Dissociation of Angiogenesis and Tumorigenesis in Follistatin- and Activin-Expressing Tumors

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Abstract
The transforming growth factor-β superfamily member activin and its antagonist, follistatin, act as a pleiotropic growth factor system that controls cell proliferation, differentiation, and apoptosis. Activin inhibits fibroblast growth factor 2–induced sprouting angiogenesis in vitro (spheroidal angiogenesis assay) and in vivo (Matrigel assay). To further study the role of the activin/follistatin system during angiogenesis and tumor progression, activin- and follistatin-expressing R30C mammary carcinoma cells were studied in mouse tumor experiments. Surprisingly, activin-expressing tumors grew much faster than follistatin-expressing tumors although they failed to induce increased angiogenesis (as evidenced by low microvessel density counts). Conversely, follistatin-expressing tumors were much smaller but had a dense network of small-diameter capillaries. Qualitative angioarchitectural analyses (mural cell recruitment, perfusion) revealed no major functional differences of the tumor neovasculature. Analysis of activin- and follistatin-expressing R30C cells identified a cell autonomous role of this system in controlling tumor cell growth. Whereas proliferation of R30C cells was not altered, follistatin-expressing R30C cells had an enhanced susceptibility to undergo apoptosis. These findings in experimental tumors are complemented by an intriguing case report of a human renal cell carcinoma that similarly shows a dissociation of angiogenesis and tumorigenesis during tumor progression. Collectively, the data shed further light into the dichotomous stimulating and inhibiting roles that the activin/follistatin system can exert during angiogenesis and tumor progression. Furthermore, the experiments provide a critical proof-of-principle example for the dissociation of angiogenesis and tumorigenesis, supporting the concept that tumor growth may not be dependent on increased angiogenesis as long as a minimal intratumoral microvessel density is maintained. (Cancer Res 2006; 66(11): 5686-95)

Introduction
Activins are members of the transforming growth factor β (TGF-β) superfamily of growth factors and the three best studied isoforms are activin A, activin B, and activin AB. Similar to TGF-β and bone morphogenetic protein, activin signaling involves two types of cell-surface receptors with serine/threonine kinase activity. The activated activin type I receptor phosphorylates Smad2 and Smad3, which in turn form a complex with the co-Smad, Smad4 (common mediator Smad4). The resulting Smad complex translocates to the nucleus, binds to the promoter region of target genes, and regulates their expression in conjunction with DNA-binding transcription factors and coactivators or corepressors (1). Activin signaling is extracellularly regulated by a high-affinity binding protein, follistatin (2). Follistatin is a single-chain glycoprotein that exists in two isoforms with different potencies to neutralize the effects of activin (3). The two isoforms of follistatin are generated by alternative splicing and contain 288 (follistatin 288) and 315 (follistatin 315) amino acids (4–6). Activin and follistatin were first isolated from the ovary based on their ability to stimulate and inhibit follicle-stimulating hormone release from the pituitary, respectively (7). However, their earlier supposed endocrine function is now questioned and recent evidence indicates that they may rather act as pleiotropic growth regulatory cytokines (8). Activin is synthesized in many adult tissues and cell types and activin receptors are coexpressed in those same tissues (8). Follistatin is also widely distributed and expressed in many tissues that synthesize activin (9, 10). Therefore, the activin/follistatin system is thought to primarily act as a local growth regulating system controlling proliferation, differentiation, and apoptosis of many cell types in an autocrine and paracrine manner (8, 11).

Activin A has been implicated in the control of endothelial cell functions with the discovery of type II activin receptor expression in human umbilical vein endothelial cells (HUVEC; ref. 12). Correspondingly, recombinant activin A inhibits proliferation of cultured endothelial cells (13). Likewise, capillary endothelial cells express the activin βA subunit in a number of human tissues (13). As such, activin seems to control endothelial cell functions in an autocrine manner, contributing to the maintenance of the quiescent endothelial cell phenotype (14). Endothelial cells express not only activin but also its functional antagonist follistatin. Yet, in contrast to the largely constitutive endothelial expression of activin, endothelial cell follistatin expression is dynamically regulated. Angiogenic endothelial cells express abundant levels of follistatin. Conversely, follistatin expression in quiescent, growth-arrested endothelial cells is down-regulated (14, 15). These differing functions and profiles of expression support a model whereby follistatin acts as an autocrine enhancer of endothelial cell proliferation by neutralizing the activin-mediated growth arrest (14). This model has also important biological implications as it conceptualizes the vascular endothelium not as a bradytrophic cell population capable of responding to exogenous angiogenic activation. Instead, the quiescent endothelial cell phenotype is...
actively maintained by endogenous growth inhibitory activity, which is neutralized during angiogenic activation.

The role of the activin/follistatin system during tumor growth and tumor angiogenesis has been noted but its implications have not been widely delineated (16, 17). Activin inhibits cell growth of many normal cell types as well as in some tumor types such as prostate cancer, breast cancer, B-cell leukemia, and in the prostate tumor cell line LNCaP (18). Likewise, activin induces programmed cell death in a number of different cell populations and over-expression of activin in LNCaP cells decreased the tumorigenicity of these cells (18). Yet, activin functions are more complex and stimulating effects on cell proliferation have also been reported for a number of cell populations including germ cells (19), fibroblasts (20), osteoblasts (21), trophoblast stem cells (22), synoviocytes (23), airway smooth muscle cells (24), and thymocytes (25).

Given the angiogenesis-inhibiting functions of activin and its proliferation-inhibiting effects on many cell types, we hypothesized that manipulation of the activin/follistatin system in tumors may be exploited as a potential therapeutic target to interfere with tumor growth and tumor angiogenesis. To further validate this system as a potential therapeutic target, we set out experiments aimed at studying the effect of follistatin and activin expression on the growth of human R30C mammary carcinoma cells grown as tumors in immunocompromised mice. Surprisingly, these experiments identified a dissociation of angiogenesis and tumorigenesis in activin- and follistatin-expressing R30C tumors. Tumors expressing the angiogenesis inhibitor activin were poorly vascularized but grew much faster than follistatin-expressing tumors. In turn, follistatin-expressing tumors grew very slowly despite a dense network of small capillaries, indicative of active ongoing angiogenesis.

Materials and Methods

Antibodies, Growth Factors, Cells, and Media

The following antibodies were used for this study: anti–phospho-Smad2 antibody (kindly provided by Prof. Carl-Henrik Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden), monoclonal rat anti-mouse CD34 antibody (Sanbio), rabbit polyclonal anti-Desmin antibody (Dianova), rabbit polyclonal anti-Ki67 antibody (Dako), and rat monoclonal antibody to mouse CD31 (clone MEC13.3; BD Bioscience). Human recombinant activin A and follistatin were purchased from R&D Systems and human recombinant fibroblast growth factor-2 (FGF-2) was purchased from Promega. FITC-labeled Bandeiraea simplicifolia lectin was from Sigma. R30C cells (human mammary tumor cell line) were kindly provided by Dr. Ralf Bargou (Max Delbrück Center Berlin for Molecular Medicine, Berlin, Germany) and transfected with Follistatin (FS-288) and activin under the control of the cytomegalovirus promoter or the empty vector (mock) using Lipofectamine (Invitrogen) according to the instructions of the manufacturer. Transfected cells were maintained in RPMI 1640 with Glutamax-I, with 25 mmol/L HEPES supplemented with 10% heat-inactivated FCS, 0.5 mmol/L sodium pyruvate, 60 units/mL penicillin, 60 μg/mL streptomycin, and 300 μg/mL hygromycin (Merck). HUVECs were freshly isolated from human umbilical veins of newborns by collagenase digestion. Cells were cultured at 37°C in 75-cm² tissue culture dishes in endothelial cell growth medium (PromoCell) containing 10% heat-inactivated FCS.

In vitro Angiogenesis Assay

Endothelial cell spheroids of defined cell number were generated and embedded as previously described (26). To quantitate in-collagen-gel angiogenesis, the cumulative length of all capillary-like sprouts originating from the central plain of an individual spheroid was measured at ×4 magnification using a digitized imaging system (DP-Soft, Olympus) connected to an inverted microscope (IX50, Olympus). Ten spheroids per experimental group per experiment were analyzed.

Matrigel Implant Assay

Matrigel (BD Biosciences) was thawed on ice and a concentration of 11.1 mg/mL and volume of 0.3 mL per plug were used. FGF-2 (300 ng/mL) and human recombinant activin A (100 ng/mL) were mixed into the Matrigel. Matrigel plugs were s.c. injected into nude mice. Mice were sacrificed 5 days postimplantation and plugs were fixed in 4% paraformaldehyde, embedded in paraffin, and stained for CD34.

Animals

Fox Chase severe combined immunodeficient (SCID) mice (C.B-17/scid/scid) were obtained from Taconic. Nude mice (NMRI) were obtained from Harlan-Winkelmann. Mice were housed in sterile cages in a temperature-controlled room and were fed with autoclaved food and water. All animals were handled in accordance with institutional guidelines for the care and use of experimental animals. Mice at the age of 5 to 6 weeks were chosen for experiments.

Tumorigenesis Assay

R30C cells (2 × 10⁶) stably transfected with activin, follistatin, or mock were suspended in 200 μL of PBS and s.c. injected into both flanks of SCID mice (C.B-17/scid/scid). Tumor size was measured twice weekly and tumor volume (V) was calculated according to the formula V = 1/6(abc)/π. Mice were sacrificed between 30 and 40 days of tumor growth or earlier when the tumor volume exceeded 2 cm³. Tumors were dissected and aliquots fixed in 4% paraformaldehyde for subsequent paraffin embedding and immunohistology or rinsed several times with PBS and embedded in tissue freezing medium for frozen tissue specimens (frozen on dry ice and then stored at −80°C). Aliquots were also quick-frozen in liquid nitrogen and stored at −80°C for activin A and follistatin immunoassays. Two activin-overexpressing clones, two follistatin-overexpressing clones, one mock-transfected control clone, and the parental R30C cell line were used in all experiments. All animal studies were approved by the Bezirksregierung Braunschweig, Germany.

Volumetric Computed Tomography Studies

Noninvasive imaging of tumors in mice was done on a prototype laboratory animal fP/VCT from GE Global Research with a resolution of 200 μm at 10% modulation transfer function (27). Anesthetized mice were scanned at 80 kV and 100 mA with 1,000 images per rotation (8 seconds per rotation). The contrast agent Isovist 300 (200 μL; Schering) was injected into the tail vein ~30 seconds before imaging.

Lectin Staining and Perfusion Fixation of Vasculature

FITC-labeled B. simplicifolia lectin (150 μL, 2 mg/mL; Sigma) was injected i.v. and allowed to circulate for 5 minutes. Thereafter, mice were sacrificed and tumors dissected, rinsed several times with PBS, and embedded in tissue freezing medium for frozen tissue specimen. Specimens were stored at −80°C. Sections of 10-μm thickness were cut with a cryostat, dried for several minutes, and fixed with 4% paraformaldehyde for 10 minutes.
Specimens were incubated with a rat monoclonal antibody to mouse CD31 and visualized with a biotinylated polyclonal goat anti-rat immunoglobulin secondary antibody and streptavidin-7-aminomethylcoumarin-3-acetic acid (Dianova).

**Immunohistochemistry**

Standard immunohistochemistry studies were done on paraffin-embedded tumor sections or cryosections using the different antibodies.

**Cell Cycle Analysis by Fluorescence-Activated Cell Sorting**

Cultured cells. Equal numbers of cells were seeded for all tested clones. All cells from six-well plates were harvested at the indicated time points either by trypsinization (living cells) or by collecting the cells in the supernatant (dead cells). Cells were resuspended in 0.5-mL PBS. For fixation, 5 mL of 100% methanol (−20°C) were added dropwise while mixing. Cells were left at 4°C overnight or at −20°C for 2 hours. Cells were then collected by centrifugation and resuspended in 500-μL PBS for 30 minutes. The cells were centrifuged again and resuspended in 500-μL PBS containing 100 μg/mL RNase A and 25 μg/mL propidium iodide. Cells were left for 30 minutes in the dark and then analyzed by fluorescence-activated cell sorting (FACS).

**Tumors.** Tumors were cut into small pieces and incubated in collagenase solution (0.5%) at 37°C for 90 minutes. Isolated cells were collected by centrifugation, resuspended in 1-mL PBS, counted, and 1 × 10⁶ cells were fixed and stained with propidium iodide (see above). Flow cytometric analysis was done on a Becton Dickinson FACSCalibur. Data from 10,000 cells per sample were analyzed with the CellQuest Cell Cycle Analysis software.

**Cell Proliferation**

Cells (1.5 × 10⁵) were seeded in six-well plates, collected by trypsinization on days 1, 3, 5, and 6 after seeding, and counted with a hemocytometer.

**Quantification of Intratumoral Microvessel Density**

CD34 stained sections were analyzed for the number of blood vessel at low power (×100) to assess the uniformity of vessel staining. Microvessels were then counted at ×400 magnification. To express microvessel densities (MVD), microscope-independent counts were transformed and expressed as the number of microvessels per square millimeter (1 microscopic field = 0.196 mm²). Five different fields in two sections for each tumor were examined to quantitate average MVD. Six different fields in three sections were analyzed in Matrigel assays.

**Statistical Analysis**

To detect differences between data sets, a Student’s t test (two sided, unpaired) was applied in which P < 0.05 was considered statistically significant.

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**Results**

**Effect of activin and follistatin on sprouting angiogenesis in vitro and in vivo.** Activin has previously been shown to inhibit endothelial cell proliferation and angiogenesis (13, 16, 17). Conversely, the activin inhibitor follistatin has been shown to induce endothelial cell proliferation and angiogenesis (14). Yet,
it has also been reported that activin induces the expression of vascular endothelial growth factor (VEGF; ref. 30) and that it controls tubulogenesis of endothelial cells (31, 32). To clarify the role of activin and follistatin during angiogenesis, we did in vitro sprouting angiogenesis assays and in vivo Matrigel plug assays.

Three-dimensional in vitro sprouting angiogenesis assays were done by embedding endothelial cell spheroids in a collagen matrix in which the spheroids serve as focal starting point for the growth of lumenerized capillary-like sprouts (26). Follistatin stimulated sprouting angiogenesis in this assay with a similar potency to FGF-2 (Fig. 1A) and VEGF (data not shown). Follistatin-induced sprouting angiogenesis was dose-dependently inhibited by human recombinant activin (Fig. 1A and Supplementary Fig. S1). Activin did not inhibit capillary sprouting below baseline levels but partially inhibited FGF-2-induced angiogenesis (Fig. 1A and Supplementary Fig. S1).

To assess the effect of activin on angiogenesis in vivo, Matrigel plugs containing FGF-2, human recombinant activin A, or a combination of both were s.c. implanted in mice. Ingrowth of vessels was quantitated 5 days later by staining for the pan-endothelial cell marker CD34 (Fig. 1B). Activin-containing Matrigel plugs had fewer microvessels than control plugs (Fig. 1B and C). Implantation of FGF-2-containing Matrigel plugs induced a strong angiogenic response (Fig. 1B and C). FGF-2-induced sprouting angiogenesis was significantly inhibited by coinoculation with activin (Fig. 1B and C).

Effect of activin and follistatin on R30C tumor growth in vivo. To study the effect of activin and follistatin on tumor growth and tumor angiogenesis, we generated clones of activin- and follistatin-overexpressing human R30C mammary carcinoma cells. Expression of activin and follistatin was confirmed by Northern blot (Fig. 2A and B, left) as well as by detecting follistatin and activin protein in the supernatant of transfected R30C cells by ELISA and RIA, respectively (Fig. 2A and B, left). The angiogenesis-inhibiting activity of follistatin and the angiogenesis-inhibiting activity of activin were confirmed by testing tumor cell supernatants in the three-dimensional in-collagen-gel sprouting angiogenesis assay (data not shown).

Two independent clones each of activin-expressing R30C cells (AC-1 and AC-2) and of follistatin-expressing R30C cells (FS-1 and FS-2) as well as mock-transfected cells were inoculated s.c. in SCID mice. Surprisingly, tumors expressing the angiogenesis inhibitor activin gave rise to rapidly growing tumors that progressed significantly faster than control follistatin-transfected cells and the parental cells (Fig. 2C). In turn, tumors expressing the angiogenesis stimulator follistatin grew significantly slower (Fig. 2C). Analysis of the concentrations of activin and follistatin in extracts of the tumors confirmed that the activin-expressing tumors contained 10-fold higher levels of activin compared with mock-transfected and parental cell-derived tumors (Fig. 2A, right).
Likewise, follistatin-expressing tumors contained 40-fold increased concentrations of follistatin compared with mock-transfected and parental cell–derived tumors (Fig. 2B, right).

The above experiments suggested that activin and follistatin exert opposing functions on the cells of the tumor compartment and of the vessel compartment. To confirm activin-mediated signaling in the tumor cell compartment, we assessed the activation status of the downstream intracellular mediator Smad2, which is phosphorylated by TGF-β and activin using a phospho-Smad2 antibody (33). R30C cells in activin-expressing tumors stained uniformly positive for nuclear phospho-Smad2 (Fig. 2D). In contrast, no phospho-Smad2–positive cells were detectable in follistatin-expressing tumors. Mock-transfected control tumors contained intermediate numbers of phospho-Smad2–positive cells (Fig. 2D). These data indicate that activin, but not TGF-β, is the primary cause for the Smad2 phosphorylation because all tumors were weakly positive for TGF-β (data not shown).

**Morphologic and functional characterization of activin- and follistatin-expressing tumors.** Vascularization of R30C tumors in mice was analyzed in vivo noninvasively by volumetric computed tomography (27) as well as by immunohistochemical analysis. Follistatin-expressing tumors grew smaller than mock-transfected or activin-expressing clones (Fig. 3A). Yet, they had a significantly higher density of microvessels (Fig. 3B and C). Quantification of average cross-sectional vessel area revealed that follistatin tumors contained a higher percentage of vessels with a surface area of <25 μm² (Fig. 3B and D). In contrast, activin-expressing tumors and mock-transfected tumors had, on average, larger vessel areas (Fig. 3B and D). Large-diameter vessels were also readily detectable by volumetric computed tomography scans of activin-expressing tumors (Fig. 3A).

To analyze the functional status of the tumor vasculature, we assessed the recruitment of mural cells (pericytes) to the tumor neovasculature and did perfusion experiments. Double immunofluorescence staining of endothelial cells with an antibody to CD31 and of mural cells with an antibody to Desmin identified a uniformly high coverage of microvessels with mural cells in all experimental groups (Fig. 4A and B). For the analysis of tumor vessel perfusion, mice were i.v. injected with FITC-conjugated BS1 lectin. The lectin was allowed to circulate for 5 minutes to label perfused vessels in situ. Perfused tumors were collected and processed for CD31 immunohistochemistry. The perfused vessels were expressed as the percentage of BS1-positive vessels to the total number of CD31-positive vessels. On average, between 70% and 80% of tumor vessels were perfused. However, there was no significant difference in tumor perfusion in the different experimental groups (Fig. 4C).

**Effect of activin and follistatin expression in R30C cells on cell proliferation, apoptosis, and cell cycle profile.** To assess the functional consequences of altered activin signaling in the different tumor cell transfectants, we analyzed proliferation and apoptosis of the different R30C cell populations. Immunohistochemical staining with a Ki67 antibody was done to detect proliferating cells (Fig. 5A). No difference in the percentage of Ki67-positive
tumor cells was detected in the different experimental groups (Fig. 5B). The analysis of apoptosis in activin- and follistatin-expressing tumors as well as in mock-transfected control tumors was done by TUNEL analysis (Fig. 5A). TUNEL-positive cells were equally distributed in all different tumor sections; however, follistatin-expressing tumors had a significantly higher rate of apoptosis as compared with activin-expressing tumors and mock-transfected tumors (Fig. 5C).

The above experiments suggested an imbalance of proliferation and apoptosis in follistatin-expressing R30C tumors. To analyze the cell cycle profiles of R30C cells in activin- and follistatin-expressing tumors as well as in mock-transfected control tumors was done by TUNEL analysis (Fig. 5A). TUNEL-positive cells were equally distributed in all different tumor sections; however, follistatin-expressing tumors had a significantly higher rate of apoptosis as compared with activin-expressing tumors and mock-transfected tumors (Fig. 5C).

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The above experiments have identified a dissociation of angiogenesis and tumorigenesis in activin- and follistatin-expressing R30C mammary tumors. The much faster growing activin tumors were capable of growing without significant necrosis despite the fact that these tumors failed to induce increased angiogenesis. Apparently, a density of 40 vessel/mm² (Fig. 3C), of which 80% were perfused (Fig. 4C), was sufficient to nourish these tumors. We consequently asked if a similar dissociation of angiogenesis and tumorigenesis may also occur in human tumors. Figure 7 depicts a case report showing an intriguing example of a renal cell carcinoma (RCC). Normal kidney, grade 1 RCC, and grade 3 RCC were found adjacent to each other within the same patient. The grade 1 and grade 3 tumors may have arisen independently or may originate from the same tumor and progressed independently through clonal dominance phenomena. Likewise, the observed tumor phenotype much more likely involves mutations of the von-Hippel-Lindau tumor suppressor gene (34) rather than a direct involvement of the activin/follistatin system. Whatever the cause for these two tumors in the same patient may be, the well-differentiated grade 1 RCC seems to be highly angiogenic with an average MVD of 740/mm², which corresponds

Figure 4. Mural cell recruitment and perfusion in activin-expressing, follistatin-expressing, and mock-transfected R30C tumors. A, tumor sections were double stained for CD31 expression detecting endothelial cells (left, green) and for Desmin expression detecting mural cells (pericytes, smooth muscle cells; middle, red). Right, extensive colocalization of the endothelial cell marker CD31 and the mural cell marker Desmin. Bar, 100 μm. B, quantitative analysis of mural cell recruitment was done by expressing the number of Desmin-positive vessels to the total number of CD31-positive vessels. Columns, mean; bars, SD. At least six tumors per experimental group were analyzed. C, quantitative analysis of vessel perfusion was done by expressing the number of BS-1-FITC-positive vessels to the total number of CD31-positive vessels. Columns, mean; bars, SD. At least five tumors per experimental group were analyzed.
well to previous reports on MVD in highly angiogenic RCC (35). In contrast, although the highly anaplastic grade 3 RCC had a much lower MVD of 44/mm², the tumor is not necrotic, suggesting that this MVD was sufficient to nourish the tumor.

Discussion

The TGF-β superfamily member activin and its antagonist follistatin act as a pleiotropic growth factor system that controls proliferation, differentiation, and apoptosis of numerous cell types in an autocrine and paracrine manner (1, 8, 11). Activin seems to primarily act as an inhibitor of cell proliferation. Yet, the proliferation of a number of different cell populations is also stimulated by this factor (19–25). As such, activins seem to be able to exert context-dependent and cell type–specific inhibiting and activating functions. Dichotomous inhibiting and stimulating cellular functions are likely mediated by differential downstream Smad molecule engagement, which is also observed for other TGF-β superfamily members, including TGF-β itself (36–38).

To shed further light into the role of the activin/follistatin system during tumor progression and tumor angiogenesis, we have generated activin- and follistatin-expressing human R30C mammary carcinoma cells and studied the tumor growth properties of these cells on inoculation in SCID mice. Collectively, the experiments have shown that (a) activin inhibits angiogenesis whereas the activin antagonist follistatin stimulates angiogenesis; (b) activin-expressing R30C tumors grow much faster than follistatin-expressing R30C tumors; (c) follistatin-expressing tumors have a significantly higher MVD than activin-expressing tumors; (d) activin-expressing and follistatin-expressing tumors have similar vessel maturation and perfusion properties despite distinct angioarchitectural differences in vessel diameter; and (e) follistatin-expressing tumor cells have a higher susceptibility to undergo serum starvation-induced apoptosis, which limits their tumor progression properties. Taken together, the data establish an important proof-of-principle for the dissociation of angiogenesis and tumor progression of which the clinical relevance was corroborated by a case report of an unusual human RCC, which similarly shows such a dissociation of angiogenesis and tumor progression.

The results of this study further validate and extend previous work showing an angiogenesis-inhibiting role of activin and an angiogenesis-stimulating role of follistatin. Activin has been shown to inhibit endothelial cell proliferation (13). Likewise, the activin antagonist follistatin has been shown to induce angiogenesis in the rabbit cornea assay (14). Yet, other authors have reported that activin induces the expression of VEGF (30) and that it controls tubulogenesis of endothelial cells (31, 32). Employing a three-dimensional spheroidal in vitro angiogenesis assay as well as an in vivo Matrigel plug assay, this study has in two independent experimental set-ups validated activin as an inhibitor of angiogenesis.

The angiogenesis-inhibiting functions of activin led us to hypothesize that activin may be able to act as a potent inhibitor of tumor growth. Surprisingly, activin-expressing R30C tumors grew much faster than follistatin-expressing R30C tumors. The analysis of the tumor cell transfectants identified an enhanced susceptibility of follistatin-expressing R30C cells for apoptosis and, correspondingly, a higher rate of apoptotic cells in follistatin-expressing R30C tumors. The detailed molecular mechanisms of increased apoptosis of follistatin-expressing tumors and the increased tumor growth of activin tumors await further analysis. Based on vascular pattern, such as MVD, vessel maturation, and

![Figure 5](https://example.com/figure5.jpg)

Figure 5. Proliferation and apoptosis of activin-expressing, follistatin-expressing, and mock-transfected R30C tumor cells grown as s.c. tumors in SCID mice. A, top, tumor sections were stained with an antibody against human Ki67 for the in situ assessment of proliferation (brown 3,3’-diaminobenzidine staining with hematoxylin counterstaining). Bar, 50 μm. Bottom, TUNEL staining for the in situ detection of apoptotic cells in tumor sections (green fluorescence staining resulting from the enzymatic incorporation of FITC-UTP into DNA strand breaks). Bar, 100 μm. B, quantitative analysis of cell proliferation was done by expressing the number of Ki67-positive tumor cells to the total number of tumor cells. CD31-positive vessels. C, quantitative analysis of apoptosis was done by expressing the number of TUNEL-positive tumor cells to the total number of tumor cells. B and C, columns, mean; bars, SD. At least 1,000 cells were counted for each tumor. Four tumors of each experimental group were analyzed. *P < 0.01, follistatin compared with mock and activin.
A, analyzed by FACS. At least 2,000 cells per tumor were analyzed by FACS follistatin-expressing, activin-expressing, and mock-transfected R30C cells. Cells iodide and examined by FACS analysis.

B, by collagenase treatment. Single cells were fixed and stained with propidium cell cycle distribution in tumors, tumor fragments were enzymatically dissociated to assess long-term proliferative potential of the different tumor cell transfectants, equal numbers of follistatin-expressing, activin-expressing, and mock transfected R30C cells were seeded and the cells were allowed to grow to confluence for 6 days. The number of cells was counted on days 1, 3, 5, and 6. Points, mean of duplicate analyses. The image shows one of three independent experiments with similar results. *, P < 0.01, follistatin compared with mock.

Figure 6. Cell cycle analysis and long-term proliferation experiments of activin- and follistatin-expressing tumors and cultured R30C cells. A, to assess cell cycle distribution in tumors, tumor fragments were enzymatically dissociated by collagenase treatment. Single cells were fixed and stained with propidium iodide and examined by FACS analysis. B, cell cycle distribution of cultured follistatin-expressing, activin-expressing, and mock-transfected R30C cells. Cells were serum starved for 24 hours, fixed, stained with propidium iodide, and analyzed by FACS. At least 2,000 cells per tumor were analyzed by FACS analysis. A and B, representative FACS scans of two independent experiments. C, to assess long-term proliferative potential of the different tumor cell transfectants, equal numbers of follistatin-expressing, activin-expressing, and mock transfected R30C cells were seeded and the cells were allowed to grow to confluence for 6 days. The number of cells was counted on days 1, 3, 5, and 6. Points, mean of duplicate analyses. The image shows one of three independent experiments with similar results. *, P < 0.01, follistatin compared with mock.

phospho-Smad2 antibody (41) allowed the detection of activated Smad2 in tumor sections, which revealed intense nuclear phospho-Smad2 staining in activin-expressing tumors but not in follistatin-expressing tumors, confirming the inhibitory role of follistatin on activin signaling.

The most surprising finding of the study is the observed dissociation of angiogenesis and tumorigenesis in activin- and follistatin-expressing R30C tumors. Angiogenesis is a prerequisite for tumor progression and metastasis (42). Correspondingly, the intratumoral MVD correlates in most human tumors with poor prognosis (43, 44). More than 1,000 MVD counting studies have been published thus far. A meta-analysis of all published MVD studies has determined MVDs in the most frequent human tumors to range between 75/mm² and 110/mm². MVD is an important angioarchitectural variable as it reflects the average intercapillary distance. MVDs in experimental tumors vary widely and range from <50/mm² to several hundred microvessels. Experimental tumors are prone to extensive necrosis if the MVD is significantly below 50/mm². This vessel density reflects the maximum intercapillary distance that is compatible with sufficient nourishment of the tumor cells by diffusion from the nearest blood vessel. Activin-expressing tumors had an average intratumoral MVD of ~40/mm². Likewise, the grade 3 RCC shown in Fig. 7C had an average intratumoral MVD of 44/mm². Neither the activin-expressing tumors nor the grade 3 RCC shown in Fig. 7C had extensive areas of necrosis. Thus, the intratumoral MVD in these tumors seemed to be compatible with the survival and growth of the tumor cells.

A similar dissociation of angiogenesis and tumorigenesis as observed in the present study has recently been reported in hypoxia-inducible factor (HIF)-2α- and dominant-negative HIF-2α-expressing gliomas (45) and in HIF-1α+/− embryonic stem cell-derived tumors (46, 47). HIF-2α-expressing tumors are strongly angiogenic. Yet, the tumors grow much slower than corresponding weakly angiogenic HIF-2α small interfering RNA-silenced or dominant-negative HIF-2α-expressing tumors. These authors report 190 vessels/mm² in HIF-2α-expressing tumors and 70 vessels/mm² in dominant-negative HIF-2α-expressing tumors. Survival of dominant-negative HIF-2α-expressing tumor cells in these weakly angiogenic tumors is enhanced by an increased resistance to undergo apoptosis. Similar findings have been reported in tumors derived from HIF-1α−/− embryonic stem cells. Loss of HIF-1α−/− prevents in these tumors formation of large vessels and impaired vascular function leading to a hypoxic microenvironment. Surprisingly, growth of HIF-1α−/− derived tumors was accelerated as compared with HIF-1α+/− embryonic stem cells (46, 47). These observations are also compatible with experimental findings suggesting that an increased resistance of tumor cells to apoptosis in p53−/− tumors allows the tumor cells to survive increased intercapillary distances induced by an antiangiogenic therapy. As a result, p53−/− tumors are less responsive to antiangiogenic intervention (48). Besides these experimental tumors, dissociation between MVD and survival of patients has already been reported in colorectal cancer patients (49). In this study, patients with an increased number of microvessels survived longer than those with a low density of microvessels. Moreover,

23 H.G. Augustin, unpublished data.
it has been suggested that some non–small-cell carcinomas can grow without inducing neoangiogenesis, suggesting that some tumors are capable of preying on the host vascular bed through mechanisms of vessel cooption (50).

Taken together, the present study further establishes the activin/follistatin system as a potent regulator of angiogenesis with activin acting as an angiogenesis inhibitor and follistatin acting as an angiogenesis stimulator. The observed dissociation of angiogenesis and tumorigenesis in activin- and follistatin-expressing tumors points towards an important role of tumor cell resistance to undergo apoptosis in balancing the consequences of an increased intercapillary distance that is associated with low MVDs. The findings also support the concept of a minimum MVD that a tumor needs to ensure to support its growth and survival. Conversely, the findings may also have important implications for antiangiogenic tumor therapies because the individual intratumoral MVD may determine the therapeutic window and efficacy of an antiangiogenic intervention.

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References

20. Sakurai T, Abe Y, Kasuya Y, et al. Activin A stimulates angiogenesis in a human RCC. Top, an overview of a histologic section of an unusual RCC in which a highly anaplastic grade 3 tumor (right) is adjacent to a well-differentiated grade 1 tumor (middle). Left, normal tissue of the kidney. The histologic H&E stainings show the architecture of the normal kidney with glomeruli and tubuli (A), a well-differentiated grade 1 RCC (B), and a highly anaplastic grade 3 RCC (C). CD34 staining of the tumor section identified a >15-fold higher MVD in the grade 1 tumor (B) than in the grade 3 tumor (C). Yet, this poorly vascularized tumor contains numerous pyknotic nuclei (C) but it is not necrotic, suggesting that this low MVD is sufficient to nourish the tumor. Bar, 100 µm (middle) and 50 µm (bottom).
Dissociation of Angiogenesis and Tumorigenesis in Follistatin- and Activin-Expressing Tumors

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