Chapter 4

MicroRNA in Breast Cancer — Gene Regulators and Targets for Novel Therapies

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Additional information is available at the end of the chapter

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1. Introduction

MicroRNAs (miRNAs) are a recently discovered class of endogenously expressed, single stranded, non-protein coding RNAs of about 19-25 nucleotides in length, that bind to the 3` un-translated regions (UTR) of target messenger RNAs (mRNAs) through complementarity with the first 2-8 nucleotides at the 5` end of the miRNA. They play important roles in diverse biological and pathological processes, through the regulation of gene expression at both transcriptional and post-transcriptional level [1,2]. The miRNA-mediated gene regulation is part of a larger mechanism known as RNA interference which involves other regulatory RNAs; small interfering RNAs (siRNA), which induce silencing of specific mRNA through complementary nucleotide sequences, and piwi-interfering RNAs (piRNAs) which, through similar mechanisms, induce silencing of active mobile elements to maintain germ line integrity and fertility. To date, over 1000 miRNAs and 16,228,619 predicted mRNA target sites have been identified, affecting over 30% of the human genome [3]. What makes them important players in regulating protein expression is the ability of a single miRNA to interact with more than one target gene (due to the imperfect matching between miRNA and its target which still produces a functional effect). In addition, a single gene can be regulated by multiple miRNAs. Currently, around 200 human transcription factor-miRNA relationships have been described and collated into the TransmiR database [4]. These include transcription factors such as Nanog and Oct3/4, [5], hormones such as estradiol [6], and tumor suppressor genes such as p53 [7]. Since the initial reports of the miRNAs *lin-4* and *let-7* as developmental regulators of *Caenorhabditis elegans* (*C. elegans*) [8,9], there have been numerous studies describing the involvement of miRNAs in normal cellular function as well as in various disease conditions, such as cardiac arrhythmias [10,11], fibrosis [12,13], remodeling [14,15], metabolic disorders [16], diabetes [17],

Alzheimer and Parkinson`s disease [18], autoimmune disorders (e.g. systemic lupus erythematous, rheumatoid arthritis and multiple sclerosis) [19], stroke [20] and schizophrenia [21].

This chapter will summarise and discuss recent evidence elucidating the role of these newly discovered regulators of gene expression in cancer pathogenesis, with particular emphasis on breast cancer.

1.1. Biogenesis of miRNAs

The biogenesis of mature miRNA is a multistep process which requires the contribution of various enzymes, binding proteins and transporters. In the nucleus, miRNAs are transcribed from either intra-or inter-genic regions by RNA polymerase II to form the primary miRNAs (pri-miRNAs), structures of approximately 1-3 kb in length [22]. These are initially cleaved by RNase III enzyme Drosha, and the double-stranded RNA-binding partner protein Pasha (DiGeorge Syndrome Critical Region 8 Protein), into stem-loop structures of approximately 50-70 nucleotides, with a 3` overhang of a few nucleotides, termed precursor-miRNAs (pre-miRNAs) [23,24]. These pre-miRNAs are then transported into the cytoplasm by a nuclear exporter protein termed Exportin-5 (RanGTP-dependent dsRNA-binding protein) [25]. Once in the cytoplasm, the pre-miRNAs are further cleaved from their terminal loops into double-stranded oligonucleotides of approximately 18-24 bp in length into mature miRNA, by RNase-III Dicer: miRNA* duplexes [26]. These strands are then separated and one of them becomes a mature miRNA molecule to be incorporated with several argonaute (AGO) and other proteins into an RNA-induced silencing complex (RISC) which either perfectly or imperfectly hybridises with its target mRNA. In case of near-perfect to perfect matching, this results in mRNA cleavage and degradation by the action of mRNA processing bodies [27,28,29,30], while translational inhibition or sequestration of mRNA from the translational machinery results in the case of imperfect matching [27,31]. In either event, the end result is ultimately reduced protein levels. In vertebrates, most of the miRNA-mRNA interactions are of imperfect complementarity at the 5` end seed sequence [32], unlike the plant miRNA-mRNA interactions which generally target via perfect complementarity [33]. It has been suggested that the miRNA star strand is often degraded, but some evidence suggests that it plays a role in the regulation of miRNA homeostasis and other downstream effects [34,35]. For example, ectopic expression of miRNA-24-2 star strand in the estrogen receptor (ER) positive breast cancer cell line MCF-7, results in suppression of cell survival, through the targeted suppression of protein kinase Cα (PKCα), and decreased tumor formation when injected into nude mice [36]. Recent evidence suggests the existence of alternative miRNA biogenesis pathways not involving Drosha activity, from introns that bear hairpin structures similar to Drosha processed pre-miRNAs. These miRNAs are termed mitrons [37,38,39]. Both pathways merge at the point of cytoplasmic transfer via Exportin-5. In addition, another new group of miRNAs (termed smitrons) has been described as splicing-independent mitron-like miRNA, which require Drosha activity but not splicing, DGCR8 or Dicer activity [40,41,42,43]. Their subsequent mechanism of processing into the RISC is unclear. These events are illustrated in Figure 1.

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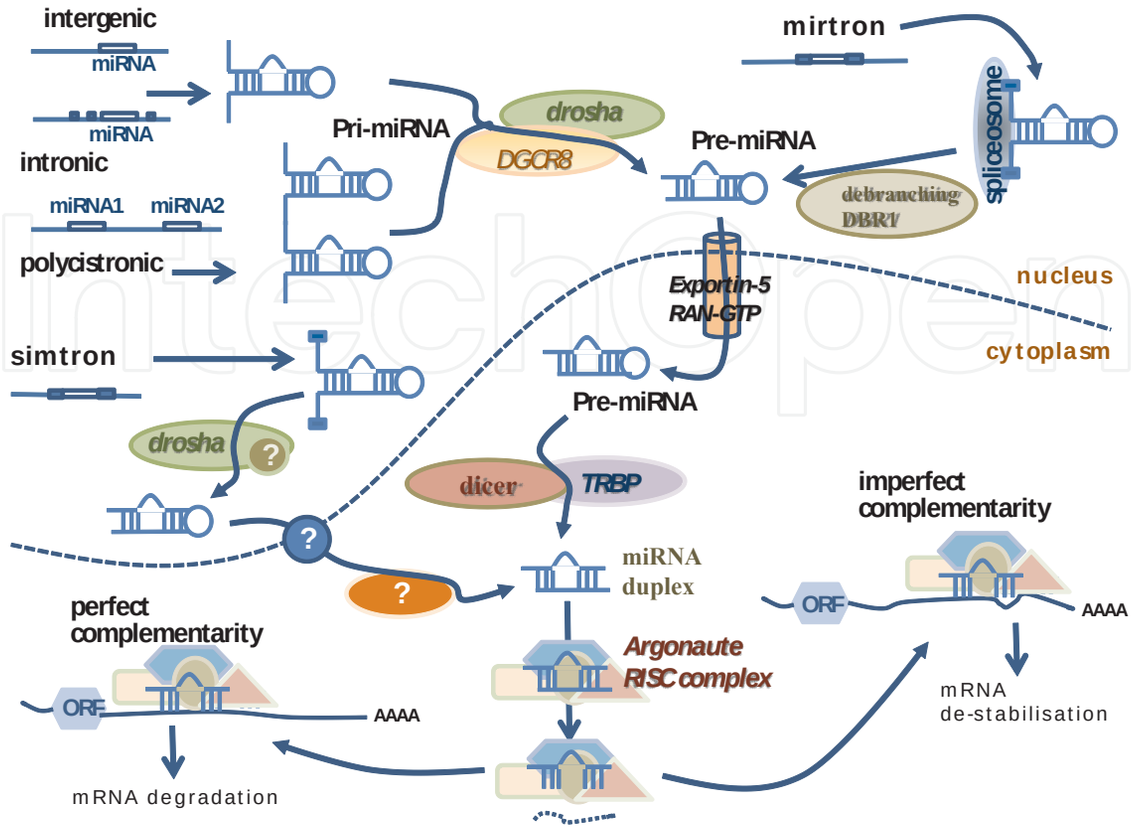

Figure 1. miRNA biogenesis. Following transcription by RNA polymerase II from intergenic, intronic or polycistronic regions, the primary transcripts (pri-miRNA) are processed by drosha/DGCR8 enzyme complex into approximately 70 nucleotide pre-miRNA hairpin structures. Two other pathways involving short intronic hairpins have been described; mirtrons that are spliced and processed via a debranching enzyme (DRB1), and simtrons that are processed by drosha in cooperation with an unknown factor. All enter the cytoplasm through Exportin-5/RAN-GTP activity (except for the simtron-derived molecule which is processed by other undefined mechanisms) and are further processed by dicer/TRBP into a duplex form which then associates into an RNA induced silencing complex (RISC). Within this, RNase H activity degrades the passenger strand and the antisense strand guides the complex to its target mRNA sequence in the 3′ UTR sequence downstream of the open reading frame (ORF). Perfect base pairing results in mRNA degradation, and permitted imperfect matching, in mRNA de-stabilisation, both of which result in translational blockade.

1.2. Physiological role of miRNAs

Aside from their major function of inducing mRNA target gene degradation or translational inhibition, several specific actions of miRNAs have been reported. One such is in senescence-associated transcriptional gene silencing; an event triggered by cancer-initiating or promoting events, through repression of proliferation promoting genes regulated by a retinoblastoma protein (RB)/E2F repressor complex. For example, AGO2, RB1 and *let-7* interact to repress RB1/E2F-target genes in senescence in premalignant cancer cell lines, which may contribute to tumor suppression [44]. In addition, miRNA can *positively* regulate gene expression by targeting promoter elements of protein coding genes, a phenomenon known as RNA activation (RNAa). For example, transfection of miRNA-373 and its precursor (pre-miRNA-373) into the prostate cancer cell line PC-3, resulted in the induction of E-cadherin and cold-shock domain-containing protein C2 (CSDC2) expression [45]. Also, miRNA-744,-1186 and-466d-3p can

induce the expression of cyclin B1 in mouse cell lines and lead to chromosomal instability and tumor suppression *in vivo* [46].

A diverse range of biological processes appear to be at least partly regulated by miRNAs. These include early development and developmental timing [47,48], hematopoietic lineage differentiation [49], cellular differentiation, proliferation and apoptosis [50,51,52], development and function of innate [53,54,55,56] and adaptive [57,58,59,60] immune response, neurotransmitter synthesis [61], viral replication [62], insulin secretion [63] and cardiac rhythm [64].

1.2.1. Development

Genetic deletion studies indicate that some developmental processes are absolutely dependent on miRNAs. In *C. elegans*, miRNAs play a role in locomotion, body size and egg laying [65]. For example, *lin-4* and *let-7* control the timing of larva development [8,66]; severe defects in germline development and embryonic morphogenesis was evident in *dcr-1* mutant worms [67]. In Drosophila, cell division defects were evident in *dicer-1* mutant germline stem cells, with marked reduction in the rate of germline cyst production and cell cycle control (delayed G1 to S transition) [68]. Mice null in Direc-1 and AGO-2, did not survive beyond embryonic day (ED) 7.5 [69]. Some miRNAs show temporal expression profiles during mouse prenatal development (at ED 9.5, 10.5 and 11.5); miRNA-2 and-193 showed specific expression in mouse embryo at ED 10.5 suggesting a role in developmental transitions [70]. In human embryonic stem cells, about 14 miRNAs were found to interact with developmental transcription factors such as POU class 5 homeobox 1 (OCT4), sex determining region Y (SRY)-Box 2 (SOX2), and Nanog Homeobox (NANOG) [70]. Some miRNAs, such as 142 and 181, were shown to be specifically expressed in hematopoietic tissues, suggesting a role in morphogenesis [71], while Dicer and AGO gene family (mainly AGO-1 and 2) transcripts were restrictively expressed at ED 11.5 and 14.5 in specific organs including brain, neural tube, limb, lungs and hair follicles, with significant expression in lung tissues undergoing branching morphogenesis [72]. Dicer-1 deficient mouse lungs exhibit defective morphology, with significant apoptosis in the epithelium [73].

Involvement of miRNAs in the development of the cardiovascular system [74] is reflected by variable expression/activity of miRNAs such as-126,-143,-145, and-218 [75]. miRNA-1 has a unique expression profile in cardiac myocytes and plays a critical role in heart development, by influencing cardiac morphogenesis, electrical conduction and cell-cycle control [76]. It is strongly expressed during heart development between ED 8.5-11.5 and represses the expression of heart and neural crest derivatives expressed-2 (Hand-2) transcription factors, which are responsible for ventricular cardiomyocyte differentiation [77]. In the nervous system, Dicer deficient zebra fish show defects in neuronal cell differentiation and development [78]. In mammals, miRNA-124 and-128 are highly expressed in neuronal progenitor cells and mature neurons, and are considered to be the main regulators of neuronal development [79,80,81]. miRNA-124a is thought to constitute 25-50% of the total brain miRNA population, and is implicated in switching brain progenitor cells into a neuron lineage [82]. miRNA-134 plays a role in central synaptic function [83,84]. miRNAs are also implicated in the development of skeletal muscles; miRNA-1 is abundantly expressed in the muscle progenitor cells and

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differentiating muscle [77], and facilitates myotube formation [85] by interacting with muscle differentiating factors such as serum response factor (SRF), myocyte enhancer factor-2 (MEF-2) and myogenic regulatory factors (MRFs) [77].

1.2.2. Differentiation

miRNAs have been shown to play an important role in the differentiation of pluripotent embryonic stem (ES) cells which gives rise to more than 200 cell types in the adult body. Differential expression has been observed during ES cell differentiation, with decrease in miRNA 290-295 cluster and-296, and increase in miRNA-21 and-22 [86]. Mice oocytes with targeted deletion of Dicer, failed to produce any miRNA, resulting in failure of cell division, in part due to disorganized spindle formation, reflecting the importance of maternal miRNAs in the earliest stages of embryonic development [87]. Over-expression of miRNAs 290-295/302 could overcome the proliferation defects of Dgcr8 mutant mouse ES cells, whereas over-expression of *Let-7* could rescue them from their differentiation defects [79]. Reduced expression of *Let-7* was seen in breast tumor initiating cells (BT-IC), and its forced expression markedly reduced BT-IC proliferation and the proportion of undifferentiated cells, with subsequent reduction in tumor size and metastasis through reduced expression of its targets, H-RAS and HMGA2 [88].

2. Involvement of miRNAs in etiology of cancer

Numerous miRNAs are involved in controlling the activity of intracellular signaling molecules (e.g. MAPK, PI3K/PTEN, NFκB, TGFβ, Notch, and Hedgehog) which are critical in regulating multiple processes linked to cancer pathogenesis such as proliferation, apoptosis, angiogenesis and immune function, emphasizing their potential value in cancer classification, as diagnostic biomarkers for staging, predictive markers of prognosis and response to therapy, and as therapeutic targets [89,90,91]. For example, miRNA-21 is described as a positive-feedback regulator of MAPK/ERK1/2 pathways. Its own expression is induced by the activation of ERK1/2, whose activity it then increases by repressing negative regulators of ERK/MAPK. Stimulation of HER2/neu signaling enhances MAPK/ERK phosphorylation, which results in enhanced miRNA-21 levels and increased invasive capacity of HER2/neu expressing breast cancer cells, by repression of the metastasis suppressor protein; programmed cell death 4 (PDCD4) [92]. The miRNA-200 family have been reported to target the downstream mediators of the TGF-β pathway, ZEB-1 and-2, resulting in inhibition of the epithelial to mesenchymal transition process (EMT), with subsequent suppression of metastasis in various cancer cell lines including those of the breast [93,94]. Interestingly, ZEB1 reduces the expression of the miRNA-200 cluster and hence promotes EMT in a feed-forward manner [95]. *Let-7* directly targets the Ras proto-oncogene which plays a major role in cancer pathogenesis [96]. Phosphatase and tensin homolog (PTEN), considered one of the main negative regulators of the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) pathway, is targeted by various miRNAs including miRNA-21,-26a,-221, and-222 [90]. miRNA-21 enhances proliferation, survival and migration in cancer cells through targeting of PTEN, leading to enhanced PI3K

activity [97]. The role of the transcription factor, NFκB, is well established in cancer pathogenesis, and some evidence suggests that several miRNAs, such as-301,-146,-155, and-9, indirectly activate it by inhibiting the NFκB repressing factor (NKRF) [98,99].

A multiplicity of factors, that include chromosomal instability, genomic mutations and polymorphisms, epigenetic changes, alterations in synthetic pathways, promoter methylation, or changes in the activity of their transcriptional factors, modify the expression of miRNAs [29,86]. For example, single nucleotide polymorphism (SNP) type mutations in miRNA-146a can predispose for development of various tumors [100,101,102] including those of the breast [103]. Specific G/C polymorphisms (rs2910164) in miRNA-146a precursor leads to increased production of the mature form, which binds to and modulates the BRCA1 and BRCA2 genes, whose activity is a predisposing factor for early onset familial breast cancer [103].

Although miRNAs have been found to be both over-as well as under-expressed in cancer cells as compared with normal tissues, the more frequent observation is one of decreased expression. For example, whereas miRNA-21 is elevated, miRNAs-126,-143, and-145 are all decreased in most (~ 80%) types of tumors [104]. This means that miRNAs can function both as oncogenes (e.g.-9,-17-92 cluster,-21,-27a,-103,-106,-107,-125b, and-155) or more often, as tumor suppressor genes (e.g. *let-7*,-15a, 16-1, 23b, 29a/b/c, 34a, 124, 133, 137, 143, 145, 192, and 215) [102,105,106,107,108,109,110,111].

Analysis of dysregulated miRNA expression may also have prognostic relevance in many cancers; for example, metastatic breast tumors show elevated miRNA-10b and reduced miRNA-126,-206, and-335 levels [112,113]. A recent report suggested that higher expression of miRNA-126 and-10a in breast cancer patients was associated with longer relapse-free survival [114]. The detection of circulating miRNA in plasma and serum also presents these molecules as potential novel biomarkers for cancer and other diseases. A pilot study showed that miRNA-155 serum levels could be a significant index to distinguish patients with breast cancer from healthy individuals, and serum levels of miRNA-34 could indicate disease prognosis [115]. Another report [116] suggested that four miRNAs,-215,-299-5p,-411, and-452, that were differentially expressed between serum samples from patients with metastatic breast cancer and healthy volunteers, could be used as biomarkers for detection and staging; requiring only a blood sample. Heneghan *et al* [117] demonstrated increased serum levels of miRNA-195 in breast cancer patients (as compared with healthy control subjects), which were then decreased (together with *let-7a*) after curative tumor resection. Molecular classification of non-BRCA1/2 hereditary breast tumors into four distinct subgroups, on the basis of their miRNA expression profiles, was used in a recent report to search for novel susceptibility pathways in hereditary breast cancer [118]. Furthermore, high expression of *let-7*, miRNAs-21,-23, and-27a has been linked with drug resistance in ovarian cancer [119]. miRNA-452 was shown to be significantly down-regulated in adriamycin-resistant, as compared with the parental MCF-7 breast cancer cells; modulating its level partially reversed the adriamycin-resistance, by targeting insulin-like growth factor-1 receptor (IGF-1R) [120].

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3. Role of miRNAs in EMT

3.1. Pathways of the EMT process

Cellular transition from epithelial to mesenchymal phenotype (EMT) and *vice versa* (MET) was first identified as a physiological event occurring during embryonic development [121]. Currently, it is well established that the EMT process is a hallmark event occurring in a number of disease conditions including breast cancer [122]. In our laboratory, it has been demonstrated that EMT can be induced in breast cells *in vitro,* in parallel with development of endocrine resistance induced by blockade of ERα function, and this results in enhanced cellular proliferative and invasive capacity [123,124]. During the EMT process *in vivo*, individual epithelial cells lose their cell-cell and cell-matrix contacts and apico-basolateral polarity, and gain a mesenchymal phenotype which enables them to dissociate from the tumour mass, invade into and interact with the extracellular matrix (ECM) before entering blood and lymphatic vessels. Many phenotypic changes occur during this process; these include loss of cell-cell adhesion as a result of reduced E-cadherin and catenins expression in adherens junctions, reduced claudins and occludins expression at tight junctions and reduced expression of various epithelial cytokeratins such as KRT8, 18 and 19, which presumably aids in disruption of cytoskeletal connections that maintain tissue architecture [123]. Various transcription factors such as WNT, NOTCH, TWIST, ZEB1/TCF8, ZEB2, SNAIL, SLUG, GOOSECOID, FOXC1/2, E12/E47 and TCF3, and downstream mediators of several growth factor receptors such as TGFβ, IGF1R, epidermal growth factor receptor (EGFR), platelet derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR), and signaling molecules PI3K/AKT, mTOR, ERK/MEK, and MAPK all play important roles in the EMT process, which has been described in detail previously [122,123,125]. In addition, the stroma of neoplastic tissues and hypoxia can also induce EMT through the production of hypoxia induced factor (HIF-1α) [123].

3.2. miRNAs implicated in the EMT process in breast cancer

miRNA can either induce or inhibit the EMT process by modulating various target genes (Figure 2)

3.2.1. Inducers of EMT

miRNA-9: miRNA-9 is up-regulated in breast cancers relative to normal tissues [126]. It showed a thousand-fold increase seen in c-*myc*-induced mouse mammary tumours [127], and its level was significantly elevated in primary breast tumours from patients with diagnosed metastases, in comparison with those from metastasis-free patients [128]. This is consistent with its higher expression in HER2+and triple-negative (ERα-ve/progesterone receptor (PR)-ve and HER2-ve) tumours, in comparison to luminal subtypes, and in tumours with advanced T stage, high histologic grade, p53 over-expression and high proliferation index, as well as in tumors with mesenchymal-like phenotype (high vimentin, low E-cadherin) [129]. Ectopic expression of miRNA-9 leads to an EMT-like conversion in human mammary epithelial cells

in vitro. These become scattered with spindle-like morphology and exhibit a significant decrease in E-cadherin and increase in vimentin [128].

miRNA-24: TGF-β acts both as a tumor suppressor in early-stage adenomas, through its ability to inhibit cell growth and, as an important promoter of the EMT process during late stages of cancer progression [130]. Expression of miRNA-24 was significantly increased in breast cancer cell lines which had undergone TGF-β-induced EMT through targeting the guanine nucleotide exchange factor Net1A; an important activator of Rho kinase [131,132]. In addition, Papadimitriou *et al* [131] showed that miRNA-24 levels are up-regulated in metastatic compared with primary breast tumor samples with mesenchymal phenotype.

miRNA-29: N-myc interactor (NMI) is a cytokine-inducible protein that interacts with several transcription factors important in tumour progression such as STATs, myc, BRCA1, TIP60 and SOX10 [133,134], and loss of NMI expression promotes EMT by the activation of TGFβ signaling pathway [135]. A recent report by Rostas *et al* [136] showed increased levels of miRNA-29 in the highly invasive mesenchymal-like breast cancer cell lines, and its over-expression in breast cancer cells expressing robust level of NMI resulted in decreased NMI expression and increased invasion, whereas treating cells with miRNA-29 antagonist increased NMI expression, reversed EMT, and decreased invasion, suggesting a novel inverse regulatory relationship of NMI and miRNA-29 in breast cancer.

miRNA-29a: enhanced miRNA-29a and reduced tristetraprolin (TTP, a protein involved in the degradation of mRNAs with AU-rich 3' UTRs) was observed in breast cancer patient samples with invasive phenotype. Over-expression of miRNA-29a induced EMT and metastasis in Ras-transformed mouse mammary epithelial cells through suppression of TTP [137].

miRNA-103/107: enhanced miRNA-103/107, and reduced expression of the RNase III endonuclease Dicer, was observed in breast cancer cell lines with highly invasive mesenchymal phenotype. Over-expression of miRNA103/107 induced Dicer down-regulation and induction of EMT, with subsequent enhancement in invasive capacity. Furthermore, miRNA103/107 could induce EMT by decreasing miRNA-200 (which negatively regulates EMT), and controlling the levels of ZEB1/2 in a miRNA-200-dependent manner [138,139].

miRNA-106b-25 cluster: the expression profile of this miRNA cluster in human breast cancer patients significantly correlates with metastatic phenotype and shorter relapse free survival. Over-expression induced EMT in breast cancer cell lines, with reduced E-cadherin and increased expression of mesenchymal markers such as β-catenin, Jag1, MMP-9 and vimentin, as well as increasing the percentage of cells with tumor initiating characteristics ($CD24^{low}$ $CD44^{+}$); typical of mesenchymal cells. This cluster also induced EMT by enhancing the action of the metastatic regulator Six1 (a major mediator of the TGF-β-initiated EMT promoting pathway) and by targeting the inhibitory Smad7 protein, which results in increased levels of the TGF-β type I receptor and downstream activation of TGF-β signaling [140].

miRNA-155: Johansson *et al* [141] have demonstrated that miRNA-155 could mediate a switch in TGFβ effect, from tumor suppression to induction of EMT both, in breast cancer cell lines, and in MMTV-PyMT mice. Treatment of mouse mammary gland epithelial cells with a synthetic miRNA-155 mimic repressed the level of the mammary epithelium differentiation

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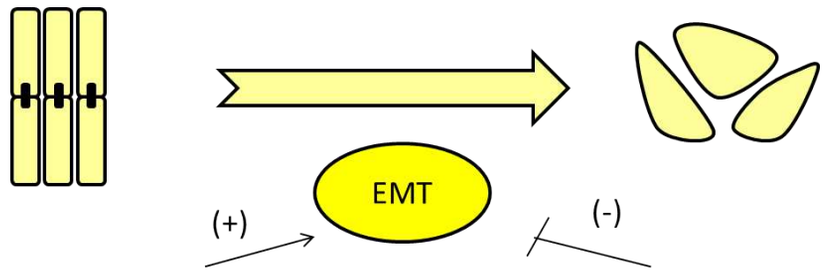
factor CCAAT-enhancer binding protein beta (C/EBPβ) and induced EMT in response to TGFβ treatment; loss of E-cadherin expression, induction of vimentin, and enhanced metastasis and invasion, both *in vitro* and *in vivo*.

miRNA 221/222: miRNA-221/222 induces EMT and subsequent enhancement in invasion by decreasing expression of epithelial-specific genes while increasing expression of mesenchymal-specific genes, in part through stimulation of the transcription factor FOSL1 (Fra-1) and reduction of adiponectin receptor 1 (ADIPOR1). miRNA-221/222-mediated reduction of E-cadherin was effected through targeting of the 3' UTR of the GATA family transcriptional repressor TRPS1 (tricho-rhino-phalangeal syndrome type 1), and modulating ZEB2 levels [142,143,144]. Lambertini *et al* [145] showed that miRNA-221can induce EMT in MDA-MB-231 cells by directly targeting SLUG, a master regulator of the EMT process. Another recent report showed that the secreted form of miRNA221/222 serves as a signaling molecule which plays a pivotal role in the induction of tamoxifen resistance in the ER+ve breast cancer cell line MCF-7; this can be blocked by anti-miR221/222 treatment [146].



miRNA	Target (s)
9	E-cadherin, vimentin
24	TGFβ, Net1A, Rho
29	NMI
29a	TTP, dicer
103/107	dicer, miRNA-200, ZEB1/2
106b-25 cluster	E-cadherin, β-catenin, Jag1, MMP-9, vimentin, TGFβ, Six1
155	E-cadherin, C/EBPβ, vimentin
221/222	E-cadherin, Fra-1, ZEB2/Slug

miRNA	Target (s)
7	SETDB1, STAT3
124	Slug, vimentin, fibronectin, E-cadherin
145	Snail, ZEB1/2, OCT4, fibronectin, E-cadherin
200 family & 205	ZEB1/2, PI3K/AKT
206	IL-6, NFkB, Notch3
375	MTDH
448	SATB1, amphiregulin, Twist, NFkB

Figure 2. Regulation of EMT in breast cancer cells. miRNAs can act as either inducers or inhibitors of EMT by increasing (blue) or decreasing (red) the expression of various target genes as indicated.

3.2.2. Inhibitors of EMT

miRNA-7: Zhang *et al* [147] showed that miRNA-7 expression was significantly reduced in cancer stem cells isolated from MDA-MB-231 and MCF-7 cell lines, and down-regulates the

oncogene SETDB1 by targeting the 3`UTR of the mRNA. Over-expression of miRNA-7 suppressed the EMT-like characteristics of MDA-MB-231 cells, as reflected in the observation that these cells became less scattered and lost their spindle-like morphology, increased E-cadherin and reduced vimentin expression. This was in part due to reduced expression and activity of STAT3.

miRNA-124: as mentioned previously, this miRNA is highly expressed in the brain and plays a crucial role in neural development. Recent evidence suggests that it is also involved in cancer pathogenesis, with reduced expression seen in various cancers, including the breast. Its expression was inversely correlated to histological grade. Its over-expression could repress many of the mesenchymal characteristics of highly metastatic breast cancer cell lines (e.g. MDA-MB-231 and B-549) [148] by reducing SLUG expression, through direct interaction with its 3′-UTR region.

miRNA-145: Hu *et al* [149] have demonstrated that the expression of miRNA-145 was decreased in breast tumor tissues with invasive phenotype, and its over-expression in various breast cancer cell lines leads to enhanced E-cadherin expression, reduced expression of fibronectin, ZEB1/2 and SNAIL, and inhibition of EMT by targeting Oct4.

miRNA-200 family and miRNA-205: expression of the miRNA-200 family (miRNA-200a,-200b,-200c,-141 and-429) and miRNA-205 were found to be significantly reduced in breast cancer cell lines with mesenchymal phenotype, but high in E-cadherin expressing cells with epithelial characteristics. Similarly, their expression was lost in regions of metaplastic breast tumours with mesenchymal characteristics and lacking E-cadherin expression [94]. Enforced expression of miRNA-200 alone was sufficient to prevent EMT induced by TGFβ stimulation [94]. ZEB-1 (TCF8/deltaEF1) and ZEB-2 (SMAD-interacting protein 1 [SIP1]/ ZFXH1B), which are able to initiate EMT by binding to E-boxes within the E-cadherin promoter, repressing its transcription, were the main target genes for these miRNAs [150]. Over-expression of miRNA-200 could induce MET in both normal and cancer cell lines and reduce the motility and invasiveness of MDA-MB-231 by enhancing E-cadherin expression [151,152]. Chen *et al* [153] demonstrated that the loss of miRNA-200c in breast cancer cells was correlated with both EMT and acquired resistance to doxorubicin. In addition, decreased levels of E-cadherin and PTEN, and increased levels of ZEB1 and phospho-Akt were seen in these cells, which correlated with loss of miRNA-200c. Ectopic expression of miRNA-200c reversed all of these changes, suggesting that miRNA-200c inhibits the acquired resistance of breast cancer cells against doxorubicin through inactivation of the PI3K/Akt signaling pathway.

miRNA-375: this was reported to be significantly down-regulated in tamoxifen-resistant (TamR) MCF-7 cells which had acquired a mesenchymal phenotype. Its re-expression re-sensitized the TamR cells to tamoxifen, reversed the EMT process, and reduced invasiveness by targeting the metadherin (MTDH) gene [154].

miRNA-448: suppression of miRNA-448 induced EMT in MCF7 cells, with characteristic acquisition of a fibroblast-like cell morphology, dissolution of tight junctions (ZO-1), formation of F-actin stress fibers, severe E-cadherin suppression and enhanced vimentin expression. In addition, enhanced invasive capacity was also observed upon miRNA-448 inhibition *in vitro*.

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These effects were due to direct targeting of specific AT-rich sequence-binding protein-1 (SATB1) mRNA, leading to elevated levels of amphiregulin and EGFR-mediated TWIST1 expression, as well as NF-κB activation through the MAPK and PI3K/Akt pathways. On the other hand, over-expression of miRNA-448 in MDA-MB-231 cells (which otherwise express very low levels), leads to MET and decreases cell migration and invasion. Similar effects were also observed *in vivo* where miRNA-448-silenced MCF-7 cells (which are usually poorly invasive) showed a spindle-like morphology, with islands of cancer cells that had invaded the muscle and lung tissues when injected into the left flank of nude mice [155].

3.2.3. Role of p53 in EMT and miRNA expression

Deletions and/or mutations in p53 are frequently involved in the pathogenesis of many human cancers including those of the breast (mutated in 25-30% of breast cancers) [156,157]. Kim *et al* [158] observed that p53 prevented EMT in primary hepatocellular carcinomas by repressing ZEB1 and 2 in a 3'UTR-dependent manner. Furthermore, p53-induced ZEB1/2 repression was mediated through up-regulation of various miRNAs; -141, -192, -193b, -194, -200b, -200c, -215, -224, and -34a). p53 is able to positively modulate miRNA-205 expression in triple negative breast cancer cell lines, through regulation of two newly identified target genes, E2F1 and LAMC1, resulting in reduced cellular proliferation [159]. In addition, p53 knockdown can increase proliferation of both luminal and basal-like breast cancer cell lines, in part through up-regulation of miRNA-134,-146a, and-181b. Over-expression of miRNA-146a leads to decreased NF-kB expression and inhibition of the NF-kB-dependent extrinsic apoptotic pathway (TNF, FADD, and TRADD) in basal-like cells expressing mutant p53, suggesting that targeting miR-146a expression may have potential therapeutic value for reducing the aggressiveness of such tumors [160].

4. ER signalling, EMT and miRNA

Estrogen (E_2), acting through ERα, plays a major role in controlling the normal growth and development of mammary epithelial cells, as well as in the pathogenesis of breast cancer. E_2 binding induces ERα activation by the dissociation of the inactive ER-heat shock protein complex, leading to conformational changes, dimerization and autophosphorylation. The activated dimer complex binds to either estrogen response elements (EREs) or to other promoters such as the AP1/SP1 sites in target genes, to initiate events culminating in cellular proliferation. Other target genes may include transcriptional repressors or initiate anti-proliferative or pro-apoptotic function [161].

The mammary ducts are composed of an inner layer of luminal epithelial cells and an outer layer of basal or myoepithelial cells. The majority of breast cancers arise from the luminal epithelium of small mammary ducts, and are classified as luminal-A subtype, characterized as low grade, weakly proliferative and invasive. These express ERα, PR, luminal associated transcription factors such as GATA-3 and FOXA1, and epithelial markers such as E-cadherin [162]. Luminal-A cancers can progress into more aggressive and metastatic forms through the

EMT process. Although ERα plays a critical role in enhancing cellular proliferation, where anti-estrogen therapy (e.g. tamoxifen) are the preferred treatment options, E_2/ERα signaling also promotes the differentiation of mammary epithelial cells along the luminal/epithelial lineage and thereby *opposes* the EMT process. ERα stimulates the transcription factors required for luminal differentiation such as GATA-3 and FOXA1 [163,164]. In fact, forced GATA-3 expression in mesenchymal-like breast cancer cells reduces their metastatic capabilities by inducing MET [165]. ER signaling also suppresses EMT-promoting transcription factors such as SLUG and SNAIL [166,167]. Furthermore, ER antagonizes signaling pathways that lead to EMT, such as those of TGFβ and NFκB. E_2/ERα signaling has been shown to oppose the action of TGFβ in promoting EMT, by initiating formation of ternary complexes of Smad2/3 and SmadE3 ubiquitin ligase smurf, thereby increasing the proteosomal degradation of Smad proteins [168]. The NFκB subunit, RELB, is needed to maintain the mesenchymal phenotype; ectopic expression of ERα in the presence of E_2 was shown to decrease RELB expression in ER-ve cell lines [169,170].

In our laboratory, we have established several endocrine resistant breast cancer cell lines that exhibit an ERα-depleted phenotype induced by shRNA transfection of the ER+ve MCF-7 cells. Such cells have all gained estrogen independence and exhibit a series of changes in morphology and enhanced motility and invasiveness accompanied by a modified gene expression profile indicative of EMT. Microarray and real time-PCR analysis have confirmed the loss of genes associated with epithelial cells such as E-cadherin, catenin, occludins, claudins, and enhanced gene expression associated with mesenchymal cells such as N-cadherin, vimentin, fibronectin, integrin β4 and α5, and various metalloproteinases [124,171,172]. This model of endocrine resistance induced by ERα loss was also confirmed by others. Moreover, ectopic ERα over-expression in ER–ve breast cancer cell lines reverses the EMT process through enhanced E-cadherin and reduced SLUG expression [173].

The ERα mRNA has a long 3' UTR of about 4.3 kb which has been reported to reduce mRNA stability and which bears evolutionarily conserved miRNA target sites, suggesting that it might be regulated by miRNAs. Overall, ER–ve cells display generally lower levels of miRNA expression. Of the miRNAs that are up-regulated there are distinct differences between ER-ve and ER+ve cells (Figure 3).

Of note, miRNA-10b,-125, and-145 were significantly down-regulated in the majority of breast cancer samples and cell lines, whereas miRNA-21,-17-5b,-29b-2,-146,-155, and 181b-1 were up-regulated [126,174]. Estrogen has been shown to induce Dicer expression; loss of ERα may contribute to reduced Dicer and consequently lower miRNAs levels in ER–ve cells. In addition, some miRNA such as miRNA-29a, 103/107 and-200c, and *let-7* inhibit Dicer expression and thereby promote the EMT process [175,176,177,178]. Restoration of miRNA-200c in triple negative breast cancer cells causes an increase in Dicer levels [176]. The expression of both AGO-1 and-2 was reported to be significantly elevated in ER–ve cells [179]; forced expression of AGO-2 enhanced breast cancer cell motility through reduced E-cadherin expression [180]. Understanding how miRNAs modulate ERα and its signaling pathway may offer new therapeutic approaches to restore endocrine sensitivity and responsiveness to anti-estrogen therapies, and reverse the EMT process, thereby reducing metastasis. The following section

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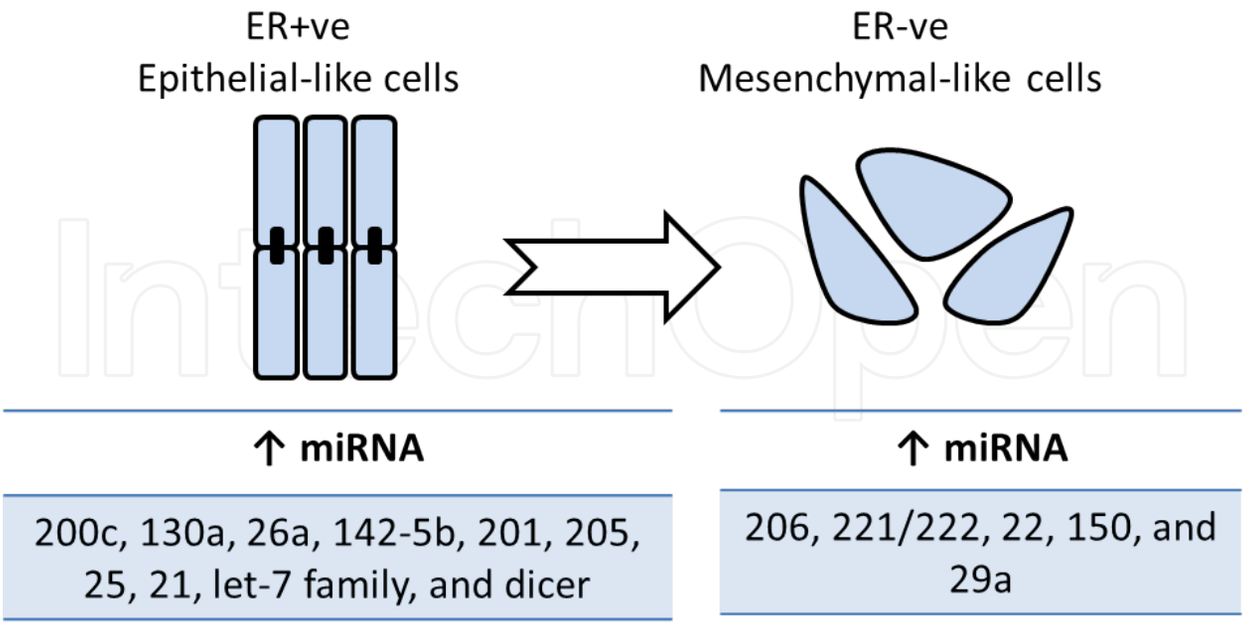

Figure 3. Up-regulation of miRNA expression in relation to ER status of breast tumours

describes individual miRNAs that have been shown to modulate ER*α* expression and hence the EMT process (Figure 4).

miRNA-22: over-expression of miRNA-22 represses ER*α* expression through the 3`UTR leading to reduction in estrogen signaling. This also leads to impaired estrogen-induced proliferation of MCF7 breast cancer cells to an extent similar to that of shRNA directed at the ER*α* mRNA [181]. Similar findings were also reported by Xiong *et al* [182].

miRNA-145: transfection of miRNA-145 into ER+ve breast cancer cells significantly reduced ER*α* protein levels through interaction of two miRNA-145 target sites within the coding region of ER*α* mRNA, reducing the levels of its downstream target cyclin D1 [183].

miRNA-206: E_2/ER*α* directly suppresses miRNA-206 levels [184], while the miRNA-206 itself directly targets the mRNAs encoding components of ER*α* signaling molecules such as the nuclear receptor co-activator proteins steroid receptor co-activator-1 (SRC-1) and-3 as well as GATA-3 [184,185]. In addition, ectopic expression of miRNA-206 in ER+ve breast cancer cells reduces endogenous ER*α* at both mRNA and protein levels and leads to enhanced invasive capacity [186,187]. Moreover, miRNA-206 can also decrease the expression of DNA polymerase A1 subunits as well as the oncogenic receptor c-MET, while increasing the expression of the tumor suppressor forkhead box O3 (FOXO3). Consequently, this leads to the inhibition of cell proliferation, suggesting a role for miRNA-206 in repressing proliferation of ER+ve breast cancer by enhancing myoepithelial differentiation and ER*α* silencing [30,184,188]. Enhanced level/activity of EGF add its receptor (EGFR/HER1) is seen in mesenchymal type breast cancer cells and may contribute to ER silencing though enhancement of miRNA-206 levels [184]. Ectopic expression of miRNA-206 in MCF-7 cells enhances IL-6 expression, which is known

to induce EMT through STAT signaling. Also, it maintains its level by autocrine positive feedback loops that involve NFκB or NOTCH3 [189,190].

miRNA-221/222: expression of this pair of miRNAs was found to be higher in ER-ve compared with ER+ve breast cancer cells. In this context, miRNA-221 and-222 can inhibit the translation of the ERα mRNA [191]. Several studies have also suggested that ERα directly represses the gene promoter region of both miRNAs by recruiting the co-repressors NCoR and SMRT [187].

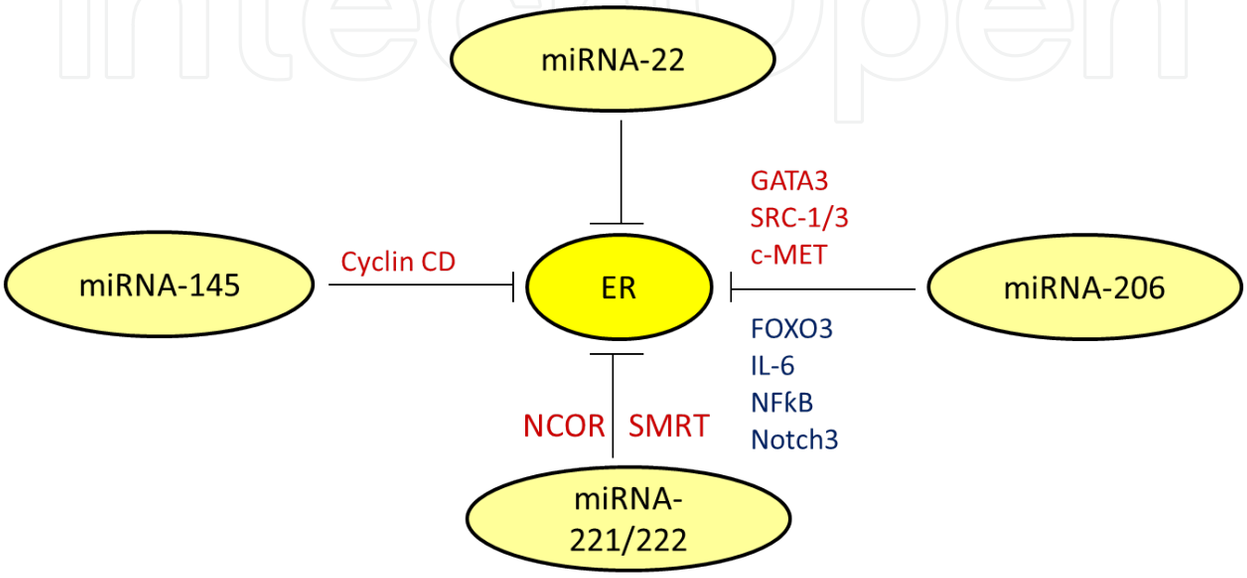

Figure 4. miRNAs that negatively regulate ER expression in breast cancer. These act either directly (miRNA-22), or through up-(blue) or down-(red) regulation of other target mRNAs.

5. miRNAs and cell invasion

Cancer cell invasion is a multi-step process which involves dissociation of extracellular matrix components by the action of various proteases and the subsequent movement of detached tumor cells from the original tumor site to distinct organs, and is associated with poor clinical outcome and reduced survival rates. Several miRNAs have been implicated in either enhancing or reducing cellular invasion by targeting various mRNAs that encode proteins crucial to the process (Figure 5).

5.1. miRNAs with anti-metastatic actions

miRNA-7: expression of miRNA-7 was significantly reduced in cancer stem cells (CSCs) isolated from breast cancer cell lines which demonstrated significant metastatic migration to the bone and the brain. It attenuated the invasion and self-renewal of CSCs by enhancing the expression of KLF4 [192]. A recent report also confirmed the anti-metastatic properties of miRNA-7 both *in vitro* and *in vivo* by targeting the oncogene SETDB1 and showing decrease in expression and activity of STAT3 in MDA-MB-231 cells [147].

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miRNA-18a: a recent report showed that over-expression of miRNA-18a in MDA-MB-231 cells reduced cell invasiveness and sensitivity to anoikis and hypoxia *in vitro*, and primary tumor growth and lung metastasis *in vivo*. On the other hand, its inhibition leads to a pro-metastatic effect by targeting of the HIF1A gene [193].

miRNA-31: expression of miRNA-31 is reduced in several metastatic breast cancer cell lines, and correlates inversely with metastasis in human breast cancer patients. Over-expression of this miRNA in otherwise-aggressive breast tumor cells suppresses metastasis, whereas inhibition of miRNA-31, by miRNA sponge strategy, induced metastasis in non-aggressive breast cancer cells both *in vitro* and *in vivo* by enhancing the expression of several metastasis-promoting genes including Fzd3, ITGA5, RDX, and RhoA [194].

miRNA-107: over-expression of this miRNA in MDA-MB-231 cells significantly inhibited cell migration and invasion by targeting of the cyclin-dependent kinase 8 (CDK8) gene [195].

miRNA-124: expression of miRNA-124 was significantly reduced in MDA-MB-231 compared to MCF-7 cells. Induced over-expression in MDA-MB-231 significantly inhibited cell migration and invasion *in vitro*, in part through reduced SLUG and enhanced E-cadherin expression [148,196]. In addition, reduced tumor formation and lung metastasis was seen in MDA-MB-231 cells over-expressing miRNA-124 when injected into the tail vein of nude mice [148]. Another report confirmed its anti-metastatic role through its ectopic expression in MDA-MB-231 and T47D cells, which significantly reduced their invasive capacity through targeting of flotillin-1 (FLOT1) [197]. Other evidence suggests that its anti-metastatic properties are exerted through the suppression of several pro-metastatic genes such as connective tissue growth factor (CTGF), Ras homolog family member G (RhoG), ITGB1 and ROCK1 [196].

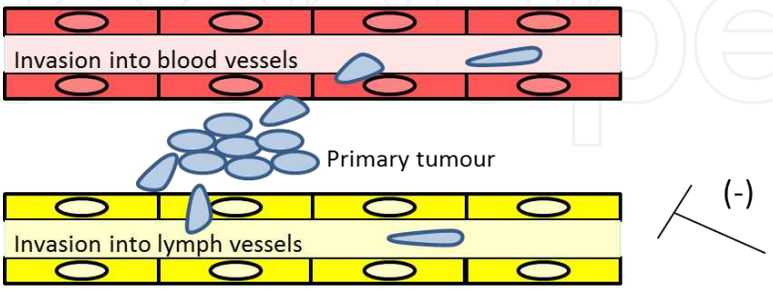
miRNA-145: the expression of miRNA-145 was found to be reduced in breast cancer cells compared to normal tissue, and its over-expression in various breast cancer cell lines (MDA-MB-231, MCF-7, MDA-MB-468 and SK-BR-3) significantly reduced motility and invasiveness. This effect was due to down-regulation of the cell–cell adhesion protein JAM-A and the actin bundling protein fascin [198], and silencing of the metastasis gene mucin 1 (MUC1), with subsequent reduction of beta-catenin as well as the oncogenic cadherin 11 [199].

miRNA-146a/b: over-expression of miRNA146a/b in MDA-MB-231 resulted in marked inhibition of migration and invasion due to reduced NF-κB activity. This was through miRNA146a/b-induced down-regulation of two key regulators of this signaling pathway; interleukin (IL)-1 receptor-associated kinase and TNF receptor-associated factor 6 [200].

miRNA-149: a recent report [201], showed reduced expression of miRNA-149 in basal compared with luminal A/B, erbB2/HER2 positive and normal-like cancers and cell lines. It was also found to be inversely correlated with higher tumor stage. Over-expression of a mature miRNA-149 mimic in MDA-MB-231 cells significantly reduced their spreading in culture; the cells exhibited a depolarized actin cytoskeleton and failed to establish prominent cell protrusions and lamellipodia. Moreover, significant reduction in migration and invasion towards a serum plus EGF gradient was observed in cells over-expressing miRNA-149. These effects were due to decreased phosphorylation levels of src and rac, and to targeting of the small GTPases rap1a and rap1b, the downstream effectors of the integrin receptor.

miRNA-195-5p: a recent study demonstrated that over-expression of miRNA-195-5p significantly inhibited MDA-MB-231 and MCF-7 breast cancer cell invasion by targeting cyclin E1 and *raf*-1/Ccdn1 genes respectively [202,203].

miRNA-223: over-expression of miRNA-223 in MDA-MB-231 significantly decreased cell migration and invasion by down-regulating STAT5A [204].



miRNA	Target (s)
9	E-cadherin, β-catenin, VGEFA
10b	RHOC, TIAM1
18b	NLRP7, KLK3, OLFM3, POSTN, MAGED4B, KIR3DL3, CRX, SEMG1, CEACAM5
21	PDCD4, TPM1, TIMP3, PTEN, PI3K, MARK, ERK1/2
24	TGFβ, Net1A
Has-30c	NOV, CCN3
155	C/CBPβ
221/222	E-cadherin, Fra-1, uPA
301a	PTEN, Wnt, PI3K, β-catenin
373/520	E-cadherin, CD44
495	JAM-A

miRNA	Target (s)
7	STAT3, SETDB1
18a	HIF1A
31	Fzd3, ITGA5, RDX, RhoA
107	CDK8
124	Slug, FLOT1, CTGF, RhoG, ROCK1, E-cadherin
145	JAM-A, fascin, MUC1, cadherin-11, β-catenin
146a/b	NFkB, IRAK, TRAF6
149	SRC, Rac, Rap1 a/b
195-5p	cyclin E1, Raf1, Ccdn1
200	ZEB1/2
223	STAT5
302	CXCR4
335	SOX4, tenascin-C, MERTK, PTPRN2
448	STAB1, amphiregulin, Twist, NFkB

Figure 5. miRNAs involved in breast cancer cell invasion. miRNAs can act as either inducers or inhibitors of invasion by increasing (blue) or decreasing (red) the expression of various target genes as indicated.

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miRNA-302a: expression levels of this miRNA was significantly decreased in metastatic breast cancer cell lines and tumor tissues, and enforced expression of miRNA302a significantly inhibited both *in vitro* and *in vivo* cell invasion, by inhibiting the CXCR4 gene [205].

miRNA-335: the expression of miRNA-335 and-126 was lost in human breast cancer tissues from patients who developed metastasis and relapse. Restoring miRNA-335 expression in highly metastatic breast cancer cell lines suppresses lung and bone metastasis *in vivo* through targeting of the progenitor cell transcription factor SOX4, the extracellular matrix component tenascin C, the c-Mer tyrosine kinase MERTK, and the receptor protein tyrosine phosphatase PTPRN2 [112].

5.2. miRNAs with pro-metastatic actions

miRNA-9: over-expression of miRNA-9 in human mammary epithelial cells and the human breast cancer cell line SUM149 resulted in significant increase in their motility and invasiveness *in vitro*, through E-cadherin suppression. In addition, it led to increased β-catenin activity as well as vascular endothelial growth factor (VEGFA) expression in MCF7-RAS breast carcinoma cells, with subsequent enhancement in their invasive capacity. Furthermore, miRNA-9 knockdown in the highly metastatic 4T1 mouse mammary tumour cells, inhibited lung metastasis formation when injected into the mammary fat pads of syngeneic immunocompetent mice *in vivo* [128].

miRNA-10b: the transcription factor TWIST induces the expression of miRNA-10b [206], which is highly expressed in metastatic breast cancer cells and correlates with poor clinical progression in patients with breast cancer. Transfection of the antisense inhibitor for miRNA-10b in MDA-MB-231 cells caused a significant reduction in the invasive properties of these cells. Moreover, over-expression of this miRNA in otherwise non-metastatic breast tumour cells (SUM149) initiates robust invasion and lung metastasis *in vivo* when injected into the mammary fat pads of immunodeficient mice, by enhancing the expression of the pro-metastatic gene RHOC [113]. In addition, miRNA-10b also targets another pro-metastatic gene which influences breast cancer cells; the guanidine exchanger factor for rac activation [T-lymphoma invasion and metastasis (TIAM1)] [207]. Furthermore, Ahmad *et al* [208] demonstrated increased expression of miRNA-10b in the primary breast cancer specimens of patients who subsequently developed brain metastasis compared to those who did not, suggesting that miRNA-10b could serve as a prognostic factor for brain metastasis in breast cancer patients and a potential target for anti-metastatic therapy.

miRNA-18b: expression of miRNA-18b was shown to be up-regulated in various breast cancer cell lines and in clinical specimens of breast tumors. Inhibition of miRNA18b in breast cancer cell lines significantly suppressed their invasive capacity by modulating several target genes including NLRP7, KLK3, OLFM3, POSTN, MAGED4B, KIR3DL3, CRX, SEMG1, and CEACAM5 [209].

miRNA-21: suppression of the oncogenic miRNA-21 in MDA-MB-231 significantly reduced invasion *in vitro* and lung metastasis *in vivo* through targeting of the tumour suppressor gene tropomyosin 1 (TPM1), maspin, tissue inhibitor of metalloproteinase 3 (TIMP3), and the

programmed cell death 4 (PDCD4) [92,210,211]. In addition, miRNA-21 is involved in HER2/neu-induced cell invasion, which is mediated by the MAPK pathway [92].

miRNA-24: TGF-β treatment of breast cancer cell lines results in EMT and enhanced invasive capacity. Down-regulation of miRNA-24 expression resulted in suppression of the TGF-β-induced cell invasiveness through Net1A regulation [131].

Has-miRNA-30c: enhanced expression of has-miRNA-30c was observed in MDA-MB-231 compared to the poorly metastatic MCF-7 cells. Transfection of has-miRNA-30c into MDA-MB-231 cells significantly enhanced their invasive capacity towards conditioned osteoblast media, while transfection with anti-miRNA-30c had the opposite effect. This effect was due to targeting and inhibiting of NOV/CCN3, which has been described as an inhibitor of invasion [212].

miRNA-221/222: up-regulation of miRNA-221/222 in breast cancer is associated with malignancy and poor clinical outcome, while down-regulation of this miRNA is inversely correlated with metastasis [112,213,214,215]. Falkenberg *et al* [216] reported that miRN-221/222 is a significant prognostic marker for distinguishing sub-groups, particularly in advanced nodal (LN+) and HER2+breast tumors, and its over-expression in T47D, MDA-MB-231 and SKBR3 cell lines markedly enhanced their invasive capacity through targeting of the serine protease urokinase-type plasminogen activator (uPA).

miRNA-301a: a study by Ma *et al* [217] demonstrated that miRNA-301a was significantly up-regulated in primary tumor samples with a metastatic phenotype, as well as in metastatic breast cancer cell lines. *In vitro* over-expression of miRNA-301a in the non-invasive MCF-7 cells leads to significant enhancement in migration and invasion through targeting of PTEN and activation of the wnt/β-catenin signaling pathway, while its inhibition in the invasive MDA-MB-231 significantly decreased cell invasion. Furthermore, mice injected with miRNA-103a over-expressing MCF-7 cells displayed prominent lung metastasis, while mice injected with MDA-MB-231 cells pre-treated with miRNA-103a inhibitor reduced the degree of lung metastasis.

miRNA-373 and-520c: the expression of both these miRNAs (which are members of the same family and share similar seed sequence [218]) was significantly up-regulated in clinical breast cancer metastasis samples. Stable over-expression of miRNA-373 and-520c stimulated breast cancer cell migration *in vitro* and *in vivo* by the suppression of the cell surface glycoprotein CD44 through binding to the 3` UTR region of its mRNA [219]. In addition, miRNA-373 promotes cell invasion through targeting of sites in the promoter of E-cadherin mRNA [45].

miRNA-495: expression of miRNA-495 was significantly increased in both clinical breast cancer tissue samples compared to adjacent normal breast tissue as well as in MDA-MB-231 compared to MCF-7. Over-expression of miRNA-495 significantly enhanced invasive capacity of both cell lines, while its knockdown by miRNA-495 inhibitor showed the opposite effects. Its pro-metastatic effect was due to targeting and inhibiting of the JAM-A gene [220].

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6. miRNAs implicated in breast cancer proliferation

Several miRNAs have been implicated in either enhancing or reducing cellular proliferation by targeting various mRNAs that encode proteins crucial to the process (Figure 6).

miRNA-21: this was found to be highly expressed in breast tumors compared to normal breast tissue biopsies. Suppression of miRNA-21 levels (using anti-miR-21 oligonucleotides) in MCF-7 suppressed cell growth in vitro as well as the tumor growth in the xenograft mouse model *in vivo*. This effect was associated with increased apoptosis, down-regulation of the anti-apoptotic protein bcl-2 [221], and modulation of several survival-related genes including ACTA2, APAF1, BTG2, FAS, p21, PDCD4, and SESN1 [211].

miRNA-22: it is highly expressed in ER-ve breast cancer cell lines and in clinical samples with mesenchymal phenotype. miRNA-22-mediates growth repression of ER+ve breast cancer cells and it might serve as a potential therapeutic agent in the treatment of ER+ve cancers [182].

miRNA-26a/b: a recent study by Tan *et al* [222] demonstrated that forced expression of miRNA-26a/b markedly inhibited E_2-stimulated proliferation of ER+ve breast cancer cells *in vitro* by modulating CHD1, GREB1 and KPNA2 target genes. miRNA26a/b depletion enhanced their proliferative capacity. In addition, injecting miRNA26a or by over-expressing MCF-7 breast cancer cells into nude mice, resulted in the formation of slower growing and significantly smaller tumors compared with tumors derived from untreated MCF-7 injected mice.

miRNA-27a: it has been suggested that miRNA-27a enhances the proliferation of breast cancer cell lines through targeting of genes that regulate the specificity protein transcription factors (Sp) which are often over-expressed in tumors and associated with enhanced proliferative and angiogenic capacity. Suppression of miRNA-27a (using anti-sense miRNA-27a) in MDA-MB-231 resulted in growth suppression through increased expression of Myt-1 and the zinc finger ZBTB10 gene (a putative Sp repressor), and increased levels of Sp1, Sp3, and Sp4. In addition, decreased expression of Sp-dependent survival and angiogenic genes, including survivin, VEGF and VEGF receptor 1 (VEGFR1) was also seen after miRNA-27a suppression [65].

miRNA-34c: the expression of miRNA-34c was significantly decreased in basal-like breast cancer cells (MDA-MB-231, MDA-MB-468 and BT-549) and was associated with poor prognosis. Its over-expression resulted in suppressed proliferation and increased cell death by influencing the cell cycle mainly by inducing an arrest in the G2/M phase and down-regulation of various cell cycle-regulators such as CCND1, CDK4 and CDK6. Furthermore, CDC23 was identified as an miRNA-34c-regulated target that could be responsible for the induction of cell cycle arrest [223].

miRNA-93: this miRNA induced MET in claudin-low SUM159 cells, and reduced their proliferation level through down-regulation of TGFβ signaling and multiple stem cell regulatory genes such as JAK1, STAT3, AKT3, SOX4, EZH1, and HMGA2. On the other hand, it enhanced the CSC population in MCF7 cultures that display a more differentiated phenotype, suggesting different effects based on cellular differentiation state [224].

miRNA-107: the expression of miRNA-107 was decreased in breast cancer specimens compared with adjacent normal tissues, and its over-expression significantly suppressed proliferative capacity, and induced arrest at G0/G1 phase in MDA-MB-231 cells. These effects were due to down-regulation of CDK8 target gene by miRNA-107 [195].

miRNA-124: expression of miRNA-124 was reduced in breast cancer tissues and inversely correlated with TNM stage and lymph node metastasis. Its over-expression in MDA-MB-231 and T47D cells significantly inhibited their growth and proliferative capacity. This was due to increased number of cells in the G0 and G1 phase and decreased number in the S, G2 and M phases [197]. The anti-proliferative function of miRNA-124 in MDA-MB-231 and MCF-7 cells was seen as a consequence of targeting and inhibiting the E26 transformation specific-1 (Ets-1) gene [225].

miRNA-145: expression of this miRNA was significantly lower in breast cancer cell lines, as well as in primary human breast tumors as compared with normal breast tissues. Transfection with a synthetic miRNA-145 precursor into several breast cancer cell lines produced a pro-apoptotic effect, which was dependent on p53-mediated transactivation of PUMA [183].

miRNA-195-5p: this miRNA was significantly down-regulated in breast cancer tissues compared to adjacent normal tissues, and over-expression of miRNA-195-5p in MDA-MB-231 cells inhibited their proliferative capacity and ability to form colonies, and caused G1 phase arrest by targeting of cyclin E1 (CCNE1) [202]. In addition, its over-expression also inhibited the proliferative capacity of MCF-7 cells by targeting of raf-1 and Ccdn1 genes [203].

miRNA-196a: over-expression of miRNA196a in various breast cancer cell lines led to reduction in their proliferative capacity by suppressing annexin A1 (ANXA1), a mediator of apoptosis and inhibitor of cell proliferation [226].

miRNA-206: introduction of miRNA-206 into MCF-7 cells inhibited cell growth in a dose-and time-dependent manner [188], in part through inducing a significant block in G1phase [191].

miRNA-221/222: by targeting the cell cycle inhibitor p27(Kip1) [216,227], miRNA-221/222 enhanced ER–ve breast cancer cell proliferation. In addition, it also increased ER+ve cell proliferation by stimulating cell transition from G1 to S phase [191]. Other reports have suggested that miRNA-221/222 is involved in the EGFR-RAS-RAF-MEK signaling pathway and down-regulates PTEN, leading to enhanced cell proliferation [228,229].

miRNA-486-5p: expression of miRNA-486-5p was reduced in breast cancer biopsies compared to adjacent non-neoplastic tissues, as well as in various breast cancer cell lines. Its over-expression in MDA-MB-231 and T47D significantly reduced their proliferative capacity *in vitro* by inducing G0/G1 arrest and promotion of apoptosis. Furthermore, its over-expression in MDA-MB-231 cells significantly inhibited xenograft tumor growth when injected subcutaneously into the right flank of nude mice. The oncogene PIM-1 was identified as a direct target of miRN-486-5p, suggesting that the miRNA-486-5p/PIM-1 axis might be a useful therapeutic target for prevention or treatment of breast cancer [230]. Zhao *et al* [231] demonstrated that over-expression of miRNA-486-5p in SKBR3 cells inhibits HER3 expression and lowers its downstream mediators, inhibits clonogenic potential, and enhances their sensitivity to

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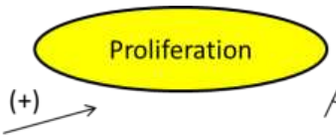
trastuzumab or doxorubicin by repressing proliferative signal pathways mediated by HER3/HER2/PI3K/AKT.

miRNA-769-3p: Luo *et al* (2014a) have shown that culturing MCF-7 cells under hypoxic conditions followed by re-oxygenation enhanced the expression levels of various miRNAs such as miRNA-769-3p,-501-3p,-2276, and-1282. Over-expression of miRNA-769-3p significantly inhibited cell proliferation and enhanced apoptosis by targeting and inhibiting the expression of the NDRG1 gene, suggesting that miRNA-769-3p can functionally regulate NDRG1 during changes in oxygen concentration in breast cancer cells.



miRNA	Target (s)
21	PTEN, PI3K, Bcl2, ACTA2, APAF1, BTG2, Fas, p21, PDCD4, SESN1
27a	SP, myt-1, ZBTB10, survivin, VGED, VGEFR1
221/222	Kip1, PTEN, EGFR, RAS, RAF, MEK

miRNA	Target (s)
22	
24-2* strand	PCKα
26a/b	CHD1, GREB1, KPNA2
34c	CCND1, CDK4/6, CDC23
93	TGFβ, JAK1, STAT3, AKT3, SOX3, EZH1, HMGA2
107	CDK8
124	Ets-1
145	PUMA
195-5p	CCNE1, Raf1, Ccdn1
196a	ANXA1
206	G1-phase
486-5p	PIM1, HER2/3, PI3K/AKT
769-3p	NDRG1
Let-7	RB1, E2F, HRAS, HMGA2

Figure 6. miRNAs involved in breast cancer cell proliferation. miRNAs can act as either inducers or inhibitors of proliferation by increasing (blue) or decreasing (red) the expression of various target genes as indicated.

7. miRNAs controlling the biosynthesis of other miRNAs

An example of miRNAs regulating the expression levels of other miRNAs was reported by Martello *et al* [139]. They showed that miRNA-103/107 attenuated the global biosynthesis of other miRNAs through targeting of the RNase-III Dicer. miRNA103-107 also specifically down-regulated miRNA-200, which led to EMT, and subsequent enhancement in breast cancer cell invasion, but without major impact on primary tumor growth.

8. miRNAs as therapeutic agents

Several studies, as outlined above, have highlighted the contribution of various miRNAs in multiple processes of tumor pathogenesis (proliferation, invasion, EMT and endocrine/chemotherapy resistance), making them potential tools for applications as indicators in breast cancer diagnosis and staging, as markers of response to therapy and as therapeutic agents/targets for treatment. Unlike mRNAs, miRNAs (presumably due to their smaller size) are relatively stable in formalin fixed tissue specimens and in the blood stream, which facilitates their detection/measurement. There is opportunity to develop miRNA-based drugs that target specific oncomiRs or replace down-regulated miRNAs which have tumor suppressor properties. Unlike other nucleic acid, protein or small molecule drugs, many miRNAs (such as miRNA-31) have pleiotropic actions by which they can affect several related target genes; this can have a desirable cumulative effect that has obvious advantages in treating multifactorial diseases like cancer [194]. Of course it could also produce deleterious effects, so it is important to identify all the potential targets of particular miRNAs.

The value of using miRNAs as diagnostic and/or prognostic signature in breast cancer is currently receiving some attention. For example, miRNA-7,-128a, 210, and 516-3p can be used as markers for distant metastases of ER+ve, lymph node-ve breast cancer cases [214], while miRNA-210 can be used as marker for distant metastasis in triple –ve breast tumours [214,232,233]. Some miRNAs could be used as markers for disease survival in ER+ve (e.g. miRNA-128a,-135a, 767-3p) [234], ER–ve (e.g. miRNA-27b,-30c,-144,-150,-210,-342) [234], as well as in triple negative breast tumours (e.g. miRNA-21,-205,-210,-221,-222) [235,236]. Also, some miRNA such as-30a-3p,-30c, and-182 can be used as markers for response to adjuvant tamoxifen treatment in advanced ER+ve cases [237], while miRNA-21can predict the response to neoadjuvant trastuzumab treatment in breast cancer [213,238,239].

8.1. miRNA mimics

miRNA mimics, or replacement therapy, aims to restore normal levels of certain miRNAs that are down-regulated. They usually carry the same sequence as the missing or deficient naturally occurring miRNA. Introduction would be via viral or liposomal delivery [240].

8.2. Antagomirs

These are oligonucleotides that are chemically engineered to bind to a specific miRNA to prevent it from interacting with its mRNA target. To increase their stability, they can be chemically modified through the inclusion of 2′-O-methyl modified ribose sugars, 2′-O-methyl ribose sugars with the addition of an extra 2′-O, 4′C methylene bridge sugar [241,242,243,244]. An example of an important miRNA in breast cancer pathogenesis is miRNA-21, which is significantly up-regulated in breast tumors compared to normal tissue. Treatment of both ER +ve and ER-ve breast cancer cells with anti-miRNA-21 oligonucleotides suppressed both cell growth and migration *in vitro* and tumor growth in the xenograft mouse model *in vivo* [213,221]. Furthermore, miRNA-21 has the capacity to sensitize breast cancer cells to some anti-cancer agents such as topotecan and taxol [221]. Combination treatment of taxol with miR-

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NA-21 inhibitor significantly decreased the 50% inhibitory concentration (IC50) of taxol in breast cancer cells when compared with taxol monotherapy. In addition, treatment of the miRNA-21 inhibitor-transfected cells with taxol resulted in significantly reduced cell viability and invasiveness compared with control cells [213]. Of note, the most developed miRNA-based agent to date is the miRNA-122 inhibitor for the treatment of hepatitis C virus [245], but before reaching clinical usage its interaction with other clinically used drugs should be extensively studied.

8.3. miR masks

Another range of compounds that are under development are known as target masks [246], of which there are several types. A target mask is conceptually an oligonucleotide whose sequence has been designed to bind either to an endogenous miRNA (miR sponge) or to its target on the mRNA (sponge miR mask). Whilst the binding of the sponge miR mask will prevent the binding of all miRNA belonging to the same seed family [247], (and is therefore miRNA seed specific and not gene specific), the miR mask blocks only a particular miRNA from interacting with its target mRNA.

These interactions are illustrated in Figure 7.

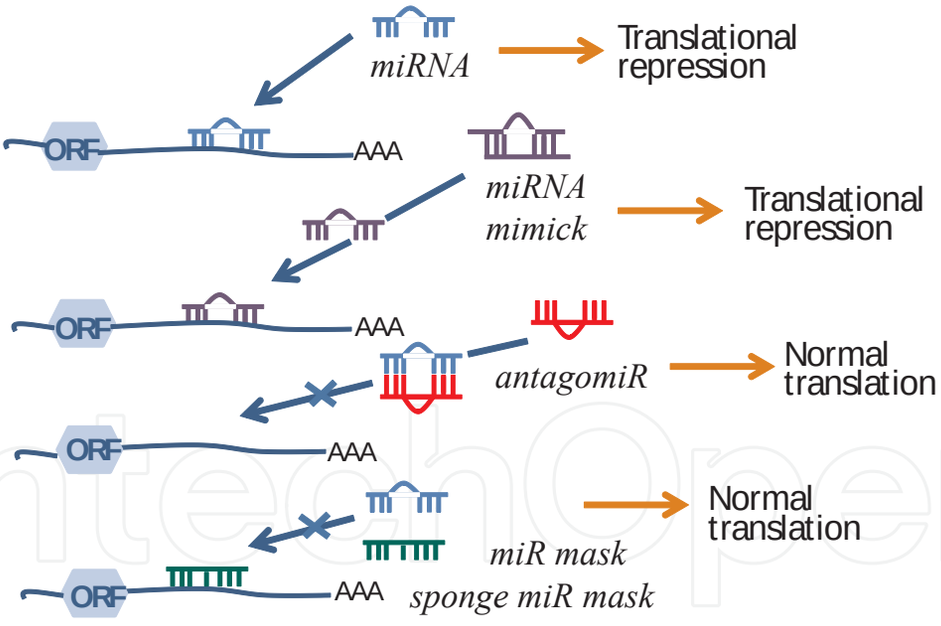

Figure 7. Manipulation of miRNA function. Endogenous miRNAs bind to target sequences in the 3′ UTR regions of their target mRNA, downstream of the open reading frame (ORF), to produce translational arrest. miRNA mimics are synthetic oligonucleotide duplexes that have the same sequence as the endogenous miRNA and also produce the same effect. Antagomirs (also called antimiRs) are oligonucleotides that have complementarity with the miRNA and bind to it, preventing it from interacting with its target mRNA, thereby allowing normal mRNA translation. An miR mask is a construct that is complementary to a sequence in the mRNA; this binding does not initiate mRNA degradation or translational inhibition but prevents the endogenous miRNA from binding. The sponge miR mask differs from the miR mask in that it binds to any mRNA with a similar target sequence and is therefore miRNA seed specific and not gene specific.

9. Summary

- miRNAs are endogenously synthesized single stranded RNA molecules that are 19-25 nucleotides in length, which play a vital role in the regulation of gene expression.
- Their exact mechanism of regulating gene expression is determined by the degree of complementarity with their target mRNAs; perfect complementarity usually results in mRNA degradation while permitted imperfect complementarity results in translational inhibition.
- miRNA biogenesis takes place in two phases; nuclear and cytoplasmic, both of which include events mainly carried out by Drosha, Argonaute and Dicer.
- The expression level of miRNA is mostly found to be down-regulated in cancers, and miRNA-155 was the first to be found to actually induce tumorigenesis.
- miRNAs are differently expressed in ER-ve *vs* ER+ve breast cancer cells; enhanced expression of miRNA-206,-221/222,-22, 150, and-29a was seen in ER-ve cells while enhanced expression of miRNA-200c,-130a-26a,-142-5b,-201,-205,-25,-21 and *let-7* family was seen in ER+ve cells.
- miRNA-221/222 targets and down-regulates ERα, induces EMT, and enhances breast cancer cell invasion and proliferation.
- miRNA-9,-24, and-155 induce EMT and enhance cell invasion.
- miRNA-7 and-44 inhibit EMT and decrease cell invasion.
- miRNA-124 and-145 inhibit EMT, and decrease cell invasion and proliferation.
- miRNAs could be used as non-invasive biomarkers for the diagnosis and prognosis, and as a promising therapeutic target for breast cancer.
- miRNA mimicks, antagomiRs and miR masks are being developed as new ways to interfere with miRNA regulation of gene translation in cancer cells.

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