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INTEGRIN β1 IS CRITICAL FOR GASTRIN-RELEASING PEPTIDE RECEPTOR-MEDIATED NEUROBLASTOMA CELL MIGRATION AND INVASION

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Abstract

Background—Gastrin-releasing peptide (GRP) and its receptor, GRP-R, are critically involved in neuroblastoma tumorigenesis; however, the molecular mechanisms and signaling pathways that are responsible for GRP/GRP-R-induced cell migration and invasion remain unclear. Here, we sought to determine the cell signals involved in GRP/GRP-R-mediated neuroblastoma cell migration and invasion.

Methods—Human neuroblastoma cell lines, SK-N-SH, LAN-1 and IMR-32, were used for our study. Transwell migration and invasion assays were performed after GRP (10^{-7} M) stimulation. cDNA GEArray® Microarray kit was used to determine GRP-R-induced gene expression changes. Protein and membrane expression of integrin subunits were confirmed by Western blotting and flow cytometry analysis. siRNA transfection was performed using Lipofectamine 2000. For scratch assay, a confluent monolayer of cells in 6-well plate were wounded with micropipette tip and observed microscopically at 24 to 72 h.

Results—GRP increased neuroblastoma cell migration and expressions of MMP-2 while TIMP-1 level decreased. GRP-R overexpression stimulated SK-N-SH cell migration, and upregulated integrin α2, α3, and β1 protein as well as mRNA expression. Targeted silencing of integrin β1 inhibited cell migration.

Conclusions—GRP/GRP-R signaling contributes to neuroblastoma cell migration and invasion. Moreover, integrin β1 subunit critically regulates GRP-R-mediated neuroblastoma cell migration and invasion.

Keywords

Integrin; GRP-R; Migration; Neuroblastoma

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INTRODUCTION

Neuroblastoma, a neural crest cell-derived tumor, is the most common extracranial solid tumor of infants and children, accounting for approximately 10% of all childhood-related death.[1] Since roughly 50% of patients will have metastatic disease at the time of diagnosis,[2] there have been considerable efforts aimed at delineating the cellular mechanisms that contribute to metastatic progression in neuroblastoma, leading to poor overall survival. Clinicopathologic data has shown that older age at diagnosis (> 18 months), *MYCN* amplification, unfavorable histology, and DNA ploidy are factors that portend a 'high-risk' classification and are associated with poor prognosis.[1] The poor survival seen in patients with metastatic or refractory neuroblastoma underscores the need for improved understanding of the molecular mechanisms that govern tumor cell migration and invasion.

Gastrin-releasing peptide (GRP) is a gut neuropeptide with mitogenic properties that is secreted by neuroblastoma in an autocrine/paracrine fashion.[3] We have previously shown that GRP binds to its cell surface receptor, GRP-R, to stimulate neuroblastoma growth, and that GRP-R overexpression increases tumorigenicity and metastatic potential in neuroblastoma cells.[4, 5] Further, increased expression of GRP-R is found in more undifferentiated neuroblastoma that are associated with metastatic disease and dismal patient outcomes. Conversely, we have also reported that targeted silencing of GRP-R through stable transfection can inhibit tumor growth and metastasis *in vivo*;[6] however, the exact cellular mechanisms responsible for distant organ metastasis in neuroblastoma are not clearly defined.

A multitude of intra- and extra-cellular signaling pathways coordinate to exert molecular changes that ultimately allow cancer cells to progress beyond the primary tumor. Integrins are a group of heterodimeric transmembrane surface glycoproteins that play a key role in the regulation of cell-to-cell and cell-to-extracellular matrix (ECM) attachments, yet also possess the capacity to transmit information from the extracellular milieu that can regulate cellular growth, survival, and migration.[7] As key mediators of tumor progression, integrins allow cells to adapt to changing microenvironments during metastatic progression, and it is not surprising that changes in membrane integrin expression have been identified in various types of cancer.[8] The association between integrin expression and GRP/GRP-R signaling in neuroblastoma is heretofore undefined. In this study, we examine the effects of GRP/GRP-R signaling on neuroblastoma cell tumorigenicity, and determine the role of integrin as it relates to GRP-R-mediated neuroblastoma cell migration and invasion.

MATERIALS AND METHODS

Cell culture and transfection assays

Human neuroblastoma cell lines, SK-N-SH, LAN-1 and IMR-32, were purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 media (Cellgro Mediatech Inc., Herndon, VA) supplemented with 10% fetal bovine serum (FBS) (Sigma Aldrich) in a humidified atmosphere of 5% CO_2 at 37°C. The plasmid pEGFP-GRP-R construct was previously described.[5] Briefly, pEGFP-GRP-R was constructed by inserting GRP-R cDNA fragment to pEGFP (N3) at restriction enzyme NheI

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and EcoR I site. For transfections, 5×10^5 cells were seeded per well in a 6-well plate and transfected the next day with Lipofectamine 2000 (Life Technology). Stably transfected cells were established by selection with G418 at 300 μg/ml for 2 weeks. Cells were seeded on culture plates, serum-starved for 24 h and then treated with GRP (100 nM). Small interference (si) RNAs against integrin α2, α3 and β1 were from Santa Cruz Biotechnology, Inc., along with non-targeting scrambled sequences that were used as controls.

Migration, invasion, and scratch assays

For transwell migration, transwell filters (8 μm; Corning, Lowell, MA) were coated on the lower chamber with 5 μg/ml collagen type I (BD Biosciences) overnight and then blocked with 2.5% BSA/PBS for 1 h. 1×10^5 cells in serum-free media added to the upper chamber and incubated for 6 h. Cells were fixed with 4% paraformaldehyde, stained with DAPI, and counted. Assay was performed in duplicate, and counting was from five randomly selected microscopic fields (200X magnification). For invasion assay, the transwell filters were coated with 1/30 diluted Matrigel (BD Biosciences). Cells (1.5×10^5) in serum-free media were added to the upper chamber and GRP (100 nM) was added to the lower chamber. After 48 h incubation, cells were fixed with 4% paraformaldehyde, stained with DAPI, and counted. For wound healing, confluent monolayer of cells in 6-well plate was wounded using 200 μL tip and incubated and observed microscopically at 24 to 72 h. Wound closure was calculated by measuring the remaining space in the microscopic images.

Flow cytometry

Cell surface expression of integrins was measured using the integrin antibodies as previously described.[9] Cells were trypsinized, washed once by adding 0.1%BSA/PBS and spun down. Cell pellets were resuspended in 0.1%BSA/PBS and adjusted to 1×10^6 cells/mL. Integrin antibody (1 μg) was added to 1 mL of cell solution and incubated for 1 h at 4°C on a shaker, then the cells were washed with 0.1%BSA/PBS three time by centrifugation at 300g for 3 min. Allophycocyanin (APC)-conjugated secondary anti-mouse antibody (1:250) was added and incubated for 30 min at 4°C (excitation at 633 nm). Cells were washed three times and resuspended for flow cytometry analysis. Cells without primary antibody incubation was used as negative control. Flow cytometry was performed using a FACSCalibur System (BD Bioscience), and data were collected for viable cells according to side and forward scatter. Integrin expression on the cell surface was quantitated with fluorescence intensity.

Western blot analysis

Whole cell lysates were collected using cell lysis buffer (Cell Signaling) supplemented with proteinase inhibitors (Roche, Indianapolis, Indiana). Protein concentrations were quantified using a Protein Assay kit from Bio-Rad (Hercules, CA). Protein (30 μg) was fractionated by electrophoresis on 4–12% NuPAGE Novex Bis-Tris gels (Life Technology), transferred to polyvinylidene difluoride membranes, and probed with antibodies. The bands were visualized by an enhanced chemiluminescent detection system according to the manufacturer's instructions (Amersham Inc.).

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Materials

Primary antibody for GRP-R was purchased from Abcam (Cambridge, MA). Matrix metalloproteinase (MMP)-2 antibody was purchased from Calbiochem (Darmstadt, Germany), tissue inhibitor of metalloproteinase (TIMP)-1 from Bethyl Laboratories (Montgomery, TX) and ERK1/2 from Cell Signaling Technology (Danvers, MA). β-actin antibody was from Sigma-Aldrich (St. Louis, MO). Integrin antibodies and all secondary antibodies against mouse and rabbit IgG were purchased either from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) or from Dr. Kathleen L. O'Connor (Markey Cancer Center, University of Kentucky at Lexington). GRP was obtained from TOCRIS Bioscience (Bristol, United Kingdom) and Matrigel was purchased from BD Bioscience (San Jose, CA). RNA isolation kit was from Life Technology (Grand Island, NY) and cDNA GEArray® Microarray was from SuperArray Bioscience Corporation (Qiagen, Valencia, CA).

Statistical analysis

All results are shown as mean ± SEM. Statistical analysis was performed with Student's t-test. p values <0.05 were considered to be statistically significant.

RESULTS

GRP stimulated neuroblastoma cell migration and invasion

To assess the ability of GRP to stimulate cell motility, serum-starved SK-N-SH and LAN-1 cells were cultured with or without GRP. Cell migration and invasion assays were independently performed on each cell line using transwell system and relative values were calculated, as well as cell count quantification to measure invasion. GRP treatment significantly increased SK-N-SH cell migration (Fig. 1A). Similar results were observed with LAN-1 cells (data not shown). Furthermore, cell counts after GRP-induced invasion were significantly higher when compared to controls (Fig. 1B). Given the important role of MMP and TIMP in modulating the ECM during cell movement, we next wanted to determine whether GRP stimulation leads to altered expression of MMP or TIMP.[10] GRP significantly upregulated MMP-2 expression in SK-N-SH cells when compared to controls, while the expression of TIMP-1, a known inhibitor of both MMP-2, was decreased after GRP treatment (Fig. 1C). Upregulation of MMP-2 was also noted after GRP in IMR-32 cells (data not shown). Taken together, these results suggest a critical role for GRP-mediated neuroblastoma metastasis.

GRP-R overexpression increased integrin α2, α3, and β1 expression in SK-N-SH cells

GRP stimulation is known to stimulate neuroblastoma cell growth and proliferation;[11] however, its relationship to tumor progression and cell motility is less defined. In order to better understand the cellular mechanisms underlying GRP/GRP-R mediated neuroblastoma cell motility, we established stably transfected GRP-R overexpressing SK-N-SH cells. We then performed gene expression analysis using a cDNA GEArray® Microarray kit to identify target genes that may be altered as a result of GRP-R signaling. We found that GRP-R overexpressing SK-N-SH human neuroblastoma cells showed increased mRNA levels of integrin α2, α3, and β1 (Fig. 2A). Correlative to mRNA, integrin α2, α3, and β1

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protein levels were also increased as measured by Western blotting when compared to controls (Fig. 2B). In addition, these increased expression of integrin α2, α3, and β1 on cell membrane of GRP-R overexpressing SK-N-SH cells were further confirmed using flow cytometry (Fig. 2C), indicating the functional level of integrin α2, α3, and β1 were increased. Consistent with GRP-induced cell migration in Figure 1A, stable transfection of GRP-R overexpressing SK-N-SH cells also resulted in a concomitant increase in cell migration (Fig. 2D). Hence, our results support a positive correlation between GRP-R and integrin expression, indicating that GRP-R is important for a cellular function of cell migration in neuroblastoma cells by regulating integrin.

Integrin β1 regulates SK-N-SH cell migration

Since GRP-R overexpression induced SK-N-SH cell migration and increased integrin expression, we sought to better characterize this cellular process. First, we used siRNA to silence integrin α2, α3, and β1 expression in SK-N-SH cells, then performed wound healing assays at 24, 48, and 72 h to determine the effects on cell migration (Fig. 3A). Wound unclosure was measured microscopically and relative values determined. Targeted silencing of integrin β1 (siIntegrin β1) significantly decreased cell migration at 72 h after wounding when compared to siRNA targeted at integrin α2 and α3 (Fig. 3B). Western blot analysis was used to confirm the specificity of siRNA (Fig. 3C).

Integrin β1 is a critical regulator of cell migration in GRP-R overexpressing SK-N-SH cells

Given the measurable result of integrin β1 silencing on cell migration in SK-N-SH cells, we then sought to identify the effect of integrin β1 knockdown in GRP-R overexpressing SK-N-SH cells. Scratch assays were established with siIntegrin β1 in GRP-R overexpressing cells and measured at 24, 48, and 72 h for wound unclosure as a function of cell migration (Fig. 4A). siRNA inhibition of integrin β1 decreased cell migration in GRP-R overexpressing SK-N-SH cells at 72 h after wounding compared with the non-targeted control GRP-R overexpressing cells (Fig. 4B). Western blot analysis was used to confirm the specificity of siIntegrin β1 (Fig. 4C).

DISCUSSION

In this study, we found that GRP treatment led to increased migration and invasion of neuroblastoma cells. GRP-R overexpression not only stimulated neuroblastoma cell migration, but also led to the upregulation of mRNA, protein, and membrane expression of integrin subunits α2, α3, and β1. Moreover, silencing of integrin β1 led to inhibition of cell migration suggesting its role as a potential key regulator of GRP-R-mediated neuroblastoma cell migration and invasion.

As a neuroendocrine tumor, neuroblastoma exhibits mitogenic response to neuroendocrine peptides such as GRP. In fact, GRP/GRP-R signaling has been shown to induce neuroblastoma cell growth through the PI3K/Akt pathway.[12] The ‘high-risk’ group of neuroblastoma remains difficult to cure due to its refractoriness to treatment protocols, propensity to metastasize and disease relapse. Hence, discerning molecular mechanisms involved in GRP-R-mediated neuroblastoma cell migration and invasion may shed novel

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insights to clinically aggressive phenotype. In this study we found that signaling through the GRP/GRP-R axis results in neuroblastoma cell migration and invasion through upregulation of α2, α3, and β1 integrin subunits. Further, we also show that integrin β1 expression is particularly critical to GRP-R-mediated cell migration as evidenced by the significant decrease observed in neuroblastoma cell migration upon inhibition of integrin β1 with siRNA.

It has been reported that GRP can stimulate cell migration in prostate[13] and breast cancers[14] through binding to its cognate receptor, GRP-R. Similar studies using the GRP analog, bombesin, have shown that bombesin/GRP-R signaling enhances the migratory and invasive capacity of colorectal carcinoma cells in a dose-dependent fashion,[15] albeit the exact molecular mechanisms still remain unclear. While the role of GRP-R signaling in neuroblastoma tumorigenesis has been established,[6] the effects of such signaling events as they relate to cell migration and invasion have not been examined. Here, we show that both GRP treatment and GRP-R overexpression led to increased motility of SK-N-SH neuroblastoma cells. The ability of a cancer cell to negotiate the ECM is paramount for tumor progression. Neoplastic cells take advantage of enzymatic digestion of the ECM by MMPs to accomplish these tasks. Interestingly, treatment of SK-N-SH cells with GRP led to increased expression of MMP-2 while expression of the MMP-inhibiting enzyme TIMP-1 was downregulated, suggesting that GRP may play a secondary role in modulating the extracellular environment to allow for tumor cell motility through the ECM. In fact, it is known that MMPs play a crucial role in tumor progression through regulation of the tumor microenvironment,[16] and that dysregulation of the balance between MMP proteolysis and TIMP expression is linked to cancer cell invasion.[17] Further studies examining the relationship between GRP signaling and regulation of the MMP/TIMP balance in neuroblastoma tumor progression will be needed.

Since cell migration and invasion, as well as intravasation and extravasation, are fundamental processes of cancer cells during metastasis, we sought to determine genes that may reflect cellular changes in tumor progression. As such, our data showed that GRP-R overexpression led to upregulation of integrin α2, α3, and β1 mRNA; these were further corroborated with Western blotting and flow cytometry. Collectively, our findings show that GRP-R overexpression increases the expression of integrin α2, α3, and β1 in SK-N-SH cells, and outlines a potential relationship between GRP/GRP-R-induced cell migration/invasion and integrin expression. It further emphasizes the role of neuroendocrine signaling in neuroblastoma tumor progression, which underscore the notion that cell-ECM interactions can regulate molecular signaling in response to mitogenic factors, such as FAK.[18]

As the prominent receptor for the ECM, integrins function beyond simple cell adhesion and can influence numerous cellular functions in response to signals from the environment. Previous studies have suggested that changes in integrin expression by tumor cells can modulate growth and survival, as well as cell adhesion and migration.[19] Of particular interest in the present study is the relationship of GRP/GRP-R-induced integrin expression to cell migration and invasion. We found that stable knockdown of integrin α2, α3, and β1 subunits in SK-N-SH cells resulted in a marked decrease in cell migration. In fact, the decrease in cell movement observed on scratch assay was most significant after integrin β1

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silencing. These effects could not be rescued with GRP-R overexpression, suggesting the crucial role of integrin β1 in GRP-R-mediated cell motility. Dysregulation of cell adhesion molecules, particularly integrins, is well established in human cancers, and overexpression of the integrin β1 subunit is associated with increased metastatic potential in breast[20] and lung cancers.[21] Although previous reports have suggested that decreased integrin β1 in neuroblastoma leads to increased cell migration *in vivo*,[22] our study findings do not corroborate such.

In conclusion, our data show that GRP and its receptor, GRP-R, provide a significant molecular access point for the induction and stimulation of neuroblastoma tumor cell motility through cellular mechanisms that likely involve the modulation of integrin subunit expression, particularly integrin β1. The role of β1 integrin expression in cell attachment and invasion has been implicated in other cancers.[23] Further studies will be needed to determine the exact molecular mechanisms underlying GRP-mediated integrin expression and how these interactions contribute to the metastatic potential of neuroblastoma.

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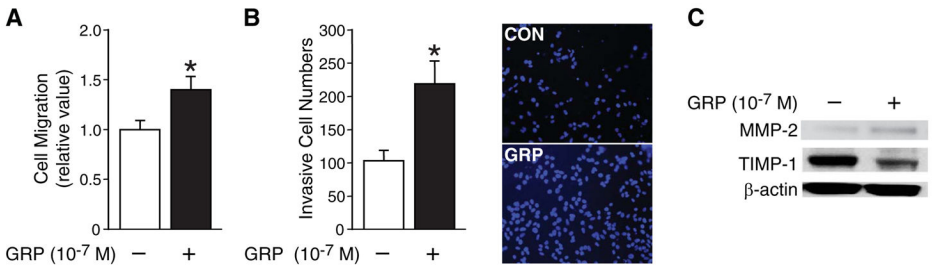

Figure 1. GRP increased cell migration and invasion by differential expression of MMP-2 and TIMP-1

(A) GRP treatment (10^{-7} M) for 24 h increased SK-N-SH cell migration in transwell plates (*= p <0.05 vs. control). **(B)** GRP stimulated the number of SK-N-SH cells in Matrigel-coated transwell plates (*= p <0.05 vs. control). **(C)** Increased MMP-2 but decreased TIMP-1 expressions were observed in SK-N-SH cells after GRP treatment. β-actin was used to demonstrate equal protein loading.

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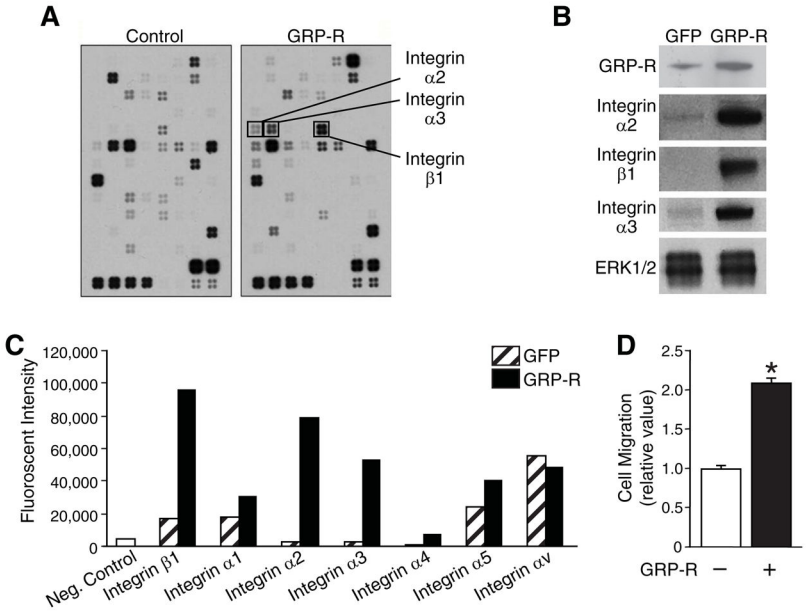

Figure 2. GRP-R overexpression upregulated integrin α2, α3, and β1 expression in SK-N-SH cells and stimulated constitutive cell migration

(A) GRP-R overexpression resulted in increased mRNA levels of integrin α2, α3, and β1 (*boxed areas)* as measured by SuperArray. **(B)** Western blot analysis was performed with indicated antibodies in GRP-R overexpressing cells. Total ERK1/2 expression was probed to demonstrate equal protein loading. **(C)** Increased levels of integrin α2, α3, and β1 membrane expression in GRP-R overexpressing cells as measured by flow cytometry. **(D)** GRP-R overexpressing cells with stimulated cell migration rate (*= p <0.05 vs. control).

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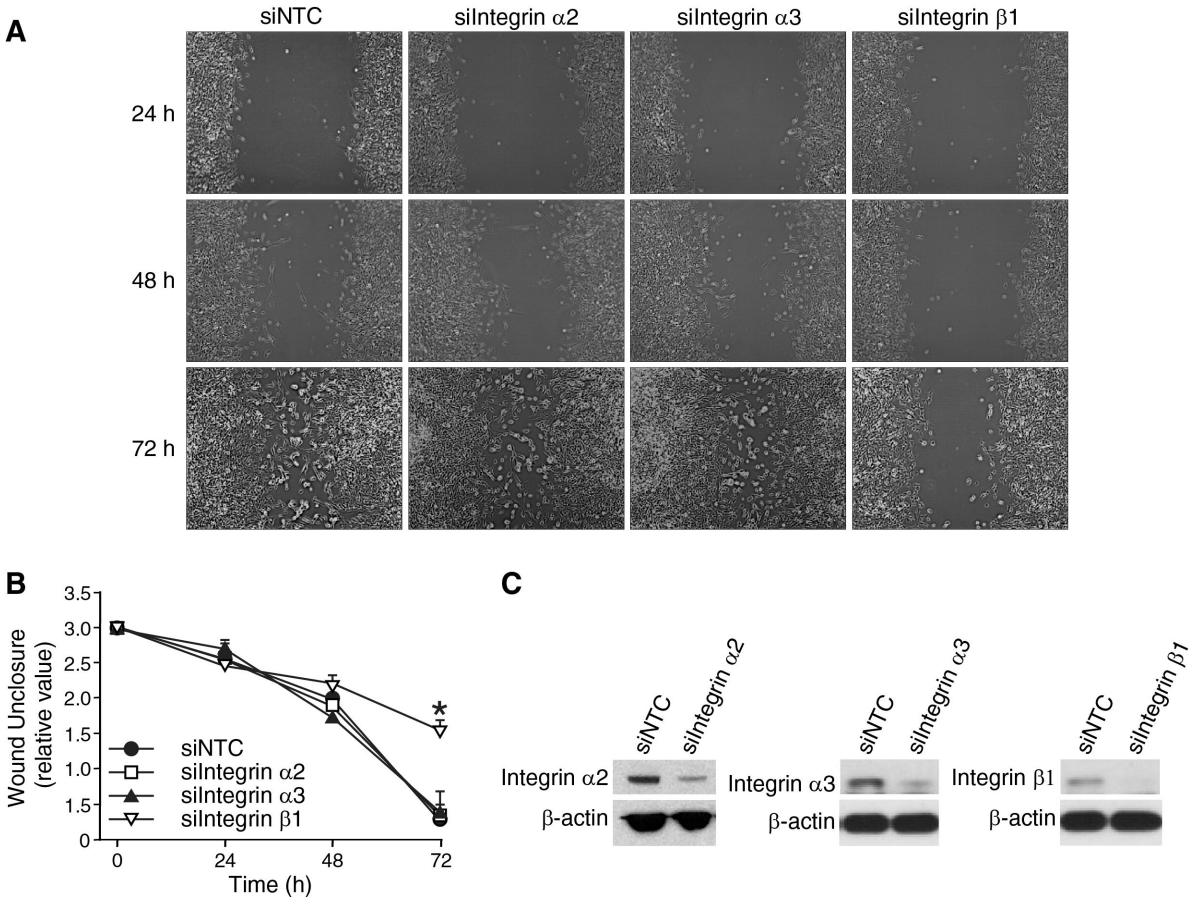

Figure 3. Silencing of integrin α2, α3, and β1 in SK-N-SH cells

(A) SK-N-SH cells were transfected with siRNA against integrin α2, α3, and β1. After 48 h, scratches were made using 200 μl tips. Wound closure was measured from microscopic images at 24, 48, and 72 h after wounding (100X magnification). **(B)** Data are representative of the mean distance of unclosure from three independent experiments (*= p <0.05 vs. siNTC). **(C)** Western blot analysis was performed with indicated antibodies in the cells. β-actin was used to demonstrate equal protein loading.

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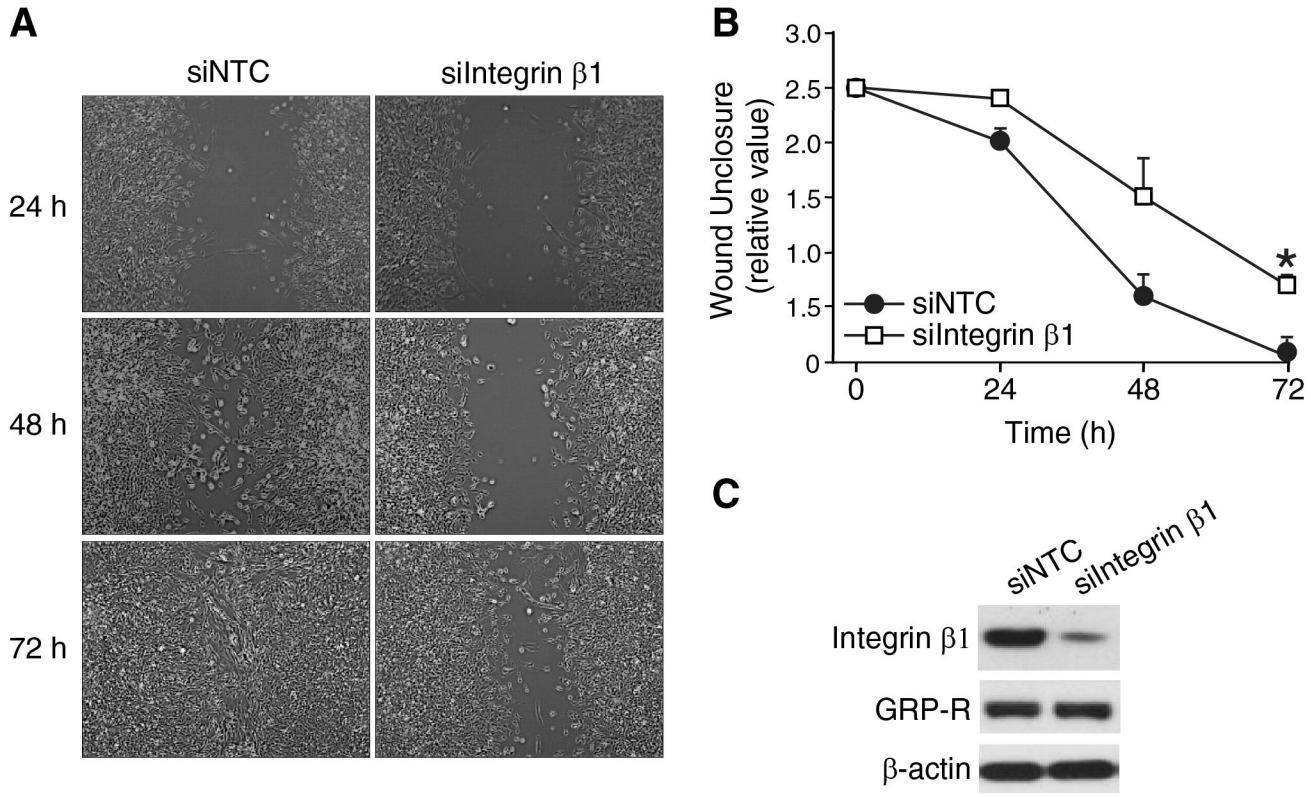

Figure 4. Silencing of integrin β1 decreased cell migration in GRP-R overexpressing SK-N-SH cells

(A) GRP-R overexpressing SK-N-SH cells were transfected with siRNA against integrin β1. After 48 h, scratches were made using 200 μl tips. Wound closure was measured from microscopic images at 24, 48, and 72 h after wounding (100X magnification) **(B)** Data are representative of the mean distance of unclosure from three independent experiments (*= p <0.05 vs. siNTC). **(C)** Western blot analysis was performed with indicated antibodies in the cells. β-actin was used to demonstrate equal protein loading.