

Review

The Role of micro RNAs in Breast Cancer Metastasis: Preclinical Validation and Potential Therapeutic Targets

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Abstract. *Despite the approval of several molecular therapies in the last years, breast cancer-associated death ranks as the second highest in women. This is due to metastatic disease, which represents a challenge for treatment. A better understanding of the molecular mechanisms of metastasis is, therefore, of paramount importance. In this review we summarize the role of micro RNAs (miRs) involved in metastasis of breast cancer. We present an overview on metastasis-promoting, -suppressing and context-dependent miRs with both activities. We have categorized the corresponding miRs according to their target classes, interaction with stromal cells or exosomes. The pathways affected by individual miRs are outlined in regard to *in vitro* properties, activity in metastasis-related *in vivo* models and clinical significance. Current approaches that may be suitable for therapeutic inhibition or restoration of miR activity are outlined. Finally, we discuss the delivery bottlenecks which present as a major challenge in nucleic acid (miR)-based therapies.*

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Key Words: Colonization of distinct organs, epithelial-mesenchymal transition (EMT), exosomes, metastasis-related *in vivo* models, mesenchymal-epithelial transition (MET), migration and invasion, tumor cell/stromal cell interactions, review.

Breast Cancer (BC) is the second leading cause of death in women with a death toll of 40,000 in the US in 2015 (1). In recent years there have been a number of advances in targeted therapies for BC, for example the combination of aromatase inhibitors with everolimus (afinitor) and cyclin-dependent kinases 4/6 (cdk4/6) inhibitors such as palbociclib (ibrance) in estrogen-receptor-positive (ER⁺) tumors, the use of trastuzumab (herceptin), pertuzumab (perjeta) and adotrastuzumab emtansine (kadcyla) in patients with human epidermal growth factor 2 overexpressing (HER2⁺) tumors and the identification of subgroups of BC patients which respond to Platin-based chemotherapy and poly (adenosine-diphosphate-ribose polymerase) (PARP) inhibitors (2)). Yet despite these improvements, a high death toll remains and is due to the difficult tractability of metastatic disease, which is only poorly affected by these agents. A further complicating issue is the fact that the frequency of mutated drivers such as ER, HER2, phosphatidylinositol 3-kinase catalytic α polypeptide (PIK3CA) and AKR mouse thymoma kinase 1 (AKT1) is low in BC metastases (3). Another critical issue is the heterogeneity of the disease as manifested by the occurrence of several histological and molecular subtypes (4). Whereas targeting the seeds of metastasis is difficult, progress has been made by targeting the soil in prevention and treatment of BC-derived bone metastasis. Treatment with bisphosphonates, receptor activator of nuclear factor κ B (RANK)-monoclonal antibody denosumab and transforming growth factor β (TGF β) inhibitors such as LY157299 inhibit osteoclasts, which contribute to the pathogenesis of metastatic colonization (5).

The invasion-metastasis cascade is defined by distinct steps such as invasion, intravasation, survival in the circulation, arrest at distant organ sites, formation of

micrometastases and metastatic colonization (6, 7). Several promising targets for inhibition of BC metastases have been identified, but their clinical validation is still pending (6, 7). One has to keep in mind that BC can disseminate to different organs such as the bones, lungs and brain, making use of organ-tropism specific mechanisms. In addition to these pathways, shared mechanisms seem to be activated in metastatic BC (8).

In this review we focus on the role of micro RNAs (miRs) as modulators of BC metastasis. As promoters or suppressors, such miRs may also serve as targets for therapeutic intervention.

miRs – General Aspects and Roles in Cancer

MiRs are small non-coding RNAs with lengths varying between 18 and 25 nucleotides (nts). In contrast to long-noncoding RNAs they are evolutionary conserved (9, 10) and involved in post-transcriptional regulation of gene expression. Their molecular mode of action is targeting specific mRNAs *via* complementary sequences resulting in their degradation by RNases or inhibition of translation (11). More than 1000 miR genes have been identified in the human genome, the vast majority of them being transcribed by RNA polymerase II. This generates primary transcripts referred to as pri-miRNA which contain a 7-methyl-guanosin cap, a polyA-tail and may additionally contain introns (12, 13). Pri-miRs can be transcribed from intergenic regions or from introns and can encode a single miR or clusters of two or more miRs (13). The pre-miRs are cleaved by the Microprocessor complex which is composed of DROSHA, a double-stranded RNase III enzyme and its co-factor double-stranded RNA binding DiGeorge syndrome critical region 8 (DGCR8) (14). Each strand of the double-stranded pri-miR is cleaved, resulting in the generation of a hairpin shaped precursor miR (pre-miRNA) comprised of 60-70 nts. This precursor is exported by double-stranded RNA binding protein exportin 5 (XPO5) (15) to the cytoplasm. MiRs with mRNA degradation functionality interact in the cytoplasm with the RNase DICER and finally form the RNA-induced silencing complex (RISC) (16). The latter consists of the transactivation-responsive RNA binding protein (TRBP) and Argonaute 2 (Ago2) which together with DICER give rise to a mature miR-miR* 18-25 nts duplex. Finally, the two strands are separated: the mature miR strand (guide strand) is incorporated into the RISC, whereas the passenger miR* strand can be loaded into the RISC or be degraded. A 6-8 nts sequence at the 5' end of the miR is crucial for mRNA target selection, preferentially located in the 3'-untranslated region (3'-UTR) of the target mRNA (17, 18). After base pairing, potential functional consequences are degradation of the corresponding mRNA or inhibition of its translation. Overall, one should keep in mind that a miR can target several different mRNAs and individual mRNAs can be suppressed

by multiple miRs, underlining the important role of miRs in gene regulatory networks. It is expected that the functional outcome of inhibition or activation of a specific miR will be context-dependent based on the transcriptional program of an individual cell. It is expected that 30-50% of human genes are post-transcriptionally regulated by miRs (9, 11).

An important impact of miRs covering all stages of pathogenesis has been noted in cancer (9, 19, 20). Oncogenic and tumor-suppressive functions or in some cases both roles have been assigned to defined miRs (18, 21). These findings rely on proof-of-concept experiments in B-cell chronic lymphocytic leukemia (B-CLL) (22). Tumor suppressors frequently deleted in B-CLL were identified on chromosome 13q14, a locus containing two miR genes, miR15a and miR-16-1 (22). Their function could be delineated to repression of B-cell lymphoma 2 (Bcl-2), an anti-apoptotic protein overexpressed in B-CLL and many types of solid tumors (23, 24). Noteworthy, deletion of the miR-15 and miR-16-1 cluster in mice recapitulated the human disease phenotype of B-CLL (25).

In this review we specifically focus on the role of miRs in metastasis of BC. We discuss metastasis-promoting and metastasis-suppressing miRs as well as miRs with context-dependent metastasis-related function in BC. For interpretation of the results presented it should be considered that BC molecular subtypes can be defined based on their transcriptional profile which might be responsible for subtype specific function of defined miRs. The major subtypes identified are: luminal A (ER high, HER2 low), prevalence 40%; luminal B (ER low, HER2 low), prevalence 20%; HER2-enriched (HER2 high, ER⁻), prevalence 10-15%; basal-like, triple-negative breast cancer (ER⁻, progesterone receptor negative (PR⁻), HER2⁻), prevalence 10-15% and claudin-low (claudin 3,4,7 low, E-cadherin low, vimentin+ and zinc finger E-box-binding homeobox 1 (zeb1)+), prevalence 12-14% (26, 27). Different breast cancer-derived cell lines represent and serve as *in vitro* models for different subtypes of BC. These will therefore also be addressed when discussing the role of specific miRs in metastasis (27).

Breast Cancer Metastasis-promoting miRs

In this chapter we are alluding to miRs with validated mode of action *in vitro* and *in vivo* with respect to metastasis-related functions and documented correlation to clinical features of metastatic BC. Metastasis-promoting miRs can be expressed by tumor cells, or they can be activated by interaction between stromal cells and tumor cells and prometastatic miRs transferred by exosomes. In the first paragraphs we summarize miRs expressed by tumor cells which down-regulate metastasis-suppressing genes, Rho-ROCK signaling related miRs and miRs with an impact on a variety of other signaling pathways.

miRs targeting metastasis-suppressing genes.

miR-21: miR-21 is a BC-related target with an impact on tumorigenesis as well as metastasis. The tumorigenesis-related proteins tumor suppressor phosphatase and tensin homolog (PTEN) (28) and anti-apoptotic protein bcl-2 have been identified as targets (29). Here we focus on the metastasis-related functions of miR-21. The following have been identified as anti-invasive targets of miR-21: metastasis-suppressors programmed cell death 4 (PDCD4) (30, 31), maspin (30, 32), tumor suppressor gene tropomyosin (30, 33, 34) and tissue inhibitor of metalloproteinase 3 (35). Interestingly, HER2-induced motility of BC cells is mediated by E26 transformation specific-1 (Ets-1) induced miR-21 transcription and inhibition of its downstream effector PDCD4 (36). Suppression of miR-21 in MDA-MB-231 (basal-type BC cells) is associated with a 10-fold decrease of invasion *in vitro* and lung metastasis after tail vein injection (30). Regarding the relevance of miR-21 as a prognostic parameter, it has been shown that miR-21 overexpression in human BC is associated with clinical stage, lymph node metastasis and patient poor prognosis (37).

miR-93: miR-93 was identified as a miR up-regulated in BC specimens in comparison to benign breast tissue (38). MT-1 BC cells (human, ER-, PR-, HER2+) transfected with miR-93 gave rise to tumors with increased density of blood vessels in non-obese diabetic-severe combined immunodeficiency (NOD-SCID) mice and enhanced lung metastasis after tail vein injection (38). Large tumor suppressor, homology 2 (LATS2) was identified as a direct target of miR-93 (38-41). Ectopic expression of LATS2 decreased survival and invasion of MT-1 cells (38). Moreover, when miR-93 is overexpressed in the marginally invasive MCF-7 cells, proliferation and invasion of these cells is increased (42). *In situ*-hybridization of miR-93 in BC tissues indicated elevated expression levels especially in triple negative breast cancer (TNBC) pointing to a possible role of miR-93 as a biomarker in this subtype of BC (42).

Metastasis-promoting miRs targeting the Rho-ROCK signaling pathway. Rho-associated kinases (ROCKs) regulate morphology, adhesion, stress fiber formation and motility through their serine-threonine kinase activity (43, 44). ROCK signaling has been implicated with BC metastasis and ROCK expression in late stage tumors and metastases is negatively correlated with survival (43).

miR-10b: miR-10b was identified as a miR up-regulated in several metastatic BC cells and is located in the intergenic region between homeobox domain 4 (HOXD4) and HOXD8 genes (45, 46). miR-10b is induced by the epithelial mesenchymal transition (EMT)-promoting transcription factor Twist and inhibits translation of the transcriptional repressor HOXD10 (45). As a consequence, tumor-spreading promoting

targets such as GTPase RhoC, urokinase plasminogen activator receptor (uPAR) and matrix metalloprotease 14 (MMP-14) are overexpressed (45). Independently, syndecan, functioning as a transmembrane co-receptor for growth factors, chemokines and angiogenic factors as well as a matrix adhesion receptor (47, 48) was identified as a target of miR-10a. Ectopic expression of miR-10b in immortalized human mammary epithelial cells (HMEC) and non-metastatic SUM149 BC cells (basal subtype) (49) had no effect on proliferation *in vitro*, but mediated a 4-6 fold increase in cell motility and invasiveness (45). Ectopic expression of miR-10b in the non-invasive SUM149 cells and in the invasive, but non-metastatic SUM159 BC cells (basal subtype) (49), had no impact on tumor growth after fat pad implantation, but mediated appearance of lung metastases and macroscopic peritoneal metastases (45). Therapeutic proof-of-concept (POC) experiments were performed in the syngeneic 4T1 mouse BC model (subtype TNBC) based on a transplantable cell line which produces metastatic tumors in lung, liver, lymph nodes and brain (50). In this model dissemination of fat pad implanted 4T1 cells to the lungs was suppressed after systemic administration of anti-miR-10b antagomirs, chemically modified anti-miR oligonucleotides (51). Similar results were obtained by expression of a miR-10b sponge containing multiple 3'UTR tandem binding sites for miR-10b, in 4T1 cells. Interestingly, the miR-10b antagomir did not affect lung colonization of 4T1 cells after tail vein injection, pointing to a role of miR-10b in earlier stages of the metastatic process in this system (51). Regarding clinical correlation, the expression of miR-10b was lower in breast carcinomas from metastasis-free patients (n=5) and 50% of metastasis-positive patients (9/18) had elevated miR-10b levels in their primary tumors (45).

miR-548j: miR-548j was identified in a genetic screen by transducing a lenti-miR virus library into MCF-7 (BC subtype luminal A) cells followed by transwell invasion assays (52). Endogenous levels of miR-548j in BC cells correlate with invasiveness and miR-548j overexpression stimulates BC cell invasion *in vitro* in MCF-7, SKBr3 and MDA-MB-231 BC cells. MDA-MB-231 cells transfected with a miR-548j mimic metastasize to the lungs after tail vein injection, without affecting proliferation (52). Tensin-1, a protein which localizes to focal adhesions and is involved in cell migration (53) was identified as a direct target for miR-548j (52). miR-548j mediated inhibition of tensin-1 relieves inhibition of cell division cycle protein 42 homolog (cdc42), a small GTPase of the ras homologue (Rho) family which is involved in control of pathways mediating morphology, migration, endocytosis, cell-cycle progression and invasion (54). Migration of BC cells as outlined above could be inhibited by ML141, a small molecule cdc42 inhibitor (52). Using two sets of clinical samples, a strong correlation between the expression level of miR-548j and lymph node metastasis and survival has been observed in BC patients (52).

miRs-125b, -182 and -1792: For miR-125b, STAR-related lipid transfer domain protein 13 (STARD13) (55-57) has been identified as a target. STARD13 inhibits RhoA activity and thus activates ROCK signaling resulting in up-regulation of the EMT markers vimentin and α smooth muscle actin and reorganization of the cytoskeleton. miR-125b mimics induce migration in MCF7 and MDA-MB-231 cells (claudin-low subtype). MCF-7 cells with up-regulated expression of miR-125b give rise to metastases in the kidney, lungs and eyes after injection into the lateral vein (55). A clinical correlation between expression of miR-125b and BC metastasis has not yet been worked out. miR-182 targets Missing in Metastasis (MIM) (58, 59) resulting in activation of RhoA and stress fiber formation (60). Ectopic expression of miR-182 enhances BC cell mobility and invasion and increases pulmonary colonization of BC cells (60). In BC specimens, miR-182 induction is linked to down-regulation of MIM, RhoA activation and poor prognosis (60).

miR-1792 is a downstream effector of ROCK signaling. ROCK expression (43, 44) is enhanced in metastatic human BC and BC cell lines in comparison to non-metastatic BC cell lines (60). Overexpression of ROCK mediates cell migration and proliferation of non-metastatic MCF-7 cells and their metastasis to the bones (60). Concomitantly miR-1792 is induced and its expression is diminished by treatment with ROCK inhibitor Y27632 (60). miR-1792 is overexpressed in metastatic BC and is involved in proliferation and motility of BC cells (60). Intratumorally injected anti-miR-1792 agents do not affect the growth of primary MDA-MB-231 cells, but the frequency of metastasis to the bones was reduced by 50% compared to a scrambled control (60). The role of miR-1792 as a downstream effector of ROCK signaling and other not yet identified pathways in BC metastasis warrants further investigations. Figure 1 displays the MOA of BC-metastasis promoting miRs-10b, -126, -182, -1792 and -5485j, which all promote activation of ROCK.

miRs interfering with other signaling pathways. miR-9: miR-9 was identified by screening mammary carcinoma cells for up-regulated miRs through real-time RT-PCR (61). miR-9 expression is up-regulated by MYC and N-MYC. E-cadherin has been identified as one of the crucial targets of miR-9. E-cadherin associates with intracellular proteins that link E-cadherin to the actin cytoskeleton and its down-regulation dissociates carcinoma cells from each other (62, 63). In addition, loss of E-cadherin leads to movement of β -catenin into the nucleus to activate pro-metastatic genes (64). Vascular endothelial growth factor (VEGF) has been identified as one of the target genes of β -catenin in SUM149 cells *in vitro* and *in vivo*, resulting in a 10-fold increase of the density of intratumoral microvessels (61). Ectopic expression of miR-9 in immortalized human mammary

luminal epithelial cells (HMLE) results in EMT-like conversion, but not in SUM 149 cells (basal subtype), pointing to a context-dependent phenomenon (61). Ectopic expression of miR-9 in SUM149 cells mediated the establishment of lung metastases after fat pad implantation, in contrast to the non-transfected cells (61). In the previously described 4T1 mammary carcinoma model, expression of a miR-9 sponge in 4T1 cells reduced the number of lung metastases by 50% after fat pad implantation in immunocompetent Balb/c mice (61). A clinical correlation of miR-9 expression and prognosis in BC patients revealed elevated expression of miR-9 in primary breast tumors with diagnosed metastases compared to those from metastasis-free patients (61). Earlier reports (65) indicated up-regulation of miR-9 in clinical breast cancers.

miR-24: miR-24 was identified as a miR with increased expression in BC tissue compared to benign breast tissue (66). Ectopic expression promotes invasion and migration of the BC cells 4T1 and MT-1 (66). miR-24 transfected MT-1 cells promote increased tumor growth and formation of lung metastases after tail vein injection (66). Protein tyrosine phosphatase non-receptor 9 (PTPN9) and protein tyrosine phosphatase receptor F (PTPRF) (67, 68) have been identified as direct targets of miR-24 (66) resulting in up-regulation of phosphorylated epidermal growth factor receptor (pEGFR). miR-24 promotes the increased expression of downstream effectors such as MMP-2, MMP-11 and a disintegrin and metalloprotease 15 (ADAM15), mediators of cell invasion, migration and metastasis (66). pEGFR was found to be more abundant in BC tissues than in normal breast tissue and immuno-histochemistry (IHC) revealed that PTPN9 and PTPRF gave rise to much weaker signals in tumor tissues than in normal breast tissue (66). Studies addressing the inverse relationship between expression of PTPN9, PTPRF and pEGFR in BC *versus* normal breast tissue have to be extended to more patients.

miR-181a: Late-stage BC metastasis is driven by dysregulated transforming growth factor β (TGF β) signaling (69). In 3D organotypic cultures, miR-181a is up-regulated by TGF β and inactivation of miR-181a attenuates TGF β -mediated EMT, invasion and migration (70). Pro-apoptotic protein Bim was identified as one of the targets of miR-181a, resulting in decrease of anoikis after inhibition by miR-181a (71). Inactivation of miR-181a in 4T1 cells impaired their ability to grow as 3D organotypic cultures (70). In an experimental metastasis model, inhibition of miR-181a in 4T1 cells decreased pulmonary tumor burden and increased survival time (70). In an orthotopic fat pad injection-based metastasis model, miR-181a was essential for pulmonary metastatic outgrowth of 4T1 cells (70). Regarding clinical prognostic relevance, miR-181a was shown to be selectively up-regulated in metastatic BC, particularly in TNBC and up-regulation was highly predictive for overall survival in BC

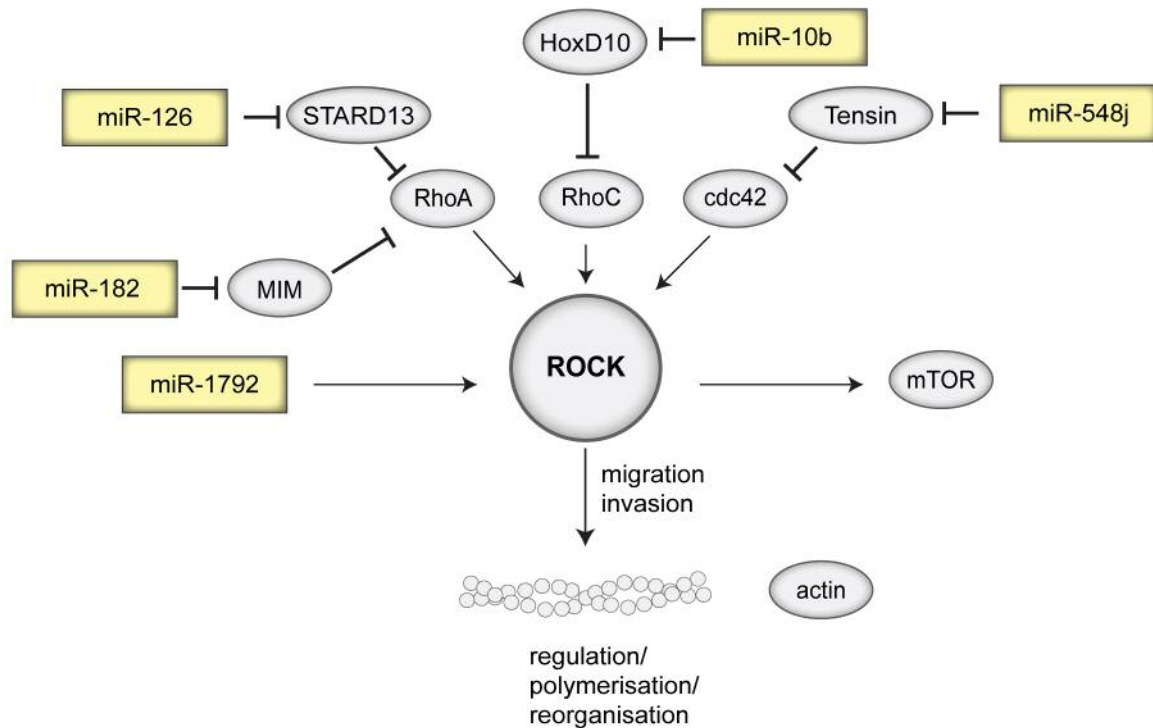


Figure 1. miRs promoting breast cancer metastasis by activation of ROCK signaling. miRs 10b, -126, -182, -548j and -1792 activate several small GTPases resulting in activation of ROCK signaling followed by modification of actin and migration and invasion of breast cancer cells. Cdc 42: Cell division cycle protein 42; HoxD10: homeobox domain 10; MIM: missing in metastasis; mTOR: mechanistic target of rapamycin; RhoA: ras homologue A; Rho C: ras homologue C; ROCK: Rho-associated kinase; STARD13: StAR-related lipid transfer domain protein 13.

patients (70). Figure 2 shows the MOA of BC-metastasis promoting miRs -9, -21, -24, -93 and -181.

miRs-199a and -494 involved in interactions between tumor cells and stromal cells. miR199a: The interaction between mesenchymal stem/stromal cells (MSC) and BC cells is an important driver of BC metastasis (72, 73). Recently, involvement of miR-199a primed in BC cells after interaction with MSC was reported to play an important role in this process (74). MDA-MB-231 cells transfected with miR-199a exhibit increased cancer stem cell (CSC)-related traits such as tumor-initiating capabilities in mice, improved capability to grow as mammospheres and elevated expression of CSC marker aldehyde dehydrogenase 1 (ALDH1) (74). From a mechanistic point of view, down-regulation of speech gene, transcription factor forkhead box P2 (FoxP2) (74, 75), a common target of a converging set of MSC-regulated miRs was identified as a target of miR199a (74). Down-regulation of FoxP2 results in tumor-initiating properties and metastasis. Although elevated miR-199a and depleted FOXP2 have been found in clinical BC specimens, this needs to be further investigated before a clinical impact can be determined (74).

miR-494: miR-494 was identified as a BC-metastasis mediating miR expressed in myeloid-derived suppressor cells (MDSC) after induction by tumor-derived TGF β (76). miR-494 targets PTEN and thereby activates the Akt pathway (76). Akt mediates mechanistic target of rapamycin (mTOR) and nuclear factor κ B (NF κ B) signaling, which leads to the expression of MMPs by MDSCs and promotes metastases of BC cells. In addition, enhanced expression of C-X-C chemokine receptor 4 (CXCR4) on MDSCs mediated chemotaxis and subsequent accumulation of MDSCs in tumors (76-78). miR-494 is highly expressed in tumor-expanded MDSCs and knock-down of miR-494 by a lentivirus-based sponge attenuates primary tumor growth and metastasis of 4T1 cells (76). Data regarding clinical significance of these findings with respect to BC metastasis are not yet available.

miRs transferred by exosomes: miR-19a, -105, -122 and -223. miR-19a: miR19a is transferred *via* exosomes from astrocytes to mouse (4T1) and human (HCC 1954, BT474, MDA-MB-231) BC cells and mediates adaptive loss of tumor suppressor PTEN in brain metastatic tumor cells (79). Blockage of astrocyte exosome secretion rescues PTEN loss and suppresses metastasis of a subclone of MDA-MB-231

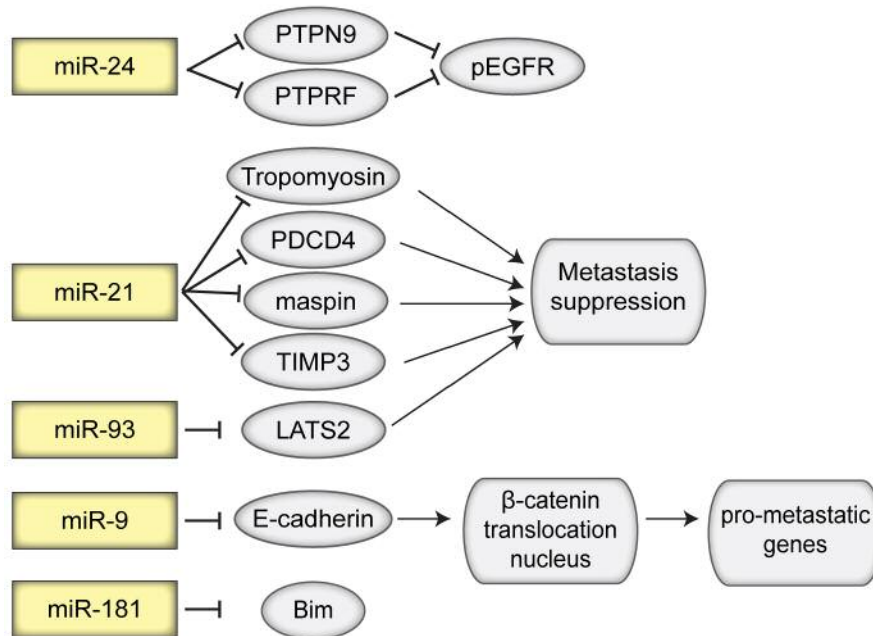


Figure 2. Mode of action of selected BC-promoting miRs. The left-hand panel displays selected miRs. The corresponding targets are shown in the middle panel and the physiological consequences of target inhibition are shown in the right-hand panel. LATS2: Large tumor suppressor, homology 2; PDCD4: programmed cell death 4; pEGFR: phosphorylated epidermal growth factor receptor; PTPN9: tyrosine-protein phosphatase non-receptor 9; PTPRF: receptor tyrosine phosphatase F.

cells with high tropism of metastasis to the brain (79). Elimination of PTEN is brain-specific and does not occur in BC cells disseminating to other organs. Loss of PTEN in BC cells leads to increased secretion of CC-chemokine ligand 2 (CCL2) (79-82), a recruiter of IBA-1-expressing myeloid cells which enhance outgrowth of metastatic tumor cells in the brain *via* enhanced proliferation and reduced apoptosis. IHC staining of human primary BC and brain metastases indicated higher CCL2 expression in brain metastases than in primary tumors (79). These findings suggest CCL2 targeting for therapeutic intervention of life-threatening BC-derived brain metastases.

miR-105: miR-105 is expressed by metastatic BC cells and can also be transferred by exosomes (83). *In vitro*, exosomes harbouring miR-105 have been shown to target endothelial monolayers by inhibition of tight junction protein zonula occludens-1 protein (ZO-1) (83, 84). Pre-treatment of mice with exosomes exhibiting high levels of miR-105 derived from MDA-MB-231 cells, but not those derived from MCF-7 cells, mediated metastasis of MDA-MB-231 cells to the lungs and the brain after intracardiac injection (83). High vascular permeability and reduced levels of ZO-1 were observed in MCFDCIS (a tumorigenic cell line derived from the non-cancerous mammary epithelial MCF-10A cell line) orthotopic xenografts overexpressing miR-105 (83). In patients who later developed distant metastases

higher levels of tumor miR-105 and lower levels of tumor and vascular ZO-1 were observed (83). miR-105 has potential as a blood-based marker for prediction or early diagnosis of BC metastases (83).

miR-122: miR-122 has been identified as a miR with the capability to reprogram glucose metabolism in the pre-metastatic niche to promote metastasis (85). miR-122 is secreted in vesicles by BC cells and down-regulates the glycolytic enzyme pyruvate kinase M (PKM) to suppress glucose uptake by stromal cells of the metastatic niche and cells located in distant organs (86-88). Metastatic colonization in brain and lungs was observed after intracardiac injection of MDA-MB-231 cells into mice pretreated with endocytic vesicles containing miR-122, whereas no metastases were detected in controls (85). From a clinical point of view, it has been noted that miR-122 levels in the circulation correlate with metastatic progression in BC patients (89). Figure 3 shows the MOA of exosome-transferred BC promoting miRs-19a, -105, -122 and -223.

miR-223: It has been shown previously that interleukin 4 (IL4) released by CD4+ T-cells can induce tumor-associated macrophages, which can promote BC metastasis (90, 91). In order to resolve the MOA underlying these observations, it was shown that co-culture of IL4-activated macrophages and BC cells is associated with transfer of miR-223 containing exosomes to BC cells (SKBr and MDA-MB-231) leading to

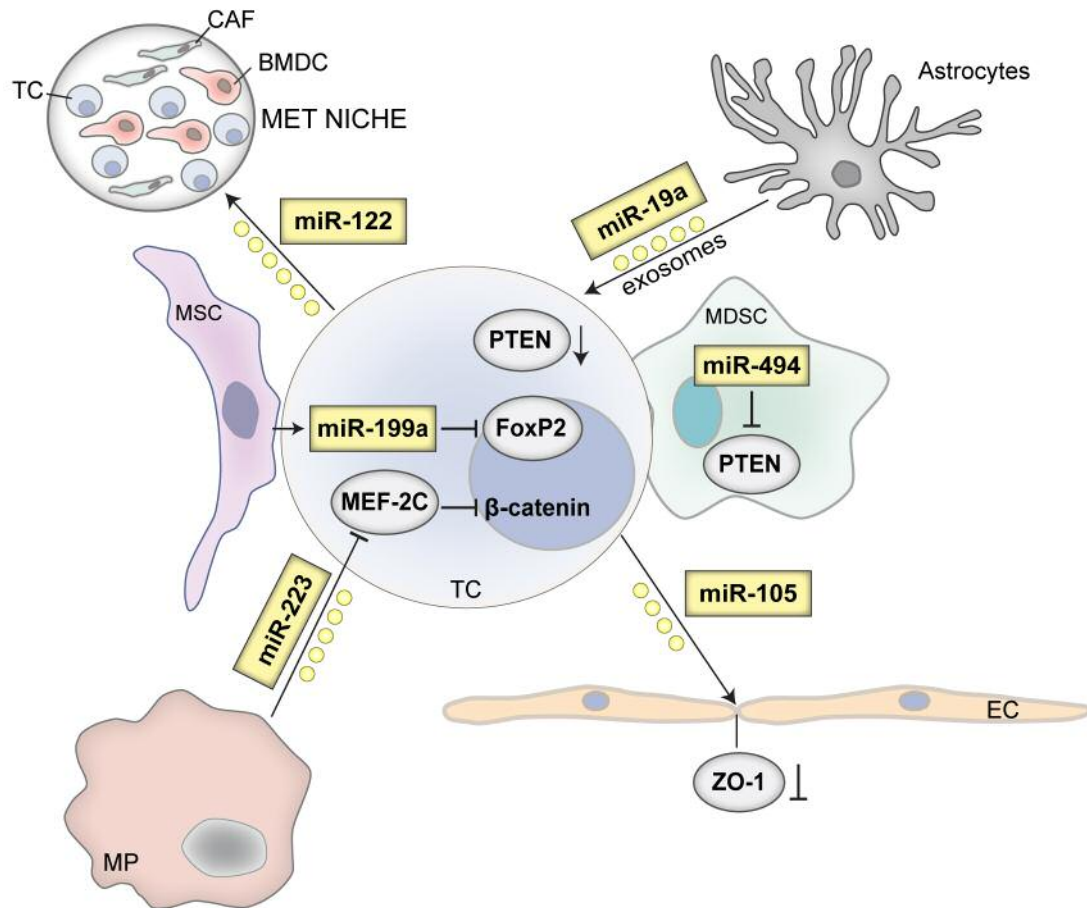


Figure 3. miRs promoting BC- metastasis by interaction of tumor cells with stromal cells or by miR-based exosome transfer between tumor cells and stromal cells or vice versa. Exosomes are displayed as small circles. BMDC: Bone marrow-derived cell; CAF: cancer-associated fibroblast; EC: endothelial cell; FoxP2: forkhead box P2; MDSC: myeloid-derived suppressor cell; MEF-2C: monocyte enhancer factor 2C; MET NICHE: metastatic niche; MP: macrophage; MSC: mesenchymal/stromal stem cell; PTEN: phosphatase and tensin homolog; TC: tumor cell; ZO-1: zonula occludens-1.

their increased invasiveness (92). Treatment of macrophages with ASO directed against IL4 decreased invasiveness of SKBr and MDA-MB-231 cells (92). miR-223 promotes invasiveness by targeting monocyte enhancer factor 2C (MEF-2C) (92). Reduction of MEF-2C has been linked to nuclear accumulation of β -catenin, which promotes cell migration (93). *In vivo* POC metastasis-related data as well as a correlation between the expression status of miR-223 and BC metastasis are not yet available.

Breast Cancer Metastasis Suppressing miRs

In this chapter we discuss different classes of miRs. According to the selected categories they interfere with diverse signaling pathways, the cytoskeleton and its modulators, with a network of metastatic regulators in BC cells, or are involved in tumor cell/stromal interactions.

Finally, we discuss additional promising miRs which warrant further validation regarding their anti-metastatic role in BC.

miRs which target diverse signaling pathways. miR-148a was identified by bioinformatic analysis of BC patient databases. There is a correlation between the low expression of miR-148a in higher grade tumors and a higher likelihood to develop metastases and poor prognosis especially in BC patients with basal and luminal B subtypes (94). In 4T1 and MDA-MB-231 BC cells, ectopic expression of miR-148a did not affect cell growth, viability in culture or *in vitro* migration (94). However, for both cell lines there was a reduction of lung metastases ($\geq 50\%$) observed after fat pad implantation of cells ectopically expressing miR-148a. It was shown that the extravasation process of circulating tumor cells (CTC) and therefore the access to lung parenchyma was suppressed by miR-148a (94). Wingless/integrated1 (Wnt1)

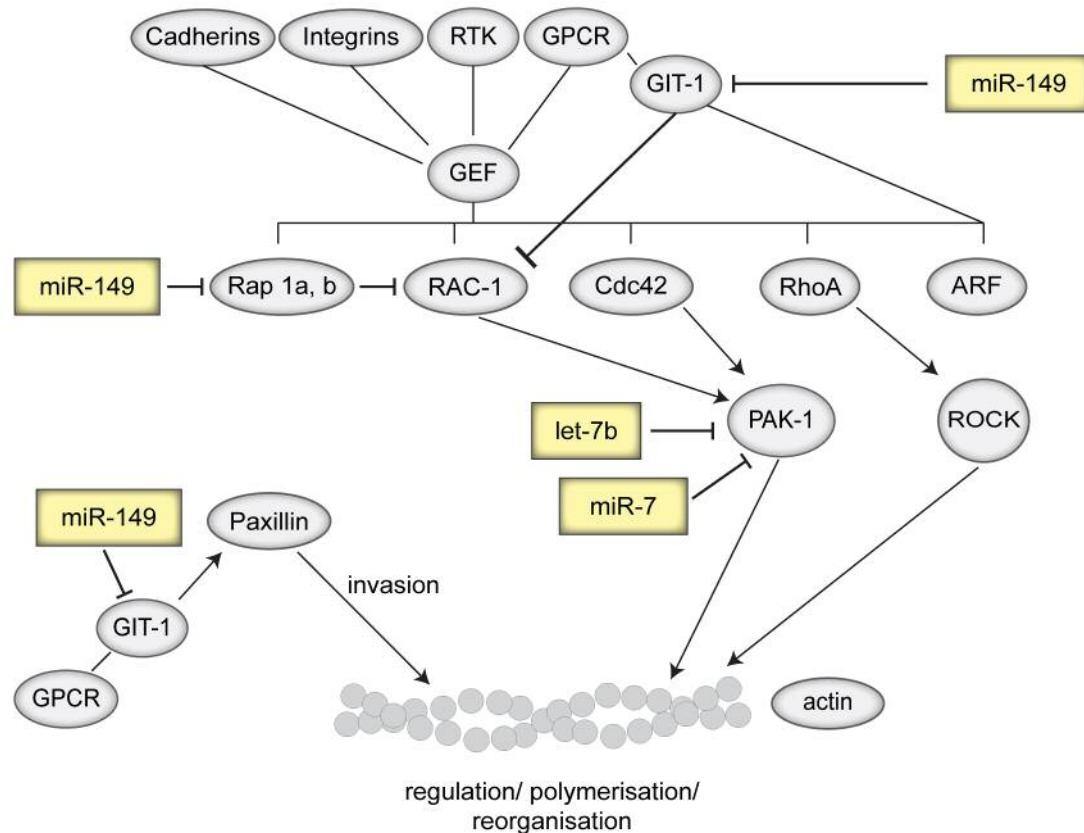


Figure 4. miRs suppressing breast cancer metastasis by modulation of cytoskeletal components. Arrows indicate activation. Actin is displayed as the major downstream effector of miRs let-7b, miR-7 and miR-149. ARF: ADP-ribosylation factor GTPase activating factor; cdc 42: cell division cycle 42 homolog; GEF: guanine nucleotide exchange factor; GIT-1: G-protein coupled receptor interacting protein-1; GPCR: G-protein coupled receptor; PAK-1: p21-activated protein kinase; RAC-1: Rac-related C3 botulinum toxin substrate 1; Rap 1a, b: ras related protein 1a, b; RhoA: ras homologue A; ROCK: Rho-associated kinase; RTK: receptor tyrosine kinase.

and neuropilin-1 (NRP1) were identified as direct targets for miR-148a (94). Wnt-1 induced Wnt signaling can promote EMT and metastasis (95, 96) and NRP1 functions as mediator of cell growth, survival and metastasis (97, 98).

miR-148b: In a retrospective study, miR-148b and additional 15 miRNAs were found to be associated with relapse in BC patients (99). Increased miR-148b levels mediate stronger adhesion, anoikis and reduce invasion of BC cells *in vitro* (99). miR-148 does not affect tumor cell growth *in vitro*, however *in vivo* tumor growth and lung metastasis are suppressed due to alteration of survival and extravasation (99). Direct targets affected by miR-148b include metastasis-related mRNAs encoding integrin subunit $\alpha 5$ (100, 101), ROCK1, PIK3CA, NRAS and colony-stimulating factor-1 (CSF-1).

miR-335: miR-335 was identified by screening MDA-MB-231 cells (BC subtype Her2⁺) and subclones that are highly metastatic to the bones or lungs for decreased expression of miRNAs in all metastatic sublines (102). miR-335 reduces

invasion, but does not affect proliferation of BC cell lines (102). Restoring miR-335 expression in LM2 cells (subline of MDA-MB-231 highly metastatic to the lungs) through retroviral transduction reduced lung colonizing activity of these cells by more than fivefold (102). Tenascin C and SRY-box containing transcription factor 4 (SOX4) have been identified as invasion-related direct targets of miR-335 (102). Tenascin C is a glycoprotein of the extracellular matrix (ECM) highly expressed during development, tissue repair and in the stroma of some tumors (103). SOX4 is a transcription factor which regulates progenitor cell development and migration (104) and functions as a master regulator of EMT by controlling expression of histone-lysine-N-methyltransferase enhancer of zeste homolog 2 (EZH2) and epigenetic reprogramming (105). miR-335 can also inhibit migration of BC cells through targeting the oncoprotein c-MET (106). In human BC, miR-335 is silenced through genetic and epigenetic mechanisms (107) and is inversely correlated with relapse in distant organs (102).

miRs interfering with cytoskeletal targets and their modulators. Let-7: In humans, the lethal 7 (let-7) family of miRs consists of 13 members with both overlapping and distinct functions (108). In BC, let-7 functions as a regulator of self-renewal and tumorigenicity (109). Let-7 inhibits BC cell migration and invasion as well as lung metastasis of tail vein injected MDA-MB-231 cells expressing transfected let-7 in comparison to controls (110). Genes involved in the actin cytoskeletal pathway and disassembly of focal adhesions such as p21-activated protein kinase-1 (PAK1) (Figure 4), diaphanous homolog 2 (DIAPH2), radixin (RDX) and integrin subunit $\beta 8$ have been identified as direct targets of let-7 (110). Further studies have revealed a raf kinase inhibitory related protein (RKIP) based pathway for induction of let-7 with subsequent induction of invasion and metastasis promoting genes (111, 112). RKIP has been identified as an inducer of let-7 which inhibits the leucine zipper family transcription factor BTB domain and CNC homolog 1 (BACH1) (113) and the DNA-binding protein high-mobility group A2 (HMGA2) (114). Down-regulation of let-7b results in expression of BACH1- and HMGA2-suppressed metastasis-promoting genes such as the zinc-finger transcription factor SNAIL homolog 1 (SNAIL1), MMP-1, CXCR4 and osteopontin (OPN) (111, 112). Depletion of BACH1, HMGA2 or both decreased the metastasis of 1833 cells, a subline of MDA-MB-231 cells specifically metastatic to the bones (112). Patients with high activity of the pathway outlined above have a markedly greater risk of metastases compared to those with less active pathway (111, 112).

In addition, a role for let-7a related to BC metastasis has been defined (115). Let-7a suppresses BC migration and invasion through down-regulation of C-C chemokine receptor 7 (CCR7) (115) and transfection of synthetic let-7a decreases BC cell invasion in zebrafish embryo animal models (115). An inverse correlation between expression of CCR7 and let-7a has been noted in BC patients (115).

miR-149: Two groups have identified miR-149 as a miR, which inhibits BC metastasis by modulating downstream targets of integrin signaling (116, 117) (Figure 4). MDA-MB-231 cells transfected with miR-149 exhibited severely impaired spreading on collagen gels and haptotactic cell migration (116). The small GTPases Rap1a and Rap1b (118, 119) as well as the G-protein coupled receptor interacting protein-1 (GIT-1) (120), an ADP-ribosylation factor GTPase activating protein (ArfGAP), were identified as direct targets of miR-149 (116, 117). Inhibition of Rap1a and Rap1b by miR-149 results in aberrant src and RAC-1 activation, thereby promoting invasion (116). Low levels of miR-149 leads to high levels of GIT-1 expression, which stabilizes integrin $\alpha 5 \beta 1$ and paxillin proteins, thus inhibiting adhesion, migration and metastasis (117). MDA-MB-231 cells transfected with miR-149 exhibited decreased lung colonization after tail vein injection (116, 117). From a clinical perspective, low levels of

miR-149 are associated with advanced stages of BC and lymph node metastasis (116, 117).

miRs that target networks of pro-metastatic regulators. miR-7: miR-7 has been identified as a BC invasion and metastasis inhibitory miR, which regulates multiple targets and pathways (121). The following have been shown to be negatively regulated by miR-7: transcription factor homeobox protein B3 (HoxB3) (122), PAK-1 (Figure 4) (123), focal adhesion kinase (FAK) (124), krüppel-like factor 4 (KLF4) (125), histone methyltransferase SET domain containing 8 (SET8) (126) and EGFR (127), which function as promoters of tumor growth, migration, invasion and metastasis and are highly interconnected. Inhibition of HoxB3 by miR-7, by targeting the 3'-UTR of its mRNA, leads to the expression of tumor suppressors Ras associated domain family 1A (RASSF1A) and claudin 6, due to decreased promoter methylation (122). Down-regulation of SET8, a histone H4lysine 20 (H4K20) specific monomethyltransferase (126) by miR-7 is an important aspect of the epigenetic function of miR-7. Down-regulation of the well-documented promoters of motility PAK1, FAK and EGFR enables miR-7 to decrease metastasis (128, 129). Ectopic expression of miR-7 in BC cell lines such as MDA-MB-435 and MBA-MB-231 inhibits growth, migration and invasion *in vitro* (121, 123). Forced expression of miR-7 in MDA-MB-435 cells decreased primary tumor growth 1.5 fold after fat pad implantation and no invasive lesions were observed, due to encapsulation (124). In addition, miR-7 functions as a suppressor of metastasis of BC-derived CSC to the brain, but not to the bones, through down-regulation of KLF4 (125), an essential gene product for pluripotent stem cells promoting their self-renewal (130). KLF4 also correlates with an aggressive phenotype in early BC (131). The prognostic relevance of miR-7 in BC patients remains to be investigated.

miR-29b: miR-29b is induced by GATA-family of transcription factors 3 (GATA3) (132), which is involved in mammary gland morphogenesis and luminal cell differentiation (133, 134). GATA3 is expressed at higher levels in luminal *versus* basal A and B subtypes of BC consistent with its luminal localization. A correlation was found between higher levels of GATA3 and better survival in BC patients (135, 136). miR-29b promotes luminal differentiation and targets a network of angiogenic and pro-metastatic regulators such as vascular endothelial growth factor isoform A (VEGFA), angiopoietin-like 4 (ANGPTL4), platelet-derived growth factor (PDGF), lysyl-oxidase (LOX), MMP-9, TGF β and integrin subunits $\alpha 6$ and $\beta 1$ (132), which modify the tumor microenvironment. After orthotopic implantation of 4T1 cells ectopically expressing miR-29b, fewer and smaller lung metastasis were noted (132). miR-29b is enriched in more differentiated BC and normal mammary epithelial cells (132).

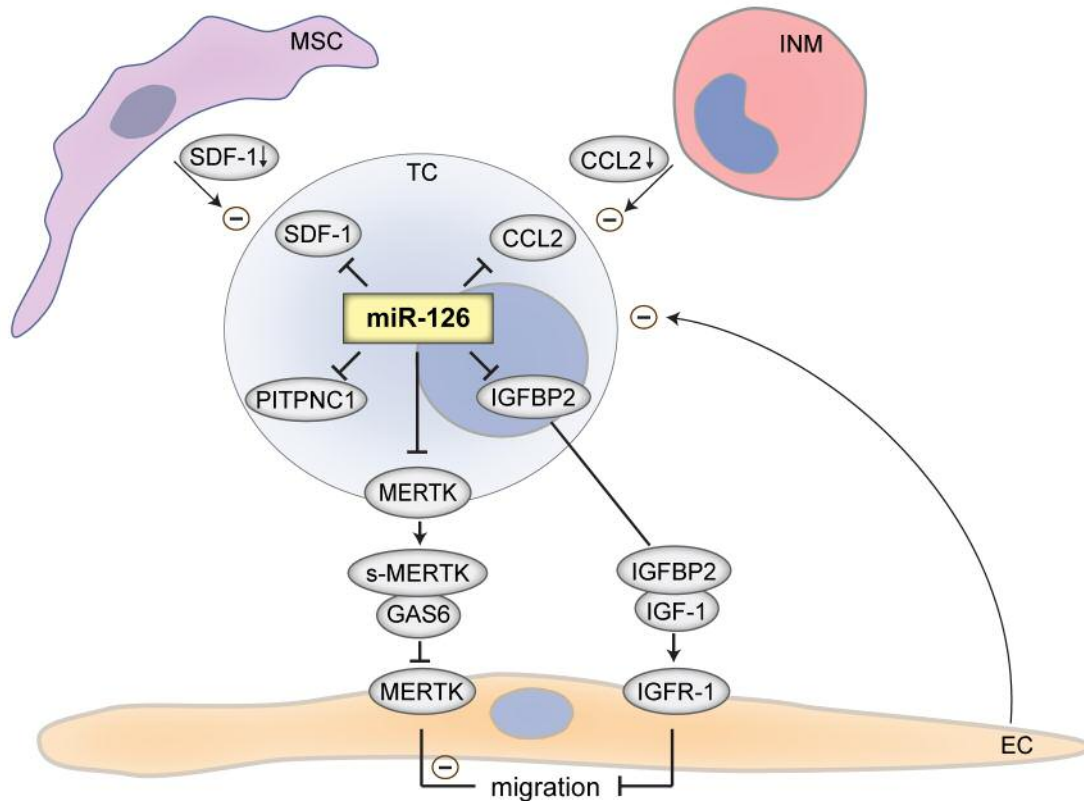


Figure 5. Anti-metastatic function of miR-126 is mediated by inhibition of recruitment of stromal cells by breast cancer cells. miR-126 inhibits recruitment of MSC, INM and EC cells by tumor cells through inhibition of SDF-1 and CCL2 as well as inhibition of IGF-1/IGF-1R and MERTK signaling. CCL2: CC chemokine ligand 2; EC: endothelial cell; IGFBP2: insulin-like growth factor binding protein 2; IGF-1: insulin-like growth factor-1; IGFR-1: insulin-like growth factor receptor-1; INM: inflammatory monocyte; MERTK: c-MER tyrosine kinase; s-MERTK: soluble-MERTK; MSC: mesenchymal stem cell; PITPNC1: phosphoinositol transfer protein, cytoplasmic 1; SDF-1: stromal-derived factor-1; TC: tumor cell.

miRs Involved in Tumor Cell/Stromal Cell Interaction

miR-126: miR-126 was identified as a miR down-regulated in BC cells according to their metastatic potential (137). miR-126 exerts its anti-metastatic function by suppressing the recruitment of endothelial cells (ECs) (137), MSCs and inflammatory monocytes (138) (Figure 5).

In the Boyden chamber system, miR-126 overexpressing BC cells inhibit recruitment of human umbilical vein endothelial cells (HUVECs) (137). miR-126 silencing in MDA-MB-231 cells increased lung metastatic colonization and systemic colonization of multiple organs such as liver, bone and brain (137). Direct targets of miR-126 include insulin-like growth factor binding protein 2 (IGFBP2), c-MER tyrosine kinase (MERTK) and phosphoinositol transfer protein, cytoplasmic 1 (PITPNC1) (137). Individually and in combination they were shown to suppress the ability of LM2

and CN34-LM1A BC cells (a highly metastatic subline derived from ER- CN34 cells, which originate from pleural BC cells) to recruit ECs (137). In the extracellular space IGFBP2 forms a complex with insulin-like growth factors 1 and 2 (IGF1 and 2) and enhances IGF-type 1 receptor activation on ECs promoting chemotaxis by increasing extracellular levels of IGF-1 (139). MERTK cleaved from metastatic BC cells promotes EC recruitment by competitively antagonizing its ligand growth-arrest specific 6 (GAS6) as a decoy receptor to endothelial MERTK receptors preventing GAS6 from inhibiting EC migration mediated by binding to the MERTK receptor (140). Neutralization of negative chemotactic factor GAS6 leads to enhanced endothelial cell chemotaxis. PITPNC1 is a promoter of EC migration by enhancing IGFBP2 levels (141). Patients whose primary BC displayed overexpression of these genes were significantly more likely to develop distal metastases and displayed shorter metastases-free

survival (137) and reduced expression of miR-126 correlates with poor metastasis-free survival of BC cells.

Another property of miR-126 expressed in BC cells is the recruitment of MSCs and inflammatory monocytes, both promoting BC metastasis (138). 4T1 cells transfected with miR-126 did not exhibit a difference in proliferation and migration in comparison to non-transfected 4T1 cells, but rather showed suppressed formation of lung metastases after surgical removal of the primary tumor from fat pads (138). Stromal-derived factor 1 (SDF-1), the ligand of CXCR4, was identified as a direct target of miR-126. Down-regulation of SDF-1 results in suppression of MSC recruitment (142). Recruitment of inflammatory monocytes is inhibited by miR-126 indirectly by down-regulation of CCL2 (138). Recruitment of MSC and formation of a paracrine loop with tumor cells enhances tumor cell invasion and metastasis (72), additionally inflammatory monocytes might promote intravasation later in tumor progression (81). miR-126 is located in an intron of host gene *Egfl7* and is down-regulated in cancer cells by methylation (138).

Further Breast Cancer Metastasis-suppressive miRs

In the following we summarize briefly BC metastasis-suppressing miRs which inhibit migration, invasion and metastasis of BC cells with pending or early clinical validation with respect to prognosis of BC patients and are awaiting broader confirmation of their relevance in this context as well as extension of MOA studies.

miR-22 induces cellular senescence in BC cells. It is down-regulated in BC specimens and mediates translational inhibition of genes counteracting senescence such as CDK6, sirtuin1 (*SIRT1*) and transcription factor specificity protein 1 (*Sp1*) (143). In a fat-pad injection based MDA-MB-231 tumor model, lung metastases are inhibited by ectopical expression of miR-22 (143).

miR-30 is reduced in BC-tumor-initiating cells allowing them to maintain their self-renewal and inhibiting their apoptosis (144). miR-30 inhibition leads to up-regulation of its targets integrin subunit $\beta 3$ and ubiquitin-conjugating enzyme 9 (*ubc9*) (144). Ectopic expression of miR-30 in SK-BR derived mammospheres (subtype HER2+) inhibits lung metastases (144).

miR-34a blocks osteoporosis and bone metastasis by inhibition of osteoclastogenesis and TGF $\beta 2$ as shown in a miR-34 knock-out transgenic mouse model (145). It also represses EMT by targeting tumor protein D52 (TPD52), an oncogene expressed in BC (146).

miR-124 targets transcription factor SNAIL homolog 2 (SNAIL-2) (147) leading to up-regulation of E-cadherin (148). Lentivirus-based delivery of miR-124 into MDA-MB-231 cells suppresses colony formation *in vitro* and metastases *in vivo*.

miR-145 targets cell surface glycoprotein mucin-1 (MUC-1) (149) and has no impact on the growth of BC cells. miR-145 silencing of MUC-1 suppresses lung metastases in an experimental metastases model (150).

miR-193 targets urokinase plasminogen activator (uPA) (151), a component of the plasminogenolytic system with a well documented role in BC metastasis (152). Ectopic expression of miR-193b in MDA-MB-231 cells inhibits orthotopic tumor growth and formation of spontaneous pulmonary metastases (152). miR-193 is associated with clinical BC metastases (152).

miR-205 targets VEGF and human epidermal growth factor receptor 3 (HER3). Ectopic expression of miR-205 in MDA-MB-231 cells inhibits cell proliferation, invasion, anchorage independent growth and experimental metastases to the lungs (153).

miR-320a acts by suppressing metadherin (MTDH) expression and is down-regulated in BC tissues and cell lines (154). MTDH expression is related to breast cancer progression (155). miR-320a suppresses lung metastases of MDA-MB-231 cells (154).

miR-421 is down-regulated in BC and down-regulation correlates with lymph node metastasis, recurrence and metastasis (156). miR-421 targets metastasis-associated protein-1 (MTA-1), which is involved in chromatin remodeling (157). miR-421 knock-down leads to invasion and metastasis of MCF-7 cells and overexpression in MDA-MB-231 cells suppresses invasion and metastasis (156).

miRs with Context-dependent Breast Cancer Metastasis-promoting or -suppressive Properties

miR-200: The miR-200 family consists of miRs-200 a,b,c, miR-429 and miR-141 which share the same seed sequence (158). miR-200 can inhibit EMT by targeting the E-cadherin repressors zinc finger E-box binding homeobox 1 and 2 (ZEB1 and ZEB2) (159), but can also promote EMT *via* exosomes by mechanisms yet to be resolved (160). It is able to enhance mesenchymal epithelial transition (MET) (160). In the following we comment on the context-dependent pro- and anti-metastatic function of miR-200 in BC (Figure 6).

Pro-metastatic function of miR-200: It has been observed that endocytic vesicles containing miR-200 are secreted from metastatic murine and human BC cell lines and their transfer to non-metastatic BC cells alters gene expression and promotes EMT. *In vivo* experiments were performed with syngeneic mammary tumor cell lines derived from a single spontaneous mammary tumor with different metastatic performance (160). Incubation of 4T1-derived exosomes enhances lung colonization of 4T07 cells, which are unable to colonize distant organs after tail vein injection (160). miR-200 secreted from CA1a (Her2+ subtype, highly metastatic) primary mammary tumors promote lung colonization of tail-

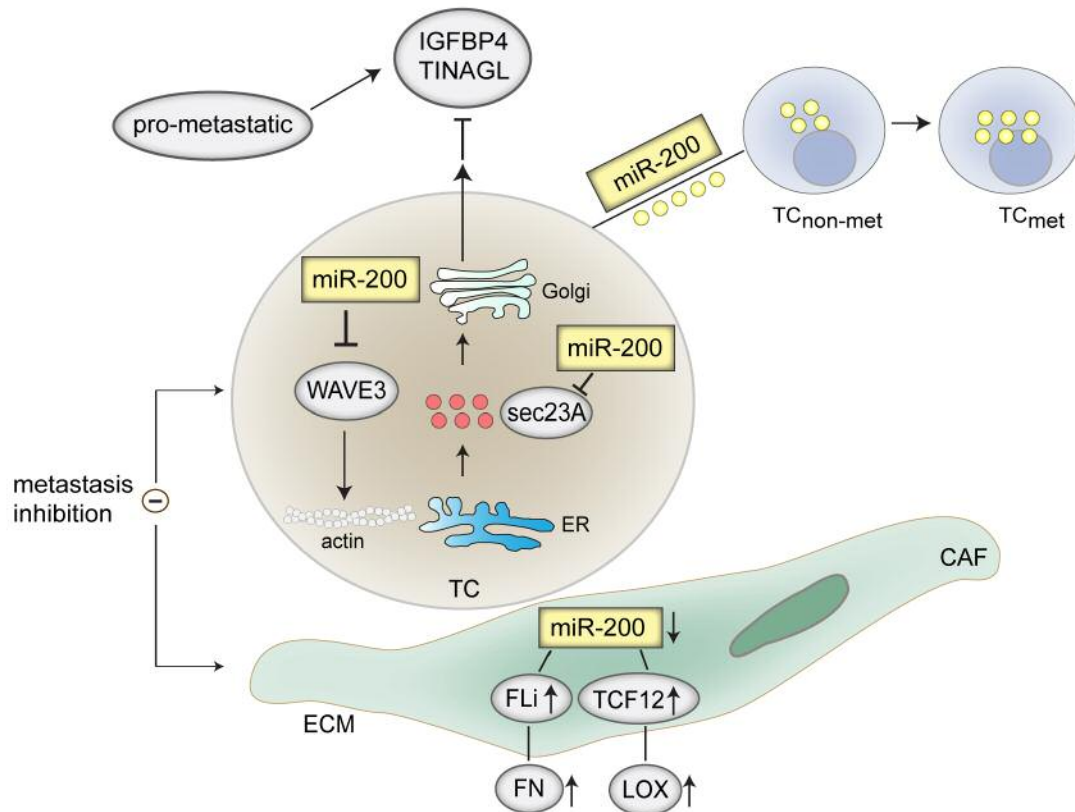


Figure 6. miR-200 promotes or inhibits metastasis of breast cancer cells in a context-dependent manner. Pro-metastatic actions of miR-200 can be mediated by exosomes or through inhibition of secretion of anti-metastatic proteins and inhibition of metastasis is based on modulation of actin interaction with WAVE3 and re-organisation of the ECM in CAFs. Exosomes and secretory vesicles are displayed as green or red small circles. CAF: Cancer-associated fibroblast; ECM: extracellular matrix; ER: endoplasmic reticulum; Fli-1: Friend leukemia integration-1; FN: fibronectin; IGFBP4: insulin-like growth factor binding protein 4; LOX: lysyl oxidase; sec 23A: sec 23 homolog A (*S. cerevisiae*); TC: tumor cell; TCF12: transcription factor 12; TINAGL: tubuli interstitial nephritis antigen-like 1; WASP: Wiskott Aldrich Protein; WAVE3: WASP verprolin homologue 3.

vein injected MDA-MB-231 cells (160). miR-200 was shown to target sec 23 homolog a (sec 23a), an essential component of vesicles transporting proteins from the endoplasmic reticulum (ER) to the Golgi apparatus and thus influencing the BC secretome (161, 162). Sec 23a mediates secretion of metastasis-suppressive proteins including insulin-like growth factor binding protein 4 (IGFBP4) and tubulo interstitial nephritis antigen-like 1 (TINAGL1) (163, 164). IGFBP4 and TINAGL1 are associated with metastasis-free survival in a BC-related clinical data set (165).

Metastasis-inhibiting function of miR-200: It has been reported that down-regulation of WASP verprolin homologous 3 (WAVE3), a member of Wiskott-Aldrich syndrome protein (WASP) actin cytoskeleton remodeling family of proteins by miR-200 inhibits the invasive phenotype of MDA-MB-231 cells (166). WAVE mediates remodeling of actin-cytoskeleton interactions critical for motility, migration and invasion of transformed cells (167). Another role of miR-200 in

modulation of BC metastasis was found in the context of cancer-associated fibroblasts (CAF)-BC cell interactions (168). Down-regulation of miR-200 was shown to activate normal fibroblasts to CAFs (168), resulting in up-regulation of transcription factors Friend leukemia integration (Fli-1) (169) and transcription factor 12 (TCF12) (13). These factors induce remodeling of the ECM by up-regulating fibronectin and LOX (170) thus promoting metastasis. Ectopic expression of miR-200 in CAFs significantly decreased lung metastatic burden after co-injection with MDA-MB-231 BC cells in orthotopic models (168).

Overexpression of miR-200 has been shown to be associated with increased risk of metastasis in BC (161, 171). For the opposite correlation, no clinical data are available. These seemingly counterintuitive results, as outlined above might be due to cell-type (context)-dependent functions of miR-200 and/or due to its dichotomous role in metastasis. miR-200 might counteract early steps of invasion and

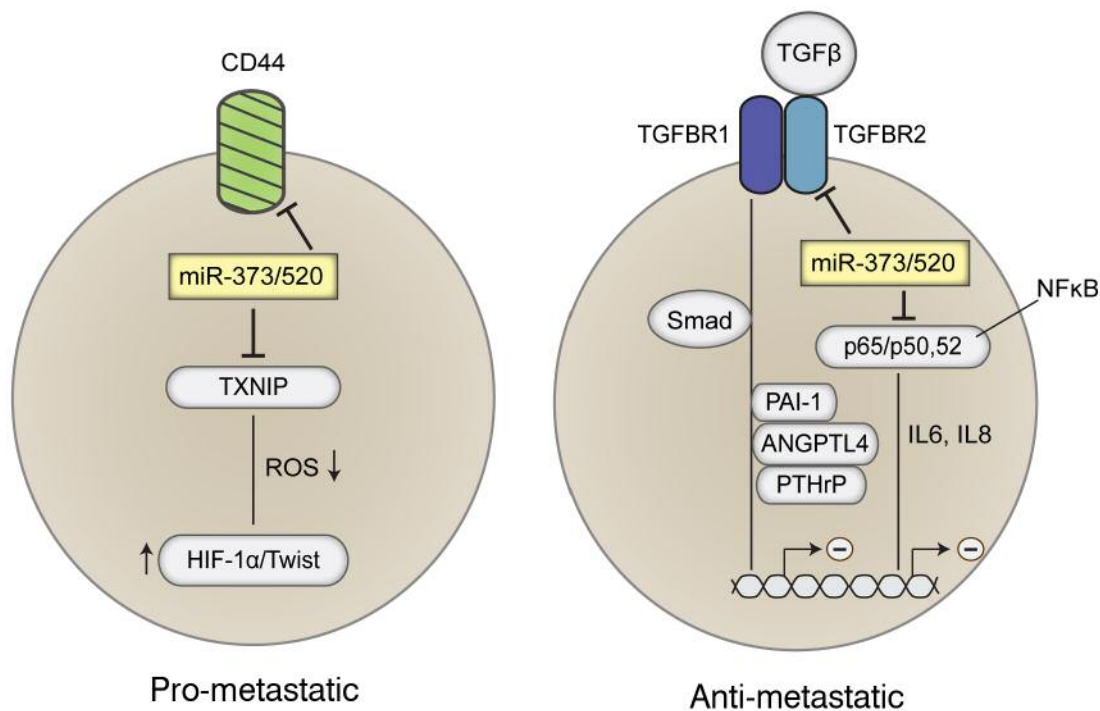


Figure 7. Pro- and anti-metastatic functions of miR-373/520. Pro-metastatic function of miR-373/520 in breast cancer cells is mediated by degradation of CD44 and activation of HIF-1 α /Twist signaling. Anti-metastatic function of miR-373/520 is based on inhibition of TGF β - and NF κ B signaling. ANGPTL4: Angiopoietin-like 4; CD44: cluster of differentiation 44; HIF-1 α : hypoxia-inducible factor 1 α ; interleukin 6,8: interleukin 6 or 8; PAI-1: plasminogen activator inhibitor-1; PTHrP: parathyroid hormone-related protein; ROS: reactive oxygen species; Smad: contraction of Sma and Mad (mothers of decapentaplegic); TGF β : transforming growth factor β ; TGFBR1,2: transforming growth factor β receptor 1,2; TXNIP: thioredoxin-interacting protein.

migration and promote colonization of the parenchyma of distinct organs by enhancing MET (160, 161). On the other hand miR-200 might be down-regulated at the invasive front of breast tumors and up-regulated in the resulting metastases (172). More work addressing the MOA of miR-200 in BC metastasis is needed to resolve these issues.

miR-373/520c: The miR-373/520 family consists of three different clusters possessing an identical seed region (173). In BC-related experimental systems miR-373/520c can act as an oncogene or as a tumor suppressor in a context-dependent manner (174-176) (Figure 7). Ectopic expression of miR-373/520 confers migratory properties to non-migratory MCF-7 cells (174, 175). In MDA-MB-231 cells (ER-, aggressively invasive) overexpression of miR-373 blunts invasive capacity (174) and on the other hand in MDA-MB-435 cells, which express endogenous miR-373/520, down-regulation of miR-373/520 inhibits migration and invasion (176). MCF-7 cells ectopically expressing miR-373/520 give rise to metastatic nodules in the lungs and bone metastasis in the skull after tail vein injection (174, 176). There are no *in vivo* metastasis-related data available for the metastasis suppressive function of miR-373/520. Two basic

mechanisms have been shown to be connected to the metastasis-promoting function of miR-373/520. One is based on down-regulation of cell surface receptor cluster of differentiation 44 (CD44), a direct target of miR-373/520 (174). The other one is based on down-regulation of thioredoxin-interacting protein (TXNIP) and subsequent activation of the hypoxia-inducible factor 1 α (HIF-1 α)/Twist signaling pathway (175). The tumor-suppressive function of miR-373/520 is mediated by inhibition of NF κ B signaling through direct targeting of NF κ B subunit p65 (176) and down-regulation of TGF β signaling due to direct suppression of transforming growth factor β receptor 2 (TGFBR2) (176). The modulation of these pathways leads to decreased secretion of pro-inflammatory molecules such as interleukin 6 and 8 (IL6 and IL8) and TGF β -dependent molecules such as angiopoietin-like 4 (ANGPTL4), parathyroid hormone-related protein (PTHrP) and plasminogen activator inhibitor-1 (PAI-1) (176). In line with the preclinical data, controversial clinical correlations have been reported. Expression of miR-373/520 in BC specimens correlated with a higher probability of metastasis in one study (174), in another study an inverse correlation between miR-373/520

levels and lymph node metastases has been noted, especially in ER- patients (4). However, due to the heterogeneity of BC with respect to subtypes as outlined previously, the small sample size in both studies might not be sufficient for correlation analysis.

Steady-state RNA Levels of Selected miR in Breast Cancer and Corresponding Normal Tissues

The data were derived from The Cancer Genome Atlas (TCGA) and are shown in Figure 8. Prometastatic miRs -105, -182, -21 and -9 are up-regulated in breast cancer tissues in comparison to matching normal tissues (Figure 8A), whereas anti-metastatic miRs -126, -145, -205 and -335 are down-regulated (Figure 8B).

Potential Relevance and Current Bottlenecks of miR-directed and/or -based Therapies

For the treatment of BC metastasis several scenarios can be envisaged: (i) therapy initiation prior to dissemination (clinically uncommon), (ii) therapy initiation after dissemination prior to formation of overt metastases, or (iii) initiation of therapy after formation of overt metastases. Significant numbers of disseminated tumor cells are present in blood, bone marrow and distant organs of BC patients upon initial presentation in the clinic (177, 178). Therefore, effective anti-metastatic therapy should, in addition to interference with migration and invasion, also affect proliferation and survival of disseminated tumor cells. Anti-metastatic therapy also needs to target established metastases otherwise its utility would be restricted to (rather unlikely) long-term prophylactic settings.

In contrast to therapies focusing on one defined target, miR-directed therapies are able to concurrently target multiple pathways involved in dissemination, proliferation and survival. In addition, modulation of miRs also can affect epigenomic modifications, which modulate a variety of different cancer-related processes.

The potential of miR-related agents to be efficacious against metastatic disease has been described in the preceding chapters of this review. These include on one side application of agents that interfere with tumor/metastasis promoting miRs. Furthermore, miRs or nucleic acid derivatives with miR functionality may be applied on the other side to elicit functionalities of tumor/metastasis suppressing or preventing functionalities. Experimental data targeting established metastases in preclinical *in vivo* models by modulation of miRs are not yet available. Possible modes of intervention, dependent on the function of the specific miR under consideration are inhibition, degradation or reconstitution of its function by replacement therapy.

Inhibition of miR functionalities can be achieved by nucleic acid -derived therapeutics. Examples for such entities

include antisense oligonucleotides (ASOs), siRNAs, gapmers, locked nucleic acids (LNA) and antagomirs to name a few. Common features of such 'small' nucleic-acid based entities are chemical modifications introduced to enhance biophysical properties, stability and/or nuclease resistance. Further modifications may include 5' or 3' additions of entities to enhance tissue targeting (*e.g.* cholesterol (179-183)). Other miR-inhibitory agents are anti-miR-MASK single-strand ASOs which are complementary to the miR-binding nucleotides in the 3'-UTR of the target RNA. Or miR sponges that harbor multiple (4 to 16) seed binding sites as decoys and duplex RNAs which induce degradation due to RNA interference (RNAi) and hairpin RNAs, which mediate miR degradation (179-183). Finally, small molecule inhibitors may be able to prevent binding of miRs to 3'-UTR seed sequences.

Replacement therapy can *in vitro* be 'mimicked' by transfection or transduction of pri-miRNA, pre-miRNA or mature miRs, *e.g. via* liposomes, targeted liposomes or viral transduction. Although such approaches are feasible for proof-of-concept experiments in cultured cells, they cannot be applied for therapeutic approaches. This is mainly because of systemic toxicity and major side effects.

The *in vivo* delivery of nucleic acids is one (still unsolved) major bottleneck that has hampered nucleic acids from being safely applicable as therapeutic compounds. After the administration of RNAs (or RNA-derivatives/conjugates) the molecules face several physiological barriers: i) serum stability, ii) renal excretion, iii) immunogenicity, iv) extravasation, v) entry into the target cell, and vi) endosomal escape.

To avoid a fast renal clearance, RNAs can be conjugated to polyethylene glycol (PEG) to increase the molecular weight. Furthermore, a conjugation to cholesterol or albumin triggers the binding to lipoproteins in the circulation which enhances the serum half-life (184-186). Another possibility is the formulation into large nanoparticle structures. An advantageous effect of these modifications is that they can also protect the RNA from nuclease-mediated degradation in the circulation and prevent immunogenicity and cytokine release syndrome (187, 188).

In contrast to small molecules, RNAs cannot passively cross the cell membrane due to their hydrophilic character and size. Thus, conjugate delivery systems were used to shield the charged nucleic acids and allow a fusion with the cell membrane. Lipid Nanoparticles (LNP) are small lipoplexes that are broadly used for cell culture transfections. In the meanwhile, they were also tested for their suitability as *in vivo* transfectants (189, 190). However, those compounds strongly accumulate in the liver and other filtering organs and are rather cell-type unspecific which makes them likely to cause side effects and toxicity issues (191).

Dynamic PolyConjugates (DPC) consist of different components that can change their mode of action upon milieu

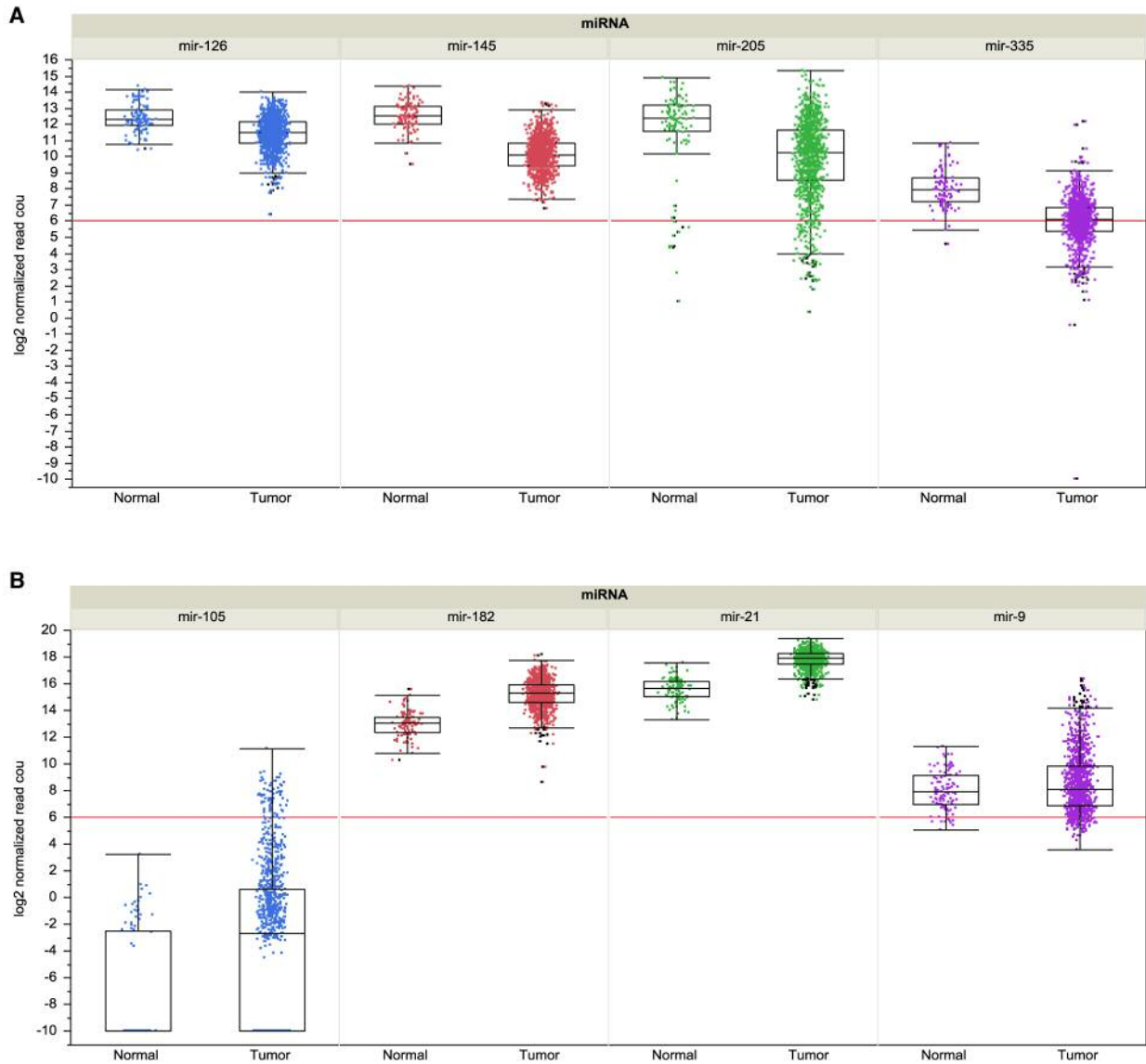


Figure 8. Expression of selected microRNAs in breast cancer in comparison to matched normal tissues. A: Pro-metastatic miRNAs: miRNAs -105, -182, -21 and -9; B: Anti-metastatic miRNAs: miRNAs -126, -145, -205 and -335. Steady-state RNA levels corresponding to miR-105, miR-181c and miR-210 based on 1,084 invasive breast cancer samples and 104 matched normal samples derived from cohorts of The Cancer Genome Atlas (TCGA) are shown. Expression was quantified by RNA sequencing and is shown as \log_2 of normalized read counts. The red lines indicate low versus higher expression. Expression data are shown as box plots. The line in the middle of the box represents the data median, the rectangles show the upper and lower 25% quartile and 50% of all data points are included in the rectangle. All other data points, except for outliers are located within the upper and lower whiskers.

changes. The nucleic acid is incorporated into lipophilic polymers which are again shielded by PEG. Thus, the membrane-disrupting agents are covered within the circulation. Upon entry into the endosome, the acidic conditions make the PEG molecules dissociate and unleash the endosomal escape capacity of the lipid polymers. Thereby, the nucleic acid payload is released into the cytosol in a prodrug-like manner (192, 193).

The problem that goes along with LNP and DPC synthesis/assembly is, that these structures are often not well defined and rather heterogeneous pools containing molecules of different size and composition. To ensure a population of molecular identical shape, oligonucleotide nanoparticles (ONP) might be a promising solution. RNA molecules are extended at the 3' end with complementary DNA fragments that hybrid into predefined three-dimensional structures (194-196).

As mentioned earlier the majority of conjugates end up in the liver as the primary filtering organ. To gain a higher degree of tissue specificity, siRNAs were equipped with target cell binding entities. The first major advance and entry into clinical trials was achieved with the addition of N-acetylgalactosamine (GalNAc) that binds with high affinity to the asialoglycoprotein receptor (ASGPR) on hepatocytes. Various chemically stabilized siRNAs that are conjugated on their 3' end with a trivalent GalNAc entity mediate great knockdown efficiencies of respective genes in hepatocytes. Thus, these conjugates are subject to several clinical trials against different indications such as amyloidosis, haemophilia and hypercholesterolemia (197-199).

Although the liver itself provides wide therapeutic possibilities, the targeted delivery of miRNAs to a specific cell type gained more and more interest in the last years. Conjugates of RNAs and cell penetrating entities with targeting ligands, aptamers, or antibodies (-fragments) both avoid a fast excretion and facilitate a specific binding to target cells and receptor-mediated endocytosis (200).

Integrin-binding RGD peptides were successfully used to deliver nanoparticle-packed siRNA to tumor neovasculature (201), and folate-incorporated lipid-based nanoparticles guided nucleic acids to rapid dividing cancer cells that require high amounts of folate (202).

Non-protein-based targeting agents are aptamers, selected nucleic acid binding species. They can be used to bind and deliver siRNA specifically to surface antigens such as the prostate specific membrane antigen (PSMA) (203-205).

Binding entities can be both linked covalently and non-covalently to the RNA-conjugates. One popular covalent approach is to use acid-labile or protease cleavable linkers that are exclusively degraded/cut within the endosome but stable in the circulation (206).

In another concept, haptenylated siRNA is complexed in DPC and delivered with a hapten-binding bispecific antibody to target cells in mice. Thereby, a significant target gene knockdown within tumor cells could be achieved (207).

Song and coworkers used the siRNA complexing agent protamine and fused it to an antibody Fab fragment which was directed against HIV-1 envelope. This complex achieved a specific delivery of siRNA to HIV-infected cells (208).

With respect to the internalization of the bound antibody, suitable receptors were identified in different screens. Especially membrane antigen with high turnover rates and the tendency to trigger receptor-mediated endocytosis lead to a high accumulation of nucleic acid in endosomes. Intracellular routing and recycling pathways of vesicular cargo molecules are also subject to current research (209, 210). Nevertheless, the biggest challenge is the entry of the therapeutic nucleic acids to its point of action – the cytoplasm or even the nucleus. The endosomal membrane is an evolutionary conserved barrier to prevent the entry of foreign nucleic acids, *e.g.* from bacterial or viral origin. Nevertheless, not only viruses evolved mechanisms to overcome

this roadblock and accomplish endosomal release. To mimic these mechanisms, various attempts were tried such as the complexation of viral proteins as cell-penetrating entities. The most prominent example is the trans-activating transcriptional activator (TAT)-derived peptide from human immunodeficiency virus 1 (HIV-1) which was shown to promote delivery of nucleic acids (211). Other examples are penetratin (*Drosophila*), transportan and Pep-1 (HIV) (212, 213). However, immune recognition and related toxicity problems limited a significant therapeutic success since many of these compounds are not well tolerated *in vivo*. Human-derived cell penetrating peptides such as neuriturin (NRTN) displayed reduced toxicity but comparable membrane activity (214). Galenics have improved the *in vitro* transfer of cationic molecules (215). Considering the mode of action of cell penetrating peptides (CPP), many theories were discussed (*e.g.* proton sponge theory) (212, 216). Langel *et al.* and others put much effort in defining rules and characteristics of CPP and designed artificial hybrids that carry advantageous features for intracellular cargo delivery.

Despite the fact that some clinical trials show promising data, the majority of these (targeted-) approaches must be seen as “early” concepts compared to established therapeutic agents such as small molecules, peptides, proteins or combinations thereof.

Taken together, the delivery bottleneck and other still unresolved issues presently represent serious limitations for clinical applications. Examples are (in addition to delivery) potential issues of removal of complexed DNA by phagocytic immune cells from the bloodstream, off-target effects and resulting toxicity such as cytokine release syndrome and hematological toxicity such as thrombocytopenia and biological stability of these agents in biological fluids and tissues (179-183). The detailed discussion of these issues is not in the scope of this review. The responsiveness of BC cell lines representing different molecular subtypes *versus* miR antagonists or mimetics with respect to parameters such as migration and invasion, proliferation and survival has to be investigated in more detail. Several *in vivo* models should be explored to get a more comprehensive picture of the functional consequences of modulation of a specific miR. Regarding clinical evaluation of miR-related agents in cancer patients, Mirna Therapeutics has evaluated an miR-34 mimetic as a replacement therapeutic in patients with hepatocellular carcinoma in a Phase I study. However, due to toxicity issues such as cytokine-release syndrome, the study has been halted (www.mirnarx.com). Other, miR-based cancer-related and metastasis-targeting approaches will be preclinically validated and clinically explored in cancer patients in the up-coming years including their potential as biomarkers for metastatic disease.

Acknowledgements

SD is a member of the international doctoral program “i-Target: Immunotargeting of Cancer,” funded by the Elite Network of Bavaria.

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Received October 10, 2017
Revised November 5, 2017
Accepted November 6, 2017