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Schwann Cells Increase Prostate and Pancreatic Tumor Cell Invasion Using Laminin Binding A6 Integrin

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ABSTRACT

Human pancreatic and prostate cancers metastasize along nerve axons during perineural invasion. The extracellular matrix laminin class of proteins is an abundant component of both myelinated and non-myelinated nerves. Analysis of human pancreatic and prostate tissue revealed both perineural and endoneural invasion with Schwann cells surrounded or disrupted by tumor, respectively. Tumor and nerve cell co-culture conditions were used to determine if myelinating or non-myelinating Schwann cell (S16 and S16Y, respectively) phenotype was equally likely to promote integrin-dependent cancer cell invasion and migration on laminin. Conditioned medium from S16 cells increased tumor cell (DU145, PC3, and CFPAC1) invasion into laminin approximately 1.3–2.0 fold compared to fetal bovine serum (FBS) treated cells. Integrin function (e.g., ITGA6p formation) increased up to 1.5 fold in prostate (DU145, PC3, RWPE-1) and pancreatic (CFPAC1) cells, and invasion was dependent on ITGA6p formation and ITGB1 as determined by function-blocking antibodies. In contrast, conditioned medium isolated from S16Y cells (non-myelinating phenotype) decreased constitutive levels of ITGA6p in the tumor cells by 50% compared to untreated cells and decreased ITGA6p formation 3.0 fold compared to S16 treated cells. Flow cytometry and western blot analysis revealed loss of ITGA6p formation as reversible and independent of overall loss of ITGA6 expression. These results suggest that the myelinating phenotype of Schwann cells within the tumor microenvironment increased integrin-dependent tumor invasion on laminin. J. Cell. Biochem. 117: 491–499, 2016.

KEY WORDS: SCHWANN CELLS; METASTASIS; A6B1; LAMININ; MICROENVIRONMENT; PERINEURAL INVASION

The pathologically distinct migration and invasion of tumor cells along nerves is a process known as perineural invasion (PNI) [Liebig et al., 2009; Bapat et al., 2011]. Neurotropic cancers, including prostate and pancreatic cancer, utilize the complex neuroanatomy of these highly innervated organs as a primary method for metastasis [Liebig et al., 2009]. Pancreatic cancer cells invade the pancreatic parenchyma and penetrate the celiac plexus that surrounds the superior mesenteric artery as they metastasize [Loukas et al., 2010]. PNI is observed in 50–100% of pancreatic cancers and contributes significantly to the poor prognosis and extreme pain associated with pancreatic cancer progression [Liebig et al., 2009; Bapat et al., 2011]. In addition, confined and localized prostate cancer is considered curable, while invasion out of the prostate capsule is associated with poor prognosis [Villers et al., 1989]. Studies have shown that approximately 85% of prostate cancer cases demonstrate PNI along the cavernosal nerve, prostatic plexus, and neurovascular bundles [Hirai et al., 2002]. This is a major concern considering that 233,000 men will be diagnosed and 29,480 men will die from prostate cancer, and of the 46,420 newly diagnosed patients with pancreatic cancer, approximately 77% will die from the disease in 2014 [Siegel et al., 2014]. Despite the prominence of PNI as a significant metastatic route for neurotropic cancers, the mechanism(s) are poorly defined.

The laminin binding integrin A6B1 has a major role in determining the aggressive phenotype of tumor cells during cancer progression [King et al., 2008; Ports et al., 2009; Sroka et al., 2011; Landowski et al., 2014]. The receptor is a member of the heterodimeric cell surface integrin protein family of receptors that

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have unique binding specificities and function in cell migration, adhesion, and invasion [Hynes, 2002]. The A6B1 receptor is retained in prostate cancer [Schmelz et al., 2002], is expressed on prostate tumor cells undergoing PNI [Sroka et al., 2010] and is highly expressed in advanced pancreatic cancer [Zhu et al., 2011]. Previous work by our group has demonstrated that tumors produce a structural variant of A6B1, called A6pB1, via removal of the ligand binding domain while on the cell surface. The serine protease, urokinase-type plasminogen activator (uPA), and its receptor urokinase plasminogen activator receptor (uPAR), regulate this specific post translational modification of A6B1 [Demetriou and Cress, 2004; Demetriou et al., 2004; Pawar et al., 2007; Ports et al., 2009; Sroka et al., 2011]. Evidence has shown that cleavage of integrin A6B1 to form A6pB1 increases tumor cell motility, invasion, and osseous prostate cancer metastasis [King et al., 2008; Ports et al., 2009; Sroka et al., 2011; Landowski et al., 2014], although the role of A6pB1 in promoting perineural invasion in neurotropic cancers remains to be determined.

Peripheral nerves consist of three layers: the endoneurium, perineurium, and the epineurium [Sroka et al., 2010]. The innermost layer, or the endoneurium, contains Schwann cells [Dubovy, 2004] that are either myelinating or non-myelinating [Nave and Werner, 2014]. Myelin-forming Schwann cells require interaction with the laminin and collagen rich extracellular matrix around peripheral nerve axons and are responsible for the sorting and myelination of axons in the peripheral nervous system [Chernousov et al., 2008; Feltri and Wrabetz, 2005]. Tumor cells undergoing PNI invade along the perineural space or into the endoneurium [Sroka et al., 2010] and disrupt and damage nerve axons, thereby affecting Schwann cells and the overall nerve microenvironment. The role of Schwann cell factors such as the neurotrophic factor pleiotrophin and myelin-associated glycoprotein (MAG) in cancer invasion have been identified [Swanson et al., 2007; Yao et al., 2013]. However, the role of Schwann cells in regulating laminin receptors of neurotropic cancers during perineural invasion is currently unknown.

We hypothesized that Schwann cells in the tumor-nerve environment affect tumor cell migration and invasion through regulation of the laminin receptor A6B1 and its variant A6pB1. Laminin in the extracellular nerve environment is required for proper Schwann cell function and myelination of nerve axons [Sroka et al., 2010]. Invading tumor cells that breach the perineural layer and cause nerve damage may impede proper Schwann cell function and induce Schwann cells to overproduce factors such as laminins and growth factors as the cells repair the disturbed environment. The mechanisms associated with Schwann cell-mediated remodeling of injured peripheral nerve axons have been well-studied in other systems [Kidd et al., 2013]. However, the role of Schwann cell secreted factors in regulating tumor cell metastasis has not been well studied. We hypothesized that invading tumor cells may harness Schwann cell-derived factors to execute the metastatic program.

The goal of this study was to determine if the myelinating (S16 Schwann cells) or non-myelinating (S16Y Schwann cells) nerve phenotype was equally likely to promote cancer cell invasion and migration on laminin, dependent upon integrin function. Human prostate (DU145, PC3, and RWPE-1) and pancreatic (CFPAC1) tumor cells were treated with conditioned medium from immortalized S16 and S16Y cell lines. Previous groups have established that S16 cells demonstrate increased expression of multiple proteins associated with the myelination process when compared to S16Y cells [Toda et al., 1994; Sasagasako et al., 1996; Hai et al., 2002]. The influence of S16 and S16Y Schwann cells conditioned media on pancreatic and prostate tumor cell invasion, and expression changes in A6pB1, were investigated.

MATERIALS AND METHODS

CELL CULTURE CONDITIONS

All cells were cultivated at 37°C in a 5% CO_2 atmosphere with constant humidity. Human prostate cancer DU145, PC3, and RWPE-1 cells and human pancreatic cancer CFPAC1 cells were obtained from American Type Tissue Collection (ATCC, Manassas, VA) and maintained in Iscove′s modified Dulbecco′s medium (IMDM) or Dulbecco′s modified Eagle′s medium (DMEM) from Invitrogen (Grand Island, NY), supplemented with 10% fetal bovine serum (FBS) from Hyclone Laboratories (Novato, CA) and 1% penicillin/streptomycin. Immortalized rat S16 and S16Y Schwann cells were obtained from ATCC and maintained in DMEM supplemented with L-glutamine (Invitrogen) and 10% FBS. The Schwann cells were grown in flasks coated with 0.1 mg/ml poly-L-lysine (PDL) from Sigma–Aldrich (St. Louis, MO).

ANTIBODIES AND REAGENTS

The J1B5 rat monoclonal used for immunoprecipitation of the A6 integrin (ITGA6), and the AIIB2, B1 integrin (ITGB1) blocking antibody were generous gifts provided by Dr. Caroline Damsky and have been previously described [Bohnsack et al., 1990; Damsky et al., 1994]. The J8H antibody was a generous gift from Dr. Arnoud Sonnenberg and has been previously described as blocking A6p production without altering the cell adhesion function [Hogervorst et al., 1993; Ports et al., 2009; Sroka et al., 2011]. The AA6A rabbit polyclonal antibody that recognizes the c-terminus of ITGA6 and AA6NT rabbit polyclonal antibody specific for the NH_2 terminus of ITGA6 has been previously characterized [Demetriou and Cress, 2004; Demetriou et al., 2004; Pawar et al., 2007; Ports et al., 2009] and was generated and purified by Bethyl Laboratories (Montgomery, TX). The actin polyclonal antibody used for immunoblot analysis was obtained from Cytoskeleton (Denver, CO). The horseradish peroxidase–conjugated secondary antibodies used for immunoblotting were from Jackson Immuno-Research Laboratories, Inc. (West Grove, PA). The SRY (sex-determining region Y) box 10 (SOX10) antibody and amiloride was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and the S100β antibody was obtained from Millipore (Temecular, CA).

CONDITIONED MEDIUM

S16 and S16Y Schwann cell conditioned medium used in all experiments was collected after incubation for 48 h in DMEM medium supplemented with 10% FBS.

IMMUNOHISTOCHEMISTRY

Human prostate and pancreatic cancer tissues were harvested, fixed in 10% neutral buffered formalin for 24 h, processed, and embedded in paraffin using the Tissue Acquisition and Cellular/Molecular Analysis Shared Service (TACMASS) of The University of Arizona Cancer Center. Immunohistochemistry was performed using the affinity purified AA6NT antibody (1:700) and the anti-S100β antibody (1:1000) on a Discovery XT Automated Immunostainer (Ventana Medical Systems, Inc., Tucson, AZ). Antigen retrieval was performed using a borate-EDTA buffer at 100°C.

IMMUNOPRECIPITATION AND IMMUNOBLOT ANALYSIS

Cell lysates were prepared by harvesting the cells into PBS (phosphate buffer solution, pH 7.4) and centrifuging the cells (2500 rpm for 5 min at 4°C). The pellet was resuspended in ice-cold radioimmunoprecipitation assay (RIPA) buffer (50 mmol/L Tris, 150 nmol/L NaCl, 1% Triton X-100, 0.10% sodium dodecyl sulfate, and 1% deoxycholate) with complete mini protease inhibitor cocktail (Roche, Indianapolis, IN). The lysates were incubated on ice for 30 min and centrifuged (14,000 rpm for 5 min at 4°C). Protein quantification was performed using the Bradford Assay (Bio-Rad Laboratories, Hercules, CA). ITGA6 was immunoprecipitated (IP) from DU145, PC3, RWPE-1, and CFPAC1 cells using the J1B5 antibody (1mg/ml, 1:100), incubated at 4°C with continuous rotation overnight and analyzed using SDS–PAGE. The AA6A antibody (1 mg/ml, 1:10,000) was used to detect ITGA6 using immunoblot analysis. SOX10 and S100β expression were determined by lysing the Schwann S16 and S16Y cells in RIPA buffer, resolving the samples by SDS–PAGE, and immunoblotting using the SOX10 (1 mg/ml, 1:1000) and S100β (1 mg/ml, 1:1000) antibodies. The proteins were visualized using the ECL Western Blotting Detection System from Invitrogen. Quantitation of the blots was done using NIH Image J analysis to determine the area density of the bands and the corresponding ratios.

CULTREX INVASION ASSAY

The Cultrex laminin invasion assay (Trevigen Inc., Gaithersburg, MD) was performed as recommended by the manufacturer. Briefly, DU145, PC3, and CFPAC1 cells were plated in 10 cm plates, cultivated until 80% confluent, and then serum-starved overnight. The upper chambers of the Cultrex plates were coated with 50 μL of 0.2x mouse laminin 111 (5x laminin solution diluted in 1x coating buffer supplied by Trevigen Inc.) at 37°C overnight. Commercially available Laminin 111, a known ligand for ITGA6, was used as an ECM surface. The cells were harvested and 50 μL of cell suspension was added to each well. The J8H and AIIB2 blocking antibodies (1 mg/ml; 1:100) were added to the cells prior to plating. The access port was used to add 150 μL of control or conditioned medium to each well. The cells were then incubated for 24 h in a 37°C CO_2 incubator. The following day, the top and bottom chambers were aspirated and washed twice, and invaded cells were detected using calcein-AM containing cell dissociation buffer and an immunofluorescence plate reader at 485/520 nm per the manufacturer's instructions.

FLOW CYTOMETRY

Flow cytometry analysis was used to quantitate integrin ITGA6 cell surface expression on DU145, PC3, and CFPAC1 cells cultured in the presence of S16Y Schwann cell conditioned medium. The tumor cells were harvested using EDTA/PBS (5 mmol/L) and 1.5×10^6 cells were used per experimental condition. The cells were washed in 0.2% BSA in PBS and centrifuged at 1,000 rpm at 4°C for 2 min and then suspended in 500 μL of 0.2% BSA in PBS with the J1B5 ITGA6-specific antibody diluted 1:200. Antibody binding was detected using a goat anti-mouse IgG PE conjugated antibody diluted 1:500 (Invitrogen). All antibody reactions were incubated on ice for 30 min with gentle vortexing every 5 min and were analyzed by the Flow Cytometry core service at The University of Arizona Cancer Center.

RESULTS

SCHWANN CELLS IN PNI: HUMAN TISSUE

In order to identify whether Schwann cells were present within prostate and pancreatic tumors, formalin-fixed paraffin-embedded human prostate and pancreatic tissues were probed with the Schwann cell specific marker S100β (Fig. 1). Perineural invasion (PNI) of prostate cancer tissue (Fig. 1A) and pancreatic cancer tissue (Fig. 1C) shows an intact nerve sheath with Schwann cells separated from tumor cells by the perineurium. Endoneural invasion of cancer cells beyond the perineural space (Fig. 1B) demonstrates Schwann cells interspersed throughout invading tumor cells. Prostate tumor cells undergoing PNI express ITGA6 protein on the cell surfaces [Sroka et al., 2010] and Fig. 1D shows expression of ITGA6 on cell–cell surfaces and polarized in pancreatic tumor cells undergoing PNI. Notably, ITGA6 is also expressed in Schwann cells as expected [Niessen et al., 1994].

CHARACTERIZATION OF IMMORTALIZED SCHWANN CELL LINES AND CO-CULTURE CONDITIONS

The goal was to determine whether Schwann cells of the myelinating or non-myelinating phenotype affect tumor cell invasion. Immortalized S16 and S16Y cells were derived from primary Schwann cells isolated from the sciatic nerve of neonatal rats as previously described [Toda et al., 1994; Hai et al., 2002]. Hai et al. [2002] established that S16 cells express proteins required for myelination while the S16Y cells do not express these critical myelination specific proteins. Previous studies have determined the requirement of SOX10 and S100β expression in Schwann cell lineages to confirm Schwann cell identity and progression into the mature Schwann cell lineage [Finzsch et al., 2010]. Therefore, we determined whether SOX10 and S100β were expressed in S16 and S16Y Schwann cells using immunoblot analysis (Fig. 2). The results demonstrate that DU145 and PC3 cells did not express Schwann cell specific markers as expected and S16Y cells express higher levels of SOX10 and S100β than S16 cells (Fig. 2).

S16 CONDITIONED MEDIUM INCREASED TUMOR CELL INVASION, DEPENDENT ON A6B1

We next determined whether treatment of tumor cells with S16 and S16Y cell conditioned medium affected tumor cell invasion and

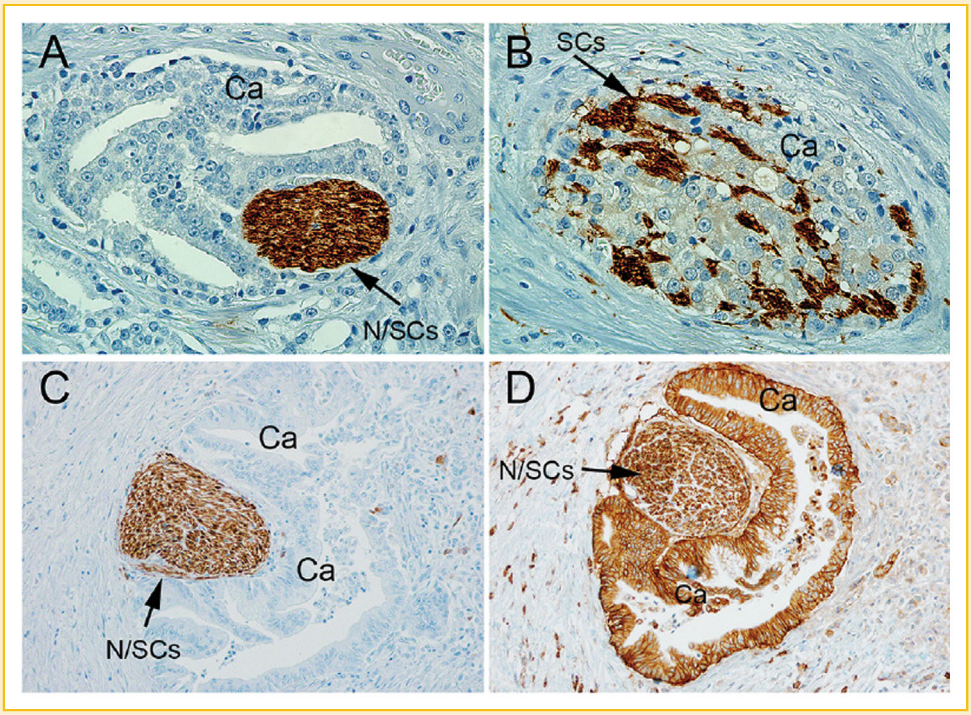

Fig. 1. Perineural and endoneural tumor cell invasion in human prostate and pancreatic cancer. (A) Prostate cancer cells (Ca) invade around the perineurium of the nerve (N) near Schwann cells (SCs, arrow; stained with S100 antibody; magnification x 20). (B) Endoneural invasion shows SCs stained with S100 antibody; interspersed throughout the invaded nerve (arrow) and in contact with prostate tumor cells (x 40). (C) Pancreatic cancer tissue stained with the S100 antibody indicating the presence of SCs in the nerve (arrow) with cancer invading the perineurium (x 20). (D) ITGA6 expression (dark brown) on pancreatic cancer cells (x 20) invading the perineurium of the same nerve depicted in part C and within SCs as expected.

whether this was integrin dependent. Previous work by our group established that A6B1 integrin and production of A6pB1 promoted tumor cell migration, invasion, and metastasis. The J8H antibody blocks A6pB1 production without altering the cell adhesion function. Prior studies have established that B1 blocking antibody can prevent DU145 and PC3 from integrin dependent attachment to laminin 111 without affecting integrin independent attachment [King et al., 2008; Ports et al., 2009; Sroka et al., 2011; Landowski et al., 2014]. Figure 3A, B, C demonstrated a 1.6–2.0 fold increase in invasion of DU145, PC3 prostate, and CFPAC1 pancreatic cell lines ($P < 0.05$) with S16 conditioned medium, respectively. In contrast, S16Y conditioned medium decreased invasion by 50–60% of the FBS control ($P < 0.05$). Blocking ITGA6p formation using the J8H antibody or ITGB1 function using the AIIB2 antibody (Fig. 3A, B, C) eliminated or significantly decreased S16 induced migration of DU145, PC3, and CFPAC1 cells ($P < 0.05$).

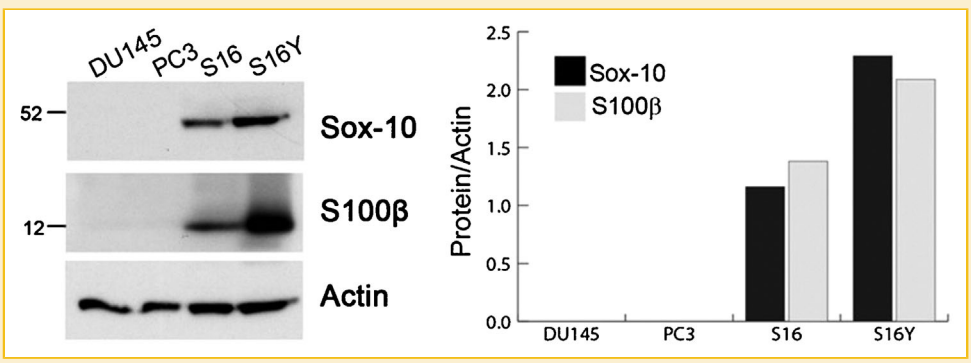

Fig. 2. Immortalized S16 and S16Y rat Schwann cell lines expressed Schwann cell specific markers. Immunoblot analysis was used to identify expression of the Schwann cell specific transcription factor SOX10 and the S100β protein. Actin was used as a loading control. The results are representative of three independent experiments.

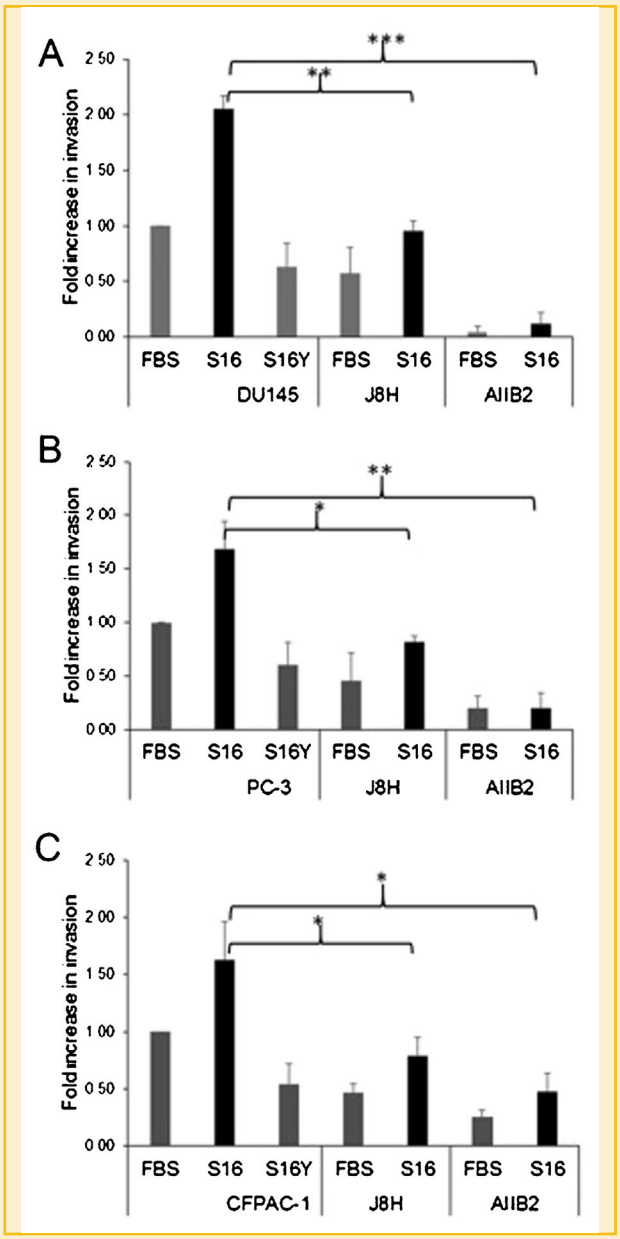

Fig. 3. S16 conditioned media increased tumor cell invasion dependent on A6B1. (A) DU145, (B) PC3, and (C) CFPAC1 cells were analyzed using the Cultrex modified Boyden chamber invasion assay with laminin 111. The fold-increase in invasion was determined under conditions of either FBS, S16 (black bars), or S16Y (gray bars) conditioned media, FBS+J8H (ITGA6 blocking antibody), S16+J8H (ITGA6 blocking antibody), FBS+AIIB2 (ITGB1 blocking antibody), or S16+AIIB2 (ITGB1 blocking antibody). The cells were treated with the blocking antibodies during the invasion assay. The results are expressed as mean values ± SD of three independent experiments. The asterisks denote a significant difference ($^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0001$, unpaired Student Test) comparing the samples as indicated by the brackets.

S16 AND S16Y SCHWANN CELL CONDITIONED MEDIA ALTERED ITGA6p PRODUCTION

DU145 and PC3 prostate tumor cells, CFPAC1 pancreatic tumor cells, and RWPE-1 cells were cultured in the presence of S16 and S16Y conditioned medium for 24 h, followed by immunoprecipitation of ITGA6 and immunoblot analysis. Incubation of the cells with S16 conditioned medium increased production of ITGA6p as compared to S16Y conditioned medium in all four cell lines (Fig. 4A, B). The S16 induced production of ITGA6p was inhibited in both RWPE-1 and DU145 cells by amiloride, a uPA inhibitor (Fig. 4 C), consistent with our earlier work [Ports et al., 2009; Sroka et al., 2011].

REVERSIBLE SUPPRESSION OF TUMOR CELL ITGA6p PRODUCTION BY S16Y CONDITIONED MEDIUM

We next determined whether the decreased cleavage of ITGA6 observed in the presence of S16Y (non-myelinating phenotype) conditioned medium was reversible. DU145, PC3, and CFPAC1 cells were treated with S16Y conditioned medium for 24 h followed by cultivation in either S16Y conditioned medium or IMDM for an additional 24, 48, or 72 h. The cells grown in IMDM demonstrated increased ITGA6NT, indicating recovery of cleavage of the receptor. Flow cytometric analysis following 24 h of S16 and S16Y conditioned medium treatment of DU145 (Fig. 5Ac), PC3 (Fig. 5Bc), and CFPAC1 (Fig. 5Cc) showed that ITGA6 cell surface levels are unchanged in tumor cells treated with the conditioned medium. These results indicate that altered cleavage of the receptor mediated by Schwann cell conditioned medium occurs on the cell surface and is not an effect related to altered expression of the receptor on the cell surface.

DISCUSSION

Invading tumor cells damage nerve axons as they invade [Nagakawa et al., 1992; Liu and Lu, 2002; Li et al., 2011], and it has been well-characterized that myelinating Schwann cells secrete a myriad of trophic and adhesive factors including neurotrophins, cytokines, and laminin extracellular matrix proteins as they execute the regeneration program [Stoll and Muller, 1999; Jessen and Mirsky, 2005; Campana, 2007]. Studies have identified the role of reciprocal signaling between tumor cells and the nerve environment or stromal cells as promoters of perineural invasion [Dai et al., 2007; Ceyhan et al., 2008; He et al., 2014b; Li et al., 2014]. Others have shown that Schwann cells expressing MAG increase pancreatic tumor cell adhesion and perineural invasion by binding to MUC1 on tumor cells [Swanson et al., 2007] or tumor-specific pleiotrophin attraction to N-syndecan produced by Schwann cells [Yao et al., 2013].

However, the role of secreted factors produced by myelinating Schwann cells and the role of laminin adhesion in PNI has not been studied. Expression levels of the laminin receptor, A6B1, and cleavage to form the variant A6pB1 are associated with increased tumor cell invasion and enhanced metastatic potential [King et al., 2008; Ports et al., 2009; Sroka et al., 2010; Sroka et al., 2011; Landowski et al., 2014]. We have shown that ITGA6 is highly expressed in neurotropic cancer cells undergoing perinerual invasion in both prostate [Sroka et al., 2010] and pancreatic cancer (Fig. 1B and C) and in tumor cells invading the endoneurium where Schwann cells reside (Fig. 1B). We hypothesized that tumor cells require A6B1 and A6pB1 dependent migration and invasion along the laminin rich nerve axons

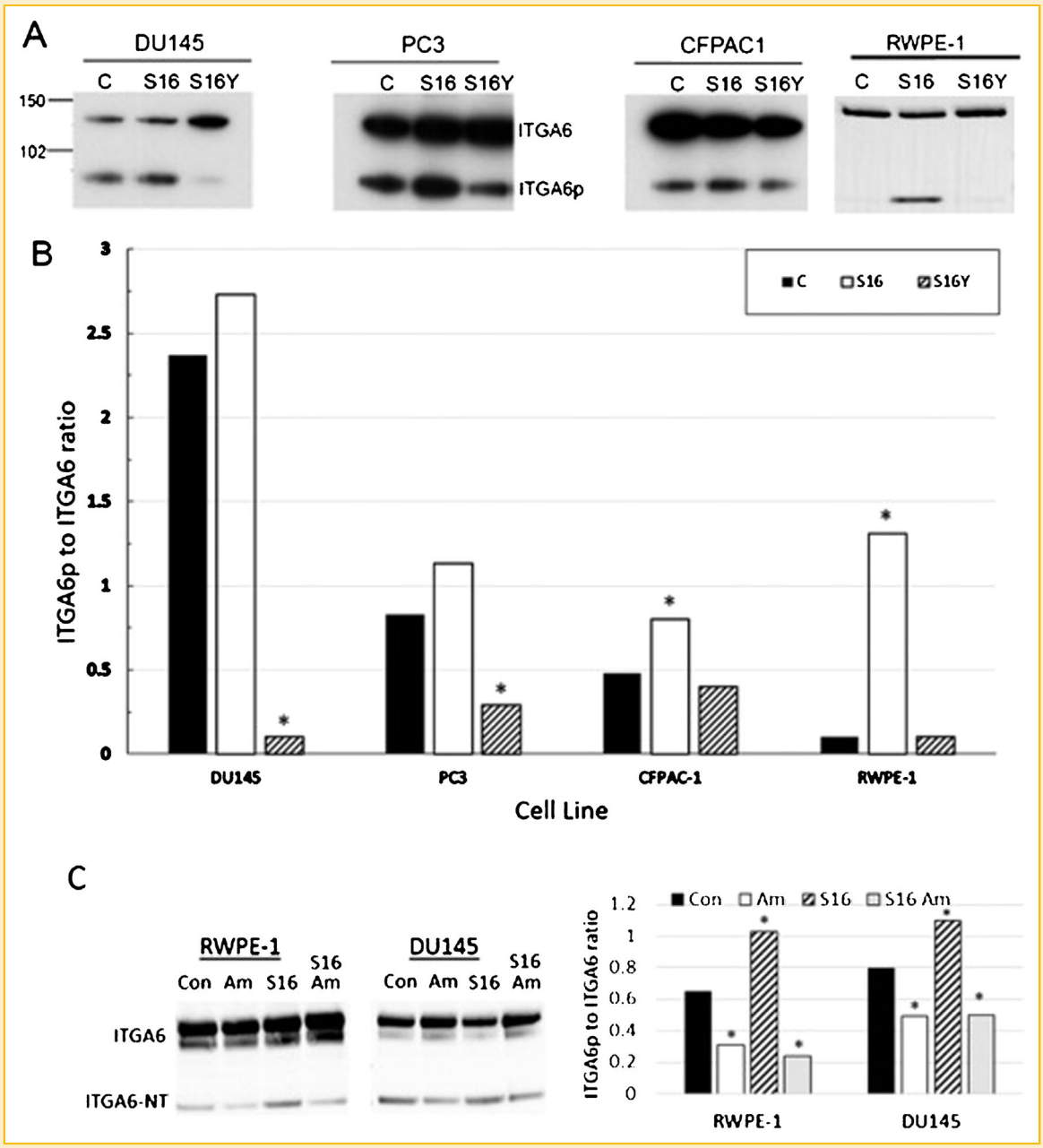

Fig. 4. Suppression of ITGA6p production in tumor cells by S16Y cell (non-myelinating phenotype) conditioned media. (A) DU145, PC3, CFPAC1 tumor, and normal prostate (RWPE-1) cells were treated with DMEM control media (C), or S16 and S16Y conditioned media for 24 h. Integrin A6 (ITGA6) and A6p (ITGA6p) were immunoprecipitated using the J1B5 antibody and detected by immuno blot. (B) Quantitative analysis of the immuno blot experiments in part A for DU145, PC3, CFPAC1, and RWPE-1 cell lines. NIH Image J analysis determined the ratio of area density of ITGA6p to ITGA6 as shown. The results are representative of three independent experiments and the asterisk denotes a significant difference ($P < 0.05$, unpaired Student *T*-test) as compared to the control media (C) sample. (C) RWPE and DU145 cells were treated with DMEM control media (C) or 100 uM amiloride (Am) or S16 conditioned media alone (S16) or S16 conditioned media containing amiloride (S16 Am) for 24 h. Immunoblot analysis of whole cell lysates was used to detect integrin A6 (ITGA6) or the ITGA6-NT fragment as a surrogate marker for ITGA6p production. NIH Image J analysis determined the ratio of area density of ITGA6p to ITGA6 as shown. The results are representative of three independent experiments and the asterisk denotes a significant difference ($P < 0.05$, unpaired Student *T*-test) as compared to the signal in the control sample of each group.

[Feltri and Wrabetz, 2005; Chernousov et al., 2008] as they invade along the perineurium and into the endoneural space in response to Schwann cell signals.

In order to understand the role of A6B1 integrin in modulating the aggressive tumor phenotype in response to Schwann cells during PNI, we used the immortalized rat Schwann S16 and S16Y cell lines in model systems (in vitro) with pancreatic and prostate tumor cell lines. Previous work established that the S16 cells exhibit characteristics of Schwann cells that are in the early phase of the myelination process, including decreased proliferation, clustering around neurons in culture, and

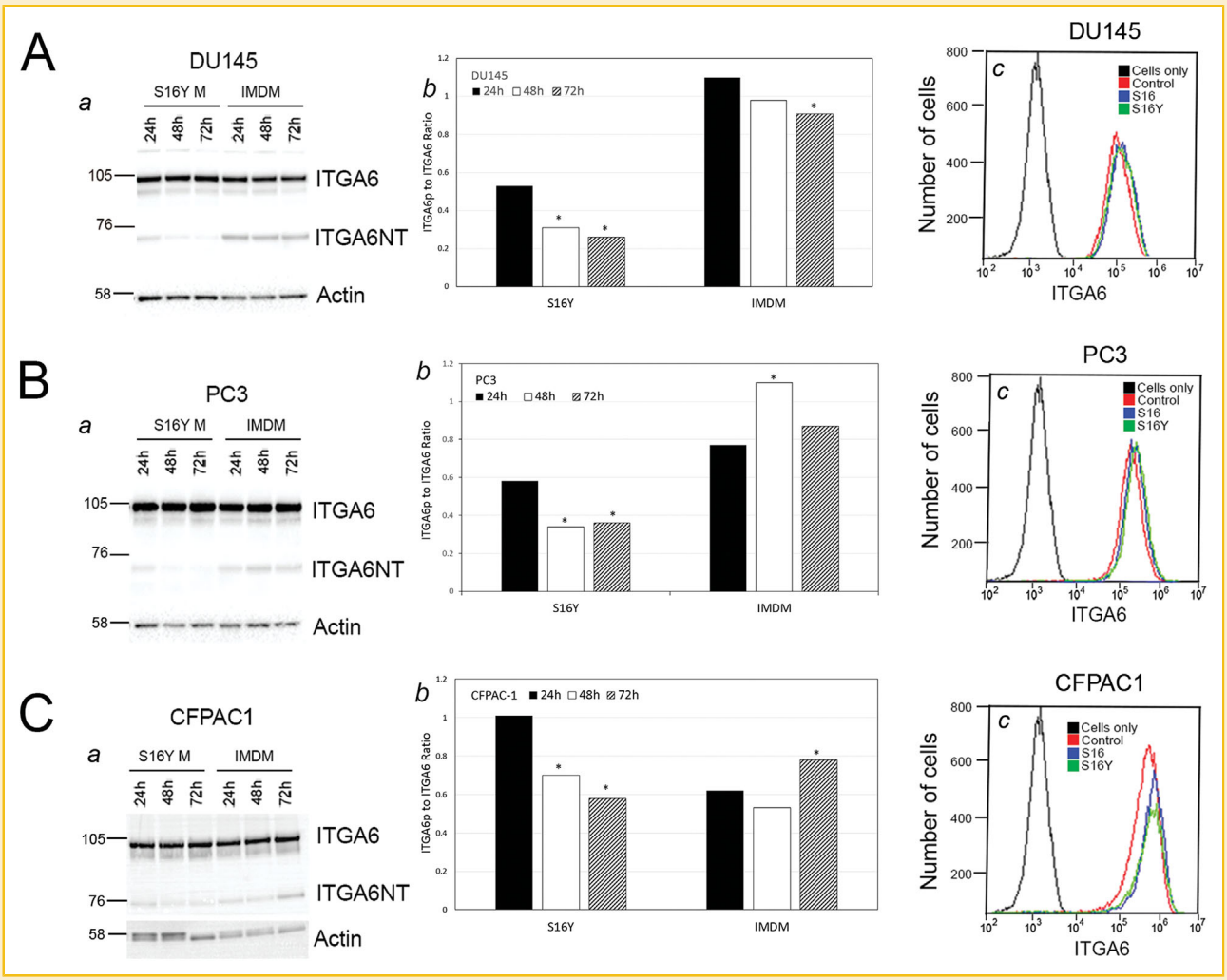

Fig. 5. Recovery of ITGA6p expression in tumor cell lines treated with S16Y (non-myelinating phenotype) conditioned medium. (A) The DU145, the (B) PC3, and (C) CFPAC1 cancer cell lines were treated with S16Y conditioned medium for 24 h to decrease ITGA6p expression. The ITGA6NT fragment is a surrogate marker for ITGA6p production. The cells were then treated with either S16Y medium (S16Y M) or control medium (IMDM) for an additional 24, 48, or 72 h. Immunoblot analysis of whole cell lysates was used to detect A6 (ITGA6) and ITGA6NT with actin used as a loading control (A, B, and C, panel a). Quantitative analysis of the immunoblots A, B, and C is shown in (b). NIH Image J analysis determined the ratio of area density of ITGA6p to ITGA6 as shown. The results are representative of three independent experiments and the asterisk denotes a significant difference ($P < 0.05$, unpaired Student *T*-test) as compared to the signal in the 24 h sample of each group. Panel c of A, B, and C is total cell surface levels of ITGA6 as detected by flow analysis on the cell lines treated with control media, S16, or S16Y conditioned media for 24 h. The black peak = cells only control, red peak = cells treated with control media, blue = cells treated with S16 media, and green = cells treated S16Y conditioned media. All results are representative of three independent experiments.

increased expression of the myelin specific proteins MAG, P0, MBP, and laminin glycoproteins [Toda et al., 1994], even in the absence of axons that are normally required for Schwann cells to express these proteins [Jessen and Mirsky, 2005]. Conversely, the S16Y cells, which are a spontaneous clone of the S16 cells, exhibit rapid proliferation, decreased expression of MAG, P0, and laminin proteins, and are unable to cluster around neurons in vitro, which are characteristics of Schwann cells that are not actively myelinating [Toda et al., 1994; Stoll and Muller, 1999]. It is not known whether the S16 and S16Y cells represent the glial cell lineages of myelinating or non-myelinating Schwann cells specifically; therefore, we determined whether the S16 and S16Y cells are Schwann cells by detecting expression of SOX10, a transcription factor required for Schwann cell differentiation, and S100β, a marker for mature Schwann cells [Jessen and Mirsky, 2005] (Fig. 2). These results indicate that although the S16 and S16Y cells demonstrate differences in Schwann cell characteristics (i.e., expression of myelin specific proteins), they are indeed mature Schwann cells.

Studies have shown that a reciprocal relationship exists between tumor cells and the nerve environment during PNI [Dai et al., 2007; Ceyhan et al., 2008], and other researchers have shown that nerve growth-like proteins secreted from prostate stromal cells and tumor cell exposure to NGF increased tumor cell invasion [Djakiew et al., 1993; Geldof et al., 1997]. Recent work by Gao et al. [2014] established that the neurotrophic factor artemin expressed in nerve fibers and stroma increased pancreatic cancer PNI, and Li et al.

[2014] showed that sonic hedgehog paracrine signaling activated stromal cells and increased tumor cell PNI. Other studies have shown that DRG devoid of the chemokine CCL2 harvested from knockout mice decreased tumor cell PNI and that soluble glial cell derived neurotrophic factor (GDNF) family receptor (GFR) alpha1 released from DRG increased PNI [He et al., 2014a, 2014b]. Based on these studies demonstrating the role of paracrine factors in the nerve environment in regulating PNI, we hypothesized that secretion of Schwann cell factors may increase tumor PNI.

Results from this study show that S16 Schwann cell conditioned medium significantly increased cleavage of ITGA6 to produce ITGA6p integrin in both prostate (DU145, PC3, RWPE-1) and pancreatic (CFPAC1) cells. In tumor cell lines, total cell surface levels of ITGA6 remained constant while increased levels of ITGA6p were observed as well as significantly increased tumor cell invasion. These results suggest that S16 cells secrete factors that promote cleavage of ITGA6 integrin to the more motile and aggressive variant. Likely molecular candidates in the secretome include the multiple components of the uPA:uPAR axis since it regulates ITGA6 integrin cleavage [Ports et al., 2009; Sroka et al., 2011]. A uPA inhibitor, amiloride, will significantly decrease ITGA6p production mediated by S16 cells (Fig. 4C). A recent study by Rivellini et al. [2012] established that uPAR is required for nerve axon repair and that uPA levels increase significantly following nerve damage. An understanding of the nerve uPA/PAI secretome to include the level and activity of uPA and its many forms, along with forms of uPAR and known secreted inhibitors, would be very informative. Considering the decrease in cleavage was reversible, we hypothesize that either continuous exposure to a soluble factor or reversal of a tumor cell signaling response mediates down-regulated cleavage of the receptor. Endogenous inhibitors of the uPA:uPAR axis, such as plasminogen activator inhibitor-1 [Mekkawy et al., 2014] may be of interest for future studies.

In summary, the results indicated that in human tumor tissue specimens, the tumor cells undergoing PNI breach the perineurium and contact Schwann cells in the endoneural space, suggesting that Schwann cells may play a role in promoting the metastatic program. The conditioned medium from S16 and S16Y Schwann cells significantly altered both the cleavage of the A6B1 integrin laminin receptor and tumor cell invasion. The decreased cleavage observed in the presence of S16Y conditioned medium was reversible and suggests that a dynamic relationship likely exists between Schwann cell subtypes and tumor cells. Future work in proteomics with modern mass spectrometry approaches will likely identify factors secreted by Schwann cells that elicit differential tumor responses and mechanisms of PNI during metastasis. Understanding the factors that stimulate or impede tumor cell invasion may offer new therapeutic opportunities and provide hope for individuals diagnosed with aggressive forms of neurotropic cancers.

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REFERENCES

Bapat AA, Hostetter G, Von Hoff DD, Han H. 2011. Perineural invasion and associated pain in pancreatic cancer. Nat Rev Cancer 11(10): 695–707.

Bohnsack JF, Akiyama SK, Damsky CH, Knape WA, Zimmerman GA. 1990. Human neutrophil adherence to laminin in vitro. Evidence for a distinct neutrophil integrin receptor for laminin. J Exp Med 171(4):1221–1237.

Campana WM. 2007. Schwann cells: Activated peripheral glia and their role in neuropathic pain. Brain, behavior, and immunity 21(5):522–527.

Ceyhan GO, Demir IE, Altintas B, Rauch U, Thiel G, Muller MW, Giese NA, Friess H, Schafer KH. 2008. Neural invasion in pancreatic cancer: A mutual tropism between neurons and cancer cells. Biochemi Biophys Res Commun 374(3):442–447.

Chernousov MA, Yu WM, Chen ZL, Carey DJ, Strickland S. 2008. Regulation of Schwann cell function by the extracellular matrix. Glia 56(14):1498–1507.

Dai H, Li R, Wheeler T, Ozen M, Ittmann M, Anderson M, Wang Y, Rowley D, Younes M, Ayala GE. 2007. Enhanced survival in perineural invasion of pancreatic cancer: An in vitro approach. Hum pathol 38(2):299–307.

Damsky CH, Librach C, Lim KH, Fitzgerald ML, McMaster MT, Janatpour M, Zhou Y, Logan SK, Fisher SJ. 1994. Integrin switching regulates normal trophoblast invasion. Development 120(12):3657–3666.

Demetriou MC, Cress AE. 2004. Integrin clipping: A novel adhesion switch?. J Cell Biochem 91(1):26–35.

Demetriou MC, Pennington ME, Nagle RB, Cress AE. 2004. Extracellular alpha 6 integrin cleavage by urokinase-type plasminogen activator in human prostate cancer. Exp Cell Res 294(2):550–558.

Djakiew D, Pflug BR, Delsite R, Onoda M, Lynch JH, Arand G, Thompson EW. 1993. Chemotaxis and chemokinesis of human prostate tumor cell lines in response to human prostate stromal cell secretory proteins containing a nerve growth factor-like protein. Cancer Res 53(6):1416–1420.

Dubovy P. 2004. Schwann cells and endoneurial extracellular matrix molecules as potential cues for sorting of regenerated axons: A review. Anat Sci Int 79(4):198–208.

Feltri ML, Wrabetz L. 2005. Laminins and their receptors in Schwann cells and hereditary neuropathies. J Peripher Nerv Syst: JPNS 10(2):128–143.

Finzsch M, Schreiner S, Kichko T, Reeh P, Tamm ER, Bosl MR, Meijer D, Wegner M. 2010. Sox10 is required for Schwann cell identity and progression beyond the immature Schwann cell stage. J Cell Biol 189(4):701–712.

Gao L, Bo H, Wang Y, Zhang J, Zhu M. 2014. Neurotrophic factor artemin promotes invasiveness and neurotrophic function of pancreatic adenocarcinoma in vivo and in vitro. Pancreas.

Geldof AA, De Kleijn MA, Rao BR, Newling DW. 1997. Nerve growth factor stimulates in vitro invasive capacity of DU145 human prostatic cancer cells. J Cancer Res Clin Oncol 123(2):107–112.

Hai M, Muja N, DeVries GH, Quarles RH, Patel PI. 2002. Comparative analysis of Schwann cell lines as model systems for myelin gene transcription studies. J Neurosci Res 69(4):497–508.

He S, Chen CH, Chernichenko N, He S, Bakst RL, Barajas F, Deborde S, Allen PJ, Vakiani E, Yu Z, Wong RJ. 2014a. GFRalpha1 released by nerves enhances cancer cell perineural invasion through GDNF-RET signaling. Proc Nat Acad Sci USA 111(19):E2008–E2017.

He S, He S, Chen CH, Deborde S, Bakst RL, Chernichenko N, McNamara WF, Lee SY, Barajas F, Yu Z, Al-Ahmadie HA, Wong RJ. 2014b. The chemokine (CCL2-CCR2) signaling axis mediates perineural invasion. Mol Cancer Res: MCR.

Hirai I, Kimura W, Ozawa K, Kudo S, Suto K, Kuzu H, Fuse A. 2002. Perineural invasion in pancreatic cancer. Pancreas 24(1):15–25.

Hogervorst F, Admiraal LG, Niessen C, Kuikman I, Janssen H, Daams H, Sonnenberg A. 1993. Biochemical characterization and tissue distribution of the A and B variants of the integrin alpha 6 subunit. J Cell Bbiol 121(1):179–191.

Hynes RO. 2002. Integrins: Bidirectional, allosteric signaling machines. Cell 110(6):673–687.

Jessen KR, Mirsky R. 2005. The origin and development of glial cells in peripheral nerves. Nat Rev Neurosci 6(9):671–682.

Kidd GJ, Ohno N, Trapp BD. 2013. Biology of Schwann cells. Handb Clin Neurol 115:55–79.

King TE, Pawar SC, Majuta L, Sroka IC, Wynn D, Demetriou MC, Nagle RB, Porreca F, Cress AE. 2008. The role of alpha 6 integrin in prostate cancer migration and bone pain in a novel xenograft model. PLoS ONE 3(10):-e535.

Landowski TH, Gard J, Pond E, Pond GD, Nagle RB, Geffre CP, Cress AE. 2014. Targeting integrin alpha6 stimulates curative-type bone metastasis lesions in a xenograft model. Mol Cancer Ther 13(6):1558–1566.

Li J, Ma Q, Liu H, Guo K, Li F, Li W, Han L, Wang F, Wu E. 2011. Relationship between neural alteration and perineural invasion in pancreatic cancer patients with hyperglycemia. PLoS ONE 6(2):e17385.

Li X, Wang Z, Ma Q, Xu Q, Liu H, Duan W, Lei J, Ma J, Wang X, Lv S, Han L, Li W, Guo J, Guo K, Zhang D, Wu E, Xie K. 2014. Sonic hedgehog paracrine signaling activates stromal cells to promote perineural invasion in pancreatic cancer. Clin Cancer Res 20(16):4326–4338.

Liebig C, Ayala G, Wilks JA, Berger DH, Albo D. 2009. Perineural invasion in cancer: A review of the literature. Cancer 115(15):3379–3391.

Liu B, Lu KY. 2002. Neural invasion in pancreatic carcinoma. Hepato Pancreatic Dis Int: HBPD INT 1(3):469–476.

Loukas M, Klaassen Z, Merbs W, Tubbs RS, Gielecki J, Zurada A. 2010. A review of the thoracic splanchnic nerves and celiac ganglia. Clin Anat 23(5):512–522.

Mekkawy AH, Pourgholami MH, Morris DL. 2014. Involvement of urokinase-type plasminogen activator system in cancer: An overview. Med Res Rev 34(5):918–956.

Nagakawa T, Kayahara M, Ueno K, Ohta T, Konishi I, Miyazaki I. 1992. Clinicopathological study on neural invasion to the extrapancreatic nerve plexus in pancreatic cancer. Hepato-Gastroenterology 39(1):51–55.

Nave KA, Werner HB. 2014. Myelination of the nervous system: Mechanisms and functions. Ann Rev Cell Dev Biol 30:503–533.

Niessen CM, Cremona O, Daams H, Ferraresi S, Sonnenberg A, Marchisio PC. 1994. Expression of the integrin alpha 6 beta 4 in peripheral nerves: Localization in Schwann and perineural cells and different variants of the beta 4 subunit. J Cell Sci 107(Pt2):543–552.

Pawar SC, Demetriou MC, Nagle RB, Bowden GT, Cress AE. 2007. Integrin alpha6 cleavage: A novel modification to modulate cell migration. Exp Cell Res 313(6):1080–1089.

Ports MO, Nagle RB, Pond GD, Cress AE. 2009. Extracellular engagement of alpha6 integrin inhibited urokinase-type plasminogen activator-mediated cleavage and delayed human prostate bone metastasis. Cancer Res 69(12):5007–5014.

Rivellini C, Dina G, Porrello E, Cerri F, Scarlato M, Domi T, Ungaro D, Del Carro U, Bolino A, Quattrini A, Comi G, Previtali SC. 2012. Urokinase plasminogen receptor and the fibrinolytic complex play a role in nerve repair after nerve crush in mice, and in human neuropathies. PLoS ONE 7(2):e32059.

Sasagasako N, Toda K, Hollis M, Quarles RH. 1996. Myelin gene expression in immortalized Schwann cells: Relationship to cell density and proliferation. J Neurochem 66(4):1432–1439.

Schmelz M, Cress AE, Scott KM, Burger F, Cui H, Sallam K, McDaniel KM, Dalkin BL, Nagle RB. 2002. Different phenotypes in human prostate cancer: Alpha6 or alpha3 integrin in cell-extracellular adhesion sites. Neoplasia 4(3):243–254.

Siegel R, Ma J, Zou Z, Jemal A. 2014. Cancer statistics. CA: Cancer J Clin 64(1):9–29.

Sroka IC, Anderson TA, McDaniel KM, Nagle RB, Gretzer MB, Cress AE. 2010. The laminin binding integrin alpha6beta1 in prostate cancer perineural invasion. J Cell Phys 224(2):283–288.

Sroka IC, Sandoval CP, Chopra H, Gard JM, Pawar SC, Cress AE. 2011. Macrophage-dependent cleavage of the laminin receptor alpha6beta1 in prostate cancer. Mol Cancer Res: MCR 9(10):1319–1328.

Stoll G, Muller HW. 1999. Nerve injury, axonal degeneration and neural regeneration: Basic insights. Brain pathology 9(2):313–325.

Swanson BJ, McDermott KM, Singh PK, Eggers JP, Crocker PR, Hollingsworth MA. 2007. MUC1 is a counter-receptor for myelin-associated glycoprotein (Siglec-4a) and their interaction contributes to adhesion in pancreatic cancer perineural invasion. Cancer Res 67(21):10222–10229.

Toda K, Small JA, Goda S, Quarles RH. 1994. Biochemical and cellular properties of three immortalized Schwann cell lines expressing different levels of the myelin-associated glycoprotein. J Neurochem 63(5):1646–1657.

Villers A, McNeal JE, Redwine EA, Freiha FS, Stamey TA. 1989. The role of perineural space invasion in the local spread of prostatic adenocarcinoma. J Urol 142(3):763–768.

Yao J, Hu XF, Feng XS, Gao SG. 2013. Pleiotrophin promotes perineural invasion in pancreatic cancer. World J Gastroenterol: WJG 19(39):6555–6558.

Zhu GH, Huang C, Qiu ZJ, Liu J, Zhang ZH, Zhao N, Feng ZZ, Lv XH. 2011. Expression and prognostic significance of CD151, c-Met, and integrin alpha3/alpha6 in pancreatic ductal adenocarcinoma. Digest Dis Sci 56(4):1090–1098.