ZEB2 Represses the Epithelial Phenotype and Facilitates Metastasis in Ewing Sarcoma

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Abstract

The vast majority of cancer-related deaths are attributable to metastasis. Effective treatment of metastatic disease will be improved by a better understanding of the molecular mechanisms contributing to this phenomenon. Much of the work in this field has focused on metastasis of carcinomas, tumors of epithelial origin, while metastasis of sarcomas, tumors of mesenchymal origin, remains poorly understood. Experimental evidence from studies in carcinomas, coupled with clinical observations, highlights the importance of both epithelial and mesenchymal characteristics in these cancer cells that make them competent for metastasis. We set out to test if similar cellular plasticity contributes to sarcoma metastasis. We found that the transcription factor, ZEB2, repressed epithelial gene expression in Ewing sarcoma cells, and this, in turn, repressed the epithelial phenotype. When ZEB2 was experimentally reduced in these cells, epithelial characteristics including decreased migratory ability and cytoskeleton rearrangements were observed. Furthermore, ZEB2 reduction in Ewing sarcoma cells resulted in a decreased metastatic potential using a mouse metastasis model. Our data show that Ewing sarcoma cells may have more epithelial plasticity than previously appreciated. This coupled with previous data demonstrating Ewing sarcoma cells also have mesenchymal features primes these cells to successfully metastasize. This is clinically relevant for 2 important reasons. First, this may offer a therapeutic opportunity to induce characteristics of one cell type or the other depending on the stage of the disease. Second, and more broadly, this raises questions about the cell of origin in Ewing sarcoma and may inform future animal models of the disease.

Keywords

Ewing sarcoma, EVS/FLI, EMT, metastasis, ZEB2

Introduction

Cancer metastasis is a major clinical problem with >90% of all cancer deaths attributed to metastatic disease.¹ Metastasis is a complex, multistep process that results in the dissemination of cells from the site of the primary tumor to distant organs where these cells are able to colonize and form a secondary lesion.² Recent experimental evidence suggests that successful metastasis requires a tumor cell to possess both epithelial and mesenchymal characteristics.^{3,4} Epithelial features promote cell growth at both the primary and secondary sites, while mesenchymal features contribute a migratory capacity to these cells facilitating escape from the tumor, the ability to survive in the circulatory system, and extravasate at distant sites. The vast majority of our knowledge regarding the metastatic cascade comes from the study of carcinomas, tumors originating from epithelial cells. In this setting, epithelial tumor cells are thought to undergo an epithelial-mesenchymal transition (EMT) in order to disseminate from the bulk tumor, and emerging evidence also supports the idea that a mesenchymal-epithelial transition (MET) is required for colonization of distant sites.³⁻⁶

Not all cancer metastases conform to this biological paradigm. Sarcomas, for example, are thought to arise from

mesenchymal tissues such as bone, cartilage, and muscle,⁷ thus making it unlikely that an EMT-MET is a requirement in sarcoma metastasis. Several sarcomas are well differentiated and closely resemble what is thought to be the cell of origin (but could also be aberrantly differentiated), whereas others are poorly differentiated and the cell of origin remains uncertain.⁸ A diverse group of sarcomas display features reminiscent of an EMT-MET,⁹⁻¹² suggesting that sarcomas may have some phenotypic plasticity.

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Ewing sarcoma is an undifferentiated sarcoma characterized by the oncogenic fusion protein and transcription factor, EWS/FLI, and its small round blue cell histological presentation.¹³ Due to its poorly differentiated nature, the cell of origin for this tumor is unknown. Neural crest stem cells (NCSC),¹⁴ mesenchymal stem cells (MSC),^{15,16} or perhaps neural crest-derived mesenchymal stem cells¹⁷⁻¹⁹ are currently the most widely accepted cell of origin candidates. Further complicating the cellular histogenesis are the diverse locations including bone, most commonly the pelvis and long bones, and a variety of soft tissues such as kidney and pancreas, in which these tumors arise.⁸ Patients generally present with this disease in the second decade of life, and approximately 20% to 25% have detectable metastatic spread at diagnosis.¹³ Patients lacking overt metastasis likely harbor micrometastases as indicated by the high rate of relapse at distant sites following surgical resection of the primary tumor in the absence of systemic chemotherapy.^{20,21} The presence of metastatic disease comes with a poor prognosis, bringing the overall survival rate from 70% to 75% for localized disease to less than 20% for those with metastasis.²² The aim of this study is to better understand the cellular plasticity in Ewing sarcoma and how that contributes to the highly metastatic phenotype in this disease.

Results

ZEB2 is highly expressed in Ewing sarcoma. Metazoans are primarily composed of 2 cell types, epithelia and mesenchyme.²³ Epithelial cells have an organized structure marked by intercellular adhesions while mesenchymal cells lack these cell-cell contacts and are characterized by their ability to migrate as single cells.²³ It is well established that Ewing sarcoma cells have mesenchymal features, which becomes more pronounced with EWS/FLI knock-down.^{24,25} Histological examination has also demonstrated that a subset of Ewing sarcoma patient tumor samples display epithelial features.²⁶⁻²⁸ Despite retaining remnants of these 2 cell types, Ewing sarcoma cells are overwhelmingly considered to be undifferentiated. Experimental evidence has made clear that EWS/FLI prevents the mesenchymal phenotype in Ewing sarcoma,^{24,25} but the factor(s) preventing an epithelial phenotype are unknown.

Using the Baird *et al.* data set that contains gene expression profiling of 19 Ewing sarcoma tumors,²⁹ we confirmed variant expression of well-accepted epithelial and mesenchymal markers.³⁰ We next investigated the expression of the classic EMT-inducing transcription factors (EMT-TF)³¹ (Fig. 1A), reasoning that they represent good candidates for repressors of the epithelial phenotype in Ewing sarcoma. Strikingly, we found that zinc finger E-box binding homeobox 2 (ZEB2) (encoded by *ZFHX1B*, and also known as SMAD interacting protein 1, SIP1) was the only EMT-TF³² that was consistently expressed in Ewing sarcoma patient

tumors (Fig. 1A) and cell lines (Fig. 1B). Many highly expressed genes in Ewing sarcoma are induced by EWS/FLI; however, when EWS/FLI is knocked-down, ZEB2 expression levels do not change consistently in the Ewing sarcoma cell lines tested (A673, SKNMC, TC71) (Fig. 1C and D). This indicates that ZEB2 levels are not dependent on the translocation and thus ZEB2 may be expressed in the cell of origin. Accordingly, we show that ZEB2 is expressed in both proposed cells of origin, BM-MSCs (bone marrow derived-MSCs) and NCSCs (Fig. 1E).¹⁴ Much of the work in the Ewing sarcoma field has focused on EWS/FLI transcriptional targets, but a permissive cellular environment is crucial for EWS/FLI's oncogenic ability,³³ necessitating a better understanding of relevant gene expression in the cell before the translocation event.

ZEB2 represses epithelial gene expression in Ewing sarcoma. To determine if the expression of ZEB2 in Ewing sarcoma was indeed repressing epithelial gene expression, we performed genome-wide gene expression profiling using RNA-sequencing (RNA-seq) in A673 cells expressing a control siRNA (siControl) or 2 unique siRNAs targeting ZEB2 (siZEB2-5 or siZEB2-6) (Fig. 2A). We found 46 genes were up-regulated and 124 genes were down-regulated by ZEB2 using significance cutoffs set at a 2-fold change and a false discovery rate (FDR) of 10% (Suppl. Table S1). We validated the RNAseq profile by qRT-PCR of select genes (Suppl. Fig. S1). Of note, several ZEB2 up-regulated genes have previously been shown to contribute to metastasis in a variety of solid tumors (COL1A1, COL1A2)³⁴ as well as in Ewing sarcoma specifically (MMP2, SPARC).³⁵ To identify functional classes that were enriched in our ZEB2 repressed gene list, we used the DAVID analysis functional annotation clustering algorithm.^{36,37} The most enriched term representing this list was epithelial cell differentiation, followed by actin binding and cell junction, supporting our hypothesis that ZEB2 is repressing epithelial gene expression in Ewing sarcoma cells. E-cadherin is notably absent from our repressed gene list, as its expression does not increase even in the context of ZEB2 knock-down in Ewing sarcoma cells. E-cadherin is a hallmark of epithelial cells; however, its absence in 97% and 100% of tumor samples, respectively, in 2 independent studies,^{28,38} suggests that it may be in a more permanently repressed state and that Ewing sarcoma cells cannot achieve full epithelial status.

We compared the gene expression profile generated in Ewing sarcoma cells with reduced ZEB2 expression to gene expression data sets generated in cells undergoing EMT induced by different mechanisms. We first compared our ZEB2 gene expression profile to EMT gene expression profiles derived from human mammary luminal epithelial cells (HMLE) expressing TGF- β 1 or EMT-TFs, Twist, Snail, or Goosecoid (GSC).³⁹ As expected, heat maps display an

Figure 1. ZEB2 is expressed in Ewing sarcoma cells. **(A)** Gene expression data from Ewing sarcoma patient tumors from the Baird *et al.*²⁹ dataset showing the expression of epithelial markers, E-cadherin (CDH1), tight junction protein 1 (ZO-1), laminin beta 1 (LAMB1), and mucin 1 (MUC1), mesenchymal markers, N-cadherin (CDH2), vimentin (VIM), fibronectin (FN), and integrin alpha 5 (ITGA5), and EMT-TF, Snail1 (SNAI1), Snail2/Slug (SNAI2), TWIST1, ZEB1 and ZEB2. Horizontal line indicates mean. **(B)** qRT-PCR data showing the expression of EMT-TF in a panel of Ewing sarcoma cell lines—A673, RDES, SKNMC, TC71, TC32, CHLA10, and CHLA258. **(C)** Western blot showing EWS/FLI and ZEB2 expression in control shRNA and EWS/FLI shRNA infected Ewing sarcoma cells lines, A673, SKNMC, and TC71. **(D)** qRT-PCR data showing EWS/FLI and ZEB2 expression levels in control shRNA and EWS/FLI shRNA infected Ewing sarcoma cell lines A673, SKNMC, and TC71. Errors bars represent standard deviation (SD) from 3 technical repeats. **(E)** ZEB2 gene expression in hBM-MSC and hNCSC from the von Levietz *et al.*¹⁴ data set. Error bars represent SD.

inverse expression pattern in ZEB2 knock-down cells undergoing some degree of MET compared to the HMLE cells undergoing EMT. Unsupervised hierarchical clustering also clusters the EMT cells apart from the siZEB2

Ewing sarcoma cells (Fig. 2C; Suppl. Table S2). We confirmed this finding using a distinct data set that induced EMT in HMLE cells by suppressing E-cadherin expression with shRNA. In this study, HMLE cells expressing an

Figure 2. ZEB2 represses epithelial gene expression in Ewing sarcoma cells. **(A)** qRT-PCR data and western blot showing ZEB2 expression levels in A673 cells transfected with control siRNA or 2 different siRNAs targeting ZEB2 (siZEB2-5 and siZEB2-6). Tubulin is shown as a loading control. **(B)** Graph showing the top 10 enriched terms by DAVID analysis in the ZEB2 repressed gene set from our RNAseq analysis. Enrichment score equals $-\log(\text{mean } P\text{-value})$. **(C)** Heat map and hierarchical clustering of the ZEB2 RNAseq data with Taube *et al.*³⁹ EMT data in HMLE cells. **(D)** Heat map and hierarchical clustering of the ZEB2 RNAseq data set compared to the Onder *et al.*⁴¹ EMT data set in HMLE cells. **(E)** Heat map of the ZEB2 RNAseq data with Keshamouni *et al.* EMT time course data in A549 lung carcinoma cells. Color key represents log₂ FC.

shRNA targeting E-cadherin displayed increased migration and invasion *in vitro* and metastatic ability in mouse models. This increased metastatic potential was mediated, at least in part, by β -catenin. When cells were infected with shRNAs targeting both E-cadherin and β -catenin, they were no longer metastatic, suggesting a more epithelial state.⁴⁰ Consistent with these experimentally verified cell states, Ewing sarcoma cells with reduced ZEB2 clustered with the double knock-down HMLE gene expression profile (epithelial), whereas the HMLEs that had transitioned to a mesenchymal status by expressing only the shRNA targeting E-cadherin had an inverse gene expression profile and clustered separately (Fig. 2D; Suppl. Table S3).

To validate these findings using a different cell type, we used an EMT gene expression profile derived from A549 lung adenocarcinoma cells stimulated with TGF- β and followed over a 72-hour time course.^{42,43} In this model of EMT, western blot analysis shows that at 16 hours post-TGF- β treatment mesenchymal markers, vimentin and N-cadherin, increase in expression while E-cadherin expression begins to decrease, indicating the start of the EMT.⁴⁴ Strikingly, our ZEB2 knock-down gene expression data display an expression signature similar to the early time points (0.5–4 hours), which represents an epithelial cellular state. At 8 hours, the A549 gene expression profile starts to shift, and at 16 hours, a time point when the EMT has occurred based on the western blot analysis, the gene expression pattern is completely reversed (compared to our ZEB2 knock-down data set)—representing a mesenchymal cellular state (Fig. 2E; Suppl. Table S4). These transcriptional profiling comparisons demonstrate that Ewing sarcoma cells and epithelial cells regulate a similar panel of genes to achieve cellular plasticity.

ZEB2 represses the epithelial phenotype in Ewing sarcoma. To further study the gene expression changes seen by RNA-seq, we designed a retroviral shRNA targeting the 3'UTR of ZEB2 to achieve stable knock-down in Ewing sarcoma cell lines. This construct reduced ZEB2 RNA and protein levels (Fig. 3A and B). Using this shRNA, we examined the transcript changes of several genes identified as ZEB2 repressed targets. It has been suggested that total ZEB (ZEB1 and ZEB2) levels in a cell are interdependent and that there is some ability to compensate between ZEB1 and ZEB2.⁴⁵ In agreement with this hypothesis, we saw that knock-down of ZEB2 led to an increase in ZEB1 expression in 3 Ewing sarcoma cell lines (A673, SKNMC, and TC71). We also verified the ZEB2 mediated repression of several epithelial genes in A673 cells including desmosome components, desmoplakin (DSP) and plakophilin 2 (PKP2), an intermediate filament characteristic of simple epithelia, keratin 8 (KRT8), the tight junction protein, F11 receptor (F11R, also known as junctional adhesion molecule A, JAM-A), and a facilitator of cytoskeletal

remodeling, Rho guanine nucleotide exchange factor 5 (ARHGEF5). ZEB2 repressed these genes to a lesser degree and not as consistently in 2 other Ewing sarcoma cell lines, SKNMC and TC71 (Fig. 3A). KRT8 and DSP protein levels changed consistently with what was seen at the RNA level (Fig. 3B). The concomitant increase in ZEB1 expression with ZEB2 knock-down may be responsible for the lack of expression of some epithelial genes in other cell lines—for example, KRT8 in SKNMC cells (see below, Fig. 4B). ZEB1 and ZEB2 can bind similar DNA sequences and have some redundant function⁴⁶; however, ZEB1 does not appear to function as a repressor of the epithelial phenotype to the same extent as ZEB2 in Ewing sarcoma. Despite the increase in ZEB1 RNA levels with ZEB2 knock-down, ZEB1 protein remains undetectable in Ewing sarcoma cell lines after reduction of ZEB2 (Suppl. Fig. S2A). ZEB1 knock-down does not result in an increase in the expression of KRT8 or DSP, suggesting that ZEB2 is sufficient for this repression even when ZEB1 knock-down (Suppl. Fig. S2B).

In vitro functional assays were performed to ask whether the increase in epithelial gene expression resulting from ZEB2 knock-down led to a more epithelial phenotype in Ewing sarcoma cell lines. Boyden chamber assays (Fig. 3C) and wound-healing assays (Fig. 3D) were used to define the migratory capacity of Ewing sarcoma cells. In both settings, Ewing sarcoma cells with ZEB2 knock-down displayed reduced migratory ability. Western blot for cleaved caspase-7 and cleaved PARP determined that ZEB2 knock-down did not increase apoptosis as compared to the control knock-down cells (Suppl. Fig. S3A). For the Boyden chamber assay cells were plated in serum-free media in the upper chamber, and induced to migrate toward serum containing media in the bottom chamber. As a specific control for this assay, cells from each condition were plated in serum-free media and counted 24 hours later to eliminate the possibility that differences in the cell's ability to survive under serum-free conditions contributed to the phenotype (Suppl. Fig. S3B). The wound healing invasion pattern of cells with reduced ZEB2 shows a slower and more cohesive movement, which is characteristic of epithelial cells. In contrast, A673 and SKNMC control cells demonstrate a more mesenchymal migration pattern marked by single cells invading the wounded area and faster wound closure.

Epithelial and mesenchymal cells have unique cytoskeletal arrangements. Using phalloidin to label F-actin, we show that A673 cells expressing an siRNA targeting ZEB2 traded a more diffuse actin cytoskeleton for more compact actin rings with a cobblestone morphology typical of epithelial cells. We also confirm the knock-down by the absence of ZEB2 nuclear staining in the siZEB2 transfected cells (Fig. 3E). Taken together, the increase in epithelial gene expression when ZEB2 is reduced allows for phenotypic and morphological changes consistent with an epithelial shift.

Figure 3. ZEB2 represses the epithelial phenotype in Ewing sarcoma cells. **(A)** qRT-PCR data showing ZEB2 knock-down in A673, SKNMC, and TC71 cells infected with control shRNA or shRNA targeting ZEB2 and the corresponding change of expression in the indicated genes. Error bars represent SD from technical triplicates. **(B)** Western blot showing expression of ZEB2, KRT8, and DSP in A673, SKNMC, and TC71 cells expressing a control or ZEB2-targeting shRNA. Tubulin is shown as a loading control. **(C)** Boyden chamber cell migration assays in A673, SKNMC, and TC71 cells infected with a control shRNA or ZEB2 targeting shRNA. Data are from 2 independent experiments each done in triplicate. Each point represents cells counted in one field—5 fields were counted per chamber. Significance was determined by Student's *t*-test and the mean is shown. Horizontal bar represents mean. **(D)** Representative images from 3 time points of a wound healing assay using control or ZEB2 shRNA infected A673 and SKNMC cells (left). Scale bar = 250 μ m. Quantification of the area of the wound healed measured every 24 hours (right). Data are the average of 2 independent experiments each done in triplicate ($n = 6$). *P*-values indicated above each condition as determined by Student's *t*-test and error bars show SD. **(E)** Immunofluorescence images showing ZEB2 expression levels (red—568 nm) and actin cytoskeleton stained with phalloidin (green—488 nm) in A673 cells transfected with control siRNA or siRNA targeting ZEB2 (siZEB2-5). Scale bar = 50 μ m.

Ectopic expression of miR-200 family epithelializes Ewing sarcoma cells. The relationship between ZEB1 and ZEB2 and the miR-200 family of micro-RNAs (miRNA) (miR-200a, miR-200b, miR-200c, miR-141, and miR429) is well established.^{45,47} These miRNAs bind to the 3'UTR of both ZEB1 and ZEB2 to repress translation; conversely, ZEB1 and ZEB2 can bind to the promoters of miR-200s to repress transcription. This double-negative feedback loop is thought to serve as a switch to regulate EMT—when ZEB levels are high and miR-200 levels are low, then cells are phenotypically mesenchymal, and when the reverse is true, then cells are more epithelial.⁴⁸ We used this phenomenon to investigate whether Ewing sarcoma cells could be induced to epithelialize by a mechanism that was distinct from RNAi methods previously tested, yet still dependent on ZEB2 levels.

We ectopically expressed the polycistronic miR-200 gene cluster from chromosome 1 (miR-200b, miR200a, and miR429) in A673 and SKNMC Ewing sarcoma cells (Fig. 4A). As predicted, expression of miR-200 family members reduced the expression of ZEB2 protein. Epithelial proteins, DSP and KRT8, were also increased in miR-200 expressing A673 and SKNMC cells. Notably, KRT8 expression was not observed when ZEB2 was specifically knocked-down in SKNMC cells (Fig. 3B). This discrepancy may result from the unremitting repression of ZEB1 by the miR-200 family despite reduction in ZEB2 (Suppl. Fig. S2C), in contrast to the possibility of compensation by ZEB1 when ZEB2 is specifically reduced. Furthermore, Ewing sarcoma cells expressing miR-200s display a migration defect in wound-healing assays (Fig. 4C), similar to that of cells with specific reduction of ZEB2 using shRNA (Fig. 3D). The epithelial plasticity of Ewing sarcoma cells

Figure 4. Expression of miR-200 family members epithelializes Ewing sarcoma cells. **(A)** qRT-PCR data showing the expression of the indicated miRNA in A673 and SKNMC cells infected with a lentivirus expressing a control miRNA or miR-200 family cluster from chromosome I (miR-200b, miR-200a, miR-429). Error bars represent SD from 3 technical repeats. **(B)** Western blot showing expression of ZEB2, KRT8, and DSP in A673 and SKNMC cells expressing a control miRNA or miR-200 family cluster from chromosome I (miR-200b, miR-200a, miR-429). Tubulin is shown as a loading control. **(C)** Representative images from 3 time points of a wound healing assay using control miRNA or miR-200 family cluster from chromosome I (miR-200b, miR-200a, miR-429) infected A673 and SKNMC cells (**left**). Scale bar = 250 μ m. Quantification of the area of the wound healed measured every 24 hours (**right**). Data are the average of 2 independent experiments each done in triplicate ($n = 6$). P-values indicated above each condition as determined by Student's *t*-test and error bars show SD.

expressing miR-200 family members complements our findings implicating ZEB2 in the repression of the epithelial phenotype.

ZEB2 facilitates metastasis in Ewing sarcoma. We have established the ability of ZEB2 to repress epithelial features in Ewing sarcoma cells and the reemergence of such features with ZEB2 knock-down. We hypothesized that this

innate epithelial repression primes Ewing sarcoma cells for metastasis, and thus if ZEB2 levels were reduced, Ewing sarcoma cells would lose the ability to metastasize. To test this hypothesis, we used an orthotopic mouse metastasis model whereby Ewing sarcoma cells are injected intratibially and cells spontaneously metastasize to the lung.⁴⁹ This model accounts for all steps in the metastatic cascade from escaping the primary tumor to colonizing the secondary

site, making it superior to the tail vein injection metastasis model. Two doses of A673 cells (25K or 100K) infected with a retroviral shRNA targeting ZEB2 or control shRNA were injected into the right tibia of male NOD-SCID mice. Tumor growth was monitored weekly by measuring the right tibia using calipers. Mice were sacrificed at 6 weeks postinjection, and the lungs were harvested. ZEB2 knock-down had no effect on tumor growth (Fig. 5A). Fewer mice injected with A673 cells expressing the ZEB2 shRNA showed macroscopic pulmonary metastases at both doses; however, this binary comparison did not yield statistical significance (Fig. 5B). It was clear by gross observation that the metastatic burden was much greater in the control shRNA injected animals compared to the ZEB2 shRNA injected animals (Fig. 5C). When the area of the lung with metastatic lesions was quantified, ZEB2 knock-down cells showed significantly less metastatic burden (Fig. 5D, Suppl. Fig. S4). Importantly, we saw no difference in tumor size and no correlation between tumor size and propensity to metastasize (Fig. 5E), indicating ZEB2 does not play a role in primary tumor growth, but specifically contributes to the metastatic ability of Ewing sarcoma cells.

Accordingly, we found that metastases derived from control shRNA injected A673 cells had strong ZEB2 staining by immunohistochemistry. Metastatic lesions derived from ZEB2 knock-down injected cells also showed areas of ZEB2 positive staining (Fig. 5E), which could indicate the need for these knock-down cells to re-express ZEB2 in order to metastasize. Further characterization of ZEB2 expression, both temporally and spatially, in the primary tumor and metastatic nodules is necessary to better understand the relationship between ZEB2 and the capacity of Ewing sarcoma cells to spread.

ZEB2 levels correlate with patient metastasis. These data suggest that low ZEB2 levels in patient tumors would decrease the occurrence of metastatic disease. We tested this hypothesis by staining for ZEB2 by immunofluorescence on tissue microarrays containing Ewing sarcoma tumor specimens. The staining results were read in a semiquantitative manner using an AQUA (Automatic Quantitative Analysis) system.⁵⁰ Consistent with our hypothesis, tumors from patients with no metastasis expressed lower levels of ZEB2 than those from patients with metastatic disease ($P = 0.0198$) (Fig. 6A). Since presence of metastasis is a poor prognostic indicator, we tested if low ZEB2 levels also correlated with an increase in overall survival. We generated a Kaplan-Meier curve by stratifying the patients into 2 groups: tumors with high ZEB2 expression (values above the median) and tumors with low ZEB2 expression (values below the median). Patients with low ZEB2 had a slight, but statistically insignificant ($P = 0.3364$ by Log-rank Mantel Cox test) increase in overall survival (Fig. 6B). Many factors influence patient outcome, and therefore it is not surprising that expression levels of one protein involved in metastasis, ZEB2, does not predict overall patient survival.

Discussion

Sarcoma metastasis is not well understood. Sarcomas are thought to arise from mesenchymal cells and thus do not have a baseline epithelial differentiation state that is seen in many carcinomas. This fact excludes sarcomas from the EMT-MET metastasis paradigm whereby tumor cells in carcinomas must lose their epithelial features to escape the primary tumor, but regain them in order to colonize the secondary site. However, it is likely that the concept of cellular plasticity used to metastasize can be applied to sarcoma metastasis. Undifferentiated Ewing sarcoma cells may innately have the right balance of epithelial and mesenchymal features that allow for successful growth at the primary and secondary sites, as well as a high propensity for metastasis. Providing support for this intrinsic ability, micrometastatic spread is nearly ubiquitous in Ewing sarcoma patients and occurs at early stages of the disease,⁵¹ suggesting a parallel progression of tumor and metastases.⁵² This is in stark contrast to some carcinomas such as colorectal cancer where a slow progression and accumulation of mutations eventually leads to an invasive malignancy.⁵³ A “passive metastasis” model has recently been proposed for Ewing sarcoma dissemination to contrast the deliberate steps necessary in carcinoma metastasis.²⁴

The passive metastasis model predicts that Ewing sarcoma cells have weak intercellular adhesions and ample access to the bone marrow and circulatory system due to their bone-associated locations. This puts tumor cells in a prime position to enter the blood stream and colonize the secondary site, most frequently lung and bone. This is consistent with our data showing that reduction of ZEB2 increases epithelial features and cell-cell adhesion. Ewing’s tumors are then in a less passive state and therefore a decrease in metastatic potential is achieved. The fundamental ability of Ewing sarcoma cells to proliferate as well as metastasize may also contribute to the limited latency (72% of relapse occur within 2 years of diagnosis and 94% within 5 years) seen in Ewing sarcoma.⁵⁴ According to the EMT-MET metastasis model, colonization (the ability to form a macroscopic secondary lesion) is the limiting step in the multistep metastatic cascade. In this setting, circulating tumor cells are plentiful, and some are able to seed the secondary site; however, these cells are quiescent, marked by the absence of Ki-67 staining.^{55,56} A subsequent event, the proposed MET, is then necessary for these cells to reenter into the cell cycle. This has been used to explain the long latency often seen in breast cancer recurrence.^{57,58}

Genetic Type II Metastasis was proposed by Brabletz to explain undifferentiated carcinoma metastases.⁵⁹ In this model, poorly differentiated primary tumors that arise in primitive cell types give rise to undifferentiated metastases. These malignancies do not rely on EMT-MET for metastasis because their primitive state endows them with an intrinsic EMT phenotype. Other characteristics of cancers of this type include a high

Figure 5. Reduction of ZEB2 decreases metastatic potential in Ewing sarcoma cells. **(A)** Volume of the right tibia from mice 6 weeks postinjection with either 25K or 100K control or ZEB2 shRNA (as indicated) infected A673 cells. Horizontal bar indicates mean. **(B)** Pie charts showing the total number of mice with macroscopic metastatic lung lesions at the time of sacrifice (6 weeks postinjection). Combined data includes both 25K and 100K conditions. **(C)** Images of lungs (front and back) from the animal from each condition bearing the highest metastatic burden. Scale bar = 5 mm. **(D)** Graph showing the percentage of the lung containing macroscopic pulmonary lesions in each condition, as indicated. P-value determined by Wilcoxon Mann-Whitney test. Horizontal bar indicates mean. **(E)** Correlation of metastatic burden (y axis) and tumor size (x axis). Pearson's correlation coefficient for shControl = -0.2892 and shZEB2 = -0.4414. **(F)** Immunohistochemical staining for ZEB2 on lung metastases from indicated conditions. No primary antibody control is shown for control shRNA 25K sample's adjacent section. Scale bar = 250 μ m.

Figure 6. ZEB2 levels correlate with patient metastasis. **(A)** ZEB2 expression levels determined by immunofluorescence intensity read by the AQUA system in tumor samples from patients with and without metastasis. Horizontal bar indicates the mean. *P*-value was determined by Student's *t*-test. **(B)** Kaplan-Meier plot of overall patient survival. Patients were stratified based on ZEB2 expression levels—high ZEB2 represents values above the median and low ZEB2 represents values below the median.

propensity for metastasis at early stages of the disease, limited latency, and the presence of a genetic alteration that contributes to a fairly fixed cell state that is compatible with tumor growth and metastasis. Ewing sarcoma metastasis conforms to this model. Ewing sarcoma is thought to occur in a primitive cell type where the genetic alteration, EWS/FLI, prevents mesenchymal differentiation and ZEB2 prevents epithelial differentiation, as demonstrated here. Metastasis is a frequent and early event in Ewing sarcoma compatible with the idea that EMT is not needed. The presence of overt metastasis at presentation coupled with the limited latency seen in this disease also supports a model where MET is not a limiting factor in colonization of the secondary site.

The cell of origin for Ewing sarcoma is unknown, but it is thought to be an NCSC or MSC. We show that ZEB2 is expressed in both of these primitive cells. It should be noted that these 2 cell types may not be mutually exclusive. Experimental evidence has described the isolation of NCSCs from human embryonic stem cells that can be directed to differentiate along mesenchymal lineages in addition to neurons and Schwann cells.¹⁸ Studies show that experimental reduction of EWS/FLI causes Ewing sarcoma cells to resemble MSCs both in gene expression and differentiation capacity.²⁵ This coupled with evidence that ectopic expression of EWS/FLI in human MSCs leads to a Ewing sarcoma gene expression signature has supported MSCs as the cell of origin. However, hMSCs expressing EWS/FLI are not transformed.¹⁶ Ewing sarcomas have a relatively low frequency of mutations in known tumor suppressors and oncogenes, supporting the concept that EWS/FLI is the main oncogenic driver in this malignancy.^{60,61} If this is true, EWS/FLI alone should induce transformation if expressed in the correct cell of origin.

We propose an alternate, yet integrative, hypothesis for tumorigenesis in Ewing sarcoma: the EWS/FLI translocation occurs in a cell that is undergoing a normal developmental EMT. In this model, ZEB2 is expressed in a neural crest or neuroepithelia-derived progenitor cell to induce a developmental EMT. The EWS/FLI translocation can be acquired at various stages of this transition and blocks further mesenchymal differentiation, resulting in an undifferentiated Ewing sarcoma cell. Support for this model comes from the role of ZEB2 during development. ZEB2 is expressed in the developing neural crest and neural epithelium. The homozygous ZEB2 knock-out mouse is embryonic lethal due to defects in neural crest migration seen on embryonic day 8.5.⁶² This model is also consistent with the well-characterized ability of EWS/FLI to prevent mesenchymal differentiation,^{24,25,63} and may help reconcile the observation that Ewing sarcoma cell lines can only differentiate along mesenchymal lineages in the absence of EWS/FLI,²⁵ while MSCs, being further along the differentiation spectrum than the Ewing sarcoma cell of origin, retain this ability even when expressing EWS/FLI.¹⁶ Expression of EWS/FLI in human pediatric bone marrow derived MSCs⁶⁴ as well as neural crest derived MSCs¹⁴ results in repression of MSC genes while increasing expression of NCSC genes, which could reflect a de-differentiation, in line with our proposal that the cell of origin is a precursor to an MSC.

Strong evidence to support this concept also comes from the finding that neural crest cells and neuroepithelial cells are the initial source of MSCs from mouse embryonic stem cells *in vitro*.¹⁹ This group went on to show that *P0*⁺ neural crest cells and *Sox1*⁺ neuroepithelial cells gave rise to MSCs during normal mouse development. Interestingly, in lineage

Figure 7. Model for ZEB2 in Ewing sarcoma. ZEB2 is expressed in Ewing sarcoma cells and acts to repress epithelial differentiation. This works in conjunction with EWS/FLI, which represses mesenchymal differentiation, to keep Ewing sarcoma cells in an undifferentiated state. Ewing sarcoma cells retain access to features of both epithelial and mesenchymal cells, which positions them for successful growth and metastasis.

tracing studies, these neural crest and neuroepithelial derived MSCs were present at low abundance in bone cell preparations from femoral and tibial bones of neonates, 4-week-old and 12-week-old mice, and this population of cells decreased with age.¹⁹ This places these cells in the proper space and time to serve as the cell of origin for Ewing sarcoma. Of relevance to the present study, desmosomes are abundant in neuroepithelium,⁶⁵ and KRT8 is also expressed in early embryonic epithelial tissue.⁶⁶⁻⁶⁸

A mouse model for Ewing sarcoma has remained elusive, despite several attempts.⁶⁹⁻⁷¹ One possible reason for this is that EWS/FLI has not been expressed in the appropriate precursor cell. A prediction based on the model presented in this article is that perhaps expression of EWS/FLI driven by the *Zeb2* promoter in conjunction with the *Sox1* promoter would allow for relevant spatial and temporal expression of this translocation. Based on the mouse expression studies of ZEB2 and its role as an EMT-TF, ZEB2 expression would mark the EMT of a neural crest cell or neuroepithelial cell and thus provide a cellular intermediate to an NCSC and an MSC.

Here we show that Ewing sarcoma cells express high levels of the EMT-TF, ZEB2, and that this transcription factor represses epithelial gene expression and epithelial phenotypes in Ewing sarcoma cells. Reduced ZEB2 expression results in the re-expression of genes involved in epithelial differentiation and the formation of intercellular junctions concomitant with a decrease in cell migration *in vitro* and decreased metastatic potential *in vivo*. We have proposed a model for Ewing's sarcomagenesis whereby the EWS/FLI translocation occurs in a cell undergoing a ZEB2 induced developmental EMT. This leaves the cell "stuck" in between an epithelial differentiation state (prevented by ZEB2 expression) and a mesenchymal differentiation state (prevented by EWS/FLI expression), but with access to features of both cell types. This poorly

differentiated cancer cell is now poised for successful growth in the primary and secondary sites, owing to its epithelial features, while at the same time made competent for metastasis by its mesenchymal features (see model in Fig. 7). In the future it will be interesting to investigate the ability of Ewing sarcoma cells to "toggle" expression of EWS/FLI and ZEB2 depending on their stage in the metastatic process to accentuate either epithelial or mesenchymal traits, reminiscent of what has been proposed to achieve EMT-MET in carcinoma metastasis. This cellular plasticity may offer a therapeutic opportunity, if cells can be forced toward one lineage or the other depending on the stage of the disease.

Materials and Methods

Constructs. shRNA targeting EWS/FLI (EF2 shRNA) or control (control shRNA) targeting luciferase or ERG (which is not expressed in these cells) have been previously described (Smith *et al.*, 2006).⁷² shRNA was designed targeting the 3'UTR of ZEB2 (Suppl. Table S4) and cloned into pMKO.1 puro retroviral vector (Masutomi *et al.*, 2003).⁷³ ON-TARGETplus siRNA targeting the ORF of ZEB2 (siZEB2-5, siZEB2-6), SMARTpool targeting ZEB1 (siZEB1), and non-targeting siRNA (siControl) were purchased from Thermo Scientific (Waltham, MA). Lentiviral microRNA precursor constructs (miR-control, miR-200a,b,429) were purchased from System Biosciences (Mountain View, CA). These constructs were also modified by subcloning a CMV promoter and puromycin resistance cassette downstream of the miRNA precursor to allow for antibiotic selection.

Cell culture. Ewing sarcoma cell lines A673 (ATCC), SKNMC (ATCC), and TC71 (a gift from Timothy Triche, Children's Hospital Los Angeles) were grown as previously

described.⁷⁴ MG-63 osteosarcoma cell line (a gift from Timothy Triche, Children's Hospital Los Angeles) were used as a positive control for ZEB1 expression and grown in RPMI plus 10% FBS (fetal bovine serum) and 1% PSQ (penicillin-streptomycin-glutamine). For retroviral plasmids, control shRNA (LUC or ERG) and ZEB2 shRNA, cells were infected and selected in the appropriate antibiotic selection media resulting in a polyclonal population. For lentiviral vectors, miR-control and miR-200 family, cells were infected and selected with the appropriate antibiotic selection media or sorted for GFP+ cells using a FACS Aria (BD Biosciences, Franklin Lakes, NJ). Transfections with siRNA were preformed according to the manufacturer's instructions (Thermo Scientific) at final concentration of 25 nM. As a positive control for apoptosis, A673 cells were treated with DMSO or etoposide (Sigma-Aldrich, St Louis, MO) at the indicated concentrations for 24h.

Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). Total RNA was extracted with an RNAeasy kit (Qiagen, Venlo, The Netherlands). mRNA (30 ng) was quantitated by SYBR green (BIO-RAD, Hercules, CA) using one-step qRT-PCR with gene specific primers. Unpublished primers are listed in Supplementary Information (Suppl. Table S5). Messenger RNA was reverse-transcribed at 50°C for 10 minutes followed by a 5-minute denaturation at 95°C and then 45 cycles of PCR (95°C for 30 seconds, 57°C for 30 seconds, 72°C for 30 seconds). For miRNA quantification, total RNA was extracted using the mirVana miRNA isolation kit (Ambion, Austin, TX). RNA (10 ng) was reverse-transcribed using the Taqman Micro-RNA Reverse Transcription Kit and miRNA specific primers (Applied Biosystems, Foster City, CA) at 16°C for 30 minutes, 42°C for 30 minutes, followed by 5 minutes at 85°C in a BIO-RAD DNA engine Peltier thermal cycler. The reverse-transcribed DNA was then used for qPCR with Taqman small RNA assay miRNA specific probes (Applied Biosystems) with the following cycling parameters: 95°C 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Fold change was determined using the $\Delta\Delta C_t$ method comparing all samples to the control after normalizing to GAPDH (for mRNA) or snRNA U6 (for miRNA) or expression was determined as a percentage of GAPDH expression, as indicated. qPCR and qRT-PCR were performed using the BIO-RAD MyiQ single color real-time PCR detection system.

RNA sequencing. RNA from A673 cells transfected with siZEB2-5, siZEB2-6, or siControl 72 hours post-transfection was extracted with the RNAeasy kit (Qiagen) and treated with DNase. Two biological replicates per condition were used to construct libraries for high-throughput sequencing according to the manufacturer's instructions (Illumina, San Diego, CA) and sequenced on the Illumina Hi-Seq with 50 cycles of single end reads. Sequences were aligned to the human genome

build hg19. The RNA-seq data from this publication have been submitted to the US National Center for Biotechnology Information-Sequence Read Archive (NCBI-SRA) and assigned the identifier #SUB139166. Differential gene expression was determined using the publically available USeq package (useq.sourceforge.net). Significance parameters were set at an FDR of 10% and 2-fold change.

Comparison of RNA-seq with microarray data. Microarray data from Sartor *et al.* (2010)⁴³ (PMID 20007254, GEO accession GSE17708, platform hgu133plus2), Onder *et al.* (2008) (PMID 18483246, GEO accession GSE9691, platform ht_hgu133a), and Taube *et al.* (2010) (PMID 20713713, GEO accession GSE24202, platform ht_hgu133a) were downloaded as raw CEL files from GEO (www.ncbi.nlm.nih.gov/geo). Fluorescent intensities were extracted and normalized by RMA as implemented in the Bioconductor package affy⁴⁰ and cdf and annotation packages appropriate to their platform. Differential gene expression (DGE) among published conditions was determined using the Bioconductor package limma⁷⁵ and obtaining the log₂ fold change (log₂FC) from the top statistically significant differentially expressed genes. Genes from these sets were intersected with log₂FC values of corresponding genes from the ZEB2 knock-down RNA-seq. The only genes allowed from the ZEB2 knock-down RNA-seq were those having a *P*-value for DGE less than 0.05. Final sets were derived by further requiring that the absolute log₂FC DGE be greater than 1 for at least one of the conditions in the comparison. For the purposes of DGE comparison, heat maps were made from the results of standardizing column values followed by hierarchical clustering using Euclidean distance and complete linkage. Both row and column clustering were performed except in the case of Sartor *et al.* where the sample order follows a time course.

Migration assays. Boyden chambers were performed using cell inserts with 8- μ m pores (BD Biosciences). Fifty-thousand cells were seeded in serum-free media in the upper compartment of a fibronectin (1 μ g/mL) coated chamber, which was placed into a well containing media with 10% serum. Cells were allowed to migrate for 24 hours before fixation in methanol and staining with modified Giesma stain (1:10). Each condition was done in triplicate, and 5 fields from each chamber were imaged at 10 $\times$ using an Olympus IX81 microscope with CCD camera and DP Controller imaging software. Wound healing assays were performed by plating each condition in triplicate in 6-well plates and growing to ~90% confluence. Monolayer wound was created with a micropipette tip, washed 4 $\times$ with PBS, and grown in appropriate media containing 5% serum. Cells were washed twice with PBS and given fresh media before imaging. Phase-contrast images were taken at 0, 24, 48, 72, and 96 hours on a Zeiss Axiovert100 at 10 $\times$ magnification with a Q imaging MicroPublisher 5.0 RTV camera

using QCapture Pro7.0 imaging software. The area of the wound at each time point was measured using the magic wand tool in Adobe Photoshop.

Immunodetection. Western blots were performed using standard protocols with 4% to 15% gels, nitrocellulose membranes, and developed with home-made enhanced chemiluminescence (ECL). The following antibodies were used: DSPI+II (Abcam 71690), KRT8 (Abcam 9023), Tubulin (Calbiochem CP06), ZEB1 (Cell Signaling 3396), ZEB2 (Active Motif 61095), Caspase-7 (Cell Signaling 9492), and Cleaved PARP (Cell Signaling 9541).

Immunofluorescence. A673 cells were plated on fibronectin (10 µg/mL) coated coverslips 48 hours post-transfection. Twenty-four hours after plating, cells were fixed in 3.7% formaldehyde. Fixed cells were blocked and permeabilized with PBST-BSA (0.1% triton-x100 and 0.5% BSA). Cells were incubated for 1 hour at 37°C in a moist chamber with ZEB2 antibody (Active Motif 61095) (1:100), washed for 15 minutes in PBST-BSA, followed by incubation with AlexaFluor-568 secondary antibody (1:100), AlexaFluor-488 phalloidin (1:50) (Invitrogen), and DAPI (1 µg/mL) for 1 hour at 37°C in a moist chamber. Cells were washed and mounted in FluoromountG (Southern Biotech). Cell images were captured using a Zeiss Axioskop2 mot plus microscope with a 40× plan NA 0.75 NeoFluor objective, Zeiss AxioCam MR camera, and Zeiss Axiovision v4.8.1 software (Carl Zeiss MicroImaging, Inc.). The following exposure times were used for the displayed images: 405 nm (DAPI)—10 ms, 488 nm (Phalloidin)—400 ms, 568 nm (ZEB2)—600 ms.

Tibial injection metastasis model. All animal studies were performed in accordance with protocols approved by the University of Utah Institutional Animal Care and Use Committee. Male NOD-SCID mice (kindly provided by Alana Welm) were 6 to 7 weeks old at the time of injection. A673 cells were retrovirally infected with either a control shRNA or ZEB2 shRNA and selected in puromycin. Cells were counted and resuspended in growth factor reduced Matrigel matrix (BD Biosciences). After boring a hole in the tibia with a 26-gauge needle, 10 µL of Matrigel containing 25K or 100K cells were injected into the right tibia using a glass Hamilton syringe. Tibial measurements using calipers were performed weekly to follow the growth of the tumors. Mice were sacrificed at 6 weeks and the lungs were harvested to evaluate for metastases. Mice that were sacrificed before the 6 week endpoint of the study due to a primary tumor size >2 cm in one direction and mice with no detectable primary tumor were excluded from the study.

Tissue processing and immunohistochemistry. The lungs were washed in PBS and stored in 10% formalin immediately after harvesting. Samples were fixed in 10% formalin at 4°C for 24 hours. Lungs were then washed 3× with PBS and

stored in 70% ethanol at 4°C. Whole lung images were acquired using an Olympus MVX10 dissecting microscope at 0.63×, Spot insight Firewire 4 camera, and Spot Alias imaging software. Area of whole lung and metastasis (front and back) was measured using ImageJ software. Fixed lungs were embedded in paraffin blocks and sectioned into 5-µm sections on glass slides. Antigen retrieval was performed using 10 mM sodium citrate. Mouse on mouse elite peroxidase kit and DAB peroxidase substrate kit (Vector Labs) with ZEB2 antibody (Active Motif 61095) were used for immunohistochemistry. Sections were imaged using a Zeiss Axiovert100 at 10× magnification with a Q imaging MicroPublisher 5.0 RTV camera using QCapture Pro7.0 imaging software.

Tissue microarray. Analysis was performed following the institutional review board approval. Fifty-four cases of morphologically confirmed, pretreatment, primary Ewing sarcoma tumor samples (from the University of Michigan Medical Center Department of Pathology) included on a tissue microarray (TMA) were analyzed by double immunofluorescence staining for both CD99 (which marks Ewing sarcoma tumor cells; Abcam, Ab-27271, rabbit polyclonal antibody, 1:100) and ZEB2 (Active Motif 61095, mouse antibody, 1:400). The AQUA system (HistoRx) was used for the automated image acquisition and analysis as previously described (Luo *et al.*, 2012).⁷⁶

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Declaration of Conflicting Interests

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