RESEARCH ARTICLE

Tumor Angiogenesis in the Absence of Fibronectin or Its Cognate Integrin Receptors

Patrick A. Murphy, Shahinoor Begum, Richard O. Hynes*

Howard Hughes Medical Institute, David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA, United States of America

* rohynes@mit.edu

Abstract

Binding of α5β1 and αvβ3/β5 integrin receptors on the endothelium to their fibronectin substrate in the extracellular matrix has been targeted as a possible means of blocking tumor angiogenesis and tumor growth. However, clinical trials of blocking antibodies and peptides have been disappointing despite promising preclinical results, leading to questions about the mechanism of the inhibitors and the reasons for their failure. Here, using tissue-specific and inducible genetics to delete the α5 and αv receptors in the endothelium or their fibronectin substrate, either in the endothelium or globally, we show that both are dispensable for tumor growth, in transplanted tumors as well as spontaneous and angiogenesis-dependent RIP-Tag-driven pancreatic adenocarcinomas. In the nearly complete absence of fibronectin, no differences in vascular density or the deposition of basement membrane laminins, ColIV, Nid1, Nid2, or the TGFβ binding matrix proteins, fibrillin-1 and -2, could be observed. Our results reveal that fibronectin and the endothelial fibronectin receptor subunits, α5 and αv, are dispensable for tumor angiogenesis, suggesting that the inhibition of angiogenesis induced by antibodies or small molecules may occur through a dominant negative effect, rather than a simple functional block.

Introduction

Extracellular matrix proteins and their adhesion receptors are enticing targets for the regulation of tumor angiogenesis. The recruitment of new blood vessels by tumors is an important bottleneck in tumor development, without which tumors fail to grow. Thus, targeting tumor angiogenesis has been a therapeutic goal. Endothelial cell migration and survival is strongly regulated *in vitro* by adhesion to extracellular matrix, mediated by integrin receptors on the endothelium. Since the endothelium and its underlying matrix are readily targeted with small molecules and antibodies, disrupting matrix-integrin interactions would seem to be a useful method of inhibiting tumor angiogenesis.

Interactions between the extracellular matrix protein Fibronectin (FN) and its integrin receptors were some of the first such proposed targets, since FN and its receptors are strongly expressed around the tumor vasculature, and both are essential for developmental angiogenesis. Embryos and embryoid bodies deficient in FN fail to form vascular networks, despite proper

endothelial cell specification and vasculogenesis of the dorsal aorta and cardinal vein [1–3]. The FN binding integrins include α5β1, α4β1, α8β1, α9β1, αvβ1, αvβ3, αvβ5, αvβ6 and αvβ8 [4]. Embryos deficient in the α5 subunit (Itga5) of α5β1, considered the primary FN receptor, are embryonic lethal with vascular defects [5]. Combined deletion of integrin αv (Itgav) and α5 results in a more severe phenotype than deletion of αv alone, yielding a spectrum of defects resembling the FN-null embryos and suggesting that these two alpha subunits contribute to the primary FN receptors in embryonic vascular development [6]. Indeed, mutating the RGD motif in FN critical for binding of both α5β1 and αvβ/3β5 integrin receptors also results in embryonic lethality with vascular phenotypes [7]. Thus, several lines of genetic evidence suggest that binding of FN by α5- and αv-based integrins is critical for mammalian angiogenesis.

One of the critical processes regulated by the FN-binding integrins is the assembly of soluble FN into insoluble FN fibrils [8]. *In vitro* experiments suggest this is an essential step in incorporation of other matrix proteins, such as the fibrillins, latent-TGFβ-binding proteins, collagens, and elastin, and the subsequent development of the endothelial basement membrane [8]. Blocking FN assembly also disrupts vascular network formation *in vitro* and in collagen plugs *in vivo*, suggesting that establishment of the proper, FN-based basement membrane is essential for angiogenesis [9]. Although FN assembly increases in co-cultured endothelial and mural cells, which cell type is responsible for the assembly remains unclear [10]. Both are independently capable of FN production and assembly *in vitro*, and the same may be true *in vivo*. The complete endothelial deletion of α5 and αv did not significantly interfere with developmental angiogenesis or FN assembly *in vivo*, although isolated endothelial cells exhibited major defects in FN assembly *in vitro*, and most of these mice eventually succumbed to embryonic defects in the remodeling of the great vessels [11]. Likewise, deletion of α5 in smooth muscle cells and pericytes did not obviously affect vascular development, although it did cause defects in lymphatic valve remodeling [12]. Thus, FN assembly appears to be essential for the formation of the basement membrane and angiogenesis, but the cells type(s) critical for *in vivo* assembly during angiogenesis remain unclear.

Although early preclinical studies supported the utility of inhibitors of the FN- α5β1 and FN- αvβ3/β5 interactions, the clinical results thus far have been disappointing. The most advanced study to date, a Phase III clinical trial of the selective αvβ3 and αvβ5 integrin inhibitor Cilengitide revealed no treatment benefit [13]. A competitive inhibitor of the α5β1 synergy site important in FN binding, ATN-161, also moved to Phase II clinical trials, but there are no ongoing studies with this drug [14]. Antibodies targeting α5β1 more specifically have been no more successful. Volociximab, designed to bind α5β1 and block interactions with FN, did not result in significant therapeutic benefits in several clinical trials—some of which were discontinued for failing to reach primary thresholds [14]. PF-04605412, also designed to bind α5β1, failed to reach primary thresholds, despite effective suppression of tumor growth when used in preclinical xenografts [15]. It is difficult to know whether such treatments would have worked if the inhibition obtained were complete. In fact, low doses of Cilengitide have been shown to promote, rather than suppress, tumor angiogenesis [16]. Higher and more consistent doses are possible in pre-clinical models, suggesting the possibility that the level of inhibition achieved, rather than the target, may be the reason behind the potent pre-clinical effects and disappointing clinical results. Genetic mutation of the genes involved would help to resolve these questions, but due to the embryonic lethality of the α5 knockout, studies to date have involved teratocarcinomas (which recruit host vasculature) [17] and heterozygous or mosaic deletion of α5, neither of which fully addresses the requirement for this integrin in the vasculature of tumors [18]. Likewise, while portions of the FN protein [19] or plasma pool [20] have been genetically removed, the effect of complete FN ablation on tumor angiogenesis and matrix deposition has not been examined.

Here, to determine the absolute requirement for the FN-integrin interactions in tumor angiogenesis, we used genetic tools to remove the FN-binding integrin subunits α5 and αv from the endothelium, prior to tumor growth, in two transplant models and the RIP-Tag model of angiogenesis-dependent pancreatic cancer. We further tested the requirement for FN, from any source, in RIP-Tag tumors in mice with global post-natal deletion of FN.

Materials and Methods

Mice

Integrin *Itga5*$^{f/f}$; *Itgav*$^{f/f}$ (also called α5$^{f/f}$; αv$^{f/f}$) mice were generated from an intercross of our existing *Itga5*$^{f/f}$ and *Itgav*$^{f/f}$ lines, as we previously reported [11, 21]. *FN*$^{f/f}$ mice were obtained from R. Fassler [22]. To obtain endothelial deletion of integrins α5 and αv, or FN, the respective lines were crossed with *Cdh5-CreER*^{T2} (obtained from R. Adams [23]) or *ROSA-CreER*^{T2} (obtained from T. Jacks [24]) mice. Cre-mediated excision efficiency was monitored with the fluorescent *mT/mG* Cre-reporter mice [25], crossed with the *FN*$^{f/f}$ mice. The reporter switches from red to green when activated by Cre. *RIP1-Tag2* mice were obtained from the National Cancer Institute [26], and intercrossed with the α5$^{f/f}$; αv$^{f/f}$, *FN*$^{f/f}$, *Cdh5-CreER*^{T2}, *ROSA-CreER*^{T2}, and *mT/mG* mice.

In experiments with *Cdh5-CreER*^{T2} mice, excision was induced with 3 x 1mg Tamoxifen by intraperitoneal injection one week before tumor transplant, or at weeks 5–6 in RIP-Tag mice. Excision in *ROSA-CreER*^{T2} mice was induced with 5 x 1mg Tamoxifen by intraperitoneal injection at weeks 5–6 in RIP-Tag mice, and then either continued with 1 x 1mg weekly, or not.

All mice were housed and handled in accordance with approved Massachusetts Institute of Technology Division of Comparative Medicine protocols (IACUC approval 0412-033-15).

In brief, animals are monitored daily by animal care staff and laboratory personnel and appropriate measures are taken if infection, inflammation or distress occur. If body condition score is <2 or if aggregate tumor burden exceeds 1 cm in diameter, mice are euthanized by CO2 narcosis. We use a regulated flow valve (in addition to cylinder regulator) that restricts the CO2 flow rate to 20% of chamber volume per minute. These methods were chosen because they cause minimal stress to the animal. All methods of euthanasia are consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

Tumor transplant and quantitation

Lewis lung carcinoma (LL2, ATCC) or melanoma (B16/F10, ATCC) cells were injected subcutaneously on each dorsal flank, delivering either $1x10^5$ or $5x10^5$ cells to littermate mutant and control mice. Tumors were harvested 12 days (LL/2) or 18 days later (B16/F10) and weighed.

RIP-Tag tumor quantitation

Blood-filled angiogenic tumors were dissected from the pancreas of RIP-Tag mice at 10–11 weeks or 12–13 weeks of age and counted (10–11 weeks) or weighed (12–13 weeks).

RNA isolation and quantitation

RNA was extracted using the Qiagen RNAeasy mini-column kit, after combining the chloroform extract from Trizol 1:1 with 70% ethanol. A Promega kit was used to random prime a cDNA library from each tumor before expression analysis using the Bio-Rad iQ sybr mix (Table 1).

Table 1. Primers for quantitative PCR.

Gene	Forward	Reverse
Murine 18s	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
Murine FN	CTTTGTGGTCTCATGGGTCTC	AGCAGGTCAGGAATGTTCAC
Murine αv	TGGAACCTGCTTTCTTCAGG	CTACCAGGACCACCGAGAAG
Murine α5	TTCTCCGTGGAGTTTTACCG	GGTGGTGCACTGGATAGGAC
Genomic Murine Control	AACAAGAAAGGCCTCACTTCAG	GGTGTTCTCTGACTTATACATGCAC
Genomic Murine FN *No amplification product upon Cre-mediated excision*	GTGTGAGCCGGACAACTTC	TCACGCTTGCTCTGACTGAC

doi:10.1371/journal.pone.0120872.t001

Table 2. Antibodies used for immunofluorescence staining.

Antibody	Source	Dilution
Anti-FN	Rabbit polyclonal, Richard Hynes Lab	1:100
Anti-α5	BD (553319, clone 5H10-27)	1:200
Anti-ColIV	Abcam (ab6586)	1:100
Anti-Nid-1	Abcam (ab14511)	1:200
Anti-Nid-2	Abcam (ab14513)	1:200
Anti-laminin	Abcam (ab11575)	1:200
Anti-Fibrillin-1	Rabbit polyclonal, Lynn Sakai Lab (9543)	1:200
Anti-Fibrillin-2	Rabbit polyclonal, Lynn Sakai Lab (868)	1:200
Andi-CD31	BD (550274, clone MEC 13.3)	1:100

doi:10.1371/journal.pone.0120872.t002

Staining and fluorescence quantification

For quantification of fluorescence, tumors were fixed in 4% PFA, sucrose infused and snap frozen in Optimal cutting temperature compound (OCT, Tissue Tek) on a metal block cooled by liquid nitrogen, and then sectioned at 10μm thickness. Sections blocked in 2% BSA, 0.1% TritonX-100 in PBS were stained using the primary antibodies indicated (Table 2). All samples were stained and imaged in parallel for experiments in which comparisons are made.

Quantitation of staining area was performed in ImageJ. An arbitrary threshold was set, and the percentage of area above this threshold was quantified.

Statistics

Student's t test was used to compare means of two independent groups to each other.

Results

Tumor growth is not affected by the absence of endothelial integrin subunits Alpha5 and AlphaV

To determine the requirement for $\alpha5$ integrins in tumor angiogenesis, *Tie2-cre* mice were crossed with $\alpha5^{f/f}$ mice [11]. We previously found that this results in efficient deletion of endothelial $\alpha5$ by embryonic day 10.5 [11]. Despite the absence of endothelial $\alpha5$, we observed no defects in the final tumor mass of either subcutaneous Lewis Lung tumors, or B16 melanoma tumors (Fig. 1A&1B).

Since developmental deletion of $\alpha5$ was tolerated, but we had previously found that the combined *Tie2-Cre* mediated deletion of $\alpha5$ and αv in the endothelium resulted in vascular remodeling defects in the embryo, we hypothesized that αv might similarly compensate for the
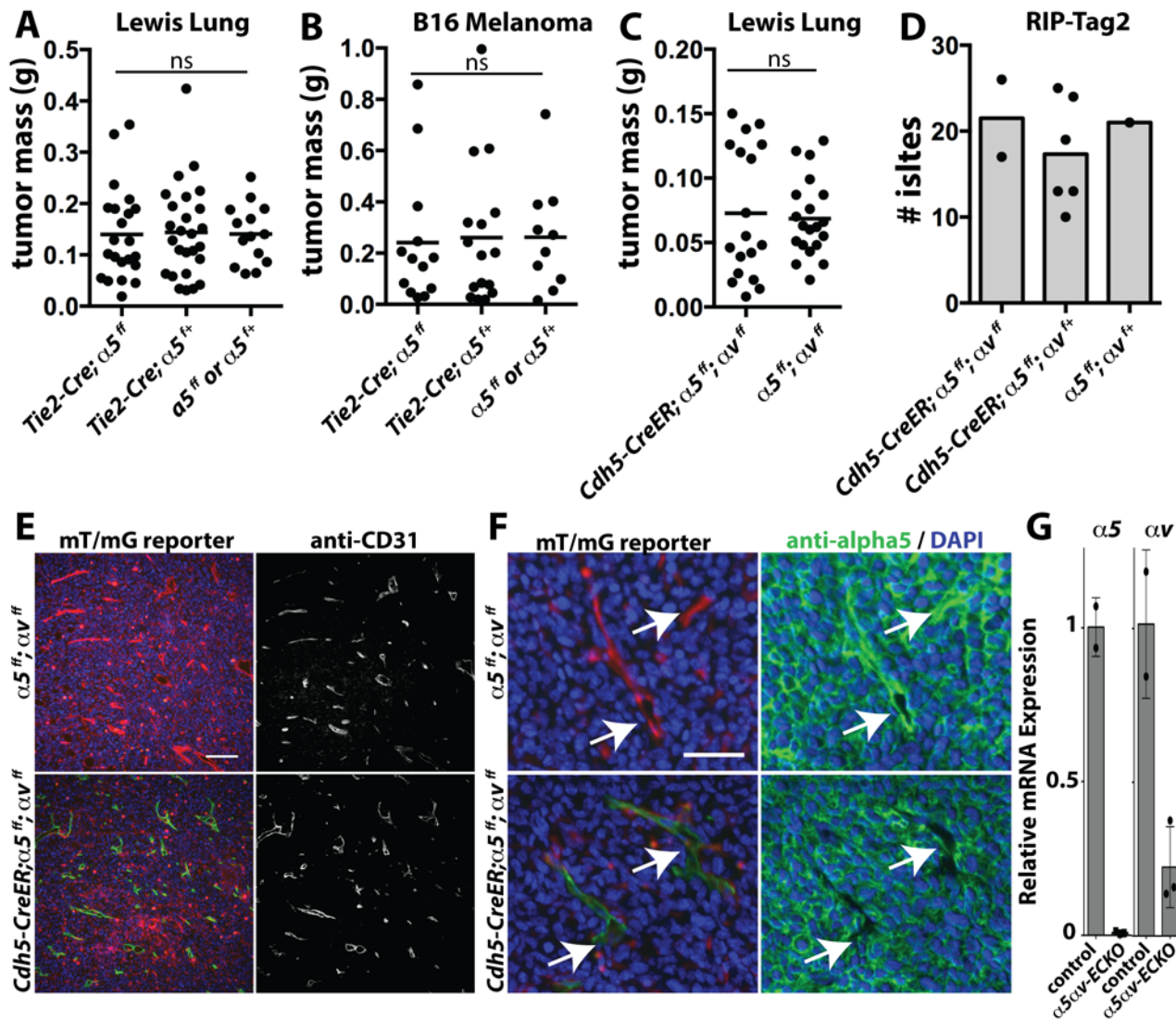
Fig 1. Tumor growth following deletion of integrins Alpha5 and Alphav in the endothelium. (A&B) Tumor mass 12 days after subcutaneous implantation of Lewis Lung (LL2) or 18 days after B16-F10 melanoma cells in the dorsal flank. Each point represents a single tumor, from paired injections into individual mice. (C) Tumor mass 12 days after subcutaneous implantation of Lewis Lung (LL2) cells in mice treated with 3x 1mg tamoxifen 1 week prior to tumor cell implantation. (D) Total number of angiogenic islets harvested from mice with pancreatic neuroendocrine tumours induced by RIP-Tag. (E) Immunofluorescence staining of endothelial marker CD31 or (F) integrin α5 in frozen sections of LL2 tumors from *Cdh5-CreER; mT/mG; α5 $^{f/f}$; αv $^{f/f}$* or *mT/mG; α5 $^{f/f}$; αv $^{f/f}$* mice. (G) Quantitative PCR analysis of RNA isolated from the aortic endothelium of *Cdh5-CreER* (control) or *Cdh5-CreER; α5 $^{f/f}$; αv $^{f/f}$* (α5αv-ECKO) mice treated one week earlier with 2x 1mg Tamoxifen. Expression of the indicated integrin mRNA, relative to 18s, is normalized to the genetic controls. Points indicate results in individual mice. Scale bars (E) = 100µm, (F) = 50µm.

doi:10.1371/journal.pone.0120872.g001

absence of *α5* in tumor angiogenesis [11]. To test this hypothesis, we generated *α5 $^{f/f}$; αv $^{f/f}$* mice, and crossed them with *Cdh5(PAC)-CreERT2* (hereafter referred to as *Cdh5-CreER*) mice allowing inducible deletion in the endothelium. In these mice, Cre was activated by tamoxifen treatment 1 week prior to tumor inoculation. We found no defect in the growth of the implanted Lewis Lung tumors (Fig. 1C).

Transplant models are highly selected and aggressive, therefore we also examined the requirement for endothelial *α5* and *αv* in the *RIP1-Tag2* (hereafter referred to as RIP-Tag) model of pancreatic cancer [27]. Transformation of the beta cells of the pancreas in this model is induced by insulin-promoter-driven SV40 expression. Cancer growth follows a well-

characterized progression from hyperplastic islets at 4–5 weeks of age, to angiogenic islets at about 7 weeks of age, and encapsulated adenomas by 10 weeks of age, and then finally to invasive adenomas by 14 weeks of age [28]. This is a selective process, and of the initial ~400 islets, 50% become proliferative, ~10% become angiogenic and only 1–2% go on to become tumors [29]. The model allows the genetic detection of defects in the angiogenic stage [30] as well as a later growth stage [31].

We treated mice with Tamoxifen at 5–6 weeks of age, and counted the number of angiogenic islets at 10 weeks of age. Here again, we saw no obvious reduction in the number of angiogenic islets, a measure of tumor angiogenesis (Fig. 1D).

We confirmed that *Cdh5-CreER* effectively excises endothelial $\alpha 5$ and αv by several methods. First, we crossed the *mT/mG* Cre reporter line with the *Cdh5-CreER; $\alpha 5^{f/f}$; $\alpha v^{f/f}$* mice. The reporter expresses membrane RFP in all cells from the ROSA locus, and converts to membrane GFP with Cre activation. We induced Cre activity by tamoxifen treatment 1 week prior to Lewis Lung tumor cell inoculation and examined tumor sections for Cre reporter activity, with co-staining for CD31 to confirm the location of endothelial cells. We found that *Cdh5-CreER* induced strong deletion (green) within the endothelium of Lewis Lung tumors (Fig. 1E). Cre activation strongly overlapped with CD31-labeled endothelium, whether or not the floxed $\alpha 5$ and αv alleles were included in the cross (Fig. 1E), suggesting that excised endothelial cells are not selected against in tumor growth. Staining of sections of these tumors for $\alpha 5$ showed strong $\alpha 5$ staining in the endothelium of controls, but very little staining in the marked endothelial cells of *Cdh5-CreER; mT/mG; $\alpha 5^{f/f}$; $\alpha v^{f/f}$* mice (Fig. 1F). Furthermore, endothelial expression of $\alpha 5$ and αv mRNA was effectively depleted, as measured in RNA collected from the aortic endothelium of similarly treated mice (Fig. 1G).

Thus, we conclude that $\alpha 5$ and αv are dispensable for tumor angiogenesis. Other integrins ($\alpha 4 \beta 1$, $\alpha 8 \beta 1$ and $\alpha 9 \beta 1$) are able to bind FN, and both $\alpha 4 \beta 1$ and $\alpha 9 \beta 1$ are expressed on the endothelium, albeit at lower levels. Thus, we cannot exclude the possibility that they are able to compensate for the loss of endothelial $\alpha 5$ and αv.

Endothelial deletion of Fibronectin does not inhibit tumor growth

Given the practical limitations of targeting all of the FN-binding integrins, we turned our attention to FN itself. To determine the requirement for endothelial FN in tumor growth, we used *Cdh5-CreER* to excise *FN* in the endothelium one-week prior to transplant of Lewis Lung tumor cells. We have previously found this results in >90% deletion of endothelial FN [32]. The absence of endothelial FN did not significantly affect the final Lewis Lung tumor mass (Fig. 2A).

Similarly, when we treated *RIP-Tag* mice with Tamoxifen at 6 weeks of age, we observed no reduction in the number of angiogenic, red islets in *Cdh5-CreER; FN^{f/f}* mice at 10 weeks of age (Fig. 2B).

Therefore, we conclude that endothelial *FN* is also not required for tumor angiogenesis. This, of course, does not exclude contributions of FN from other sources, since FN is produced by almost all of the cells in the tumor, and is also abundant in plasma.

Global deletion of Fibronectin delays the formation of angiogenic islets but does not affect final tumor mass

Total *FN* deletion is embryonic lethal, therefore we used *Rosa-CreER* to bypass embryonic lethality and excise *FN* globally in the post-natal mouse. We examined the requirement for *FN* in the *RIP-Tag* model system.

We deleted *FN* from the *RIP-Tag* tumors at the angiogenic stage (5–6 weeks) by tamoxifen treatment. To determine the effect of *FN* deletion at the angiogenic stage, we counted the
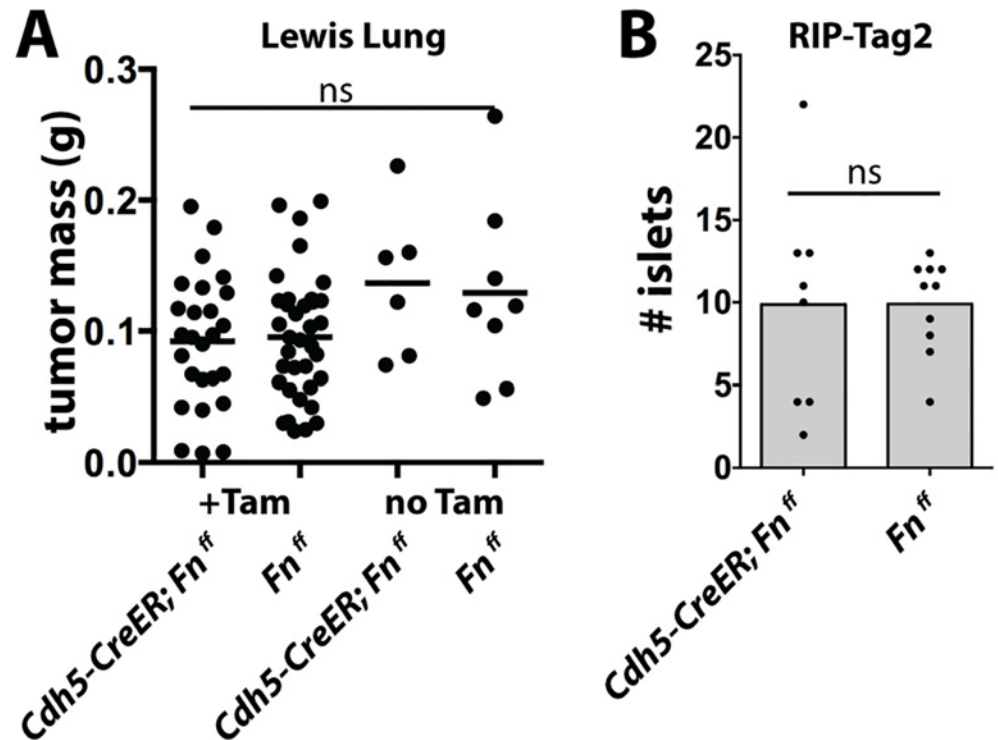
Fig 2. Tumor growth after deletion of endothelial Fibronectin. (A) Tumor mass 12 days after subcutaneous implantation of Lewis Lung (LL2) tumor cells into mice treated with 3x 1mg tamoxifen 1 week prior to tumor cell implantation. (B) Total number of angiogenic islets harvested from RIP-Tag mice at week 10–11.

number of angiogenic tumors at 10–11 weeks of age. We found that deletion of *FN* reduced (~30%) the total number of angiogenic islets ([Fig. 3A-3C](), P<0.004). To determine the effect on final tumor mass, we weighed all of the tumors from the pancreas of each individual mouse at 12–13 weeks. Surprisingly, we saw no significant effect on final tumor mass ([Fig. 3D]()).

To confirm the efficiency of gene deletion, we examined the *mT/mG* Cre reporter in *Rosa-CreER; mT/mG; FN^f/f; RIP-Tag* mice. We found that excision in the islets and adjacent pancreas was robust 1 week after the first tamoxifen treatment ([Fig. 3E]()). Initially, we had performed analysis with continuous weekly tamoxifen treatment, to prevent outgrowth of cells with intact FN floxed sites. However, analysis of the largest tumors from mice with either a single (5x 1mg, over one week) or continuous (5x 1mg + 1mg weekly thereafter) tamoxifen treatments showed no significant difference in final tumor mass ([Fig. 3D]()), or *mT/mG* Cre-reporter activation at endpoint ([Fig. 3E]()), suggesting that outgrowth of cells with intact *FN* floxed sites does not explain the maintenance of tumor growth.

To confirm the ablation of tumor FN, we examined sections of tumors from *Rosa-CreER; mT/mG; FN^f/f; RIP-Tag* mice and their Cre-negative or tamoxifen-negative controls at 12–13 weeks. We found the characteristic vascular pattern of FN staining was absent in the *Rosa-CreER; mT/mG; FN^f/f; RIP-Tag* mice with either single or continuous Tam treatment ([Fig. 3E]()). Quantification of the stained areas revealed a nearly complete ablation of FN in the *Rosa-CreER; mT/mG; FN^f/f; RIP-Tag* tumors ([Fig. 3F]()).

We asked whether there was any difference in the depletion of DOC-soluble FN versus the DOC-insoluble, or "fibrillar" FN. We found that, consistent with the histological immunofluorescence staining, both total and soluble FN were depleted by 12 days after the first tamoxifen
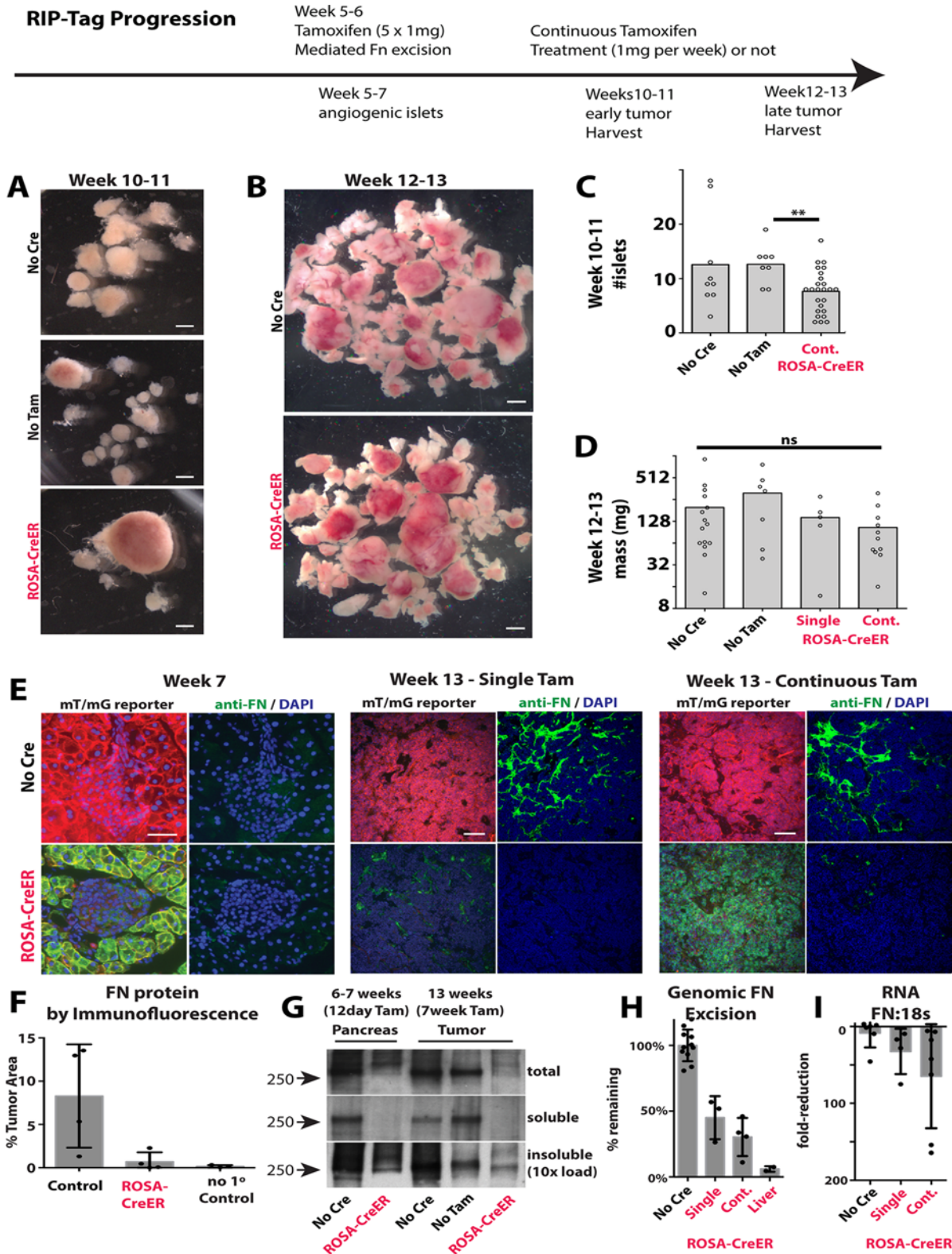
Fig 3. RIP-Tag Tumor growth in the absence of Fibronectin. (A&B) Isolated pancreatic tumors from individual mice. (C) Numbers of angiogenic islets isolated from individual mice. (D) Total mass of tumors isolated from individual mice. (E) Immunofluorescence staining for FN in the pancreas or in tumors in mT/mG reporter mice. (F) Percentage of the total tumor area covered by FN staining in individual mice. Each dot represents the average of three fields from a single mouse. Non-specific control represents the staining from pre-immune serum. (G) Western blot of equal tissue loads (by wet mass) of total pancreas or

tumor, and 1% DOC-soluble and insoluble protein. (H) Remaining FN genomic DNA after Cre-mediated deletion, as determined by qPCR relative to an unaffected genomic location. (I) Fold-reduction in FN mRNA expression relative to 18s. Each point represents the results from the largest tumor in each mouse. Scale bars (A&B) = 1mm, (E) 7 week = 50μm, 13 week = 100μm. ** p value <0.01.

treatment, and that the levels of total and soluble FN remained low to undetectable in most *Rosa-CreER; FN^{f/f}; RIP-Tag* tumors up to 13 weeks (N = 3) (Fig. 3G). FN was not entirely removed from the insoluble pool. It is not clear whether the remaining insoluble FN is derived from existing matrix, prior to FN excision, or FN deposited subsequent to FN excision and tumor growth.

We next asked whether FN expression by the tumor cells themselves was also reduced. First, we examined the efficiency in the deletion of *FN* in the tumors by quantitative PCR. We found that Cre activity resulted in a reduction of intact FN alleles by 40–75% with a single period of Tamoxifen treatment and 50–90% with continuous Tamoxifen treatments (Fig. 3H). RIP-Tag tumors were targeted less efficiently than the liver, which yielded a consistent >90% reduction in intact FN alleles with a single Tamoxifen treatment period (Fig. 3H, "liver"). There was no obvious increase in excision frequency with continuous Tamoxifen treatments.

We examined the levels of FN RNA, relative to 18s RNA in the largest tumors of individual mice. We found that FN expression was variable, but that several of the largest tumors isolated from *Rosa-CreER; FN^{f/f}* mice had >150-fold depletion of FN transcript (Fig. 3I).

Thus, we conclude that the genetic deletion of almost all FN in *RIP-Tag* tumors slightly reduces the number of initial angiogenic islets, but does not significantly suppress final tumor mass.

Angiogenesis and deposition of vascular basement membrane is not suppressed by Fibronectin deletion

In vitro studies [9] and *in vivo* developmental studies [1, 2] had suggested FN was essential for angiogenesis, yet apparently normal tumor growth occurred in the near absence of FN. Therefore, we examined tumor angiogenesis closely.

To determine whether the deletion of FN suppressed angiogenesis in the tumors of *Rosa-CreER; FN^{ff}; RIP-TAg* mice, we examined CD31 staining by immunofluorescence staining of histological sections of 12–13 week tumors from *Rosa-CreER; FN^{f/f}* mice and *FN^{f/f}* controls. We found no significant reduction in CD31-stained tumor area in the *Rosa-CreER; FN^{f/f}; RIP-Tag* mice (Fig. 4A-4C).

FN is typically found adjacent to CD31-stained blood vessels in RIP-Tag tumors, but perivascular FN staining is absent in *Rosa-CreER; FN^{f/f}; RIP-Tag* mice (Fig. 4D). Since FN assembly has been shown to be important in basement membrane assembly *in vitro* [10], we tested whether the absence of FN impaired the assembly of the basement membrane in tumors *in vivo*. However, despite the nearly complete absence of FN in the tumor, basement membrane components ColIV, laminin, Nid-1 and Nid-2 appeared to be properly assembled (Fig. 4E).

Thus, we conclude that nearly complete absence of FN in *Rosa-CreER; FN^{ff}; RIP-Tag* tumors does not reduce vessel density or appreciably alter the composition of basement membrane components.

No significant defects in the recruitment of a FN-linked regulator of TGF-beta signaling

In vitro studies have shown that FN plays a critical role in the deposition of other ECM proteins, including collagens, fibrillins, fibulins, latent TGFβ-binding proteins (ltbps), and
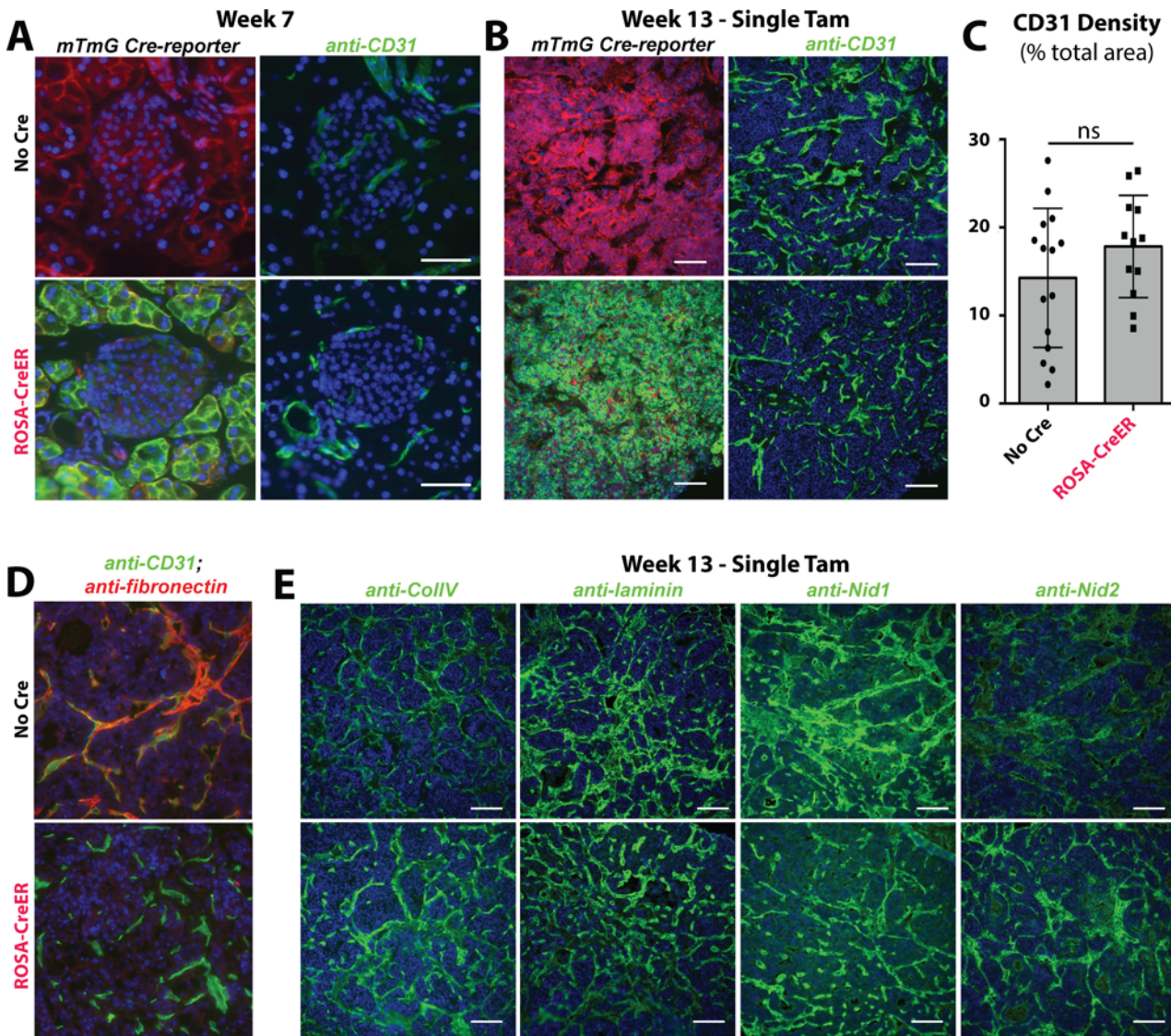
Fig 4. Angiogenesis in RIP-Tag tumors without Fibronectin. (A&B) Immunofluorescence staining of CD31+ vessels in pancreatic islets at 7 weeks and 12–13 weeks after activation of Cre. mT/mG reporter is shown in the same sections, showing the efficiency of Cre activity. Fig. 4A shows the adjacent section to Fig. 3E. (C) Area of the week 12–13 tumors that stained for CD31. The area of each individual field is shown, 3–4 fields per animal (N = 4 per group). (D) Immunofluorescence staining showing co-localization of CD31 and FN in sections from week 12–13 tumors. Each point represents results from a single tumor measurement. (E) Immunofluorescence staining for basement membrane proteins ColIV, laminin, Nid-1 and Nid-2. Scale bars (A) = 50μm, (B&E) = 100μm.

doi:10.1371/journal.pone.0120872.g004

Tenascin-C [8]. However, *in vivo* studies are not as clear. In mice with Mx-Cre-mediated excision of floxed FN in the liver, injury still induces a robust increase in ColI, ColIII, Ltbp-1, Ltbp-3, and Ltbp-4 incorporation into the extracellular matrix [33, 34].

We examined the expression of Fibrillin-1, Fibrillin-2, Ltbp-1, Ltbp-3 and Ltbp-4 by immunofluorescence in FN-deleted RIP-Tag tumors. We found abundant Fibrillin-1 expression, and no reduction in the percentage of tumor area with Fibrillin1 staining, relative to control tumors (Fig. 5A&5B). Only minimal levels of Fibrillin-2, Ltbp-1, Ltbp-3 and Ltbp-4 could be detected in tumors of either type, and were not obviously different (Fig. 5A and data not shown). Staining patterns for Fibrillin-1 were similar when detergent was excluded from the staining
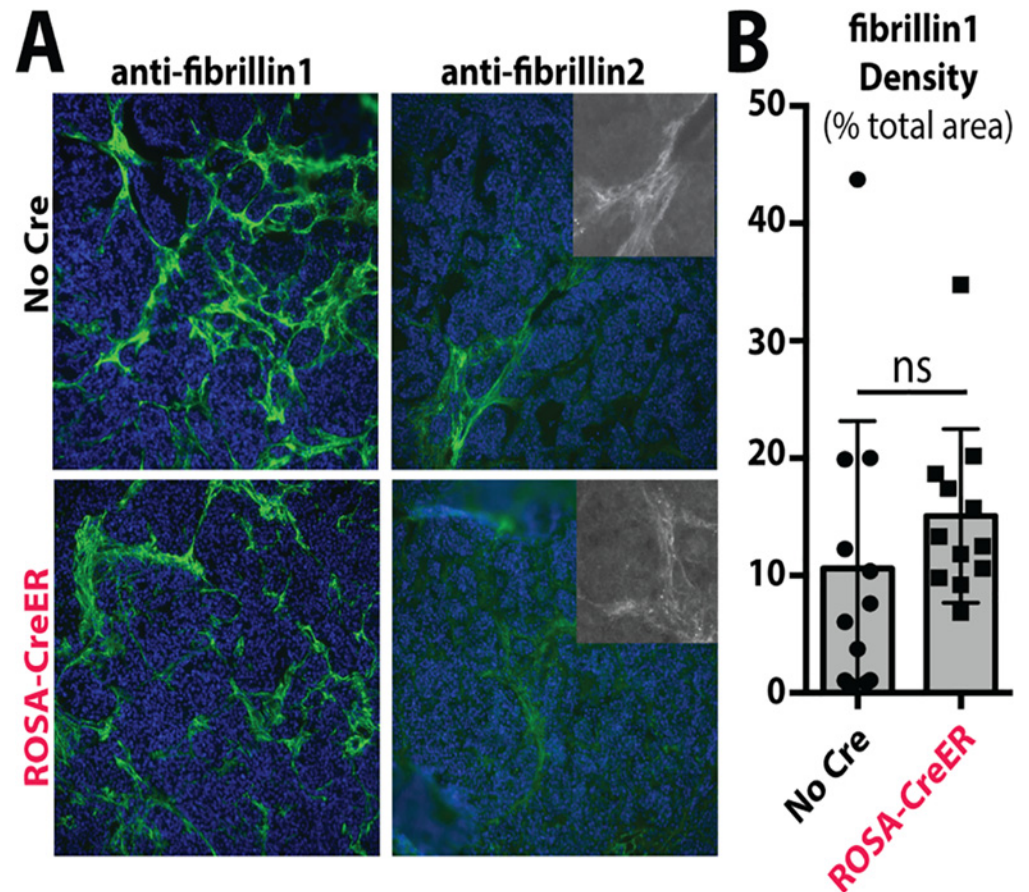
Fig 5. Matrix incorporation of other ECM proteins in the absence of Fibronectin. (A) Immunofluorescence staining of Fibrillin-1 and -2 in 12–13 week RIP-Tag tumors. Insets show fibrils in Fibrillin-2 staining. (B) Quantitation of total tumor area positive for Fibrillin-1 staining. Each point represents results from a single tumor measurement.

doi:10.1371/journal.pone.0120872.g005

buffer, suggesting that the fibrillar staining is extracellular matrix and not intracellular deposits (S1 Fig.).

Thus, we conclude that nearly complete deletion of *FN* in *Rosa-CreER; FN^{f/f}; RIP-Tag* tumors does not appreciably affect Fibrillin-1 incorporation into the extracellular matrix.

Discussion

FN and the FN integrin receptors α5β1 and αvβ3/β5 have been thought to be critical for new angiogenesis. Although antibody and small molecule targeting of the adhesion of these receptors to their FN ligand was a promising target for anti-angiogenic therapies, early optimism has been tempered by a series of disappointing clinical trials. However, since lower doses of FN-integrin blocking peptides have been shown to promote, rather than suppress angiogenesis [16], it has not been clear whether the initial pre-clinical success and subsequent clinical failures reflect targeting difficulties or true biological redundancy. Here, using temporally regulated and tissue-specific genetic deletion, we demonstrate that endothelial expression of α5 and αv is dispensable for tumor growth in two transplant models as well as a "spontaneous," *RIP-Tag*-driven model. Furthermore, we demonstrate that endothelial FN is not required for tumor growth. In fact, nearly complete ablation of FN by *ROSA-CreER*-mediated global deletion does

not significantly impede tumor angiogenesis, deposition of basement membrane proteins, or recruitment of matrix-linked proteins Fibrillin-1 and -2 (Figs. 4 & 5). Together, our results demonstrate that the growth of these tumor models is not significantly dependent on FN or its α5β1 and αvβ3/β5 integrin receptors, and suggest that other mechanisms may explain the effects of high-dose RGD inhibitors and integrin-blocking antibodies on angiogenesis observed in pre-clinical studies.

Our data suggest that the antibodies targeting the interaction between FN and its receptors may be doing more than simply blocking this interaction. Blockage of α5β1 binding to FN is a common feature of blocking antibodies—but is not necessarily their only function. Early pre-clinical work showed that antibodies blocking α5β1 binding to FN interfered with adhesion and migration of HUVECs *in vitro*, and potently inhibited FGF-induced angiogenesis in a chick CAM assay [35]. Volociximab, a humanized antibody that interferes with the binding of α5β1 to FN, blocks endothelial cell binding to FN *in vitro* and inhibits angiogenesis in animal models [36, 37]. A similar antibody, designed to bind to murine α5β1 and block its adhesion to FN, blocked angiogenesis in xenografted human tumors, suggesting that effects were mediated by interfering with murine host α5β1, rather than the human α5β1, which was not recognized by the antibody [38]. Yet, our results show that genetic deletion of either endothelial α5 or total FN has little effect on tumor angiogenesis. Differences in models may explain the differential effects. However, a more interesting possibility is that antibodies to α5β1 antagonize tumor angiogenesis not only by a simple block of the interaction between Fn and the α5β1 receptor, but rather by inducing some anti-angiogenic function in the integrin. The genetic deletion of β3 integrin (Itgb3) results in increased angiogenesis and tumor growth [39], while antibody targeting of αvβ3/β5 integrins or mutations of a tyrosine phosphorylation site that leave the β3 receptor intact but disrupt downstream signaling suppresses angiogenesis and tumor growth, suggesting a similar antagonistic effect [40]. While the findings in the RIP-Tag and transplant tumors we describe may not necessarily reflect the requirement for α5β1 and αvβ3/β5 integrins in human tumors, our results suggest that a better understanding of the effects of the blocking antibodies to α5β1 and αvβ3/β5 integrin receptors might be a fruitful path forward towards inhibiting tumor angiogenesis [41].

That robustly vascularized tumors grow in the near absence of FN was surprising, since embryos in which FN has been completely deleted have severe defects in developmental angiogenesis and die by embryonic day 8.5 to 9.5 [1, 2]. Subsequent work showed a requirement for FN in vascular network formation in embryoid bodies [3], and a requirement for the assembly of soluble FN in angiogenesis [9]. Why are the pancreatic tumors, but not the embryo, able to establish vascular networks in the absence of FN? One potential explanation is that FN is not entirely deleted from the tumors, and that the remaining FN is sufficient. Analysis of genomic DNA revealed that deletion ranged from 40–90% in isolated tumors, and reduced DOC-insoluble FN could be detected in Western blot analyses. However, there is effectively no detectable extravascular FN in the tumors, suggesting that if FN is playing a role, it does so as an organizer, acting at low concentrations, rather than simply a "building-block" of the fibrils. Alternatively, the longer time-course (several weeks for tumor angiogenesis, versus several days for embryo angiogenesis) and growing genetic heterogeneity may allow tumors time to compensate for the loss of FN in ways that the developing embryo cannot. This hypothesis is supported by an initial delay (at 10 weeks) in tumor growth in mice deleted for FN that is eventually overcome (Fig. 3). Potential compensating RGD-containing extracellular matrix proteins include the collagens, fibrillins and nidogens, all expressed around RIP-Tag tumor vessels, as we show here. Which, if any, are important in compensating for the loss of FN remains unclear.

In vitro studies have suggested a critical function for FN as an organizer of the extracellular matrix, necessary for the integration of other matrix proteins, including ColIV, Laminin, Fibrillin-1 and -2 [8, 10], yet we observed no significant reduction or alteration in the vascular deposition pattern in any of these matrix components in RIP-Tag tumors with nearly complete FN deletion. It is possible that, as with the requirement for FN in angiogenesis, the FN requirement in fibril organization can be achieved at a low concentration, or that its absence only slows, but does not halt matrix assembly. Subtle differences in assembly speed would be more clearly appreciated *in vitro* than in the weeks-long development of RIP-Tag tumors. Another possible explanation is that other matrix proteins could compensate for the function of FN in the deposition of other matrix proteins, as suggested by the initial delay in tumor growth that is overcome at later stages. The ECM produced by growing tumors is diverse, with dynamic changes in the ECM repertoire according to tumor phenotype [42–46]. In liver fibrosis, Col5 has been suggested to compensate for the deletion of liver FN [33]. Whether Col5, or other matrix proteins are able to compensate for the absence of FN in RIP-Tag tumor growth is unclear. Alternatively, compensation may be through changes in ECM-linked signaling pathways, such as Hippo [47]. Further analysis of compensatory measures in this model may reveal new modifiers of vascular matrix assembly.

Conclusions

Taken together, our results demonstrate that FN and the FN receptors are dispensable for tumor angiogenesis, raising new questions about the mechanisms underlying the anti-angiogenic activities of the blocking antibodies to the FN receptors and underscoring the importance of *in vivo* genetic studies to test the complex regulation of endothelial basement membrane assembly.

Supporting Information

S1 Fig. Staining of Fibronectin and Fibillin-1 in RIP-Tag tumors in the absence of detergent. Immunofluorescence staining of CD31 (green) and Fibronectin or Fibrillin-1 (red) in 12–13 week RIP-Tag tumors in the absence of detergent. Blocking buffer used was 3% Fn-depleted goat serum in PBS. Antibodies and concentrations were as reported in the methods section. (TIF)

Acknowledgments

We thank members of the Hynes lab for advice and discussions, especially Alexandra Naba, John Lamar and Chris Turner. We thank the Swanson Biotechnology Center at the Koch Institute/MIT, especially the Hope Babette Tang (1983) Histology Facility, and the Microscopy Facility, and Denise Crowley and Eliza Vasile for exceptional technical support. The authors wish to dedicate this paper to the memory of Officer Sean Collier for his caring service to the MIT community.

Author Contributions

Conceived and designed the experiments: PAM ROH. Performed the experiments: PAM SB. Analyzed the data: PAM ROH. Wrote the paper: PAM ROH.

References

1. George EL, Georges-Labouesse EN, Patel-King RS, Rayburn H, Hynes RO. Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin. Development. 1993 Dec; 119(4):1079–91. PMID: 8306876

2. George EL, Baldwin HS, Hynes RO. Fibronectins are essential for heart and blood vessel morphogenesis but are dispensable for initial specification of precursor cells. Blood. 1997 Oct 15; 90(8):3073–81. PMID: 9376588

3. Francis SE, Goh KL, Hodivala-Dilke K, Bader BL, Stark M, Davidson D, et al. Central roles of alpha5-beta1 integrin and fibronectin in vascular development in mouse embryos and embryoid bodies. Arteriosclerosis, thrombosis, and vascular biology. 2002 Jun 1; 22(6):927–33. PMID: 12067900

4. Astrof S, Hynes RO. Fibronectins in vascular morphogenesis. Angiogenesis. 2009; 12(2):165–75. doi: 10.1007/s10456-009-9136-6 PMID: 19219555

5. Goh KL, Yang JT, Hynes RO. Mesodermal defects and cranial neural crest apoptosis in alpha5 integrin-null embryos. Development. 1997 Nov; 124(21):4309–19. PMID: 9334279

6. Yang JT, Bader BL, Kreidberg JA, Ullman-Cullere M, Trevithick JE, Hynes RO. Overlapping and independent functions of fibronectin receptor integrins in early mesodermal development. Developmental biology. 1999 Nov 15; 215(2):264–77. PMID: 10545236

7. Takahashi S, Leiss M, Moser M, Ohashi T, Kitao T, Heckmann D, et al. The RGD motif in fibronectin is essential for development but dispensable for fibril assembly. The Journal of cell biology. 2007 Jul 2; 178(1):167–78. PMID: 17591922

8. Singh P, Carraher C, Schwarzbauer JE. Assembly of fibronectin extracellular matrix. Annual review of cell and developmental biology. 2010; 26:397–419. doi: 10.1146/annurev-cellbio-100109-104020 PMID: 20690820

9. Zhou X, Rowe RG, Hiraoka N, George JP, Wirtz D, Mosher DF, et al. Fibronectin fibrillogenesis regulates three-dimensional neovessel formation. Genes & development. 2008 May 1; 22(9):1231–43.

10. Stratman AN, Malotte KM, Mahan RD, Davis MJ, Davis GE. Pericyte recruitment during vasculogenic tube assembly stimulates endothelial basement membrane matrix formation. Blood. 2009 Dec 3; 114(24):5091–101. doi: 10.1182/blood-2009-05-222364 PMID: 19822899

11. van der Flier A, Badu-Nkansah K, Whittaker CA, Crowley D, Bronson RT, Lacy-Hulbert A, et al. Endothelial alpha5 and alphav integrins cooperate in remodeling of the vasculature during development. Development. 2010 Jul; 137(14):2439–49. doi: 10.1242/dev.049551 PMID: 20570943

12. Turner CJ, Badu-Nkansah K, Crowley D, van der Flier A, Hynes RO. Integrin-alpha5beta1 is not required for mural cell functions during development of blood vessels but is required for lymphatic-blood vessel separation and lymphovenous valve formation. Developmental biology. 2014 Aug 15; 392 (2):381–92. doi: 10.1016/j.ydbio.2014.05.006 PMID: 24858485

13. Stupp R, Hegi ME, Gorlia T, Erridge SC, Perry J, Hong YK, et al. Cilengitide combined with standard treatment for patients with newly diagnosed glioblastoma with methylated MGMT promoter (CENTRIC EORTC 26071–22072 study): a multicentre, randomised, open-label, phase 3 trial. The Lancet Oncology. 2014 Sep; 15(10):1100–8. doi: 10.1016/S1470-2045(14)70379-1 PMID: 25163906

14. Schaffner F, Ray AM, Dontenwill M. Integrin alpha5beta1, the Fibronectin Receptor, as a Pertinent Therapeutic Target in Solid Tumors. Cancers. 2013; 5(1):27–47. doi: 10.3390/cancers5010027 PMID: 24216697

15. Mateo J, Berlin J, de Bono JS, Cohen RB, Keedy V, Mugundu G, et al. A first-in-human study of the anti-alpha5beta1 integrin monoclonal antibody PF-04605412 administered intravenously to patients with advanced solid tumors. Cancer chemotherapy and pharmacology. 2014 Sep 12.

16. Reynolds AR, Hart IR, Watson AR, Welti JC, Silva RG, Robinson SD, et al. Stimulation of tumor growth and angiogenesis by low concentrations of RGD-mimetic integrin inhibitors. Nature medicine. 2009 Apr; 15(4):392–400. doi: 10.1038/nm.1941 PMID: 19305413

17. Taverna D, Hynes RO. Reduced blood vessel formation and tumor growth in alpha5-integrin-negative teratocarcinomas and embryoid bodies. Cancer research. 2001 Jul 1; 61(13):5255–61. PMID: 11431367

18. Taverna D, Ullman-Cullere M, Rayburn H, Bronson RT, Hynes RO. A test of the role of alpha5 integrin/fibronectin interactions in tumorigenesis. Cancer research. 1998 Feb 15; 58(4):848–53. PMID: 9485045

19. Astrof S, Crowley D, George EL, Fukuda T, Sekiguchi K, Hanahan D, et al. Direct test of potential roles of EIIIA and EIIIB alternatively spliced segments of fibronectin in physiological and tumor angiogenesis. Molecular and cellular biology. 2004 Oct; 24(19):8662–70. PMID: 15367684

20. von Au A, Vasel M, Kraft S, Sens C, Hackl N, Marx A, et al. Circulating fibronectin controls tumor growth. Neoplasia. 2013 Aug; 15(8):925–38. PMID: 23908593

21. Lacy-Hulbert A, Smith AM, Tissire H, Barry M, Crowley D, Bronson RT, et al. Ulcerative colitis and auto-immunity induced by loss of myeloid alphav integrins. Proceedings of the National Academy of Sciences of the United States of America. 2007 Oct 2; 104(40):15823–8. PMID: 17895374

22. Sakai T, Johnson KJ, Murozono M, Sakai K, Magnuson MA, Wieloch T, et al. Plasma fibronectin supports neuronal survival and reduces brain injury following transient focal cerebral ischemia but is not essential for skin-wound healing and hemostasis. Nature medicine. 2001 Mar; 7(3):324–30. Epub 2001/03/07. eng. PMID: 11231631

23. Wang Y, Nakayama M, Pitulescu ME, Schmidt TS, Bochenek ML, Sakakibara A, et al. Ephrin-B2 controls VEGF-induced angiogenesis and lymphangiogenesis. Nature. 2010 May 27; 465(7297):483–6. doi: 10.1038/nature09002 PMID: 20445537

24. Ventura A, Kirsch DG, McLaughlin ME, Tuveson DA, Grimm J, Lintault L, et al. Restoration of p53 function leads to tumour regression in vivo. Nature. 2007 Feb 8; 445(7128):661–5. PMID: 17251932

25. Muzumdar MD, Tasic B, Miyamichi K, Li L, Luo L. A global double-fluorescent Cre reporter mouse. Genesis. 2007 Sep; 45(9):593–605. PMID: 17868096

26. Adams TE, Alpert S, Hanahan D. Non-tolerance and autoantibodies to a transgenic self antigen expressed in pancreatic beta cells. Nature. 1987 Jan 15–21; 325(6101):223–8. PMID: 3543686

27. Hanahan D. Heritable formation of pancreatic beta-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes. Nature. 1985 May 9–15; 315(6015):115–22. PMID: 2986015

28. Hager JH, Hodgson JG, Fridlyand J, Hariono S, Gray JW, Hanahan D. Oncogene expression and genetic background influence the frequency of DNA copy number abnormalities in mouse pancreatic islet cell carcinomas. Cancer research. 2004 Apr 1; 64(7):2406–10. PMID: 15059892

29. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell. 1996 Aug 9; 86(3):353–64. PMID: 8756718

30. Inoue M, Hager JH, Ferrara N, Gerber HP, Hanahan D. VEGF-A has a critical, nonredundant role in angiogenic switching and pancreatic beta cell carcinogenesis. Cancer cell. 2002 Mar; 1(2):193–202. PMID: 12086877

31. Xie L, Duncan MB, Pahler J, Sugimoto H, Martino M, Lively J, et al. Counterbalancing angiogenic regulatory factors control the rate of cancer progression and survival in a stage-specific manner. Proceedings of the National Academy of Sciences of the United States of America. 2011 Jun 14; 108(24): 9939–44. doi: 10.1073/pnas.1105041108 PMID: 21622854

32. Murphy PA, Hynes RO. Alternative Splicing of Endothelial Fibronectin Is Induced by Disturbed Hemodynamics and Protects Against Hemorrhage of the Vessel Wall. Arteriosclerosis, thrombosis, and vascular biology. 2014 Jun 5.

33. Moriya K, Bae E, Honda K, Sakai K, Sakaguchi T, Tsujimoto I, et al. A fibronectin-independent mechanism of collagen fibrillogenesis in adult liver remodeling. Gastroenterology. 2011 May; 140(5):1653–63. doi: 10.1053/j.gastro.2011.02.005 PMID: 21320502

34. Kawelke N, Vasel M, Sens C, Au A, Dooley S, Nakchbandi IA. Fibronectin protects from excessive liver fibrosis by modulating the availability of and responsiveness of stellate cells to active TGF-beta. PloS one. 2011; 6(11):e28181. doi: 10.1371/journal.pone.0028181 PMID: 22140539

35. Kim S, Bell K, Mousa SA, Varner JA. Regulation of angiogenesis in vivo by ligation of integrin alpha5-beta1 with the central cell-binding domain of fibronectin. The American journal of pathology. 2000 Apr; 156(4):1345–62. PMID: 10751360

36. Bhaskar V, Fox M, Breinberg D, Wong MH, Wales PE, Rhodes S, et al. Volociximab, a chimeric integrin alpha5beta1 antibody, inhibits the growth of VX2 tumors in rabbits. Investigational new drugs. 2008 Feb; 26(1):7–12. PMID: 17786386

37. Avraamides CJ, Garmy-Susini B, Varner JA. Integrins in angiogenesis and lymphangiogenesis. Nature reviews Cancer. 2008 Aug; 8(8):604–17. doi: 10.1038/nrc2353 PMID: 18497750

38. Bhaskar V, Zhang D, Fox M, Seto P, Wong MH, Wales PE, et al. A function blocking anti-mouse integrin alpha5beta1 antibody inhibits angiogenesis and impedes tumor growth in vivo. Journal of translational medicine. 2007; 5:61. PMID: 18042290

39. Reynolds LE, Wyder L, Lively JC, Taverna D, Robinson SD, Huang X, et al. Enhanced pathological angiogenesis in mice lacking beta3 integrin or beta3 and beta5 integrins. Nature medicine. 2002 Jan; 8(1):27–34. PMID: 11786903

40. Hodivala-Dilke K. alphavbeta3 integrin and angiogenesis: a moody integrin in a changing environment. Current opinion in cell biology. 2008 Oct; 20(5):514–9. doi: 10.1016/j.ceb.2008.06.007 PMID: 18638550

41. Hynes RO. A reevaluation of integrins as regulators of angiogenesis. Nature medicine. 2002 Sep; 8(9): 918–21. PMID: 12205444

42. Naba A, Clauser KR, Whittaker CA, Carr SA, Tanabe KK, Hynes RO. Extracellular matrix signatures of human primary metastatic colon cancers and their metastases to liver. BMC cancer. 2014; 14:518. doi: 10.1186/1471-2407-14-518 PMID: 25037231

43. Naba A, Clauser KR, Lamar JM, Carr SA, Hynes RO. Extracellular matrix signatures of human mammary carcinoma identify novel metastasis promoters. eLife. 2014; 3:e01308. doi: 10.7554/eLife.01308 PMID: 24618895

44. Naba A, Hoersch S, Hynes RO. Towards definition of an ECM parts list: an advance on GO categories. Matrix biology: journal of the International Society for Matrix Biology. 2012 Sep-Oct; 31(7–8):371–2.

45. Naba A, Clauser KR, Hoersch S, Liu H, Carr SA, Hynes RO. The matrisome: in silico definition and in vivo characterization by proteomics of normal and tumor extracellular matrices. Molecular & cellular proteomics: MCP. 2012 Apr; 11(4):M111 014647.

46. Hynes RO, Naba A. Overview of the matrisome—an inventory of extracellular matrix constituents and functions. Cold Spring Harbor perspectives in biology. 2012 Jan; 4(1):a004903. doi: 10.1101/cshperspect.a004903 PMID: 21937732

47. Lamar JM, Stern P, Liu H, Schindler JW, Jiang ZG, Hynes RO. The Hippo pathway target, YAP, promotes metastasis through its TEAD-interaction domain. Proceedings of the National Academy of Sciences of the United States of America. 2012 Sep 11; 109(37):E2441–50. doi: 10.1073/pnas.1212021109 PMID: 22891335