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ZEB1 promotes invasiveness of colorectal carcinoma cells through the opposing regulation of uPA and PAI-1

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Abbreviations used in this paper: CRC: colorectal carcinoma; EMT: epithelial-to-mesenchymal transition; LMB: leptomycin B; PAI-1: plasminogen activator inhibitor-1; qRT-PCR: quantitative real-time polymerase chain reaction; uPA: urokinase plasminogen activator.

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ABSTRACT

Purpose: Carcinoma cells enhance their invasive capacity through dedifferentiation and dissolution of intercellular adhesions. A key activator of this process is the ZEB1 transcription factor, which is induced in invading cancer cells by canonical Wnt signaling (β-catenin/TCF4). Tumor invasiveness also entails proteolytic remodeling of the peritumoral stroma. This study aimed to investigate the potential regulation by ZEB1 of the plasminogen proteolytic system constituted by the urokinase plasminogen activator (uPA), and its inhibitor, plasminogen activator inhibitor-1 (PAI-1).

Experimental Design: Through multiple experimental approaches, colorectal carcinoma (CRC) cell lines and samples from human primary CRC and ZEB1 (-/-) mice were used to examine ZEB1-mediated regulation of uPA and PAI-1 at the protein, mRNA and transcriptional level.

Results: ZEB1 regulates uPA and PAI-1 in opposite directions: induces uPA and inhibits PAI-1. In vivo expression of uPA depends on ZEB1 as it is severely reduced in the developing intestine of ZEB1 null (-/-) mice. Optimal induction of uPA by Wnt signaling requires of ZEB1 expression. ZEB1 binds to the uPA promoter and activates its transcription through a mechanism implicating the histone acetyltransferase p300. In contrast, inhibition of PAI-1 by ZEB1 does not involve transcriptional repression but rather downregulation of mRNA stability. ZEB1-mediated tumor cell migration and invasion depends on its induction of uPA. ZEB1 coexpresses with uPA in cancer cells at the invasive front of CRCs.

Conclusions: ZEB1 promotes tumor invasiveness not only via induction in cancer cells of a motile dedifferentiated phenotype but also by differential regulation of genes involved in stroma remodeling.
TRANSLATIONAL RELEVANCE

ZEB1 is expressed by invading carcinoma cells at the tumor front where represses epithelial adhesion genes and induces a migratory and dedifferentiated phenotype. Consequently, ZEB1 promotes tumorigenesis and metastasis in mouse models and correlates with a poorer clinical prognosis in human cancers. We identified here a novel mechanism for the promotion of tumor invasiveness by ZEB1. Using cell lines and samples from primary human carcinomas and mice knocked out for ZEB1, we show that ZEB1 regulates genes involved in proteolytic remodeling of the peritumoral stroma: activating uPA and inhibiting PAI-1. In fact, our results indicate that uPA is responsible for most of ZEB1-mediated cancer cell migration and invasion and that both proteins are coexpressed in tumor cells at the invasive front of primary carcinomas. Our results set ZEB1 as a potential biomarker of prognosis and a potential therapeutic target in colorectal carcinomas and probably other carcinomas.
INTRODUCTION

During carcinoma progression, cancer cells enhance their migratory and invasive capacity through the downregulation of epithelial markers involved in the maintenance of cell polarity and intercellular adhesion—chiefly the inhibition of E-cadherin—and the acquisition of a motile dedifferentiated phenotype as part of the epithelial-to-mesenchymal transition (EMT) (reviewed in 1-3). At the transcriptional level, E-cadherin is repressed by factors of the ZEB, Snail and Twist families, with ZEB1 (also known as δEF1) as final downstream effector and having the most consistent inverse correlation with E-cadherin across carcinomas (1-5). In addition to repress epithelial polarity and adhesion genes, ZEB1 activates mesenchymal and stemness markers (2). Consequently, ZEB1 expression promotes tumorigenesis and metastasis in mouse models and correlates with a poorer prognosis in human cancers, including colorectal carcinomas (CRCs) (2,3,6,7).

ZEB1 is induced by multiple signaling pathways including TGFβ, Notch and canonical Wnt (β-catenin/TCF4) (reviewed in 2,3,8; 9-11). As a repressor of E-cadherin, ZEB1 is not expressed in normal epithelium or the tumor center of well-differentiated carcinomas (12). ZEB1 is rather upregulated at the invasive front of tumors in dedifferentiated cancer cells that have translocated β-catenin to the nucleus and therefore display active Wnt signaling (7,11-14).

However, tumor invasiveness does not depend exclusively on intrinsic cancer cell traits and often entails a deregulation of proteolytic systems operating in the stroma (15). Proteases produced at the tumor invasive front by cancer and stromal cells remodel the peritumoral matrix modifying cancer cell adhesions and releasing active peptides that promote tumor invasion. A key protease cascade involved in tumor invasiveness is the plasminogen activation system formed by the urokinase plasminogen activator (uPA) and its inhibitor, the plasminogen activator inhibitor-1 (PAI-1) (16). uPA processes
plasminogen into plasmin, which in turn cleaves extracellular matrix components (16). uPA is upregulated in cancer and stromal cells at the invasive front of colorectal and other carcinomas, where high expression by invading cancer correlates with metastasis and an adverse prognosis (17,18). In turn, elimination of uPA in either cancer or stromal cells reduces tumor growth and the incidence of metastasis in mouse models (19,20).

PAI-1 plays distinct roles in tumor invasion depending on the cell type expressing it and the tumor model. At the invasive front of tumors, including CRCs, PAI-1 is induced in cancer cells, but it is especially upregulated in stromal cells (17,18). Paradoxically, considering its inhibitory function over uPA activity, PAI-1 expression at the tumor front of colorectal and other carcinomas correlates with an adverse clinical course (17,18,21). While results in mouse models vary with the experimental setting, evidence indicates that expression of PAI-1 by myofibroblasts and endothelial cells has a pro-invasive effect whereas PAI-1 expressed by cancer cells inhibits tumor growth and invasion (21). Thus, malignant keratinocytes and fibrosarcoma cells produce less invasive tumors when injected in PAI-1-deficient mice than in normal counterparts (19,22). By contrast, compared to parental cells, overexpression of PAI-1 in prostate carcinoma cells decreases their invasiveness when xenotransplanted in nude mice (23).

If unchecked by PAI-1, excessive proteolysis by uPA can potentially inhibit tumor invasiveness as tumor cells need sufficient stromal matrix for traction. At the same time, PAI-1 has pro-invasive effects that are independent of its role as enzymatic inhibitor of uPA but rather related to its regulation of cell adhesion (21). Efficient tumor invasiveness requires therefore a tight control of uPA and PAI-1 expression in cancer cells. uPA is a downstream target of Wnt (β-catenin/TCF4) and Notch pathways (24,25) and PAI-1 is transcriptionally activated or repressed by multiple signals (e.g. p53, TGFβ, hypoxia, insulin, KLF2) (reviewed in 26). Expression of uPA by cancer cells at the invasive tumor
front of CRCs—where ZEB1 is also induced in response to aberrant activation of Wnt signaling (11)—prompted us to investigate both the potential regulation of the uPA/PAI-1 system by ZEB1 and its contribution to ZEB1-mediated tumor invasiveness.

In this study, we show that ZEB1 controls both components of the plasminogen activation system in CRC cells, but in opposing ways; inducing uPA and inhibiting PAI-1. ZEB1 upregulates uPA through direct binding to its promoter and activation of transcription via a mechanism involving the histone acetyltransferase p300. Meantime, ZEB1 repressive effect on PAI-1 entails a downregulation of PAI-1 mRNA stability. Importantly, the ability of ZEB1 to promote migration and invasion of CRC cells depends on its induction effect over uPA. In vivo relevance of these findings is confirmed through two approaches. In vivo expression of uPA is critically dependent on ZEB1 as is virtually abrogated in the developing intestine of embryos from ZEB1 null (-/-) mice. uPA also requires of ZEB1 for its optimal induction by Wnt, which is hindered in ZEB1-deficient cells from these mice or CRC cells knocked down for ZEB1. In addition, ZEB1 coexpresses with uPA in cancer cells at the invasive front of primary CRCs but inversely correlates with PAI-1.
MATERIALS AND METHODS

Antibodies, plasmids, siRNAs and shRNAs

Description and source of antibodies, plasmids, siRNAs oligonucleotides and shRNA lentivirus used in the study are detailed in Supplementary Information.

Cells

SW480 and HCT116 cells originated as in (14) and Colo320 cells from the Cancer Cell Line Repository (RTICCC-PRBB, Barcelona, Spain). Mouse embryo fibroblasts (MEFs) were obtained from E15.5 C57BL/6J wild-type (+/+), and δEF1 null (-/-) embryos (27). Mouse Wnt3a-containing and control conditioned media were produced from L-cells stably carrying either an expression vector for mouse Wnt3a (L-Wnt3a cells) or its corresponding empty vector (L-Ctl cells), respectively. Details regarding cell culture, transfections and transduction are provided in Supplementary Information.

Mouse and human tissues

Mouse tissue samples corresponded to 4 μm sections from E18.5 C57BL/6J wild-type (+/+), and δEF1 null (-/-) embryos (27). Paraffin-embedded sections of human primary CRCs, either as individual samples or as an array, were obtained from IDIBAPS’ Tumor Bank. The tissue array contained 36 separate cores for the CRC invasive front from 12 independent patients in triplicate. Use of mouse and human samples was approved by the corresponding research ethics committees. Tissue immunostaining was performed as described in Supplementary Information.
Determination of protein and mRNA expression and transcriptional assays

Western blots, immunostaining, qRT-PCR, ChIP and transcriptional assays were performed as described in Supplementary Information.

Migration and invasion assays

The migration and invasion capacity of CRC cells was performed as per standard protocols and detailed in Supplementary Information.

Statistical Analysis

Statistical significance ($p \leq 0.01$) was determined by t- and Mann-Whitney-U tests.
RESULTS

ZEB1 regulates endogenous uPA and PAI-1 in opposite directions

Of the transcription factors repressing E-cadherin, ZEB1 has the most consistent inverse correlation with across carcinomas (2-4,8). Since ZEB1 is induced by Wnt signaling, nuclear accumulation of β-catenin in cells of the CRC cell line SW480 results in higher levels of ZEB1 and lower E-cadherin with respect to HCT116 cells, another CRC cell line where β-catenin is mostly membranous/cytoplasmic (11). Here, we found that, as compared to HCT116 cells, higher ZEB1 expression in SW480 cells was associated not only with lower E-cadherin but also with higher uPA expression (Supplementary Figure S1A). This observation, along the reported expression of uPA in cancer cells at the invasive front of carcinomas (24), prompted us to question whether ZEB1 could also promote tumor invasion by regulating the uPA/PAI-1 system.

To test this hypothesis, we explored the effect of manipulating endogenous ZEB1 expression on endogenous uPA and PAI-1 protein levels. We found that knockdown of ZEB1 in SW480 cells with two different siRNA (hereafter referred as si1ZEB1 and si2ZEB1), but not by transfection of a siRNA control (siCtl), downregulated endogenous uPA protein (Figure 1A). Likewise, SW480 cells where ZEB1 had been stably knocked down with a pool of specific shRNAs against ZEB1 (shZEB1)—whose target sequences differ from those in si1ZEB1 and si2ZEB1—displayed lower uPA expression than SW480 cells stably transfected with control shRNA (shCtl) (Supplementary Figure S1B). Interestingly, both transient and stable downregulation of ZEB1 in SW480 cells resulted in the opposite effect on PAI-1—lowering of ZEB1 expression increased endogenous levels of PAI-1 protein (Figure 1A and Supplementary Figure S1C).
Next, we investigated ZEB1 regulation of uPA and PAI-1 at the mRNA level by quantitative real time PCR (qRT-PCR). SW480 cells were again transiently transfected with si1ZEB1, si2ZEB1 or siCtl. Knockdown of ZEB1, but not transfection with siCtl, downregulated uPA mRNA levels while upregulating those for PAI-1 (Figure 1B). Likewise, transient knockdown of ZEB1 in Colo320 cells—a CRC cell line that, like SW480, displays nuclear accumulation of β-catenin and high endogenous Wnt signaling activity (28)—also resulted in downregulation of uPA mRNA (Supplementary Figure S1D). Opposite regulation of uPA and PAI-1 mRNA by ZEB1 was also observed when ZEB1 was stably knocked down in SW480 cells with shZEB1 (Supplementary Figure S1E).

As indicated above, and contrary to SW480 cells, β-catenin in CRC HCT116 cells remains mostly at the plasma membrane and in the cytoplasm, thus preventing activation of Wnt signaling and β-catenin-mediated transcription (11,28). Leptomycin-B (LMB)—a drug that specifically blocks CRM1/exportin-dependent nuclear export—triggers the nuclear translocation of β-catenin in HCT116 cells and the induction of ZEB1 (11). We found here that induction of ZEB1 mRNA upon short-time exposure of HCT116 cells to LMB also increased uPA mRNA and downregulated PAI-1 mRNA (Supplementary Figure S1F). Similar results were obtained when HCT116 cells were treated with the human recombinant Wnt3a ligand (Supplementary Figure S1G). Altogether, these results show that ZEB1 has opposing effects on the two arms of the plasminogen activation system, inducing uPA expression and repressing PAI-1.
ZEB1 induces uPA through direct binding and transcriptional activation of its promoter via a mechanism involving p300

ZEB1 represses epithelial markers and activates mesenchymal and stemness genes by either direct binding to their regulatory regions and repression/activation of their transcription or through repression of an expanding set of microRNAs (2,3,8). We therefore decided to investigate the mechanism of ZEB1-mediated induction of uPA by examining the effect on uPA transcription of overexpressing or knocking down ZEB1.

Since SW480 and Colo320 cells exhibit strong β-catenin-mediated transcription and high levels of endogenous ZEB1 (11), the basal activity of a 1.9 kb fragment of the human uPA promoter proved to be relatively high when transfected in both cell types (Figure 2A and Supplementary Figure S2A). Still, overexpression of exogenous ZEB1 was able to further activate uPA transcription (Figure 2A and Supplementary Figure S2A). As found earlier for uPA protein and mRNA, transient and stable knockdown of ZEB1 in SW480 cells downregulated the basal transcriptional activity of the uPA promoter (Figure 2B). uPA is also a direct transcriptional target of β-catenin/TCF4 (24) and overexpression or knockdown of β-catenin had similar effects on uPA transcription than those obtained for ZEB1 (Supplementary Figure S2B and S2C). We therefore hypothesized that Wnt-mediated activation of the uPA promoter occurs not only by direct β-catenin/TCF4-mediated transcription (24) but also via ZEB1 (that is, indirectly via Wnt-mediated induction of ZEB1) (11). Accordingly, knockdown of ZEB1 not only reduced basal uPA promoter activity but also its optimal response to soluble Wnt3a ligand (Figure 2C).

We next wondered whether the ability of ZEB1 to activate uPA transcription was mediated by direct binding of ZEB1 protein to its promoter. ZEB1 binds to a subset of E-box and E-box-like sequences on the regulatory regions of target genes (29). Examination of the first 1.9 kb of the human uPA promoter revealed the existence of several E-box sites,
three of which (located at -1624, -1480 and -230 bp from the transcriptional start site) conforming to the reported ZEB1’s optimal recognition sequence (CACCTG/CAGGTG) (29) (Figure 2D). We therefore tested by chromatin immunoprecipitation (ChIP) assays if endogenous ZEB1 binds to any of these sites on the uPA promoter. Indeed, it was found that an antibody against ZEB1, but not its respective control IgG, immunoprecipitated regions of the uPA promoter containing E-boxes at -1624 bp and -1480 bp (Figure 2E). ZEB1 failed to bind, however, to the region containing the ZEB1 consensus binding site at -230 bp (Figure 2E). Likewise, ZEB1 did not bind to a region of the uPA promoter lacking consensus binding sites (-1376/-1223, referred as NCBS in Figure 2D) or to the human GAPDH promoter, whose expression is not regulated by ZEB1 (Figure 2E).

To test the functionality of ZEB1 binding sites at -1624 bp and -1480 bp, both E-boxes were mutated to sequences known to not bind ZEB1 (29) and the resulted mutant uPA promoter tested for its induction by ZEB1. As shown in Figure 2F, mutation of both sites was sufficient to abrogate uPA promoter response to ZEB1 overexpression. As in the ChIP assays we only tested sites with optimal ZEB1 consensus binding sequence (CACCTG/CAGGTG), we could not rule out that other E-boxes in the mutant uPA promoter, apart from -1624 bp and -1480 bp, could still bind ZEB1 and maintain its basal transcriptional activity. Together with the ChIP assays, these results demonstrate that endogenous ZEB1 binds to E-boxes at -1624 bp and -1480 bp in the uPA promoter to directly drive its transcription.

Mutation of ZEB1 sites in the uPA promoter did not impede direct Wnt-mediated transcription (by overexpression of TCF4) of the uPA promoter (Figure 2F). The lower response of the mutant uPA promoter to TCF4 was likely due to the elimination of the ZEB1 component of Wnt-mediated induction of uPA: ZEB1 that is induced in response to
TCF4 overexpression would be unable to bind to the mutant uPA promoter (see also below).

ZEB1 activates transcription through recruitment of p300 and p/CAF histone acetyltransferases to its N-terminal region (4,30,31). We therefore sought to investigate the involvement of p300 in ZEB1-mediated activation of uPA. Transfection of a version of p300 where its cDNA is fused to the activation domain of the herpes simplex virus VP16 protein (p300-VP16AD), synergized with ZEB1 in the transcriptional induction of uPA (Figure 2G). In contrast, deletion of the N-terminal region of ZEB1 (ZEB1\textsubscript{ΔNT}) eliminated most of the transcriptional activation effect of ZEB1 on the uPA promoter and failed to synergize with p300-VP16AD (Figure 2G). We could therefore conclude that ZEB1 induces uPA expression through direct binding and transcriptional activation of its promoter via a mechanism involving p300.

**ZEB1 inhibits PAI-1 expression by reducing the stability of its mRNA**

Increased PAI-1 protein and mRNA expression upon ZEB1 knockdown indicates that, contrary to uPA, PAI-1 is under negative regulation by ZEB1. In fact, most known ZEB1 targets are transcriptionally inhibited rather than activated (2-4). We therefore proceeded to test the ability of ZEB1 to repress the human PAI-1 promoter at the transcriptional level. As shown in Figure 3A, overexpression of ZEB1 in SW480 cells did not affect the basal transcriptional activity of a reporter containing a 0.8 kb fragment of the human PAI-1 promoter harboring most known PAI-1 regulatory elements (reviewed in 26). ZEB1 also failed to alter the basal activity of a larger 3.4 kb fragment of the PAI-1 promoter (Figure 3A). We also found that neither transient nor stable knockdown of ZEB1 affected PAI-1 promoter transcription (Figure 3B).
Although it cannot be ruled out that ZEB1 could transcriptionally repress PAI-1 via binding to more upstream or downstream regulatory regions, we decided to explore the potential regulation of PAI-1 by ZEB1 at an alternate level and examined whether ZEB1 expression affects PAI-1 mRNA stability. SW480 cells transiently or stably knocked down for ZEB1 were treated with Actinomycin D (Act D) to inhibit RNA elongation and PAI-1 mRNA levels examined by qRT-PCR (Figure 3C and Supplementary Figure S3). We found that following treatment with Act D, PAI-1 mRNA remained more stable in SW480 cells where ZEB1 had been knocked down than in counterpart control cells (Figure 3C and Supplementary Figure S3).

Similar to parental SW480 cells, SW480 cells transfected with siCtl displayed very low levels of E-cadherin mRNA that, as expected, were drastically upregulated upon ZEB1 knockdown with si1ZEB1 (Figure 3C). In contrast to PAI-1, E-cadherin expression is inhibited by ZEB1 through transcriptional repression of its promoter (2-4) and incubation of SW480 cells with ActD resulted in a decline of E-cadherin mRNA levels irrespective of ZEB1 knockdown with si1ZEB1 (Figure 3C). At the incubation periods examined here, mRNA levels of the housekeeping gene β-actin—included as negative control—remained relatively stable to ActD treatment and, given that is not regulated by ZEB1, unaffected by ZEB1 knockdown (Figure 3C). Altogether these results indicate that ZEB1 controls PAI-1 expression, at least in part, through regulation of its mRNA stability.

**uPA expression mediates ZEB1-dependent CRC cell migration and invasion**

Expression of ZEB1 and uPA by CRC cells promotes tumor cell migration, invasion and metastasis (2,6,17,18,32). We therefore questioned whether upregulation of uPA by ZEB1 contributes to ZEB1-mediated tumor cell migration and invasion. To that effect, the SW480 cells stably infected with control (shCtl) and ZEB1-specific (shZEB1) shRNAs used in
previous experiments were also stably transfected with either a cytomegalovirus (CMV) expression vector encoding the uPA cDNA (to restore loss of uPA expression due to ZEB1 knockdown) or the corresponding empty vector (Figure 4A). The resulting stable pools were then assessed for their capacity to either actively migrate through an 8 μm pore polycarbonate membrane or invade through a Matrigel matrix. We reasoned that since ZEB1 upregulates uPA via transcriptional activation of the endogenous uPA promoter, ZEB1 knockdown should not affect uPA expression when driven by the CMV promoter.

As expected, cells knocked down for ZEB1 and expressing the empty CMV vector (“shZEB1 + vect” cells) displayed significantly lower migratory and invasive capacity than counterpart cells stably transfected with shRNA control and the empty vector (“shCtl + vect” cells) (Figures 4B and 4C). To ascertain whether lower levels of uPA in ZEB1 knockdown cells (Figure 4A) contributed to their lower migratory and invasive capacities, we also tested their counterpart cells overexpressing exogenous uPA (“shCtl + uPA” and “shZEB1 + uPA” cells) (Figure 4A). We found that in cells where ZEB1 has been knocked down, forced restitution of uPA expression restored virtually all their migratory capacity and a significant part of their invasive behaviour (Figures 4B and 4C, and below in Supplementary Figure S4A).

Given the central role of the plasminogen activation system in cancer progression a number of specific inhibitors of uPA have been identified and/or developed (33). To confirm the involvement of uPA in ZEB1-mediated invasiveness of SW480 CRC cells, we tested the effect of the uPA inhibitor amiloride in Matrigel invasion. Treatment with amiloride reduced uPA levels and inhibit cell invasiveness in all four stable cell lines of Figure 5A, inhibition that, as expected, was not rescued by exogenous overexpression of uPA (Supplementary Figures S4A and S4B). Altogether, these results indicate that the
well-established role of ZEB1 promoting tumor migration and invasion involves its upregulation effect over uPA.

Several reports have shown that intracellular signaling upon binding of uPA to its cell surface receptor triggers an EMT phenotype in breast and squamous carcinoma cells (e.g., 34,35). In that line, we observed that overexpression of uPA in ZEB1 knockdown cells upregulated ZEB1 expression (Figure 4A) while uPA inhibition by amiloride decreased it (Supplementary Figure S4B).

**ZEB1 is required for in vivo expression of uPA and its optimal induction by Wnt**

We decided then to test the *in vivo* relevance of the above findings by examining uPA and PAI-1 expression in ZEB1 (δEF1) null (-/-) mice. As these mice die around birth (27), we examined their expression in the developing intestine of late stage embryos (embryonic day 18.5) (Figure 5A and Supplementary Figure S5A). We found that uPA was significantly reduced in the developing intestine of these mice indicating that *in vivo* expression of uPA requires of ZEB1 (Figure 5A). Although in carcinomas uPA is mostly located at the cytoplasm or plasma membrane level, a large share of the uPA staining observed in the developing intestine was nuclear, finding that is in line with evidence about uPA nuclear localization in other cell systems and its binding to transcription factors (e.g., 36,37). In contrast, expression of PAI-1 in ZEB1 null (-/-) embryos was not altered or only slightly upregulated (Figure 5A). It could be argued that being uPA and PAI-1 expressed at high levels in the developing intestine of wild type embryos, it is more difficult to detect an increase in PAI-1 immunostaining upon loss of ZEB1. Overall, these results demonstrate that ZEB1 is critically required for the regulation of the plasminogen activation system *in vivo*. 
As indicated earlier, expression of both ZEB1 and uPA is induced by Wnt signaling (11,24). We therefore wondered if ZEB1 expression is also required for uPA induction by Wnt in cells from ZEB1 null (-/-) mice. To that effect, we tested the ability of conditioned medium containing recombinant mouse Wnt3a ligand to activate uPA expression in mouse embryo fibroblasts (MEFs) from the ZEB1 (-/-) mice. Importantly, we found that induction of uPA by Wnt3a was significantly reduced in ZEB1 null (-/-) MEFs (Figure 5B). However, Wnt3a-mediated induction of Id2, a target of canonical Wnt signaling in CRCs (38) but that is not regulated by ZEB1, was not altered by the loss of ZEB1 expression (Figure 5B). Lastly, no effect of Wnt3a-conditioned medium or of ZEB1 loss was observed in the expression of housekeeping gene β-actin (Figure 5B). Therefore, and in line with cell line-based results above (Figures 2C and 2F), we conclude that endogenous ZEB1 expression is also required for optimal Wnt-mediated induction of uPA in vivo—via induction of ZEB1 by Wnt and subsequent activation of uPA by ZEB1—but not for direct β-catenin/TCF4-mediated activation of uPA or of other ZEB1-independent Wnt targets.

**ZEB1 coexpresses *in vivo* with uPA but not PAI-1 at the invasive front of human primary CRCs**

While the tumor center of typical CRCs is relatively well differentiated, their invasive front contains progressively less-differentiated open tubular structures, small cell clusters and eventually isolated dedifferentiated cancer cells (13). ZEB1 is never expressed by epithelial cells of the normal colonic mucosa of the tumor center of CRCs. Instead, ZEB1 is induced in CRC cells at the invasive front that have accumulated nuclear β-catenin (7,11,12,14). uPA and PAI-1 are also expressed, albeit at different levels, by cancer cells at the invasive tumor front of CRCs (17,18,24). We therefore tested whether the expression of these genes correlates in cancer cells at the invasive front using an array of primary human CRCs. As
shown in Figure 5C, ZEB1, nuclear β-catenin and uPA were expressed by a higher number of cancer cells than PAI-1. Statistical correlation indicated that ZEB1 expression significantly associates with uPA, but not with PAI-1, in cancer cells at the tumor front of CRCs (Figure 5C).

To confirm the correlation between ZEB1 and uPA in the tissue array, we also examined whether both proteins coexpressed in confocal immunofluorescence analysis. High levels of ZEB1 (red) and uPA (blue) were found coexpressed by cancer cells at the invasive front of CRCs (Figure 5D and Supplementary Figures S5B, S5D and S5E). These cancer cells also co-expressed nuclear β-catenin (green). In contrast, cancer cells in central areas of the same CRCs were negative for ZEB1 and uPA while β-catenin was mostly membranous/cytoplasmic (Figure 5D and Supplementary Figure S5C). At the tumor center, as in normal areas, ZEB1 and uPA were only found in non-epithelial cells of diverse origin at the stromal compartment Altogether these data show that, in line with earlier results in this study, ZEB1 and uPA are coexpressed in vivo in CRC cells at the invasive front.
DISCUSSION

Efficient tumor invasiveness requires not only the loss of intercellular adhesions and the acquisition of a dedifferentiated phenotype by cancer cells, as part of the EMT, but also a remodeling of the surrounding extracellular matrix through the concerted action of cytokines, proteases and their inhibitors. Coordinated regulation of both processes involves reciprocal and dynamic influences between the tumor and its microenvironment. In this study, we showed that ZEB1, a key inducer of EMT during cancer progression, regulates the expression of both components of the plasminogen activation system, inducing uPA and repressing PAI-1. We found that ZEB1 induces uPA by direct binding and transcriptional activation of its promoter through a mechanism involving p300. The relevance of ZEB1 in the regulation of uPA is evidenced in vivo by its virtual loss in the developing intestine of ZEB1 null (-/-) mouse embryos and its suboptimal induction by Wnt in ZEB1-deficient cells from these mice. Meantime, inhibition of PAI-1 by ZEB1 does not involve a transcriptional mechanism but rather the downregulation of PAI-1 mRNA stability. We also found that ZEB1-mediated motility of CRC cells depends on uPA and that exogenous reconstitution of uPA restores the motility of CRC cells where uPA expression had been downregulated following ZEB1 knockdown. Finally, we showed that ZEB1 is coexpressed with uPA, but not PAI-1, in cancer cells at the invasive front of primary CRCs.

This study has identified ZEB1 as a regulator of the plasminogen activation system that participates in the remodeling of the tumoral stroma in several human cancers, including CRCs (16,21,32). ZEB1 would thus promote tumor invasion not only by enhancing the motility of cancer cells—via dissolution of intercellular adhesions—but also creating a path for their migration through restructuring of the tumor microenvironment. In addition, pericellular degradation of the basement membrane and stromal matrix generates
proteolytic fragments that foster tumor invasion. In CRCs, ZEB1 regulates the expression of components of the epithelial basement membrane, disruption of which is a crucial step preceding tumor invasion into the peritumoral stroma (12). Proteolysis of extracellular matrix proteins by uPA-generated plasmin also creates pro-migratory peptides. Cleavage by plasmin of fibronectin and binding of its fragments to $\alpha_5\beta_1$ integrin mediate the tumor invasion- and hematogenous dissemination-promoting effects of uPA (39). Interestingly, ZEB factors induce $\alpha_5\beta_1$ integrin expression (40). It is therefore possible that in promoting cancer cell migration, ZEB1 not only induces the generation of fibronectin pro-migratory peptides through activation of the uPA/plasmin system but also activates expression of their integrin receptors.

While proteolytic remodeling of the stroma is required for tumor invasion, excessive matrix degradation could actually hinder invasion as cancer cells need sufficient substrate to sustain their traction and motility. A tight regulation of uPA and PAI-1 levels is therefore needed for an optimal proteolytic balance. In that regard, joint but differential regulation of uPA and PAI-1 by a single gene, ZEB1, seems particularly relevant. Likewise, separate and distinct mechanisms of control—direct transcriptional activation and likely indirect inhibition of mRNA stability, respectively—allow ZEB1 to finely tune the expression of both genes during tumor progression. uPA and PAI-1 also regulate cancer cell migration and invasion through mechanisms independent of their regulatory role over proteolysis. For instance, PAI-1 binding to vitronectin, which releases uPA activity, modulates successive cycles of adhesion/de-adhesion of cancer cells as they migrate across the stroma (reviewed in 21). By regulating their expression, ZEB1 also modulates these non-proteolytic activities of uPA and PAI-1. In addition, increased production of vitronectin by cancer cells adds another level of functional regulation of uPA/PAI-1 by ZEB1.
ZEB1 binds to selected E-box and E-box-like sequences in the regulatory regions of its targets showing higher affinity for the CACCTG/CAGGTG core sequence (29). We found that ZEB1 activates uPA expression by direct binding and activation of its promoter in a mechanism involving the histone acetyltransferase p300. Although we identified three optimal ZEB1 sites in the first 1.9 kb of the human uPA promoter, one of them (-230 bp) failed to recruit ZEB1, potentially suggesting that binding of ZEB1 to its target genes is also modulated by the identity of the nucleotides surrounding the core E-box. As in this study we only checked by ChIP assays binding of ZEB1 to optimal consensus sites (CACCTG/CAGGTG ) it is possible that ZEB1 still binds to lower affinity sites in the mutant uPA promoter to maintain its basal transcriptional activity. In contrast, regulation of PAI-1 by ZEB1 in CRC cells occurs at the post-transcriptional level. Knockdown of ZEB1 reduced and/or delayed the effect of Actinomycin D over PAI-1 mRNA indicating that ZEB1 inhibits PAI-1 expression, at least in part, by reducing the stability of its mRNA. PAI-1 mRNA is known to have a very short half-life and its stability to be modulated in response to multiple stimuli (e.g. hypoxia, growth factors) by the interaction of mRNA binding proteins, some still to be identified and characterized, to its 3’UTR and protect or degrade PAI-1 mRNA (e.g., 41,42). It remains to be elucidated whether inhibition of PAI-1 expression by ZEB1 involves regulation of PAI-1 mRNA binding proteins. In this manner, ZEB1 may not only act as both a transcriptional repressor and an activator (depending on the target gene) (4), but also regulate gene expression through different mechanisms.

In breast and squamous carcinoma cells, binding of uPA to its receptor triggers an EMT phenotype, including the expression of Snai1 (34,35). In this study, we found that uPA overexpression upregulates ZEB1 while inhibition of uPA expression by amiloride reduces ZEB1. Since ZEB1 is a downstream target of Snai1 (reviewed in 3), uPA induction of ZEB1 could be mediated by Snai1, although direct activation cannot be
excluded given that uPA triggers signaling via Ras-ERK, JAK-STAT and PI3K-AKT (42), all of which are direct upstream inducers of ZEB1 (2). ZEB1 has been suggested to maintain the EMT process initiated by Snail1 (3,9), making possible that ZEB1 mediates uPA’s EMT-promoting effects. The induction of uPA by ZEB1 reported here could thus create a feed forward loop between both proteins, reinforcing their expression and a mesenchymal and dedifferentiated phenotype among invading cancer cells, thus promoting tumorigenesis and tumor invasion across the stroma. Of note, only migrating cancer cells that have lost E-cadherin expression and undergone an EMT, could intravasate and disseminate through blood circulation (reviewed in 44), a process that, as noted earlier, depends on uPA expression (39). In that regard, the loop between ZEB1 and uPA could be important in maintaining repression of E-cadherin and promoting ulterior intravasation of cancer cells.

Over the last few years our understanding of ZEB1 has evolved from being a repressor of E-cadherin that promotes EMT and tumor invasiveness to a factor that induces most of the hallmarks that cancer cells need to acquire during tumor progression: thus, inter alia, ZEB1 contributes to overriding safeguard programs against cancer like senescence, promotes tumor angiogenesis and cooperates with oncogenic signals (reviewed in 2,3,8). Likewise, a number of reports indicate that uPA and PAI-1 also participate in an expanding set of functions during cancer progression beyond tumor invasiveness (32), many in line with their coordinated regulation by ZEB1. In addition, evidence of the nuclear localization of uPA and its binding to transcription factors (36,37), supports that the uPA and/or PAI-1 may be playing roles in gene regulation still to be elucidated. It is therefore tempting to speculate whether uPA/PAI-1 could mediate some of the new ZEB1 roles in cancer progression or vice versa.
In sum, this work has shown that ZEB1 promotes tumor invasiveness not only by inducing a more motile dedifferentiated phenotype in cancer cells but also through the opposing regulation of uPA and PAI-1. By expanding the mechanisms by which ZEB1 regulates tumor invasiveness, the results presented here reinforce its role as a potential therapeutic target in CRCs.

*****
CONFLICT OF INTEREST

Authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

As per CCR instructions, author contributions to the study are as follows:

EST and OdB participated equally in all parts of the study.

EST, OdB and AP designed experiments and interpreted data.

EST, OdB, LS and PGA performed experiments.

DSD provided mouse tissue samples.

MC diagnosed human samples and interpreted staining of the tissue microarray.

AC identified and diagnosed patients and collected clinical data.

AP wrote the paper with critical comments by all authors, particularly from EST and OdB.
REFERENCES


FIGURE LEGENDS

FIGURE 1
Opposing regulation of endogenous uPA and PAI-1 by endogenous ZEB1. (A) Transient knock down of ZEB1 differentially regulates uPA and PAI-1 proteins. SW480 cells were transfected with 100 nM of a control siRNA (siCtl), or specific siRNAs for ZEB1 (si1ZEB1 and si2ZEB1). Cell lysates were immunoblotted for uPA (3689/HD-UK1), PAI-1 (H-135), ZEB1 (H-102), along α-tubulin (B5-1-2) as loading control. (B) Transient knock down of ZEB1 differentially regulates uPA and PAI-1 mRNAs. SW480 cells transfected as in (A) were assessed for relative mRNA levels of uPA, PAI-1 and ZEB1 by quantitative real time PCR (qRT-PCR) respect to GAPDH.

FIGURE 2
ZEB1 transcriptionally activates uPA in CRC cells by direct binding to its promoter. (A) ZEB1 activates uPA transcription. SW480 cells were transfected with 0.5 μg of a luciferase reporter containing 1.9 kb of the human uPA promoter along with 1 μg of an empty expression vector or the corresponding equal molar amount of a ZEB1 expression vector. Transcriptional assays and relative luciferase activity (RLU) in panels (A-C,F,G) were performed as described in Supplementary Materials and Methods. (B) Transient and stable knockdown of ZEB1 inhibits uPA transcription. SW480 cells were transiently transfected with 100 nM of a siRNA control (siCtl) or a specific siRNA for ZEB1 (si1ZEB1) or stably transfected with control (shCtl) or ZEB1-specific (shZEB1) shRNAs. (C) Downregulation of ZEB1 hinders Wnt-mediated transcription of uPA. SW480 cells transiently transfected as in (B) with siCtl or si1ZEB1 were either untreated (Unt) or exposed to 100 ng/ml of recombinant human Wnt3a during 48 h. (D) Schematic representation of E-box
(CANNTG) and E-box-like sequences (E1 to E9) and TCF4 (T1 and T2) consensus binding sites in the first 1.9 kb of the human uPA promoter. E1 (-1792/-1787: CACGTT), E2 (-1665/-1660: CAGCTC), E3 (-1624/-1619: CACCTG), E4 (-1575/-1570:CAGCTG), E5 (-1480/-1475:CAGGTG), T1 (-737/-731:CTTTGTT), E6 (709-704: CACCTA), T2 (-562/-556: CTTTGTT), E7 (422-417: CAGCTC), E8 (413-408:CAGCTG), E9 (230-225:CAGGTG). NCBS refers to a region of the uPA (-1376/-1223) that contains no consensus binding sites for ZEB1. (E) ZEB1 binds to the uPA promoter. qRT-PCR of fragments of the uPA and GAPDH promoters immunoprecipitated in ChiP assays from SW480 cells with ZEB1 antibody (E-20X), goat IgG or with no antibody. Regions of the uPA promoter lacking consensus binding sites for ZEB1 (NCBS, -1376/-1223) or containing consensus ZEB1 binding sites at –1624 bp, –1480 bp and -230 bp or from the GAPDH promoter were amplified by qRT-PCR as described in Supplementary Material and Methods. Values represent average relative binding in relation to input from three independent experiments performed in triplicate. (F) ZEB1 binding sites are required for ZEB1- but not TCF4-mediated induction of uPA transcription. SW480 cells were transfected with 0.5 μg of a luciferase reporter containing 1.9 kb of the human uPA promoter either wild type (-1.9 kb uPA) or mutated for ZEB binding sites at -1624 and –1480 bp (-1.9 kb uPA mut) along with 1 μg of an empty expression vector or the corresponding equal molar amount of either ZEB1 or TCF4 expression vectors. (G) Induction of uPA transcription by ZEB1 involves the histone acetyltransferase p300. As in (A) SW480 cells were transfected with 0.5 μg of the human uPA promoter along with 0.5 μg of empty expression vector or the corresponding equal molar amount of full length ZEB1 or a version of ZEB1 lacking the N-terminal region (ZEB1\textsubscript{NT}) along with 0.5 μg p300-VP16AD.
FIGURE 3

ZEB1 regulates PAI-1 at the post-transcriptional level. (A) ZEB1 overexpression does not affect the transcription of the human PAI-1 promoter. SW480 cells were transfected with 0.5 μg of two different fragments of the PAI-1 promoter (-0.8 kb and -3.4 kb) along with 1 μg of an empty expression vector or the corresponding equal molar amount of a ZEB1 expression vector. (B) Transient and stable knockdown of ZEB1 does not alter PAI-1 transcription. As in Figure 2B, but using instead -0.8 and -3.4 kb PAI-1 promoter luciferase reporters. (C) ZEB1 expression decreases PAI-1 mRNA stability. SW480 cells transfected for 48 h with either siRNA control (siCtl) or a siRNA against ZEB1 (si1ZEB1) were incubated for the indicated periods with 10 μg of Actinomycin D (Act D) before relative mRNA levels of PAI-1, E-cadherin and β-actin were assessed by qRT-PCR.

FIGURE 4

ZEB1 promotes migration and invasiveness of CRC cells in an uPA-dependent manner. (A) SW480 cells were stably transfected with shRNA control (shCtl) or shRNA against ZEB1 (shZEB1) plus either an empty expression vector (“shCtl + vect” and “shZEB1 + vect” cells, respectively) or an expression vector encoding human uPA cDNA (“shCtl + uPA” cells and “sh1ZEB1 + uPA” cells, respectively). Levels of ZEB1 (H-102) and uPA (3689/HD-UK1) were determined by Western blot along α-tubulin (B5-1-2) as loading control. (B) Stable pooled cell lines as in (A) were tested for their ability to migrate during 6 h through a Transwell polycarbonate membrane (migration assays) as described in Materials and Methods. A t-test for significance of means difference was used. (C) As in (B) but cells were examined for their ability to invade during 24 h through a Matrigel matrix (invasion assays).
**FIGURE 5**

*In vivo* expression of uPA depends on ZEB1 and both proteins are coexpressed in invading tumor cells at the leading front of CRCs. (A) *In vivo* expression of uPA depends on ZEB1. Immunohistochemistry for uPA (H-140) and PAI-1 (H-135) of the developing intestine from ZEB1 wild type (+/+) and null (-/-) E18.5 mouse embryos. Immunostaining was performed as described in Supplementary Materials and Methods (B) Optimal induction of uPA by Wnt signaling depends on ZEB1. Relative mRNA levels of uPA, Id2 and β-actin in ZEB1 wild type (+/+) or null (-/-) mouse embryo fibroblasts under either basal conditions (Unt) or upon incubation for 48 h with conditioned media from either L-cells stably transfected with an expression vector for mouse Wnt3a (L-Wnt3a cm) or its corresponding empty vector (L-Ctl cm). Relative mRNA levels were assessed by qRT-PCR respect to GAPDH. The experiment is the average of three different experiments. (C) ZEB1 correlates with uPA and nuclear β-catenin, but not with PAI-1, in invading cancer cells at the leading front of CRCs. A tissue array of primary CRCs was stained by DAB method for ZEB1 (H-102), β-catenin (C2206), uPA (3689/HD-UK1) and PAI-1 (H-135). Samples were scored for number of positively stained tumor cells at the invasive front as indicated in Supplementary Materials and Methods. Values represented in a quartile boxplot were assessed for significance using a non-parametric Whitney-Mann-U test. (D) ZEB1 colocalizes with uPA in invading cancer cells at the tumor front of CRCs but neither protein is expressed in the tumor center. At the tumor front, ZEB1 and uPA also colocalize with nuclear β-catenin. β-catenin is mostly membranous/cytoplasmic at the tumor center. Sections of the tumor front and tumor center of a sporadic CRC were immunostained for ZEB1 (red, E-20), uPA (blue, 3689/HD-UK1), and β-catenin (green, Ab5302). Representative merge pictures are shown. Single staining for uPA, ZEB1 and β-catenin along with DAPI is shown in Supplementary Figures S5B and S5C. Staining of a more
differentiated area of the tumor front is shown in Supplementary Figures S5D and S5E. uPA was originally detected with Dylight™ 649 (far red) but converted to blue for representation. Scale bars represent 25 μm.
Figure 1

A

B

siCtl  si1ZEB1  si2ZEB1

uPA
PAI-1
ZEB1
α-tubulin

Relative mRNA expression

siCtl  si1ZEB1  si2ZEB1

uPA  PAI-1  ZEB1
Figure 2

**A**

![Diagram](./figure2A.png)

**B**

![Diagram](./figure2B.png)

**C**

![Diagram](./figure2C.png)
Figure 2

D

uPA promoter

E1 E2E3 E4 E5 NCBS

-1624 / -1619
CACCTG

-1480 / -1475
CAGGTG

E6

T1 T2

-230 / -225
CAGGTG

1.9kb

E

E

120

No Ab

Goat IgG

ZEB1 Ab

80

Site

- 1624 bp

- 1480 bp

-230 bp

NCBS

GAPDH

40

0

Relative binding / input
**Figure 2**

**F**

- **ZEB1**
- **uPA mut**
- **Luc**

- **TCF4**
- **ZEB1**
- **Luc**

**Promoter**
- -1.9 kb **uPA**
- -1.9 kb **uPA mut**

**RU**
- 0
- 40
- 80
- 120

- **vect**
- **ZEB1**
- **TCF4**

- **RLL**
- +
- -
- -

**G**

- **p300**
- **ZEB1**
- **uPA**

- **Luc**
- **ZEB1**
- **uPA mut**

- **p300**
- **ZEB1**
- **uPA**

- **Luc**

**Promoter**
- -1.9 kb **uPA**
- -1.9 kb **uPA mut**

**RU**
- 0
- 40
- 80
- 120

- **Vect**
- **ZEB1**
- **ZEB1\text{\tiny{\text{\Delta NT}}}**

- **p300-VP16AD**
- +
- -

**Research.**
Figure 3

A

B

ZEB1

PAI-1

Luc

0
40
80
120

RLU

vect ZEB1
vect ZEB1

- 0.8 kb PAI-1
- 3.4 kb PAI-1

Promoter

ZEB1

PAI-1

Luc

0
40
80
120

RLU

siCtl si1ZEB1 shCtl shZEB1

-0.8 kb PAI-1
-3.4 kb PAI-1

Promoter
Figure 4

A

B

C

% of migration

% of invasion

shCtl + vect  shZEB1 + vect  shCtl + uPA  shZEB1 + uPA

shCtl + vect  shZEB1 + vect  shCtl + uPA  shZEB1 + uPA

p < 0.001

p < 0.001

uPA  shZEB1  α-tubulin
Figure 5

A

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<th>uPA</th>
<th>PAI-1</th>
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<td><img src="image2.png" alt="Image" /></td>
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<tr>
<td>ZEB1 (-/-)</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
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Figure 5

C

MW p > 0.05
MW p > 0.05
MW p > 0.05

MW p < 0.001

Tumor Cell Number Score

ZEB1  Nuclear \(\beta\)-cat  uPA  PAI-1
Figure 5

D

<table>
<thead>
<tr>
<th>Tumor Front</th>
<th>Tumor Center</th>
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<td>ZEB1 uPA</td>
<td>ZEB1 β-catenin</td>
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<td>uPA β-catenin</td>
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ZEB1 promotes invasiveness of colorectal carcinoma cells through the opposing regulation of uPA and PAI-1

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