Effect of β4 Integrin Knockdown by RNA Interference in Anaplastic Thyroid Carcinoma

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Abstract. *Background*: *Integrin α6β4 is a known tumor antigen; however, its function in different subtypes of thyroid cancer is not known. This study reports that α6β4 expression is selectively up-regulated in anaplastic thyroid cancer (ATC) cells, the most malignant subtype of human thyroid cancer. Materials and Methods: To assess the contribution of α6β4 in ATC progression, cell proliferation, motility and soft agar assay were performed in vitro and a xenograft tumor growth assay was performed in vivo. Results: Knockdown of β4 integrin subunit expression by shRNA in ATC cells reduced the proliferation, migration, and anchorage-independent growth of ATC cells in vitro and xenograft tumor growth in vivo. Conclusion: These data suggest that integrin α6β4 contributes to the development of aggressive forms of thyroid cancer with poor prognostic potential, such as ATC, and thus may be a novel therapeutic target for the treatment for this subtype of thyroid cancer.*

Thyroid carcinoma is the most common malignancy of the endocrine system (1). Long-term survivors of anaplastic thyroid carcinoma (ATC) are rare (2-4) and have extremely low 5-year survival rates (5, 6). Metastasis to cervical lymph nodes is common, and more than half of ATC patients present with metastasis (2, 3, 7, 8). Initial treatment options are limited to palliation of asphyxiation by tracheostomy,

which is invariably associated with a poor outcome. Although ATC is radiation resistant, radiotherapy (RT) is commonly added to the treatment regimen to help relieve these airway obstructions.

Common types of differentiated follicular-cell derived thyroid tumors include papillary (PTC) and follicular (FTC) subtypes. Poorly differentiated thyroid carcinomas (PDTC), including ATC, are less common but represent the highest grades of malignancy (2, 3, 7-15). Patients with differentiated thyroid tumors have good long-term survival rates, while those with the less differentiated subtypes of thyroid tumors, such as ATC, have a poor prognosis (2, 3, 7-17). This poor clinical outcome is due to the rapid proliferation and metastasis of these tumor subtypes (2, 3, 7, 8). The loss of the sodium/iodide symporter (NIS) expression that imports and concentrates iodine in thyroid cells, and which is essential for diagnosis and treatment of both tumor remnants and distant metastases, has been considered to be one of the major causes of poor prognosis (18). There have been many attempts to re-express NIS to re-establish iodide uptake function in tumor cells (19-23). New treatment strategies, such as chemotherapy agents (24, 25) bovine serum ribonuclease (26), bone morphogenic protein (27), *p53* gene therapy (28, 29), and re-differentiation gene therapy (19-23), have been attempted to alter the course of the disease. However, the results of these trials were disappointing and have not resulted in clinical application. Therefore, to develop novel target-specific therapies, it is necessary to understand the molecular events responsible for the aggressive behavior of ATC.

In this study, it was hypothesized that α6β4 integrin is a candidate target for thyroid cancer therapy based on its established role in breast and other cancer progression (30, 31). α6β4 Integrin is a laminin receptor and is ubiquitously expressed in most epithelial cells (30, 31). Due to its expression in epithelia, the primary role of α6β4 was previously thought to maintain the tissue integrity (30, 31). However, recent reports suggest that α6β4 integrin also plays

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a pivotal role in carcinoma progression, suggesting that α6β4 may exist in "two different functional states" depending on the surrounding microenvironment (32, 33). In normal epithelia, α6β4 is mainly localized in hemidesmosomes (HDs) without having any signaling functions (33). In the tumor microenvironment, α6β4 is mobilized from HDs to actin filament (F-actin)-rich structures such as lamellipodia and filopodia in a PKC-dependent manner (34, 35). It is thought that this re-localization of α6β4 from HDs to the leading edge enhances its signaling function in cancer cells (34). Once α6β4 becomes signaling competent, it enhances the ability of carcinoma cells to invade (35, 36), as well as survive (37, 38), under stress conditions.

In the current study, the expression of α6β4 integrin was evaluated in various thyroid cancer cell lines that represent different subtypes, and it was found that α6β4 expression is up-regulated in ATC cells compared to other subtypes of thyroid cancer. Knockdown of β4 integrin expression in ATC cells efficiently blocked their ability to proliferate, migrate, and grow in an anchorage-independent manner. This finding was further extended *in vivo* by performing xenograft studies using nude mice. Injection of α6β4-deficient ATC cells formed dramatically smaller tumor masses than did wild-type ATC cells. These studies suggest that α6β4 is critical for the aggressive behavior and tumor progression of ATC, and could provide a basis for the development of targeted therapy for the treatment of ATC.

Materials and Methods

Cell lines and reagents. MDA-MB-435 human cancer cells were obtained from the Lombardi Breast Cancer Depository at Georgetown University (Washington, DC, USA). MDA-MB-435 subclones [MDA-MB-435/mock (vector only, clone 6D2) and MDA-MB-435/β4 (β4 integrin, clone 3A7)] were generated as previously described (35, 37, 39) and cultured in low glucose Dulbecco's modified Eagle's medium (DMEM) with L-glutamine, sodium pyruvate, 10% fetal bovine serum (FBS), and 100 U/ml penicillin and streptomycin. FTC, NPA, TPC-1, and FTC236 human thyroid cancer cell lines were grown in DMEM with 10% FBS and 1% antibiotic-antimycotic (Gibco BRL, Grand Island, NY, USA) in 5% CO_2 at 37°C. ARO and FRO human ATC cell lines were grown in RPMI-1640 with 10% FBS and 1% antibiotic-antimycotic (Gibco BRL) in 5% CO_2 at 37°C. Integrin β4 (clone H-101) and actin (clone C-11) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and Akt and p-Akt (Ser473 and Thr308) antibodies were from Cell Signaling Technology (Beverly, MA, USA). Lentivirus expressing shRNA against β4 integrin was from Sigma (St. Louis, MO, USA), and infection was performed according to the manufacturer's protocol.

Western blot analysis. Cells were lysed in 50 mM Tris buffer, pH 7.4, containing 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM sodium orthovanadate, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1% protease inhibitor (Pierce, Rockford, IL, USA), scraped

with a rubber policeman, and collected in 1.5-ml tubes. Protein concentration was determined using the BCA protein assay kit (Pierce). Total cellular protein was resolved by 4-20% gradient SDS-PAGE, transferred to polyvinylidene fluoride membranes, and incubated with primary antibody. After three 10-minute washes in 50 mM Tris buffer, pH 7.5, containing 0.15 M NaCl and 0.1% Tween-20, protein was detected with peroxidase-conjugated secondary antibody and visualized using Luminol and Oxidizing solutions (Boston Bioproducts, Worcester, MA, USA).

Flow cytometry. Adherent cells were collected in ice cold phosphate-buffered saline (PBS) and stained with rat anti-human integrin β4 (clone CD104; BD Biosciences) for 30 min. After washing in PBS, they were stained with Alexa Fluor 488 Goat anti-rat IgG (1:100; Invitrogen, Carlsbad, CA, USA) on ice for 30 min and washed with PBS. Samples were analyzed on a FACScan flow cytometer (BD Biosciences).

Soft agar assay. FRO cells (1×10^3) expressing GFP or S100A4 shRNA were suspended in serum (2.5% FBS) with DMEM (2 ml) containing 0.35% low melt agarose (ISC BioExpress, Kaysville, UT, USA) and overlaid on a 1 ml layer of 0.75% agar in six-well plates. Soft agar was overlaid with complete medium (0.5 ml/well), which was changed every other day. After 14 days, the number of colonies was quantified by counting 50 fields per well using bright-field microscopy.

Cell motility assay. The upper chambers (8-μm pore size) of transwells (Costar, Cambridge, MA, USA) were coated with collagen at 4°C. Matrigel (0.5 μg, Collaborative Research, Bedford, MA, USA) was diluted in cold water and dried onto filters overnight at room temperature. After washing in PBS, cells were added to the upper chamber in serum-free DMEM/BSA, and 100 nM lysophosphatidic acid (Sigma) was added to the lower chamber as a chemo-attractant. After incubation for 2 h at 37°C in 10% CO_2, cells attached to the bottom of the membrane were stained and counted using crystal violet. Assays were performed in triplicate and repeated five times.

Xenograft studies. FRO cells, wild-type, treated with shRNA for GFP or integrin β4 were grown to ~90–95% confluency in 100 mm petri dishes, collected, washed twice with PBS, resuspended in medium, and injected subcutaneously (2×10^6 cells) into the flanks of 9-week-old athymic female nude mice (Harlan-Sprague Dawley, Indianapolis, IN, USA). Mice were divided into three groups: group A, wild-type FRO cell line; group B, FRO treated with shRNA to GFP; and group C, FRO treated with shRNA to integrin β4. Tumor size was measured every three days with calipers in three dimensions. Tumor size (mm^3) was calculated as (3.14 × length × width × depth)/6. The experiment was terminated after 21 days because mice injected with wild-type FRO cells exhibited morbidity. All studies involving mice were approved by the Yonsei University College of Medicine Animal Care and Use Committee.

Results

α6β4 is selectively expressed in ATC cell line. To assess the relationship between α6β4 expression and thyroid carcinoma progression, the level of β4 integrin expression in thyroid

Figure 1. *Analysis of α6β4 integrin expression levels in thyroid cancer cell subtypes. Follicular (FTC236), papillary (TPC1, NPA), anaplastic thyroid cancer cell lines (ARO, FRO), MDA-MB-435 mock and β4 integrin transfectants were lysed in RIPA buffer and equal amounts of protein immunoblotted for β4 integrin and actin.*
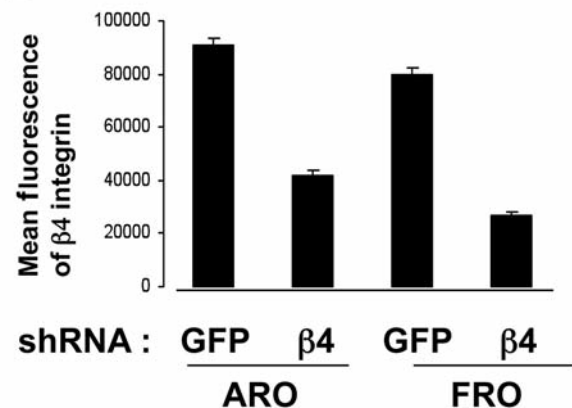

Figure 2. *Reduction of integrin β4 expression by shRNA in anaplastic thyroid carcinoma cell lines. A: Lysates from GFP and β4 integrin shRNA-transfected anaplastic thyroid cancer cell lines (ARO and FRO) were immunoblotted for β4 integrin and actin. B: Cell surface expression of β4 integrin in ARO, FRO cells, and MDA-MB-435 cell lines was determined by flow cytometry.*

cancer cell lines representing different subtypes and prognoses was monitored (Figure 1). β4 Integrin only pairs with the α6 integrin subunit and therefore represents α6β4 integrin. The thyroid carcinoma cell lines were derived from follicular (clone: FTC), papillary (clones: TPC1 and NPA), and anaplastic (clones: ARO and FRO) subtypes. The ten-year overall relative survival rates of patients with PTC and FTC are longer than those with undif ferentiated/ATC (40). On the other hand, ATC is one of the most lethal human malignancies (42, 43) with no known targeted therapy. It is notable that β4 integrin expression was selectively detectable

and up-regulated in the ATC cell lines (clones ARO and FRO), but at background levels in FTC and PTC cell lines. MDA-MB-435 mock and β4 integrin transfectants were used as negative and positive controls for this experiment as this cell line lack endogenous β4 expression. These studies suggest that malignant behavior and poor prognosis of ATC may be functionally linked to α6β4 expression.

Generation of ATC cell lines deficient in β4 integrin expression. Based on the data that β4 integrin is up-regulated in ATC cell lines, shRNAs that encode either GFP or β4 integrin using lenti
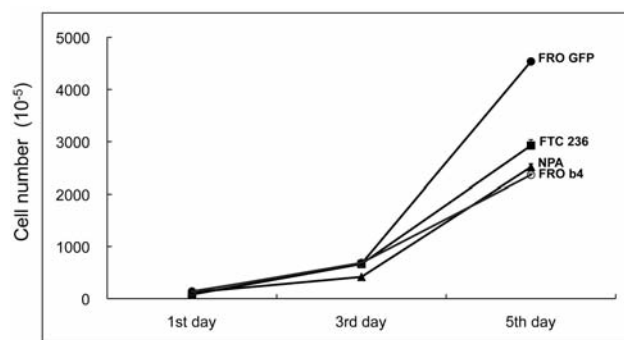
Figure 3. *β4 Integrin shRNA reduces the proliferation of anaplastic cancer (FRO) cells. Proliferation of follicular (FTC236), papillary (NPA) and GFP or β4 integrin shRNA-transfected anaplastic cancer cells (FRO) was measured on days 1, 3 and 5 post-transfection.*

virus in ARO and FRO ATC cell lines were stably expressed to selectively knockdown β4 integrin expression. Compared to control GFP shRNA, β4 integrin shRNA effectively reduced the expression of β4 integrin in FRO cells more than 70% as confirmed by Western blot (Figure 2A) and flow cytometry (Figure 2B). β4 integrin shRNA had no impact on actin expression (Figure 2A). The specificity of β4 integrin knockdown by shRNA was confirmed by measuring cell surface expression of other integrin subunits such as α5, α3 and β1, which showed no significant difference in cell surface expression by β4 integrin shRNA expression (data not shown).

α6β4 loss in ATC cells results in reduced cell proliferation, migration, and anchorage-independent growth. To assess the role of α6β4 in ATC cell function, first the proliferation of FRO cells expressing GFP or β4 integrin shRNA was monitored. Knockdown of β4 integrin expression in FRO cells dramatically reduced the rate of proliferation up to approximately 60% by day five compared to GFP shRNA-expressing control cells (Figure 3). It is notable that the growth rate of β4 shRNA FRO cells was quite similar to that of the other differentiated subtype of thyroid carcinoma cell line (FTC and NPA), that endogenously lacked β4 expression. Next, the impact of β4 integrin knockdown on colony formation in soft agar was investigated, because anchorage-independent growth is necessary for metastasis. FRO cells that stably express β4 shRNA formed fewer colonies that were less than 3-fold smaller in size than the colonies formed by cells that expressed control GFP shRNA (Figure 4). Finally, the role of α6β4 in FRO cell motility, which is also critical for metastasis, was tested. MDA-MB-435 cells were used as a control because this cell line endogenously lacks α6β4, and stable ectopic expression of β4 integrin dramatically enhances their motility (Figure 5) (33). Loss of β4 integrin expression induced 60% less migratory capacity

in FRO cells than the GFP shRNA-expressing cells towards the lysophosphatidic acid (LPA) chemoattractant (Figure 5). Taken together, these data indicate that α6β4 is essential for anchorage-independent growth and migration of ATC cells, which are important aspects of tumor progression.

Effects of integrin α6β4 knockdown on anaplastic thyroid tumor growth in nude mice. Based on the findings that knockdown of integrin α6β4 by shRNA expression inhibited ATC cell growth and migration *in vitro*, it was hypothesized that integrin α6β4 plays a crucial role in ATC tumor formation. Wild-type FRO cells, FRO cells expressing GFP shRNA and integrin β4 shRNA were injected subcutaneously into female athymic nude mice. Tumors formed rapidly within three days but were of variable size. Tumors formed by FRO cells expressing integrin β4 shRNA were significantly smaller than tumors formed by wild-type FRO cells and FRO cells expressing GFP shRNA. Even more strikingly, there was decrement of tumor mass 18 days after injection with FRO/integrin β4 shRNA (Figure 6). These data suggest that integrin α6β4 plays a pivotal role in ATC progression *in vivo*.

Discussion

While the role of α6β4 in breast cancer progression is well established, its functions in different subtypes of thyroid cancer is not known. This study evaluated the expression of integrin α6β4 in various subtypes of human thyroid cancer tissue by Western blot analysis. It was found that α6β4 is selectively expressed in ATC and is important for ATC cell growth, migration, and invasion. These data suggest a potential correlation of α6β4 with the dedifferentiation and metastatic phenotypes of thyroid cancer, and that α6β4 may be a promising candidate for the development of new ATC treatment strategies

Higher expression of α6β4 in the ATC cell line supports the hypothesis that α6β4 expression is related to the poor prognosis of patients with dedifferentiated ATC. The data that ATC cell functions are efficiently blocked by β4 shRNA further support this hypothesis. It was recently shown that curcumin, a phytochemical compound, selectively inhibits α6β4 functions in breast carcinoma cells (44). Thus, multimodality approaches targeting α6β4 with curcumin and inhibitors of other signaling receptors known to be up regulated in ATC (such as abnormal p53, p-glycoprotein, Cdk activity) may be an effective treatment for ATC (27, 41, 42).

Several previous studies suggest that some cases of ATC may be derived from well-differentiated thyroid carcinoma (WDTC) (43, 45, 46). This proposition is based on the co-existence of ATC or PDTC within an area of WDTC tissue, and the fact that some cases of treated WDTC have recurred as ATC (47). Moreover, a subset of ATC may be present within a component of a larger WDTC or may contain microscopic foci of differentiated carcinoma (48). These
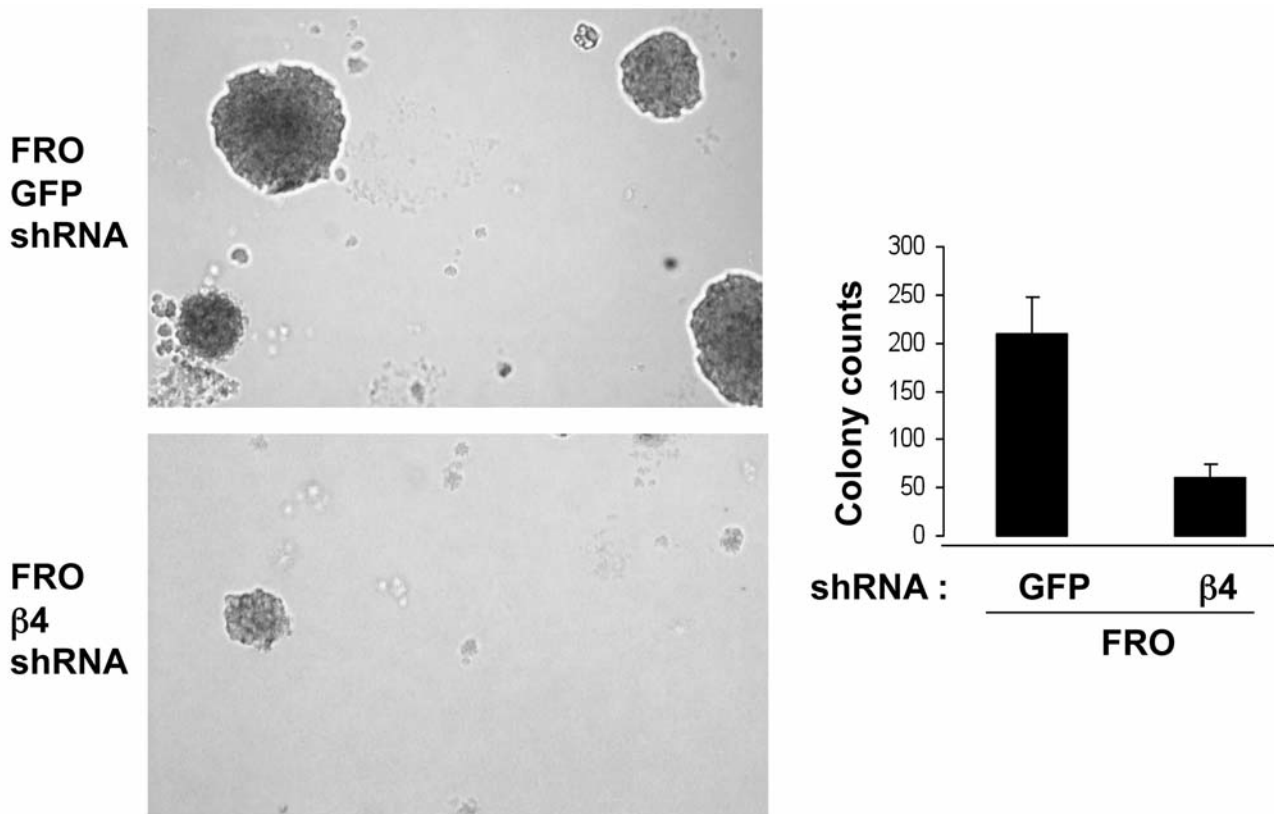
Figure 4. *α6β4 is essential for anchorage-independent growth of FRO cells. Representative bright-field images captured at ×10 magnification of FRO anaplastic thyroid cancer cells expressing either GFP or β4 integrin shRNA. Cells were grown in 0.3% agar with culture medium containing 2.5% FBS for 2 weeks. Columns, mean of three representative experiments performed in triplicate; bars, SE. Fifty fields per well were counted for each assay.*

findings suggest that dedifferentiation of WDTC may occur and cause progression to ATC (43, 45-49), but little is known about the pathophysiological mechanisms of this process. The current study indicates that the dedifferentiated subtypes of thyroid cancer may be linked to elevated α6β4 expression. It will be interesting to evaluate the role of α6β4 in the process of dedifferentiation of thyroid cancer. α6β4 could be a key molecule in the differentiation and metastatic switch during thyroid cancer progression.

In conclusion, the aggressiveness of ATC is closely related to the expression of α6β4, and the suppression of α6β4 expression effectively blocks the proliferation, migration, and tumor formation of ATC cells. Therefore, α6β4 is a potential novel target for ATC therapy.
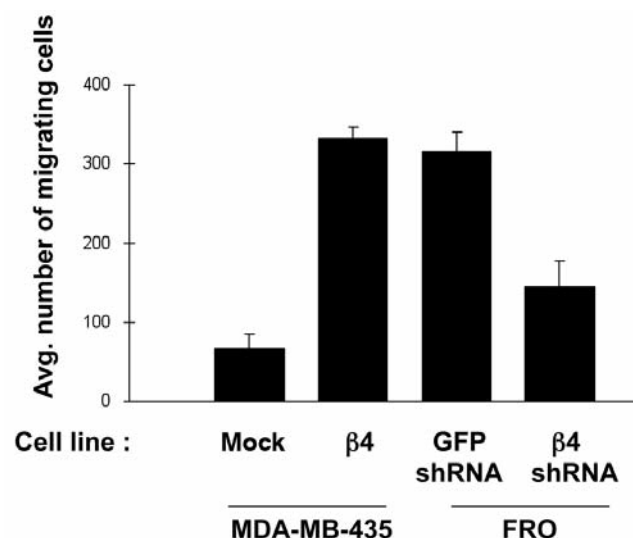
Acknowledgements

Figure 5. *Efficient migration of FRO cells towards LPA requires α6β4. The ability of MDA-MB-435 (mock or β4 integrin-transfected) and FRO (GFP or β4 integrin shRNA-transfected) to migrate toward 100 nM LPA was measured using a transwell cell motility assay.*
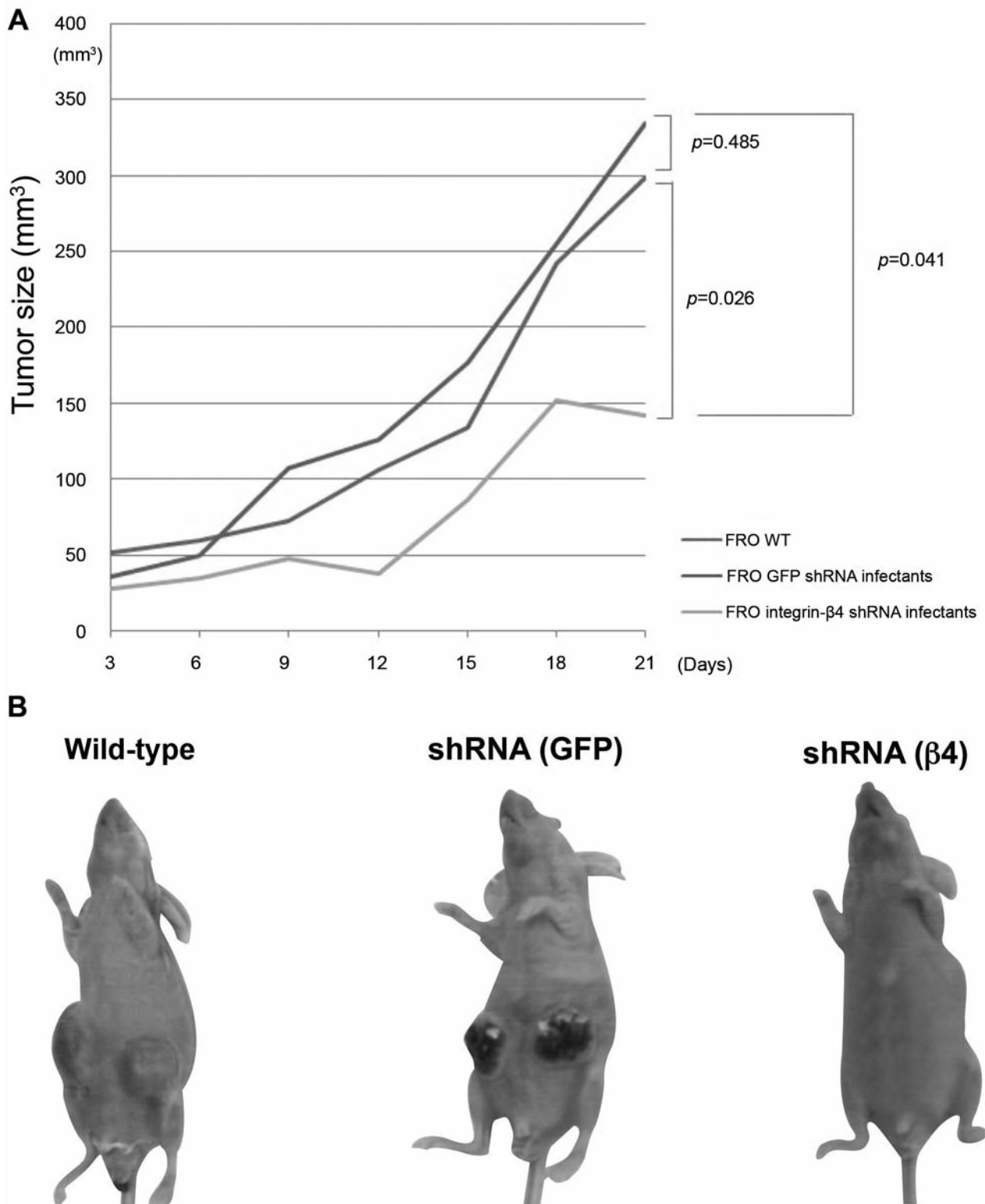
Figure 6. *Knockdown of integrin β4 expression by shRNA reduced tumor growth in vivo. Tumor formation capacity of tumor cell lines (wild-type, GFP shRNA-, or β4 shRNA-transfected) was measured 21 days after subcutaneous injection. Tumor size = (3.14 × length × width × depth)/6. A: Size of tumors, B: Images of tumors.*

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