The Transcriptional Repressor ZEB1 Promotes Metastasis and Loss of Cell Polarity in Cancer

Simone Spaderna,[1] Otto Schmalhofer,[1] Mandy Wahlbuhl,[2] Arno Dimmler,[2] Katja Bauer,[3] Aneesa Sultan,[4] Falk Hlubek,[5] Andreas Jung,[5] Dennis Strand,[6] Andreas Eger,[4] Thomas Kirchner,[5] Jürgen Behrens,[3] and Thomas Brabletz[1]

[1]Department of Visceral Surgery, University of Freiburg, Freiburg, Germany; [2]Department of Pathology and [3]Nikolaus-Fiebiger-Center, University of Erlangen, Erlangen, Germany; [4]Max F. Perutz Laboratories, Medical University Vienna, Vienna, Austria; [5]Department of Pathology, University of Munchen, Munich, Germany; and [6]First Department of Internal Medicine, University of Mainz, Mainz, Germany

Abstract

Invasion and metastasis are the hallmarks of malignant tumor progression and the main cause of death in cancer. The embryonic program "epithelial-mesenchymal transition" (EMT) is thought to trigger invasion by allowing tumor cell dissemination. Here, we describe that the EMT-inducing transcriptional repressor ZEB1 promotes colorectal cancer cell metastasis and loss of cell polarity. Thereby, ZEB1 suppresses the expression of cell polarity factors, in particular of Lgl2, which we found reduced in colorectal and breast cancers. We further show that retention of Lgl2 expression is critical for the epithelial phenotype and that its loss might be involved in metastasis. Thus, by linking EMT, loss of polarity, and metastasis, ZEB1 is a crucial promoter of malignant tumor progression. [Cancer Res 2008;68(2):537–44]

Introduction

Invasion and metastasis are the hallmarks of malignant tumor progression. There is increasing evidence that aberrant activation of the embryonic program termed epithelial-mesenchymal transition (EMT) is crucially involved in tumor cell invasion (1, 2). EMT allows the detachment of cells from each other and increases cell mobility, both necessary for tumor cell dissemination. EMT is triggered by the inductive environment and is mediated intracellularly through different transcriptional repressors. These EMT inducers are aberrantly expressed in various types of carcinomas and typically suppress the transcription of the E-cadherin gene, an important marker of epithelial differentiation. EMT inducers include members of the snail family (Snail; refs. 3, 4, and Slug; ref. 5), Twist (6), E12/47 (7), Goosecoid (8) and members of the ZFH family of repressors (ZFHX1a, also called ZEB1 or δEF1; ref. 9, and ZFHX1b, also called SIP1 or ZEB2; ref. 10). We described an EMT at the invasive front of colorectal adenocarcinomas, but strikingly detected a mesenchymal to epithelial retransition in metastases, which in most cases, again show the same differentiated phenotype as the primary tumor (11, 12).

Recently, we detected that ZEB1 is a crucial EMT inducer in human colorectal carcinomas (CRC) and suppresses the expression of basement membrane components (13). Importantly, expression of ZEB1 associated with EMT and selective loss of basement membrane in invasive tumor regions was a strong predictor of poor patient survival and metachronous distant metastasis, but a direct key role of ZEB1 in metastasis has not yet been shown (13). However, recent data indicate a direct connection between EMT and metastasis, e.g., the mesenchymal differentiation factor FOXC2, which is activated by EMT inducers, was shown to promote metastasis in breast cancer (14).

Based on previous results, the aim of this study was to prove a crucial function of ZEB1 for metastasis and to identify new molecular links for ZEB1 in the context of malignant tumor progression.

Materials and Methods

Tissue specimens and immunohistochemistry. Formalin-fixed, paraffin-embedded as well as fresh-frozen samples of colorectal and breast carcinomas from patients who underwent surgery without additional treatments were retrieved from the archives of the Department of Pathology, University of Erlangen-Nürnberg. Immunohistochemistry on human tumors and on cultured cells was done as previously described (15).

DNA constructs and cell clones. For stable knockdown of ZEB1 (shZEB1) and for control (shGFP), the following sequences were annealed and cloned into pRETRO-SUPER (16): shZEB1 for, 5′-gatccccagatgatgaatgcgagtcgttcaagagatgactcgcattcatcatctttttggaaa-3′; shZEB1 rev, 5′-agcttttccaaaaaagatgatgaatgcgagtcatctcttgaacgactcgcattcatcatctggg-3′; shGFP for, 5′-gatccccgctacctgttccatggccattcaagagatggccatggaacaggtagctttttggaaa-3′; shGFP rev, 5′-agcttttccaaaaagctacctgttccatggccatctcttgaatggccatggaacaggtagcggg-3′. Constructs were transfected into the indicated cell lines and stable clones were selected using 2.5 μg/mL of puromycin (Calbiochem). HEK293 Lgl2 were constructed by stable overexpression of the human Lgl2 expression vector *pIND-Hugl2*.

Cell culture and various assays. All cell lines were purchased from American Type Culture Collection. Cell culture, transient transfections, reporter assays, electromobility shift assays, immunoblots, transient short interfering RNA–mediated knockdown, RNA isolation, and real-time reverse transcription-PCR for quantification of mRNA expression were done as previously described (13, 15–17). mRNA expression values were measured in triplicate using the ABI PRISM 7700 and a Sequence Detector V1.7a program (PE Biosystems) and normalized to β-actin expression as a housekeeping control. To determine the absolute copy number of the target transcript, cloned plasmid DNAs were used to produce a standard curve.

Statistics. Data are the means ± SD of the number of experiments (minimum of three). The statistical significance between experimental values was assessed by the unpaired Student's t test using SPSS software, and $P < 0.05$ was considered to be statistically significant.

Immunofluorescence and confocal laser scanning microscopy. Fluorescence immunocytochemistry on cultured cells was done as previously described (15). Confocal laser scanning microscopy was performed using a Leica TCS SP2 microscope (Leica Microsystems).

Nude mice xenografts. Male, athymic nude mice (BALB/c *nu/nu*) were used for s.c. injection of 2×10^6 tumor cells, which however did not result in

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Thomas Brabletz, Department of Visceral Surgery, University of Freiburg, Hugstetter Str. 55, 79106 Freiburg, Germany. Phone: 49-0761270-2577; Fax: 49-0761270-2779; E-mail: thomas.brabletz@uniklinik-freiburg.de.

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metastasis for the cell lines used. Therefore, to study lung metastasis, 1×10^6 tumor cells were injected into the tail vein. For liver metastasis, mice were anesthetized by i.p. injection of both Ketanest and Rompune and 1×10^6 tumor cells were injected into the exteriorized spleen after abdominal incision. The abdominal wound was closed in one layer with single sutures. After 6 weeks, mice were sacrificed by cervical dislocation after i.p. anesthesia with Ketanest and Rompune. The tumors were removed, fixed in 5% formaldehyde, and stained with H&E (for more methods, see supplementary online data).

Results

ZEB1 is crucial for the metastasis of colorectal cancer cells. In accordance with our data from human CRCs (13), ZEB1 is overexpressed in various colorectal and breast cancer cell lines (13, 18). In order to prove the central role of ZEB1 in malignant tumor progression, we stably knocked down ZEB1 by short hairpin RNA transfer in tumor cells expressing high levels of ZEB1 (see Supplementary data, Fig. S1*A*), leading to a restitution of the epithelial phenotype as indicated by enhanced E-cadherin and reduced Vimentin expression. Loss of ZEB1 led to reduced tumor cell migration in SW480 and HCT116 colorectal cancer cells as well as in undifferentiated MDA-MB231 breast cancer cells (Fig. 1*A*; Supplementary data, Fig. S1*B*). Moreover, invasion in collagen gels was also reduced (Fig. 1*B*). Most importantly, knockdown of ZEB1 strongly affected the formation of metastasis in nude mice xenografts (Table 1; Fig. 1*C*): Intrasplenic injection of HCT116 control transfectants or wild-type cells (data not shown) rapidly led to metastases in liver and pancreas. ZEB1 knockdown (two independent clones: shZEB1 Z*4* and shZEB1 Z6; data not shown) completely abolished metastasis formation, although the primary tumors in the spleen grew to a similar size. After tail vein injection, the number of lung metastases was strongly reduced (10 versus 1) by ZEB1 knockdown. Moreover, the growth of metastasis following ZEB1 knockdown was much smaller. SW480 control transfectants and wild-type cells did not promote metastases. These data suggest that ZEB1 is crucial for the formation of metastasis without affecting the growth of the primary tumor. All experiments were done with at least two independent clones from each stable transfected cell line.

ZEB1 is crucial for loss of basal-apical cell polarity. To further investigate how aberrant expression of ZEB1 triggers the processes which are involved in malignant progression, we further analyzed the behavior of ZEB1 knockdown clones in cell cultures. All wild-type cell lines or control transfectants tested grow in a

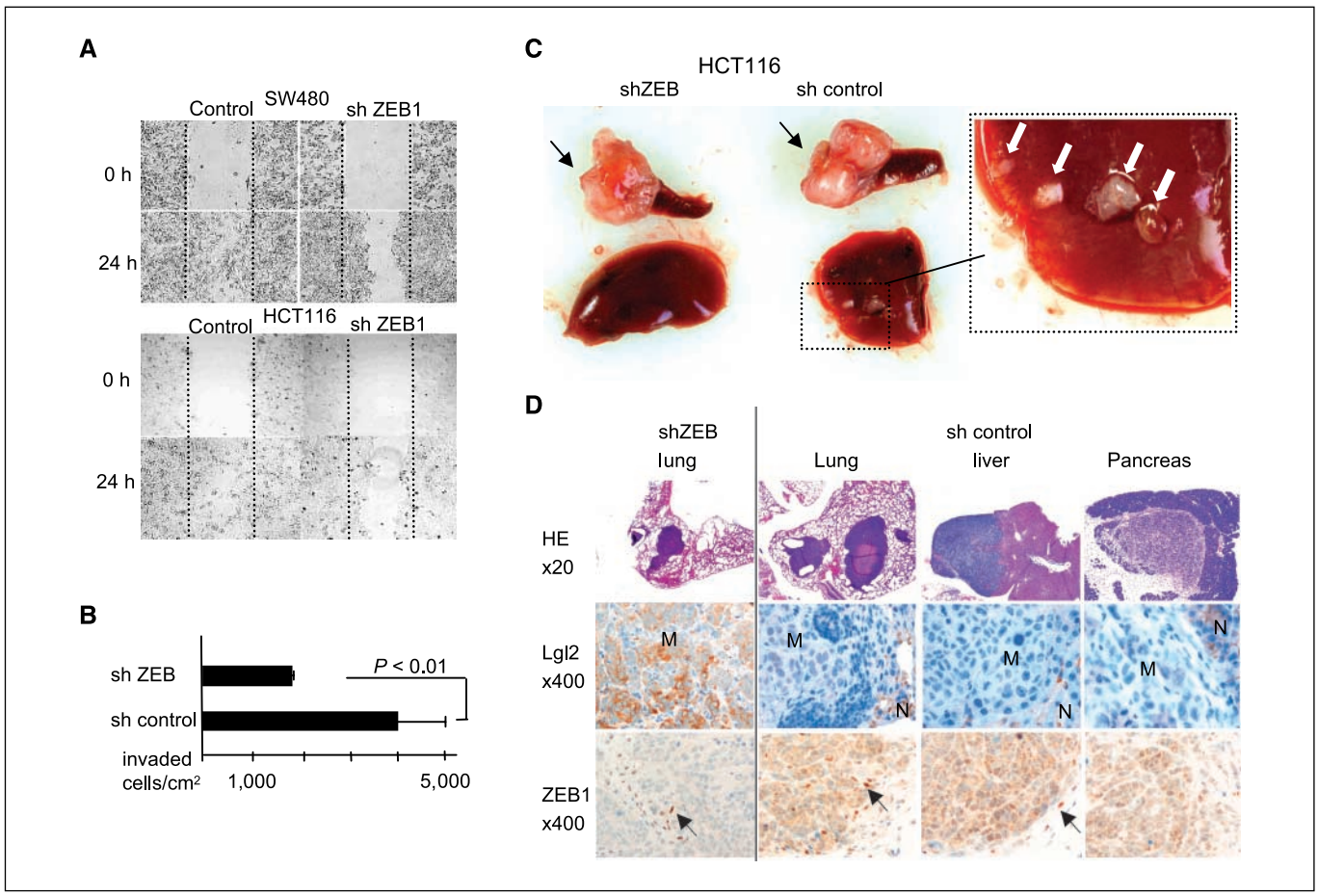

Figure 1. ZEB1 is important for metastasis. *A,* ZEB1 knockdown results in the reduced migratory potential of SW480 and HCT116 cells in a wounding assay ($P < 0.01$). *B,* ZEB1 knockdown inhibits the invasion of MDA-MB231 cells into collagen type 1 gel. *C,* ZEB1 knockdown leads to the complete suppression of liver metastasis after intrasplenic injection (an example of liver metastases). Note that the primary spleen tumors (*black arrows*) have grown to the same size, although only the control-transfected clone produced liver metastases (*white arrows*). *D,* histology of the metastases, showing that the only small lung metastases from shZEB cells consists of tumor cells expressing Lgl2 and lacking ZEB1. In contrast, tumor cells of all metastases (*M*) to different organs of sh control cells retain ZEB1 and lack Lgl2. Normal tissues (*N*) express Lgl2 and tumor stroma cells (*arrow*) express ZEB1.

Table 1. Incidence of metastases produced by the HCT116 sh control and shZEB1 cell clones after tail vein or intrasplenic injection to nude mice

HCT116 clone	Injection procedure	Primary tumors		Metastases			
		No. of mice	Average size (mm)	No. of mice	Total no.	Location	Average size (mm)
sh control C1	Tail vein	—	—	4/6	10	Lung	1.2
	Intrasplenic	5/5	8.1	4/5	14	Liver (11)	1.8
						Pancreas (3)	0.9
shZEB1 Z4	Tail vein	—	—	1/6	1	Lung	0.5
	Intrasplenic	5/5	7.9	0/6	0	—	—

NOTE: $P < 0.01$ (for metastasis, number and size).

fibroblastoid spindle-shaped morphology, and show low cytoplasmic, perinuclear expression of E-cadherin or cytoplasmic expression of β-catenin (Fig. 2*A*; Supplementary data, Fig. S1*C*). Knockdown of ZEB1 led to a change in morphology resembling a mesenchymal to epithelial retransition: cells grow cobblestone-like with clear cell-cell contacts, and increased amounts of E-cadherin and cytoplasmic β-catenin translocate to the cell membrane. Moreover, as indicated by confocal microscopy using the *z*-axis mode, ZEB1 knockdown leads to a partial restitution of a basal-apical polarity in a cell monolayer, in contrast to control transfectants, which grow as unconnected, unpolarized and rounded cells.

Basal-apical cell polarity is an important hallmark of epithelial differentiation. In glandular tissues such as intestinal mucosa or breast epithelium, epithelial cell polarity is necessary to build up luminal structures and to organize the asymmetrical distribution of proteins along the basal-apical axis, which for instance, is necessary for a directed luminal transport of mucins or milk proteins. In order to further investigate if aberrant expression of ZEB1 is crucial for loss of cell polarity, we grew the cells in three-dimensional Matrigel or collagen gel culture. In contrast to control transfectants, which grow completely scattered or highly invasive, knock down of ZEB1 induced spheroidal growth (Fig. 2*A*; Supplementary data, Fig. S1*C*). In particular, in the normally undifferentiated colorectal cancer cell line, SW480, a central lumen was often formed. Lumen formation requires a functional apoptotic cascade. All the cancer cell lines used here have activating *K-ras* gene mutations affecting apoptosis, which might explain why only a partial lumen formation is seen after ZEB1 knockdown.

ZEB1 suppresses the expression of the polarity factor Lgl2. Loss of basal-apical polarity is a hallmark of poor tumor differentiation, and accordingly, high tumor grade (Supplementary data, Fig. S1*D–G*). Because loss of polarity/high grading is associated with metastasis and poor prognosis, and thus, might be an important aspect for the prometastatic effect of ZEB1, we further analyzed the ZEB1-associated loss of polarity in tumor cells.

Basal-apical polarity is mediated by polarity factors, which are broadly grouped as the Crumbs, the Par, and the Scribble complex (19). Knockdown of ZEB1 in colorectal cancer cells resulted in increased expression of polarity factors, in particular, we detected factors of the Crumbs complex (Crumbs3 and INADL), the Scribble complex (Lgl2; ref. 19), as well as the basolateral sorting factor AP1M2 (20) and the apical sorting factor Mal2 (ref. 21; Fig. 2*B*). Interestingly, the Par complex factors were not affected. All genes from this factor had potential binding sites for ZEB1 or other EMT repressors, like Snail and Slug, within their promoter regions and binding of endogenous ZEB1 to their native promoters was confirmed by chromatin immunoprecipitation (Supplementary data, Fig. S2*A,B*).

Lethal giant larvae 2 (Lgl2) is a vertebrate homologue of the cell polarity factor *lgl*, initially discovered in *Drosophila* (22), in which it was classified as a tumor suppressor because its loss leads to metastatic tumors (23). Therefore, we further focused on the analysis of Lgl2 expression in cell culture and human tumors. We detected two highly conserved E-boxes as potential ZEB1 binding sites in the *Lgl2* promoter (Fig. 2*C*), and by using gel shift and reporter assays, we could show that *Lgl2* is a direct target gene of ZEB1-mediated transcriptional repression in colorectal cancer cells. Overexpression of other EMT-associated transcriptional repressors, such as Snail1 and Snail2/Slug also led to a reduction of *Lgl2* promoter activity (Supplementary data, Fig. S2*C–G*), and transient siRNA-mediated knockdown of Slug also led to the up-regulation of Lgl2 in SW480 and MDA-MB231 cells, although to a lower extent compared with ZEB1 knockdown (data not shown). These data were confirmed in the stable shZEB1 knockdown clones, showing a consistent up-regulation of Lgl2 at both mRNA and protein levels (Fig. 2*D*; Supplementary data, Fig. S1*A*). Moreover, Lgl2 expression was detectable at the membrane and was associated with the restitution of basal-apical polarity of all shZEB1 clones in two-dimensional and three-dimensional cultures (Fig. 2*A*; Supplementary data, Fig. S1*C*). Furthermore, after nude mice xenografting, all metastases from control transfectants had a complete lack of Lgl2, in contrast with the only grown metastasis (lung) from ZEB1 knockdown cells, which expressed membranous Lgl2 and was much smaller (Fig. 1*D*).

The role of ZEB1 as a repressor of *Lgl2* gene transcription and as an EMT inducer was further indicated by the inverse correlation of ZEB1 and Lgl2 expression in various cancer cell lines (Supplementary data, Fig. S3*A*). High ZEB1 expression, e.g., in the related colorectal cancer cell lines, SW620 and SW480, was associated with low Lgl2 expression. These cells grow in a fibroblastoid pattern, similar to mesenchymal cells such as human fibroblasts, which expressed the highest levels of ZEB1, and consequently, no Lgl2. In contrast, T84 colorectal cancer cells, expressing almost no ZEB1, showed the highest levels of Lgl2 expression. Moreover, in nude mice xenografts, SW620 tumors are completely undifferentiated, lacking any sign of polar architecture. In contrast, T84 tumors are

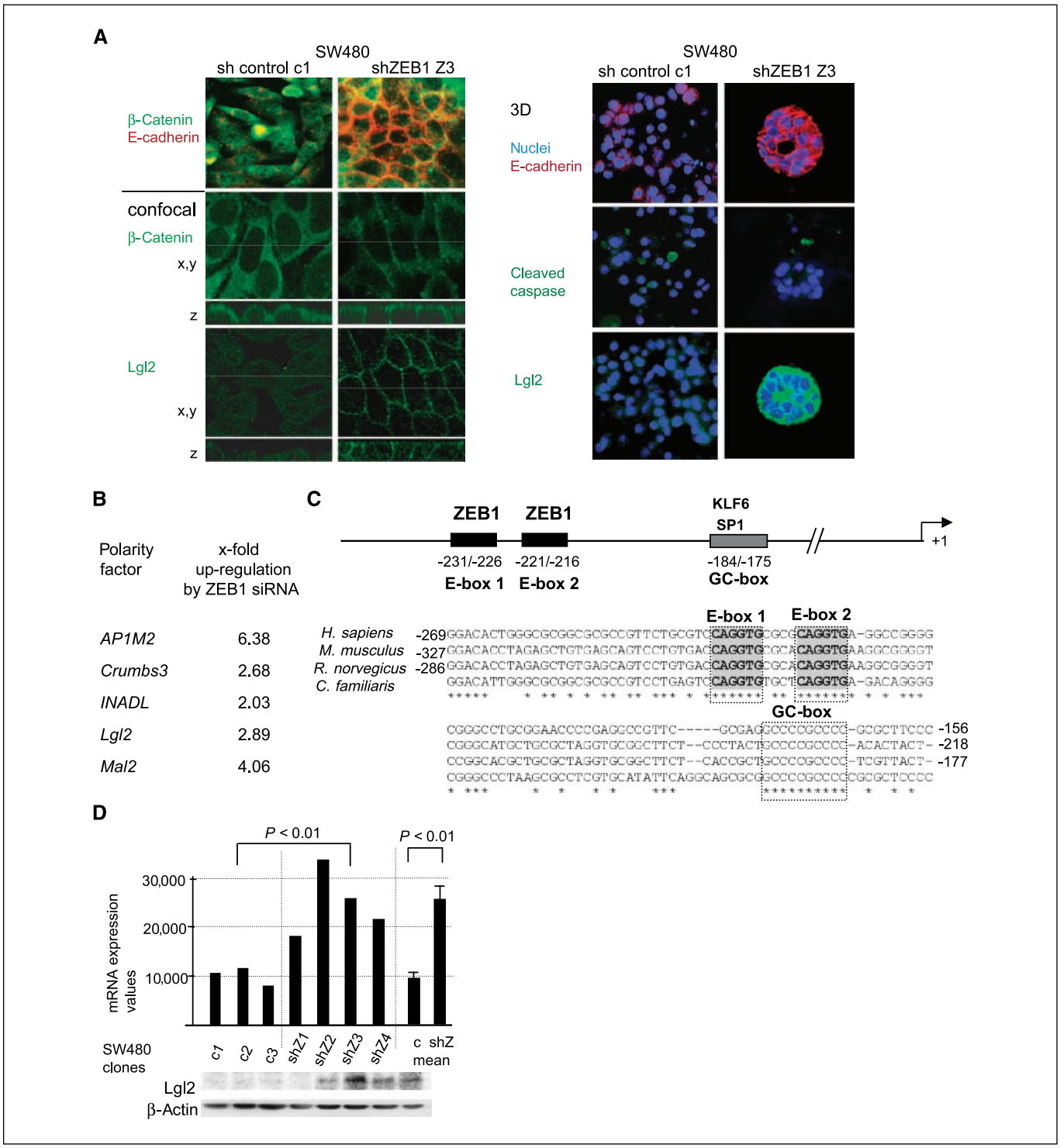

Figure 2. ZEB1 is crucial for cellular polarity and the regulation of Lgl2 expression. *A, left,* stable ZEB1 knockdown leads to a dramatic change in the cellular growth pattern of SW480 cells from a fibroblastoid towards an epithelial phenotype: increased expression of E-cadherin and colocalization with β-catenin translocated from the nucleus to the membrane. Confocal laser scanning fluorescence shows restoration of membranous Lgl2 expression: in the *z*-axis mode, these clones show a cobblestone-like phenotype, membranous expression of β-catenin and reexpressed Lgl2, and restoration of basal-apical polarity. In contrast, the control-transfected cells grew without fixed cell-cell contacts in a round, unstructured phenotype (*dotted line* in the *x,y*-mode indicates *z*-axis plane), and lack Lgl2. *Right,* ZEB1 knockdown allows a spheroid growth of SW480 cells in three-dimensional Matrigel. Cells expressed membranous E-cadherin and Lgl2, show beginning polarization, and in some cases, the formation of a central lumen. However, no luminal apoptosis was detectable, as shown by the lack of cleaved caspase staining. In contrast, control transfectants grew scattered and lacked any sign of organization or spheroid formation in three-dimensional cultures. *B,* up-regulation of polarity factor mRNAs after stable knockdown of ZEB1 in SW480. *C,* structure of the human *Lgl2* promoter and conserved sequences of the relevant promoter region (*stars,* evolutionarily conserved nucleotides in all four species; numbers are relative to the transcription start sites, for the dog lgl2 gene, the conserved region is in intron 2). The human promoter contains two adjacent E-boxes as potential binding sites for EMT-associated transcriptional repressors like ZEB1, which are highly conserved throughout evolution. *D,* stable knockdown of ZEB1 (*clones shZ1–4*) leads to an increase of Lgl2 expression both at the mRNA and protein level (*immunoblots, bottom*), compared with stable control transfectants (*clones c1–3*). *Columns,* mean expression values for all clones tested.

differentiated and characterized by tubular structures, built-up by polarized tumor cells. Caco-2, showing intermediate levels of ZEB1 and Lgl2, grow in a mixed tumor phenotype with more differentiated, polar areas, as well as undifferentiated areas.

The polarity factor Lgl2 is lost in human colorectal and breast cancers. We wanted to know if EMT and expression of ZEB1 was associated with loss of Lgl2 and polarity in human carcinomas. By performing real-time reverse transcription-PCR on

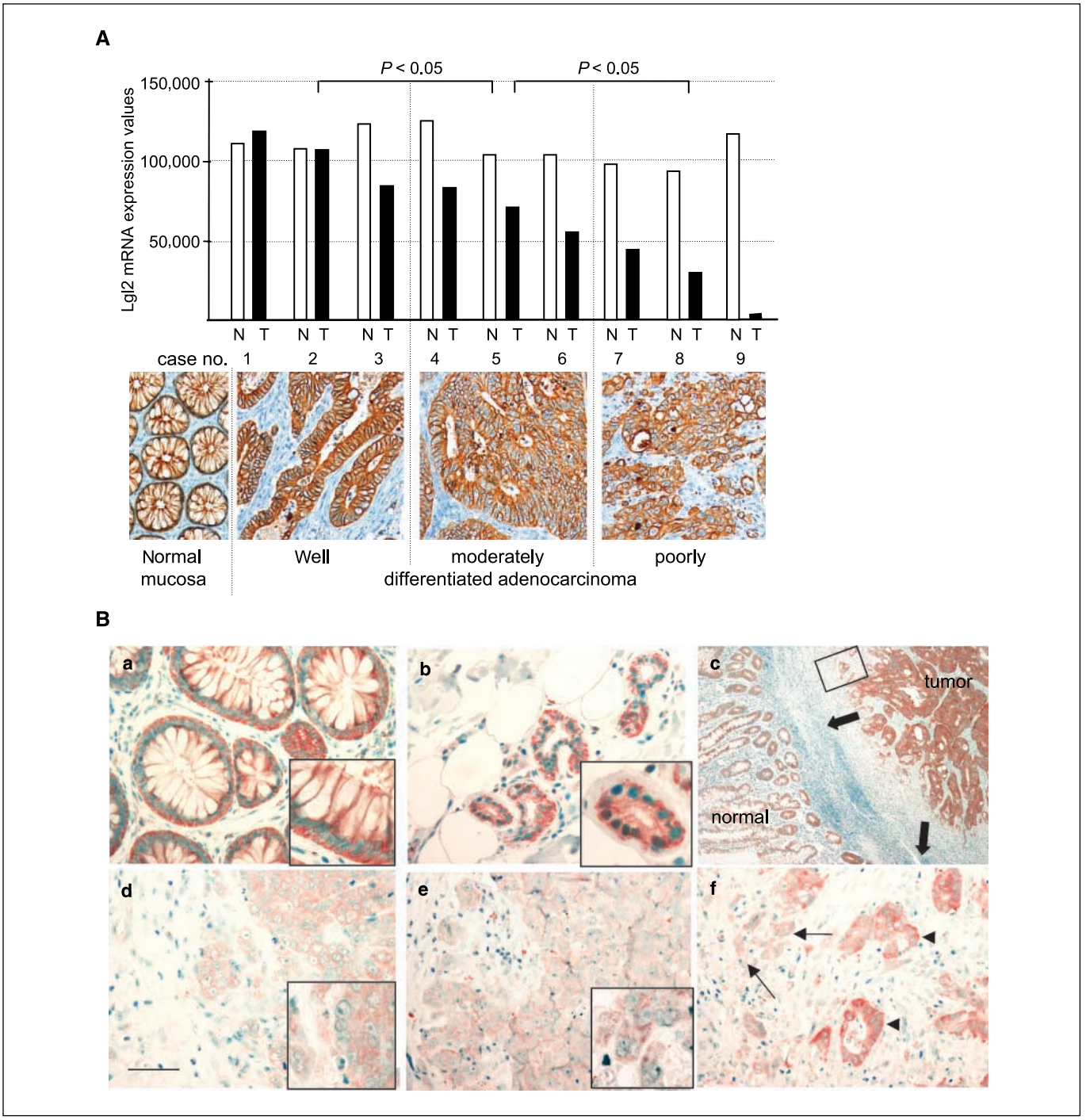

Figure 3. Reduction of Lgl2 expression is associated with increased malignancy. *A,* real-time reverse transcription-PCR comparing Lgl2 expression in CRCs (*black columns*) and adjacent normal mucosa (*white columns*) of various gradings, showing increasing loss of Lgl2 associated with reduced cellular polarity and increased malignancy (*1–3,* well differentiated; *4–6,* moderately differentiated; and *7–9,* poorly differentiated tumors; *bottom,* the corresponding typical histology using keratin-18 staining). *B,* normal colorectal mucosa (*a*) and normal breast epithelium (*b*) are polarized, characterized by tubuli with central lumen and show basolateral expression of Lgl2 (*red*). Undifferentiated CRCs of the medullary type (*d*) and lobular breast cancers (*e*) have a nonpolar structure and strongly reduced expression of Lgl2; (*c*) overview of a typical well to moderately differentiated colorectal adenocarcinoma with central differentiated areas, indicated by tubuli. Differentiation is lost at the invasive front (*big arrow,* direction of invasion). *Inset,* invasive area magnified in *f*. More differentiated tubuli still express Lgl2 (*arrowheads*), whereas it is lost in undifferentiated, detaching tumor cells (*arrows*), lacking polarity. *Bars,* 50 μm and 20 μm (*insets, a, b, d, e,* and *f*); *bar,* 250 μm (*c*).

Table 2. Knockdown of Lgl2 in shZEB1 knockdown clones leads to a reduced expression of E-cadherin and stable overexpression of Lgl2 results in an increase of E-cadherin expression in HEK293 cells

Cell clone	siRNA	Percentage relat. (Lgl2)	mRNA expression (E-cadherin)
SW480shZEB Z3	si control	100	100
SW480shZEB Z3	si Lgl2 1	13	78
SW480shZEB Z3	si Lgl2 2	7	44
MDAshZEB Z13	si control	100	100
MDAshZEB Z13	si Lgl2 1	35	74
MDAshZEB Z13	si Lgl2 2	5	38
HEK 293 control	—	100	100
HEK 293 Lgl2	—	681	394

laser-microdissected CRCs, we found a reduction of Lgl2 expression and cellular polarity, which was associated with poor differentiation and high tumor grading (Fig. 3*A*). Lgl2 mRNA expression was also reduced in breast cancer tissue compared with normal breast epithelium (Supplementary data, Fig. S3*B*). Using immunohistochemistry, we detected basolateral expression of Lgl2 in tubules of normal colorectal and breast epithelium, but a strong reduction in undifferentiated CRCs and lobular breast cancers, lacking any signs of polarity (Fig. 3*B*). However, typical colorectal adenocarcinomas are well to moderately differentiated and exhibit a heterogeneous morphology with differentiated, tubular areas in central tumor regions, and loss of differentiation in invasive tumor cells. We have previously shown that these invasive tumor cells are associated with metastasis formation and poor prognosis, have undergone EMT, and aberrantly overexpress nuclear β-catenin as well as ZEB1 (11, 24). Similar to normal mucosa, Lgl2 expression was retained in the main, differentiated tumor mass (Fig. 3*B*C), with no aberrant ZEB1 expression (Supplementary data, Fig. S3*C*). However, Lgl2 was strongly reduced in EMT-associated invasive tumor cells without signs of polarity (Fig. 3*B*F) and an aberrant nuclear expression of ZEB1 (Supplementary data, Fig. S3*C*).

Loss of Lgl2 is associated with EMT and increased metastasis. All metastases from control transfectant xenografts had a complete loss of Lgl2 compared with the only grown metastasis (lung) from ZEB1 knockdown cells, which was much smaller, more differentiated and expressed membranous Lgl2 (Fig. 1*D*). In order to further elucidate the importance of Lgl2 for the epithelial phenotype, and thus, for a potential role in suppressing metastasis, we manipulated its expression. On top of the stable ZEB1 knockdown clones, which up-regulated Lgl2 and gained an epithelial, polarized phenotype (see Fig. 2*A*), we additionally knocked down Lgl2 using different specific siRNAs. This not only led to reduced Lgl2, but interestingly, also to reduced E-cadherin expression in comparison to controls (Table 2; Fig. 4*A*; Supplementary data, Fig. S3*D*). Moreover, the cells lost their epithelial phenotype and polarity (regained after ZEB1 knockdown), flattened even more than the wild-type cells (compare Fig. 4*A*–Fig. 2*A, left*), and β-catenin translocated from the membrane to the cytoplasm and nucleus. Accordingly, stable Lgl2 overexpression in HEK293 cells, which express high levels of ZEB1,

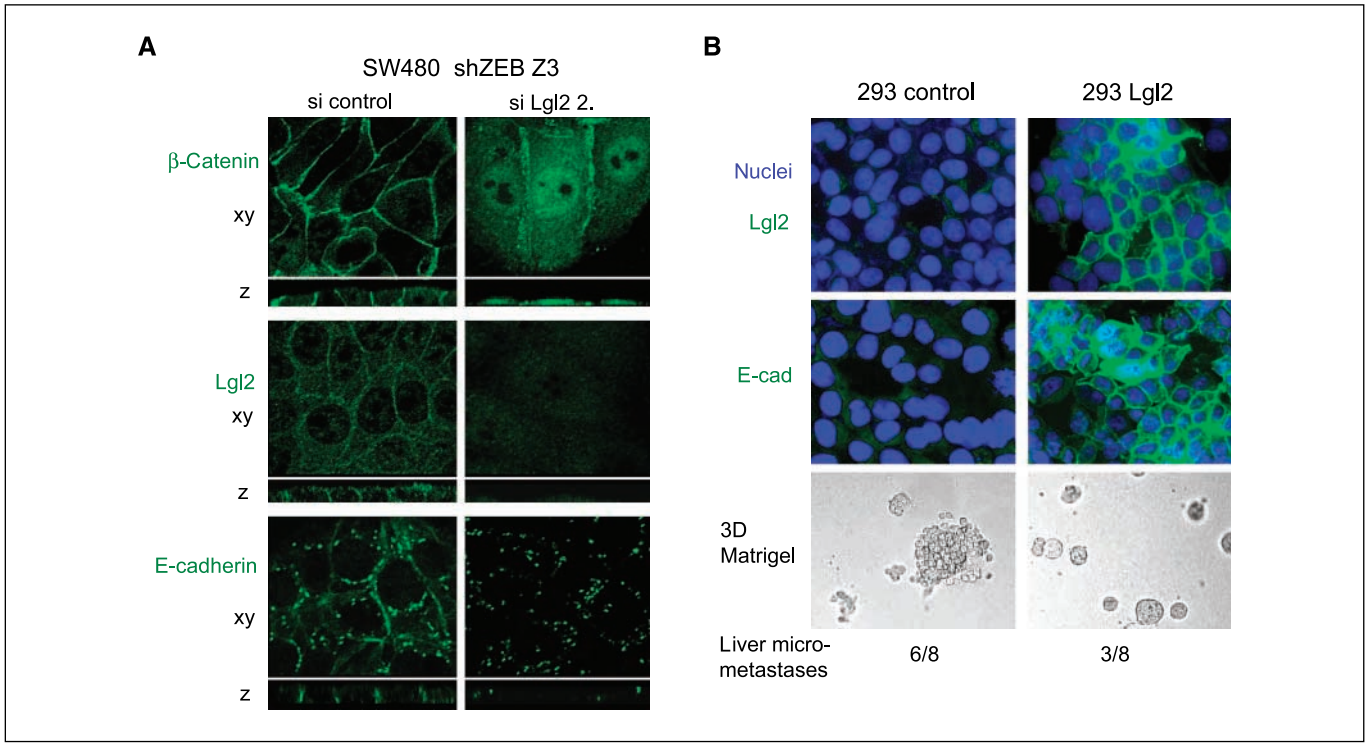

Figure 4. Lgl2 knockdown reverts epithelial phenotype and polarity. *A,* transient knockdown of Lgl2 on top of a stable shZEB1 knockdown reverts the epithelial phenotype induced by ZEB1 knockdown in SW480 colon cancer cells as shown by confocal staining: cells lose polarity, show reduced expression of Lgl2 and E-cadherin, and translocate β-catenin to the cytoplasm and nucleus. As shown in the *z*-axis mode for β-catenin staining, Lgl2 knockdown leads to a flattening of the cells and reduced apical-basal polarity, which is even more prominent than in the sh control cell clones (see Fig. 2*A, left*). *B,* Lgl2-overexpressing HEK293 cells up-regulate E-cadherin, gain an epithelial phenotype with membranous Lgl2 and E-cadherin, grow as spheroids in three-dimensional Matrigel, and show a reduced metastatic potential after tail vein injection in nude mice as assessed by the number of mice bearing micrometastases ($P < 0.05$).

resulted in the overexpression of E-cadherin, cell-cell attachment, and allowed spheroid growth in three-dimensional Matrigel culture (Fig. 4*B*). Moreover, the metastatic potential of Lgl2-overexpressing cells was reduced after tail vein injection in nude mice as assessed by the number of mice bearing micrometastases (Fig. 4*B*). These data suggest that Lgl2 is not only important for polarity but also for the epithelial cell phenotype and that its down-regulation by aberrant ZEB1 expression may be important for tumor progression. Further experiments are under way to prove this aspect.

Discussion

By selectively reducing its expression, we have shown that ZEB1 is crucial for the metastasis of colorectal cancer cells. A stable knockdown of ZEB1 completely reduced metastasis formation of HCT116 colorectal cancer cells after intrasplenic injection, without affecting growth of the primary tumors. The only metastasis in shZEB1 HCT116 cells (versus 10 metastases in control cells) was found after tail vein injection, which could reflect that tail vein injection—in comparison to intrasplenic injection—does not need the detachment of tumor cells from the primary tumor and, therefore, is a bit less dependent on ZEB1. In the course of EMT, ZEB1 triggers the loss of basal-apical polarity by directly suppressing the activation of polarity factor genes. In particular, Lgl2 is lost in colorectal and breast cancer. Moreover, we have shown that retention of Lgl2 expression is important for the epithelial phenotype and that its loss might be directly involved in metastasis.

We have already described ZEB1 as a crucial EMT inducer in human CRCs and that it suppresses the expression of basement membrane components. Importantly, expression of ZEB1, associated with EMT and selective loss of basement membrane in invasive tumor regions, was a strong predictor of poor patient survival and metachronous distant metastasis (13). Recently goosecoid, an EMT activator during gastrulation, was shown to promote metastasis in breast cancer (8), and together with this finding, our data now support a central role for EMT in malignant tumor progression by showing that ZEB1 is directly involved in the metastasis of colorectal cancer cells.

Epithelial tissues are surrounded by a basement membrane, which is necessary to induce epithelial differentiation and to define basal-apical polarity. We could show that, in the course of malignant progression, ZEB1 targets these crucial hallmarks of epithelia by reducing both basement membrane components (13) and intracellular effectors of cell polarity. Our results confirm recent data describing the repression of polarity factors by ZEB1 in breast cancer (25) and extend these findings by showing the direct role of ZEB1 and loss of the polarity factor Lgl2 in metastasis. In contrast to Crumbs and Scribble complex factors, ZEB1 did not affect the expression of Par complex polarity factors and aPKC. Recently, the function of Par complex polarity factors was shown to be inhibited by the activation of ErbB2 in breast tumors (26), and thus, our data suggest that different pathways synergize to fully suppress cellular polarity in carcinomas.

We focused on the Lgl2 polarity factor because its *Drosophila* homologue *lgl* is classified as a tumor suppressor and its loss leads to metastatic tumors (23). There are two vertebrate homologues, Lgl1 and Lgl2, and Lgl1 was already shown to be often reduced in human colorectal (27) and breast cancers (28). However, Lgl1 expression was not affected by ZEB1 (data not shown). We found that loss of Lgl2 is inversely coupled with differentiation and invasion in colorectal and breast cancer. Thereby, *Lgl2* transcription is directly suppressed by ZEB1 through two highly conserved E-boxes. However, we do not want to exclude that other EMT inducers, such as Snail factors, cooperate with ZEB1 in suppressing the expression of polarity factors, as indicated by an increase of Lgl2 expression after transient knockdown of Slug (data not shown). Moreover, the role of Lgl2-loss for malignant tumor progression is further suggested by preliminary experiments showing the reduced metastatic potential of Lgl2 overexpressing HEK293 cells in nude mice xenografts, which however have to be confirmed using colorectal cancer cells. Thereby, in addition to its direct role in cell polarity, the effect of Lgl2 on E-cadherin expression and β-catenin localization (see Fig. 4*A*) could be an important molecular link. A possible molecular connection is also indicated in human CRCs, in which invasive tumor cells, characterized by loss of E-cadherin and nuclear accumulation of β-catenin, also lost the expression of Lgl2 (see Fig. 3*B*F).

Polarity factors are not only involved in basal-apical polarity of epithelial cells, but also in asymmetrical division of stem cells, whereby they direct the asymmetrical separation of cell fate determinants into the two different daughter cells. Lee et al. have shown that Lgl mutants increase the symmetrical division and self-renewal of neuronal stem cells in *Drosophila*, resulting in the accumulation of undifferentiated neuroblasts (29). Based on these results, it is tempting to speculate that loss of cell polarity factors, like Lgl2, might also be involved in the formation and accumulation of cancer stem cells. We recently proposed the concept that the EMT-associated tumor cells at the invasive front might include migrating cancer stem cells (30). Interestingly, these cells lost polarity and aberrantly express ZEB1 (see Supplementary data, Fig. S3*C*), and further work will show if both are associated with increased stemness. Thus, besides a direct effect in reduced cellular polarity, a ZEB1-induced loss of Lgl2 might have additional consequences for malignant tumor progression including metastasis, and therefore, targeting ZEB1 could be a therapeutic option for its prevention.

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