HHS Public Access

Author manuscript

*Exp Cell Res*. Author manuscript; available in PMC 2021 May 15.

Published in final edited form as:

*Exp Cell Res*. 2020 May 15; 390(2): 111960. doi:10.1016/j.yexcr.2020.111960.

# HLA-B Influences Integrin Beta-1 Expression and Pancreatic Cancer Cell Migration

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## Abstract

Human leukocyte antigen (HLA) class I molecules present antigenic peptides to cytotoxic T cells, causing lysis of malignant cells. Transplantation biology studies have implicated HLA class I molecules in cell migration, but there has been little evidence presented that they influence cancer cell migration, a contributing factor in metastasis. In this study, we examined the effect of HLA-B on pancreatic cancer cell migration. HLA-B siRNA transfection increased the migration of the S2–013 pancreatic cancer cells but, in contrast, reduced migration of the PANC-1 and MIA PaCa-2 pancreatic cancer cell lines. Integrin molecules have previously been implicated in the upregulation of pancreatic cancer cell migration, and knockdown of HLA-B in S2–013 cells heightened the expression of integrin beta 1 (ITGB1), but in the PANC-1 and MIA PaCa-2 cells HLA-B knockdown diminished ITGB1 expression. A transmembrane sequence in an S2–013 HLA-B heavy chain matches a corresponding sequence in HLA-B in the BxPC-3 pancreatic cancer cell line, and knockdown of BxPC-3 HLA-B mimics the effect of S2–013 HLA-B

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

knockdown on migration. In total, our findings indicate that HLA-B influences the expression of ITGB1 in pancreatic cancer cells, with concurrent distinctions in transmembrane sequences and effects on the migration of the cells.

### Keywords

HLA; integrin; major histocompatibility complex class I; migration; pancreatic cancer

## Introduction

HLA class I molecules are composed of a heavy chain, the light chain beta 2-microglobulin, and endogenous peptide.[1] These heavy chains are encoded by three separate genetic loci that encode for three similar yet not completely identical isotypes: HLA-A, -B, and - C.[2] HLA class I molecules are best known for their contribution to the adaptive immune system by presenting antigenic peptides to cytotoxic T cells. Peptide presentation to T cells leads to lysis of the target cell and this allows for protection against viral infection or malignant transformation.[3]

In the field of transplantation biology, HLA class I molecules have also been implicated in processes such as cell migration.[4] Furthermore, although HLA class I molecules can be down-regulated as a form of immune escape by cancer cells, there have been studies in various types of malignancies (including breast, gastric, and lung cancer) that have shown high expression of HLA class I molecules to be positively correlated with poor prognosis.[5–7] Additionally, HLA class I molecules were found to be more highly expressed by a set of medulloblastoma tumor samples that had accompanying phenotypic markers of poor prognosis (although clinical data were not available to verify the survival outcomes for all the patients).[8] HLA class I molecules were demonstrated to promote increased medulloblastoma cell migration upon addition of exogenous $\beta_2$m (which stabilizes cell-surface HLA class I heavy chains that have released $\beta_2$m after arrival at the plasma membrane) via an ERK1/2-mediated mechanism.[8–9] However, even though pancreatic cancer is known for its high rate of metastasis, an investigation of the role HLA class I molecules may play in cell migration in pancreatic cancer has not yet been reported. Moreover, the influence of specific HLA class I isotypes and allotypes on the migration of any type of cancer cells has never been fully addressed.

Integrins are cell-surface molecules that function as heterodimers and are present on nearly all nucleated cells. There are 8 α subunits and 18 β subunits that can pair together to form 24 disparate functional heterodimers. A specific range of heterodimers can exist on particular cell types, and they function mainly to bind to certain components of the extracellular matrix.[10–12] The α2β1 heterodimer (composed of integrin β1 [ITGB1] and integrin α2 [ITGA2]) binds mainly to collagen but also has the ability to bind to laminin, fibronectin, and E-cadherin.[13–18] This heterodimer is overexpressed in many cancer types, including pancreatic cancer, and is associated with an aggressive disease phenotype.[19–25] The α2β1 heterodimer has been shown to promote the migration of pancreatic cancer cells, as well as various other cancer cell types such as gastric cancer and prostate cancer.[20,26–30]

A few previous transplantation biology studies have indicated that integrins interact with HLA class I molecules and that this interaction can contribute to cell migration in transplantation models.[4,31,32] Whether an interaction exists between integrins and HLA class I molecules in pancreatic cancer cells, and whether this interaction correlates with pancreatic cancer cell migration have not previously been analyzed.

Thus, the purpose of this study was to investigate whether the HLA-B isotype influences the migration of pancreatic cancer cells, and, if so, to assess the potential involvement of ITGB1 and ITGA2 in this mechanism. Our findings, as described below, indicate that HLA-B is amply expressed in pancreatic cancer cells, though with variation in expression, and influences the migration of pancreatic cancer cells. Furthermore, we demonstrated that HLA-B regulates the expression of ITGB1, which has been previously established to affect the migration of pancreatic cancer cells. However, our co-immunoprecipitation data suggests that this regulation is not dependent on HLA-B association with ITGB1. Comparison of the sequences for HLA-B heavy chains that have differing effects on pancreatic cancer cell migration revealed sequences in the HLA-B transmembrane region that correspond with ability to decrease ITGB1 expression and migration. Therefore, our findings indicate that HLA-B has unexpected and important effects on pancreatic cancer cells beyond its immunological role, and that an HLA-B transmembrane sequence may govern HLA-B effects on pancreatic cancer cell migration.

## Materials and Methods

### Cell lines and transfections

The S2–013, PANC-1, MIA PaCa-2, and BxPC-3 pancreatic cancer cell lines[33–55] were used in this study. The S2–013 cell line (a gift from Dr. Michael A. Hollingsworth, University of Nebraska Medical Center, Omaha, NE) is a SUIT-2 subclone that has been used previously in many pancreatic cancer studies.[33–53] The BxPC-3 cell line was also a gift from Dr. Hollingsworth.[33] PANC-1 cells were a gift from Dr. Michel Ouellette (University of Nebraska Medical Center, Omaha, NE), and MIA PaCa-2 cells were recently purchased from the American Type Culture Collection.[54,55] Both PANC-1 and MIA PaCa-2 cells were cultured in supplemented DMEM medium, and S2–013 and BxPC-3 cells were cultured in supplemented RPMI 1640 medium. The media supplementation included 10% fetal bovine serum (Atlantic Biologics S11550, heat inactivated for 30 minutes at 56°C), 1 mM sodium pyruvate (11360–070), 2 mM L-glutamine (25030–081), 10 mM HEPES (15630–080), 1X non-essential amino acids (11140–050), 100 units/ml penicillin and 100 μg/mL streptomycin (from Penicillin-Streptomycin 10,000 U/ml stock 15140–122). All the aforementioned media additives were obtained from Thermo Fisher Scientific with the exception of the fetal bovine serum. The S2–013, PANC-1, and BxPC-3 cells were authenticated by short tandem repeat deoxyribonucleic acid profiling analysis (UNMC Molecular Diagnostics Facility) and the MIA PaCa-2 cells were authenticated by the American Type Culture Collection. MycoAlert Mycoplasma Detection Kit (Lonza LT07–118) was used to determine that all pancreatic cancer cell lines used in this study were free of mycoplasma.

The HLA-B heavy chain was experimentally knocked down by transient transfection with siRNA specific to HLA-B. The ON-TARGETplus SMARTpool siRNA (containing four

separate HLA-B-specific siRNAs), purchased from Dharmacon/Horizon Discovery, was used to downregulate HLA-B expression in all three pancreatic cancer cell lines. The OnTARGETplus SMARTpool non-targeting siRNA pool (Dharmacon D-001810-10-50) was used as a negative control. Briefly, pancreatic cancer cells were plated in a 6-well plate at $1\times10^5$ cells/well, and on the following morning the cells were starved in base maintenance medium for 3 hours. DharmaFECT Transfection Reagent No. 1 (Dharmacon T-2001–03) was incubated with siRNA (at 10 nM) for 30 minutes and the mixture was added to respective wells dropwise. Afterward, the cells were incubated for 4 hours, and serum-containing medium was then added. Knockdown of HLA-B was confirmed by immunoblot analysis.

## Antibodies

The HC-10 monoclonal antibody (which recognizes principally HLA-B and HLA-C isotypes) was used to monitor HLA-B levels.[56,57] Little to no HLA-C is expressed in the pancreatic cancer cell lines utilized in this study, so immunoblotting of lysates of these cell lines with the HC10 antibody yields a band that almost entirely consists of HLA-B (as shown by the reduction in the band's density when the cells are transfected with HLA-B siRNA). The HC-10 antibody was kindly provided by Dr. Ted Hansen (Washington University at St. Louis). For detection of ITGB1 and ITGA2 by immunoblot, antibodies specific to ITGB1 (A303–735A-T) and ITGA2 (A305–211A-T) were obtained from Bethyl Laboratories. The pFAK (Y397)-specific antibody (8556P) and total FAK-specific antibody (130095) were purchased from Cell Signaling Technologies. A rat monoclonal antibody for Hsc70 was bought from Enzo Life Sciences (ADI-SPA-815B-F).

## Assessment of pancreatic cancer cell migration

Transwell assays were used to assess changes in pancreatic cancer cell migration. Pancreatic cancer cells were seeded in 6-well plates at a density of $1\times10^5$ cells/well, and 24 hours later were transfected with siRNA for 72 hours to decrease the expression of the protein of interest. Cells were then re-seeded at $1\times10^5$ cells in base maintenance media into inserts with 8-μm pores (Falcon/Thermo Fisher Scientific 353097). Cells were incubated for 24 hours at 37°C in 5% $CO_2$, and then the inserts were stained with Hema 3 Stat Pack (Thermo Fisher Scientific 123–869) and mounted. Three random fields per well were photographed and all the cells in each field were counted. Student's two-tailed t-test was used to determine the level of significance for migration results, and the criterion used for significance was $p<0.05$.

## Immunoblotting

Cells were washed once with cold phosphate-buffered saline (PBS) and collected in cell lysis buffer (1 mM EGTA [Sigma E-3889], 1 mM EDTA [Sigma E-9884], 50 mM Tris-HCl pH 7.5 [made with Trizma base Sigma T-8524], 1% Triton X-100 [Sigma T-8532], 1 mM $Na_3VO_4$ [Thermo Fisher Scientific AA8110414], 2 mM DTT [Sigma D0632], 0.1 mM PMSF [Sigma 10837091001], and 1 g/ml Halt Cocktail [Thermo Fisher Scientific 78430]). Cell lysates were stored at −80°C overnight then thawed on ice, followed by centrifugation at 13,000 rpm for 30 min at 4°C. On the day of immunoblotting, aliquots of the lysate supernatants were combined with 5X sodium dodecyl sulfate loading dye (250 mM Tris-HCl

pH 6.8, 10% w/v sodium dodecyl sulfate [Tokyo Chemical Industry Company D0996], 30% v/v glycerol [Sigma G-5516], 5% v/v β-mercaptoethanol [Sigma M-7522], 0.02% w/v bromophenol blue [Sigma B-7021]), and boiled for 5 minutes before loading.

Cell lysate samples were loaded on 4–20% Invitrogen Novex Tris-glycine polyacrylamide pre-cast gels (Thermo Fisher Scientific XP04205BOX) and electrophoresis was performed at 90 V at room temperature. The proteins were transferred at 40 V for 2.5 hours at room temperature to polyvinylidene difluoride Immobilon-P Millipore membranes (PIVH00010). For 1 hour, membranes were blocked in a 5% w/v solution of nonfat dry milk and were then incubated overnight at 4°C with primary antibodies. After primary antibody incubation, 3 washes with 1% Tween-20 (Thermo Fisher Scientific BP-337) in PBS were performed for 5 minutes each. Subsequently, membranes were incubated for 1 hour in secondary antibodies, and washed 3 times for 5 minutes with 1% Tween-20 in PBS. The membranes were incubated in Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific 32106) and exposed to CareStream BioMax MR film (8941114) for protein visualization.

### RNA-Seq analysis

Epithelial cells (229 samples: 203 from primary tumors+26 from low-grade PanINs) and stromal cells (125 samples, with matched epithelium: 102 from primary tumors+23 from low-grade PanINs) were microdissected. Cells were isolated to produce paired samples for each patient (>1000 cells/sample). Libraries were made with the NuGEN Ovation RNA-Seq System V2 Kit and cDNAs were sequenced with an Illumina HiSeq (3500 to 30 million 100 base-pair single-end reads). The y axis shows log2 Transcripts Per Million (TPM), calculated by dividing the read counts for HLA-B by the length of the HLA-B genetic sequence in kb, and then dividing by a scaling factor (total reads per kb for all genes in the dataset/1,000,000). The TPM unit allows comparison of the proportion of reads mapping to a particular gene between samples.

## Results

### HLA-B is expressed in pancreatic cancer cell lines and pancreatic tumor epithelial cells

To determine the expression of the HLA-B heavy chain in a panel of human pancreatic cancer cell lines, we immunoblotted lysates of S2–013, PANC-1, and MIA PaCa-2 pancreatic cancer cell lines in comparison with hTERT-HPNE, an immortalized but not transformed human pancreas ductal cell line that serves as a model for non-malignant pancreatic ductal cells. As shown in Figure 1A, all of the pancreatic cancer cell lines tested have ample expression of HLA-B, as does the non-transformed hTERT-HPNE cells. Our findings suggest that despite the anti-cancer immunological function of this molecule, the HLA-B isotype is not necessarily diminished in human pancreatic cancer cell lines. Figure 1B displays data acquired using samples derived from pancreatic adenocarcinoma patients. These data were obtained by laser capture microdissection of epithelial and adjacent stromal cells, respectively, followed by RNA-Seq analysis. These data indicate that, for the majority of pancreatic cancer patients, primary tumor epithelial cell expression of HLA-B expression is not downregulated, although some patients have especially low (and others particularly high) HLA-B expression by their tumor cells.

### HLA-B knockdown influences pancreatic cancer cell migration

To investigate whether the HLA-B isotype affects pancreatic cancer cell migration, we transfected S2–013, PANC-1, and MIA PaCa-2 pancreatic cancer cell lines with pan-HLA-B-specific siRNA (or with control scrambled siRNA). After siRNA transfection, the reduction of HLA-B protein levels was confirmed in the pancreatic cancer cell lines tested (Figure 2A). By transwell assay, the migration of the S2–013 cell line was assessed as significantly increased when HLA-B expression was knocked down (Figure 2B). In contrast, when we down-regulated the expression of HLA-B in PANC-1 and MIA PaCa-2 cells, the migration of those two cell lines was significantly impaired (Figure 2B). Thus, these findings indicate clearly that HLA-B affects the migration of pancreatic cancer cells.

### Interference with HLA-B expression alters ITGB1 total expression but not ITGA2 expression

To examine what might be mediating the differential effects that HLA-B exerts on pancreatic cancer cell migration, integrin expression was investigated, as integrins have a major role in cancer cell migration, including the migration of pancreatic cancer cells.[20,26–30] ITGB1 and ITGA2 were chosen for analysis because of their high prevalence in heterodimeric form (i.e., as integrin α2β1) in pancreatic cancer.[19–25] First, we assessed the expression levels of ITGB1 and ITGA2 in pancreatic cancer cell lines. Expression of both ITGB1 and ITGA2 was confirmed in all of the pancreatic cancer cell lines tested (S2–013, PANC-1, and MIA PaCa-2) with the exception that MIA PaCa-2 cells were found to lack expression of ITGA2 (Figure 3). The expression levels for the ITGB1 and ITGA2 integrins were then assessed after HLA-B siRNA transfection of each of the cell lines. For the S2–013 cells, when HLA-B was knocked down there was little or no change in ITGA2 protein expression, but ITGB1 expression substantially increased (Figure 3A). Conversely, in PANC-1 and MIA PaCa-2 cells, there was a decrease in ITGB1 expression when HLA-B was knocked down (Figure 3B,C). ITGA2 also remained unchanged in the PANC-1 cells when HLA-B was knocked down (Figure 3B). Although the S2–013 and PANC-1 pancreatic cancer cells express ITGA2 at a higher level than the non-transformed hTERT-HPNE cells, the MIA PaCa-2 cells do not express detectable ITGA2 (Figure 3D). Notably, for each of these pancreatic cancer cell lines, the impact of HLA-B expression on ITGB1 expression exactly parallels the effect of HLA-B expression on migration (compare Figure 3 to Figure 2).

To evaluate whether HLA-B associates with ITGB1 in pancreatic cancer cells, we tried immunoprecipitating ITGB1 from lysates of S2–013 and PANC-1 cells and probing for co-immunoprecipitated HLA-B. Unfortunately, these experiments were indeterminate, because although there was a faint band (indicating possible weak co-immunoprecipitation), the band was not substantially darker than the weak band in the negative control obtained by performing an immunoprecipitation assay with anti-mouse IgG and immunoblotting for HLA-B (data not shown). Thus, our results do not support association of HLA-B with ITGB1 as the mechanism whereby HLA-B influences ITGB1 expression and pancreatic cancer cell migration.

## FAK expression is altered in S2–013 and PANC-1 pancreatic cancer cells upon HLA-B knockdown

A major, well-studied signaling molecule downstream of integrins is FAK, a non-receptor cytoplasmic tyrosine kinase.[10,58] Ligand binding leads to clustering of integrins, at which point activation of FAK by autophosphorylation at Tyr397 can occur. The Tyr397 position on FAK is a binding site for other kinases, including Src and PI3K.[59–61] The integrin-FAK pathway has been associated with many aspects of pancreatic cancer metastasis, such as adhesion, migration, and invasion.[62–65] To examine further downstream signaling controlled by the HLA-B class I molecule and the α2β1 heterodimer, the expression of pFAK (Y397) and total FAK was investigated in pancreatic cancer cell lines following knockdown of HLA-B. HLA-B knockdown in S2–013 pancreatic cancer cells caused an increase in pFAK (Y397) (Figure 4A), corresponding to the rise in ITGB1 expression that was observed as a result of HLA-B knockdown (Figure 3A). PANC-1 cells, which had a decrease in ITGB1 following HLA-B siRNA transfection (Figure 3B), also had a decline in pFAK (Y397) expression (Figure 4B). In S2–013 and PANC-1, the changes that occurred in the total FAK expression levels after HLA-B siRNA transfection followed the same pattern as the pFAK Y397 levels (Figure 4A,B). Total FAK protein expression has been found to be upregulated in many different cancer types (including pancreatic cancer), and diminished expression of this protein can contribute to decreased cell migration through reduction in the amount of FAK available for activation.[62–66] In contrast, MIA PaCa-2 pFAK (Y397) expression was not altered by HLA-B knockdown (Figure 4C). It is possible that the absence of ITGA2 in MIA PaCa-2 cells (Figure 3D) prevents regulation of FAK phosphorylation by HLA-B in these cells. Therefore, for S2–013 and PANC-1, though not for MIA PaCa-2, the FAK protein may play a role in the migration changes resulting from HLA-B knockdown.

## A sequence in the HLA-B transmembrane domain correlates with effects on migration

To discern what sequences in these HLA-B heavy chains might be responsible for the effects on migration, as well as the changes in ITGB1, pFAK, and total FAK expression, we compared the full sequences of the HLA-B heavy chains expressed in S2–013, MIA PaCa-2, and PANC-1. The allotypes of the HLA-B heavy chains in these cell lines were found in the TRON Cell Line Portal (www.celllines.tron-mainz.de), and the sequences for each of the allotypes were obtained from the IPD-IMGT/HLA database (www.ebi.ac.uk/ipd/imgt/hla). In this analysis, we noted that the two HLA-B heavy chains in S2–013 differed from each of the single HLA-B heavy chains expressed by MIA PaCa-2 and PANC-1 by amino acid residues in the transmembrane/cytoplasmic domain region (Figure 5). The S2–013 cell line expresses an HLA-B7 allotype (HLA-B*07:02) that has a cytoplasmic region cysteine residue at position 325 rather than a serine (the serine is present at this position in the other HLA-B allotypes shown in Figure 5). In addition to HLA-B*07:02, the S2–013 cell line also expresses HLA-B*59:01, which in its transmembrane domain has an isoleucine instead of a valine at position 282, and a threonine in place of an alanine at position 305, as compared to the HLA-B allotypes expressed by MIA PaCa-2 (HLA-B*14:02) and PANC-1 (HLA-B*38:01) (Figure 5). Since down-regulation of HLA-B in S2–013 cells has an effect on migration and ITGB1 expression that is opposite to that of HLA-B down-regulation in PANC-1 and MIA PaCa-2 cells, these disparities in the transmembrane/cytoplasmic domain sequences were considered as potential mediators of the differences.

In searching for a pancreatic cancer cell line expressing an endogenous HLA-B heavy chain matching the sequence of an S2–013 HLA-B heavy chain in the transmembrane domain or the cytoplasmic domain, we found BxPC-3, a human pancreatic cancer cell line that expresses only HLA-B★37:01 as a single HLA-B heavy chain (Figure 5). The transmembrane sequence of HLA-B★37:01 is completely identical to the HLA-B★59:01 transmembrane sequence, sharing the isoleucine at position 282 and the threonine at position 305. In assessing the effects of downregulating HLA-B in this cell line, we found that transfection of HLA-B siRNA greatly facilitated the migration of BxPC-3 cells, and it increased the expression of ITGB1 but not ITGA2 (Figure 6A,B,C). The effect of HLA-B siRNA transfection of BxPC-3 cells on pFAK expression was also tested, and pFAK was demonstrated to rise in the transfected BxPC-3 cells (Figure 6D). Therefore, the influence of HLA-B downregulation on BxPC-3 migration, ITGB1, and pFAK was the same as the effect of HLA-B knockdown on S2–013 cells, consistent with the possible involvement of the matched transmembrane sequence in HLA-B★37:01 and HLA-B★59:01 in the observed phenotypic alterations in both S2–013 and BxPC-3.

## Discussion

Our findings suggest that HLA-B, even though having the capability of presenting tumor antigens to cytotoxic T cells, remains highly (yet variably) expressed in human pancreatic cancer cell lines (Figure 1A) and human pancreatic cancer tissue samples (Figure 1B). Studies investigating HLA class I molecule expression in pancreatic cancer have mainly interrogated total HLA class I expression and not assessed differences in expression among HLA class I isotypes in normal tissue as compared to malignant tissue.[67–70] Our findings indicate that the HLA-B isotype is not consistently down-regulated in pancreatic cancer (Figure 1).

Additionally, we found that HLA-B slows the migration of the S2–013 pancreatic cancer cell line while it increases the migration of the PANC-1 and MIA PaCa-2 cell lines (Figure 2). In our analysis of the mechanism by which HLA-B exerts these effects on pancreatic cancer cell migration, we assessed the possible role of integrins, since some integrin molecules have been demonstrated to interact with HLA class I molecules and contribute to cell migration in an endothelial cell model of transplantation biology.[4] ITGB1 was chosen, since the integrin α2β1 heterodimer is commonly expressed in pancreatic cancer.[20–21] Consistent with our theory that ITGB1 is linked to HLA-B's effect on pancreatic cancer cell migration, we observed that ITGB1 protein expression is induced by HLA-B knockdown in S2–013 cells, yet diminished upon knockdown of HLA-B in PANC-1 and MIA PaCa-2 cells (Figure 3). The expression of the downstream effector molecule FAK (pFAK and total FAK) also followed these same trends in S2–013 and PANC-1 (but not in MIA PaCa-2, which as mentioned above may be due to its lack of ITGA2, as shown in Figure 3D).

Pancreatic cancer cells express other integrin heterodimers in addition to integrin α2β1, and therefore certain integrin heterodimers not yet explored in this study could also be contributing to the differential effects of HLA-B on pancreatic cancer cell migration. The potential influence of other integrins is particularly relevant in the case of the MIA PaCa-2 cell line, which does not express ITGA2. ITGA3, another integrin alpha chain found to

heterodimerize in pancreatic cancer,[71] might also be mediating effects on migration under the influence of HLA-B in MIA PaCa-2 cells. Furthermore, ITGA6, which binds to both ITGB1 and ITGB4,[72–73] could also have a role in conjunction with HLA-B class I molecules in pancreatic cancer cells.

In the S2–013 cell line, HLA-B represses cell migration, as compared to the PANC-1 and MIA PaCa-2 cell lines, for which HLA-B was found to induce cell migration. S2–013 cells express two distinct allotypes of HLA-B, while PANC-1 and MIA PaCa-2 cells express only one allotype, likely having lost the other allotype during oncogenic transformation. The sequences of the particular HLA-B allotypes expressed by these pancreatic cancer cell lines must be responsible, either directly or indirectly, for differential effects on migration and ITGB1 expression.

A previous study indicated that an interaction in endothelial cells between ITGB4 and HLA class I molecules is mediated by the cytoplasmic tail of the HLA class I molecule.[4] A few allotypes of HLA-B, such as HLA-B27, have previously been found to be able to form interchain disulfide bonds that cause homodimerization via cysteine residues in their extracellular sequences, and formation of “redox-induced dimers” of certain HLA class I molecules can occur that involve cysteine residues in the cytoplasmic portions of those HLA class I molecules.[74–76] HLA-B★07:02, which is expressed by the S2–013 cell line (Figure 5), has not been reported to form interchain disulfide bonds, although it does share the cytoplasmic domain cysteine residue that in HLA-B27 is able to form such bonds.

In addition to HLA-B★07:02, the S2–013 cell line also expresses the HLA-B★59:01 heavy chain (Figure 5). The single HLA-B allotype expressed by the MIA PaCa-2 cell line is HLA-B★14:02, and the Panc1 cell line expresses only HLA-B★38:01 (Figure 5). HLA-B★14:02 and HLA-B★38:01 are identical throughout their transmembrane and cytoplasmic domains, and HLA-B★59:01 is also identical both to HLA-B★14:02 and to HLA-B★38:01 in the cytoplasmic domain. However, HLA-B★59:01 is different from HLA-B★14:02 and to HLA-B★38:01 at two amino acid positions (282 and 305) in the transmembrane domains (Figure 5). The HLA-B★37:01 heavy chain expressed by the BxPC-3 pancreatic cancer cell line shares with HLA-B★59:01 the isoleucine and threonine, respectively, at positions 282 and 305. Therefore, these particular sequences in the transmembrane domain of the HLA-B allotypes could be potential mediators of effects on migration and ITGB1 expression.

In seeking to determine by co-immunoprecipitation experiments whether the HLA-B heavy chains expressed by S2–013 associate with ITGB1, we found minimal to no association in comparison with control immunoprecipitation with irrelevant antibody (data not shown). It is possible that under different conditions of solubilization, or with other modifications in the immunoprecipitation parameters, association might yet be detectable; however, at least with the immunoprecipitation procedures that we have used to date we have not observed association. Thus, the identity of the protein(s) with which these transmembrane domain residues interact was not found to be ITGB1 in our study.

An interesting possibility is presented by the identification of the HLA-B transmembrane sequence 304–308, which encompasses position 305 (threonine in B★59:01 and B★37:01,

but alanine in B*07:02, B*14:02, and B*38:01) as a putative PDZ ligand by Frietze et al.[77] PDZ ligands are very short sequences that are bound by longer sequences called PDZ domains (which exist in hundreds of human proteins), and phosphorylation of a PDZ ligand by a serine or threonine kinase can affect the interaction of a PDZ ligand with a PDZ domain.[78] Future studies can explore whether this transmembrane sequence in the HLA-B heavy chain (which, in the case of B*59:01 and B*37:01, contains a threonine) associates with a PDZ domain-containing protein in pancreatic cancer cell lines.

Together, these findings suggest that the HLA class I heavy chain isotype HLA-B influences pancreatic cancer cell migration, possibly through effects on the expression of ITGB1. By influencing cell migration, HLA-B may be playing a role in pancreatic cancer metastasis. Due to the high metastatic rate of pancreatic cancer and its persistently dismal 5-year survival rate, garnering information about factors that contribute to the migration of pancreatic cancer cells from studies such as this one will aid in the comprehension of the pathogenesis characteristic of this disease, and may potentially contribute to the generation of better treatments for pancreatic cancer and/or improved prognostic strategies based on HLA typing of patients.

## Acknowledgments

This research was funded by the National Institutes of Health (R21 CA223429, Specialized Program of Research Excellence P50 CA127297, and the Fred and Pamela Buffett Cancer Center Support Grant P30 CA036727). B.H.S. received fellowships from the National Institutes of Health-funded Cancer Biology Training Program (T32 CA009476) and the UNMC Graduate Studies Office.

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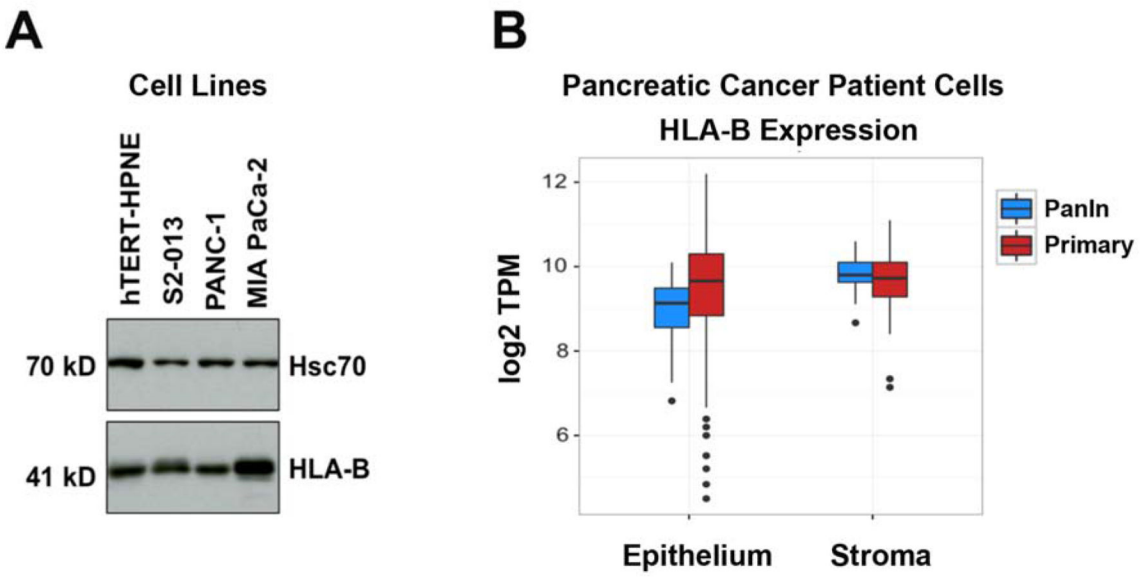


**Figure 1.**

The HLA class I heavy chain isotype HLA-B is abundantly expressed in human pancreatic cancer cells. (A) Immunoblots to detect HLA-B were performed on lysates of an untransformed pancreas duct cell line (hTERT-HPNE) and S2–013, PANC-1, and MIA PaCa-2 pancreatic cancer cell lines. Hsc70 immunoblotting was included as an internal control. The results shown are representative of 3 experimental replicates to assess HLA-B expression in lysates of these cell lines. (B) HLA-B is expressed at comparable levels in primary pancreatic cancer cells from patients versus stromal cells and versus PanIn lesions from patients, as demonstrated by RNA-Seq.

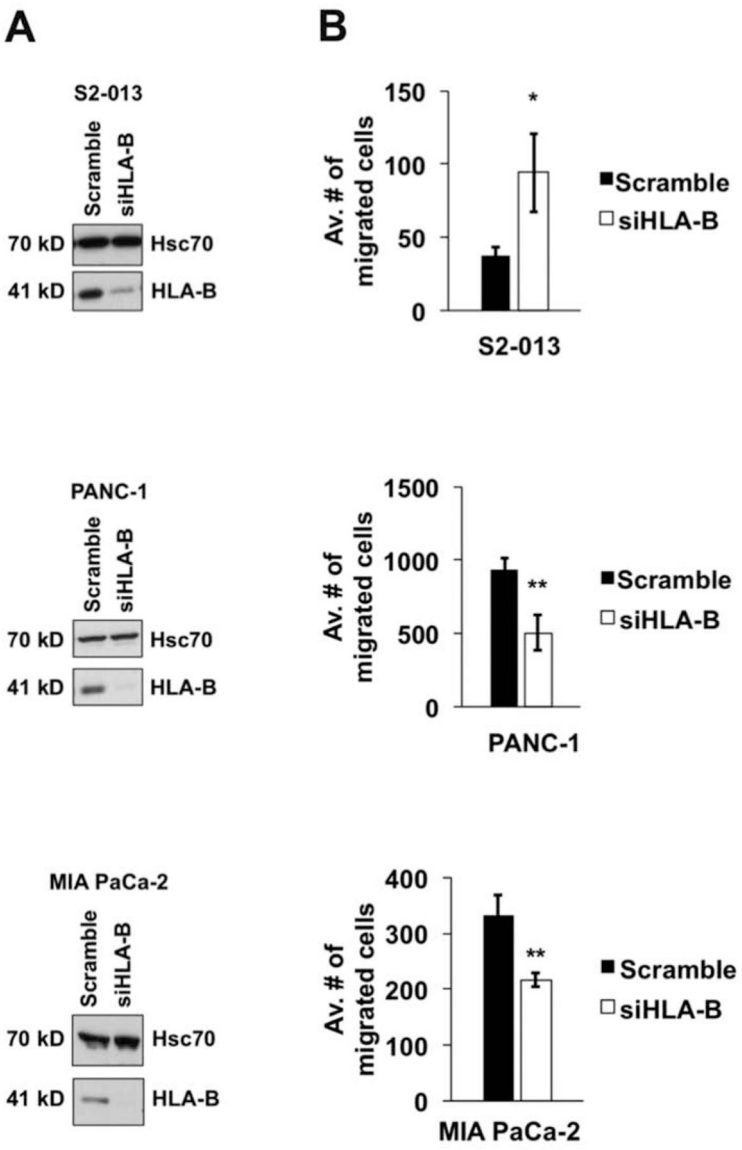


**Figure 2.**

HLA-B influences pancreatic cancer cell migration rate. Upon knockdown of HLA-B expression, the migration rate of S2–013 cells was increased, and the migration of PANC-1 and MIA PaCa-2 cells was diminished. (A) S2–013, PANC-1, and MIA PaCa-2 pancreatic cancer cell lines were transfected with siRNA for pan-HLA-B (or scrambled control siRNA) and the knockdown of the HLA-B heavy chain was verified by immunoblotting (the results shown are representative of 5 repetitions for each cell line). Hsc70 was included as a loading control. (B) The migration of each pancreatic cancer cell line was assessed by transwell assay. At 72 h post-transfection, the cells were replated into 8-μm inserts and incubated for 24 h. Photographs were taken of 3 random fields, the cells in each of the fields were counted, and the results were graphed as average number of cell migrated. For each cell line, 5 repetitions were performed. The error bars represent the standard error of the mean.

Student's two-tailed t-test was used for assessment of statistical significance. The symbol * indicates $p<0.05$, ** indicates $p<0.01$, and *** indicates $p<0.001$.

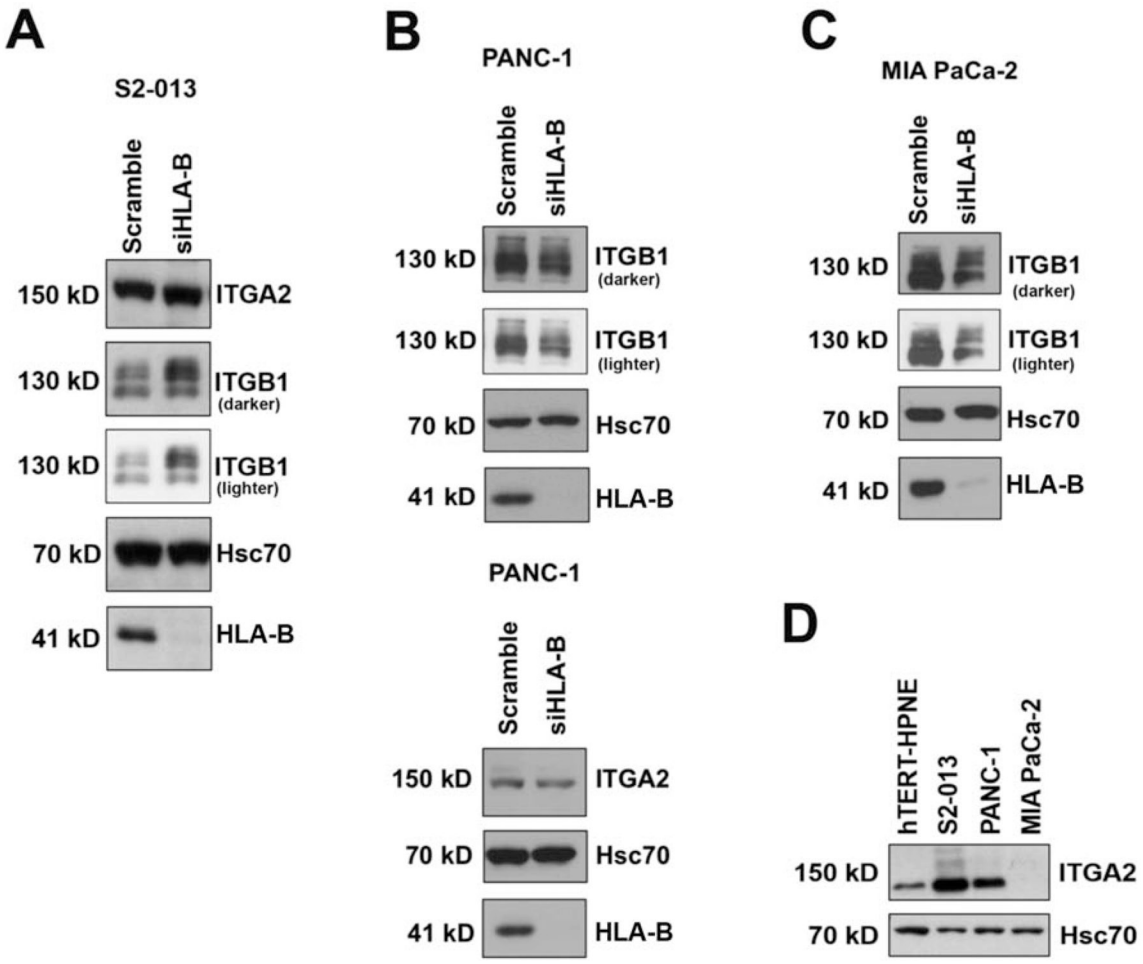


**Figure 3.**

HLA-B affects the expression of ITGB1 but not ITGA2 in pancreatic cancer cell lines. HLA-B knockdown after siRNA transfection for 72 h was verified by immunoblotting with the HC-10 antibody. Control transfections with scrambled siRNA were also performed. Hsc70 immunoblotting was included as an internal control. (A) Knockdown of HLA-B in S2–013 cells increased ITGB1 expression, but did not affect ITGA2 expression. (B, top) In contrast, knockdown of HLA-B expression in PANC-1 caused a decline in ITGB1 expression. (B, bottom) ITGA2 expression was not affected by HLA-B knockdown in PANC-1 cells. (C) The knockdown of HLA-B expression in MIA PaCa-2 cells also caused a decrease in ITGB1 expression. (D) MIA PaCa-2 cells do not express ITGA2). (A,B,C) Both darker and lighter exposures of the same ITGB1 blots are shown. (A,B,D) The results shown are representative of 2 separate experiments. (C) For MIA PaCa-2, a single ITGB1 immunoblotting experiment was performed.

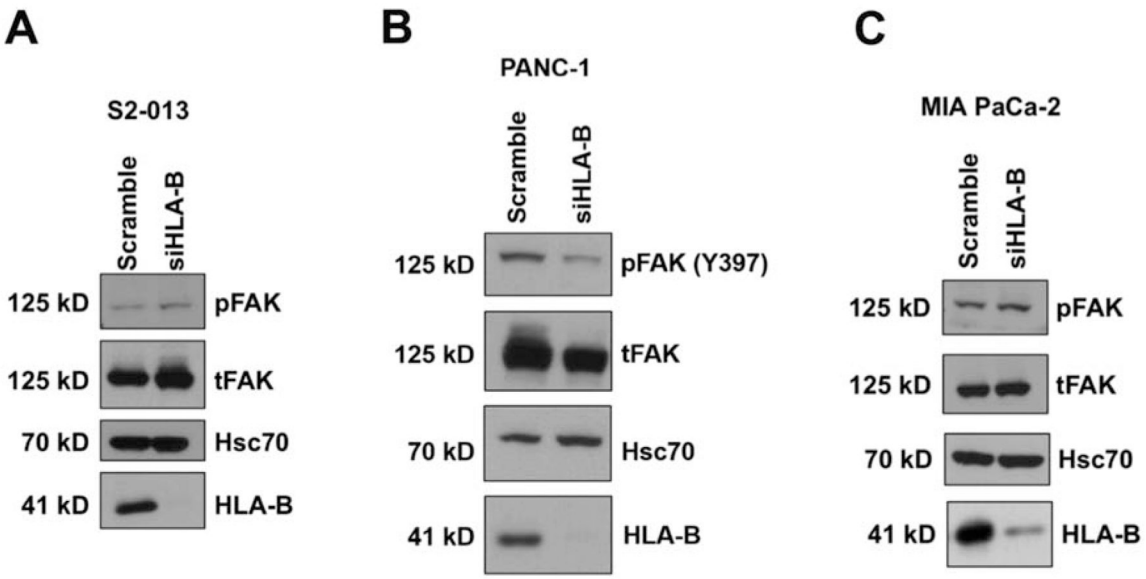


**Figure 4.**

Upon HLA-B knockdown, total FAK and pFAK (Y397) expression levels were (A) increased in S2–013 cells, (B) decreased in PANC-1, and (C) unchanged in MIA PaCa-2. Cell lysates were collected from the aforementioned pancreatic cancer cell lines at 72 h post-transfection with control siRNA or HLA-B siRNA. The lysates were immunoblotted for pFAK (Y397), total FAK, Hsc70 (loading control), and HLA-B (to confirm knockdown). The results displayed are representative of multiple experiments done with 7 separate lysates for the S2–013 cell line, 2 separate lysates for the PANC-1 cell line, and 2 separate lysates for the MIA PaCa-2 cell line.

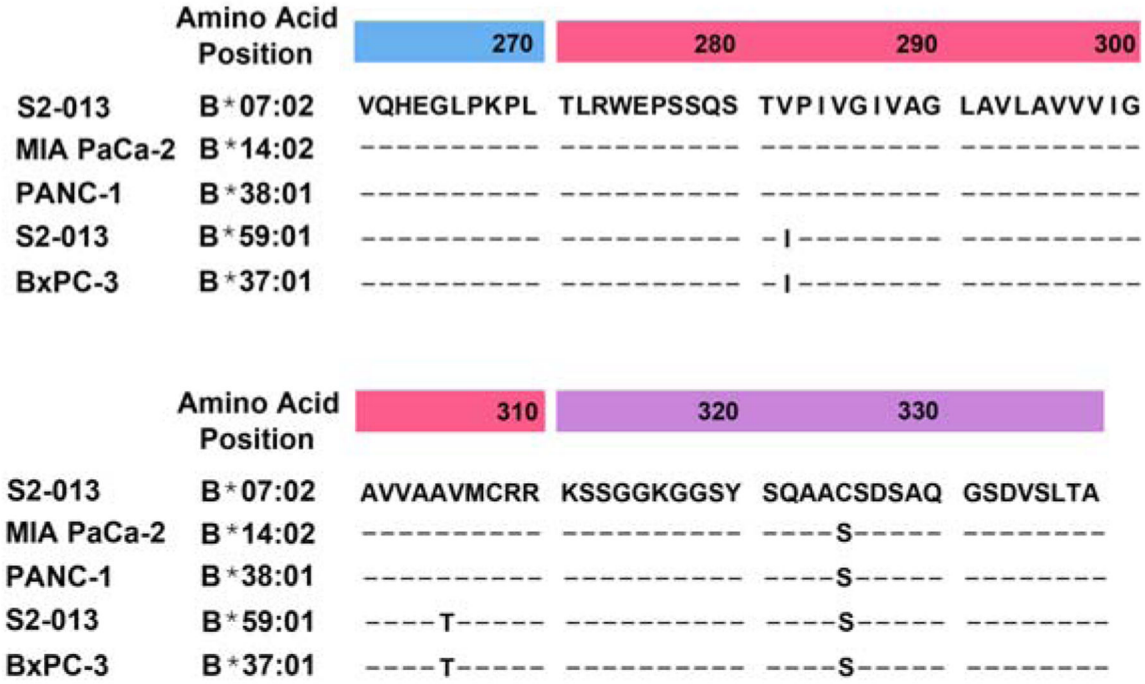


**Figure 5.**

The transmembrane/cytoplasmic regions of the HLA-B heavy chains expressed by the S2–013 pancreatic cancer cell line differ from those of the MIA PaCa-2 and PANC-1 cell lines, but the transmembrane region of one of the S2–013 HLA-B heavy chains matches the transmembrane region of the HLA-B heavy chain expressed by the BxPC-3 pancreatic cancer cell line. The sequences shown from the HLA-B heavy chains expressed in the S2–013, MIA PaCa-2, PANC-1, and BxPC-3 include the final 10 amino acids in the α3 domains (positions 261–270, blue heading), the amino acids in the transmembrane domains (positions 271–310, red heading), and the amino acids in the cytoplasmic domain (positions 311–338, purple heading). Position 325 in the S2–013 B★07:02 cytoplasmic domain is a cysteine, whereas the other HLA-B heavy chains displayed have a serine at that location. Positions 282 and 305 are valine and alanine, respectively, in B★07:02 (expressed by S2–013), B★14:02 (expressed by MIA PaCa-2), and B★38:01 (expressed by PANC-1). Positions 282 and 305 are isoleucine and threonine, respectively, in B★59:01 (expressed by S2–013) and B★37:01 (expressed by BxPC-3). The sequences shown are from IPD-IMGT/HLA Release 3.38.0 2019-10-17 (https://www.ebi.ac.uk/ipd/imgt/hla/).

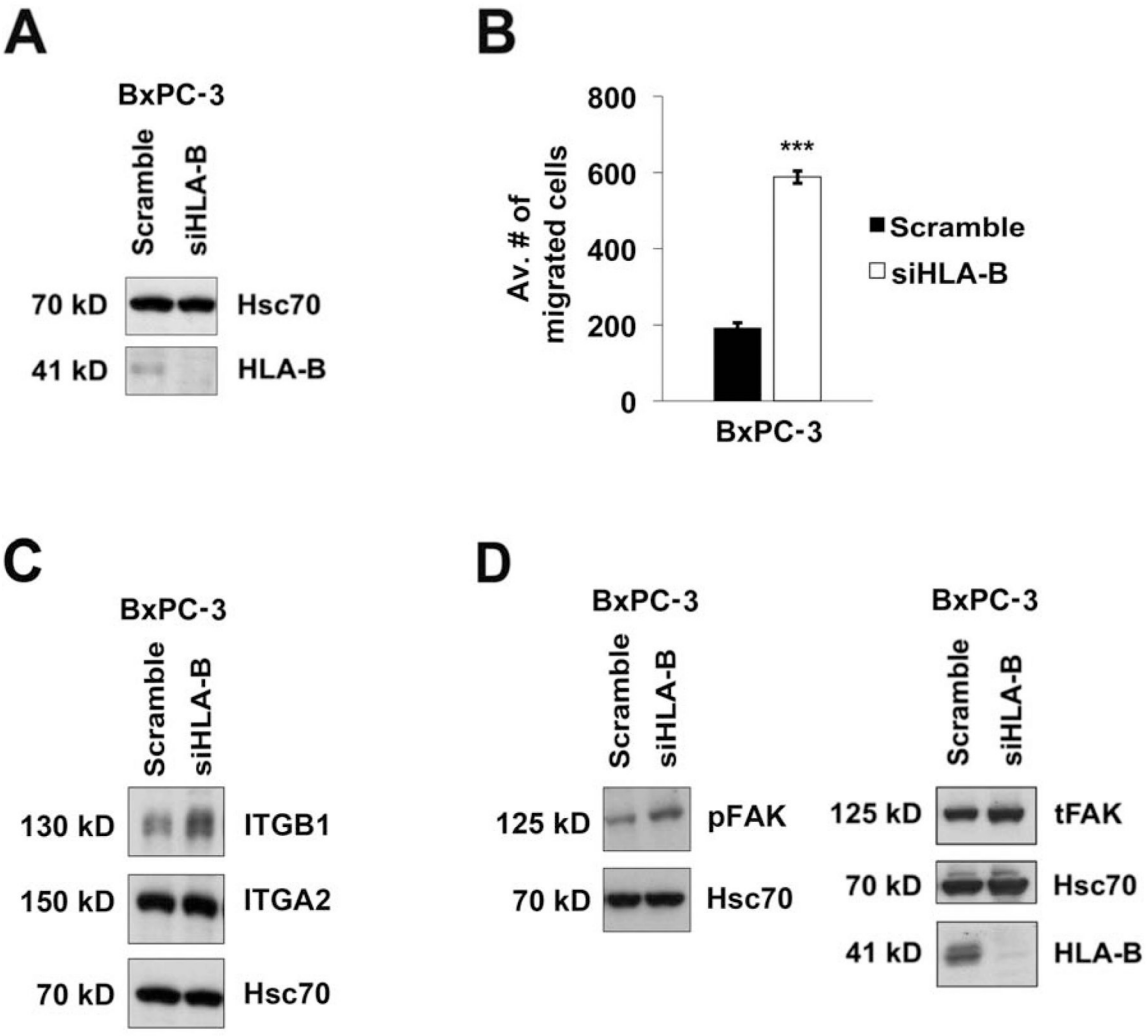


**Figure 6.**

Knockdown of HLA-B*37:01 expression in BxPC-3 cells results in increased migration of BxPC-3 and elevated expression of ITGB1 (but not ITGA2). (A) BxPC-3 pancreatic cancer cells were transfected for 72 h either with siRNA to downregulate HLA-B or with scrambled control siRNA. HLA-B heavy chain knockdown was confirmed by immunoblotting, and probing the blot with antibody for Hsc70 was included as a loading control. (B) The migration of the BxPC-3 cells, transfected either with scramble control siRNA or HLA-B siRNA, was monitored by transwell assay. At the 72 h time point after transfection, the cells were plated in 8-μm inserts. After 24 h of incubation, 3 random fields were photographed. The cells in each of the photographic fields were counted, and the average numbers of cells migrated were graphed. The data shown in the graph was compiled from 4 repetitions of the experiment. The error bars indicate the standard error of the mean. Student's two-tailed t-test was used to determine statistical significance. The symbol *** represents $p<0.001$. (C) Knockdown of HLA-B in BxPC-3 cells increased ITGB1 expression, but did not affect ITGA2 expression, as shown by immunoblotting for ITGB1 and ITGA2. Transfection with scramble control siRNA was performed as a negative control. Immunoblotting with an anti-

Hsc70 antibody was included as a loading control. The results shown are representative of 3 experiments that included ITGB1 blots and 2 experiments that included ITGA2 blots. (D) The pFAK (Y397) and total FAK expression levels were increased in BxPC-3 cells upon transfection of HLA-B-specific siRNA, as compared to BxPC-3 cells transfected with scramble control siRNA. Hsc70 was immunoblotted as a control, and HLA-B knockdown was also confirmed by immunoblotting. The results displayed are representative of those demonstrated in 3 separate experiments.