EFFECT OF INTEGRIN α5 ON CANCER CELL MOTILITY IN 2D AND 3D MATRICES

by
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ABSTRACT

The main cause of cancer related deaths is results from metastasis of cancer, or the spread of cancer to secondary sites. In order to metastasize, cancer cell migration is necessary. Numerous studies in cancer biology have examined mechanisms of cancer cell migration possible target molecules to prevent metastasis of cancer cells. During the early stages of metastasis, cancer cells penetrate through the basement membrane and invade into the extracellular matrix (ECM). During cell migration, integrins which are transmembrane receptors bind to extracellular matrix (ECM) molecules which mediate cell attachment and the formation of focal adhesions. Among many integrins, integrin α5β1 is often overexpressed in many cancer cells.

We utilized 2D and 3D assays with an APRW model analysis to investigate the effect of downregulation of integrin α5 on cell motility using the metastatic breast cancer cell line, MDA-MB-231. We decreased integrin α5 expression using shRNA. Cells were plated on top of fibronectin, fibrinogen and collagen 2D substrates. Then, they were also embedded in a 3D collagen matrix and 3D collagen matrices with fibronectin. 2D and 3D cell movements were analyzed using an APRW model, saying cell movements are highly anisotropic. Furthermore, we performed focal adhesion staining on 2D cells to test correlation with cell migration

Comparing 2D and 3D cell motility, we were able to observe integrin α5 had a remarkable effect on cell motility for 3D but showed less of an effect for 2D. We tested the correlation between 2D motility and focal adhesion and concluded that focal adhesion is not a predictor for 2D migration. With an increasing amount of fibronectin in 3D collagen matrices, the cell migration has decreased possibly due to the gel structure alteration. By investigating different parameters for cell motilities, such as diffusivity and

persistence time, we were able to test effect of downregulation of integrin α5 on cell motility in 3D. Finally, the APRW model provided better characterization of cell movement than measuring cell velocities from cell trajectories data. Downregulation of integrin α5 does not alter cell speed but decreases diffusivity and persistence of metastatic cancer cells.

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DEFINITION OF SYMBOLS

APRW	Anisotropic persistent random walk
ECM	Extracellular Matrix
D	Diffusivity
FN	Fibronectin
KD	Knock down
MMP	Matrix Metalloproteinase
MSD	Mean squared displacement
NTC	Non target control
P	Persistence time
PI3K	Phosphatidylinositide 3-kinase
PRW	Persistent random walk
S	Speed of cell movement

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INTRODUCTION

Metastasis overview

Metastasis is responsible for most cancer deaths. Primary tumors are often successfully removed by surgery or treated by irradiation. However, once tumor cells disseminate to other organs, it is very difficult to treat and ultimately leads to death. Moreover, for many patients, metastasis will occur years or even decades after initial diagnosis and primary treatment (Chambers et al., 2002). Multimodal therapy, combination of systemic therapy, surgery and radiotherapy, is used to treat cancers. Initial treatment of cancer often includes using chemotherapy and/or targeted agents interfering with selected signaling pathways (Tryfonidis et al., 2015).

Metastasis involves migration and proliferation processes (Gupta et al., 2010). Initially, cancer cell proliferation is essential at primary tumor sites. Then, cancer cells gain the ability to degrade, remodel and migrate through the extracellular matrix following penetration through the basement membrane. Once cells invade through the basement membrane and extracellular matrix, cancer cells enter the bloodstream and travel to distant sites via the vascular and lymphatic systems. Then, they colonize at secondary tumor sites which is the final stage of metastasis (Guo et al., 2004) (see Figure 1).

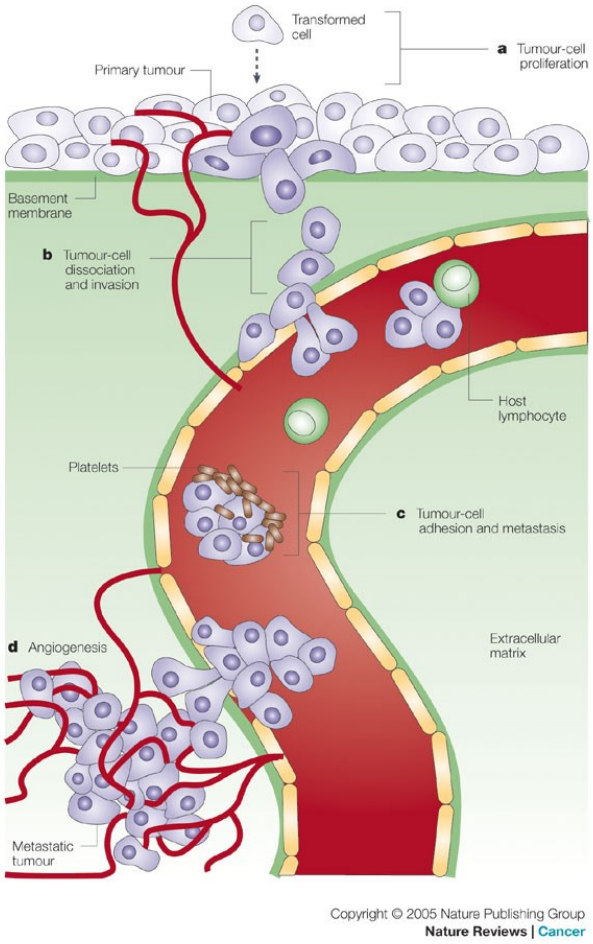

Figure 1: Stages of metastasis. (A) Cancer cells grow at primary site with altered adhesion capacity onto surrounding cells and their microenvironment. (B) Once they acquire migratory ability, they penetrate through the basal membrane and invade the extracellular matrix. (C) Cancer cells enter and circulate blood stream as a form of small aggregates. (D) Then, they exit bloodstream and undergo local expansion via angiogenesis. (Fuster et al., 2005)

During metastasis, migration is a crucial process for cells to invade through the extracellular matrix (Yamazaki et al., 2005). Alteration of adhesion capacity to extracellular matrix enables migration and invasion (Hood et al., 2002). Among several factors for migration, changes in integrin expression occur to loosen the adhesion or to foster cancer cells' migration and proliferation during migration (Hood et al., 2002).

Cell migration process

During active migration, cells form actin dependent protrusions to pull themselves forward. They first extend filopodia, finger like structures filled with parallel f-actin and then merge into lamellipodia, branched networks of actin, when adhering to the ECM (Mattila et al., 2008). In order to initiate migration, Rac and Cdc42 activation is by phosphatidylinositide 3-kinase (PI3K) is required. PI3K plays a major role in the amplification of internal signaling asymmetry and induces cell polarity to promote directional cell movements (Yamazaki et al., 2005). Rac generates protrusive forces by local polymerization of actin. Cdc42, which can regulate direction of migration, induces actin polymerization to generate filopodia (Raftopoulou et al., 2004). Then, using clusters of integrins such as integrins with β1 and integrin αvβ3 (Desgrosellier et al. 2010), cells attach onto the extracellular matrix (ECM). Formation of focal adhesion within the lamellipodia occurs when cells make contact on ECM (Guo et al., 2004). Rho is associated with focal adhesion assembly and contractility. It is also responsible for cell body contraction by targeting actin: myosin filament assembly (Raftopoulou et al., 2004). Thus, through rho activation, stress fibers composed of actin and myosin at rear sites pull the nucleus and cell body forward. Rho is involved in contraction and retraction forces. Rac inhibits rho activity at the leading edge of migrating cells. Finally, adhesions at the

back end of cells are released by several mechanisms involving simple dissociation of integrins or by fracturing the cell-ECM linkage (see Figure 2) (Hood et al., 2002).

Cancer cells have different types of migration. When cell-cell junctions are present, epithelial cancer cells move as sheet like structures showing collective behavior during wound healing or angiogenesis (Yamazaki et al., 2005). When they lose cell to cell adhesion by suppressing E-cadherin, a cell-to-cell junction protein, they move as single cells. Single cell migrations show two types of morphology. First, mesenchymal migration depends on integrin mediated adhesion where cells display an elongated morphology. For mesenchymal migration, cancer cells need to degrade ECM. Thus, cancer cells often upregulate matrix metalloproteinases (MMPs) and cathepsins, which are ECM-degrading enzymes. MMPs are also accumulated in an integrin dependent manner. They remodel surrounding ECMs at the leading edge of migratory cells and help elongated cells form a path and overcoming tissue barriers (Friedl et al., 2003; Yamazaki et al., 2005). On the other hand, amoeboid migration where cells maintain a rounded morphology is not dependent on integrin function. Since cell-ECM adhesion is weak, cancer cells keep their round morphology and squeeze into gaps in the ECM while migrating. Cancer cells are able to shift their migration pattern from mesenchymal to amoeboid types. Thus, to regulate cancer cell movements, both types of migration need to be suppressed (Yamazaki et al., 2005).

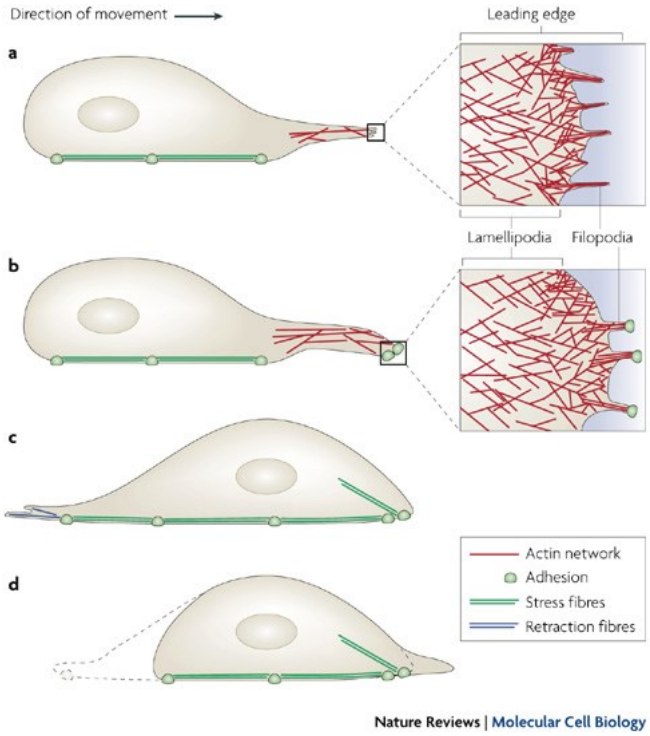

Figure 2: Steps of cell migration. (A) Migrating cells extend lamellipodia and filopodia in the direction of migration. (B) Focal adhesion by integrins formed by binding to ECM. (C) Stress fiber contracts to pull cell body and nucleus forward. (D) Cells break adhesion at trailing edge of cells. (Mattila et al., 2008)

Integrins in cancer cells

Cancer cells require integrin mediated adhesion for their directed migration (Yamazaki et al., 2005). Integrins are heterodimeric transmembrane receptors for ECM molecules expressed on many cell types. There are 24 distinct integrin heterodimers expressed in mammals and formed by a combination of 18 α subunits and 8 β subunits (see Figure 3). Integrins directly bind components of the ECM and provide the traction necessary for cell motility and invasion (Desgrosellier et al. 2010). From direct interaction with the ECM, integrins sense the tumor microenvironment and regulate

intracellular signaling and cellular responses including proliferation, migration, invasion and differentiation. Ligands for integrin include fibronectin, fibrinogen, vitronectin, collagen and laminin which are all components of ECM. As integrins form adhesion necessary for migration, they cluster into focal contacts containing many different actin-associated proteins, such as α-actinin, vinculin, tensin and paxillin connecting integrins and the cytoskeleton (Hood et al. 2002).

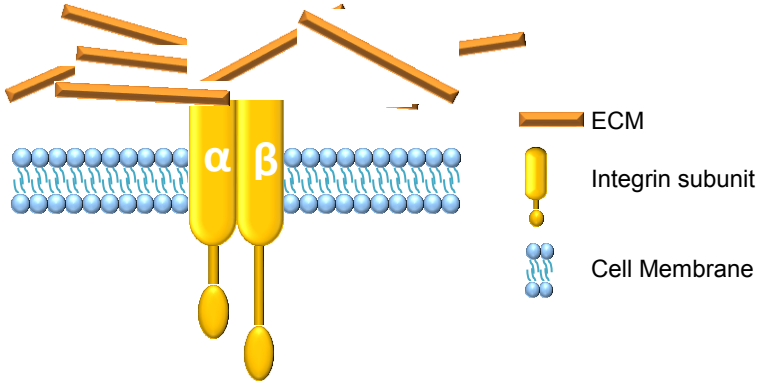

Figure 3: Schematic of integrin. Integrin is a transmembrane heterodimeric receptor directly binds to ECM.

Cancer cells often show drastic alterations in expression levels of integrin and integrin affinity for ECM substrates. For example, most adult epithelial cells show very low expression levels of integrins αvβ3, α5β1 and αvβ6 whereas tumor cells often upregulate expressions of them (Hood et al. 2002). Integrin αvβ3 binds several ECM components like fibronectin, fibrinogen, von Willebrand factor, vitronectin and proteolysed forms of collagen and laminin and is upregulated in invasive melanoma cells and angiogenic blood vessels. Inducing αv or β3 subunit expression showed highly increased metastatic potential for melanoma cells in mice or chick embryo model, respectively (Felding-Habermann et al., 1992; Hood et al., 2002). Integrin α6β4, a

laminin-binding receptor, is not expressed in normal thyroid cells but increased expression is positively correlated to invasion of thyroid carcinoma. Some integrins show decreased expression level in tumor cells (Hood et al., 2002). Integrin α2β1 had lower expression level, possibly inducing dissemination of cancer cells (Kren et al., 2007). Re-expression of integrin α2β1 in breast cancer cells altered malignant properties of breast cancer cells (Desgrosellier et al., 2010).

Among many integrins, integrin α5β1 is often overexpressed in many cancers including colon, ovarian, lung, and breast cancer, as well as in melanomas and gliomas (Schaffner et al., 2013). Previous studies have shown an effect of integrin α5β1 expression level on cancer metastasis. Using a mouse model of ovarian cancer cells which metastasize to kidneys, one study showed that cells overexpressing integrin α5 metastasized more than cells expressing native levels of integrin α5 or cells with expressing a defective integrin α5 mutant (Tani et al., 2002). Furthermore, from node-negative non-small cell lung cancer patients, overexpression of integrin α5 was observed in 50% of patients and integrin α5 overexpressing tumors may serve as a marker of potential micro metastasis (Masashi et al., 2000). However, the effect of integrin α5β1 on cancer is controversial as it has shown both tumor suppressive and protumoral behavior. Overexpression of integrin α5β1 has been shown to negatively regulate colon cancer cell growth (Schmidt et al., 1998). However, other studies have shown that upregulation of α5 integrin subunit caused upregulation of cell invasion (Nam et al., 2012). Furthermore, suppression of integrin α5β1 by lunasin, a peptide having an RGD motif that integrin α5β1 can bind, prevented the outgrowth of colon cancer cells (Dia et al., 2011). This study showed the effect of lunasin on colon cancer metastasis by studying human colon

cancer cell lines *in vitro* and a liver metastasis mice model *in vivo*. The study observed that lunasin internalized into nucleus by immunofluorescence microscopy and it interacted with integrin α5β1 in human colon cancer cells through co-immunoprecipitation of lunasin-integrin α5β1. Furthermore, in mice models, lunasin treated mice injected with human colon cancer cells exhibited decreased liver metastasis. Similar to colon cancer, initial study on integrin α5β1 in breast cancer showed a tumor suppressive effect (Seftor et al., 1998). This study tested the effect of Maspin, a serine protease inhibitor/non-inhibitor superfamily, on a highly invasive breast cancer cell line, MDA-MB-435. Maspin suppressed the invasive phenotype of MDA-MB-435 in invasion assays performed with MICS chamber pore coated with fibronectin. Treatment of Maspin also exhibited increased expression of integrin α5, αv and β1 at both mRNA and protein level indicating effect of increased expression of integrin on cell invasion (Seftor et al., 1998). However, later studies challenged this result. Invasive breast cancer cell, MDA-MB-231, with a higher expression level of integrin α5β1 showed increased invasion into 3D collagen matrix through enhanced contractile force (Mierke et al., 2011). Integrin α5 was proposed to be positively involved in lung metastasis of human breast cancer cells, MDA-MB-231, in mice model by studying effect of nischarin, integrin α5 binding protein (Baranwal et al., 2011). When nischarin- or control vector- expressing MDA-MB-231 cells were injected into mice, control cells showed increased lung metastasis with increased tumor growth and higher integrin α5 expression levels. However, the role of integrin a5 on metastasis was not directly tested in this study.

Two dimensional and three dimensional models for in vitro testing

The extracellular matrix (ECM) is a complex array of secreted molecules such as collagen, fibronectin, elastin, fibrinogen, vitronectin and laminin assembled into diverse structures to constitute the cell microenvironment (Kim et al., 2011). Previously to investigate cell signaling, migration, adhesion, and even cytoskeletal function, many studies were performed on planar 2D ECM substrates. Conventional 2D model consisted of seeding cells on top of a culture plate coated with ECM components. This poorly mimics the extracellular conditions of living organisms (Hess et al., 2010), whereas3D cell culture models provide a microenvironment which simulates *in vivo* conditions and promotes morphology, motility, signaling and polarity that more closely resembles cells *in vivo* (See Figure 4).

To develop a 3D gel, type I collagen is often used. Among many components in ECM, collagen is the most abundant protein that constitutes up to 30% of the total protein mass. It is a main structural element of ECM providing tensile strength, regulating cell adhesion, support chemotaxis and migration and tissue development (Frantz et al., 2010). Moreover, type I collagen is easily accessible and able to polymerize. Additionally, to study the effect of integrin on cell migration or invasion, fibronectin or vitronectin is added depending on specific ligand of integrin prior to collagen polymerization (Mierke et al., 2011). For migration or invasion, vertical gel 3D assay can be used by seeding cancer cells on top of collagen gels. The vertical 3D assay is used to monitor 3D cell migration by counting the cell numbers. However, 3D vertical assay cannot measure how cells move into the gel and how fast cells move. It is hard to set the border line of determining invasive or non-invasive cells with normal bright field microscopy.

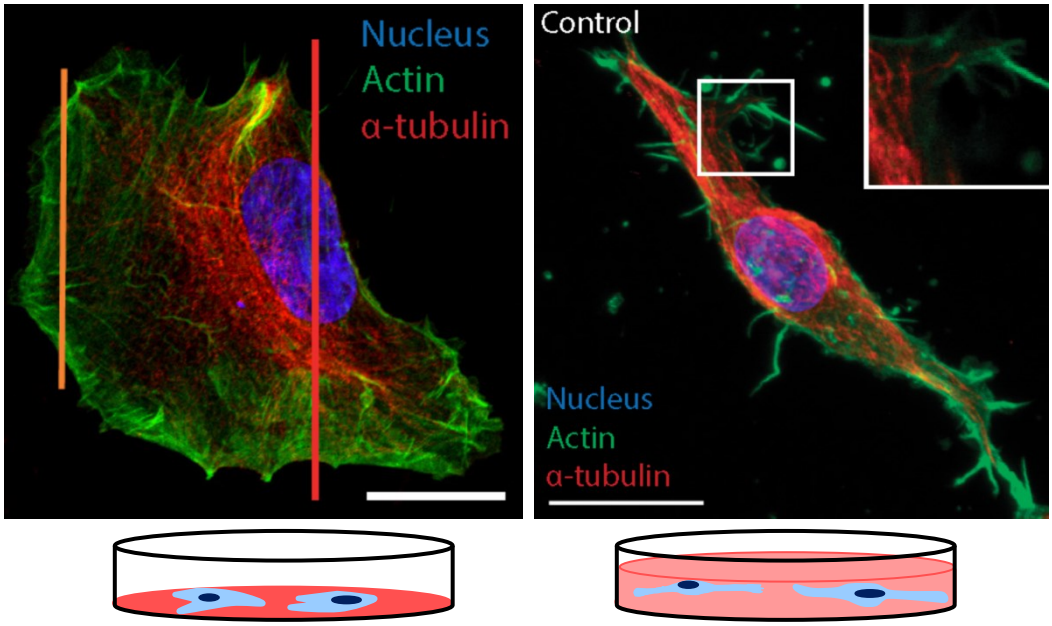

Figure 4: Cell morphology in 2D and 3D matrices. (A) Cell in 2D culture is very flat. (B) Cell in 3D culture is more round mimicking *in vivo* tumor microenvironment. (Giri et al. Not published)

We utilized both 2D and 3D models to study the effect of integrin α5 on cell motility. However, as we do not fully understand how cells move in ECM while migrating to secondary tumor sites, cell movements in ECM and effects of integrin α5 on cell motility need to be investigated more. We investigated cell movements using a traditional 2D cell migration model by coating plates with different ECM components. As vertical 3D model only provide how many cells migrate into 3D matrix, we monitored cell movements by embedding cells in 3D gel to provide *in vivo* like environment. From 2D and 3D model, we confirmed that 3D model provide better characterization of cell motility than 2D and ECM structure also affects cell motility. By using metastatic breast cancer cell, MDA-MB-231, we confirmed that higher expression level of integrin α5 also induces migration of cancer cells in 3D collagen matrices.

Anisotropic persistent random walk (APRW) model

Cell motility on 2D has been described by a persistent random walk (PRW) model which indicates that cell movements are highly isotropic in the absence of gradients and assumes a Gaussian distribution of cell velocities. Speed and persistence of cell movements can be obtained from fitting mean squared displacements (MSD) of cell movements with a PRW model (Stokes et al., 1991). However, since mechanisms of 2D and 3D cell migration are different, 3D cell movement does not follow PRW model but rather displays a highly anisotropic pattern (Wu et al., 2015). As described earlier, cells in 3D can alternate its movement between mesenchymal and amoeboid migration. Unlike cell migration in 2D, they do not display lamellipodia or filopodia when embedded in a 3D matrix. Human fibrosarcoma (HT1080) cells embedded in a 3D collagen matrix, display an exponential-like distribution not the predicted Gaussian distribution. Considering angular displacement and velocity profiles over different orientations, cell movements in 3D are highly anisotropic and more closely follow the APRW model. When movements of several cell lines in both 2D and 3D matrices were fitted with PRW and APRW models, APRW model did describe 3D migration well with a higher goodness of fit (see Table 1) (Wu et al., 2015). By fitting cell trajectories obtained with the APRW model, speed and persistence along the primary and non-primary axis of cell movements can be obtained. From speed and persistence along the primary and non-primary axis, we can calculate diffusivity ($D = S^2P/4$) to describe how fast cells diffuse into a matrix. After calculating primary and non-primary diffusivities, a strong correlation between diffusivity and persistence was observed from cell migration in 3D collagen matrix (Wu et al., 2014).

EXPERIMENTAL PROCEDURE

Cell Culture

Human breast carcinoma cells MDA-MB-231 were cultured in Dulbecco's modified Eagle's medium, high glucose (4.5g/L) (DMEM, Mediatech), supplemented with 10% fetal bovine serum (Hyclone Laboratoreis) and 1% Pen/Strep (Sigma). Cells were maintained in an incubator with 5% CO_2 at 37°C. MDA-MB-231 cells with non-target control (NTC) and integrin α5 knock down (shITGA5 KD) by shRNA were established with viral transduction of shITGA5 expressing lentriviral constructs (Sigma Aldrich). Cells were culture in Dulbecco's modified Eagle's medium, high glucose (4.5g/L) (Mediatech), supplemented with 10% fetal bovine serum (Hyclone), 1% Pen/Strep (Sigma) and 1μg/ml puromycin.

Cell Proliferation PrestoBlue Assay

To generate the standard curve of proliferation rate of MDA-MB231-cells, different numbers of cells (500, 1,000, 5,000, 10,000, 50,000, 100,000, 200000, and 250,000) were seeded in 24 well plates and cultured overnight. Each well was then incubated with 100μl PrestoBlue reagent (Invitrogen) for 1 hr or 2 hr with 5% CO_2 at 37°C. 200μl of cell culture media with PrestoBlue reagent were collected in 96 well plates. The absorbance was measured using a plate reader with excitation and emission wavelength at 560nm and 590 nm.

3000 cells were seeded in 24 well plates and cultured for 8 days. Cells were incubated with 100μl PrestoBlue reagent for 1 hr with 5% CO_2 at 37°C and 200μl cell

culture media and PrestoBlue reagent mixtures from each day were collected in 96 well plates. Fluorescence was measured.

2D cell migration assay

Two-dimensional cell-culture 24 well plates were coated with different matrices before seeding cells. Soluble rat tail type I collagen (Corning Inc.) in acetic acid were coated to achieve a final concentration of 50μg/ml and plates were washed gently three times with PBS. 50μg/ml fibrinogen in PBS and 50μg/ml fibronectin (Fisher Scientific) in sterile water were also coated and washed with PBS or sterile water, respectively. Plates were incubated for 1 h at room temperature and seeded with cells.

For 2D, cells were incubated for 1 h before time-lapse movies were acquired. Cell movements over time were imaged using a Cascade 1K CCD camera (Roper Scientific) mounted on mounted on a Nikon TE2000E phase contrast microscope equipped with a 10X objective and controlled by NIS-Elements AR imaging software. Images were taken every 5 minutes for 13 hours. Cells in the time-lapse movies were tracked using MetaMorph imaging software. The results were exported to an excel file which contained the x-, y- coordinates, time interval and speed of the tracked cells.

3D cell migration assay

3D collagen matrices were prepared with soluble rat tail type I collagen in acetic acid (Corning) to achieve a final concentration of 1mg/ml collagen. 1 M NaOH was then

added to normalize pH to about 7.0. For 3D collagen-fibronectin matrices, 10 and 50µg/ml fibronectin in sterile water for final concentration of 10 and 50µg/ml was added to collagen 3D matrices immediately after normalizing matrices with 1M NaOH. Remaining volume filled with a 1:1 ratio of reconstitution buffer [0.2 Hepes (Sigma-Aldrich), 0.26 M NaHCO3 (Sigma-Aldrich), and water as solvent] and culture medium. For 3D fibrinogen matrices, 100µl of 5mg/ml or 10mg/ml fibrinogen stock was mixed with 10µl of 100u/ml thrombin stock and 5µl 100mM $CaCl_2$ stock to achieve final concentration of 0.16mg/ml or 0.32mg/ml. To achieve total volume of 315µl, remaining volume filled with PBS. All matrices were placed in 24-well culture plates. All of gels solidified within 1h in an incubator at 5% CO_2 and 37°C, then 500µl of cell culture medium were added.

For 3D experiments, cells were incubated for 1h or overnight before time-lapse movies were acquired. Cell movements over time were imaged using a Cascade 1K CCD camera (Roper Scientific) mounted on a Nikon TE2000E phase contrast microscope equipped with a 10X objective (Nikon) and controlled by NIS-Elements AR imaging software (Nikon). Images were taken every 5 minutes for 13 hours for 3D experiments. Cells in the time-lapse movies were tracked using MetaMorph imaging software. The results were exported to an excel file which contained the x-, y- coordinates, time interval and speed of the tracked cells.

2D and 3D APRW Model Analysis

APRW model analysis was performed as described previously (Wu et al., 2015). Briefly, x- and y-coordinates from 2D and 3D cell trajectories data were exported from cell tracking Metamorph (Molecular Devices) software. Then, statistical profiling of cell motility was performed with Matlab. Statistical profiling includes MSD, correlation of cell velocities at different time lag, occurrence or probability distribution of cell displacement, occurrence of cell angular displacements and average magnitude of cell speed evaluated at different orientations after re-alignment along the primary migration direction. Cell trajectories data were then fitted to the APRW models. APRW model fitting generates speed of cells, diffusivity, persistent time and anisotropic index. Statistical profiling of fitting results was also performed with Matlab. Simulated cell trajectories based on APRW model fitting were then used to obtain MSDs.

Statistical Analysis

The mean values ± standard errors (SE) were calculated and plotted using Graphpad Prism software (GraphPad Software). One-way and two-way ANOVA test was performed to determine statistical significance, which is indicated in the graphs using the standard Michelin grade scale ***$p<0.001$, **$p<0.01$ and *$p<0.05$.

Reflection Microscopy

Reflection microscopy was performed for 3D matrices. Cells were plated in 3D matrices with collagen only and collagen with fibronectin. After waiting for overnight, gel structures were imaged using a Nikon A1 confocal microscope using 60X oil-immersion lens.

Immunofluorescence microscopy

Immunofluorescence in 2D was performed. Cells were plated on the fibronectin, fibrinogen and collagen gels. After overnight incubation, cells were fixed with 4% formaldehyde for 10 min and permeabilized with 0.1% Triton-X 100 (v/v) for 10 min. Cells were then incubated with vinculin antibody (Sigma-Aldrich) at 1:150 dilution and integrin α5 antibody (Abcam) at 1:250 for 1 hr at room temperature. Following washes in PBS cells were incubated with phalloidin (Invitrogen) at 1:150 dilution ratio and anti-mouse and rabbit antibodies at 1:200 dilution ratio for both for 1 hr at room temperature. Then, cells were incubated with hoechst 33342 at 1:200 dilution ratio. Cells at 2D were then imaged using a Nikon A1 confocal microscope using 60X oil-immersion lens.

RESULTS

The role of integrin α5 in cancer is controversial. Some literature suggests a tumor suppressive effects of integrin α5β1 (Seftor et al., 1998) while other study shows a positive correlation with cancer metastasis and tumorigenesis (Baranwal et al., 2011). Some studies report that tumor cells have appropriate integrin α5β1 expression level for metastasis (Tani et al., 2002). Too high or too low expression level had negative effect on the metastasis by observing kidney metastasis of ovarian cancer cells in mice injected with ovarian cancer cells expressing different levels of integrin α5 (Tani et al., 2002). In our work, we have tested the effect of integrin α5 on cancer cell migration on different types of matrices as integrin is a receptor for cell-ECM interactions.

Downregulation of α5 integrin decreases cell growth in 2D cultures

To test the effect of downregulation of α5 integrins on breast cancer cell proliferation, we compared the growth of MDA-MB-231 metastatic breast cancer cells expressing a shRNA targeting integrin α5 (shITGA5) or a non-target control (NTC) on different 2D matrices including fibronectin, fibrinogen and collagen. We observed NTC cells grew faster than shITGA5 cells (See Figure 5 A, B). For shITGA5 cells, proliferation curves were quite similar on all types of coatings. *In vitro* proliferation was decreased when expression of integrin α5 was lowered. We confirmed reduced expression of α5 integrin mRNA and protein by RT-PCR and immunoblotting (See Figure 6 A, B).

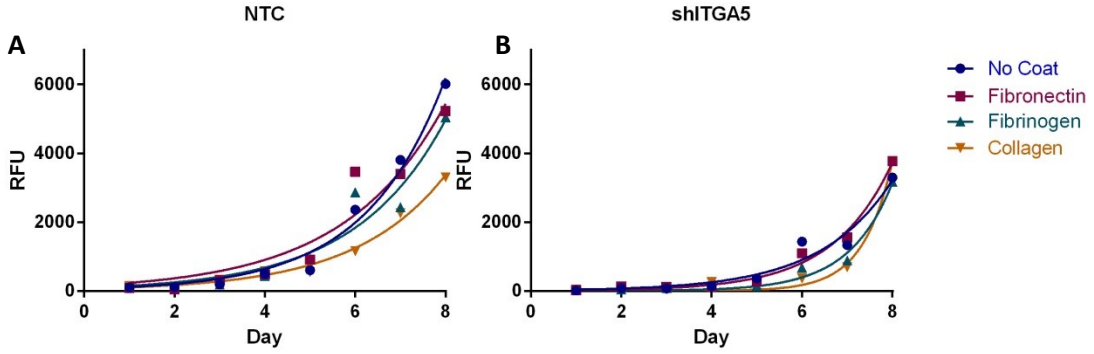

Figure 5: Proliferation rate of NTC and shITGA5 cells. (A) NTC cells on collagen showed slower cell growth rate compared to other coatings. (B) Cell growth rates of shITGA5 cells on all 2D coatings are reduced compared to NTC cells. Y axis presents relative fluorescence unit representing cell numbers.

Figure 6: RT-PCR and western blots to detect mRNA and protein levels of integrin α5. (A) RT-PCR results showed around 8 fold decrease in ITGA5 mRNA levels between NTC cells and integrin α5 knockdown cells in integrin α5 (ITGA5) expression level. (B) The protein levels of integrin α5 were reduced in knockdown cells.

Downregulation of integrin α5 did not have drastic effects on 2D cell motility

To test cancer cell motility on different matrices, we plated breast cancer cells MDA-MB-231, on fibronectin, fibrinogen and collagen coated plates. Cell motilities were monitored every 5min for 13h using live-cell phase-contrast microscopy. Compared to cells plated without coating, we observed cells are remarkably both NTC and shITGA5 cells were more motile on collagen coated matrices (See Figure 7 A). We also expected cells migration speed on collagen would not be affected. While the migration of shITGA5 cells on collagen coated matrices are decreased compared to controls, cell speeds on fibronectin coating did not change as a function of ITGA5 expression. (See Figure 7 B)

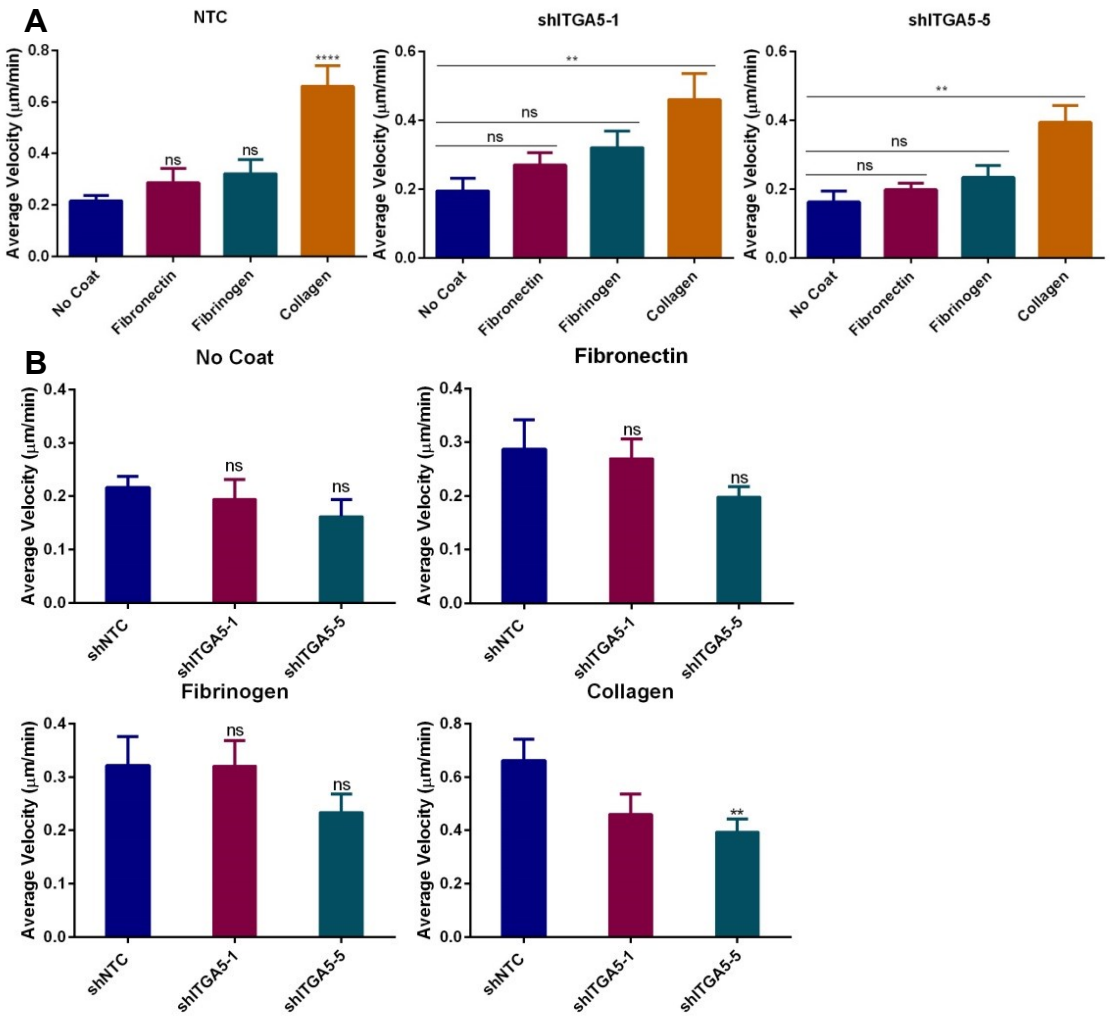

Figure 7: The effects of integrin α5 downregulation on 2D coatings. (A) Average cell velocity by cell type. Cells on collagen coating showed significantly increased average cell velocities for NTC, shITGA5-1 and shITGA5-5 cells. Average cell velocities on no coating, fibronectin and fibrinogen were not affected by expression of integrin α5. (B) Average velocities compared by coatings. Only average velocity on collagen for shITGA5-5 cells reduced.

To ensure that fibronectin concentration does not affect cell speed, we also plated NTC and shITGA5 cells on different fibronectin concentration coatings. In a previous study, the speed of cell migration depends on the substrate extracellular matrix concentration (Palecek et al. 1997). We did not observe remarkable differences of cell migration speeds on different concentrations of fibronectin coating. The correlation between cell speeds and fibronectin concentrations was similar on both NTC and shITGA5 cells (see Figure 8).

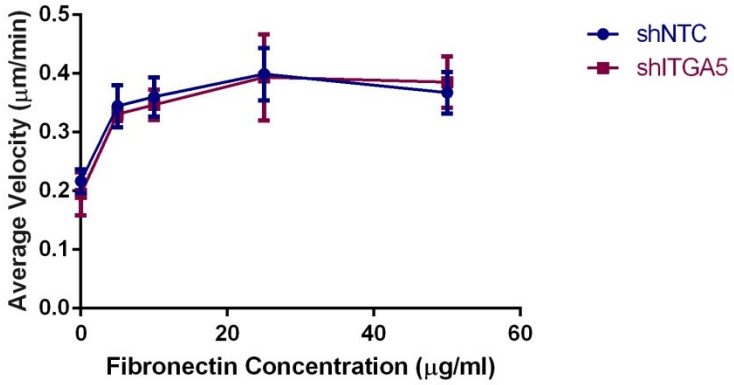

Figure 8: Average velocity on different fibronectin concentrations. No significant change of average velocity on different fibronectin concentration coatings was observed for both NTC and shITGA5 cells.

For MDA-MB-231 cells on 2D coated substrates, APRW model provides a better fit for motility data compared to the PRW model (Wu et al. 2015). Furthermore, from NTC cells on no coating, orientation of the velocities of cell migration relative to the primary axis indicated that even movements of NTC cells without any coating are intrinsically anisotropic (see Figure 9).

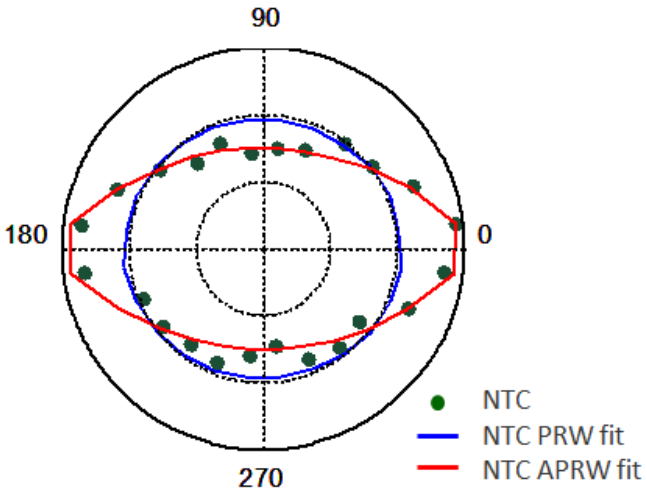

Figure 9: Orientation of the velocities of cell migration relative to the primary axis. NTC cell motility on no coating (in green dots) showed that it followed APRW model (in red line) rather than PRW model (in blue line). NTC cell movements on 2D were highly anisotropic, not following PRW model.

We performed the APRW model fitting for NTC and shITGA5 cells on no coating, fibronectin, fibrinogen and collagen. As MSD graphs indicate, the APRW model fitted 2D cell motilities with R-squared value higher than 0.95 for all cell types and coatings (see Figure 10). From MSD values for cells plated on no coating, fibrinogen and collagen, NTC cells moved slightly faster than shITGA5 cells. Consistent will cell motilities calculated from average velocity measurements (see Figure 7 B), cell movements on fibronectin coatings indicated no difference between NTC and shITGA5 cells.

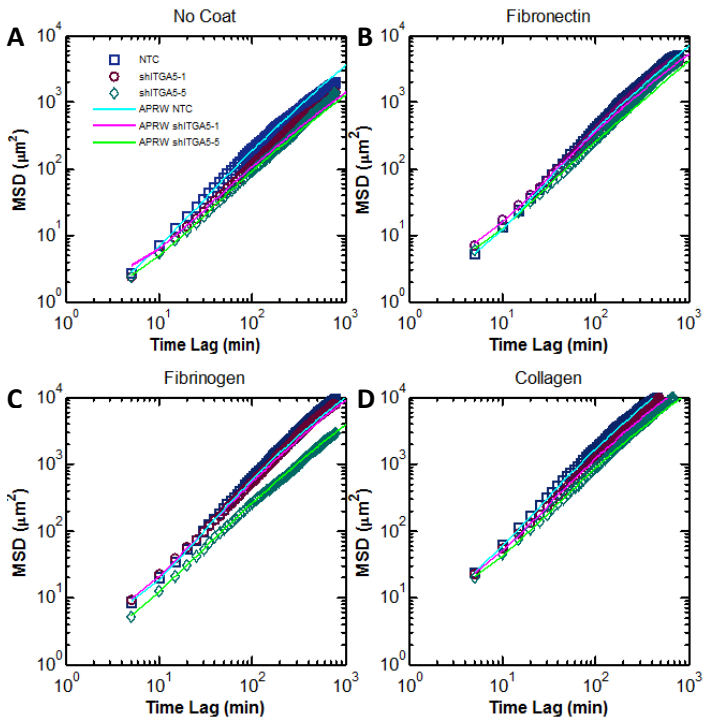

Figure 10: Mean squared displacement from cell trajectories and APRW fitting. (A) MSD from no coat (B) from fibronectin coating (C) from fibrinogen coating and (D) collagen coatings with R-squared values higher than 0.95.

Since 2D cell motilities follow an APRW model, we can obtain different cell motility parameters such as total diffusivity and persistent time. Total diffusivities and primary persistence times of NTC cells on no coating, fibrinogen and collagen were greater than those of shITGA5 cells. On the other hand, 2D cell movements on fibronectin were not affected by expression of integrin α5. Moreover, primary persistent times on fibronectin coating were not affected (see Figure 11). Integrin α5 expression levels did not have remarkable effects on 2D cell motilities.

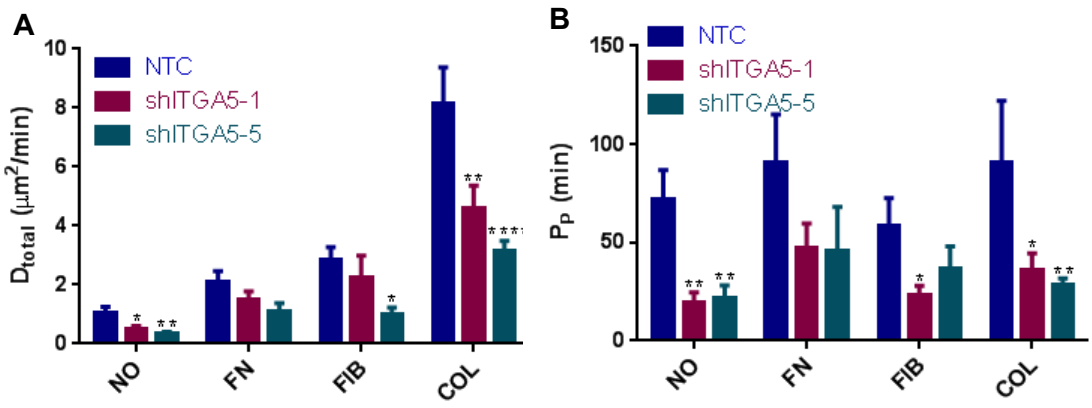

Figure 11: Total Diffusivity and primary persistent time on 2D coatings. (A) Total diffusivity, sum of primary diffusivity and non-primary diffusivity, showed that NTC cells on no coating (NO), fibrinogen (FIB) and collagen (COL) are moved faster than shITGA5 cells. However, cell movements on fibronectin (FN) were not influenced by integrin α5 expression level. (B) Primary persistent time on 2D coatings showed similar trend as total diffusivity.

To evaluate the correlation between focal adhesion formation and cell motility, we plated NTC and shITGA5 cells on the glass bottom plate coated with different matrices. Then, we fixed cells with 4% formaldehyde for 10 min at room temperature. We stained cells with integrin α5, vinculin and actin. By visual inspection, we observed more focal adhesions for NTC cells than for shITGA5 cells (see Table 1). For NTC cells, focal adhesion staining overlapped with staining for integrin α5. This was observed from cells plated on fibronectin, fibrinogen, no coating (see Figure 12). For shITGA5 cells, focal adhesions still formed on all of 2D coatings with downregulation of integrin α5. For

both NTC and shITGA5 cells, less focal adhesions were formed on collagen. Integrin expression might affect focal adhesion and cell motility.

Table 1: Focal adhesion numbers per cell on 2D coatings

	NTC	shITGA5
No Coat	++++	+++
Fibronectin	+++	++
Fibrinogen	+++	+
Collagen	++	++

Number of + indicates intensity of focal adhesions

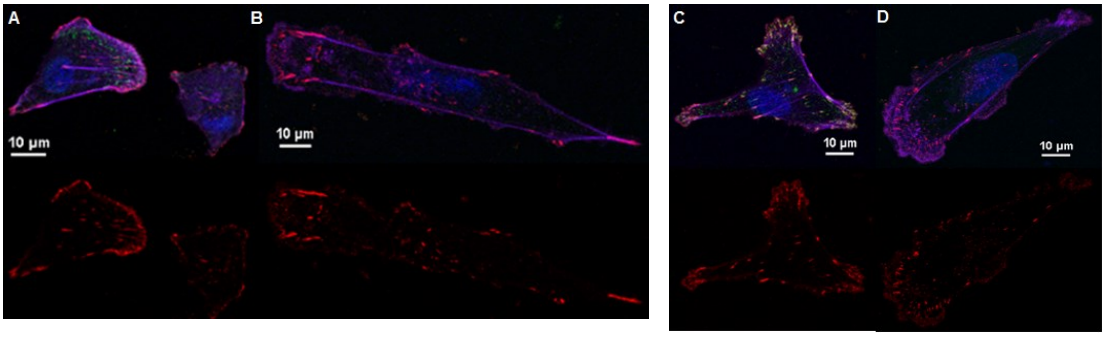

Figure 12: Focal adhesion of cells on 2D coating from immunostaining. (A) NTC cells on no coating showed vinculin (a marker of focal adhesions) (in red) and integrin α5 (in green). (B) shITGA5 cells on no coating still displayed vinculin staining focal adhesion. (C) NTC cells on fibronectin coating showed vinculin staining overlaps with integrin α5. (D) shITGA5 cells on fibronectin still formed vinculin focal adhesions. Nucleus (in blue), and actin (in pink).

To observe 3D matrices structures, we performed reflection microscopy for gels with collagen only, collagen with 10µg/ml fibronectin and collagen with 50µg/ml fibronectin. With increasing amounts of fibronectin in collagen matrices, a tighter gel structure was observed (see Figure 13 A, B, C). Pore sizes for each matrices indicated that amounts of fibronectin in gel altered gel structure with decreasing sizes of pore (see Figure 13D).

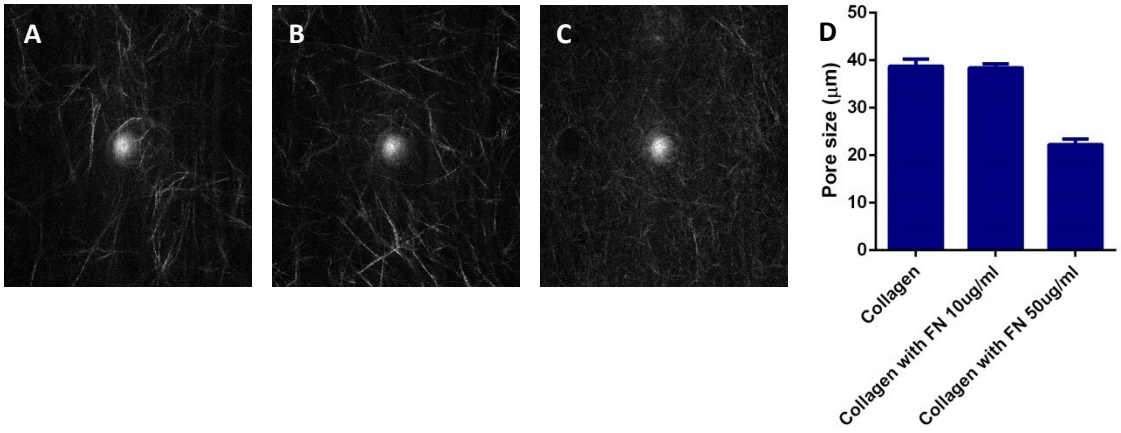

Figure 13: Gel structures from reflection microscopy. (A) Collagen 1mg/ml, (B) Collagen 1mg/ml with fibronectin 10µg/ml showed similar gel structure with collagen only 3D matrix, (C) Collagen 1mg/ml with fibronectin 50µg/ml. We observed tighter fiber formation for increasing amount fibronectin in collagen matrices. (D) Pore size of 3D matrices. Collagen with fibronectin (FN) 50µg/ml had smaller pore size than collagen only and collagen with fibronectin (FN) 10µg/ml.

Downregulation of integrin α5 remarkably decreased cell motility in 3D

To test the effect of downregulation of integrin α5 on cell motility in 3D matrices, we cultured NTC and shITGA5 cells embedded in collagen alone and collagen-fibronectin matrices. We monitored cell migration for 13 h using live cell phase contrast microscopy. Unlike 2D cell culture, cell migration speeds of NTC and shITGA5 cells had no difference in 3D collagen. Even with fibronectin added in matrices, cell speeds were not affected (see Figure 14).

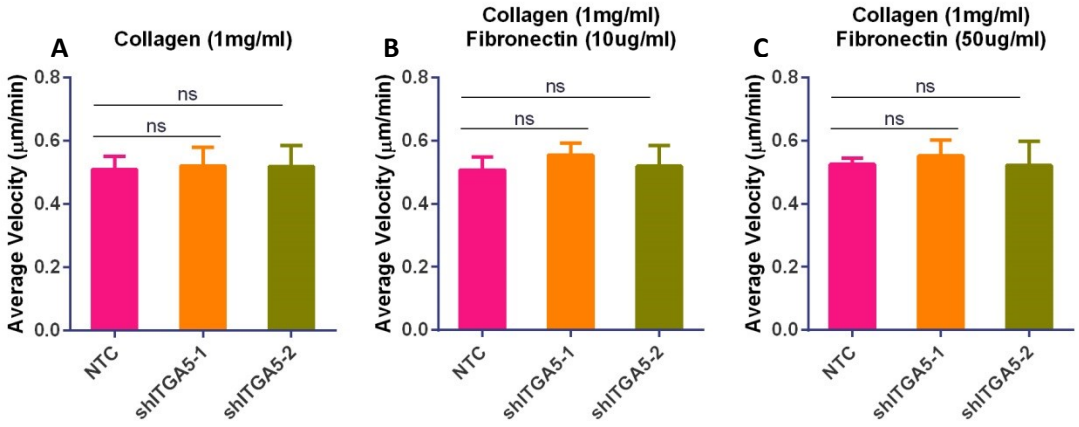

Figure 14: Average velocity in 3D matrices. (A) Average velocity of cells in collagen, (B) Average velocity of cells in collagen with fibronectin 10μg/ml, (C) Average velocity of cells in collagen with fibronectin 50μg/ml. Average velocities were not affected by integrin α5 expression level or 3D matrices composition.

Thus, we performed APRW model fitting for cell migration trajectory data for more accurate statistical analysis. Cell movements in 3D matrices were fitted with APRW model with R-squared values around 0.99 showing high goodness of fit (see Figure 15).

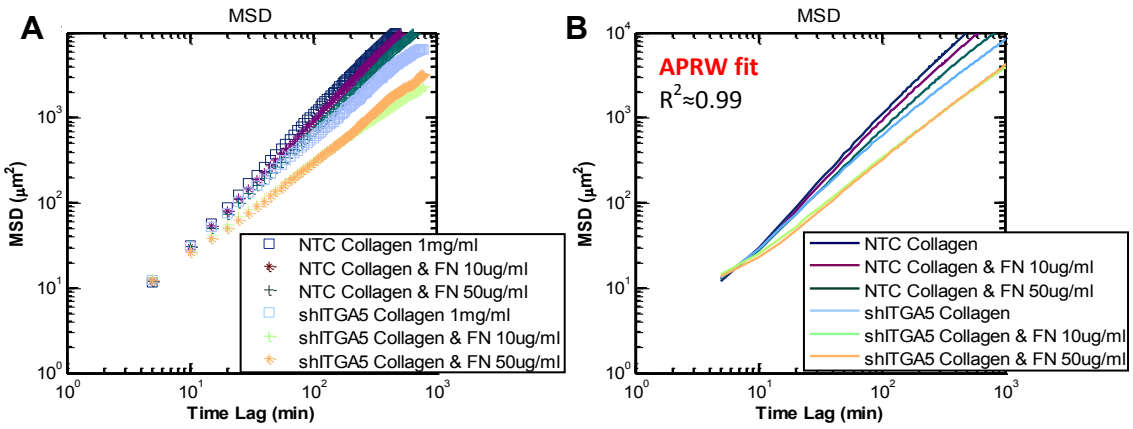

Figure 15: Mean squared displacement from cell trajectories and APRW model. (A) MSD from cell trajectories. (B) MSD from APRW model with R-squared values around 0.99 showing 3D cell motilities follow APRW model.

For more accurate characterization of cell motilities in 3D matrices, we measured primary persistent time, total diffusivities and anisotropic indexes. Primary persistent times for shITGA5 cells remarkably decreased compared to NTC cells. In addition to persistent time, total diffusivities that can be calculated from cell speed and persistent time also drastically decreased for shITGA5 cells unlike average velocities (see Figure 16 A, B). Both diffusivities for primary and non-primary axis remarkably decreased (see TABLE 2). NTC cells moved dramatically faster in 3D matrices showing effect of integrin α5 expression level. Furthermore, increasing amounts of fibronectin in gels decreased cell movements in 3D matrices for both NTC and shITGA5 cells potentially

due to changes of gel structures. According to anisotropic index, in 3D collagen matrices with fibronectin, shITGA5 cells showed more isotropic cell movements (see Figure 16 C). Moreover, shITGA5-2 cells have higher expression of integrin compared to shITGA5-1 (see Figure 6 B). shITGA5-2 cells showed higher persistent time total diffusivity confirming that integrin α5 expression affect cell motility. Average velocities obtained from cell trajectories were not affected but total diffusivities and persistent time altered due to downregulation of integrin α5. As total diffusivity is calculated from cell speed and persistent time, correlation between total diffusivity and persistent time shows that persistent time is major factor that affects cell motility (see Figure 16 D).

Cell motility increased with decreasing expression level of integrin α5 for 3D fibrinogen

To investigate the effect of integrin α5 on cell motility in 3D fibrinogen, we plated cells in 3D fibrinogen matrices. From both average velocities and total diffusivity obtained from APRW model, cells with lower expression of integrin α5 moved faster regardless of concentration of fibrinogen (see Figure 17). For NTC cells, cell motility decreased with increasing amount of fibrinogen potentially due to stiffness of gel matrix. However, shITGA5 cells were not affected by the concentration of fibrinogen. While total diffusivities for shITGA5 cells in fibrinogen remained similar to diffusivities of cells in a collagen only matrix, total diffusivity for NTC cells in fibrinogen was decreased by 84% compared to cells in 3D collagen only matrix.

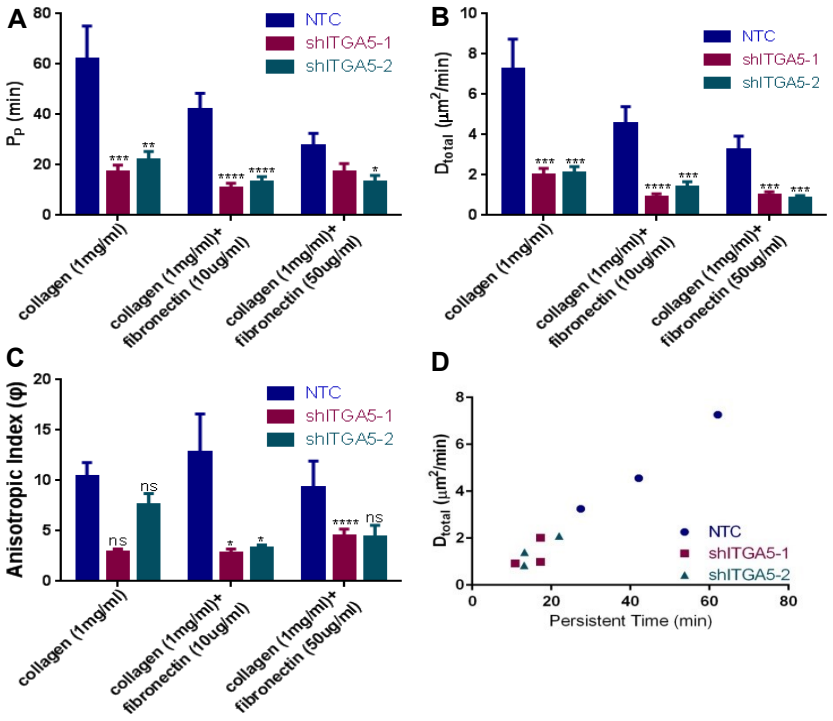

Figure 16: Cell motility parameters from APRW model fitting. (A) Primary persistent time. Primary persistence decreased with downregulation of integrin α5. (B) Total diffusivity. With increasing amounts of fibronectin in 3D matrices, cell movements decreased potentially due to change of gel structure. (C) Anisotropic index. Anisotropic index showed that in 3D matrices with fibronectin, shITGA5 cells moved more in isotropic manner. (D) Correlation between primary persistence and total diffusivity. Positive correlation between total diffusivity and persistent time shows that persistence is the factor determining cell motility in 3D collagen matrix.

Table 2: Cell motility parameters from APRW

cell type	condition		APRW					
			Pp	Pnp	Dp	Dnp	Dtotal	φ
NTC	3D	Collagen(1mg/ml)	62.09	20.49	6.49	0.77	7.26	10.35
shITGA5-1	3D	Collagen(1mg/ml)	17.33	15.42	1.35	0.68	2.02	2.92
shITGA5-2	3D	Collagen(1mg/ml)	21.99	11.12	1.65	0.44	2.09	7.57
NTC	3D	Collagen(1mg/ml) & Fibronectin(10ug/ml)	42.09	16.88	3.78	0.78	4.56	12.80
shITGA5-1	3D	Collagen(1mg/ml) & Fibronectin(10ug/ml)	10.85	10.69	0.64	0.28	0.92	2.83
shITGA5-2	3D	Collagen(1mg/ml) & Fibronectin(10ug/ml)	13.21	14.88	0.92	0.48	1.40	3.26
NTC	3D	Collagen(1mg/ml) & Fibronectin(50ug/ml)	27.44	12.46	2.72	0.54	3.25	9.34
shITGA5-1	3D	Collagen(1mg/ml) & Fibronectin(50ug/ml)	17.25	17.41	0.71	0.27	0.99	4.47
shITGA5-2	3D	Collagen(1mg/ml) & Fibronectin(50ug/ml)	13.15	10.54	0.61	0.23	0.84	4.36

Pp: primary persistent time (min), Pnp: non-primary persistent time (min), Dp: primary diffusivity (μm^2/min), Dnp: non-primary diffusivity (μm^2/min), Dtotal: toal diffusivity = Dp + Dnp (μm^2/min) and φ: anisotropic index = Dp/Dnp

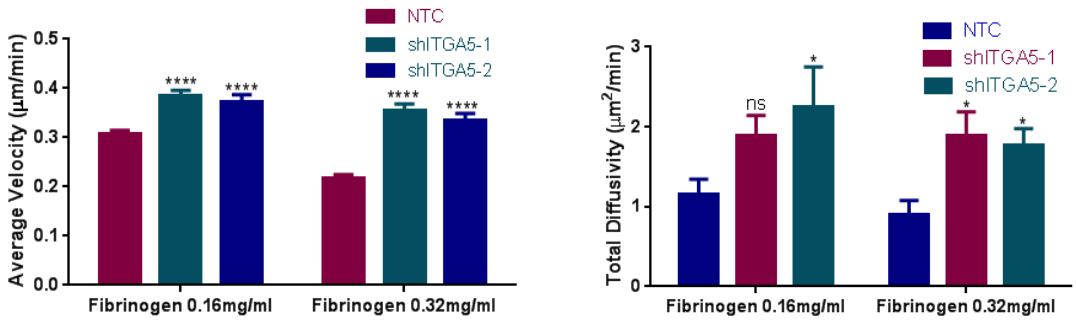

Figure 17: Average velocity and total diffusivity for 3D fibrinogen. shITGA5 cell movements increased in fibrinogen matrices unlike in collagen matrices.

DISCUSSION

As integrin plays important role in cancer migration, proliferation invasion and angiogenesis, many studies on integrin as therapeutic target were performed (Schaffner et al., 2013). Our work agrees with several studies suggesting integrin α5 plays major role in breast cancer metastasis involving migration, invasion and proliferation. Integrins regulate not only cell migration and invasion but also proliferation and/or survival of cancer cells. Previous studies have shown that integrin α5β1binding to fibronectin inhibits drug induced apoptosis and upregulation of integrin α5β1 promotes tumor cell survival (Aoudjit et al., 2001). Our work is consistent with these findings. Integrin α5 knock down led to a decrease in cell growth, indicating integrin α5β1 plays an important role for cancer cell growth. As overexpression of nischarin, integrin α5 binding protein, also decreased tumor growth (Baranwal et al., 2011), suggesting that targeting integrin α5 as potential therapeutic solution would inhibit breast cancer growth.

Studies have shown that on fibronectin coated plates, breast cancer cells transfected with siRNA targeting integrin α5 showed less migration area exhibiting decreased cell motility and suggesting elevated integrin α5 resulted from steroid receptor coactivator-1 in tumors is important for breast cancer cell migration (Qin et al., 2011). However, other studies have shown that when endothelial cells were cultured on fibronectin coated with antibody blocking integrin α5, percentage of motile cells were decreased but cell speeds were not affected (Chon et al., 1998). The discordance among studies may be a result of the different cell types that were studied. As fibronectin is main ligand for integrin α5β1, we expected downregulation of integrin α5β1 would affect cancer cell motility on fibronectin coating. However, as our work exhibited, effect of

integrin α5β1 in cancer cell motility on collagen was greater than any other type of coating. Secretion of fibronectin by cells and binding of fibronectin onto collagen may affect cancer cell motility on collagen coating as well. As integrin α5β1 is mainly known for fibronectin receptor, we evaluated the effect of fibronectin concentration on cell motility. Consistent to previous study (Chon et al., 1998), we did not observe significant differences between cell speeds on various concentrations.

Our work exhibited that expression level of integrin α5 as well as ECM component affects 3D cancer cell motility in collagen including total diffusivity and primary persistence from APRW model. We further need to investigate how ECM components affect cell motility in different 3D matrices. Other studies have shown that metastatic breast cancer cells with higher expression of integrin α5β1 invaded more into 3D collagen gel (Mierke et al., 2011). Decrease of cell motility in 3D model suggests integrin α5 as potential therapeutic target for inhibiting cancer cell migration and invasion. Baranwal et al. identified nischarin, a protein binding to proximal transmembrane region of integrin α5 cytoplasmic tail and observed that nischarin reduced proliferation and lung metastasis. However, the mechanism of how nischarin regulates cancer migration and invasion is not known. In preclinical studies, integrin α5β1 function-blocking murine antibody, IIA1, was able to promote apoptosis of breast cancer cells in 3D culture (Nam et al., 2010). ATN-161 developed by Attenuon LLC is actylated amidated PHSCN peptide and it blocks cancer growth and metastasis in preclinical mouse models. ATN-161 with radiotherapy also induced apoptosis of breast cancer growth in 3D culture (Schaffner et al., 2013). PHSCN dendrimers were able to inhibit

human prostate cancer invasion, lung metastasis, breast cancer invasion *in vivo*. (Schaffner et al., 2013).

CONCLUSION

Our work here focuses on the effect of integrin α5 on cell motility for 2D coatings and 3D matrices. To test the effect of integrin α5 on 2D cell migration, cell movements on fibronectin, fibrinogen and collagen were observed. According to average velocity analysis, which measures cell velocity from cell trajectories, we observed cell motility was not affected. However, we were able to observe cell movements on no coating, fibrinogen and collagen were affected by expression of integrin α5 through APRW model. Total diffusivities and persistence times for cells with downregulation of integrin α5 were decreased for no coating, fibrinogen and collagen. Cell migration on fibronectin was not affected by integrin α5 expression levels. We studied the effect of focal adhesion on 2D cell migration. However, we were not able to establish any correlation between the expression level of integrin α5 and cell motility.

To further study effect of cell motility, we studied cell migration in 3D matrices. As 3D cell culture simulates *in vivo* tumor microenvironment, we were able to perform better characterization on effect of integrin α5 on 3D cell migration. Initially, from average velocity, we did not observe any effect of integrin α5. We also did not observe any effect of fibronectin in 3D collagen matrices. However, interestingly, by APRW model fitting, we confirmed that decreased expression level of integrin α5 drastically reduced cell migration in 3D. We observed remarkable decreases on total diffusivity and persistent time showing strong correlation between two cell motility parameters. We also studied the effect of fibronectin in 3D collagen matrices and confirmed that with an increasing amount of fibronectin in 3D collagen, the gel structure was altered and tighter fiber formation occurred. Potentially due to structure changes in the gel, we observed 3D

cell motility decreased with more amounts of fibronectin in matrices. We also investigated cell motility in 3D fibrinogen matrix. We observed a negative correlation between integrin α5 expression level and cell migration was observed. With decreasing expression of integrin α5, cells moved faster and diffused faster in the 3D fibrinogen matrices. Furthermore, fibrinogen concentration did not affect shITGA5 cells but did decrease cell motility for NTC cells.

Our work confirmed that APRW model provided better characterization of cell migration and persistence as compared to measuring the average speed from cell trajectories. Furthermore, downregulation of integrin α5 had a more drastic effect on 3D cell migration than 2D cell motility. Integrin α5 showed opposite effects on 3D cell migration in collagen matrices with fibronectin and fibrinogen matrices. 2D cell motility had different movement patterns compared to 3D cell motility. Moreover, for 3D, extracellular matrix affects cell motility showing that ECM plays an important role in 3D cell movements.

FUTURE WORK

For future work, we will quantify focal adhesion and test correlation between cell motility and focal adhesion to confirm our results that focal adhesion was not a predictor for 2D cell migration. Moreover, to ensure that other types of integrin α were not affected by knocking down integrin α5, we will perform flow cytometry, RT-PCR or immunoblotting for other integrin α subunits.

We propose to further investigate correlation between focal adhesion and cell motility in 3D matrices. As we observed remarkable difference in 3D migration, focal adhesion, involved in cell migration, in 3D gels might be affected as well. Moreover for 3D cell motility, we plan to manipulate gel composition by altering collagen and fibronectin concentrations. As different cell invasion rates into 3D matrices with various concentrations of collagen matrices were observed, we propose to further investigate relationship between gel structures and 3D cell migration. To further study cell motility in 3D fibrinogen matrix, we plan to examine effects of fibrinogen gel structure, stiffness of gels and alignment of fibers on cell motility.

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Samsung Biologics, Fill & Finish Team – Songdo, Korea Summer 2013
Intern Associate

- Assist writing SOP and performed GWP and GMP
- Provide analysis on lyophilization in biopharmaceutical industry
- Create work schedule management system for the plant
- Conduct plant maintenance before production

Elisseeff Lab at Johns Hopkins University Medical Campus - Baltimore, MD Fall 2013
Undergraduate Researcher
Maintain the growth of Mesenchymal stem cells and conduct 3D culture.

- Accomplish the differentiation of Mesenchymal stem cells

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Assistant Researcher

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- Manage the growth of breast cancer cells and distribution of experimental equipment and chemicals
- Conduct experiment for the research such as western blotting, DNA/RNA extraction, ELISA, and PCR, MTT assay
- Conduct the presentation of result and process of experiment every week

BLSA (Baltimore life scientists association) 4th annual conference – Baltimore MD November 2012
Volunteer

- Assist the preparation for the conference

NHN Corporation – Sungnam, Korea December 2011
CEO supporting department

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SKILLS

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