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Translational Research on Breast Cancer: miRNA, siRNA and Immunoconjugates in Conjugation with Nanotechnology for Clinical Studies

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1. Introduction

Currently, breast cancer is a global public health issue**.** However, recent progress in phenotyping and expression profiling of human cancers have greatly enhanced the diagnosis and biological classification of several tumors, in particular breast cancers. Despite significant advances in cytotoxic chemotherapy, endocrine therapy and novel targeted agents, metastatic breast cancer remains an incurable disease (Ocana et al., 2006; Ocana & Pandiella, 2008). The lack of curative potential is partially explained by the heterogeneous biology of this disease which exhibits both *de novo* and acquired resistance to many treatment modalities (Ocana & Pandiella, 2008).

Breast cancers are classified into distinct subtypes using microarray-based gene expression signatures identification; these are largely based on their (Estrogen) ER, progesterone (PR), and HER2 receptor status (Perou et al., 2000; Sorlie et al., 2001; Sorlie et al., 2006). Subtypes were designated *Luminal A*, which strongly expressed ER and/or PR, but not HER2; *Luminal B*, which were ER, PR and HER2 positive; *Basal* tumors which were ER, PR, and HER negative, preferentially affecting young women and women of African origin, usually of high histological grade and more aggressive clinical behavior (Yehiely et al., 2006) .

Genomic tumor profiling has provided us with important insights to mechanisms of tumorigenesis and translational data for clinical advances (Bauer et al.). Relative to some cancer types, there is tremendous genomic information available for breast cancers, which includes tumor DNA copy number (Adelaide et al., 2007; Bergamaschi et al., 2006; Chin et al., 2006; Han et al., 2008; Neve et al., 2006) DNA sequence and mutations (Leary et al., 2008; Nikolsky et al., 2008; Shah et al., 2009; Sjoblom et al., 2006; Stephens et al., 2009; Wood et al., 2007), gene expression and protein profiles(Boyd et al., 2008; Hennessy et al., 2009), as well as epigenetics (Andrews et al.; Ruike et al.) and microRNAs (Iorio et al., 2005a; Mattie et al., 2006a).

Promising new targeted agents, such as small molecule tyrosine kinase inhibitors and monoclonal antibodies such as trastuzumab, which targets breast cancer cells

overexpressing her2neu, are rapidly making their way into general oncology practice in high-resource countries. These "smart bombs" of the oncologywill, no doubt, have increasing utility either as stand-alone or adjunct drugs in the armamentarium of anti-neoplastics. However, given the experience to date with cost of these agents, and the likelihood that any of these drugs will be off-patent and "genericized" in the coming decade, it is presently entirely beyond the reach of low and middle-resource countries to even consider their use.

According to the WHO, a drug may be considered cost-effective if the annual cost per quality-adjusted life year saved, or QALY, is no greater than a country's per capita GDP (gross domestic product) ("https://www.cia.gov/library/publications/the-world-factbook/,"). Although major share of drug development budgets are focused on targeted agents, which escalates the cost of the drug. Hence to decrease drug cost, drug activity should be improved, and treatment failures need to avoided, to achieve this identification of common 'drugable' oncogenic mechanisms is an important area to focus research.

Many strategies have been developed to treat cancer by targeting the cancer cells without affecting normal cells. Recent research on gene delivery such as, siRNAs, miRNAs which have been proven to control breast cancer tumors can be introduced into breast tissue through the use of nanoparticle technology and breast cancer targeting immunoconjugates. Hence, in this chapter we will focus mainly translational research on novel formulation that contains miRNA or siRNA or immunoconjugate modified to nanoparticles for specific delivery to breast cancer tissues. Thus this chapter will divides into four sub sections, first three will be briefing current updates on miRNA, siRNA, immunoconjugates, relevant to breast cancer research ;and the fourth one is clinical applications on nanoparticles modified with targeting agents to deliver into breast cancer cells. Hence this book chapter will be an unique one to all breast cancer researchers with the following expertise; gene delivery, nanotechnology, immunologists, clinicians, and clinical translational scientists.

2. MicroRNAs

MicroRNAs (miRNAs) are a family of endogenous, noncoding, small single stranded RNAs (21-25 nucleotides) that regulate the gene expression at post-transcriptional levels (Venugopal et al., 2009; Yoon et al., 2010). miRNAs bind to the target sequences by partial or complete binding to the 3' untranslated region (UTR) of their target mRNAs, and thereby mediate mRNA degradation or translational inhibition, which depends on the degree of complementarity between miRNAs and their targets (Carthew, 2006). They appear to function via several mechanisms in repressing gene expression and regulating cellular activities, such as development (Ambros, 2004), cell proliferation (Zhang et al., 2006), differentiation (Esau et al., 2004), apoptosis (Cimmino et al., 2005), glucose metabolism (Poy et al., 2004), stress resistance (Dresios et al., 2005), and cancer (Calin et al., 2002). One of the miRNAs, which is abundantly expressed in the liver, and appears to affect hepatic function, is microRNA-122a (miR-122a)(Krutzfeldt et al., 2005). Using antagomiR-122a, when miR-122a expression was silenced, there was a 44% decrease in cholesterol synthesis in hepatocytes (Krutzfeldt et al., 2005). The mechanism of this effect appears to be that inhibition of miR-122a caused the activation of a transcriptional repressor protein involved in cholesterol biosynthesis (Krutzfeldt et al., 2005). Another study reported that inhibition of miR-122a in the liver caused a marked loss of hepatitis C viral RNAs, and that miR-122a may represent a target for antiviral intervention (Jopling et al., 2005). Several miRNAs have

been implicated in the process of apoptosis and cell proliferation in non-liver systems (Cimmino et al., 2005; Xu et al., 2004). For example, miR-21 has been shown to inhibit caspase activation, thereby promoting anti-apoptosis (Chan et al., 2005). miR-15 and miR-16 were shown to promote apoptosis by interacting with Bcl-2 (Cimmino et al., 2005), and down-regulation of miR-27b caused apoptosis (Scott et al., 2006). Another study shows that miR-126 increased the growth and proliferation of megakaryocytes (Garzon et al., 2006), and that miR-451 enhanced the differentiation of erythroid cells (Rathjen et al., 2006). The miRNAs, which are secreted to the blood stream, can be detected and used as diagnostic markers, one such example is miR-21 released to the blood stream. It has shown by various investigators that several of these miRNAs including miR-21, miR-126 have been shown to be involved in the progression of breast cancer. Anti-cancer therapies are also focused in modulating the expression of miRNAs to inhibit cell growth, proliferation and invasion.

The biogenesis of miRNA occurs in nucleus, like other genes, **(Figure 1)** and a longer primary miRNA is transcribed by RNA polymerase II or III. The primary miRNA is processed by the RNAse III endonuclease Drosha and DGCR8 to form stem-loop structure

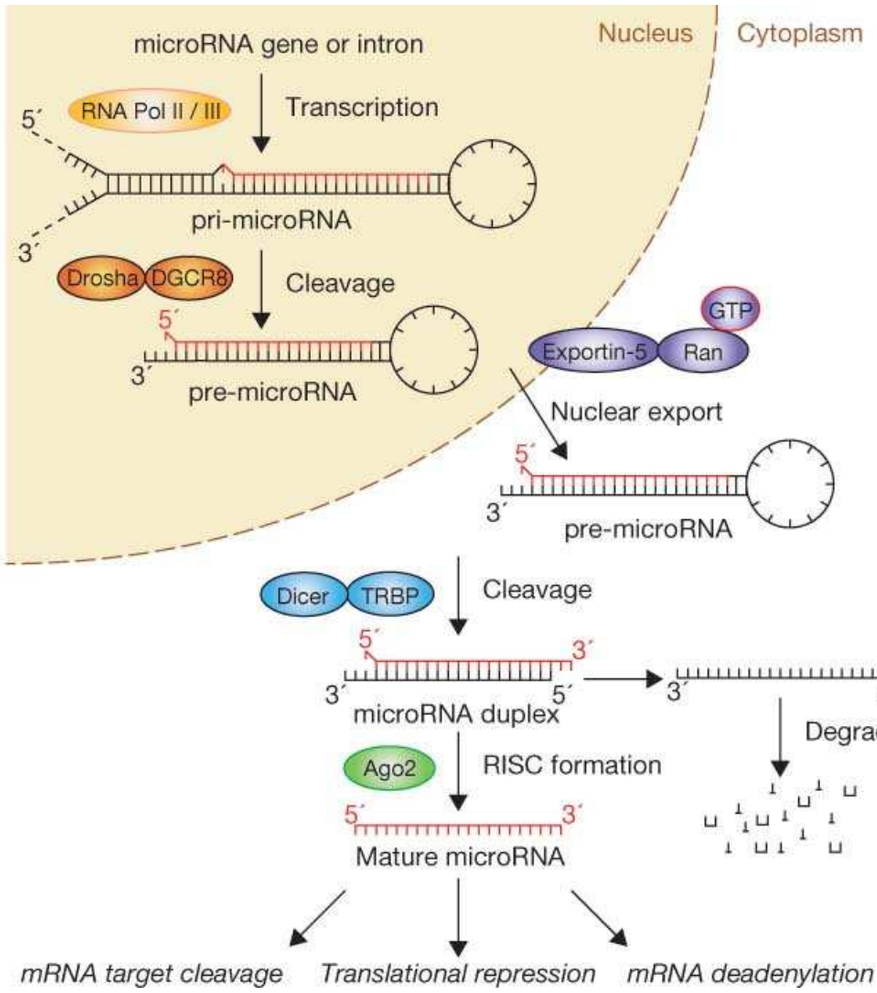

Fig. 1. Schematic diagram of the biogenesis of microRNA in eukaryotic cells. (Reproduced with permission from Nature Cell Biology, 2009)

of approximately 50 nculeotides long called "precursor miRNA" (pre-miRNA)(Borchert et al., 2006; Gregory et al., 2004; Han et al., 2004; Lee et al., 2004). The pre-miRNA is transported from the nucleus to the cytoplasm with the help of exportin 5, where this pre-miRNA will undergo further processing by another RNase III endonuclease, Dicer (Lee et al., 2003; Lund et al., 2004). Dicer removes the loop structure of the pre-miRNA to produce an imperfect duplex made up of the mature miRNA and a fragment of similar size (miRNA*), which is derived from the opposing arm of the pre-miRNA. The miRNA strand of the duplex is loaded onto the RNA-induced silencing complex (RISC); the miRNA* gets separated from the duplex and is degraded (Lee et al., 2002).

Regulation of gene expression by miRNAs occurs either by degrading the mRNA or by inhibiting the translation of the mRNA. The mechanisms by which the miRNAs inhibit the translation depends on the degree of complimentarity between the particular miRNA and the target mRNA. If there is a perfect complementarity, the target mRNA sequence is degraded and if there is an imperfect complementarity, the translation of the mRNA is blocked (Hutvagner & Zamore, 2002).

These miRNAs can bind to a seed sequence (about 6-8 bases) of the approximately 22 nucleotides aligns perfectly with the target mRNA's 3′ untranslated region and can bind more than one target sequence. Hence, finding the target sequences for these miRNAs has been difficult for researchers. Using bioinformatic approaches, we can identify the putative targets of these miRNAs, but one needs to conduct confirmatory laboratory experiments to establish the target genes.

2.1 miRNA and cancer

In most of the cancers, expression of miRNA is generally decreased, as most of them may act as tumor suppressors (Lu et al., 2005). Furthermore, poorly differentiated tumors have lower miRNA levels compared to more differentiated tumors, indicating that global changes in miRNA expression may indicate the degree of cellular differentiation. Recently it has been shown that several miRNAs were expressed at lower levels in human tumor-derived cell lines compared to the corresponding tissue (Gaur et al., 2007). Another study has also shown that a loss in the expression of miRNA in cancer resulted in tumorigenesis (Kumar et al., 2007). When drosha and dicer were knocked down, which are needed for the miRNA biogenesis, there was a complete loss of miRNA expression. When these cells were injected in nude mice, they formed accelerated growth with increased invasive properties. These results suggest that the loss of miRNA expression enhances tumorigenesis.

2.2 miRNA in breast cancer

Several studies have been conducted in the recent past on profiling miRNAs and found that several of them were aberrantly expressed in breast cancer tissues. Although several of the functions of these miRNAs were studied, still an array of experiments needs to be conducted for utilizing them as diagnostic markers or therapeutic agents. In addition, tumor metastasis may be promoted by enhanced expression of prooncogenic/prometastatic and/or downregulation of antioncogenic/antimetastatic miRNAs.

Several miRNAs have shown to be deregulated in breast cancer, suggesting that these miRNAs may modulate oncogenesis (Iorio et al., 2005b; Lu et al., 2005; Yu et al., 2010). Lu et al. (Lu et al., 2005) had reported that miRNA expression is downregulated in tumors, including breast cancer. Several miRNAs, such as miR-10b, miR-125b and miR-145 were down regulated, while miR-21 and miR-155 were up regulated, suggesting that these

miRNAs may play an important role as either tumor suppressor genes or oncogenes (Iorio et al., 2005b). A recent study has shown that miR-7, miR-128a, miR-210 and miR-51-3p were involved in breast cancer progression (Foekens et al., 2008). Upregulation of miR-373 and miR-520c promotes metastasis by inhibiting CD44 expression (Huang et al., 2008). Ma et al. (Ma et al., 2007) showed that a transcription factor, twist1, is involved in the upregulation of miR-10b. Suppression of miR-21 led to an increase in apoptosis and inhibition of bcl-2 (Si et al., 2007). miR-21 also has been shown to inhibit tropomyosin 1, which plays an important role in antioncogenic function including binding microfilaments and regulating cytosekleton miRNAs (Zhu et al., 2007). miR-27a is reported to inhibit the transcription factor ZBTB10/RINZF, which is a putative suppressor of specificity protein (Sp) (Mertens-Talcott et al., 2007). Overexpression of Sp by miR-27a contributes to the increased expression of Sp-dependent survival and angiogenic genes, including survivin, vascular endothelial growth factor (VEGF) and VEGF receptor 1. miR-175p is inhibited in breast cancer and it regulates AIB1 (Amplified in Breast Cancer-1 protein) (Hossain et al., 2006). AIB1 is a coactivator for nuclear receptors, such as estrogen receptor, and is overexpressed in breast cancer. Both miR-125a and miR-125b are down-regulated in breast cancer and these miRNAs suppress the HER2 and HER3, the two tyrosine kinase receptors frequently deregulated in breast cancer (Scott et al., 2007). miR-206 has been found to target the 3'UTR of the estrogen receptor-α protein, leading to an inverse correlation between miR-206 concentration and estrogen receptor-α status (Adams et al., 2007; Kondo et al., 2008). Some of the key miRNAs, which are shown to be involved in modulating tumor growth and metastasis, is described below.

2.3 Oncogenic miRNAs

miR-21

There have been several studies showing that miR-21 is consistently overexpressed in many tumors, including breast cancer (Iorio et al., 2005b; Volinia et al., 2006). It also has been shown that miR-21 was highly upregulated in breast tumors compared to the matched normal breast tissues among 157 human miRNAs analyzed by real-time RT-PCR arrays (Si et al., 2007), suggesting that miR-21 may function as an oncomiR. In the later study the authors have also shown that knocking down miR-21 resulted in a marginal decrease in cell survival (25% decrease), and the authors found that this growth inhibition increased when the transfected MCF-7 cells were treated with the anticancer drug topotecan, suggesting that suppression of miR-21 can sensitize tumor cells to anticancer agents. The target prediction using bioinformatics approaches followed by experimental validation showed that tropomyosin 1 (TPM1), which is known to be downregulated in breast cancer epithelial cell lines, was a target of miR-21 (Zhu et al., 2007). miR-21 has also been shown to inhibit the expression of the tumor suppressor PDCD4 (programmed cell death-4) (Frankel et al., 2008). Another important target of miR-21 is the tumor suppressor gene phosphatase and tensin homolog (*PTEN*) (Qi et al., 2009). These genes were shown to reduce invasiveness of a metastatic breast cancer cell line. These findings further establish miR-21 as an oncogenic miRNA and suggest that miR-21 has a role not only in tumor growth but also in invasion and tumor metastasis by targeting multiple anti-metastatic genes.

miR-155

MiRNA microarray has shown that miR-155 is over-expressed in a number of human malignancies, including breast cancer (Iorio et al., 2005b; Volinia et al., 2006). A recent study

has shown that miR-155 is upregulated in normal mouse mammary gland epithelial cells (NMuMG cells) by the TGF-β/Smad4 pathway and mediates TGF-β-induced EMT and cell invasion (Kong et al., 2008). miR-155 directly inhibits the expression of *RhoA*, a gene that regulates many cellular processes, including cell adhesion, motility, and polarity, and is an important modulator of cell junction formation and stability. It also has been shown that miR-155 is highly expressed in invasive tumors but not in noninvasive cancer tissues.

miR-10b

miR-10b was found to influence the metastatsis of human cancer cells (Ma et al., 2007). Unlike miR-155, which is overexpressed in many breast tumors, miR-10b was highly expressed only in metastatic cancer cells and was found to promote cell migration and invasion *in vitro* and initiate tumor invasion and metastasis *in vivo*. miR-10b expression is shown to be regulated by a transcription factor Twist, and miR-10b inhibits the translation of the transcription factor homeobox D10 (HOXD10), resulting in a cascade of cellular alterations that include expression of the prometastatic gene *RHOC* (ras homologue gene family member C), a gene that promotes cancer cell migration and invasion.

miR-373/520c family

miRNAs of the miR-373/520c family were identified (Kato et al., 2009) as prometastatic using a forward genetic screen involving overexpression of almost 450 miRNAs in a nonmetastatic human breast cancer cell line (MCF-7 cells). MCF-7 cells were transduced with these miRNAs and subjected to a trans-well cell migration assay to identify miRNAs that stimulate cell migration. The authors found that miR-373 and miR-520c promoted cancer cell migration and invasion *in vitro* and *in vivo*. It has been shown that the downregulation of CD44 (a metastasis repressor) was found when miR-373 or miR-520c were overexpressed in MCF-7 cells.

2.4 Tumor suppressor miRNAs

miR-206

Using miRNA microarrays it was found that miR-206 is involved in suppressing breast cancer cells (Iorio et al., 2005b). miR-206 was upregulated in estrogen receptor (ER)α-negative breast cancers, suggesting a role of miR-206 in regulation of the estrogen receptor gene *ERa* (*ESR1*). Indeed, miR-206 was recently shown to inhibit the expression of *ESR1* mRNA through two binding sites in the *ESR1* 3' UTR (Kumar et al., 2007). The latter study also showed that miR-206 expression was strongly repressed by ERα agonists, but not by an ERβ agonist or progesterone, suggesting the existence of a feedback loop. Another study had shown that miR-206 expression decreased in ERα-positive human breast cancer tissues and that miR-206 suppresses ESR1 expression and inhibits growth of MCF7 breast cancer cells. In addition to miR-206, the authors found that *ESR1* mRNA was a direct target of miR-18a, miR-18b, miR-193b and miR-302c in breast cancer cells (Leivonen et al., 2009), most of which were shown to induce cell cycle arrest and to inhibit estrogen-induced proliferation. Bioinformatics approach had revealed that the direct targets of miR-335 are *PTPRN2*, *MERTK*, *TNC* and *SOX4*; among them, *TNC* (encoding an extracellular matrix component) and *SOX4* (encoding a transcription factor that is involved in tumorigenesis) were shown to be functional targets implicated in metastasis. Restoring miR-206 expression in metastatic cells did not influence their proliferation or sensitivity to apoptosis, but altered cellular morphology, possibly contributing to a decrease in cell motility that could limit the

migration of metastatic cells (Tavazoie et al., 2008). These findings suggest that miR-206 could be a novel candidate for breast cancer therapy.

miR-17-5p

miR-17-5p, also known as miR-91, is located on chromosome 13q31, a genomic region that undergoes loss of heterozygosity in multiple cancers, including breast cancer (Eiriksdottir et al., 1998). The oncogene *AIB1* (amplified in breast cancer) is a direct target of miR-17-5p (Hossain et al., 2006). The protein encoded by the *AIB1* gene is a steroid receptor co-activator that enhances the transcriptional activity of *ERa*, *E2F1* (which is also directly regulated by miR-17-5p) and other transcription factor genes. Downregulation of AIB1 by miR-17-5p results in the suppression of estrogen-stimulated proliferation and estrogen/ER-independent breast cancer cell proliferation (Hossain et al., 2006). In breast cancer cells, the gene cyclin D1 (*CCND1*), which is overexpressed in approximately 50% of human cancers, was recently identified as a direct target of miR-17-5p (Yu et al., 2008). miR-17-5p inhibits the proliferation of breast cancer cells by suppressing cyclin D1 protein synthesis. *miR-125a and miR-125b*

miR-125a and miR-125b are downregulated in HER2-amplified and HER2-overexpressing breast cancers (Mattie et al., 2006b). These two miRNAs are potential tumor suppressors and their overexpression in SKBR3 cells (a HER2-dependent human breast cancer cell line) suppresses *HER2* and *HER3* mRNA and protein levels, leading to a reduction in anchorage-dependent growth, cell motility, and invasiveness (Scott et al., 2007). However, this influence was subtle in non-transformed HER2-independent breast cancer cells (MCF10A).

miR-200 family

Overexpression of miR-200 induced epithelial differentiation in undifferentiated breast cancer cells (MDA-MB231) whereas antagonizing it induced an EMT phenotype in the colorectal cancer cell line HCT116. *ZEB1* and *ZEB2* were again detected as targets of the miR-200 family members. These and other recent reports on the miR-200 family (Hurteau et al., 2009; Korpal et al., 2008) add important miRNAs to the growing list of tumor-associated miRNAs. Furthermore, a recent study showed that the expression of the miR-200 family was decreased by Akt2, suggesting that, in many cases, breast cancer metastasis may be under the control of the Akt-miR-200-E-cadherin pathway (Iliopoulos et al., 2009). Interestingly, a recent study showed that expression of miR-200 unexpectedly enhanced macroscopic metastases in mouse breast cancer cell lines (Dykxhoorn et al., 2009). Their results suggest that, for some tumors, tumor colonization at metastatic sites might be enhanced by mesenchymal-to-epithelial transition (MET). MET is a reversible biological process that involves the transition from motile, multipolar or spindle-shaped mesenchymal cells to planar arrays of polarized cells called epithelia. Thus the epithelial nature of a tumor would be difficult to predict metastatic outcome. Taken together, these studies suggest that miRNAs of the miR-200 family play important roles in regulating tumor progression and metastasis.

let-7 family

let-7 is poorly expressed or deleted in many human cancers. Recent data from both hematologic malignancies and solid tumors suggest that each includes minor populations of cells that are capable of tumor initiation (Clarke & Fuller, 2006). A recent study compared miRNA expression in self-renewing and differentiated cells from breast cancer lines and found that the expression of let-7 was strongly reduced in breast tumor initiating cells (BT-

ICs) and increased with differentiation (Yu et al., 2007). Introducing let-7 in BT-ICs reduced their proliferative capacity, their ability to form mammospheres and tumor formation and metastasis *in vivo*. Conversely, knocking down let-7 enhanced self-renewal of non-T-ICs *in vitro*. Known oncogenic targets of let-7, such as *H-RAS* and *HMGA2*, were downregulated by let-7 overexpression. Silencing *H-RAS* in a BT-IC-enriched cell line reduced self-renewal but had no effect on differentiation, while knocking down *HMGA2* enhanced differentiation but did not affect self-renewal. Their results suggest that let-7 regulates multiple BT-IC stem cell-like properties and that let-7 may offer a unique opportunity to attack tumor stem cells using therapeutic RNA. Delivery of the *let-7* miRNA to tumors could potentially deplete stem cells by inducing cellular differentiation.

miR-34a

miR-34a is one of several miRNAs that are downregulated in multiple cancers (Gaur et al., 2007) and has been shown to be transcriptionally regulated by p53. In the context of breast cancer, only one study (Kato et al., 2009) has shown that miR-34a levels were lower in triple negative and mesenchymal breast cancer cell lines compared with normal epithelial lines and HER-2+ lines. Increasing the levels of miR-34a protected the MDA-MB-231 cells from radiation-induced cell death, and downregulating it had the converse effect. These results show that miR-34a is necessary for the survival of MDA-MB-231 cells from non-apoptotic cell death and suggest that miR-34a may have therapeutic potential in breast cancers, since antagonizing miR-34a increases the sensitivity of breast cancer cells towards radiation.

miR-31

miR-31 was recently shown to prevent metastasis at multiple steps by inhibiting the expression of prometastatic genes (Valastyan et al., 2009). miR-31 is expressed in normal breast cells and its abundance was shown to be dependent on the metastatic state of the tumor. It is moderately decreased in non-metastatic breast cancer cell lines and is almost undetectable in metastatic mouse and human breast cancer cell lines. Importantly, the authors demonstrated that introducing miR-31 in metastatic breast cancer cells suppressed metastasis-related functions (motility, invasion and resistance to anoikis) *in vitro* and metastasis *in vivo*. Injecting miR-31 overexpressing cells directly into the circulation impeded the ability of the cells to survive and form secondary tumors in the lung, suggesting that miR-31 inhibits metastasis at multiple steps of the metastatic cascade. Conversely, inhibition of miR-31 function increased invasiveness and promoted metastasis *in vivo*. The targets have been identified for this miRNA is frizzled3 (*Fzd3*), integrin α-5 (*ITGA5*), myosin phosphatase-Rho-interacting protein (*M-RIP*), matrix metallopeptidase 16 (*MMP16*), radixin (*RDX*) and the ras homolog gene family member A (*RhoA*). Taken together, their findings demonstrate that miR-31 may also be an attractive therapeutic target for breast cancer as it exerts its antimetastatic effect by targeting multiple prometastatic genes of the metastasis cascade.

3. Nanoparticles in breast cancer translational research

Nanoparticles (NP) have been widely utilized in biomedical applications; such as drug delivery, transfection agents, molecular imaging, and as bioprobes. The size and surface area of NP provide unique features to modify various functions using organic/inorganic compounds. Thus NPs provide an excellent template for multimodality agents such as imaging, tracking, and sensitive detection of biomarkers specific to disease (Medarova et al.,

2007; Phillips et al., 2008; Sonnichsen et al., 2005; Yang et al., 2007). Several classes of NPs have been developed and utilized for various applications. For example, oligonucleotide-functionalized nanoparticles have been introduced as an effective bioassay tool, referred to as a bio-barcode assay, which allows for sensing small biomolecules such as β- amyloid related to Alzheimer's disease and small DNA fragments associated with various diseases such as human immunodeficiency virus (HIV) (Keating, 2005; Stoeva et al., 2006). Furthermore, NPs linked to antibodies have demonstrated selective binding of rare stem cells, immune cells and cancer cells presenting specific receptors on the cell surface (Pissuwan et al., 2007). Several study reports have been showed that antibodies linked to NP can detect or target cells with high specificity (Yang et al., 2007). In our recent study, we have used gold nanoparticles (AuNP) of a small size (10 nm) coated with streptavidin (SA) for conjugation of molecules to create a nanoimmunogene conjugate (Yoon et al., 2010). This conjugates includes an apoptosis-inducing miRNA (miRNA-491), breast cancer targeting MAb, and quantum dots to target breast cancer selectively and to induce apoptosis (Natarajan et al., 2009). This novel approach of making multifunctional nanoparticles, combining NP, miRNA and antibody, will target cell surface receptors, and subsequently nanoparticles will enhance the transfection of apoptosis-inducing miRNA into the cell to enhance the apoptosis **(Figure 2)**.

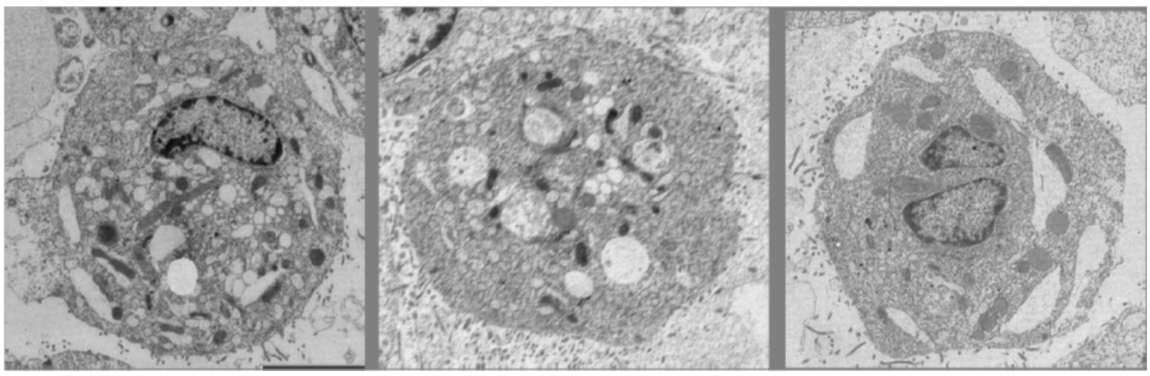

Fig. 2. Images left to right shows evidence of apoptosis in HBT3477 human breast cancer cells treated with miR-491-AuNP 10nm for 24h. The apoptotic effect was analyzed by Transmission Electron microscopy to confirm the morphological changes at a cellular level. Morphological changes show irregular nuclear contours, smudged chromatin (left panel), and focal breakdown of the nuclear membrane (center panel) as well as; marginated and clumped chromatin at the periphery (right panel).

3.1 Targeted nanoparticles

Nanomaterials or nanoparticles (NPs) are currently, synthesized by various polymers and lipids; iron, gold and silica particles. These particles are typically < 200 nm size used for drug delivery vehicles that can carry multiple drugs and/or imaging agents. Due to high surface-area-to-volume ratio, it is possible to achieve high ligand density on the surface for targeting purposes. Further, NPs can also be used to increase local drug concentration by carrying the drug within and control-releasing it when bound to the targets.

Targeted nanoparticles (NPs) conjugates are created by linking to targeting agents such as proteins (mainly antibodies and their fragments), nucleic acids (aptamers), or other receptor

ligands (peptides, vitamins, and carbohydrates). These NPs that are chemically conjugated with drugs are often considered new chemical entities owing to a distinct pharmacokinetic profile from that of the parent drug.

In spite of variety of novel drug targets and sophisticated chemistries available, only four drugs (doxorubicin, camptothecin, paclitaxel, and platinate) and few polymers (N-(2-hydroxylpropyl)methacrylamide (HPMA) copolymer, poly-*L*-glutamic acid, poly(ethylene glycol) (PEG), and Dextran) have been reported quite often used to develop NP–drug conjugates(Duncan, 2006).

Many polymer based NP–drug conjugates have entered Phase I and II clinical trials and these are especially useful for targeting blood vessels in tumors. Examples include anti-endothelial immunoconjugates, fusion proteins (Arap et al., 1998; Halin et al., 2002; Schraa et al., 2002), and caplostatin, the first polymer-angiogenesis inhibitor conjugates (Satchi-Fainaro et al., 2004).

3.2 miRNA-NPs

The key to success of RNA-based therapeutics is to have effective strategies for the delivery of siRNA or miRNA in vivo (Hammond, 2006). For example, modification of antisense RNA with a cholesteryl functionality results in enhanced stability in the serum, improved cellular uptake and inhibition of target mRNA (Krutzfeldt et al., 2005). If the delivery of miRNAs through nanoparticles is proven to be an effective breast cancer therapeutic, this could revolutionize breast cancer treatment. In contrast to chemotherapy, targeted miRNA-nanoplatform is only the tumor would be targeted for treatment, preventing serious side-effects. Thus delivering siRNA and / or miRNA with nanoparticles simultaneously targeting several different oncogenic pathways is definitely an important advantage of the current approach.

Chen et al first developed an approach for co-delivery of siRNA and miRNA using liposome-polycation-hyaluronic acid particles with tumor targeting single-chain antibody fragment to target murine B16F10 melanoma (Chen et al., 2010). Targeted nanoparticles with miR-34a induced apoptosis inhibited survivin expression, in the metastatic tumor and reduced tumor load in the lung. This study clearly demonstrated this novel therapeutics enhanced the antitumor effect. Similarly in another study by Anand et al demonstrated mir-132 linked nanoparticles were able target $\alpha_V\beta_3$ integrins to manuplate miRNA levels in the endothelium to control pathological neovascularization (Anand et al., 2010).

Natarajan et al delivered miR491 to induce apoptosis in human breast cancer cells (HT3477) using quantum dots, gold nanoparticles, and HBT3477 targeting Mab. The transmission electron microscopy (Fig 2) clearly showed the apoptotic effect on cells due to delivery of Au-NP-miR491, compared to control cells which were not delivered miR-491.

3.3 SiRNA-NPs

Gene silencing using short interfering RNA (siRNA) is an attractive approach to probe gene function, and to silence cancer genes in mammalian cells. Recently some success in the delivery of siRNA using various methods; here we mainly discuss their delivery through non viral vectors using nanoparticles for breast cancer imaging and therapy. Kumar et al synthesized (Kumar et al., 2010) a tumor-targeting nanodrug of Iron oxide nanoparticles linked to peptide and small interfering RNA (siRNA), (MN-EPPT-siBIRC5) to specifically shuttle siRNA to human breast tumors. The nanodrug binds the tumor-specific antigen

uMUC-1, which is found in >90% of human breast adenocarcinomas. MN-EPPT-siBIRC5 consists of superparamagnetic iron oxide nanoparticles [for magnetic resonance imaging (MRI)], the dye Cy 5.5 (for near-IR optical imaging), peptides (EPPT) that specifically target uMUC-1, and a synthetic siRNA that target the tumor-specific anti apoptotic gene BIRC5. Nanodrug uptake by human breast adeno carcinoma cells resulted in a significant down regulation of BIRC5. Following i.v. delivery into subcutaneous mouse models of breast cancer, the nanodrug showed a preferential tumor uptake, which could be visualized by MRI and near-IR optical imaging. Intravenous injection of the agent once a week over 2 weeks resulted in the induction of considerable levels of necrosis and apoptosis in the tumors, translating into a significant decrease in tumor growth rate. This strategy permits the simultaneous tumor-specific delivery of siRNA to tumors and the imaging of the delivery process.

Bouclier et al studied suppression (Bouclier et al., 2010) of oestrogen receptor alpha (ERalpha) functions by silencing RNAs in association with or not with anti-oestrogens (AEs) both in vitro and in breast cancer cell xenografts was assessed. In vitro, a prolonged decrease in ERalpha protein expression and an enhanced AE-induced inhibition of ERalpha-mediated transcription, together with antiproliferative activity, were observed. Incorporation of ERalpha-siRNAs in PEGylated nanocapsules (NC) was achieved; and their intravenous injections in MCF-7 xenografts, in contrast to scramble siRNA containing NCs, lead to decrease in ERalpha protein content and Ki67 labeling in tumour cells. In this study, co-injection of the two nanocarriers potentiated the decrease in ERalpha protein, concomitantly with decreasing tumour vasculature and glucose transporter-1. These data support that the targeted delivery of ERalpha-siRNA in breast tumors potentiates the inhibition of E(2)-induced proliferative activity by encapsulated AE through enhanced anti-vascular activity. The study findings suggest that the anti-oestrogen activity of RU as well as that of targeted ERalpha-siRNA leads to anti-angiogenic activity. Their delivery in "stealth" nanocarriers may constitute a new anti-cancer therapeutic strategy in solid tumors. For example to create "stealth" (sneaky) characteristics for nanoparticles the surface of the particles were coated with polyethylene glycol (PEG), a procedure called PEGylation to protect nanocarriers from the reticulo-endothelial system.

Tan et al (Tan et al., 2007) synthesized chitosan NPs with encapsulated quantum dots (QDs) to deliver HER2/neu siRNA. These constructs were tested for the delivery and track the siRNA by monitoring the presence of fluorescent QDs in the chitosan NPs. Chitosan is a linear polysaccharide compound composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). It is a fiber isolated from shellfish. It has a unique property that allows it to bind with fat. Targeted delivery of HER2 siRNA to HER2-overexpressing SKBR3 breast cancer cells was shown to be specific with chitosan /QD NP surface labeled with HER2 antibody targeting the HER2 receptors on SKBR3 cells. In this study gene-silencing effects of the conjugated siRNA was established using the luciferase and HER2 ELISA assays. These self-tracking siRNA delivery NPs could be useful in the monitoring of future gene silencing studies both in vitro and in vivo.

3.4 Immunoconjugate linked NPs

DeNardo et al studied (DeNardo et al., 2005) radioisotope conjugated-chimeric L6 mAb-linked iron oxide nanoparticles (bioprobes) studied in athymic mice bearing human breast cancer HBT 3477 xenografts. These NP based bioprobes were given to i.v., escapes into the

extravascular space and bind to cancer cell membrane antigen, later these bioprobes can be used in concert with externally applied alternating magnetic field (AMF) to deliver thermoablative cancer therapy. The pharmacokinetics, tumor uptake, and the therapeutic effect of inductively heating by bioprobes indicated these probes were capable of killing breast cancer cells selectively.

In another study Gao et al developed (Gao et al., 2009) PE38KDEL-loaded poly(lactic-co-glycolic acid) (PLGA) nanoparticles conjugated to Fab' fragments of a humanized anti-HER2 monoclonal antibody (rhuMAbHER2). The PE38KDEL-loaded nanoparticles-anti-HER2 Fab' bioconjugates (PE-NP-HER) were constructed modularly with Fab' fragments of rhuMAbHER2 covalently linked to PLGA nanoparticles containing PE38KDEL. Compared with nontargeted nanoparticles that lack anti-HER2 Fab', PE-NP-HER specifically bound to and were sequentially internalized into HER2 overexpressing breast cancer cells, which resulted in significant cytotoxicity in vitro.

Recently Chattopadhyay et al reported (Chattopadhyay et al., 2010) an immunoconjugate linked 30nm AuNPs for HER-2 targeting NPs. This nanotechnology-based radiosensitizer was tested on SK-BR-3 breast cancer cells *in vitro*. This newly designed trastuzumab-AuNPs described in this study could be useful to for breast cancer treatment.

4. Nanoparticles in clinical studies for breast cancer therapy

Ranson et al, published the treatment outcome of Caelyx (Doxil; Sequus Pharmaceuticals Inc, Menlo Park, CA), the PEGylated liposomal doxorubicin NP in advanced breast cancer (Ranson et al., 1997). In advanced breast cancer, standard therapy regimens yield infrequent complete responses, and continued therapy has been associated with an improved quality of life (Coates et al., 1987) and favorable time to progression (Muss et al., 1991) over intermittent therapy or observation, respectively. This study reported median survival of 7 months and the 9-month time to progression in the responding patients and it proposed if the majority of patients that completed six cycles had received additional Caelyx, the survival and time to progression might have been extended further.

Metastatic breast cancer is still incurable. Taxanes represent an important class of antitumor agents, which have proven to be fundamental in the treatment of advanced and early-stage breast cancer, but the clinical advances of taxanes have been limited by their highly hydrophobic molecular status. To overcome this poor water solubility, lipid-based solvents have been used as a vehicle, and new systemic formulations have been developed, mostly for paclitaxel, which are Cremophor-free and increase the circulation time of the drug. ABI-007 is a novel, albumin-bound, 130-nm particle formulation of paclitaxel, free from any kind of solvent. It has been demonstrated to be superior to an equitoxic dose of standard paclitaxel with a significantly lower incidence of toxicities in a large, international, randomized phase III trial. The availability of new drugs, such as Abraxane, in association with other traditional and non-traditional drugs (new antineoplastic agents and targeted molecules), will give the oncologist many different effective treatment options for patients in this setting (Miele et al., 2009).

In another study metastatic breast cancer (MBC) phase II study evaluated the efficacy and safety of weekly administration of nanoparticles with albumin-bound paclitaxel as a first-line treatment (Mirtsching et al.). The overall response rate (ORR) was 42.2% (95% CI, 30%-55%); 5 patients had a complete response (CR) and 22 patients had a partial response (PR). Additionally, 17 patients experienced stable disease (SD), providing an overall benefit (CR +

PR + SD) of 68.8%. These findings demonstrated that weekly NP- paclitaxel had a favorable safety profile and is well tolerated as a first-line treatment for MBC. An ORR of 42% and an overall benefit of 69% is extremely encouraging, particularly in the HER2-positive population where 52% of patients responded. Clinically approved NP-conjugates for breast cancer are listed in Table 1.

NP type /material (Brand Name)	**Drug entrapped or linked**	**Current stage of development**	**References**
PEG-liposomes (Doxil/Caelyx)	Doxorubicin	Phase I/II	(Coates et al., 1987; Ranson et al., 1997)
Albumin (Abraxane)	Paclitaxel	Phase II/III	(Lobo et al., 2007; Miele et al., 2009; Moreno-Aspitia & Perez, 2005)

Table 1. Nanoparticles and their current stage of development for use in breast cancer therapy

5. Summary

In conclusion, we envision that breast cancer targeting agents such as miRNAs, siRNAs, and immunoconjugates are already demonstrated that these agents could be potential molecules for molecular cancer therapy. Only few siRNAs are in clinical trials because of their off-target effects, and the efforts being made to develop miRNAs as either diagnostic or therapeutic markers, since these they are one of the natural mechanisms to control the gene expression. For example Rosetta Genomics, Israel-based microRNA Genomics Company is already actively planning for Investigational New drug (IND) study using miRNA for infectious disease and hepatocellular carcinoma the fifth most common cancer in the world. Similarly, the first in-human phase I clinical trial involving the systemic administration of siRNA to patients with solid cancers using a targeted, nanoparticle delivery system. Davis et al., (California Institute of Technology, Pasadena, California 91125, USA) study indicated the evidence of inducing an RNAi mechanism of action in a human from the delivered siRNA. Tumour biopsies from melanoma patients obtained after treatment show the presence of intracellularly localized nanoparticles in amounts that correlate with dose levels of the nanoparticles administered.

Several reports have shown that use of nanoparticles and nanotechnologies have been giving very encouraging results. One of the efficient ways to deliver these therapeutic molecules is through the use of nanoparticles. These novel technologies will help in achieving accurate early detection, prognosis, imaging and targeted therapy for breast cancer.

In spite of many positive reports on this area, very few nanoparticles linked with targeting agents are showed desired properties for clinical studies. Further studies are needed in

translational research to incorporate these molecules into nanoparticles for the cancer treatment options. Therefore active research on nanotechnology warrants as answer for a number of questions prior to translational research: example specificity, toxicity, well defined characterization, stringent quality and in vivo clearance profile. Although active researches are ongoing in this area still we need to go a long way to achieve curable treatment outcome using these agents and technology.

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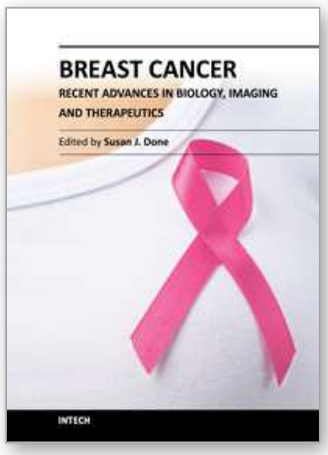

Breast Cancer - Recent Advances in Biology, Imaging and Therapeutics

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In recent years it has become clear that breast cancer is not a single disease but rather that the term encompasses a number of molecularly distinct tumors arising from the epithelial cells of the breast. There is an urgent need to better understand these distinct subtypes and develop tailored diagnostic approaches and treatments appropriate to each. This book considers breast cancer from many novel and exciting perspectives. New insights into the basic biology of breast cancer are discussed together with high throughput approaches to molecular profiling. Innovative strategies for diagnosis and imaging are presented as well as emerging perspectives on breast cancer treatment. Each of the topics in this volume is addressed by respected experts in their fields and it is hoped that readers will be stimulated and challenged by the contents.

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