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The $\alpha_2\beta_1$ integrin is a metastasis suppressor in mouse models and human cancer

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Integrins regulate cell-cell and cell-matrix adhesion and thereby play critical roles in tumor progression and metastasis. Although work in preclinical models suggests that β_1 integrins may stimulate metastasis of a number of cancers, expression of the β_1 subunit alone has not been shown to be a useful prognostic indicator in human cancer patients. Here we have demonstrated that the $\alpha_2\beta_1$ integrin suppresses metastasis in a clinically relevant spontaneous mouse model of breast cancer. These data are consistent with previous studies indicating high expression of $\alpha_2\beta_1$ integrin in normal breast epithelium and loss of $\alpha_2\beta_1$ in poorly differentiated breast cancer. They are also consistent with our systematic analysis of microarray databases of human breast and prostate cancer, which revealed that decreased expression of the gene encoding α_2 integrin, but not genes encoding α_1, α_3, or β_1 integrin, was predictive of metastatic dissemination and decreased survival. The predictive value of α_2 expression persisted within both good-risk and poor-risk cohorts defined by estrogen receptor and lymph node status. Thus, the $\alpha_2\beta_1$ integrin functionally inhibits breast tumor metastasis, and α_2 expression may serve as an important biomarker of metastatic potential and patient survival.

Introduction

Cancer progression involves fundamental alterations of cell-cell and cell-matrix interactions and the cues derived from them that contribute to the invasive and metastatic phenotype. Death from cancer is usually caused by metastatic disease rather than the primary tumor. Tumor metastasis is not a single event but requires a series of steps that allow tumor cells to detach and invade through the basement membrane (1, 2). The detached cells then intravasate into the circulation via blood vessels or lymphatics where they are transported throughout the body, arrest, and survive (3).

Hynes first argued that defects in cell surface adhesive receptors, in addition to altered expression of matrix components in the microenvironment, could lead to the altered cell behavior that typifies cancer progression and metastasis (4–7). As important mediators of cell-adhesive behavior, integrins play a critical role in tumor progression and metastasis. Based on these observations, integrins have become attractive therapeutic targets (8–13). A selective inhibitor targeting the ligand-binding RGD sequence within the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins is in phase II and phase III clinical trials for glioblastoma multiforme and lung and breast cancer. These α_v integrins are expressed both by some tumor cells and by the tumor-associated neovasculature and therefore have an impact on tumor growth and progression in 2 ways. Not only does the inhibitor suppress tumor growth and invasion, but it inhibits tumor angiogenesis.

Much attention has been devoted to the β_1 family of integrins. Early work by Weaver et al. demonstrated that inhibition of β_1 integrin ligation produced reversion of the malignant phenotype of a human breast tumor cell line to a normal, growth-arrested phenotype (9). Work using numerous in vitro and in vivo orthotopic or xenograft models indicated that the β_1 integrins mediate drug resistance and stimulate metastasis in gastric, ovarian, and lung cancer (14, 15). Similarly, animals with targeted dele-

tion of the β_1 gene in mammary epithelium failed to develop cancer, suggesting that the β_1 integrins are essential for cancer initiation (16). Strategies to target the β_1 integrin subunit for therapy have not progressed rapidly, in part due to the heterogeneity of the β_1 integrin family.

The β_1 integrin subunit pairs with numerous α subunits to produce heterodimers that mediate adhesion to a wide variety of ligands including matrix molecules (collagen, laminin, fibronectin, etc.) and cell surface proteins such as E-cadherin and vascular cell adhesion molecule (6). Despite the impressive experimental evidence described above demonstrating that collectively the family of β_1 integrins is essential for tumor initiation and progression, a well-defined role for any single $\alpha\beta$-heterodimer in tumor initiation and cancer metastasis is not known.

The $\alpha_2\beta_1$ integrin, a receptor for collagen and other matrix molecules, is highly expressed on normal breast epithelium (17). Early studies provided the initial correlative suggestion that loss of $\alpha_2\beta_1$ integrin expression might play an important role in cancer progression (18). Alternatively, loss of $\alpha_2\beta_1$ integrin expression may simply be a consequence of malignant progression. Other studies, however, using cell lines either in vivo or in vitro, suggest that the $\alpha_2\beta_1$ integrin may serve to enhance metastasis to different organs (19–22).

The results of in vitro and in vivo studies of various designs may be subject to conflicting interpretation. Therefore, we have explored the role of the $\alpha_2\beta_1$ integrin in cancer initiation and progression using a clinically relevant, spontaneous mouse model, the mouse mammary tumor virus–*Neu* (MMTV-Neu) model of breast cancer progression and metastasis. Furthermore, we have validated and extended the conclusions of our model system with a detailed analysis of α_2 integrin gene expression and its significance in human breast and prostate cancer. We demonstrate that the $\alpha_2\beta_1$ integrin is a metastasis suppressor of breast cancer, and we identify key mechanistic steps in the metastatic cascade enhanced by loss of $\alpha_2\beta_1$ integrin expression.
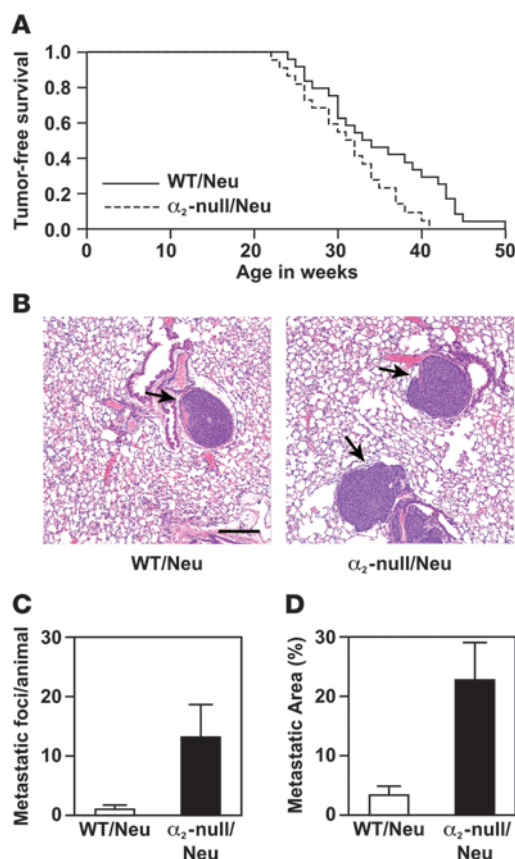
Figure 1
Loss of the $\alpha_2\beta_1$ integrin promotes breast cancer metastasis in vivo. (**A**) Kaplan-Meier plots of tumor-free survival in WT/Neu and α_2-null/Neu mice. Tumor development was recorded when a tumor nodule reached 1 cm³. Tumor latency (time to tumor development) was significantly shorter in the α_2-null/Neu relative to WT/Neu mice ($P = 0.02$). (**B**) Representative tissue sections of the lung stained with H&E demonstrate metastatic foci in WT/Neu and α_2-null/Neu mice. Arrows (→) denote metastatic foci in the lung. Scale bar: 100 μm. (**C**) The number of metastatic foci per lung was significantly increased in the α_2-null/Neu ($n = 15$) mice compared with the WT/Neu ($n = 10$) mice, at the time of sacrifice ($P = 0.02$). (**D**) The percentage of lung area occupied by metastatic tumor was significantly greater in the α_2-null/Neu ($n = 15$) mice than in the WT/Neu ($n = 10$) mice at the time of sacrifice ($P = 0.02$). Error bars represent SEMs.

Results

Loss of the $\alpha_2\beta_1$ integrin increases breast cancer metastasis in vivo. To determine the role of the $\alpha_2\beta_1$ integrin in breast cancer initiation, progression, and metastasis, we crossed the $\alpha_2\beta_1$ integrin–deficient mouse with transgenic animals carrying the MMTV-*c-erbB2/*Neu oncogene to generate WT/MMTV-*Neu* (WT/Neu) and α_2-null/MMTV-*Neu* (α_2-null/Neu) animals (23–25). Tumor latency was significantly reduced in α_2-null/Neu mice when compared with WT/Neu animals; however, the difference in latency was quite small ($P = 0.02$, Figure 1A). Tumors were first identified at 22 weeks of age in the α_2-null/Neu mice ($n = 22$) and at 24 weeks of age in the WT/Neu ($n = 24$). The median time to palpable tumor development was 31 and 33 weeks in α_2-null/Neu and WT/Neu mice, respectively. The hazard ratio for developing tumors in the α_2-null/Neu animals relative to WT/Neu mice was 2.09 (95% confidence interval 1.1–4.0).

Spontaneous metastasis to the lungs was evaluated when the tumors reached 10% of the body weight of the animal; therefore, tumor metastasis was evaluated when the tumors in α_2-null/Neu and WT/Neu mice were of similar size. The number and size of metastatic foci in the lungs were significantly increased in the α_2-null/Neu mice (Figure 1B). The metastatic lesions remained within the vasculature of both α_2-null/Neu and WT/Neu lungs and did not extravasate, a feature characteristic of the MMTV-Neu mouse model of tumor metastasis. As expected from previous studies, 60% of the WT/Neu mice ($n = 10$) developed grossly visible or microscopic metastasis. In contrast, 80% of the α_2-null/Neu animals ($n = 15$) developed metastases in the same time frame. Animals lacking the $\alpha_2\beta_1$ integrin developed a significantly greater number

of metastatic nodules per animal ($P = 0.02$), and the metastatic foci were larger in the α_2-null/Neu animals compared with the WT/Neu mice ($P = 0.02$) (Figure 1, C and D). These data demonstrate that the $\alpha_2\beta_1$ integrin plays a critical role not only in regulating tumor latency but also in determining metastatic tumor cell dissemination to the lung (26).
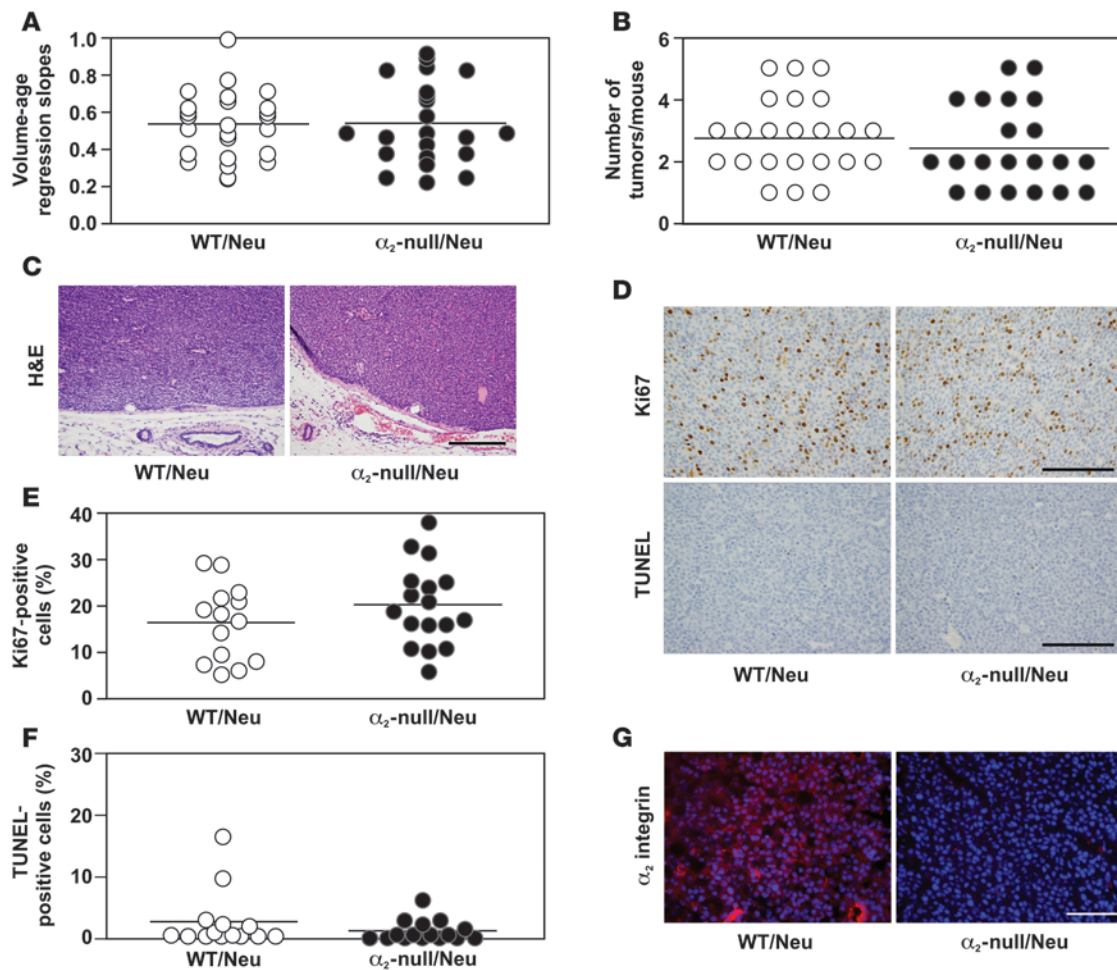
The increased rate of metastasis in α_2-null/Neu mice was not a result of increased primary tumor growth because the rates of growth of primary tumors spontaneously arising in WT/Neu and α_2-null/Neu animals were similar (Figure 2A and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI42328DS1). Thus, the increased rate of metastasis was not a result of primary tumor growth. The number of tumors per animal also did not differ significantly between the 2 genotypes (Figure 2B). Consistent with the similar growth kinetics of tumors in the WT/Neu and α_2-null/Neu mice, histologic and immunohistochemical analyses of the primary tumors demonstrated similar morphology (Figure 2C), comparable rates of proliferation and apoptosis, as revealed by the number of Ki67 (Figure 2, D and E) and TUNEL-positive (Figure 2, D and F) cells, and insignificant differences in collagen deposition (Supplemental Figure 2A and Supplemental Table 1). In addition, recruitment of B cells, T cells, macrophages, and granulocytes to WT/Neu and α_2-null/Neu tumors was similar (Supplemental Figure 2B). As expected, the tumors expressed the $\alpha_2\beta_1$ integrin in WT/Neu mice, but not in α_2-null/Neu mice (Figure 2G). Expression of the α_1, α_4, α_5, α_6, β_3, and β_4 integrin subunits by WT/Neu and α_2-null/Neu tumor cells was similar (Supplemental Figure 2C and Supplemental Table 2). Together these data demonstrate that the loss of $\alpha_2\beta_1$ integrin increases the rate of breast cancer metastasis without altering tumor growth, collagen deposition, or the immune response in vivo.

Loss of $\alpha_2\beta_1$ integrin expression results in increased intravasation. Since the in vivo data suggested that $\alpha_2\beta_1$ integrin expression regulates one or more steps in tumor metastasis, we next focused on tumor cell intravasation. To determine the ability of tumor cells from WT/Neu and α_2-null/Neu to intravasate, blood was collected by cardiac puncture at the time of sacrifice. The colony-forming ability of tumor cells in the cellular fraction of 1 ml of blood was analyzed ex vivo (Figure 3, A and B) (27). As shown in Figure 3, A and B, the number of tumor colonies generated from the blood of α_2-null/Neu mice ($18^{+/-}$ colonies) was significantly greater than from the blood of WT/Neu mice ($5^{+/-}$ colonies) ($P = 0.002$). The increased number of viable, circulating tumor cells correlated with the number of lung metastases.
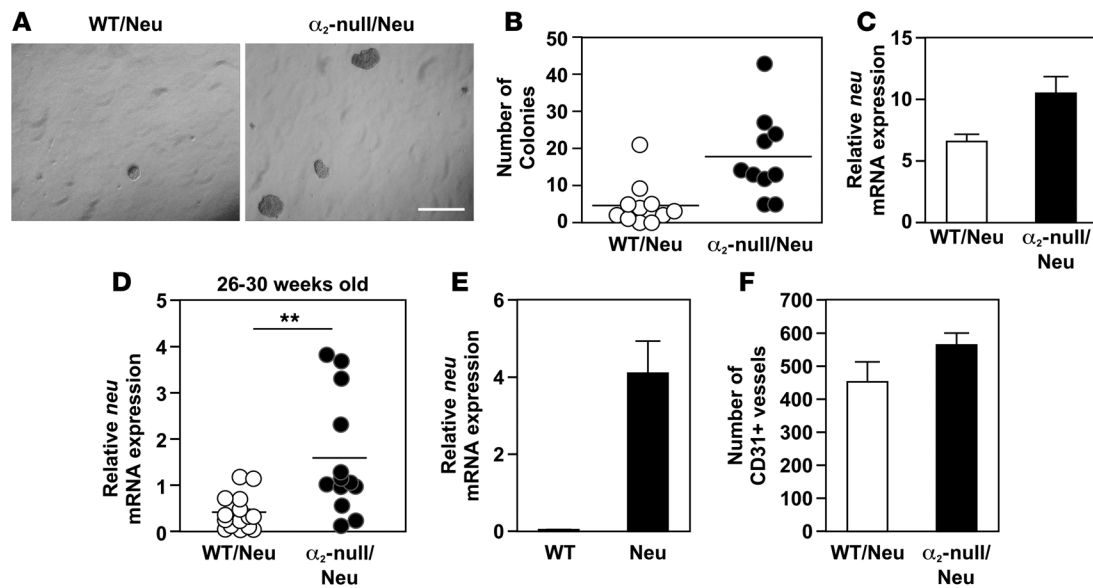
Figure 2
$\alpha_2\beta_1$ integrin expression does not alter tumor growth in vivo. (**A**) The rate of tumor growth over time was calculated from the regression slopes of tumor volume as a function of time, measured by calipers once a week ($P > 0.86$). (**B**) Total tumor burden in WT/Neu and α_2-null/Neu mice was quantitated as the total number of tumors per mouse at the time of sacrifice and ranged from 1 to 5 tumors per animal. No significant difference between the 2 genotypes was observed for either tumor growth rates ($P > 0.86$) or number of tumors per mouse ($P = 0.3$). (**C**) Representative H&E-stained sections of primary mammary tumors from WT/Neu and α_2-null/Neu animals. Scale bar: 200 μm. (**D**) Representative sections of primary WT/Neu and α_2-null/Neu mammary tumors were analyzed by immunohistochemistry for Ki67 or TUNEL staining to define proliferation or apoptosis, respectively. Scale bar: 100 μm. (**E** and **F**) Number of Ki67-positive (**E**) or TUNEL-positive (**F**) cells in WT/Neu and α_2-null/Neu primary mammary tumors was quantitated in 10 high-power fields. There was no significant difference between the cohorts for either Ki67-positive ($P > 0.27$) or TUNEL-positive ($P > 0.32$) cells. (**G**) Immunofluorescence analysis demonstrated $\alpha_2\beta_1$ integrin expression (red) by tumors from WT/Neu animals, but not α_2-null/Neu mice. Nuclei are stained with DAPI (blue). Scale bar: 60 μm.

Increased colony growth ex vivo may result from increased numbers of viable, circulating tumor cells or from an increased ability of isolated tumor cells to proliferate ex vivo. To differentiate between these possibilities, we determined the relative number of circulating, *Neu* mRNA–positive cells in the blood by quantitative real-time PCR (qRT-PCR). Figure 3C shows the ratio of circulating Neu-expressing cells relative to GAPDH in the blood at the time of sacrifice when the primary tumors in the WT/Neu and α_2-null/Neu mice were of similar size; α_2-null/Neu mice had a significantly larger proportion of these cells than WT/Neu mice ($P = 0.005$). Since c-ErbB2/Her2 is expressed in normal hematopoietic cells, the number of circulating tumor cells was also characterized by qRT-PCR for cytokeratin 19 (28). As shown in Supplemental Figure 3, increased numbers of circulating, cytokeratin 19–expressing cells were identified in the peripheral blood

of tumor-bearing, α_2-null/Neu in comparison with WT/Neu mice at the time of sacrifice. These results imply that the greater number of tumor colonies arising from blood of α_2-null/Neu mice was due to increased numbers of circulating tumor cells in these mice. At the time of sacrifice, when the tumors were of similar size, tumor cell dissemination was significantly increased in mice lacking the $\alpha_2\beta_1$ integrin.

We hypothesized that differences in tumor cell dissemination occurred not only at the time of sacrifice but also at earlier time points in α_2-null/Neu mice. To determine the time course of tumor cell dissemination, the number of circulating tumor cells was quantitated at approximately 10 to 11 weeks of age and every other week thereafter. There was no difference between the genotypes in the proportion of Neu-positive cells between 10 and 25 weeks of age, a time prior to the development of palpable

Figure 3
$\alpha_2\beta_1$ integrin expression inhibits tumor cell intravasation. (**A**) Ex vivo colonies were derived from circulating tumor cells within the peripheral blood of WT/Neu and α_2-null/Neu mice at the time of sacrifice. Scale bar: 100 μm. (**B**) The number of colonies arising from the blood after 12 days of ex vivo culture is depicted. The mean number (denoted by the black line) of ex vivo colonies was significantly greater in α_2-null/Neu mice than in WT/Neu mice. $P = 0.002$. (**C**) The relative ratio of *Neu* mRNA to *GAPDH* mRNA in the peripheral blood at the time of sacrifice was determined by qRT-PCR. The level of *Neu* mRNA–expressing cells was significantly increased in the peripheral blood of α_2-null/Neu mice ($n = 12$) compared with WT/Neu mice ($n = 12$) ($P = 0.005$). (**D**) The relative level of *Neu* mRNA to *GAPDH* mRNA in the peripheral blood of WT/Neu and α_2-null/Neu mice at 26–30 weeks of age when the tumors were first palpable. The α_2-null/Neu mice demonstrated significantly increased numbers of circulating cells relative to WT/Neu mice ($P = 0.002$). (**E**) The relative number of *Neu* mRNA–expressing tumor cells in the blood of WT nontransgenic control mice and WT/Neu mice was evaluated. Mice lacking the transgene failed to show evidence of circulating *Neu* mRNA ($P = 0.0003$). (**F**) The number of CD31-positive vessels in 10 high-power fields was quantitated. There was no difference in the vascular density within the tumors of WT/Neu ($n = 5$) and α_2-null/Neu ($n = 5$) animals ($P = 0.55$). Error bars represent SEMs in **C**, **E**, and **F**.

tumors (data not shown). However, between 26 and 30 weeks of age, the time that tumors first became palpable in both the WT/Neu and α_2-null/Neu mice, the number of circulating Neu-positive tumor cells was significantly increased in α_2-null/Neu mice relative to WT/Neu mice (Figure 3D) ($P = 0.002$). These results demonstrate that not only was tumor cell dissemination significantly enhanced in the absence of the $\alpha_2\beta_1$ integrin, but that tumor cell intravasation occurred at an earlier time point in α_2-null/Neu mice. Although we cannot exclude a contribution of enhanced tumor cell survival, the data support the hypothesis that enhanced metastasis in the α_2-null/Neu mice is, in part, due to increased tumor cell intravasation.

The level of circulating Neu-positive tumor cells was elevated in both WT/Neu and α_2-null/Neu mice. The large number of Neu-expressing cells in the circulation of both young WT/Neu and α_2-null/Neu mice was unexpected. Therefore, the level of detectable circulating Neu-expressing cells in nontransgenic littermates was determined. In comparison with WT/Neu mice, there was no detectable *Neu* mRNA in the circulation of nontransgenic animals, an important negative control (Figure 3E).

Since the $\alpha_2\beta_1$ integrin is expressed on activated endothelial cells and lack of the $\alpha_2\beta_1$ integrin can alter tumor angiogenesis, enhanced intravasation could be due to changes in the host tumor vessels. To examine this possibility, expression of CD31-positive vessels in WT/Neu and α_2-null/Neu tumors was evaluated. As shown in Figure 3F and Supplemental Figure 4, there was no difference in vascular density between the 2 subgroups.

Tumor cell expression of $\alpha_2\beta_1$ integrin does not impact extravasation or colonization in the lung. To further evaluate the contribution of enhanced tumor cell intravasation to the metastatic phenotype observed in vivo, we employed the classic experimental metastasis model, tail vein injection. This model examines the later stages of the metastatic cascade. Primary neoplastic cells were isolated from the tumors of WT/Neu and α_2-null/Neu mice and reinjected i.v. into the tail veins of either WT/Neu or α_2-null/Neu animals, thereby bypassing the intravasation step. The lungs were evaluated histologically and the number of metastatic foci within the lungs was quantitated. As shown in Figure 4, A and B, there was no difference in the percentage of WT animals that developed metastases ($P = 0.34$) and no difference in the number of metastases ($P = 0.35$) per animal when WT mice were injected with WT/Neu or α_2-null/Neu tumor cells. Similarly, when the α_2-null animals were used as the host, there was also no difference in the percentage of α_2-null animals that developed metastases ($P = 0.72$) or number of metastatic foci ($P = 0.85$) (Figure 4, C and D). These data from the i.v. tail vein injection model indicate that the later stages of the metastatic cascade are not altered by tumor cell expression of the $\alpha_2\beta_1$ integrin and that there is no difference in the ability of the α_2-null/Neu cells to colonize the lung. Extravasation into the lung is not evaluable in the MMTV-*Neu* mouse model. These data provide additional support that the $\alpha_2\beta_1$ integrin regulates tumor metastasis at the step of tumor cell intravasation.

$\alpha_2\beta_1$ integrin expression inhibits anchorage-independent growth and transendothelial cell intravasation in vitro. Intravasation requires tumor cells to migrate and invade through the basement membrane and into
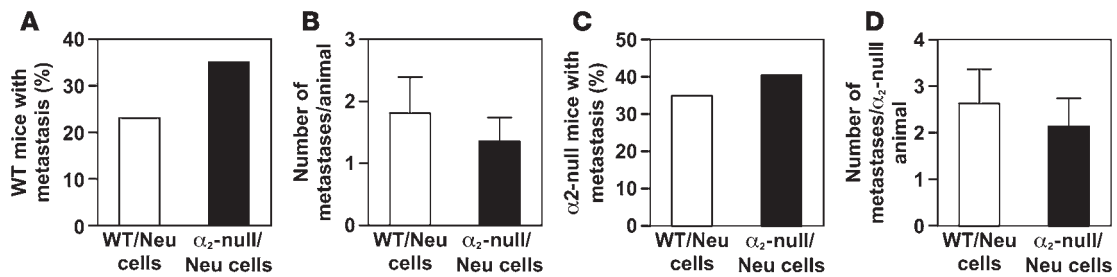
Figure 4
$\alpha_2\beta_1$ integrin expression does not regulate later stages of tumor metastasis. Equal numbers of primary, WT/Neu, or α_2-null/Neu tumor cells were injected i.v. via the tail vein into WT, nontransgenic animals (**A** and **B**) or α_2-null, nontransgenic mice (**C** and **D**). Similar numbers of WT animals injected with either WT/Neu tumor cells ($n = 23$) or α_2-null/Neu tumor cells ($n = 20$) developed metastatic disease ($P = 0.34$) (**A**). The number of metastatic foci per animal following i.v. tail vein injection of WT/Neu or α_2-null/Neu tumor cells was also similar ($P = 0.35$) (**B**). There was also no significant difference in the percentage of animals developing metastases ($P = 0.72$) or the number of metastatic foci ($P = 0.85$) when primary, WT/Neu ($n = 23$), or α_2-null/Neu ($n = 20$) tumor cells were injected into α_2-null, nontransgenic mice. Error bars represent SEM.

the vasculature. To compare the malignant behavior of α_2-null/ Neu and WT/Neu tumor cells, primary tumor cells were isolated. In monolayer culture, primary WT/Neu tumor cells exhibited an epithelial morphology with cobblestone-appearing colonies and cell-cell junctions. In contrast, the α_2-null/Neu tumor cells were mesenchymal in shape and failed to cluster into epithelioid colonies (Figure 5A). To determine whether cells lacking $\alpha_2\beta_1$ integrin expression were more motile, migration of WT/Neu cells and α_2-null/Neu cell was evaluated in an in vitro wound closure assay. The α_2-null/Neu tumor cells migrated more rapidly than the WT/Neu cells (Figure 5, B and C, and Supplemental Figure 5). The mesenchymal morphology of the α_2-null/Neu cells was apparent in migrating cells.

Morphogenesis of primary, WT/Neu, and α_2-null/Neu tumor cells was evaluated in 3D gels of reconstituted basement membrane (Matrigel). In this system, nontransformed and Neu-transformed mammary epithelial cells undergo rapid reorganization into acinar structures that recapitulate mammary morphogenesis in vivo (29). Primary tumor cells from WT/Neu animals formed typical acinar structures with small, well-circumscribed cell aggregates, as previously described by several investigators. In contrast, the tumor cells from the α_2-null/Neu mice formed large, disorganized, and irregular-shaped acini with protrusions that extended into the surrounding matrix. Such protrusions were entirely lacking from WT/Neu acini (Figure 5D).

The α_2-null/Neu acini also appeared slightly larger than the WT/Neu acini. Malignant potential is associated with enhanced proliferation under conditions of both anchorage dependence and anchorage independence. Proliferation of WT/Neu and α_2-null/ Neu tumor cells was not different when tumor cells were plated on plastic in either serum-free or complete media (Supplemental Figure 6). The α_2-null/Neu tumor cells demonstrated a decreased proliferative potential when plated on type I collagen.

To assess anchorage-independent growth, the ability of primary WT/Neu and α_2-null/Neu tumor cells to form colonies in soft agar was determined. After 3 weeks in culture, the number of colonies equal to or larger than 50 mm was enumerated. Tumor cells from α_2-null/Neu mice generated significantly greater numbers of colonies than tumor cells isolated from WT/Neu mice (Figure 5E). Therefore, although there was no difference in tumor growth in vivo (Figure 2), tumor cells lacking the $\alpha_2\beta_1$ integrin demonstrate enhanced anchorage-independent, but not anchorage-dependent, growth in vitro.

In order to disseminate into the blood, tumor cells must transit across the endothelial cell barrier. We compared the ability of α_2-null/ Neu and WT/Neu tumor cells to intravasate in vitro by migrating across a barrier of WT endothelial cells in a Transwell chamber. The α_2-null/Neu cells more effectively intravasated through the endothelial cell barrier than WT/Neu cells. The number of α_2-null/ Neu cells that successfully intravasated through the endothelial cell barrier was significantly increased in comparison with the number of WT/Neu tumor cells ($P = 0.0003$) (Figure 5, F and G). Therefore, lack of $\alpha_2\beta_1$ integrin expression may contribute to the metastatic phenotype by potentially 2 separate mechanisms. Although the α_2-null/Neu tumors did not demonstrate a growth advantage in vivo, enhanced anchorage-independent growth in vitro raises the possibility that the tumor cells survive in the circulation or once lodged in the lung vasculature, leading to enhanced metastasis. In contrast, increased α_2-null/Neu tumor cell intravasation both in vitro and in vivo is well supported by the data.
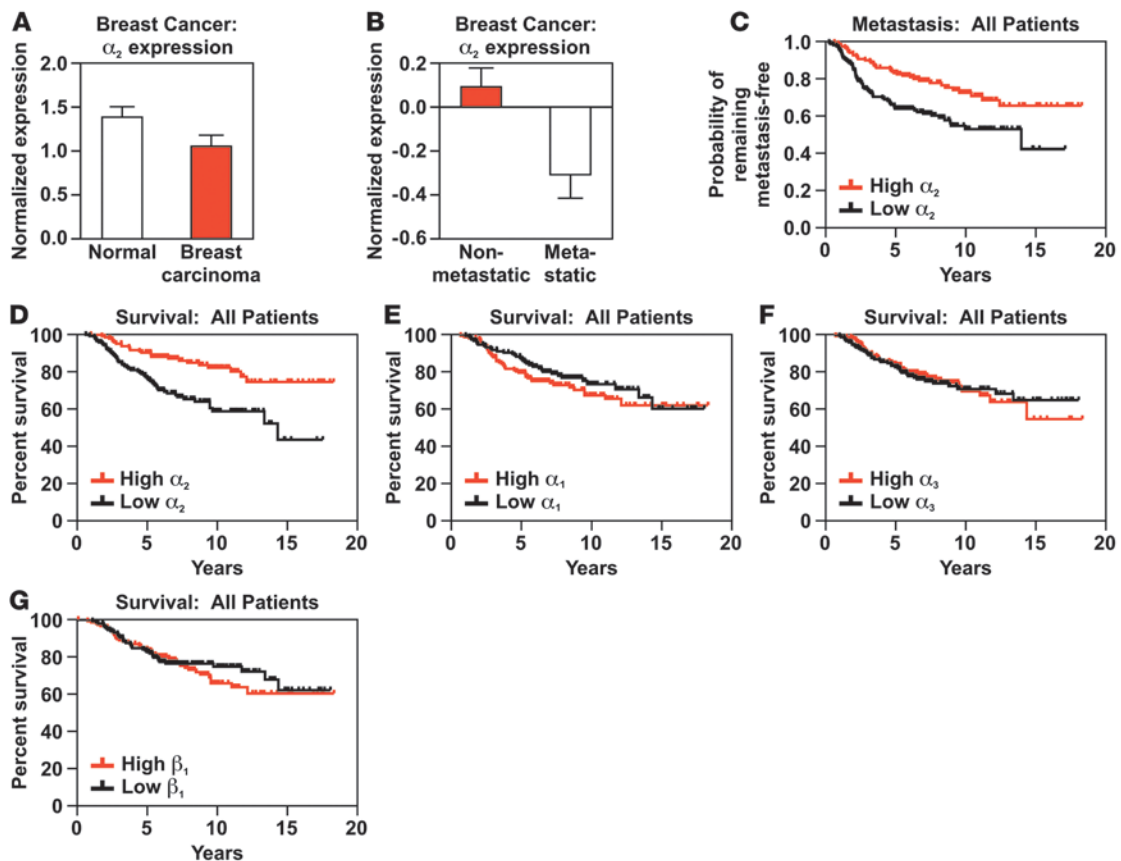
Loss of $\alpha_2\beta_1$ integrin expression predicts metastasis and decreased survival in human breast and prostate cancer. To validate our findings from the murine breast cancer model and to extend the relevance to human breast cancer, publicly accessible microarray data were analyzed for a correlation between α_2 integrin gene expression and clinically relevant parameters. Expression of the α_2 integrin was significantly reduced in breast cancer versus normal breast tissue in the Richardson cohort ($P = 0.038$; Figure 6A) (30). Not only was the loss of α_2 integrin gene expression highly associated with malignant disease, the decrement in α_2 integrin expression consistently correlated with disease progression in 2 different public data sets for which stage-specific data were available ($P \leq 0.01$) (31, 32). All data sets are shown in Supplemental Table 3.

To determine whether the loss of α_2 integrin expression was an indicator of prognosis, the correlation between α_2 integrin expression, metastasis, and patient survival was analyzed in publicly accessible breast cancer cohort NKI-295 organized by van de Vijver (33). The expression of α_2 integrin was greatly reduced in the patients with distant metastasis when compared with patients without distant metastases ($P < 0.0084$; Figure 6B). To further evaluate its predictive value, the correlation between α_2 integrin expression, metastasis-free survival, and overall survival was determined using log-rank analysis. The expression of α_2 integrin correlated significantly with improved metastasis-free survival ($P = 0.0022$) and overall patient survival ($P < 0.0001$) as depicted in Kaplan-
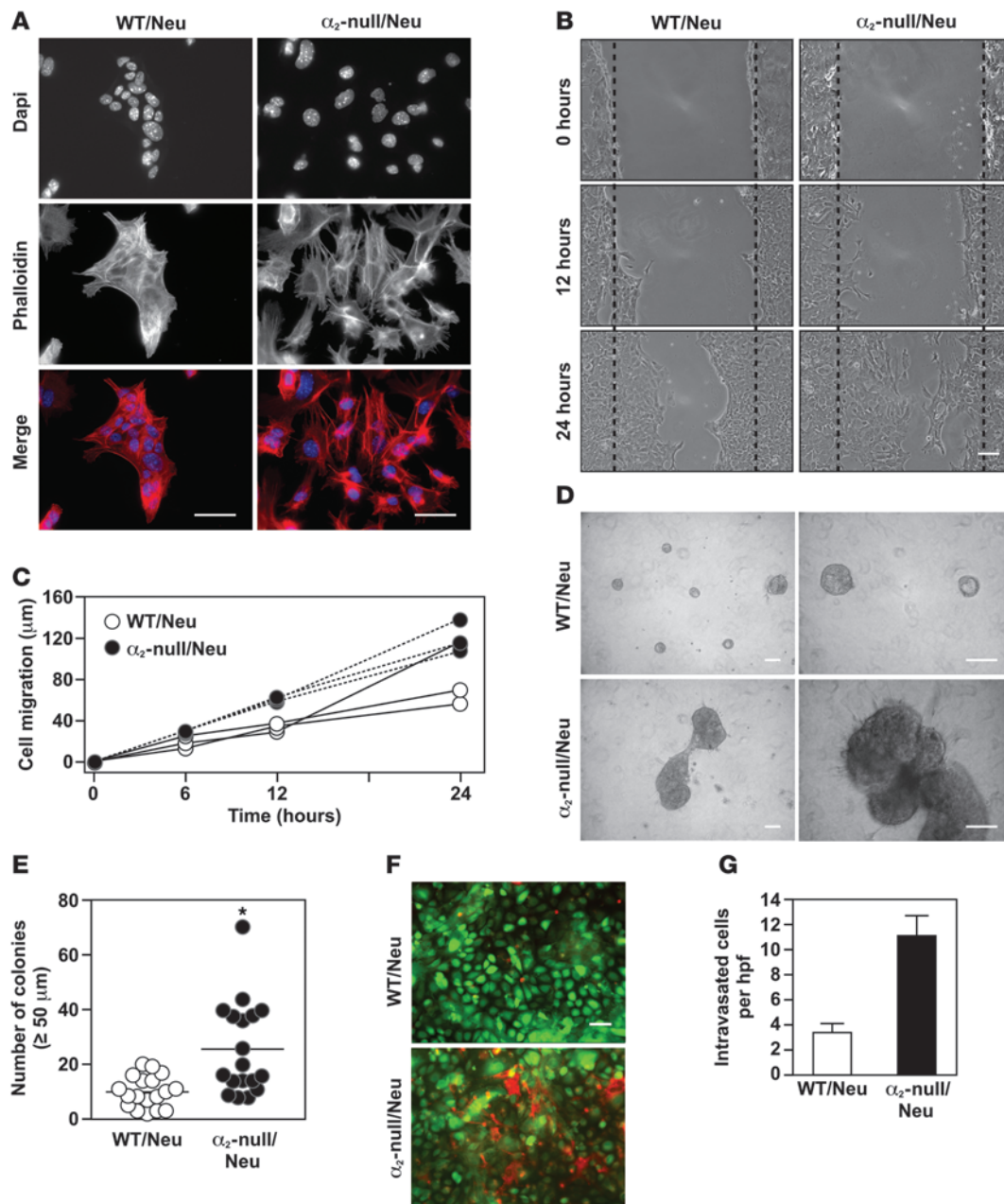
Figure 5

$\alpha_2\beta_1$ integrin expression inhibits migration, intravasation, and anchorage-independent growth in vitro. (**A**) Primary WT/Neu or α_2-null/Neu tumor cells stained with rhodamine-phalloidin (red) and DAPI (blue) were examined by immunofluorescence microscopy. Primary WT/Neu tumor cells exhibit an epithelial morphology with cobblestone-appearing colonies. In contrast, the α_2-null/Neu tumor cells are mesenchymal in shape. Scale bar: 100 μm. (**B** and **C**) α_2-null/Neu tumor cells exhibited significantly enhanced migration at 12 and 24 hours in a scratch assay. The straight lines in part **C** connect data points on cells from the same mouse. The rate of cell migration was significantly greater in cells from α_2-null/Neu mice than WT/Neu mice ($P = 0.05$). (**D**) Morphogenesis of WT/Neu and α_2-null/Neu tumor cells into 3D matrigel cultures. Scale bar: 100 μm. (**E**) Anchorage-independent colony assays were conducted in soft-agar. α_2-null/Neu tumor cells generated significantly greater numbers of colonies than WT/Neu tumor cells. Each point represents the number of colonies 50 μm or greater in diameter generated from 10,000 primary tumor cells from an individual mouse. (**F** and **G**) α_2-null/Neu tumor cells exhibit enhanced intravasation in comparison with WT/Neu tumor cells in vitro through a barrier of WT endothelial cells. Primary WT endothelial cells, stimulated with TNF, were labeled with CellTracer Vybrant CFDA SE (green) and seeded onto the bottom of a Transwell filter. The ability of WT/Neu or α_2-null/Neu tumor cells, labeled with CellTracker Red CMTPX (red), to intravasate through the endothelial barrier in 8 hours was determined. The number of intravasated (red) cells was quantitated as mean $\pm$ SEM of 3 separate experiments carried out with primary cells from 3 different animals ($P < 0.0003$ by Mann-Whitney analysis).

Figure 6
Loss of $\alpha_2\beta_1$ integrin expression predicts metastasis and decreased survival in breast cancer patients. (**A**) Using the publicly available data from the Richardson cohort (30), expression of the α_2 integrin was significantly decreased in breast carcinomas ($n = 40$) compared with normal breast tissue ($n = 7$) ($P = 0.038$). (**B–G**) Analysis of the publicly available NKI-295 cohort was used to correlate integrin expression with metastasis (**B** and **C**) and patient survival (**D–G**). The α_2 integrin expression was substantially reduced in patients with metastases ($n = 101$) when compared with nonmetastatic patients ($n = 194$, $P = 0.0038$) (**B**). In log-rank analyses, high-level expression of the α_2 integrin correlated with the probability of both remaining metastasis-free (**C**, $P = 0.0022$) and with improved long-term survival (**D**, $P < 0.0001$). In contrast, expression of the α_1 (**E**, $P = 0.2639$), $\alpha3$ (**F**, $P = 0.9509$), and β_1 (**G**, $P = 0.5$) integrin subunits did not correlate with patient survival.

Meier plots of 295 patients divided evenly according to high-versus-low α_2 integrin expression (Figure 6, C and D). Further subdivision of the cohort into terciles demonstrated that increasing α_2 integrin expression is strongly associated with improved patient survival (log-rank test for trend $P = 0.0008$; Supplemental Figure 7A). In contrast, expression of the integrin subunits α_1, α_3, and β_1 did not correlate with survival (Figure 6, E, F, and G; $P = 0.2639$, 0.9509, and 0.5, respectively).

Both lymph node–negative and lymph node–positive patients were included in the NKI-295 cohort. In accordance to the standard of care, the majority of node-positive patients had received systemic adjuvant treatment while node-negative patients had not. These 2 classes of patients were analyzed separately to evaluate any correlation with α_2 integrin expression. Elevated expression of the integrin subunit continued to correlate strongly with improved survival in both node-positive and node-negative patients (Figure 7, A and B; $P = 0.0049$ and 0.0084 respectively).

The ability of α_2 integrin expression to predict distant metastasis was further assessed in the context of estrogen receptor (ER) status. ER status has been particularly important in the treatment of breast cancer because ER-negative status corresponds with poor patient prognosis (34–36). In 15 independent studies in which ER status was available, decreased α_2 integrin expression correlated with decreased ER expression. The individual studies analyzed are outlined in Supplemental Table 3. A detailed survival analysis was performed on the NKI-295 patients. As shown in Figure 7, C and D, high and low α_2 integrin expression subdivided both the ER-positive patients and the high risk, ER-negative patients into prognostically distinct categories. High α_2 integrin expression was significantly associated with survival in the ER-positive patient cohort ($P = 0.001$). A similar difference in estimated survival between high and low α_2 expression was also observed in ER-negative patients. However, this difference did not achieve statistical significance ($P = 0.15$) due to the modest number of ER-negative women in these studies. In patients with known metastatic disease at presentation, survival was also shortened in patients with decreased α_2 integrin expression ($P = 0.004$; Figure 7E).

To validate the clinical findings from the NKI-295 cohort, the correlation between α_2 integrin expression and improved clinical performance of the patients was confirmed in a separate clinical investigation of 286 lymph node–negative patients with invasive breast carcinoma in the Wang cohort (37). Disease-free survival in
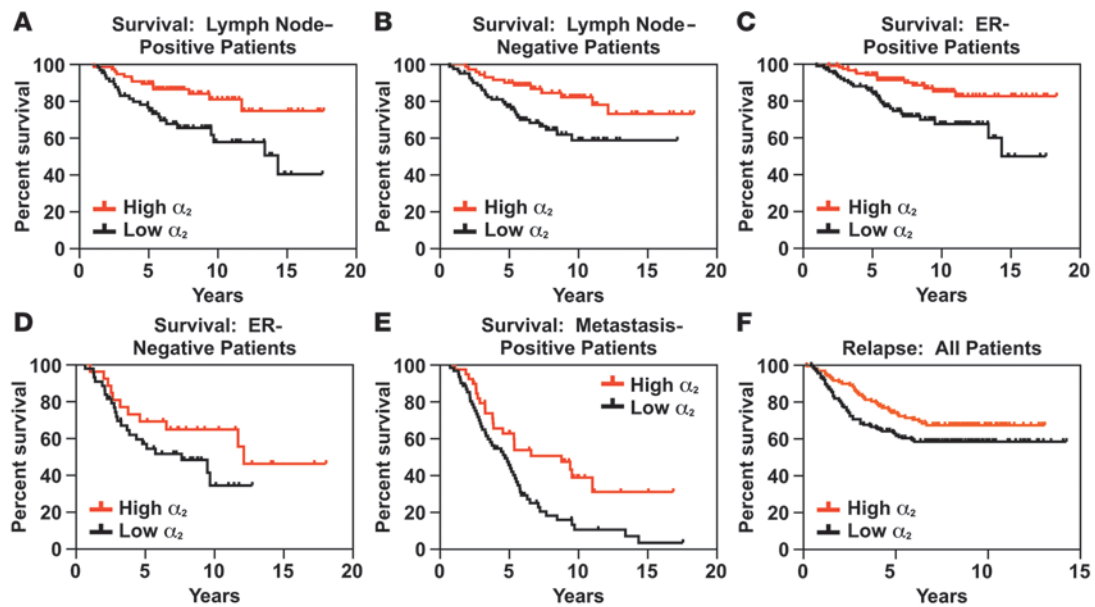
Figure 7
Expression of the $\alpha_2\beta_1$ integrin predicts metastasis and survival in high- and low-risk cohorts. (**A–D**). The clinical importance of $\alpha_2\beta_1$ integrin expression levels in good-risk or poor-risk subpopulations was determined using a log-rank analysis of the NKI-295 cohort. Expression of high levels of α_2 integrin correlated with decreased metastasis and increased survival in both high-risk and low-risk subgroups (**A**, lymph node positive, $P = 0.0049$; **B**, lymph node negative, $P = 0.0084$; **C**, ER+, $P = 0.001$; **D**, ER–, $P = 0.1482$). (**E**) For patients in this cohort with known metastatic disease at presentation, decreased survival significantly correlated with decreased α_2 integrin expression (**E**, $P = 0.004$). (**F**) To validate the correlation between α_2 integrin expression and outcome among lymph node–negative patients a separate publicly available microarray analysis of 286 breast cancer patients (Wang cohort) was analyzed. In this separate cohort, α_2 integrin expression correlated with increased disease-free survival in a second lymph node–negative cohort (**F**, $P = 0.0317$).

this lymph node–negative cohort was significantly decreased in patients with reduced α_2 integrin expression ($P = 0.0317$; Figure 7F). In addition, among all patients with metastatic disease, disease relapse involving brain metastasis occurred only in patients with reduced α_2 integrin expression ($P = 0.0049$; Supplemental Figure 7B). Overall survival information was not available for this study.

The strong clinical association between loss of α_2 integrin expression and breast cancer progression and metastasis suggested that α_2 integrin expression may be a metastasis suppressor in other models of cancer. To assess this possibility, we evaluated α_2 integrin expression in normal prostate, prostate intraepithelial neoplasia (PIN), prostate cancer, and prostate cancer metastasis. As shown in Figure 8, A and B, the α_2 integrin was expressed at high levels in normal prostate (38). The level of α_2 integrin gene expression was diminished in PIN and prostate cancer and was more greatly decreased in metastatic prostate cancer. These findings in a second model of human cancer further validate the role of the α_2 integrin as a metastasis suppressor in many subtypes of human cancer.

Discussion
The studies reported in this manuscript identify the $\alpha_2\beta_1$ integrin as the first integrin known to serve as a metastasis suppressor in breast cancer and prostate cancer. We found this to be the case in both a spontaneous, clinically relevant mouse model of breast cancer and in human breast cancer. In MMTV-*Neu* transgenic mice, lack of $\alpha_2\beta_1$ integrin expression resulted in modestly decreased mammary tumor latency and markedly increased cancer metastasis. Deletion of the $\alpha_2\beta_1$ integrin enhanced tumor cell intravasation both in vivo and in vitro. The increased tumor cell dissemi-

nation detected in the peripheral blood of the α_2-null/Neu, but not the WT/Neu mice, at both early time points when the tumors were just barely palpable and later when the primary tumors were of comparable size at sacrifice suggests that intravasation is a key step in the metastatic cascade where loss of integrin expression acts to enhance metastasis. Loss of the $\alpha_2\beta_1$ integrin does not affect tumor growth or tumor cell proliferation in vivo or in vitro. However, in vitro, the α_2-null/Neu tumor cells demonstrate enhanced anchorage-independent growth. We therefore propose that enhanced anchorage-independent growth and the slightly shorter latency period in mice lacking the α_2 subunit contribute to the metastatic phenotype. It was surprising that a difference in metastasis to the lung was not observed in the classic experimental metastasis model. Although we did not see a difference between the ability of WT and α_2-null tumor cells to colonize the lung, there was a slight trend in favor of colonization by the α_2-null tumor cells. However, our sample size was too small to show that this difference was significant. The primary tumor cells when reinjected were not highly metastatic, and increasing the number of injected cells was limited by animal viability.

The $\alpha_2\beta_1$ integrin was globally deleted in all cells of the α_2-null mouse, not just in the mammary gland. Therefore, although our in vitro and in vivo data strongly support the important role that $\alpha_2\beta_1$ integrin expression by the tumor cells plays in tumor metastasis, the possibility exists that $\alpha_2\beta_1$ integrin expression by cells of the tumor microenvironment, i.e., endothelial cells, fibroblasts, or inflammatory cells, and not just by the tumor cells themselves, also plays a role in inhibiting tumor metastasis in vivo. Although we cannot exclude the role of the $\alpha_2\beta_1$ integrin in the tumor microenviron-
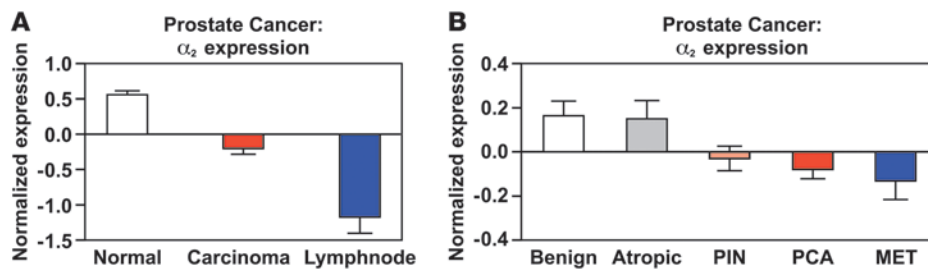
Figure 8
Expression of $\alpha_2\beta_1$ integrin expression correlates with prostate cancer progression and metastasis. The α_2 integrin was expressed at high levels in normal prostate. The level of α_2 integrin gene expression was progressively diminished in PIN, prostate cancer, and metastatic prostate cancer. Loss of α_2 integrin expression predicts for metastasis in patients with prostate cancer. (**A**) The Lapointe data set (59) demonstrated a significant reduction in α_2 integrin expression among normal prostate, prostate cancer, and lymph node metastases (ANOVA, $P = 0.0016$; linear trend, $P < 0.0001$). (**B**) Similar analysis of the Tomlins dataset (38) revealed the same correlation. The expression of α_2 integrin diminished as prostate cancer progressed (ANOVA, $P < 0.0001$; linear trend, $P < 0.0001$). Error bars represent SEM.

ment, our studies define the critically important role that $\alpha_2\beta_1$ integrin expression by the tumor cells plays in controlling tumor cell metastasis. However, the biologic studies and the human expression data focus on integrin expression or lack of integrin expression by the mammary epithelial cells. All in vitro experiments, including cell migration, epithelial morphogenesis, and tumor cell intravasation, solely define the importance of integrin expression by the tumor cells to the malignant phenotype. In addition, the in vivo classic metastasis model using primary tumor cells also supports the role of the integrin in modulating tumor cell intravasation. Finally, the extensive microarray analysis further supports the significance of $\alpha_2\beta_1$ integrin loss from the malignant tumor in tumor cell progression, metastasis, and patient outcome.

Previous studies by White et al. demonstrate that animals lacking the β_1 integrin due to targeted deletion of the gene in the mammary epithelium fail to develop cancer (14, 16). Since the β_1 integrin subunit forms heterodimers with at least 11 α subunits that mediate adhesion to multiple different matrix molecules and other cell adhesion receptors, it is not surprising that the suppressive phenotype of any individual heterodimer may be masked. Our finding that decreased α_2 integrin gene expression is associated with increased metastatic disease and decreased patient survival does not exclude additional specific roles for other heterodimers. However, expression of the α_1, α_3, or β_1 integrin genes did not correlate with metastasis or patient survival in the same cohort of breast cancer patients. Since integrins have become attractive targets for adjuvant therapy, understanding the influence of each specific heterodimer is critical to understanding the potential ramification of such therapy (14). Indeed, the biologic consequences of genetic deletion versus inhibition of a specific integrin leave a number of open questions regarding the applicability of anti–β_1 integrin therapy in breast cancer. More selective agents for specific heterodimers deemed biologically relevant in human disease may have greater efficacy.

In patients with breast cancer, α_2 integrin expression correlated with ER positivity. In both the ER-positive patient subgroup and the ER-negative cohort, α_2 integrin expression was associated with a survival advantage. Therefore, α_2 integrin expression is not simply a surrogate marker of ER expression. Although ER-negative breast cancer accounts for only 15%–20% of cases (34), this subgroup is very important clinically because these patients have a poorer prognosis and there are fewer therapeutic targets. Expression of the α_2 integrin subunit may serve as an important

biomarker and clinical determinant for future clinical trials in ER-negative breast cancer patients by identifying the subset likely to benefit from novel or more aggressive therapies.

The identification of the $\alpha_2\beta_1$ integrin as a metastasis suppressor opens up a large number of exciting and unanswered questions for future basic and clinical studies. It remains to be determined whether occupancy of the integrin with ligand is required for the metastasis suppressor effect. The potential role for the collagenous matrix in regulating tumor progression is an exciting avenue of current investigation (39–41). The mechanism by which α_2 integrin subunit gene expression is diminished during oncogenesis and tumor progression is unknown. The classic metastasis suppressor genes are lost due to genetic and epigenetic changes (3, 42–44). Loss of metastasis suppressors augments tumor cell procession through the metastatic cascade. In the mouse model, loss of $\alpha_2\beta_1$ integrin expression was accomplished by targeted gene deletion. In human disease, the mechanisms by which α_2 integrin subunit gene expression is decreased in cancer are unknown. The human α_2 integrin gene lies on chromosome 5q23, a site of frequent deletions in breast cancer (45). However, deletion of the α_2 integrin gene in cancer has not been reported. Genetic polymorphisms in the α_2 gene that potentially alter expression have been associated with prostate cancer susceptibility (46). Epigenetic modification of the α_2 gene promoter and regulation by microRNA are possibilities, since there is a progressive loss of expression in humans as tumor cells become less differentiated. Since reexpression of the α_2 subunit in patients with cancer may be beneficial, defining the mechanisms of α_2 gene silencing may lead to novel therapeutic strategies to restore gene expression in tumor cells.

Hunter and colleagues have suggested that constitutional genetic heterogeneity modulates tumor efficiency and determines those individuals at increased risk of metastasis once a primary cancer arises (47). It is well recognized that genetic polymorphisms within the α_2 gene regulate $\alpha_2\beta_1$ integrin expression by platelets and perhaps other cell types (reviewed in ref. 48). The variable number of $\alpha_2\beta_1$ integrin molecules in different individuals correlates with the ability of platelets to adhere to type I collagen (49–51). Individuals with low baseline levels of cellular $\alpha_2\beta_1$ integrin expression due to allelic differences in the α_2 integrin gene may be at increased risk for metastatic disease or earlier age of tumor onset.

In conclusion, we demonstrate that the $\alpha_2\beta_1$ integrin, which serves as a receptor for collagens, laminins, and other matrix and nonmatrix proteins, functions as a metastasis suppressor in part

by regulating tumor cell intravasation. The impact of deleting the α_2 integrin subunit, hence $\alpha_2\beta_1$ integrin heterodimer expression in cancer biology, is distinctly different and in some ways counter to the consequences of deleting the β_1 subunit and thus all the heterodimers associated with the β_1 integrins. We provide both experimental evidence and analysis of gene-profiling data that establish the contribution of the $\alpha_2\beta_1$ integrin in regulating tumor progression and the metastatic phenotype. Future studies will evaluate the genetic and epigenetic modifications of the gene that lead to loss of integrin expression in breast cancer. In addition, it will be critical to determine whether similar changes in $\alpha_2\beta_1$ integrin expression are seen in subtypes of cancer other than breast and prostate and whether they are predictive of metastasis and/or poor prognosis in other tumor types.

Methods

Mice. Animals were housed in pathogen-free conditions at Vanderbilt University Medical Center in compliance with IACUC regulations. All animal studies were approved by the Vanderbilt Institution Animal Care and Use Committee at Vanderbilt Medical Center. The $\alpha_2\beta_1$ integrin–deficient mice (52) were backcrossed 10 generations onto the FVB/N background to obtain animals that were 99% genetically FVB/N and crossed with transgenic mice carrying the *Neu* (*c-neu*) gene under the transcriptional control of the MMTV promoter, obtained from Jackson Laboratories (24, 25) to generate WT/Neu and α_2-null/Neu mice. Mice positive for the transgene were identified by PCR following Jackson Laboratories' recommendations. Female mice were examined after birth for tumor development by palpation. Once detected, primary tumors were measured weekly with digital calipers. Total tumor volume was calculated using the equation: volume = $a \times b^2 \times 0.52$, where a is the longest dimension and b is the shortest. Blood was collected every 2 weeks after 6 weeks of age by saphenous vein or at the time of sacrifice by cardiac puncture. Animals were sacrificed when total tumor volume reached 10% of their body weight. The lungs were perfused with formalin. Blood, tumor tissue, and lung were collected. Tumor nodules in lung were quantitated.

Histology, immunohistochemistry, and immunofluorescence. Tumor and lung morphology were evaluated on formalin-fixed, paraffin-embedded, H&E-stained sections. Immunohistochemical identification of Ki67, TUNEL, type IV collagen, B220, CD3, and F4/80 was performed on paraffin-embedded tissues with a biotin-conjugated secondary antibody and diaminobenzidine substrate (Vectastain ABC Kit; Vector Laboratories). The number of Ki67- and TUNEL-positive cells was determined quantitatively using the Metamorph Imaging System (Molecular Devices). Primary antibodies included CD45/B220 (BD Biosciences), Ki67 and collagen IV (Abcam), TUNEL assay (R&D Systems), CD3 (Santa Cruz Biotechnology Inc.), and F4/80 (Novus Biologicals). Immunofluorescence analysis was conducted on 7-μm frozen sections with the primary antibodies, anti-mouse CD31, anti-α_2 integrin subunit (BD Biosciences — Pharmingen), and secondary antibodies Alexa Fluor 594– or Alexa Fluor 488–conjugated IgG and DAPI, all from Molecular Probes. Quantification of the CD31$^+$ vessels was carried out using the Scion Image system. The number of CD31$^+$ structures in 5 high-power fields/tumor with 5 animals in each group were elucidated. Tumor cells were also analyzed by flow cytometry using the following antibodies (all from BD Biosciences): hamster anti-rat/mouse CD49a, APC anti-mouse CD49b, FITC anti-mouse CD49d, PE anti-mouse CD49e, and anti-mouse CD104. Additional antibodies included anti-mouse CD29 and PE anti-mouse CD61 from Pharmingen.

Cell culture. Primary WT/Neu and α_2-null/Neu tumor cells were isolated, as previously described (53), with modifications. Briefly, primary tumors were dissected under sterile conditions and incubated overnight at 4°C in growth media (DMEM/F12, 10% FBS, 20 ng/ml EGF, 0.5 μg/ml hydrocortisone, 0.1 μg/ml cholera toxin, 10 μg/ml insulin, 1× Pen/Strep solution) supplemented with 2 mg/ml collagenase type I, 100 units/ml hyaluronidase, and 50 μg/ml gentamicin. Minced tumors were digested at 37°C for 2 hours. After digestion, cells were centrifuged for 10 minutes at 500 g, followed by differential centrifugation (5 times for 45 seconds at 500 g). Cells were cultured in serum-free growth media and incubated at 37°C, 5% CO_2 for 3–4 days. The subsequent generations were cultured in growth media with 5% FBS. Primary tumor cells in 3D Matrigel cultures were established as previously published (29). For 3D gel culture, 10,000 cells were overlain on growth factor–reduced Matrigel (BD Biosciences) with complete growth media containing 2% Matrigel and incubated at 37°C.

Circulating tumor cells were cultured as described by Biswas et al. (27). 500 μl of blood supplemented with DMEM, 10% FBS, was added onto 6-well plates coated with growth factor-–reduced Matrigel and incubated at 37°C, 5% CO_2. The next day, wells were washed with lysis buffer (4.15 g NH_4Cl, 0.5 g $NaHCO_3$, 0.0186 g disodium EDTA in 200 ml water) and PBS. At 12 days, colonies of 50 μm or greater were counted.

Cell migration, intravasation, and anchorage-independent growth. WT/Neu or α_2-null/Neu primary tumor cells were cultured in 24-well plates with complete growth media until confluence. The cell monolayers were scratched with a P200 pipette tip and incubated with serum-free media at 37°C. Wound closure was quantitated by digital imaging using T-Scratch (54).

Intravasation assays were carried out with WT primary pulmonary endothelial cells, prepared as previously described (55). Endothelial cells (5×10^4) were seeded on the bottom side of a Transwell filter, precoated with growth factor–reduced Matrigel (1:20), and cultured in growth medium overnight. On day 2, primary WT/Neu or α_2-null/Neu tumor cells were labeled with CellTracker Red CMTPX (Invitrogen), and the endothelial cells were labeled with CellTracer Vybrant CFDA SE. Both tumor and endothelial cells were starved in DMEM medium with 1% FBS overnight. On day 3, the endothelial cells were stimulated with TNF-α (20 ng/ml) for 2 hours and then washed in DMEM medium with 1% FBS. WT/Neu or α_2-null/Neu primary tumor cells (5×10^4) in DMEM plus 1% FBS were placed on the upper membrane of the Transwell filter. DMEM with 10% FBS was placed in the bottom well. After 8 hours, all remaining tumor cells in the upper chamber of Transwell were removed. Endothelial cells and intravasated tumor cells on the lower surface of the Transwell were fixed with 4% paraformaldehyde. The membrane was analyzed and the number of intravasated tumor cells was quantitated using a Nikon Eclipse 80i fluorescence microscope.

For evaluation of anchorage-independent growth, 10,000 primary tumor cells were plated in 1 ml growth media with 0.4% agarose in 35-mm petri dishes coated with 0.8% agarose. Plates were incubated at 37°C and colonies 50 μm or greater were counted at 21 days of culture. All assays were carried out in triplicate.

qRT-PCR. Total RNA was extracted from blood using the RiboPure-Blood Kit (Ambion) following the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA using iScript cDNA Synthesis kit (Bio-Rad). qRT-PCR analysis was performed on an iCycler system (Bio-Rad) using mouse Neu (sense, CAGTGTCTTCCAGAACCTTCG; anti-sense, CGGTGAATGAGAGCCAATCC) or cytokeratin 19 (sense, CCCAGCT-CAGCATGAAAGC; anti-sense, CCTGGTTCTGGCGCTCTATG) cDNA primers. Number of circulating tumor cells expressing Neu or cytokeratin 19 mRNA was quantitated by external standard curve. Serial dilutions of blood containing known number of tumor cells were carried out in duplicate in a decreasing range of 1×10^7 to 1 to generate the standard curve. Samples were normalized using GAPDH.

Total RNA was isolated from primary tumors, reverse transcribed using oligo-dT primers, and analyzed by qRT-PCR for gene expression of the following genes: collagen I, α_1 (sense, GTATCACCAAACT-

CAGAAG; anti-sense, ATCATAGCCATAGGACATC) and α_2 (sense, AGTATTCTGACAAAGGAGTTTC; anti-sense, CCAGGAAGTCCAG-GAGTC) chains, collagen IV (sense, AAAGGGTCGGTGGGAGAG; anti-sense, CGGGAAAGCCAGGGTTAC), and integrin α_1 (sense, CACCTTTCAAACTGAGCCCGCCA; anti-sense, GCTGCCCAGCGAT-GTAGAGCACAT), α_5 (sense, TGCTACCTCTCCACAGAAAAC; anti-sense, GCCAGTCTTGGTGAACTCAG), α_6 (sense, CTGGCTTCCTC-GTTTGGCTATG; anti-sense, TGCCTTGCTGGTTAATGTAGACGT), α_v (sense, TTGGGGACGACAACCCTCTGACAC; anti-sense, TGCG-GCGGGATAGAAACGATGAG), β_1 (sense, TTCAGACTTCCGCATTG-GCTTTGG; anti-sense, TGGGCTGGTGCAGTTTTGTTCAC), β_3 (sense, CACGGATGCCAAGACCCATATTG; anti-sense, GTGGAGGCAGAG-TAGTGGYYGTC), and β_4 (sense, CGTCCCACAGACAGACATGAG; anti-sense, GCGTTCTCCTTGCAGTTTGTTC) subunits. Expression of each gene was normalized to the expression of GAPDH.

Tail vein injections. Eight- to twelve-week-old female WT or α_2-null FVB mice received i.v. tail vein injections of either 5×10^5 WT/Neu or α_2-null/Neu primary tumor cells. The animals were sacrificed at 120 days after tumor cell injections, and their lungs were fixed in Bouin solution. After 24 hours of fixation, lungs were transferred to 70% ethanol and tumors visible on the lung surface were counted. In addition to gross examination, lungs were subjected to H&E staining to confirm the presence of metastatic tumors.

Statistics. Survival for both mice and women are depicted with Kaplan-Meier curves, and log-rank tests were used to compare both relapse-free and overall survival in groups defined by genotype or α_2 integrin expression levels (56). Proportional hazards regression analysis was used to derive the hazard rate ratio for α_2-null/Neu mice relative to WT/Neu mice (56). Tests of continuous response measures in mice grouped by genotype were performed using the Wilcoxon rank sum test (56). A response feature approach was taken for the analysis of longitudinal data (56, 57). Differences in the rate of growth of tumor volume by genotype were assessed by fitting separate linear regression curves to the volume-age plots for each animal. This provided a separate slope estimate for each animal; these slopes were compared in mice with different genotypes using the Wilcoxon rank-sum test. A similar approach was used to analyze the wound closer assay data. In these analyses, linear regression lines through the origin were fit to the extent of wound closure for the cells from each animal as a function of time since the start of the assay. The difference in these slopes for mice with WT/Neu and α_2-null/Neu was assessed using a Wilcoxon rank-sum test. Graphs of longitudinal data were depicted by spaghetti plots in which observations from the same animal are connected by straight lines. Longitudinal measures of mRNA expression were analyzed by first averaging the expression from each mouse over time and then performing Wilcoxon rank-sum tests on these averages to compare expression in the WT/Neu and α_2-null/Neu genotypes. Statistical analyses were performed using GraphPad Prism Software and Stata (58). All *P* values were derived with respect to 2-sides alternative hypotheses.

The NKI-295 cohort published by van de Vijver et al. and the Wang cohort were analyzed for patient survival and disease-free survival, respectively (33, 37). Expression and clinical data for the NKI-205 cohort and the Wang cohort were obtained from Rosetta InPharmatics (33) and NCBI Gene Expression Omnibus (GEO datasets GSE2034), respectively. In metastasis-free survival analyses, patients who died of causes other than breast cancer were censored at the time of their death. In the original studies, gene expression analysis was performed on RNA obtained from frozen samples taken within 1 hour of the initial surgery. The level of α_2 integrin expression was set as a dichotomous variable with a "high" α_2 integrin expression level defined to be twice the average expression level for all patients in the cohort. All other patients were classified as having "low" expression levels. Trend analysis was performed by dividing the cohort into terciles. Additional gene expression profiling studies analyzed for a correlation among α_2 integrin expression, tumor grade, ER status, and metastasis (Supplemental Table 3) were obtained from Oncomine (www.oncomine.org) and Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (NCBI). Statistical analyses including unpaired Student's *t* test, ANOVA, log-rank test, and Kaplan-Meier were performed using GraphPad Prism Software.

Gene expression profiling studies analyzed for the prostate cancer data were obtained from the Stanford Microarray Database or NCBI GEO datasets (Lapointe, ref. 59; Tomlins, ref. 38). Prostate patients in the Lapointe and Tomlins datasets (*n* = 109 and 87 respectively) were categorized according to their clinical diagnosis and analyzed by ANOVA with post-test analysis for linear trend.

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