Tie2-dependent deletion of α6 integrin subunit in mice reduces tumor growth and angiogenesis

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Abstract. The α6 integrin subunit (α6) has been implicated in cancer cell migration and in the progression of several malignancies, but its role in tumor angiogenesis is unclear. In mice, anti-α6 blocking antibodies reduce tumor angiogenesis, whereas Tie1-dependent α6 gene deletion enhances neovessel formation in melanoma and lung carcinoma. To clarify the discrepancy in these results we used the cre-lox system to generate a mouse line, α6fl/fl-Tie2Cre^{+}, with α6 gene deletion specifically in Tie2-lineage cells: endothelial cells, pericytes, subsets of hematopoietic stem cells, and Tie2-expressing monocytes/macrophages (TEMs), known for their proangiogenic properties. Loss of α6 expression in α6fl/fl-Tie2Cre^{+} mice reduced tumor growth in a murine B16F10 melanoma model. Immunohistological analysis of the tumors showed that Tie2-dependent α6 gene deletion was associated with reduced tumor vascularization and with reduced infiltration of proangiogenic Tie2-expressing macrophages. These findings demonstrate that α6 integrin subunit plays a major role in tumor angiogenesis and TEM infiltration. Targeting α6 could be used as a strategy to reduce tumor growth.

Introduction

The extracellular matrix and integrins, their cellular receptors, play key roles in tumor progression and tumor angiogenesis, and blocking antibodies against αvβ3, αvβ5 and α5β1 are currently under development as potential cancer therapies (1,2). The α6 integrin subunit is expressed by endothelial cells, platelets, monocytes/macrophages, neutrophils, epithelial cells and Schwann cells (3-6). It can associate with β1 or β4 subunits to form receptors for laminins, which are major components of the extracellular matrix and endothelial basement membrane (7). A role of α6 integrin subunit has been demonstrated in the progression of several malignancies such as breast cancer, prostate cancer, glioblastoma and pancreatic cancer. α6 integrin overexpression or *de novo* expression by tumor cells (depending on the type of cancer) is associated with a poor prognosis. Indeed, α6 expression increases cell motility and adhesion, which confers invasive properties to tumor cells (8-12). α6 is also strongly expressed on endothelial cells, and its expression is enhanced by proangiogenic growth factors such as VEGF and FGF2 (13-17). We have previously shown that α6 is involved in endothelial cell adhesion, migration, pseudotube formation and post-ischemic vascular repair (13,14,18,19). Moreover, α6 binds laminin 411 (laminin 8) and laminin 511 (laminin 10) (7), expression of which is upregulated in the basement membrane of tumor blood vessels in invasive brain (20) and breast (21) carcinomas. α6 targeting could potentially have therapeutic benefits by disrupting tumor angiogenesis and growth, but the role of α6 in tumor angiogenesis is controversial. In a breast carcinoma xenograft model (MDA-MB-231), Lee *et al* (16) found that an anti-α6 blocking antibody reduced tumor volume, tumor weight and blood vessel abundance. However, these effects might have resulted from either an antitumoral effect or anti-angiogenic activity or both effects. To resolve this issue, Primo *et al* (17) used Rip-Tag2 mice, which spontaneously develop pancreatic tumors that do not express α6. They found that α6 expression on tumor blood vessels was increased during the angiogenic stage and that administration of an anti-α6 blocking antibody reduced tumor vascularization. However, other authors obtained opposite results with a genetically manipulated mouse model. Germain *et al* (22) generated mice in which α6 gene deletion was restricted to endothelial cells, by using

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the cre-lox system under the control of the Tie1 promoter. However, Tie1-dependent α6 deletion was counterbalanced by VEGFR2 overexpression, resulting in enhanced tumor growth and angiogenesis. Further investigation was thus required to clarify the role for α6 in tumor angiogenesis.

To understand the discrepancy in the results reported in the different studies cited above we generated a mouse model in which the gene coding for α6 was deleted by using the cre-lox system under the control of the Tie2 promoter, leading to α6 gene deletion only in Tie2-lineage cells (endothelial cells, subsets of hematopoietic stem cells, pericytes and monocytes/macrophages). We investigated the effect of this Tie2-dependent α6 gene deletion on B16F10 melanoma growth, tumor angiogenesis, macrophage infiltration and pericyte coverage by comparing α6fl/fl-Tie2Cre$^+$ and α6fl/fl-Tie2Cre$^-$ mice.

Materials and methods

Animals. We generated α6 floxed mice [Itga6^{tm2Egl}, MGI:4439081] as previously described and bred them with mice expressing Cre recombinase under the control of the Tie2 promoter [B6.Cg-Tg(Tek-cre)12Flv/J], purchased from Jackson Laboratory (Bar Harbor, ME, USA), in order to generate α6fl/fl-Tie2Cre$^+$ and α6fl/fl-Tie2Cre$^-$ mice (on a C57BL/6 background) (19). All the protocols were approved by the Regional Ethics Committee on Animal Experimentation (protocol CEEA34.CB.041.11) and all the experiments complied with European Parliament Directive 2010/63/EU. The adequacy of anesthesia was confirmed by the lack of the tail pinch response.

Tumor growth assay. One million B16F10 melanoma cells (syngeneic to C57BL/6 mice) were suspended in 100 μl of PBS and injected subcutaneously in the right flank of 8-week-old α6fl/fl-Tie2Cre$^+$ and α6fl/fl-Tie2Cre$^-$ male mice. Tumor growth was quantified by Vernier caliper measurements every 2 or 3 days. Tumor volume was calculated as 0.5 x length x width^2. At the end of the experiment the mice were anesthetized with a single intraperitoneal injection of ketamine (80 mg/kg) and xylazine (16 mg/kg), then sacrificed by cervical dislocation. Tumors were harvested, weighed and frozen in isopentane solution cooled in liquid nitrogen before being stored at -80˚C until immunohistological analysis. Two different independent experiments were performed. For one experiment all the tumors were harvested at Day 12 (n=7 to 9 mice/genotype), and for another experiment the tumors were harvested at different time points in order to analyze size-matched tumors (n=10 per genotype).

Immunohistological tumor analysis. For all immunofluorescence experiments, frozen 10-μm-thick sections of tumors from α6fl/fl-Tie2Cre$^+$ and α6fl/fl-Tie2Cre$^-$ mice were fixed in ice-cold acetone for 10 min, stained as described below and examined by an observer in a blinded manner using a confocal microscope (TCS SP2, Leica, Wetzlar, Germany).

Study of α6 integrin subunit co-expression with laminin chains α4 and α5. Tumor sections were incubated for 1 h with the following primary antibodies: rabbit anti-mouse laminin α4 (23), rabbit anti-mouse laminin α5 (24) (both generous gifts from Sorokin LM, University of Muenster, Germany) or rat anti-human α6 (clone GoH3, BD Biosciences, Franklin Lakes, NJ, USA), then further incubated for 1 h with the following secondary antibodies: goat anti-rabbit Alexa555 (Invitrogen, Carlsbad, CA, USA) or goat anti-rat FITC (Abcam, Cambridge, MA, USA). Nuclei were stained with TOPRO3-Iodide (Thermo Fischer Scientific, Waltham, MA, USA).

Analysis of tumor vascularization. Tumor sections were incubated for 1 h with a rat anti-mouse CD31 monoclonal antibody (clone MEC 13.3, BD Biosciences), then with a goat anti-rat secondary antibody coupled to FITC (Abcam). Ten fields were examined per section. The vessel surface area, the number of vessels and vessel diameter were quantified with Histolab™ software (Microvision Instruments, Evry, France). Results were expressed as the vessel surface area (%), the number of vessels/mm^2 and mean vessel diameter.

Quantification of Tie2-expressing macrophages. Tumor sections were sequentially incubated with the following antibodies: rat anti-mouse F4/80 (Abd Serotec, Düsseldorf, Germany), goat anti-rat FITC, biotinylated rat anti-mouse Tie2 (eBiosciences, San Diego, CA, USA) and streptavidin-Alexa 555 (Invitrogen). Ten fields were examined per section and the numbers of Tie2-expressing macrophages (TEMs) (F4/80$^+$ Tie2$^+$) and total macrophages (F4/80$^+$) were determined with Image J and Histolab™ (Microvision) software. Results were expressed as the number of TEMs and total macrophages/mm^2 and as the percentage of TEMs in the total macrophage population.

Analysis of pericyte coverage. Tumor sections were incubated with the following primary antibodies: biotinylated mouse anti-smooth muscle actin (αSMA) (Clone 1A4, Thermo Fischer Scientific), rat anti-mouse CD31 (clone mec13.3, BD Biosciences), and then with streptavidin Cy3 (Amersham, Little Chalfont, UK) and goat anti-rat Alexa 488 (Invitrogen). Ten fields were examined per section. The number of blood vessels positive for αSMA, and the surface areas stained for CD31 (endothelial cells) and αSMA (pericytes) were determined with Image J software. Results were expressed as a percentage of blood vessels positive for αSMA and as a ratio of αSMA/CD31 surface areas.

Blood sampling and analysis of monocyte α6 expression. Eight-week-old α6fl/fl-Tie2Cre$^+$ and α6fl/fl-Tie2Cre$^-$ mice were anesthetized with a single intraperitoneal injection of ketamine (80 mg/kg) and xylazine (16 mg/kg). Peripheral blood was collected by cardiac puncture and the mice were sacrificed by cervical dislocation. Peripheral blood samples were incubated with CD45-PerCP and CD49f-PE (clone GoH3) or isotype-matched irrelevant antibodies (BD Biosciences). After a lysis step (FACS lysing solution, BD Biosciences), monocyte α6 expression was analyzed on a FACS Calibur flow cytometer using Cell Quest Pro software (BD Biosciences). A SSC/CD45-PerCP dot plot was used for monocyte gating. Results were expressed as the percentage of monocytes positive or negative for α6 expression among the total monocyte population.

Statistical analysis. Results are expressed as means ± SEM. Data were analyzed using ANOVA followed by Fisher's PLSD

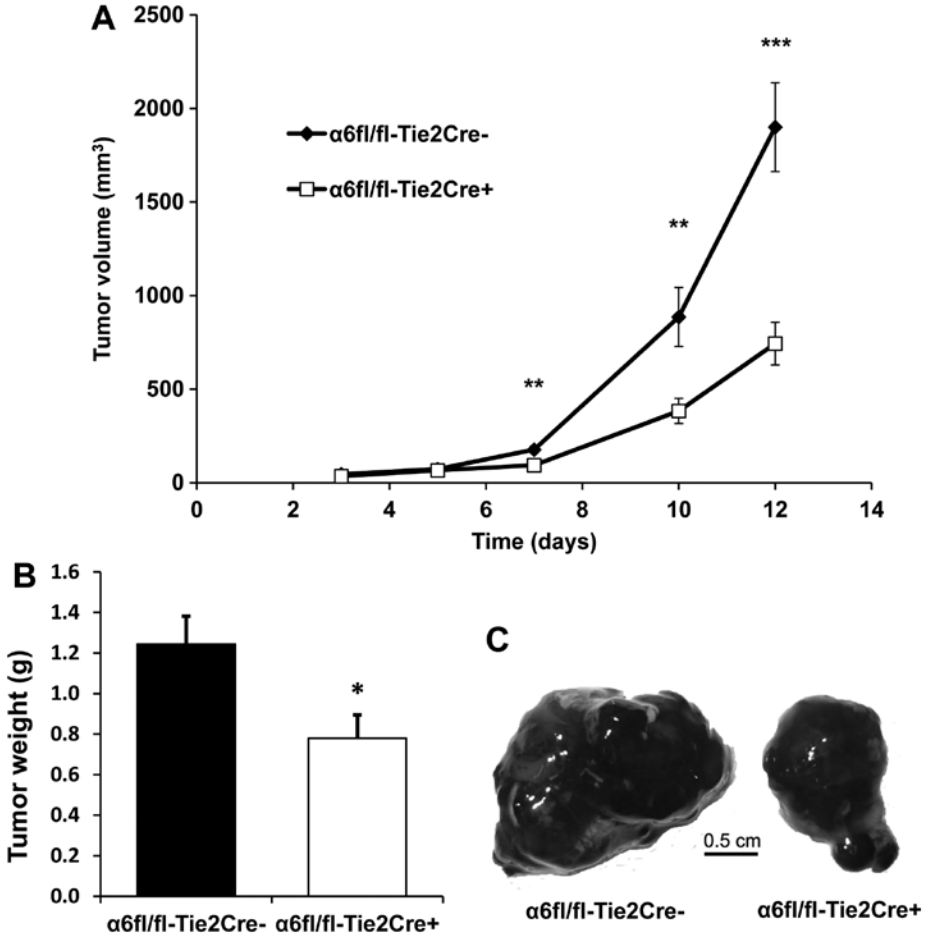

Figure 1. Tie2-dependent α6 deletion reduces tumor growth. Kinetics of tumor growth (A) assessed by Vernier caliper measurements in α6fl/fl-Tie2Cre$^-$ (◆, n=7) and α6fl/fl-Tie2Cre$^+$ (□, n=9) mice. Tumor weight (B) and representative photomicrographs (C) of B16F10 tumors from α6fl/fl-Tie2Cre$^-$ (■, n=7) and α6fl/fl-Tie2Cre$^+$ (□, n=9) mice 12 days after tumor implantation. Scale bar, 0.5 cm. Data are means ± SEM. *P<0.05, **P<0.01 and ***P<0.001.

post hoc test implemented with StatView software (SAS, Cary, NC, USA). Significance was assumed at P<0.05.

Results

Tie2-dependent deletion of α6 integrin subunit reduces tumor growth. The growth of subcutaneous B16F10 tumors was significantly reduced in α6fl/fl-Tie2Cre$^+$ mice (n=9) compared to α6fl/fl-Tie2Cre$^-$ mice (n=7): tumor volume was reduced by 56% at Day 10 (P<0.01) and by 60% at Day 12 (P<0.001) (Fig. 1A), while tumor weight was reduced by 37% at Day 12 (P<0.05) (Fig. 1B).

Tie2-dependent deletion of α6 integrin subunit reduces tumor angiogenesis. Immunofluorescence experiments showed that α6 was expressed on tumor blood vessels from α6fl/fl-Tie2Cre$^-$ mice and was associated with laminin α4 and α5 chains. Endothelial α6 deletion was complete in α6fl/fl-Tie2Cre$^+$ mice (Fig. 2).

For tumors harvested at Day 12, vessel density was reduced in tumors from α6fl/fl-Tie2Cre$^+$ mice (n=9) compared to α6fl/fl-Tie2Cre$^-$ mice (n=7): both vessel number/mm^2 and vessel surface area were reduced by 58% (P<0.001). The average diameter of tumor blood vessels was significantly larger in α6fl/fl-Tie2Cre$^+$ mice than in α6fl/fl-Tie2Cre$^-$ mice (P<0.05) (Fig. 3A and B).

To rule out an effect of tumor size on angiogenesis we also analyzed tumor vascularization in size-matched tumors from α6fl/fl-Tie2Cre$^+$ and α6fl/fl-Tie2Cre$^-$ mice, harvested at varying time points. Vessel density was reduced in tumors from α6fl/fl-Tie2Cre$^+$ mice (n=10) compared to α6fl/fl-Tie2Cre$^-$ mice (n=10): the number of vessels/mm^2 was reduced by 41% (P<0.001) and vessel surface area by 48% (P<0.01) (Fig. 3C).

Tie2-dependent deletion of α6 integrin subunit reduces infiltration by Tie2-expressing macrophages. For tumors harvested at Day 12, the number of TEMs per square millimeter was reduced by 55% in tumors from α6fl/fl-Tie2Cre$^+$ mice (n=9) compared to α6fl/fl-Tie2Cre$^-$ mice (n=7) (P<0.05). Tie2-dependent deletion of α6 also led to a 32% reduction in the percentage of TEMs among the total macrophage population (P<0.001). The total number of macrophages in tumors from α6fl/fl-Tie2Cre$^+$ mice was also reduced, but the difference with α6fl/fl-Tie2Cre$^-$ mice was not statistically significant (Fig. 4A and B). The analysis of size-matched tumors (n=10 mice/genotype) led to the same results with no significant difference observed between size-matched tumors and tumors harvested at Day 12 (Fig. 4C).

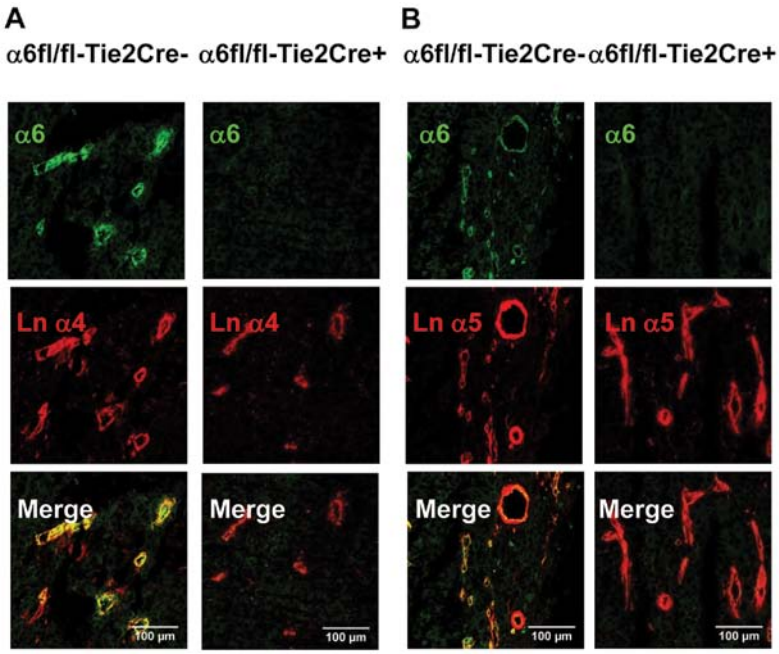

Figure 2. Laminin α4 and α5 chains colocalize with integrin α6. Representative photomicrographs of cryosections of B16F10 tumors from α6fl/fl-Tie2Cre$^-$ and α6fl/fl-Tie2Cre$^+$ mice. Integrin α6 was immunostained in green with antibody GoH3 and with a secondary antibody coupled to FITC. Laminin α4 (A) and α5 chains (B) were stained in red with rabbit antibodies and with a secondary antibody coupled to Alexa 555. Scale bars, 100 μm.

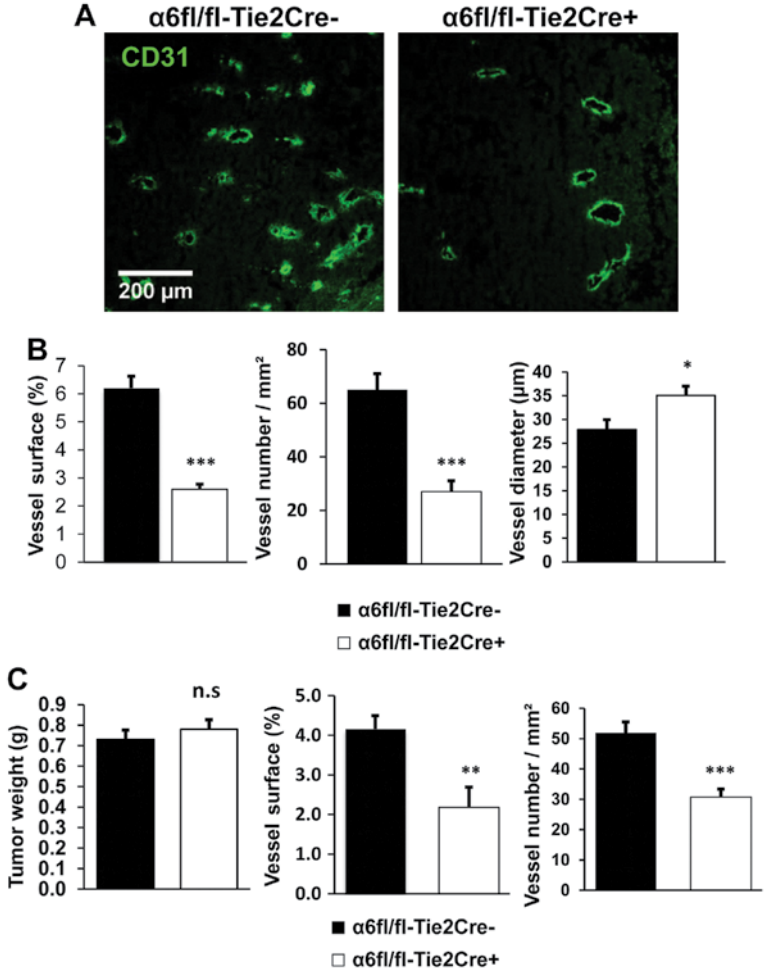

Figure 3. Tie2-dependent α6 deletion reduces tumor vascularization. Representative photomicrographs (A) and quantitative analysis of the vessel surface area (%), the number of vessels/mm^2, and mean vessel diameter on cryosections of B16F10 tumors from α6fl/fl-Tie2Cre$^-$ (■, n=7) and α6fl/fl-Tie2Cre$^+$ (□, n=9) mice, 12 days after tumor implantation (B) or when tumors have identical size in the 2 groups (n=10 in each group) (C). Vessels were stained green with an anti-CD31 antibody. Scale bar, 200 μm. Data are means ± SEM. *P<0.05 and **P<0.01.

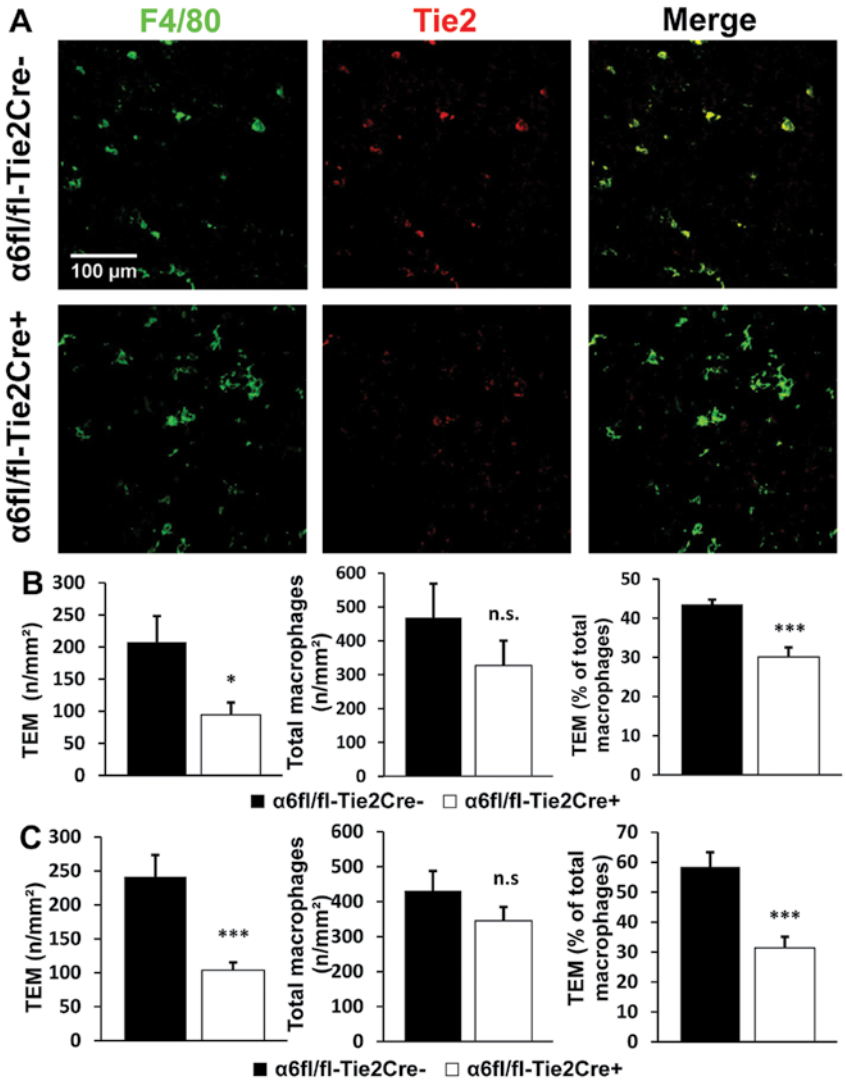

Figure 4. Tie2-dependent α6 deletion reduces infiltration by Tie2-expressing macrophages. Representative photomicrographs (A) and quantitative analysis of TEMs and total macrophages in cryosections of B16F10 tumors from α6fl/fl-Tie2Cre$^-$ (■, n=7) and α6fl/fl-Tie2Cre$^+$ (□, n=9) mice, 12 days after tumor implantation (B) or when tumors have identical size in the 2 groups (n=10 in each group) (C). Sections were stained green with an anti-F4/80 antibody and red with an anti-Tie2 antibody. Scale bar, 100 μm. Data are means ± SEM. *P<0.05 and ***P<0.001.

Tie2-dependent deletion of α6 integrin subunit on peripheral blood monocytes. The proportion of peripheral blood monocytes positive for α6 was 52.5±7.6% in α6fl/fl-Tie2Cre$^-$ mice and only 1.85±0.60% in α6fl/fl-Tie2Cre$^+$ mice (mean ± SEM, n=5 mice/genotype, P<0.01).

Tie2-dependent deletion of α6 integrin subunit does not significantly change pericyte coverage of tumor blood vessels. The percentage of blood vessels that were positive for αSMA was 38.7±6.2% in α6fl/fl-Tie2Cre$^-$ mice (n=5) and 33.25±6.5% in α6fl/fl-Tie2Cre$^+$ mice (n=8) (mean ± SEM) (Fig. 5A and B). The ratio of αSMA/CD31 surface areas was 0.19±0.03 in α6fl/fl-Tie2Cre$^-$ mice (n=5) and 0.21±0.03 in α6fl/fl-Tie2Cre$^+$ mice (n=8) (mean ± SEM) (Fig. 5A and C). There was no statistically significant difference between the two genotypes.

Discussion

Tie2-dependent α6 deletion significantly reduced tumor angiogenesis and, consequently, tumor growth in a B16F10 mouse melanoma model. The number of tumor blood vessels was significantly lower in α6fl/fl-Tie2Cre$^+$ mice than in α6fl/fl-Tie2Cre$^-$ mice. This difference is not due to a difference in tumor size as the analysis of size-matched tumors led to the same conclusion. These latter results are in keeping with those reported by Lee *et al* (16) and Primo *et al* (17), who used an anti-α6 blocking antibody (GoH3), and also with our previous results on post-ischemic angiogenesis (19). Indeed, α6 is required for endothelial cell adhesion and migration and for pseudotube formation *in vitro* (13,16,18). In contrast, however, Germain *et al* (22) observed enhanced neovessel formation in α6fl/fl-Tie1Cre$^+$ mice compared to α6fl/fl-Tie1Cre$^-$ mice. The most likely explanation for these conflicting results is VEGFR2 overexpression on endothelial cells from α6fl/fl-Tie1Cre$^+$ mice compared to α6fl/fl-Tie1Cre$^-$ mice, and the lack of this compensatory mechanism in α6fl/fl-Tie2Cre$^+$ mice. Indeed, we have shown that the loss of α6 expression on endothelial cells isolated from α6fl/fl-Tie2Cre$^+$ was not counterbalanced by other integrins or VEGFR2 overexpression (19). Another difference between the Tie1Cre and Tie2Cre

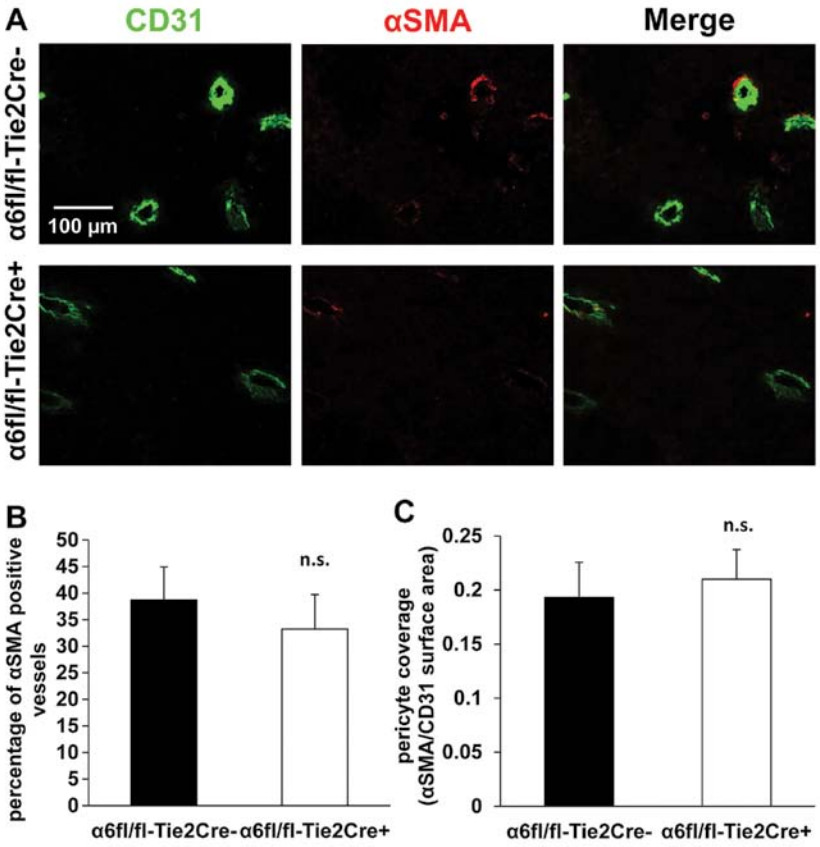

Figure 5. Analysis of pericyte coverage of tumor blood vessels. Representative photomicrographs (A) and quantitative analysis of the percentage of αSMA-positive blood vessels (B) and of the ratio of αSMA/CD31 surface areas (C) in cryosections of B16F10 tumors from α6fl/fl-Tie2Cre$^-$ (■, n=5) and α6fl/fl-Tie2Cre$^+$ (□, n=8) mice. Sections were stained green with an anti-CD31 antibody and red with an anti-αSMA antibody. Scale bar, 100 μm. Data are means ± SEM.

models is that Tie1 is restricted to endothelial cells, whereas Tie2 is expressed on endothelial cells, pericyte precursors of mesenchymal origin, subsets of hematopoietic stem cells, and also a subset of monocytes/macrophages (25). Angiopoietin 2 release by endothelial cells in tumors is upregulated by hypoxia, and its receptor, Tie2, is strongly upregulated when monocytes are recruited into the tumor and differentiate into perivascular macrophages (26). These Tie2-expressing macrophages (TEMs) are highly proangiogenic: they secrete growth factors such as VEGF and matrix metalloproteinases such as MMP9 and thereby promote neovessel formation in endometriotic lesions and tumors (26-28). TEM targeting might thus enhance anti-angiogenic therapy efficiency (29-31). About 50% of peripheral blood monocytes were positive for α6 in our α6fl/fl-Tie2Cre$^-$ mice, whereas nearly all were negative for α6 in α6fl/fl-Tie2Cre$^+$ mice, suggesting that Tie2-dependent α6 deletion also occurred efficiently in monocytes. Consequently, α6 would also be deleted from TEMs in α6fl/fl-Tie2Cre$^+$ mice, and we found that TEM tumor infiltration was significantly reduced compared to α6fl/fl-Tie2Cre$^-$ mice. In macrophages, α6 ligation on laminin triggers intracellular phosphorylation and cytoskeleton rearrangement (6). This would explain why α6 deletion in TEMs inhibits their infiltration, as TEM tumor infiltration involves laminin interaction and transendothelial migration steps. Administration of α6 blocking antibodies might also reduce TEM infiltration, a possibility not investigated by Lee *et al* (16) and Primo *et al* (17).

Interestingly, we also found fewer microvessels in tumors from α6fl/fl-Tie2Cre$^+$ mice, resulting in a slightly increased average vessel diameter. This could stem from a decreased sprouting capacity, as we have previously found that neovessel outgrowth from preexisting blood vessels in the *ex vivo* aortic ring assay is reduced in α6fl/fl-Tie2Cre$^+$ mice (19). In addition, it has been demonstrated using a three dimensional *in vitro* co-culture model, that α6 expression increases on both endothelial cells and pericytes when they are cultured together, and that the addition of an anti-α6 blocking antibody leads to an increased vessel width in pericyte-endothelial cell cocultures but not in endothelial cell monocultures (32). This suggests that α6 could play a role in pericyte-endothelial cell interactions and therefore in vessel morphogenesis and maturation. However, we did not find any significant genotype-dependent difference in the pericyte coverage of the blood vessels of B16F10 tumors from α6fl/fl-Tie2Cre$^+$ mice compared to α6fl/fl-Tie2Cre$^-$ mice, suggesting that pericyte recruitment is not impaired in this model.

Endothelial progenitor cells (EPCs) can also participate in tumor angiogenesis (33). We have previously shown that α6 is involved in EPC mobilization from bone marrow after ischemia (19). We used flow cytometry to determine the number of circulating EPCs in the peripheral blood of tumor-bearing mice, as previously described (19), and found no genotype-dependent difference (data not shown). However, it is possible that EPC recruitment to tumors may be reduced in

α6fl/fl-Tie2Cre^{+} mice compared to α6fl/fl-Tie2Cre^{-}, as we have previously shown that α6 is required for EPC recruitment to ischemic tissues (18).

This study highlights differences between Tie1Cre and Tie2Cre conditional knockout models. Our results confirm that α6 plays an important role in tumor growth and angiogenesis, by promoting neovessel formation and tumor infiltration by proangiogenic TEMs. Therapeutic targeting of α6 might affect the invasive properties of tumor cells, endothelial cells and TEMs, and could thereby reduce tumor growth and invasiveness.

Acknowledgements

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