Reduced Blood Vessel Formation and Tumor Growth in α 5-Integrin-negative Teratocarcinomas and Embryoid Bodies

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Reduced Blood Vessel Formation and Tumor Growth in α5-Integrin-negative Teratocarcinomas and Embryoid Bodies[1]

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ABSTRACT

Embryonic stem (ES) cells—wild-type, heterozygous, or null for α5-integrin—were injected ectopically into syngeneic mice to develop teratocarcinomas. α5-null-derived teratocarcinomas were significantly smaller than the wild-type or α5 heterozygous tumors. Histological analysis revealed the presence of tissues derived from all three germ layers, in all tumors. However, α5-null teratocarcinomas displayed less undifferentiated tissue than did the controls. Decreased proliferation and increased apoptosis were observed in the undifferentiated areas of the α5-null teratocarcinomas. The expression of extracellular matrix proteins, fibronectin and tenascin-C, and the basement membrane components, laminin, entactin/nidogen, and collagen IV, was similar in the different tumors, although the deposition of these molecules was more disorganized in α5-null teratocarcinomas. The absence of α5-integrin in the various tissues of the α5-null tumors was confirmed by immunohistochemistry. Many vessels, but not all, stained positively for α5-integrin, showing that they were host derived. Analysis of the area occupied by vessels revealed, on average, an 8-fold decrease in α5-null teratocarcinomas compared with control tumors. Staining for smooth muscle α-actin showed that pericytes and smooth muscle cells were recruited around the vessels in all tumors, suggesting similar vessel differentiation. Deposition of EIIIA and EIIIB and fibronectin around the vessels was observed in all tumors. The fact that some, although few, α5-integrin-negative vessels existed in α5-null tumors indicated that α5−/− ES cells could differentiate into endothelial cells. Endothelial cell differentiation and vessel formation were analyzed also *in vitro*. α5-null ES cells were differentiated into embryoid bodies, although they were delayed in growth and attachment. Differentiation into endothelial cells was achieved, but the organization into a complex vasculature was delayed compared with controls. We conclude that α5β1-integrin plays a significant role in vessel formation both in ES cell cultures and in teratocarcinomas. Reduced vascularization likely contributed to the reduced proliferation and increased apoptosis observed in α5-null teratocarcinomas.

INTRODUCTION

ECM[3] components interact with each other or with cell surface receptors called integrins. These interactions play an important role in many biological processes such as embryonic development, wound healing, tumorigenesis, angiogenesis, and many others (1–10). Integrins comprise a family of >20 heterodimers of noncovalently linked α and β subunits. Most cells express many integrins and are, therefore, able to interact with many ECM molecules. Integrins often bind to more than one ligand; however, some show selectivity. For instance, the α5β1 integrin binds specifically to FN (11). α5β1 integrin is involved in many biological processes including cell proliferation and oncogenic transformation (12–14), embryogenesis (*e.g.*, vasculogenesis; Refs. 10, 15, and 16),[4] cell survival (16, 17), cell migration (13, 18), and cell spreading (19).

A characteristic property of tumor cells is their reduced adhesion to solid substrates. In culture, many transformed cells do not spread and grow as multilayered foci. In some instances, when transplanted into animals, they can invade and colonize different organs. The role of α5-integrin in cellular transformation, tumor formation and/or progression has been studied *in vitro* and *in vivo*. In ras-transformed cells, a reduction in the level of α5β1-integrin was found (12). Transformed Chinese hamster ovary (13) and human colon carcinoma cells (16) induced to overexpress the α5β1-integrin lose the potential to form tumors in mice. Studies of osteosarcoma (20, 21) and erythroleukemia cells (22) expressing different levels of α5β1 show that cells that attach better to FN overexpress α5β1 and are less tumorigenic. The level of α5β1 is reduced in many human and murine tumors (23–26). However, we recently took several genetic approaches using mice with targeted mutations in the gene encoding α5-integrin (knock-out or chimeric mice) and found that α5β1-integrin did not contribute to tumorigenesis or metastasis in the genetic backgrounds studied (27).

ES cells, as well as pre- or early postimplantation embryos, can develop into tumors when transplanted into an ectopic location in syngeneic animals (28). These tumors differentiate into various tissues and are called teratocarcinomas. Genetically manipulated ES cells can be used to generate such tumors (29–31) and to study the role of specific genes during tissue differentiation and tumor development. Folkman and D'Amore (32) demonstrated that tumor growth is dependent on angiogenesis, *i.e.*, the sprouting of new capillary vessels from preexisting vessels. Teratocarcinomas are also useful systems to study the process of angiogenesis (30, 33).

In our study, we injected α5-integrin-null ES cells into syngeneic mice to generate teratocarcinomas. Here we present data on tissue differentiation, proliferation, cell death, ECM deposition, and blood vessel formation of α5-null teratocarcinomas in comparison with controls. Endothelial cell differentiation and vessel formation were also studied *in vitro* by inducing α5-null ES cells to differentiate into EBs in the presence of growth factors promoting endothelial differentiation.

MATERIALS AND METHODS

Cell Culture. The following ES cells were used to induce teratocarcinomas and/or to obtain differentiation into EBs: D3 (+/+; Ref. 33); 152 (α5 integrin +/−; Ref. 34); 154 and 305 (α5 integrin −/−; Ref. 34). ES cells were grown as described (35). Differentiation of ES cells was obtained by the hanging drop method as described (36). To promote endothelial cell differentiation and vessel formation, the following growth factors were added to the medium: vascular endothelial cell growth factor (Peprotech, Inc., Rocky Hill, NJ; 50 ng/ml); recombinant human basic fibroblast growth factor (Genzyme, Cambridge, MA; 100 ng/ml); recombinant mouse interleukin 6 (Genzyme; 10 ng/ml); erythropoietin (Boehringer-Mannheim, Mannheim, Germany); and insulin (Life Technologies, Inc.; 10 μg/ml).

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[3] The abbreviations used are: ECM, extracellular matrix; FN, fibronectin; EB, embryoid body; ES, embryonic stem; LM, laminin; PECAM, platelet endothelial cell adhesion molecule; SMαA, smooth muscle α-actin; TN-C, tenascin; vWF, von Willebrand factor; WT, wild type; TUNEL, terminal transferase biotinylated-dUTP nick-end labeling.

[4] S. E. Francis and R. O. Hynes, unpublished data.

Immunohistochemistry. The EBs were fixed with cold methanol for 5 min after 5, 7, or 11 days of adhesion on gelatin-coated coverslips and analyzed for the presence of PECAM, FN, and SMαA as described (36). Frozen sections (6 μm) from unfixed tumors were processed as described (34). The following primary antibodies were used at 1:100 dilution: rat monoclonal anti-PECAM (CD31; PharMingen, San Diego, CA); rabbit polyclonal anti-vWF (Diagnostica Stago, Asniere, France); rabbit 24 polyclonal anti-FN (37); mouse monoclonal anti SMαA (clone 1A4; Dako, Carpinteria, CA); rabbit polyclonal anti-LM (Sigma Chemical Co.); rat monoclonal anti-TN-C (Mtn-12; Sigma Chemical Co.); goat polyclonal anti-EIIIA-FN (38); rabbit polyclonal anti-EIIIB-FN (39); rabbit polyclonal anti-entactin/nidogen (a gift of A. Chung, University of Pittsburgh, Pittsburgh, PA); and rat monoclonal anti-α5 integrin (PharMingen). The following secondary antibodies were used at 1:200 dilution: FITC-conjugated goat antirabbit or antirat (Biosource International, Camarillo, CA); TRITC-conjugated goat antirabbit or antirat (Biosource International).

Teratocarcinoma Induction. ES cells (10^7) were trypsinized, washed twice, resuspended in 100 or 200 μl of PBS, and injected s.c. onto the backs of 8-week-old syngeneic 129/SvJae male mice. After 18, 23, 24, or 25 days, tumors were surgically removed and weighed. A portion was embedded in mounting medium (OCT compound; Miles Laboratories, Elkhart, Milwaukee, WI) and immediately frozen in liquid nitrogen-cooled isopentane. The rest of the tumor was fixed overnight in 10% formalin (3.7% formaldehyde in PBS) and paraffin embedded the day after 6-μm sections were processed for H&E staining.

Cell Proliferation and Apoptosis. Proliferation was analyzed on frozen sections using an anti-nuclear antigen Ki67 antibody (Novocastra, Newcastle, United Kingdom). Frozen sections were fixed and stained (1:500 dilution) as above. Before mounting the slides, 2 min staining with propidium iodide counterstained the nuclei. Apoptotic cells were analyzed using TUNEL on paraffin sections from formalin-fixed tumors as described (40).

Evaluation of Vessel Area. Frozen sections from WT, α5+/− or α5−/− tumors were stained with an anti-vWF or anti-PECAM antibody to label endothelial cells following the procedure described in the immunohistochemistry paragraph. The area occupied by 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 fields (×40). Ten WT and 10 α5−/− tumors were measured, and average values were compared. Many more tumors were evaluated by eye.

Statistical Analyses. Statistical analyses were assessed by the two-tailed student's *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

α5-Integrin-Null Teratocarcinomas Are Smaller Than Controls. To analyze the role of α5-integrin during the development of teratocarcinomas, α5−/−, α5+/−, or WT ES cells were injected s.c. into the backs of syngeneic 129/SvJae male mice. Three independent sets of experiments were performed (Fig. 1). Representative mice from the null and WT groups are shown in Fig. 2. All three experiments showed statistically significant differences between α5-null animals and control ES cells; the α5-null tumors grow more slowly.

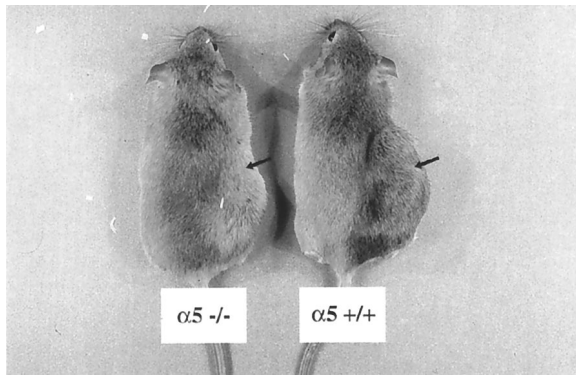

Fig. 2. Teratocarcinoma development in mice. α5−/− or WT ES cells (10^7) were inoculated in the right side of the back of 129/SvJae male mice. After 25 days of incubation, both mice developed a tumor; however, the tumor derived from the α5−/− ES cells was smaller (an example). *Arrows*, areas occupied by the tumors.

α5−/− ES-Cell Derived Teratocarcinomas Differentiate into Ecto-, Endo-, and Mesodermal Tissues and Are α5-negative except for Most of the Blood Vessels. H&E staining showed that α5-null tumors, similar to controls, are composed of ecto-, endo- and mesodermally differentiated tissues. Some examples are shown in Fig. 3. However, a higher proportion of undifferentiated cells was observed in control tumors. The expression of α5 integrin in the different tissues was analyzed using an antibody against α5-integrin. The α5-null ES cell-derived tumors were negative for α5-integrin in all of the tissues except for a majority of the blood vessels (Fig. 3*H*). The control teratocarcinomas were highly positive for α5 staining (Fig. 3*G*). Negative controls were included in each experiment (data not shown).

Reduced Proliferation and Apoptosis in α5-Null ES Cell-derived Teratocarcinomas. The proliferation index was analyzed in 8 α5-null teratocarcinomas and 5 control tumors by staining the nuclei with the Ki67 nuclear marker. Propidium iodide staining was used to counterstain all nuclei. Ten microscopic fields were counted for each tumor. High proliferation was observed in controls (positive nuclei, 30–40%), mostly in the undifferentiated areas, whereas lower proliferation was observed in α5-null teratomas (positive nuclei, 8–15%; Fig. 4*A*). Histological staining with H&E shows the appearance of highly undifferentiated areas (blue), which are less prevalent in the α5-null tumors.

Apoptosis was analyzed by TUNEL assay in 5 α5-null and 5 control tumors. Ten microscopic fields were counted for each tumor. Apoptotic cells were differentially distributed in the two groups.

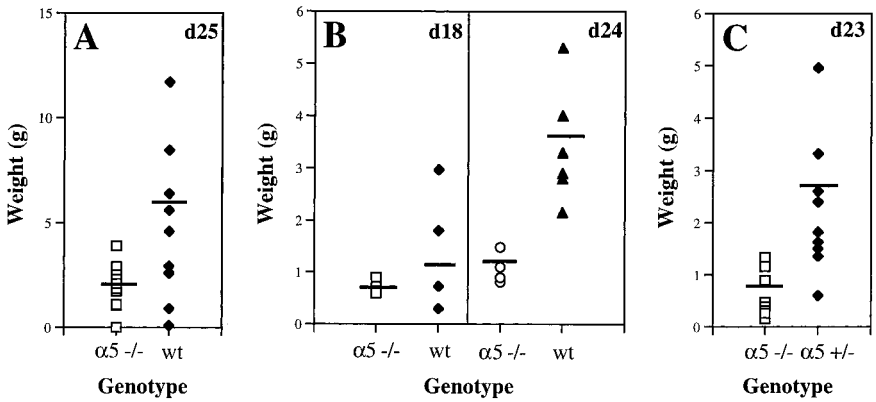

Fig. 1. Weights of teratocarcinomas obtained after injection of WT, α5+/−, or α5−/− ES cells into 129/SvJae male mice. *A*, tumors derived from WT D3 ($n = 10$) and α5−/− 154 ($n = 14$) ES cells 25 days after inoculation; $P = 0.030$. *B*, tumors derived from WT D3 and α5−/− 305 ES cells 18 days ($n = 4$ in both cases; $P = 0.105$) or 24 days ($n = 6$ and 4, respectively; $P = 0.040$) after inoculation. *C*, tumors derived from α5+/− 152 and α5−/− 154 ES cells 23 days ($n = 9$; $P = 0.006$) after inoculation. Individual values and the means are shown.

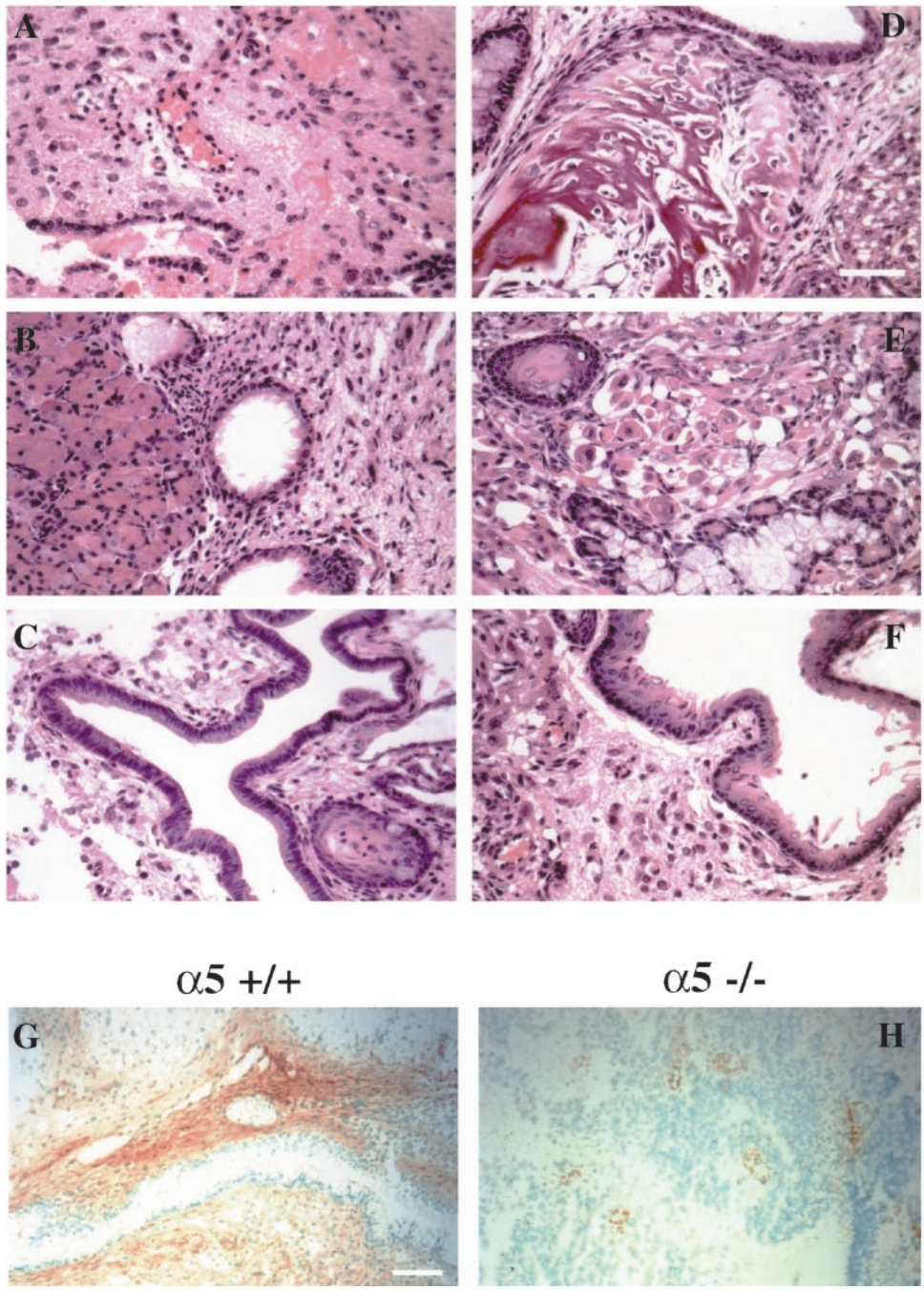

Fig. 3. Histology of α5+/+ and α5−/− teratocarcinomas; *A–F*: differentiation of α5−/− 154-derived teratomas 25 days after inoculation. *A*, nervous tissue-like and vessels; *B*, pancreas, epithelium, ciliatum, and nervous tissue-like; *C*, trachea-like tubes; *D*, bone; *E*, muscle and salivary glands; *F*, skin. *Bar*, 50 μm. *G* and *H*: staining for α5-integrin in α5+/+ or α5−/− tumors. Only the vessels stain positively for α5-integrin in the α5-null tumors.

Three % to 6% of apoptotic nuclei were found in controls, whereas <1% of the nuclei were apoptotic in α5−/− tumors. However, importantly, in control tumors apoptotic cells were mostly concentrated in areas of nervous tissue, whereas in α5-null tumors, they were mostly found in undifferentiated, proliferative zones (Fig. 4*B*). Thus, reduced proliferation and increased apoptosis in proliferative zones cause slower growth of the α5-null tumors.

α5-null tumors show a more disorganized ECM distribution. To test for the deposition of ECM molecules in the absence of α5-integrin, we analyzed the deposition of several ECM proteins in α5-null-ES cell-derived tumors. Tumors were stained for FN and TN-C or for the basement membrane components LM, entactin/nidogen, and collagen IV. Comparison showed that all of these molecules were present in the two groups of tumors, although in α5-null tumors the deposition was more disorganized (Fig. 5). Analysis of the basement membrane ultrastructural morphology was also performed with an electron microscope. The basement membranes were continuously present along the basal surface of the cells in both mutant and control tumors (data not shown). It is therefore possible that the general disorganization of ECM observed in α5-null tumors was a consequence of different tissue distribution compared with the controls.

Reduced Vascularity in α5−/− ES Cell-derived Teratocarcinomas. α5−/− or WT ES cell-derived teratocarcinomas were immunostained for α5-integrin. The same sections were costained with antibodies for endothelial cell markers (anti-PECAM or anti-vWF antibodies) to outline endothelial cells. Representative sections of α5−/− or α5+/+ ES cell-derived tumors, double-stained for α5-integrin and for the vWF antigen, are shown in Fig. 6*A*. Although most of the blood vessels present in α5-null tumors are host derived because they stain positively for the presence of α5-integrin, some

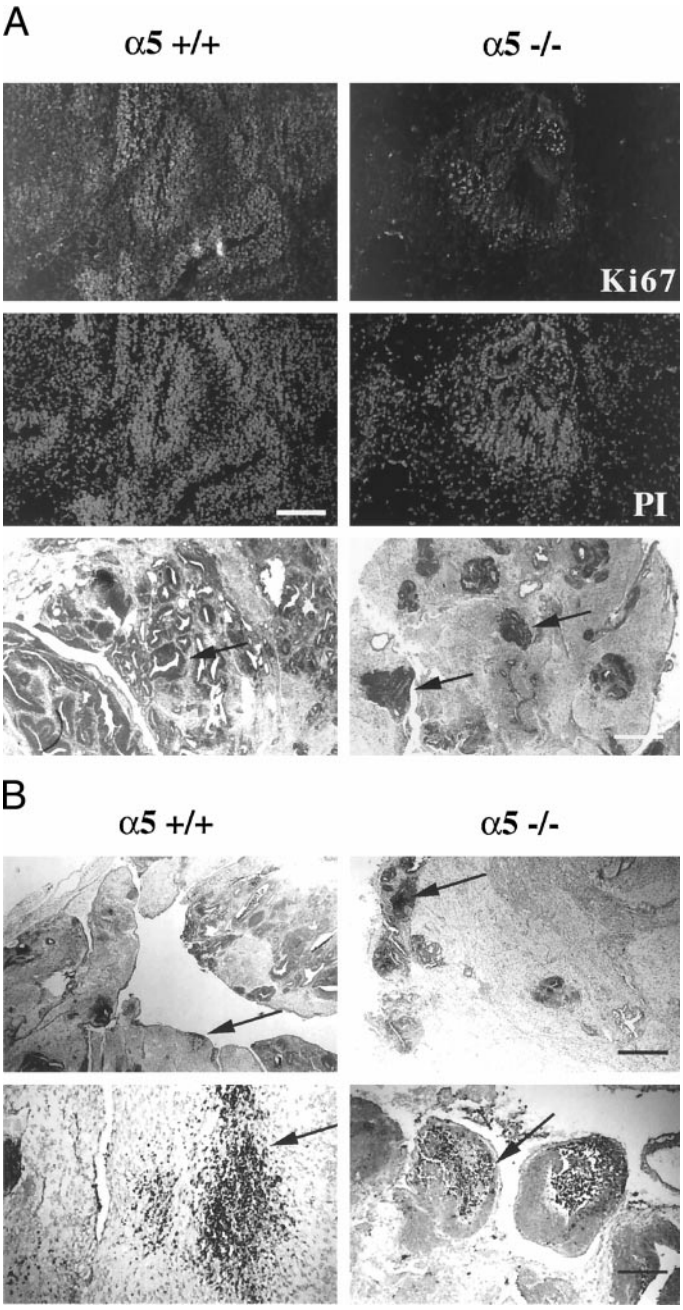

Fig. 4. Proliferation (*A*) and apoptosis (*B*) of α5+/+ and α5−/− teratocarcinomas. *A*, staining for the Ki67 nuclear antigen shows that WT tumors have a higher proliferation of cells in undifferentiated areas compared with the α5−/− derived tumors. Propidium iodide staining was used to label all of the nuclei. *Bar*, 100 μm. H&E staining for α5+/+ D3 and α5−/− 154-derived tumors shows a high number of dark, undifferentiated areas (*arrows*) in the WT. *Bar*, 400 μm. *B*, cell death measured by TUNEL method revealed a generally higher level of apoptosis in WT tumors, mostly localized in the nervous-like tissues compared with α5−/− derived tumors. Little apoptosis occurred in the undifferentiated areas of WT tumors. In the α5−/− tumors, the total cell death observed was lower than in controls; however, high levels of cell death were observed in the undifferentiated tissues. *Arrows*, areas of apoptosis. *Bars: top panel*, 400 μm; *bottom panel*, 100 μm.

vessels (<5%) are ES cell derived; the endothelial cells are, in fact, negative for α5-integrin (Fig. 6*A*). From the anti-vWF or PECAM stainings performed, it was possible to analyze the area occupied by vessels. Vessel areas were quantitated in randomly selected fields in sections from α5-null or WT cell-derived tumors as described in "Materials and Methods." Twenty fields of control teratocarcinomas and 16 fields of α5-null tumors were counted (Table 1). The values were expressed in pixels, and each value represents the mean area of all of the vessels present in one field (×40). The average vessel area in WT was 10916 pixels, and the average vessel area in α5-null was 1424 pixels.

The presence of pericytes around the vessels was studied by staining for SMαA (Fig. 6*B*). In both types of teratocarcinomas, staining for SMαA was found around the vessels (costaining for PECAM or vWF), indicating that recruitment of pericytes and/or smooth muscle cells was occurring.

The unique ligand for α5-integrin is FN. It is known that the deposition of FN-splicing variants around vessels is increased during angiogenesis (41–44). To analyze whether the deposition of FN-specific splicing variants in teratocarcinomas was affected by the absence of α5-integrin, we stained the teratocarcinomas for EIIIA-FN or EIIIB-FN. Endothelial cells were identified by PECAM expression. The stainings for EIIIB were similar in α5-null tumors and controls. Although EIIIA-positive staining was observed in all tumors analyzed, a slight decrease of expression was noted around the vessels in α5-null tumors (Fig. 6, *C* and *D*).

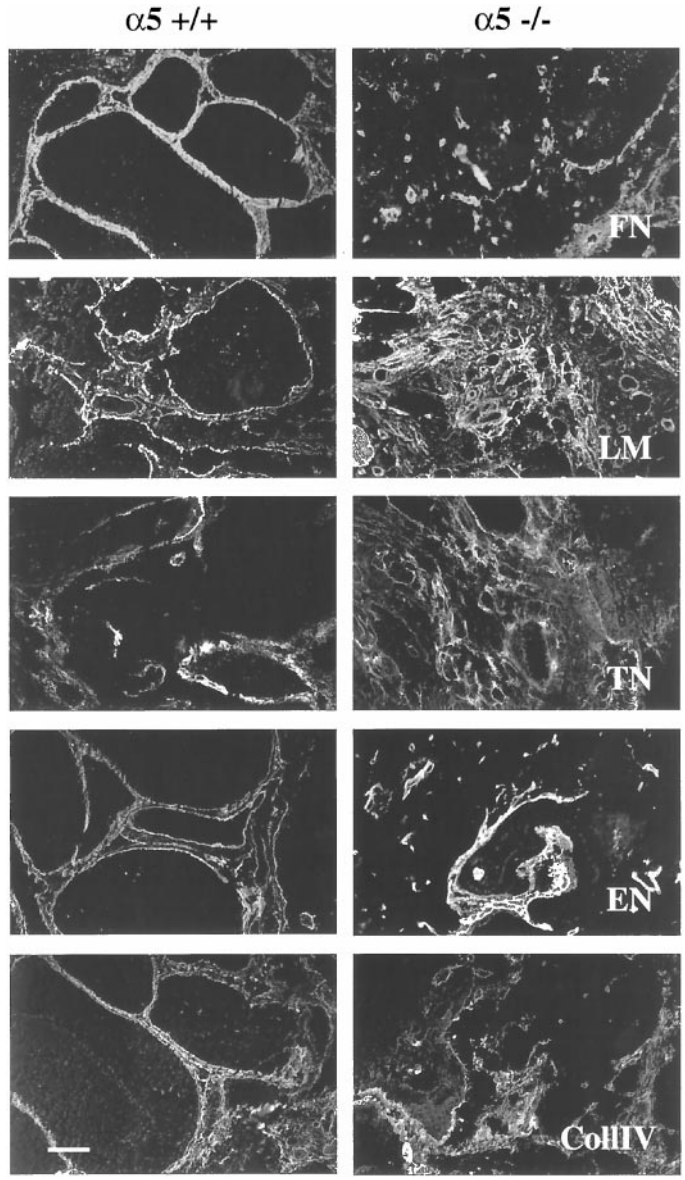

Fig. 5. ECM deposition in α5+/+ and α5−/− derived teratocarcinomas. Deposition of FN, LM, TN-C, entactin (nidogen), and collagen IV was analyzed. *Bar*, 100 μm.

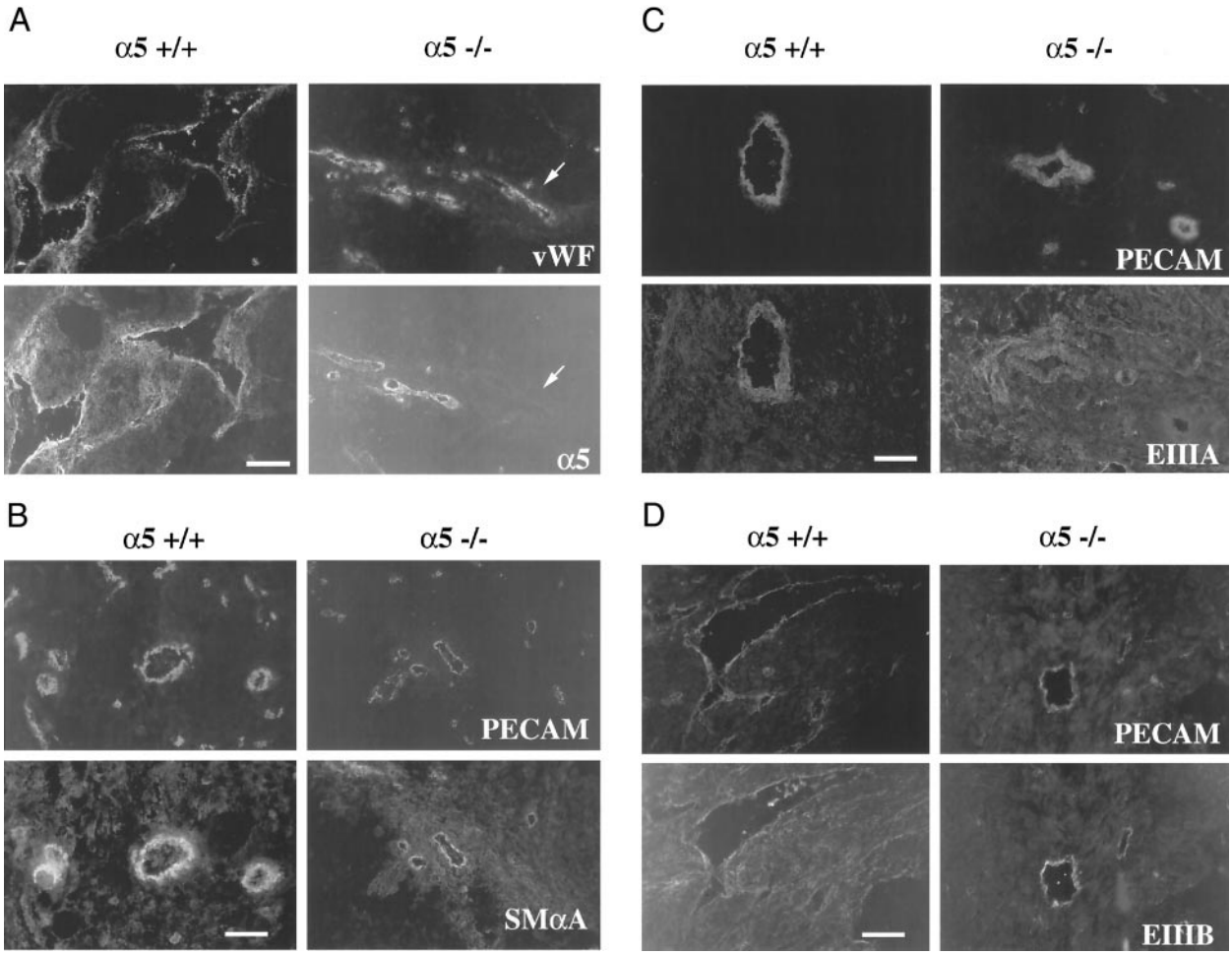

Fig. 6. Composition and complexity of vasculature in α5+/+ and α5−/− teratocarcinomas. In *A*, staining for vWF identifies the vessels. Costaining for α5 integrin shows that most of the vessels in α5-derived tumors are host derived because they are α5 positive. However, a few α5-negative vessels are present (*arrow*). In *B*, the complexity of the vessels was tested by analyzing the presence of SMαA. The vessels were costained for PECAM. In *C* and *D*, the deposition of the EIIIA (*C*) and EIIIB (*D*) FN splicing forms around the vessels was analyzed. PECAM was used to stain the vessels specifically. *Bar*, 50 μm.

***α*5-Null ES Cells Can Differentiate into Endothelial Cells *in Vitro*; However, Vessel Formation Is Delayed.** To study the differentiation of ES cells into endothelial cells *in vitro*, α5−/−, α5+/−, or WT ES cells were grown in suspension for 6 days to form EBs (36). The bodies were plated on gelatin-coated coverslips and induced to differentiate. Growth factors promoting vessel formation (vascular endothelial growth factor, basic fibroblast growth factor, erythropoietin, and interleukin 6) were added to the medium to stimulate endothelial and blood cell differentiation and vessel formation. The EBs were analyzed after 5 or 7 or 11 days of adhesion and differentiation. Fig. 7 shows that at days 5 and 7 of differentiation, the α5-null EBs have a delay in attachment and in growth, whereas by day 11, the α5-null EBs look similar to the controls. The presence of endothelial cells and vessel formation were analyzed by staining the bodies for PECAM or vWF. Endothelial cells are already present at day 5 of differentiation in all EBs; however, the organization into tubular structures (vessels) is delayed in α5-null EBs. At day 7, vessels are already present in control EBs; however, it is only at day 11 that endothelial cells organize into tubular structures in α5−/− EBs (Fig. 7*B*). The differentiation of blood cells was also delayed in α5-null; in Fig. 7*A*, a delay in the formation of dark/brown areas corresponding to blood cells can be observed.

The deposition of FN was also analyzed and was similar in the two groups of EBs, suggesting that the absence of α5-integrin does not greatly affect FN synthesis, deposition, or organization (data not shown).

DISCUSSION

The results presented here are only partially consistent with earlier publications. Despite reports that loss of α5β1-integrin enhances tumorigenesis (12, 13, 16, 23–26), we observed that α5-null teratocarcinomas grow more slowly (Figs. 1 and 2). This result is more in line with results on β1-null teratocarcinomas (30), although α5-null teratocarcinomas develop more rapidly than do β1-nulls. Furthermore, our data allow a more precise assignment of defects to a single integrin, α5β1. It is notable that α5-null cells can give rise to a wide variety of cell and tissue types (Fig. 3), consistent with earlier data on chimeric mice (34). Embryos completely lacking α5 integrin fail to progress beyond the 10–12 somite stage (15, 45). The most likely cause of abortive development of α5-null embryos and reduced growth of α5-null teratocarcinomas is defects in vascular development in the absence of α5β1. α5-null embryos exhibit defects in vessel formation, and we report here that α5-null teratocarcinomas are poorly vascularized (Table 1) and that α5-null EBs show delayed and reduced formation of tubular endothelial structures (Fig. 7). Many of the blood vessels within the α5-null teratocarcinomas are host derived (Figs. 3*H* and 6*A*). This invasion by host vessels presumably supports

Table 1 *Area occupied by vessels*

The area occupied by vessels was evaluated in pixels under the microscope as described in "Materials and Methods." Each value represents the average vessel density observed in one field.

Vessel area (pixels)	
+/+	−/−
4281	447
9735	471
3412	1452
4433	1872
7332	1548
20249	803
10967	812
2681	2019
1461	1908
2987	3816
3354	1571
18816	1792
10989	1495
10989	1259
12222	632
14153	892
12646	
21696	
19400	
26590	
Average 10916	Average 1424

the differentiation observed, which is much greater than is seen in α5-null embryos.

All of the data taken together demonstrate that α5-null endothelial cells can differentiate in embryos (15, 45), teratocarcinomas (Fig. 6*A*), and EBs (Fig. 7). However, in all of these cases, they show defects in their ability to assemble into a vascular network. Similar (indeed more severe) defects are observed in FN-null embryos (15, 35, 46). Consistent with these genetic data, it has been shown recently that inhibitors of α5β1 and FN interactions interfere with vasculogenesis and angiogenesis (47). Clearly, binding of FN to α5β1 plays important roles in blood vessel development. Even the α5-positive host-derived vessels are smaller than in controls. This could be attributable to the presence of α5-null endothelial cells derived from the tumors or to defects in other cell types (pericytes, smooth muscle, or stromal cells). It was seen previously that when CHO cells defective in α5β1-integrin were injected into nude mice, the vasculature of these tumors was abnormal, although clearly host derived (48), suggesting that the level of α5β1 in the tumor parenchymal cells can influence the formation of the host vasculature invading the tumor. This somewhat surprising result could be a consequence of failure of α5-null cells to organize an appropriate FN-rich matrix to support angiogenesis or could reflect compromised function of α5-null perivascular cells, either in their ability to induce endothelial tubes or in their ability to cooperate in vessel formation.

Returning to the question of roles for α5β1 in tumor growth *per se*, it is worth recalling that chimeric mice containing a significant proportion of α5-null cells do not develop increased numbers of tumors (34), and heterozygosity for α5 or FN does not alter the spectrum or malignancy of tumors developing in p53-deficient mouse strains (27). These results are consistent with the data presented here, which show reduced rather than increased tumor growth in the absence of α5β1 (Figs. 1 and 2). Our data suggest that both reduced proliferation and increased apoptosis of α5-null cells contribute to this slower growth. One possibility is that this is a secondary consequence of the reduced vasculature. A second possibility is that the absence of α5β1 from some cells in itself allows or induces apoptosis. This has been demonstrated for several cell types *in vitro* (17, 49–52) and for neural crest cells *in vivo* (45).

In conclusion, α5β1/FN interactions clearly play an important part in vascular development. Indeed, they appear more important than interactions of αvβ3- or αvβ5-integrins with their ligands, because mice lacking αvβ3 (53) or αvβ5 (54) are viable and fertile, and mice lacking all αv integrins show extensive vasculogenesis and angiogenesis (55), far more than mice lacking ether α5β1 or FN. In contrast with this primary role in vascular development, α5β1 and FN play less of a role in tumor development. Loss of α5β1/FN interactions in tumors do not appear to be sufficient to lead to excessive tumor growth. The reduced growth of α5-null tumors may reflect decreased proliferation or increased apoptosis of tumor cells lacking α5β1 as well as reduced vascularity. Loss of α5β1/FN interactions may still contribute to tumor progression when other alterations in oncogenes

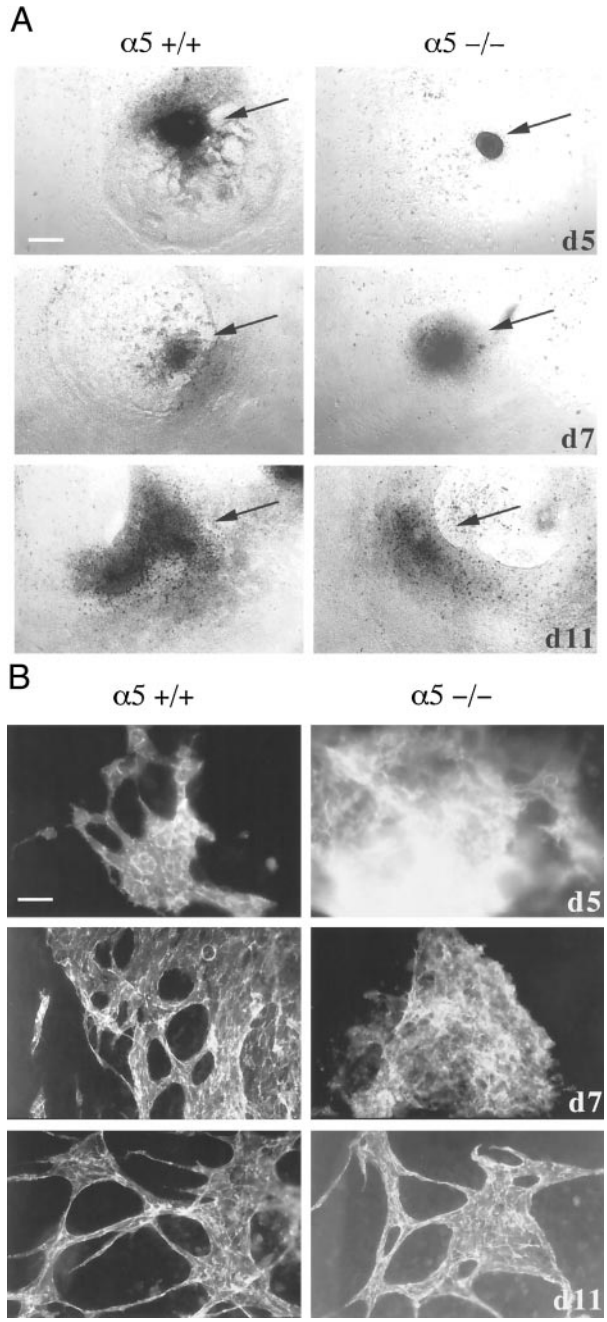

Fig. 7. D3 and α5−/− derived EBs. *A*, morphology after 5 or 7 or 11 days (*d5*, *d7*, or *d11*, respectively) of adhesion shows a delay in growth and attachment in α5−/−-derived EB. *Arrows*, attached EBs. *Bar*, 500 μm. *B*, PECAM staining of EBs after 5, 7, or 11 days of adhesion. Endothelial cells are present at day 5 in both kinds of bodies; however, vessels can be observed in α5−/− bodies only after 11 days of adhesion. *Bar*, 100 μm.

and/or tumor suppressor genes subvert controls on proliferation and survival.

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