CHAPTER FOUR

Pleiotropic Roles of AEG-1/MTDH/LYRIC in Breast Cancer

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Abstract

Since the initial discovery of AEG-1/MTDH/LYRIC, our appreciation for this novel protein's involvement in cancer has increased dramatically over the past few years. *AEG-1/MTDH/LYRIC* is a key functional target of the 8q22 genomic gain that is frequently observed in poor-prognosis breast cancer, where it plays a dual role in promoting chemoresistance and metastasis. Beyond this, growing evidence from clinical research indicates a strong correlation between AEG-1/MTDH/LYRIC expression and the pathogenesis of a large spectrum of cancer types, and multiple studies employing *in vitro* cell culture systems and *in vivo* xenograft models have revealed multifaceted roles of AEG-1/MTDH/LYRIC in cancer biology, including tumor cell proliferation, apoptosis, angiogenesis, and autophagy. With increasing mechanistic understanding of AEG-1/MTDH/LYRIC, discovery of agents that can block AEG-1/MTDH/LYRIC and its regulated pathways will be beneficial to cancer patients with aberrant expression of AEG-1/MTDH/LYRIC.

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1. INTRODUCTION

AEG-1/MTDH/LYRIC was initially reported as a novel gene induced by human immunodeficiency virus-1 in primary human fetal astrocytes (Su et al., 2002). Subsequently, four independent groups cloned *AEG-1/MTDH/LYRIC* (Britt et al., 2004; Brown & Ruoslahti, 2004; Kang et al., 2005; Sutherland, Lam, Briers, Lamond, & Bickmore, 2004). Employing *in vivo* phage display screening, Brown and colleagues identified mouse AEG-1/MTDH/LYRIC as a protein mediating specific adhesion of mouse 4T1 mammary tumor cells to lung vascular endothelium, thus giving it the name "Metadherin" (Brown & Ruoslahti, 2004). The mouse/rat orthologs of AEG-1/MTDH/LYRIC were also found as lysine-rich CEACAM1 coisolated (LYRIC) protein that is associated with tight junctions in polarized prostate epithelial cells (Britt et al., 2004), and as a novel transmembrane protein that is present in the cytoplasm, endoplasmic reticulum, perinuclear regions, and nucleolus by gene-trapping techniques (Sutherland et al., 2004).

The initial identification of AEG-1/MTDH/LYRIC raised broad controversies on the understanding of its biological functions and molecular characteristics, most of which still remain elusive. Nevertheless, some consensus features of AEG-1/MTDH/LYRIC have been recognized. Evolutionally, AEG-1/MTDH/LYRIC orthologs have been identified in most vertebrates with a high degree of evolutionary conservation but not detected in lower invertebrates, indicating that AEG-1/MTDH/LYRIC may have specialized functions that evolve only in higher organisms. At molecular level, the human *AEG-1/MTDH/LYRIC* encodes a 582-amino acid protein with no recognizable domains that could indicate its function, except for three putative lysine-rich nuclear localization signals (Thirkettle, Girling, et al., 2009). Distinct isoforms or modifications of AEG-1/MTDH/LYRIC have long been speculated based on multiple RNA/protein species detected (Britt et al., 2004; Brown & Ruoslahti, 2004; Kang et al., 2005; Sutherland et al., 2004), although the identity and function of these isoforms and/or modifications remain mysterious. The subcellular localization of AEG-1/MTDH/LYRIC is variable and dependent on the cell types examined and detection methods employed. In most cases, endogenous and

ectopic expression of AEG-1/MTDH/LYRIC is predominantly cytoplasmic (including endoplasmic reticulum and perinuclear regions) (Blanco et al., 2011; Kang et al., 2005; Li, Zhang, et al., 2008; Meng et al., 2012; Yoo et al., 2011), but it is also found in the nucleus, especially nucleolus (Emdad et al., 2006; Sutherland et al., 2004; Thirkettle, Girling, et al., 2009; Thirkettle, Mills, Whitaker, & Neal, 2009) and plasma membrane (Britt et al., 2004; Brown & Ruoslahti, 2004). How the subcelluar localization of AEG-1/MTDH/LYRIC is regulated and what effects this has on its function are largely unknown.

AEG-1/MTDH/LYRIC is expressed ubiquitously in almost all human and murine tissues at variable levels (Jeon et al., 2010; Kang et al., 2005). In cancer, the expression level of AEG-1/MTDH/LYRIC is dramatically elevated. The first piece of evidence suggesting a functional involvement of AEG-1/MTDH/LYRIC in cancer was found in mouse mammary tumor metastasis (Brown & Ruoslahti, 2004). In this study, an unbiased screen for cell surface proteins that mediate the metastasis of the 4T1 murine mammary tumor cells to the lung identified a "lung-homing domain (LHD)" belonging to AEG-1/MTDH/LYRIC. Later in 2009, the 8q22 genomic region, where *AEG-1/MTDH/LYRIC* resides, was reported to have recurrent amplification in more than 30% of breast cancer, and this genomic alteration was associated with poor clinical outcome, underscoring the potentially crucial role of this gene in breast cancer progression (Hu, Chong, et al., 2009). In addition, numerous studies over the past decade have demonstrated a multifaceted role of AEG-1/MTDH/LYRIC in regulating phenotype characteristics of malignant features, such as aberrant proliferation (Lee et al., 2009; Li et al., 2009; Yu et al., 2009), evasion of apoptosis (Kikuno et al., 2007; Lee et al., 2008), invasion (Emdad et al., 2006; Sarkar et al., 2008), angiogenesis (Emdad et al., 2009), and chemoresistance (Hu, Chong, et al., 2009; Liu et al., 2009; Yoo, Gredler, et al., 2009; Yoo et al., 2010), in multiple cancer types. With a focus on the emerging roles of AEG-1/MTDH/LYRIC in breast cancer in this chapter, we present evidence that the expression level of AEG-1/MTDH/LYRIC correlates with patient outcome, review evidence that AEG-1/MTDH/LYRIC promotes a large spectrum of tumor-related properties of cancer cells, discuss our limited understanding of its mechanisms, and finally, evaluate the potential of therapeutic targeting of AEG-1/MTDH/LYRIC.

2. ABERRATIONS OF AEG-1/MTDH/LYRIC IN BREAST CANCER

The high frequency of AEG-1/MTDH/LYRIC overexpression in many different cancer types underscores the importance of this protein in cancer biology. Compared to normal breast tissues, in which AEG-1/MTDH/LYRIC is almost undetectable by IHC staining, the level of AEG-1/MTDH/LYRIC is drastically increased in breast cancer cell lines and tumors (Brown & Ruoslahti, 2004; Hu, Chong, et al., 2009; Kang et al., 2005; Li, Zhang, et al., 2008; Su, Zhang, & Yang, 2010). In two large cohorts of archived breast cancer samples in China (Li, Zhang, et al., 2008) and the United States (Hu, Chong, et al., 2009), more than 40% of breast cancers have a higher level of AEG-1/MTDH/LYRIC compared to normal counterparts. In most cases, AEG-1/MTDH/LYRIC is observed in cytoplasm, although a minority of primary cancer cells are also stained positive for AEG-1/MTDH/LYRIC in the nucleus (Li, Zhang, et al., 2008).

Breast cancer is a heterogeneous disease that can be classified into different subtypes based on gene expression profiles and molecular markers (Perou et al., 2000; Sorlie et al., 2001). AEG-1/MTDH/LYRIC neither seems to correlate with a specific subtype of breast cancers nor is it significantly associated with other common clinicopathological parameters including age, estrogen receptor (ER), progesterone receptor, HER2, and p53 status (Hu, Chong, et al., 2009; Li, Zhang, et al., 2008). Instead, the abundance of AEG-1/MTDH/LYRIC is positively correlated with advanced clinical stages and clinicopathological features, as well as distant metastasis and poor patient survival (Dalgin et al., 2007; Hu, Chong, et al., 2009; Tokunaga et al., 2012). Multivariate Cox analysis showed that the risk of metastasis was significantly higher with AEG-1/MTDH/LYRIC expression, even when all of the other factors, including ER, PR, HER2, p53, and tumor size were considered (Hu, Chong, et al., 2009). Taken together, the clinical association studies suggest that the expression of AEG-1/MTDH/LYRIC can be used as an independent prognostic indicator for metastasis and survival of breast cancer patients.

3. VARIANTS OF *AEG-1/MTDH/LYRIC* IN BREAST CANCER

Single-nucleotide polymorphisms have emerged as useful tools to help evaluate the susceptibility, prognosis, and treatment response of cancer

(Easton et al., 2007; Orr & Chanock, 2008). Aiming to identify novel variants of *AEG-1/MTDH/LYRIC* in breast cancer, Liu et al. direct sequenced 108 breast cancer samples and 100 normal controls (Liu et al., 2011). This study led to the identification of 13 variants in the control group and 11 in the breast cancer patient group, among which 2 variants were found to be associated with increased susceptibility for breast cancer development. While larger patient populations are required to confirm the correlation of these variants to breast cancer susceptibility and further examine their prognostic value, this study provides new possibilities of how AEG-1/MTDH/LYRIC may contribute to cancer development. In addition to this finding in breast cancer, one recent study assessing *AEG-1/MTDH/LYRIC* gene polymorphisms and their potential relationship to ovarian cancer susceptibility has also been reported (Yuan et al., 2012). By comparing 145 ovarian cancer patients and 254 matched control subjects, it was found that the *AEG-1/MTDH/LYRIC* (−470G>A) polymorphism was statistically correlated with ovarian cancer risk and clinical stage. These data suggest that *AEG-1/MTDH/LYRIC* (−470G>A) could be a useful molecular marker for assessing ovarian cancer risk and for predicting ovarian cancer patient prognosis. The implication of this variant in breast cancer and other cancer types remains to be investigated in future study.

4. REGULATION OF *AEG-1/MTDH/LYRIC* IN BREAST CANCER

Cancer is a genetic disease characterized by rampant genetic instability and massive genetic/epigenetic alterations (Chin & Gray, 2008; Hanahan & Weinberg, 2011). Recurrent DNA copy number alteration is one type of genetic change that has been observed in a wide range of human cancers, and such genetic events often indicate the presence of key mediators of malignancy in the affected genomic loci (Chin & Gray, 2008). Using a computational algorithm to map minimal recurrent genomic alterations that are associated with poor-prognosis breast cancer, Hu et al. identified the poor-prognosis genomic gain of chromosome 8q22, where human *AEG-1/MTDH/LYRIC* is located (Hu, Chong, et al., 2009). Regional gain of 8q22 was further validated in an extensive collection of breast tumor samples and cell lines. As expected, AEG-1/MTDH/LYRIC was found to be overexpressed in breast tumors with 8q22 amplification (Hu, Chong, et al., 2009; Li et al., 2011). Although the elevated level of AEG-1/MTDH/LYRIC can be predominantly attributed to genomic amplification, a substantial fraction

of breast tumors with normal copies of *AEG-1/MTDH/LYRIC* also overexpresses the protein, suggesting alternative mechanisms of *AEG-1/MTDH/LYRIC* upregulation in cancer (discussed later). Nevertheless, survival analysis of breast cancer patients showed that AEG-1/MTDH/LYRIC activated by genomic gain or other means led to similar clinical outcome, highlighting the prognostic value of *AEG-1/MTDH/LYRIC* itself, rather than its association with other genes on the 8q22 genetic locus (Hu, Chong, et al., 2009; Li et al., 2011).

Studies accumulated so far have suggested alternative mechanisms for the regulation of *AEG-1/MTDH/LYRIC* in cancer (Lee, Su, Emdad, Sarkar, & Fisher, 2006; Ward et al., 2013; Zhang et al., 2011). One possibility is transcriptional regulation by oncogenic regulatory signals. For instance, in human adult astrocytes, activation of the RAS oncogene and subsequent induction of transcription factor c-Myc lead to increased binding of c-Myc to the *AEG-1/MTDH/LYRIC* promoter region, which augments the transcription level of *AEG-1/MTDH/LYRIC* (Lee et al., 2006). Notably, *c-Myc* is often amplified in breast cancer (Bergamaschi et al., 2006). Yet it remains to be tested whether or not *AEG-1/MTDH/LYRIC* is subjected to transcriptional regulation by c-Myc in human breast tumor tissues.

The second potential way to regulate *AEG-1/MTDH/LYRIC* is through microRNAs (miRNAs). miRNAs have been increasingly recognized as crucial regulators for tumorigenesis (Chen, 2005). In fact, downregulation of miRNAs that target oncogenes is frequently observed in cancer, and this remains one of the key mechanisms of oncogene overexpression. Analysis of the 3′UTR of *AEG-1/MTDH/LYRIC* reveals multiple miRNA-binding sites, and two miRNAs have recently been reported to suppress oncogenic phenotypes by targeting *AEG-1/MTDH/LYRIC* (Nohata et al., 2011; Ward et al., 2013; Zhang et al., 2011). In breast cancer, the level of miR-26a is significantly decreased compared to adjacent normal tissues (Iorio et al., 2005; Zhang et al., 2011). Expression and mutagenesis assays validated *AEG-1/MTDH/LYRIC* and *EZH2* as two of the targets of miR-26a that mediated the proapoptotic effect of miR-26a *in vitro*. Furthermore, a negative correlation between the expression level of miR-26a and MTDH/EZH2 is also observed in a few paired clinical breast cancer samples (Zhang et al., 2011). While large-scale clinical association studies have yet to be conducted, this study provides the first piece of evidence that *AEG-1/MTDH/LYRIC* may be regulated by miRNAs in breast cancer. Besides miR-26a, tumor suppressive miRNA miR-375 was also found to have inversed expression level to that of *AEG-1/MTDH/LYRIC* in breast

cancer, hepatocellular carcinoma, head and neck squamous cell carcinoma, and esophageal squamous cell carcinoma (He et al., 2012; Hui et al., 2011; Nohata et al., 2011; Ward et al., 2013). Intriguingly, miR-375 is downregulated in a tamoxifen-resistance clone of human breast cancer cell line MCF7, and this event is associated with epithelial to mesenchymal transition (EMT)-like properties. *AEG-1/MTDH/LYRIC* was shown to be one of the targets of miR-375 to mediate this effect (Ward et al., 2013). Of note, while the level of miR-375 is much higher in ER-positive breast cancer, AEG-1/MTDH/LYRIC expression does not significantly correlate with ER status. This further supports the notion that there are multiple alternative mechanisms underlying the regulation of AEG-1/MTDH/LYRIC in breast cancer, which require further investigation.

5. AEG-1/MTDH/LYRIC IN BREAST CANCER GROWTH CONTROL

AEG-1/MTDH/LYRIC has been demonstrated in different contexts to affect cancer cell growth. In some clinical breast cancer samples, AEG-1/MTDH/LYRIC expression is highly associated with proliferative marker Ki-67 (Li, Zhang, et al., 2008). Functionally, ectopic expression and RNAi silencing of *AEG-1/MTDH/LYRIC* promotes and decreases, respectively, proliferation of breast cancer cell lines (Li et al., 2009). Additionally, key cell cycle inhibitors $p27^{Kip1}$ and $p21^{Cip1}$ were found to be downregulated by AEG-1/MTDH/LYRIC via an increase of phosphorylation and subsequent cytoplasm retention of FOXO1 transcription factor (Li et al., 2009). In this study, the effect of AEG-1/MTDH/LYRIC on FOXO1 phosphorylation is likely to be mediated by PI3K/AKT signaling, as inhibitors of this pathway abolished the growth-promoting function of AEG-1/MTDH/LYRIC. Intriguingly, AEG-1/MTDH/LYRIC has been reported in prostate cancer cells to physically interact with and promote the proteasome degradation of BCCIPα, a CDKN1A and BRCA2 binding protein involved in DNA repair and cell cycle control (Ash, Yang, & Britt, 2008). It is known that BCCIPα binds to p21 and enhances its inhibitory activity toward CDK2, leading to impairment in G1/S cell cycle progression (McShea, Samuel, Eppel, Galloway, & Funk, 2000; Meng, Liu, & Shen, 2004; Meng, Lu, & Shen, 2004). While the interaction between AEG-1/MTDH/LYRIC and BCCIPα has yet to be confirmed in breast cancer, it can be speculated that AEG-1/MTDH/LYRIC may enhance proliferation in breast cancer through its negative regulation of BCCIPα.

It is of note that inconsistencies concerning the involvement of AEG-1/MTDH/LYRIC in breast cancer proliferation do exist. For example, Hu et al. revealed no difference in proliferation or cell growth caused by manipulation of AEG-1/MTDH/LYRIC in multiple breast cancer cell lines (Hu, Chong, et al., 2009). Whether these discrepancies are caused by differences in culture conditions, cell line heterogeneity, or assays employed remains to be addressed.

6. AEG-1/MTDH/LYRIC AND EMT IN BREAST CANCER

EMT, an essential embryonic program, is often aberrantly activated in cancer. This process can endow epithelial cancer cells with a highly motile mesenchymal phenotype associated with increased metastatic capability (Nieto, 2011). In carcinomas, the cancer cells at the invasive front are often stained positive for mesenchymal protein markers such as N-cadherin and Vimentin, suggesting that these cells may have undergone EMT and invaded into surrounding tissues. Two recent studies reported a possible link between AEG-1/MTDH/LYRIC and EMT in breast cancer (Li et al., 2011; Ward et al., 2013). Li and colleagues showed that transient ectopic expression of AEG-1/MTDH/LYRIC in MCF7 breast cancer cells enhanced their migratory and invasive capabilities by inducing EMT (Li et al., 2011). This was evidenced by a switch from epithelial-to-mesenchymal-like cell shape, downregulation of E-cadherin expression, and upregulation of mesenchymal markers Fibronectin and Vimentin. Moreover, transcriptional factors that are important for EMT such as Snail and Slug are also upregulated by AEG-1/MTDH/LYRIC in MCF7 cells. Mechanistically, it was showed that inhibitors of NF-κb pathway or RNAi silencing of NF-κB subunit p65 abolished the effect of AEG-1/MTDH/LYRIC on EMT. Of note, AEG-1/MTDH/LYRIC has been linked to NF-κB signaling pathway in the context of cell growth, survival, and invasion in other cancer types (Emdad et al., 2006; Kikuno et al., 2007; Liu et al., 2010; Sarkar et al., 2008). In those studies, AEG-1/MTDH/LYRIC was hypothesized to act as a transcriptional coactivator in the nucleus through its binding with the NF-κB subunit p65. Given the observation that AEG-1/MTDH/LYRIC is predominantly localized in the cytoplasm of breast cancer cells, it is important to vigorously test whether the interaction with NF-κB pathway is the main mechanism through which AEG-1/MTDH/LYRIC may enhance EMT and invasion of breast cancer cells.

Another study also using MCF7 cells revealed an unexpected connection between AEG-1/MTDH/LYRIC, EMT, and tamoxifen resistance. MCF7 cells are ER positive and thus are sensitive to tamoxifen treatment. However, it has been demonstrated that long-term treatment of MCF7 cells with tamoxifen will result in the appearance of resistant clones, recapitulating clinical observation that ER+ breast cancers often acquire endocrine resistance with prolonged treatment (Musgrove & Sutherland, 2009). Multiple studies demonstrated that tamoxifen-resistant clones of MCF7 have undergone EMT, as indicated by morphological and transcriptional changes (Hiscox et al., 2006; Kim, Choi, Cho, Kim, & Kang, 2009; Ward et al., 2013). An unbiased miRNA screening identified mir-375 as a suppressor of this EMT-associated resistance by targeting *AEG-1/MTDH/LYRIC*. Silencing of *AEG-1/MTDH/LYRIC* in the tamoxifen-resistant clones of MCF7 partially reversed EMT in this context. Endocrine therapies have become the most important treatment options for women with ER-positive breast cancer (which account for 70% of diagnosed cases); however, their efficacy is limited by intrinsic and acquired therapeutic resistance. While more thorough mechanistic studies are needed to further prove the link between AEG-1/MTDH/LYRIC and endocrine resistance, this study suggests a potential use of AEG-1/MTDH/LYRIC as both a diagnosis marker for treatment resistance and a target for combination therapy to enhance drug efficacy.

7. AEG-1/MTDH/LYRIC AND BREAST CANCER METASTASIS

Poor prognosis of breast cancer at the time of diagnosis or surgery indicates a higher probability of death, mainly as the result of metastasis to vital organs. The strong clinical correlation between AEG-1/MTDH/LYRIC expression and shorter metastasis-free survival suggests that AEG-1/MTDH/LYRIC may function as a metastasis gene with great prognostic potential and therapeutic value. Two studies so far have independently identified and demonstrated the functional involvement of AEG-1/MTDH/LYRIC in breast cancer metastasis (Brown & Ruoslahti, 2004; Hu, Chong, et al., 2009). Brown et al. used *in vivo* phage screening of cDNAs library from metastatic breast carcinoma to identify protein domains that preferentially bind to lung vasculatures. An extracellular LHD (a.a. 378–440 in mouse or 381–443 in human) in AEG-1/MTDH/LYRIC has been uncovered to mediate the adhesion of 4T1 murine mammary

tumor cells to lung endothelium and thus promote lung metastasis. Neutralizing antibodies against this LHD- or siRNA-mediated silencing of *AEG-1/MTDH/LYRIC* reduce experimental metastasis of 4T1 cells. On the contrary, ectopic expression of *AEG-1/MTDH/LYRIC* in human embryonic kidney cells HEK293 results in increased localization of these cells to the lung vasculatures. The effect of AEG-1/MTDH/LYRIC on metastasis has also been validated using the MDA-MB-231 xenograft model of breast cancer metastasis (Hu, Chong, et al., 2009). In this model system, AEG-1/MTDH/LYRIC promotes experimental metastasis not only to the lung but also to the bone, albeit to a lesser extent.

Mechanistically, it is conceivable that the LHD of AEG-1/MTDH/LYRIC may function as an adhesion molecule to direct the association of cancer cells to lung endothelia cells, as it was originally identified (Brown & Ruoslahti, 2004). In fact, it has been showed that antibodies against LHD bound to nonpermeabilized cells, confirming the presence of this LHD of AEG-1/MTDH/LYRIC at the cell surface of tumor cells where it would be able to bind to vascular targets during metastasis. However, a significant amount of AEG-1/MTDH/LYRIC was also detected in the cytoplasm when cells were permeabilized (Brown & Ruoslahti, 2004). This is consistent with subsequent studies in which AEG-1/MTDH/LYRIC has been shown to predominantly localize in the cytoplasm in breast tumor tissues and cancer cell lines (Blanco et al., 2011; Li, Zhang, et al., 2008). Therefore, a better understanding of the function of AEG-1/MTDH/LYRIC requires a higher resolution of its possible dynamic changes in subcellular localization under various pathological conditions and during cancer progression. It also remains to be tested whether AEG-1/MTDH/LYRIC may affect the lung-homing properties of cancer cells by inducing adhesion molecules through its interaction with other pathways such as NF-κB, a known pathway that has been shown to activate a cohort of adhesion genes. Alternatively, AEG-1/MTDH/LYRIC may affect different steps during metastatic cascades, such as cancer cell dissemination through EMT, rather than homing cancer cells to the lung.

8. AEG-1/MTDH/LYRIC AND CHEMORESISTANCE

Chemoresistance remains one of the biggest challenges for clinical management of breast cancer. Current standard treatment for breast cancer uses a combination of surgical removal of primary tumors and adjuvant chemotherapy to prevent systematic spreading. However, recurrent cancers

inevitably acquire resistance to treatment and often spread to distant organs, which accounts for over 90% of cancer-related deaths. This suggests that metastatic cancers not only have to overcome numerous obstacles during the multistep process of metastasis but also need to acquire the ability of survival under antineoplastic stresses such as those imposed by standard adjuvant therapy. Pharmacogenomic analysis of the NCI60 panel of cancer cell lines revealed that the genomic gains of 8q22 strongly correlate with a higher mean GI50 (the drug concentration for 50% growth inhibition) for nearly half of the drugs used in this database (Hu, Chong, et al., 2009). Moreover, 8q22 amplification, and the resulting overexpression of genes located in this region, significantly associates with cancer recurrence despite of adjuvant chemotherapy (Li et al., 2010). When individual genes on the 8q22 region were examined, only the mRNA level of *AEG-1/MTDH/LYRIC* was found to significantly correlate with higher drug resistance of NCI60 cell lines (Hu, Chong, et al., 2009), although another two genes, *LAMPTM4B* and *YWHAZ*, were demonstrated in a separate study to correlate with and functionally contribute to chemoresistance of breast cancer cell lines (Li et al., 2010). *In vitro* and *in vivo* silencing of *AEG-1/MTDH/LYRIC* sensitize human breast cancer cells to a broad spectrum of chemotherapy drugs, including paclitacxel, doxorubicin, cisplatin, and hydrogen peroxide (Hu, Chong, et al., 2009; Li et al., 2010). Importantly, in a therapeutic model, AEG-1/MTDH/LYRIC-based DNA vaccine was proved to increase chemosensitivity to doxorubicin and inhibit breast cancer lung metastasis (Qian et al., 2011). Mechanistically, AEG-1/MTDH/LYRIC does not seem to affect the uptake and/or retention of drugs in breast cancer cells but rather confers a survival advantage under antineoplastic stress (Hu, Chong, et al., 2009). This could be mediated by survival pathways such as PI3K and NF-κB, which have been connected to AEG-1/MTDH/LYRIC in other cancer types (Hu, Wei, & Kang, 2009), or by direct downstream targets of AEG-1/MTDH/LYRIC. In fact, microarray profiling in breast cancers revealed that AEG-1/MTDH/LYRIC increases drug resistance genes such as *ALDH3A1* and *MET* and downregulates proapoptotic genes such as *TRAIL*.

The chemoresistance function of AEG-1/MTDH/LYRIC has also expanded to other cancer types, including neuroblastoma (Liu et al., 2009), hepatocellular carcinoma (Yoo et al., 2010), ovarian (Li et al., 2012; Meng et al., 2011), and endometrial cancers (Meng et al., 2011). Despite accumulating evidence for the broad chemoresistance effect of AEG-1/MTDH/LYRIC, the underlying mechanisms seem to vary among

different cancer types or depend on chemotherapeutic drugs tested. For example, in contrast to what has been shown in breast cancer (Hu, Chong, et al., 2009), AEG-1/MTDH/LYRIC decreases doxorubicin uptake and retention in the hepatocellular carcinoma cells through regulation of multidrug resistance gene MDR1 at posttranscriptional level (Yoo et al., 2010). Alternatively, AEG-1/MTDH/LYRIC may confer resistance to chemotherapeutic drugs by regulating genes that are critical for drug action and catabolism, as in the case of 5-FU. It has been reported that AEG-1/MTDH/LYRIC confers resistance to 5-FU in hepatocellular carcinoma cells by augmenting the expression of the transcription factor LSF that induces thymidylate synthase, the substrate for 5-FU, and by increasing the 5-FU-catabolizing enzyme dihydropyrimidine dehydrogenase, DPYD (Yoo, Gredler, et al., 2009). Future studies are necessary to gain a better understanding of how AEG-1/MTDH/LYRIC could confer resistance to a diverse range of chemotherapeutic drugs through distinct mechanisms.

9. MOLECULAR UNDERSTANDING OF AEG-1/MTDH/LYRIC

9.1. Integration of oncogenic pathways

In sharp contrast to the rapidly growing clinical and phenotypic studies of AEG-1/MTDH/LYRIC, our molecular understanding of this novel protein remains poor. Depending on the cancer cell types tested, AEG-1/MTDH/LYRIC can activate multiple major oncogenic pathways, including PI3K/AKT, Wnt/β-catenin, and NF-κB signaling pathways, to promote different aspects of tumor malignancy (Fig. 4.1). Overexpression of AEG-1/MTDH/LYRIC inhibits serum starvation-induced cell death of human fetal astrocytes through the activation of PI3K–AKT signaling pathway and its downstream substrates, such as GSK3β/c-Myc, MDM2/p53, and Bad (Lee et al., 2008). On the other hand, silencing of *AEG-1/MTDH/LYRIC* results in apoptosis of prostate cancer cell lines through upregulation of forkhead box 3a activity, which is dependent on the reduction of AKT signaling (Kikuno et al., 2007). How AEG-1/MTDH/LYRIC regulates PI3K/AKT pathway is currently unknown. In addition, AEG-1/MTDH/LYRIC promotes anchorage-independent growth and invasion of Hela cells by enhancing the nuclear accumulation, DNA binding, and transcriptional activities of the NF-κB subunit p65 (Emdad et al., 2006). This may be achieved by directly binding of AEG-1/MTDH/LYRIC to p65 (Sarkar et al., 2008), although this model requires further elucidation, as

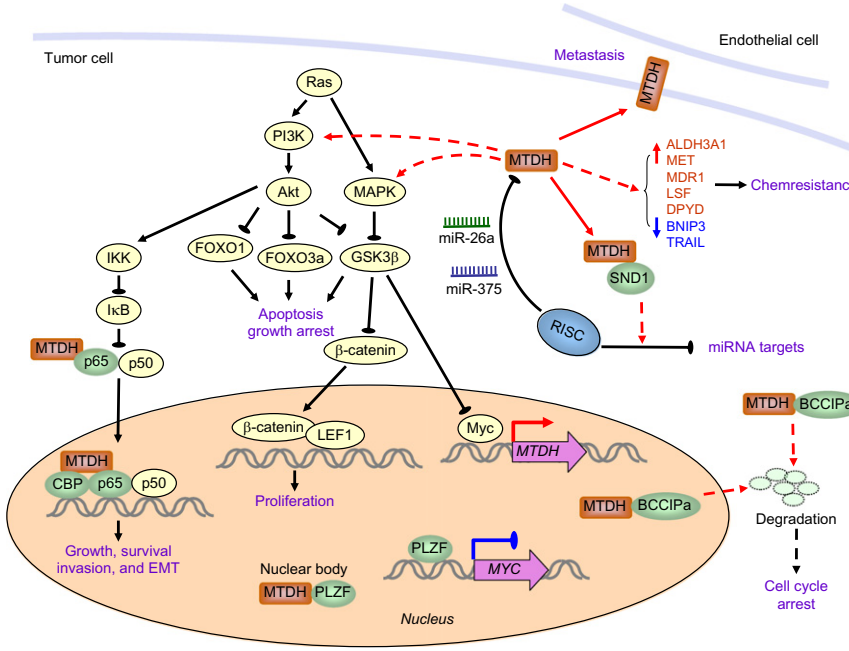

Figure 4.1 A schematic depiction of AEG-1/MTDH/LYRIC functions in cancer. AEG-1/MTDH/LYRIC can be regulated at transcriptional level by oncogenic Ha-Ras through the activation of PI3K/AKT signaling, or alternatively, at posttranscriptional level by miRNAs such as miR-375 and miR-26a. AEG-1/MTDH/LYRIC activates PI3K/AKT, Wnt/β-catenin, and NFκB pathways to promote growth, survival, EMT, and invasion under different conditions. The activation of NFκB pathway by AEG-1/MTDH/LYRIC is in part through the direct interaction of AEG-1/MTDH/LYRIC with p65 and CBP, a general transcriptional coactivator. AEG-1/MTDH/LYRIC confers resistance to a broad spectrum of chemotherapeutic agents by regulating a number of downstream genes. The prometastasis function of AEG-1/MTDH/LYRIC could be mediated by the interaction of the lung-homing domain of AEG-1/MTDH/LYRIC with an unknown receptor in endothelial cells. In the cytoplasm, AEG-1/MTDH/LYRIC binds to SND1 and thus associates with RNA-induced silencing complex (RISC). AEG-1/MTDH/LYRIC also interacts with and targets BCCIPα for proteasome-mediated degradation. Nuclear AEG-1/MTDH/LYRIC binds to PLZF and relieves PLZF-mediated repression on genes such as *c-Myc*. Proteins with direct interactions with AEG-1/MTDH/LYRIC are shown in green, while proteins with possible interactions with AEG-1/MTDH/LYRIC are shown in blue. Red-dotted line indicates pathways/mechanisms yet to be fully validated and/or characterized. Additional oncogenic phenotypes and related mechanisms of AEG-1/MTDH/LYRIC not discussed in this review are not highlighted here.

the interacting domain of AEG-1/MTDH/LYRIC to p65 failed to mediate its effect on NF-κB activation. Alternatively, AEG-1/MTDH/LYRIC may function to bridge the interaction between p65 and CBP, a transcriptional coactivator of NF-κB in glioma (Sarkar et al., 2008). In hepatocellular carcinoma, AEG-1/MTDH/LYRIC has been connected to the Wnt/β-catenin pathway through the activation of the Raf/MEK/MAPK branch of the Ras signaling pathway (Yoo, Emdad, et al., 2009).

Despite the evidence presented in these above cancer types, little is known about whether or not AEG-1/MTDH/LYRIC exerts its roles in breast cancer through cross talk with these well-known oncogenic pathways. The observation that AEG-1/MTDH/LYRIC displays diverse oncogenic phenotypes in a highly context-dependent manner may well suggest that AEG-1/MTDH/LYRIC is a multifunctional protein that works through different mechanisms.

9.2. Interacting partners of AEG-1/MTDH/LYRIC

Despite the high level of interest in AEG-1/MTDH/LYRIC due to its diverse oncogenic phenotypes, the lack of any recognizable functional domains in this protein remains as a major obstacle for a better understanding of its molecular mechanisms. Multiple groups have employed unbiased screening methods to uncover binding partners of AEG-1/MTDH/LYRIC in a variety of cell types and physiological conditions (Fig. 4.1) (Ash et al., 2008; Blanco et al., 2011; Meng et al., 2012; Thirkettle, Mills, et al., 2009; Yoo et al., 2011). AEG-1/MTDH/LYRIC is a dynamic protein that localizes in multiple subcellular compartments, and in different locations, it binds to specific proteins. In the nucleus, AEG-1/MTDH/LYRIC was first shown to bind to the p65 subunit of NF-κB, and this interaction augments the transcriptional activity of p65 (Sarkar et al., 2008). Two additional AEG-1/MTDH/LYRIC-interacting proteins, PLZF and BCCIPα, were later identified in prostate cancer cells using yeast two-hybrid screening (Ash et al., 2008; Thirkettle, Mills, et al., 2009). AEG-1/MTDH/LYRIC binds to PLZF via the NH_2-terminus (a.a. 1–285) and the COOH-terminus (a.a. 487–582) and localizes with PLZF transcriptional repressor machinery within nuclear bodies. Overexpression of AEG-1/MTDH/LYRIC reduces PLZF-mediated repression by abrogating the ability of PLZF to bind DNA, perhaps by sequestering PLZF to nuclear bodies (Thirkettle, Mills, et al., 2009). The AEG-1/MTDH/LYRIC–BCCIPα interaction was mapped to the region amino acid 72–192 of AEG-1/MTDH/LYRIC. Instead of

affecting transcriptional activities of its binding partners, AEG-1/MTDH/LYRIC targets BCCIPα for proteasomal degradation (Ash et al., 2008).

In human metastatic breast cancer cell lines, protein coimmunoprecipitation followed by mass spectrometry sequencing led to the identification of *staphylococcal* nuclease domain-containing 1 (SND1) as a major AEG-1/MTDH/LYRIC-interacting protein in the cytoplasm (Blanco et al., 2011). SND1 is a multifunctional protein that contains four N-terminal SN domain repeats, which harbor nuclease activity (Li, Yang, Chen, & Yuan, 2008), and a C-terminal Tudor-SN hybrid domain, which has been shown to interact with methylated protein substrates (Shaw et al., 2007). The multifaceted roles of SND1 depend on its subcellular localizations. When localized in the nucleus, SND1 couples transcription and splicing via interactions with key components of both processes. On the one hand, it can act as a transcriptional coactivator to enhance transcriptional activity of EBNA-2 (Tong, Drapkin, Yalamanchili, Mosialos, & Kieff, 1995), c-Myb (Leverson et al., 1998), STAT5 (Paukku, Yang, & Silvennoinen, 2003), and STAT6 (Yang et al., 2002). On the other hand, it interacts with the spliceosome machinery (Shaw et al., 2007) and thus facilitates mRNA splicing (Yang et al., 2007). In the cytoplasm, SND1 functions as a nuclease in the RNA-induced silencing complex (RISC), where it promotes cleavage of double-stranded RNA and hyperedited double-stranded RNA substrates (Caudy et al., 2003). Furthermore, SND1 has been implicated in other cytoplasmic processes such as programmed cell death (Sundstrom et al., 2009) and the formation of stress granules upon induction of various cellular stresses (Gao et al., 2010). Though various intracellular localizations have been reported for AEG-1/MTDH/LYRIC and SND1, endogenous AEG-1/MTDH/LYRIC and SND1 were each observed to predominantly localize to regions of the ER with a diffuse signal observed in the cytoplasm of human breast cancer cells (Blanco et al., 2011). Immunofluorescence analysis revealed colocalization of AEG-1/MTDH/LYRIC and SND1 in punctate patterns to the ER/cytoplasm regions. Domain-mapping experiments with a series of AEG-1/MTDH/LYRIC deletion mutant constructs demonstrated that amino acid 364–470 region of AEG-1/MTDH/LYRIC is required for its interaction with SND1, whereas both SN domains and Tudor-SN domain of SND1 are sufficient to bind to AEG-1/MTDH/LYRIC (Blanco et al., 2011). To explore the potential functionality of SND1 in the context as a novel AEG-1/MTDH/LYRIC-interacting protein in breast cancers, Blanco et al. conducted *in vivo* functional studies and demonstrated that SND1 is itself a strong promoter for

metastasis. However, similar to other AEG-1/MTDH/LYRIC-binding partners, the functional significance of the interaction remains to be elucidated.

Two other groups have also independently uncovered the AEG-1/MTDH/LYRIC–SND1 interaction in different cancer types, underscoring the potential importance of this binding (Meng et al., 2012; Yoo et al., 2011). Intriguingly, Yoo et al. mapped the domain of AEG-1/MTDH/LYRIC–SND1 interaction to amino acid 101–205 of AEG-1/MTDH/LYRIC, a region that does not overlap with what has been reported. This discrepancy remains to be further clarified using biochemical and structural studies. Given the observation that SND1 could interact with AEG-1/MTDH/LYRIC with both SN domain and Tudor-SN domains (Blanco et al., 2011), it can be speculated that more than one domain of AEG-1/MTDH/LYRIC is sufficient to interact with SND1. Functionally, AEG-1/MTDH/LYRIC was documented to associate with RISC through its physical interaction with SND1 in hepatocellular carcinoma, and AEG-1/MTDH/LYRIC-mediated activation of RISC increased degradation of tumor suppressor mRNAs that are targets of oncomiRs (Yoo et al., 2011). Still, it is unclear how AEG-1/MTDH/LYRIC affects RISC activities and how this action results in net oncogenic effects of miRNAs in hepatocellular cancer cells. Besides SND1, AEG-1/MTDH/LYRIC interacts with multiple other cytoplasmic proteins including NPM1 and RPL4 (Meng et al., 2012). Interestingly, treatment with Benzonase nuclease abolished the binding of AEG-1/MTDH/LYRIC with SND1 and RPL4, prompting the hypothesis that AEG-1/MTDH/LYRIC functions as an mRNA-binding protein.

Taken together, identification of AEG-1/MTDH/LYRIC-interacting proteins has provided interesting insights into how AEG-1/MTDH/LYRIC may orchestrate gene regulation and exert its function in a highly context-dependent manner. Future studies are urgently needed to further elucidate the functional significance of these interactions to AEG-1/MTDH/LYRIC-mediated oncogenic effects.

10. THERAPEUTIC TARGETING OF AEG-1/MTDH/LYRIC

Breast cancer patients harboring an elevated level of AEG-1/MTDH/LYRIC are more likely to suffer from metastatic recurrence and acquire resistance to chemotherapeutic treatment. Therefore, AEG-1/MTDH/LYRIC can be used as a biomarker to identify subgroups of patients that require closer monitoring for signs of relapse for early clinical intervention.

For these high-risk patients, more aggressive adjuvant treatment, coupled with molecular targeting of *AEG-1/MTDH/LYRIC*, may be required to enhance therapeutic efficacy and achieve optimal clinical outcome.

There are several possible avenues to develop novel cancer therapies through molecular targeting of AEG-1/MTDH/LYRIC. Based on the initial observation that AEG-1/MTDH/LYRIC mediates the adhesion of cancer cells to lung endothelium, one can envision that neutralizing antibodies against AEG-1/MTDH/LYRIC can be used to block the early establishment of lung metastasis. In fact, antibodies reacting to the LHD of AEG-1/MTDH/LYRIC have been reported to reduce lung metastasis when coinjected with 4T1 murine mammary tumor cells (Brown & Ruoslahti, 2004). This needs to be expanded and tested in preclinical models of human breast cancer to ensure that the feasibility and effectiveness before human monoclonal antibodies against AEG-1/MTDH/LYRIC are developed. In addition, studies are required to examine the effect of AEG-1/MTDH/LYRIC on established metastasis in order to fully explore the potential of targeting AEG-1/MTDH/LYRIC to treat metastatic diseases. Another alternative is silencing *AEG-1/MTDH/LYRIC* by RNA interference or miRNAs if high-efficiency *in vivo* delivery can be achieved and nonspecific immune response can be overcome. Indeed, previous studies have shown that RNAi silencing of *AEG-1/MTDH/LYRIC* sensitizes breast cancer cells to chemotherapeutic drugs and reduces lung metastasis (Hu, Chong, et al., 2009). More recently, an artificial miRNA engineered to target *AEG-1/MTDH/LYRIC* has been established and proved to inhibit AEG-1/MTDH/LYRIC expression and its oncogenic effect *in vitro* (Wang, Shu, Cai, Bao, & Liang, 2012). Different from directly targeting the gene of interest by neutralizing antibodies or RNA interference, immunotherapy harnesses the host immune system to attack and destroy tumor cells, and it has emerged as an attractive approach for clinical management of different cancer types. Recently gene-based vaccines have become a favored immunotherapeutic strategy to activate effective immunity against cancer (Rice, Ottensmeier, & Stevenson, 2008). An AEG-1/MTDH/LYRIC-based DNA vaccine was recently developed and tested in preclinical models of human breast cancer (Qian et al., 2011). Delivered by attenuated salmonella typhimurium, this vaccine effectively suppressed tumor growth in a prophylactic model and metastasis of 4T1 cells in both prophylactic and therapeutic models, without obvious side effects observed in the mice. Mechanistically, this vaccine evoked strong CD8+ cytotoxic T cell-mediated immune response both *in vitro* and *in vivo*. Although our knowledge about the normal

physiological function of AEG-1/MTDH/LYRIC is currently lacking, and uncertainties exist regarding to the safety of AEG-1/MTDH/LYRIC-based DNA vaccines, this study offers new possibilities to target AEG-1/MTDH/LYRIC. Finally, identification and functional characterization of interactions between AEG-1/MTDH/LYRIC and its partners may lead to the discovery of small molecules that can effectively block the functionality of AEG-1/MTDH/LYRIC. A better understanding of the molecular mechanisms of AEG-1/MTDH/LYRIC will also help reveal more alternative targets that are mediators of AEG-1/MTDH/LYRIC's oncogenic effects.

11. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Accumulated clinical and functional studies have demonstrated AEG-1/MTDH/LYRIC as a strong indicator and promoter of tumor malignancy in multiple organs, including the breast. Notably, AEG-1/MTDH/LYRIC exerts its multifaceted roles in a highly context-dependent manner, and the underlying mechanisms remain poorly understood. Future investigations are urgently needed to clarify the following questions. Although a great abundance of evidence points to important roles of AEG-1/MTDH/LYRIC in cancer, little is known about its biological significance in normal physiological conditions. Studies employing relevant animal models are essential to provide insights into the role of this conserved molecule in development. At the molecular level, while recent progress has been made to elucidate the subcellular localization of AEG-1/MTDH/LYRIC, it remains a mystery what signaling events lead to the distribution of AEG-1/MTDH/LYRIC and whether subcellular localizations contribute to the function of AEG-1/MTDH/LYRIC. In addition, it has long been speculated that AEG-1/MTDH/LYRIC harbors many different isoforms/modifications based on sequence prediction and multiple RNA/Protein detected; however, none of them have been adequately examined. Another layer of complexity within our understanding of AEG-1/MTDH/LYRIC is the board range of proteins it interacts with in different cellular components. Evidence is urgently needed to illustrate whether AEG-1/MTDH/LYRIC exerts its diverse functions through interacting and modifying its binding partners, or whether it functions as a scaffold protein in a signaling complex. Only by the understanding of these basic cellular and biochemical properties of AEG-1/MTDH/LYRIC can we gain better understanding of its actions in cancer and develop effective inhibitors.

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