METASTASIS

A Splicing Twist on Metastasis

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An oncogenic splice variant of the transcription factor KLF6 is associated with metastasis and poor survival in node-negative breast cancer patients and promotes the epithelial-to-mesenchymal transition by regulating Twist.

TREATING LYMPH NODE–NEGATIVE BREAST CANCER PATIENTS

Early detection of cancer dramatically improves patient outcome. For example, a breast cancer patient with lymph node involvement has a 10-year risk of recurrence approaching 70%; however, if that same patient has no lymph node involvement, their risk of recurrence drops to nearly 15% (*1*). Systemic adjuvant chemotherapy is associated with high toxicities. Therefore, the recommended therapeutic course may vary depending on the relative risk of these different patient populations. Tools such as the epidemiological database *Adjuvant! Online* and the gene expression assay OncotypeDX™ aim to guide this decision-making process through improved risk assessment (*2*). Nonetheless, there remains a high degree of uncertainty in how to treat the lower-risk patient population, and there is a clear need for additional prognostic indicators to identify those lymph node–negative (LNN) patients who will derive benefit from chemotherapy. Now, Hatami *et al.* (*3*) have identified Krüppel-like factor 6 splice variant 1 (KLF6-SV1) as a prognostic for poor survival in annotated clinical samples from 671 LNN breast cancer patients. This work not only provides insight into the biology of breast cancer metastasis but also has important translational potential for more precise risk assessment in LNN patients.

EMT IN METASTASIS

The epithelial-to-mesenchymal transition (EMT)—the process by which epithelial cells revert to their differentiated phenotypes and acquire a mesenchymal character (*4*)—is not only critical for embryonic development but also now understood to be involved in cancer progression. EMT provides a mechanistic explanation for cancer invasion and dissemination to distant sites. When cells undergo EMT, they lose cell-cell attachments, become more invasive, and are increasingly resistant to apoptotic stimuli. Furthermore, these cells are also considered to possess more stemlike characteristics (*4*). Through these phenotypic changes, EMT is thought to drive metastatic spread and the development of therapeutic resistance (*4*). Many diverse signals, often of a stromal origin, integrate to induce the activity of a core group of EMT-inducing transcription factors, including Snail, Slug, Zeb 1 and 2, and Twist. Indeed, Twist has been shown to be critical for EMT-induced metastasis in experimental models of breast cancer.

KRÜPPEL-LIKE FACTOR 6 IN CANCER

Krüppel-like zinc finger transcription factors (KLFs) are a large family of evolutionarily conserved proteins that have pleiotropic roles related to cell proliferation, signaling, and differentiation (*5*). KLF6 has been shown to have tumor-suppressive abilities through its direct transcriptional targets epithelial (E)–cadherin and p21, as well as the capacity to induce apoptosis. Supporting its role as a tumor suppressor, KLF6 is often functionally suppressed in cancer through somatic mutation and loss of heterozygosity, or through alternative splicing (*5*). Intriguingly, the splice variant KLF6-SV1 has been shown to have oncogenic properties. It is not uncommon for splice variants to have such antagonistic functions, and misregulation of alternative splicing is thought to play an important role in oncogenesis (*6*).

It is not fully understood exactly how alternative splicing of the KLF6 gene is regulated during breast cancer progression, but recent findings in other cancer types provide important clues. For example, a germline DNA polymorphism in the KLF6 gene created a functional binding site for the serine/arginine–rich splicing factor 5 (SRSF5), which resulted in both production of KLF6-SV1 and an increased risk of prostate cancer (*5*). In another example, hepatocyte growth factor has been implicated in KLF6 alternative splicing through Akt-mediated de-repression of the splicing factor SRSF1 (*7*). SRSF1 is implicated in numerous examples of protumorigenic alternative splicing and is considered to be a proto-oncogene (*6*). Indeed, SRSF1 is frequently up-regulated in breast cancer and has demonstrated roles in the transformation of mammary epithelium.

The KLF6-SV1 protein is structurally distinct from its wild-type (WT) KLF6 counterpart. The splice variant lacks the three zinc finger DNA binding domains, contains a novel C-terminal region, and does not localize to the nucleus in the manner of the WT protein (Fig. 1). KLF6-SV1 has been shown to directly antagonize the function of WT KLF6 by binding the transcription factor and targeting it for proteasome-mediated degradation (*8*). Additionally, KLF6-SV1 has de novo functions independent of WT KLF6. The splice variant can directly bind the proapoptotic protein NOXA and target it for degradation, which results in decreased sensitivity to platinum-based agents in preclinical models of ovarian cancer (*9*). However, our understanding of the functional relationship between KLF6-SV1 and the WT protein remains incomplete. It is insufficiently clear the extent to which KL6-SV1 exerts its function through direct antagonism of WT KLF6 or as an example of de novo functionality, and this remains an open question within the field.

KLF6-SV1 AND BREAST CANCER METASTASIS

Hatami *et al.* now provide a potential mechanistic explanation for the enhanced metastatic potential in tumors that express high levels of KLF6-SV1. KLF6-SV1 mRNA levels positively correlated with multiple EMT markers in 294 primary breast tumors from LNN patients subjected to microarray gene expression analysis (*3*). Interestingly, this association with EMT markers was greatest in estrogen receptor (ER)–positive tumors. Most notably, the EMT-inducing transcription factor TWIST1 significantly associated with mRNA levels of KLF6-SV1. The authors then validated this positive correlation in the entire 671 LNN sample set. To determine the functional relevance of the correlation between KLF6-SV1 and EMT, the authors ectopically expressed the splice variant in two nontumorigenic mammary epithelial cell lines and one tumorigenic line with an epithelial phenotype. In each cell line, expression of KLF6-SV1 caused the cells to gain expression of the EMT marker, neural (N)–

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cadherin, and display increased migratory and invasive characteristics. Furthermore, ectopic expression significantly increased colony formation in the nontumorigenic lines. KLF6-SV1 caused three-dimensional acinar structures to adopt an abnormal morphology with disrupted basolateral polarity as indicated by integrin α-6 staining. Examination of cleaved caspase 3 levels within the acini revealed a significant reduction in apoptosis. Taken together, these data clearly implicate KLF6-SV1 as an important regulatory component of the EMT program. In a reciprocal series of experiments, small interfering RNA (siRNA) knockdown in a cell line expressing high levels of KLF6-SV1 (MDA-MB-231) reverted the cells to a more epithelial phenotype with increased expression of E-cadherin and decreased migratory and invasive capability. Thus, sustained expression of KLF6-SV1 is critical to the maintenance of a mesenchymal phenotype in this cell line.

To understand the role that KLF6-SV1–mediated EMT might play in promoting metastasis of the primary tumor, the authors used the human cancer cell line BT474, which expresses low levels of the KLF6-SV1 splice variant and is poorly metastatic in a subcutaneous xenograft mouse model. KLF6-SV1 was expressed ectopically in these cells, which resulted in subsequent widespread metastasis in 100% of implanted animals as compared with 0% in animals implanted with the vector control cell line. Increased tumor burden did not account for the enhanced metastasis, as no difference was observed in "primary" tumor burden. In a more relevant orthotopic model of spontaneous metastasis, MDA-MB-231 cells were implanted in the mammary fat pad of immunodeficient mice. Overexpression of KLF6-SV1 also significantly enhanced the capacity of these tumors to metastasize to the lung, demonstrating a linear relationship between expression level and metastatic phenotype.

STIMULATORY SIGNALS FROM THE MICROENVIRONMENT

The authors report that KLF6-SV1 expression was greatest in those LNN tumors with high stromal involvement (*3*). These data raise the intriguing possibility of a role for KLF6-SV1 in heterotypic signaling within the tumor microenvironment. KLF6-SV1 may be up-regulated in response to microenvironmental cues, or alternatively, the splice variant could have a functional role in the recruitment of stromal cells and their subsequent education. There is the additional possibility that KLF6-SV1 is expressed in cancer-associated fibroblasts and infiltrating lymphocytes and may contribute to their tumor-promoting functions. It remains unclear to what extent cancer cells within the tumor mass exhibit phenotypic heterogeneity with regards to the expression of KLF6-SV1.

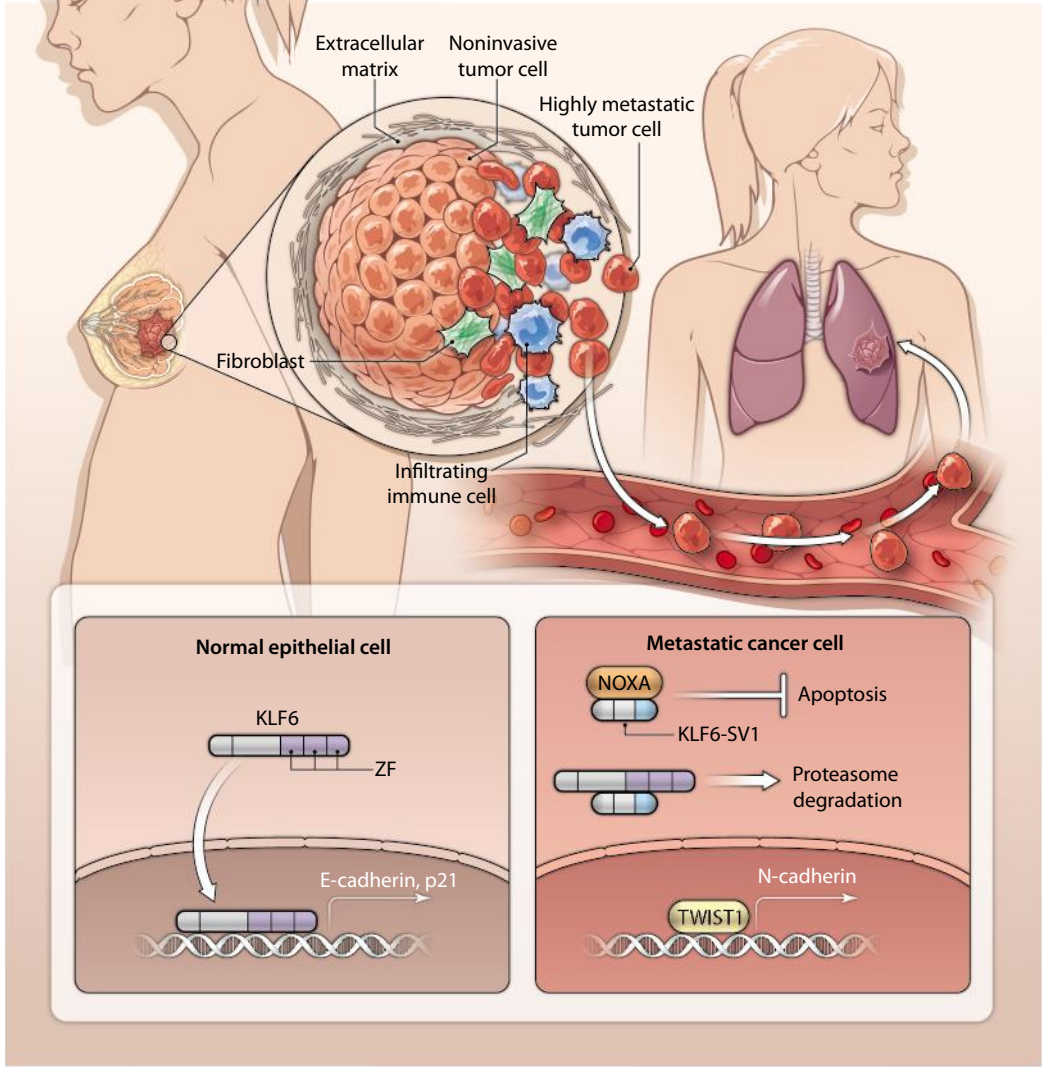

Fig. 1. Model of KLF6-SV1–driven metastasis. Breast cancer metastasis is a multistep process by which invasive cancer cells in the primary tumor intravasate into the vasculature, travel through the circulation, and extravasate at a distant organ site and establish metastases. In a normal epithelial cell, WT KLF6, which contains three zinc factor (ZF) DNA binding domains, suppresses tumorigenesis through direct regulation of E-cadherin and p21. The alternative splicing product KLF6-SV1, which lacks the ZF domains and contains a novel C-terminal region (blue), directly antagonizes WT KLF6, as well as the apoptotic protein NOXA, by targeting them for proteasome-mediated degradation. Additionally, KLF6-SV1 drives EMT through increased expression of TWIST1, causing breast cancer cells to transition from E-cadherin to N-cadherin expression and adopt a highly metastatic phenotype. High expression of KLF6-SV1 in the primary tumors of LNN breast cancer patients is prognostic for a high risk of metastasis and poor survival.

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Because of its demonstrated role in breast cancer cell EMT, one might hypothesize increased expression near the invasive edge of the tumor. Additionally, expression of the splice variant might identify the regions within a heterogeneous primary tumor that will give rise to metastases, allowing for more precise sampling and molecular characterization. Answering these questions with regards to KLF6-SV1 and the microenvironmental and cellular heterogeneity represents an avenue of further research with exciting translational potential.

KLF6 has also been found to play an important role in transforming growth factor–β (TGF-β)–induced EMT in the proximal renal tubule cells. Hatami and colleagues show that KLF6-SV1 is a substantial mediator of EMT in breast cancer, and through siRNA knockdown experiments, they demonstrate that this function is dependent on Twist (*4*). It is surprising that these two variants of the same protein, which are generally thought to have antagonistic functions, both play a role in promoting the same biological process. Understanding which cell context–specific factors dictate functionality for KLF6 and its splice variants will be an important advance in the field. Regardless, the multiple examples of this gene acting as a regulator of EMT strongly argue for further research into its mechanistic role in this important biological process.

KLF6-SV1 AS A POTENTIAL THERAPEUTIC TARGET

In addition to the prognostic and biological findings, this work also demonstrates the potential of KLF6-SV1 as a therapeutic target in the adjuvant setting. The authors suggest that with the advancement of RNA interference therapeutic technologies, or through the identification of small molecules capable of enhancing degradation, the splice variant could be targeted directly. Alternatively, splice-switching oligonucleotides can be particularly effective in targeting pathogenic splice variants (*10*). This approach has been used in Duchenne muscular dystrophy, where splicing reversion is able to restore some WT function, while removing the pathogenic form of the dystrophin protein (*10*). Considering the purported tumor-suppressive role of WT KLF6, targeting the splicing process itself might thus have a doubly beneficial outcome. Furthermore, if KLF6-SV1 can be placed in a larger program of protumorigenic alternative splicing in breast cancer, targeting the upstream splicing regulators might represent an ideal therapeutic option by allowing for the reversion of multiple tumor-promoting genes. Last, compounds have been developed to inhibit CDC2-like kinases (CLK1/CLK4), which phosphorylate and activate SRSF1 and other SRSF proteins, and these may have utility in suppressing the alternative splice variants of KLF6.

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