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Integrin $\alpha 9\beta 1$ promotes malignant tumor growth and metastasis by potentiating epithelial-mesenchymal transition

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Abstract

The integrin $\alpha 9\beta 1$ binds a number of extracellular matrix components to mediate cell adhesion, migration and tissue invasion. Although expressed in variety of normal human cells including endothelium, it is also expressed in cancer cells. We have previously shown that $\alpha 9\beta 1$ binds VEGF-A to facilitate angiogenesis, an important component of the tumor microenvironment. Since $\alpha 9\beta 1$ induces accelerated cancer cell migration, we wished to determine what role it played in cancer growth and metastasis. In this study we show that $\alpha 9\beta 1$ expression induces molecular changes consistent with epithelial-mesenchymal transition. In addition, we found that $\alpha 9\beta 1$ forms a tri-partite protein complex with β -catenin and E-cadherin, which dissociates following integrin activation and subsequent src and β -catenin phosphorylation. These findings were consistent in cells in which: $\alpha 9\beta 1$ was exogenously over-expressed, or when its expression was suppressed in cancer cells endogenously expressing $\alpha 9\beta 1$. These in vitro results are biologically significant since $\alpha 9\beta 1$ expressing cancer cells induce greater tumor growth and metastases in mice as compared to cells without $\alpha 9\beta 1$ expression or when integrin expression is suppressed. Furthermore, integrin $\alpha 9\beta 1$ is expressed in primary human small cell lung cancer and patients having a high expression of $\alpha 9\beta 1$ demonstrated significantly worse long term survival compared to patients with low $\alpha 9\beta 1$ expression. These findings highlight a novel mechanism of integrin $\alpha 9\beta 1$ function in human cancer.

Keywords

EMT; $\alpha 9\beta 1$ integrin; lung cancer; metastasis; cell migration

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Introduction

Epithelial-mesenchymal transition (EMT) represents a well co-ordinated series of molecular changes in epithelial cells including loss of E-cadherin expression, increased vimentin and N-cadherin¹ and rearrangement of cortical actin, imparting an increased migratory capacity to the resulting mesenchymal cells.² The timing and extent of EMT is essential for fetal development but is also associated with diseases such as fibrosis and cancer metastasis.^{3,4} In tumors, EMT imparts a more aggressive phenotype, ultimately resulting in metastasis by direct local tissue invasion or distantly through blood vessels and lymphatics.⁵ The prototypic stimulus for EMT is TGF- β , which induces phosphorylation of β -catenin and subsequent activation of transcription factors including Snail.⁶ TGF- β is not alone in its ability to induce EMT and various other growth factors and transmembrane receptors have been implicated, including the integrins.^{1,5,7}

Integrins are matrix adhesion receptors that can transduce bi-directional signals to regulate cancer cell migration and invasion facilitating mechanisms such as EMT, depending upon available ligand and their interactions with membrane receptors.⁸⁻¹² Integrins $\alpha 3\beta 1$, $\alpha 5\beta 1$, and $\alpha v\beta 6$ have all been shown to modulate TGF- β induced EMT through distinct mechanisms in a variety of tumor cell lines.^{3,13-15} EMT induces an increase in both $\alpha v\beta 6$ and $\alpha 5\beta 1$ expression to facilitate cell migration: $\alpha v\beta 6$ through autocrine TGF- β activation,¹³ and $\alpha 5\beta 1$ through binding of autocrine fibronectin.¹⁵ Integrin $\alpha 3\beta 1$ facilitates cell-cell adhesion by formation of a complex with E-cadherin and β -catenin.¹⁶ In kidney and lung epithelium $\alpha 3\beta 1$ complexes with E-cadherin and TGF- β R1 and its activation is required for β -catenin phosphorylation.^{3,14}

Integrin $\alpha 9\beta 1$ is expressed in human cancers and appears to correlate with higher grade tumors.¹⁷⁻¹⁹ Although we have recently shown that integrin $\alpha 9\beta 1$ plays a role in lymphangiogenesis,²⁰⁻²³ and angiogenesis^{24,25} the exact mechanisms by which it transduces a pro-cancerous effect is unclear. Since integrins have been implicated in EMT⁴ and having shown that $\alpha 9\beta 1$ induces accelerated cancer cell migration through src, a signaling intermediate implicated in cancer metastasis,^{26,27} we postulated that $\alpha 9\beta 1$ might promote carcinoma growth and metastasis via src mediated EMT.

Results

Exogenously expressed $\alpha 9\beta 1$ promotes molecular and cytoskeletal changes consistent with EMT

We have previously used SW480 colon carcinoma cells⁷ transfected with integrin $\alpha 9\beta 1$ ²⁵ and observed that, compared to mock cells, $\alpha 9$ -transfected SW480 (Fig 1A) displayed a more spindle shape, suggestive of a mesenchymal phenotype. Immunofluorescent staining (Fig 1A) and immunoblots of cell lysates (Fig 1B) showed that compared to mock, SW480- $\alpha 9$ expressed less E-cadherin, more α -SMA and vimentin and demonstrated loss of cortical F-actin; all molecular changes consistent with EMT. These molecular changes increased significantly with $\alpha 9\beta 1$ activation, and were associated with increased expression of the transcriptional repressor Snail (Fig. 1C), further supporting the notion that $\alpha 9\beta 1$ -induces EMT. In contrast, SW480-mock did not demonstrate these changes when activated by the

common integrin ligand, plasma fibronectin (pFN). Transient $\alpha 9$ -overexpression in other cancer cell lines also resulted in similar EMT changes measured by immunoblotting (Suppl. Fig-S1A) and immunocytochemistry (Suppl. Fig-S1B); helping to rule out the confounding effect of cell type.

$\alpha 9\beta 1$ expressing cancer cells demonstrate increased migration and invasion in vitro

The functional consequences of EMT include increased cell migration, invasion and anchorage independent growth; all of which were increased with expression of $\alpha 9\beta 1$. Figure 1D shows that $\alpha 9\beta 1$ expression increases both haptotaxis and chemotaxis of SW-480 cells; which were associated with an increased cellular expression of N-cadherin and loss of cortical actin, both markers of increased migratory capacity (Figure 1C). Furthermore, expression of $\alpha 9\beta 1$ was associated with a time dependent increase in invasion, increasing further when cells were treated with $\alpha 9\beta 1$ ligand and significantly decreased by $\alpha 9$ -specific inhibitors (Fig 1E). Lending further support to the notion that $\alpha 9\beta 1$ induces a transformed phenotype, SW480- $\alpha 9$ were found to have increased anchorage independent growth (Suppl. Fig S1-C). Taken together, these results suggest that $\alpha 9\beta 1$ can induce a more aggressive cancer cell phenotype by inducing EMT.

Activation of $\alpha 9\beta 1$ results in src dependent phosphorylation of β -catenin

Having recently shown src is essential for $\alpha 9\beta 1$ signaling²⁸ and knowing src is implicated in EMT²⁹ we investigated its role in $\alpha 9\beta 1$ -associated EMT. Fig 2A shows that association of src with integrin $\alpha 9\beta 1$ is facilitated by $\alpha 9\beta 1$ ligand and depends on the $\alpha 9$ subunit and not $\beta 1$ (Fig 2B). During EMT there is loss of cell-cell contact resulting from β -catenin phosphorylation and disruption of the β -catenin/E-cadherin complex at the adherens junction.¹³ Fig 2C shows that ligation of $\alpha 9\beta 1$ induces phosphorylation of src-Y416 and also β -catenin-Y654, the tyrosine phosphorylation site associated with EMT.¹⁴ Src also mediated $\alpha 9\beta 1$ -induced β -catenin phosphorylation and changes in E-cadherin and vimentin expression consistent with EMT (Suppl. Fig S2-A). In non- $\alpha 9$ expressing SW480 neither src nor β -catenin phosphorylation were significantly induced by pFN, suggesting other $\beta 1$ -integrins are not playing a role (Fig 2C).

$\alpha 9\beta 1$ forms a complex with E-cadherin and β -catenin, which dissociates following $\alpha 9\beta 1$ activation

Similar to other integrins,^{3,14} we found that $\alpha 9\beta 1$ co-localizes with both β -catenin and E-cadherin on the cell surface (Fig 2D). This co-localization is disrupted following $\alpha 9\beta 1$ activation in SW480- $\alpha 9$ cells (Fig 2E) and appears to be mediated by src (Suppl. Fig S2-C). Although the dissociation of this tripartite protein complex may be secondary to loss of E-cadherin function, when taken together our results appear to support a direct effect of $\alpha 9\beta 1$ -associated EMT. Furthermore, targeted knockdown of β -catenin in SW480- $\alpha 9$ cells decreased E-cadherin and increased vimentin expression and were associated with functional changes consistent with EMT: increased cell migration, invasiveness and decreased proliferation (Fig 2F). In contrast, expression of E-cadherin and vimentin did not change significantly when cells were exposed to src-targeted siRNA, and migration and invasion were significantly less (Fig 2F, left panel).

β -catenin is also a component of the Wnt signaling pathway which is associated with EMT.^{30,31} Our data shows that wnt signaling does not appear to play a role in $\alpha 9\beta 1$ -associated EMT (Suppl Fig S3). Blocking wnt signaling either by Dickkopf1 (Dkk1), a Wnt signaling antagonist³² or by siRNA-mediated knockdown of lipoprotein receptor-related protein-6 (LRP6), the major wnt co-receptor, did not show any effect on $\alpha 9\beta 1$ mediated pro-EMT changes in E-cadherin or vimentin (Suppl Fig S3A,B,D) or on the invasiveness of cancer cells (Suppl Fig S3C,E).

$\alpha 9\beta 1$ -associated EMT is not modulated by other EMT-associated integrins or TGF- β

Since integrins $\alpha 3\beta 1$ and $\alpha v\beta 6$ are known to regulate EMT through β -catenin phosphorylation^{13,14} we next determined if these integrins might influence $\alpha 9\beta 1$ -associated EMT. Using flow cytometry we found minimal expression of $\alpha v\beta 6$, no differential expression between SW480-mock and $\alpha 9$ cells (Suppl. Fig S4-A) and that expression was unaltered by culture conditions, type of matrix (Suppl. Fig S4-A) or cell culture time (data not shown). Integrins $\alpha 3\beta 1$ and $\alpha v\beta 6$ modulate EMT through their interactions with TGF- β , and modulating downstream SMAD2 signaling.^{13,14} However, we found no difference in SMAD2 phosphorylation in SW480-mock or SW480- $\alpha 9$ at baseline or following stimulation by ligand: pFN (common ligand to $\alpha 3\beta 1$ and $\alpha v\beta 6$) or $\alpha 9$ -specific ligand, TnfnRAA (Suppl. Fig S4-B). Furthermore, $\alpha 9\beta 1$ expression in cancer cells did not alter TGF- β induced EMT (Suppl Fig S4-C) nor did TGF- β change cellular expression of $\alpha 9\beta 1$ (Suppl Fig 4D). Taken together these results suggest that unique to the integrins, $\alpha 9\beta 1$ can induce EMT independent of TGF- β .

In cells expressing $\alpha 9\beta 1$, total β -catenin is significantly decreased (Fig 2C) suggesting that β -catenin is either trafficking to the nucleus or undergoing proteasomal degradation. Supporting this notion, we found proportionally greater accumulation of nuclear β -catenin in SW480- $\alpha 9$ compared to mock cells suggesting that at least in part, there is increased transcriptional activity (Suppl. Fig S5-A). This is further supported by the increased proliferative capacity of SW480- $\alpha 9$, which can be blocked by inhibiting $\alpha 9\beta 1$ (Suppl. Fig S5-B,C).

$\alpha 9\beta 1$ promotes tumor growth, vasculogenesis, and metastasis

We next performed tumor experiments to determine the pro-carcinogenic effects of $\alpha 9\beta 1$ in-vivo. Tumors formed in mice from flank injection of SW480- $\alpha 9$ cells were up to 3 times larger than tumors formed from SW480-mock cells (Fig 3A), were associated with increased intra-tumoral angiogenesis and lymphangiogenesis (Fig 3B) and displayed altered cadherin and vimentin expression consistent with EMT (Fig 3C).

Also consistent with an EMT phenotype, animals injected with SW480- $\alpha 9$ cells demonstrated significantly greater metastasis with marked inguinal lymphadenopathy and widespread lung and liver metastases (Fig 3D). In contrast, only infrequent metastases could be detected in mice injected with SW480-mock cells (data not shown).

Endogenous $\alpha 9\beta 1$ mediates EMT in lung carcinoma cells and increases cell migration and invasiveness

Having shown that EMT and metastatic cell behavior can be induced by the exogenous over-expression of $\alpha 9\beta 1$, we next determined whether endogenously expressed integrin in lung cancer cells had a comparable action. Using flow cytometry (Fig 4A) and immunoblotting (Fig 4B) we found that $\alpha 9\beta 1$ expression was variable but appeared more robust in human SCLC compared to NSCLC. In addition, LLC1, a mouse model cell line for lung carcinoma, also expressed integrin $\alpha 9\beta 1$. Compared to normal bronchial epithelium (NHBE), these cancer cell lines showed evidence of EMT, as measured by E and N-cadherin expression. However, those cells expressing $\alpha 9\beta 1$ demonstrated relatively less expression of E-cadherin and higher expression of N-cadherin and the extent of colony formation by these cancer cells on soft agar was dependent on the extent of $\alpha 9\beta 1$ expression (Suppl. Fig S6-A,B). Integrin $\alpha 9\beta 1$ appears to induce a functionally relevant EMT phenotype in these lung cancer cells as demonstrated by increased $\alpha 9\beta 1$ -dependent adhesion (Fig 4C), migration (Fig 4D) and Matrigel invasion (Fig 4E). Although the overall extent of the response varied, they were mostly proportional to the magnitude of $\alpha 9\beta 1$ expression.

$\alpha 9\beta 1$ -associated EMT was dependent on the expression level of the integrin. The change in expression of E-cadherin and vimentin following $\alpha 9\beta 1$ activation was comparable to that induced by TGF- β in high $\alpha 9\beta 1$ -expressing LLC-1, intermediate in the minimally expressing A549, while NHBE or H69 cells showed no significant change in expression of E-cadherin and vimentin (Fig 4F). Taken together these findings support a physiologically relevant role for endogenous $\alpha 9\beta 1$ in the induction of EMT in human cancer cells in vitro.

Suppression of $\alpha 9$ expression decreases tumor growth and metastasis

Having shown that endogenously expressed $\alpha 9\beta 1$ confers an EMT phenotype and more aggressive cancer attributes, we next assessed EMT when $\alpha 9$ expression was suppressed using $\alpha 9$ -targeted lentiviral shRNA (Fig 5A). As expected $\alpha 9$ depleted LLC-1 demonstrated significantly decreased adhesion on TfnRAA (Fig 5B) whereas cell adhesion to pFN, a non- $\alpha 9\beta 1$ ligand, was not affected. Similarly, migration and invasion (Fig 5C, D) were significantly decreased in cells expressing $\alpha 9$ -targeted shRNA suggesting an inhibitory effect on EMT.

This inhibition of adhesion, migration and invasion was accompanied by changes in EMT markers. Compared to control LLC-1, $\alpha 9$ -depleted cells grown on $\alpha 9\beta 1$ -specific ligand, demonstrated neither a decrease in E-cadherin nor an increase in vimentin, α SMA or Snail, suggesting that the process of $\alpha 9\beta 1$ induced EMT was blocked (Fig 5E). Furthermore, the $\alpha 9\beta 1$ specific ligand, TfnRAA, did not significantly activate the pro-EMT signaling intermediates, Src or β -catenin. Taken together these findings provide further support for $\alpha 9\beta 1$ inducing physiologically relevant EMT through src signaling.

The relevance of $\alpha 9\beta 1$ -associated EMT in vitro was confirmed by in vivo tumor studies using shRNA to deplete LLC1 cells of $\alpha 9$. Fig 5F demonstrates that tumors formed by $\alpha 9$ -depleted cells (shRNA- $\alpha 9$), were at least half the size of those formed from cells with endogenous expression of $\alpha 9\beta 1$ (shRNA-Cont). Furthermore, cancer cell metastasis was

negligible in mice injected with $\alpha 9$ -depleted LLC-1 compared to those injected with cells expressing $\alpha 9\beta 1$ (Fig 5G).

High expression of integrin $\alpha 9\beta 1$ in human small cell lung cancer (SCLC) is a prognostic indicator of poor survival

Based on our in vitro and in vivo data, we postulated that the extent of $\alpha 9\beta 1$ expression in SCLC might correlate with the severity of disease. We analyzed SCLC tissue from fifteen patients and found variable $\alpha 9\beta 1$ expression both by immunoblot (Fig 6A) and RT-PCR (data not shown). Tumors from three separate patients, were also used for immunohistochemistry which confirmed the expression of $\alpha 9\beta 1$ on the surface of SCLC cells (Fig 6B). Subsequently, we were able to separate patients based on the extent of $\alpha 9\beta 1$ expression in their tumor tissue: eleven high expressers and four low expressers. Fig 6C tables the characteristics of the patients in relation to the degree of $\alpha 9\beta 1$ activity and is highlighted by significant differences in stage of disease and survival time. The average survival for patients with high $\alpha 9\beta 1$ was 19 mo compared to 30 mo in patients with low $\alpha 9\beta 1$ expression ($p < 0.001$), (Fig 6D).

Discussion

The cellular mechanisms that induce EMT are complex^{3,5,26} and a role of integrins has been well established under the paradigm of TGF- β induced EMT.³³ In contrast, our findings show that in cancer cells, $\alpha 9\beta 1$ expressed exogenously or endogenously can trigger EMT, in a TGF- β independent fashion and that expression of $\alpha 9\beta 1$ and its pro-EMT effects are not modulated by TGF- β . These results suggest there is at least one distinct integrin pathway regulating EMT; which is not surprising since tumor stroma provides a rich supply of integrin ligands.³⁴ Although we did not examine expression of all integrins, our findings also suggest that $\alpha 9\beta 1$ does not require or promote the increased expression of other known pro-EMT integrins such as $\alpha v\beta 6$ or $\alpha 3\beta 1$. Other transmembrane receptors are known to partner with integrins to induce EMT without a growth factor stimulus⁷ such as the serine protease TMPRSS4, which requires the synergistic action of active integrin $\alpha 5\beta 1$ (ref. 35). Whether $\alpha 9\beta 1$ functions in concert with other transmembrane proteins to facilitate EMT is yet to be determined.

Integrin $\alpha 9\beta 1$ also distinguishes itself from other integrins such as $\alpha 5\beta 1$ and $\alpha 3\beta 1$ since its pro-EMT effect is transduced without a requirement for a change in expression. Following ras transformation of mammary epithelial cells the resulting increased expression of $\alpha 5\beta 1$ does not initiate EMT,¹⁵ and expression of $\alpha 3\beta 1$ alone is not sufficient to induce EMT as suggested by $\alpha 3$ -null alveolar cells which express similar amounts of E-cadherin to $\alpha 3$ expressing cells.¹⁴ Although TGF- β does induce increased expression of $\alpha v\beta 6$, it is unknown whether the exogenous expression of $\alpha v\beta 6$ might itself induce EMT. However, this would seem unlikely since the function of increased $\alpha v\beta 6$ is to activate autocrine derived TGF- β .¹³ Whether integrin $\alpha 9\beta 1$ induces autocrine TGF- β production and presumably activation of TGF- β receptor is yet to be determined but seems unlikely since we found no significant SMAD2 activation under our experimental conditions.

Our findings implicate src as a signaling intermediate for $\alpha 9\beta 1$ -associated EMT acting by facilitating disassociation of E-cadherin and β -catenin, which is consistent with previous reports showing src induced effects on the cytoskeleton and E-cadherin/ β -catenin.²⁹ The relevance of these molecular effects was confirmed by the inhibition of cancer cell migration and invasion, both functional EMT effects. In human carcinomas the role of src is well established with more than half of lung carcinomas demonstrating aberrantly activated src.³⁶ Further cancer research should focus on determining whether co-expression of $\alpha 9\beta 1$ directly contributes to the deregulation of src activity or if $\alpha 9\beta 1$ can induce EMT in cells lacking activated src.

The importance of $\alpha 9\beta 1$ in human cancer is supported by reports of its expression in breast, brain and lung cancer.^{18,37,38} We show for the first time a clinical relevance for high expression of $\alpha 9\beta 1$ in SCLC namely a decreased patient survival by 11 months. These findings are tempered by the low number of patients included in our study which reflects clinical practice in which few patients undergo surgical procedures for diagnosis or treatment. This notwithstanding, our results suggest that future research should focus on confirming these findings. Taken together with our previous work it appears $\alpha 9\beta 1$ may promote the cancer process through multiple mechanisms including effects on cell migration and invasion, induction of nitric oxide production,²⁸ angiogenesis²⁴ and lymphangiogenesis^{20,22} and now promotion of EMT. Our findings present cancer researchers with novel approaches to not only deciphering the carcinogenic trigger but also to better understand the interplay between the seemingly separate EMT mechanisms of TGF- β and integrin $\alpha 9\beta 1$. Furthermore, our findings highlight the need for developing novel therapies targeting more than one pathogenic pathway and identify integrin $\alpha 9\beta 1$ as a strong candidate for pharmacotherapy in human cancer.

MATERIALS AND METHODS

Materials

Mouse monoclonal antibodies to $\alpha 9\beta 1$: Y9A2, NC4, polyclonal anti $\alpha 9$ -serum and TNfnRAA (gift from Dr. Dean Sheppard, UCSF); mAb against murine $\alpha 9\beta 1$ (R&D), β -Actin and GAPDH (Sigma), E-cadherin, N-cadherin and β -catenin (BD Biosciences), β -catenin-Y654 (Invitrogen). Rabbit polyclonal antibodies: Src-Y416, SMAD2-ser245/250/255, Src, vimentin, Snail, HDAC1, Erk2 and LRP6 (Cell Signaling), anti $\alpha 3\beta 1$, $\alpha v\beta 6$, anti-SMAD2 (Millipore); anti- α SMA (Santa Cruz Biotechnology), anti-CD31 (Dako), anti-podoplanin (D2-40, Covance). Phycoerythrin conjugated goat anti-mouse (Jackson Immunoresearch); Horseradish peroxidase conjugated anti-mouse and anti-rabbit antibodies (Amersham Biosciences). Plasma fibronectin (Chemicon), wnt3A and Dkk1 (R&D); TGF β (Dr. E. Leof, Mayo Clinic), src inhibitor, PP1 (BioMol International), VLO5 (Dr. Cezary Marcinkiewicz, Temple University).

Cell culture

SW480- $\alpha 9$ or mock cells were cultured as previously described.^{24,28} A549 and Lewis lung carcinoma cells (LLC-1) cultured in F12K/DMEM medium with 10% FBS. All other human

lung carcinoma cell lines (ATCC) cultured in RPMI with 10% FBS. Primary NHBE cells: specialized medium (Lonza).

siRNA, plasmids and cell transfection

Integrin α 9-EGFP-N3 expression vector (Addgene, Cambridge-MA), siRNA oligonucleotides, negative control (Ambion) and siRNA to human β -Catenin, c-Src (Santa Cruz Biotechnology) and LRP6 (Cell Signaling) performed as described previously.²⁸ Lentivirus packaging; ViraPower Lentiviral Expression System (Invitrogen, Life Technologies). Using Lipofectamine-2000, 293FT cells were co-transfected with ViraPower DNA mix and plasmid (pLKO.1-puro) encoding shRNA targeting murine α 9-subunit (Mayo Clinic RNA Resource) and supernatants collected 48-72 h after transfection. LLC-1 cells were transduced in the presence of 6 μ g/mL polybrene (Sigma-Aldrich), and stable clones selected in 10 μ g/mL puromycin.

Immunoprecipitation, SDS-PAGE and immunoblot analysis

Serum starved cells were trypsinized, washed with PBS and suspended in DMEM. In a 6-well plate, 1×10^6 cells were added to wells coated or not with 10 μ g/ml TnfrAA or pFN, incubated for various times and lysed in RIPA buffer as previously described.²⁸ Protein lysates from fresh frozen tissue ~50mg were obtained by pulverizing in RIPA buffer. After 20 min incubation on ice, lysates were cleared by centrifugation and immunoprecipitation and immunoblotting performed as previously described.²⁸

To detect nuclear translocation of β -catenin, nuclear and cytoplasmic proteins were extracted using 'NE-PER Extraction Reagents (Thermo Scientific) and immunoblotted for β -catenin. Extraction purity was determined by immunoblotting for Erk2 and HDAC1.

Fluorescence microscopy

Cells cultured on sterile glass cover slips were washed with PBS, fixed, and incubated with antibody in 5% normal goat serum (Upstate) at 4°C overnight. Cells were washed, incubated with anti mouse Alexa Fluor-594 (or -488) antibody (BD Biosciences) for 1 hr. The cytoskeleton of fixed cells was immunostained with Texas-Red phalloidin and coverslips mounted with Slowfade containing DAPI (Invitrogen). Images were acquired as previously described.²⁸ Co-localization of integrin α 9 β 1 with β -catenin (or E-cadherin) was determined using appropriate primary antibodies followed by incubation with Alexa-Fluor labeled secondary antibodies. Immunostained cells were analyzed by confocal microscopy (Zeiss/SM510); 100x oil DIC objective with ~0.5 μ m optical sectioning.

Flow cytometry

Single cell suspensions were rinsed and suspended in PBS at a density of $0.5-1 \times 10^6$ per ml, incubated with appropriate primary antibody for 30 min. Subsequent staining with phycoerythrin conjugated secondary antibody, acquisition and data analysis was performed as previously described.²⁸

Cell adhesion assays

Cell adhesion assays were performed as previously described²⁸ in flat bottom 96 well microtiter plates (ICN, Linbro/Titertek) coated with 10 µg/ml TnfnRAA or FN at 37°C for 1 hr, blocked with 1% BSA (Sigma) for 30 min. 5×10^4 cells suspended in basal medium were plated in each well and when indicated incubated with $\alpha 9\beta 1$ inhibitors; Y9A2 (20 µg/ml) or 50 µM VLO5 for 30 min.

Cell migration and invasion assays

Cell migration assays were performed as previously described.^{24,28} Haptotaxis: 10 µg/ml of TnfnRAA or FN for 1 hr at 37°C; chemotaxis: medium with 10% FBS. 5×10^4 cells were added to the top chamber, with 30min inhibitor incubation. Migration time of 12-16 hrs at 37°C. Invasion assays were performed using BD BioCoat (8 µm pore size) Matrigel chambers (Becton Dickinson-BD) as per the manufacturer's instructions. Briefly, cells treated with or without inhibitors were placed in the top chamber and 10% FBS for chemoattractant. After 14-16-hrs (A549 and LLC-1) or 24-36 hrs (SW480, SCLC lines), invaded cells were fixed, stained (Hema-3 kit, Fisher Scientific) and counted in four separate view fields by light microscopy (Axiostar, Zeiss).

Cell proliferation assay

2×10^3 SW480-mock or SW480- $\alpha 9$ cells in full growth medium were added to 12-well plates (Costar), trypsinized and counted using a hemocytometer. For BrdU assays cells were grown overnight in 96-well plates, in the absence or presence of 50 µM VLO5 for 16 hrs. Medium containing BrdU was added for 2 hrs and uptake measured with ELISA (RPN 250, Amersham Biosciences) as per the manufacturer's instructions.

Soft agar assay

To prevent cell settling, bottom plugs (0.5 mL, 0.8% Sea Plaque-agarose, 10% FBS/DMEM; FMC Bioproducts) were casted in 12-well plates (Costar). Top plugs (1 mL, 0.4% agarose, 10% FBS/DMEM), and 10^3 cells with or without 50 µM VLO5 were incubated for 14 d at 37°C. The number of colonies >50 µm diameter were scored using Gelcount (Oxford Optronics).

Animal tumor studies

All experiments were IACUC approved and animals cared for in accordance with IACUC guidelines. 1×10^6 SW480-mock or $\alpha 9$ cells were injected subcutaneously in the flank of Nude mice (n=5). After 35 days, tumor volume was measured ($l \times w \times h / 2$). To study metastasis, cells were injected through the tail vein and on day 45 tissues harvested for histology. In other experiments 0.5×10^6 LLC-1 cells were transduced with lentivirus carrying control or $\alpha 9$ -specific shRNA were injected subcutaneously in the flank (n=5) or tail vein (n=3) of nude mice.

Human lung cancer tissues

The Mayo Clinic IRB approved all human studies. Fresh frozen lung cancer specimens were from the Mayo Clinic Lung Cancer Specimen Registry. Fifteen SCLC samples were

analyzed for expression of $\alpha 9\beta 1$ and patient clinical characteristics. Histology was verified by a Board Certified lung pathologist (MCA). The patient clinical data was analyzed in a blinded fashion (BPW). Survival (months) was from diagnosis to death.

Histology and Immunohistochemistry

Mouse tissue was fixed in 10% formalin, embedded in paraffin and 5 μm sections stained with H&E or immunostained for PECAM1 (CD31) or podoplanin (D2-40). 5 Human SCLC samples and matched normal lung were immunostained with $\alpha 9\beta 1$ specific rabbit antibody (1:250) overnight at 4°C followed by biotinylated goat anti-rabbit secondary antibody (1:200), streptavidin-peroxidase (1:100), then 3,3'-diaminobenzidine (Amresco) and peroxide. Slides were rinsed, counterstained with hematoxylin, dehydrated and mounted. Images were obtained by scanning laser microscopy (Axiostar, Zeiss) or with NanoZoomer Digital Pathology software (Hamamatsu).

Statistical methods

Unless otherwise indicated data presented as mean \pm SE from 3 or more experiments; p values determined using paired student's T-tests. Immunoblots were performed at least 3 times and representative examples presented. Statistical analysis of patient survival and clinical characteristics was performed using multivariate analysis with a p-value <0.05 .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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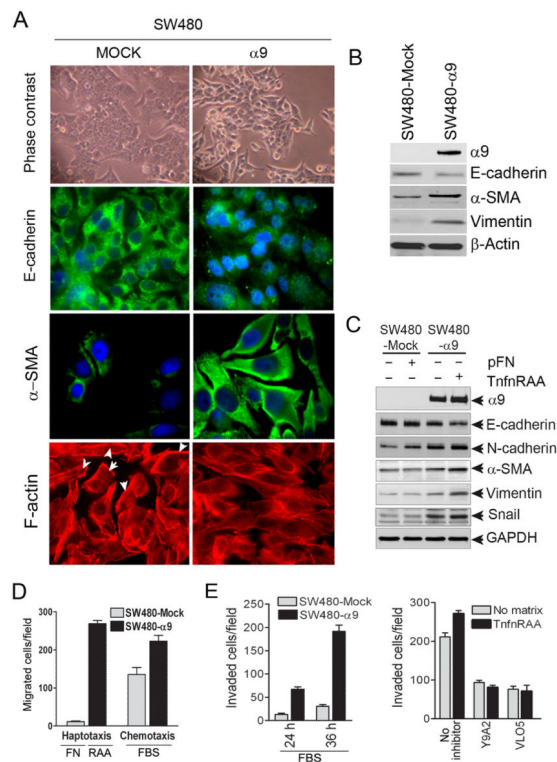


Figure-1. $\alpha 9\beta 1$ promotes phenotypic and functional changes of EMT

A, Top: Phase contrast photomicrographs comparing SW480-Mock and SW480- $\alpha 9$ cells following 36h of serum depletion; 2nd-4th panels: immunofluorescent photomicrographs of SW480-Mock and $\alpha 9$ cells for E-cadherin, α -SMA or cytoskeletal F-actin. Cell nuclei stained blue with DAPI. B, Immunoblots of SW480-mock and $\alpha 9$ lysates for $\alpha 9$ and markers of EMT: E-cadherin, α -SMA and vimentin. C, Immunoblots of SW480-mock and $\alpha 9$ lysates grown in the absence or presence of activating matrix, as indicated, demonstrating the relative expression of the $\alpha 9$ integrin subunit and markers of EMT: E-cadherin, N-cadherin, α -SMA, vimentin and Snail. D, Chemotaxis and haptotaxis assays with activating matrices: pFN or TnfnRAA; using SW480-mock (grey) or SW480- $\alpha 9$ (black). E, Invasion assays: Left panel, SW480-mock or $\alpha 9$ cells after 24h or 36h growth; Right panel, SW480- $\alpha 9$ cells in absence (grey) or presence of TnfnRAA (black), with or without $\alpha 9\beta 1$ inhibitors Y9A2 or VLO5.

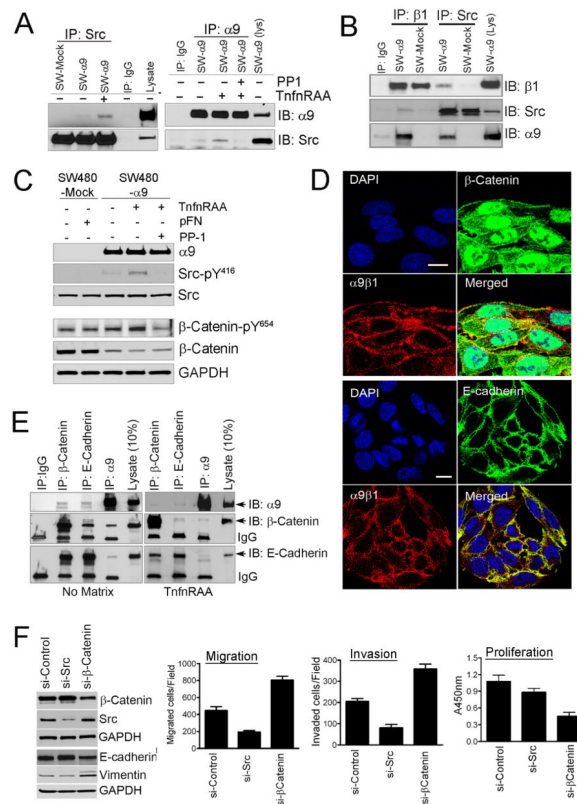


Figure-2. $\alpha 9\beta 1$ co-associates with E-cadherin and β -catenin inducing EMT through src signaling
 A, Immunoblots for $\alpha 9$ or src from immunoprecipitates of SW480 cells cultured with or without the $\alpha 9\beta 1$ specific ligand, TnfrRAA and/or the src inhibitor, PP1. B, Immunoblots for $\beta 1$, $\alpha 9$ or Src from immunoprecipitates of matrix activated SW-480-mock (pFN) or SW480- $\alpha 9$ cells (TnfrRAA). C, Immunoblots for $\alpha 9$, Src or β -catenin in lysates from SW480-mock or $\alpha 9$ cells grown with pFN or TnfrRAA and/or PP1. D, Confocal images of SW480- $\alpha 9$ cells to determine co-localization of $\alpha 9\beta 1$ (red) with β -catenin (green, top panel) or E-cadherin (green, bottom panel); nuclei stained blue with DAPI. E, Immunoblots, of immunoprecipitates from SW480- $\alpha 9$ grown on plastic (no matrix) or TnfrRAA (right panel), to determine co-association of $\alpha 9$ with β -catenin or E-cadherin. F, Left panel, Immunoblots for β -catenin, src, E-cadherin or vimentin in lysates from SW480- $\alpha 9$ cells transfected with non-targeted siRNA or siRNA targeted to src or β -catenin; Right panel: Migration (left), invasion (middle) or proliferation (right) assays in SW480- $\alpha 9$ cells following transfection with siRNA as indicated.

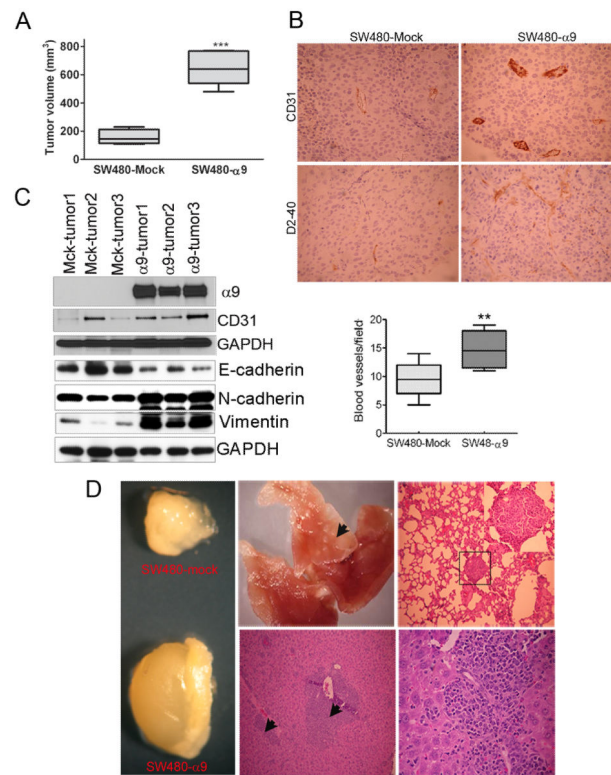


Figure-3. $\alpha 9\beta 1$ is associated with increased tumor growth, EMT and cancer metastasis

A, Volume of mouse flank tumors formed following subcutaneous injection of SW480-mock (n=5) or SW480- $\alpha 9$ (n=5) cells. B, Top panel: representative photomicrographs of tumor sections stained for the endothelial cell marker CD31 or the lymphatic marker, D2-40; Bottom panel: quantitative analysis of intra-tumoral blood vessels counted in 1 view field (10X) (n=3 per cell type). C, Immunoblots showing relative expression of $\alpha 9$, CD31, EMT-associated cadherin proteins and vimentin in 3 separate tumors derived from SW480-mock or $\alpha 9$ cells. D, Left panel: representative images of lymph nodes from mice injected with SW480-mock or $\alpha 9$ cells; Right top panels: photomicrographs showing lung metastases grossly (arrow) or H&E stained; Bottom right panels: H&E stained liver sections demonstrating metastases at low (40x) and high power (400X).

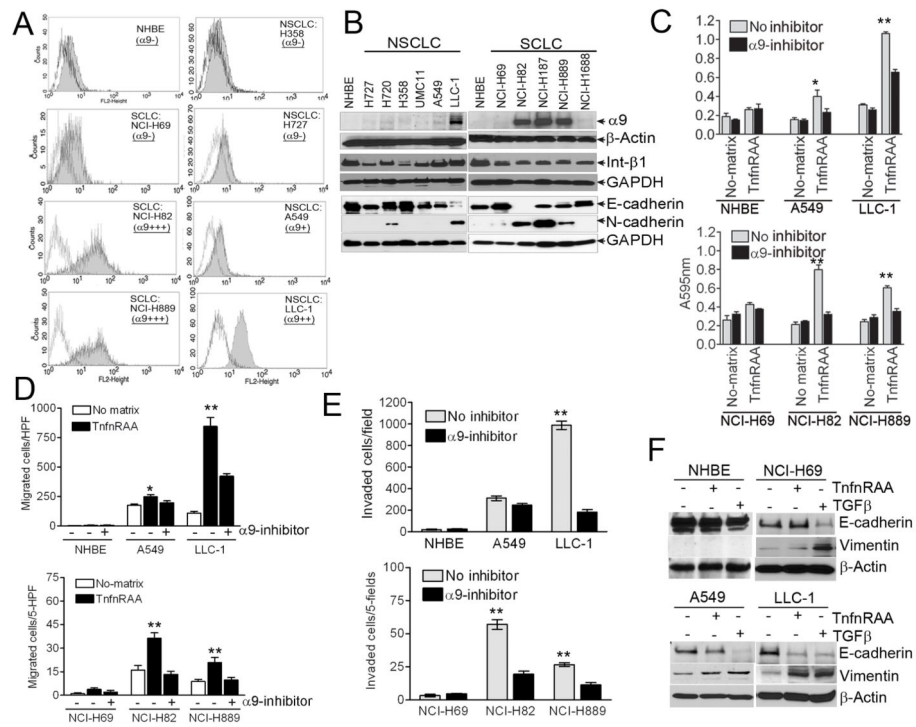


Figure-4. $\alpha 9\beta 1$ induces cellular changes of EMT and enhanced cancer cell migration and invasion

A, Flow cytometry analysis showing the extent of $\alpha 9\beta 1$ surface expression (shaded histograms) on cells as indicated; and isotype antibody control (clear histogram). B, Immunoblots for $\alpha 9$, $\beta 1$, E-cadherin or N-cadherin in lysates from mouse (LLC-1) or human NSCLC and SCLC cell lines. C, Cell adhesion on plastic (no matrix) or TnfnRAA with (black) or without (grey) $\alpha 9\beta 1$ inhibitor, VLO5. D, Cell migration in the presence (black) or absence (grey) of TnfnRAA and/or VLO5. E, Cell invasion using cells stimulated by TnfnRAA and treated with (black) or without (grey) VLO5. F, Immunoblots to detect the EMT markers E-cadherin and vimentin in lysates from cells grown on plastic (no matrix) or TnfnRAA or with TGF- β .

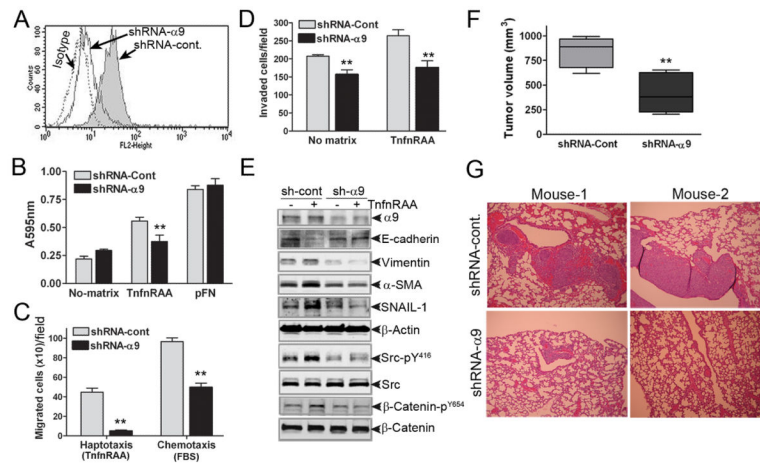


Figure-5. $\alpha 9\beta 1$ -induced malignant tumor growth and metastasis is inhibited following $\alpha 9$ knockdown

A, Flow cytometry for expression of $\alpha 9\beta 1$ in LLC-1 cells transduced with non-targeted (shRNA-Cont) or $\alpha 9\beta 1$ -targeted (shRNA- $\alpha 9$) shRNA. B, Cell adhesion C, Haptotaxis (using $\alpha 9\beta 1$ -specific ligand, TfnfRAA) and chemotaxis (using 10% FBS) and D, Cell invasion assays in shRNA-Cont (grey bars) or shRNA- $\alpha 9$ (black bars) LLC-1 grown on plastic (no matrix) or TfnfRAA or pFN as indicated. E, Immunoblots to detect EMT and signaling proteins as indicated in lysates from LLC-1 cells expressing shRNA-cont (grey) or shRNA- $\alpha 9$, and grown with (+) or without (-) TfnfRAA. F, Volume of mouse flank tumors from subcutaneous injection of LLC-1 cells expressing shRNA-Cont (grey) or shRNA- $\alpha 9$ (black) ** $p < 0.01$. G, Representative photomicrographs of H&E stained lung (2 mice) showing metastatic lesions derived from LLC-1 cells expressing shRNA-cont or shRNA- $\alpha 9$.

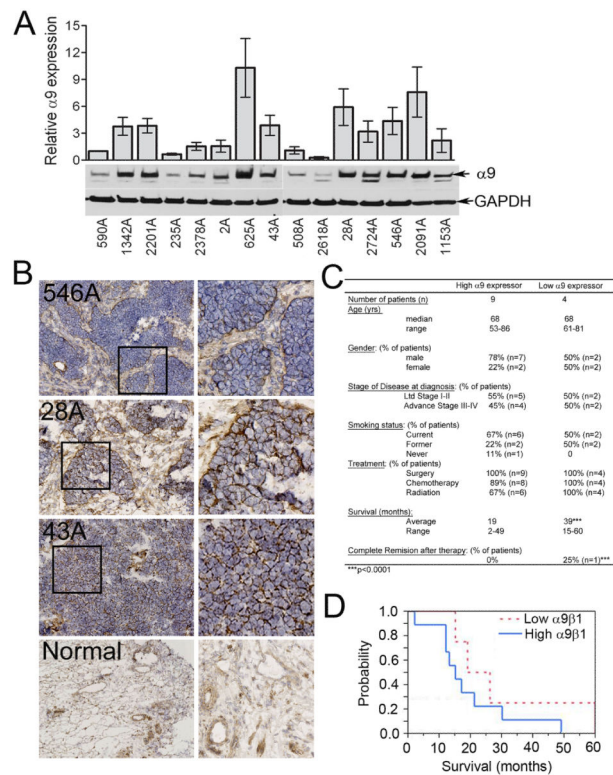


Figure-6. High expression of $\alpha 9\beta 1$ in human SCLC predicts shorter patient survival

A, Immunoblot of lysates from fresh frozen SCLC samples for detection of $\alpha 9\beta 1$; densitometric quantification from 3 separate immunoblots. B, Representative photomicrographs (20X) of SCLC samples (3 patients) stained to detect $\alpha 9\beta 1$. Bottom panels are normal lung controls. C, Patient characteristics of tumors with high or low $\alpha 9\beta 1$ expression. D, Kaplan-Meier plot comparing survival in patients with high or low $\alpha 9\beta 1$ expression.