ZEB1 Coordinate Regulates Laminin-332 and β4 Integrin Expression Altering the Invasive Phenotype of Prostate Cancer Cells*§

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Justin M. Drake†1, J. Matthew Barnes†1, Joshua M. Madsen§, Frederick E. Domann†¶, Christopher S. Stipp†‖, and Michael D. Henry†¶‡1

From the Departments of †Molecular Physiology and Biophysics, ‡Pathology, and † Radiation Oncology, Roy J. and Lucille A. Carver College of Medicine, the † Department of Biology, and the † Holden Comprehensive Cancer Center, University of Iowa, Iowa City, Iowa 52242

Metastasis involves the invasion of cancer cells across both the extracellular matrix and cellular barriers, and an evolving theme is that epithelial-to-mesenchymal transition (EMT) may mediate invasive cellular behavior. Previously, we isolated and analyzed a subpopulation of PC-3 prostate cancer cells, TEM4-18, and found that these cells both invaded an endothelial barrier more efficiently and exhibited enhanced metastatic colonization in vivo. Transendothelial migration of these cells depended on expression of ZEB1, a known regulator of EMT. Surprisingly, these cells were much less invasive than parental PC-3 cells in assays that involve matrix barriers. Here, we report that TEM4-18 cells express significantly reduced levels of two subunits of laminin-332 (β3 and γ2) and that exogenous laminin-332, or co-culture with laminin-332-expressing cells, rescues the in vitro invasion phenotype in these cells. Stable knockdown of ZEB1 in prostate cancer cells up-regulated LAMC2 and ITGB4 mRNA and protein and resulted in a concomitant increase in Transwell migration. Using chromatin immunoprecipitation (ChIP), we show that ZEB1 directly interacts with the promoters of LAMC2 and ITGB4. These results provide a novel molecular basis for reduced laminin-332 observed in clinical prostate cancer specimens and demonstrate a context-dependent role for EMT in invasive cellular behavior.

Metastasis involves the invasion of cancer cells across natural barriers such as basement membranes, interstitial matrix, and the endothelium. Considerable effort over the past decades has defined a number of mechanisms that contribute to the invasive behavior of metastatic cancer cells such as elevated secretion of matrix-degrading enzymes, altered expression of matrix components or their receptors, and increased motility in response to tumor microenvironmental cues. Epithelial-to-mesenchymal transition (EMT) is a mechanism involved in multiple aspects of mammalian development, such as gastrulation and formation of the neural crest, whereby cells lose epithelial identity and gain the ability to move to distant sites in the organism and thus is an attractive paradigm for understanding metastasis. Although abundant experimental and some clinical evidence for EMT in cancer exists, the extent to which this mechanism contributes to metastasis remains controversial. Although EMT has been most thoroughly investigated in relation to its role in invasion of basement membranes and interstitial matrix, less is known about how it might be involved in later steps of metastasis such as extravasation and colonization (survival and proliferation at distant sites).

We recently isolated a subpopulation of the PC-3 prostate cancer cell line, TEM4-18, that was proficient in transendothelial migration and displayed hallmarks of EMT. We found that TEM4-18 cells differentially express a dual zinc finger homeodomain transcription factor ZEB1 (also known as ZFHX1a, δEF1, or TCF-8), which represses a variety of epithelial genes in TEM4-18 cells and has been implicated in EMT in both developmental and pathological contexts, including cancer. Here we describe the molecular basis for this paradoxical finding. We show that the reduction of Transwell migration observed in TEM4-18 cells is due to the loss of expression of laminin-332, a molecule well known to promote cancer cell migration. Further, we show that both β3 and γ2 subunits of laminin-332 as well as β4 integrin, a receptor for laminin-332, are repressed by ZEB1 through direct association with the LAMC2 and ITGB4 promoter regions. Loss of laminin-332 expression and integrin β4 in clinical prostate cancer specimens is a longstanding observation. Our study presents a novel molecular basis for reduced laminin-332 expression in TEM4-18 cells.

The abbreviations used are: EMT, epithelial-to-mesenchymal transition; CMFDA, 5-chloromethylfluorescein diacetate; HMVEC-L, human microvascular endothelial cells from lung; qRT-PCR, quantitative RT-PCR.
new mechanism that may underlie this observation. Moreover, this study shows that EMT may not always be associated with changes that are cell-autonomously advantageous for invasive behavior and points to cooperative interactions among different cancer cell populations, or with the stroma, to support fully the invasive cellular behavior observed in tumors.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Extracellular Matrix Proteins**

Laminin-332 was purified from SCC-25 squamous carcinoma cells as described previously (10). Laminin-332 antibody, clone 6F12, (which recognizes the β3 chain and also known as K140) (11) was used for immunofluorescence. Laminin-332 antibody, clone E-6, (which recognizes the γ2 chain) was purchased from Santa Cruz Biotechnology. Laminin from the Engelbreth-Holm-Swarm mouse tumor, consisting primarily of laminin-111, was purchased from BD Biosciences. Collagen IV was purchased from Sigma.

**Cell Lines**

PC-3 prostate adenocarcinoma (American Type Culture Collection (ATCC)) cells were stably transduced with a luciferase-expressing retroviral vector and were grown in the ATCC recommended medium (Invitrogen) supplemented with 10% FBS (Hyclone) and 1 mM nonessential amino acids (Invitrogen) as described previously (12). Primary human microvascular endothelial cells from the lung (HMVEC-L) (Lonza) were grown in endothelial growth medium-2MV medium (Lonza) supplemented as indicated by the manufacturer. All cells were grown at 37 °C and 5% CO₂. TEM4-18 cells were grown in DMEM/F12 medium supplemented with 400 μg/ml G418. TEM4-18 cells expressing ZEB1 shRNAs were described previously (3).

**Migration Assays**

**Preparation of Transwell Inserts**—24-well Transwell inserts were coated with 1 μg/ml, in 0.005% Tween 20, either laminin-111 or laminin-332 for 1 h at room temperature. The inserts were then washed with PBS with 0.005% Tween 20 twice before placing in endothelial growth medium (Lonza) for the experiment. Collagen IV coating was performed as described previously (3). For PC-3- and HMVEC-L-conditioned membranes, 1 × 10⁵ nonluminescent parental PC-3 or 4 × 10⁵ HMVEC-L cells, respectively, were plated onto 24-well Transwell inserts for 24 h. After 24 h, the cells were washed in PBS and treated with Versene to detach the cells without digesting the extracellular matrix deposited on the inserts.

**Transwell Migration Assays**—Prior to plating onto the Transwell inserts, PC-3 or TEM4-18 cells were detached with 0.48 mM Versene (Invitrogen) for 10–15 min. Cells were then resuspended in complete DMEM/F12 medium and resuspended in endothelial growth medium at a concentration of 5 × 10⁵ cells/ml. Prostate cancer cells (1 × 10⁵, 200 μl) were added onto the Transwell inserts and allowed to incubate for 18 h prior to analysis of migration. A standard curve was performed by serial dilution of prostate cancer cells (10,000 to 20 cells) in a 96-well dish followed by bioluminescence imaging in a Xenogen IVIS100 imaging system (Caliper Life Sciences). To assay migration using bioluminescence imaging, Transwell inserts were placed into a new 24-well dish containing trypsin (400 μl, 10 min at 37 °C) to remove only the cells that had migrated. After 10 min, trypsin was neutralized with 600 μl of serum-containing DMEM/F12 medium, and each insert was washed with medium. 100 μl of sample, in duplicate, from each well was then added to a black 96-well dish (Corning) followed by the addition of 100 μl of luciferin (0.3 mg/ml). Bioluminescence imaging was determined following a 5-min luciferin incubation. Cell quantitation was performed by converting the bioluminescence imaging signal from the sample into the standard curve to derive the number and percent of total cells migrated. Experiments were performed in triplicate, and the data presented herein represent one of three individual experiments.

**Single Cell Motility Assay**—Prior to plating tumor cells, laminin-332-coated dishes were prepared as described above. After coating, 2.3 × 10⁵ cells were plated in serum-free medium onto the dish and allowed to incubate at 37 °C for 30 min. After incubation, the dish was transferred to a microscope stage incubator (20/20 Technology, Inc.) to maintain a humidified, 5% CO₂, 37 °C environment. Images were collected and analyzed as described previously (10).

**Cooperative Migration Analysis**—TEM4-18 cells grown to ∼75% confluence were stained with 10 μM CMFDA (CellTracker Green; Invitrogen) for 30 min in serum-free medium followed by a PBS wash and 30-min incubation in complete medium. Labeled cells were mixed 1:1 with unlabeled TEM4-18 or PC-3E cells and brought to a final concentration of 5 × 10⁵ cells/ml. Co-cultures were then subjected to Transwell migration on collagen IV-coated membranes as described above. After 18 h, transmigrated cells were removed from the lower chamber as described above, pooled from triplicate wells, and pelleted at 700 × g for 5 min. Pellets were resuspended in 350 μl of FACS buffer containing 1 × 10⁵ counting beads (Caltag Laboratories). Live, single cells were gated on forward and side scatter on a BD Biosciences LSR. Stop gates were set on CMFDA cells. The volume of each sample analyzed was calculated by dividing the number of counting beads collected by the total number of counting beads per tube. By dividing the number of CMFDA-expressing cells by the volume analyzed, we derived the number of CMFDA-positive cells per tube. This experiment was repeated three times, and the results presented are averaged values.

**Quantitative RT-PCR (qRT-PCR)**

Primer to human ZEB1, LAMA3a, LAMA4, LAMAS, LAMB3, and LAMC2 are listed in Table 1. Total RNA was

| Gene | Forward primer | Reverse primer |
|------|----------------|----------------|
| ZEB1 | GCACTTGAAGAGGCCAGAG | TGACTCTGTGTTCCTAATTTT |
| LAMAS | GCTGACAGGTTCTCTACAG | AGCTGCAATGCTTTCAACCTG |
| LAMB3 | CGGAGCTGAGACCTACTGC | GAGGTCCCAGACGGCTTAT |
| LAMC2 | GCCTTTGGACCTTATTC | CAAGGTTCCTGATCCCTGAA |
| LAMA4 | CGGAAGACTGTTGAAGAAG | CAAGTCTCCAGAGCGACTTAT |
| LAMAS | CTTCAAGTGTGCAACTCACC | GCCCTAGACGTCCCTTGT |

**TABLE 1**

Human primer sequences for qRT-PCR
extracted from low passage PC-3, TEM4-18, and ZEB1 knock-down cells using an RNeasy mRNA isolation kit (Qiagen). Reverse transcription was carried out using iScript cDNA synthesis kit (Bio-Rad). The resulting cDNAs were used as PCR template using CYBR Green I (Invitrogen), and data were collected on iCycler thermal cycler (Bio-Rad). Experimental values were normalized to GAPDH values. Relative expression values were calculated using a comparative Ct method (13).

**Flow Cytometry**

Cells were detached using 2 ml of 0.48 mM Versene/10-cm dish and allowed to incubate for 5–10 min. The cells were then harvested, resuspended in 10 ml of serum-containing DMEM/F12 medium, and pelleted at 700 × g for 5 min. 5 × 10⁵ cells/tube were placed into 1.5-ml Eppendorf tubes and spun down at 700 × g, 4 °C, for 5 min. Supernatant was removed, and 50 µl of FACS buffer (PBS + 0.02% sodium azide + 5% BSA) + integrin α3 (1:200, A3-X8; Ref. 14), α6 (1:200, A6-EL; Ref. 15), β1 (1:200, TS2/16; Ref. 16), or β4 (1:200, clone 450-9D, GenTex) antibodies were added to the cells. The cells were then incubated for 20 min on ice in the dark, washed with 1 ml of FACS buffer, and pelleted for 5 min at 700 × g, 4 °C. 50 µl of FACS buffer + secondary antibody (1:100, goat anti-mouse FITC, Chemicon) was added to the cells and incubated for 20 min on ice in the dark. Cells were washed again with 1 ml of FACS buffer followed by a 5-min spin at 700 × g, 4 °C, resuspended in 400 µl of FACS buffer, and transferred to a 12 × 75-mm polystyrene FACS tube (BD Biosciences). Samples were analyzed using the BD Biosciences FACSCan at the University of Iowa Flow Cytometry Core Facility using the WinMDI software (Purdue University).

**Western Blotting**

To obtain whole cell extracts, 2% SDS protein lysates were collected. Following protein estimation, whole cell lysates (30 µg) were separated by SDS-PAGE and then transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was blocked in 5% milk with 0.1% Tween 20 in PBS for 1 h at room temperature followed by incubation of laminin γ2, clone E-6 (1:500), primary antibody overnight at 4 °C in blocking solution. The membrane was washed three times for 5 min in blocking solution followed by incubation with donkey anti-mouse (1:2,500) horseradish peroxidase secondary antibody for 1 h at room temperature. The membrane was then rinsed three times for 5 min in TBS with 0.1% Tween 20 followed by incubation with SuperSignal West Pico Chemiluminescent substrate (Pierce) for 5 min at room temperature. The membrane was then exposed on Classic Blue autoradiography film BX (Midwest Scientific).

**Chromatin Immunoprecipitation (ChIP)**

ChIP was carried out using an antibody against ZEB1 (clone E-20; Santa Cruz Biotechnology). ChIP analyses were performed according to the manufacturer’s instructions in the EZ-Chip Assay kit (Upstate Biotechnology). 5–10 × 10⁶ cells were sonicated for 20 s at 50% amplitude 10 times, with a 1-min rest between sonications using a Vibra Cell 150 µl–5 ml stepped tip (Sonics, Newtown, CT) in 500 µl of lysis buffer to generate fragments of DNA between 200 and 1,000 bp. Purified DNA was used as template to amplify human CDH1, LAMC2, and ITGB4 promoters with the primers listed in Table 2. 2 M betaine (Sigma) was added to the β4 integrin-specific primer set due to its high GC content. All samples were analyzed using qRT-PCR as mentioned above with the fold change measured as 2²(ΔΔCt of IgG – ΔΔCt of ZEB1).

## RESULTS

**Metastatic Subpopulation of PC-3 Cells Does Not Express Pro-migratory Laminin-332**—Previously we found that although TEM4-18 cells more efficiently crossed an endothelial monolayer and colonized tissues in mice following intravenous injection than the bulk PC-3 population from which they were isolated, they were less invasive than PC-3 cells in other in vivo invasion assays (3). To explore these seemingly inconsistent findings, we hypothesized that PC-3 cells might secrete a matrix-associated component that facilitates cell invasion in Transwell assays. To test this, we plated nonluminescent PC-3 cells onto uncoated Transwell membranes allowing them to “condition” the membrane before nonenzymatically removing the cells and plating luciferase-expressing PC-3 or TEM4-18 cells onto the conditioned Transwell membranes (Fig. 1A). Consistent with our previous observations, TEM4-18 cells migrated considerably less well compared with PC-3 on unconditioned membranes. In contrast, when plated on PC-3-conditioned membranes; TEM4-18 cells exhibited dramatically enhanced migration, greater than PC-3 cells which also showed a modest increase when plated onto conditioned membranes. This suggests that the bulk PC-3 population secretes an insoluble matrix or matrix-associated component that enhances the migratory activity of TEM4-18 cells, a constituent subpopulation of PC-3 cells.

To gain possible insight into the identity of this factor, we reviewed our previously published microarray data (3). We noted a much lower expression of LAMB3 and LAMC2 subunits of laminin-332, a matrix molecule well known to be involved in cancer cell motility, in TEM4-18 cells relative to PC-3 cells (data not shown). Because we had shown that TEM4-18 cells are present as an E-cadherin-negative subpop-
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The lack of laminin-332 expression in TEM4-18 prostate cancer cells might repress laminin-332 in TEM4-18 prostate cancer cells. We previously showed that TEM4-18 cells express the transcription factor ZEB1 at a high level relative to the bulk PC-3 population and that ZEB1 both repressed the epithelial phenotype and promoted transendothelial migration of TEM4-18 cells (3). Another study implicated ZEB1 in the regulation of laminin-332 expression in colorectal cancer cells; however, in this context ZEB1 repressed LAMA3 expression and activated LAMC2 expression (17). Although these details were at odds with our observations in TEM-18 cells because LAMA3a mRNA is unchanged and LAMC2 mRNA is reduced relative to PC-3 cells, we wished to determine whether ZEB1 might repress laminin-332 in TEM4-18 prostate cancer cells. We previously silenced ZEB1 in TEM4-18 cells and were able to achieve ~60–70% reduction in ZEB1 protein levels (supplemental Fig. 2) (3). Using these lines, we measured the mRNA expression levels of all three laminin-332 chains, LAMA3a, LAMB3, and LAMC2, using qRT-PCR. We detected a significant increase in mRNA of all three subunits of laminin-332, LAMA3a, LAMB3, and LAMC2 after stable ZEB1 silencing (Fig. 3A). The effect on LAMA3a mRNA, however, was of lower magnitude than LAMB3 or LAMC2 and did not reach significance in one of the ZEB1 shRNA constructs (Fig. 3A). Furthermore, laminin γ2 protein expression was increased in the ZEB1 knockdown cells compared with the TEM4-18 or shControl cell lines, although it did not reach the levels detected in PC-3 cells (Fig. 3B). The latter result is concordant with our findings on the effect of ZEB1 silencing in TEM4-18 cells on E-cadherin expression, which was only partially restored, suggesting that we either have not achieved sufficient inhibition of ZEB1 for a complete rescue of laminin γ2 expression or other factors contribute to the full repression of these genes (3).

FIGURE 1. Extracellular matrix deposition by PC-3 cells enhances TEM4-18 cell migration. A, migration of TEM4-18 cells was measured after allowing nonluminescent PC-3 cells to condition the Transwell membrane for 24 h. This treatment enhanced TEM4-18 cell migration significantly over nonconditioned Transwell inserts. **, p < 0.01 (paired t test) versus nonconditioned Transwell insert for each cell type. Error bars, S.E. B, qRT-PCR analysis reveals reduced expression of the laminin β3 and γ2 chains, but not the α3 chain in the TEM-18 cells compared with PC-3 and E-cadherin-positive PC-3E cells **, p < 0.01;***, p < 0.001 (one-way ANOVA, Bonferroni post test) versus PC-3E for each gene. C, Western blot analysis also confirmed the lack of the laminin γ2 subunit (LAMC2) in TEM-18 cells compared with PC-3 and PC-3E cells.

The expression of LAMA3, LAMB3, and LAMC2 subunits in PC-3 parental cells, E-cadherin-positive PC-3 cells (PC-3E) and TEM4-18 cells (Fig. 1, B and C), qRT-PCR analysis showed that the steady-state mRNA levels of both LAMB3 and, to a greater extent, LAMC2 (Fig. 1B) were reduced in TEM-18 cells, whereas the levels of LAMA3 where laminin heterotrimer includes laminin-332, were not significantly different between the cell lines. Loss of the laminin γ2 protein was also confirmed by Western blotting (Fig. 1C) because this subunit was expressed at a much higher level in both the PC-3 and PC-3E cells compared with TEM-4-18 cells (Fig. 1C).

To test the hypothesis that lack of laminin-332 expression was responsible for the reduced migration of TEM4-18 cells, we coated the Transwell inserts with various matrix proteins and measured Transwell migration. TEM4-18 cells showed significantly increased Transwell migration on laminin-332-coated Transwell inserts compared with other matrix components such as collagen IV and laminin-111 (Fig. 2A). Laminin-332 restored Transwell migration of TEM4-18 cells to levels comparable with PC-3 cells. To determine whether integrins known to support cell motility on laminin-332 are involved in Transwell migration of TEM4-18 cells, we performed antibody inhibition experiments. This showed that α3 integrin, but not α6 integrin, was involved in Transwell migration of TEM4-18 cells (supplemental Fig. 1). We also evaluated the effect of laminin-332 on migration of isolated PC-3 and TEM4-18 cells. When plated onto laminin-332, the average velocity (Fig. 2B), persistence (Fig. 2C), and distribution of individual cellular velocities (Fig. 2D) were indistinguishable. Taken together, these studies indicate that the TEM4-18 cells do not have an intrinsic deficit in either Transwell migration or planar cell motility, but rather they lack the ability to produce laminin-332, which acts as a potent, pro-migratory molecule for these cells.

Laminin-332 and β4 Integrin Expression Are Repressed by ZEB1—We showed previously that TEM4-18 cells express the transcription factor ZEB1 at a high level relative to the bulk PC-3 population and that ZEB1 both repressed the epithelial phenotype and promoted transendothelial migration of TEM4-18 cells (3). The latter result is concordant with our findings in TEM-18 cells because LAMA3a mRNA is unchanged and LAMC2 mRNA is reduced relative to PC-3 cells, which we wished to determine whether ZEB1 might repress laminin-332 in TEM4-18 prostate cancer cells. We previously silenced ZEB1 in TEM4-18 cells and were able to achieve ~60–70% reduction in ZEB1 protein levels (supplemental Fig. 2) (3). Using these lines, we measured the mRNA expression levels of all three laminin-332 chains, LAMA3a, LAMB3, and LAMC2, using qRT-PCR. We detected a significant increase in mRNA of all three subunits of laminin-332, LAMA3a, LAMB3, and LAMC2 after stable ZEB1 silencing (Fig. 3A). The effect on LAMA3a mRNA, however, was of lower magnitude than LAMB3 or LAMC2 and did not reach significance in one of the ZEB1 shRNA constructs (Fig. 3A). Furthermore, laminin γ2 protein expression was increased in the ZEB1 knockdown cells compared with the TEM4-18 or shControl cell lines, although it did not reach the levels detected in PC-3 cells (Fig. 3B). The latter result is concordant with our findings on the effect of ZEB1 silencing in TEM4-18 cells on E-cadherin expression, which was only partially restored, suggesting that we either have not achieved sufficient inhibition of ZEB1 for a complete rescue of laminin γ2 expression or other factors contribute to the full repression of these genes (3).
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FIGURE 3. Silencing ZEB1 restores laminin β3 and γ2 chains of laminin-332 and β4 integrin. A, qRT-PCR analysis reveals increased expression of laminin β3 and laminin γ2 chains after stable ZEB1 silencing. ***, p < 0.001 (one-way ANOVA, Bonferroni post test) versus shControl for each gene. Error bars, S.E. B, Western blot analysis confirms up-regulation of laminin γ2 protein (both the full-length 150-kDa and 100-kDa form) in the ZEB1 knockdown cells, although levels are not restored to the PC-3 parental cell line. C-F, flow cytometry analysis reveals increased cell surface protein expression of β4 integrin after stable ZEB1 silencing (F), whereas other laminin 332 receptors are moderately decreased or unchanged (C-E).

FIGURE 2. Laminin-332 restores TEM4-18 Transwell migration. A, TEM4-18 cells were plated onto various extracellular matrix components, and migration across Transwell inserts was measured. Laminin-332-coated Transwell inserts enhanced migration of TEM4-18 to levels similar to parental PC-3 cells on uncoated inserts (PC-3 untreated versus TEM4-18 laminin-332-coated). The values are compared with PC-3 migration on untreated Transwell inserts. *, p < 0.05 (one-way ANOVA, Bonferroni post test) versus untreated for each cell type. Error bars, S.E. B-D, PC-3 parental cells and TEM4-18 cells were plated on purified laminin-332. After 30 min for cell attachment and spreading, cell motility was monitored by time-lapse video microscopy every 2 min for 5 h. B, mean velocities of PC-3 cells and TEM4-18 cells were similar (p = 0.072, unpaired t test). C, directional persistence (the net hourly distance traveled divided by the total hourly distance traveled) was also indistinguishable for PC-3 cells and TEM4-18 cells (p = 0.239, unpaired t test). D, velocity distributions of the two cell types were very similar as well (p = 0.386, Kolmogorov-Smirnov test). For PC-3 and TEM4-18 cells, n = 41 and 56, respectively.

We have previously measured the cell surface expression of the three integrin receptors of laminin-332: α3β1, α6β1, and α6β4 integrins in the PC-3 and TEM4-18 cell lines (3). Because we found that β4 integrin was down-regulated in TEM4-18 cells compared with PC-3 cells, this suggested that ZEB1 might coordinate regulate both laminin-332 and β4 integrin. We investigated this possibility using the ZEB1 knockdown TEM4-18 cells. Cell surface β4 integrin expression increased ~2-fold in ZEB1 knockdown TEM4-18 cells, whereas α3 and β1 integrin expression was modestly reduced and integrin α6 was unchanged (Fig. 3C-F). This suggests that ZEB1 represses β4 integrin expression and confers a shift in laminin-332 receptor expression on the surface of TEM4-18 cells. Thus, ZEB1 coordinate regulates both laminin-332 and its receptor α4β6 integrin.

Because we have shown above that ZEB1 repressed endogenous laminin-332 and that exogenous laminin-332 could rescue Transwell migration, we tested the effects of ZEB1 knockdown on Transwell migration. ZEB1 knockdown increases Transwell migration of TEM4-18 cells 6–7-fold (Fig. 4A) to a level about half that of parental PC-3 cells (data not shown), consistent with the observed partial restoration of laminin-332 expression (Fig. 3, A and B). These data support the idea that ZEB1 represses laminin-332 expression in TEM4-18 cells and thereby results in poor Transwell migration of these cells.

To extend these findings to other prostate cancer cells, we knocked down ZEB1 expression in Du145, another prostate cancer cell line that we had previously shown to express high levels of ZEB1 (3). In accord with the findings in TEM4-18 cells, ZEB1 knockdown in Du145 resulted in ~2-fold increases in laminin γ2 and β4 integrin expression (supplemental Fig. 3, A and B). However, in contrast to our findings in TEM4-18 cells, ZEB1 knockdown results in decreased cell migration in the Transwell assay as has been shown in other cell lines (Fig. 4B) (5, 18, 19). To reconcile the disparate effects of ZEB1 knockdown on Transwell migration in these two prostate cancer cell lines, we considered the possibility that ZEB1 may exert both pro- and anti-migratory effects in cells. Because we showed that ZEB1 represses the expression of laminin-332, a pro-migratory molecule, this is in essence an anti-migratory effect of ZEB-1. To test this idea, we plated ZEB1 knockdown TEM4-18 cells on laminin-332-coated Transwells. In this context, ZEB1 knockdown in TEM4-18 cells was similar to the effects observed in
promoters of other target genes, including CDH1 (E-cadherin) and LAMA3a (laminin α3a) (17, 18, 20). Therefore, we proceeded to investigate the promoter regions of CDH1, LAMC2 (laminin γ2), and ITGB4 (integrin β4) for E/Z-box elements. We identified regions of clustered E-box or Z-box elements located in the proximal promoter regions of all three genes, including the already published ZEB1 target gene, E-cadherin, which we used as a positive control (Fig. 5A).

We determined that ZEB1 binds specifically to tandem E/Z-boxes within the proximal promoter of all three genes investigated, with enrichment values ranging from 2.5- to 4-fold compared with the isotype IgG control (Fig. 5, B–D, primer sets #1 and supplemental Fig. 4). Further, when we amplified regions significantly upstream from the proximal promoter that lack E-boxes (~3–5 kb), we did not see any enrichment of ZEB1 binding (Fig. 5, B–D, primer sets #1 and supplemental Fig. 4), nor did we observe an enrichment of ZEB1 binding to tandem E-boxes within LAMC2 located ~1 kb downstream of the transcriptional start site (Fig. 5, A and C, primer set #3 and supplemental Fig. 4). These findings indicate that ZEB1 may directly repress laminin γ2 (and thereby laminin-332) and β4 integrin expression by binding to the promoter elements of these genes.

**Laminins and Cooperative Cancer Cell Migration**—Because TEM4-18 cells failed to express laminin-332, and yet laminin-332 facilitates the migratory and invasive phenotype of those cells, we asked whether laminin-332-expressing cells within the bulk PC-3 population can support Transwell migration of TEM4-18 cells. Indeed, PC-3E cells, which express laminin-332 (see Fig. 1B), exhibit even greater Transwell migration than parental PC-3 cells, perhaps due to the lack of the poorly migrating TEM4-18 cells (Fig. 6A). To test whether PC-3E cells could support the Transwell migration of co-cultured TEM4-18 cells, we mixed CMFDA-labeled TEM4-18 cells with unlabeled PC-3E or TEM4-18 cells and assayed migration of the labeled cell population. When mixed with PC-3E cells, TEM4-18 cells were capable of efficient Transwell migration (Fig. 6B). These results are consistent with the notion that PC-3E cells supply laminin-332, which is a limiting factor for cell migration in this context, to TEM4-18 cells.

With the finding that laminin-332 effectively promoted Transwell migration of TEM4-18 cells, we considered the possibility that a component of the subendothelial matrix might be involved in the enhanced transendothelial migration that we previously documented in these cells (3). Indeed, relative migration of PC-3E cells and TEM4-18 cells across collagen IV-treated Transwell filters and HMVEC-L monolayers is consistent with our previous findings, with TEM4-18 cells showing considerably enhanced transendothelial migration (Fig. 6C).

Further, to test whether a component of the subendothelial matrix contributes to this enhanced migration, we nonenzymatically removed HMVEC-L cells from the Transwell insert and plated PC-3E and TEM4-18 cells on these “conditioned” membranes. This showed that both PC-3E and TEM4-18 cells migrated equally well on the HMVEC-L matrix (Fig. 6C). Thus, interaction with the subendothelial matrix does not explain the difference in transendothelial migration between PC-3E and TEM4-18 cells. Nevertheless, the HMVEC-L matrix did augment migration of TEM4-18 cells compared with collagen IV-
FIGURE 5. **ZEB1** binds specifically to E-boxes within the 5′ regulatory regions of laminin-γ2 and β4 integrin. **A**, promoters of E-cadherin (CDH1), laminin-γ2 (LAMC2), and β4 integrin (ITGB4) are depicted from +1 kb to −5.5 kb of the transcription start site. Exons are shown as light gray boxes, and vertical black bars represent E/Z-box elements. Primers used for ChIP analysis are indicated. Primer set #3 for LAMC2 is an area with E-box elements, but shows no enrichment, revealing the resolution of the sonicated DNA to be under 1 kb (**A**, primer set #1 (regions lacking E/Z-box elements). Extensive efforts to detect laminin-332 and its receptor, β3 integrin, in prostate cancer cells. Although notable for its pro-migratory properties and its up-regulation in invasive regions of a number of tumor types, laminin-332 and β4 integrin expression is typically reduced in prostate cancer (8, 9, 22). This has led to the proposition that loss of these hemidesmosomal cell adhesion components may facilitate dispersion of prostate cancer cells from the primary site. In prostate cancer, specific reduction of the β3 and γ2 polypeptide chains, but not the α3 chain, has been observed (8). However, the mechanism by which this occurs remains unclear. Hao et al. reported that LAMB3 and LAMC2 mRNA could be detected in prostate cancer tissue, suggesting that the mechanism is post-transcriptional (23). Cleavage of β3 and γ2 proteins by matrix metalloproteases and hepsin has also been described (7, 24, 25). These results may appear at odds with those reported here; however, more than one mechanism may account for the reduction of laminin-332 immunoreactivity in prostate cancer. The continued expression of LAMB3 mRNA by prostate cancer cells has been demonstrated (7). In this study, we demonstrate that ZEB1 represses the expression of laminin-332 and its receptor, β3 integrin, in prostate cancer cells. The expression of laminin-332 is down-regulated in prostate cancer tissues (8), and it has been suggested that the reduction of laminin-332 may account for the reduced immunoreactivity in prostate cancer tissue. This has led to the proposition that loss of these hemidesmosomal cell adhesion components may facilitate dispersion of prostate cancer cells from the primary site. In prostate cancer, specific reduction of the β3 and γ2 polypeptide chains, but not the α3 chain, has been observed (8). However, the mechanism by which this occurs remains unclear. Hao et al. reported that LAMB3 and LAMC2 mRNA could be detected in prostate cancer tissue, suggesting that the mechanism is post-transcriptional (23). Cleavage of β3 and γ2 proteins by matrix metalloproteases and hepsin has also been described (7, 24, 25). These results may appear at odds with those reported here; however, more than one mechanism may account for the reduction of laminin-332 immunoreactivity in prostate cancer. The continued expression of LAMB3 mRNA by prostate cancer cells has been demonstrated (7). In this study, we demonstrate that ZEB1 represses the expression of laminin-332 and its receptor, β3 integrin, in prostate cancer cells. The expression of laminin-332 is down-regulated in prostate cancer tissues (8), and it has been suggested that the reduction of laminin-332 may account for the reduced immunoreactivity in prostate cancer tissue.
and LAMC2 mRNA in most tumor tissue does not exclude the existence of a less abundant population of cells that may exhibit ZEB1-dependent repression of these mRNAs (5). Moreover, proteolysis of laminin-332 might contribute primarily to the generation of pro-migratory forms of this molecule, rather than its decreased abundance from the extracellular matrix (7).

Prior studies have implicated ZEB1 in the regulation of laminin-332 subunits. Spaderna et al. found in colorectal carcinoma that ZEB1 repressed LAMA3a and either directly or indirectly activated LAMC2 (17). The latter finding is consistent with up-regulation of laminin γ2 protein at the invasive front of colorectal cancers (26–28). Loss of the laminin-332 heterotrimer is consistent with the loss of basement membrane structure at the invasive front of colorectal tumors (17). Our findings in prostate cancer are different in that although ZEB1 has some effect on LAMA3a expression, it is clearly associated with repression, not activation, of LAMC2. Together, these results indicate that ZEB1 affects transcription of LAMC2 in a manner that depends on tissue context. Because colorectal cancer is generally regarded as a more rapidly progressive disease than prostate cancer and laminin γ2 may possess intrinsic motility-promoting properties independent of the laminin-332 heterotrimer, it is interesting to speculate that the differential function of ZEB1 in these two tissues may contribute to the pace of disease progression. ZEB1 is a dual zinc finger homeodomain transcription factor that has been shown to recruit both co-repressors including CtBP and histone-modifying enzymes and co-activators such as p300 (29–31). The differential levels or recruitment of these components, possibly involving post-translational modifications to ZEB1 itself, may underlie the apparent ability of ZEB1 to act as a transcriptional repressor of LAMC2 in one context and an activator in another consistent with other targets of ZEB1 (31, 32). Finally, transfection of other cell types with SNAIL1 resulted in decreased expression of laminin-332 and/or β4 integrin, suggesting that other master regulators of EMT may control these genes similarly, perhaps explaining why ZEB1 knockdown only partially restores their expression in our studies (33, 34).

Here, we have shown that in prostate cancer cells, ZEB1 coordinates repression both laminin-332 and its receptor integrin β4. Laminin-332 binds preferentially to three integrin receptors, α3β1, α6β1, and α6β4 (35–37). Although both α6β4 integrin and laminin-322 function in stable adhesion complexes in hemidesmosomes, α6β4 integrin is involved in invasion and motility in some cancers (38, 39). However, in mammary epithelial cells, the ITGB4 promoter is methylated in association with TGFβ-induced EMT, and β4 integrin expression is reduced in invasive prostatic carcinoma compared with the normal glands or early prostatic intraepithelial neoplasia lesions (40, 41). Therefore, α3β1 and α6β1 integrins remain as receptors for laminin-332 in TEM4-18 cells as has been noted in prostate cancer specimens. Indeed, here we show that α3 integrin supports Transwell migration of prostate cancer cells on laminin-332. Interestingly, in keratinocytes, whether α6β4 integrin binds to laminin-332 may determine whether these cells migrate as collective sheets or in a more mesenchymal mode in response to epidermal growth factor (42). Thus, ZEB1, by repressing ITGB4, as well as CDH1, may contribute to a change in the mode of cell migration from collective to single cell.

EMT is generally associated with invasive cellular behavior and is mediated by a host of transcription factors, such as ZEB1, which promote this state. Unlike their role in repressing epithelial identity such as inhibiting E-cadherin and polarity gene expression, the detailed mechanisms by which EMT-promoting transcription factors drive invasive behavior remain largely unclear. In fact, here we show that one consequence of ZEB1 activity is repression of laminin-332, a potent pro-migratory molecule. Demonstrating this, ZEB1 knockdown in TEM4-18 cells showed increased migration. Thus, ZEB1 may exert both pro- and anti-migratory effects. In contrast to TEM4-18 cells, in Du145 cells, as with other epithelial cell lines, ZEB1 silencing decreased cell migration, demonstrating its pro-migratory function (5, 18, 19). When we plated ZEB1 knockdown TEM4-18 cells on laminin-332, this revealed its pro-migratory function. Thus, the pro-migratory function of ZEB1 is dependent on the presence of laminin-332 in the cellular microenvironment, which in tumors may be supplied by cells other than the invasive cancer cells themselves. In this regard, possibilities include normal prostate basal epithelial cells or perhaps other, less invasive cancer cell populations (43). Indeed, our studies here experimentally demonstrate cooperation between PC-3E and TEM4-18 cells. Cooperation among distinct tumor cell populations or between cancer and stromal cells during cancer progression has a solid theoretical basis and emerging experimental support, including a recent demonstration of cooperative interactions relative to the role of EMT in an experimental metastasis model (44–46). However, as mentioned above, loss of laminin-332 from the epithelial basement membrane is consistently observed in primary prostate tumors; suggesting that local invasion of prostate cancer does not depend on laminin-332. Interestingly, we show that a matrix preparation from endothelial cells also promotes prostate cancer cell migration and lacks laminin-332. Because endothelial cells express laminin-411 and -511, which are also ligands for α3 integrins, these proteins may support prostate cancer cell migration during intravasation or extravasation following transendothelial migration (21).

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