Roles for miRNAs in endocrine resistance in breast cancer

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Abstract

Therapies targeting estrogen receptor alpha (ER α), including selective ER modulators such as tamoxifen, selective ER downregulators such as fulvestrant (ICI 182 780), and aromatase inhibitors such as letrozole, are successfully used in treating breast cancer patients whose initial tumor expresses ER α . Unfortunately, the effectiveness of endocrine therapies is limited by acquired resistance. The role of microRNAs (miRNAs) in the progression of endocrine-resistant breast cancer is of keen interest in developing biomarkers and therapies to counter metastatic disease. This review focuses on miRNAs implicated as disruptors of antiestrogen therapies, their *bona fide* gene targets and associated pathways promoting endocrine resistance.

Key Words

- ▶ antiestrogen
- ▶ aromatase inhibitor
- ▶ breast cancer
- ▶ endocrine-resistance
- ▶ estrogen receptor
- ▶ miRNA
- ▶ tamoxifen

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Introduction

The sustained exposure to endogenous estrogens is involved in the initiation and progression of breast cancer (Colditz 1998). The cellular effects of estrogens are mediated by estrogen receptors alpha and beta (ER α and ER β) and their splice variants (Herynk & Fuqua 2004). Approximately 70% of primary breast tumors express ER α (Clark *et al.* 1984, Ring & Dowsett 2004), providing the rationale for the successful use of targeted endocrine therapies in breast cancer progression (reviewed in Jordan *et al.* (2014)).

Endocrine therapies including selective ER modulators (SERMs) such as tamoxifen (TAM); selective ER downregulators (SERDs) such as fulvestrant (ICI 182 780) and aromatase (CYP19A) inhibitors (AIs) such as anastrozole and letrozole, are the frontline adjuvant therapies in treatment of women with ER α + breast tumors (Regan *et al.* 2011). These therapies have resulted in substantial improvements in outcomes and quality of life of breast cancer survivors (Jordan *et al.* 2011). Adjuvant TAM therapy

with TAM was the mainstay for ER α + breast cancer management until clinical trials comparing TAM with AIs that block the conversion of androgens to estrogens (Santen *et al.* 2009) were proven to provide a significant increase in disease-free survival (Cuzick *et al.* 2010, Regan *et al.* 2011). Unfortunately, the effectiveness of TAM and AI therapy is limited, as seen in the relapse of ~40% of patients (1998). When resistance occurs, it is unclear which subsequent endocrine therapy is most appropriate (Choi *et al.* 2015). Fulvestrant is used as a second-line therapy for patients with metastatic breast cancer, after developing AI or TAM resistance (Johnston *et al.* 2005, Perey *et al.* 2007). Endocrine resistance can be intrinsic (*de novo*) or acquired (reviewed in Clarke *et al.* (2003)). In intrinsic resistance, patients are initially unresponsive to endocrine therapies due to lack of ER α , while in acquired resistance, patients become unresponsive after the initial 5-year treatment, even though ER α is still expressed (Dowsett *et al.* 2010). Biological mechanisms underlying *de novo* and acquired

resistance are therefore of considerable clinical significance. Overall, the mechanisms of endocrine resistance are many and include amplification of multiple growth factor signaling pathways (Ring & Dowsett 2004, Riggins *et al.* 2005, Bedard *et al.* 2008, O'Brien *et al.* 2009, Palmieri *et al.* 2014). Recently, ER α ligand-binding domain (LBD) mutants that are intrinsically active in the absence of ligand were identified in AI-resistant metastatic disease, but not TAM-resistant (TAM-R) metastases (Li *et al.* 2013a, Robinson *et al.* 2013, Toy *et al.* 2013), providing a new impetus for understanding ER α 's role in driving metastatic disease (Jordan *et al.* 2015). This review will focus on the role of microRNAs (miRNAs) in acquired endocrine-resistant breast cancer.

miRNAs or miRs are small (22 nt), non-protein coding RNAs first identified over a decade ago (Iorio & Croce 2012). Their dysregulation has been implicated in many diseases, including breast cancer (Iorio & Croce 2012). Post-transcriptionally, miRNAs regulate the expression of target genes and are novel candidates for clinical development as therapeutic targets and biomarkers. The role of noncoding RNAs and miRNAs in breast cancer and endocrine-resistant breast cancers have been recently reviewed (Hayes & Lewis-Wambi 2015, van Schooneveld *et al.* 2015). Here we will review studies demonstrating dysregulation of miRNAs linked to endocrine resistance that result in breast cancer progression. We also describe the *bone fide* targets of these miRNAs and the molecular pathways dysregulated in conferring resistance. We will summarize miRNAs with predictive and prognostic potential in endocrine-resistant breast cancer.

Overview of ER pathways in breast tumors

The biological effects of estrogens, including estradiol (E₂), are mediated by binding to nuclear receptors ER α and ER β and their splice variants, such as ER α 36 and ER α 46 and G-protein-coupled ER (GPER). The ER α activation initiated by E₂ binding and consequent conformational changes results in 'nuclear/genomic' or 'non-genomic/membrane-initiated' responses (Watson *et al.* 2012, Levin 2014). ER α is upregulated in breast tumors (Clark & McGuire 1988). It is the target for therapeutic agents originally termed antiestrogens because they compete with E₂ for ER α binding, but now termed SERMs and SERDs because of our greater understanding of their molecular actions (Jordan *et al.* 2014). Because the role of ER β in breast cancer remains to be clearly established (reviewed in Thomas & Gustafsson (2011)) and because ER α expression is higher than ER β in breast tumors and is thus the target

of therapeutic intervention, this review will focus primarily on ER α activities related to miRNA expression and activity in endocrine resistance.

In the non-nuclear/membrane-initiated ER pathways, E₂ rapidly alters intracellular signaling pathways culminating in changes in gene transcription by processes mediated by plasma membrane (PM)-associated ER α , ER β , or GPER/GPR30 (reviewed in Riggins *et al.* (2005), Arpino *et al.* (2008), Filardo *et al.* (2008), Watson *et al.* (2012), and Levin (2014)). These rapid responses include E₂ activation of PI3K and Src in the PM, which then activate mTOR through PI3K-mediated AKT phosphorylation. Membrane ER and GPER activate epidermal growth factor receptor (EGFR) with downstream signaling through Ras/Raf and MAPK (Razandi *et al.* 2003). E₂ activation of EGFR can increase ER α phosphorylation (reviewed in Arpino *et al.* (2008), Johnston (2010), and Renoir *et al.* (2013)). These signaling pathways subsequently regulate ER transcriptional activity. Despite the numerous studies on ER α , the events and overall processes, including their regulated gene targets, are not completely understood.

Mechanisms of endocrine therapy in breast cancer

Antiestrogen therapies function by two main mechanisms: targeting ER α activity and/or stability. SERMs, e.g., TAM, raloxifene (RAL), and toremifene, compete with E₂ for binding the LBD of ER α and inhibit ER α transcriptional activity in a gene- and cell-specific manner. SERMs can be agonists or antagonists depending on the tissue and gene. For example, SERMs are agonists for ER α in the endometrium and increase endometrial tumor incidence (Gottardis *et al.* 1988, Fornander *et al.* 1989). As antagonists, SERMs are used in the treatment of breast cancer patients with ER α + breast tumors (Baum *et al.* 1983). SERDs, i.e., fulvestrant (ICI 182 780, Faslodex), not only alter the ER α conformation, but stimulate ER protein degradation (Jordan & Brodie 2007, Osborne & Schiff 2011, Zhao & Ramaswamy 2014). AIs inhibit the activity of aromatase (CYP19A1), thus reducing estrogen synthesis in peripheral adipose tissues and within the tumor (Zhao & Ramaswamy 2014). Examples of AIs include letrozole and anastrozole, which are steroidal/irreversible inhibitors, and exemestane, a non-steroidal/reversible inhibitor (Zhao & Ramaswamy 2014).

For postmenopausal women with ER α + primary tumors, ASCO guidelines recommend AI therapy and for premenopausal women TAM for 10 years (Smith 2014). Adjuvant therapy with TAM for postmenopausal women

with endocrine-responsive breast tumors effectively reduced the odds of recurrence by 40% and death from breast cancer after 5 years by 20% (1998). Unfortunately, 40–50% of patients initially responsive to TAM develop TAM resistance (Ring & Dowsett 2004). Likewise, a similar proportion of patients develop AI-resistance (Johnston *et al.* 2005, Hayashi & Kimura 2015). This indicates that additional mechanisms evolve to promote breast cancer progression in the absence of estrogen signaling.

Overview of mechanisms of endocrine resistance

A number of molecular mechanisms have been implicated in promoting endocrine resistance (Fig. 1; Ring & Dowsett 2004, Musgrove & Sutherland 2009, Nagaraj & Ma 2015). For example, ER α expression is silenced by methylation, resulting in reduced ER α (Martinez-Galan *et al.* 2014). Mutations in ER α (Herynk & Fuqua 2004) or increased expression of truncated forms of ER α , including ER α 36 (Deng *et al.* 2014), are potential mechanisms in acquired resistance. Alterations in ER α coregulators, e.g., increased expression of AP1 and nuclear factor kappa B (NF κ B), are associated with endocrine resistance (Johnston *et al.* 1999, Zhou *et al.* 2007). Crosstalk between ER α and amplification or activation of receptor tyrosine kinases (RTKs), including EGFR and insulin-like growth factor receptor, have been implicated in endocrine resistance (Arpino *et al.* 2008). Overexpression of HER2 (ERBB2) can elicit TAM resistance (Arpino *et al.* 2008), although HER2+ tumors are of a distinct molecular genotype from luminal A/ER α + breast tumors (Sorlie *et al.* 2001). Apoptotic and cell survival signals are also dysregulated in TAM-R cells (Riggins *et al.* 2005). A more extensive review of mechanisms promoting endocrine resistance can be found in several reports (Musgrove & Sutherland 2009, Hasson *et al.* 2013, Zhao & Ramaswamy 2014). Additional factors are continually identified as playing roles in endocrine resistance.

miRNA biogenesis

miRNAs are evolutionarily conserved, small, non-coding, 22 nt RNAs that post-transcriptionally regulate gene expression by binding to the 3'-UTR of mRNAs to repress transcription or promote degradation (Iorio & Croce 2012). There are an estimated 2588 miRNAs arising from intragenic or intergenic regions of the human genome (June 2014; <http://www.mirbase.org/>; Kozomara & Griffiths-Jones 2014). Intragenic, i.e., intronic or exonic, miRNAs, which constitute about half of all miRNAs (Berillo *et al.* 2013),

originate from within protein coding genes and can therefore have a shared promoter (and/or transcriptional start site (TSS)) and are expressed simultaneously with their host protein-coding transcript (Rodriguez *et al.* 2004, Baskerville & Bartel 2005). However, later findings suggest this may not be the case and the regulation of intronic miRNA transcription can occur independent from the host gene (Gennarino *et al.* 2009, Marsico *et al.* 2013). Intergenic miRNAs tend to have their own promoters (Gennarino *et al.* 2009). Determining these TSSs will be essential in understanding the regulation of miRNA expression. Sixty percent of all human protein coding genes are regulated by miRNAs (Friedman *et al.* 2009), and because miRNAs regulate multiple mRNAs, they are implicated as key regulators in a variety of cellular processes, including cell differentiation, cell death development, proliferation, and metabolism (Bartel 2004).

miRNA biogenesis occurs through canonical and non-canonical pathways. In the canonical pathway of miRNA biogenesis, the primary RNA transcript (pri-miRNA) is transcribed from DNA by RNA polymerase II. Pri-miRNA is further processed to a hairpin precursor transcript (pre-miRNA; ~70 nt) by a microprocessor complex comprised of Drosha (RNase III enzyme) and associated DiGeorge syndrome critical region gene 8 (DGR8) (Han *et al.* 2004). DGR8 anchors and recognizes the miRNA region for endonuclease cleavage by Drosha (Kim *et al.* 2009, Fukunaga *et al.* 2012). The Drosha microprocessor complex is also implicated in miRNA-independent functions including regulation of heteronuclear ribonucleoproteins and alternative splicing (Macias *et al.* 2013). Pre-miRNA is exported from the nucleus by Exportin 5 (a RanGTP-dependent dsRNA-binding protein (Bohnsack *et al.* 2004)) to the cytoplasm where it is further processed by another RNase III enzyme, DICER, in conjugation with trans-activation response RNA-binding protein and protein activator of the interferon-induced protein kinase (PACT; also known as PRKRA) results in a small dsRNA duplex (~22 nt) (Chendrimada *et al.* 2005, Kim *et al.* 2009). One of the duplex strands is included in the RNA-induced silencing complex (RISC), where it recognizes and binds mRNA, resulting in mRNA degradation or translational repression depending on the extent of complementarity (Huntzinger & Izaurralde 2011). The core RISC is composed of four Argonaute (Ago) proteins with AGO2 endonuclease activated upon recruitment of target mRNAs (Meister *et al.* 2004).

In the non-canonical pathway, miRNAs (miRtrons) are processed by spliceosomes in an RNase III (Drosha)-independent manner (reviewed in Yang & Lai (2011)). The intermediate generated is further processed by lariat

Figure 1

Summary of the molecular mechanisms promoting acquired endocrine resistance. Activation and/or amplification of receptor RTKs, including insulin-like growth factor receptor (IGFR), epidermal growth factor receptor (EGFR), and HER2 have been detected in tamoxifen-resistant breast cancer cells and endocrine-resistant patient tumors. PM-associated GPER and ER α , including splice variants ER α 36/ER α 46, are increased in endocrine-resistant breast cancer cells and tumors. Activation of these receptors activate intracellular signaling cascades, including MAPK and PI3K/AKT pathways, that ultimately increase transcription of genes that promote growth and survival and resistance to apoptosis. Additionally,

these pathways increase ligand-independent ER α activation by phosphorylation. Alternatively, MAPK and PI3K/AKT can directly promote expression of non-ERE responsive genes by activating other transcription factors, e.g., AP1, not shown here. Promoter methylation of CpG islands and histone deacetylation has been shown to repress ER α expression and promote endocrine resistance. ER α , estrogen receptor α ; ERE, estrogen response element; GPER, G protein-coupled estrogen receptor; PM, plasma membrane; RB, retinoblastoma; MYC, v-myc avian myelocytomatosis viral; BCL, B-cell lymphoma; BAK, homologous antagonist killer; BIK, BCL2-interacting killer.

debranching enzyme resulting in products that appear as pre-miRNA mimics. These mimics then enter the canonical pathway as Exportin 5 or DICER substrates.

miRNAs are considered to be key players in cellular transformation and in the initiation and progression of cancer (Wiemer 2007). Selected miRNA signatures have been recognized in categorizing developmental lineages and differentiation states of different tumors (Lu *et al.*

2005, Rosenfeld *et al.* 2008). In these roles, miRNAs may function as oncogenic miRNAs (oncomiRs) or oncosuppressor miRNAs, although there is an overall down-regulation of miRNAs in tumors compared to normal tissues (Lu *et al.* 2005). Substantial effort is currently underway to understand the molecular mechanisms associated with miRNA dysregulation to assist in early diagnosis and management of breast cancer patients.

miRNA and breast cancer

Since 2005, when miRNA deregulation was first reported in breast cancer (Iorio *et al.* 2005), over 1000 studies have been published identifying and examining the role of miRNAs in breast cancer. Some of these miRNAs are regulated by E₂ and/or influence expression of estrogen-responsive genes (Ferraro *et al.* 2012, Klinge 2012, 2015). miRNAs have been implicated in regulating hallmarks of breast cancer (reviewed in Goh *et al.* (2015)), including cell proliferation, cell death, apoptosis, immune response, cell cycle energetics, metabolism, replicative immortality, including senescence, invasion, metastasis (reviewed in Negrini & Calin (2008), O'Day & Lal (2010), Singh & Mo (2013), and McGuire *et al.* (2015)), and angiogenesis (reviewed in Cortes-Sempere & Ibanez de Caceres (2011) and Goh *et al.* (2015)).

Models for miRNA investigation

The process of investigating miRNAs involved in endocrine resistance usually begins with an initial profiling of miRNA differences between endocrine-sensitive vs -resistant breast cancer cell lines or between breast tumors from patients responsive and non-responsive to endocrine therapies. Methods used in these studies have included microarrays, RNA sequencing, and the relatively recent high-throughput sequencing of RNA isolated by cross-linking immunoprecipitation (HITS–CLIP) method with confirmation by quantitative real-time PCR (qPCR). These methods result in the acquisition of huge amounts of data that require integrative analysis and further confirmation. Computational approaches are then utilized to predict possible targets and signaling pathways that are aberrantly regulated by the identified miRNAs. Functional analysis utilizing ectopic expression and forced repression of miRNA expression are performed to validate the role of deregulated miRNAs in tumorigenesis and/or endocrine resistance *in vivo* and *in vitro*. The targets of miRNAs are further confirmed by cloning the target 3'-UTR downstream of a luciferase reporter, transfecting a cell line with this reporter plasmid to validate direct inhibition, and western blots and qPCR. Target validation in clinical samples provides human significance to the study. These models have identified miRNAs involved in endocrine resistance that are summarized in Tables 1 and 2.

There are limitations to these approaches. For example, integrative analysis for identifying aberrantly expressed miRNAs is limited by the set of computational parameters used in the study and rarely are these

parameters applied to other studies. Functional investigations need to be performed for each miRNA and its target. The acquisition of massive amounts of information, which, though relevant, do not often translate to physiological significance, necessitates further studies within clinical settings. HITS–CLIP is technically challenging and complex, requiring great skill, but has the advantage of capturing interactions 'frozen' by u.v.-crosslinking under physiological conditions without the use of exogenous crosslinking agents, which can lead to artificial interactions (Moore *et al.* 2014).

miRNAs regulating ER α protein and signaling

The role of miRNAs in promoting endocrine resistance is exemplified by, but not limited to, their involvement in regulating ER α (Fig. 2). Decreased ER α expression is involved in endocrine-resistant breast cancer progression. miRNAs, including miR-221/222 (Zhao *et al.* 2008), miR-342-3p (He *et al.* 2013), miR-873 (Rothe *et al.* 2011), and Let7b/Let-7i (Zhao *et al.* 2011), downregulate ER α protein expression (Table 1). miR-221 and miR-222 are over-expressed in TAM-R and ER α – breast cancer cell lines and tumors (Miller *et al.* 2008, Zhao *et al.* 2008, Manavalan *et al.* 2011). The 3'-UTR of ER α is a direct target of miR-221/222 decreasing ER α protein but not mRNA (Zhao *et al.* 2008). Transient overexpression of miR-221/222 in TAM-sensitive (TAM-S) MCF7 and T47D cells resulted in TAM resistance, whereas the downregulation of miR-221/222 in ER α –/TAM-R MDA-MB-468 cells restored ER α expression and sensitized cells to TAM-induced cell cycle arrest and apoptosis (Zhao *et al.* 2008). ER α is not a direct target of miR-342-3p, but loss of miR-342-3p was associated with a concomitant loss in ER α expression and resulted in TAM resistance (He *et al.* 2013). Conversely, forced overexpression of miR-342-3p sensitized MCF7 cells to TAM-induced apoptosis (He *et al.* 2013). The exact mechanism promoting loss of ER α expression upon downregulation of miR-342-3p is yet to be determined (van Schooneveld *et al.* 2015).

Increased expression of ER α splice variants has also been reported to be associated with poor prognosis and contribute to endocrine resistance (Shi *et al.* 2009, Li *et al.* 2013b). ER α 36 is an N-terminal truncated 36 kDa variant of full-length ER α (ER α 66; Wang *et al.* 2006). ER α 36 lacks AF1 and AF2 of ER α 66, retains DNA binding and dimerization domains and binds E₂, but is not inhibited by TAM or fulvestrant (Wang *et al.* 2006). ER α 36 is expressed in ER α – breast cancer cells and tumors (Zhang *et al.* 2012) and overexpressed in TAM-R breast cancer cells (Li *et al.* 2013b). Increased ER α 36 protein expression is

Table 1 miRNA associated with antiestrogen resistance in breast cancer. Experimentally proven *bona fide* targets and method used in confirming target are indicated. Proposed pathways associated with endocrine resistance are included. Method of identification of targets: 3'-UTR luciferase reporter assay (L), downregulation of protein shown by western blot (W), and downregulation of target in quantitative real-time PCR assay (Q)

miRNA	Treatment/human cell line/tissue	Comments	Targets (method of identification)	Pathway
Putative oncomiRs based on experimental data				
miR-101	0, 1, 2, 3, and 4 μ M 4-OHT 4 days in MCF7 cells	miR-101 infected cells promote growth stimulatory activity in medium lacking E_2 and TAM-R. miR-101 has growth-inhibitory activity in E_2 -containing medium (Sachdeva <i>et al.</i> 2011)	MAGI2 (L, W) (Sachdeva <i>et al.</i> 2011)	Growth factor receptor (GFR) cytoplasmic signaling
miR125b-5p	0.001–10 μ M anastrozole or letrozole in LET-R MCF7 ANA-R MCF7 vs MCF7aro cells Primary breast tumors	Upregulated in LET-R MCF7 cells, ANA-R MCF7 compared to MCF7aro; high miR-125b-5p correlated with earlier relapse in ER+/PR+ patients (Vilquin <i>et al.</i> 2015)		
miR-128a	TAM+LET-R MCF7 cells, MCF7aro cells	Upregulated in TAM+LET-R MCF7 cells vs MCF7aro cells (Masri <i>et al.</i> 2010)	TGF β R1 (L, W) (Masri <i>et al.</i> 2010)	GFR cytoplasmic signaling
miR-181b	Human breast samples 1 μ M 4-OHT, for 0, 24, 48, and 72 h; 50 nM 4-OHT, 0, 3, and 5 min T47D, TAM-R MCF7 vs TAM-S MCF7	Enhanced expression in TAM-R MCF7 cells (Lu <i>et al.</i> 2011) Anti-miR-181b suppressed TAM-R xenograft tumor growth in TAM treated mice	TIMP3 (L, W, Q) (Lu <i>et al.</i> 2011)	GFR cytoplasmic signalling
miR-205-5p	1, 10, 100 nM, 1, 10 μ M anastrozole; 1, 10, 100 nM, 1, 10 μ M letrozole; LET-R MCF7 cells, ANA-R MCF7 vs MCF7aro Primary breast tumors	Upregulated in LET-R MCF7 cells, ANA-R MCF7 compared to MCF7aro cells; high miR-125b-5p correlated with earlier relapse in ER+/PR+ patients (Vilquin <i>et al.</i> 2015)		
miR-210	Patient samples 100 nM 4-OHT, 0, 1, 2, 3, and 4 days, MCF7, MDA-MB-231	Increased miR-210 with breast tumor histological grade Higher in MDA-MB-231 compared to MCF7 cells (Rothe <i>et al.</i> 2011)		
miR-222	Human breast samples 1 μ M 4-OHT, for 0, 24, 48, and 72 h; 50 nM 4-OHT, 0, 3, and 5 min T47D, TAM-R MCF7 vs TAM-S MCF7	Anti-miR-222 suppressed TAM-R xenograft tumor growth in TAM treated mice (Lu <i>et al.</i> 2011)	TIMP3 (L, W, Q) (Lu <i>et al.</i> 2011)	GFR cytoplasmic signaling
miR-221/222	0–65 μ M 4-OHT, 6 days MCF7/TAM-R vs MCF7 measured in conditioned media (Wei <i>et al.</i> 2014)	Sixfold increase in exosomes in MCF7/TAM-S vs MCF7/TAM-R cells (Wei <i>et al.</i> 2014)	P27 and ER α (W, Q) (Wei <i>et al.</i> 2014)	ER α signalling/cell cycle
	0, 15, and 20 μ M TAM, 16 h TAM-S vs TAM-R MCF7 cells, HER2/neu (+) vs HER2/neu (–) human breast tissue	TAM increased miR-221/222 in TAM-R cells and HER2/neu (+) breast tumors compared to TAM-S MCF7 and HER2/neu (–) tumors respectively (Miller <i>et al.</i> 2008)	p27(Kip1) (W, Q) (Miller <i>et al.</i> 2008)	GFR cytoplasmic signaling/cell cycle
	0, 5, 10, and 20 μ M 4-OHT, 12, 24, and 48 h, MCF7 and MDA-MB-231		TIMP3 (W, Q) (Gan <i>et al.</i> 2014)	GFR cytoplasmic signaling

Table 1 Continued

miRNA	Treatment/human cell line/tissue	Comments	Targets (method of identification)	Pathway
	10 μ M 4-OHT, $\pm$ 1 nM E ₂ , 48 h MCF7 mammosphere transformed vs MCF7 cells as xenografts in SCID mice	Higher in MCF7 mammospheres compared to MCF7 cells (Guttilla <i>et al.</i> 2012)	ER α (W)	EMT
	100 nM fulvestrant TAM-R MCF7, Ful-R MCF7, MCF7, MCF7-Mek5, MCF7 TNR	Overexpressed in Ful-R MCF7 cells. Indirect activation of β -catenin Repression of TGF β mediated growth inhibition (Rao <i>et al.</i> 2011)		GFR cytoplasmic signaling
	0, 5, 10, 20 μ M, 10, 20 nM 4-OHT, 72 h MCF7, T47D vs MDA-MB-468 cells Breast tumor tissue	Higher in ER α — MDA-MB-468 breast cancer cells and primary breast tumors vs ER α + MCF7 and T47D cells (Zhao <i>et al.</i> 2008)	ER α (L, W) (Zhao <i>et al.</i> 2008)	ER α signaling
	100 nM 4-OHT and 100 nM fulvestrant, 6 h, 2 days, TAM-S MCF7 vs TAM-R LY2 cells	4-OHT upregulates miR-221/222 in LY2 TAM-R cells and downregulates miR-221/222 in MCF7 cells (Manavalan <i>et al.</i> 2011)	ERBB3, ER α (Q) (Manavalan <i>et al.</i> 2011)	ER α signaling/GFR cytoplasmic signaling
miR-301	300 nM 4-OHT, 24, 48, and 72 h MCF7, MDA-MB-231	Higher in MCF7, T47D, MDA-MB-231, and MDA-MB-231 compared to MCF7 10A. Higher expression in lymph node negative (LNN) invasive ductal breast cancer (Shi <i>et al.</i> 2011)	FOXF2, BBC3, PTEN (L, W, Q), and COL2A1 (L, Q) (Shi <i>et al.</i> 2011)	GFR cytoplasmic signaling
miR-519a	0, 5, and 10 μ M 4-OHT, 3 and 72 h TAM-R MCF7 vs MCF7, HEK 293FT, breast cancer patient datasets	Upregulated in TAM-R MCF7 cells compared to TAM-S MCF7 cells (Ward <i>et al.</i> 2014)	CDKN1A, RB1, and PTEN (L, W, Q) (Ward <i>et al.</i> 2014)	Cell cycle
miR-1280	Patient blood samples	Higher in blood from patients with metastatic breast cancer after cytotoxic chemotherapy or undefined endocrine therapy (Park <i>et al.</i> 2014)		
Putative oncosuppressor miRNAs based on experimental data				
Let7b/Let7i	TAM-R MCF7, MCF7, MDA-MB-231 Breast cancer tissues	Overexpression of Let7b/Let7i enhanced sensitivity of TAM-R MCF7 cells to TAM only in hormonal withdrawal medium and not in normal growth medium (Zhao <i>et al.</i> 2011)	ER α 36 (L, W, Q) (Zhao <i>et al.</i> 2011)	ER α signaling
Let7i	5, 10, 15, and 20 μ M 4-OHT, 48 h, ZR-75-1 cells	Overexpression of Let7i increased TAM-S in ZR-75-1 cells Inverse correlation of Let7i and TNF receptor associated factor 1 (TRAF1; Weng <i>et al.</i> 2014)		Apoptosis/cell survival signaling
miR-10a	Primary breast tumors	Higher expression in patient tumors was associated with longer relapse-free time. Increased expression predicted tumor relapse in TAM-treated ER+ postmenopausal breast cancer patients (Hoppe <i>et al.</i> 2013)		
miR-15a/16	100 pM E ₂ , 1 μ M 4-OHT, and 100 nM fulvestrant, E ₂ +4-OHT, E ₂ +ICI, for 24, 24, and 72 h TAM-R MCF7/HER2 Δ 16 vs MCF7/HER2 cells (Cittelly <i>et al.</i> 2010a)	Suppressed expression of miR-15a and miR-16 in HER2 Δ 16 mutant cells associated with inverse expression of BCL2 protein and mRNA and decreased sensitivity to TAM and ICI (Cittelly <i>et al.</i> 2010a)		Apoptosis/cell survival signaling

Table 1 Continued

miRNA	Treatment/human cell line/tissue	Comments	Targets (method of identification)	Pathway
miR-30a-3p	Patient samples (Rodriguez-Gonzalez <i>et al.</i> 2011)	Increased expression in ER+ primary breast tumors of patients who received TAM and showed longer progression-free survival; inverse correlation with HER2 and RAC1 cell motility signaling pathways (Rodriguez-Gonzalez <i>et al.</i> 2011)		
miR-126	Primary breast tumors	Higher expression in patient tumors was associated with longer relapse-free time. Increased expression predicted tumor relapse in TAM-treated ER+ postmenopausal breast cancer patients (Hoppe <i>et al.</i> 2013)		
miR-200b/200c	100 nM 4-OHT and 100 nM fulvestrant, 6 h, 2 days, TAM-S MCF7 vs TAM-R LY2 (Manavalan <i>et al.</i> 2013)	Decreases in TAM-R LCC1, LCC2, LCC9, and LY2 cells vs MCF7 cells (Manavalan <i>et al.</i> 2013)	ZEB1/2 (W, Q) (Manavalan <i>et al.</i> 2013)	EMT
	100 nM 4-OHT and 100 nM ICI, 6 h, 2 days, TAM-S MCF7 vs TAM-R LY2	Increased in TAM-S MCF7 and decreased in TAM-R LY2 cells (Manavalan <i>et al.</i> 2011)	CYP1B1 (Q) (Manavalan <i>et al.</i> 2011)	
miR-342-3p	24 h 100 pM E ₂ , 1 μM 4-OHT, E ₂ +4-OHT in TAM-R MCF7/HER2Δ16, MCF7/HER2, TAM-R1, LCC2 cells, breast tumors (Cittelly <i>et al.</i> 2010b)	Downregulated in TAM-R MCF7/HER2Δ16 cell, TAM-R1, LCC2 cells, and TAM refractory human breast tumors vs MCF7 cells and TAM-S tumors. TXNIP is an indirect target of miR-342 (Cittelly <i>et al.</i> 2010b)	BMP7, GEMIN4 (microarray, L, Q), and SEMAD (microarray, Q) (Cittelly <i>et al.</i> 2010b)	GFR cytoplasmic signalling
	Primary breast tumors 10 nM E ₂ , 20 μM 4-OHT, 72 h, MCF7 vs SKBR3 and MDA-MB-231 cells	Decreased in ERα– SKBR3 and MDA-MB-231 cells vs MCF7 cells Direct correlation between miRNA-342 expression and ERα expression (He <i>et al.</i> 2013)		ERα signaling
miR-375	5 μM 4-OHT TAM-R MCF7 cells vs TAM-S MCF7 cells	Lower in TAM-R MCF7 vs MCF7 cells (Ward <i>et al.</i> 2013)	MTDH (L, W, Q) (Ward <i>et al.</i> 2013)	EMT
miR-424-3p	0.001–10 μM anastrozole or letrozole; LET-R MCF7 cells, ANA-R MCF7 vs MCF7aro	Downregulated in LET-RMCF7 cells, ANA-R MCF7 compared to MCF7aro (Vilquin <i>et al.</i> 2015)		
miR-451	Primary breast tumors 1 μM 4-OHT for 0, 4, 8, and 24 h, TAM-R MCF7 vs TAM-S MCF7 cells	Reduced levels in TAM-R vs TAM-S MCF7 cells (Bergamaschi & Katzenellenbogen 2012)	14-3-3ζ (W, Q) (Bergamaschi & Katzenellenbogen 2012)	GFR cytoplasmic signaling
miR-574-3p	1 μM 4-OHT, MCF7 cells/tissue sample	Lower in TAM-R MCF7 cells and clinical breast cancer tissues compared to TAM-S MCF7 cells and adjacent normal control respectively (Ujihira <i>et al.</i> 2015)	Clathrin heavy chain (CLTC) (L, W, Q) (Ujihira <i>et al.</i> 2015)	GFR cytoplasmic signaling
miR-873	1, 10, 100 nM, 1, 5 μM 4-OHT, 7 days, MCF7/TAM-R vs MCF7 cells and xenograft tumors	Downregulated in TAM-R MCF7 and breast tumors compared to TAM-S and normal tissues respectively	Cyclin-dependent kinase 3 (CDK3) (L, W, Q) (Cui <i>et al.</i> 2014)	Repressed ERα transcriptional activity (Cui <i>et al.</i> 2014)

4-OHT, 4-hydroxytamoxifen; TAM-S, TAM-sensitive; TAM-R, TAM-resistant; LET-R, letrozole-resistant; ANA-R, anastrozole-resistant; Ful-R, fulvestrant-resistant. MCF7aro cells are MCF7 cells stably overexpressing aromatase.

proposed to be mediated by decreased let7, since transfection of let7b and let7i mimics repressed ER α 36 expression and sensitized TAM-R MCF7 cells to TAM growth inhibition (Zhao *et al.* 2011). Let7 family members are downregulated in breast cancer tissues and TAM-R MCF7 cells (Vadlamudi *et al.* 2001, Zhao *et al.* 2011).

ER α is regulated by post-translational modifications including phosphorylation, methylation, sumoylation, and palmitoylation (Li *et al.* 2003, Acconcia *et al.* 2004, Sentis *et al.* 2005, Zhang *et al.* 2013, Cui *et al.* 2014). These modifications influence ER α interaction with other molecules, including transcriptional coregulators, hence regulating gene transcription (Fig. 1). These post-translational events also contribute to endocrine resistance (Anbalagan & Rowan 2015). miRNAs are implicated in altering post-translational ER α modifications to promote TAM resistance (Cui *et al.* 2014). For example, miR-873 targets CDK3, which phosphorylates ER α at Ser104/116 and Ser118 (Cui *et al.* 2014). miR-873 expression was downregulated in TAM-R/MCF7 breast cancer cells and forced overexpression of miR-873 in these cells reversed TAM resistance and decreased xenograft tumor growth. The authors postulated that the decrease in miR-873 resulted in enhanced ER α phosphorylation and ligand-independent activity in TAM-R/MCF7 cells (Cui *et al.* 2014).

miRNA regulation of ER α protein interactors in breast cancer

ER α interacts with other transcription factors, e.g., AP1, Sp1, NF κ B, and the forkhead transcription factor (FOXO1), to regulate gene expression (Petz *et al.* 2002, Pradhan *et al.* 2010, Sanders *et al.* 2013). Increased activity of these transcription factors is associated with endocrine resistance (Johnston *et al.* 1999, Schiff *et al.* 2000, Zhou *et al.* 2007, Bergamaschi *et al.* 2014). FOXO1 is overexpressed in many cancers, including breast cancer, and its ectopic expression promotes cell invasiveness (Bergamaschi *et al.* 2014). Repression of FOXO1 was associated with increased miR-211 (Song & Zhao 2015) and miR-23a (Eissa *et al.* 2015), and repressed breast cancer cell growth, migration, and invasion in animal models.

Activation of NF κ B contributes to endocrine resistance in breast cancer (Keklikoglou *et al.* 2011). A genome-wide miRNA screen in HEK-293T cells identified 13 miRNAs regulating NF κ B transcriptional activity (Keklikoglou *et al.* 2011). Subsequent studies in MDA-MB-231 TNBC cells demonstrated that miR-570 and miR-373

inhibited TGF α -activation of NF κ B-induced transcription of pro-inflammatory cytokines, e.g., IL6, IL8, CXCL1, and ICAM1, and TGF β signaling by direct targeting of RELA and TGFBR2. The authors reported that transient overexpression of miR-520 or miR-373 inhibited TGF β -induced MDA-MB-231 cell invasion. While they did not detect miR-373 in human breast tumors, a correlation of higher miR-520c expression in ER α tumors and lower TGFBR2 transcript expression was observed, allowing the authors to suggest loss of miR-520 expression may play a role in ER α -tumor progression via altered NF κ B signaling (Keklikoglou *et al.* 2011).

The nuclear receptor co-activator proline glutamic acid leucine rich protein (PELP1) interacts with ER α (Vadlamudi *et al.* 2001) to modulate genomic (Nair *et al.* 2004) and nongenomic functions of ER α (Barletta *et al.* 2004, Vadlamudi *et al.* 2005). As a proto-oncogene, PELP1 is upregulated during breast cancer metastasis and promotes human breast tumor xenograft growth in nude mice (Vadlamudi *et al.* 2005, Rajhans *et al.* 2007, Roy *et al.* 2012). In MCF7 cells, cytoplasmic localization of PELP1 conferred resistance to TAM (Vadlamudi *et al.* 2005). Although the mechanisms of PELP1 promotion of TAM resistance is not fully known, the binding of PELP to the proximal promoters of the oncosuppressors miR-200a and miR-141 recruited histone-deacetylase 2 (HDAC2) and repressed their transcription (Roy *et al.* 2014). The attendant decrease in miR-200a and miR-141 was suggested to stimulate metastatic growth (Becker *et al.* 2015).

ER α coactivator nuclear receptor co-activator 3 (NCOA3, also known as SRC3 (Liao *et al.* 2002) and AIB1 (Anzick *et al.* 1997)), is overexpressed in 50% of breast tumors (Anzick *et al.* 1997). Targeting SRC3 is of clear clinical interest (Tien & Xu 2012). SRC3 overexpression results in constitutive activation of ER α -mediated transcription, breast tumor growth and resistance to TAM *in vivo* and in xenograft models (List *et al.* 2001, Ring & Dowsett 2004). SRC3 translation is repressed by miR-17-5p. Overexpression of miR-17-5p in MCF7 cells repressed E₂-induced proliferation and endogenous cyclin D1 transcription (Hossain *et al.* 2006). miR-195 negatively regulates SRC3 in human hepatoma cells (Jiang *et al.* 2014), but whether it does so in breast cancer is unknown.

ER α corepressors including nuclear receptor co-repressor 1 (NCoR1) influence gene transcription by recruiting HDAC complexes to promote chromatin condensation and repression of gene transcription (Lavinsky *et al.* 1998, Ring & Dowsett 2004). NCoR1 is reduced in TAM-R MCF7 xenograft tumors grown in

Table 2 Exosomal miRNAs described in breast cancer

EV miRNA composition	Direction of miRNA expression	Sample source			Time in culture prior to harvesting exosome	Comments	Targets analyzed in study	References
		Cell line	Patient	Xenograft				
miR-21, let7a, miR-100, miR-125b, miR-720, miR-1274a, and miR-1274b	↑	MCF7 MCF10A	No	No	72 h			Guzman <i>et al.</i> (2015)
miR-205	↓							
miR-10a, miR155, miR-373, miR-10b, miR-21, and miR-27a	↑	MCF7 MCF10A NmuMG	Yes	Yes	24 h 72 h	OncomiRs miR-10b and miR-21 confirmed by northern blot		Melo <i>et al.</i> (2014)
miR-10b and miR-10a, miR-218, miR10a, miR-99a, miR-142-3p; miR-32, miR-138, miR-7e, miR-106b	↑	MDA-MB-231 MCF7 MDA-MB-231 MCF10A HMLE	No	No	48 h	OncomiR	HOXD10 and KLF4 (L, W) (targets for miR-10b)	Singh <i>et al.</i> (2014)
miR-140	↓	HEK-293T MCF10DCIS	No	No	3 days			Li <i>et al.</i> (2014)
miR-29a and miR-21	↑	MDA-MB-231 HEK-293T						
miR-373, miR-101, and miR-372	↑	MCF7	Yes (serum)	No	Serum used	High in TNBC; over-expression of miR-373 promotes loss of ER and resistance to camptothecin		Eichelder <i>et al.</i> (2014)
miR-221/222	↑	MCF7 TAM-R/MCF7	No	No	72 h	Exosomal transport may be an additional mechanism by which miR-221/222 promote TAM-R	P27 (Q, W) and ER α (Q, W)	Wei <i>et al.</i> (2014)
miR-23a and miR1246	↑	MCF7 MCF7/Doc	No	No	12 h 24 h	May contribute to cisplatin resistance		Chen <i>et al.</i> (2014b)
miR-100, miR-17, miR-222, miR-342-3p, miR-451, and miR-30a	↑	MCF7 MCF7/Adr	No	No	72 h		PTEN (Q; target for miR-222)	Chen <i>et al.</i> (2014c)
Let7a, miR-328, miR-130a, miR-149, miR-602, and miR-92b	↑	MCF7/Doc MCF7 and MDA-MB-231	No	No				Kruger <i>et al.</i> (2014)
miR-198	↓							
miR-16	↑	MSCs 4T1 SVEC	No	No	48 h		VEGF (Q)	Lee <i>et al.</i> (2013)
miR-16, miR-720, miR-451, and miR-1246	↑	MCF7 MDA-MB-231	No	No	5 days			Palma <i>et al.</i> (2012)
miR-451 and miR-1246	↑	MCF7 MDA-MB-231 SKBR3 BT-20	No	No	5 days			Pigati <i>et al.</i> (2010)

Table 2 Continued

EV miRNA composition	Direction of miRNA expression	Sample source			Time in culture prior to harvesting exosome	Comments	Targets analyzed in study	References
		Cell line	Patient	Xenograft				
miR-223	↑	Macrophages SKBR3 MDA-MB-231	No	No	24–48 h	Released by macrophages and promotes breast cancer cell invasion	Mef2c (L, W)	Yang <i>et al.</i> (2011)
miR-210	↑	MCF7	No	No	48 h	Released in response to hypoxia		King <i>et al.</i> (2012)

↑, Increased expression; ↓, reduced expression. EV, extravesicular; Adr, adriamycin; Dox, doxorubicin; MSCs, mesenchymal stem cells; 4T1, mouse breast cancer cell line; SVEC, mouse endothelial cell line; NMuMG, nontumorigenic mouse mammary epithelial cells; HMLE, human mammary epithelial cell line; MCF10DCIS, MCF10 ductal carcinoma *in situ* cells.

nude mice (Lavinsky *et al.* 1998). However, both NCoR and the corepressor SMRT stimulated 4-hydroxytamoxifen (4-OHT)–ER α agonist activity on an estrogen response element-driven luciferase reporter in transiently transfected Rat-1 cells. Blockage of NCoR1 promoted the agonistic activity of TAM (Lavinsky *et al.* 1998). To our knowledge, there are no reports of miRNA regulation of NCoR1. However, inhibition of miRNA synthesis by knocking down DICER in LNCaP prostate cancer cells increased NCoR1 transcription and likewise, NCoR1 increased in the prostate of DICER^{−/−} mice (Narayanan *et al.* 2010). Conversely, ectopic expression of DICER mediates metastasis and TAM resistance in breast cancer cells (Selever *et al.* 2011).

miRNA activation of growth factor receptor signaling in endocrine resistance cancer

Endocrine-resistant breast cancer cells and tumors show increased EGFR signaling (Aiyer *et al.* 2012). Although trastuzumab is a targeted therapy widely used in patients whose breast tumors overexpress HER2, these patients also benefit from TAM (Huynh & Jones 2014). Unfortunately, overexpression of an isoform of HER2, HER2 Δ 16, which is associated with metastasis (Mitra *et al.* 2009), also promotes TAM resistance (Cittelly *et al.* 2010a,b). Decreased expression of miR-15a, miR-16, and miR-342-3p contribute to endocrine resistance in TAM-R MCF7/HER2 Δ 16 breast cancer cells (Cittelly *et al.* 2010a,b). miR-342 was also downregulated in TAM-non-responsive breast tumors and HER2-negative, TAM-R TAM-R1 and LCC2 cells (Cittelly *et al.* 2010b). Transient overexpression of miR-342 resensitized TAM-R MCF7/HER2 Δ 16 and TAM-R1 cells to TAM-induced apoptosis; decreased BMP7, GEMIN4, and SEMAD are proposed as direct miR-342 targets mediating this response. However, the role of these targets in directly promoting TAM resistance was not examined.

Decreased miR-451 also regulates mitogenic signaling to promote TAM resistance (Bergamaschi & Katzenellenbogen 2012). TAM, but not RAL or fulvestrant, downregulates miR-451 in TAM-R cells (Bergamaschi & Katzenellenbogen 2012). Downregulation of miR-451 was associated with upregulation of its target protein 14-3-3 ζ , a scaffolding protein whose high expression is correlated with early time to disease recurrence in patients treated with TAM. Overexpression of miR-451 in MCF7 cells decreased 14-3-3 ζ and reduced activation of HER2, EFGR, and MAPK signaling, resulting in decreased cell proliferation and migration and increased apoptosis.

Ectopic expression of miR-221/222 in TAM-R MCF7 and TAM-R BT474 cells increased β -catenin and relieved TGF β -mediated growth inhibition. Inhibition of β -catenin decreased estrogen-independent growth in pre-miR-221/222-transfected MCF7 cells (Rao *et al.* 2011). The TGF β signaling pathway was inhibited in letrozole-resistant, aromatase-stably transfected MCF7 (T+LET-R) breast cancer cells, and miR-128a was upregulated in these cells (Masri *et al.* 2010). miR-128a targeted and repressed TGF β receptor 1 (TGF β R1) protein expression in T+LET-R cells compared to the parental MCF7 cells stably transfected with aromatase (MCF7aro). Repression of miR-128a re-sensitized T+LET-R cells to TGF β growth inhibition.

Loss of PTEN is associated with poor outcome in HER2+ breast tumors (Stern *et al.* 2015). PTEN is downregulated by miR-101 (Sachdeva *et al.* 2011). Overexpression of miR-101 promotes MCF7 cell growth and TAM resistance in estrogen-free growth medium but suppressed cell growth in E₂-containing medium (Sachdeva *et al.* 2011). TAM resistance was mediated by Akt activation and was independent of ER α expression. miR-101 repressed its target membrane-associated guanylate kinase inverted 2 (MAGI2), a scaffolding protein required for PTEN activity, thus reducing PTEN activity leading to activation of Akt. PTEN is also a *bona fide* target of the oncomiR miR-301 (Shi *et al.* 2011). Transient repression of miR-301 in MCF7 cells decreased cell viability and sensitized cells to TAM (Shi *et al.* 2011).

miRNAs as cell cycle regulators in endocrine-resistance

SERMs can be cytostatic and cytotoxic by promoting G1-phase cell cycle arrest (Subramani *et al.* 2015). miR-221/222 (Miller *et al.* 2008) and miR-519a (Ward *et al.* 2014) have been implicated in altering expression of molecular regulators of the cell cycle to promote endocrine resistance. miR-221/222 represses p27 to promote TAM resistance in breast cancer cells (Miller *et al.* 2008, Wei *et al.* 2014).

Recently, miR-519a was reported as a novel oncomiR by increasing cell viability and cell cycle progression (Ward *et al.* 2014). miR-519a was upregulated in TAM-R MCF7 cells compared with TAM-S MCF7 cells. Elevated levels of miR-519a in primary breast tumors were associated with reduced disease-free survival in ER α + breast cancer patients and miR-519a was suggested to contribute to TAM resistance. Knockdown of miR-519a in TAM-R MCF7 cells sensitized the cells to TAM growth inhibition. Concordantly, overexpression of miR-519a

in TAM-S MCF7 cells desensitized the cells to TAM by preventing growth inhibition while promoting caspase activity and apoptosis. Tumor suppressor genes involved in PI3K signaling CDKN1A (which encodes p21), RB1, and PTEN, were reported to be *bona fide* targets of miR-519a, although the role of these targets in mediating TAM-R have not been explored.

miRNAs in epithelial-to-mesenchymal transition

Changes involved in tumor progression include acquisition of migration/invasion, gain of front-rear polarity, resistance to anoikis, and mesenchymal transition (Howe *et al.* 2012). Genetic changes that occur during epithelial-to-mesenchymal transition (EMT) include but are not limited to activation of SNAIL, increased zinc-finger E-box-binding 1 (ZEB1), reduced E-cadherin and increased vimentin and N-cadherin (Lamouille *et al.* 2014). EMT is also implicated as a mechanism by which tumors enact resistance to TAM (Steinestel *et al.* 2014).

To identify miRNAs that mediate TAM resistance, we used a microarray to identify miRNAs differentially regulated between endocrine-sensitive MCF7 cells and an endocrine-resistant MCF7 variant LY2 cells, with selected results confirmed by qPCR (Manavalan *et al.* 2011). Among these, miR-200a, miR-200b, and miR-200c were found to be downregulated in LY2 cells and other TAM-R breast cancer cell lines (LCC9) compared with parental TAM-S MCF7 cells (Manavalan *et al.* 2011, 2013). The decrease in miR-200 family expression was associated with increase in ZEB1. ZEB1 is an EMT-inducing transcription factor that represses E-cadherin (Hurteau *et al.* 2007). Ectopic expression of miR-200b and/or miR-200c altered LY2 morphology to a more epithelial-like phenotype and inhibited cell migration. These phenotypic changes were associated with repression of the mesenchymal markers N-cadherin, vimentin, and ZEB1 and an increase in the epithelial marker E-cadherin. Further, upregulation of miR-200b/200c or ZEB1 knockdown sensitized LY2 cells to TAM- and fulvestrant-induced growth inhibition. However, overexpression of miR-200b/200c in MCF7 cells did not promote resistance to TAM or fulvestrant, indicating that cellular changes in addition to down-regulation of miR-200 family members are involved in TAM resistance in LY2 cells.

In another miRNA microarray study, miR-375 was found to be downregulated in a mesenchymal TAM-R MCF7 cell line model (Ward *et al.* 2013). Re-expression of this miR-375 sensitized TAM-R cells to TAM and reduced

invasiveness by decreasing expression of mesenchymal markers fibronectin, ZEB1, and SNAIL2 while increasing the epithelial markers E-cadherin and ZO1. This resulted in partial reversal of EMT called mesenchymal-to-epithelial transformation. Metadherin (MTDH), a cell surface protein upregulated in breast tumors that mediates metastasis (Brown & Ruoslahti 2004), was identified as a direct, *bona fide* miR-375 target mediating this response. TAM-treated patients whose primary tumor showed high MTDH showed shorter disease-free survival and a higher risk of relapse. This study exemplifies the role of miRNAs in mediating cellular transformations that foster tumor progression and TAM resistance.

EMT allows the emergence of cancer stem cells (CSCs) that have properties including self-renewal potential and tumorigenicity (Singh & Settleman 2010, Ward *et al.* 2013). Mammosphere culture is widely utilized to enrich the population of mammary epithelial stem cells and breast CSCs *in vitro* (Dontu *et al.* 2003, Charafe-Jauffret *et al.* 2009). Mammosphere culture of MCF7 cells (MCF7_M cells) resulted in permanent EMT with increased miR-221/222 and loss of their target ER α mRNA expression (Guttilla *et al.* 2012). MCF7_M cells were also characterized by downregulation of epithelial-associated tumor suppressor miRNAs including miR-200c, miR-203, and miR-205. MCF7_M cells were resistant to TAM-induced cell death. These data reinforce other studies discussed above demonstrating a role for increased expression of miR-221/222 in driving TAM-R.

miRNAs as regulators of apoptosis/ cell survival signaling

Tumor growth reflects a balance between cell growth and cell death. Endocrine inhibitors activate apoptotic and stress signals to inhibit breast cancer cell growth (Mandlekar & Kong 2001, Riggins *et al.* 2005, Musgrove & Sutherland 2009). However, the molecular mechanisms behind these observations are yet to be fully defined. Activation of antiapoptotic proteins such as BCL2, cross-talk between apoptotic effects of antiestrogens and the TNF α pathway and promotion of survival signals including PI3k/Akt and NF κ B have been documented to promote resistance endocrine therapy (Riggins *et al.* 2005). miRNAs can directly target antiapoptotic transcripts or regulate mediators of the survival signaling pathways. For example, low miR-15a/16 expression correlated with upregulation of BCL2 in TAM- and fulvestrant-treated TAM-R MCF7/HER2 Δ 16 cells and xenograft tumor promotion *in vivo* (Cittelly *et al.* 2010a). RNAi targeting of BCL2 or

reintroduction of miR-15a/16 decreased TAM-induced BCL2 expression in TAM-R MCF7/HER2 Δ 16 cells resulting in TAM-induced decrease in cell growth and promotion of apoptosis. Conversely, repression of miR-15a/16 in TAM-S MCF7/vector or MCF7/HER2 increased BCL2 expression and promoted resistance to TAM by inhibiting apoptosis and preventing growth inhibition.

To identify potentially ethnic group-specific TAM-S biomarkers, Weng *et al.* performed an integrative genomic analysis on 58 African-derived HapMap YRI lymphoblastoid cell lines (YRI LCLs; breast cancer cells; Bradley & Pober 2001). Genetic variants (including 50 SNPs with effects on 34 genes and 30 miRNAs) were identified to be sensitive to endoxifen, an active metabolite of TAM. Among the genes identified, increased TNF receptor-associated factor 1 (TRAF1) and decreased let7i expression correlated with endoxifen resistance in 44 YRI LCLs. TRAFs are intracellular signal transducers for death receptor superfamily TNF receptor (TNFR; Bradley & Pober 2001). TRAF1 associates with TRAF2 to form a protein complex that interacts with inhibitor-of-apoptosis protein (IAP) to mediate anti-apoptotic signals (MAPK8/JNK and NF κ B) from the TNFR (Wang *et al.* 1998, Wajant *et al.* 2003). Repression of TRAF1 or overexpression of let7i in ZR-75-1 luminal breast cancer cells enhanced sensitivity to TAM and decreased the number of viable cells. These data show that by regulating death signals, miRNAs can also mediate response to antiestrogens.

Extracellular vesicular (exosomes) transport of miRNAs in endocrine resistance

Extracellular vesicles (EVs), produced by outward budding of the PM, contain proteins and nucleic acids that can be transported in blood between tissues and cells (Chiba *et al.* 2012, Gong *et al.* 2012). The contents of EVs can facilitate tumor growth including angiogenesis, invasion, metastasis, and immune suppression. They also play a role in reducing effectiveness of drugs (Chen *et al.* 2014a). Exosomes are smaller EVs of endosomal origin and formed by the fusion of multivesicular bodies with PMs (Johnstone *et al.* 1987, Jung *et al.* 2012). Exosomes transport miRNAs in circulation. Mechanisms of exosomal formation and delivery are cell-specific and display proteins from their tissue of origin and are specific to the target cells (Clayton *et al.* 2001, Simpson *et al.* 2009, Braicu *et al.* 2015). It was initially reported that miRNAs were randomly packaged into exosomes with no specific miRNA preferentially incorporated (Valadi *et al.* 2007, Skog *et al.* 2008). Studies now indicate different miRNAs

are associated with specific customized exosomes (Palma *et al.* 2012). However, the selection mechanism for incorporating miRNAs into exosomes is yet to be elucidated.

EVs or exosomal delivery of miRNAs is thought to play roles in breast tumorigenesis and metastasis (Table 2). Cell culture studies showed that exosomes secreted by TAM-S MCF7 cells were larger in size and number compared to TAM-R MCF7 cells and were taken up by the TAM-S cells (Wei *et al.* 2014). This study showed that miR-221/222 were released from exosomes into TAM-S MCF7 cells, resulting in the reduction of their target genes p27 and ER α and enhanced TAM resistance. Transfection of a miR-221/222 inhibitor in MCF7 cells treated with TAM-R MCF7 exosomes reduced TAM resistance. These data again support the importance of miR-221/222 in TAM resistance in MCF7 cells.

The potential use of exosomal miRNAs as candidate biomarkers for cancer diagnosis and prognosis remains to be definitively proven. The manipulation of exosomal miRNAs suggests a new therapeutic approach for drug delivery, but requires further research.

miRNAs of unknown function in endocrine resistance

Other miRNAs identified by microarray or library screen and confirmed by qPCR to promote antiestrogen resistance in breast cancers, but having undetermined functional roles include: miR-10a, miR-21, miR-22, miR-29a, miR-181a, miR-125b, miR-205, which mediate resistance to TAM (Manavalan *et al.* 2011), and miR-125a and miR-877, which also mediate TAM resistance (Ujihira *et al.* 2015). Other miRNAs identified by integrative analysis to make up network clusters that contribute to antiestrogen resistance include: miR-146a, miR-27a, miR-145, miR-21, miR-155, miR-125b, and let7s (Xin *et al.* 2009).

Roles of Drosha, DICER, and AGO2 in endocrine resistance

As described earlier in this review, Drosha and DICER function in miRNA processing. The role of Drosha in endocrine resistance has not been ascertained, despite the observation that reduced cytoplasmic Drosha is predictive of better endocrine therapy response (Khoshnaw *et al.* 2013).

Loss of DICER is predictive of a better response to endocrine therapy (Khoshnaw *et al.* 2012). Elevated DICER

was associated with TAM resistance in metastatic breast tumors and tumor xenografts (Selever *et al.* 2011). DICER-overexpressing cells were enriched with the breast cancer resistance protein (BCRP), a member of the ATP-binding cassette transporter superfamily that causes resistance to several chemotherapeutic agents (Doyle & Ross 2003). Increased BCRP resulted in a more efficient efflux of TAM in DICER-overexpressing cells compared to control cells. Inhibition of BCRP inhibited TAM efflux and restored TAM-S in DICER-overexpressing cells. In ER α - breast cancer cells, DICER is targeted by oncomiRs including miR-103/107 (Martello *et al.* 2010), let7, miR-222/221,

Figure 3
Higher expression of AGO2 is statistically associated with decreased relapse-free survival in all breast cancer cases and in patients whose primary tumors are ER α + / PR+. The Kaplan-Meier plots of AGO2 expression in breast tumors with breast cancer survival were generated using <http://kmplot.com/analysis/index.php?p=service> (Gyorffy *et al.* 2010). (A) All breast tumors, $n = 3557$ and (B) ER α + / PR+, $n = 701$.

and miR-29a (Cochrane *et al.* 2010). Whether repression of DICER by these miRNAs mediates endocrine resistance is yet to be determined.

AGO2 recruits mRNA and miRNA into the RISC and is the catalytic component of the RISC. AGO2 is elevated in ER α – compared to ER α + breast cancer cell lines and tumors (Adams *et al.* 2009). Expression of AGO2 is mediated by ER α /estrogen signaling and EGFR/MAPK signaling pathways (Adams *et al.* 2009). Ectopic expression of full length AGO2 in MCF7 cells promoted cell proliferation, reduced cell–cell adhesion, and increased cell migration (Adams *et al.* 2009). Whether AGO2 plays a role in endocrine resistance is yet to be determined.

We examined the association of AGO2 expression and overall survival rate in breast cancer patients using the online survival analysis tool, Kaplan–Meier plotter (<http://kmplot.com/backup/breast>; Györffy *et al.* 2013). It assesses the association gene expression on breast cancer prognosis using microarray data from 3554 patients. The patient data are from GEO. Higher AGO2 expression correlates with reduced relapse-free survival in all breast cancer patients and those whose primary tumors are ER+/PR+ (Fig. 3).

Conclusion

miRNAs are dysregulated in endocrine-resistant breast cancer and these miRNAs regulate specific genes in growth-promoting, apoptosis-resistant, and EMT pathways that result in TAM and AI resistance (Tables 1 and 2). The involvement of exosomes containing miRNAs in mediating endocrine resistance provides a new target for biomarker identification and therapeutic intervention to block metastatic spread. Although identifying new miRNAs mediating endocrine resistance is important, research effort is needed to determine the mechanisms and functional roles of already identified miRNAs with unknown roles in endocrine resistance and to develop targeted therapeutics to counter miRNA dysregulation and enhance hormonal sensitivity.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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