T-cadherin Supports Angiogenesis and Adiponectin Association with the Vasculature in a Mouse Mammary Tumor Model

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Abstract

T-cadherin delineates endothelial, myoepithelial, and ductal epithelial cells in the normal mouse mammary gland, and becomes progressively restricted to the vasculature during mammary tumorigenesis. To test the function of T-cadherin in breast cancer, we inactivated the T-cadherin (Cdhl13) gene in mice and evaluated tumor development and pathology after crossing the mutation into the mouse mammary tumor virus (MMTV)-polyoma virus middle T (PyV-mT) transgenic model. We report that T-cadherin deficiency limits mammary tumor vascularization and reduces tumor growth. Tumor transplantation experiments confirm the stromal role of T-cadherin in tumorigenesis. In comparison with wild-type MMTV-PyV-mT controls, T-cadherin–deficient tumors are pathologically advanced and metastasize to the lungs. T-cadherin is a suggested binding partner for high molecular weight forms of the circulating, fat-secreted hormone adiponectin. We discern adiponectin in association with the T-cadherin–positive vasculature in the normal and malignant mammary glands and report that this interaction is lost in the T-cadherin null condition. This work establishes a role for T-cadherin in promoting tumor angiogenesis and raises the possibility that vascular T-cadherin–adiponectin association may contribute to the molecular cross-talk between tumor cells and the stromal compartment in breast cancer. [Cancer Res 2008;68(5):1407–16]

Introduction

The adhesive function of classical cadherins cell adhesion molecules is well known to play a major role in maintaining tissue integrity. Loss of cadherin expression or function is associated with a loss of cellular and spatial control that characterizes neoplasia (1–5). T-cadherin shares the ectodomain structure with the classical cadherins and is anchored to the membrane via a glycosyl phosphatidylinositol moiety (6, 7). Like the classical transmembrane cadherins, T-cadherin is capable of conferring calcium-dependent homophilic cell adhesion (7). T-cadherin (also called Cadherin-13 or H-cadherin in humans) is implicated in diverse types of human cancers where gene expression is silenced through methylation (8–12). Indeed, down-regulation in human mammary neoplasia, together with the observation that human breast cancer cells forced to overexpress T-cadherin show reduced growth in culture, has led to the suggestion that T-cadherin may act as a tumor suppressor (13).

T-cadherin is expressed in diverse organs and cell types during development and in adulthood. Homophilic interactions are implicated in nervous system development and blood vessel growth. In the nervous system, T-cadherin distribution and function correlate with events of decreased adhesion, such as axon branching, defasciculation, and repulsion (14, 15). Similarly, T-cadherin is suggested in regulating adhesiveness of vascular cells (16–18). We noted abundant vascular T-cadherin expression in mouse tumors, including transgenic epithelial mammary tumors expressing the Neu oncogene or both Neu and vascular endothelial growth factor (19, 20). We thus hypothesized a possible role for T-cadherin in tumor angiogenesis. T-cadherin is a suggested binding partner for hexameric and high molecular weight forms of the fat-secreted, circulating hormone adiponectin (21). Adiponectin is much discussed as a regulator of metabolic and vascular functions (22), although its mode of operation and functions at the cellular and molecular level remain incompletely understood.

The aim for this current study was to determine the role of T-cadherin in breast cancer. We generated a null allele of T-cadherin in mice and challenged the phenotype of homozygous mutants in the mouse mammary tumor virus (MMTV)-polyoma virus middle T (PyV-mT) transgenic mammary cancer model. We report that loss of T-cadherin limits angiogenesis of mammary tumors, resulting in slower tumor growth, increased hypoxia, and pulmonary metastases. This phenotype correlates with the loss of adiponectin associated with the tumor vasculature and increased levels in the circulation. This work is the first to report a function for T-cadherin in promoting tumor angiogenesis in vivo and to suggest a link between T-cadherin and the association of adiponectin with blood vessels that may influence cross-talk between tumor cells and the stromal compartment.

Materials and Methods

Animal models and tissue preparation. All experiments were done in accordance with Burnham Institute for Medical Research Animal Research Committee guidelines. The generation of the T-cadherin null mice and biochemical characterization are described elsewhere. Wild-type MMTV-PyV-mT mice, originally generated by Dr. William Muller, were obtained in the C57Bl/6 background through Dr. Leslie Ellies (University of California San Diego, San Diego, CA). T-cadherin–deficient MMTV-PyV-mT mice were derived in two mating steps: (a) heterozygous male MMTV-PyV-mT mice were crossed with T-cadherin+/+ females and (b) male MMTV-PyV-mT T-cadherin+/− progeny was crossed with T-cadherin+/− females. T-cadherin−/− females were crossed with female MMTV-PyV-mT T-cadherin+/− and PyV-mT T-cadherin−/− mice. Genotypes were determined by PCR. TcadFOR 5′-CTCTGAACGTTATCGATGCAAGACAGAC-3′ and TcadREV
5'-CGGAGACACTGCCTGTGTTCTCATTG-3' amplified a 120-bp DNA fragment representing the wild-type allele. In the same reaction, the TcadFOR primer and the neomycin cassette primer 5'-GCATCGCCTTC-TATCGCCTTCTG-3' amplified the 350-bp mutant DNA fragment (Fig. 1). Tumor development was checked by palpitation thrice a week from 60 days of life and measured with digital calipers. Tumor volume was calculated as (length × width²)/2, and appearance, survival, and growth curves were derived in Prism by log-rank test and linear regression analyses. Wild-type MMTV-PyV-mT³³⁵/³²²F tumors were transplanted into the number 4 mammary glands as previously described (23, 24). The T-cadherin mutation was back-bred onto the FVB background for seven generations and 13 female mice of each genotype were used as hosts. Mice were sacrificed when T-cadherin+/+ and T-cadherin⁻/⁻ MMTV-PyV-mT tumors or the T-cadherin⁺⁺/⁻ recipients of MMTV-PyV-mTY³³⁵/³²²F tumors reached the institutionally set limit, or the mice were moribund. Hypoxia was induced as previously described (25). The animals were housed in the Institute's vivarium in compliance with the Animal Research Committee rules.

**Histology, immunohistochemistry, and image analysis.** Mice were sacrificed by CO₂ inhalation before removing mammary fat pads or tumors. One half of the tissue was fixed in 4% paraformaldehyde in PBS overnight, dehydrated, and embedded in paraffin. Sections were cut at 10 μm and stained with H&E. For pathologic evaluation, 88 tumors from 28 mice (14 from each genotype) were examined by Dr. Cardiff. The other half was snap-frozen in liquid nitrogen and sectioned frozen at 10 μm for immunohistochemical analysis. Sections were collected on slides, fixed in ice-cold acetone for 10 min, and air-dried. Unspecific binding sites were blocked with TBS with 0.05% Tween 20 (TBST) buffer containing 10% FCS.

**Figure 1.** T-cadherin expression in the mouse mammary gland. **A,** in 7-wk-old virgin mouse mammary glands, T-cadherin (a and e) colocalizes with CD31 on endothelial cells (b and c) and with smooth muscle actin (SMA) on the myoepithelium (f and g). T-cadherin also delineates the mammary ductal epithelium where it is primarily localized apically. d and h, representative cross sections of ductal epithelium. Bar, 50 μm. **B,** the gene-targeting vector for generic ablation of T-cadherin in mice. The neomycin cassette was inserted into the XhoI site (asterisk) introduced into exon 5 by point mutation. **C,** genotypes were distinguished by PCR using forward and reverse primers indicated by small arrows in B to amplify the 120-bp wild-type and the 350-bp mutant DNA fragments, respectively. **D,** Western blot analysis of three wild-type mammary fat pads identifies a doublet representing the mature 100-kDa T-cadherin protein and the 130-kDa precursor containing the proprotein region. No protein is detected in T-cadherin null mice. Equal loading is confirmed with anti-β-tubulin antibodies.
The generation and specificity of T-cadherin antibodies are described elsewhere. Antibodies to CD31 (clone 390, PharMingen), SMA (clone 1A4, Sigma), E-cadherin (clone EC2D-2, Zymed), phospho-histone H3 (Ser10) (Upstate Cell Signaling Solutions), Acrp30 (adiponectin, PA1-054, Affinity Bioreagents), and β-tubulin [clone E7, derived by Michael Klymkowsky (University of Colorado, Boulder, CO) from the Developmental Studies Hybridoma Bank (Iowa City, IA)] were purchased from the indicated commercial sources. Hypoxia analyses were done with a commercial kit (HypoxyProbe, Chemicon). Sections were incubated with primary antibodies in TBST overnight. For immunofluorescence, donkey anti-rabbit streptavidin-Alexa 488 conjugate (Molecular Probes) was used to detect T-cadherin and phospho-histone H3 antibodies, and anti-rat streptavidin-Alexa 594 conjugate (Molecular Probes) to detect CD31 and E-cadherin antibodies. Secondary antibodies were applied for 30 min. Apoptotic cells were identified with a commercial terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) immunofluorescence staining kit (ApopAlert DNA fragmentation assay kit, BD Biosciences). For bromodeoxyuridine (BrdUrd) incorporation, mice were injected with 0.01 mL/g BrdUrd (Amersham, GE Healthcare) 2 h before sacrifice. Frozen tissue sections were treated with 2 mol/L HCl, neutralized with 0.1 mol/L borate buffer (pH 8.5), and processed for detection with a BrdUrd-specific antibody [clone BU1/75 (ICRI), Oxford Biotechnology]. Sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes) for 10 min and mounted in Fluorescent Mounting Medium (DAKO). Staining signals were analyzed by confocal microscopy (MRC-1024 MP, Bio-Rad) or by capturing images of constant exposure with a Spot camera (Diagnostic Incorporated) on a Zeiss Axiosverter 405M microscope for analyses in Photoshop. For tumor statistics, four random images of solid tumor from two sections of the largest tumor from each mouse were analyzed. CD31 and HypoxyProbe stainings were related to tumor area. Vessel branch points were identified in CD31-stained sections and related to tumor surface area. Apoptotic and phospho-histone H3–positive cells were related to the total number of DAPI-stained nuclei using ImagePro software. Statistical analyses were done with Prism software using the Student t test on all data sets. Statistics are expressed as mean ± SE.

Mammary gland whole mounts. Number 4 mammary glands were processed overnight in Carnoy's fixative and stained with carmine for several hours. After dehydration in xylene, glands were mounted with Permount (Fisher Scientific). Images were collected using a Olympus dissecting microscope and ductal branching and neoplastic area were analyzed using ImagePro software.

Retinal staining. Retinal neovascularization was induced by sequential exposure of P7 old mice to 75% oxygen followed by normoxic conditions for several hours. After dehydration in xylene, glands were mounted with Permount (Fisher Scientific). Images were collected using a Olympus dissecting microscope and ductal branching and neoplastic area were analyzed using ImagePro software.

Immunoblotting. Tissues were lysed in radioimmunoprecipitation assay buffer [50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mmol/L sodium orthovanadate, 50 mmol/L NaF, 1 mmol/L sodium molybdate, 40 mmol/L β-glycerophosphate, 1 mmol/L phenylmethylsulfonyl fluoride, and 1/100 protease inhibitor cocktail, Sigma, P8340], mechanically dissociated by sonication, and centrifuged at 14,000 rpm for 10 min at 4°C. Thirty-microgram supernatant protein was loaded per lane and separated on SDSPAGE gels under reducing conditions.

Results

T-cadherin delineates the vasculature in the mammary gland and mammary tumors. As we used the mammary gland as our experimental model, we first established the distribution of T-cadherin in wild-type mouse virgin breast tissue. Immunohistochemistry distinguished prominent T-cadherin expression on the apical surfaces of the polarized ductal mammary epithelium (Fig. 1A, a and e) and on CD31-positive endothelial cells (Fig. 1A, b and c). T-cadherin was also observed on the myoepithelium identified by smooth muscle actin expression (Fig. 1A, f and g). The histologic structure of the glands can be appreciated in Fig. 1A, d and h. Thus, T-cadherin delineates the myoepithelium, epithelium, and endothelium of virgin mouse mammary glands.

Generation and overall phenotype of T-cadherin–deficient mice. To test the in vivo functions of T-cadherin, we generated mice deficient for T-cadherin gene expression. The generic targeting vector was created by insertion of the neomycin cassette into the XhoI restriction site created within exon 5 of the T-cadherin (Cdh13) gene by site-directed mutagenesis (Fig. 1B). Exon 5 encodes amino acids 161 to 210 within the T-cadherin extracellular domain 1. After homologous recombination in embryonic stem cells, germ-line chimeras were generated. Southern blotting (data not shown) and amplification of mutant DNA by the PCR (Fig. 1C) verified the homologous recombination event. The T-cadherin null mice were viable and fertile. On first inspection, their phenotype was indistinguishable from their wild-type counterparts, including the lack of spontaneous tumor formation over a normal life span. Western blot analysis confirmed abundance of both the mature 100-kDa T-cadherin protein and the 130-kDa unprocessed precursor in the normal virgin mammary gland and deletion in the null mutant (Fig. 1D). To address if T-cadherin is required for normal mammary gland development, we examined the ductal patterning of virgin female T-cadherin+/− T-cadherin−/− fat pads by carmine staining of whole-mounted glands. We observed no overt differences between genotypes in the degree of mammary ductal growth and branching (Supplementary Fig. S1). Moreover, sexually mature females deficient for T-cadherin produced and nourished multiple rounds of normal-sized litters (data not shown). Thus, mammary gland development and function seem to proceed normally in the absence of T-cadherin.

T-cadherin deficiency restricts tumor growth in the MMTV-PyV-mT transgenic model. Because T-cadherin has been implicated in breast cancer in humans, we sought to establish a mouse model for examining the role of T-cadherin in mammary cancer. To accomplish that, we generated T-cadherin–deficient, tumor-bearing mice by crossing the T-cadherin mutation into syngeneic C57Bl/6 transgenic mice expressing the PyV-mT antigen from the mouse mammary tumor virus promoter (MTTV-PyV-mT; ref. 26). The MMTV-PyV-mT mouse model is fast and reliable. Females form hyperplasias with 100% penetrance and display four identifiable mammary tumor stages classified as benign in situ proliferative lesions to invasive carcinomas with a high frequency of distant metastases (27, 28). These stages mimic the expression of biomarkers characteristic of human mammary tumors with poor prognosis (29). We first examined the expression of T-cadherin during epithelial-mesenchymal transition in wild-type MMTV-PyV-mT tumors. At 85 days of life, T-cadherin was strongly expressed in the CD31-positive endothelium (data not shown) and in E-cadherin–labeled, polarized ductal epithelial cells and developing neoplasias (Fig. 2A, a–c). Contrastingly, developed...
cancers displayed decreased or no T-cadherin tumor cell expression. In both early and advanced tumors, T-cadherin delineated the CD31-positive vasculature (Fig. 2A, e–g). Fig. 2A (d and h) depicts the histologic structure of the normal ductal, hyperplastic epithelium and the appearance of advanced MMTV-PyV-mT tumors, respectively. Western blot analysis confirmed the significant reduction in T-cadherin levels in wild-type MMTV-PyV-mT tumors, respectively. Western blot analysis confirmed the significant reduction in T-cadherin levels in wild-type MMTV-PyV-mT tumors, respectively. Western blot analysis confirmed the significant reduction in T-cadherin levels in wild-type MMTV-PyV-mT tumors, respectively. Western blot analysis confirmed the significant reduction in T-cadherin levels in wild-type MMTV-PyV-mT tumors, respectively.

To test if the mutation affected overall tumor onset, growth, and progression, we examined T-cadherin−deficient and wild-type MMTV-PyV-mT transgenic mice for palpable tumors thrice a week, starting at 60 days of age. Mammary tumors were detected in wild-type MMTV-PyV-mT mice with a median onset of 96.5 days. In the T-cadherin mutants, tumor growth was delayed by 10 days showing a median onset of 106.5 days (P = 0.0268; Fig. 3A). Monitoring the degree of neoplastic growth in both genotypes in whole mounts of number 4 fat pads at 85 days of life (i.e., before tumors were palpable in the wild-type condition; Fig. 3B, a and b) revealed a 3-fold reduction of the area covered by neoplastic growth in the T-cadherin+/− MMTV-PyV-mT mice in comparison with the wild-type (16.74 ± 2.254% for T-cadherin+/+ and 5.413 ± 1.627% for T-cadherin+/−; n = 9 for both genotypes, P = 0.0009; Fig. 3B, c). T-cadherin−deficient MMTV-PyV-mT mice also survived their wild-type counterparts by an average of 18.5 days: The median survival time was 151 days for the wild-type and 169.5 days for the T-cadherin null mice (P = 0.0008; Fig. 3C). Consistently, the growth kinetics for the two largest tumors in each animal differed significantly between genotypes (P < 0.0001; Fig. 3D). These data establish that T-cadherin deficiency delays onset and restricts growth of mouse mammary tumors.

**Limited vascularization in T-cadherin−deficient mammary tumors.** To define the cellular events leading to restricted tumor growth in the T-cadherin−deficient mice, we examined mutant and wild-type MMTV-PyV-mT tumors for differences in cell proliferation, blood vessel density, apoptotic rates, and hypoxia. First, because T-cadherin can affect cell cycle progression (30), we examined the proliferative potential of wild-type and T-cadherin−deficient tumors. BrdUrd incorporation (data not shown) and immunostaining for phosphorylated histone revealed no overt changes in the proliferative potential of tumor cells between genotypes (Fig. 4A, a–c). Second, because T-cadherin is prominent in the tumor vasculature (19, 20), we investigated the vascular coverage of MMTV-PyV-mT tumors in both genotypes. CD31 immunostaining detected a 31% reduction in endothelial cell density in T-cadherin+/− MMTV-PyV-mT tumors as compared with the wild-type (Fig. 4B, a–c; 5.816 ± 0.4326% for T-cadherin+/− versus 4.004 ± 0.3110% for T-cadherin+/+; P = 0.0022). Vessel branching was reduced by 45% in the null condition (Fig. 4B, a, b, and d; 77.57 ± 3.446 branch points for T-cadherin+/− and 42.28 ± 2.531 branch points for T-cadherin+/+; P < 0.0001). These results indicate a function for T-cadherin in tumor neovascularization. To obtain further evidence for the suggested proangiogenic role of T-cadherin, we assessed neovascularization in the retina after hypoxia (25). Flat-mounted retinas from wild-type and T-cadherin−deficient MMTV-PyV-mT mice were stained with CD31 antibodies (Fig. 4C, a–c). The branching index was 1.627% for T-cadherin+/+, 0.3110% for T-cadherin+/−, 3.446% for T-cadherin+/+; and 2.531% for T-cadherin+/−. These data indicate a function for T-cadherin in tumor neovascularization. To establish that T-cadherin deficiency delays onset and restricts growth of mouse mammary tumors.

![Figure 2](cancerres.aacrjournals.org/article-fig2)

*Figure 2.* Expression of T-cadherin in the neoplastic mammary gland. A, in wild-type MMTV-PyV-mT mammary fat pads at P85, T-cadherin (a) and E-cadherin (b and c; white arrows) delineate the normal ductal epithelium. Reduced levels of T-cadherin are detected on developing neoplasias (central white box and magnified image box; a–c). For comparison, the histologies of a normal duct (asterisk) and developing neoplasias (black arrows) are illustrated (d). Bar, 50 μm. In developed wild-type MMTV-PyV-mT tumors, T-cadherin (e) is detected only on the CD31-positive vasculature (f and g). h, pathology of a T-cadherin−/− MMTV-PyV-mT tumor. Bar, 100 μm. I, Western blotting shows T-cadherin in the fat pads of T-cadherin+/− female mice and its down-regulation in the MMTV-PyV-mT transgenic mammary gland. No T-cadherin protein is detected in breast tissue from T-cadherin null mice. Equal loading is confirmed with anti-β-tubulin antibodies.
null mice exposed to hypoxia were examined for vascular glomeruli (highly proliferative clusters of tortuous vessels produced in response to angiogenic stimuli) and vessel branching. Quantification of blood vessels labeled with endothelial cell–specific BSL1-B4 revealed a 63% reduction of vascular glomeruli in T-cadherin−/− retinae as compared with the wild-type (Supplementary Fig. S2A–C; 95.0 ± 7.0 glomeruli for T-cadherin+/+ and 35.5 ± 4.5 glomeruli for T-cadherin−/−; P < 0.0001). Furthermore, we observed a 53% reduction of vascular branch points in the T-cadherin null condition (Supplementary Fig. S2D; 639.3 ± 29.07 branch points for T-cadherin+/+ and 299.3 ± 11.72 branch points for T-cadherin−/−; P < 0.0001). These data further support a role for T-cadherin in promoting neovascularization in vivo. Lastly, because limited blood supply may starve MMTV-PyV-mT tumors, we examined apoptosis by staining for DNA strand breaks by TUNEL. Our analyses revealed a 6-fold increase in apoptotic nuclei in T-cadherin−/− MMTV-PyV-mT tumors (10.24 ± 3.105%) over the corresponding wild-type (1.588 ± 0.4696%; P = 0.0106; Fig. 4C, a–c). Consistent with the increase of apoptosis, staining with HypoxyProbe indicated a 3-fold significant increase in oxygen-deprived areas in T-cadherin−/− MMTV-PyV-mT tumors (7.002 ± 2.090%) versus wild-type (2.064 ± 0.7181%; Fig. 4D, a–c; P = 0.0401; d and e represent respective examples of the T-cadherin+/+ and T-cadherin−/− MMTV-PyV-mT tumor pathology). These results, combined with the observation that T-cadherin deficiency limits hypoxia-induced retinal angiogenesis, suggest that loss of T-cadherin limits tumor neovascularization and causes hypoxia, which in turn restricts tumor cell survival.

Altered pathology and metastatic potential of T-cadherin–deficient MMTV-PyV-mT tumors. To characterize the effects of the T-cadherin mutation on mammary tumors, we compared the tumor pathology between genotypes. Histology of H&E-stained sections revealed an unexpected and dramatic change in aggressiveness and metastatic rate of T-cadherin–deficient tumors. Wild-type MMTV-PyV-mT tumors showed the established range of PyV-mT–induced mammary hyperplasias and tumor phenotypes in the C57Bl/6 background (31) with a predominance of adenocarcinomas (44 of 45 tumors) with a mixture of papillary and adenosquamous variants (Fig. 5A, a and c). One myoepithelioma was found. The MMTV-PyV-mT–induced tumors in the T-cadherin null condition differed from the wild-type cohort with the appearance of a unique poorly differentiated tumor phenotype (Fig. 5A, b and d) in 8 of the 14 animals sampled, and in 13 of the 43 (30%) tumor samples (Supplementary Table S1). The
Figure 4. T-cadherin−/− MMTV-PyV-mT tumors show unchanged proliferation, reduced endothelial density, increased tumor cell apoptosis, and enhanced hypoxia. A, phospho-histone H3 labeling reveals no statistical differences in cell proliferation between T-cadherin+/+ (a) and T-cadherin−/− (b) tumors (c; P = 0.50). B, analysis of CD31 staining from T-cadherin−/− (a) and T-cadherin+/+ (b) tumors staining shows a 31% reduction of endothelial cell coverage (c; P = 0.0022) and a 45% reduction of vessel branching (d; P < 0.0001). C, TUNEL staining reveals a 6-fold increase in apoptotic tumor nuclei in T-cadherin−/− (a) over T-cadherin+/+ (b) tumors (c; P = 0.0106). D, immunofluorescence with a FITC-labeled Hypoxyprobe-1 antibody exhibits a significant 3-fold increase in hypoxic area from T-cadherin−/− (a) to T-cadherin+/+ (b) tumors (c; P = 0.0401). Representative images of T-cadherin−/− (d) and T-cadherin+/+ (e) tumors. Bar, 50 μm.
poorly differentiated tumors were composed of expansile nodular and solid cell masses with large pleomorphic hyperchromatic nuclei and scanty amorphophilic cytoplasm. These cells did not form glands, papillae, or squamous areas that are characteristic of the better differentiated, wild-type MMTV-PyV-mT tumors. T-cadherin–deficient tumors often displayed juxtaposed regions of high mitotic rates and tumor necrosis, in line with the documented increase in hypoxia and decrease in tumor vascularization.

All of the T-cadherin−/− MMTV-PyV-mT tumors developed pulmonary metastases in the C57Bl/6 background (Fig. 5A, e and f). In contrast, no metastatic growth was discernable in the wild-type condition (Supplementary Table S2). The lung metastatic rate in the MMTV-PyV-mT mouse model is known to depend on the genetic background, and our observation that wild-type MMTV-PyV-mT tumors show few or no metastases in the C57Bl/6 strain is in agreement with previous reports (28, 31). T-cadherin null MMTV-PyV-mT tumors acquired definitive metastatic properties as the perimeter of the pulmonary metastases was devoid of an endothelial border, and metastases were not lodged in the vasculature (Fig. 5A, g). In further support of the altered tumor phenotype, the hematoxylin/eosin ratio in sections from T-cadherin−/− and T-cadherin+/− MMTV-PyV-mT tumors showed a significant shift in aggressive pathology (increase of eosin-stained areas) in the T-cadherin−/− deficient condition (Fig. 5A, h; 65.35 ± 2.435% for T-cadherin+/− and 35.96 ± 2.556 for T-cadherin−/−; P < 0.0001).

**T-cadherin affects tumor growth as a stromal factor.** With the reduction of pathologic neovascularization in T-cadherin−/− deficient MMTV-PyV-mT tumors and the concomitant increase in tumor dedifferentiation, we next distinguished if T-cadherin exerts cell autonomous and nonautonomous effects on tumor growth. To accomplish that, we transplanted wild-type MMTV-PyV-mT tumors into mammary fat pads of FVB syngeneic wild-type and T-cadherin−/− deficient hosts. The switch of background was necessary because C57Bl/6 MMTV-PyV-mT tumors grow poorly after transplantation into syngeneic hosts. Moreover, wild-type MMTV-PyV-mT tumors in the FVB background are more invasive than in the C57Bl/6 mouse strain. Thus, we used the nonmetastatic variant MMTV-PyV-mT+/−/C0 (23) for transplantation into mammary fat pads. Transplanted tumors were palpable in wild-type hosts at 23 days, and with a 1-week delay, at 30 days, in T-cadherin null recipients. Tumor growth kinetics was significantly reduced in the T-cadherin−/− deficient background (Fig. 5B; P = 0.000166 by linear regression analysis). Accordingly, the final MMTV-PyV-mT+/−/C0 tumor weight in T-cadherin null mice was reduced by a factor of 3 in comparison with the wild-type (Fig. 5C; 956.5 ± 237.4 mg, n = 22 for T-cadherin+/− and 345.3 ± 58.45 mg, n = 20 for T-cadherin−/− hosts; P = 0.0215). Pathologic analysis established that the donor papillary adenocarcinoma tumor pathology was preserved in hosts of both genotypes, albeit tumors remained small and often formed papillary cysts in the absence of T-cadherin (Fig. 5D, a and b; Supplementary Table S3). These data support a role of T-cadherin in the tumor microenvironment in line with the concept that T-cadherin regulates tumor vascularization.

**T-cadherin ablation disrupts the association of adiponectin with the vasculature.** With T-cadherin regulating neovascularization *in vivo*, the primary question arising from our studies is “How does T-cadherin regulate blood vessel growth?” T-cadherin is a binding partner for the hexameric and high molecular weight forms of adiponectin (21) and adiponectin is implicated in vascular functions (32, 33). To gain initial insights into the possible crosstalk between these molecules *in vivo*, we examined the expression of adiponectin in wild-type and T-cadherin−/− deficient mouse mammary tumors by immunohistochemistry. In wild-type virgin mammary glands (data not shown) and MMTV-PyV-mT tumors, adiponectin was detected in association with the CD31-positive vasculature (Fig. 6A, a–c). In contrast, in T-cadherin null tumors, no specific signal was evident for adiponectin in any structure (Fig. 6A, e–g). Examination of the serum from mice of both genotypes revealed a dramatic up-regulation of adiponectin levels in the T-cadherin null condition (Fig. 6B). Taken together, these *first in vivo* observations link the expression of T-cadherin to a role in sequestering adiponectin to the vasculature where this interaction could contribute to regulating vascular functions.

**Discussion**

By generating and analyzing a T-cadherin−/− deficient mouse model of mammary cancer, we have revealed an unprecedented role for T-cadherin in tumor angiogenesis. T-cadherin is expressed on epithelial, myoepithelial, and endothelial cells in virgin mouse mammary tissue, and down-regulated in epithelial cells during development of luminal epithelial tumors. Thus, T-cadherin becomes progressively confined to the vasculature during tumorigenesis. The restricted vascular expression of T-cadherin in the MMTV-PyV-mT model replicates our observations in MMTV-Neu–induced mouse tumors (19) and is similar to the situation in human cancers, including those of the breast (8–13). As T-cadherin expression is progressively lost from epithelial cells during mammary tumorigenesis, the T-cadherin−/− PyV-mT model can only assess a limited range of changes and leaves open the role of T-cadherin in the tumor cells. We report that genetic inactivation of T-cadherin does not accelerate tumorigenesis or result in formation of spontaneous tumors as might be expected if T-cadherin acted as a bona fide tumor suppressor. Rather, the loss of T-cadherin delays MMTV-PyV-mT tumor formation, retards growth, and increases metastases. We attribute this phenotype to a major function of T-cadherin in tumor angiogenesis. The T-cadherin−/− MMTV-PyV-mT tumors are significantly less vascularized than those from corresponding wild-type mice. The null mice develop no life-threatening vascular defects during embryogenesis, but show specific impairments in pathologic neovascularization of mammary tumors and hypoxia-induced blood vessel remodeling of the retina. Reduced endothelial cell density and increased hypoxia in T-cadherin−/− MMTV-PyV-mT tumors, together with the observation that T-cadherin in the host microenvironment is needed for supporting growth of MMTV-PyV-mT tumors after transplantation, suggest an important role for T-cadherin in pathologic neovascularization. We correlate the association of adiponectin with the tumor vasculature with the proangiogenic role of T-cadherin, and suggest a link between T-cadherin vascular expression, adiponectin binding capability, and tumor neovascularization.

An unexpected outcome of the current study was that T-cadherin−/− MMTV-PyV-mT tumors attain a more malignant pathology and metastatic potential although overall tumor growth is limited. The metastatic rate of MMTV-PyV-mT mouse tumors is known to be influenced by the genetic background (28) and is low in the C57Bl/6 strain used for the current study (See Supplementary Table S2). As T-cadherin is progressively lost from the ductal epithelium during normal tumorigenesis, how can the ablation of T-cadherin affect tumor cell invasion and metastasis? Two explanations are possible. First, the increased metastatic potential results from the disruption of T-cadherin–mediated adhesive
Figure 5. Poor differentiation and high metastatic potential of transgenic T-cadherin−/− MMTV-PyV-mT tumors, and limited MMTV-PyV-mT/MMTV-PyV-mT tumors growth after transplantation into T-cadherin−/− hosts. A, examples of gross tumor pathology. a, T-cadherin+/+ MMTV-PyV-mT tumor with complex solid and papillary carcinomas with prominent vessels; b, three poorly differentiated, partially necrotic T-cadherin−/− MMTV-PyV-mT carcinomas and papillary tumors. Bar, 1 mm. Magnified images of these tumors display glandular forms in differentiated T-cadherin+/+ tumors (c), and the poorly differentiated pathology of necrotic T-cadherin−/− tumors (d). Note the apoptosed cells on either side of the central blood vessel (arrow) in d. Bar, 50 μm. Supplementary Table S1 presents the summary of tumor pathologies. Evaluation of the metastatic potential revealed that T-cadherin+/+ MMTV-PyV-mT tumors metastasize poorly to the lungs (e; 14 mice), whereas all T-cadherin−/− tumor–bearing animals (f; 14 mice) show lung metastases. Magnified images of the lungs illustrate the invasive phenotype of T-cadherin−/− MMTV-PyV-mT tumor metastasis (g). Statistics in Supplementary Table S2 show 6.1 ± 4.2 metastases per T-cadherin−/− MMTV-PyV-mT mouse and none in the wild-type. Quantitative evaluation of the hematoxylin/eosin ratio from mammary tumors shows that T-cadherin−/− MMTV-PyV-mT tumors exhibit poorly differentiated pathology as compared with the T-cadherin+/+ MMTV-PyV-mT condition (h; 65.35 ± 2.435% for wild-type and 35.96 ± 2.556% for mutant tumors; P < 0.0001). B, MMTV-PyV-mT/MMTV-PyV-mT tumors show delayed appearance and retarded growth after transplantation into T-cadherin−/− hosts (WT, n = 22; KO, n = 20). Linear regression analysis shows significant differences in MMTV-PyV-mT/MMTV-PyV-mT growth kinetics between the T-cadherin+/+ and T-cadherin−/− host environment (P = 0.000196). C, comparison of final tumor weights confirms a significant difference between genotypes (P = 0.0215). Supplementary Table S3 summarizes the pathology of T-cadherin+/+ MMTV-PyV-mT/MMTV-PyV-mT tumors after transplantation into T-cadherin+/+ and T-cadherin−/− mice. Donor pathology was maintained in both genotypes. D, examples of gross tumor pathology in T-cadherin+/+ (a) and T-cadherin−/− (b). Bar, 1 mm. Asterisk, lymph node.
interactions that prevent straying of neoplastic epithelial cells. This model assumes that low T-cadherin levels normally present on PyV-mT tumor cells are sufficient to restrain cells to the primary tumor mass and inhibit metastatic spreading. This suggestion would need to be tested in a gain-of-function genetic model. Alternatively, the limited blood supply of T-cadherin–deficient tumors increases hypoxia and changes in the tumor pathology (34, 35). Hypoxia is well known to be associated with a poor clinical outcome of invasive human breast carcinoma (36), and T-cadherin–deficient tumors present with reduced blood vessel density, enhanced apoptosis, and enlarged hypoxic and necrotic regions. The metastases in T-cadherin−/− MMTV-PyV-mT mice are not associated with endothelial cells and thus may represent selection for an epithelial-mesenchymal type transition.

T-cadherin has at least two activities that may influence angiogenesis. First, T-cadherin confers homophilic binding between cells (7), and this engagement is reported to decrease adhesion, enhance migration, and induce proliferation of endothelial cells (16, 17). Moreover, ectopic T-cadherin expression in the capillary microenvironment in vivo repulses blood vessels and stops their growth (37). Thus, inactivation of T-cadherin in vivo might be expected to increase vascularization. However, the data presented here from the mouse null model do not support a restrictive role for T-cadherin in blood vessel growth in vivo. Rather, the T-cadherin null mice show limited angiogenic responses, suggesting a function for T-cadherin in supporting angiogenesis. Our work thus leaves open if the repulsive, homophilic binding function of T-cadherin plays into the complex interactions during angiogenesis in vivo. Second, T-cadherin is a binding protein for the hexameric and high molecular weight forms of adiponectin (21), the predominant active forms in serum (38). We find that adiponectin is sequestered to the vasculature in a T-cadherin–dependent manner and levels are dramatically increased in serum. Thus, T-cadherin may serve as a major adiponectin repository. The functions of adiponectin in the vasculature remain controversial; both positive and negative actions on blood vessel growth are reported (32, 33). Linking T-cadherin and adiponectin functions at a mechanistic level is thus a primary research task. Because of its membrane attachment through a glycosylphosphatidylinositol moiety, T-cadherin alone is insufficient to act as a receptor that transduces signals elicited by adiponectin binding. Thus, we favor a model in which T-cadherin signals through associated molecules

Figure 6. Adiponectin is displaced from the T-cadherin+/+ MMTV-PyV-mT tumor vasculature and is up-regulated in the serum. A, confocal analysis of T-cadherin+/+ MMTV-PyV-mT tumors after staining for adiponectin (APN; a), and CD31 (b) shows colocalization to the vasculature (c). In T-cadherin−/− MMTV-PyV-mT tumors, adiponectin is not associated with the vasculature (e–g). Identical exposure times were used. Representative images of T-cadherin+/+ (d) and T-cadherin−/− (h) MMTV-PyV-mT tumors. Bar, 50 µm. B, Western blot analysis for adiponectin. High molecular weight adiponectin is detected in 1-µL serum from each of three T-cadherin+/+ and three T-cadherin−/− mice. Levels are dramatically elevated in T-cadherin null mice. Staining for Amido black is used as a loading control.
that perhaps provide a link with adiponectin receptors or other vascular receptors. One conceivable role for T-cadherin as an adiponectin binding protein includes sequestering adiponectin-associated growth factors such as platelet-derived growth factor-BB, basic fibroblast growth factor, heparin-binding epidermal growth factor, and thrombospordin 1 (39, 40). These factors exert important roles in establishing functional and stable vascular networks (41–43), and the T-cadherin-dependent accumulation of adiponectin may regulate their availability upon signals eliciting vascular responses. Irrespective of the mechanism, the work reported here establishes that T-cadherin regulates retinal and tumor angiogenesis and is responsible for sequestering adiponectin to the vasculature. These studies thus open new avenues for unraveling the molecular complexity of the vascular response under challenging physiologic conditions.

References


Correction: T-cadherin Regulates Angiogenesis

In the article on how T-cadherin regulates angiogenesis in the March 1, 2008 issue of Cancer Research (1), a footnote should have been included indicating that Lionel W. Hebbard and Michèle Garlatti contributed equally to the work.


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T-cadherin Supports Angiogenesis and Adiponectin Association with the Vasculature in a Mouse Mammary Tumor Model

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