti-fascin-1 antibody (red) and anti-F-actin antibody (green). Magnification is 100 X.

dual positive for both F-actin and fascin. In contrast, the fascin-1 siRNA treated U251 cells lack this internal cytoskeleton as the control cells displayed. The only fluorescent staining appeared along the various edges of the cells. Only a few microspikes/microvilli are seen. These membrane structures are dramatically shortened when compared to the filopodia on the normal cells.

Using the shRNA constructs, we initially observed about a 50% knock-down of the early bulk transduced cells. After cloning these transfected cells, we obtained stable cell clones that possessed between 50-70% knock-down as determined by qRT-PCR. The best knock-down (70%) clone was then used for all future work. The protein content of fascin-1 was confirmed to be lost by intracellular flow cytometry (data not shown). The stable fascin-1 knock-down

also appeared to have a rounded morphology lacking membrane protrusions (**Figure 2**, bottom row of panels). As a result of stable fascin-1 knock-down, there was also a reorganization of the actin cytoskeleton, so that the cells assumed a rounded phenotype with very few microspikes. The untreated cells showed membrane protrusions and polarity. These two characteristics were largely absent in the fascin-1 knock-down cells.

Atomic force microscopy (AFM) confirms there is a loss of microvilli/microspikes on the fascin-1 knock-down U251 cells

We showed that human U251 cells possess multiple microvilli/micro-spikes by AFM, which images the topography of cell surfaces [17]. These genetic knock-down cells were also evaluated by AFM to confirm that their cell-surface
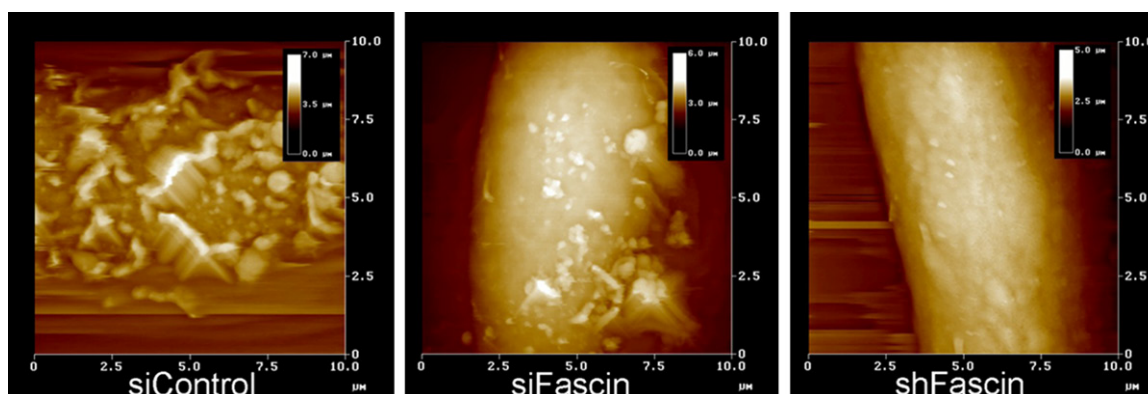
Figure 3. Atomic force microscopy reveals that fascin-1 knock-down results in loss of microvilli/microspikes. The U251, siRNA control, siRNA fascin and shRNA fascin, cells were allowed to adhere overnight on sterile cover-slips. The cells were fixed in 2% paraformaldehyde and imaged by atomic force microscopy. Left Panel: the siRNA controls U251 cells with multiple microspikes/microvilli. While Middle Panels (siRNA fascin treated) and Right Panel (shRNA fascin treated) displays a smoothed cell-surface topography.
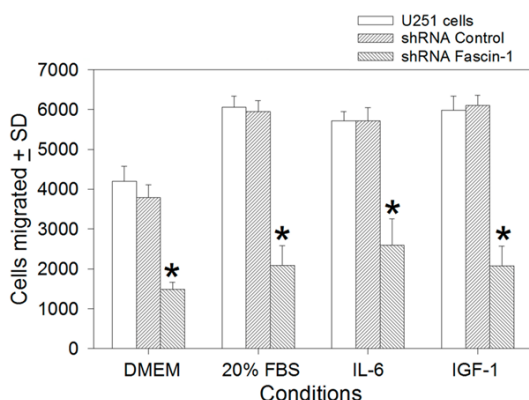

Figure 4. Fascin-1 knock-down cells loss of transmigration ability by the U251. Ten thousand cells (U251 untreated, shRNA-control U251 and fascin-1 knock-down U251 cells) were placed in the top chamber of the NeuroProbe Invasive unit. The chemo-attractants (20% FBS, 100 ng/ml rh-IL-6 or rhIGF-1) were placed in their respective bottom chambers. After 5 hours, the number of invading cells that penetrated through the 8 μm pore was measured and then calculated for the number of cells. Data is expressed as the mean of sextuplicate cultures ± standard deviation (SD). The asterisk denotes a significant difference by the student's t test (P<0.05) of the fascin-1 knock cells compared to both the untreated U251 or shRNA control U251 cells.

Figure 5. LAK cells kill siRNA knock-down cells better than control siRNA treated cells. Human lymphokine activated killer cells were tested against adherent U251 non-treated, shRNA control U251 and U251 fascin-1 knock-down cells. The non-adherent U251 cells which lack microvilli served as the maximum killing. Data is expressed as the mean of specific killing of quadruplicate cultures ± SD. The asterisk denotes a significant difference by the student's t test (P<0.05) from the shRNA-fascin-1 knock-down cells compared to the adherent U251 or shRNA-control U251 cells.

topography was changed. **Figure 3**, Left Panel shows siRNA control-treated U251 cell that possess many membrane protrusions classified as microspikes, microvilli or microridges. The Middle Panel shows the siRNA fascin-1 treated U251 cells are relatively smooth and relatively devoid of microvilli and micro-spikes.
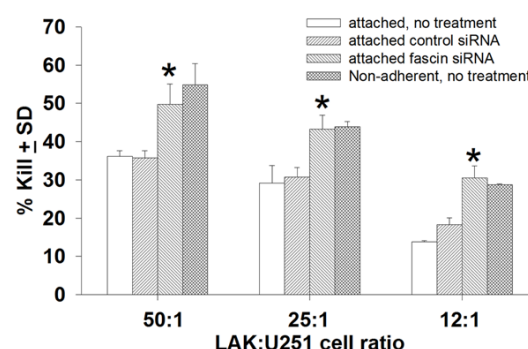
We also saw identical results using our stable shRNA knock-down U251 cells (Right Panel). Thus, both silencing techniques cause a dramatic reduction in membrane protrusions.

Fascin-1 knock-down slows U251 growth

One empirical finding we quickly noted was that the shRNA-fascin-1 knock-down clone grew at a slower rate than the shRNA control cells. The growth kinetics of the U251 shRNA control and fascin-1 knock-down cells was examined over
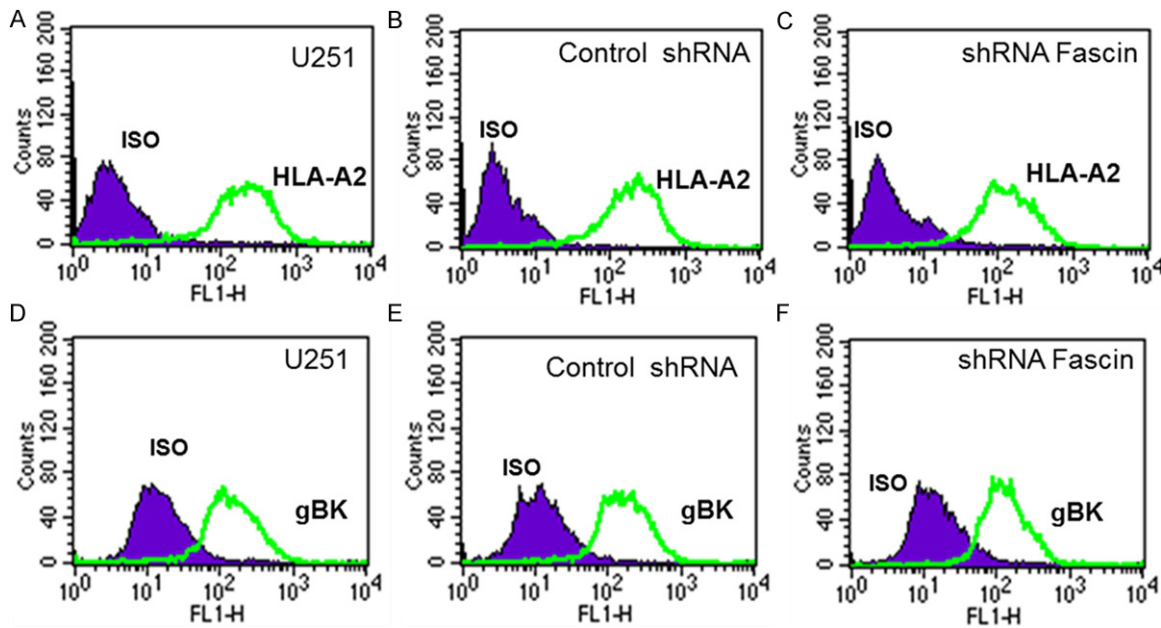
Figure 6. shRNA knock-down of fascin-1 does not alter expression of HLA-A2 or gBK antigens. A-C. The U251 untreated, shRNA control or fascin-1 shRNA treated cells were stained for the presence of membrane HLA-A2 (Top Row) or the cells were fixed, permeabilized and stained for gBK (Bottom Row). D-F. Ten thousand were analyzed by flow cytometry.

the next 3 days. These cells were grown in 24 well plates, and the cell counts were done daily. The doubling rate was next calculated from this data. The shRNA-control cells had a doubling time of 17.4 ± 1.0 hours, while the fascin-1 knock-down was 33.8 ± 0.7 hours. This delayed doubling time represented nearly a doubling of the time; it was significantly different from the shRNA control cells by using a student's t test (P<0.05). Thus, the fascin-1 knock-down unexpectedly slowed down the growth of U251 glioma cells, besides having the cosmetic loss of microvilli and loss of polarity.

U251 fascin-1 knock-down cells had decreased transmigration properties

The U251-shRNA control and U251-shRNA fascin-knock-down cells were tested in transmigration assays using the NeuroProbe chemotaxis chamber with 8 μm pores. One of two reproduced experiments is presented here. The U251 cells, (non-modified, shRNA control and fascin knock-down cells) were placed in the upper chambers, while the various chemo-attractants: 20% fetal bovine serum, recombinant interleukin-6 or recombinant insulin-like growth factor-1 was placed in their respective bottom chambers. **Figure 4** shows that all three

of the attractants tested allowed the U251 and shRNA control cells to migrate faster through the 8 micron pores than when no chemo-attractant was added. The U251 fascin-1 knock-down cells displayed a significant inhibition (P<0.05) towards all of the chemo-attractions that were tested. The repeated experiment confirmed these results.

Adherent fascin-1 knock-down cells are killed better than are non-treated U251 cells

Adherent U251 cells are more resistant to being killed by a variety of lymphocyte effectors than are non-adherent cells *in vitro* [17]. We postulated that the microvilli act analogous to sea urchins spins by providing a physical barrier to protect the main cell body from the lymphocyte effectors' molecules like perforin or granzymes.

Using the siRNA fascin-1 knock-down cells, we showed that these fascin-1 knock-down cells lost their ability to defend themselves from the cytolytic effects of non-specific cytolytic lymphokine-activated killer cell (also known as LAK cells). **Figure 5** shows the results of one representative experiment of two different experiments done with human LAK cells. U251, U251 siRNA control cells and fascin-1 knock-down
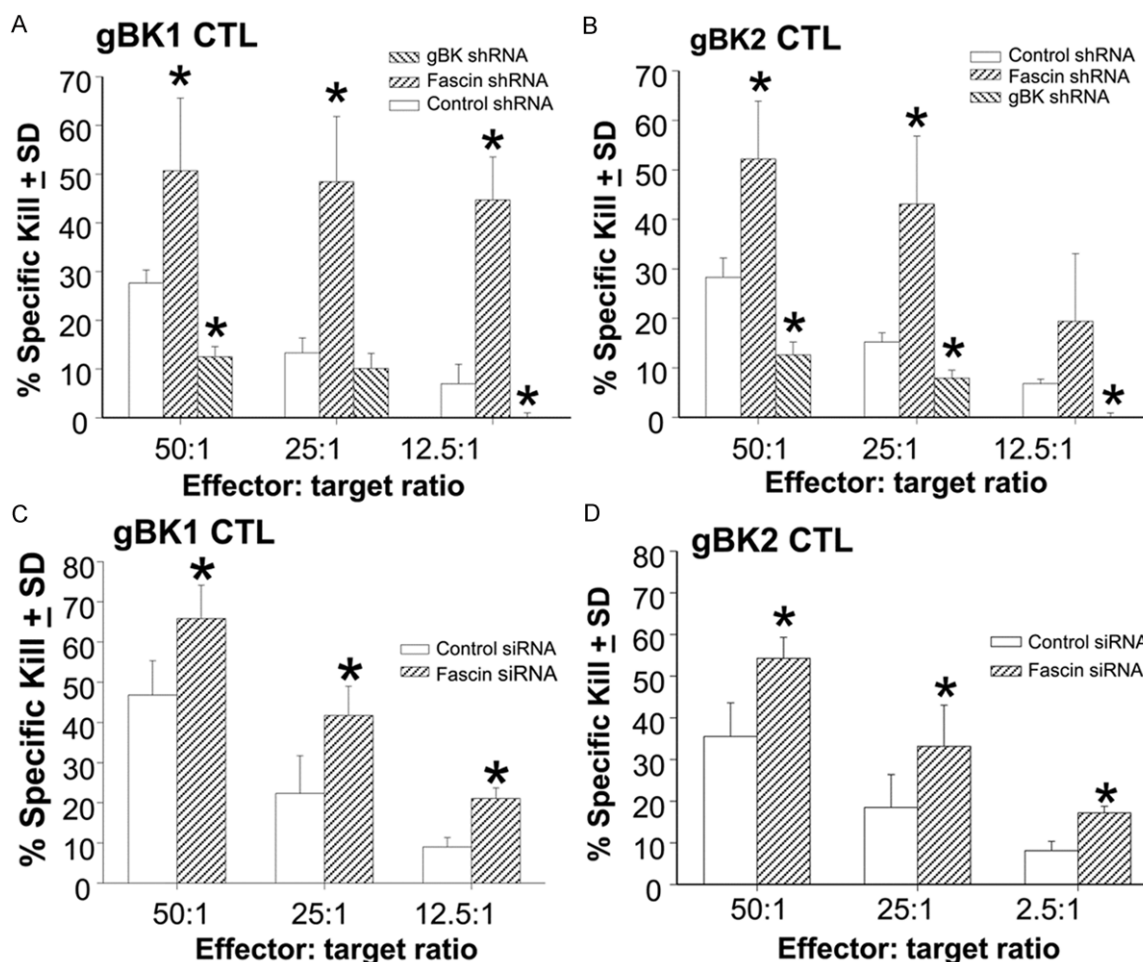
Figure 7. CTL-mediated cytotoxicity is enhanced on fascin-1 knocked-down U251 cells. HLA-A*0201+ human CTLs sensitized to gBK1 or gBK2 peptides were tested against adherent U251 shRNA control U251, U251 fascin-1 knock-down and gBK knock-down cells. The top panels show the representative results of one experiment done out of three different assays. The Bottom Panel shows the results when adherent siRNA control or siRNA fascin-1 U251 cells were used. A, C. The gBK1 specific CTLs are shown in the left panels while the gBK2 CTLs are displayed on the right panels B, D. Data is expressed as the mean of specific killing of quadruplicate cultures ± SD. The asterisk denotes a significant difference by the student's t test (P<0.05) from the fascin-1 knock-down cells compared to the adherent control U251 cells. In the Top Panel, the asterisk also indicates that the gBK shRNA knock-down U251 cells were significantly killed less than the shRNA control U251 cells.

cells were allowed to adhere to plastic and were labeled directly with Cr[51] *in situ*, and LAK cells were then added to the U251 cells. As a control, non-adherent U251 cells which lack microvilli were used to achieve maximal killing. Both adherent U251 and adherent control-siRNA cells showed significantly lowered killing (P< 0.05) when compared to the adherent fascin-1 knock-down U251 or the non-adherent U251 cells. Since the fascin-1 knock-down cells lack the microvilli as the non-attached U251, they were killed at the maximal amount.

Besides LAK cells, we next used cytolytic T lymphocytes (CTLs) to show that other lymphocyte

killers could reproduce this previous effect. We generated HLA-A*0201 restricted CTLs towards the two glioma Big Potassium (gBK) epitopes (gBK1 and gBK2), as previously described [52, 53] and then used these CTLs as effector cells towards U251 control cells and U251-fascin knock-down cells. The expression of either cell surface of HLA-A2 molecules (**Figure 6**, Panels A-C) or the internal target antigen precursor protein, gBK (Panels D-B), was unaffected by fascin-1 knock-down within the shRNA treated U251 cells.

The anti-gBK1 or anti-gBK2 specific CTLs were tested against adherent control-treated (shRNA

or siRNA) U251 and adherent U251 fascin-1 knock-down (shRNA or siRNA) cells (**Figure 7**). When the shRNA-treated matched U251 cells were tested (Top Panels), both sets of CTLs (gBK1 specific CTLs-left panel or gBK2 specific CTLs-right panel) killed the shRNA fascin-1 treated cells better than the shRNA control cells, since these fascin-1 knock-down cells lack the microvilli/microspikes, which prevented optimal killing. As a further specificity control, we also tested a shRNA gBK knock-down (with 70% knock-down) that reduced the amount of targeted antigenic epitopes that the CTLs recognize in the context of HLA-A*0201. The CTLs showed significantly less killing against these gBK knock-down cells (P<0.05) than the shRNA control cells.

In a separate experiment, we showed identical results occurred when we used the fascin-1 siRNA treated cells (**Figure 7**, Bottom Panels), too. Again, the adherent siRNA control U251 cells were killed by both sets of CTLs. But the adherent siRNA Fascin knock-down cells were killed significantly better by both sets of the gBK-specific CTLs (P<0.05). Thus, the loss of microvilli/microspikes by fascin-down strategies provides a better target cell for CTLs.

Discussion

Previously, we reported that glioma cells possess a cell surface that was covered in microspikes, microridges and microvilli, which prevented lymphocyte effector cells from optimal killing of the glioma cells *in vitro*. These micro projections can also be seen within *in situ* gliomas [17-19]. Fascin is an important actin bundling protein, which provides cytoskeletal support for the microspikes, microvilli and filopodia by cross-linking individual actin filaments together. There are three different members of this family of proteins, fascins-1 through 3. Fascin-1 over-expression is reported in glioma, colon, breast, lung, kidney, ovarian, cervix and ovarian cancers [20, 39-50]. Most of these previous studies also correlated fascin-1 expression with the increased grade and/or malignancy of the cancers and with a poorer prognosis when it was over-expressed. Yamashiro and colleagues [42] took rounded epithelial cells (LLC-PK1 pig epithelial cells or rat embryonic REF-52 cells) and by either gene transduction or by direct intracellular micro-injection of purified fascin-1 protein induced major cellular morphological changes. These differences included increased numbers of microspikes, longer and thicker microvilli, and more extensive lammelipodia. These modified cells also improved their transmigratory ability to penetrate through 8 micron pores. With regards to human glioma biology, fascin-1 is concluded to play active roles in tumor cell migration and invasion. Hwang, *et al.*, [20] showed that U87, U251 and SNB19 glioma cells treated with siRNA knock-down constructs had less adhesiveness towards laminin, collagen IV and vitronectin coated substrates. Fascin-1 also was concentrated at the leading edge of the migrating glioma cell. Fascin-1 knock-down cells using siRNA possessed less migration potential. Vignjevic and colleagues concluded that fascin provides the rigidity necessary to push the membrane forward [56]. This biophysical property, therefore, allowed the cell to move in that direction that was supplying a source of chemo attractants. The filopodia thereby provides the cell with a sense of polarity and promotes cell migration [25, 57-59].

We genetically knocked-down fascin-1 either by using current siRNA or shRNA technologies in U251 cells. Previously, all glioma-based work done with fascin knock-down employed siRNA, so our studies prove that shRNA technology is easily amendable with fascin-1 silencing and alters cell morphology, although the siRNA appeared more efficient *in vitro* transfection. With both transient siRNA technology and stable shRNA knock-down, the expression of fascin-1 mRNA was shown by using qRT-PCR and the proteins were confirmed to be reduced by using a monoclonal antibody in conjunction with intracellular flow cytometry (**Figures 1** and **2**). The morphology of these cells changed from fibroblastic-looking cells to a more rounded squamous looking one with minimal membrane protrusions (**Figures 2** and **3**). When we used shRNA technology, the best shRNA knock-down clone produced 70% knock-down. By atomic force microscopy, we saw the loss of microvilli, microridges and microspikes on these knock-down cells, but not on the control siRNA or shRNA (data not shown), and the glioma cells assumed a smoother surface (**Figure 3**). One empirical observation we noticed was that our knock-down cells had slower growth rates. Our best shRNA fascin-1 knocked-down clone had a doubling time of 33.8 ± 0.7 hours. The control shRNA U251 cells had faster doubling time of 17.4 ± 1.0 hour. Since previous work used

siRNA, which is a transient system, previous researchers probably didn't recognize the overall effect on long term cell proliferation rates, as we saw with the stable knock-down of fascin-1. These fascin-1 shRNA silenced cells also failed to transmigrate through 8 μm pores in response to three different chemo-attractants, fetal bovine serum, interleukin-6 and insulin-like growth factor-1 (**Figure 4**). The last two chemo-attractants induce migration of various cancer cells [55, 60]. The expression of fascin and fascin-containing microspikes has been reported to be controlled by IL-6 and IGF-1, too [55, 60].

Finally, when these knock-down cells were used in cytolytic assays *in vitro*, the adherent siRNA fascin-1 knock-down cells were better targets for LAK cells (**Figure 5**), since they lack their defensive microspikes/microvilli. Identical results occurred when two sets of gBK-specific cytolytic T lymphocytes were used. HLA-A2 restricted CTLs generated towards the gBK ion channel, which had HLA-A2 restricted peptides [52, 53]. The fascin-1 knock-down cells did not have altered levels of either HLA-A2 or gBK expression (**Figure 6**). As a result of either knock-down strategy, the fascin-1 knock-down cells were optimally killed by the gBK-specific CTLs (**Figure 7**), because the defensive microvilli/microspikes were lost and the amount of HLA-A2 or the tumor antigen was unchanged. Thus, the conditions are right, so that the loss of these defensive membrane protrusions permit a direct attack upon the glioma's cell body. Our ultimate goal is to disrupt the microvilli of human glioma cells by genetic silencing to improve the cytotoxic capability of human cytolytic lymphocytes and may be a universally applicable to improve immunotherapy towards brain cancers, in general.

Our work illustrates that we can improve immunotherapy, especially cytolytic lymphocyte-based therapies towards glioma by the judicious use of a fascin-1 knock-down strategy. If fascin-1 down-down can be delivered by some genetic delivery system (virus, continuous infusion of si/shRNA constructs or targeted drugs) the glioma cells should express less microvilli/filopodia. Knock-down should make glioma cells less invasive *in situ*, due to their loss of invadopodia-requiring membrane projections. On a second level, the fascin-1 knock-down should also slow down glioma cell division, by interfering with cell division. Finally, if the glioma cells lose their defensive microvilli/microspikes then this glioma defense should be reduced and allow the maximal effects of cytolytic lymphocytes to occur. Thus, immunotherapy, as a whole may be augmented on three different levels by a fascin-1 knock-down strategy.

Disclosure of conflict of interest

None.

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