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ZEB1: At the crossroads of epithelial-mesenchymal transition, metastasis and therapy resistance

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Zinc finger E-box binding homeobox 1 (ZEB1) is a transcription factor that promotes tumor invasion and metastasis by inducing epithelial-mesenchymal transition (EMT) in carcinoma cells. EMT not only plays an important role in embryonic development and malignant progression, but is also implicated in cancer therapy resistance. It has been hypothesized that carcinoma cells that have undergone EMT acquire cancer stem cell properties including self-renewal, chemoresistance and radioresistance. However, our recent data indicate that ZEB1 regulates radioresistance in breast cancer cells through an EMT-independent mechanism. In this Perspective, we review different mechanisms by which ZEB1 regulates tumor progression and treatment resistance. Based on studies by us and others, we propose that it is specific EMT inducers like ZEB1, but not the epithelial or mesenchymal state itself, that dictate cancer stem cell properties.

Keywords: epithelial-mesenchymal transition, drug resistance, metastasis, radioresistance, ZEB1

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Introduction

Epithelial-mesenchymal transition (EMT), originally observed during embryogenesis,[1] plays important roles in early developmental processes, including neural crest development, heart valve development, mesoderm formation and secondary palate formation.[2-4] It has been recognized that the EMT program can be resurrected in adult tissues under several disease conditions, including wound healing, fibrosis and cancer.[2-4] EMT and its reverse process, mesenchymal-epithelial transition (MET), are involved in different stages of metastasis. The induction of EMT in epithelial cancer cells promotes migration, invasion and dissemination, whereas MET facilitates metastatic colonization of distant sites by disseminated tumor cells.[5-7] Recently, mesenchymal-like tumor cells generated by EMT have been shown to exhibit characteristics of cancer stem cells, including self-renewal, radioresistance and drug resistance.[8-12] Thus, research on EMT and MET will not only advance our understanding of tumor progression, but also shed light on improving cancer treatment.

Over the past decade, extensive studies have been performed to investigate the role and regulation of EMT and MET in tumor progression.[5-7,13] A growing list of EMT regulators has been identified, including extracellular factors (such as TGF-β, HGF, FGF, IGF and Notch ligands), transcription factors (such as Twist, Snail, Slug and ZEB1), microRNAs (such as the miR-200 family, miR-205 and miR-9) and microenvironment.[5,13-15] Although the association between EMT and cancer stem cell properties has been recognized by many recent studies, new evidence suggests that EMT-independent mechanisms exist and that all EMT inducers are not equal.[16,17] For instance, while the transcription factor ZEB1 can promote migration and invasion by inducing EMT, this protein enhances tumor radioresistance independent of EMT.[16] In this article, we review EMT-dependent and EMT-independent mechanisms by which ZEB1 regulates development, tumor progression and therapy resistance.

ZEB1 Protein and Its Physiological Functions

Expression of ZEB1 (also named TCF8 or DeltaEF1) is regulated by multiple signaling pathways, such as WNT,[18] NF-κB,[19] TGF-β,[20] COX2,[21] HIF signaling[22] and microRNAs.[23-25] ZEB1 belongs to the ZEB family of transcription factors characterized by the presence of 2 zinc finger clusters, which are responsible for DNA binding, and a centrally located homeodomain. In addition, ZEB1 contains other protein binding domains including the Smad interaction domain (SID), CtBP interaction domain (CID) and p300-P/CAF binding domain (CBD)[26-29] (**Fig. 1A**). Through zinc finger clusters, ZEB1 can bind to specific DNA sequences named E-boxes.[28] By recruiting co-suppressors or co-activators through CID, SID or CBD, ZEB1 can either downregulate or upregulate the expression of its target genes.[27,30] For instance, ZEB1 directly binds to the E-box located in the promoter of *CDH1,* the gene encoding E-cadherin,[30] and recruits the CtBP transcriptional co-repressors[31] and/or the SWI/SNF chromatin-remodeling protein BRG1,[32] leading to repression of *CDH1* transcription and induction of EMT. On the other hand, by recruiting p300-P/CAF and Smad proteins, ZEB1 can activate the transcription of TGF-β-responsive genes and promote osteoblastic differentiation.[26,27] In MDA-MB-231 human breast cancer cells, knockdown of ZEB1 resulted in upregulation of ~200 genes and downregulation of ~30 genes, most of which are determinants of epithelial differentiation and cell-cell adhesion.

When Zeb1 was first identified as a repressor of δ1-crystallin enhancer in the chicken embryo in early 1990s, it was thought to be involved in embryogenesis because Zeb1 is specifically expressed in mesodermal tissues, the nerve system and the lens in the chicken embryo.[34] Later, mouse models with *Zeb1* deletion revealed the role of Zeb1 in mammalian development. While heterozygous *Zeb1* mouse mutants are viable and fertile, *Zeb1* null mice die perinatally exhibiting respiratory failure, severe T cell deficiency of the thymus, and various skeletal defects including craniofacial abnormalities, limb and sternum defects, and malformed ribs.[35] Interestingly, these developmental defects are associated with a mesenchymal-epithelial transition, as evidenced by re-expression of E-cadherin and loss of vimentin in several embryonic tissues (including the palate and nasal mesenchyme, the perichondrial region of forming cartilage, the embryonic eye and the ventricular zone of the brain) and in embryonic fibroblasts.[36] This finding underscores the critical role of Zeb1 in developmental EMT. In addition, either heterozygous or homozygous inactivation of *Zeb1* in mouse embryos results in corneal defects, which mimics human posterior polymorphous corneal dystrophy.[37] In humans, *ZEB1* mRNA expression levels vary significantly among different adult tissues, from barely detectable expression in the pancreas and liver, to moderate expression in the mammary gland and ovary, and to high expression in the bladder and uterus.[38] However, the function of ZEB1 in adult tissues is still largely unknown. Therefore, mouse models with conditional *Zeb1* deletion or overexpression will not only shed light on the physiological functions of Zeb1 in adult tissues, but also provide tools to investigate the role of Zeb1 in cancer and other diseases.

ZEB1 in Tumor Progression and Metastasis

E-cadherin, a major cell-cell adhesion molecule, has long been recognized as a tumor suppressor protein.[39] Loss of E-cadherin leads to tumor cell dissociation and enhanced ability to migrate, invade and metastasize, which is associated with poor prognosis in human cancer patients.[5,40-42] Due to the pivotal role of ZEB1 in the downregulation of E-cadherin, ZEB1 acts as a driver of EMT and cancer progression.[16,28,33,34,44] Aberrant expression of ZEB1 has been observed in many human cancers, such as uterine cancer,[45] pancreatic cancer,[29,46] osteosarcoma,[47] lung cancer,[48,49] liver cancer,[50] gastric cancer,[51,52] colon cancer[53] and breast cancer.[30] In these tumors, ZEB1 expression correlates with loss of E-cadherin and is associated with advanced diseases or metastasis, which indicates the relevance of ZEB1 induction of EMT and tumor progression in clinical cancers. This notion is further supported by functional studies using human cancer cell lines, in which overexpression of ZEB1 can induce EMT and promote metastasis in multiple cell lines, such as breast cancer,[30] colon cancer[43] and lung cancer[54] cell lines.

In addition to repressing E-cadherin expression, ZEB1 also regulates other target genes involved in tumor progression (**Fig. 1B**). For example, ZEB1 binds to the promoters of epithelial polarity genes including Crumbs3, HUGL2 and PATJ (Pals1-associated tight junction) and represses their transcription, leading to reduced adhesion and increased invasiveness of breast cancer cells.[33] In colorectal cancer cells, ZEB1 promotes metastasis and loss of cell polarity by repressing the expression of Lgl2 (lethal giant larvae homolog 2),[43] a cell polarity factor. Moreover, ZEB1 enhances the tumorigenicity of pancreatic cancer cells by inhibiting the expression of stemness-repressing microRNAs, including miR-203 and the miR-200 family.[55] It is not clear, however, what relationship these various ZEB1 target genes have: synergistic, additive or independent?

ZEB1 in Cancer Therapy Resistance

Cancer therapeutic resistance, including radioresistance and drug resistance, is a major challenge in cancer research and treatment.[9,56] Drug resistance has become a key obstacle in developing targeted therapies and limited the efficacy of these therapies in treating cancer patients.[56-58] Cancer stem cells, a small subpopulation of cancer cells with tumor-seeding and self-renewing ability, have been found to contribute to therapy resistance in different cancer types through preferential activation of the DNA damage checkpoint response and repair, thereby protecting cells from DNA damage, cleaning reactive oxygen species (ROS) and activating the cell survival signaling pathways.[9,56,59] Since tumor cells that have gone EMT can acquire cancer stem cell properties,[8] the EMT program has been implicated in

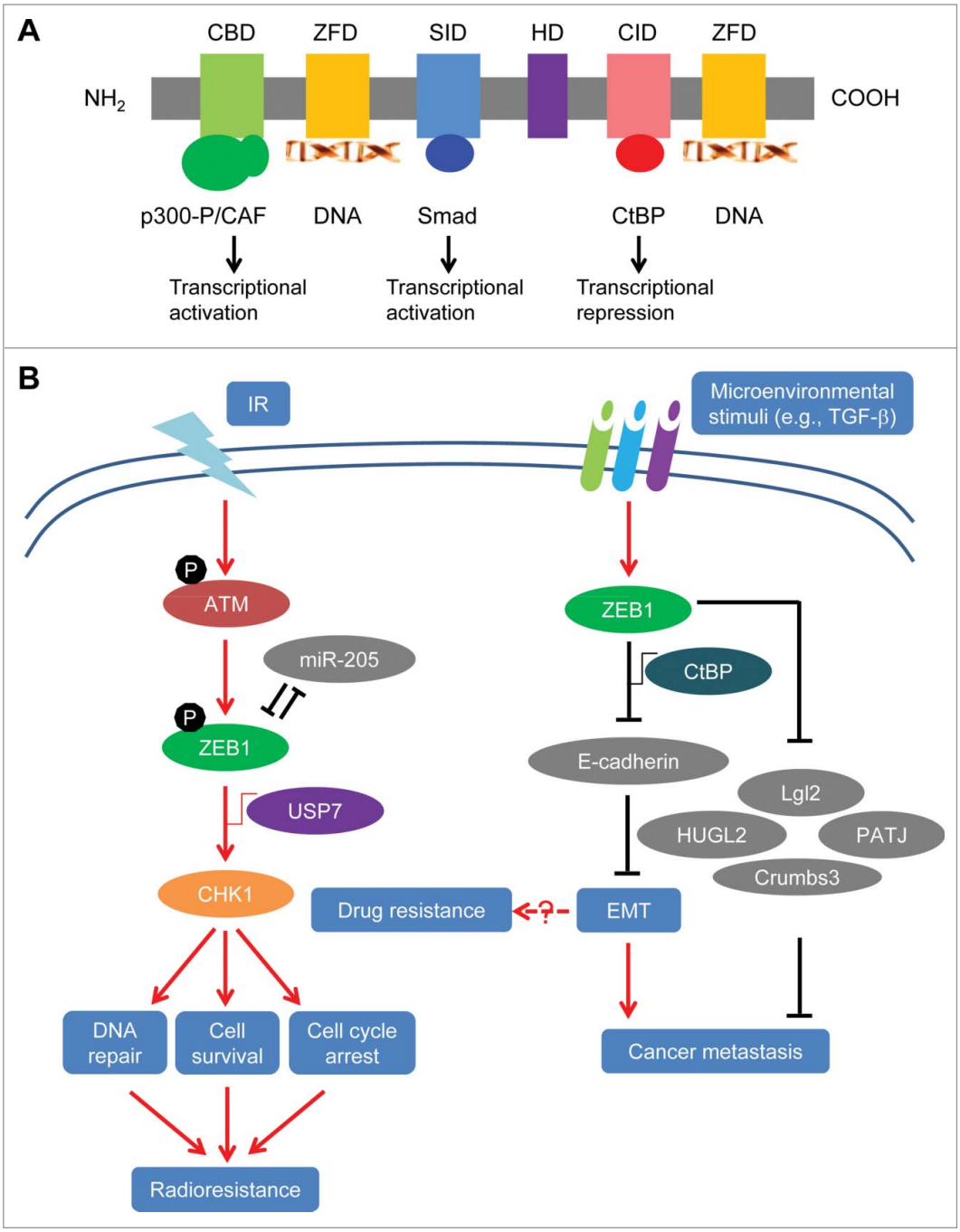

Figure 1. ZEB1 protein domains and mechanisms of action. (**A**) Schematic representation of ZEB1 protein. CBD: coactivator p300 and P/CAF binding domain; ZFD: zinc finger domain; SID: Smad interaction domain; HD: homeodomain; CID: CtBP interaction domain. (**B**) The working model of regulation of EMT, metastasis and therapy resistance by ZEB1. (i) Upon ionizing radiation (IR), ZEB1 is phosphorylated and stabilized by activated ATM kinase, and ZEB1 in turn recruits USP7 which deubiquitinates and stabilizes CHK1, leading to induction of DNA repair, cell survival and radioresistance. In parallel, ZEB1 represses its own negative regulator, miR-205. (ii) Microenvironmental stimuli such as TGF-β upregulate ZEB1 expression, and ZEB1 can promote cancer metastasis by repressing E-cadherin expression and inducing EMT, or by repressing other target genes (such as Lgl2, PATJ, HUGL2 and Crumbs3). In addition, ZEB1 can promote drug resistance through EMT-dependent and EMT-independent mechanisms. Gray color indicates ZEB1's transcriptional targets.

therapy resistance in cancer, including radioresistance, chemoresistance, HER2, EGFR, endocrine and other targeted therapy resistance.[11,12,44,60-64] A provocative study by Weinberg and colleagues demonstrated that basal, but not luminal breast cancer cells can readily convert from non-cancer stem cell state to cancer stem cell state in response to microenvironmental stimuli such as TGF-β; such conversion is driven by activated expression of ZEB1.[65] Interestingly, ZEB1 overexpression is

frequently observed in mesenchymal-like carcinoma cells. These findings underscore the importance of elucidating the functional role that ZEB1 plays in the acquisition of cancer stem cell properties. Moreover, what is the mechanism by which these mesenchymal-like carcinoma cells acquire treatment resistance: EMT-dependent or EMT-independent?

ZEB1 regulates radioresistance through an ATM−ZEB1−CHK1 signaling axis

Recently, we and others reported that ZEB1 plays a critical role in regulating tumor radiosensitivity.[16,66] Knockdown of ZEB1 in SUM159-P2 (a radioresistant subline of the SUM159 breast cancer cells derived from ionizing radiation), U2OS (osteosarcoma), H460 (non-small cell lung cancer) and H1299 (non-small cell lung cancer) cell lines increased radiosensitivity, while ectopic expression of ZEB1 in radiosensitive mammary epithelial cell lines, HMLE and MCF7, led to elevated radioresistance.[16,66] Since ZEB1 is a well-established EMT-inducing transcription factor, we were led to question whether ZEB1 regulates radiosensitivity through EMT. First, we overexpressed Snail, Twist or ZEB1 in HMLE cells. Each of these transcription factors induced EMT and increased radioresistance, and in each case, expression of Snail, Twist and ZEB1 was upregulated, regardless of which factor was used to induce EMT.[16] These findings demonstrate an association between EMT and radioresistance, but do not reveal whether it is EMT itself or a specific EMT inducer that causes radioresistance. When we silenced each of the 3 transcription factors in HMLE cells overexpressing Snail, Twist or ZEB1, it did not cause reversal of EMT. Notably, only knockdown of ZEB1 increased radiosensitivity.[16] We also overexpressed these 3 transcription factors in the MCF7 human breast cancer cell line, and none of them induced EMT due to the expression of intact p53 protein, a barrier to EMT induction. Interestingly, only ZEB1, but not Snail or Twist, conferred radioresistance on MCF7 cells even without inducing EMT.[16] These results suggest that it is a specific EMT regulator, ZEB1, but not EMT itself, that induces radioresistance.

Mechanistically, ZEB1 regulates radiosensitivity in cancer cells by promoting homologous recombination (HR)-mediated DNA damage repair and the clearance of DNA breaks (**Fig. 1B**).[16] CHK1 (but not CHK2) mediates, at least in part, the effect of ZEB1 on DNA damage repair and radioresistance. Interestingly, ZEB1 regulates CHK1 levels by interacting with the deubiquitinase USP7. This interaction promotes USP7's ability to deubiquitinate and stabilize CHK1 protein without affecting *CHK1* gene transcription.[16]

Upon ionizing radiation, ZEB1, but not Twist and Snail, is upregulated in SUM159 cells.[16] Radiation causes DNA damage which leads to activation of ATM and ATR kinases. Activated ATM, but not ATR, phosphorylates ZEB1 at serine 585 and stabilizes ZEB1, resulting in elevated ZEB1 protein levels.[16] How ATM-mediated phosphorylation of ZEB1 leads to ZEB1 stabilization remains to be determined. Similarly, others also reported that radiation causes upregulation of ZEB1 in the A549 lung cancer cell line[67] and in the CNE1 and CNE2 nasopharyngeal cancer cell lines.[68] Taken together, these findings by us and others suggest that radiation treatment can give rise to increased ZEB1 levels and therapy-induced radioresistance. Cancer cells with treatment resistance including radioresistance are most likely to be the source of local and distant recurrence. Indeed, in a cohort of 286 human breast cancer patients (87% of them received radiotherapy), patients with high ZEB1 expression in their mammary tumors had much worse distant relapse-free survival compared with those with low ZEB1 expression.[16]

Since silencing ZEB1 expression in cancer cells leads to increased tumor radiosensitivity *in vitro* and *in vivo*,[16,66] microRNAs targeting ZEB1, such as miR-205 and the miR-200 family, have the potential to be used as radiosensitizers in cancer treatment. Recently, we found that miR-205 can radiosensitize SUM159-P2 (breast cancer), MDA-MB-231 (breast cancer), A549 (lung cancer) and U2OS (osteosarcoma) cells by targeting ZEB1 and Ubc13, leading to inhibition of DNA damage repair; we also demonstrated the therapeutic utility of nanoparticle-encapsulated miR-205 mimics as a tumor radiosensitizer in a preclinical model.[69] Similarly, another group reported that therapeutic delivery of a miR-200 family member, miR-200c, can sensitize lung cancer cells to radiation in xenograft models.[66] Therefore, ZEB1-targeting agents like miR-205 and miR-200c microRNAs represent a new class of radiosensitizers.

ZEB1 regulates drug resistance: EMT-dependent or EMT-independent?

Over the past few years, emerging evidence has implicated ZEB1 in drug resistance in multiple cancers, but it remains unclear whether ZEB1-induced drug resistance is EMT-dependent or EMT-independent. In glioblastoma (GBM), ZEB1 is preferentially expressed at the invasion front of xenografts generated by cell lines derived from primary GBM specimens. Silencing ZEB1 expression in these cell lines reduced both invasion and the resistance to temozolomide (TMZ), a standard of care chemotherapeutic drug for glioblastoma.[63] Mechanistically, ZEB1 regulates the expression of MGMT, a gene promoting TMZ resistance, through a ZEB1−miR-200c−c-MYB loop.[63] In pancreatic cells, the EMT status (gauged by E-cadherin and vimentin expression levels) and the expression level of ZEB1 correlate with the resistance to chemotherapeutic agents including gemcitabine, 5-fluorouracil (5-FU) and cisplatin.[61] Interestingly, knockdown of ZEB1 in mesenchymal-like pancreatic cancer cell lines not only reversed the EMT phenotype, but also sensitized these cells to gemcitabine, 5-FU and cisplatin treatment.[61] Similarly, ZEB1, but not other EMT-inducing transcription factors including Snail, Slug and Twist, is upregulated in docetaxel-resistant human lung adenocarcinoma cells.[44] Ectopic expression of ZEB1 conferred docetaxel resistance on the SPC-A1 human lung adenocarcinoma cell line, while silencing ZEB1 in SPC-A1/DTX cells, which are mesenchymal-like and docetaxel-resistant cells, reversed EMT and chemoresistance.[44] These studies led the authors to conclude that ZEB1-induced EMT contributes to drug resistance. However, an alternative interpretation is that ZEB1 induces EMT and treatment resistance separately. Despite

the association, it may not be the EMT status itself that dictates drug sensitivity.

Additional studies provided further insight into ZEB1-induced drug resistance. In a Kras/p53-mutant lung adenocarcinoma mouse model, high expression of ZEB1 drives metastasis which can be suppressed by a PI3K inhibitor.[70] Mechanistically, ZEB1 activates PI3K by derepressing miR-200 and miR-183 targets, including amphiregulin (AREG), betacellulin (BTC), the transcription factor GATA6 and friend of GATA 2 (FOG2).[70] FOG2 mediates ZEB1-induced expression of the p110α catalytic subunit of PI3K, but surprisingly, FOG2 is not required for ZEB1-induced EMT,[70] suggesting that ZEB1-mediated activation of PI3K and sensitization to the PI3K inhibitor is independent of EMT. On the other hand, knockdown of ZEB1 in erlotinib (an EGFR inhibitor)-resistant head and neck squamous cell carcinoma cell lines (1386LN and UMSCC1) increased erlotinib sensitivity in an E-cadherin-dependent manner,[64] indicating that ZEB1's effect on erlotinib resistance may be EMT-dependent.

Conclusions

As a driver of EMT, ZEB1 plays an important role in tumor progression and metastasis and correlates with poor clinical outcomes in cancer patients. In addition, ZEB1 is involved in therapy resistance in multiple cancers through both EMT-dependent and EMT-independent mechanisms. We propose that ZEB1 employs multiple different mechanisms to regulate therapy resistance, depending on the specific cancer type and treatment type. This can be mediated by distinct transcriptional targets and different interacting proteins of ZEB1. Therefore, the underlying mechanisms of therapy resistance should be carefully examined in individual situations. Despite the apparent association between EMT and therapy resistance, we propose that it is not the epithelial or mesenchymal state itself that dictates cancer stem properties such as radioresistance and drug resistance; instead, it depends on the functions and mechanisms of action of specific EMT regulators. All EMT inducers are not equal.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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