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Disruption of Tumor Cell Adhesion Promotes Angiogenic Switch and Progression to Micrometastasis in RAF-Driven Murine Lung Cancer

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SUMMARY

Progression of non-small-cell lung cancer (NSCLC) to metastasis is poorly understood. Two genetic approaches were used to evaluate the role of adherens junctions in a C-RAF driven mouse model for NSCLC: conditional ablation of the *cdh1* gene and expression of dominant-negative (dn) E-cadherin. Disruption of E-cadherin caused massive formation of intratumoral vessels that was reversible in the early phase of induction. Vascularized tumors grew more rapidly, developed invasive fronts, and gave rise to micrometastasis. β-catenin was identified as a critical effector of E-cadherin disruption leading to upregulation of VEGF-A and VEGF-C. In vivo, lung tumor cells with disrupted E-cadherin expressed β-catenin target genes normally found in other endodermal lineages suggesting that reprogramming may be involved in metastatic progression.

INTRODUCTION

Non-small-cell lung cancer (NSCLC) is the most frequent type of lung cancer with high metastatic potential and low cure rate (American Cancer Society, 2006). This high metastatic potential may be due to active angiogenesis (Folkman et al., 1989). Vascularized NSCLC is well known for its ability to metastasize into regional lymph nodes even at early tumor stages, and tumor lymphangiogenesis is directly correlated with lymph node metastasis (Renyi-Vamos et al., 2005). Therefore, prevention of early NSCLC tumor metastasis is a primary therapeutic goal.

Escape of cells from the primary tumor is considered as a rate-limiting step and requires block of adhesion molecules (Bremnes et al., 2002). Among various adhesion molecules, cadherin family members were consistently shown to be involved in the progression of NSCLC (Nakashima et al., 2003; Kim et al., 2005). Cadherins are transmembrane proteins and components of adherence junctions (Takeichi, 1995). Their extracellular domains form calcium-dependent, homophilic transdimers that mediate cell-cell adhesions. The cytoplasmic tails of cadherins bind to several potential signaling proteins, most notably β-catenin, a transcriptional cofactor in Wnt signaling (Clevers, 2006). Overexpression of positive—and suppression of negative—regulators of Wnt signaling has been reported for NSCLC (Uematsu et al., 2003; You et al., 2004; Mazieres et al., 2004). Epithelial (E)-cadherin is the major mediator of intercellular adhesion in epithelial tissues. Aberrant expression of E-cadherin was shown to be highly correlated with invasion and spread of NSCLC (Shibanuma et al., 1998). Moreover,

SIGNIFICANCE

Prevention of metastasis of NSCLC, the most frequent type of human cancer is a primary therapeutic goal. A rate limiting step in metastasis is disruption of cell-cell contacts. We describe an approach to reversibly disrupt cell-cell adhesion in C-RAF driven lung adenomas and promote conversion to micrometastasis by induction of *cdh1* gene ablation or expression of dn E-cadherin. Micrometastasis was preceded by massive formation of blood and lymph vessels. E-cadherin dysfunction is a frequent event in human cancer giving these findings a broader significance. Insight into the mechanisms of angiogenic switch induction may help in developing novel strategies for prevention of malignant progression.

reduced or absent expression of E-cadherin was reported to be a significant prognostic factor in patients with NSCLC (Liu et al., 2001; Huang et al., 2005). However, how disruption of E-cadherin complexes may cause lung tumor progression remains largely unresolved.

In order to elucidate the role of E-cadherin in NSCLC, we took advantage of a transgenic mouse model of NSCLC, *SP-C C-RAF BXB*, that expresses oncogenic C-RAF in type II alveolar pneumocytes (Kerkhoff et al., 2000). *SP-C C-RAF BXB* mice reproducibly develop multiple lung adenomas early in postnatal life. Adenomas consist of cuboidal cells that grow continuously without signs of apoptosis (Fedorov et al., 2002; Götz et al., 2004) and eventually kill the host by elimination of gas exchange surface. Progression of the *SP-C C-RAF BXB* adenomas to metastasis was neither observed in aged animals (Kerkhoff et al., 2000) nor after removal of p53 (Fedorov et al., 2003), making this transgenic model an ideal system for identification of NSCLC progression factors (Rapp et al., 2003). We hypothesized that oncogenic C-RAF might stabilize cell-cell interaction of the adenoma cells that might in turn contribute to the benign nature of the tumor cells. We therefore investigated whether disruption of E-cadherin might cause progression of *SP-C C-RAF BXB* adenomas. For disruption, we used two approaches, doxycycline (DOX)-inducible Cre recombinase-mediated E-cadherin (*cdh1*) gene inactivation or expression of dominant-negative (dn) E-cadherin (Dahl et al., 1996).

Transgenic mice conditionally disrupting E-cadherin function in type II alveolar epithelial cells were crossed with *SP-C C-RAF BXB* mice. In normal lung, inhibition of E-cadherin expression did not lead to overt tumor formation, but diffuse hyperplasia was observed. Disruption of E-cadherin complexes in adenoma cells induced invasive carcinoma that metastasized into regional lymph nodes and bone marrow. Tumor progression was reversible and strictly correlated with the induction of angiogenic switch presumably via β-catenin-mediated VEGF-A and VEGF-C upregulation. Taken together, these results reveal a novel and unanticipated function of E-cadherin as an important mediator of angiogenesis which might shed light on the missing link between E-cadherin loss and tumor progression.

RESULTS

Angiogenic Switch Induction in *C-RAF BXB* Lung Adenomas by Ablation of E-Cadherin

A hallmark of *SP-C C-RAF BXB* lung adenomas is tight cell-cell association maintained over the lifetime of the animal (Fedorov et al., 2003; Houben et al., 2004). To examine whether *SP-C C-RAF BXB* epithelial tumors express E-cadherin, individual adenomas were microdissected from frozen lung sections of adult *SP-C C-RAF BXB* transgenic mice. We readily detected *cdh1* transcripts by RT-PCR (see Figure S1A in the Supplemental Data available with this article online). For localization of endogenous E-cadherin, lung sections were stained with an antibody to the extracellular domain of E-cadherin. All cuboidal tumor cells expressed functional E-cadherin in the membrane (Figure S1B and S1C).

To examine whether E-cadherin ablation would affect tumor progression, we first employed conditional *cdh1* gene inactivation using a DOX inducible Cre/*loxP* site-specific recombination system (Figure S2A). For this, Cre recombinase was induced in 6-week-old compound mice (*SP-C rtTA/Tet-O-cre/cdh1*$^{flox/flox}$) by DOX treatment, and lungs were analyzed after a 2 month period. In the presence of DOX, no tumors were formed, though there was diffuse hyperplasia accompanied by enlargement of alveolar spaces in the absence of inflammation (Figure S4A). These hyperplastic lesions showed scattered cells of unpolarized morphology and a marked increase in proliferation (Figures S4A and S4C). Further, evaluation of these mice for periods up to 10 months did not reveal overt tumor formation. To evaluate the effect of *cdh1* loss on tumorigenesis, tumor-bearing mice (*SP-C C-RAF BXB/SP-C rtTA/Tet-O-cre/cdh1*$^{flox/flox}$) of 6 weeks of age were treated with DOX for 4 months and subsequently examined histologically. Tumor foci showed cell separation and partial loss of cuboidal morphology (Figure 1A). Notably, ablation of E-cadherin did not occur in all cells, consistent with scattered Cre staining in only 35–50 percent of cells in tumor foci (Figures 1A, 1B and Figure S2B). The most dramatic finding came from analysis of blood vessel density by CD31 staining which revealed massive intratumoral invasion after *cdh1* inactivation, whereas adenomas harboring intact *cdh1* alleles were poorly vascularized (Figures 1A and 1D). Vessel induction outside of tumors was difficult to evaluate because of the abundance of vessels in normal lung. To examine whether *cdh1* loss had any effect on intratumoral lymphangiogenesis, we stained tumors with a lymphatic-specific marker, LYVE-1. Whereas tumors from untreated mice revealed poor lymphatic vessels in the center of adenomas, their number and size have significantly increased by 4 months of DOX induction (Figures 1A and 1E). In search of potential mediators of angiogenesis, we have stained the sections for β-catenin. There was a significant loss of membrane staining (data not shown) and an increased nuclear accumulation of β-catenin in a small fraction of tumor cells (Figures 1A–1C).

As further dissection of this process requires a system for reversible inactivation of E-cadherin, which Cre-mediated ablation does not allow, we turned to the well established dn E-cadherin approach (Dahl et al., 1996; Cavallaro and Christofori, 2004) and generated mice carrying a DOX-regulated dn E-cadherin transgene (Figure S3A). Consistently in compound mice (*SP-C rtTA/Tet-O dn E-cadherin*), the expression of dn E-cadherin by DOX treatment for different periods led to hyperplasia, but again no tumors formed (Figure S4A). The extent of diffuse hyperplasia and alveolar space enlargement was comparable to *cdh1* gene ablation (Figure S4A). These hyperplastic lesions showed a marked increase in proliferation (Figures S4A and S4C). Silencing of dn E-cadherin expression by withdrawal of DOX for 2 months led to a pronounced regression of the hyperplastic regions, coincident with a return to physiological cell-cycle status

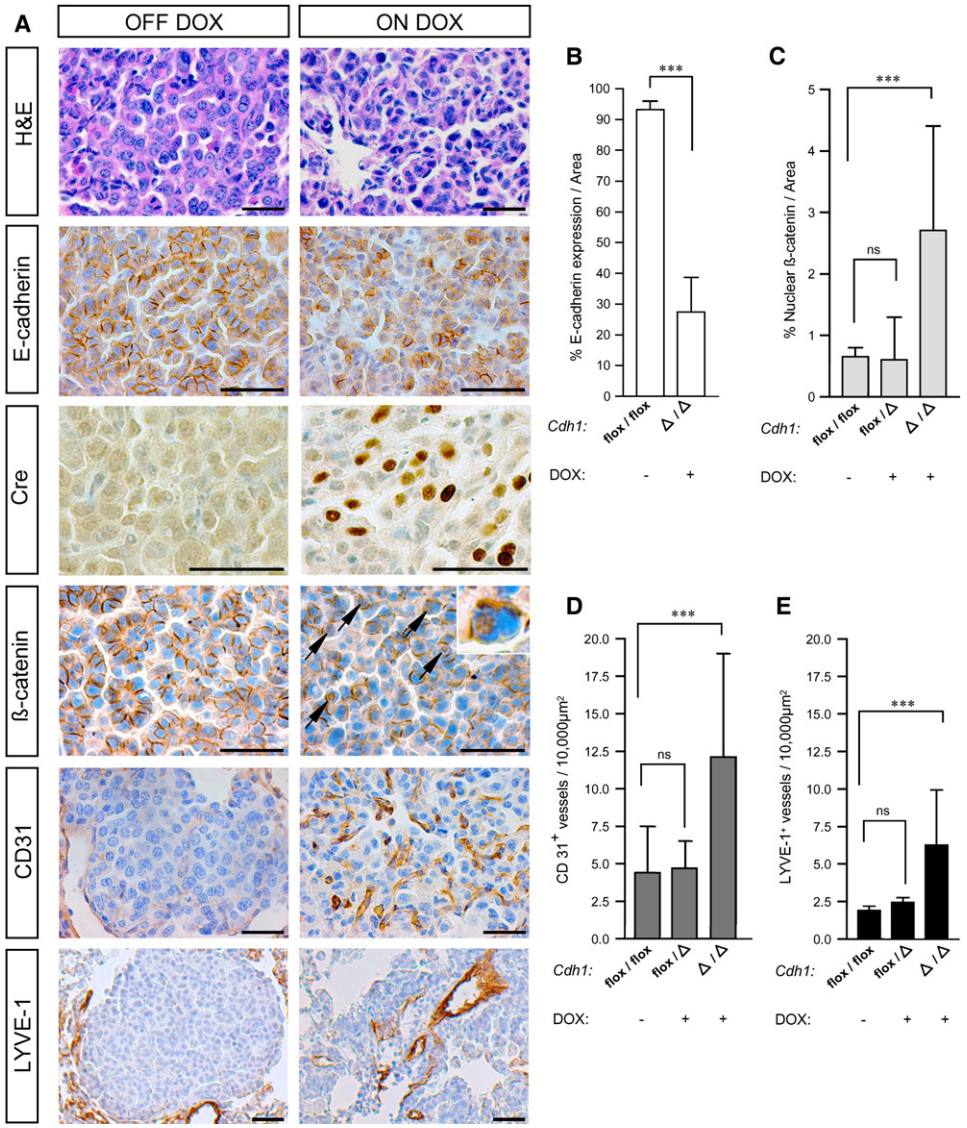


Figure 1. Conditional Deletion of *cdh1* in *SP-C C-RAF BXB* Lung Adenomas Provokes Vascular Invasion

(A) Representative paraffin embedded sections of lung tumors from compound mice (*SP-C C-RAF BXB/SP-C rtTA/Tet-O-cre/cdh1*$^{flox/flox}$) were stained as indicated. For Cre-mediated deletion of *cdh1*, mice were treated with DOX for 4 months starting at 6 weeks of age (ON DOX) and compared with untreated controls (OFF DOX). Membrane loss of E-cadherin leads to cell-cell separation and is paralleled by relocalization of β-catenin to the cytoplasm and cell nucleus (arrows and inset). This is accompanied by massive invasion of blood and lymph vessels.

(B–E) Quantification of E-cadherin in the membrane of intratumoral cells, of β-catenin relocalization and of intratumoral blood and lymph vessels. Ten tumor areas (approximately 200 cells per area) for each of five mice were evaluated for E-cadherin and β-catenin. To enumerate vessel density, 10 tumor areas (10,000 μm^2) per mouse were counted in eight mice. All values are mean ± SD (***$p < 0.001$; ns = nonsignificant). Scale bars, 50 μm.

(Figures S4A and S4C). To determine whether regression of the hyperplastic lesions was caused by apoptosis, we performed active caspase-3 staining. We observed a significant increase in cells that contained activated caspase-3 following restoration of E-cadherin membrane expression. Search for compensatory upregulation of P-cadherin was negative in all genotypes (Figure S4D).

To assess whether dn E-cadherin-mediated disruption of cell adhesion leads to malignancy in benign *SP-C C-RAF BXB* adenomas, triple transgenic mice (*SP-C C-RAF BXB/SP-C rtTA/Tet-O dn E-cadherin*) were treated with DOX for different periods. Already after 1 week of DOX administration, loss of cell-cell contact between tumor cells was observed (Figure 2A). Using an antibody directed against the ectodomain of E-cadherin that only detects membrane inserted E-cadherin, we observed almost complete removal from the membrane 4 months after induction (Figures 2A and 2B). Displacement from the cell surface was not paralleled by a corresponding decrease in E-cadherin protein that was detected at only a slightly

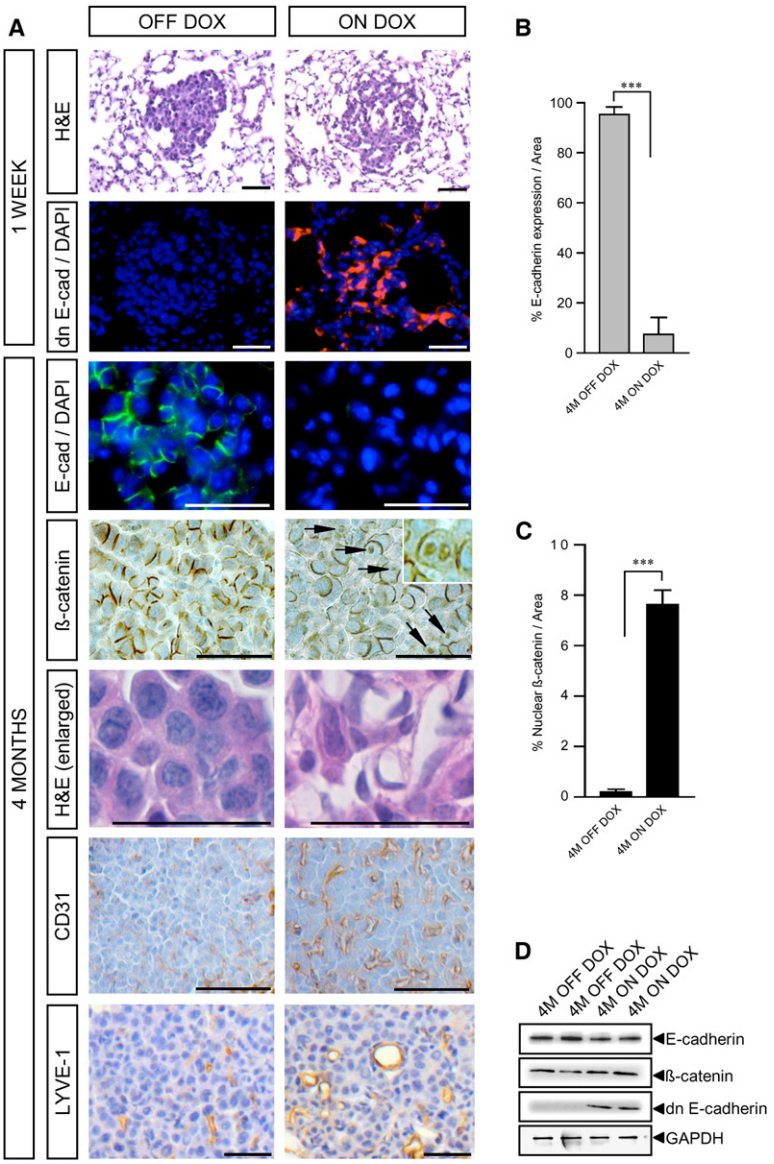


Figure 2. Expression of dn E-Cadherin Induces Tumor Vasculature

(A) Triple transgenic mice (*SP-C C-RAF BXB*/*SP-C rtTA*/*Tet-O dn E-cadherin*) at 6 weeks of age were treated with Dox for 1 week and compared with age-matched controls. Note pronounced cell separation in the H&E staining that correlates with the fraction of cells showing transgene expression. After 4 months induction, loss of membrane E-cadherin and relocalization of β-catenin (see arrows and inset) is apparent. At higher magnification, H&E staining suggests emergence of microvessels in tumors. Staining for blood (CD31) and lymph (LYVE-1) endothelial cells demonstrates angiogenic switch. Scale bars, 50 μm.

(B and C) Quantification of E-cadherin expression in membranes and of β-catenin relocalization was as described in Figure 1; ***$p < 0.001$.

(D) Immunoblot of isolated tumor cells from triple transgenic mice treated as indicated were reacted with antibodies against E-cadherin, β-catenin, myc-epitope, (dn E-cadherin) or GAPDH as loading control. M = month.

decreased level (Figures 2A and 2D). Removal of E-cadherin from the plasma membrane was, however, accompanied by nuclear relocalization of β-catenin in tumor cells expressing dn E-cadherin (Figures 2A and 2C). Nuclear β-catenin-positive cells were only a fraction (approximately 8%) of E-cadherin downregulated cells demonstrating heterogeneity in the induced tumor cell population. Consistent with the observations made in the course of *cdh1* gene inactivation, 2 months of dn E-cadherin expression led to uniform penetration of tumors by vessels of increased size that was even more pronounced after 4 months (Figure 2A) and continued to increase in density by 8 months of induction (Figure 3D and data not shown). Staining of lung sections for CD31 confirmed strong induction of intratumoral vessels (Figure 2A and Figure 3D). There was a 3-fold increase in vessel number and a 4-fold increase in vessel size (Figure 3D) indicating an enormous level of neoangiogenesis. As lymphangiogenesis may be most relevant for metastasis, we examined presence of vessels positive for the lymphangiogenesis marker LYVE-1. We observed that tumors from untreated mice revealed poor lymphatic vessels in the center of adenomas (Figures 2A and 3D). After four months of dn E-cadherin induction, their number and size had increased significantly (Figure 2A and Figure 3D) and increased even further when treatment was continued for 8 months (Figure 3D). To confirm the lymphatic nature of the vessels, Prox1 staining was additionally used (Figure S5A). Similar data were obtained with tissues from induced triple transgenic or *cdh1*-ablated quadruple transgenic mice (Figure S5A and S5B). We conclude that E-cadherin ablation induces the angiogenic switch that has previously been reported as a requirement for tumor progression (Folkman et al., 1989).

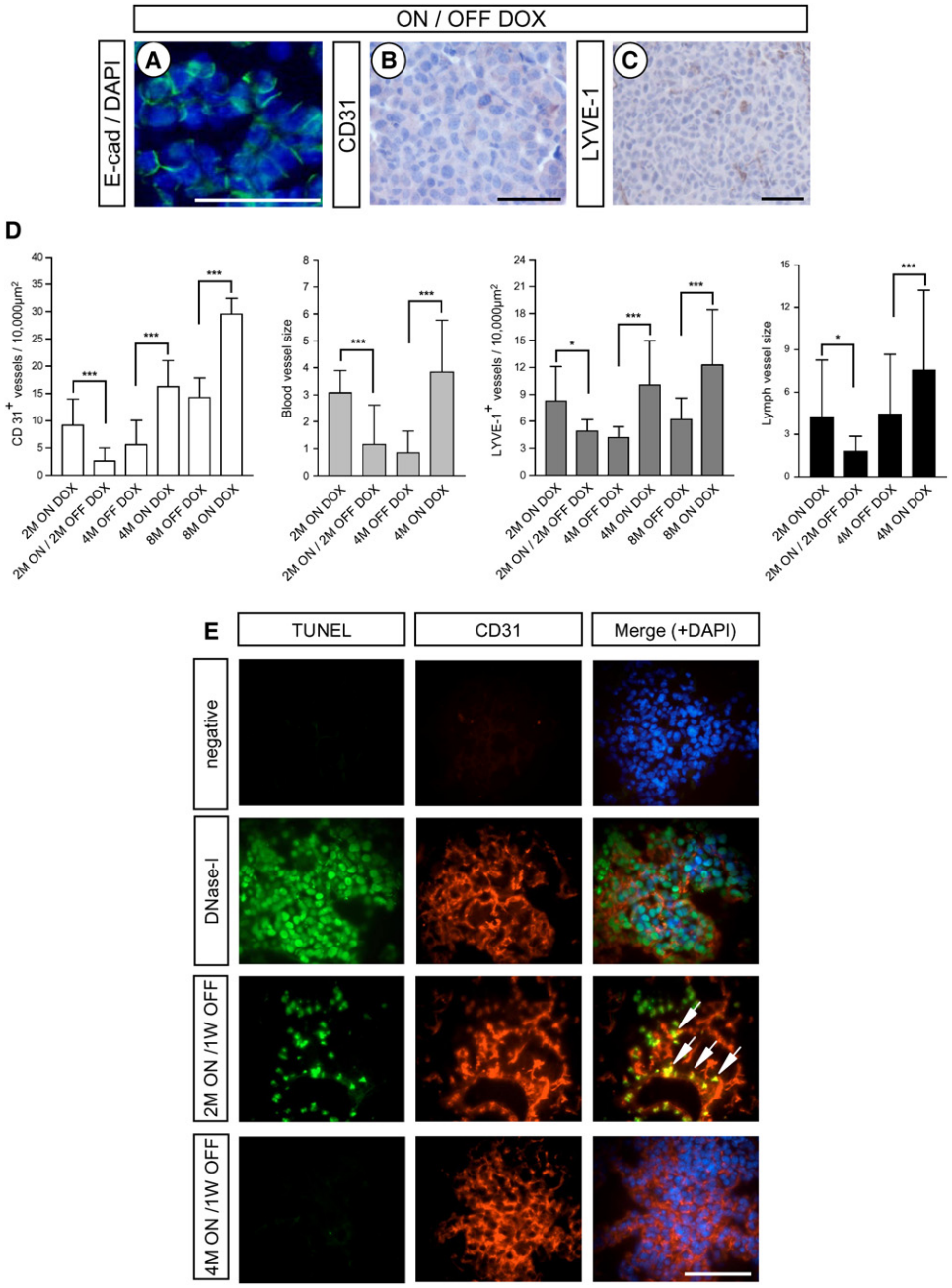


Figure 3. Reversal of the Angiogenic Switch via Endothelial Cell Apoptosis after Restoration of E-Cadherin Expression

(A) Reexpression of membrane E-cadherin in tumor cells of triple transgenic mice (*SP-C C-RAF BXB/SP-C rtTA/Tet-O dn E-cadherin*) that were kept on DOX for 2 months followed by 2 months DOX withdrawal.

(B and C) Restoration of E-cadherin expression is correlated with disappearance of blood (CD31) and lymph (LYVE-1) vessels.

(D) Quantification of blood and lymph vessels in number and size µm. Ten tumor areas (10,000 μm^2) per mouse were counted in 10 mice except in the ON/OFF category for which 5 mice were evaluated. M = month. All values are mean ± SD (*$p < 0.05$; ***$p < 0.001$).

(E) Detection of apoptosis of CD31 positive endothelial cells (see arrows) in frozen tumor sections after DOX withdrawal. Controls include TUNEL assay without terminal transferase (top panel left), antibody specificity (CD31, top panel middle) and apoptosis induction by DNaseI (second panel). Note that tumor vasculature in 4-months-induced mice is no longer DOX dependent. For each treatment, three mice and at least 5 tumors per mouse were evaluated. Scale bars, 50 µm.

Restoration of Intratumoral E-cadherin Expression Reverses the Angiogenic Switch

To determine whether restoration of E-cadherin membrane expression leads to suppression of vessel growth, dn E-cadherin transgene expression was switched off by DOX withdrawal for 2 months. E-cadherin expression at the cell surface of tumor cells was restored demonstrating reversibility (Figure 3A). CD31 staining after 2 months of DOX removal revealed blood vessel regression in both number and size (Figures 3B and 3D). To examine whether vessel regression was caused by endothelial cell apoptosis, we performed CD31/TUNEL double staining. Sections treated without primary antibody or enzyme were used as negative control (Figure 3E, upper panel). DNase-I treatment served as a positive control (Figure 3E, second panel). Endothelial intratumoral apoptosis occurred in 2 month ON DOX/1 week OFF DOX mice (Figure 3E, third panel). Additionally there may have been tumor cell apoptosis as we also see CD31 negative apoptotic cells in the same tumor section. Interestingly, using a 4 month ON DOX/1 week OFF DOX schedule, almost no TUNEL positive endothelial cells were detected (Figure 3E, bottom panel), indicating that long-term loss-of-membrane E-cadherin expression led to neovascularization that was resistant to re-expression of E-cadherin. Similar observations were made regarding vessel size (data not shown). As in the case of neoangiogenesis, we tested reversibility of lymphangiogenesis using a 2 month ON DOX/2 month OFF DOX schedule. The pattern of reversibility of lymphangiogenesis was similar to blood angiogenesis as complete reversibility was seen only after 2 months treatment (Figures 3C and 3D and data not shown).

Tumor Progression in *SP-C C-RAF BXB* Lung Tumors after E-cadherin Disruption

To investigate long-term effects of *cdh1* deletion or dn E-cadherin expression, compound mice were kept under continuous DOX treatment for up to 13 months. After 4 months, we observed large invasive tumors that were diffusely scattered throughout the lung parenchyma (Figure 4A) as well as loss of cuboidal cell morphology within tumors (Figure 2A, high magnification H&E pictures). Tumor volume was increased 5-fold (Figure 4B). Prolongation of induction to 8 months generated macroscopic solid tumors that were diagnosed as adenocarcinomas (Figure 4A). Cooperation between dn E-cadherin and C-RAF BXB in accelerating tumor growth required continued induction, as a 4 month ON DOX/4 month OFF DOX schedule reduced tumor size to the level of 8 month OFF DOX C-RAF BXB only (Figure 4B). Macroscopic tumors retained expression of SP-C and thyroid transcription factor-1 (TTF-1), a marker of human adenocarcinomas (data not shown). Tumor progression frequently involves processes such as epithelial-mesenchymal transition (EMT) and cadherin switch (loss of E-cadherin and gain of N-cadherin) (Huber et al., 2005). For detection of EMT, lung tumor sections were stained with vimentin and N-cadherin antibodies. There were scattered vimentin or N-cadherin positive cells in the induced tumors that most likely represent vessel associated cells as no double-positive (pro SP-C/Vimentin; pro SP-C/N-cadherin) cells were detected (Figures S6A and 6B). To confirm that the increased N-cadherin staining resulted from endothelial rather than tumor cells, we have isolated tumor and endothelial cell fractions by panning from triple transgenic mice and performed western blotting. There was no N-cadherin protein detectable in tumor cells from untreated or treated mice (Figure S6C), whereas the endothelial fraction showed positive staining independent of DOX treatment (Figure S6D). These data suggested that there was no cadherin switch in our lung tumors that has been frequently observed in EMT (Birchmeier, 2005). Finally, tumor cells from induced mice had stable epithelial features as cytokeratin staining was retained (Figure S6A). We conclude that, as in case of *cdh1* gene deletion in quadruple compound mice (Figure 4A and data not shown), dn E-cadherin-induced tumor progression of *SP-C C-RAF BXB* driven lung adenomas may not involve classical EMT. To examine how disruption of cell-cell contacts induced tumor progression, we determined the proliferation rate of tumor cells. PCNA staining of tumor cells in *SP-C C-RAF BXB/SP-C rtTA/Tet-O dn E-cadherin* mice and Ki67 staining in *SP-C C-RAF BXB/SP-C rtTA/Tet-O-cre/cdh1*$^{flox/flox}$ mice were highly increased, compared to animals in the absence of DOX (Figures 4C–4E). Taken together, these results demonstrate that inactivation of E-cadherin in benign *SP-C C-RAF BXB* adenomas not only promotes tumor growth, but also leads to progression to adenocarcinomas.

E-Cadherin Disruption Promotes Progression of *SP-C C-RAF BXB* Adenomas to Micrometastasis

To examine whether inhibition of E-cadherin function in benign *SP-C C-RAF BXB*-driven lung adenomas is necessary and sufficient for the metastatic spread of tumor cells, we conducted a large scale search for ectopic cells expressing SP-C or cytokeratin before and after induction. We detected both cytokeratin (Figure 5A, upper panel)- and SP-C (Figure 5A, lower panel)-positive cells in the lymph nodes after *cdh1* deletion or dn E-cadherin expression. The metastatic cells were not growing into tumors but formed small clusters of one to five cells identifying them as micrometastasis (Schardt et al., 2005) in mediastinal and axillary lymph nodes. Neither SP-C nor cytokeratin positive cells were visible in lymph nodes from untreated age-matched control mice (Figure 5A). Although we were not able to detect any distant metastasis in the preferred target tissues of NSCLC such as liver and brain, we infrequently observed SP-C positive cells in the bone marrow of triple transgenic mice induced for dn E-cadherin (Figure 5A, lower panel on the right). Dissemination of tumor cells from primary tumors was a late event, as we never detected metastasis in transgenic mice younger than 9 months (Figure 5B). Moreover, even in older mice, despite long-term inactivation of E-cadherin, only 43% of all triple transgenic mice and 25% of quadruple compound mice developed lymph node micrometastasis.

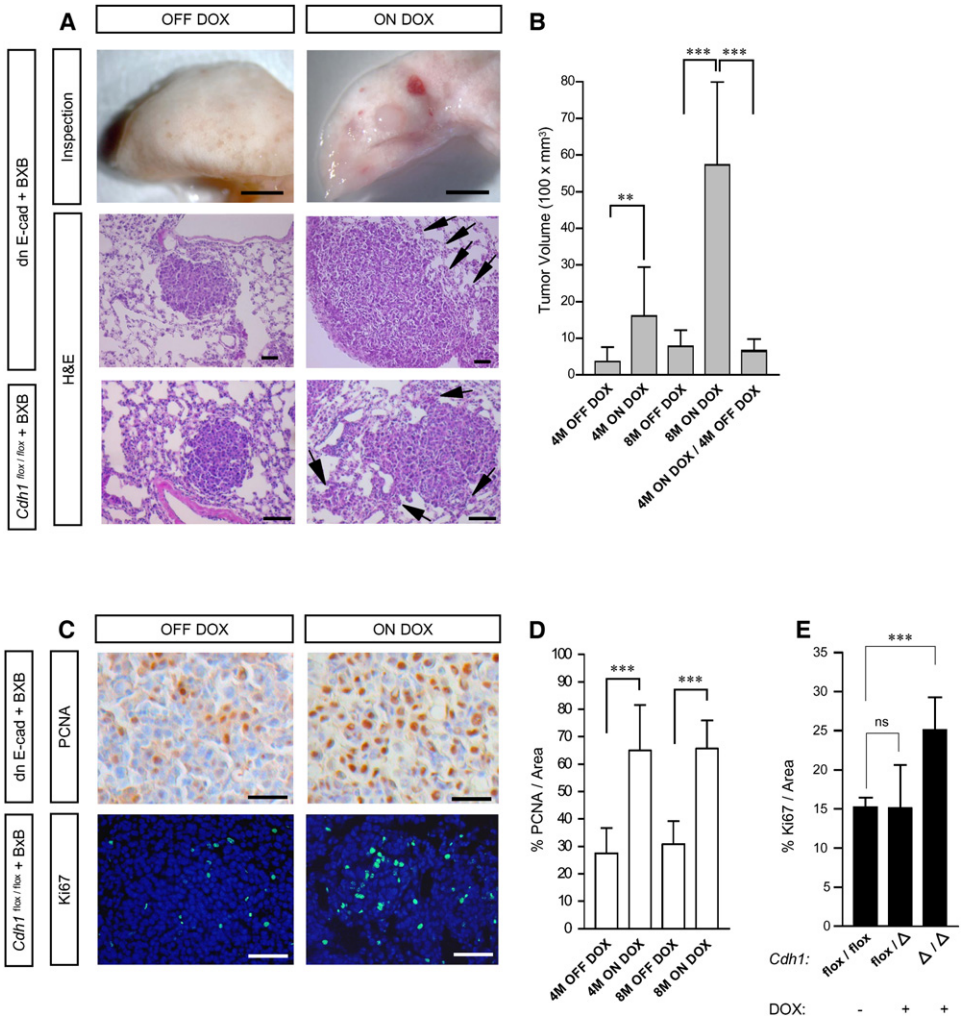


Figure 4. Oncogenic C-RAF and E-Cadherin Ablation Cooperate in Acceleration of Tumor Growth and Progression to Invasive Adenocarcinoma

(A) Macroscopic tumors are visible after 8 months DOX induction. Accelerated tumor growth and formation of invasive fronts (indicated by arrows) are already apparent after 4 months induction of either Cre recombinase to delete the *cdh1* gene or of dn E-cadherin expression.

(B) Quantification of tumor volume (n = 10 animals; for details see supplemental experimental procedures). Note that the difference in tumor volume between 4M ON DOX and 4M ON DOX/4M OFF DOX is not significant (p = 0.126). The difference between 8M OFF DOX and 4M ON DOX/4M OFF DOX is also not significant (p = 0.60).

(C) PCNA (brown) and Ki67 (green) staining show increased proliferation rates after 4 months induction of either Cre recombinase to delete the *cdh1* gene or of dn E-cadherin expression.

(D) Quantification of PCNA-positive cells (n = 5 animals).

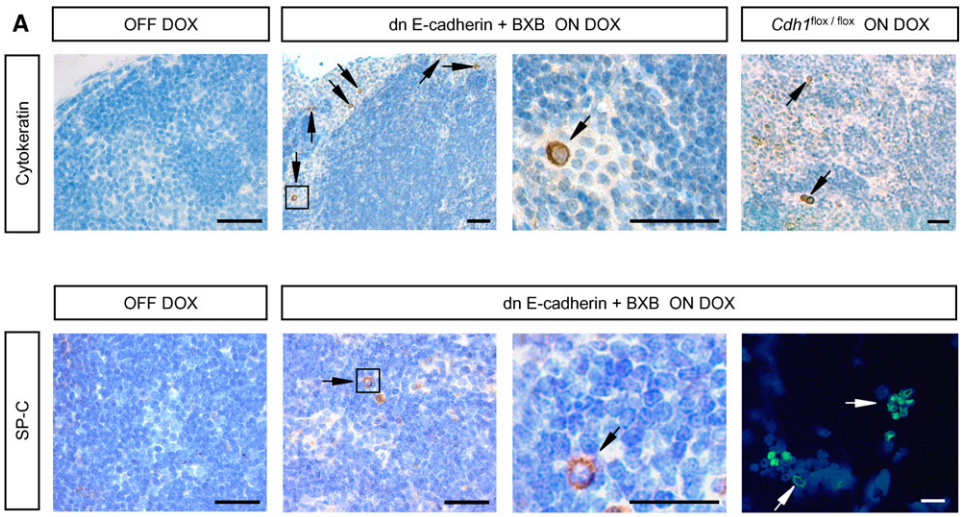
(E) Quantification of Ki67-positive cells (n = 5 animals).

Values represent mean ± SD (**p < 0.01; ***p < 0.001). Scale bars, 50 μm except for intact lung which is 2 mm.

Induction of dn E-cadherin or ablation of *cdh1* alone did not result in metastasis (data not shown). As in the case of angiogenic switch induction by dn E-cadherin, we wanted to examine whether continuous induction was required for progression to metastasis. Toward this end, we performed histological analysis of tissues from mice that were kept on 4 months ON DOX/4–9 months OFF DOX schedules (Figure 5A). This experiment revealed that none of the transgenic mice displayed lymph node or distant metastasis after DOX removal (Figure 5B). We conclude that there is a direct correlation between continuous angiogenesis induction and micrometastasis of *C-RAF BXB*-driven lung tumors.

Role of β-Catenin Signaling in dn E-Cadherin-Mediated Tumor Progression

To further resolve by which mechanism dn E-cadherin provokes tumor angiogenesis and micrometastasis during *SP-C C-RAF BXB* lung tumor progression, we analyzed expression of a panel of candidate genes using real time RT-PCR analysis. To avoid secondary gene expression changes due to tissue disaggregation, we chose to focus



B

DOX treatment	N	Metastasis	%Metastasis
4M OFF	12	0	0
4M ON	10	0	0
2M ON / 2M OFF	10	0	0
4M ON *Cdh1*$^{flox / flox}$	12	0	0
9-13 M OFF	14	0	0
9-13 M ON	14	6	43
9-10 M ON *Cdh1*$^{flox / flox}$	8	2	25
4M ON / 4-9M OFF DOX	14	0	0

N = Number of animals
In case of dn E-cadherin: DOX: treatment from E 0.0 on
In case of *Cdh1*$^{flox / flox}$: DOX: treatment after 1.5M

Figure 5. Oncogenic *C-RAF* and *E-cadherin* Ablation Cooperate in Progression to Micrometastasis
(A) Paraffin-embedded lymph node sections from untreated mice or mice treated with DOX for 10 months were screened for regional and long-distance metastasis by staining for pancytokeratin and pro SP-C (brown). Sections shown in higher magnifictions are boxed. Arrows mark positive cells. pro SP-C positivity (green) in cytospin preparations of bone marrow. Scale bars, 50 μm.
(B) Incidence and latency of lymph node micrometastasis. M = month.

on early events taking place during tumor progression. Adult triple transgenic mice were treated with DOX for 36 hr. Increased luciferase expression was observed and confirmed that transgene induction had occurred (data not shown). Short term induction typically led to hemorrhage at multiple sites, presumably as a result of vessel leakage (Figure 6A). Because VEGF family members were shown to be primary regulators of angiogenesis in NSCLC (Nakashima et al., 2004), we first evaluated VEGF mRNA expression levels. Interestingly, in comparison to the untreated control mice, we consistently observed a 1.8-fold increase in the level of VEGF-A and a 1.9-fold increase VEGF-C but not VEGF-D expression (Figure 6B and Table S2). Additionally, we noted a 3-fold increase in the level of VEGFR-3 (or Flt-4) (Figure 6B), a tyrosine kinase receptor for VEGF-C, suggesting that an autocrine loop may contribute to the higher proliferation observed in induced tumor cells (Figures 4C-E). Transcripts for the endothelial cell adhesion molecules VE-cadherin and PECAM-1 were significantly amplified, indicating a dynamic angiogenesis process in *SP-C C-RAF BXB* lung tumors in response to dn E-cadherin expression (Table S2). These data demonstrate that the onset of angiogenesis in triple transgenic lung tumors may be a direct effect of dn E-cadherin signaling via VEGF-A and VEGF-C.

Since E-cadherin competes with Apc for the binding of β-catenin, we examined the potential involvement of Wnt/β-catenin signaling in the cooperation of oncogenic C-RAF in conjunction with dn E-cadherin induction. Comparison of β-catenin mRNA levels in *SP-C C-RAF BXB* lung tumors revealed increased expression of β-catenin after 36 hr DOX treatment (Figure 6B and Table S2). Among various β-catenin target genes investigated, cyclin D1 was significantly elevated (Figure 6B). Thus, β-catenin may be an important effector of dn-E-cadherin induction. This possibility was further examined using two lung epithelial cell lines derived from NSCLC-type tumors, the mouse 3041 line (Rizzino et al., 1982) and the human A549 cell line. Transfection of *Tet-O dn E-cadherin* and

A

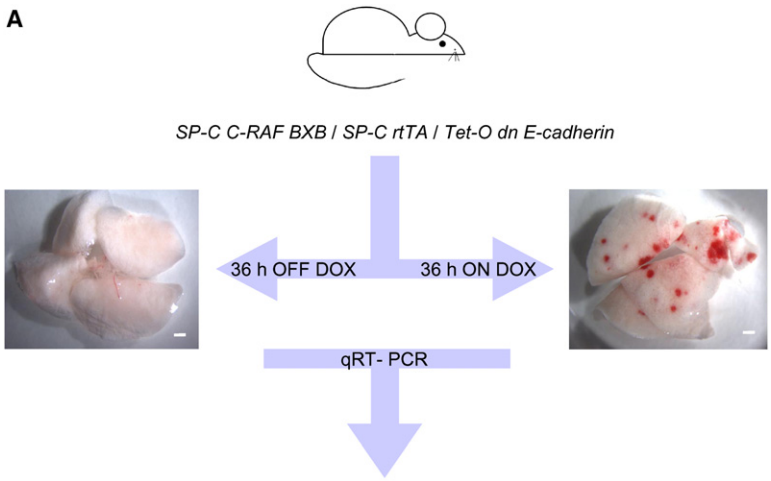


Figure 6. Acute Induction of dn E-Cadherin Leads to Elevated Expression of Target Genes for Angiogenesis

(A) Strategy for detection of transcripts during angiogenic switch induction. Six-week old triple transgenic mice were induced (36hr DOX pulse) or left untreated; note vessel leakage in the induced lung. Scale Bars, 2 mm.

(B) Quantitative real time RT-PCR from total lung. Transcripts included candidate angiogenic switch genes as well as β-catenin target genes that have been functionally linked to angiogenesis and cell cycle control. Values represent the average of triplicate samples of six mice for each condition. hr = hour.

B

Genes	ON DOX	OFF DOX	Fold Change	p values
VEGF-A	1.31	0.74	1.8	0.0041
VEGF-C	4.41	2.36	1.9	0.0113
Flt-4	0.22	0.07	3.1	0.0010
Cyclin D1	3.52	1.70	2.1	0.0005
ß-catenin	0.61	0.30	2.0	0.0276

a *SP-C rtTA* expressing activator plasmid alone or in conjunction with *SP-C C-RAF BXB* showed elevated VEGF staining 24 hr after transfection (Figures 7A and 7B). By transfection of a constitutively active β-catenin, β-catenin 4S, a similar level of VEGF induction was achieved (Figures 7A and 7B). Notably, neither in 3041 nor in A549 cells did *SP-C C-RAF BXB* induce VEGF expression (Figures 7A and 7B). After dn E-cadherin transfection, β-catenin was relocalized from the membrane and presumably made available to the cytoplasmic signaling pool similar to the constitutively active β-catenin 4S (Herzig et al., 2007) (Figures 7A and 7B, Figures S8A and S8B). To unravel the signaling cascade between dn E-cadherin and VEGF induction, we next investigated the role of β-catenin by addition of β-catenin siRNA. Notably, β-catenin siRNA suppressed VEGF expression in both 3041 and A549 tumor cell lines (Figures 7A and 7B). To further evaluate these observations, we determined the mRNA levels of VEGF-A and VEGF-C expression by real-time PCR analysis. Expression of dn E-cadherin increased the level of VEGF-A transcripts in a β-catenin dependent fashion, irrespective of C-RAF BXB coexpression in either cell line (Figures S7A and S7B). Different results were observed when we analyzed VEGF-C mRNA expression that was β-catenin independent (Figure S7B), even though β-catenin 4S was a powerful inducer of VEGF–C in both cell lines. These differences suggest that, for VEGF-C induction by dn E-cadherin, other pathways exist besides β-catenin-mediated signaling. There was also suggestive evidence for a suppression of VEGF-C by β-catenin in both cell lines although the effect was significant only in A549 cells (Figure S7B). In summary, in our transgenic NSCLC model, β-catenin plays a key role in the induction of cell-cycle progression of tumor cells and angiogenesis.

Chronic Expression of dn E-Cadherin in SP-C RAFBXB-Expressing Lung Epithelial Cells Upregulates β-Catenin-Responsive Genes that Include Endodermal and Other Lineage Markers

Previous data on SP-C-driven β-catenin signaling in embryonic lung, using expression of a fusion protein containing the aminoterminus of LEF-1 linked to the transactivation domain of β-catenin, had highlighted increased expression of genes associated with other endodermal lineages (Okubo and Hogan, 2004). We therefore extended our RT-PCR analysis to lung tumors from 4 months induced mice. Importantly, six of six genes selected from the list found to be ectopically expressed during embryogenesis (Okubo and Hogan, 2004) were upregulated in alveolar type II cells in adult mice to various extents (Figure 8). Most prominent was ectopic expression of the transcriptional regulators *Tcf4* and *Tcf1* and high mobility group box transcription factor *Cdx1*. Moreover, *Wnt-2* was induced, which is normally expressed preferentially in mesenchymal cells and has a role in early development of lung and intestine but has also been shown to

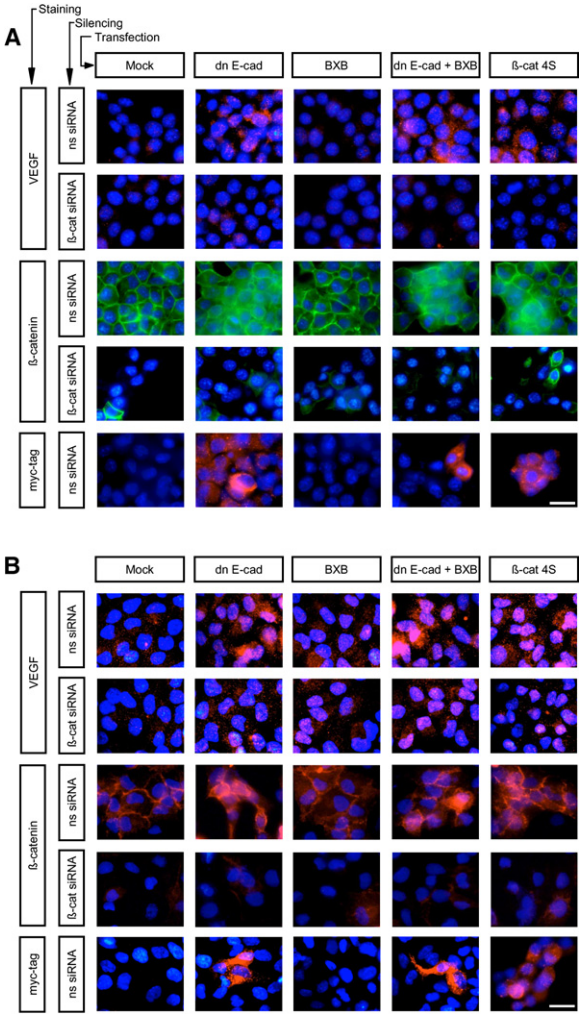


Figure 7. β-Catenin Dependence of dn E-Cadherin-Mediated VEGF Induction

Epithelial mouse lung 3041 and human NSCLC A549 cells were transfected with non specific siRNA (ns siRNA), β-catenin specific siRNA (β-cat siRNA) and DNA constructs as indicated. A constitutively active mutant of β-catenin, β-catenin 4S, was included.

(A) 3041 cells. Representative immmunocytochemistry staining identifies VEGF expression (red) in tumor cells transfected with dn E-cadherin in the presence or absence of *C-RAF BXB*. Expression of VEGF is strongly reduced by β-catenin siRNA. β-catenin siRNA also strongly reduced endogenous β-catenin (green) in transfected tumor cells. Note the redistribution of β-catenin from membrane to cytosol in cells expressing dn E-cadherin or β-catenin 4S. Scale Bars, 10 μm.

(B) A549 cells. Details as in (A), except for β-catenin staining (red).

be upregulated in NSCLC as well as other human carcinomas (Mazieres et al., 2004). As in the case of lung-targeted expression of constitutive β-catenin signaling during embryogenesis, ectopic expression was not confined to genes of the intestinal endoderm but included for example *DCPP* that is normally active in sublingual and salivary glands. Considering that we have constitutive β-catenin signaling in the DOX-treated triple transgenic mice, we also asked whether negative feed-back regulators of the Wnt pathway (*Dkk4*, *Nkd1*, *Wif1*, and *Axin-2*) might be upregulated (Dequéant et al., 2006). Of these, *Wif1* and *Nkd1* were clearly upregulated whereas *Axin-2* showed weekly increased levels. Taken together, these data support the notion that β-catenin is a major effector of dn E-cadherin and raise the possibility that forced simultaneous Wnt/RAF signaling generates phenotypic heterogeneity in tumors that may contribute to metastatic conversion.

DISCUSSION

Two strategies were followed to disrupt E-cadherin function in normal and *SP-C C-RAF BXB* transgenic adenomatous alveolar type II cells, DOX inducible *cdh1* gene ablation and expression of dn E-cadherin. Use of this transgenic strain with its discrete premalignant lesion allowed us to study NSCLC progression and to evaluate the role of cell-cell adhesion in progression of NSCLC-like lung adenomas to metastatic adenocarcinoma. The striking finding was that interference with E-cadherin function not only broke cell-cell contacts but contributed to tumor progression by massive induction of angiogenesis. Attempts to delineate the mechanism of angiogenesis induction highlighted β-catenin as a critical effector of VEGF induction by abrogation of E-cadherin. Disruption of cell-cell contacts and induction of angiogenesis was sufficient to promote micrometastasis.

Is Interference with E-Cadherin Function Oncogenic?

Both strategies to genetically disrupt E-cadherin mediated cell-cell contacts, ablation of *cdh 1* or dn E-cadherin induction failed to induce tumor formation in normal lung. Similar observations about lack of tumor formation upon loss of function of E-cadherin has been reported previously (Boussadia et al., 2002) by conditional knockout of *cdh1* in alveolar epithelial cells of the mammary gland using *MMTV-Cre* mice. More recently, the effects of E-cadherin loss on mammary tumorigenesis were studied by use of *K14cre* mice leading to E-cadherin loss in ductal and alveolar epithelium (Derksen et al., 2006). No tumor formation was observed in the mammary gland or in the skin which also expresses Cre in epithelium (Jonkers et al., 2001; Derksen et al., 2006). These data on skin were similar to an earlier report by Tinkle et al. who, in addition, observed compensatory upregulation of P-cadherin resulting in a hyperproliferative response (Tinkle et al., 2004). Our data in the lung are comparable to those of Tinkle et al., as we observe diffuse hyperplasia in mice with either genetic condition to disable E-cadherin in lung alveolar type II cells. In contrast to skin, we did not observe a compensatory P-cadherin upregulation. N-cadherin expression also remained unchanged. We conclude that *cdh1* knockout or dn E-cadherin induction is not sufficient for lung tumorigenesis.

Induction of Angiogenic Switch by Disruption of E-Cadherin

Lung adenomas induced by C-RAF BXB are notoriously stable, a feature that may result from high E-cadherin

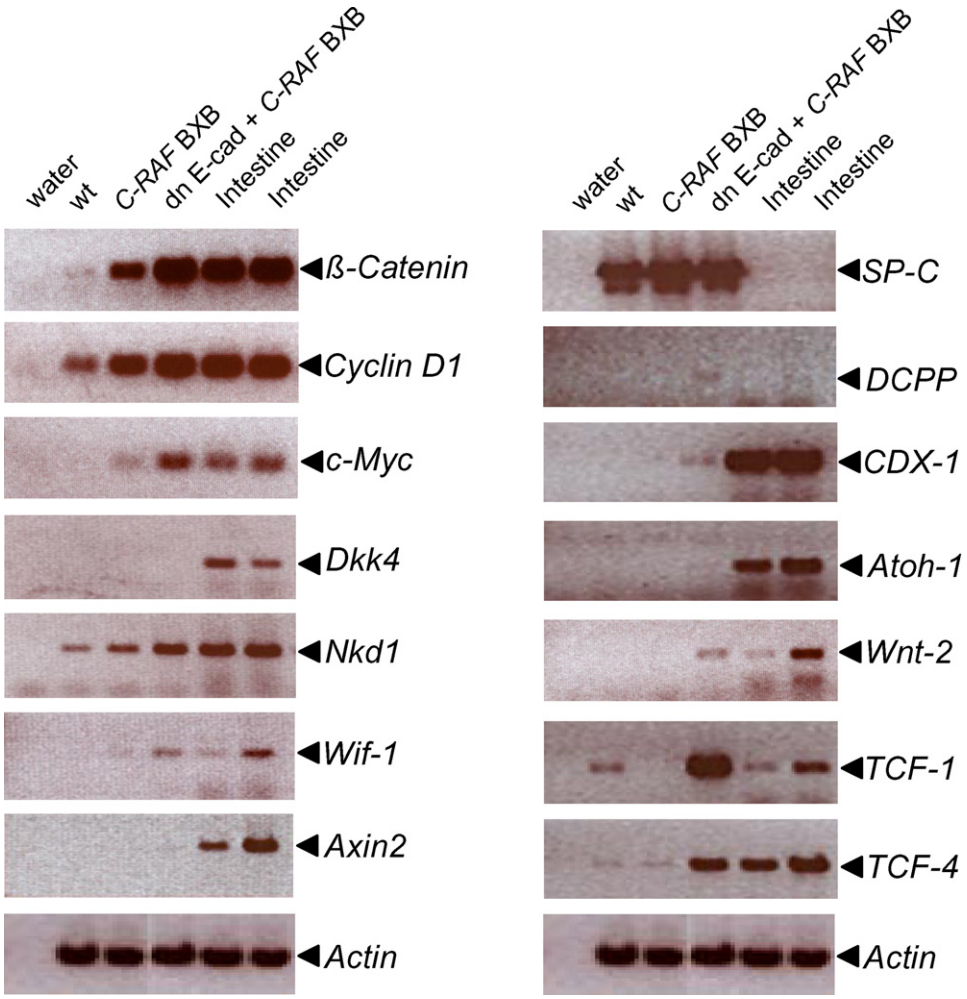


Figure 8. Expression of β-Catenin Responsive Genes in Chronically Induced Mice Reveals Reprogramming
Type II pneumocytes were prepared from mice of different genotypes as indicated. Triple transgenic mice were induced for 4 months and used for RT-PCR. The panel of β-catenin responsive target genes includes components of the Wnt pathways (*Wnt-2*, *Tcf-1*, *Tcf-4*, *Dkk4*, *Nkd1*, *Wif-1*, and *Axin2*), cell-cycle regulators (*cyclin D1*, *c-myc*), and previously identified endodermal selector genes (*Atoh-1*, *Cdx-1*), as well as secretory lineage of the trachea (*DCPP*). *SP-C* was used to confirm the identity of the type II epithelial cells, and *β-actin* was used as control for RNA integrity.

expression. One way by which cadherin junctions may contribute to stability of C-RAF BXB adenomas is by suppression of angiogenesis. Evidence for suppression was obtained by our findings that disruption of cell-cell contact by *cdh1* gene ablation or expression of dn E-cadherin in C-RAF BXB adenomas was followed by massive angiogenesis correlating with appearance of nuclear β-catenin in a fraction of tumor cells and their invasive behavior. These data demonstrate for the first time that the disruption of E-cadherin leads to the induction of the angiogenic switch in a preexistent adenoma and consequent progression to adenocarcinomas. Consistent data regarding blood angiogenesis were presented in a recent publication that examined effects of Cdh1 loss in a model for invasive lobular breast cancer (Derksen et al., 2006). However, no data on lymphangiogenesis were included in this study. In the pioneering studies of Folkman et al., large T antigen was expressed under the control of rat insulin promoter (Folkman et al., 1989). Large T antigen was sufficient by itself to promote the full course of tumor progression from hyperplasia of pancreatic β-cells to carcinoma. Emergence of carcinoma was preceded by angiogenesis in a small fraction of hyperplastic islands that correlated with carcinoma incidence. This phenomenon was called the angiogenic switch. In subsequent studies Perl et al. used crosses between Rip1Tag2 and Rip1 dn E-cadherin mice and suggested that dn E-cadherin can induce tumor progression. Double transgenic mice showed doubling of carcinoma incidence and low level of lymph node metastasis (Perl et al., 1998). In their study, no data on angiogenesis were reported. Moreover, causal evidence for the role of dn E-cadherin at specific steps of tumor progression was not provided and would have required use of reversible expression systems. We have used such a conditional expression system and demonstrated that formation of blood and lymph vessels in tumors entirely depends on

disruption of E-cadherin expression. Reversibility was maintained for up to 2 months after induction and involved apoptosis of endothelial cells upon restoration of E-cadherin expression. Appearance of blood and lymph vessels in the tumors correlated with increased size of individual tumor nodules some of which reached macroscopic scale. As in the case of angiogenesis, the dn E-cadherin induced increase in tumor volume required continuous presence of DOX. Long-term treatment did uncover another important aspect of tumor vasculogenesis relating to reversibility. After 4 months induction, vessels self-stabilized, presumably due to acquisition of a quiescent differentiated phenotype and recruitment of pericytes (Risau, 1997; Nikolopoulos et al., 2004). As a consequence, DOX withdrawal no longer leads endothelial cells to apoptosis. Although these findings support the concept of tumor therapy with angiogenesis inhibitors (Folkman, 2006), they also point to a limitation. It may be that treatment of patients with established tumors with a mature vasculature will only induce stasis but not tumor regression.

E-Cadherin Disruption Promotes Progression of Angiogenic Tumors to Invasive Growth and Micrometastasis

Besides induction of angiogenesis, *cdh1* deletion or dn E-cadherin induction also cooperates with C-RAF BXB in driving proliferation and invasive growth. The increase in proliferation rates was in the order of 50% and led to appearance of macroscopic tumors on the surface of lungs by the age of 8 months. Cooperation in growth stimulation required continuous induction of dn E-cadherin as a 4 month ON DOX/4 month OFF DOX schedule reduced tumor volume to the level C-RAF BXB. These data may suggest a continuous need for neoangiogenesis or result from cell-autonomous effects of E-cadherin loss on cell-cycle control. In addition to cooperation in proliferation, loss of E-cadherin function changed the growth pattern of tumor cells in tumor foci. Tumors in compound mice were more dispersed and expanded into the lung parenchyma without signs of classical EMT. This finding is consistent with a previous study where expression of dn E-cadherin in Rip1Tag2 transgenic mice also led to invasive growth and lymph node metastasis without complete EMT conversion (Perl et al., 1998). Furthermore, our data on E-cadherin gene ablation in *SP-C C-RAF BXB* mice are in agreement with a large body of literature that links loss of E-cadherin expression with adenoma-carcinoma progression (Birchmeier, 2005; Berx and Van Roy, 2001; Cavallaro and Christofori, 2004) and with a recent publication by Derksen et al. who did not observe classical EMT in invasive lobular breast cancer upon *cdh1* loss (Derksen et al., 2006). Our data on EMT agree well with those of Perl and Derksen (Perl et al., 1998; Derksen et al., 2006). While we cannot exclude that dispersed cells in tumor foci of induced compound mice have a transient EMT in their history, we have no evidence for such an event as we did not find cells double-positive for vimentin/proSPC or N-cadherin/proSPC expression. On the other hand, as cells with disrupted E-cadherin appear spindle-shaped, this may be considered as a partial EMT.

Angiogenic switch followed by progression of induced tumors to invasive adenocarcinomas was not in itself sufficient to promote metastatic spread. This required additional time (at least 5 months) and led to micrometastasis into regional lymph nodes and occasionally into bone marrow. Micrometastasis was completely dependent on continuous DOX treatment. One explanation for the time requirement might be increase in tumor mass that would raise the probability for an infrequent event such as generation of metastatic cells to take place. The fact that micrometastasis occurred late whereas angiogenesis and cell dispersion were early events is consistent with the concept that additional genetic changes are required in the tumor cells before they express (mutated) metastasis gene(s) (Hanahan and Weinberg, 2000). Tumor cells equipped with a "metastatic signature" should be able to grow into new tumors at distant sites. That is not the case in micrometastasis (Schardt et al., 2005) that may spread dormant tumor cells. Dormant tumor cells are initiated cells using the language of two-stage carcinogenesis (Rous and Kidd, 1941; Berenblum, 1954). These cryptic transformants require a tumor-promoting condition which may utilize regenerative signaling pathways (Maurange et al., 2006). The dependence of micrometastasis on continued dn E-cadherin expression suggests that the ability of tumor cells to induce angiogenesis may be required for seeding at distant sites but may not be sufficient for clonal expansion at that site. The combined data suggest that the step in tumor progression that is being induced by disruption of E-cadherin may be limited to acquisition of a self-seeding phenotype that includes micrometastasis (Norton and Massaqué, 2006). It will be interesting to determine whether strengthening the growth engine by combination of *RAF* with other oncogenes would overcome this limitation. We are currently exploring this possibility in transplantation experiments with fractionated tumor cells.

Disruption of E-Cadherin Activates β-Catenin Signaling

Quantitative RT-PCR of acutely induced compound mice identified 3 classes of angiogenic factors that showed a 2-fold increase. These were VEGF-A, VEGF-C and its receptor Flt-4, as well as Tie1, endothelial cell adhesion molecule VE-cadherin and β-catenin together with cyclin D1. VEGF-A and VEGF-C were also induced in vitro in transient transfection experiments with a dn E-cadherin expression vector in mouse and human cell lines derived from NSCLC. The simplest explanation for the combined in vivo and in vitro findings is a signaling cascade connecting dn E-cadherin or *cdh1* inactivation via β-catenin with angiogenesis target genes. Such a chain is supported by in vitro siRNA experiments against β-catenin and identifies E-cadherin disruption for the first time as a signaling condition. Induction of VEGF-A, not VEGF-C was β-catenin-dependent, consistent with an earlier report which described VEGF-A as a direct β-catenin target gene in

human colon cancer cell lines (Easwaran et al., 2003). In human endothelial cells (HUVECs), β-catenin was described to mediate both VEGF-A and VEGF-C expression (Skurk et al., 2005) which parallels the pattern we found in our lungs from DOX-induced triple transgenic mice. VEGF-A and VEGF-C have prognostic value for NSCLC (Nakashima et al., 2004), and the VEGF-C/Flt-4 axis has been shown to promote invasion and metastasis of lung A549 and other cancer cell lines (Su et al., 2006). On the basis of our and these literature data we conclude that the mechanism of angiogenic switch induction and subsequent tumor progression induced by disruption of E-cadherin involves signaling via β-catenin and VEGF-A/VEGF-C.

Upregulation of β-Catenin Targets by Chronic Disruption of E-Cadherin in Alveolar Epithelial Cells Includes Genes from Endodermal Lineages

Further evidence that β-catenin functions as a critical effector of chronic E-cadherin disruption was obtained by RT-PCR experiments using RNA from purified type II cells of lung tumors. These experiments revealed upregulation of a set of genes that was previously reported to be induced in embryonic lungs with a SP-C promoter-driven constitutively active β-catenin transgene (Okubo and Hogan, 2004). Selected candidate genes included endodermal and other lineage markers and Wnt pathway and cell-cycle regulators. Our data suggest reprogramming of C-RAF-transformed type II cells and raise the possibility that constitutive signaling by the Wnt pathway simultaneously with mitogenic cascade signaling may erode phenotypic stability. There is evidence that these two pathways normally function in an alternating fashion and thus constitute the core element of a developmental oscillator (Dequéant et al., 2006). As combined signaling is a prerequisite for micrometastasis in our model, it is tempting to speculate that ontogenetically younger programs are used as a resource for acquisition of metastatic behavior.

EXPERIMENTAL PROCEDURES

Animals

Generation of transgenic mice conditionally expressing dn E-cadherin is described in detail in Supplemental Experimental Procedures. All animal studies were approved by the Bavarian State authorities for animal experimentation.

RNA Isolation and RT-PCR Analysis

Protocols for RNA isolation from microdissected mouse lung tumors, 3041, A549 lung tumor cells and total mouse lungs, RT-PCR and real-time RT-PCR procedures are given in Supplemental Experimental Procedures.

Cell Culture and Transfections

Mouse 3041 alveologenic lung adenocarcinoma (Rizzino et al., 1982) and human lung carcinoma A549 epithelial cells (ATCC CCL-185) were maintained in DMEM supplemented with 10% calf serum. Cells were seeded into 12-well plates 24 hr before transfection. For DNA transfection, Lipofectamine 2000 reagent (Invitrogen) was used. siRNA against mouse β-catenin (sc-29210, Santa Cruz) and human β-catenin (CTNNB1, M-003482, Upstate) were transfected using transfection reagent (sc-29528, Santa Cruz) according to the manufacturer's descriptions. Validated nonsilencing control siRNA (Alexa Fluor 488 Labeled, QIAGEN) was always included for control cells (mock) and for siRNA transfection efficiency. Transfected cells were starved for 5 hr in the absence of serum and antibiotics followed by recovery in complete medium for 20 hr. Cells were subsequently transfected with plasmids *Tet-O dn E-cadherin*, *SP-C rtTA*, *SP-C C-RAF BXB* alone or combination, and *β-catenin 4S* plasmid (Herzig et al., 2007) using lipofectamine reagent. Cells were harvested 24 hr later and RNA extracted using TRIzol reagent (Invitrogen). Knockdown efficiency of β-catenin was determined by β-catenin immunostaining and by qRT-PCR. Transfection efficiency of DNA constructs was monitored by qRT-PCR, staining, and luciferase assay (Promega).

Luciferase Activity Assays

Mice were sacrificed by lethal injection of ketanest (Parke-Davis) together with rompun (Bayer). After lung dissection, the tissue was homogenized in lysis buffer containing 0.25 M Tris, pH 7.6, 1% Triton X-100. After centrifugation at 11,000 rpm for 10 min, the luciferase activity in the supernatant was measured in a luciferase assay according to the manufacturer's instructions (Promega). Protein concentrations were determined with the Bradford protein assay. Luciferase activity was normalized to protein concentration to calculate relative light units (RLU) per mg protein.

Terminal Deoxynucleotidyltransferase-Mediated dUTP-Biotin Nick End Labeling Assay

Frozen lung sections of triple transgenic mice were stained according to the Dead End Fluorometric TUNEL System instructions (Promega). Immunofluorescence Double Staining for CD31/PECAM-1 and TUNEL was as described (Solorzano et al., 2001).

Immunoblotting and Immunoprecipitation

Procedures were as detailed in Supplemental Experimental Procedures.

Immunofluorescence and Immunohistochemistry Microscopy

Animals were sacrificed and lungs were fixed under 25 cm water pressure with 4% PBS buffered formalin. Histology was done on formalin-fixed, paraffin-embedded lung specimen. 6 μm-cut sections were deparaffinized, rehydrated in graded alcohols and Hematoxylin and eosin stained. Details of staining protocols are given in Supplemental Experimental Procedures.

Histopathology

Preparation of embedded tissues and quantitative assays for lung tumor development are described in Supplemental Experimental Procedures.

The Graphpad Prism version 4.00 (Graphpad Software, Inc, San Diego, CA) software programs were used for all statistical analysis and graphing. For all tests, statistical significance was considered to be at the $p < 0.05$ level.

Supplemental Data

The Supplemental Data include Supplemental Experimental Procedures, eight supplemental figures, and two supplemental tables and can be found with this article online at http://www.cancercell.org/cgi/content/full/12/2/145/DC1/.

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