High Lung-metastatic Variant of Human Osteosarcoma Cells, Selected by Passage of Lung Metastasis in Nude Mice, Is Associated with Increased Expression of $\alpha_v\beta_3$ Integrin

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Abstract. *Altered expression of $\alpha_v\beta_3$ integrin is associated with tumor progression and metastasis in several types of cancer, including metastatic osteosarcoma. In this study, we demonstrate that in vivo passaging of lung metastasis in nude mice can generate an aggressive variant of human osteosarcoma cells. Experimental metastases were established by injecting 143B human osteosarcoma cells, expressing green fluorescent protein (GFP) in the nucleus and red fluorescent protein (RFP) in the cytoplasm, in the tail vein of nude mice. Lung metastases were harvested under fluorescence microscopy from nude mice to establish cell lines which were then injected via the tail vein of additional nude mice. This procedure was repeated for four passages in order to isolate highly metastatic variant sublines. When the parental and metastatic variants were transplanted orthotopically into the tibia of nude mice, the 143B-LM4 variant had the highest metastatic rate, approximately 18-fold higher than the parent ($p<0.01$). $\alpha_v\beta_3$ integrin expression was increased approximately 5.6-fold in 143B-LM4 compared to parental cells ($p<0.05$). Thus, serial passage of lung metastases created a highly metastatic variant of human osteosarcoma cells which had increased expression of $\alpha_v\beta_3$ integrin, suggesting that $\alpha_v\beta_3$ integrin plays an essential role in osteosarcoma metastasis. With this highly metastatic variant overexpressing $\alpha_v\beta_3$ integrin, it will now be possible to further investigate the mechanism by which $\alpha_v\beta_3$ integrin facilitates metastasis.*

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Osteosarcoma is the most common, primary malignant bone tumor in children and adolescents (1, 2). Despite aggressive chemotherapy (3, 4) and surgical excision of the primary tumor (5, 6), patients with metastatic or recurrent disease still have poor prognosis (7-9). Therefore, novel therapeutic targets are needed to prevent recurrence or metastasis to improve the disease-free survival rate.

A family of integrins, which are structurally similar heterodimers composed of α- and β-subunits, regulates diverse cellular processes, such as cell proliferation, migration, attachment, and angiogenesis (10-12). $\alpha_v\beta_3$ integrin is minimally expressed on resting or normal blood vessels, but is significantly up-regulated on vascular cells within human tumors. Many types of cancer including melanoma (13), breast cancer (14), prostate cancer (15), colon cancer (16), and glioma (17), overexpress $\alpha_v\beta_3$ integrin, whose expression has been shown to be associated with tumor progression and metastasis.

We previously stably knocked down β1 integrin subunit expression in human FG-red fluorescent protein (RFP) pancreatic cancer cells using lentiviral-based RNA interference. Knockdown of the β_1 integrin subunit inhibited cell adhesion, migration and proliferation on types I and IV collagen, fibronectin and laminin *in vitro* and reduced primary tumor growth by 50% and completely inhibited spontaneously-occurring metastasis (18).

We also previously used a powerful subcellular *in vivo* imaging model to demonstrate how an anti-integrin antibody affects seeding and growth of osteosarcoma cells on the lung. The 143B human osteosarcoma cell line, expressing RFP in the cytoplasm and green fluorescent protein (GFP) in the nucleus, was established. Such double-labeled cells enable imaging of apoptosis and mitosis and other nuclear-cytoplasmic dynamics. Using the double-labeled osteosarcoma cells, single cancer-cell

seeding in the lung after *i.v.* injection of osteosarcoma cells was imaged. The monoclonal antibody against β_1 integrin, AIIB2, greatly inhibited the seeding of cancer cells on the lung. AIIB2 also inhibited spontaneous lung metastasis but not primary tumor growth, possibly due to inhibition of lung seeding of the cancer cells as imaged in the experimental metastasis study. AIIB2 treatment also increased survival of mice with orthotopically-growing 143B-RFP tumors (19).

In the present study, a highly metastatic variant of human osteosarcoma with elevated levels of $\alpha_v\beta_3$ integrin was isolated from the 143B human osteosarcoma by serial passage of lung metastases in nude mice, suggesting an important role for $\alpha_v\beta_3$ integrin in osteosarcoma metastasis.

Materials and Methods

Transformation of 143B human osteosarcoma cells to express GFP in the nucleus and RFP in the cytoplasm (dual-color cells). To establish 143B human osteosarcoma expressing GFP in the nucleus and RFP in the cytoplasm, the cells were transfected with retroviral DsRed2 and histone H2B-GFP vectors and selected by antibiotic resistance as previously described (20-23).

Establishment of highly lung-metastatic variant. Mice were injected *via* the tail vein with a suspension of 143B dual-color cells (1.5×10^6). Three weeks after injection, mice were sacrificed and the lungs were harvested. Under fluorescence microscopy, the fluorescent pulmonary metastases were readily identified and precisely harvested. Harvested lung metastases were dissected aseptically and rinsed three times with phosphate buffered saline (PBS)-penicillin and minced into small fragments using sterile scalpel blades. Tumor tissue was dissociated by treatment with collagenase type I (200 units/ml) for 4 h at 37˚C. After centrifugation, the pellet was resuspended in cell culture medium and seeded in cell culture dishes. The first selected subline, termed 143B-LM1, was maintained in RPMI-1640 medium containing 15% fetal bovine serum (FBS) medium and re-injected *via* the tail vein in nude mice to develop lung metastases from which the 143B-LM2 cell line was isolated three weeks after inoculation. This process was repeated two additional times to establish the 143B-LM3 and 143B-LM4 sublines. The 143B parental cells and sublines were maintained with RPMI-1640 medium containing 15% FBS and 1% penicillin/streptomycin at 37˚C in 5% CO_2.

Doubling time of 143B parental and LM sublines. 143B dual-color cells and LM sublines were seeded at density of 2.0×10^5 cells/dish in 100-mm culture dishes with RPMI-1640 with 15% FBS. The dishes were kept in an incubator at 37˚C and 5% CO_2. Every 24 h for a total of 96 h, three dishes for each subline were used for cell counting. Resuspended cells, collected after trypsinization, were stained with trypan blue (Sigma-Aldrich, St. Louis, MO, USA). Only viable cells were counted using a hemocytometer (Hausser Scientific, Horsham, PA, USA). The doubling time was calculated with the following formula: $(t\text{-}t_0)\times\log 2/\log(n/n_0)$, in which t_0 and n_0 are the time and the number of cells incubated for 24 h, respectively, and t and n are the time and the counts of cells at 48, 72 or 96 h, respectively. The assays were performed in triplicate and at least twice independently.

Mice. Athymic nu/nu (nude) mice (AntiCancer Inc., San Diego, CA, USA), 4 to 5 weeks old, were used in this study. Mice were kept in a barrier facility in HEPA-filtered isolators. Mice were fed with autoclaved laboratory rodent diet. All animal studies were conducted with an AntiCancer Institutional Animal Care and Use Committee (IACUC)-protocol approved for this study and in accordance with the principals and procedures outlined in the National Institute of Health Guide for the Care and Use of Animals under Assurance Number A3873-1.

Spontaneous lung metastasis assay. 143B parental cells (5×10^5) or LM sublines were transplanted into the tibia of nude mice as previously described (23). Ten mice were transplanted for each subline tested. Five weeks after intratibial transplantation, all mice were sacrificed and lungs were harvested as previously described (23).

Imaging. The OV100 Small Animal Imaging System (Olympus Corp., Tokyo, Japan) (24) was used to identify fluorescent pulmonary metastases. Images were captured directly on a PC (Fujitu Siemens, Munich, Germany). The total number of metastases per lung was counted and averaged among all the mice.

Immunoprecipitation and densitometry. The monoclonal antibody for $\alpha_v\beta_3$ integrin, LM609 (Millipore, Temecula, CA, USA), has been previously described (18). Immunoprecipitations (IPs) were conducted as previously described using 4 μg of LM609 absorbed onto 25 μl packed goat anti-mouse IgG-agarose (Sigma-Aldrich, St. Louis, MO, USA) and 400 μg protein from cell surface-biotinylated cell lysates (18). IP results were quantitatively analyzed with NIH Image J software (National Institutes of Health, Bethesda, MD, USA). Band intensities were assigned integrated density values, which represent the sum of all pixels in the box. Bands were quantified in equal area boxes and compared to β-actin immunoblotting protein loading controls to generate a ratio.

Statistical analyses. Statistical analysis method used was ANOVA for multiple data sets. Values of $p<0.05$ were considered statistically significant.

Results and Discussion

Proliferation of parental and LM sublines. The dual-color 143B cells have a strikingly bright GFP in the nucleus and RFP in the cytoplasm *in vitro* (Figure 1). *In vitro* proliferation rates of parental and passage-selected cell lines were similar (Table I).

Spontaneous lung metastases of 143B parental cell and LM sublines. When the 143B parental and the LM sublines were transplanted orthotopically into the tibia of nude mice, all cell lines had a higher metastatic frequency than the parental, with the 143B-LM4 cells having the highest (Figure 2, Table I). The number of lung metastases was significantly higher in 143B-LM4 cells compared with 143B parental cells ($p<0.05$).

Expression of $\alpha_v\beta_3$ integrin in 143B parental and LM sublines. IP and subsequent densitometoric analyses indicated that the protein expression level for $\alpha_v\beta_3$ integrin

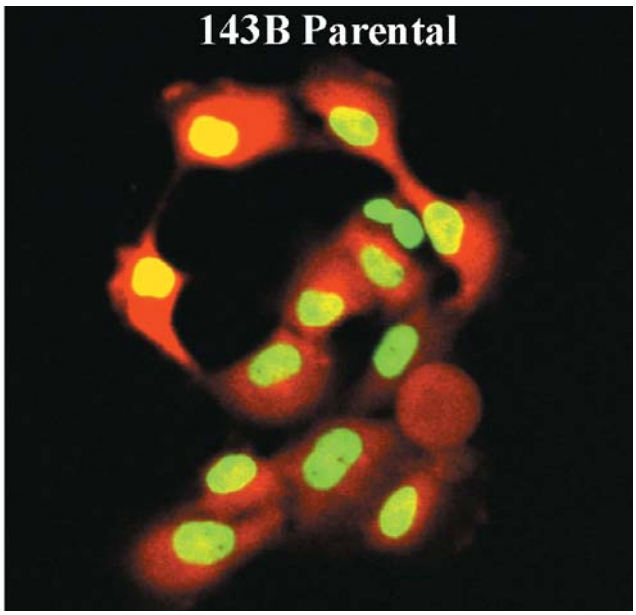

Figure 1. *Dual-color 143B human osteosarcoma cells expressing GFP in the nucleus and RFP in the cytoplasm in vitro.*

Table I. *Proliferation and metastatic characteristics of 143B parental and LM sublines. The doubling time was calculated as described in the Materials and Methods.*

Cell line	Doubling time (hours ± S.D.	Lung metastases	
		Incidence[a]	Average number of lung metastases per mouse ± S.D.
143B Parental	17.9 ± 2.8	10/10	19.8 ± 18.2
143B-LM1	21.9 ± 2.4	10/10	186.3 ± 221.5
143B-LM2	18.9 ± 2.6	10/10	90.6 ± 72.3
143B-LM3	16.9 ± 2.0	10/10	73.2 ± 44.7
143B-LM4	16.0 ± 2.9	10/10	352.4 ± 381.2*

143B parental and the indicated LM cells (5×10^5 cells) were transplanted into the tibia of nude mice. Mice were sacrificed at five weeks. [a]Number of mice with metastasis/number of tumor-implanted mice. *$p<0.05$ compared to 143B parental cell lines.

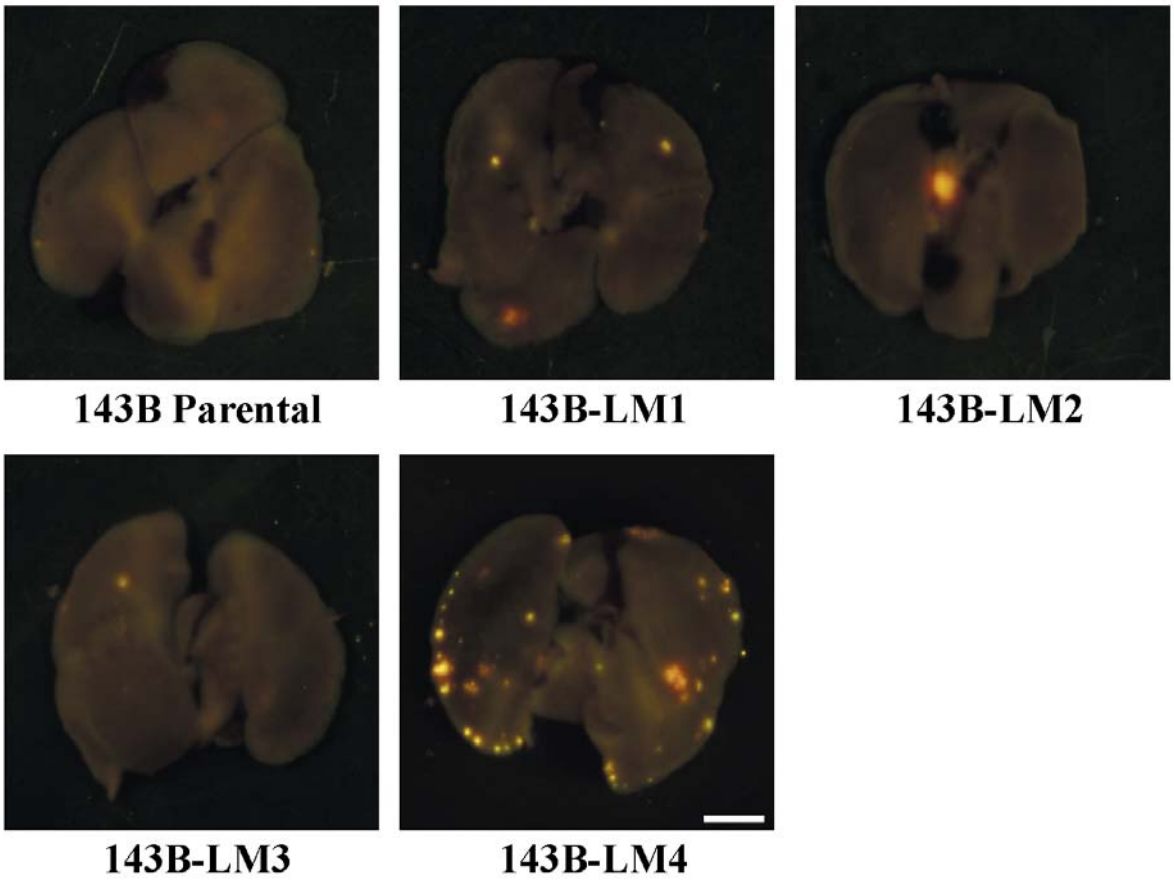

Figure 2. *Imaging of spontaneous lung metastasis in 143B parental and LM sublines. Visualization of lung metastases five weeks after 143B parental or LM sublines were transplanted into the tibia. The OV100 variable-magnification imaging system (Olympus) was used. Bar: 2 mm.*

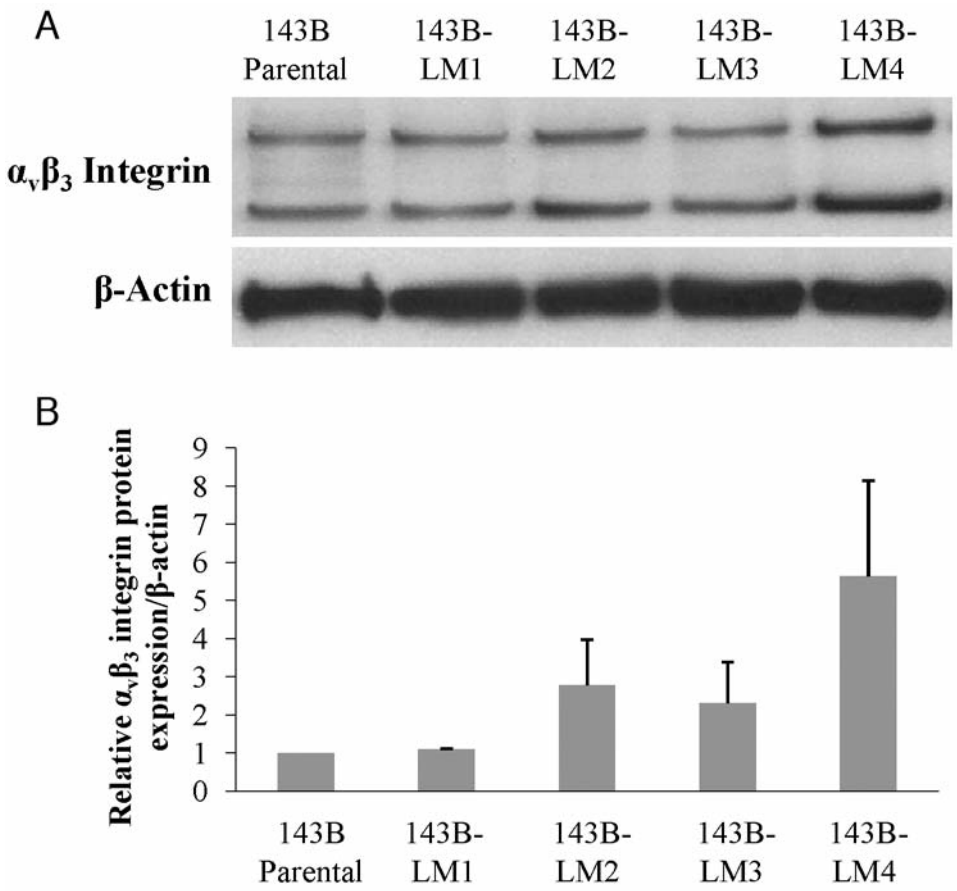

Figure 3. *Protein expression of $\alpha_v\beta_3$ integrin in the 143B parental and LM sublines. Immunoprecipitations (IPs) were conducted as described in the Materials and Methods. A: Representative immunoblot results for the $\alpha_v\beta_3$ integrin are shown for each cell line, compared to an independent immunoblot for β-actin from each corresponding cell line or subline. B: Densitometry results from IP data for $\alpha_v\beta_3$ integrin expression are shown for each cell line. Data represent the mean ± SEM from at least three independent experiments.*

was relatively high in 143B-LM4, 143B-LM3 and 143B-LM2 compared with 143B parental cells. The $\alpha_v\beta_3$ integrin protein level in 143B-LM4 cells was significantly higher than in parental line ($p<0.05$) (Figure 3).

Previously, implantation of human pancreatic cancer cells into the pancreas of nude mice was used to select variants with increasing metastatic potential. Hepatic metastases were harvested. This cycle was repeated several times to yield highly metastatic variant sublines. The variant sublines produced significantly higher numbers of lymph node and liver metastases than the parental cells. Their increased metastatic potential was associated with increased expression (mRNA and protein) of the proangiogenic molecules including basic fibroblast growth factor, vascular endothelial growth factor, and interleukin-8 (25).

The present study has demonstrated that variants of a human osteosarcoma cell line can be generated that are more lung-metastatic than the parental line by serial passage of the metastatic cells. The parental cell line and variant sublines have similar doubling times *in vitro*. 143B-LM4 had an 18-fold higher metastatic potential compared to the parental line ($p<0.05$) and overexpressed $\alpha_v\beta_3$ integrin by 5.6-fold compared to the parental line ($p<0.05$).

Thus, passage of metastasis of osteosarcoma cancer cells in nude mice is a relevant model with which to select variant cell lines with enhanced metastatic potential and study the relationship of $\alpha_v\beta_3$ integrin to the biology of metastasis.

The importance of the expression of $\alpha_v\beta_3$ integrin in osteosarcoma for metastasis has been suggested (13,15,17). In the present study, we showed that the metastatic potential and expression of $\alpha_v\beta_3$ integrin in 143B-LM4 cells were both significantly increased compared to 143B parental cells. These results suggest that $\alpha_v\beta_3$ integrin expression plays an important role in metastasis. With this highly metastatic variant overexpressing $\alpha_v\beta_3$ integrin, it will now be possible to further investigate the mechanism by which $\alpha_v\beta_3$ integrin facilitates and/or enhances metastasis.

Conflict of Interest

None of the Authors have any conflict of interest in regard to this study.

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