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β_1 integrins mediate cell proliferation in three-dimensional cultures by regulating expression of the sonic hedgehog effector protein, GLI1

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Abstract

The β_1 integrins play an important role in the modulation of cancer cell proliferation and tumor growth. We have previously shown that β_1 integrins associate with insulin-like growth factor type 1 receptor (IGF-IR) and regulate IGF-stimulated prostate cancer cell proliferation. In the present study, we find that downregulation of β_1 in prostate cancer cells inhibits IGF-IR and AKT activation. We also show that β_1 downregulation in prostate cancer two- and three-dimensional (3-D) cell cultures significantly reduces expression of GLI1, a transcription factor known to be regulated by the IGF/AKT signaling pathway and to be a downstream effector of sonic hedgehog. Re-expression of GLI1 rescues the inhibitory effect of β_1 downregulation on prostate cancer cell proliferation in 3-D cultures. We find that downregulation of β_1 reduces surface expression of associated α integrin subunits, predominantly α_5 and at a lower extent: α_2, α_3 and α_4. Our results indicate that the β_1/IGF-IR complex regulates expression of GLI1, which in turn promotes cancer cell proliferation in 3-D cultures.

Keywords

IGF-IR; GLI1; prostate cancer; β_1 integrins; AKT

Introduction

Extracellular matrix (ECM) proteins and growth factors activate several signaling pathways via, respectively, integrins and growth factor receptors. The cross-talk between these pathways is known to regulate several physiological functions including cell adhesion, migration, proliferation and differentiation (Comoglio et al., 2003; Eliceiri, 2001).

Integrins are heterodimers consisting of α and β subunits. Currently, 24 heterodimers of the integrin family, consisting of 18 α and 8 β subunits, have been described (Alam et al., 2007; Hynes, 2002) and their ability to activate specific signaling pathways has been investigated (Alam et al., 2007). Integrin signaling plays a key role in the alteration of cellular growth and tumor progression through the regulation of gene expression, apoptosis, cell adhesion, proliferation and migration (Felding-Habermann, 2003). Several studies have reported the association between deregulation of integrin, ECM or growth factor expression with the

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progression of prostate cancer to an advanced stage (Culig et al., 2005; Goel et al., 2008). Among the β subunits, β_1 is the predominant subunit expressed by prostatic epithelium. Five β_1 variant subunits, β_{1A}, β_{1B}, β_{1C}, $\beta_{1C\text{-}2}$, and β_{1D}, generated by alternative splicing, have been described. Two variants, β_{1C} and β_{1A}, are shown to be expressed in normal prostatic epithelium. β_{1A} is upregulated in prostate cancer, whereas β_{1C} is markedly downregulated in adenocarcinoma (Fornaro et al., 1999; Goel et al., 2008). We have shown upregulation of β_1 expression in a mouse model designated TRAMP (transgenic adenocarcinoma of mouse prostate) (Goel et al., 2005). The findings that the expression of the β_{1A} integrin variant is upregulated (Goel et al., 2005) and is necessary for cells' ability to form tumors *in vivo* (Goel et al., 2009b) pinpoint an important role of the β_{1A} integrin during prostate cancer progression.

β_1 integrins play an important role in disrupting the formation of normal acini structure. β_1 inhibition causes formation of polarized acini of malignant human breast cancer cells mediated by anti-proliferative and pro-apoptotic signaling (Weaver et al., 1997). Previously, our group and others have shown a direct interaction between β_1 integrins and IGF-IR (Goel et al., 2004; Tai et al., 2003). Downregulation of β_1 blocks IGF-stimulated cell proliferation and transformation of prostate cancer cells (Goel et al., 2005), but the effect of β_1 downregulation on activation of IGF-IR and tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1) has never been described. Expression of wild-type, but not kinase-inactive, IGF-IR in non-transformed breast epithelial cells (MCF-10A) causes formation of large abnormal structures (Irie et al., 2005). Over-expression of IGF-IR in the same cells also results in disruption of apical basal polarization (Yanochko and Eckhart, 2006). Recently, Kim et al. expressed a constitutively active form of IGF-IR in non-transformed MCF-10A cells and found larger and disrupted acini with protrusions (Kim et al., 2007). Similar to β_1 inhibition, an IGF-IR blocker reduces cell proliferation, and resulted in the formation of hollow polarized lumen in MCF7 breast cancer cells (Litzenburger et al., 2009). All these studies point to a possible involvement of β_1-IGF-IR complex in the regulation of cell proliferation in 3-D cultures.

The sonic hedgehog (SHH)/GLI signal transduction pathway controls a variety of developmental processes involved in embryogenesis. Besides embyrogenesis, aberrant activation of SHH pathways have been implicated in several malignancies like lung, pancreatic and prostate cancer (Kasper et al., 2006). The expression of SHH and GLI1, a downstream effector of SHH, is upregulated in human prostate cancer as compared to normal prostatic epithelia (Sanchez et al., 2004). Treatment with cyclopamine, a SHH inhibitor, blocks proliferation of prostate cancer cell lines (PC3, DU145 and 22RV1) as well as of primary prostate tumor cultures expressing GLI1 (Karhadkar et al., 2004; Sanchez et al., 2004). This inhibitory effect of cyclopamine is bypassed by over-expression of GLI1 (Karhadkar et al., 2004). Ectopic expression of GLI1 increases β_1 levels along with increased proliferation and invasiveness in ovarian cancer cells and these effects are reverted by cyclopamine (Liao et al., 2009). These results suggest a possible interaction between β_1 integrins and SHH pathway in cancer.

In the present study, we show that β_1 downregulation reduces IGF1-stimulated tyrosine phosphorylation of IGF-IR, activation of AKT as well as expression of GLI1. We, then, demonstrate that β_1 integrins regulate proliferation of prostate cancer cells in 3-D cultures in a GLI1-dependent manner.

Materials and Methods

Reagents and antibodies

Reagents used for this study include: lipofectamine 2000, oligofectamine (OLF, Invitrogen) and Matrigel (BD Bioscience). The following monoclonal antibodies (mAbs) were used: to human β_1, clone-18 (BD Bioscience) and TS2/16 (ATCC); to α_2, P1H5 (Life Technologies); to α_3, P1B5; to α_4, P4C2 (both were kindly provided by Dr. Elizabeth Wayner); to α_5, P1D6 (Life Technologies); to α_6, clone GoH3 (kindly provided by Dr. Arnoud Sonnenberg); to α_v, L230 (ATCC); to mouse α_5, 5H10 (BD Bioscience); to hemagglutinin, 12CA5 (ATCC) and to phosphotyrosine, PY20 (Santa Cruz). The following rabbit polyclonal Abs were used: to ERK1 (C-16, this Ab also cross-reacts with ERK2); to phospho-AKT (Ser 473); to AKT; to GLI1; to FAK; to IGF-IRβ; to IRS-1 (all these Abs were purchased from Santa Cruz) and to the cytoplasmic domains of α_v or α_5 (kindly provided by Dr. Erkki Ruoslahti). We also used rat IgG (Pierce) and mouse IgG (Sigma) as a control.

Cell lines and transfectants

PC3, DU145 and TRAMP-C2 cells were cultured as described (Goel et al., 2005; Goel et al., 2009b). PC3 and DU145 cells were stably transfected with plasmids containing either pEGFP (vector) or pEGFP-β_1-shRNA using lipofectamine 2000 (Goel et al., 2009b). G418-resistant clones were pooled to generate populations. PC3 cells were stably transfected with pLKO-β_6-shRNA (Open Biosystem, clone ID TRCN0000057704) and these stable transfectants were used as control cell lines (designated as PC3-cont-shRNA).

3-D cultures

PC3 and DU145 stable transfectants (PC3-β_1-shRNA, PC3-vector, PC3-cont-shRNA, DU145-β_1-shRNA and DU145-vector) were embedded in Matrigel as single cells and cultured for 12 days (Weaver et al., 1997). Colonies in 3-D cultures were observed under a phase contrast microscope and images were captured using an Olympus IX71 inverted microscope with IPLab V3.55 (Scanalytics, Inc). Colony size was measured using a grid by phase contrast microscopy. In some experiments, PC3-cont-shRNA and PC3-β_1-shRNA cells were transiently transfected with either EGFP or GLI1-GFP (kindly provided by Dr. Junhao Mao) before embedding and culturing in Matrigel.

Embedded multicellular structures were scooped with the Matrigel included and placed into cold 2.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4 and fixed for 3 hours at room temperature. The samples were washed in the same buffer at 4°C for several days, then post-fixed in 1% osmium tetroxide in 0.1M cacodylate buffer, pH 7.4 at 4°C for 30 minutes, dehydrated in graded ethanols and embedded in Epon 812 with propylene oxide used as the transitional solvent (Underwood et al., 2006). Semi-thin sections were prepared and stained with 1% toluidine blue in 1% borax and imaged with a Zeiss Axioscope microscope. These images were used to count the number of cells per colony. A minimum of 20 cross-sections was used.

Immunoblotting (IB)

PC3-β_1-shRNA, PC3-vector, DU145-β_1-shRNA and DU145-vector transfectants were lysed and immunoblotted as previously described (Fornaro et al., 2000). Colonies of PC3-β_1-shRNA and PC3-cont-shRNA transfectants cultured in Matrigel were isolated using PBS-EDTA as described (Weaver et al., 1997); cells were lysed and immunoblotted using Abs to GLI1, β_1 or ERK.

TRAMP-C2 cells were transiently transfected with siRNA to either β_{1A} or β_{1C} (used as a control) or treated with OLF alone (Goel et al., 2005). Cells were lysed and immunoblotted as previously described (Fornaro et al., 2000).

PC3-cont-shRNA and PC3-β_1-shRNA cells, transiently transfected with either EGFP or GLI1-GFP, were lysed 48 hours after transfection and IB was performed using Abs to GLI1 or ERK, as a loading control.

Immunoprecipitation (IP)

PC3-β_1-shRNA or PC3-cont-shRNA transfectants were cultured in serum-free medium (SFM) for 24 hours. Cells were incubated in the presence or absence of IGF-1 (100 ng/ml) for 10 minutes. Cells were lysed and proteins were immunoprecipitated using Abs to IGF-IRβ or IRS-1 and protein A-Sepharose. Immunoprecipitated proteins were separated using 10% SDS-PAGE and immunoblotted using Abs to phosphotyrosine (p-Tyr), IGF-IRβ or IRS-1.

FACS analysis

PC3-β_1-shRNA, PC3-cont-shRNA and PC3-vector transfectants were detached and analyzed by FACS using P1H5 (a-α_2), P1B5 (a-α_3), P4C2 (a-α_4), P1D6 (a-α_5), GoH3 (a-α_6), L230 (a-α_v), TS2/16 (a-β_1), mIgG or 12CA5 (cont IgG). TRAMP-C2 cells were transiently transfected with siRNA to either β_{1A} or β_{1C} (used as a control). Cells were detached and FACS analysis was performed to detect surface expression of α_6 (GoH3), α_5 (5H10), or, as negative control, 12CA5 or rat IgG (Goel et al., 2009b).

GLI1 luciferase assay

PC3 parental, PC3-vector, PC3-cont-shRNA or PC3-β_1-shRNA cells were transfected with pCMV-β-galactosidase (Dr. Michael Lu) and 8×3'GLI-BS pδ51*Luc*II reporter construct [eight directly repeated copies of 3'GLI-BS into plasmid pδ=51*Luc*II, kindly provided by Dr. Hirosh Sasaki (Sasaki et al., 1997)]. Cell lysates were prepared with the reporter lysis buffer and GLI luciferase activity was assayed using Luciferase reagent (Promega). β-galactosidase activity was assayed using the Galacto-Lite Plus reagent (Tropix).

Statistical analysis

Statistical analysis was conducted using the Student's t-test except in one instance, when colony size in 3-D cultures was determined and summarized in ordered categories of increasing size. In this case, the colony size was compared among different groups using a Chi-square test for linear trend with 1 degree of freedom.

All p-values were based on two-tailed tests.

Results

β_1 integrins stimulate IGF-IR signaling and GLI1 expression

We have previously shown that β_1 integrins regulate IGF-IR's mitogenic and transforming ability in prostate cancer cells (Goel et al., 2005). To study the effect of β_1 downregulation on IGF-IR signaling pathway, we measured activation of IGF-IR and its downstream effectors, IRS-1 and AKT, in PC3 cells. β_1 downregulation shows significant inhibition of IGF-1 dependent tyrosine phosphorylation of IGF-IR (Fig. 1 part A, left) and IRS-1 (data not shown) as well as serine phosphorylation of AKT (Fig. 1 part A, right). Since it has been shown that IGF-1 potentiates GLI activity induced by low levels of SHH and this is mediated by activation of PI 3-kinase/AKT pathway (Riobo et al., 2006), we analyzed the

effect of β_1 downregulation on expression and activity of GLI1. Our results show that transfection of β_1 integrin shRNA reduces expression of GLI1 as compared to vector (Fig. 1 part B, left) or control shRNA (Fig. 1 part B, right) transfected cells, as evaluated by immunoblotting. As a consequence, GLI1 activity measured by reporter gene assay is also decreased in cells expressing β_1 integrin shRNA (Fig. 1 part C). These data indicate that β_1 downregulation reduces expression and activity of GLI1.

Downregulation of β_1 integrins by shRNA reduces cell proliferation in 3-D cultures

To study the role of β_1 downregulation in 3-D cultures, we used PC3 and DU145 prostate cancer cells stably expressing either shRNA to β_1 integrins (PC3-β_1-shRNA) or vector (PC3-vector) alone. We performed immunoblotting to confirm the downregulation of β_1 integrin protein levels in β_1-shRNA expressing cells in monolayer cultures (data not shown). When cultured in Matrigel as shown in Figure 2, part A, vector-transfected PC3 cells still expressing β_1 integrins are organized into large, loosely connected masses with an irregular morphology, whereas cells expressing β_1-shRNA form smaller, more organized structures with a more glandular morphology. We obtained similar results using DU145 cells cultured in Matrigel (Fig. 2 part A). PC3-β_1-shRNA 3-D cultures show increased cell-cell contacts (Fig. 2 part B, left) and contribute to a more normal morphology compared to cells expressing β_1 integrins. We counted the number of cells in the multicellular structures formed by vector or PC3-β_1-shRNA transfectants after 12 days in Matrigel cultures. There is a significantly reduced number of cells per structure upon β_1 integrin downregulation (Fig. 2 part B, right). The colony dimensions are decreased (Fig. 2 part C) consistent with both the decreased cell number and the increased cell-cell contacts observed after β_1 integrin downregulation. We confirmed by immunoblotting that β_1 was downregulated in the 3-D cultures of PC3-β_1-shRNA cells up to 12 days (Fig. 2 part D). We conclude that β_1 integrin downregulation decreases cell proliferation and reverts PC3 prostate cancer cells towards a more normal phenotype in 3-D reconstituted basement membrane cultures.

GLI1 increases proliferation of β_1-shRNA expressing PC3 cells in 3-D cultures

To study if GLI1 expression can rescue the inhibitory effect on cell proliferation in 3-D cultures due to β_1 downregulation, PC3-cont-shRNA and PC3-β_1-shRNA cells were transiently transfected with human GLI1. Expression of GLI1, but not of control DNA, causes increased colony size in PC3-β_1-shRNA cells cultured in 3-D Matrigel (Fig. 3 part A). No significant change in colony size is observed in PC3-cont-shRNA cells in 3-D cultures upon expression of either GLI1 or of control DNA (Fig. 3 part B). We confirmed by immunoblotting that the transfected GLI1 is expressed up to 12 days (Fig. 3 part C). These results suggest that GLI1 mediates β_1-dependent cell proliferation in 3-D cultures.

β_1 integrin downregulation affects the expression of the associated α subunits

β_1 integrins heterodimerize with several α subunits. To study the effect of β_1 downregulation on expression of various α subunits known to associate with β_1 integrins, we performed FACS using PC3 transfectants. As shown in Figure 4, β_1 downregulation causes reduced expression of α_2, α_3, α_4, and α_5 subunits. The most significant effect is observed on the expression of α_5 subunits, suggesting that α_5 is a major partner for β_1 in prostate cancer cells. In contrast, expression of α_6 and α_v integrin subunits is not changed. These results can be explained by the fact that PC3 cells express endogenous β_3, β_4 and β_5 integrins (data not shown), which can heterodimerize with α_6 and α_v subunits. These observations were further confirmed by using TRAMP-C2 cells, a mouse prostate cancer cell line. In these cells, downregulation of β_1 integrins reduces expression of α_5 subunit, but not α_v or α_6 subunit (data not shown). These data indicate that the $\alpha_5\beta_1$ is the predominant integrin heterodimer that regulates GLI1 expression and proliferation of prostate cancer cells in 3-D cultures.

Discussion

The novel finding described in this study is the discovery of a new function for β_1 integrins in regulating the expression of GLI1 and, consequently, GLI-dependent cancer cell proliferation in 3-D cultures.

Several studies have shown that GLI1 expression can be regulated by either classical or non-classical pathways (Lauth and Toftgard, 2007). The classical pathway is mediated by binding of SHH to Patched, a transmembrane protein. Binding of SHH to Patched suppresses the inhibitory activity of Patched on Smoothened, as a consequence stimulates expression of GLI1 (Sanchez et al., 2005). Although an indirect involvement of β_1 integrins in the classical pathway has been demonstrated by Blaess et al., who have shown that SHH binds laminin and promotes cell proliferation in a β_1-dependent manner (Blaess et al., 2004), integrins have not been shown to directly regulate SHH expression or activity. While it is unlikely that the classical pathway mediates GLI1 regulation by β_1 integrins observed in this study, it is likely that non-classical pathways mediated by PI 3-kinase/AKT, K-Ras/Raf/MEK/MAPK and TGF-β/Smad are involved. These pathways are known to potentiate SHH/GLI signaling (Lauth and Toftgard, 2007) and to be activated by integrins (Goel et al., 2009a; Munger et al., 1999). Among others, the IGF-1/AKT pathway is relevant to this study since it has been shown to increase GLI1 activity via increased expression of GLI2 (Riobo et al., 2006). Based on the results described in this study, we hypothesize that reduced PI 3-kinase/AKT activity due to deregulated IGF-IR signaling upon β_1 downregulation may cause reduced expression of GLI2, and as a consequence, reduced expression of GLI1. This hypothesis remains to be investigated.

The present study shows that downregulation of β_1 integrins in prostate cancer cells reduces colony size in 3-D Matrigel cultures and results in the formation of regular glandular structures, whereas irregularly-shaped structures are formed by control cells. We conclude that β_1 integrins may promote a transformed phenotype in prostate epithelial cells. Indeed, our findings are relevant to prostate cancer, since the SHH pathway has been shown to regulate prostate cancer progression (Karhadkar et al., 2004; Stecca et al., 2005). Using a similar 3-D system, it has also been shown that inhibition of β_1 causes phenotypic reversion of malignant human breast cancer cells via cell proliferation (Park et al., 2006; Park et al., 2008; Weaver et al., 1997; Zhang et al., 2009). Therefore, given the ability of GLI1 to rescue this effect, it is conceivable that GLI1 may regulate cell proliferation and phenotypic reversion in prostate as well as in breast cancer cells.

Our data suggest that the effect of β_1 integrins on cell proliferation and GLI1 expression in 3-D cultures is mediated by signaling via the IGF-IR/AKT pathway. These findings are relevant to prostate cancer, since the IGF-IR/AKT pathway has been shown to regulate prostate cancer progression (Majumder and Sellers, 2005; Sachdev and Yee, 2007). Similarly, inhibition of IGF-IR has been reported to block proliferation of breast cancer cells in 3-D cultures (Litzenburger et al., 2009). Therefore, β_1 integrins may regulate these aberrations in a wider set of carcinomas.

Finally, it is necessary to stress that the downregulation of β_1 integrins reduces expression of associated α subunits, predominantly α_5 and at a lower extent α_2, α_3 and α_4; but not α_6 and α_v, which are also known to heterodimerize with β_1 integrins. Among others, α_5 is significantly inhibited by β_1 shRNA, thus suggesting that $\alpha_5\beta_1$ is the functionally predominant β_1 integrin heterodimer in these cells. The data also indicate that other β integrin subunits do not play a crucial role in the novel pathway analyzed in this study.

In summary, we describe a new mechanism of regulation of GLI1 expression, and consequently of cancer cell proliferation, by β_1 integrins in 3-D cultures and propose that this pathway is mediated by IGF-IR and AKT (Fig. 5).

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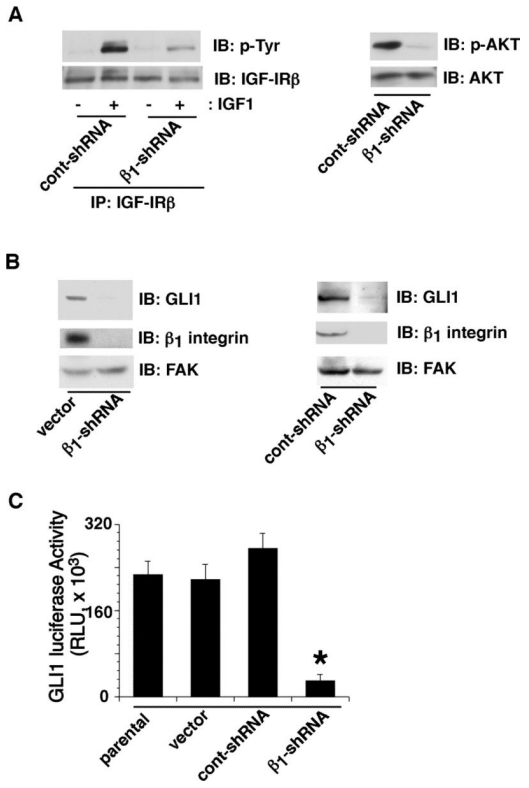

Fig. 1. β_1 integrins promote IGF-IR activation and increase GLI1 expression

A: PC3-β_1-shRNA or PC3-cont-shRNA transfectants were serum starved and stimulated with or without IGF-1. Cell lysates were immunoprecipitated using Abs to IGF-IRβ and immunoblotted using Abs to phosphotyrosine (p-Tyr, PY20) or IGF-IRβ (left panels). In some experiments, cell lysates from IGF-1 treated PC3-β_1-shRNA or PC3-cont-shRNA transfectants were immunoblotted using Abs to p-AKT or AKT (right panels). **B**: PC3-vector, PC3-β_1-shRNA or PC3-cont-shRNA were detached, lysed and immunoblotted using Abs to GLI1, β_1 (C-18) or FAK, as a loading control. **C**: PC3 parental, PC3-vector, PC3-cont-shRNA or PC3-β_1-shRNA cells were transfected with pCMV-β-galactosidase and 8×3'GLI-BS pδ51*Luc*II reporter construct. GLI reporter activity was analyzed by luciferase assay. The data were normalized using β-galactosidase activity. Data are expressed as means ±SEM (*p=0.0072).

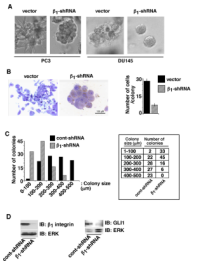

Fig. 2. β_1 integrins reduce cell proliferation in 3-D cultures

A: PC3-vector, PC3-β_1-shRNA, DU145-vector and DU145-β_1-shRNA transfectants were cultured in Matrigel. Colonies in 3-D cultures were observed under a phase contrast microscope and images were captured. **B**: PC3-vector and PC3-β_1-shRNA transfectants cultured in Matrigel were fixed and sections were stained with 1% toluidine blue (left panels). Number of cells in each colony was counted and shown as average with SEM (n=20, right panel, $p<0.00001$). **C**: PC3-cont-shRNA and PC3-β_1-shRNA transfectants were cultured in Matrigel for 12 days. Colonies in 3-D cultures were observed and the size of each colony was measured using a phase contrast microscope. Chi-square for linear trend with 1 degree of freedom is 73.462 ($p<0.00001$). **D**: PC3-cont-shRNA and PC3-β_1-shRNA transfectants were cultured in Matrigel for 12 days. Cells were isolated from Matrigel using PBS-EDTA and then lysed. Lysates were separated and immunoblotted using Abs to β_1 (C-18), GLI1 or ERK, as a loading control.

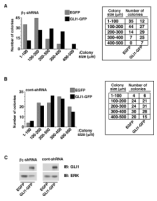

Fig. 3. GLI1 expression increases proliferation of PC3-β_1-shRNA cells in 3-D cultures

PC3-β_1-shRNA (**A**) and PC3-cont-shRNA (**B**) cells were transfected with either EGFP or GLI1-GFP. Cells were cultured in Matrigel for 12 days. Colonies in 3-D cultures were observed and the size of each colony was measured using a phase contrast microscope. Expression of GLI1 shows statistically significant differences in colony sizes in PC3-β_1-shRNA (Chi-square for linear trend with 1 degree of freedom is 36.181, $p<0.00001$), but not in PC3-cont-shRNA cells (Chi-square for linear trend with 1 degree of freedom is 0.608, $p=0.44$). **C**: PC3-β_1-shRNA cells were transiently transfected with either EGFP or GLI1-GFP. Cells were lysed and immunoblotted using Abs to GLI1 or ERK, as a loading control.

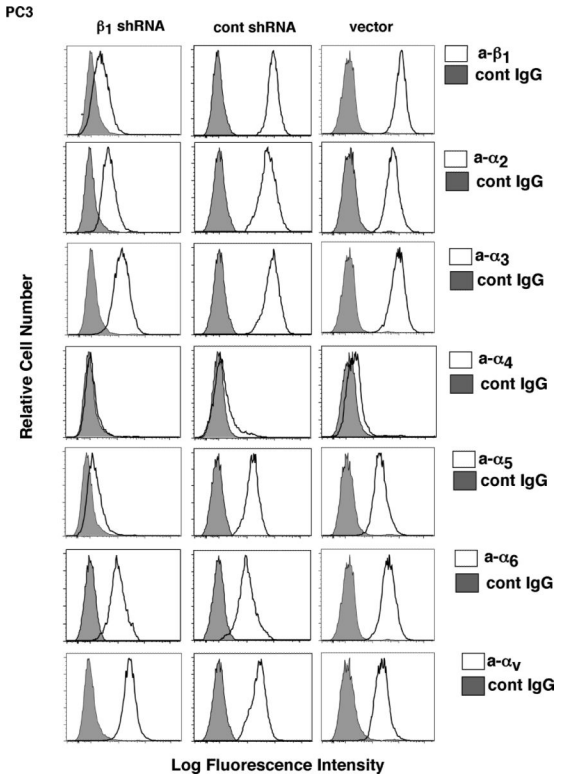

Fig. 4. β_1 integrin downregulation affects the expression of the associated α subunits

PC3-β_1-shRNA, PC3-cont-shRNA and PC3-vector transfectants were detached and analyzed by FACS using TS2/16 (a-β_1), P1H5 (a-α_2), P1B5 (a-α_3), P4C2 (a-α_4), P1D6 (a-α_5), GoH3 (a-α_6), L230 (a-α_v), or 12CA5 (cont IgG).

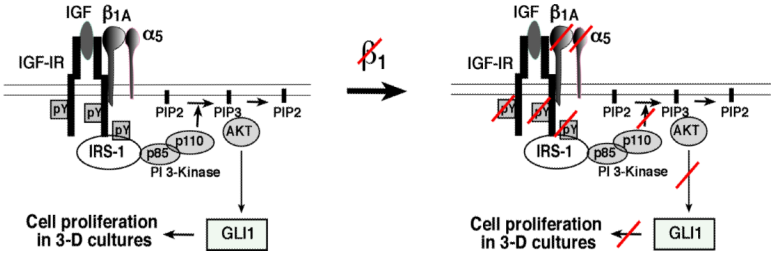

Fig. 5. β_1/IGF-IR complex regulates prostate cancer cell proliferation in 3-D cultures through GLI1 expression

A model for the interaction between β_1 integrins and IGF-IR regulating cell proliferation of prostate cancer cells in 3-D cultures in an AKT/GLI1-dependent manner is shown.