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Review:

Estrogen receptor coactivator Mediator Subunit 1 (MED1) as a tissue-specific therapeutic target in breast cancer*

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Abstract: Breast cancer, one of the most frequent cancer types, is a leading cause of death in women worldwide. Estrogen receptor (ER) α is a nuclear hormone receptor that plays key roles in mammary gland development and breast cancer. About 75% of breast cancer cases are diagnosed as ER-positive; however, nearly half of these cancers are either intrinsically or inherently resistant to the current anti-estrogen therapies. Recent studies have identified an ER coactivator, Mediator Subunit 1 (MED1), as a unique, tissue-specific cofactor that mediates breast cancer metastasis and treatment resistance. MED1 is overexpressed in over 50% of human breast cancer cases and co-amplifies with another important breast cancer gene, receptor tyrosine kinase HER2. Clinically, MED1 expression highly correlates with poor disease-free survival of breast cancer patients, and recent studies have reported an increased frequency of MED1 mutations in the circulating tumor cells of patients after treatment. In this review, we discuss the biochemical characterization of MED1 and its associated MED1/Mediator complex, its crosstalk with HER2 in anti-estrogen resistance, breast cancer stem cell formation, and metastasis both in vitro and in vivo. Furthermore, we elaborate on the current advancements in targeting MED1 using state-of-the-art RNA nanotechnology and discuss the future perspectives as well.

Key words: Mediator Subunit 1 (MED1); Mediator; Estrogen receptor; Breast cancer; Endocrine resistance; RNA nanotechnology
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1 Introduction

Despite decades of pre-clinical and clinical research (including a number of US Food and Drug Administration (FDA)-approved treatment modalities), breast cancer has remained a leading source of cancer-related deaths in women globally (Ferlay et al., 2015; Reinert et al., 2019). Based on gene expression profiles, breast cancers are commonly categorized into at least four major subtypes: estrogen receptor (ER)-positive luminal A and luminal B, HER2-positive, and triple negative breast cancers (Perou et al., 2000; Parker et al., 2009; The Cancer Genome Atlas Network, 2012). ER-positive breast cancers are the most prevalent and account for approximately 75% of breast cancer cases. Endocrine therapies, such as selective estrogen receptor modulators (SERMs) and selective estrogen receptor degraders (SERDs), have been developed. These therapies are being widely used in clinics to target and inhibit ER functions for breast cancer treatment (Dutertre and Smith, 2000; Jensen and Jordan, 2003; McDonnell and Wardell, 2010; Abderrahman and Jordan, 2019a, 2019b). Unfortunately, therapeutic resistance frequently

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occurs in these patients. Nearly half of all the ER-positive breast cancer patients experience de novo or acquired resistance to the treatment (Shou et al., 2004; Fu et al., 2019). In addition, the unwanted side effects on other estrogen-responsive tissues of the patients pose a major hindrance for treatment effectiveness (Kedar et al., 1994; Mourits et al., 2001; Eastell et al., 2008). Thus, a better understanding of the molecular mechanisms underlying ER functions and therapeutic resistance is urgently required for the development of safer and more effective strategies for future treatments. Recently, studies have identified ER transcriptional coactivator MED1 as a key tissue-specific Mediator for both breast cancer metastasis and therapeutic resistance. In this review, we will discuss the most recent advancements in our current understanding of the molecular mechanisms of MED1 functions in these processes and its role as a potential therapeutic target.

2 Estrogen receptor

ERα is a ligand-dependent transcription factor that belongs to a superfamily of nuclear hormone receptors with homologous structures (Kumar et al., 1987; Mangelsdorf et al., 1995; Bick et al., 2019). The structure of ERα comprises multiple, distinct functional domains: the ligand-independent transcriptional activation function-1 (AF-1) domain at the N-terminal, followed by a highly conserved central zinc-finger DNA binding domain (DBD), a hinge domain, the ligand binding domain (LBD) that contains a ligand-dependent activation function-2 (AF-2), and a C-terminal F domain. Once bound to its ligand, estradiol-activated ERα can bind either directly to estrogen response elements (EREs) or, in a "non-classical" binding mode through tethering with other transcription factors (e.g., AP-1, SP-1) to elicit targeted gene transcription (Bick et al., 2019). It is now known that pioneer factors like FOXA1, TLE, PBX1, etc., that directly interact with compact chromatin, are also playing a key role in opening the active sites to facilitate such bindings (Hurtado et al., 2011; Magnani et al., 2011; Holmes et al., 2012; Bick et al., 2019).

The DNA-bound ERα functions through an ordered recruitment of various transcriptional cofactors in a dynamic and sequential manner to ultimately regulate targeted gene transcription (Fig. 1) (Glass and Rosenfeld, 2000; Shang et al., 2000; McKenna and O'Malley, 2002; Bick et al., 2019; Vasquez and Lee Kraus, 2019). These cofactors include chromatin remodeling factors like the SWI/SNF complex, followed by histone acetyltransferases, e.g., p160/SRC family members (SRC1-3) and CBP/p300, and arginine methyltransferases like CARM1, etc. These coactivators collectively work to further facilitate chromatin accessibility and allow for RNA polymerase II and general transcription factors (GTFs) to assemble at the transcription-initiating site (Roeder, 1998, 2003). It is now clear that another type of transcription cofactor, the multi-subunit Mediator complex, is required to act as a bridge to relay signal(s) from ligand-bound ER to RNA polymerase II to elicit targeted gene transcription (Myers and Kornberg, 2000; Malik and Roeder, 2005). The ER-binding sites are now known to be generally located at the distal enhancers that are often tens of kilo-bases away from the transcription initiation sites. Recently, the Mediator complex has also been implicated in chromosome looping, which is a process that brings the distal enhancers and promoters in proximity to one another in 3-dimensional space (Bick et al., 2019)

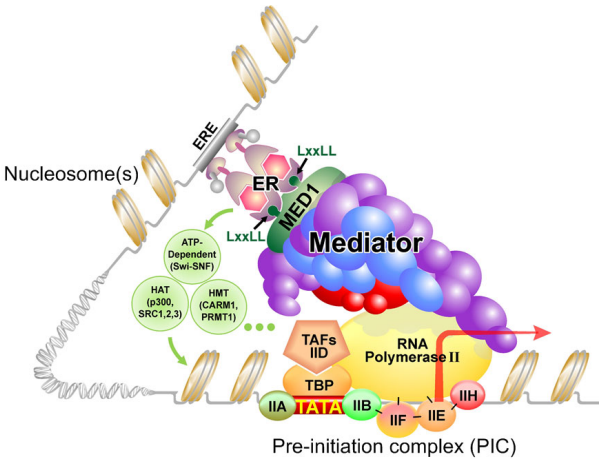

Fig. 1 MED1/Mediator complex in estrogen receptor (ER)-mediated gene transcription
Ligand-bound ER recruits diverse transcriptional co-factors, such as chromatin remodeling factors, histone modifying enzymes, and ultimately the MED1/Mediator complex, to relay signal(s) to RNA polymerase II and general transcription factors (GTFs) to initiate targeted gene transcription. The ER directly interacts with Mediator through MED1 and its two classical LxxLL motifs/NR-boxes

3 MED1/Mediator

Mediator complex is a multi-subunit complex of 25–30 subunits that regulates the activity of many transcription factors through direct-paired interactions with its distinct subunits (Fondell et al., 1996; Myers and Kornberg, 2000; Boube et al., 2002; Blazek et al., 2005). The Mediator complex in mammalian cells was first identified through its ligand-dependent interaction with the thyroid hormone receptor (TR) and is evolutionarily conserved from the previously identified yeast Mediator complex. Evidence from multiple laboratories has confirmed the direct interactions between ER and the Mediator complex through the MED1 subunit and a requirement (of MED1) for ER-dependent gene transcription in both in vitro transcription assays and cultured cells (Wärnmark et al., 2001; Kang et al., 2002; Zhang et al., 2005). Importantly, knockdown of MED1 expression abolishes the expression of ER-dependent reporter genes and the endogenous genes, but not the expression of the genes that are controlled by other transcriptional activators, such as p53 (Zhang et al., 2005). Notably, knockdown of MED1 impairs not only the expression of these ER-target genes, but also the estrogen-dependent growth of breast cancer cells (Zhang et al., 2005).

Interestingly, it was found that MED1 does not exist in all Mediator complexes, rather, in a small sub-population of less than 20% of the total complexes (Zhang et al., 2005). Importantly, it was found that MED1/Mediator complex is selectively recruited to endogenous ER-target gene promoters over the Mediator complexes that lack the MED1 subunit, upon estrogen stimulation (Zhang et al., 2005). Thus, ultraviolet (UV) stimulation led to increased recruitment of core Mediator complexes but not MED1 levels on p21 promoter of the target gene, p53 (Zhang et al., 2005). Further, mass spectrometry analysis of the MED1-containing Mediator complex revealed the presence of at least eight additional subunits and interacting proteins, along with RNA polymerase II enrichment (Zhang et al., 2005). Subsequent studies have identified one such new protein, arginine and glutamate-rich 1 (Arglu1), that not only co-localizes with MED1, but also interacts directly with the not-well-characterized C-terminal domain of MED1 (Zhang et al., 2011). Importantly, Arglu1 knockdown led to significant inhibition of breast cancer cell growth and anchorage-dependent and -independent colony formation (Zhang et al., 2011).

4 Tissue-specific role of MED1 and its LxxLL motifs in vivo

ERs directly interact with the two classical LxxLL motifs, or "NR-Boxes," of MED1 in a ligand-dependent fashion (Fig. 1) (Heery et al., 1997; Kang et al., 2002; Savkur and Burris, 2004; Plevin et al., 2005). These LxxLL motifs are also found in a number of other ER coactivators such as SRCs/p160 family members (SRC1, 2, 3), CBP/p300, PGC-1, etc. (Heery et al., 1997; Savkur and Burris, 2004; Plevin et al., 2005). While the LxxLL sequence primarily mediates interactions with ER, the sequences flanking the LxxLL motifs play key roles in determining binding specificity. Importantly, studies have demonstrated that a mutation in the AF-2 domain of ER results in the loss of interaction only with SRC1 and not MED1, suggesting that these LxxLL motifs could potentially serve as a therapeutic target to block specific pairing of ER-coactivator interactions for tissue- and gene-specific inhibition of the ER pathway (Acevedo et al., 2004; Hall and McDonnell, 2005; Jiang et al., 2010). Several studies have supported the role of LxxLL motifs in nuclear receptor-mediated transcription and other functions in vitro and in cultured cells; however, little is known about their in vivo role and function(s).

To examine the in vivo function and the underlying molecular mechanisms of MED1 LxxLL motifs, we generated MED1 LxxLL motif-mutant knock-in mice (Jiang et al., 2010). These mice have MED1 LxxLL motifs mutated to LxxAA, which has previously been confirmed to disrupt the interaction between ER and MED1/Mediator in vitro. Surprisingly, the resultant MED1-mutant progeny were both viable and fertile, with most apparent defects found only during pubertal mammary gland development (Jiang et al., 2010). Importantly, these mutations did not affect the protein expression level of MED1 or estrogen production. Further studies revealed that the MED1 LxxLL motif mutations resulted in a loss of sensitivity of mammary epithelial cells to estrogen stimulation and an impaired expression of selective

ER-target genes. Importantly, estrogen-stimulated mammary duct growth in these mutant knock-in mice was severely blunted in vivo when compared to that of the wild-type mice. However, estrogen-responsive weight gain of uteri was unaffected in these mice, demonstrating a tissue-specific function of MED1 and its LxxLL motifs in mediating ER functions in vivo. Moreover, it was observed that MED1 is expressed only in luminal, and not basal mammary epithelial cells and MED1 LxxLL motifs are required for the maintenance and differentiation of the mammary luminal progenitor cell (Jiang et al., 2010). Together, the data strongly supported a previously unexpected tissue-, cell-, and gene-specific function(s) of MED1 in regulating ER-signaling in vivo.

5 Roles of MED1 in breast cancer metastasis and stem cell formation

MED1 was found to be overexpressed in about 50% of all primary breast cancers and breast cancer cell lines (Zhu et al., 1999; Leonard et al., 2019). In addition, *MED1* gene is located within 17q12 region of the chromosome 17, also known as the "*HER2* amplicon" that is amplified in approximately 20%–25% of the human breast tumors (Zhu et al., 1999; Luoh, 2002). Fluorescence in situ hybridization and subsequent studies further confirmed that *MED1* co-amplifies with *HER2* in essentially all instances examined. Moreover, tissue microarray (TMA) analysis of human breast cancer samples indicated that the expression level of MED1 strongly correlated with *HER2* status (Cui et al., 2012). However, it is still unknown if *MED1* or any other gene(s) that co-amplified with *HER2*, participates in *HER2*-mediated breast tumorigenesis in vivo. To examine that, Yang et al. (2018) crossed the MED1 LxxLL-mutant knock-in mice with mammary tumor-prone MMTV-HER2 mice whose tumorigenesis was driven by the oncogene, *HER2*, under the control of an MMTV promoter.

This cross revealed that the MED1 LxxLL motif mutations significantly delayed mammary tumor onset by an average of about 16 weeks when compared to the wild-type control mice (Yang et al., 2018). Furthermore, tumors of MED1-mutant mice grew much slower with an overall lower tumor weight as compared to the controls. Importantly, hematoxylin and eosin (H&E) staining of the lung tumors of these MED1-mutant mice displayed a significant loss in the number of metastatic lung nodules. Transwell assays of the isolated tumor cells indicated disrupted migration and invasion capabilities of MED1 LxxLL motif-mutant cells, whereas RT-PCR analysis showed a loss in EMT-related gene expression. Furthermore, the MED1 LxxLL motif-mutant tumor cells were less capable of forming mammospheres and the flow cytometry analyses revealed reduced cancer stem cell (CSC) content. Further mechanistic analyses demonstrated that the MED1-mutant tumor cells had a lower expression of both traditional ER-target genes including *IGF-1* and *cyclin D1*, and *HER2* activated ER-target genes like *LIF* and *ACP6* (Lupien et al., 2010; Yang et al., 2018). Importantly, supplementation of IGF-1 can largely restore the above mutant phenotypes in both normal mammary gland development and tumorigenesis. Taken together, the findings of this extensive study elucidated MED1 LxxLL motifs as a key determinant in *HER2*-mediated breast tumorigenesis, and further highlighted MED1 and its LxxLL motifs as promising tissue-specific therapeutic targets.

6 MED1 in anti-estrogen resistance

HER2 amplification and activation have been recognized as major contributors to anti-estrogen therapy resistance; however, the underlying molecular mechanisms are not fully understood (Shou et al., 2004; Fu et al., 2019). Given the co-amplification and correlation between *HER2* status and the expression of MED1 in breast cancers, the role of MED1 in anti-estrogen resistance was further examined. Previous studies have reported MED1 to be phosphorylated and activated by the MAP kinase pathway at two key threonine residues (T1032 and T1457) (Pandey et al., 2005). Since mitogen-activated protein (MAP) kinase pathway is a key downstream pathway in the HER2 signaling cascade, Cui et al. (2012) performed the experiments to examine if *HER2* overexpression and activation could activate MED1. It was found that phosphor-MED1 levels are significantly higher in HER2-positive, BT474 breast cancer cells than in MCF-7 cells. In addition, overexpression of *HER2* in MCF-7 cells is sufficient to increase MED1

phosphorylation. Importantly, both HER2 and MAP kinase inhibitors were able to disrupt this HER2-mediated phosphorylation of MED1. Of note, MED1 knockdown re-sensitized these HER2-positive breast cancer cells to tamoxifen treatment. Mechanistically, it was observed that in HER2-overexpressing cell lines, phosphorylated MED1, instead of the transcriptional co-repressors (N-CoR and SMRT), was recruited to the gene promoter of ER-responsive gene (*TFF1*) in the presence of tamoxifen (Fig. 2). Conversely, mutation of the MED1 phosphorylation sites (T>A) resulted in restored tamoxifen-induced N-CoR and SMRT recruitment (Cui et al., 2012).

Similarly, subsequent study reported that MED1 knockdown rendered the otherwise resistant breast cancer cells sensitive to another anti-estrogen, fulvestrant, both in vitro and in orthotopic xenograft mouse models (Zhang et al., 2013). Taken together, these findings support a major role of MED1 as a point of crosstalk between HER2- and ER-signaling pathways and for MED1's function in HER2-mediated anti-estrogen resistance in breast cancer. Consistent with that, *MED1* has been identified as a gene associated with poor treatment outcome, and high MED1 expression correlates with poor survival of breast cancer patients that have undergone anti-estrogen therapy (Nagalingam et al., 2012; Ross-Innes et al., 2012). Intriguingly, recent studies have revealed an increased frequency of MED1 mutations in the circulating tumor DNA in breast cancer patients following anti-estrogen and anti-HER2 treatments (Murtaza et al., 2013). Together, data from these studies demonstrate how MED1 could serve as a potential target to overcome the therapeutic resistance (prevalent in breast cancer) and to improve the treatment outcomes of these patients.

7 Targeting MED1 by RNA nanotechnology

With the recognition of MED1 as a key determinant in the emergence of therapeutic resistance of human breast cancers, recent studies have been set forth to investigate its role as a potential target to overcome such resistance using novel RNA nanotechnology (Germer et al., 2013; Zhang et al., 2017). RNA nanotechnology is a burgeoning field of nanoscale RNA-based moieties with a diverse array of diagnostic and therapeutic capabilities (Guo, 2010; Jasinski et al., 2017). Recently, 3-way junction (3-WJ) packaging RNA (pRNA) nanoparticles, derived from bacteriophage Phi29 DNA packaging motors, have been extensively characterized (Shu et al., 2011; Leonard et al., 2019). These RNA nanoparticles range in size between 10 and 100 nm. These size(s) allow the nanoparticles to avoid renal clearance while maintaining the ability to effectively target and penetrate tumor tissues. RNA nanoparticles are highly modifiable and provide incorporation of different therapeutic and diagnostic agents including imaging probes, riboswitches, non-coding RNAs (small interfering RNAs (siRNAs), microRNAs (miRNAs)), RNA aptamers, etc. They are highly stable due to the use of 2'-fluorination of the RNA bases and have even shown resistance to even direct RNase exposure. Additionally, RNA nanoparticles have demonstrated significant bioavailability and favorable pharmacokinetic profiles in both pre-clinical and clinical models, while eliciting little or no immune response and toxicity. These advantages highlight the promising use of RNA nanoparticles as multi-functional targeted delivery systems for specific and selective delivery of anti-cancer agents.

In their study, Zhang et al. (2017) employed the 3-WJ RNA nanodelivery system and incorporated a HER2-targeting RNA aptamer, along with two different MED1-targeting siRNAs (Fig. 3). Atomic force microscopy demonstrated the homogenous formation of nanoparticles as expected. Biochemical characterization of the pRNA-HER2apt-siMED1 nanoparticles revealed high thermo-stability with a melting temperature (T_m) of approximately 70 °C. These RNA nanoparticles displayed resistance to RNase A, serum, and 8 mol/L urea exposures. In vitro analyses of the pRNA-HER2apt-siMED1 nanoparticles demonstrated their successful uptake by HER2-overexpressing BT474 breast cancer cells (and not the triple negative MDA-MB-231 cells). However, HER2 aptamer mutations resulted in a loss of the nanoparticle uptake by BT474 cells. In vivo distribution analyses indicated a significant accumulation of pRNA-HER2apt-siMED1 nanoparticles in the orthotopically xenografted BT474 tumors after systemic administration. Further, this study revealed that most of the nanoparticles were enriched in HER2-overexpressing tumors with only residual levels of nanoparticles in liver and kidneys.

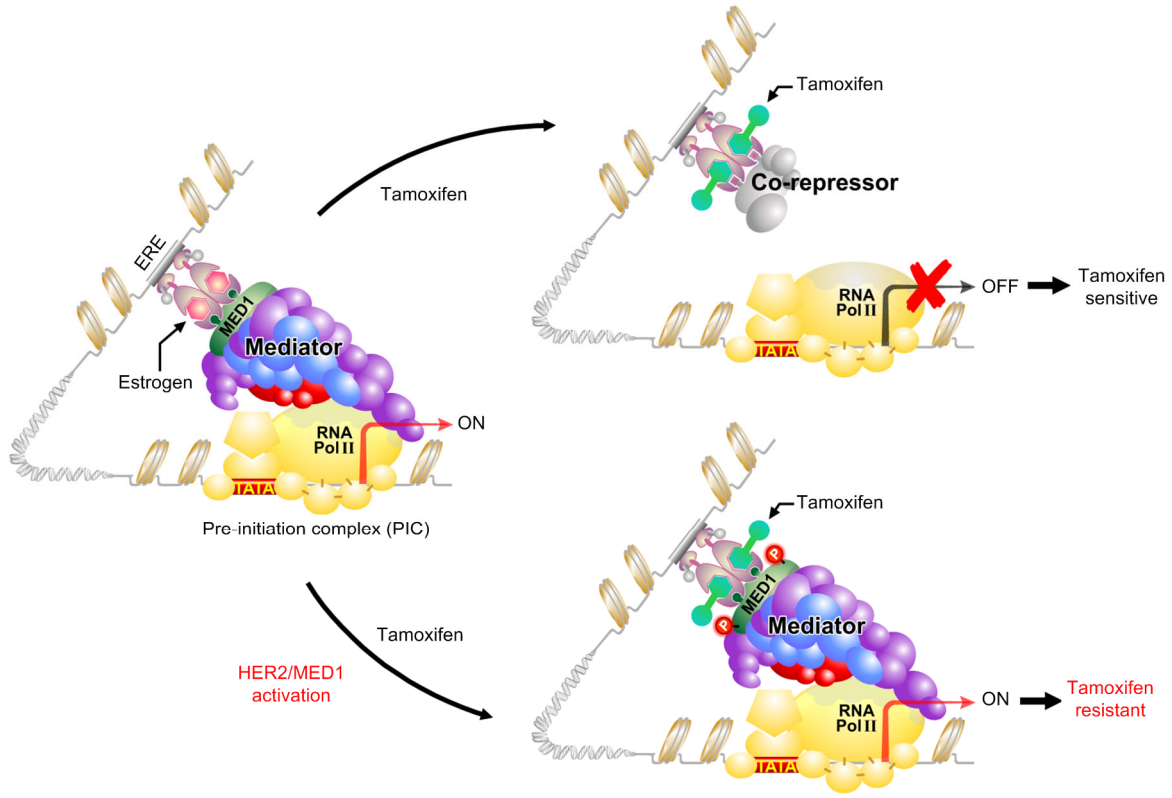

Fig. 2 Molecular mechanisms of HER2/MED1 crosstalk in anti-estrogen therapy resistance

HER2-mediated phosphorylation and MED1 activation led to the recruitment of phosphor-MED1 and MED1/Mediator complex, instead of transcriptional co-repressors (N-CoR/SMRT), to the target gene promoter even in the presence of anti-estrogens such as tamoxifen

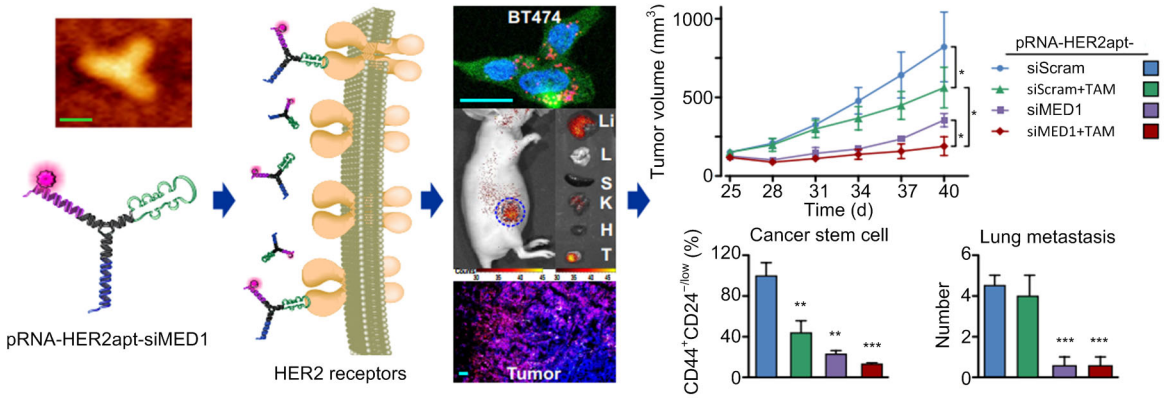

Fig. 3 Targeting MED1 by pRNA nanoparticles to overcome anti-estrogen resistance of human breast cancer cells

pRNA nanoparticles harboring a HER2-specific RNA aptamer and two siRNAs against MED1 were constructed and tested for efficacy both in vitro and in vivo in orthotopic xenograft models. These highly stable and bio-safe nanoparticles not only targeted the breast cancer cells specifically both in vitro and in vivo, but also immensely inhibited tumor growth, cancer stem cell formation, lung metastasis, and re-sensitized human breast cancer cells to anti-estrogen tamoxifen treatment. Scale bars: green, 10 nm; blue, 25 μm. Adapted with permission from Zhang et al. (2017), copyright (2017) American Chemical Society

These pRNA-HER2apt-siMED1 nanoparticles diminished MED1 expression in HER2-overexpressing breast cancer cells as compared to the controls both in vitro and in vivo (Zhang et al., 2017). Transwell assays displayed that the migration and invasion capabilities of BT474 cells were disrupted. Also, reverse transcription-polymerase chain reaction (RT-PCR) analyses demonstrated a loss in the expression of key ER-responsive genes like *TFF-1*, *c-Myc*, and *cyclin D1*. Importantly, combinational treatment of both tamoxifen and pRNA-HER2apt-siMED1 nanoparticles led to improved inhibitory effects of tamoxifen along with an enhanced disruption of the metastatic potential of these cells. Significantly, systemic injection of pRNA-HER2apt-siMED1 nanoparticles in the orthotopic xenograft mouse models led to a dramatic decrease in tumor growth, metastasis, and cancer stem cell formation (Fig. 3). These effects were even greater when the MED1-targeting RNA nanoparticles were combined with tamoxifen treatment in vivo. Importantly, these RNA nanoparticles demonstrated highly desirable biosafety with no significant effect on the overall viability, body weight, or the histopathology of major organs including heart, spleen, lung, liver, etc.

8 Conclusions and future perspective

The studies discussed above firmly establish MED1 as a key regulator of the cellular processes involved in breast cancer tumor growth, metastasis, cancer stem cell formation, and therapeutic resistance. Although the current studies focus on MED1 and its crosstalk with HER2-mediated signaling due to their co-amplification, MED1 is known to be overexpressed in about 50% of breast cancers, and its interactions with other signaling pathways could play important roles in other cancer subtypes as well. Future studies on these interactions and the molecular mechanism underlying MED1 overexpression in these cancer subtypes could provide novel insights. Given the highly tissue-specific roles of MED1 LxxLL motifs in vivo and its potent requirement for breast cancer tumorigenesis, future development of innovative approaches to target this motif and ER/MED1 interaction could provide unique therapeutic agents for breast cancer treatment and avoiding unwanted side effects on other vital estrogen-responsive tissues. With rapid development of genome-wide analysis methods, including single cell approaches, understanding the molecular pathways (upstream and downstream) and functions of MED1 at a genome level could also provide additional potential therapeutic targets. Combining these prospects with our current development of RNA nanotechnology for clinical applications, we expect to move rapidly toward the safer and more effective next generation therapies to overcome these major obstacles in current breast cancer treatment.

Contributors

Marissa LEONARD and Xiaoting ZHANG wrote and revised this review.

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Compliance with ethics guidelines

Marissa LEONARD and Xiaoting ZHANG declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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中文概要

题　目：乳腺癌组织特异性治疗靶标 MED1

概　要：在这篇综述中，我们讨论了雌激素受体共激活子转录中介体亚基 1（MED1）作为独特的组织特异性辅因子在乳腺癌干细胞形成、转移和治疗抗性中的关键作用。此外，我们详细介绍了目前使用最先进的 RNA 纳米技术靶标 MED1 的成果，并提出了未来的展望。

关键词：转录中介体亚基 1（MED1）；中介体；雌激素受体；乳腺癌；内分泌治疗抗性；RNA 纳米技术