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Expression and Function of CD9 in Melanoma Cells

Jun Fan¹, Guo-Zhang Zhu², and Richard M. Niles¹

¹ Department of Biochemistry and Microbiology, Marshall University, Huntington, WV 25755

² Department of Biological Sciences, Marshall University, Huntington, WV 25755

Abstract

CD9, a member of the tetraspanin family, functions as an organizer in “tetraspanin webs”, through interacting with other cell adhesion molecules. It plays a role in differentiation, fertilization and cell migration. We investigated the expression and function of CD9 in melanoma. CD9 protein expression in B16 mouse melanoma and six human melanoma cell lines was decreased compared to normal melanocytes. B16F1 clones stably over-expressing CD9 had reduced ability to form colonies in soft agar; however paradoxically these over-expressing clones had increased ability to invade Matrigel. Similarly, transient over-expression of CD9 in the human metastatic melanoma cell line WM9 dramatically decreased anchorage-independent growth, while transient over-expression of CD9 in the radial growth phase cell line SbCl2 resulted in the gain of Matrigel invasion activity. DNA sequencing of CD9 cDNA from all six human melanoma cell lines did not show deletions, insertions or mutations. Treatment of all six human melanoma cell lines with the histone deacetylase inhibitor trichostatin A increased CD9 levels. The DNA methylation inhibitor 5-aza-cytidine also increased CD9 protein levels with greater increases seen in cell lines derived from more malignant melanomas.

Keywords

CD9; tetraspanins; human melanoma; anchorage-independent growth; matrigel invasion

Introduction

Melanoma is the most deadly form of skin cancer and its incidence is increasing, especially in the under 40 year old population [1]. It arises from the malignant transformation of pigment-producing cells called melanocytes. Melanoma is thought to progress through a radial growth phase (RGP), vertical growth phase (VGP), and then to a metastatic phenotype [2–3]. Molecular changes associated with these transitions play a key role in the evolution of melanoma. Key alterations include B-RAF and N-ras mutations, resulting in the activation of the Erk1/2 MAPK and PI-3 Kinase/AKT pathways. Also changes in the expression or function of cell cycle regulators are required in order to bypass senescence [4].

CD9 is a member of the tetraspanin family of integral membrane proteins. These proteins span the membrane four times, and have small and large extracellular domains. In mammals, there are at least 32 members of the tetraspanin family [5,6]. Members of this family are involved in regulating diverse functions such as fertilization, infectious disease & immunity, cell migration, cell growth, and cell differentiation, and malignancy [6,7]. Tetraspanins accumulate in membrane microdomains, distinct from lipid rafts. Here they associate with

other proteins in either a direct or indirect fashion. This entire complex of interactions has been termed the “tetraspanin web” [5,8,9].

A variety of experimental and clinical studies have found that down-regulation of CD9 is associated with increased malignancy of solid tumors. An early study by Ikeyama et al [10] demonstrated that overexpression of CD9 in a variety of tumor cells decreased their motility. Examination of CD9 expression in breast tumors [11], colonic adenocarcinoma [12] and prostatic adenocarcinoma [13] showed that metastases had reduced CD9 levels compared to primary tumors. Longo et al [14] reported that endothelial cells had an active redistribution of CD9 to points of tumor cell insertion suggesting a role of CD9 in the extravasation phase of tumor metastasis. In a seminal study, La Nour et al [15] used proteomic technology to identify proteins that associated with CD9 in primary colon tumor cells and two metastases from the same patient. While there were a number of common CD9-associated proteins, the laminin receptor Lutheran/B-cell adhesion molecule was expressed only on the primary tumor cells, whereas CD26/dipeptidyl peptidase IV and tetraspanin Co-029 were observed only on metastatic cells. However, CD9 is not strictly classifiable as a metastasis suppressor because in some tumor types increased CD9 levels are associated with metastases. Such opposing activities probably depend on the proteins that associate with CD9

Si and Hersey [16] first demonstrated that CD9 expression was inversely related to the thickness of the primary melanoma tumor and that it was expressed predominantly on primary rather than on metastatic tumors. One of the physiological functions of CD9 in melanocytes appears to be inhibition of migration [17]. Thus low levels of CD9 in melanoma may allow these cells to migrate, a prerequisite for invasion. A recent study of CD9 expression in human nevi and cutaneous melanoma samples [18] found somewhat different results than reported by Si and Hersey [16]. CD9 was present in all nevi, but lost in 20/28 primary melanomas (including thin lesions) and 24/52 metastatic melanomas. Interestingly, Rambow et al [19] found CD9 expression was restored during spontaneous regression and differentiation of melanomas in the MeLiM swine model. Immunohistochemistry studies have limitations of antibody specificity and investigator subjective analysis of degree of reactivity. Therefore we used non-malignant mouse melanocytes, normal human cutaneous melanocytes, B16 mouse melanoma and a series of human melanoma cell lines to examine CD9 protein and RNA expression. To gain an understanding of CD9 function, we manipulated its expression in the melanoma cells and determined how this affected growth (anchorage-dependent and independent) and Matrigel invasion. Lastly, we initiated studies to examine the mechanism for altered CD9 expression in human melanoma.

Materials and Methods

Chemicals and reagents

All chemicals were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise stated.

Cell Culture and Treatments

All cells except Melan-a cells were maintained in 5% CO₂/95% air, humidified cell culture incubator at 37°C. B16F1 cells were grown in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 ug/ml streptomycin (all reagents from Invitrogen, Carlsbad, CA). Melan-a cells (non-malignant mouse melanocytes [20]) were a generous gift from Dr. Dorothy Bennett, St. Georges Hospital, London, England. They were grown in RPMI 1640 with 5% fetal bovine serum supplemented with 200 nM phorbol 12-myristate 13-acetate plus antibiotics, and cultured in 10% CO₂/90% air, 37°C condition. Human melanoma cells SBcl2, WM3211, WM 1366, WM3248, WM9 and WM239 were kindly

provided by Dr. Meenhard Herlyn, Wistar Institute, Philadelphia, PA. All human melanoma cell lines except SbCl2, were grown in DMEM plus 10% fetal bovine serum and antibiotics. The latter cells were grown in MCDB153:L15 (4:1) supplemented with 2% FBS and 5 ug/ml insulin, (Pierce, Rockford, IL).

Neonatal human epidermal melanocytes (lightly pigmented passage 2) were maintained in 254 medium supplemented with PSA (Penicillin, Streptomycin, and Amphotericin B) and human melanocyte growth supplement. The cells and supplements were obtained from Cascade Biologics, Portland, OR. Several new stocks of human melanocytes were used for the experiments described in this report. For certain experiments, cells were treated with 25ng/ml, 50ng/ml, 100ng/ml Trichostatin A (TSA) or 0.5, 1.0, 5.0 umol/L 5-aza-2'-Deoxycytidine (5-aza-dC) for 5 days. DMSO (vehicle for TSA and 5-aza-dC) was used as a control for these experiments.

RNA isolation and RT-PCR

Total RNAs from B16F1 and melan-a cells were extracted by TRI Reagent (Sigma Aldrich, St. Louis, MO), while Qiagen RNeasy Midi Kit was used to isolate total RNAs from human melanocytes and melanoma cells, according to the protocols as provided by the manufacturers. The purified RNA samples were dissolved in RNase-free water and quantified by a Nanodrop spectrophotometer (NanoDrop Technology, Inc., Wilmington, DE). Each RNA sample had an A260/A280 ratio of 1.8 or higher. Prior to synthesis of cDNA, the quality of RNA was determined on an Agilent 2100 Bioanalyzer, using the RNA 6000 Nano Assay kit (Agilent Technologies, Wilmington, DE). Synthesis of cDNA was performed by the Advantage RT-for-PCR kit (Clontech, Mountain View, CA), according to the manufacturer's instructions.

For PCR : The forward and reverse primers used to amplify the cDNA encoding the extracellular large loop of CD9 were forward primer, 5'-CCCACAAGGATGAGGTGATTA-3' and reverse primer, 5'-ATGTGGAAGTGTGTTGAAG-3'. PCR was performed using thermocycler (Biometra Tgradient, Goettingen, Germany) conditions of 94°C for 2 min; 30 cycles of 20sec at 94°C, 20sec at 60°C, 1min at 72°C; and 72°C for 7 min. β -actin amplification was used as an internal PCR control with 5'-GTGGGCCGCTCTAGGCACCAA-3' as the sense primer and 5'-CTCTTTGATGTCACGCACGATTTC-3' as the antisense primer.

The PCR products used for sequencing were modified by pfu polymerase (Clontech, CA), and then purified by the Qiagen PCR Purification Kit. These purified samples were then sent to our Genomics Lab for sequencing. The cDNAs used for quantitative RT-PCR were synthesized by a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA), and quantitative-PCR was performed using the ABI PRISM 7000 Sequence Detection System instrument, with CD9 TaqMan primers. Gene Expression Assays (Applied Biosystems, Foster City, CA). The amount of CD9 mRNA relative to 18s rRNA was calculated and corrected for efficiency based on the $2^{-\Delta\Delta CT}$ method. These assays were performed on samples from three separate cell cultures.

Transfection and Isolation of B16F1 clones over-expressing CD9

The full-length mouse/human CD9 and CD9-GFP cDNAs containing an extra four base pair sequences (CACC) were generated by PCR. The PCR template for mouse CD9 was the plasmid pEGFP-N1-CD9 as reported previously [21], while the human CD9 cDNA clone was purchased from ATCC. These PCR products were inserted into the pLenti-based expression vector pLenti6/V5-D-TOPO (Invitrogen, Carlsbad, CA). Lentivirus stock expressing lacZ as a control virus was also generated as described below. The plasmids were

co-transfected with the ViraPower Packaging Mix into 293FT cells using Lipofectamine 2000 to produce Lentivirus stock (all reagents from Invitrogen), according to the manufacturer's instructions.

Generation of clones stably expressing CD9—A panel of B16F1 cell clones overexpressing CD9 was established by infecting the Lentivirus stocks expressing mouse CD9 into B16F1 cells at an MOI of ~1. After 14 days of selection in 4ug/ml of blasticidin, resistant colonies were subcloned and characterized for expression of ectopic CD9 by Western blotting and immunofluorescence (CD9-GFP). The subclones B16F1-CD9 and B16F1-lacZ overexpressed CD9 and beta-galactosidase respectively.

Transient transfection and expression of CD9—Lentivirus stocks expressing human CD9 were infected into SBcl2 cells or WM9 cells at an MOI of ~1. Cells were harvested after 72 hours, assayed for CD9 expression by Western blots and used for Matrigel invasion or soft agar colony forming assays.

Western blotting

Cells were lysed in 1X TNT buffer (25mM Tris HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 and protease inhibitor cocktail [Complete™, Roche Diagnostics, Indianapolis, IN]). Twenty ug of total protein from each sample was separated on SDS-PAGE using 10% separating and 4% stacking gels with 100 volts for 120 min under non-reducing conditions. After separation, the proteins were electrophoretically transferred to nitrocellulose membranes (0.2 um, Bio-Rad, Hercules, CA) using 100 volts for one hour. Membranes were incubated in a blocking solution consisting of 3% non-fat dry milk and 2% BSA in 1X TBST (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween, pH 7.5) for 1 h at room temperature. Membranes were then incubated overnight at 4° C in the appropriate antibody (5ug/ml anti-mouse CD9 antibody from BD Bioscience Pharmingen, San Diego, CA; 1ug/ml of anti-human CD9 antibody from Abcam, Cambridge, MA). Membranes were then probed with the secondary antibody conjugated with HRP followed by the ECL detection kit or ECL advance detection kit (Amersham Bioscience, Piscataway, NJ) according to the manufacturer's instructions. Nuclei and nuclear proteins were isolated using the NE-PER kit from Pierce (Rockford, IL). A total of 100 ug of protein from the nuclear and cytoplasmic/membrane fractions was used for detection of CD9 by Western blots.

Matrigel invasion assay

Matrigel invasion chambers and corresponding control chambers (BD Bioscience, San Jose, CA) were seeded with 8×10^4 cells per chamber in 2 ml of appropriate growth media and the chambers added to the 6-well plate. After three days of incubation, the non-invading cells were removed from the upper surface of the membrane and the cells on the lower surface were fixed in absolute methanol for 5 min and then stained with 0.5% crystal violet for 5 min. The air-dried membranes were dissolved in 1 ml of 10% acetic acid, and 200 ul of this solution was transferred to one well of a 96 well plate and absorbance measured at 570 nm (Kinetic microplate reader, Molecular Devices, Sunnyvale, CA). Data from this assay were expressed as the percent invasion defined as A_{570} of the cells invading through the Matrigel membrane divided by the A_{570} of the cells migrating through the control membranes $\times 100$.

Anchorage independent growth assay

Cytoselect™ 96 well Cell Transformation Assay Kits (Cell Biolabs, San Diego, CA) were used for the soft agar colony formation assay. Briefly, 1×10^3 cells in a mixture of DMEM containing 10% FBS and 0.4% agar were seeded into each well of a 96 well plate containing

0.6% agar bed in DMEM with 10% FBS. Wells were incubated for 6 days at 37°C and 5% CO₂. Colony formation was determined with a light microscope and quantitated by CyQuant GR fluorescent dye (Cell Biolabs, San Diego, CA) according to the manufacturer's instruction.

Cell proliferation

B16-F1 wild type and clones were seeded at 3×10^3 cells/well in a 96-well plate. Plates were incubated at 37°C, 5% CO₂, 95% air for 24, 48 and 72 h. Viable cells were counted by the MTT assay at A570 using Promega Cell Titer 96 Non-Radioactive Proliferation Assay Kit (Promega, Madison, WI). For each data point, six individual wells were assayed.

Transfection of siRNA

SMART pool siRNA for CD9 and control siRNA were purchased from Dharmacon (Lafayette, CO). Transfection of siRNA was performed using RNAiFect Transfect Reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. B16F1 or MW9 cells (1×10^5) were seeded in 6 well plates and allowed to reach 50–80% confluence. The final siRNA concentration used for transfection was 100 nM. Cells were harvested 48 h after siRNA transfection for use in the various assays described in Results.

Immunofluorescence

Cells were cultured on sterile cover slips overnight, washed with PBS (pH 7.2) three times, and fixed in 1.8% formaldehyde/PBS for 10 min at room temperature. Fixed cells were washed with PBS, permeabilized with 0.25% Triton X-100/PBS for 10 min at room temperature (RT) and then blocked in PBS with 2.5% bovine serum albumin (BSA) for 30 min at RT. Blocked cells were incubated with primary antibodies diluted in blocking buffer--monoclonal anti-CD9 antibody (1:300) alone or combined with polyclonal anti-GPR56 (1:300). After 1 hour at RT, cells were rinsed with PBS for 5 times and incubated with fluorescein isothiocyanate (FITC) or rhodamine-conjugated secondary antibodies, which were diluted in blocking buffer (1:200). Cells were then stained with 4',6'-diamidino-2-phenylindole (DAPI) at 0.5 µg/ml. To prevent bleaching, coverslips were mounted on slides using Fluoromount-G. Images were recorded by an Olympus BX51 microscope equipped with epifluorescence optics using a 60x oil immersion lens (NA=1.4) and an Olympus CC12 camera (Olympus America Inc, Center Valley, PA). Image analysis was performed using MicroSuite software, Basic edition.

Statistical analysis

Error bars in all figures represent SEM. The Student's t-test was performed to evaluate pairwise differences with a $p < 0.05$ being considered significant.

Results

Expression of CD9 in melanocytes and melanoma cells

We initially compared the expression of CD9 in the non-malignant mouse melanocyte cell line melan-a and B16 mouse melanoma cells, both of which are derived from C57Bl6 mice. Melan-a cells had 11-fold higher levels of CD9 protein and 5-fold greater level of CD9 mRNA relative to B16 melanoma cells (Fig. 1A & B). To determine if these findings also applied to human cells, we compared CD9 expression in normal human melanocytes and a series of human melanoma cell lines derived from different stages of tumor progression. All of the human melanoma cell lines had a lower level of CD9 protein relative to normal human melanocytes (Fig. 1C). There was no correlation between the degree of loss of CD9 protein expression and the stage of melanoma progression. All of the human melanoma cell

lines, except WM3248, also had lower levels of CD9 mRNA relative to normal human melanocytes (Fig. 1D). Although the SbCl2 cells were grown in a different medium than the other human melanoma cells, incubation of this cell line in DMEM (medium used for other cell lines) for 48 h did not change the expression of CD9. In addition, incubation of melan-a and normal human melanocytes in DMEM for 48 h also did not change their level of CD9.

In addition to expression levels, we also examined the subcellular localization of endogenous CD9 in the human melanoma cell lines using indirect immunofluorescence staining (Fig. 2). In SbCl2 cells, derived from a radial growth phase melanoma, CD9 (green fluorescence) is located predominantly at the plasma membrane. To confirm this finding, we used rhodamine-labeled mouse anti-human IgG to detect GPR56 which has a documented expression and plasma membrane location in all cells that have been examined for the expression of this protein [22,23]. Merging of the CD9 and GPR56 images shows areas of yellow-orange indicating co-localization. One of the melanoma cells, WM239, derived from a metastatic melanoma, appears to have nuclear localization of CD9 evidenced by staining the exact same nuclear structures as DAPI, a nuclear-specific stain (Fig. 2B). To confirm this finding we examined nuclear extracts of SbCl2 and WM239 for presence of CD9 by Western blots. Using enhanced chemiluminescence detection, CD9 was found predominantly in the cytoplasmic/membrane fraction in SbCl2 cells. There is a small amount of CD9 in the nucleus, but this may be due to the 10% cross-contamination that is inherent using this nuclear isolation kit (NE-). WM239 cells had little or no CD9 in the cytoplasmic/membrane fraction, but an immunoreactive 30,000 MW protein was found in the nuclear fraction. A similar MW protein was also detected in the nuclear fraction from SbCl2 cells. It is not clear whether this is a modified form of CD9 or whether this is a cross-reacting protein. Immunofluorescent CD9 staining of other human melanoma cells, showed either very little specific signal, or membrane staining similar to SbCl2.

Stable expression of tagged CD9 in B16 mouse melanoma cells

To determine the biological significance of decreased CD9 expression in melanoma cells, we established a panel of B16F1 clones that stably expressed ectopic CD9. These clones are designated as B16F1-CD9-GP and B16F1-CD9-V5 according to their fusion tag. We also generated B16F1 clones expressing LacZ-V5 to use as a control in addition to wild-type cells subjected to mock transfection. Fig. 3A shows the endogenous and CD9 fusion protein expression for two of these clones. It should be noted that in these Western blots, as well as all the Western blots in subsequent figures, an enhanced development agent (ECL-Advanced from GE HealthCare) was used in order to visualize the low protein level of CD9 in the B16-F1 and human melanoma cells.

The anchorage-dependent growth rate of B16F1 wild type, B16F1-LacZ-V5, B16F1-CD9-GFP and B16F1-CD9-V5 was determined by measuring cell number at 24, 48 and 72 h after seeding equal numbers of cells. These experiments showed that the doubling time was similar among the wild type cells and the different transfected clones (data not shown).

The ability to survive and grow without attachment to a substrate is a defining characteristic of somatic tumor cells. B16F1 cells (low CD9 expression) form a large number of colonies in soft agar, while melan-a cells (high CD9 expression) lack the ability to form colonies under these conditions. B16F1 clones ectopically expressing CD9, had a 40–60% reduction in soft agar colony formation relative to either wild-type cells or a clone expressing LacZ-V5 (Fig. 3B). Continual passaging of clones ectopically expressing a selected gene can lead to loss of expression of the selected gene. This phenomenon is shown in Fig. 3B where CD9-GFP clone 5 at low passages has high expression of the fusion construct and loss of anchorage-independent growth, while at high passages, the expression of the CD9 fusion

construct is markedly reduced and soft agar colony formation approaches wild type B16F1 levels.

We also measured the effect of ectopic expression of CD9 on the ability of B16F1 cells to invade and migrate through Matrigel. In two clones of B16F1 cells expressing GFP and V5 tagged CD9 respectively, there was a greater than 5-fold increase in invasion relative to either wild-type cells or a B16F1 clone expressing LacZ-V5 (Fig. 3C). These results were surprising given the inverse correlation between CD9 expression and metastatic potential of melanoma cells [16].

Ectopic expression of CD9 in human melanoma cells

CD9 protein expression was also markedly decreased in human melanoma cells relative to normal human melanocytes (Fig. 1C). WM9, derived from a metastatic melanoma forms large numbers of colonies in soft agar (Fig. 4A). Transient overexpression of CD9 in these cells, resulted in almost a 90% decrease in colony formation (Fig. 4A). SbCl2 cells, derived from a radial growth phase tumor, form very few colonies in soft agar and are weakly invasive through Matrigel. Transient overexpression of CD9 in these cells resulted in a two-fold increase in Matrigel invasive activity (Fig. 4B). Overexpression of CD9 did not increase soft agar colony formation in SbCl2 cells (data not shown). To further explore these findings, we treated WM9 cells, which express a moderate amount of CD9 (but still significantly less than normal human melanocytes), with CD9 siRNA to further decrease the amount of CD9 protein. This decrease in CD9 resulted in a small, but statistically significant decrease in Matrigel invasive activity relative to WM9 cells treated with the universal control siRNA (Fig. 4C).

Mechanism for decreased CD9 expression in melanoma cells relative to normal melanocytes

We explored the reason for decreased expression of CD9 in human melanoma cells vs. melanocytes. First we determined whether there were any large insertion, deletions or mutations in the coding region. There were no changes in the cDNA sequence of CD9 between the melanocytes and the six melanoma cell lines used in this study (data not shown).

Next we investigated whether the epigenetic modifiers, 5-aza-2'-deoxy cytidine, which inhibits DNA methylation, and trichostatin A (TSA), a histone deacetylase inhibitor, affected the expression of CD9 protein in six different human melanoma cell lines. TSA at concentrations ranging from 25–100 ng/ml was effective at inducing CD9 expression in all melanoma cell lines, although the degree of induction was less in the metastatic melanoma cell lines WM9 and WM239. In these two metastatic melanoma cell lines, 5-aza-2'-deoxycytidine was more effective in reactivating CD9 expression than TSA (Fig. 5).

Discussion

We found that CD9 protein expression was dramatically reduced in all melanoma cell lines examined relative to non-malignant melanocytes. CD9 mRNA levels, as measured by qRT-PCR were also decreased in all the melanoma cell lines relative to the melanocytes, although in some cells (WM9 and WM3248) the decrease in RNA was not as pronounced as the decrease in CD9 protein. This suggests that there may be additional translational/post-translational alterations of CD9 expression in melanoma cells, such as accelerated ubiquitination/proteasomal degradation. Altered cellular localization might contribute to rapid CD9 proteolysis resulting in low levels of the protein despite near normal levels of the transcript.

Mischiati et. al. [18] found that CD9 expression, in three separate clinical cases, was down-regulated in early passage cultures from metastatic melanomas compared with uninvolved skin melanocytes from the same patient. They also reported that CD9 was expressed in 18/18 nevi, but was lost in 20/28 (71%) primary melanomas and 24/52 (46%) metastatic melanomas. In addition, cDNA microarray profiling of changes in gene expression during spontaneous melanoma regression in the MeLiM swine model identified CD9 as a gene whose expression significantly increased during the regression phase [17]. In contrast to these and other reports of loss or decreased expression of CD9 in melanoma [11–13], cDNA expression profiling showed an up-regulation of CD9 expression in human gastric carcinoma relative to normal gastric mucosa [24]. Thus, changes in CD9 expression as a function of tumorigenesis appear to be tumor-type specific.

In addition to CD9 expression, we also examined its subcellular location in melanocytes and melanoma cells. We found that in SbCl2 cells, derived from a radial growth phase melanoma and human melanocytes, CD9 was located in the plasma membrane. However in the WM238 metastatic melanoma cell line, there was apparent nuclear localization of CD9. Western blot analysis of nuclear extracts from WM238 cells identified a 30,000 MW protein. This same MW protein, however, was found in nuclear extracts from SbCl2 cells. However, these cells had a much stronger 25,000 MW band in the cytoplasmic/membrane extract while WM239 cells had barely detectable 25,000 MW CD9 protein in their cytoplasmic/membrane fraction. Additional investigation will be required to determine if the 30,000 MW protein is a modified nuclear form of CD9 or whether it is a non-CD9 cross-reacting protein.

Ectopic overexpression of CD9 in B16-F1 melanoma cells did not alter anchorage-dependent growth, but did inhibit soft agar colony formation (anchorage-independent growth) relative to wild-type cells and LacZ expressing clones. One of the overexpressing clones subsequently increased its anchorage-independent growth with continued in vitro passage. This phenotype was tightly correlated with loss of ectopic CD9 expression. We could find only one other report in the literature that indirectly implicates CD9 in the regulation of anchorage-independent growth. Mitsuzuka et. al. [25] reported that mouse fibroblast expressing c-jun, had increased soft agar colony formation with decreased amounts of a complex containing GM3, CD9 and alpha-5-beta-1 integrin. Re-expression of GM3 led to increased amounts of this complex and loss of anchorage-independent growth.

Ectopic overexpression of either tagged version of CD9 led to a five-fold increase in Matrigel invasion compared to either wild type cells or a clone of B16-F1 cells expressing LacZ. This was a surprising finding since CD9 has been characterized as a metastasis suppressor gene. For example, Ikeyama et.al. [10] showed overexpression of CD9 in BL6 melanoma decreased metastatic potential relative to parental BL6 cells. Also Miyake and colleagues [11] reported that intra-tumor injection of adenovirus encoding CD9 results in a marked reduction in the number of pulmonary metastases relative to tumor injected with adenovirus encoding beta-galactosidase. Both of these studies measured in vivo metastasis which involves a number of steps in addition to the invasion potential quantitated in our assay.

Similar to mouse melanoma, we showed that human melanoma cells also had reduced CD9 expression relative to normal human melanocytes. We also found that increased CD9 expression suppressed anchorage-independent growth in WM9 metastatic melanoma, while it stimulated Matrigel invasion in low invasive SbCl2 melanoma cells. Knockdown of CD9 in WM9 metastatic melanoma with high Matrigel invasive melanoma cells resulted in small, but statistically significant decrease in invasion. One clue to explaining these seemingly contradictory results is found in the study of Garcia-Lopez et al. [17]. Using blocking

antibodies and siRNA to CD9, they found that CD9 enhances motility of melanocytes. Since motility is an important component of tumor cell invasiveness, we suggest that overexpression CD9 in melanoma increases motility, which may be responsible in part for the increased Matrigel invasion activity. Based on our results and those of Mischiati et al [18], we hypothesize that suppression of CD9 levels may be important in the transition from melanocytes to melanoma. However, in some melanomas, re-expression of CD9 within the altered microenvironment of the tumor may lead to enhanced invasion.

What is the cause for decreased expression of CD9 in the melanoma cells? Our DNA sequencing data did not reveal any differences between coding sequence of CD9 between normal human melanocytes and all of the human melanoma cell lines that have low levels of CD9 protein. This rules out major gene deletions as a cause for the decrease in CD9 expression. However, we have not examined the CD9 promoter sequence and there remains the possibility that mutations or deletions in this region could lead to decreased CD9 mRNA expression. In myeloma cells lines there is increased promoter methylation of the CD9 gene relative to bone marrow samples. Treatment of the myeloma cell lines with the demethylating agent 5'-aza-deoxycytidine reactivated CD9 expression [26]. Zhang, et al. [27] found that CD9, which is frequently down-regulated in lung tumors, is dominantly regulated by histone deacetylation, since the histone deacetylase inhibitor, trichostatin A, increased CD9 expression. However, in macrophages, histone deacetylase inhibition results in decreased CD9 expression [28]. These reports suggest that tumor cells silence CD9 expression either through restricting access of transcriptional regulators to DNA (deacetylation of histone) or by methylating sites in the CD9 gene. We examined the ability of trichostatin A an inhibitor of class I & II histone deacetylases, as well as 5'-aza-deoxycytidine, a DNA methylation inhibitor, for their ability to reactivate CD9 protein expression in six different human melanoma cell lines. TSA was effective in reactivating expression of CD9 in all melanoma cell lines except WM239 and WM9, where 5-aza-2-deoxycytidine was more effective than TSA. Based on these results, DNA methylation appears to play a larger role in silencing CD9 expression in cell lines derived from vertical and metastatic melanoma and much less of a role in the decreased expression found in cell lines derived from radial growth phase melanomas.

Overall, our results complement the studies on CD9 expression in clinical specimens from patients with melanoma [16,18] and suggest that decreased CD9 expression occurs in the early stage of melanoma development. Further study is needed to determine how lack of CD9 expression could contribute to the development of a primary melanoma.

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Abbreviations

RGP	radial growth phase
VGP	vertical growth phase
SCID	severe combined immunodeficiency

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Fig. 1.

Expression of CD9 in melanocytes and melanoma cells. Non-malignant melan-1 mouse melanocytes and B16-F1 mouse melanoma cells were assayed for the level of CD9 protein (A) or RNA (B), by Western blotting and RT-PCR respectively. CD9 protein level (Western blots) was also examined in normal human melanocytes and a series of human melanoma cell lines isolated from different stages of melanoma progression (D). CD9 RNA levels (D) were also examined in the human melanocytes/melanoma cells using qRT-PCR. The data in D is expressed $\Delta/\Delta C_T$ relative to the normal human melanocyte level set as 1.0.

A **B**

Fig. 2.

Sub-cellular localization of CD9 in human melanoma cells. Cells were grown, fixed and stained for indirect immunofluorescence as described in Methods. A. SBcl2 human melanoma cells derived from a primary, cutaneous radial growth phase melanoma. Green fluorescence is CD9; red fluorescence is GPR56, a plasma membrane marker; yellow/orange are areas of co-localization of these two proteins. B. WM239 human melanoma cells derived from a metastatic melanoma. Green fluorescence is CD9; blue fluorescence is DAPI (nuclear). C. Expression of CD9 in nuclear and cytoplasmic/membrane fractions from WM239 and SBcl2 cells. Nuclei were isolated and extracted using the NE-PER kit from Pierce. Equal amounts of protein (100 ug) from each fraction and each cell line was used for Western blots.

Fig. 3.

Ectopic expression of CD9 in B16-F1 mouse melanoma cells inhibits anchorage-independent growth, but stimulates Matrigel invasion. B16-F1 cells were infected with lentiviruses containing either GFP-tagged CD9 or V5 epitope-tagged CD9. Following selection in blastocidin, resistant clones were examined for ectopic CD9 expression by Western blotting using ECL-Advanced for detection of the low levels of CD9 expressed in these cells as compared to melanocytes (A). Several clones were examined for anchorage-independent growth (B). Equal numbers of cells from the various clones, or wild-type cells were seeded in the soft agar system described in Methods. The data is presented as the relative colony formation compared to wild type cells. Representative clones expressing CD9-GFP or CD9-V5 were tested for invasion through Matrigel-coated wells and

corresponding control wells (C). The data (B & C) is presented as the mean $\pm$ the SEM (error bars) of triplicate dishes or wells. Similar results were obtained in several additional experiments. * $p < 0.01$.

Fig. 4.

Transient ectopic expression or siRNA-mediated knockdown of CD9 alters anchorage-independent growth and Matrigel invasion in human melanoma cells. WM9 cells (A), derived from a human metastatic melanoma, or SbCl2 cells (B) derived from a radial growth phase human melanoma, were infected with lentiviruses containing CD9. Cells were harvested 72 h after infection and tested for soft agar colony formation by the rapid Cytoselect Cell Transformation Assay Kit (Cell Biolabs, San Diego, CA). A. The top panel shows colonies in mock transfected (left) or CD9 transfected (right) cells. The bottom panel shows the relative fluorescence units representing the number of colonies from control lentivirus vs CD9-lentivirus infected cells. B. SbCl2 cells infected with control or CD9-lentiviruses were assayed for their ability to invade Matrigel as described in Material and

Methods. C. WM9 cells were treated with a universal control siRNA or CD9 siRNA (both from Dharmacon, Lafayette, CO) for 48 h. The top panel shows a Western blot of the treated cells using ECL-Advanced to detect CD9. The expression of CD9 protein was decreased by ~80% relative to the two controls. Cells were then assayed for Matrigel invasion (bottom panel), and the invasion units calculated as described in Methods. All data is expressed as the mean $\pm$ SEM (error bars) of triplicate samples. Experiments were repeated two additional times with similar results. * $p < 0.05$

Fig. 5.

Inhibition of histone deacetylase and DNA methylation increases CD9 expression. Human melanoma cells were treated with the indicated concentrations of trichostatin A (TSA) or 5'-aza-2'-deoxycytidine for 5 days. Control cells received DMSO, the solubilization vehicle for these compounds. CD9 protein levels were measured by Western blotting using GAPDH as a loading control. No data is shown for 100 ng/ml in TSA-treated SBcl2 cells since this concentration was toxic to the cells. This experiment was repeated several times with similar results.