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Research Article

# A Direct Test of Potential Roles for β3 and β5 Integrins in Growth and Metastasis of Murine Mammary Carcinomas

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## Abstract

**αvβ3 or αvβ5 integrins are widely expressed on blood and endothelial cells. Inhibition of the functions of these integrins has been reported to suppress neovascularization and tumor growth, suggesting that they may be critical modulators of angiogenesis. However, mice lacking these integrins exhibit extensive angiogenesis. Tumors arising from s.c. injections of tumor cells into mice lacking one or both integrins show enhanced tumor growth compared with growth in control mice due to both increased angiogenesis and to altered innate immune response. Other data suggest additional roles for these integrins, on either platelets or the tumor cells themselves, in enhancing tumor progression and metastasis. Here, we investigate the involvement of β3 and β5 integrins in the development and progression of mammary carcinomas. We intercrossed mouse mammary tumor virus (MMTV)-c-neu transgenic mice with β3 or β5 or β3β5 integrin-deficient mice and observed that multiple, large mammary tumors developed in 100% of mice on all genetic backgrounds. A statistically significant earlier onset of tumor growth was observed in the MMTV-c-neu/β3β5 integrin-null females compared with control mice. No major differences were observed in tumor size or number, vessel number or vessel structure and lung metastases were observed with similar frequency and size in all strains. MMTV-c-neu/β3β5 integrin-null mice had higher numbers of mammary acini, which may account for the earlier onset of tumors in this strain. These data indicate that αvβ3 or αvβ5 integrins are not essential for tumor growth and progression, although they might play some role in mammary gland development.** (Cancer Res 2005; 65(22): 10324-9)

## Introduction

We have previously investigated the growth of transplanted tumors in mice lacking αv integrins (αvβ3 and αvβ5) and observed that tumors grow larger and this was accompanied by enhanced tumor angiogenesis but not by impairments in vessel morphology or pericyte recruitment (1, 2). Bone marrow transplants suggested that the absence of β3 integrins on bone marrow–derived host cells also contributes to the enhanced tumor growth in β3-deficient mice, although few, if any, bone marrow–derived endothelial cells were found in the tumor vasculature (2). Here, we analyze tumor growth and metastasis in a model of endogenous tumorigenesis; the mammary carcinomas that develop in mouse mammary tumor virus (MMTV)-c-neu-transgenic female mice (3).

In several malignancies, the tumor cells express αvβ3 and this expression correlates with tumor progression in melanomas, gliomas, ovarian, and breast cancer (4–8). In particular, in breast cancer, αvβ3 characterizes the metastatic phenotype and this integrin is up-regulated in invasive tumors and distant metastases (9). A potential mechanism for tumor cells to arrest within the vasculature is an interaction of circulating tumor cells with platelets (10). During blood flow, shear forces oppose cell attachment. Therefore, cells must be equipped with adhesive mechanisms that support cellular arrest within the vasculature (10). There is evidence that activation of αvβ3 integrins promotes breast cancer cell arrest during blood flow via platelet interactions (4). Similarly, a highly specific inhibitor of activated αIIbβ3 and platelet aggregation prevented B16 melanoma metastasis (11).

αIIbβ3 is solely expressed by platelets and megakaryocytes and is required for platelet aggregation and hemostasis (12–17). Interactions between tumor cells and platelets could form aggregates and promote metastatic arrest in the capillary networks, for example, in the lungs (18–22). The MMTV-c-neu mice develop mammary carcinomas that metastasize to the lungs (3). β3 integrin-null mice have dysfunctional platelet aggregation (17) and also lack αvβ3 integrin on all other cells.

Thus, this system provides an excellent one in which to investigate the potential roles of αvβ3, αIIbβ3, and αvβ5 in multiple stages of tumorigenesis, including roles both on the tumor cells themselves as well as on possible supporting cells such as platelets, endothelial, and immune cells. We present data showing that mammary gland tumors form in MMTV-c-neu-transgenic females also in the absence of β3 or β5 or both integrins. The tumor size and number are similar in all genetic backgrounds and all tumors are well vascularized with no obvious differences in vessel structure. Moreover, the frequency of metastasis to the lungs is similar in the various mouse lines. However, mice null for both β3 and β5 integrins developed mammary gland tumors and died somewhat earlier than control mice. The mammary glands of these females contained a higher number of acini, which may increase the chance of earlier tumor formation.

## Materials and Methods

**Mice.** FVB/N-Tg MMTV-c-neu (3) mice were purchased from Charles River Laboratories, Wilmington, MA. These mice were intercrossed in our laboratory with β3 integrin-null mice (17) and with mice lacking β5 integrin

**Note:** Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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(23) to generate MMTV-c-neu/β3, MMTV-c-neu/β5, or MMTV-c-neu/β3β5 integrin-deficient mice. All the mice used in the experiments described here were on a mixed C57BL/6 × 129S4 × FVB background. Because of the complexities of breeding, experimental MMTV-c-neu mice having or lacking individual integrin genes were cousins, not siblings. However, the effects seen were consistent among diverse experiments. All the mice were viable and fertile. The mice were sacrificed either after 1 week from tumor detection or when the tumor was impairing the animal's quality of life (the tumor was very big or the animal was suffering or was lethargic). Many of our mice developed tumors in more than one mammary gland. Moreover, we found animals with more than one tumor arising from the same mammary gland but no multilobulated tumors were noticed during the dissections.

**Antibodies.** Monoclonal antibodies used for immunohistochemistry were: rat anti–platelet/endothelial cell adhesion molecule 1 (PECAM-1)/CD31 and biotinylated hamster anti-β3 integrin (PharMingen, San Diego, CA); mouse anti-smooth muscle α-actin (Sigma, St. Louis, MO); rat anti-F4/80 (Serotec, Kidlington, United Kingdom); rabbit anti-NG2 polyclonal antiserum (Chemicon International, Inc., Temecula, CA); all were used at a 1:100 dilution. Secondary antibodies for immunofluorescence were Alexa-conjugated goat anti-rabbit IgG, goat anti-mouse IgG, or goat anti-rat IgG, all from Molecular Probes (Eugene, OR). Biotin-conjugated goat anti-rat immunoglobulin antibody used for chromogenic analysis was from PharMingen.

**Immunohistochemistry and histology.** Five-micrometer frozen sections of tumors were used for immunohistochemistry. Chromogenic visualization followed the Vectastain avidin-biotin complex kit protocol (Vector Laboratories, Burlingame, CA). For immunofluorescence, the conjugated secondary antibodies were used at a 1:400 dilution. Histology of tumors or lungs or normal mammary glands was done on 5 μm paraffin-embedded sections counterstained with H&E. To investigate whether the tumors metastasized to the lungs, we did microscopic analyses of the lungs at the time of sacrifice following the criteria listed above. Lungs were cut up into small pieces and embedded in paraffin. We then analyzed three H&E-stained step sections. Some microscopic metastases were already visible as macroscopic growths during the dissection. The lung metastases we detected were formed by at least 10 cells and were never trapped in the blood vessels. Histology of normal mammary glands was done on the right inguinal gland. One section of the gland, containing the lymph node, was analyzed for each animal. The acini present in two fields, near the lymph node, were counted and the average was calculated. Two observers, blinded to the genotype of the specimen, quantitated the number of acini (buds). All images were visualized with a Zeiss Axiophot photoscope and were processed with NIH Scion Image and Adobe Photoshop (Adobe, Mountain View, CA).

**Statistical analysis.** The two-tailed Student's *t* test was used to validate the data regarding tumor size, number of tumors, metastases or acini, tumor growth rate, percentage of tumor-free animals, and percentage of mice with lung metastases; $P < 0.05$ was considered statistically significant. The number of lung metastases per animal were analyzed using a program available at http://www.physics.csbsju.edu/stats/. Lung metastasis distributions were represented by box and whisker plots, where the box represents the interquartile range (central 50% of the data points), horizontal lines in the boxes represent the median, and vertical bars represent a spread of 1.5 × interquartile range, whereas dots represent outliers, which were included in calculations of significance. *n* indicates the number of samples, *P* indicates probability of identity of the distributions.

**Evaluation of vessel areas.** Frozen tumor sections from the various genetic backgrounds were stained with anti-PECAM-1 antibody to label endothelial cells following the procedure described in Immunohistochemistry and histology. The area occupied by the vessels was evaluated with a microscope connected to a computer, and NIH/SCION image camera software was used to measure the area occupied by vessels present in randomly selected ×40 fields.

## Results

**Tumor incidence occurs earlier in MMTV-c-neu/β3β5 integrin-null females.** We compared the percentage of tumor-free MMTV-c-neu/β3 or β5, or β3β5 integrin-null virgin (Fig. 1*A*) or parous (Fig. 1*B*) female mice with control MMTV-c-neu mice >15 months (Fig. 1*B*) or >18 months (Fig. 1*A*). We observed a statistically significant reduction in the number of tumor-free MMTV-c-neu/β3β5 ($P = 0.0001$) or MMTV-c-neu/β5 ($P = 0.0007$) integrin-null virgin females (Fig. 1*A*). No difference was observed in the occurrence of tumors for MMTV-c-neu/β3 integrin-null virgin animals ($P = 0.97$). A statistically significant reduction in the number of tumor-free females was also observed for MMTV-c-neu/β3β5 integrin-null parous females (Fig. 1*B*; $P = 0.0006$). The MMTV-c-neu/β3- or β5-null parous females (Fig. 1*B*) did not show any change in tumor development ($P = 0.3$ and $P = 0.81$, respectively). Thus, absence of both integrins, but not of either one alone, consistently allows the earlier appearance or growth of mammary tumors.

**Tumor burden is generally not reduced in MMTV-c-neu/β3 or β5 or β3β5 integrin-null females compared with controls.** The sizes of the tumors were evaluated by weighing the total mammary gland tumors arising from virgin females 1 week after tumor detection (Fig. 2*A*) or at the time of sacrifice (Fig. 2*B*). From the values shown in Fig. 2*A* and *B*, no statistically significant differences were observed. In Fig. 2*C*, the same comparisons were

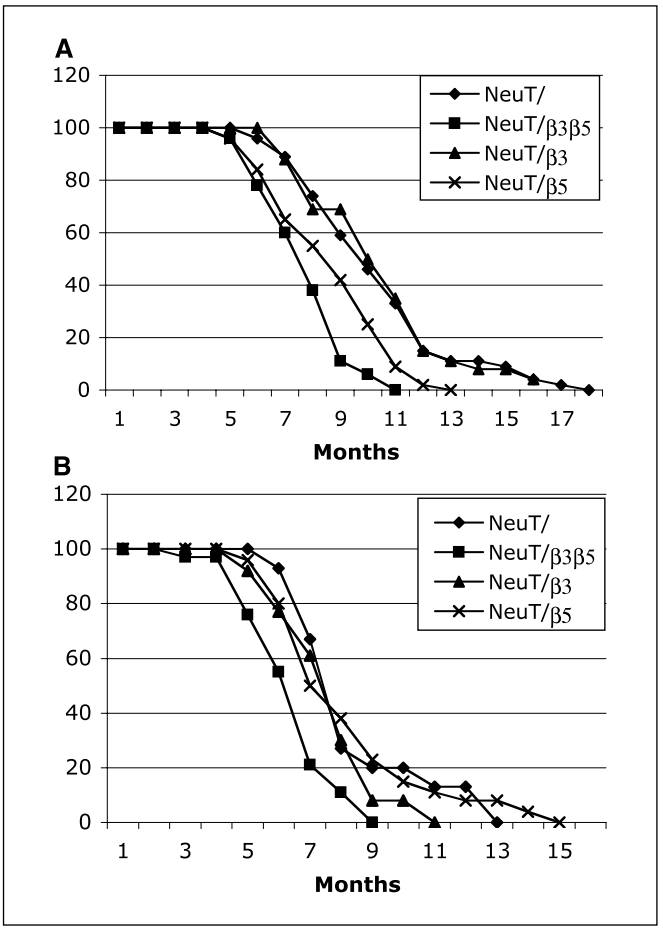


**Figure 1.** Accelerated tumor incidence in MMTV-c-neu/β3β5 integrin-deficient female mice compared with control animals. Virgin (*A*) or (*B*) parous females. *Points,* percentage of tumor-free animals; *n,* number of females in each group; *m,* mean time for onset of tumor growth expressed in months. *A,* MMTV-c-neu, $n = 46$, $m = 10.5$; MMTV-c-neu/β3, $n = 26$, $m = 10.5$, $P = 0.97$; MMTV-c-neu/β5, $n = 55$, $m = 8.8$, $P = 0.0007$; MMTV-c-neu/β3β5, $n = 47$, $m = 7.9$, $P = 0.0001$. *B,* MMTV-c-neu, $n = 15$, $m = 8.5$; MMTV-c-neu/β3, $n = 13$, $m = 7.8$, $P = 0.3$; MMTV-c-neu/β5, $n = 26$, $m = 8.3$, $P = 0.81$; MMTV-c-neu/β3β5, $n = 29$, $m = 6.6$, $P = 0.0006$. The average weights of all the tumors combined per mouse at sacrifice are given in Fig. 2.

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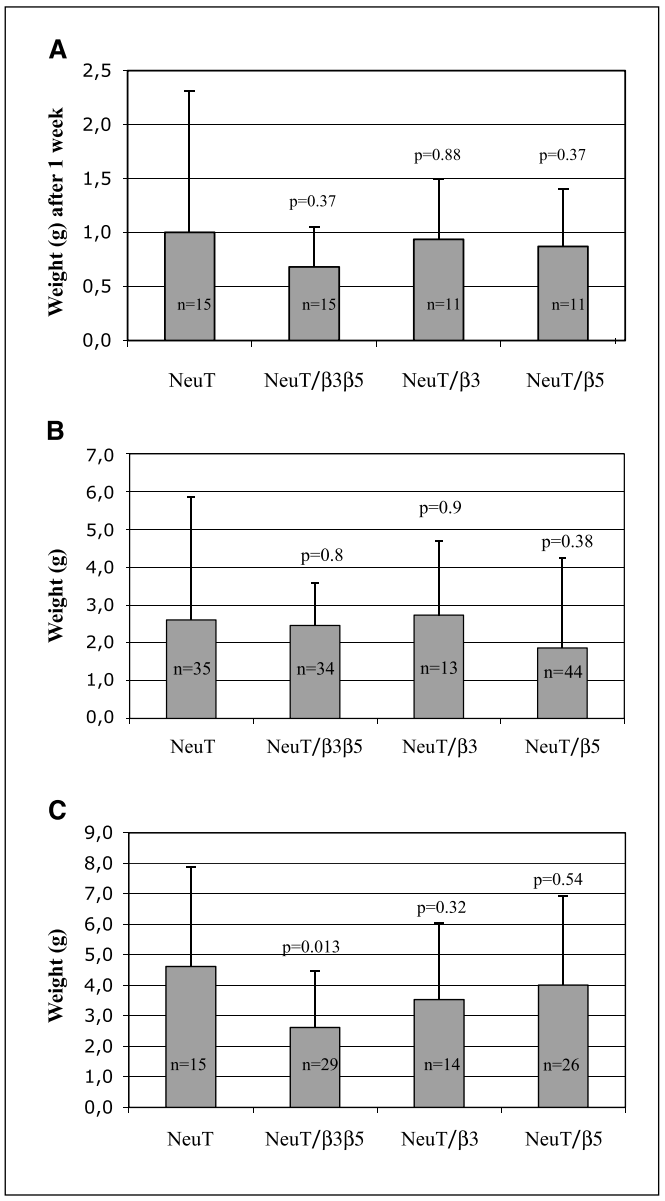


**Figure 2.** Total body tumor burden and rate of tumor growth in MMTV-c-neu/β3 or β5, or β3β5 integrin-null female mice compared with control animals. Tumor size is expressed as weight (g) after 1 week from tumor detection (*A*) or at time of sacrifice (*B* and *C*), in virgin (*A* and *B*) or parous (*C*) females. *n,* number of animals in each group; *P* values as indicated. *Columns,* average weight of all the tumors combined per mouse; *bars,* SD.

done for tumors that formed in parous females. In this case, a small but significant reduction was observed in MMTV-c-neu/β3β5 integrin-deficient females ($P = 0.013$). In the case of MMTV-c-neu/β3β5 integrin-null parous females, the mean time from initial detection of a tumor to sacrifice was somewhat decreased (16.6 days compared with 25.6 days in controls and similar durations in the other strains; $P = 0.047$) The reasons for the earlier onset of morbidity in the MMTV-c-neu/β3β5 integrin-deficient animals despite slightly lower tumor burden remain unclear and could have led to an underestimation of the eventual size of their tumors as compared with controls (Fig. 2*C*). Their earlier demise could be related to issues of general health rather than as a direct consequence of tumor burden (see Discussion). No differences in tumor number were seen when the tumors were counted 1 week after detection of the first tumor (Fig. 3*A*) or at the time of sacrifice (Fig. 3*B* and *C*) for either virgin (Fig. 3*A* and *B*) or parous females (Fig. 3*C*).

**Angiogenesis is not blocked in the absence of β3 or β5 or β3β5 integrins.** Sections of differently sized tumors were analyzed by histology and no obvious differences were observed among the various genetic backgrounds (data not shown). Immunohistochemical analysis with anti-PECAM-1, an endothelial cell marker, revealed no clear differences in blood vessel density in the absence of integrins (Fig. 4*A* and *B*). Tumors of different sizes (see Fig. 4*A*) were analyzed and large and small vessels were present in all the tumors. Vessel areas were quantitated in randomly selected fields in PECAM-1-stained tumor sections from tumors of different sizes for all genetic backgrounds (data not shown) as described in Materials and Methods. No significant differences were observed.

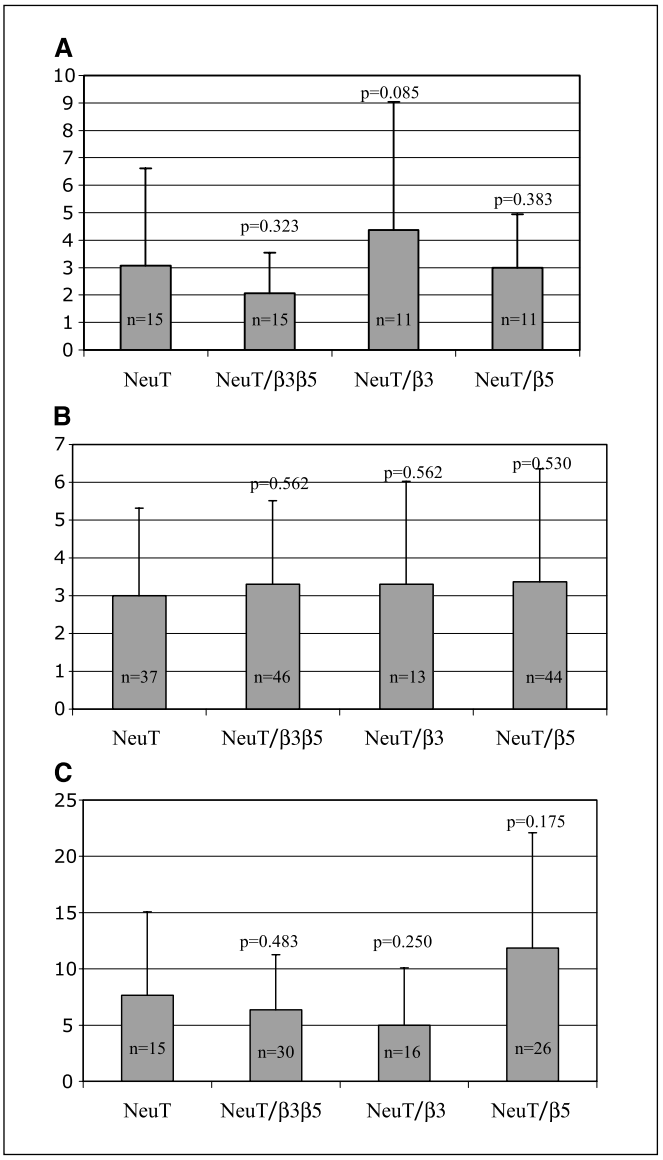


**Figure 3.** Total numbers of tumors in each animal in MMTV-c-neu/β3 or β5 or β3β5 integrin-null female mice compared with control animals. The tumors were counted 1 week after detection of the first tumor (*A*) or at the time of sacrifice (*B*) (*C*) in virgin (*A*) (*B*) or parous (*C*) females. *n,* number of animals in each group; *P* values as indicated. *Columns,* average number of all the tumors combined per mouse; *bars,* SD.

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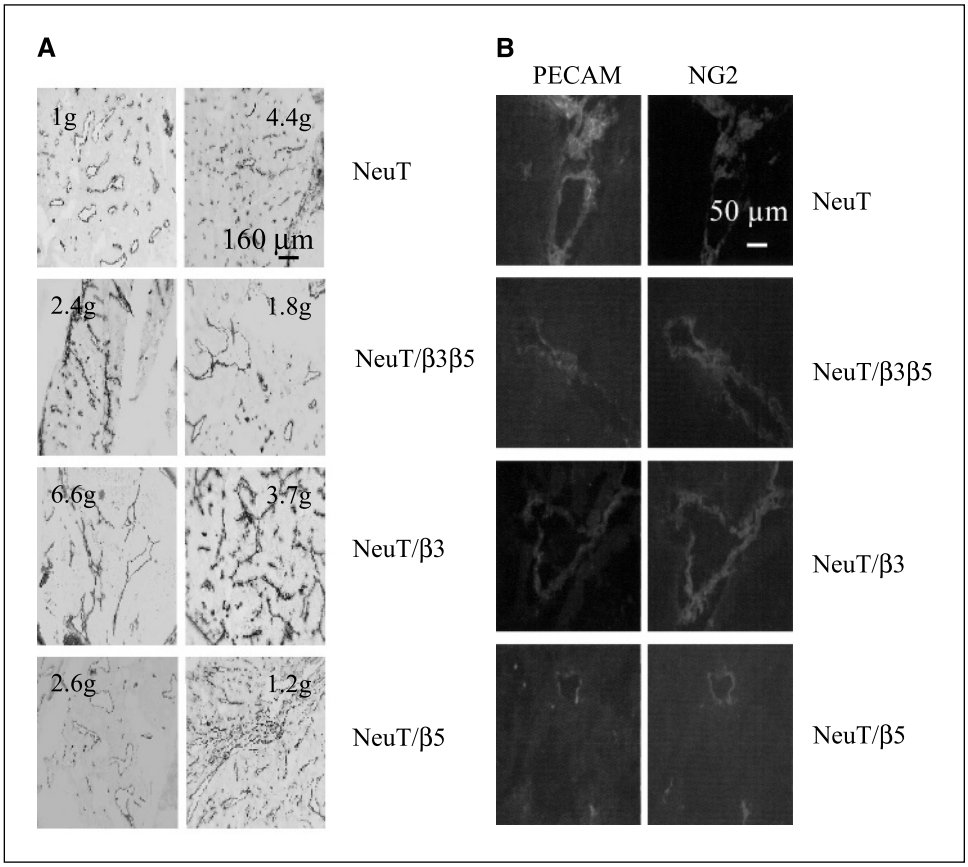


**Figure 4.** Characterization of vasculature in mammary gland tumors developed in MMTV-c-neu/β3 or β5 or β3β5 integrin-null virgin females compared with control animals. *A,* tumors of the indicated sizes were stained with an anti-PECAM-1 antibody. Vessels of different sizes are present in the various tumor areas without correlation with the mouse genotype. *B,* PECAM-1 and NG-2 double-staining of 1-week-old tumors. PECAM and NG-2 stainings are present around the same vessels. Similar results were obtained with smooth muscle α-actin staining for pericytes (data not shown).

To investigate possible differences in the recruitment of mural cells (pericytes and smooth muscle cells), sections of tumors were stained with antibodies against NG2 (Fig. 4*B, right*) or smooth muscle α-actin (data not shown), two markers for pericytes, and costained with anti-PECAM-1 antibody to reveal the endothelial cells (Fig. 4*B, left*). Similar staining for NG2 or smooth muscle α-actin was observed around the vessels of tumors grown in control or integrin-deficient females. Thus, the absence of these integrins does not seem to block vessel development as compared with control tumors. The presence of β3 integrin in the cells of control tumors was investigated by immunohistochemistry; both tumor cells and blood vessels were positive in wild-type mice and negative in β3-null animals (Supplementary Fig. S1).

**Lung metastases can form in the absence of β3 or β5 or β3β5 integrins.** To determine whether lung metastases could form in the absence of β3 or β5 or of both integrins, we analyzed the lungs histologically by performing step sections through both lungs. Lung micrometastases were found with similar frequency in every genetic background analyzed (see Table 1) and the numbers of lung metastases (Fig. 5) or the area of the lung occupied by metastasis (data not shown) present in each animal did not differ among the various genetic backgrounds. Therefore, we conclude that the absence of β3 or β5 or of both integrins does not block the formation of metastases. No correlation was found between tumor size or mean time from tumor detection to sacrifice and presence or extent of metastases (numbers or area).

**MMTV-c-neu/β3β5 integrin-null females show increased numbers of acini in their mammary glands compared with control animals.** The histology of the right inguinal mammary glands of virgin MMTV-c-neu/β3β5 integrin-null females 2 to 4, 4 to 6, 6 to 8, or 8 to 10 months old, were analyzed and compared with control animals. An increased number of acini were observed in the mammary glands of integrin-null females at every time point analyzed (Table 2; Fig. 6). Thus, the absence of β3 and β5 integrins influences the normal mammary gland development. The presence of a higher number of acini could favor tumor development. In fact, that group of animals does develop tumors at an earlier age (Fig. 1*A* and *B*).

**Table 1.** Incidence of lung metastases

| | NeuT (%) | NeuT/ β3-null (%) | NeuT/ β5-null (%) | NeuT/ β3β5-null (%) |
|---|---|---|---|---|
| Virgin | 50 (7 of 16) | 56 (5 of 9) | 60 (12 of 20) | 63 (5 of 8) |
| Parous | 50 (6 of 12) | 36 (4 of 11) | 57 (8 of 14) | 56 (9 of 16) |
| Total | 46 (13 of 28) | 45 (9 of 20) | 62 (21 of 34) | 58 (14 of 24) |
| *t* Test — total | | $P = 0.924$ | $P = 0.275$ | $P = 0.401$ |

NOTE: The percentage of virgin or parous females (or both) with lung metastases in MMTV-c-neu/β3 or β5 or β3β5 integrin-null virgin females compared with control animals.
The number of animals with metastases over the total number of animals per group are shown next to the percentages. The *P* values are calculated for the total number of females with lung metastases. The number of metastases in those mice which had them were also counted (see Fig. 5).

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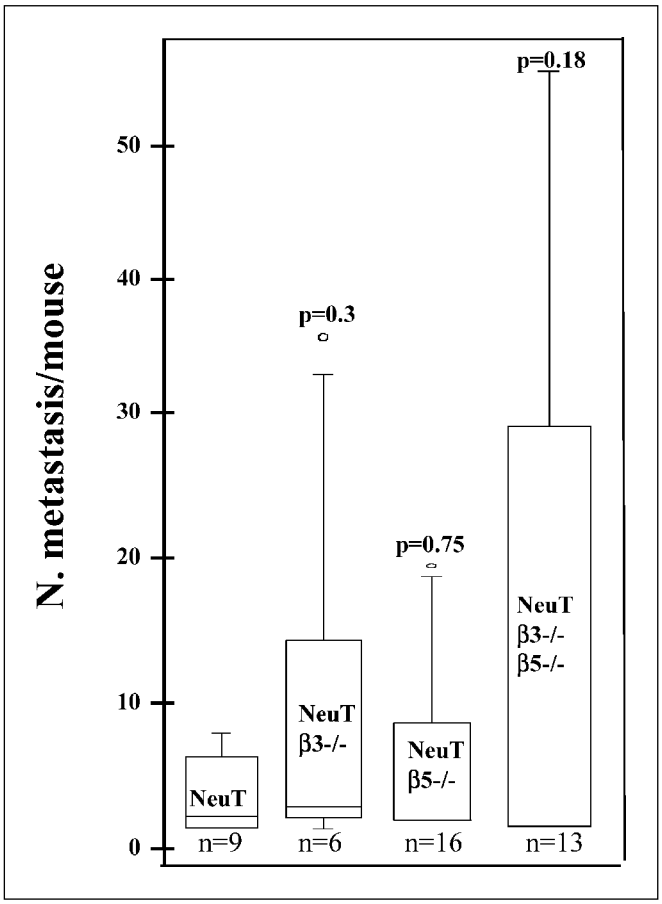


**Figure 5.** Numbers of metastases in MMTV-c-neu/β3 or β5 or β3β5 integrin-null virgin and parous female mice compared with control animals. The lung sections of animals developing tumors were analyzed to count the number of metastases present. The distributions for the various genetic backgrounds are shown. *n,* number of animals in each group. The *P* values are indicated. The median values are: MMTV-c-neu (2), MMTV-c-neu/β3 (2.5), MMTV-c-neu/β5 (1), MMTV-c-neu/β3β5 (1).

## Discussion

The data presented here clearly show that mammary gland tumors can form in the absence of β3 or β5 or of both integrins. These tumors are generally similar to the controls in terms of tumor burden and vascularization. A complex network of capillaries was found in all tumors, with normal appearance and investiture by pericytes. Previous studies with models of transplanted tumors suggested that αv integrins (αvβ3 and αvβ5) could act as negative regulators of angiogenesis. In fact, enhanced vascularization was observed in transplantable tumors grown in mice lacking these integrins (1, 2). In the current model of endogenous mammary carcinomas, we did not observe any obvious increased vascularization, but the fact that the absence of β3 or β5 integrins does not block vascularization confirms the nonessential role of β3 and β5 in tumor angiogenesis. Moreover, it is in conflict, as are the results for the transplanted models, with data obtained by using integrin antagonists (24, 25).

The increased tumor size that we observed in the transplanted models was also due to the absence of β3 integrins on bone marrow–derived host cells (2). A reduced infiltration of macrophages was observed in tumors grown in immunodeficient mice lacking β3 integrins (2). In the current study, we did not observe any difference in macrophage infiltration (data not shown). However, the fact that we did not see increased tumor growth for most of the animals might suggest that, if there is an alteration of bone marrow–derived cells involved in the innate immune

**Table 2.** Numbers of mammary gland acini

| Age (mo) | NeuT | NeuT/β3β5 | *t* Test (*P*) |
|---|---|---|---|
| 2-4 | 37 ± 10.6 (*n* = 5) | 72 ± 12.3 (*n* = 7) | 0.0004 |
| 4-6 | 27 ± 11.7 (*n* = 7) | 50 ± 25.6 (*n* = 7) | 0.048 |
| 6-8 | 14 ± 3.8 (*n* = 12) | 36 ± 8.9 (*n* = 9) | 0.0000003 |

NOTE: The numbers of acini in the right inguinal mammary gland were counted for each animal, in two fields in the area near the lymph node (not the total mammary gland). The numbers of acini shown are the average of acini scored in *n* animals ± SD. Only tumor-free mammary glands were scored. The single knockout mice were not evaluated here. An increased number of acini were observed in the doubly deficient mice. The *P* values are shown and they are statistically significant (see Fig. 6).

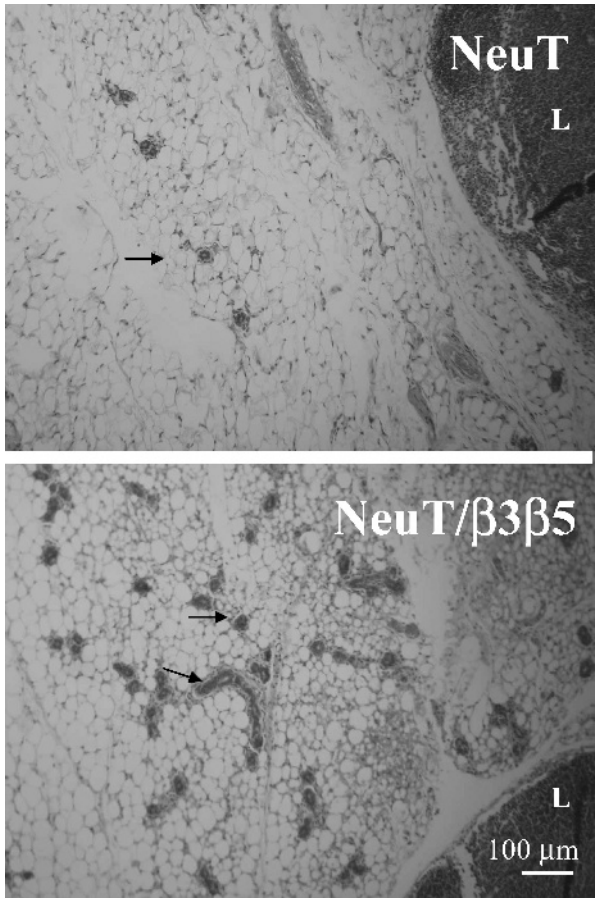


**Figure 6.** Histology of the mammary glands in MMTV-c-neu/β3β5 integrin-null virgin females compared with control mice. A portion of the inguinal mammary gland near the lymph node (*L*) is shown for 2- to 4-month-old females; *arrows,* acini. A higher number of acini is present in the mammary glands of MMTV-c-neu/β3β5 integrin-null virgin females.

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response here, this alteration is compensated by a correct adaptive immune response, because the mice used in this study have a complete immune system.

Another somewhat unexpected result obtained in our study concerns the presence of lung metastases in MMTV-c-neu/β3 or β5, or β3β5 integrin-deficient females. As stated in the Introduction, several malignant cells express αvβ3 and this expression correlates with tumor progression in melanomas, gliomas, ovarian, and breast cancer (4–8). A potential mechanism for tumor cells to arrest within the vasculature is an interaction of circulating tumor cells with platelets (10). We hypothesized that tumor cells might require the presence of β3 integrins, either on invading tumor cells or on platelets or on both. In our experiments, a similar number of mice developed lung metastases in the presence or absence of β3 integrin. Moreover, no differences in the number of lung metastases that each animal developed (or area occupied by metastases) were found in β3-positive or null animals. This could be because the process of invasion and metastasis formation starting from an endogenous tumor occurs by a mechanism that is different from the transplantable models mentioned above.

The tumor burden and the vascularization generally do not differ among the various genetic backgrounds analyzed. However, we did observe slightly reduced survival for the MMTV-c-neu/β3β5 integrin-null females, perhaps because tumors arose earlier in this background (Fig. 1) or because of generally reduced health of those animals. In fact, the size of tumors in this strain at the time of harvest was somewhat smaller than in the other strains (Fig. 2*C*), and it is possible that their full potential for tumor growth was underestimated because of their earlier sacrifice. The mammary glands of these females show differences in development; i.e., they contain more acini. It is intriguing to observe that the number of acini is generally reduced in the older animals, although still higher in the double knockout mice, which might be due to a remodeling of the gland. The presence of higher numbers of acini in the double-null mice means that more epithelial cells could undergo transformation; therefore, these females have a higher possibility of developing tumors. These data reveal some of the roles for β3 and β5 integrins in proper mammary gland development.

In conclusion, the absence of β3 or β5 integrins or of both has very little effect on tumor progression in the mammary tumor–prone genetic background studied here. Primary tumor incidence, size, and vascularity are largely unaffected as are the number and sizes of metastases. These results lend little support for hypothesized central roles of these integrins in tumor progression in this model system, whether on the tumor cells themselves, or on various stromal and vascular cells.

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