Universidade de Lisboa

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Circulating MicroRNAs as Biomarkers for Breast Cancer Brain Metastasis

Marta Bruno Soares de Melo Sereno

Dissertation supervised by Professora Doutora Maria Alexandra Brito and co-supervised by Professora Doutora Mafalda Videira

Master course in Biopharmaceutical Sciences

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Para a Linda.

Abstract

With the recent developments in the treatment of breast cancer (BC), that have improved prognosis of BC patients, metastatic disease is presently a major concern and the leading cause of death from BC. Particularly brain metastasis represent a serious oncologic problem, not only due to their high incidence in BC, poor prognosis and low survival but also because the brain microenvironment is considered a sanctuary for metastatic growth. While the blood brain barrier (BBB) restricts the entrance of most chemotherapeutics into the brain, brain cells can also contribute for tumor growth by exchanging factors with malignant cells. Altogether, this makes breast cancer brain metastases (BCBM) an uprising concern. Thus, the finding of reliable biomarkers that allow the early detection of BCBM, as well as new potential therapeutic targets, is in order. Recently, microRNAs (miRNAs or miR-) have arisen as powerful and specific biomarkers for different types of cancer and metastasis, having unique expression profiles depending not only on the type of cancer but also on the stage of the disease. Circulating miRNAs can be of special interest, due to their stability and easy quantification in biological fluids, allowing their usage in liquid biopsies for diagnosis and prognosis of oncologic diseases. Based on this, we hypothesized that miRNAs are aberrantly expressed in plasma prior to the detection of brain metastasis. So, the aim of this work was to identify miRNAs that could work as predictive biomarkers of BCBM in liquid biopsies. To this end, brain, lungs, kidneys, liver and plasma samples were collected from vehicle (control) and 4T1-injected female mice, at different timepoints during metastatic progression (5 hours, 3 days, 7 days and 10 days). Human samples of resected brain metastasis from triple negative BC patients were also studied. Analysis of brain and peripheral organs of the mouse model revealed that metastasis are particularly relevant in the brain, mainly in the hippocampus and that well established metastasis are only detectable from 7 days onwards. An initial miRNA screening by next generation sequencing (NGS) showed that plasma miRNAs are altered during brain metastasis development and that at 3 days, no metastasis were detected but a set of 8 miRNAs was deregulated. By real time PCR (RT-PCR), we could validate the downregulation of plasma miR-802-5p and miR-194-5p at three days and could see a trend for miR-92a-1-5p upregulation, and so, we propose that these miRNAs are potential biomarkers for the early detection of BCBM. Moreover, using bioinformatical tools, we predicted targets for these miRNAs, not only for a better understanding of the underlying mechanisms of these

miRNAs but also to identify potential new targets for modulation. *Matrix metalloproteinase-9* (*MMP9*) was a predicted target for miR-92a-1-5p. Immunofluorescence analysis of mouse brain sections revealed that MMP9 protein is expressed not only in malignant cells but also in the brain vasculature and in metastasis-associated vessels, pointing to a proangiogenic role of MMP9 *Myocyte enhancer factor 2C* (*MEF2C*) was predicted as a common target for both miR-802-5p and miR-194-5p. It was also observed that MEF2C is highly expressed in BCBM and that its expression increases with metastatic progression. Moreover, a nuclear translocation of MEF2C was observed in later stages of tumor progression, which indicates a higher activation of this transcription factor. Furthermore, MEF2C expression was detected in peritumoral astrocytes, pointing to a potential role of MEF2C in the cross-talk between astrocytes and malignant cells to promote tumor growth. High expression of MEF2C was further confirmed in human brain metastases from triple negative BC. In summary, this work revealed the potential in using circulating miRNAs as biomarkers for BCBM, pointing to miR-802-5p, miR-194-5p and miR-92a-1-5p as candidates to be used in liquid biopsies for the early detection of BCBM. It also revealed MEF2C as a new target for modulation and abrogation of BCBM.

Keywords: Breast Cancer Brain Metastases, MicroRNAs, Liquid Biopsies, MEF2C

Resumo

Com os desenvolvimentos recentes no tratamento do cancro da mama que têm vindo a melhorar o prognóstico dos doentes, o cancro metastático é uma preocupação e a principal causa de morte nos doentes de cancro da mama. Particularmente as metástases cerebrais, representam um problema na área da oncologia, não só devido à sua alta incidência no cancro da mama, mau prognóstico e baixa taxa de sobrevivência, mas também porque o microambiente do cérebro é bastante favorável ao desenvolvimento de metástases. Enquanto que a barreira hematoencefálica restringe a passagem da maioria dos medicamentos quimioterapêuticos, as células do cérebro também podem contribuir para o crescimento metastático através da troca de factores com as células malignas. Assim, as metástases cerebrais provenientes do cancro da mama são, cada vez mais, consideradas uma preocupação no tratamento do cancro e a descoberta de novos biomarcadores que permitam a sua detecção precoce e de novos alvos terapêuticos, é necessária. Recentemente, os microRNAs (miRNAs ou miR-) têm sido estudados como biomarcadores específicos para diferentes tipos de cancro e de metástases, tendo perfis de expressão únicos, dependendo não só do tipo de cancro, mas também do estadio da doença. Os miRNAs circulantes podem ter especial interesse para este efeito, devido à sua grande estabilidade e fácil quantificação em fluidos biológicos, que permite o seu uso em biópsias líquidas para o diagnóstico e prognóstico de doenças oncológicas. Sendo assim, neste trabalho, foi proposto que os miRNAs circulantes podem funcionar como biomarcadores eficientes para metástases cerebrais de cancro da mama, tendo como objectivo a selecção de miRNAs específicos que pudessem funcionar como biomarcardores precoces para este tipo de metástases. Para isto, inocularam-se ratinhos com células 4T1 ou com solução salina, como controlo e colheram-se os encéfalos, rins, pulmões e fígados e amostras de plasma a diferentes tempos depois da inoculação (3 dias, 7 dias e 10 dias). Também foram usadas amostravas humanas de metástases cerebrais de cancro da mama. A análise dos encéfalos e dos órgãos periféricos de ratinho demonstrou que este modelo metastiza fortemente para o encéfalo, ao contrário dos outros órgãos e que metástases bem estabelecidas só são detectáveis a partir dos 7 dias. Resultados de sequenciamento de nova geração mostraram os níveis expressão dos miRNAs no plasma estão alterados durante a formação e que aos 3 dias após a inoculação, apesar de ainda não serem detectáveis metástases, há um conjunto de 8 miRNAs que estão alterados no plasma. Por PCR em tempo real, foi possível validar a diminuição do miR-802-5p e do

miR-194-5p aos 3 dias após inoculação, relativamente ao controlo. Foi também observada uma tendência do miR-92a-1-5p para estar aumentado. Assim, propõe-se estes três miRNAs como potenciais candidatos a biomarcadores precoces para a detecção de metástases cerebrais de cancro da mama. Através do uso de ferramentas bioinformáticas, foi feita uma previsão de possíveis alvos para estes miRNAs, não só para se ter uma melhor compreensão dos mecanismos subjacentes dos miRNAs, mas também como um processo de triagem para selecionar novos alvos para modulação em metástases cerebrais. A metaloproteinase da matriz-9 (MMP9) foi um dos alvos previstos para o miR-92a-1-5p. Análise por imunofluorescência em secções de encéfalo de ratinho, revelou que a MMP9 é expressa, não só em células malignas no encéfalo, mas também na vasculatura cerebral e em vasos associados às metástases, apontando para um papel pro-angiogénico da MMP9. Por sua vez, o factor potenciador de miócitos 2C (MEF2C) foi previsto como alvo comum para o miR-802-5p e para o miR-194-5p. Através de análise por imunofluorescência, também em secções de encéfalo de ratinho, mostrou-se pela primeira vez que o MEF2C tem uma expressão elevada em metástases cerebrais de cancro da mama e que a sua expressão aumenta com a progressão das metástases. Foi também observada uma translocação nuclear do MEF2C em fases mais avançadas do tumor, o que pode indicar uma maior activação deste factor de transcrição. Além disso, a expressão do MEF2C foi também detectada, especificamente em astrócitos peritumorais, apontando para um papel do MEF2C na interacção entre as células malignas e os astrócitos, para potenciar o crescimento tumoral. A expressão elevada do MEF2C foi também confirmada em metástases cerebrais humanas provenientes de cancro da mama triplo negativo. Em suma, este trabalho demonstrou o potencial do uso dos miRNAs circulantes como biomarcadores para metástases cerebrais de cancro da mama, apontando o miR-802-5p, o miR-194-5p e o miR-92a-1-5p como candidatos para serem usados nas biópsias líquidas para a detecção precoce deste tipo de metástases. Revelou também o potencial do uso do MEF2C como um novo alvo para modulação em metástases cerebrais de cancro da mama.

Palavras-chave: Metástases Cerebrais de Cancro da Mama; MicroRNAs, Biópsias Líquidas, MEF2C

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Abbreviations

3' UTR	3' Untranslated Region
5'UTR	5' Untranslated region
AGO	Argonaute proteins
AJ	Adherens junctions
BBB	Blood Brain Barrier
BC	Breast Cancer
BCBM	Breast Cancer Brain Metastases
BCC	Breast Cancer Cell
B-CLL	B-cell chronic lymphocytic leukemia
BM	Basement Membrane
BSA	Bovine serum albumin
COX2	Cyclooxigenase-2
CSC	Cancer stem-like cell
CTC	Circulating tumor cells
CXCL	Cysteine-X amino acid-cysteine ligand
CXCR	Cysteine-X amino acid-cysteine receptor
DGCR8	DiGeorge syndrome critical region 8
DTC	Disseminating tumor cell
E-Cadherin	Epithelial cadherin
ECM	Extracellular matrix
ECmiRNA	Extracellular miRNA
EDTA	Ethylenediamine tetraacetic acid
EGFR	Epidermal growth factor receptor
EMT	Epithelial-Mesenchymal Transition
ER	Estrogen Receptor
Exp5	Exportin-5
FC	Foldchange
GFAP	Glial fibrillary acidic protein
HER2	Human epidermal growth factor receptor
HPSE	Heparanase
Iba-1	Ionized calcium binding adaptor molecule 1
IF	Immunofluorescence

IL-1β	Interleukin-1β
MEF2C	Myocyte Enhancer Factor 2C
MET	Mesenchymal-Epithelial Transition
MiRNA or miR-	MicroRNAs
miRNP	Microribonuclear protein complex
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
N-Cadherin	Neuronal cadherin
ncRNAs	Noncoding RNAS
NGS	Next Generation Sequencing
NK	Natural killers
PACT	Protein activator of PKR
PBS	Phosphate-Buffered saline
Pol II	Polymerase II
Pre-miRNA	Precursor miRNa
Pri-miRNA	Primary miRNA transcript
PTEN	Phosphatase and tensin homolog
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
ROCK	Rho-Associated protein kinases
TEM	Transendothelial migration
TGF-β	Transforming growth factor-β
TJ	Tight Junctions
TNRC6A	Trinucleotide repeat containing gene 6A
TRBP	Transactivation response RNA binding protein
VEGF	Vascular endothelial growth factor
YAP1	Yes associated protein-1
ZEB	Zinc finger E-box-binding homeobox
ZO	Zonula occludens

Chapter I

Dissecting the Relevance of miRNAs in Breast Cancer:
A Focus on Brain Metastasization

Abstract

MicroRNAs (miRNAs or miR-) are small non-coding RNAs that mainly act by binding to the 3'-untranslated regions (3'UTR) of target genes to negatively regulate their expression. Due to the large variety of genes that miRNAs can regulate, these non-coding RNAs have been subject to extensive research in the past few years. This research has revealed, not only that miRNAs are powerful regulators of physiological processes but also that when aberrantly expressed, miRNAs are associated with several diseases, including cancer. Moreover, different miRNAs are related with specific types of cancer and differential metastasis, where they can influence several steps of tumor progression. So, miRNAs have been proposed as efficient biomarkers and potential therapeutic targets for cancer. Breast cancer (BC) is the most frequently diagnosed cancer and one of the leading causes of death from cancer in women, worldwide and although the treatments for BC have improved, metastatic BC is still a major concern. Thus, in this review, we summarize the role of miRNAs in metastatic BC, particularly in the miRNAs that have been studied to regulate the different steps of the metastatic cascade. We also depict the miRNAs that have been specifically known to regular breast cancer brain metastases (BCBM), due to the high incidence, poor prognosis and low survival of this type of metastases, that requires the finding of new reliable biomarkers and/or therapeutic targets.

1. Introduction

MicroRNAs (miRNAs or miR) are small non-coding ribonucleic acids (RNAs) that up until recently were fairly unknown, but that in the past few years have received great attention by the scientific community, due to their unique properties and roles in many diseases.[1,2] The first miRNA, *lin-4*, was discovered in *Caenorhabditis elegans* (*C. elegans*) by Lee and colleagues, in 1993. It was identified as a small noncoding RNA(ncRNA) affecting development through regulating the expression of the protein LIN-*14*.[3] Seven years later, *Reinhart et al.*[4] reported another miRNA in *C. elegans*, *let-7*, which negatively regulated the expression of the LIN-41 gene through sequence-specific RNA–RNA interactions with the 3′-untranslated region (3'-UTR) of its mRNA. Subsequently, miRNAs were found to be abundant in both invertebrates and vertebrates and currently, more than 1000 miRNAs are annotated in the human genome.[5] Although little is known about the specific targets and biological functions of miRNAs, it is evident that miRNAs have crucial roles in the regulation of gene expression by controlling diverse

cellular and metabolic pathways.[6] It is also known changes in the expression of miRNAs can have major cellular effects, so that nowadays the aberrant expression of miRNAs has been associated with multiple human diseases.[1,2] miRNAs have shown to have a prominent role in cancer, so the dysregulation of miRNAs in this type of disease has been the most well studied and numerous authors have shown that specific miRNAs are up or down regulated in different types of cancer.[7] This has led to the creation of miRNAs expression profiles that specifically correlate to each type of cancer even in early stages, allowing the detection and classification of poorly differentiated tumors and disclosing numerous possibilities in terms of diagnosis, prognosis and treatment.[8,9] In this review, we give special emphasis to the roles of miRNAs in cancer and, particularly to the miRNAs that have been recently found to play an active role in breast cancer metastasis to the brain and on how miRNAs can be used in the future to prognosis and early diagnose breast cancer brain metastases.

2. microRNAs – Biogenesis and Processing

It is predicted that up to 98% of the transcriptional output of the human genome could represent RNA that does not code for protein but that can fulfill a wide range of structural, enzymatic and regulatory functions in both eukaryotic and prokaryotic biology. The transcripts, commonly termed ncRNAs, fall into several classes based on their length, biogenesis, polarity (sense or antisense), and putative functions. A basic classification criterion is size. Accordingly, long ncRNAs are typically >200 nucleotide-long and function without major prior processing. whereas, small ncRNAs have to be processed from longer precursors.[6,10] miRNAs are a subclass of single stranded small ncRNAs with about 21-25 nucleotides long that are endogenously produced and found in diverse organisms, including humans, and that can play important gene-regulatory roles by pairing to the 3'-UTR of mRNAs of protein-coding genes to direct their posttranscriptional repression, thus regulating many cellular functions.[11,12] It is common that only a partial pairing between the miRNA and the target mRNA is enough to direct gene silencing. Therefore, a single miRNA can regulate several genes, while one single gene can be targeted by more than one miRNA.[13] Thus, it is predicted that approximately 1100 miRNA genes exist in the human genome,[14] and that about one third of the human genome is regulated by miRNAs.[1] Still, the location of the miRNAs in the genome varies widely and two types of miRNA have been identified, according to their location: the ones located in intergenic regions and the ones located within protein coding regions,

either in exons or in introns. Most of miRNA genes are found in intergenic regions or in antisense orientation to annotated genes, indicating that they are transcribed as independent units. However, many intragenic miRNAs are located in intronic regions and can be transcribed as part of the annotated genes. This suggests that the maturation of miRNAs is a highly complex process.[15,16] Moreover, approximately half of the known miRNAs are found in close proximity to other miRNAs in the genome. These clustered miRNAs are suggested to be transcribed in a polycistronic manner, similar to the operon regulation systems of prokaryotes. Likewise, miRNAs in the same cluster have been hypothesized to regulate functionally related genes.[17]

The processing and biogenesis of miRNAs is an intricate process that is not fully understood yet but that involves several different steps and intervenient proteins, as depicted in Figure 1. Most miRNAs are initially transcribed similarly to protein coding genes, in a process catalyzed by polymerase II (Pol II).[18] According to the different genomic contexts of the miRNAs, the transcriptional regulation processes might be different. The miRNAs in the same cluster are usually co-transcribed but the individual miRNAs can be additionally regulated at a post-transcriptional level. The miRNAs that reside within introns of protein coding genes generally share the promoter with their host.[19,20] Besides being carried out by Pol II, miRNAs' transcription can be controlled by RNA-Pol II associated transcription factors and epigenetic regulators, including p53, c-MYC, ZEB1, ZEB2 and myoblast determination protein 1. These factors can positively or negatively regulate miRNA expression, by interacting with the promoters, in a tissue or development-specific manner.[21] The products originated from transcription are designated primary miRNA (pri-miRNAs). These are long transcripts, about 400 nucleotides long, that contain a stem loop structure in which the mature miRNA is embedded. It usually also comprises a 33-35 nucleotides stem, a terminal loop and single stranded RNA segments at both 5' and 3' ends. When miRNAs are part of a cluster, and so transcribed in a polycistronic manner, one pri-miRNA contains several loop structures, one for each mature miRNA.[15,22]

The pri-miRNAs, then undergo several steps of maturation. The first step is catalyzed, still in the nucleus, by Drosha, an enzyme with a highly conserved RNase III domain that cleaves the pri-miRNAs to originate precursor miRNAs (pre-miRNA). Although Drosha is the main responsible for slicing the pri-miRNAs, i*n vitro* studies show that purified recombinant Drosha fails to generate pre-miRNAs, indicating that other cofactors might be required to enhance the catalytic activity of Drosha and, actually, mass

spectrometry confirm that this enzyme associates with at least 20 distinct polypeptides during miRNA processing.[23] Drosha also dimerizes with a double-stranded RNA binding protein called DiGeorge syndrome critical region gene 8 (DGCR8), forming an enzymatic structure commonly called microprocessor.[24] Whereas Drosha cleaves the pri-miRNA substrates, DGCR8 is responsible for binding the pri-miRNAs thanks to its unique molecular structure that contains two RNA-binding domains.[25] Accordingly, Landthaler *et al*[26] showed that a knockdown of DGCR8 results in accumulation of pri-miRNAs and reduction of pre-miRNAs and mature miRNAs.[26] Besides Drosha and DGCR8, two other proteins have been characterized as being present in the microprocessor: p68 and p72.[27] Their function in the microprocessor is not fully understood yet but they are known to ease the activity of Drosha, as studies have shown that the knockout of these genes is responsible for a significant reduction in the levels of some pre-miRNAs and mature miRNAs.[28] Altogether, the microprocessor cleaves the miRNA and releases, in the nucleus, an hairpin structured pre-miRNA of ~60-70 nucleotides, bearing a 5' phosphate and a 2 nucleotides 3' overhang, characteristic of Drosha's RNAse III activity.[29,30]

The pre-miRNA is then exported to the cytoplasm, a process mediated by a nucleocytoplasmic transport factor, exportin-5 (Exp5), that recognizes the 3' overhang of the pre-miRNA. Exp5 also protects the pre-miRNAs from digestion by cellular exonucleases.[31,32] Being a RanGTP-dependent dsRNA-binding protein, Exp5 possesses a RanGTP binding site and the RanGTP gradient across the nuclear membrane regulates the interaction with the pre-miRNAs in a compartment-specific way. Thus, Exp5 binds the pre-miRNAs at a high Ran-GTP level in the nucleus and is then translocated through a nuclear pore as a trimer (pre-miRNA-Exportin-RanGTP). When the complex reaches the cytoplasm, the GTP suffers hydrolysis, forming GDP and releasing of the pre-miRNA and Exp5's cofactor, Ran.[33-35]

In the cytoplasm, the pre-miRNAs are finally processed into mature ~22-25 nucleotides miRNAs duplexes by another RNase III, Dicer-1, that associates with ds-RNA-binding proteins, including transactivation response RNA binding protein (TRBP) and protein activator of PKR (PACT).[36] Although their roles are not well defined yet, TRBP and PACT have been shown to influence substrate binding and product length and to increase the stability of Dicer-1.[37,38] Once the mature duplex is formed, the two strands are separated, depending on factors like the thermodynamic asymmetry of the duplex and the stability and strength of binding at the 5' end. One of the strands, denominated the

guide strand, along with TRBP, PACT and other RNA binding proteins that include trinucleotide repeat containing gene 6A (TNRC6A), associates with catalytic Argonaute proteins (AGO), forming a microribonuclear protein complex (miRNP) called RNA-induced silencing complex (RISC). The other strand of the duplex, termed passenger strand is degraded. Although the mechanisms by which this process of strand selection are done are still largely unknown, the miRNA strand with the most unstable base pairing at the 5′ end usually acts as the guide strand, while the strand with stable base pairing at the 5′ end acts as the passenger strand.[39-41] In some cases the passenger strand, instead of being degraded can associate with AGO proteins enabling both strands to become active miRNAs.[42]

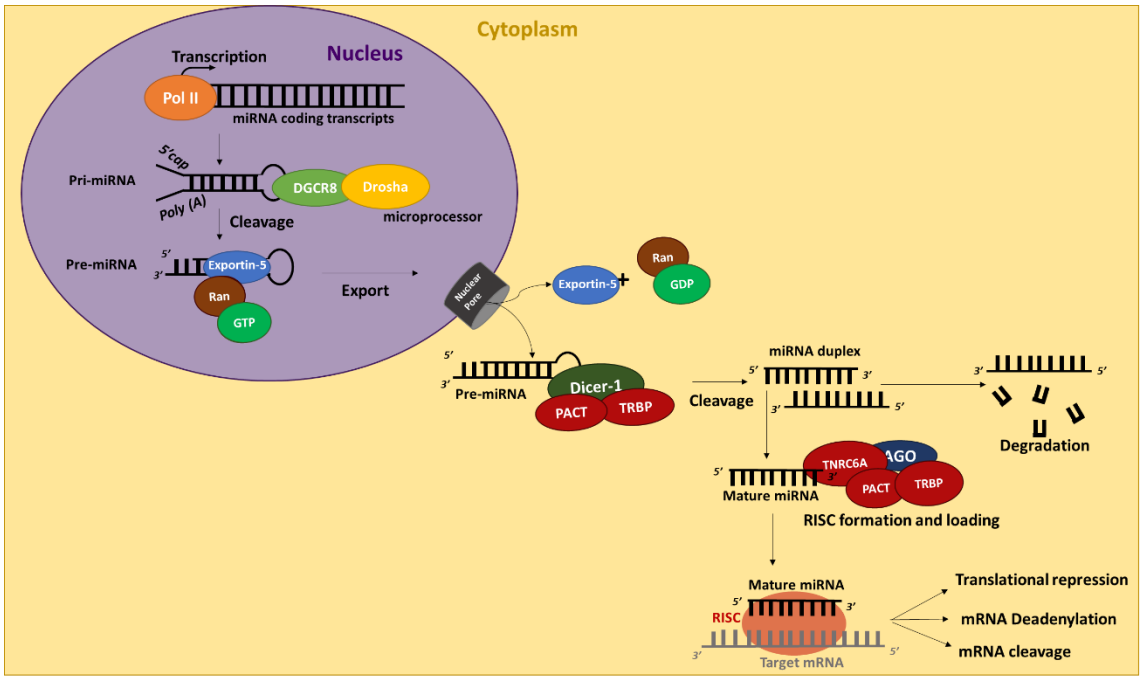

Figure 1 - Schematic representation of the canonical pathway of miRNA biogenesis. miRNAS are usually transcribed by RNA polymerase II (Pol II) to produce large primary transcripts (Pri-miRNA), about 400 nucleotides long which are cleaved by a microprocessor complex composed by a nuclear RNase III called Drosha dimerized with a double-stranded RNA binding protein called DiGeorge syndrome critical region gene 8(DGCR8) into ~70 nucleotides the precursor miRNA (pre-miRNA) , loop structures with a 3'- 2 nucleotide overhang that are transported to the cytosol, due to the interaction with exportin-5 and its cofactors Ran-GTP, through a nuclear pore. In the cytosol, GTP suffers hydrolysis, forming GDP and releasing the pre-miRNAs that are, then, cleaved by Dicer-1 to a ~20-25 nucleotide miRNA duplex that is unwound to give rise to the mature miRNA that, along with several RNA binding proteins, including the transactivation response RNA binding protein (TRBP), protein activator of PKR (PACT) and trinucleotide repeat containing gene 6A (TNRC6A), associates with catalytic argonaut (AGO) proteins forming a microribonuclear protein complex called RNA-induced silencing complex (RISC). Once the RISC is formed and the miRNA is loaded into it, miRNAs guide the complex to specifically recognize the target mRNA and downregulate gene expression by translational repression, mRNA deadenylation or mRNA cleavage.

Once the RISC is formed and the miRNA is loaded into it, miRNAs guide the complex to specifically recognize the target mRNA and downregulate gene expression mainly by one of two posttranslational mechanisms: translational repression and RNA decay via

deadenylation or mRNA cleavage, both requiring the recognition of the target and binding of the miRNA to partially complementary sequences of the 3'-UTR of the target gene through Watson-Crick base-pairing.[12,43] The most important determinant in target recognition by miRNA is the "seed sequence", which consists in the nucleotides 2-7 in the 5' region of the miRNA. The seed region is complementary to the target mRNA in the binding site and plays an important role in the interaction of miRNA with target mRNA.[44] So far, four canonical types of seed have been described: 6mer; 7mer-A1; 7mer-m8 and 8mer (Fig. 2), but the 8mer is the most effective seed type for target recognition.[11,45] Although the seed sequence is the most important for the recognition and downregulation of target mRNAs, it does not guarantee efficient downregulation of target genes, suggesting important roles for the middle and 3' regions.[44]

Until recently, miRNAs were believed to have an universal role on gene expression by exclusively downregulating the target genes.[46] Nevertheless, recent published evidences have shown that miRNAs can alternate between downregulation or upregulation of target mRNAs, according to specific conditions, including cellular microenvironment, sequences and cofactors. MiRNPs, can directly activate gene expression by recognizing target sequences in the gene promoter or, instead, can indirectly relieve mRNA repression by the miRNPs as a consequence of being disengaged from previously repressed mRNAs.[47,48] Moreover, given the complexity of miR-mediated gene regulation, one single miRNA can act both in repression or stimulation of mRNAs, as can a single gene be up or downregulated by different miRNAs. MiR-145 has been described to upregulate myocardin during smooth muscle differentiation and proliferation,[49] while, the same miRNA is known to downregulate Rho-associated protein kinase-1 (ROCK1) in cases of osteosarcoma.[50] These are very exciting new discoveries, which render more difficult to understand the mechanisms underlying miRNA-mediated

gene regulation.

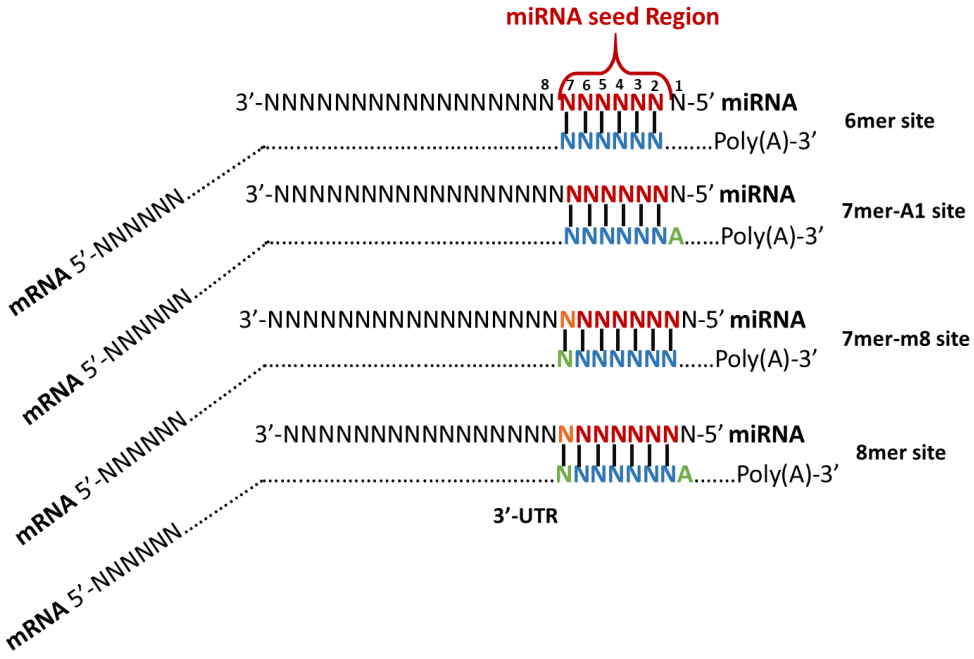

Figure 2 – Different types of miRNA-mRNA seed matches. To direct gene repression, miRNAs bind to the target gene in its 3'-UTR. The most important determinant in target recognition is the seed region match which consists in the nucleotides 2-7 in the 5' region of the miRNA, which presents full or partial Watson-Crick complementarity with the target. Four main types of seed matches are known: 6mer (perfect match to the target mRNA between nucleotides 2-7 of the miRNA); 7mer-A1 (6mer match plus an adenosine on target mRNA opposite miRNA nucleotide 1); 7mer-m8 (6mer match plus a match at nucleotide 8 of miRNA) and 8mer (6mer match plus both an adenosine opposite miRNA nucleotide 1 and a match at nucleotide 8). Currently it is known that the most common type of seed match is the 8mer.

3. miRNAs in health and disease

MiRNAs play an evolutionarily conserved role in regulating numerous genes involved in several developmental and vital process,[51,52] and are eventually involved in every biological process in multicellular organisms.[53] In fact, several studies showed that animals that do not express miRNAs or have a deficient miRNA biogenesis, fail to develop and survive.[54,55]

Given their importance as regulators of physiological processes, the high number of miRNA genes, the diverse expression patterns and the abundance of potential miRNA targets, miRNAs have recently been the subject of many studies. Such studies have shown that small variations in the levels of miRNAs can have major cellular effects and how their aberrant expression due to mutations, dysregulation or dysfunction of miRNA biogenesis can lead to the blockade of physiological and biochemical pathways and result or be involved in pathological outcomes.[56-58] Accordingly, these ncRNAs are currently associated with a wide variety of human diseases, ranging from myocardial infarction to neurodegenerative diseases, among many others, as well as in cancer, addressed below.[59-

[61] In general, the loss or gain of function of miRNAs can result from chromosomal abnormalities, inherited mutations and single nucleotides polymorphisms in either the miRNAs or their targets, as well as from epigenetic silencing of primary miRNA transcription units, or can be a consequence of defects in the miRNA biogenesis machinery.[62,63] MiRNAs processing can also be affected by other miRNAs as previously described. MiR-709 can directly bind to its recognition element that is present on pri-miR-15a/16-1, interfering with the biogenesis and thus suppressing the maturation of miR-15a/16-1.[64] This leads to the possibility of a "microRNA hierarchy system" with a complex level of mutual interaction and regulation between miRNAs that may also be involved in pathological events.

Both in physiological and pathological conditions, miRNAs expression and their targets are, in many cases, tissue specific. Thus, different miRNAs are expressed in different tissues at different levels, having characteristic and specific expression patterns. that play an important role in tissue identity, differentiation and function. The same applies to pathologies, meaning that specific miRNAs are aberrantly up or downregulated in different diseases and can influence or even determine the pathological phenotypes of cells.[65,66] Nowadays there are several papers and databases that describe the distribution of several miRNAs across the human organism, as well as the associations between miRNAs and diseases.[56,67,68] So, knowing the expression profiles of miRNAs specific to each disease allows more personalized therapies with improved rates of success. Since this is a very recent subject, there are still no miRNA-based therapeutics approved. However, more than 20 clinical trials are in course. In fact, the tumor suppressor miRNA miR-34 encapsulated by lipid nanoparticles, has reached phase I clinical trials for treating cancer and has shown to cause significant tumor reduction, while antimiRs targeting miR-122 are now in phase II trials for treating hepatitis and also show a great reduction in virus titres.[69,70]

Although most miRNAs are found inside cells, they can also circulate in a cell-free form and a surprisingly high number of miRNAs, commonly known as circulating miRNAs or extracellular miRNAs (ECmiRNAs), has been found in several body fluids, including blood, plasma, urine, cerebrospinal fluid, saliva and semen.[71] ECmiRNAs, in contrast to cellular miRNAs, are extremely stable and can in survive extreme conditions, including boiling, high or low pH, prolonged storage time and multiple freeze-thaw cycles. ECmiRNAs are also resistant to RNases, which are abundant in the extracellular microenvironment.[72,73] Hunter *et al*[74] suggested that ECmiRNAs are protected by

encapsulation into membrane-vesicles.[75] This led to the groundbreaking hypothesis of the existence of an intercellular crosstalk and inter-organ communication system mediated by extracellular vesicles, like exosomes that can carry mRNA and miRNAs, among other molecules.[76] The mechanisms for the sorting and upload of miRNAs into exosomes remain largely unknow; however, it is known that some miRNAs are preferentially loaded into exosomes, while others remain in the mother cells, indicating a selectiveness in the sorting process. Sanchez-Madrid *et al*[77] demonstrated that miRNAs present in exosomes from primary T lymphoblast share a GGAG motif that is able to modulate directly the miRNAs loaded into exosomes.[78] The theory that exosomes and extracellular vesicles transport miRNAs to mediate intercellular and interorgan communication is interesting and widely accepted, despite the studies showing that around 90% of circulating miRNAs circulate in a vesicle-free state.[79,80] Accordingly, cell free miRNAs can also be shaded from extracellular environment by associating with high density lipoprotein or with Ago-2 in a ribonucleo-protein complex. Still, it remains to understand if and how these miRNAs facilitate cell–cell communications.[81] Nonetheless, the unique properties of these miRNAs fulfill all the characteristic of an ideal biomarker as they are: specific and able to differentiate pathologies; sensitive because there is a quick and significant release upon the appearing of a pathology; predictive as they should have a long half-life and be proportional to the severity of the disease; robust and easily detectable by minimally invasive techniques; and finally, translatable.[82] This, together with the fact that some ECmiRNAs have already been associated with several diseases[83] raise in the interest in further studies, related to physiological or pathological processes, as well as for their usefulness as reliable biomarkers to be used in liquid biopsies.

3.1. miRNAs in human cancer

Though the roles of miRNAs have been well studied in several diseases since the early stages of miRNA research, cancer has been the most prominent of human diseases with a clear role for miRNAs regulation.[59] In fact, more than 50% of human miRNA genes are thought to be located in regions of chromosomal instability, more prone to gene deletion, amplification and mutations, or nearby chromosomal breakpoints, all of which are considered cancer-associated regions or fragile sites of the genome.[84] The earliest evidence of miRNA involvement in human cancer came from a study by Calin *et al* [85] demonstrating a frequent deletion of the miRNA genes miR-15 and miR-16 among 65% of B-cell chronic lymphocytic leukemia (B-CLL) patients. Down-regulation of miR-15

and miR-16 expression was also observed among B-CLL patients without the deletion, suggesting that the pathogenesis of B-CLL may be affected by the intracellular abundance of the two miRNAs. Studies of the same authors further demonstrated that miR-15 and miR-16 act as tumor suppressors by repressing Bcl-2, an anti-apoptotic protein overexpressed in malignant nondividing B cells.[86,87] Encouraged by these findings, this group applied a systemic search on the complete human genome and established correlations of miRNAs with various cancers, confirming that certain miRNAs can be related to specific cancers.[84] In the last few years, miRNA profiling and deep sequencing showed that miRNA expression is dysregulated in cancer and that different tumors have specific miRNAs signatures that could be used for tumor classification, diagnosis and prognosis, or even as therapeutic targets or agents.[88,89]

Depending on their targets, miRNAs may play a role as oncogenes (oncomiRs) by down-regulating tumor suppressor genes and/or genes controlling cell differentiation or apoptosis, whereas the down regulated miRNAs act as tumor suppressor by negatively regulating oncogenes.[90] However, the most common type of miRNAs with aberrant expression in cancer are downregulated and, therefore, are tumor suppressors.[91] In some cases, miRNAs can function as tumor suppressors or oncogenes depending on the type of tumor or the stage of the tumor progression. Costa-Pinheiro *et al*[92] transfected two different prostate cancer cell lines with either miR-375 or anti-miR-375. While in PC-3 cells, forced expression of miR-375 attenuated the malignant phenotypes, in 22Rv1 cells inhibition of miR-375 expression resulted in the same effect, suggesting a dual role for miR-375 in prostate carcinogenesis, acting as either an oncomiR or as a tumor suppressor, depending on the cellular context.

The mechanisms of miRNA dysregulation in cancer are still not figured out and differ from miRNA to miRNA. However it has been proposed that they can include chromosomal abnormalities when copy numbers and gene locations of the miRNAs are altered (by amplification, deletion or translocation), transcriptional control changes when the transcription factors that control miRNA's expression like c-MYC or p53 are dysregulated, epigenetic changes, including DNA hypomethylation, hypermethylation and disruption of the histone modification patterns, and defects in the miRNA biogenesis machinery.[93]

3.2. miRNAs in metastatic cancer

Metastasis, one of the hallmarks of cancer, is an intricate process by which cancer cells spread from a primary tumor to distant organs and tissues, forming viable secondary tumors. Recent studies showed not only that miRNAs have an active role in regulating metastasis by being up or downregulated and, consequently, by decreasing or enhancing the expression of target genes related with the metastatic process,[94] but also that there are specific patterns of miRNA's expression in different cancers and, within the same cancer, in different organs to each it metastasizes. Importantly, in 2008, *Rosenfeld et al*[95] established a set of signatures in primary cancers and metastatic tumors in 336 tumor samples by using a microarray technique. Interestingly, several miRNAs have been shown to be universally expressed in multiple different cancers, while some of them are only expressed or aberrantly expressed in primary or metastatic sites, indicating an organ and tumor type-dependent manner of miRNA expression. These findings suggest that miRNAs can constitute a good metastases biomarker.

Circulating miRNAs have been considered very good biomarkers for cancer and metastasis. Not only because of their previously mentioned unique features but also because different types of cancer have different and specific circulating miRNA signatures that may be useful for early cancer detection and its classification.[96-99] Cell-free miRNAs can be useful not only for cancer diagnosis but also for prognosis. In fact, Alhasan *et al*[100] demonstrated that in human patients, very high risk prostate cancer has a unique circulating miRNA signature, different from the low risk forms of the disease. This work was a proof-of principle that ECmiRNAs can identify different grades of prostate cancer. The same principle applies and has been described in other types of cancer, such as breast and lung cancer.[101,102] Furthermore, tumor cells often release higher numbers of microvesicles than other cells, indeed, cancer patients present a high quantity of serum exosomes, when compared to healthy individuals.[103] Accumulating evidence supports that horizontal transfer of exosomal factors, including miRNAs can functionally influence stromal cells at distant sites, thereby facilitating tumor-stroma interactions and promoting the formation of a supporting metastatic niche in distant organs.[104] It has been proposed that the cell free miRNAs are initially produced and secreted by the primary tumor and reflect its level of expression of each miRNA. After the exosomes reach the target cells, these can uptake them via endocytosis and also start producing the miRNAs on their own, by mechanisms are still quite unknown.[105] As tumor-secreted miRNAs can reach distant sites and regulate various cellular components of the pre-metastatic and

metastatic microenvironments, they might be of particular importance in understanding, detecting and targeting metastatic progression. Additionally, the presence of miRNAs that are associated with the process of metastasis by regulating one or several of its steps might identify those patients that already have distant micrometastases that are too small to diagnose otherwise. Hence, cell-free miRNAs might be valuable as prognostic markers and targets for therapeutic intervention for specific types of metastasis.

4. MiRNAs in Breast Cancer

4.1. Breast Cancer

Breast cancer (BC) is the most frequently diagnosed cancer in women worldwide, and only in 2012 almost 1.7 million new cases were registered and 521,900 deaths occurred and it represents about 12% of all new cancer cases and 25% of cancers in women, being the fifth most common cause of death from cancer in women, according to the International Agency for Research on Cancer.[106,107] It is a malignant type of tumor that initiates in the epithelial cells of the mammary duct.[108] Currently available treatments for early stage BC involve either a mastectomy or a lumpectomy, which are, respectively, a complete removal of the breast or a removal of only the tumor and some of the normal surrounding tissue, followed or not by radiation therapy. Adjuvant treatments like chemotherapy or hormonal therapy are also used.[109] Although these approaches prevent the recurrence of BC , if the maligancy is not early diagnosed, it becomes more aggressive and there is a high chance of relapse in distant organs.[110] In fact, metastatic BC is often incurable, with up to 5% of patients presenting distal metastasis at time of diagnosis and up to 15% within the first 3 years.[111,112] Additionally, growing evidence suggests that depending on the histopathological and biological features, BC exhibits distinct behaviors that result in different responses to treatment, pointing to the relevance of personalized therapeutic strategies.[113] Thus, besides the classical classification that stratifies tumors into four stages according to tumor size, regional nodal involvement and distant metastasis, other types of classification that consider non-anatomical characteristics like biomarkers have been widely used.[110] Nowadays, the most used classification, subdivides breast tumors according to receptors expression, namely, human epidermal growth factor receptor 2 (HER2), progesterone receptor and estrogen receptor (ER). This way, four subtypes are considered $ER^+/HER2^-$, $ER^+/HER2^+$, $ER^-/HER2^+$, and triple negative, which does not express any of the aforementioned receptors.[114,115] Triple negative BC accounts for approximately 15-20% of all BCs and is

usually associated with the worst prognosis, due to its high rate of relapse, tendency to metastasize to visceral organs and current lack of targeted therapies.[116,117] Since miRNAs aberrant expression in BC was first described in 2005,[86] both tissue and circulating miRNAs have been widely proposed for this role and several miRNAs profiling studies have led to the identification of miRNAs that are deregulated during the several stages of BC metastasis. Such studies have demonstrated the potential of miRNAs as biomarkers not only for diagnosis but also for prognosis and tumor identification of BC.[118,119] Accordingly, it has been demonstrated that restoring the expression of certain miRNAs that are usually downregulated in BC models can suppress metastasis *in vivo*.[120,121]

4.2. miRNAs throughout the metastatic cascade in breast cancer

In order to spread from the breast to different organs, BC cells(BCCs) need to undergo a series of steps, commonly named as the metastatic cascade.[110,122] This multi-step process comprises: (1) local infiltration of malignant cells into the surrounding tissue; (2) intravasion which is the transendothelial migration (TEM) of BCC into vessels to reach the circulation; (3) circulating and surviving in the blood stream, (4) arrest and extravasation to the target organ and (5) proliferation and colonization of competent organs.[122,123] In this section, we dissect the current knowledge about the several steps of the metastatic process and the evidences pointing to miRNAs involvement, which are summarized in Figure 3.

4.2.1. Local infiltration of malignant cells into the surrounding tissue

Under normal conditions, architecture of the mammary epithelium is ensured by cell-cell and cell-basement membrane (BM) interactions. Cell-cell interactions are established through intercellular junctions, including tight junctions (TJ), adherens junctions (AJ).[110] During transformation of normal epithelial cells into BCC cell-cell and cell-BM adhesion are disrupted allowing tumor cells detachment from and invasion of the surrounding tissue. These processes simultaneously require cell plasticity, increased motility, and ability to remodel the extracellular matrix (ECM).[124] This is partially achieved by the epithelial-mesenchymal transition (EMT), a process characterized by a phenotypical change from typical cuboidal to an elongated spindle shape, with loss of cell-cell and cell-BM adhesion and gain of migratory capacity.[125,126] During EMT, E-Cadherin, a trans-membrane glycoprotein that forms the core of the AJ between adjacent epithelial cells that maintain cell-cell adhesion and considered an epithelial marker, is downregulated.[127,128] This happens concomitantly with the loss of other epithelial markers

and the increased expression of mesenchymal markers, including N-cadherin, vimentin, smooth-muscle actin and cadherin-11.[129] The switch between E-cadherin and N-cadherin has been widely used to monitor the progress of EMT and besides increasing BCCs motility and invasiveness.[126] While tumor cells become more motile, due to the described alterations, increased migratory and invasive potential is also facilitated by changes in the ECM surrounding tumor cells.[130]

During metastatic initiation, miRNAs can be involved in the detachment and local invasion by BCCs. miR-373 and miR-520c promote detachment of BCCs by mediating loss of cell-ECM interactions by downregulating CD44.[131] CD44 is a cell surface receptor for hyaluronan, one of the major components of the ECM.[132] miR-21 also promotes the remodeling of the ECM by inhibiting TIMP3, which is a tissue inhibitor of MMP3. In that way, MMP3 is secreted in larger quantities by BCCs and degrades the ECM, facilitating local invasion and migration of malignant cells.[133] Accordingly, high expression of miR-21 had been considered a risk factor and indicator of bad prognosis in BC patients.[134] miR-10b has also been associated with BC invasion and metastasis initiation. The high expression of miR-10b in metastatic BC is induced by the transcription factor Twist, which binds directly to the putative promoter of mir-10b and leads to the inhibition of translation of the messenger RNA encoding homeobox D10, resulting in increased expression of a well-characterized pro-metastatic gene, *RHOC*. Thus, Twist-mediated miR-10b upregulation induces local invasion and migration of BCCs.[135] By its turn, miR-9, which is upregulated in BCCs, promotes loss of cell-cell interactions by targeting CDH1, the gene that encodes for the epithelial cell adhesion molecule E-cadherin. The oncoproteins MYC and MYCN are responsible for the upstream regulation of miR-9 by acting on the mir-9-3 locus, causing activation of miR-9 expression in tumor cells.[136] By downregulating E-cadherin, miR-9 can also be involved in the regulation of EMT.

Besides miR-9, nowadays numerous miRNAs have been described to regulate EMT.[137] Particularly in BC, the miR-200 family (miR-141, miR-429, miR-200a, miR-200b and miR-200c) has shown to have a relevant role in this process. The miR-200 family promotes epithelial state of cells by downregulating ZEB1/ZEB2 epithelial gene transcriptional repressors.[138] Both ZEB1 and ZEB2 are known to induce EMT by strongly suppressing the expression of E-cadherin, while ZEB2 has been shown to directly activate vimentin.[139] Thus, miR-200 family members have shown to be powerful regulators of EMT, by being highly expressed in epithelial cells and downregulated in cells with

mesenchymal phenotype.[140] Several other miRNAs, including, miR-155, miR-10b, miR-21 and miR-125b have been shown to act as promoters or repressors of EMT in BC, through diverse mechanisms and signaling pathways. Besides affecting BC's invasion, these miRNAs can be determinant to the patient's prognosis, according to their level of expression.[141]

4.2.2. Intravasation

Once malignant cells undergo EMT and are able to detach from the primary tumor and invade the surrounding tissue, to reach distant organs, the cells need to enter the circulatory or lymphatic system in a process named intravasation.[142,143] Whether tumor cells undergo hematogenous or lymphatic intravasation is dependent on a number of factors; however, the hematogenous route is the most common one, mainly because of the higher accessibility of blood vessels, since tumor angiogenesis creates a network of microvasculature that is accessible to malignant cells.[144] Similarly to epithelial cells, the endothelial cells that line the blood vessels are also connected by cell-cell junctions, namely, TJ.[145] Thus, for intravasation to occur, BCCs must induce molecular and cellular changes to overcome these blockades and cross the endothelial barrier. Although some studies showed that TEM can occur by a transcellular route, in which the BCCs transmigrate through individual cells, the preferred route for TEM seems to be the paracellular route, in which BCCs transmigrate through interendothelial junctions, by disrupting their integrity.[146,147] In BC, miR-105 has a relevant role in this disruption, as Zhou *et al*[148] demonstrated that in endothelial monolayers, exosome-mediated transfer of cancer-secreted miR-105 efficiently destroys TJ and the integrity of vascular endothelial barrier, thus promoting metastasis. miR-105 directly targets zonula occludens-1 (ZO-1), one of the integrating proteins of TJ.[149] Accordingly, overexpressing miR-105 in non-metastatic BC cell lines, induces metastasis and vascular permeability in distant organs.[148] Furthermore, the expression of the miR-520c/miR-373 family negatively correlates with lymph node metastasis of BC.[150] This family of miRNAs, particularly miR-520c and miR-373 inhibit *in vivo* intravasation of BC by directly suppressing *TGFBR2* and *RELA*, leading to a downregulation of transforming growth factor β (TGF-β) and NF-κB, respectively. TGF-β reduction leads to a decrease in angiopoietin-like 4, a protein known to disrupt vascular integrity through targeting vascular endothelial cadherin and claudin-5, well known components of AJ and TJ, respectively.[131,150]

Another molecule that plays a pivotal role in intravasation of BC is the vascular endothelial growth factor (VEGF), a powerful angiogenic factor. Thus, the several

members of the VEGF family (VEGF-A, -B, -C, -D and -E and placental growth factor) and its receptors promote metastasis and intravasation, through augmenting tumor microvasculature availability.[144,151] Moreover, VEGF-A is released by tumor associated macrophages to disrupt cell-cell interactions, mainly TJ, thus increasing vascular permeability.[152,153] Lu *et al*[154] showed that besides being downregulated in BC tissues overexpressing miR-140-5p, both in *in vitro* and *in vivo* models of BC directly target VEGF-A. This is simultaneous with an inhibition of angiogenesis and metastatic potential, as well as a decrease in MMP9 expression, indicating that miR-140-5p works as a tumor suppressor in BC and prevents intravasation. In turn, miR-9, a well-known oncomiR, was shown to be highly expressed in cell lines. This overexpression is induced by MYCN transcription factor and leads to the direct inhibition of E-cadherin. miR-9-mediated E-cadherin downregulation results in the translocation of β-catenin to the nucleus, where it promotes the transcription of the gene encoding for VEGF-A; this leads, in turn, to increased tumor angiogenesis.[136] Such results suggest a dual role of miR-9 by inducing EMT and facilitating intravasation. Other miRNAs have been suggested to modulate endothelial cells activities and be involved in BC angiogenesis, including miR-216a, miR-330, miR-608, miR-10b, miR-196b, miR-27a and miR-19.[155]

4.2.3. Survival in Circulation/ Circulation

BC metastatic cells that succeed in intravasation, become circulating tumor cells (CTCs) either via the blood or lymphatic circulation. Throughout their path, CTCs can encounter many obstacles, including sheer forces of the circulation, collision with host cells and attack of the immune system. All these factors influence CTC's survival and limit their ability to establish metastasis in distant sites. In fact, millions of metastatic cells are shedded by the tumor to the bloodstream, but only a small percentage reaches the target and very few clinically relevant metastases are formed, compared to the number of cells released by the tumor. Thus, it is expected that a selection of the most resistant and aggressive tumor cells, occurs.[156,157] Although there is a lack of studies describing the role of miRNAs in the survival in circulation of CTCs from BC, it is predictable that miRNAs that have an active role in proliferation, apoptosis or resistance to anoikis and in the regulation of the immune system, which are all factors that are relevant in the survival of BCCs in the blood stream. The first and most relevant immune response against the CTCs is played by natural killers (NKs), despite the fact that tumor development is accompanied by a disfunction and reduction of cytotoxicity by NKs,

induced by BC CTCs.[158,159] Breunig *et al*[160] have previously demonstrated that besides conferring resistance of BCCs by targeting TRAIL, FasL and granzyme B, miR-519a-3p can efficiently impair tumor cell killing by NKs. The authors further showed that this is achieved via the downregulation of UL16-binding protein 2 (ULBP2) and MHC class I chain-related protein A (MICA) by miR-519a-3p in the surface of tumor cells. ULBP2 and MICA are natural killer group 2 member D (NKG2D) ligands and are crucial for recognition of BCCs by NK cells. Accordingly, high levels of miR-519a-3p are associated with poor survival of BC patients, possibly by increasing the survival of CTCs in circulation.[161] MiRNAs belonging to the miR-17-92 cluster, especially miR-20a, have also shown to decrease the expression of MICA and ULBP2 by targeting the MICA 3'-UTR and by inhibiting the MAPK/ERK signaling pathway, respectively. Likewise, the silencing of NKG2DL-targeting miRNAs, including miR-20a in BCCs increased NK cell-mediated cytotoxicity *in vitro* and inhibited immune escape *in vivo*.[162] Another important feature that malignant cells must acquire to survive within blood and lymphatic circulation is the resistance to anoikis, a particular form of cell death occurring by loss of correct interactions between epithelial cells and ECM, thus preventing the colonization of cells outside their usual anatomic location.[163] Interestingly, an increased sensitivity of BCCs to anoikis is determined by one of the members of the miR-200 family, miR200c, which is usually downregulated in BCCs with a mesenchymal phenotype. Neurotrophic tyrosine receptor kinase type 2 was suggested as the direct target that mediates this effect.[164] Contrarily, Yu *et al*[165] have shown that a member of the same family, mir-200a, promotes resistance to anoikis and, consequently, lymph node and distant metastasis in BCCs. The proposed mechanism is through direct targeting of the 3'-UTR of the gene that encodes for Yes-associated protein 1 (YAP1) by miR-200a. YAP1 is a key node for Hippo signaling pathway that had been previously described as a tumor suppressor in BC and as its inhibition as a protective mechanism for anoikis in an *in vitro* model of BC.[166] Altogether these findings suggest that the family of miR-200, that is highly associated with EMT in BC and the promotion of distant metastasis,[140,167] can also have a relevant role in the survival in circulation of CTCs through the regulation BCCs resistance to anoikis.

4.2.4. Extravasation

The cells that survive the hostile intravascular environment, then need to cross the vascular endothelium to extravasate into the surrounding tissue. The extravasation

process involves adhesion to the endothelium, modulation of the endothelial barrier and, finally, TEM to reach the surrounding tissues.[168] Once the cells have reached the tissues, they can start proliferating again to form new solid tumors.[169,170] Extravasation of BCCs, usually takes place in the microvasculature near the target site, in a process similar to the mechanism proposed for leucocyte extravasation during inflammatory response, from the luminal to the abluminal side of the endothelium.[110,171] Similarly to intravasation, the preferred and most well studied route for cancer cells to transmigrate through the endothelial barrier seems to be the paracellular route.[172,173] The adhesion and arrest of BCCs at the endothelium is a crucial step in the extravasation, which is mediated by the interaction of numerous ligands and receptors, including selectins, cadherins, integrins, the cells surface adhesion receptor CD44, and immunoglobulin superfamily receptors but also, chemokine receptors like CXCR-4 and CXCR-7.[174,175] miR-19b is a member of the miR-17-92 cluster is as a key oncomiR in BC by regulating the PI3K/Akt pathway and leading to the downregulation of several tumor suppressor genes, including PTEN.[176] Recently, miR-19b was found to be upregulated during BC metastasis. This event was directly related with the downregulation miR19b's target, myosin regulatory light chain interacting protein, which was found to be an upstream event for the downregulation of E-cadherin and upregulation of ICAM-1 and integrinβ1, all molecules involved in the adhesion process. This study suggests a potential role for miR-19b in adhesion of BCCs to the endothelium, during extravasation.[177] As previously mentioned, CD44 expression strongly correlates with cancer cell adhesion to endothelial cells and with cancer metastasis. Specific glycosylated forms of CD44 present in BCCs binds to the vascular adhesion molecule E-selectin present in endothelial cells, promoting adhesion and TEM of $ER^-/CD44^+$ BCCs, as demonstrated by Kang *et al*.[178] Recently, miR-143 was shown to inhibit tumor progression of BC metastatic cells, both *in vivo* and *in vitro* by directly targeting CD44. Accordingly, miR-143 was subsequently shown to work a tumor suppressor, through its interaction with CD44, despite the role of CD44 in the maintenance of cancer stem cells properties.[179] Regarding miR-302a, it was found to be downregulated in highly metastatic BCCs both *in vitro* and *in vivo* and its upregulation inhibited BC metastasis. Although no concrete mechanism was proposed, this tumor suppressor activity of miR-302a was correlated with the downregulation of CXCR4.[180] CXCR4 is known to be involved in extravasation of metastatic BCCs to their target organs through the binding of its ligand CXCL12, which is produced by endothelial cells of the microvasculature and stroma of certain target organs, including lung, liver and brain, thus

having a chemoattracting role and promoting the adhesion of BCCs to the endothelium.[181,182] The CXCR4/CXCL12 axis has also been implicated in vascular permeability, endothelial cell patterning and morphology and TEM of BCCs.[183-185] miR-105 not only destroys endothelial barriers in primary sites but when secreted by BCCs can also promote extravasation by downregulating ZO-1 and consequently, disrupting TJ in secondary sites. The disruption of TJ facilitates the TEM of BCCs, through paracellular route and consequently extravasation. In fact, the overexpression of miR-105 in non-metastatic BCCs augments vascular permeability and distant metastasis, while the inhibition of the same miRNA in highly metastatic tumors lessens these effects.[148] Both miR-7 and miR-218 produced by BCCs were shown to inhibit the expression of another TJ protein, claudin-6, thus suggesting a possible role for miR-7 and miR-218 in promoting the modulation of endothelial barriers and the paracellular route of TEM in BC.[186,187] Still, for CTCs to transmigrate, they need to display a certain degree of deformability. In a triple negative model of BC this was shown to be directly correlated with the overexpression of transient receptor potential vanilloid subtype 4, a calcium permeable channel, capable of inducing reorganization of the actin cytoskeleton, and, consequently, reducing cell rigidity and promoting motility and extravasation of metastatic BCCs.[188] Some miRNAs can be involved in the remodeling of the actin cytoskeleton during extravasation. miR-31, which inhibits cancer cell detachment and invasion through repressing several metastasis-promoting genes (Fzd3, integrin α5, MMP16, radixin and RhoA) can also impair extravasation of GFP-labeled BCCs.[189] This ability was later on related with miR-31-mediated inhibition of WAVE3, an actin cytoskeleton remodeling protein that is highly expressed in advanced stages of BC and influences cancer cells motility, invasion and metastasis.[190] WAVE3 also has an established role in earlier stages of the metastatic cascade due to its regulation of EMT, where miR-200c is responsible for its regulation.[191] RhoGTPases, including RhoA, Rac1, and Cdc42 and ROCK are also needed for cancer cells to cross endothelial barriers. These are important regulators of actin and can modulate the assembly and disassembly of actin filaments. Their activation leads to stress fibers formation, lamellipodial protrusions, membrane ruffling and directed cell movement.[192,193] miR-146 is downregulated in metastatic BC cell lines and its upregulation resulted in decreased RhoA protein levels and in a consequent inhibition of cell migration and invasiveness.[194]

4.2.5. Colonization of the Target Organ

The last step of the metastatic cascade is the metastatic colonization and establishment of macroscopic secondary tumors at distant organs.[122,195] CTCs that can successfully cross endothelial barriers and infiltrate to the target organs are called disseminating tumor cells (DTCs). Although these cells were successful in entering a secondary organ, they still face some obstacles to adapt to a new microenvironment. Indeed, organ colonization is a key rate-limiting step of the metastatic process.[196] In fact, there is a period commonly referred to as metastatic dormancy that corresponds to the time of adaptation of the DTCs to the new microenvironment. During this period, DTCs remain trapped in the target organ but proliferation and apoptosis in micrometastatic lesions occur at similar rates or single-infiltrated cells are blocked in the G0 phase of cell division cycle to stay in a state of proliferative quiescence.[197,198] It has been proposed that for remaining in this state, DTCs are in a stemness state and have similar characteristics to adult stem cells that reside within the organs.[199] These cells regain the ability to proliferate upon a certain stimuli. Indeed, signals from the microenvironment influence the behavior of DTCs and determine whether they stay dormant or if proliferation pathways are activated for the cells to form macrometastases.[200,201]

MiRNAs have shown to have an important role in the switch between dormant and activated BCCs. Exosomes released by bone marrow stem cells induce dormant phenotypes of BC metastatic cells by releasing miR-23a, which suppresses the target gene MARCKS that encodes for a protein responsible for the promotion of cell cycling and motility. Thus, exosomal transfer of miRNAs can promote BCC dormancy in a metastatic niche.[202] A study by Gao *et al*[203], using an *in vivo* model of BC lung metastasization, also showed that miR-138 and miR-346 when overexpressed in BC DTCs regulate metastatic reactivation of breast DTCs, indicating that these miRNAs can promote exit from dormancy in the lung. However, no mechanism was proposed for this regulation. MiR-600 is capable of regulating BC stem cells fate, indicating a possible role for this miRNA in the dormancy of DTCs in metastatic sties. While the silencing o miR-600 results in BC stem cells phenotype, its overexpression reduces their proliferation and self-renewal by blocking Wnt signaling.[204] Aberrant Wnt signaling is observed in many types of cancer and has been implicated in triple negative BC tumorigenesis and metastasis. Indeed, triple negative BC patients that display dysregulated Wnt signaling are more prone to develop lung and brain metastases.[205]

Once DTCs have adapted to the microenvironment and established an accommodating metastatic niche they start proliferating again to colonize and invade the secondary sites. In this step of the metastatic cascade, the production of MMPs and cathepsins is essential to promote the rearrangement of the ECM and drive cell invasion and migration through the stroma.[206,207] To support metastatic growth, several growth and survival pathways are activated, including PI3K/Akt, MAPK, Notch and Wnt signaling pathways.[208,209] Finally, to complete colonization of the target organ, BCCs must reacquire an epithelial phenotype, so they undergo the opposite process of EMT, known as mesenchymal-epithelial transition (MET). Comparing to EMT, MET is far less investigated, although it substantially for the colonization of secondary organs.[210] During this process, BCCs regain the expression of epithelial markers like E-cadherin and downregulate the expression of mesenchymal markers like N-cadherin and vimentin, thus switching from an EMT/ non-differentiated state, characterized by proliferative quiescence, to a MET/differentiated state, characterized by active proliferation.[199,211]

As in all stages of the metastatic cascade, miRNAs can have an active role in promoting or inhibiting the colonization of distant organs. miR-335 is a well-known tumor suppressor that is usually deleted in BC. miR-335 was found to inhibit migration, invasion and metastatic colonization of BCCs without diminishing the overall growth of the tumor by targeting the progenitor transcription factor Sox4 but also Tenascin-C,[120] a component of the ECM that can be produced by BCCs in the lung and promotes survival and outgrowth of metastasis by activating pro-tumorigenic pathways, including Notch and Wnt signaling.[212] Furthermore, it was described that the downregulation of miR-335 in metastatic BC is epigenetically regulated, particularly by the hypermethylation of a specific CpG island upstream of the transcriptional site of miR-335 promoter.[213] miR-182 is downregulated in earlier stages of BC metastasis due to its role as a suppressor of EMT as it is directly repressed by Snail to promote EMT, however, in more advanced stages of metastasis development, miR-182 is upregulated and inhibits Snail to reestablish epithelial identity of BCCs for colonization and macrometastases formation in the lung, indicating that miR-182 can have a dual role during BC metastasis, through a dynamic reciprocal suppression with Snail.[214] The miR-200 family members have relevant roles along several steps of the metastatic cascade in BC, as previously mentioned. During colonization of BC in secondary sites, miR-200c is overexpressed and promotes metastatic colonization by directly targeting Sec23a, which mediates secretion of metastasis suppressive proteins, including insulin-like growth factor-binding protein and

Tinagl1. Moreover, miR-200s family members also promote BC metastatic colonization by inducing MET, through the targeting of ZEB-1 and ZEB-2 that are repressors for the epithelial marker E-cadherin.[140,215] Altogether, these findings indicate that miRNAs are involved in the development of well-established metastasis. However, when the secondary tumor reaches a certain dimension, nutrients and oxygen become scarce. To overcome this problem, new blood vessels are originated through the sprouting of preexisting vessels surrounding the tumor and miRNAs can also be involved in the regulation of angiogenesis in cancer.[216]

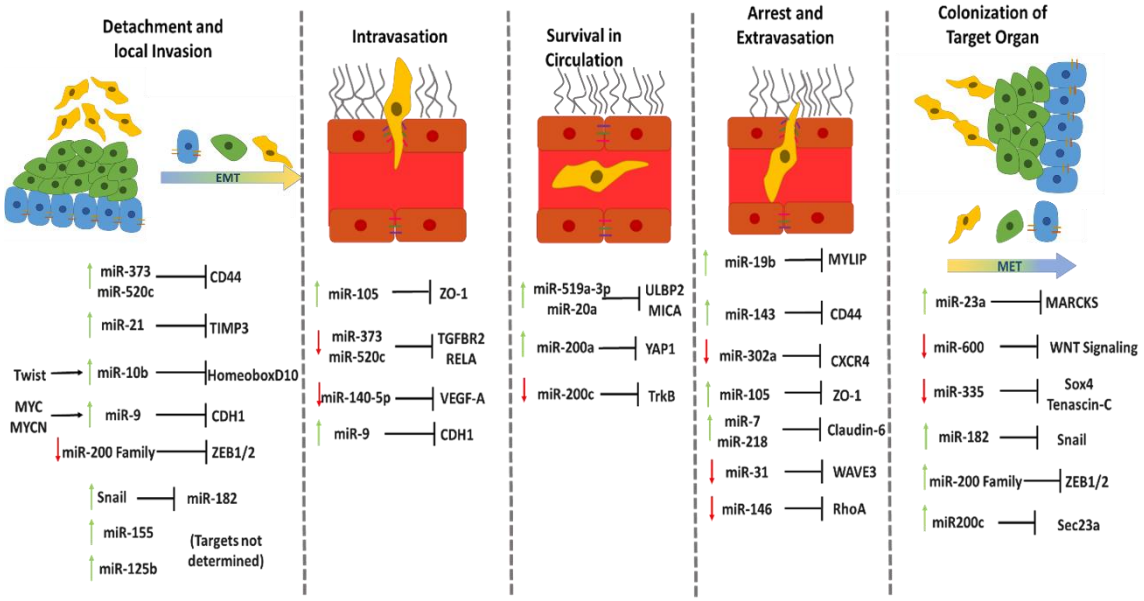

Figure 3 - Summary scheme of the miRNAs involved the metastatic cascade in breast cancer. miRNAs depicted in this article have been studied as regulators of the several steps of the metastatic cascade in breast cancer. The microRNAs that work as oncogenes are usually upregulated (green arrows), while the miRNAs that work as tumor suppressors are usually downregulated (green arrows). The studied mechanisms of the miRNAs usually relate them with their downstream targets, though some authors have studied miRNAs upstream regulators.

5. MiRNAs in Breast Cancer Brain Metastasis

5.1. Breast Cancer Brain Metastases

The development of high-resolution imaging techniques in the past few years has allowed an early detection of primary tumors, increasing the survival of cancer patients.[217,218] Nevertheless, the appearing of metastasis considerably worsens the prognosis of the patients and with estimates that metastases are still responsible for 90% of human cancer deaths.[219] Brain metastases have been associated with the worst prognosis since there is still no cure or effective treatment. Although studies reveal that surgery can improve life expectancy of patients with brain metastasis, it has been reported

that the median overall survival of patients after brain metastases diagnosis is approximately 1.33 years.[220,221] It is predicted that 10-16% of patients with stage IV BC will develop brain metastases, plus, an additional 10% of asymptomatic patients, as revealed by autopsy studies, being the second most frequent cause of brain metastases, next to lung cancer and followed by melanoma.[222,223] Importantly, given the fact that BC has a much higher incidence than lung cancer,[107] brain metastases from BC assume a greater relevance than brain metastases from lung cancer. The consequences of this type of metastasis are usually devastating, and have been associated with the worse survival and quality of life.[224,225] The medium survival after diagnosis of brain metastasis is different from study to study but ranges from 2 to 16 months, depending on the involvement of the central nervous system, the extent of the extra-cranial metastatic disease, and the treatment applied.[226] Current treatment for brain metastasis includes open surgical resection, gammaknife or cyberknife stereotactic radiosurgery, focused external beam radiotherapy, whole-brain radiotherapy, traditional chemotherapy, and newer targeted biological agents personalized for tumor type, although none of them has shown to be completely effective and only help to diminish symptoms and prolong the patients' life expectancy.[227,228] The most common symptoms of brain metastases comprise constant headache, seizures, motor weakness, ataxia, altered mental status and dysphasia which drastically impair the quality of life of both patients and their families.[229,230] It is also worth noticing that there are several risk factors that increase a BC patient's probability to develop brain metastasis, including younger age, ductal histology, hormone-receptor negativity, increased tumor size, nodal metastasis, lung and liver metastasis, overexpression of HER2, higher tumor grade, increased lactate dehydrogenase level, overexpression of epidermal growth factor receptor (EGFR), and mutations in the *BRCA1* gene. Among the several types of BC, HER2-positive and mainly triple negative tumors, are the ones presenting the highest predisposition to brain metastasis and are thus considered the most aggressive types and the ones with the lowest survival rates, as aforementioned.[225,231]

Despite its clinical importance, the molecular mechanism of brain metastasis is still poorly understood. Brain metastasization is a complex process, that comprises all the steps of the metastatic cascade plus the colonization and growth in the brain parenchyma, which takes, averagely, 32 months from the initial cancer diagnosis.[221] Thus, BC brain metastases (BCBM) are considered a late event because cancer cells, have to develop the ability to penetrate through the blood-brain barrier (BBB) and colonize the brain.[225] This

contributes to the poor efficiency of treatments targeting brain metastasis because most patients have already received several rounds of chemotherapy before its detection, which allows cancer cells to accumulate enough mutations to become resistant to new approaches.[218,232]

Once BCCs enter into the brain, they encounter an ideal microenvironment for metastatic growth, since the BBB provides protection against immune surveillance, chemotherapeutic agents and other harmful substances.[233] The anatomic basis of the BBB consists of brain microvascular endothelial cells that, not only form elaborate TJ but also use active efflux transport mechanisms that restrict the entrance of molecules into the brain,[234] thus blocking chemodrugs from reaching metastatic sites. Malignant cells can cross the BBB by the paracellular pathway with disruption of TJ, whereas the transcellular pathways remains to be well established.[110,235] Still, it is well accepted that the presence of brain lesions compromise the BBB, structurally and functionally, increasing its permeability.[236,237] In a genomewide comparative study, several proteins have been proposed as mediators of the TEM of malignant BCCs through the BBB, including the EGFR ligand HBEGF, cyclooxygenase-2 (COX2), and α-2,6-sialyltransferase 5. The latter is the most specific one for the BBB, since EGFR ligands and COX2 had been previously related to the infiltration of BCCs to the lung.[238-240] Besides, other proteins that are commonly involved in extravasation have shown to have a relevant role in the interactions between malignant cells from BC with the brain endothelium, including TJ proteins, selectins, integrins, cadherins, RhoGTPases and VEGF.[241] Another important factor for brain metastasis development is that although the brain microenvironment can be very hostile and kill most of the metastatic cells in a first response,[169] the few cells that survive can benefit from the shielding and the cells present in the brain microenvironment can switch to a supporting role. Indeed, there is a crosstalk between malignant cells and brain cells and the brain becomes a sanctuary against anti-tumor strategies.[242,243] In the brain, this tumor microenvironment consists of a complex network of interactions between a large variety of tumor-associated stromal cells, including, astrocytes, endothelial cells, and infiltrating inflammatory cells like microglia.[244] Astrocytes are the first brain cells to encounter extravasated malignant cells and the main determinants of their fate. Indeed, active astrocytes are found in close proximity to BCCs even before the extravasation process is finished.[245] Astrocytes have been shown to contribute to brain metastases from BC by producing factors, including ERK1/2 and TIMP2 that activate MAPK signaling pathways in malignant cells. The overactivation of MAPK leads to the

increased expression of MMP2 by tumor cells.[246] A different study showed that metastatic BCCs release interleukin-1β (IL-1β) which activates the surrounding astrocytes. The activation by IL-1β augmented the production of JAG1 which stimulated notch signaling by BC stem cells, promoting their self-renewal.[247] . In contrast, it was reported that plasmin from the reactive brain stroma can be toxic for malignant cells by converting membrane-bound astrocytic FasL into a paracrine death signal for breast and lung cancer cells. The metastatic cells fight the production of plasmin by expressing high levels of anti-PA serpins, including neuroserpin and serpin B2.[248] Altogether, these findings reinforce the idea of a crosstalk between brain cells, namely astrocytes and malignant cells to support metastatic growth.

Microglia, along with astrocytes, are the glial cell types most associated with brain metastasis.[249] Microglia are the resident immune cells of the central nervous system and are non-proliferative in normal adult brain, but upon injury can be rapidly activated.[250] When in contact with tumor cells, microglia cells are known to secrete a multitude of factors that can modulate the tumor microenvironment and enhance the colonization of tumor cells.[251] Among the secretome of microglia, there are mRNAs and miRNAs that can regulate the expression of genes in other cells, including tumor cells.[252] The exchange of released factors between microglia and tumor cells activate multiple key signaling pathways. Pukrop *et al*[253] showed that activated microglia can successfully promote colonization of tumor cells from BC and identified Wnt signaling as one of the pathways that need to be active during microglia-induced invasion. In fact, microglial activation-promoting brain metastasis often depends on activation of Wnt signaling and treatment with a Wnt antagonist highly decreases microglia-induced tumor invasion. Recently, it has also been reported that metastatic BCCs have a high expression of neurotrophin-3 which has a dual function of regulating the metastatic growth of metastasizing BCCs and of reducing the activation of immune response in the brain, by decreasing the number of fully activated cytotoxic microglia.[254] Besides astrocytes and microglia, neurons can also be involved in supporting brain metastases from BC. Although this has not been fully disclosed yet, it was recently found that malignant cells from BC have a GABAergic phenotype similar to that of neurons that confers a proliferative advantage to BCCs, indicating a metastasis-promoting effect of coinhabitation of the neuronal niche in the brain.[255] There is much less information about the role of pericytes in supporting brain metastases from BC, however it has been proposed that during BCBM progression, there are different subpopulations of pericytes that can regulate the permeability of the BBB.

The subpopulations are distinguished by the presence of certain proteins like desmin and CD13. [256]

5.2. miRNAs involved in metastasizing breast cancer to the brain

Despite the difficulties in the treatment of brain metastasis, an early diagnosis increases the chances of survival. However, the resolution of current MRI techniques and the contrast agents currently used do not allow an efficient detection of small tumors or micrometastasis, which could be targeted by first-line treatment more efficiently than macrometastasis.[257] So, the finding of specific biomarkers that help the prognosis and early diagnosis of BCBM is in order. As previously mentioned, miRNAs may serve as effective new biomarkers in predicting cancer progression, given the fact that metastatic cancer cell express specific miRNAs. Due to the high incidence, poor prognosis and devastating consequences of BCBM, the use of miRNAs as biomarkers is currently being studied and is of high clinical interest. Several different miRNAs were already associated with BCBM, as outlined in Table 3. Since this is a recent subject, few studies have been performed and the downstream targets of the microRNAs and the mechanisms by which they act are still uncertain and up to discussion. Therefore, in this review, we will focus only in a few miRNAs for which there is more information available and that are summarized in .

Table 1 - miRNAs associated with breast cancer brain metastasis and their expression in cancer cells

miRNA	Type of study	Cell lines	Expression in brain metastases vs. primary tumor	Expression in metastatic tumors vs. non-metastatic tumors	Putative targets	Ref.
miR-7	*In vitro* And *in vivo*	MDA-MB-231 and ER+ MCF-7	Downregulated	---	KLF4	[258]
miR-10b	*In vitro*	MDA-MB-231 and MDA-MB-468	Upregulated	Upregulated	HOXD10 and MICB	[259]
miR-19a	*In vitro* and *in vivo*	MDA-MB-231BR	---	Downregulated	Unknown	[260]
miR-20b	*In vitro* and *in vivo*	ER^+ MCF-7 and MDA-MB-231	Upregulated	Upregulated	PTEN	[261]
miR-29	*In vitro* and *in vivo*	MDA-MB-231BR	---	Downregulated	Unknown	[260]
miR-122	*In vitro* and *in vivo*	MDA-MB-231-HM	Upregulated	---	PKM	[262]
miR-141	*In vivo*	SUM149, MDA-MB-231- BR and MDA-IBC3	Upregulated	Upregulated	Unknown	[263]
miR-146a	*In vitro* and *in vivo*	MDA-MB-435	Downregulated	---	β-catenin and HnRNPC	[264]
miR-210	*In vitro* and *in vivo*	MDA-MB-231BR	---	Upregulated	Unknown	[260]
miR-509	*In vitro* and *in vivo*	MDA-MB-231 and ER+ MCF-7	Downregulated	Downregulated	RhoC and TNF-α	[265]
miR-524-5p	*In vitro* and *in vivo*	MCF-7 and MDA-MB-231	Downregulated	---	BRI3, ERK pathway	[266]
miR-181c	*In vitro and in vivo*	MDA-MB-231	Upregulated	---	Cofilin	[267]
miR-1258	*In vitro* and *in vivo*	231BR1 and 231BR3	Downregulated	Downregulated	Heparanase	[268]

It has been demonstrated that miR-7 is significantly downregulated in metastatic breast cancer stem-like cells (CSCs).[258] CSCs have been highly associated with tumor growth and metastasis initiation due to their ability of self-renewal and invasiveness, as well as the capacity to adapt themselves to different microenvironments. On the other hand, CSCs' properties like cell growth, cell cycle and self-renewal are highly regulated by miRNAs.[269] Besides confirming the role of CSCs in BCBM, it has also been demonstrated that mir-7 is specifically down regulated in highly metastatic CSCs to brain

and bone but not in CSC isolated from the primary breast tumor. This suggests that mir-7 has a specific function in metastatic cells that might be related to one of its downstream targets, KLF4, which is one of the genes responsible for maintaining stem cells properties[270] that is upregulated in metastatic CSCs in the brain but not the bone. The promotion of BCBM by miR-7 should be related to microenvironmental factors in the brain or to downstream targets of KLF-4, like TGF-β and Notch that are known to be involved in stem cell self-renewal and tumor progression.[258,269]

Mir-1258 had previously been identified as tumor suppressor in BC by down-regulating heparanase (HPSE), a mammalian endoglycosidase with tumorigenic, angiogenic and pro-metastatic activity, which is highly expressed in cancer cells with high propensity to colonize the brain.[268,271] *Zhang et al*[271] demonstrated that mir-1258 is downregulated in BC metastatic tissues in the brain and that the ectopic expression of this miRNA, due to its action on HPSE's expression and activity, results in the inhibition of cell invasion and onset of brain metastasis. indicating that mir-1258 is a suppressor of brain metastatic BC. There is a well-established notion that HPSE plays a critical role in BC progression, particularly in cell proliferation, angiogenesis, invasion and metastasis due to the degradation of heparan sulfate that allows the release of growth factors from the cell surface and ECM.[272] HPSE-mediated promotion of BCBM can be related with its downstream targets MMP-9, COX2 and EFGR, given the fact that mir-1258-mediated inhibition of HPSE resulted in decreased expression levels of these three proteins in a BCBM model. It is worth noticing that these proteins have been recently related with brain metastasis due to their important roles in the disruption of the BBB.[273-275] Although a treatment with mir-1258 reduces BCBM by inhibiting HPSE, there are cross-talk mechanisms between tumor and normal cells of the brain microenvironment, like the production of HPSE by astrocytes,[276] that cannot be inhibited by mir-1258 since this miRNA only targets the intracellular production of HPSE by BCCs.

MiR-509 has been described to be highly expressed in primary breast tumors whereas its expression is significantly decreased in brain metastatic lesions originated from the same tumors. Moreover, the levels of this miRNA are also decreased in primary breast tumors of patients with brain metastasis when compared with BC patients without this kind of metastatic lesions. This corroborates the potential of using miRNAs to early diagnose brain metastasis in BC. The downregulation of mir-509 directly relates with the up regulation of both RhoC and TNF-α, indicating that their expression is regulated by

mir-509.[265] RhoC is a well-studied oncogene that is known to enhance the migration and invasive ability of BC stem cells by activating several pathways, thus, impacting its metastatic potential and frequency.[277,278] *Xing et al*[265] suggested that mir-509 suppresses brain metastasis by decreasing RhoC expression and consequently attenuating the transmigration and invasive ability of cancer cells. In fact, RhoC induces the sequential activation of Pyk2, FAK, MAPK and Akt pathways[279] that lead to the activation of MMP-9. Further studies showed that mir-509 indirectly inhibits TNF-α, a cytokine that is also known to increase the permeability of the BBB.[280] Thus, it can be infered that mir-509 suppresses brain metastasis, not only by targeting RhoC but also by blocking TNF-α-induced BBB penetration.

Another miRNA related with BCBM worth mentioning is miR-181. In 2014, *Tominaga et al*[267] proposed that mir-181 has an active role in destructing the BBB in the process of brain metastasizing by the BC cell line (MDA-MB-231), by modulating actin dynamics. In this study, the authors showed that inhibition of secretion of extracellular vesicles suppressed the invasiveness of malignant cells through the BBB. They also showed that BCCs-derived extracellular vesicles promote brain metastasis *in vivo* by increasing the permeability of the BBB through the disruption of TJ. Curiously, the authors found out that the expression of the TJ proteins claudin-5, occludin, ZOs and junction adhesion molecules was not altered, suggesting that one of the mechanisms responsible for BBB disruption is the release of mir-181c by the extracellular vesicles. mir-181 downregulates 3-phosphoinositide protein kinase-1, which is its main target gene in endothelial cells and is an upstream protein of cofilin phosphorylation. Cofilin is a family of actin-binding proteins that dissemble cytoskeleton actin filaments upon activation by dephosphorylation.[281] Therefore, an increase of mir-181c is responsible for the activation of cofilin that leads to the reorganization and relocation of actin filaments. Since the TJ proteins are in deep association with the actin cytoskeletal network, the dissembling of the actin cytoskeleton induces the relocation of these proteins, leading to

the disruption of TJ and consequent, increase of BBB permeability that facilitates the metastasization process.[282]

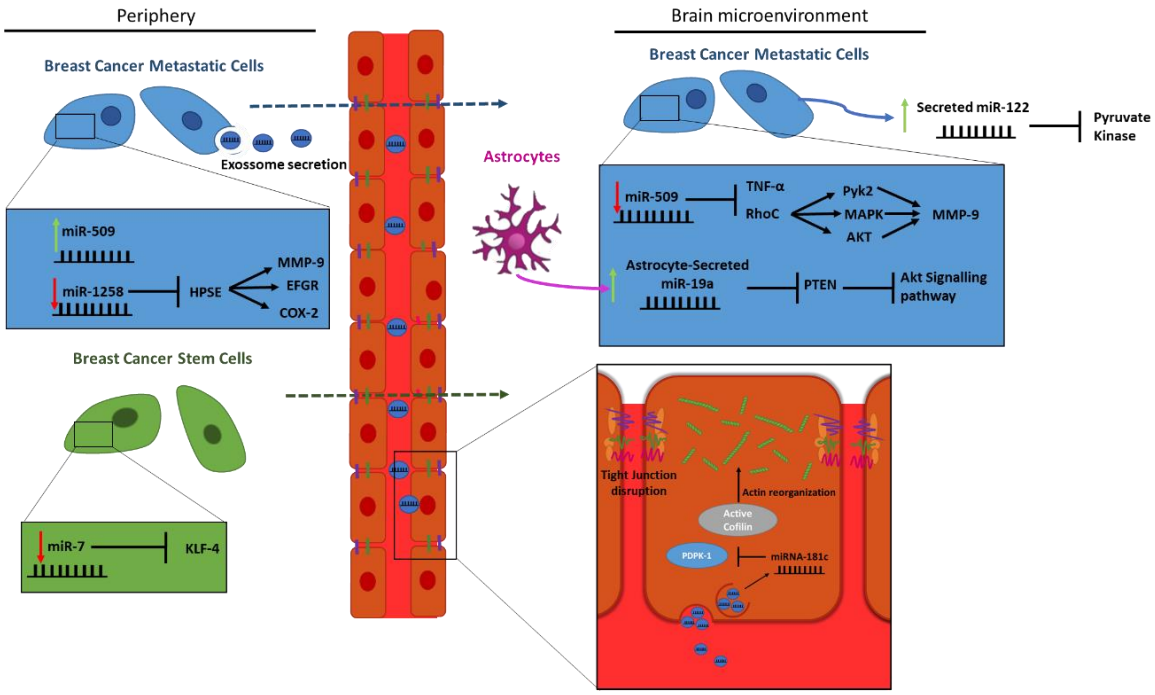

Figure 4 - miRNAs currently associated with breast cancer brain metastasis. Although there is not much information about the function of miRNAs in the metastasization process of breast cancer to the brain, changes in the expression of the miRNAs depicted in the picture have been correlated with this process, as well as their putative targets. Such changes were observed in different types of cells, including breast cancer cells prior or after metastasis have occurred, breast cancer stem cells, endothelial cells and cells in the brain microenvironment, namely astrocytes. While some miRNAs act intracellularly, other are released, either directly to the extracellular environment, or encapsulated by exosomes that can then be transported to further places. In the insets, the intracellular mechanisms are schematized. It is known that miR-122 acts in the pre-metastatic niche but the specific types of cells are not described, yet.

Another miRNA, miR-122, had been previously identified as a marker for predicting metastatic progression in early-stage BC by being highly expressed in BC patients' serum prior to metastasis.[283] More recently, it was demonstrated that miR-122 is also produced and secreted by BCCs to promote not only metastasis in the lung but also in the brain.[262] The authors found out that extracellular miR-122 downregulates the glycolytic enzyme pyruvate kinase in the pre-metastatic niche cells. This reduces glucose uptake by non-malignant cells and increases nutrient availability for cancer cells in the target organs.[262] Enhanced glucose uptake is a common feature in cancer due to the high energy demand in cancer cells and the low ATP-generating efficiency. Indeed, glycolytic enzymes have been shown to be upregulated in BC.[284,285] These findings suggest that circulating miR-122 can be a good biomarker for the early detection of BCBM and can modulate the microenvironment brain metastases from BC. Finally, another stroma-derived extracellular miRNA is capable of modulating BCBM microenvironment, namely, miR-19a by downregulating PTEN,[286] an important tumor suppressor that is frequently

deleted in triple-negative BCBM patients and associated with poor prognosis.[287] In cases of BCBM, PTEN reduces the activation of Akt signaling pathway and that has been shown to mediate the crosstalk between breast and glial cells in brain metastases leading to rapid disease progression.[251] As demonstrated by Zhang *et al*[286], both human and mouse BCCs loose PTEN expression when disseminating in the brain, but not in other organs, and its expression is restored after the cells leavethe microenvironment. The authors showed that the suppression of PTEN within the microenvironment is directly mediated by miR-19a, which is released by astrocytes. The decrease of PTEN led to an increase of chemokine C–C motif ligand 2 secretion and recruitment of myeloid cells that favor brain metastasis of BC. This study is a good example of how miRNAs can influence the crosstalk between malignant cells and brain cells to create a microenvironment that is supportive to BCBM growth.

6. Conclusion and Future directions

miRNAs have arisen as important posttranscriptional regulators of possibly all the genes present in the human genome and overwhelming amounts of data have recently linked aberrant miRNA expression to the origin and development of many, if not all types of cancer to which specific miRNAs signatures can be assigned. Moreover, some miRNAs have been specifically related to metastasization and particularly from BC to the brain. However, the role of miRNAs in the cellular mechanisms and intercellular communication underlying cancer development and metastasis progression remain mostly unmapped. Therefore, extending the current knowledge about miRNAs biology and biopathology will pave the way for the establishment of miRNAs as early biomarkers and as potential targets for modulation. Hopefully, the anticipated discoveries in the years to come will open new opportunities for a timely detection and therapeutic intervention, essential to improve the expectancies in the field of oncology and particularly of neuro-oncology.

7. Aims

Although cell-free miRNAs, have been proposed to work as efficient biomarkers for different types of metastasis, there are still very few studies that relate cell free miRNAs with the brain metastasization process from BC. So, this project aimed to: (1) understand the behavior of miRNAs, throughout metastases development; (2) establish a specific biosignature in plasma for BCBM by finding miRNAs with aberrant expression

that could work as reliable and specific biomarkers for BCBM, even before well-established metastases are detected; and (3) predict possible targets of such miRNAs, not only for a better understanding of the underlying mechanisms but also as a way to discover new targets for modulation and prevention/treatment of BCBM. To this end, we used female mice inoculated with 4T1 BCCs or vehicle (control), sacrificed at different phases of the metastatic cascade. Plasmas samples were collected to study miRNAs expression profiles, while brain samples were collected to study the development of BCBM and the expression of predicted miRNA targets.

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Chapter II

Circulating MiRNAs as Early Biomarkers of Breast Cancer Brain Metastasis and Potential Targets for Modulation

Abstract

Breast cancer brain metastasis (BCBM) are considered a serious oncologic problem due to their high incidence, poor outcome and lack of effective treatments. So, the finding of reliable biomarkers that allow the early detection of BCBM, as well as new targets for modulation, is in order. MicroRNAs (miRNAs), particularly circulating miRNAs, have recently arisen as powerful biomarkers for different types of cancer and metastasis, due to their stability, easy quantification in biological fluids and specific expression profiles. So, in this work, we proposed that circulating miRNAs could work as efficient biomarkers for the early detection of BCBM, and their targets as new potential targets for modulation. To test this hypothesis, we used an animal model of BCBM that consists in the inoculation of triple negative breast cancer cells (4T1) or vehicle (control), in female Balb/c mice and collected plasma samples and brains, lungs, livers and kidneys at different times after injection, and further analyzed a set of human resected brain metastasis of triple negative breast cancer patients. Analysis of brain and peripheral organs revealed that this model metastasis are particularly relevant in the brain, and mainly in the hippocampus. in but not to the other organs. Our results showed that miR-802-5p and miR-194-5p are downregulated in plasma, while there is a trend for miR-92a-1-5p upregulation, prior to metastases development. The expression of the transcription factor myocyte enhancer factor 2C (MEF2C), a predicted common target for both miR-802-5p and miR-194-5p, was studied in the brain along metastasis development, and the results revealed its increasingly expression by malignant cells, as well as by peritumoral astrocytes. Importantly, the expression of MEF2C was also detected in human BCBM, which validates the finding obtained in the mouse model. Altogether, our results point to plasma miR-802-5p, miR-194-5p and miR-92a-1-5p as precocious biomarkers for BCBM and to MEF2C as a new player and a potential target for modulation in BCBM.

1. Introduction

Breast cancer (BC) is the most frequently diagnosed cancer in women worldwide. It represents about 12% of all new cancer cases and 25% of cancers in women, being one of the leading causes of death from cancer in women, according to the International Agency for Research on Cancer.[1,2] BC has a high incidence and is the second most frequent cause of brain metastasis.[3] Brain metastasis are usually associated with a poor prognosis and the medium life expectancy is 1.33 years after diagnosis.[4] After

transmigrating to the brain, breast cancer cells (BCCs) encounter an ideal environment for metastatic growth, since the blood-brain barrier (BBB) restricts the entrance of chemotherapeutic agents, which renders the brain a sanctuary against anti-tumor strategies.[5] Moreover, the BBB provides protection against immune surveillance and the crosstalk between malignant and brain cells favors brain metastasis.[6,7] Altogether, this hinders the treatment of brain metastasis and none of the current therapies available is 100% effective. Thus, the finding of biomarkers that can help in the early detection of BC brain metastasis (BCBM) and improve chances of successful treatment is in order. MicroRNAs (miRNAs or miR) are a subclass of single stranded small noncoding RNAs with about 21-25 nucleotides long that are endogenously produced and found in diverse organisms, including humans. They play important gene-regulatory roles by pairing to the 3'-untranslated region (3'UTR) of mRNAs of protein-coding genes to direct their posttranscriptional repression.[8,9] Recently, miRNAs have arisen as efficient and specific biomarkers for different types of cancer and differential metastases, having specific expression profiles.[10] Particularly, cell free miRNAs can be of interest, due to their high stability and easy quantification in biofluids,[11] being key components of the so called "liquid biopsies", which are more and more replacing traditional biopsies for diagnosis and prognosis of cancer.[12]

In this study, we wanted to understand if circulating miRNAs are deregulated during BCBM progression and if there are specific miRNAs that are aberrantly expressed prior to brain metastases formation, which could work as potential predictive biomarkers for BCBM. The results presented here reveal that miRNAs are deregulated during metastatic progression and that there is a specific set of miRNAs that is aberrantly expressed prior to metastasis formation, pointing to the downregulation of miR-802-5p, miR194-5p and to the upregulation of miR-92a-1-5p as the most relevant changes, prior to detection of brain metastasis. Furthermore, by predicting targets of the aberrantly expressed miRNAs, we propose some proteins for modulation to prevent and/or treat BCBM. We give special attention to MEF2C, a predicted target for miR-802-5p and miR-194-5p, that is highly expressed in BCBM and presents a tumor supporting role.

2. Materials and Methods

2.1. Cell Culture and Animal Model for Breast Cancer Brain Metastases

The animal model used in all experiences is an animal model for BCBM, established by Doctor Istvan Krizbai and Doctor Imola Wilhelm, of the "Neurobiology and Neuropathology of the Blood-Brain Barrier of the Biophysics" group, from the Institute of Biophysics of the Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary. Samples were provided to Professor Alexandra Brito, of the "Neuron Glia Biology in Health & Disease" group, Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa under a collaborative project. This animal model relies in the use of murine mammary carcinoma triple negative 4T1 cells, purchased from ATCC (Middlesex, UK). This is a transplantable 6-thioguanine-resistant metastatic BCC line derived from the 410.4 tumor isolated from a single spontaneously arising mammary tumor of $MMTV^+$ Balb/c mouse.[13] 4T1 cells were inoculated in female Balb/c mice, purchased from Charles River Laboratories (Wilmington, MA, USA), and housed and bred in the animal facility of the Biological Research Centre of the Hungarian Academy of Sciences, Hungary. Animal experimentation was performed by certified team members at the Biological Research Centre of the Hungarian Academy of Sciences, according to the recommendations of the Declaration of Helsinki and Tokyo and were performed according to the EU Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes. The protocol was reviewed and approved by the Regional Animal Health and Food Control Station of Csongrád County (licence number: VI-I-001/2980-4/2012) of the Hungarian Academy of Sciences.

4T1 cells were maintained in RPMI 1640 medium (PAN Biotech, Aidenbach, Germany) supplemented with ultraglutamine I (Lonza, Basel, Switzerland) and 5% heat-inactivated fetal bovine serum (PAN Biotech) in a 5% CO_2 atmosphere at 37 ºC. These metastatic BCCs were xenografted in female Balb/c mice at the age of 7-8 weeks, by inoculating, under isoflurane anesthesia, 1 x 10^6 4T1 cells in the right common carotid artery in a total volume of 200 µL of Ringer-HEPES, whereas control mice were inoculated with vehicle. Plasma samples were collected (n=5) and brains were harvested (n=6), 5 hours, 3 days, 7 days or 10 days post-inoculation, as described below.

2.1.1. Plasma samples collection

Blood samples were collected for analysis of miRNAs expression in plasma by next generation sequencing (NGS) and further validation by real time polymerase chain reaction (RT-PCR). The blood samples were collected directly from the heart of alive mice under isoflurane anesthesia, which allowed a collection of approximately 500 µL of blood from each mouse. The blood samples were collected using syringes previously washed with ethylenediamine tetraacetic acid (EDTA, 0.5 M, pH 8.0) into tubes containing 40 µL of the anticoagulant. After collection, the blood samples were centrifuged for 10 minutes at 2000 RPM, at 4ºC, to remove red blood cells, white blood cells and platelets, and the plasma was collected. The plasma samples were stored at -80ºC until further analysis and shipped to the Rady Faculty of Health Sciences, University of Manitoba, Canada, for the NGS analysis, under a collaboration with Doctor Stephanie Booth.

2.1.2. Organs Collection

Anesthetized mice were perfused with 50 mL of phosphate-buffered saline (PBS), followed by 25 mL of 4% paraformaldehyde in PBS to fix the tissues. The brains, lungs, kidneys and livers were harvested and post-fixed overnight in 4% paraformaldehyde in PBS at 4ºC and afterwards were kept in PBS containing 0.1% sodium azide to prevent degradation of the tissues by microorganisms. Fixed organs were shipped to the Faculty of Pharmacy, University of Lisbon, Portugal, where the studies were performed. Brains, lungs, kidneys and livers were subjected to histological analysis of metastasis development in selected brain regions and in peripheral organs. Brain were further used for in-depth analysis of the expression of selected proteins along the metastatic process by immunofluorescence (IF) analysis.

2.2. Histology

The processing and paraffin embedding of the brains, lungs, kidneys and livers were performed at the Histology and Comparative Pathology Laboratory, headed by Doctor Tânia Carvalho, at the Institute of Molecular Medicine João Lobo Antunes. Paraffin embedded mouse organs were serially cut into 4-µm-thick sections, using a Manual Microtome (Leitz 1512 Rotary Microtome) and Microm SEC35 low profile blades purchased from ThemoFisher Scientific (Waltham, Massachussets, USA). Coronal cuts were performed to get three different regions, according to the following Bregma

coordinates: cerebellum, -6.12 mm; cranial hippocampus, -1.82 mm; and striatum, 0.5 mm. The brain slices were then mounted in microscope adhesive slides with frosted end (76 x 26 mm) bought from StarFrost® (Lowestoft, UK), and kept in the incubator at 60ºC for 1h to melt the paraffin and for the tissues to adhere to slide. The slides were stored at 4ºC until further usage.

2.2.1. Human Brain metastases samples

6-µm-thick paraffin embedded sections of resected human brain metastases were kindly provided by Dr. László Tiszlavicz, from the Department of Pathology of the University of Szeged, Szeged, Hungary. Such samples were collected from female patients (n=4), with stage IV triple negative breast cancer with well-established brain metastases.

2.2.2. Reagents and antibodies

Xylene and Quick-D Mounting Medium were purchased from Klinipath (Duiven, Netherlands). Papanicolaou's solution 1a Harris' hematoxylin solution, Eosin-Y solution 0.5% alcoholic, and Triton X-100 were obtained from Merck Millipore (Darmstadt, Germany). Bovine serum albumin (BSA), goat serum, and Tris borate-EDTA were acquired from Sigma-Aldrich (St. Louis, MO, USA). Citric acid was purchased from Chem-Lab (Zedelgem, Belgium). Hoechst 33342 and SlowFade® Diamond Antifade Mountant were obtained from Thermo Fisher Scientific (Waltham, MA, USA). All other chemicals were of analytical grade. The used primary and secondary antibodies are depicted in Table 1.

2.2.3. Hematoxylin-Eosin staining

For Hematoxylin-Eosin staining, the tissue was deparaffinized in xylene (10 minutes), rehydrated in successive ethanol solutions (100% ethanol for 3 minutes, 96% ethanol for 3 minutes, and 70% ethanol for 3 minutes), and in tap water (1 minute). The nuclei were stained with Papanicolaou's solution 1a Harris' hematoxylin solution for 10 minutes. Sections were then differentiated using a solution of 1% hydrochloric acid in 70% ethanol (20 seconds), and bluing in 1% ammonia water (10 seconds). The cytoplasm was stained with eosin Y-solution 0.5% alcoholic for 2 minutes. Finally, sections were dehydrated in a series of alcohols (70% ethanol for 3 minutes, 96% ethanol for 3 minutes, and 100% ethanol for 3 minutes) and diaphanized in xylol for 4 minutes and, finally mounted with Quick-D Mounting Medium.

Photographs of hematoxylin-eosin staining were acquired with a bright field microscope (Olympus, model BX51) with an integrated digital camera (Olympus, model DP50). Ten fields of the cerebellum, cranial hippocampus, and striatum, as well as the peripheral organs, lungs, liver and kidneys, of each animal were analyzed using the ImageJ 1.29x software (National Institutes of Health, USA), as detailed below. For analysis of metastasis extension and distribution, the area of metastases in each brain region (cerebellum, cranial hippocampus and striatum) and the other peripheral organs at each timepoint was measured, in 10 fields/organ or region. The results were presented as the ratio of tumor area/tissue area

2.2.4. Immunofluorescence

Brain sections were processed for IF analysis of the target proteins MEF2C and matrix metalloproteinase 9 (MMP9), the endothelial tight junction protein claudin-5, the epithelial marker pan-cytokeratin, as well as the astrocytic, microglial and pericytes markers, glial fibrillary acidic protein (GFAP), Ionized calcium binding adaptor molecule 1 (Iba-1) and CD13, respectively. The experimental conditions are described below and summarized in Table 1. Sections were deparaffinized in xylene (20 minutes) and rehydrated through successive immersion in 100% ethanol (20 minutes), 96% ethanol (10 minutes), 70% ethanol (10 minutes), and finally tap water (10 minutes). Heat-mediated antigen retrieval was performed with 10 mM citrate buffer pH 6.0 during 15 minutes in the microwave. A permeabilization step was performed with 0.5% Triton X-100 for 15 minutes, and tissue sections were blocked with different blocking solutions for 60 minutes. The primary antibodies were diluted in the respective blocking solutions with 0.5% Triton X-100, and sections were incubated overnight at 4 ºC. Then, the incubation with the respective fluorescent-labelled secondary antibody diluted in the respective blocking solutions with 0.5% Triton X-100, was performed during 60 minutes at room temperature. Between the several steps after the antigen retrieval treatment, washes with PBS (10 minutes) were performed. Negative controls with omission of primary antibodies were performed to exclude nonspecific binding or cross reactivity. Nuclei were labelled with Hoechst 33258 dye diluted 1:1000 in PBS for 2 minutes, followed by mounting with SlowFade® Diamond Antifade Mountant.

Table 1- Summary of the experimental conditions used for immunofluorescence in brain tissue

Marker	Blocking	Primary antibody	Dilution	Secondary Antibody	Dilution
MEF2C	10% Goat Serum/ 3%BSA	Thermo Fisher Scientific, #PA5-28247, Rabbit PC	1:100	Alexa Fluor® 555 Thermo Fisher Scientific, #A-21235 Goat anti-Rabbit	1:250
Claudin-5	10% Goat Serum	Thermo Fisher Scientific, #35-2500, Mouse MC	1:250	Alexa Fluor® 647 Thermo Fisher Scientific, #A-21235 Goat anti-mouse	1:500
Pan-cytokeratin	10% Goat Serum	Thermo Fisher Scientific, #MA5-12231, Mouse MC	1:500	Alexa Fluor® 647 Thermo Fisher Scientific, #A-21235 Goat anti-mouse	1:500
Iba-1	3%BSA	Abcam, #ab5076, Goat, PC	1:100	Alexa Fluor® 488 Thermo Fisher Scientific, #A-11055 Donkey anti-Goat	1:500
GFAP	3%BSA	Sigma Aldrich. #G3893, Mouse MC	1:1000	Alexa Fluor® 488 Thermo Fisher Scientific, #A-11001 Goat anti-mouse	1:500
MMP9	10%BSA/ 3%BSA	Thermo Fisher Scientific, #MA5-15886, Mouse MC	1:50	Alexa Fluor® 647 Thermo Fisher Scientific #A-21235 Goat anti-mouse	1:250
CD13	3%BSA	R&D, #AF2335, Goat MC	1:100	Alexa Fluor® 488 Thermo Fisher Scientific, #A-11055 Donkey anti-Goat	1:500

MEF2C – Myocyte enhancer factor 2C; PC- Policlonal; MC – Monoclonal; Iba-1- Ionized calcium binding adaptor molecule 1; GFAP – Glial Fibrillary acidic protein ; MMP9 – Matrix metalloproteinase-9

Photographs of IF labelling were acquired using a confocal microscope (Leica, model TCS SPE), equipped with 3 lasers (488, 532, and 635 nm). IF analysis relied in the cranial hippocampus, a prone region for metastasis development. So, ten fields of the cranial hippocampus of each animal were acquired under the same conditions and analyzed using the ImageJ 1.29x software (National Institutes of Health, USA). MEF2C expression was analyzed based on the evaluation of total fluorescence by tumor area determined by delimitation of each metastasis per field, using ImageJ software, and results were expressed as fluorescence intensity by μm^2 of tumor area. For evaluation of MEF2C nuclear translocation in metastasis, the number of cells with nuclear expression in each metastasis was counted and compared with the total number of cells in each metastasis. The results were presented in percentage of cells with MEF2C nuclear expression.

2.3. miRNA Analysis

2.3.1. Next-Generation Sequencing

For the NGS analysis, RNA was extracted from 50 – 200 µl of plasma using Norgen's Plasma/Serum RNA Purification kit and eluted in 10 µl. Due to small amounts of RNA, samples from the same treatment were pooled and 48 ng of RNA was used for library preparation using Illumina's TruSeq smallRNA kit. Samples were indexed accordingly for multiplexing and 15 cycles of PCR amplification were performed. To obtain desired product size a Blue Pippin isolation (Sage Science) using a 3% agarose gel and gated at 120-140 bp, followed with a clean-up using AmpureXP beads, were performed. To confirm desired product was selected the cleaned-up library prepped samples were run on Agilent's Bioanalyzer using a High Sensitivity DNA chip. These samples were run on a MiSeq v3 reagent kit flow cell from Illumina, performing 85 SR cycles. The resulting FASTQ files were uploaded into Genboree Workbench (genboree.org) where miRNA read counts were determined using the ExceRpt small RNA-seq pipeline v4.6.2 and compared against mouse genome mm10.

2.3.2. RNA extraction, cDNA preparation and RT-PCR

For the RT-PCR, we first isolated total RNA from the plasma samples using the miRCURY RNA Isolation Kit for biofluids (Exiqon Vedbaek, Denmark). The procedure was done according to manufacturer's instruction. RNA was then transcribed into cDNA, using the reverse transcription kit Universal cDNA Synthesis Kit II (Exiqon), according to manufacturer's instructions for plasma and to a final volume of reaction of 15 µL. Since miRNAs are present in plasma in very low quantities, the initial RNA volume was increased 4 times to have more concentrated cDNA and augment the yield of the subsequent procedures. Prior to the reverse transcription reaction, the synthetic RNA spike in Uni-SP6 (Exiqon, Vedbaek, Denmark), was added to the mixture to be further used as a quality control for the reverse transcription procedure. The reaction was performed on a Bio-Rad iQ5 thermocycler, using the following conditions: 42ºC for 60 minutes; 95ºC for 5 minutes to heat-inactivate the reverse transcriptase; cooling down and storage to 4ºC. The RT-PCR was performed using the same equipment and miRCURY LNA SYBR Green PCR Kit (Exiqon, Vedbaek, Denmark), according to manufacturer's instructions; however, the cDNA dilution performed was of 1:6, instead of the recommended 1:40. The following conditions were used: 50 cycles of 95ºC for 15 seconds, 56ºC for 30 seconds, 72ºC for 30 seconds and a ramp-rate of 1.6ºC/second,

followed by a melting curve analysis. Pre-designed LNA primer pairs were purchased from Exiqon for each of the selected miRNAs (mmu-miR-802-5p, mmu-miR-92a-1-5p, mmu-miR-194-5p, mmu-miR-205-5p, mmu-miR-181a-1-3p, mmu-miR-200b-3p, mmu-miR-375-3p) and for miR-16-5p that was used as an endogenous control to normalize the expression level. RT-PCR was performed in 96-well plates, with each sample performed in triplicate, and a no-template control was included for each amplificated. Determination of the threshold cycle performed using the software of the instrument (Bio-Rad iQ5 thermocycler), while the quantitation was determined by the comparative Ct (ΔΔCt) method. The results were presented as foldchange (FC), a relative measure of changes in gene expression. The FC for each target miRNA was determined according to the formula: $FC= 2^{(-\Delta\Delta Ct),}$ where ΔΔCt was calculated using the ΔCt for the control and the 4T1-injected samples. The ΔCts were calculated through the difference between the threshold cycle (Ct) of the target miRNA and the control miRNA, for each sample. For quality control, the PCR products were run by electrophoresis on a 4% agarose gel (1% Agarose + 3% Nusieve Agarose) for 1 hour at 100 mV to confirm the presence and size of the PCR products.

2.4. Bioinformatical target Prediction

To predict possible targets of each miRNA a bioinformatical analysis was performed using two different target prediction tools, available online: TargetScan v.7.2[14] and DIANA tools MicroT-CDS v.5.0.[15] The selected miRNAs were entered in both tools according to the following descriptives: mmu-miR-194-5p, mmu-miR-205-5p, mmu-miR-181a-1-3p, mmu-miR-802-5p, mmu-miR-92a-1-5p, miR – 200b-3p, miR-375-3p. TargetScan categorizes miRNAs by the state of the families conservation. While miR-92a-1-5p and miR-181a-1-3p were found in the poorly conserved miRNA families, the remaining miRNAs were part of the broadly conserved miRNA families. For DIANA tools MicroT-CDS, a threshold of 0.7 was applied as recommended by the software. The results obtained with both tools were compared and only the targets predicted by both tools were considered. Due to the high number of predicted targets for some miRNAs, a bibliographical search was done to select the targets with more relevance for further validation.

2.5. Statistical analysis

Results were analyzed using GraphPad Prism® 5.0 (GraphPad Software, San Diego, CA, USA). Immunofluorescence and hematoxylin-eosin results are expressed as mean ±

SEM. One-way ANOVA and the Dunnett post hoc test were used to compare how the parameters evolved in 4T1 injected mice at each timepoint and comparing to the vehicle injected mice, for each brain region separately. In addition, one-way ANOVA and the Bonferroni post hoc test were used to evaluate how the parameters evolved in 4T1 injected mice groups along time for each brain region separately and to determine whether there were statistically significant differences between timepoints and brain regions. RT-PCR results are expressed as mean ± SD. A two-tailed *t*-test was used to evaluate if there were significant changes in the expression of the different miRNAs in the 4T1 group, when comparing to the control group. P-values less than 0.05 were considered statistically significant.

3. Results

3.1. Well-established metastases are detected in the brain from 7-days after inoculation onwards

The 4T1 tumor is an aggressive triple negative tumor model that is highly tumorigenic and invasive. It can spontaneously metastasize from the primary tumor in the mammary gland to multiple distant sites including liver, lung, brain and bone, making it a good model for metastatic breast cancer.[16,17] To check the metastatic pattern in our animal model, in which 4T1 cells were inoculated in Balb/c mice, the tumor area was determined at different time points after injection of the tumor cells (5 hours, 3, 7 and 10 days) and in three brain regions (cerebellum, cranial hippocampus and striatum), as well as in the peripheral organs (lungs, kidney and liver) that like brain are some of the organs more prone to develop metastasis from breast cancer.[18] Observation of hematoxylin-eosin stained brain sections revealed that at 5 hours and 3 days, no metastasis were detectable in any of the regions, whereas their presence was detected at 7 days and even more at 10 days (Fig. 1 a-c). Regarding the peripheral organs, metastases were only detected in the lungs (Fig. 1 d-f). When comparing the extension of metastasis in the parenchyma, it was observed that the most affected brain region both at 7 and 10 days was the cranial hippocampus, immediately followed by the striatum, while the least affected one was the cerebellum, with a tumoral area in the lungs like that of the least affected brain region (Fig. 1 g). These results suggest that this model preferentially metastasizes to the brain, making it a good model for the study of peripheral biomarkers of metastasization to brain.

Since brain metastasis with the highest tumoral area was the cranial hippocampus, this brain region was the selected one for the subsequent brain analyses.

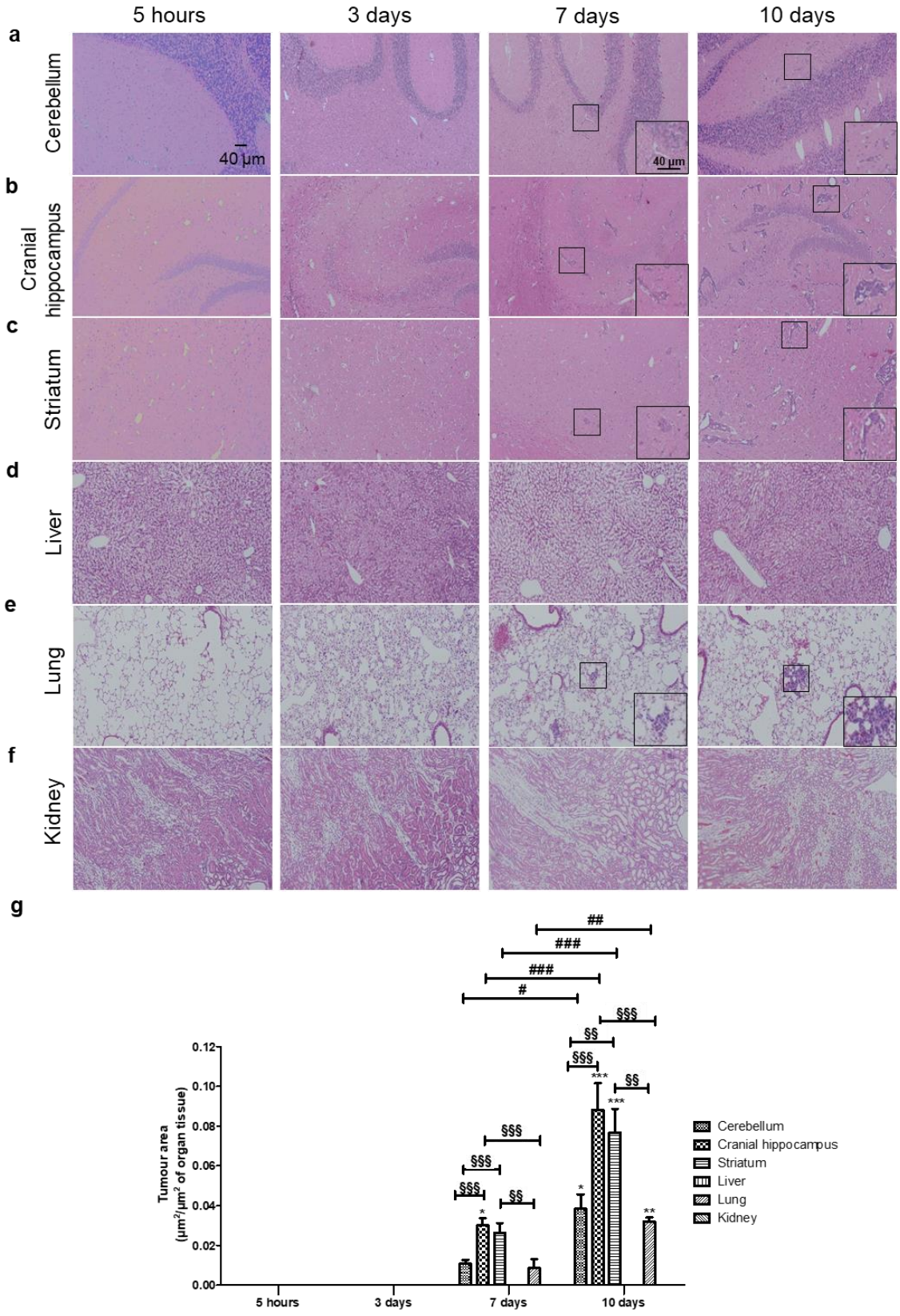

Figure 1 - Profile of breast cancer metastasis in the brain and peripheral organs. Hematoxylin-eosin staining of cerebellum (a), cranial hippocampus (b), striatum (c), liver (d), lung (e), and kidney (f) was performed and the tumour area was quantified (g) at several time points after inoculation of triple negative breast cancer cells in 7-8 weeks old female Balb/c mice. Insets show the magnification of the selected representative metastasis (inside the squares). *p<0.05, **p<0.01, ***p<0.001 vs. 5 hours; $^{\#}$p<0.05 $^{\#\#}$p<0.01, $^{\#\#\#}$p <0.001 between indicated timepoints, for the same regions; §§<0.01, §§§<0.01 between indicated regions for the same timepoint.

3.2. miRNAs are aberrantly expressed in plasma of 4T1-injected mice along the metastasization process

As previously mentioned, due to their unique characteristics circulating miRNAs can constitute reliable biomarkers for specific types of metastasis and it has been suggested that different types of metastasis can have unique circulating miRNAs signatures.[10] However, no specific miRNA profile has yet been discovered for BCBM. So, once the metastatic profile of this model was established, we wanted to identify the specific miRNA signature for BCBM by finding aberrantly expressed miRNAs that could be used as biomarkers of BCBM. With this aim, plasma samples collected from 4T1-injected mice and from controls at 3 days, 7 days and 10 days were subjected to NGS analysis. NGS or high throughput sequencing, is the catch-all term used to describe several different modern sequencing technologies that detect DNA templates randomly across the entire genome. Thus, in our study, the use of NGS allowed the detection of all the mature miRNAs annotated in miRbase v.18 that are present in the studied plasma samples. The results featured the count of reads for each miRNA in every timepoint, both for the control and for the cancer samples, which represent a relative digital measure of the miRNA expression. For each miRNA in every BCBM plasma sample the FC was calculated by dividing the read count in the BCBM samples by that in the control, to judge if the miRNA in question is unchanged, upregulated or downregulated. To render feasible the analysis of the large data ensuing from the NGS analysis, we established as arbitrary criteria the miRNAs that were upregulated or down-regulated, with FC> 2.0 or FC<0.5, respectively. Based on this criterion, we observed that for all the timepoints, the greatest part of the detected miRNAs remains unchanged and that the percentage of deregulated miRNA increases with time (Fig. 2 a). While at 3 and 7 days about 18% of the miRNAs are deregulated, at 10 days this percentage increases and about 30% of the miRNAs are deregulated. Within the deregulated miRNAs, another interesting observation is that throughout time, the number of downregulated miRNAs is decreasing, while the number of upregulated miRNAs is increasing. In fact, at 3 days 77 miRNAs were found to be altered (Fig. 2 b), 52 of which were downregulated, while the remaining 15 were upregulated; so, more than half of the deregulated miRNAs were downregulated. Similarly to the observations for the earliest time point, at 7 days 84 miRNAs were deregulated but approximately half of the miRNAs were upregulated (43 miRNAs). Contrarily, at 10 days, a total of 133 miRNAs were deregulated but only 11 of them were

downregulated, while the remaining 122 were upregulated. So, besides having more deregulated miRNAs at 10 days, the number of upregulated miRNAs suffers a dramatic increase, comparing to 3 and 7 days. Moreover, comparison between the altered miRNAs among the different timepoints revealed that they are not all the same in all the timepoints. Indeed, while most miRNAs are solely deregulated at a single timepoint, only 8 miRNAs are altered at 3, 7 and 10 days after injection of the 4T1 cells (Fig. 2 b).

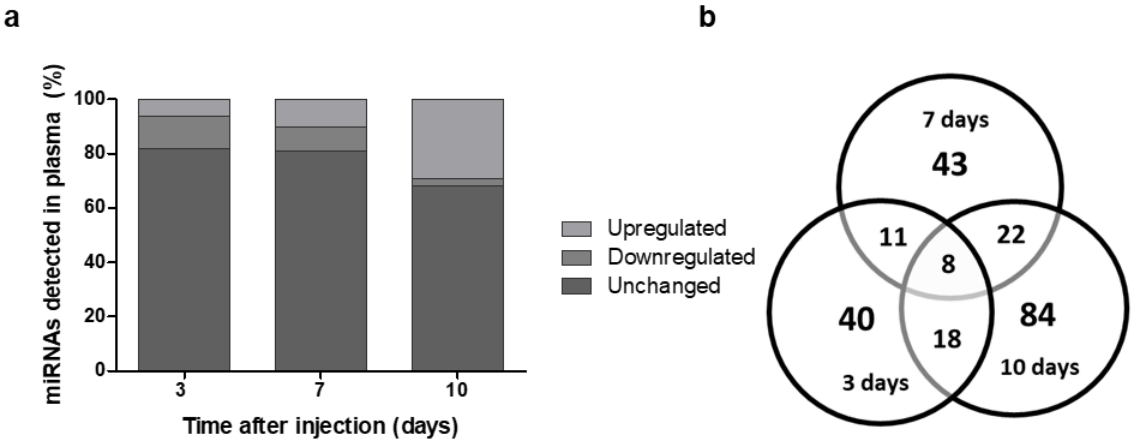

Figure 2 - Overview of the next generation sequencing (NGS) results, regarding the altered expression of miRNAs in plasma along brain metastasization of breast cancer. Plasma samples were collected at several timepoints after inoculation of triple negative breast cancer cells, or vehicle (control), in 7-8 weeks old female Balb/c mice. NGS miRNA analysis was performed, followed by analysis of the number of miRNAs whose expression was altered in comparison with the corresponding time point control. MiRNAs with fold change (FC) from control > 2.0 were considered to be significantly upregulated, while miRNAs with FC < 0.5 were considered to be downregulated. The remaining miRNAs were considered unchanged. The percentage of upregulated, downregulated or unchanged miRNAs was calculated for each timepoint (a). MiRNAs with altered expression (upregulated or downregulated) were compared between the different timepoints to check whether their expression remained altered throughout the metastatic process (b).

Altogether, these results demonstrate that the miRNA levels are changing throughout time and, so, the miRNAs that are up or downregulated prior to metastasis are not the same that are deregulated in more advanced stages of the tumor. These results also show that at more advanced stages of tumor progression miRNAs tend to be more upregulated than in earlier stages.

3.3. A specific set of miRNAs is aberrantly expressed prior to metastasis formation

3.3.1. Selection of relevant miRNAs based on NGS results

The high number of altered miRNAs, even considering only those with a FC > 2 and a FC < 0.5 (Fig. 2 b), precludes the validation of all of them. With the aim of selecting the miRNAs that are more highly expressed for further analysis, only the miRNAs with read counts higher than 20,000, either in the control samples or in 4T1-injected samples,

were considered (Fig 3 a). Since we were looking for early biomarkers for BCBM, our time point of interest is 3 days, since it is early enough that the metastasis have not appeared yet (section 3.1) but it should be late enough that some miRNAs possibly related with the early metastasizing process are already being aberrantly expressed (section 3.2.). Thus, only the miRNAs that were altered at 3 days were selected for further analysis. When considering the read counts, a set of 8 miRNAs was found to be aberrantly expressed at 3 days post-injection, 5 of which were downregulated (miR -194-5p, miR-802-5p, miR-145-5p, miR-17-3p and miR-338-3p), while 3 were upregulated (miR-205-5p, miR-181a-1-3p and miR-92a-1-5p), as indicated in Figure 3 b. A bibliographic search about the roles in cancer of the selected miRNAs revealed that, comparing with the other selected miRNAs, there is a lack of information regarding the roles of miR-17-3p and miR-338-3p roles miR-17-3p, particularly in breast cancer. So, and to improve the chances of success in the subsequent studies, we decided to replace their study by two other miRNAs, miR-200b-3p (FC=0.5031) and miR-375-3p (FC=1.809). Although these miRNAs are not within the primarily established arbitrary criteria for FC, the expression of miR-200b-3p decreased with time, becoming more downregulated (FC=0.444), while that of miR-375-3p highly increased with time (FC=4.841), becoming more upregulated at 7 days. Even if 7 days is not our timepoint of interest such altered expression could be related with these miRNAs' influence in the metastatic development, one as tumor suppressor and the other as an oncogene.

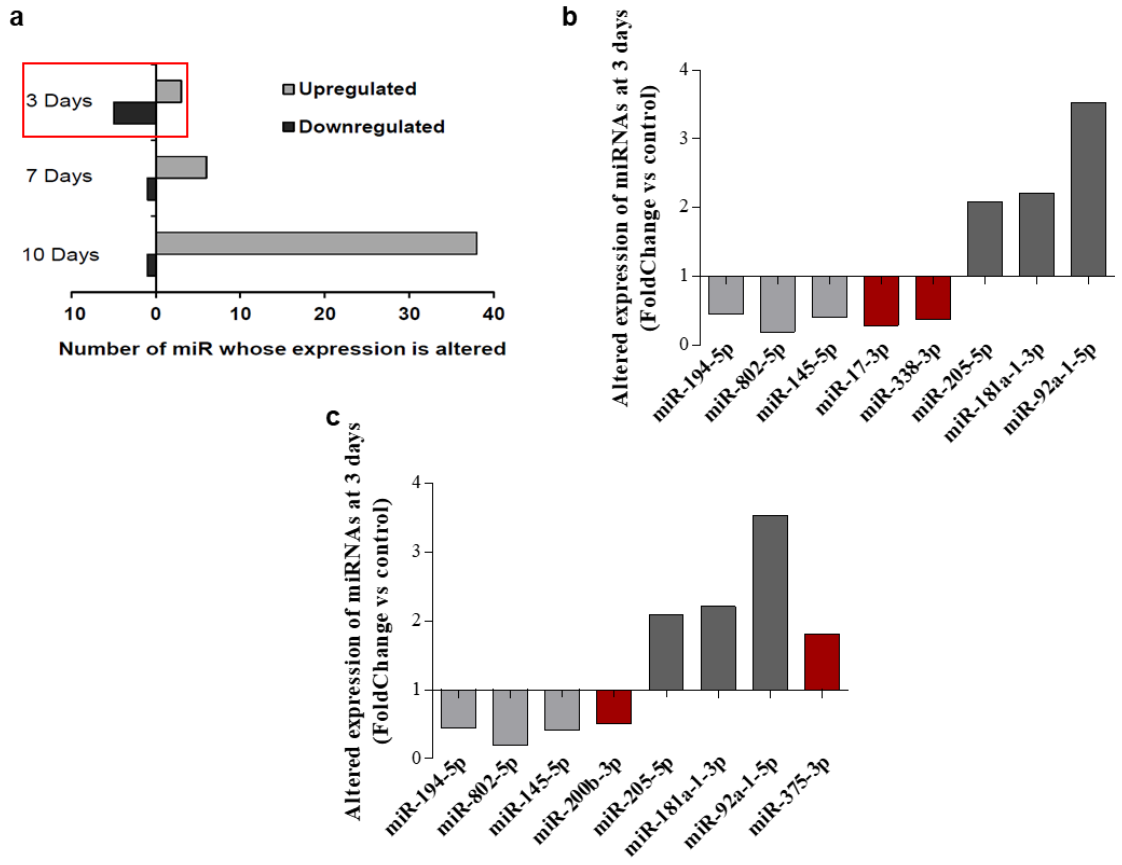

Figure 3 - Number of up and downregulated miRNAs with readcounts higher than 20,000, throughout metastatic progression and specific miRNAs that were altered prior to metastasis and selected for further studies. NGS were narrowed down based on their readcounts and only the miRNAs with readcounts higher than 20,000 were obtained for each timepoint (a). At 3 days, which is the timepoint of interest for, this study, 8 miRNAs were obtained (b). Based on the FC values and on a bibliographical research, two of the obtained miRNAs (miR-17-3p and miR-338-3p), were replaced by two others (miR-200b-3p and miR-375-3p), which, although fall out of the established criteria (0.5 > FC > 2 and readcounts> 20,000), seemed more promising for further validation.

Altogether, the miRNAs displayed in Figure 3 c are the ones identified as candidates for early biomarkers for BCBM and that should be subject to further studies.

3.3.2. Validation of the selected miRNAs by RT-PCR

NGS analysis is a very useful tool that enabled us to study the changes in the miRnome during brain metastasization. However, the results obtained by NGS are not completely reliable. NGS of miRNAs is subject to sequencing errors and the search and removal of adapter sequences can also influence the results, with risk of false positives or negatives.[19] Thus, for more reliable results, the aberrant expression levels in plasma at 3 days of the previously selected miRNAs had to be validated by RT-PCR. With this aim, RT-PCR was performed solely for plasma samples collected at 3 days after injection, using LNA primers specific for the selected miRNAs (Fig. 3 c) but also for miR-16-5p, used as an endogenous control. Although there is still no consensus about an ideal

reference gene to study miRNAs expression in plasma, we chose miR-16-5p because it has been demonstrated to be stably expressed in plasma of several different mouse strains at different ages and disease conditions.[20] Moreover, Rinnerthaler *et al*[21] have demonstrated that miR-16-5p is also stably expressed in breast cancer tissues, both from primary and metastatic sites, indicating that it should be a good housekeeping gene for our study, which was further confirmed when the RT-PCR was performed. RT-PCR results (Fig. 4) allowed to validate the downregulation of miR-802-5p ($p<0.01$) and of miR-194-5p ($p<0.05$) in plasma of 4T1-injected mice, when comparing to controls, at three days post-injection. In turn, miR-92a-1-5p showed a 1.8-fold increase in the 4T1-injected mice, though statistical significance was not achieved ($p<0.10$). The experiments should be repeated with a higher number of animals. Ideally, the experiments should also be repeated for miR-194-5p, since for this one, only 3 samples were used. Still, our validation results were in accordance with the NGS preliminary data and gave alterations in the same order of magnitude, with calculated p values that validate the results for miR-802-5p and miR-194-5p (Table 2). Due to time and logistic constrains, validation of the aberrant expression of the other selected miRNAs is still pending.

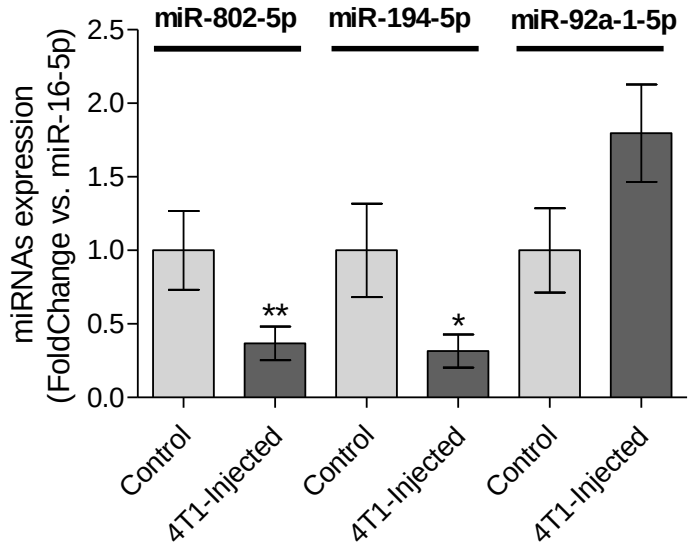

Figure 4 - Validation of the NGS result by RT-PCR for miR-802-5p, miR-194-5p and miR-92a-1-5p. Plasma samples were collected 3 days after injection of 4T1 cells or vehicle (control) in female Balb/c mice. Expression of miR-802-5p (n=5) miR-194-5p (n=3) and miR-92a1-5p (n=5) was evaluated by RT-PCR. Results are shown as mean values ± SD and expressed as fold change vs. miR-16-5p (endogenous control). *p<0. and 05 **p<0.01 vs. control, two-tailed unpaired Student's *t* test.

Table 2 - Comparison of NGS and RT-PCR data regarding miRNAs aberrant expression in 4T1-injected mice.

miRNA	NGS	RT-PCR	
	FoldChange	FoldChange	p-value
miR-802-5p	0.1890	0.3682	$p<0.01$
miR-194-5p	0.4471	0.3180	$p<0.05$
miR-92a-1-5p	3.5272	1.7970	$p<0.1$

The results of both techniques point to the downregulation of miR-802-5p and miR-194-5p and to the upregulation of miR-92a-1-5p as efficient predictors of the upcoming occurrence of brain metastasis in cases of BC, however, further experiments should be performed.

3.4. miRNAs targets can be predicted through a bioinformatical approach

MiRNAs mainly act as regulators of gene expression by binding to target mRNAs and directing posttranslational gene by one of two main mechanisms: translational repression and RNA decay, both of which require the recognition of the target and binding of the miRNA to partially complementary sequences of the 3'-UTR of the target gene through Watson-Crick base-pairing.[9,22,23] However, the miRNA-mRNA interactions are very difficult to characterize and their experimental validation is time consuming and costly. Moreover, a large number of potential target sites exist for any given miRNA.[24] Therefore, the correct identification of target genes for the miRNAs remains a challenge. Nowadays, numerous web based bioinformatical tools provide not only thousands of published miRNA sequences and annotation but also algorithms that predict potential miRNA target genes and effective miRNA-mRNA interactions, to facilitate the process of narrowing down potential targets for experimental validation, which is a critical initial step in identifying miRNA–target interactions.

Although the seed sequence is the major contributor for the target mRNA recognition, there are other factors that influence the repression by the miRNA. The available bioinformatical tools base their algorithms on these factors and, although each algorithm is unique, there are four main features that are commonly used by target prediction tools:

- Seed match;

  The seed region comprises a zone between nucleotides 2 to 8 of the 5' end of the miRNA, which has perfect Watson-Crick complementarity with the 3'UTR of the mRNA.[25] Although it is not very frequent, a mismatch in the seed can be considered, as long as it is compensated by additional extended pairing in 3' part of miRNA (3'-compensatory sites).[26] Four main different types of seed matches can be considered, which are depicted in Figure 2 of Chapter I, and it should be noted that the type of seed can influence the strength of the interaction with the target. [27]

- Conservation

  Conservation refers to the maintenance of a sequences present in the human genome across evolutionarily distant species, such as mouse, dog or fish.[28] Conservation analysis by the bioinformatical tools may focus on regions in the 3' UTR, the 5' UTR, the miRNA, or combinations of the three and recently it has also been applied to regions flanking the miRNA and the target genes, for example, to their promoter regions.[29] In general, there is higher conservation in the miRNA seed region than in the non-seed region.[30] The conservation analysis is performed using the phylogenetic and evolutionary distance calculations and it is based on the assumption that a predicted miRNA target is functional and has been kept unchanged because it is being selected by positive natural selection. In this way, a higher degree of conservation may reflect a more reliable prediction.[26]

- Free Energy

  Free energy (ΔG) is used as a measure of the stability of biological systems. Accordingly, it can be used to study the thermodynamic properties of a putative miRNA:mRNA duplex. So, by predicting how the miRNA and its candidate target hybridize, regions of high and low free energy can be inferred and the overall ΔG can be used as an indicator of how strongly bound they are.[31] Therefore, the more negative the ΔG of the duplex is, the more stable is the interaction predicted to be, increasing the likelihood that this interaction really occurs.[32] Most bioinformatical target prediction tools use the Vienna RNA package[33] to measure this parameter.

- Site Accessibility

After transcription, mRNAs undertake secondary structures, which can affect the ability of miRNAs to access and bind to certain regions of the targets. Site accessibility accounts for the ease with which a miRNA can locate and hybridize with a specific region of the 3'UTR of the target mRNA. The hybridization of the duplex involves a two-step process: first the miRNA binds to a short accessible region of the mRNA and then, the secondary structure of the mRNA unfolds, in order for the miRNA to complete the binding.[34] The amount of energy spent in this process can be calculated to evaluate the likelihood of targeting by the miRNA.[31] Usually, upstream and downstream flanking regions of miRNA binding sites have weak base-pairing to reduce energy costs of the unfolding process and make the site more accessible.[35]

Other, less common, features that are used by bioinformatical tools to predict miRNA targets, include target site abundance in the 3'UTR, local AU content of the flanking regions, GU wobbles in the seed match, 3' compensatory sites, protein binding to the RNA, methylation of RNA sites, among many others. [31,36]

3.4.1. Target prediction tools

Given the multitude of factors that can be considered for target prediction, each algorithm is distinct from the next one. Not only can they use different of the above-mentioned features, but they can also use them in different ways, as each of the target prediction features can contribute in a higher or lower percentage to the final score, according to the selected tool. With this in mind, and after studying and testing several of the available tools, two bioinformatical tools were chosen to perform a bioinformatical prediction of the targets for the selected miRNAs: TargetScan v.7.2[14] and DIANA tools MicroT-CDS v.5.0[15]. These, besides being two of the most used target prediction tools in the literature, have two very different algorithms, summarized below.

- TargetScan v.7.2;

TargetScan allows the user to search by miRNA name, gene name or broadly conserved, conserved, or poorly conserved miRNA families across several species. This algorithm ranks the targets either based on their predicted efficacy of targeting or by the probability of conserved targeting. Sites are only considered if they have full

complementarity in the seed region and the results are classified based on length of exact match and occurrence of an adenine at the first positions of the target mRNA (8mer sites).[28] Different types of seed match contribute to the final score according to their efficacy: 8mer > 7mer > 7mer-A1 > 6mer.[37] Several other parameters contribute to the final score, including pairing outside the seed region and AU content 30 nucleotide upstream and downstream of the predicted site but the conservation of the seed region among orthologous 3'UTRs within miRNA binding regions has a fundamental importance for the outcome score. Indeed, after the algorithm searches for the seed matches in the human genome and calculates free energy of binding, thus assigning each UTR a certain score, it repeats this process for the UTRs in other distant genomes, like mouse, rat or pufferfish to do a phylogenetic analysis.[31,38]

- Diana tools MicroT-CDS v.5.0;

DIANA tools microT-CDS allows the user search by miRNA name, gene name, Ensembl ID, KEGG description, or a combination of these. This algorithm uses a 38 nucleotide-long frame that is moved along 3'UTR, looking for miRNA recognition elements. The minimum energy of potential miRNA binding, that allows mismatches, is measured after every shift and compares with the energy of 100% complementary sequence bound to the 3'UTR region, and, contrary to TargetScan, is one of the features that contributes most for the final score.[39] DIANA-microT searches for sites 7, 8 or 9 nucleotide–long complementarity in 5' region of miRNA, however, it can consider 6 nucleotide-long matches within seed region or with an pairing, as long as it is compensated by additional base pairing in 3' region of miRNA. Although DIANA-microT uses conservative alignment for non-conservative sites can also be considered, but with lower scores.[28] The most important features for this algorithm, besides free energy, include binding category weight (as an estimate of the efficacy of binding mainly based on matching in an extended seed sequence), distance to the nearest end of the 3'UTR distance to an adjacent binding site, conservation, and AU content. The accessibility of the 3' UTR is also relevant and predicted using Sfold.[31] Another unique feature of this algorithm is that it accounts for the restraints that the miRNA associated proteins can have on the position and sizes of the loops and nucleotide bulges between miRNAs and their cognate miRNA recognition elements.[36]

3.4.2. Target prediction for the selected miRNAs

Although we were studying the miRNAs as early biomarkers for BCBM, we wanted to better understand of if and how the aberrant expression of the previously selected miRNAs (Fig 3 c) could be related with the metastasizing process. With this aim, we performed a bioinformatical prediction of the targets for each miRNA, using TargetScan v.7.2 and Diana tools microT-CDS v.5.0. For more reliable results, we compared the predicted targets from both tools for the different miRNAs and only the targets that were common were considered. Due to the high number of predicted targets, the results are presented in attachment (Supplementary Tables 1-7) and summarized in Table 3.

Table 3 – Summary of the results obtained for the bioinformatical target prediction, using TargetScan v.7.2. and Diana tool MicroT-CDS v.5.0

miRNA	TargetScan	DIANA tools – MicroT-CDS	Common Targets
miR-145a-5p	539	706	224
miR-194-5p	305	551	169
miR-205-5p	374	925	207
miR-181a-1-3p	479	16	1
miR-802-5p	251	666	140
miR-92a-1-5p	1982	227	76
miR-200b-3p	826	954	463
miR-375-3p	187	656	107

The results of the target prediction are sorted by the scores obtained with TargetScan (Cumulative Weighted context ++ score, Total context Score and Aggregare PCT) and with Diana tools MicroT-CDS (miTG). Total context score is the sum off the contribution of 14 features for each of the four site types, the more negative, the greater the expected repression. The cumulative weighted context score is calculated using total context scores and cumulative predicted repression at different sites existing in a target mRNA. It estimates the total repression expected from multiple sites of the same miRNA for each target. Values can be between -1 and 1 but the more negative the value, the greater the repression.[14] It is the most relevant score, regarding efficacy of repression prediction by TargetScan, which also gives a probability of preferentially conserved targeting, the aggregate PCT. This score is an estimate of the probability that a site is conserved due to the maintenance of miRNA targeting, rather than by chance or any other reason not pertinent to miRNA targeting.[26] Finally, the miTG score, calculated by DIANA tools is a general score for predicted interaction. The higher the score, the greater the confidence.[36]

Intriguingly, while for most of the miRNAs, a high number of targets was predicted, regarding miR-181a-1-3p, only one target gene was common between both bioinformatical tools, *XIRP2* that encodes for xin actin-binding repeat containing 2, which has no described role in cancer.

3.4.3. Selected targets with relevance for the metastasization process

It is evident by the results in Table 3 that this type of bioinformatical prediction of the genes that are repressed by specific miRNAs, generates huge amounts of information. And it would not be possible to validate all the targets, using laboratorial techniques. So, we performed a bibliographical search for the predicted targets for miR-802-5p, miR-194-5p and for miR-92a-1-5p, which, based on RT-PCR results (Section 3.3.2) for now seem more promising as early biomarkers for BCBM. This research focused on trying to find the proteins, encoded by the target genes, that have been related with BCBM, specifically but also with other processes that can be relevant for metastatic progression, including angiogenesis, disruption of the BBB, invasion, among others. We mainly tried to focus on proteins that have been described in BC but other types of cancers, including brain tumors were also considered. Based on this and on the scores previously obtained for the different targets, we selected, for each miRNA, some targets that would be interesting to further study in our model. The selected targets for miR-802-5p, miR-194-5p and miR-92a1-5p are depicted in Table 4, Table5 and Table 6, respectively.

Table 4– Predicted targets for miR-802-5p that were considered relevant for this study, based on a bibliographic research, also summarized in the table.

Target Gene	Name	Weighted context score	miTG
MSI1	Musashi RNA- binding protein-1	-0,43	0,8566
• MSI-1 overexpression is, for several types of cancer, including breast, associated with low differentiation, poor prognosis, lymph node invasion and metastases. MSI-1 is not only responsible for maintaining cancer stem cells populations but also for regulating the translation of proteins that operate essential oncogenic pathways;[40] • Particularly in BC, MSI-1 expression is higher in metastatic tissues than in primary tumors and is a prognostic indicator of poor survival. Its overexpression promotes proliferation by enhancing the ERK and notch signaling pathways in tumor infiltrating cells; [41,42] • The high expression of MSI-1 has been widely studied in brain tumors: MSI-1 enhances glioblastoma cell migration and cytoskeletal dynamics through translational inhibition of tensin3. It is also a central regulator of adhesion pathways by regulating integrins, influencing, therefore cell morphology, adhesion, migration and invasion;[43,44]			
RHOA	RAS homolog family member A	-0,38	0,8175
• RhoA is a well-known member of the Rho family of small GTPases, that has a well-established role in the migration and invasion of tumor cells by, though is downstream effectors, like ROCK, promoting actin polymerization and actomyosin contractibility;[45]			

• In brain metastatic cells from BC, RhoA also controls the formation and orientation of stress fibers and focal adhesions, increasing the metastatic ability of the cells;[46] • During TEM of lung cancer cells through the BBB there is an overexpression and activation of RhoA in BMVECs, which induces actin cytoskeleton remodeling, an TJ disruption, thus increasing the permeability of the BBB.[47]			
TCF4	Transcription factor 4	-0,18	0,9751
• TCF4 is described to promote initiation and progression of diverse types of cancer by binding to β-catenin and transactivate Wnt target genes. In BC, TCF-4 activates Wnt signaling through the transcriptional activation of osteopontin. The upregulation of TCF4 and osteopontin are associated with a poor prognosis; [48] • TCF4 is highly expressed in mouse and human brain metastases from lung adeno carcinoma. Concordant with previous results, a TCF-4-mediated activation of the Wnt pathway has been proposed. Wnt also promotes BCBM.[49]			
CDH11	cadherin 11, type 2, OB-cadherin (osteoblast)	-0,3	0,8580
• CDH11 belongs to a family of transmembrane adhesion molecules, the cadherins. It is overexpressed in several types of cancer, particularly in the more aggressive ones and can promote migration and metastases;[50] • CDH11 is highly expressed in invasive BC cell lines and tissues, while its expression is not detected in noninvasive types of BC and mainly in cells with a mesenchymal phenotype. It promotes BCCs migration through the activation of Rac, a small GTPase;[50,51] • During the progression of glioblastoma, endothelial cells stimulate CDH11 expression in malignant cells. CDH-11 induces TGFβ-mediated migration.[52]			
CCND2	Cyclin D2	-0,10	0,8698
• Cyclin D2 is a part of the cyclin D family. Their main function is to activate cyclin- dependent kinase 4(cdk4/cdk6), and control cell cycle progression. Cyclin D2 is overexpressed in many types of cancer where it promotes cell growth and proliferation;[53] • Cyclin D2 is the cyclin that is predominantly expressed in brain tumors. Indeed, knockdown of Cyclin D2 in glioblastoma cells, causes cell cycle arrest and tumor growth inhibition. Moreover, cyclin D2 is also highly expressed in glioblastoma stem cells and its expression decreases upon differentiation;[54]			
MEF2C	Myocyte Enhancer Factor 2C	-0,03	0,9523
• MEF2C part of the family of transcriptions factors MEF2 and has recently been proposed as an oncogene for several types of cancer and it can promote vascular mimicry, invasion and migration and angiogenesis;[55] • MEF2C has an important role in promoting angiogenesis and is involved in a feedback mechanism with VEGF: VEGF activates MEF2C through p38MAPK and calcineurin pathways. MEF2C modulates the effects of VEGF in endothelial cells but can also control VEGF at a transcriptional level.[56]			

Table 5 - Predicted targets for miR-194-5p that were considered relevant for this study, based on a bibliographic research, also summarized in the table.

Target Gene	Name	Weighted context score	miTG
STAT1	Signal Transducer and Activator of Transcription 1	-0,45	0,7627
• IFNα and TNFα activate the STAT1 pathways in brain metastatic cells, which support tumor growth and chemoresistance. The knockdown of STAT1 reduces the percentage ofbrain metastases;[57] • STAT1 is highly expressed in invasive BC and relates with poor outcome. It can promote tumor cell growth and invasiveness, immune evasion and therapy resistance;[58] • Enhanced expression of STAT1 has been correlated the disruption of the BBB by decreasing TJ proteins, as well as to modulate IL-6-induced monocyte migration, across a BBB model.[59]			
RAP2B	RAP2B, member of RAS oncogene Family	-0,44	0,8139

- Rap2B belongs to the Rap family of small GTP-binding proteins has been described to work as an oncogene is many types of cancer and has been proposed to regulate p53-mediated pro-survival functions;[60]
- Particularly in BC, Rap2B promotes cell proliferation, migration and invasion by increasing intracellular levels of calcium and upregulating the ERK1/2 signaling pathway;[61]
- It is highly expressed in BM from BC and lung cancer when comparing to primary tumors, though no exact mechanism has been proposed.[62]

HBEGF	Heparin-binding EGF-like growth factor	-0,25	0,993

- HBEGF is an EGFR ligand that promotes the transmigration of BCC through the BBB. Indeed, inhibitors of HBEGF have been shown to prevent the passage of BCCs across the BBB, in an *in vitro* model;[63,64]
- HBEGF promotes intravasation, metastasis and invasion of BC *in vivo*. The promotion of invasion by HBEGF is directly related to the downstream upregulation of metalloproteinases;[65]
- In TNBC, HBEGF plays an important proangiogenic role and its overexpression promotes proliferation of endothelial cells, tube formation, and vascular permeability in **blood vessels,** while its inhibition in vivo suppressed tumor formation, by simultaneously reducing the expression of VEGFA and angiopoietin-4.[66]

AKT2	v-akt murine thymoma viral oncogene homolog 2	-0,15	0,7733

- Akt2 is a potential therapeutic target for metastatic BC, due to its crucial involvement in multiple pathways that regulate survival, proliferation, migration and invasion of BCCs, namely the PI3K/Akt pathway;[67] Proteins related to cell motility like F-actin and vimentin have been proposed as downsteam targets of Akt2, during BC progression.[68]
- Akt2 promotes metastatic potential in brain tumors, namely neuroblastoma.[69] In gliomas, Akt2 is also related with metastatic potential and malignant transformation by regulating the β-catenin/TCF4 signaling.[70]

CHD2	cadherin 2, type 1, N-cadherin (neuronal)	-0,17	0,7967

- N-Cadherin is a crucial player in the metastatic cascade in an early stage because its overexpression promotes EMT and it defines the mesenchymal phenotype of cancer cells;[71,72]
- Particularly in BC, N-Cadherin also promotes EMT and metastasis by stimulating fibroblast growth factor receptor signaling. This leads to an activation of MAPK-Erk pathway, which, in turn promotes migration, invasion and secretion of extracellular proteases.[73,74]
- Previous studies from our group show that BCCs cross the BBB with a mesenchymal phenotype and have a high expression of N-Cadherin early in the metastasization process, a characteristic that is lost throughout time and in well-established metastasis.[75]

Table 6 - Predicted targets for miR-92a-1-5p that were considered relevant for this study, based on a bibliographic research, also summarized in the table.

Target Gene	Name	Weighted context score	miTG
FOXP1	ForkheadboxP1	-0,54	0,7947

- FOXP1 is a transcription that is known to have an ambiguous role in cancer. While in some cancers, FOXP1 overexpression promotes tumorigenesis, in other types of cancer, the expression of FOXP1 is decreased and it can exert tumor suppressive functions;[76]
- In BC, loss of FOXP1 is associated with poor prognosis, however, a recent study describes a pro-survival function for FOXP1 in BC, when activated by the PI3K/Akt signaling;[77]
- In brain tumors, FOXP1 has also been associated with tumor suppressive functions, as it inhibits cell growth, proliferation, tumor migration and invasion. The exact downstream targets of the transcriptional activity of FOXP1 that inhibit tumorigenicity are yet to be deciphered.[78,79]

ELK1	ELK1, member of ETS oncogene family	-0,28	0,8713

• ELK1 belongs to the ETS-domain family of transcriptional factors and induces immediate gene expression, in response to extracellular signals. ELK1 is a substrate for MAP kinases and for JNK protein kinases to activate multiple oncogenic pathways;[80] • In many cancers, including breast cancer, ELK1 is necessary for the activation of the proto-oncogene c-fos. C-fos is essential for BCCs growth and proliferation and metastases promotion;[80,81] • ELK1 is also known to promote EMT during BC progression. When phosphorylated by Erk1/2, ELK1 translocates to the nucleus and facilitates mitogen and stress activated protein kinase-1, which enhances Snail expression, that downregulates E-cadherin.[82]			
MMP9	Matrix metallopeptidase-9	-0,21	0,7068
• MMP9 is a zinc-dependent endopeptidase with an important role in remodeling the ECM to promote invasion by malignant cells but can also modulate tumor microenvironments regulate signaling pathways that control cell growth, inflammation, or angiogenesis;[83] • BCCs release MMP9 to promote cell motility and invasion by cleavage of ECM proteins and of cell-cell and cell-ECM attachments. BCCs derived MMP9 also promotes vascularization during the metastatic process;[84] • The expression of MMP9 has shown to be elevated both in neoplastic tissue of a rat model for BCBM and in serum of patients with BCBM and can be used as a predictive biomarker.[85,86]			
ENG	Endoglin	-0,18	0,7114
• Endoglin is a cell membrane glycoprotein that is strongly expressed in endothelial cell, particularly on tumor vasculature. It functions as an adhesion molecules for integrins but is also a receptor for TGF-β;[87,88] • Antibodies against endoglin inhibit metastatic spread of breast cancer *in vivo* by inhibiting the VEGF pathway, thus constraining angiogenesis;[89] • Brain metastatic breast tumor cells heavily express endoglin, in contrast with non-metastatic cell lines. This expression enhances invasive character of brain metastatic cells by the formation invadopodia, extracellular proteolysis, chemotaxis of TGF-β and migration.[90]			

We previously showed that miR-802-5p and miR-194-5p are downregulated in plasma samples of 4T1-injected mice at 3 days post-injection, so it is predictable that they work as tumor suppressor miRNAs by downregulating oncogenes. Thus, the expression of their targets should be increased. Indeed, many of the predicted targets for both miRNAs have been described to promote cancer progression and different types of metastasis. For miR-92a-1-5p, the results were not so conclusive. Most of the predicted targets have not been described in any type of cancer, yet and although this miRNA shows a trend to be upregulated, both by NGS and RT-PCR, most of the targets that have been previously described in cancer, have oncogenic functions (Table 6).

3.5. MMP9, a predicted target for miR-92a-1-5p is expressed in malignant and endothelial cells

MMP-9 is a well-known MMP, with important functions in the remodeling of the extracellular matrix (ECM).[91] The role of MMPs has been extensively studied in cancer, since they can modulate tumor microenvironment and contribute to tumor development and progression through diverse mechanisms.[83] Particularly, MMP-9 has been associated with the progression of BC and, when released by BCCs, promotes cell motility and

invasion by cleavage of ECM proteins and of cell-cell and cell-ECM attachments, as well as angiogenesis. [84] Moreover, the high expression of MMP-9 has been previously described to be elevated in a rat model for BCBM and in the serum of BCBM patients.[85,86]

The *MMP9* gene was predicted by bioinformatical tools as a target for miR-92a-1-5p, having a cumulative weighted context score++ = -0,21 and a miTG = 0,7068. No PCT was obtained because in the TargetScan algorithm, miR-92a-1-5p is considered within the poorly conserved families of miRNAs. MiR-92a-1-5p showed a trend to be increased in plasma, so it would be predictable that the expression of proteins encoded by its the target genes would be decreased. However, as it was previously mentioned, most of the predicted targets of this miRNA are known to have oncogenic functions. So, we wanted to understand if miR-92a-1-5p, could have any influence in the expression of MMP9. Although for the miRNA studies, we were only focusing on the 3 days, for a better understanding of MMP9 behavior, we also analyzed MMP9 expression in the brain parenchyma in the other timepoints (5 hours, 7 days and 10 days). It was observed that MMP9 is expressed in endothelial cells in all timepoints. Interestingly, at 7 and 10 days, when there are well-established metastasis, the expression of MMP9 could be detected in metastasis associated vessels (Fig. 5 a). Moreover, at the same timepoints, MMP9 expression could also be detected in well-established metastases (Fig. 5 b). In this case, no semi-quantitative analysis was possible, due to the high background, which might also be related to the extracellular presence of MMP9, but this should be further confirmed.

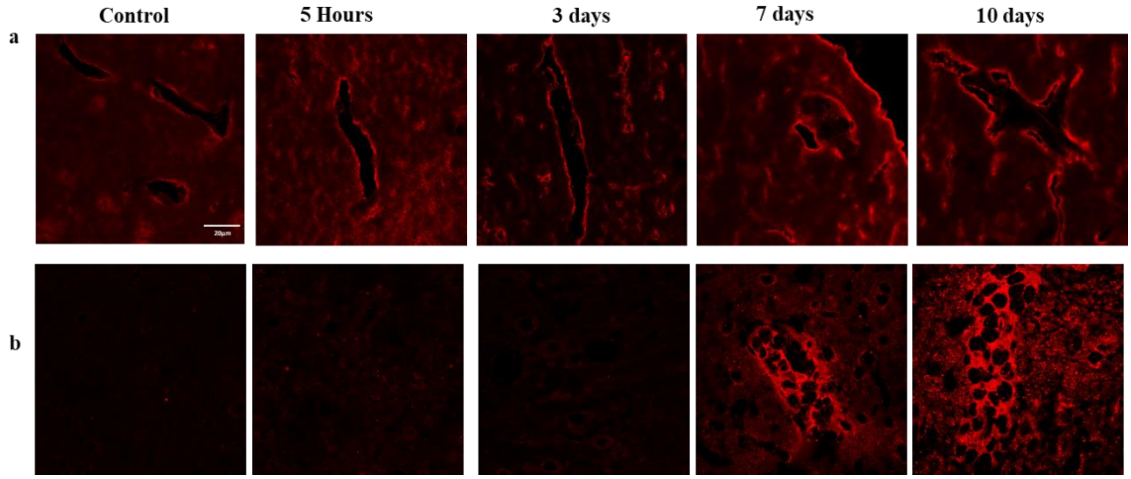

Figure 5 - MMP9 expression in the brain parenchyma, during metastasis development. Immunofluorescence labelling was performed for MMP-9 for the different timepoints after injections of 4T1 cells. MMP9 expression was detected in vessels in controls and different timepoints after 4T1 cells injection. At 7 days and at 10 days, brain metastasis-associated vessels expressed MMP9 (a). It was also observed that at 7 and 10 days, well established brain metastasis presented MMP9 cytosolic expression.

These results support the oncogenic role of MMP9, previously described in the literature and, apparently no inhibition by miR-92a-1-5p is noticeable. This could mean

that either this interaction is a false positive by bioinformatical analysis or that the action of this miRNA is not enough to inhibit the elevated expression of MMP9.

3.6. MEF2C, a predicted target for miR-802-5p and miR-194-5p is highly expressed in malignant cells

Myocyte enhancer factor 2C (MEF2C), part of the family of transcription factors MEF2, was initially described to be activated during embryogenesis to regulate tissue specific gene expression and promote organ development. Nowadays, it is widely accepted that MEF2C is also expressed during adult life in many types of cells, including skeletal, smooth and cardiac muscle cells, as well as in neuronal, chondroid and endothelial cells, and in lymphocytes.[92] There is yet no proof of any involvement of MEF2C in the brain metastasization process; however, MEF2C has a widely accepted role in angiogenesis by interacting with VEGF. MEF2C modulates the effects of VEGF in endothelial cells, including migration, but can also control VEGF at a transcriptional level. In turn, VEGF activates MEF2C, through p38MAPK and calcineurin pathways.[56,93] Moreover, MEF2C has also been proposed as a novel candidate oncogene by mediating VEGF induction of vasculogenic mimicry and angiogenesis, migration and invasion, though evidence is limited to very few types of malignancies.[55,94]

The *MEF2C* gene was predicted as a target for miR-802-5p (Table 4). Although for the repression of *MEF2C* by miR-802-5p, the cumulative weighted context score ++ was low (Cumulative weighted context score ++=-0,03), the miTG was quite high (miTG= 0,9523) and, among the predicted targets, it was the one with the highest PCT (PCT=0,71). Moreover, *MEF2C* was also a predicted target for miR-194-5p, the other miRNA that was validated as being downregulated in plasma (Cumulative weighted context score ++=-0,16; miTG= 0,74; PCT=0,55) (Supplementary Table 1). Altogether, this strengthens the possibility that MEF2C is involved in the brain metastasization process. So, we assessed MEF2C expression in the brain parenchyma, during metastatic development (Fig. 6) for all timepoints. Due to its previously described role in endothelial cells and angiogenesis, we studied its expression by immunofluorescence together with claudin-5, a protein highly expressed in brain endothelial TJs.[95] Curiously, no co-localization was detected between claudin-5 and MEF2C but MEF2C expression was found in perivascular malignant cells (Fig. 6 a). To confirm the nature of these cells, we double labelled MEF2C with an epithelial marker, pan-cytokeratin, also shown by our group to be expressed in isolated malignant cells and well-established metastases.[75]

Indeed, we observed a colocalization of MEF2C with pan-cytokeratin and could confirm that, from 3 days onwards MEF2C is highly expressed in malignant cells isolated and in well-established metastasis (Fig. 6 b). Analysis of MEF2C immunoreactivity showed an increase of MEF2C expression/ tumor area in metastasis throughout time. An increase about 24% was observed between 3 days and 10 days ($p < 0.01$) of and of 20%. between 7 days and 10 days ($p < 0.01$) (Fig. 6 c).

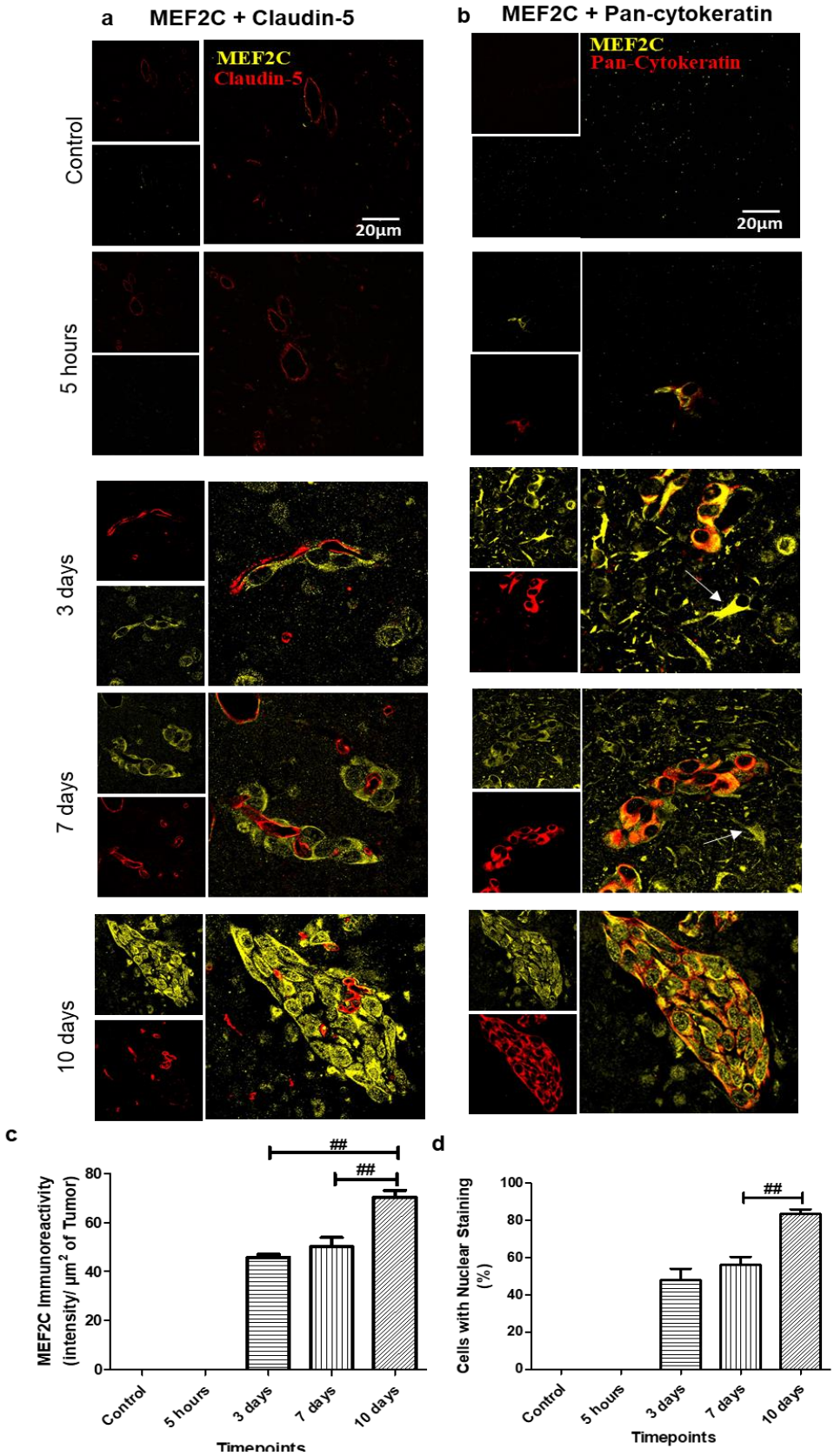

Figure 6 - MEF2C expression in the brain parenchyma along brain metastasization. Immunofluorescence analysis of MEF2C in brain sections for different timepoints after 4T1 or vehicle (control) injection. Double labeling with claudin-5 showed that MEF2C is not expressed in endothelial cells but only in perivascular cells (a). Double labeling immunofluorescence analysis of MEF2C and pan-Cytokeratin revealed that MEF2C positive cells are tumor cells (b). MEF2C labeling was also observed in other star-shaped cells of unknown origin (arrows) (b). Semi-quantitative analysis of MEF2C expression along time showed an increasing expression in tumor cells (c) and an increasing nuclear translocation of MEF2C (d). ##p<0.01 between indicated groups.

Altogether these results show that contrary to what was expected, MEF2C is not expressed in endothelial cells but it is highly expressed in brain metastatic cells and in well-established metastasis. Moreover, MEF2C is increasingly expressed by tumor cells throughout time, which supports the involvement of this transcription factor in metastatic development and the downregulation of the MEF2C gene by one or both miRNAs, miR-802-5p and miR-194-5p.

3.7. MEF2C translocates to the nucleus in advanced stages of metastasis development

Since MEF2C is known to be a transcription factor, it is synthetized in the cytoplasm, like other proteins but after certain stimuli, it can be activated and translocated from the cytoplasm into the nucleus.[96] In fact, MEF2C is mostly concentrated in the cytoplasm at 7 days, while at 10 days it is more homogenously expressed between the nucleus and the cytoplasm (Fig. 6). An analysis of the number of cells with nuclear staining per metastasis for the different timepoints showed that there is a significant increase of the percentage of cells with nuclear staining of approximately 29% between 3 and 10 days ($p < 0.005$) and of 32% between 7 and 10 days ($p < 0.01$) with no significant differences between 3 and 7 days (Fig. 6 d). These results show that there is a nuclear translocation of MEF2C in later timepoints, indicating that MEF2C is more active in later stages of the metastatic development and pointing to this transcription factor as a potential target for modulation.

3.8. MEF2C is highly expressed by peritumoral astrocytes

Cells belonging to the neurovascular unit, including microglia, astrocytes and pericytes, are known to interact with malignant cells after the transmigration through the BBB.[6] Regarding MEF2C expression in the brain parenchyma during metastatic development, another interesting observation was that, as early as 3 days, but not in controls, MEF2C is also expressed in other type of star-shaped cells close to metastasis (Fig. 6 b – Arrows),. To clarify the nature of these cells, we performed double labelings of MEF2C with markers for different cells of the neurovascular unit. Double labelings with Iba-1 and CD13, markers for microglia and pericytes, respectively, showed no colocalization with MEF2C (Fig. 7 a and b). However, double-staining with GFAP, a marker for astrocytes, showed colocalization with MEF2C in such star-shaped cells (Fig. 7 c, d). Curiously, not all GFAP-expressing cells express MEF2C as MEF2C labeling was mainly found in GFAP-expressing cells close to metastasis (Fig. 7 d -arrows),

contrary to the ones that were further away from the tumors and that only expressed GFAP.

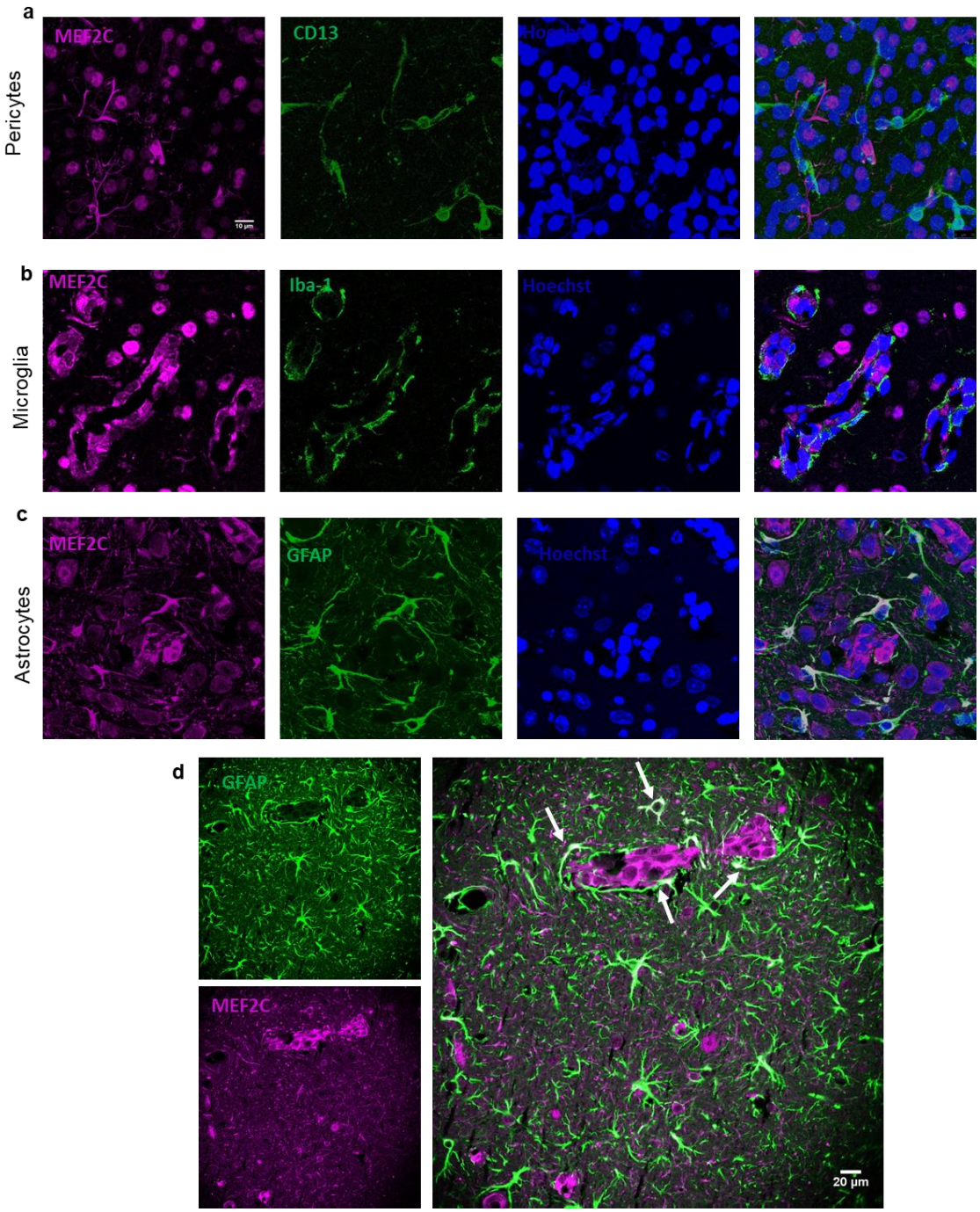

Figure 7 - MEF2C expression in the different cell of the neurovascular unit. Brain sections from 4T1-Injected mice were analysed, to study the expression of MEF2C in non-tumoral cells, in close proximity to tumour cells. Double labelings were performed for MEF2C, together with markers for pericytes (CD13) (a), microglia (Iba-1) (b) and astrocytes (GFAP) (c and d). Colocalization was only observed between MEF2C and GFAP (c) and pictures taken with a lower magnification showed that mainly astrocytes that are close to the tumour (arrows), express MEF2C, while astrocytes that are further from the tumour, only express GFAP (d). The different channels of the labelling are presented, as well as the merged pictures. Hoechst is labelling the nucleus.

These results reveal that peritumoral astrocytes, rather than distant ones or other neurovascular unit cells, express MEF2C, remaining to explore the significance of these findings in metastasis development.

3.9. MEF2C is highly expressed in brain metastases from triple-negative BC, in humans

The results obtained for the MEF2C expression were quite interesting but still, there are yet no publications that study MEF2C expression in human cancer tissues. To understand if the results previously obtained in mouse brain sections are translatable to humans, we performed immunofluorescence analysis of sections from resected brain metastases, derived from triple-negative BC in human patients. To distinguish tumoral from peripherical tissue, a double staining with pan-cytokeratin was performed. We can observe that although there are some MEF2C staining in the surroundings of the tumors, the areas where MEF2C is stronger, colocalize with the staining for pan-cytokeratin (Fig.8).

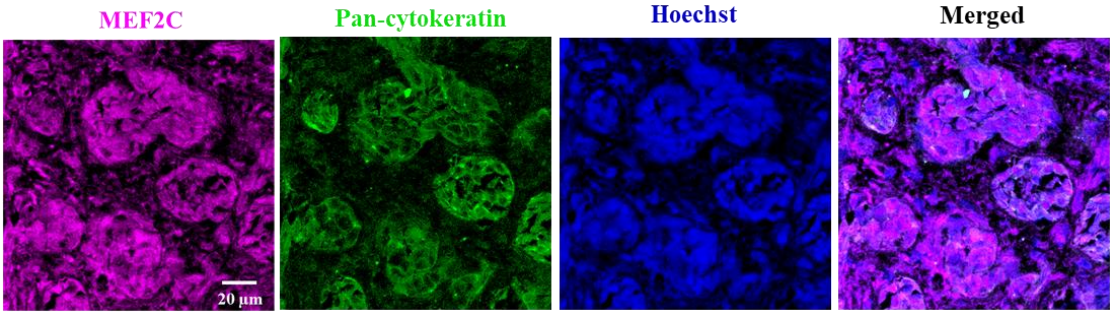

Figure 8 - MEF2C expression in human brain metastases from triple negative breast carcinoma. Double labelling immunofluorescence analysis was performed for MEF2C with pan-cytokeratin in sections from resected brain metastasis, from triple negative breast cancer patients. ME2C shows colocalization with pan-cytokeratin. The different channels of the labelling are presented, as well as the merged pictures. Hoechst is labelling the nucleus.

From these results, in accordance with the results obtained for mouse brain sections, we can conclude that MEF2C is expressed in triple negative breast carcinoma brain metastases.

4. Discussion

Due to the improved techniques for early detection of breast cancer, together with the development of therapeutic approaches, metastasis are presently the major problem in Oncology. Brain metastasis are particularly challenging since they are usually detected upon appearance of clinical manifestations, which corresponds to a stage of the disease with poor prognosis, inasmuch the BBB restricts the therapeutic options. Therefore,

discovery of early biomarkers of brain metastasization would allow a precocious intervention and improve the patients' outcome. Based on the fact that circulating miRNAs have increasingly been recognized as specific and sensitive biomarkers of different types of cancer,[97] we hypothesized that miRNAs are aberrantly expressed in plasma samples prior to the establishment of brain metastasis, which would allow their early detection by in minimally invasive liquid biopsies. Our study revealed the precocious alteration in the expression levels of several miRNAs, and further identified miR-802-5p, miR-194-5p and miR-92a-1-5p as potential biomarkers. Moreover, MEF2C emerged as an interesting miRNA target, opening new avenues for its modulation in order to abrogate BCBM.

Among the different types of BC, triple negative BC is the most aggressive and the one with the highest predisposition to develop brain metastases.[98] So, we used the murine mammary carcinoma 4T1 cell line, which is an aggressive triple negative tumor model that is highly tumorigenic and invasive and, unlike most tumor models can spontaneously metastasize to multiple distant sites including liver, lung, brain and bone, making it a good model for metastatic breast cancer.[16,17] The cells were injected in the common right carotid artery, which is frequently used for animal models of brain metastases.[99] To confirm brain specificity, metastatic progression was followed in different brain regions and peripheral organs. Whereas no metastasis were observed in kidney or liver, they were detected in the lung. Interestingly, the tumor area in the lung was similar to that of cerebellum – which was the least affected of the studied brain regions. This could be possibly justified by the high affinity of 4T1 cells for the lungs, besides the brain.[16] Still, we could confirm that this model strongly metastasizes to the brain.

Since we were interested in the role of circulating miRNAs as biomarkers for BCBM, first we did an analysis of the miRnome in plasma by NGS, which confirmed our hypothesis that cell free miRNAs are early deregulated during BCBM development and provided insights about the cell-free miRNAs behavior in plasma along metastatic progression. Based on the pattern of metastasis development, it is conceivable that the observed alterations in the plasma miRNA levels are mainly due to metastasization to brain. Moreover, the analysis of the NGS results showed that the number of deregulated miRNAs increases with tumor progression, supporting that this deregulation is somehow related with metastatic development. However, the source of this deregulation should be further studied, though it has been proposed that it can come either from malignant cells,[100] or as a response from the affected organs,[101] as conceivably the brain. Upon tumor

cell injection, the brain pre-metastatic niche can modulate the levels of the miRNAs to create a tumor favorable environment, as it has been described.[102] Another relevant observation regarding NGS results is that miRNA levels are dynamic and are changing with time and the same miRNAs that are deregulated in earlier stages of the tumor are not the same that are deregulated in more advanced stages, except for a small number of miRNAs that are deregulated throughout the metastatic development. This is supported by a recent study, showing that miRNA expression profiles can efficiently distinguish and categorize BC patients into early and advanced stages.[103]

Since we were looking for early and predictive biomarkers of BCBM, we focused on the miRNAs that are deregulated in earlier stages of metastatic progression. An early screening of the NGS results pointed to the downregulation of miR-194-5p, miR-802-5p, miR-145-5p and miR-200b-3p and the upregulation of miR-205-5p, miR-181a-1-3p, miR-92a-1-5p and miR-375-5p as a predictive miRNA expression profile for BCBM. Since miRNAs mainly act by negatively regulating gene expression, it is predictable that the downregulated miRNAs mainly act as tumors suppressors by downregulating oncogenes, while the upregulated miRNAs should work as oncogenes, mainly by downregulating tumor suppressor genes.[104] Although these miRNAs have never been described in the development of BCBM, other studies support these tumor suppressive/oncogenic roles in other BC or metastatic conditions.[105-111] Considering that NGS data needs to be validated by RT-PCR and that so far only miR-802-5p and miR-194-5p could be validated, these two are the ones that can be considered, for now, as potential predictive biomarkers for BCBM, by being downregulated prior to metastases development. RT-PCR analysis of miR-92a-1-5p revealed an increase of about 80%, although statistical significance was not achieved. So, further experiments are in progress to confirm this miRNA as another BCBM early biomarker.

The downregulation of miR-802-5p has been described for different types of cancer. Particularly, in prostate cancer, miR-802-5p was described to inhibit EMT, an essential step for metastases development, by downregulating *flotillin-2*, a known downstream gene of p53. Accordingly, forced expression of miR-802-5p, led to a decrease in mesenchymal markers and suppressed metastatic ability of cancer cells.[112] miR-802-5p downregulation has also been proposed to lead to increased Wnt activation in pancreatic adenocarcinoma.[113] As for BC, a study by Yuan *et al*[106] showed that BCCs presented a lower expression of miR-802-5p, comparing to normal breast epithelial cells and both *in vitro* and *in vivo* experiments showed that overexpressing miR-802-5p decreased BC

proliferation, through downregulation of FoxM1. These evidences support that miR-802-5p can have a tumor suppressive role and that its downregulation may play a role in the metastatic progression, reinforcing the hypothesis that this miRNA can be used as a biomarker for BCBM. Regarding miR-194-5p, more ambiguous results are found in the literature. In BC, miR-194-5p was first described as having tumor suppressive roles by inhibiting proliferation and migration *in vitro* and *in vivo*.[105] However, a recent paper demonstrated that miR-194-5p enhances cell proliferation, migration and invasion in different BC cell lines by regulating the Wnt/β-catenin pathway.[114] These contradicting results do not invalidate our results but can, instead, be a consequence of the multitude of genes and functions that one single miRNA can regulate and how they can be influenced by multiple factors, including different cellular contexts.[115] The results of the bioinformatical target prediction performed for miR-802-5p and miR-194-5p further supported the tumor suppressive roles of both miRNAs, since, many of the predicted targets have been described as oncogenes in BC, or other types of cancer and to promote metastatic development. For miR-92a-1-5p, the results were not so conclusive: we observed a trend of this miRNA to be upregulated, so, it would be expectable that miR-92a-1-5p would target tumor suppressor genes. However, many of the predicted targets for this miRNA have not been describes in cancer, yet, and the ones that have been, are mainly described as oncogenes. Moreover, there is a lack of information about the role of miR-92a-1-5p in cancer, although miR-92a has also ambiguous roles in cancer. MiR-92a has been known to be a part of the miR-17-92 cluster, whose overexpression is a key event in different cancers.[116] Mir-92a, has also been shown to have a pro-tumorigenic effect in gliomas by directly downregulating Bim, a Bcl-2 interacting mediator of cell death, and inhibiting apoptosis.[117] In BC, miR-92a was proposed to have an opposite role, since the downregulation of miR-92a has been directly related with aggressive BC features and to increase cell migration.[118] Once again these contradictory evidences do not invalidate its role as a potential biomarker for BCBM. Still, since we are studying very early stages in metastasis development, an interesting hypothesis to further study would be whether miR-92a-1-5p is being released by tumor cells or brain cells and if it works as an oncogene and promote tumor progression or if it is an immediate response from the organism, for example brain cells, as a mechanism of defense, thus having tumor suppressive functions.

MiRNAs seem very promising and useful to be used as biomarkers for different types of cancer but as the usage of miRNAs or anti-miRs as therapeutics still carries many

challenges, especially off-target effects, due to the multitude of genes that can be regulated by one single miRNA.[119] However, the dysregulation of a miRNA also implies the dysregulation of its targets, which can also be used for modulation in cancer. Thus, having proposed three miRNAs as potential early biomarkers for BCBM, the bioinformatical target prediction was performed to have better insights about the mechanisms and pathways in which these three miRNAs can be involved during brain metastases development. It should be noted that bioinformatical tools for target prediction are not 100% accurate and have a high rate of false positives.[120] So, the direct miRNA-mRNA for the predicted targets should be validated in further studies, by using luciferase assays , co-expression of the miRNA and target mRNA and studying the effects of the miRNA on the target protein.[121] Still, it allowed an initial screening of potential targets for modulation to prevent or abrogate BCBM.

The expression of MMP9, a predicted target for miR-92a-1-5p, was studied in the brain parenchyma during metastatic progression. MMP9, as most MMPs, has an established role in cancer and can be produced by BCCs to degrade the ECM, breaking cell-cell and cell-ECM interactions, thus promoting invasiveness and metastases.[84] Moreover, it has been described that tumor-secreted MMP9 can have an active role increasing the BBB permeability to promote transmigration of brain metastatic cells.[122] Accordingly, MMP9 has also been described to have a high expression in BCBM.[86] This is line with our results, that reveal that MMP9 is highly expressed in well-established BCBM, confirming that it should have a tumor promoting role. Moreover, we also detected the expression of MMP9 in brain vasculature and in tumor associated-vessels. The expression of MMP-9 in brain endothelial cells has been previously described and suggested to be related with the disruption of the BBB,[123] although we found MMP9 not only in the microvasculature but mainly in larger vessels. The expression of MMP9 in metastasis associated vessels can be related with a proangiogenic role of MMP9 that promotes tumor vascularization, by activating VEGF pathways.[124] Despite these results, no evident correlation between miR-92a-1-5p and MMP9 was found, which can indicate that this relation was a false-positive by the bioinformatical target prediction or that the repression by miR-92a-1-5p is not enough to suppress the high production of MMP9 by endothelial and tumor cells. Still, MMP9 can also be considered as a relevant target to modulate in BCBM, thus preventing, not only migration and invasion but also the neovascularization of the tumor.

Finally, we studied MEF2C, which was a predicted target for both miR-802-5p and miR-194-5p. MEF2C has recently been proposed as a new oncogene, though this has only been studied in a very restricted number of malignancies.[55,94] MEF2C expression was previously observed in primary BC tissues and to be activated by p38MAPK in metastatic breast cancer.[125] MEF2C has also been proposed to promote metastasis in pancreatic adenocarcinoma by inducing MMP10 transcription[94] and in hepatocellular carcinoma by mediating VEGF induction of vasculogenic mimicry, migration and invasion.[55] Studying its expression in the brain parenchyma revealed that MEF2C is highly expressed in BCBM and that its expression increases with tumor progression. This had never been described before but suggests an oncogenic role for MEF2C in promoting BCBM. Furthermore, a nuclear translocation of MEF2C was observed in later stages of tumor progression. Since MEF2C is a transcription factor, the natural assumption is that this translocation means a higher activation of MEF2C to promote its target genes transcription, like the aforementioned VEGF and MMP10, and support metastatic growth. However, *Bai et al*[55] postulated that cytosolic MEF2C inhibits β-catenin translocation to the nucleus, while its nuclear translocation is associated with the activation of intracellular calcium signaling induced by β-catenin. Nuclear β-catenin is essential for the activation of the Wnt signaling, which has a well-known role in promoting brain metastases from triple negative BC.[126] So, a nuclear translocation in advanced stages of BCBM can not only be related with the transcription of target oncogenes but also with an higher activation of the Wnt signaling.

Astrocytes are key components of the brain metastatic microenvironment and are determinant for malignant cells fate in the brain. Although, initially, astrocytes have an harmful role for tumor cells, in later stages astrocytes share a bidirectional communication with tumor cells, and produce proteases, growth factors and inflammatory cytokines, to support tumor growth.[6] MEF2C expression has previously been described as a regulator of the inflammatory response by microglia, during aging[127] but its expression in astrocytes has not been described, yet. Here, we show that astrocytes start expressing MEF2C after tumor cells extravasation. Interestingly, mainly peritumoral astrocytes express MEF2C, contrarily to non-peritumoral astrocytes. This can indicate that MEF2C is involved in the crosstalk between astroglial cells and tumor cells during BCBM formation, to support tumor growth. Since MEF2C was initially studied, due to its targeting by miR-802-5p and miR-194-5p, we can hypothesize that astrocytes are expressing one or both of these miRNAs, under physiological conditions and upon tumor

cells extravasation, astrocytes downregulate these miRNAs expression to relief MEF2C inhibition and promote tumor growth. This possibility should be further studied and validated. Altogether this study points to MEF2C as a key player in BCBM and as a potential target for modulation. The detection of MEF2C in human brain metastases samples from triple negative BC, further supported this statement.

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Chapter III

Concluding Remarks

Concluding Remarks

The usage of microRNAs (miRNAS or miR-) as biomarkers for cancer is an emerging field. Particularly, circulating miRNAs exhibit clinical potential as minimally invasive and specific biomarkers for different types of cancer. With this work, summarized in Figure 1, we present new insights about the deregulated expression of circulating miRNAs in metastatic breast cancer and specifically throughout progression of breast cancer brain metastases (BCBM). We showed that during BCBM formation, circulating miRNAs are deregulated in a time-dependent manner and that the miRNAs that are deregulated in earlier stages of tumor progression are not the same that are deregulated in later stages, confirming their potential as biomarkers for this condition and as indicators of tumor stage and malignancy. We observed that circulating miRNAs are deregulated even before the brain metastases are detected and so we propose that the deregulation of certain miRNAs could predict the occurrence of brain metastasis in breast cancer patients, pointing to the downregulation of miR-802-5p, miR-194-5p and the upregulation of miR-92a-1-5p, in plasma as risk factors for the development of BCBM. Although miRNAs can be very useful as biomarkers for cancer, their use as therapeutics still has many constraints. So, instead, we point to miRNA targets as new candidates for modulation and prevention or treatment of BCBM and selected some relevant targets for further studies, including matrix metalloproteinase 9 (MMP9) and myocyte enhancer factor 2C (MEF2C). Importantly, we described for the first time ever the overexpression of MEF2C in brain metastasis in mouse and human samples, and proposed a tumor supporting role for this transcription factor, possibly through the upregulation of its downstream genes and by being actively involved in the crosstalk between astrocytes and malignant cells. Our results point to MEF2C as new key player in BCBM development, thus opening new avenues in the search of therapeutic targets for the abrogation of BCBM.

Although our results are very promising and pave the way for the use of miRNAs in liquid biopsies, further studies are still needed to understand the origin and target cells of the miRNAs, as well as their specific roles in the brain metastasization process. Moreover, it would be relevant to study the selected miRNAs expression in different animal models of BCBM, to confirm their aberrant expression and specificity. The interaction of miRNAs and their proposed targets should also be validated, particularly the downregulation of MEF2C by miR-802-5p and/or miR-194-5p. Further studies are also needed to understand

the exact role of MEF2C in brain metastasis development and whether the inhibition of this transcription factor can decrease brain metastasis occurrence in breast cancer.

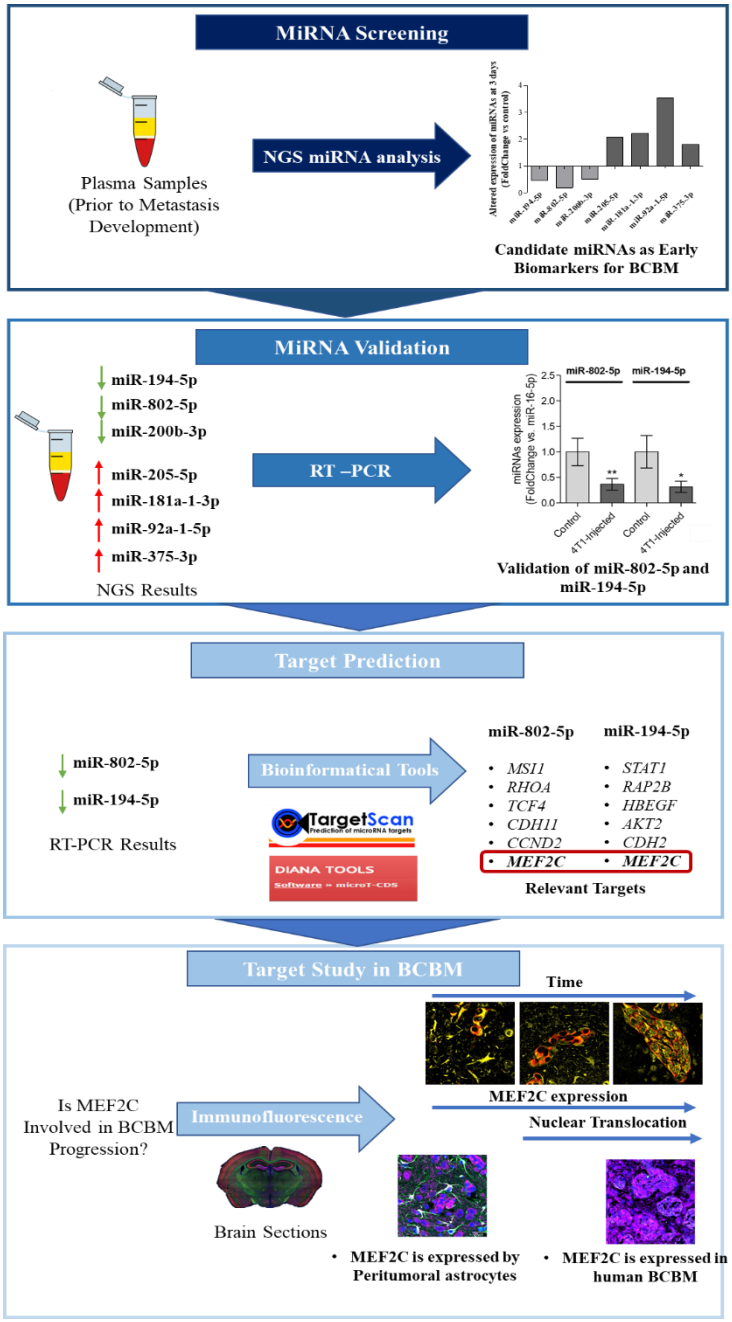

Figure 1 – Schematic representation of the major findings of this thesis. Plasma samples were collected from mice inoculated with 4T1 cells and vehicle (control), prior to metastases. An initial next generation sequencing analysis allowed us to do an initial screening of the miRNAs that are aberrantly expressed in 4T1 injected mice, thus selecting 8 miRNAs for further validation, as potential biomarkers for breast cancer brain metastases (BCBM). Using RT-PCR, the downregulation of 2 of those 8 miRNAs could be validated as early biomarkers for BCBM: miR-802-5p and miR-194-5p. From a bioinformatical target prediction for these miRNAs, followed by a bibliographical search, we selected the targets that could be relevant during metastatic progression. MEF2C was a common target gene for miR-802-5p and miR-194-5p. So we evaluated MEF2C protein expression in the brain parenchyma during BCBM formation, which showed that MEF2C is increasingly expressed in BCBM, suffering a nuclear translocation at more advanced stages of tumor progression. Moreover, MEF2C is also expressed in peritumoral astrocytes, pointing to a potential role in the crosstalk between malignant and astroglial cells. MEF2C expression was confirmed in human BCBM. Altogether, these results point to plasma miR-194-5p and miR802-5p as potential biomarker for the early detection of BCBM and to MEF2C as a new target for modulation to abrogate BCBM.

Annexes

Supplementary Table 1 – Results of the Target prediction, using Target Scan v.7.2 and Diana Tool MicroT-CDS v.5.0, for miR-194-5p

Target Gene	Cumulative weighted context++ score	Total context++ score	Aggregate PCT	MiTG	Target Gene	Cumulative weighted context++ score	Total context++ score	Aggregate PCT	MiTG
TRIM23	-0.5	-0.51	0.32	0.981011	AKT2	-0.15	-0.15	0.53	0.773286
TMED5	-0.49	-0.73	0.33	0.831892	ARHGAP24	-0.15	-0.18	0.55	0.796644
SLC10A7	-0.49	-0.7	0.14	0.920473	LPHN2	-0.15	-0.15	0.55	0.841762
ERGIC2	-0.46	-0.46	0.55	0.999352	ERBB4	-0.15	-0.18	0.55	0.903769
TEFM	-0.45	-0.47	< 0.1	0.982956	HIAT1	-0.15	-0.2	0.42	0.864159
STAT1	-0.45	-0.55	< 0.1	0.762706	AMD1	-0.14	-0.32	0.18	0.738216
RAP2B	-0.44	-0.73	0.79	0.813895	CAMK2G	-0.14	-0.14	0.55	0.835199
MID1IP1	-0.43	-0.43	0.33	0.963489	TRIP12	-0.14	-0.2	0.46	0.974147
NUDC	-0.42	-0.52	0.55	0.975467	CHD6	-0.14	-0.14	0.43	0.727125
PAIP2	-0.4	-0.4	0.55	0.954517	PSME3	-0.14	-0.14	0.34	0.720207
ATP6V1H	-0.4	-0.4	0.2	0.715332	RHEB	-0.14	-0.31	0.55	0.855686
SLC7A5	-0.39	-0.42	0.41	0.914903	DNMT3A	-0.14	-0.29	0.79	0.938058
SLK	-0.38	-0.39	0.31	0.981054	DAAM1	-0.14	-0.19	0.3	0.875616
PTPLB	-0.37	-0.37	< 0.1	0.816621	PAFAH1B1	-0.14	-0.15	0.55	0.897828
FAM107B	-0.36	-0.65	< 0.1	0.804205	SGPP2	-0.14	-0.22	< 0.1	0.765868
SNAP91	-0.36	-0.36	0.13	0.872915	SYVN1	-0.13	-0.14	0.55	0.859452
GRHL3	-0.35	-0.35	0.16	0.891536	ACHE	-0.13	-0.39	0.17	0.973065
SLTM	-0.35	-0.36	0.39	0.999316	ELF2	-0.13	-0.13	0.55	0.796334
RSBN1L	-0.35	-0.41	0.57	0.992971	OTP	-0.12	-0.12	0.55	0.710157
SEPHS1	-0.33	-0.37	0.57	0.994116	LIN28B	-0.12	-0.12	< 0.1	0.713877
SETD8	-0.33	-0.33	0.49	0.899924	PVRL4	-0.12	-0.12	< 0.1	0.722846
GOT2	-0.31	-0.43	0.46	0.999811	SETD5	-0.12	-0.2	0.47	0.998517
RFX6	-0.3	-0.3	0.27	0.943858	SNX1	-0.11	-0.16	0.69	0.781367
DUSP9	-0.29	-0.29	0.44	0.733478	PPARGC1A	-0.11	-0.11	0.55	0.811809
DCUN1D5	-0.29	-0.4	0.55	0.908211	OPCML	-0.11	-0.11	0.55	0.87958
TSPAN7	-0.29	-0.29	< 0.1	0.778829	ARID4A	-0.11	-0.11	0.55	0.8312
ARID2	-0.28	-0.28	0.4	0.82587	ITGB6	-0.11	-0.11	< 0.1	0.838143
ARF4	-0.28	-0.28	0.44	0.834818	BICD2	-0.1	-0.26	< 0.1	0.933151
RAB6B	-0.28	-0.28	0.64	0.953928	ONECUT2	-0.1	-0.11	0.62	0.930996
HNF1B	-0.28	-0.28	0.42	0.749551	SATB1	-0.1	-0.13	0.55	0.767405
FBXW7	-0.27	-0.27	0.48	0.990766	ASAP1	-0.1	-0.1	0.39	0.990011
PAK2	-0.27	-0.27	0.14	0.73961	CHD8	-0.1	-0.1	0.26	0.830213
GMFB	-0.27	-0.29	0.26	0.734658	SLC12A6	-0.09	-0.17	0.51	0.857187
KLF7	-0.26	-0.27	0.55	0.952678	MEX3A	-0.09	-0.11	0.28	0.787349
ACBD3	-0.26	-0.27	< 0.1	0.894665	SEMA6A	-0.09	-0.09	0.54	0.714767
ARHGAP21	-0.26	-0.26	0.29	0.976948	HMG20A	-0.08	-0.14	0.35	0.799471
DACH1	-0.26	-0.28	0.22	0.998087	PRKD3	-0.08	-0.33	0.55	0.894082
LPIN2	-0.25	-0.26	0.55	0.891814	RSBN1	-0.08	-0.08	< 0.1	0.733451
PHYHIPL	-0.25	-0.39	< 0.1	0.840235	CLCN5	-0.08	-0.16	0.46	0.852687
FKBP6	-0.25	-0.25	< 0.1	0.817262	MGAT4A	-0.08	-0.17	0.55	0.906412
PRSS53	-0.25	-0.25	0.54	0.928544	RFX3	-0.08	-0.26	0.37	0.904627
HBEGF	-0.25	-0.66	0.69	0.999341	E2F3	-0.08	-0.08	0.54	0.813987
PFN2	-0.23	-0.24	0.55	0.882766	PITPNM2	-0.08	-0.1	0.14	0.895725
SGCE	-0.23	-0.23	0.55	0.851101	TNPO1	-0.07	-0.11	0.55	0.78671
HNRNPA0	-0.23	-0.23	0.55	0.780024	OTUD4	-0.07	-0.07	< 0.1	0.847282
EXOC5	-0.23	-0.23	0.46	0.751349	ADAM12	-0.07	-0.08	< 0.1	0.73022
ELMSAN1	-0.23	-0.23	0.56	0.996329	STAT5B	-0.07	-0.17	0.55	0.872417
FMR1	-0.23	-0.34	< 0.1	0.994802	PPP3R1	-0.07	-0.16	0.23	0.758001
UBE2V2	-0.22	-0.54	0.55	0.787566	SDAD1	-0.06	-0.13	0.55	0.772853
VAPA	-0.22	-0.84	0.69	0.929783	FBRS	-0.06	-0.06	< 0.1	0.899171
FOXA1	-0.22	-0.22	0.36	0.816252	NUCKS1	-0.06	-0.2	0.55	0.714046
NRP1	-0.22	-0.27	0.55	0.894971	LSAMP	-0.06	-0.23	0.43	0.85762
TLN2	-0.22	-0.22	0.56	0.739586	OSBPL8	-0.05	-0.46	0.47	0.87202
SP3	-0.22	-0.22	0.55	0.719156	PTPRD	-0.05	-0.1	0.55	0.954903
KCMF1	-0.21	-0.21	0.55	0.842746	PPFIBP1	-0.05	-0.09	< 0.1	0.845036
EIF4G2	-0.2	-0.2	0.54	0.816995	TRPS1	-0.05	-0.1	0.51	0.998299
ENOX1	-0.2	-0.39	0.38	0.999234	JMJD1C	-0.05	-0.05	0.55	0.845181
NETO1	-0.2	-0.33	0.76	0.936017	ZFHX4	-0.05	-0.05	0.46	0.94843
SALL1	-0.2	-0.54	0.46	0.998889	RUNX3	-0.05	-0.09	< 0.1	0.889468
REV3L	-0.2	-0.2	0.54	0.981759	CCR10	-0.05	-0.36	0.4	0.743165
TMED9	-0.2	-0.4	0.55	0.944431	MAP2	-0.04	-0.06	0.35	0.964614
DUSP10	-0.19	-0.19	0.55	0.734887	NAA50	-0.04	-0.32	0.57	0.979536
KMT2C	-0.19	-0.19	0.61	0.864119	ITPKB	-0.04	-0.04	0.38	0.982141
DR1	-0.19	-0.25	0.55	0.773841	DMD	-0.03	-0.2	0.51	0.826378

PPP2R2C	-0.19	-0.19	0.5	0.812456	CHD1	-0.03	-0.17	0.49	0.901686
SET	-0.19	-0.19	0.55	0.83123	SOX6	-0.03	-0.09	0.52	0.889379
PRKAR1A	-0.19	-0.26	0.53	0.812713	SMURF1	-0.03	-0.11	0.55	0.915273
BCLAF1	-0.19	-0.34	0.55	0.881355	SUFU	-0.02	-0.04	< 0.1	0.787976
TTC7B	-0.18	-0.3	0.33	0.735821	CEP350	-0.02	-0.02	< 0.1	0.724434
WAPAL	-0.18	-0.22	0.33	0.9626	SLC30A10	-0.02	-0.12	0.55	0.875189
MEIS2	-0.18	-0.18	0.43	0.944928	BIRC6	-0.02	-0.03	< 0.1	0.803671
SLC30A1	-0.18	-0.2	0.2	0.870839	CAPZB	-0.02	-0.18	0.55	0.708462
COL4A3BP	-0.18	-0.29	0.43	0.92011	NFAT5	-0.02	-0.04	0.53	0.913664
CHD4	-0.18	-0.18	0.43	0.889681	UBR5	-0.01	-0.12	0.51	0.774026
CRK	-0.18	-0.18	< 0.1	0.856811	GIGYF1	-0.01	-0.01	< 0.1	0.900373
SDC4	-0.18	-0.32	0.52	0.787513	PDHB	-0.01	-0.27	0.64	0.721324
PHF21A	-0.17	-0.17	0.55	0.744362	CADM1	0	-0.24	0.9	0.972289
CDH2	-0.17	-0.17	0.55	0.796689	LRRC4C	0	-0.28	0.53	0.908255
TCF7L2	-0.17	-0.17	0.55	0.761728	ZBTB20	0	-0.2	0.61	0.939419
LHX6	-0.17	-0.17	0.55	0.801319	TSC22D2	0	-0.08	0.55	0.793327
DHX15	-0.17	-0.17	0.55	0.808113	NCL	0	-0.27	0.54	0.707306
CEP170	-0.17	-0.17	0.14	0.945352	SUMO2	0	-0.53	0.57	0.999546
NRIP1	-0.16	-0.16	0.55	0.820419	RABAC1	0	-0.76	0.22	0.961871
MEF2C	-0.16	-0.27	0.55	0.741349	UBE2D3	0	-0.3	< 0.1	0.913441
ENAH	-0.16	-0.18	0.55	0.863833					

Supplementary Table 2 – Results of the target prediction. using TargetScan v.7.2. and Diana Tools MicroT-CDS. for miR-145-5p

Target Gene	Cumulative weighted context++ score	Total context++ score	Aggregate PCT	MiTG	Taget Gene	Cumulative weighted context++ score	Total context++ score	Aggregate PCT	MiTG
FSCN1	-1.19	-1.21	> 0.99	0.990026	SMCR8	-0.19	-0.2	0.35	0.841142
ABRACL	-0.9	-0.91	0.69	0.989389	RBPMS	-0.19	-0.19	0.61	0.914387
FLI1	-0.89	-0.89	> 0.99	0.999025	MPZL1	-0.19	-0.19	0.71	0.703166
SMCP	-0.69	-0.69	0.42	0.723234	ARPC5	-0.19	-0.22	0.55	0.726186
GLIS1	-0.67	-0.67	0.89	0.990084	KLHDC10	-0.18	-0.18	0.68	0.704933
FKBP3	-0.62	-0.62	0.59	0.719159	FOXO1	-0.18	-0.18	0.58	0.700853
YTHDF2	-0.56	-0.56	0.7	0.972194	MAPK4	-0.18	-0.2	< 0.1	0.707789
PPP3CA	-0.56	-0.56	0.92	0.977589	PHACTR2	-0.18	-0.21	0.76	0.953756
KCNA4	-0.56	-0.56	0.6	0.913587	NFE2L1	-0.17	-0.17	0.79	0.878556
MYO5A	-0.53	-0.6	0.96	0.934889	RREB1	-0.17	-0.17	0.74	0.742885
DAB2	-0.53	-0.73	0.93	0.997652	RIMS1	-0.17	-0.17	0.87	0.94063
RTKN	-0.53	-0.53	0.7	0.996007	AKIRIN1	-0.17	-0.19	0.65	0.927232
NTN4	-0.52	-0.52	0.7	0.982168	ABCA1	-0.17	-0.17	0.99	0.960825
SCAMP3	-0.52	-0.62	0.39	0.795711	TBC1D14	-0.17	-0.17	0.33	0.912571
OTX2	-0.51	-0.51	0.57	0.929034	ROCK1	-0.17	-0.19	> 0.99	0.700584
RNF170	-0.51	-0.52	0.85	0.864006	GDNF	-0.17	-0.17	0.58	0.732602
UXS1	-0.5	-0.5	0.78	0.985649	ZFYVE9	-0.16	-0.42	0.93	0.942182
SEMA3A	-0.49	-0.58	0.98	0.999759	ERN1	-0.16	-0.17	0.9	0.957114
MEST	-0.49	-0.6	0.77	0.799471	ARHGAP12	-0.16	-0.16	0.38	0.931137
FAM135A	-0.47	-0.47	0.88	0.79282	ABR	-0.16	-0.16	0.61	0.715615
MPZL2	-0.47	-0.77	0.78	0.983932	CFL2	-0.15	-0.43	0.82	0.818795
ADPGK	-0.47	-0.47	0.78	0.860312	NRAS	-0.15	-0.53	0.87	0.946294
MBTD1	-0.46	-0.46	0.72	0.929347	SSBP3	-0.15	-0.21	0.69	0.755927
CAMSAP2	-0.44	-0.44	0.89	0.988622	KIF21A	-0.15	-0.15	0.53	0.924933
CASZ1	-0.44	-0.44	0.93	0.985079	CDC37L1	-0.15	-0.26	0.9	0.931664
SPSB4	-0.44	-0.44	0.88	0.818235	SPATS2	-0.15	-0.33	0.78	0.951666
UBASH3A	-0.44	-0.44	< 0.1	0.740245	AKAP12	-0.15	-0.18	0.78	0.952811
YES1	-0.43	-0.43	0.98	0.994265	EIF4A2	-0.14	-0.19	0.4	0.738121
SRGAP2	-0.43	-0.49	> 0.99	0.935824	PDGFD	-0.14	-0.43	0.18	0.716554
ACTB	-0.43	-0.45	0.76	0.985066	BACH2	-0.14	-0.14	0.78	0.831347
BTF3L4	-0.43	-0.44	0.5	0.721509	LHFPL2	-0.14	-0.14	0.71	0.76497
SPOP	-0.43	-0.43	0.35	0.96767	FAXC	-0.14	-0.14	0.65	0.834146
GABARAPL2	-0.42	-0.42	0.61	0.838191	MAP3K11	-0.14	-0.14	0.71	0.770841
DAW1	-0.41	-0.41	ORF	0.824445	ONECUT2	-0.14	-0.15	0.62	0.919817
ABHD17B	-0.41	-0.41	0.75	0.982999	CSMD3	-0.14	-0.14	0.57	0.704122
ERG	-0.41	-0.41	0.91	0.904573	NAA40	-0.13	-0.2	0.91	0.780867
MKL2	-0.41	-0.41	> 0.99	0.907962	AP1G1	-0.13	-0.14	0.49	0.897918
LDLRAD3	-0.41	-0.41	0.94	0.987876	DLC1	-0.13	-0.14	0.98	0.790943
CTNNBIP1	-0.41	-0.59	0.9	0.979857	CACHD1	-0.13	-0.13	0.53	0.770038
CAMK1D	-0.4	-0.44	0.99	0.922844	RNF216	-0.13	-0.18	0.78	0.945081
NEDD9	-0.39	-0.39	0.88	0.99835	DENND5B	-0.12	-0.13	0.77	0.776691
PPP4R2	-0.39	-0.39	0.83	0.964532	EFNA3	-0.12	-0.3	0.37	0.809025
CDO1	-0.39	-0.39	0.44	0.926782	FNDC3B	-0.12	-0.2	0.56	0.965382
CCDC25	-0.38	-0.39	0.84	0.947156	PLXNA2	-0.12	-0.12	0.66	0.726211
ELMO1	-0.38	-0.38	0.89	0.94292	PURA	-0.12	-0.23	0.78	0.945279
PAN2	-0.38	-0.38	0.78	0.963907	PLAGL2	-0.12	-0.19	0.67	0.785642
H2AFX	-0.37	-0.37	0.74	0.720457	LRRC16A	-0.12	-0.12	0.42	0.803012
EBF3	-0.37	-0.42	0.86	0.920238	UGCG	-0.12	-0.12	0.58	0.858846
CTNND1	-0.37	-0.37	0.89	0.852615	SLC25A25	-0.11	-0.12	0.65	0.779924
TNFRSF11B	-0.36	-0.36	0.43	0.872078	FNDC3A	-0.11	-0.16	0.55	0.816107
PCSK5	-0.36	-0.36	0.68	0.953307	KLHL15	-0.11	-0.11	0.69	0.724496
ERLIN1	-0.35	-0.36	0.96	0.929362	HNRNPH2	-0.11	-0.71	0.62	0.996289
DUSP6	-0.35	-0.35	0.9	0.954825	ZBTB10	-0.1	-0.15	0.94	0.917429
ZHX2	-0.34	-0.34	0.83	0.829234	TRIO	-0.1	-0.32	0.85	0.930811
CITED2	-0.34	-0.42	0.68	0.8771	RAB14	-0.1	-0.29	0.68	0.90319
GMFB	-0.33	-0.51	0.98	0.829209	BICC1	-0.1	-0.1	0.69	0.796465
NUAK1	-0.33	-0.33	0.99	0.951275	UBN2	-0.1	-0.12	0.86	0.935107
MAGI2	-0.33	-0.33	0.86	0.912266	SOS2	-0.1	-0.1	0.58	0.75894
GTPBP8	-0.32	-0.68	0.44	0.706368	ACSL4	-0.09	-0.16	0.68	0.802012
DERL2	-0.32	-0.35	0.75	0.877265	POU6F2	-0.09	-0.09	0.56	0.932484
GGT7	-0.31	-0.31	0.29	0.824598	CARF	-0.09	-0.12	0.13	0.924729
STAM	-0.31	-0.35	0.84	0.723395	ADCYAP1	-0.08	-0.73	0.31	0.946055

SRGAP1	-0.31	-0.35	0.96	0.982833	MICAL3	-0.08	-0.08	0.97	0.745035
YTHDC1	-0.3	-0.43	0.94	0.832085	ZDHHC9	-0.08	-0.08	0.45	0.720887
DACH1	-0.3	-0.3	0.76	0.850352	RBPMS2	-0.07	-0.33	0.74	0.928339
XRN1	-0.3	-0.31	0.7	0.873308	MRGBP	-0.07	-0.34	0.54	0.706744
SERINC5	-0.3	-0.32	0.81	0.799004	FLNB	-0.07	-0.08	0.75	0.788112
GRB10	-0.3	-0.3	0.7	0.968735	GPHN	-0.07	-0.07	ORF	0.760945
BBIP1	-0.29	-0.29	ORF	0.72055	NUFIP2	-0.07	-0.4	0.95	0.950969
PLCE1	-0.29	-0.29	0.88	0.949073	ASAP2	-0.07	-0.16	0.78	0.909873
SLC7A8	-0.28	-0.28	0.46	0.741942	RFX3	-0.07	-0.11	0.64	0.782173
OSBPL1A	-0.28	-0.29	0.22	0.862754	USP31	-0.06	-0.21	0.69	0.988014
NR4A2	-0.28	-0.28	0.31	0.757461	ZDHHC14	-0.06	-0.4	0.2	0.738028
UBA6	-0.27	-0.36	0.82	0.894901	MAP3K3	-0.06	-0.06	0.36	0.820314
SMAD3	-0.27	-0.35	0.85	0.779252	CLIP1	-0.06	-0.1	0.46	0.971009
ANKRD28	-0.27	-0.29	0.89	0.879218	PSD3	-0.06	-0.06	0.76	0.937893
JPH1	-0.27	-0.27	0.66	0.948413	SFXN1	-0.06	-0.17	0.63	0.773642
ADAM17	-0.26	-0.28	0.71	0.714059	DPYSL2	-0.05	-0.05	0.69	0.795619
GLIS3	-0.26	-0.26	0.92	0.984963	RBM20	-0.05	-0.07	0.81	0.778074
MTX3	-0.26	-0.28	0.65	0.956376	ABHD5	-0.05	-0.25	0.52	0.797671
PLCL2	-0.26	-0.28	0.56	0.964494	PRR14L	-0.05	-0.05	0.18	0.704938
DYRK1A	-0.26	-0.26	0.79	0.836101	HOXC11	-0.04	-0.04	ORF	0.83676
SMAD5	-0.26	-0.33	0.83	0.852461	KATNBL1	-0.04	-0.4	0.63	0.834385
UNC119B	-0.26	-0.26	0.45	0.795006	CAPRIN1	-0.04	-0.12	0.57	0.912714
ANGPT2	-0.26	-0.26	0.91	0.941583	FAM126A	-0.04	-0.19	0.74	0.982906
MIER3	-0.25	-0.26	0.72	0.846603	NFIB	-0.04	-0.14	0.72	0.96908
ZBTB46	-0.25	-0.25	0.82	0.944498	ATXN7L3	-0.03	-0.03	0.5	0.801344
PXN	-0.25	-0.25	0.62	0.846161	ATRX	-0.03	-0.05	0.65	0.731173
CYR61	-0.25	-0.25	0.72	0.75683	PLEKHH1	-0.03	-0.12	0.54	0.917543
PTGFR	-0.25	-0.25	0.8	0.941631	ADAM19	-0.03	-0.03	0.7	0.746902
TAGLN2	-0.24	-0.24	0.68	0.867969	HIC2	-0.03	-0.12	0.96	0.913012
IPMK	-0.24	-0.24	0.74	0.949723	ARHGAP19	-0.03	-0.03	0.36	0.764898
ERF	-0.24	-0.25	0.59	0.79474	SOX11	-0.03	-0.13	0.68	0.734613
HS6ST1	-0.23	-0.23	0.79	0.70318	TLN2	-0.02	-0.05	0.44	0.79691
ARHGAP24	-0.23	-0.23	0.79	0.71821	ATXN7L1	-0.02	-0.02	0.54	0.833306
CSTF3	-0.23	-0.23	0.55	0.867998	PHF21A	-0.02	-0.08	0.8	0.742215
TTC14	-0.23	-0.23	0.41	0.840476	AMOTL2	-0.02	-0.02	0.38	0.864452
SLITRK4	-0.23	-0.24	0.27	0.86923	CAPZB	-0.02	-0.23	0.82	0.866677
AP3S1	-0.23	-0.23	0.46	0.951394	SOCS7	-0.02	-0.19	0.67	0.947534
ATXN2	-0.22	-0.23	0.79	0.965054	MED13	-0.01	-0.16	0.77	0.984545
CPEB1	-0.22	-0.22	0.63	0.788947	SEMA6A	-0.01	-0.16	0.36	0.800489
EBF1	-0.22	-0.39	0.71	0.847652	INO80	-0.01	-0.28	0.82	0.745937
LOX	-0.22	-0.38	0.71	0.735396	SESN3	-0.01	-0.13	0.72	0.761828
SNX27	-0.21	-0.21	0.89	0.81629	PCBP2	-0.01	-0.33	0.45	0.907787
PAK7	-0.21	-0.21	0.26	0.942968	CDH2	0	-0.13	0.73	0.899534
SP9	-0.2	-0.2	0.46	0.7094	ORC4	0	-0.56	0.91	0.942566
CAMK2D	-0.2	-0.2	0.92	0.840552	SLC25A11	0	-0.22	0.56	0.948354
IKZF2	-0.2	-0.21	0.58	0.769321	EXT1	0	-0.18	0.45	0.801822
SLC1A2	-0.2	-0.2	0.44	0.777873	ZBTB20	0	-0.4	> 0.99	0.987385
REV3L	-0.2	-0.26	0.72	0.995828	TGFBR2	0	-0.13	0.93	0.971561
DOCK9	-0.2	-0.36	0.59	0.910929	INSIG1	0	-0.53	0.78	0.963813
TRIM2	-0.19	-0.19	0.59	0.993785					

Supplementary Table 3 – Results of the target prediction. using TargetScan v.7.2. and Diana tools MicroT-CDS v.5.0. for miR-802-5p

Target Gene	Cumulative weighted context++ score	Total context++ score	Aggregate PCT	MiTG	Target Gene	Cumulative weighted context++ score	Total context++ score	Aggregate PCT	MiTG
TMED9	-0.95	-0.96	0.7	0.999909	ZMYM2	-0.13	-0.2	< 0.1	0.952878
SREK1	-0.66	-0.76	0.42	0.985192	FAT1	-0.12	-0.15	0.7	0.981068
FOXJ3	-0.55	-0.55	0.56	0.901663	MBNL1	-0.12	-0.12	0.39	0.801453
KHDRBS1	-0.52	-0.52	0.32	0.999481	LIN54	-0.12	-0.12	< 0.1	0.782849
PSMD2	-0.52	-0.52	< 0.1	0.991373	CDV3	-0.12	-0.15	0.39	0.766561
SCAMP1	-0.47	-0.48	0.48	0.932605	DSCAM	-0.11	-0.11	0.57	0.934438
HSPD1	-0.45	-0.45	0.56	0.875645	EVI5	-0.11	-0.11	0.55	0.775588
SLC2A3	-0.44	-0.44	0.3	0.891992	MEX3B	-0.11	-0.11	0.43	0.781921
MSI1	-0.43	-0.43	0.67	0.856616	HAPLN1	-0.11	-0.11	0.51	0.781952
SCG2	-0.43	-0.43	0.43	0.972459	GABPA	-0.1	-0.1	0.34	0.815857
HNF1B	-0.42	-0.42	0.16	0.999886	DEPDC1B	-0.09	-0.1	0.28	0.755464
XKRX	-0.41	-0.41	< 0.1	0.838433	ZFYVE9	-0.09	-0.22	0.55	0.78931
DDX4	-0.39	-0.39	0.24	0.791042	PRICKLE2	-0.09	-0.09	0.51	0.865375
SMTNL2	-0.38	-0.38	0.35	0.887651	SLC9A6	-0.09	-0.09	0.13	0.742503
RHOA	-0.38	-0.38	0.34	0.817546	NR3C2	-0.09	-0.09	0.32	0.70552
RAN	-0.37	-0.43	0.55	0.922937	GNA13	-0.09	-0.12	0.54	0.854533
RAPGEF4	-0.37	-0.37	0.26	0.984161	ZMYND11	-0.08	-0.1	0.36	0.785629
ARID2	-0.36	-0.36	0.45	0.999041	YWHAE	-0.08	-0.09	0.49	0.927791
SDC4	-0.34	-0.56	0.48	0.981052	CLCN3	-0.08	-0.09	0.51	0.743844
UBE2M	-0.33	-0.33	0.22	0.913111	JMJD1C	-0.08	-0.1	0.46	0.753716
NKRF	-0.32	-0.33	0.53	0.792197	PANK1	-0.08	-0.14	0.56	0.701887
PAFAH1B1	-0.32	-0.45	0.25	0.737278	MYLIP	-0.07	-0.08	0.43	0.797326
CDH11	-0.3	-0.45	0.55	0.858018	PNPLA8	-0.07	-0.14	0.35	0.753913
KLF7	-0.29	-0.3	0.56	0.995724	CACNA1I	-0.07	-0.07	0.55	0.757567
SLC35F1	-0.29	-0.29	0.13	0.907496	DCUN1D3	-0.07	-0.07	0.53	0.77476
CSDE1	-0.28	-0.28	0.57	0.986691	EPM2AIP1	-0.07	-0.17	0.36	0.816662
CDK19	-0.27	-0.37	0.23	0.841852	PEAK1	-0.07	-0.08	0.53	0.70174
MBNL2	-0.27	-0.27	< 0.1	0.965317	SLC33A1	-0.07	-0.15	0.37	0.750557
MIER3	-0.26	-0.3	0.36	0.82333	EDEM3	-0.07	-0.07	0.37	0.75158
STX16	-0.25	-0.35	0.24	0.96191	HECW2	-0.06	-0.09	0.32	0.863959
TBC1D23	-0.25	-0.27	0.23	0.951752	ONECUT2	-0.05	-0.05	0.33	0.707787
TMEM25	-0.24	-0.24	0.18	0.703531	ATXN1	-0.05	-0.06	0.47	0.818501
GATA4	-0.23	-0.23	< 0.1	0.876993	ABAT	-0.04	-0.09	0.55	0.809815
AGFG1	-0.23	-0.23	0.56	0.897033	FZD5	-0.04	-0.25	0.54	0.834818
PPP3CA	-0.22	-0.22	0.38	0.934698	RAPGEF6	-0.04	-0.04	0.54	0.839526
ATP6V1C1	-0.22	-0.23	0.2	0.786791	NDUFB6	-0.03	-0.28	0.42	0.882873
TSHZ3	-0.21	-0.21	0.41	0.939905	MAP3K2	-0.03	-0.18	0.17	0.903692
SLC2A1	-0.2	-0.2	0.39	0.755085	MEF2C	-0.03	-0.09	0.71	0.952272
USP44	-0.19	-0.19	ORF	0.735664	TET3	-0.03	-0.03	0.2	0.732387
MATR3	-0.19	-0.28	0.21	0.9436	ASAP1	-0.03	-0.03	< 0.1	0.869298
TRAF3	-0.18	-0.24	0.42	0.722446	KMT2A	-0.03	-0.03	0.27	0.875507
TCF4	-0.18	-0.22	0.19	0.975189	PPP2CA	-0.03	-0.63	0.56	0.949455
CPEB3	-0.18	-0.18	0.43	0.824353	ATF2	-0.03	-0.03	< 0.1	0.986103
SOX6	-0.17	-0.32	0.41	0.927457	NSD1	-0.03	-0.09	0.44	0.852003
RORA	-0.16	-0.19	0.33	0.816545	KDM5A	-0.02	-0.03	0.38	0.723192
PCSK5	-0.16	-0.16	0.4	0.840574	MYO6	-0.02	-0.18	0.13	0.742437
LRRTM3	-0.16	-0.16	0.53	0.978788	HIPK1	-0.02	-0.02	0.58	0.922359
SLC25A53	-0.16	-0.33	< 0.1	0.801373	SMARCE1	-0.02	-0.11	0.47	0.802616
ZFHX4	-0.15	-0.16	0.49	0.997019	BRD3	-0.02	-0.23	0.17	0.994458
IQSEC1	-0.15	-0.25	0.54	0.745638	MED13	-0.02	-0.08	< 0.1	0.960335
SOAT1	-0.15	-0.15	0.23	0.740805	PIP4K2B	-0.01	-0.23	0.54	0.887162
HIAT1	-0.15	-0.28	0.55	0.841361	ERMP1	-0.01	-0.23	0.16	0.774486
ARMC8	-0.15	-0.15	0.45	0.74319	UFD1L	-0.01	-0.28	0.55	0.717176
NEGR1	-0.14	-0.17	0.37	0.813117	DCTN4	-0.01	-0.21	0.26	0.757104
TAB3	-0.14	-0.15	0.19	0.780968	FOXP1	-0.01	-0.08	0.47	0.903238
TOB1	-0.14	-0.14	0.48	0.895328	ARHGEF12	0	-0.01	0.55	0.915539
ZFHX3	-0.14	-0.18	0.47	0.993017	IMMT	0	-0.08	0.55	0.734088
NMT2	-0.13	-0.39	0.55	0.997604	TLK2	0	-0.13	0.12	0.847124
SETD2	-0.13	-0.14	0.45	0.924397	NFIB	0	-0.16	0.49	0.991528

Supplementary Table 4 – Results of the target prediction. using TargetScan v.7.2 and Diana Tools MicroT-CDS v.5.0. for miR-200b-3p

Target Gene	Cumulative weighted context++ score	Total context ++ score	Aggregate PCT	Target Gene	Target Gene	Cumulative weighted context++ score	Total context+ + score	Aggregate PCT	MiTG
ZEB1	-0.9	-0.92	> 0.99	0.999890928	SEMA6D	-0.14	-0.17	0.75	0.824479629
SEC23A	-0.78	-0.83	0.83	0.995839224	VLDLR	-0.14	-0.16	0.92	0.739526922
GPM6A	-0.67	-0.69	0.96	0.999725616	SLC23A2	-0.14	-0.27	0.93	0.85280862
TWISTNB	-0.66	-0.79	0.58	0.956254975	HIPK1	-0.14	-0.15	0.4	0.858830558
CRTAP	-0.62	-0.62	0.87	0.996089503	TXLNG	-0.14	-0.21	0.25	0.774129053
IMMP2L	-0.59	-0.71	< 0.1	0.975258419	ICK	-0.14	-0.14	0.85	0.739596201
APOO	-0.56	-0.56	0.64	0.900527938	ELMOD2	-0.14	-0.28	0.17	0.732711076
ZEB2	-0.55	-0.56	> 0.99	0.999995785	PCSK2	-0.14	-0.14	0.5	0.94679833
MMD	-0.54	-0.54	0.84	0.999936604	ARIH2	-0.14	-0.14	0.29	0.825607833
BAG5	-0.54	-0.63	0.88	0.826652678	OTUD4	-0.14	-0.14	0.89	0.87066196
RAP2C	-0.53	-0.54	0.91	0.99685176	CTNND2	-0.14	-0.14	0.73	0.856289034
MSN	-0.53	-0.53	0.99	0.981437195	PKD1	-0.13	-0.14	0.95	0.999494138
TCEB1	-0.52	-0.73	0.97	0.846735443	STARD13	-0.13	-0.2	0.69	0.752281248
LHFP	-0.52	-0.52	0.57	0.952212613	ESRRG	-0.13	-0.13	0.85	0.981358449
YWHAG	-0.49	-0.49	0.89	0.990019812	FMR1	-0.13	-0.13	0.43	0.874043116
RBFOX3	-0.49	-0.49	0.88	0.964080295	OCLN	-0.13	-0.13	0.83	0.808405214
NOG	-0.48	-0.48	0.85	0.996543013	HNF1B	-0.13	-0.13	0.53	0.863928497
TMEFF2	-0.48	-0.53	0.8	0.995788077	SLC4A4	-0.13	-0.13	0.82	0.809494926
FEZ2	-0.47	-0.47	0.87	0.998552068	GPATCH8	-0.13	-0.18	0.69	0.949434918
FHOD1	-0.47	-0.47	0.88	0.978609609	COL4A3	-0.12	-0.12	0.31	0.86347964
ZFPM2	-0.46	-0.46	0.91	0.996727485	ORMDL3	-0.12	-0.19	0.92	0.751725074
FLI1	-0.44	-0.44	0.87	0.94319823	FERMT2	-0.12	-0.13	0.37	0.771335759
PTPN13	-0.43	-0.43	0.65	0.900516264	ATP11C	-0.12	-0.12	< 0.1	0.796298909
MARCKS	-0.42	-0.43	0.9	0.997718337	FYN	-0.12	-0.17	0.27	0.898877347
NOVA1	-0.42	-0.42	0.96	0.995935077	NKAP	-0.12	-0.23	0.4	0.705140981
SPRYD7	-0.42	-0.88	0.9	0.943957448	REEP3	-0.12	-0.15	0.16	0.911054571
PTHLH	-0.42	-0.42	0.51	0.995270044	PPP2R2C	-0.12	-0.12	0.89	0.896928796
EFNA1	-0.41	-0.41	0.84	0.998731447	TLN2	-0.12	-0.12	0.9	0.951103225
USP27X	-0.4	-0.4	0.47	0.95906802	PPP1R10	-0.12	-0.12	0.67	0.752856656
PLCL1	-0.4	-0.4	0.94	0.928956701	SMURF2	-0.12	-0.12	0.87	0.888658532
WAPAL	-0.4	-0.44	0.9	0.993032587	PHTF2	-0.12	-0.12	0.51	0.981182444
CEP41	-0.4	-0.44	0.97	0.843732436	ATXN1	-0.11	-0.12	0.79	0.876866579
XKR8	-0.39	-0.52	0.89	0.875741562	SLC38A2	-0.11	-0.18	0.81	0.994350555
PPP4R2	-0.39	-0.39	0.91	0.972104483	MPRIP	-0.11	-0.13	0.9	0.978385928
PPM1F	-0.39	-0.45	0.98	0.986720657	ZMYM4	-0.11	-0.12	0.89	0.869066754
BAG6	-0.39	-0.39	0.59	0.798832694	CNOT6	-0.11	-0.34	0.55	0.92608497
MTFR1	-0.38	-0.4	0.39	0.829493473	WDFY3	-0.11	-0.11	0.46	0.737475649
YPEL2	-0.38	-0.38	0.94	0.999244625	PLXNA2	-0.11	-0.11	0.55	0.890226156
DUSP1	-0.38	-0.38	0.89	0.973584516	HNRNPK	-0.11	-0.11	ORF	0.882597575
LPAR1	-0.37	-0.39	0.74	0.706099255	ANKRD40	-0.11	-0.33	0.42	0.941206466
ETS1	-0.37	-0.39	0.98	0.722117488	HCCS	-0.11	-0.18	0.38	0.875406087
RANBP9	-0.37	-0.37	0.86	0.994941704	CYP1B1	-0.11	-0.11	0.42	0.787328102
RAB37	-0.37	-0.37	0.85	0.821951017	RTF1	-0.11	-0.11	0.9	0.991821658
EGLN1	-0.37	-0.37	0.8	0.976292757	GLS	-0.11	-0.11	0.43	0.739960517
FAM118B	-0.36	-0.38	0.83	0.775582938	XIAP	-0.1	-0.17	0.83	0.74517701
YIPF5	-0.36	-0.39	0.58	0.709170834	ABAT	-0.1	-0.24	0.72	0.708757844
GIT2	-0.36	-0.36	0.87	0.99630439	SEPHS1	-0.1	-0.22	0.27	0.727769266
MBLAC2	-0.36	-0.4	0.63	0.807728192	NOVA2	-0.1	-0.15	0.93	0.753402608
NAA50	-0.36	-0.51	0.88	0.840100378	MED1	-0.1	-0.12	0.48	0.720712167
NTF3	-0.36	-0.51	0.85	0.883359552	DCAF5	-0.1	-0.1	0.14	0.889213284
JUN	-0.35	-0.35	0.87	0.996636217	PUM2	-0.1	-0.2	0.54	0.967791674
LCA5	-0.34	-0.34	0.57	0.909372723	DCP2	-0.1	-0.15	0.87	0.794764277
HS3ST1	-0.34	-0.34	0.9	0.980312639	TIAL1	-0.1	-0.24	0.49	0.748045149
TFAP2A	-0.33	-0.51	0.94	0.841527079	PVRL4	-0.1	-0.1	0.9	0.906016924
DEK	-0.33	-0.38	0.86	0.823679456	DENND5B	-0.1	-0.1	0.88	0.937520822
NBR1	-0.33	-0.35	0.7	0.953704516	GOLIM4	-0.1	-0.27	0.78	0.941993288
FBXO33	-0.33	-0.33	0.87	0.996611436	TRIM62	-0.1	-0.1	0.53	0.813531779
PARD6B	-0.33	-0.34	0.91	0.777156292	SPAST	-0.09	-0.1	0.7	0.733667055
RHOA	-0.33	-0.33	0.68	0.730976746	ATP2A2	-0.09	-0.1	0.49	0.716203008
CLASP1	-0.33	-0.33	0.92	0.815633313	GJC1	-0.09	-0.61	0.95	0.988819132
ELAVL2	-0.33	-0.36	0.56	0.908466756	NCOA2	-0.09	-0.09	0.95	0.956638967
ZCCHC24	-0.32	-0.33	0.9	0.93070254	MAP2	-0.09	-0.13	0.89	0.987867569
DGKA	-0.32	-0.32	0.52	0.818350898	ATXN1L	-0.09	-0.09	0.14	0.711212309

HSPA13	-0.31	-0.33	0.86	0.96454401	KCND2	-0.09	-0.09	< 0.1	0.934322708
NFYA	-0.31	-0.31	0.84	0.987785399	COL4A3BP	-0.09	-0.26	0.73	0.981913687
FUBP3	-0.31	-0.33	0.67	0.891198159	CRYBG3	-0.09	-0.09	< 0.1	0.964907575
PTPN21	-0.31	-0.36	0.9	0.795311086	MEX3A	-0.09	-0.11	0.14	0.849098315
FOXG1	-0.31	-0.39	0.87	0.999993013	CECR2	-0.09	-0.16	0.89	0.958847792
UBE2W	-0.31	-0.31	0.9	0.997326175	TEX2	-0.08	-0.08	0.7	0.90063168
LRP1B	-0.31	-0.34	0.89	0.99880573	ASAP1	-0.08	-0.08	0.92	0.97416519
CLIC4	-0.3	-0.3	0.88	0.986115035	MBOAT2	-0.08	-0.21	0.94	0.768312578
SLC14A1	-0.3	-0.46	0.88	0.997763864	BACH2	-0.08	-0.08	0.47	0.718654082
SMARCAD1	-0.3	-0.3	0.87	0.819691522	NAB1	-0.08	-0.14	< 0.1	0.997780765
HDHD2	-0.3	-0.34	0.49	0.746291483	NRIP1	-0.08	-0.08	0.81	0.753016255
TOB1	-0.3	-0.32	0.74	0.963979674	CHD1	-0.08	-0.08	0.86	0.935042141
HNRNPD	-0.3	-0.57	> 0.99	0.983497223	TRIM2	-0.08	-0.11	0.33	0.813037346
FXR2	-0.3	-0.3	0.41	0.865111151	SEC24A	-0.08	-0.14	0.46	0.796535596
ERRFI1	-0.3	-0.31	0.85	0.948009626	ARCN1	-0.07	-0.07	0.14	0.719678023
SERINC1	-0.29	-0.31	0.93	0.861338706	MYT1	-0.07	-0.07	0.77	0.868702929
GATA4	-0.29	-0.29	0.86	0.833630173	OSR1	-0.07	-0.07	0.14	0.738483233
ST6GALNAC5	-0.29	-0.29	0.9	0.943010278	ZDHHC17	-0.07	-0.09	0.27	0.890325893
EVI5	-0.29	-0.29	0.9	0.997635245	PDS5B	-0.07	-0.12	0.73	0.926073349
ADAMTS8	-0.29	-0.29	< 0.1	0.802270864	MAFG	-0.07	-0.07	0.85	0.817644566
CNTFR	-0.28	-0.28	0.74	0.7624101	KIF13A	-0.07	-0.07	0.48	0.722771253
MAPRE1	-0.28	-0.29	0.78	0.981946051	SEC61A2	-0.07	-0.16	0.37	0.822983742
SPATS2L	-0.28	-0.28	0.81	0.748572492	SLC1A2	-0.07	-0.07	0.57	0.872066884
PSIP1	-0.27	-0.28	0.9	0.999930118	BRWD1	-0.07	-0.09	0.52	0.735472419
USP6NL	-0.27	-0.31	0.92	0.993002648	TRIO	-0.07	-0.08	0.48	0.891362073
CDK17	-0.27	-0.28	0.87	0.990267569	CSMD3	-0.07	-0.07	0.47	0.713212322
ELL2	-0.27	-0.28	0.74	0.981595646	SIK1	-0.07	-0.07	0.89	0.703968789
HSPA9	-0.27	-0.34	0.93	0.940122041	TMEM245	-0.07	-0.07	0.49	0.813679352
E2F3	-0.27	-0.27	0.87	0.897044098	CASKIN1	-0.07	-0.07	0.14	0.733943344
BASP1	-0.27	-0.27	0.58	0.949136859	SH3PXD2A	-0.07	-0.07	0.9	0.837788258
CBX4	-0.27	-0.33	0.36	0.856646278	PPP2R5E	-0.07	-0.07	0.14	0.737999987
TSC22D2	-0.27	-0.3	0.9	0.994798959	ELAVL4	-0.06	-0.06	0.14	0.938885035
RAB11FIP2	-0.27	-0.28	0.55	0.85026411	NPNT	-0.06	-0.06	< 0.1	0.701101369
PAK7	-0.27	-0.27	0.41	0.709841859	FAM168B	-0.06	-0.09	0.29	0.784784003
UBA6	-0.27	-0.37	0.96	0.943772751	VCPIP1	-0.06	-0.2	0.86	0.884873056
SOX2	-0.27	-0.27	0.69	0.778020917	GOSR2	-0.06	-0.12	< 0.1	0.703521601
BNIP3L	-0.26	-0.33	0.6	0.873621114	GNAI3	-0.06	-0.24	0.9	0.98591274
PCDH8	-0.26	-0.26	0.88	0.873526222	SOX5	-0.06	-0.18	0.96	0.999899082
KCTD10	-0.26	-0.26	0.86	0.780597698	SECISBP2L	-0.06	-0.07	0.14	0.876887264
AGFG1	-0.26	-0.26	0.93	0.933074463	PLK2	-0.06	-0.21	0.39	0.90483518
CFL2	-0.26	-0.63	0.9	0.996807032	PCDH19	-0.06	-0.06	0.27	0.946057741
RANBP10	-0.26	-0.26	0.98	0.826466544	TMPPE	-0.06	-0.22	< 0.1	0.738969184
SESN1	-0.26	-0.26	0.86	0.996469034	FBXL16	-0.06	-0.06	0.14	0.787479871
MYB	-0.26	-0.26	0.7	0.891317728	CREB1	-0.06	-0.16	0.14	0.769489025
ARIH1	-0.26	-0.43	0.99	0.934810165	MAP3K1	-0.05	-0.06	0.86	0.98871814
STX12	-0.25	-0.25	0.76	0.746677651	GAD2	-0.05	-0.05	0.14	0.85450878
SLITRK1	-0.25	-0.25	0.83	0.99643371	ARHGEF1	-0.05	-0.05	ORF	0.714190161
SCRT2	-0.25	-0.25	0.89	0.98907959	SFXN1	-0.05	-0.16	0.85	0.994374931
FBXW7	-0.24	-0.24	0.96	0.984476558	PPP1R12B	-0.05	-0.05	0.9	0.908139917
GOLGA7	-0.24	-0.47	0.55	0.911195011	SLC5A3	-0.05	-0.1	0.31	0.759946191
MKNK1	-0.24	-0.24	0.38	0.912965123	CDC73	-0.05	-0.62	0.15	0.856281558
TBK1	-0.24	-0.24	0.47	0.79557112	TLN1	-0.05	-0.07	0.27	0.955546397
NFIA	-0.24	-0.24	0.98	0.985141343	IPO8	-0.05	-0.05	< 0.1	0.975252476
SOX1	-0.24	-0.24	0.9	0.785907252	CBX5	-0.05	-0.22	0.9	0.862057321
PDIK1L	-0.24	-0.24	0.89	0.985761339	RPS6KA3	-0.05	-0.22	0.91	0.899009827
CSRNP3	-0.24	-0.24	0.86	0.86692461	GRIP1	-0.05	-0.05	0.14	0.904875971
KANK1	-0.24	-0.24	0.62	0.770896094	FRMD6	-0.05	-0.05	0.14	0.854092236
SRSF1	-0.24	-0.55	0.92	0.986362707	ZC3H4	-0.05	-0.05	0.88	0.902753463
GABPA	-0.24	-0.24	0.88	0.99517984	AGO2	-0.05	-1.1	0.9	0.979716411
OSBPL11	-0.24	-0.24	0.34	0.987019247	ATP11B	-0.05	-0.2	0.47	0.754749829
PCMTD1	-0.24	-0.24	0.47	0.782639727	ARL5A	-0.05	-0.38	0.83	0.912625601
CITED2	-0.24	-0.29	0.74	0.768179474	ACTR1A	-0.04	-0.21	0.72	0.786261763
MATR3	-0.23	-0.28	0.9	0.991509936	PEAK1	-0.04	-0.05	0.78	0.701053032
NFIB	-0.23	-0.24	0.92	0.906720281	WIPF1	-0.04	-0.37	0.94	0.834849043
S100PBP	-0.23	-0.23	0.55	0.770183899	TCF4	-0.04	-0.06	0.62	0.82444136
MARCH8	-0.23	-0.23	0.9	0.996056844	CELF1	-0.04	-0.05	< 0.1	0.719714087
KLF4	-0.23	-0.23	0.67	0.795336389	CGGBP1	-0.04	-0.04	0.14	0.945304352
GOLGA1	-0.23	-0.23	0.9	0.884364111	CLASP2	-0.04	-0.06	0.86	0.999753028
DPY19L1	-0.23	-0.5	0.98	0.966157645	MKL2	-0.04	-0.04	0.35	0.735157387
RELN	-0.22	-0.22	0.78	0.985587469	TBL1XR1	-0.04	-0.16	0.84	0.924295943
MIEF1	-0.22	-0.23	0.88	0.854822738	JMY	-0.04	-0.07	0.14	0.806389245

SLC30A4	-0.22	-0.23	0.88	0.899002988	CASK	-0.04	-0.05	0.14	0.960811147
PPP1R18	-0.22	-0.22	0.87	0.990564209	TRPV3	-0.04	-0.06	0.73	0.787449395
SLC6A15	-0.22	-0.22	0.78	0.737384704	AMBRA1	-0.04	-0.04	0.65	0.815017357
MEX3B	-0.22	-0.23	0.68	0.909686672	BBX	-0.04	-0.04	0.19	0.79913838
EFNB2	-0.22	-0.3	0.87	0.996329128	CHSY1	-0.04	-0.13	0.49	0.855639715
VEGFA	-0.22	-0.23	0.62	0.809598295	PTPN11	-0.04	-0.04	0.14	0.900670673
SCAMP1	-0.22	-0.38	0.85	0.823520182	CHD9	-0.03	-0.04	0.38	0.785942587
NR5A2	-0.22	-0.27	0.94	0.997628476	MYO9A	-0.03	-0.04	0.91	0.973600418
ADAMTS3	-0.22	-0.22	0.83	0.992607575	PRKCA	-0.03	-0.06	0.15	0.769832973
DLC1	-0.22	-0.29	0.93	0.970855048	NEGR1	-0.03	-0.19	0.98	0.807092362
NPC1	-0.22	-0.22	0.64	0.974371239	KDM3B	-0.03	-0.03	0.77	0.970553115
APAF1	-0.22	-0.22	0.5	0.760001736	FIGN	-0.03	-0.06	0.95	0.980089777
SPTSSA	-0.22	-0.43	0.92	0.858910598	PKIA	-0.03	-0.53	0.9	0.883757357
AMFR	-0.22	-0.23	0.89	0.969152684	FOXP1	-0.03	-0.08	0.47	0.832447581
ARHGAP20	-0.21	-0.21	0.56	0.895425274	UBN2	-0.03	-0.03	0.92	0.895474137
SNAI2	-0.21	-0.21	0.43	0.787907446	PLXNA4	-0.03	-0.03	0.88	0.889822971
DOT1L	-0.21	-0.25	0.89	0.771179303	ASTN1	-0.03	-0.03	0.8	0.834495622
MAP4K3	-0.21	-0.21	0.58	0.986326803	GIGYF1	-0.03	-0.03	0.79	0.924746693
MSL2	-0.21	-0.21	0.84	0.994891044	FAM179B	-0.03	-0.03	0.76	0.972541132
RPS6KA6	-0.21	-0.22	0.54	0.712477826	RFX7	-0.03	-0.03	0.64	0.817958061
FAM60A	-0.21	-0.21	0.51	0.83979062	PARD3B	-0.03	-0.03	0.64	0.821156713
PLS3	-0.21	-0.21	0.38	0.720340086	PPP2R5C	-0.03	-0.03	0.48	0.763839725
PHF6	-0.2	-0.2	0.52	0.928564153	GREB1L	-0.03	-0.03	0.37	0.983554928
TENM1	-0.2	-0.2	0.88	0.980657306	NUAK1	-0.03	-0.03	< 0.1	0.850968344
AKT3	-0.2	-0.21	0.74	0.749217038	DOCK4	-0.03	-0.03	0.44	0.981307699
DNAJC5	-0.2	-0.34	0.94	0.921571729	TJP1	-0.03	-0.16	0.27	0.872826888
NPTX1	-0.2	-0.21	0.81	0.809845324	ANK3	-0.03	-0.03	0.82	0.882809589
BAP1	-0.2	-0.21	0.87	0.969711039	DDX3X	-0.03	-0.11	0.74	0.762685442
PPP6R3	-0.2	-0.24	0.63	0.857976174	BRWD3	-0.03	-0.03	0.11	0.841143586
MTSS1L	-0.2	-0.2	0.9	0.994429659	NUP153	-0.03	-0.24	0.77	0.952677455
ERG	-0.2	-0.22	0.34	0.932515202	KDR	-0.03	-0.18	0.61	0.993856379
PHACTR3	-0.2	-0.2	0.42	0.847655279	FBXW11	-0.03	-0.03	0.6	0.916135169
RASSF8	-0.2	-0.2	0.83	0.834598177	ZFX	-0.03	-0.03	0.14	0.745977699
KCNA2	-0.2	-0.2	0.61	0.839144776	CEP350	-0.03	-0.03	< 0.1	0.986705934
VASH2	-0.19	-0.2	0.89	0.953426371	NUFIP2	-0.02	-0.15	0.78	0.83180753
LHX5	-0.19	-0.19	< 0.1	0.961572935	KCNQ4	-0.02	-0.02	0.5	0.738602761
FAM217B	-0.19	-0.19	0.56	0.964311859	SELK	-0.02	-0.27	0.14	0.793315102
NANOS1	-0.19	-0.34	0.53	0.837957888	SYNCRIP	-0.02	-0.06	< 0.1	0.884019702
MTF2	-0.19	-0.35	0.88	0.735971976	LRIG1	-0.02	-0.02	0.14	0.881732427
WDR82	-0.19	-0.2	0.98	0.858294222	ST3GAL2	-0.02	-0.23	0.87	0.712985932
ZC3H6	-0.18	-0.18	0.82	0.809198168	DESI1	-0.02	-0.1	0.14	0.765648402
PHF21B	-0.18	-0.18	0.49	0.796426901	MFN1	-0.02	-0.2	0.39	0.844789504
SYVN1	-0.18	-0.18	0.85	0.987365824	HIVEP3	-0.02	-0.02	0.91	0.724691726
SH3GL1	-0.18	-0.18	0.14	0.773259092	STX1A	-0.02	-0.02	0.43	0.75337443
SNX30	-0.18	-0.2	0.14	0.881383	SCN8A	-0.02	-0.02	0.38	0.777078086
GLI3	-0.18	-0.18	0.92	0.838074961	SCN5A	-0.02	-0.02	0.27	0.799327487
GATA2	-0.18	-0.18	0.9	0.910911536	SUV420H1	-0.02	-0.08	0.59	0.930034393
PBX3	-0.17	-0.18	0.66	0.922016177	FAM63B	-0.02	-0.16	0.84	0.97310507
ETV5	-0.17	-0.17	0.63	0.855165648	AMOTL2	-0.02	-0.19	0.82	0.89837547
ARHGAP6	-0.17	-0.17	0.41	0.790590197	FRMD4A	-0.02	-0.05	0.45	0.910995302
ADIPOR2	-0.17	-0.19	0.68	0.713370449	ETF1	-0.02	-0.02	0.14	0.868626102
NR2C2	-0.17	-0.26	0.85	0.766952751	DNAJB14	-0.01	-0.03	0.55	0.70362907
RHOT1	-0.17	-0.18	0.59	0.919931114	DNAJB5	-0.01	-0.36	0.89	0.919355861
FAM219A	-0.17	-0.17	0.57	0.78001507	SETD7	-0.01	-0.1	0.14	0.870513577
XRN1	-0.17	-0.17	0.92	0.933847504	MAP4K4	-0.01	-0.06	0.93	0.877750809
DACH1	-0.17	-0.17	0.87	0.988125214	PAK3	-0.01	-0.02	0.14	0.718596343
ATL2	-0.17	-0.17	0.7	0.926566673	RPRD1A	-0.01	-0.13	0.44	0.74998191
LRRTM3	-0.17	-0.23	0.86	0.918101818	DENND5A	-0.01	-0.25	0.83	0.990626246
MED13	-0.17	-0.18	0.79	0.992374599	SUZ12	-0.01	-0.08	0.85	0.99024925
BHLHE41	-0.16	-0.18	0.66	0.72122087	AFF1	-0.01	-0.03	0.9	0.95503571
CHD2	-0.16	-0.16	0.83	0.818917017	CDK16	-0.01	-0.04	ORF	0.7246698
CALU	-0.16	-0.18	0.7	0.759987514	RBFOX2	-0.01	-0.34	0.98	0.985455689
SCN3B	-0.16	-0.16	0.85	0.858901949	PAPD5	-0.01	-0.34	0.97	0.978228322
TRIM33	-0.16	-0.16	0.84	0.996918077	CELF2	-0.01	-0.01	0.14	0.706647692
TMEM170B	-0.16	-0.31	0.8	0.823067446	MBNL1	-0.01	-0.01	0.14	0.781069961
TBC1D22B	-0.16	-0.16	0.8	0.764539128	NRXN1	-0.01	-0.01	0.14	0.720157161
PPP1R9B	-0.16	-0.16	0.56	0.763789978	CREBBP	-0.01	-0.01	0.14	0.721417883
HS2ST1	-0.16	-0.17	0.7	0.85309054	ZFHX4	-0.01	-0.01	0.14	0.803121083
SYDE1	-0.16	-0.16	0.88	0.951140087	AFF4	-0.01	-0.01	0.14	0.827527417
SLC16A2	-0.16	-0.16	0.81	0.994084202	IRS1	-0.01	-0.01	0.14	0.763303195
GPR173	-0.16	-0.21	0.58	0.737390249	INPP4A	-0.01	-0.18	0.86	0.998404988
PHF21A	-0.16	-0.17	0.89	0.787387098	TMEM200C	-0.01	-0.06	0.14	0.944043235

TBX5	-0.16	-0.23	< 0.1	0.897642341	AGO3	-0.01	-0.07	0.34	0.718938132
WASF1	-0.16	-0.16	0.14	0.920382973	CKAP4	-0.01	-0.28	0.92	0.807669626
AFF3	-0.16	-0.17	0.83	0.975400914	XPR1	-0.01	-0.06	0.53	0.737064176
SYNJ1	-0.16	-0.17	0.56	0.999850428	FNDC3B	-0.01	-0.09	0.6	0.840931402
ULK2	-0.16	-0.17	0.9	0.858805122	HECW2	-0.01	-0.03	0.27	0.721118176
PCSK5	-0.16	-0.16	0.84	0.769937336	COPS2	-0.01	-0.22	0.66	0.990391545
CDYL2	-0.16	-0.21	0.99	0.967129682	HMGB3	-0.01	-0.34	0.9	0.993427414
ANKRD28	-0.16	-0.2	0.84	0.733967809	SPRYD4	0	-0.24	0.4	0.700306842
FOXK1	-0.15	-0.21	0.91	0.822052313	FAM107B	0	-0.12	0.14	0.800768899
OXR1	-0.15	-0.15	0.84	0.998548753	GFI1	0	-0.05	0.14	0.798658141
FOXN2	-0.15	-0.15	0.17	0.789965338	COPS8	0	-0.47	0.9	0.945355796
NR3C1	-0.15	-0.19	0.56	0.773732683	YWHAB	0	-0.19	0.68	0.783621268
ZBTB38	-0.15	-0.16	0.86	0.991980636	WNK1	0	-0.05	0.6	0.914765
ROR2	-0.15	-0.15	0.34	0.774460326	EFNA5	0	-0.04	0.14	0.863099313
PTPN14	-0.15	-0.17	0.71	0.937725147	HSPH1	0	-0.17	0.91	0.80280286
FGD1	-0.15	-0.18	0.59	0.801295205	MXD4	0	-0.13	0.14	0.780133078
CCSER1	-0.15	-0.15	0.71	0.819896067	EPS8	0	-0.39	> 0.99	0.880412151
SULF1	-0.15	-0.15	0.52	0.898849124	SLC35E2	0	-0.32	0.74	0.721011796
PI4KB	-0.15	-0.21	0.89	0.925037048	GATSL2	0	-0.26	0.92	0.867895971
REEP1	-0.15	-0.34	0.92	0.841280698	ZBTB20	0	-0.14	0.94	0.992028002
CDYL	-0.15	-0.26	0.69	0.818206253	BAZ2B	0	-0.15	0.93	0.826260733
SPAG9	-0.15	-0.26	0.88	0.930043333	CADM1	0	-0.01	0.14	0.767945637
RECK	-0.15	-0.33	0.49	0.77899381	PPAP2B	0	-0.32	0.91	0.730439648
HNRNPU	-0.15	-0.24	0.93	0.781050892	NCOA7	0	-0.15	0.75	0.710828585
SDK2	-0.15	-0.15	0.7	0.853696985	SERPINC1	0	-0.07	0.14	0.814859785
STRN	-0.14	-0.27	0.96	0.754305419	SEMA6D	-0.14	-0.17	0.75	0.824479629

Supplementary Table 5 – Results of the target prediction. using TargetScan v.7.2 an Diana Tools MicroT-CDS. for miR-375-3p

Target Gene	**Cumulative weighted context++ score**	**Total context++ score**	**Aggregate PCT**	**MiTG**	**Target Gene**	**Cumulative weighted context++ score**	**Total context++ score**	**Aggregate PCT**	**MiTG**
ELAVL4	-0.56	-0.56	0.58	0.999989	EIF1	-0.12	-0.26	0.36	0.880286
CHSY1	-0.43	-0.66	0.59	0.976388	RBM47	-0.11	-0.13	0.36	0.765032
SLC16A2	-0.37	-0.38	0.36	0.999782	LGALSL	-0.11	-0.22	0.34	0.754329
SHOX2	-0.35	-0.45	< 0.1	0.991189	TCF4	-0.1	-0.12	0.35	0.753006
YBX1	-0.33	-0.33	0.36	0.902381	HS3ST3B1	-0.1	-0.1	0.36	0.876033
PAX2	-0.32	-0.32	0.39	0.820665	SOCS4	-0.1	-0.17	0.41	0.755476
EBF3	-0.32	-0.35	0.35	0.726432	SOX21	-0.09	-0.11	0.36	0.732496
HNF1B	-0.32	-0.32	< 0.1	0.908362	ZFYVE26	-0.09	-0.13	0.36	0.706853
FZD8	-0.32	-0.32	0.54	0.863638	MYO9A	-0.09	-0.15	0.59	0.927255
MXI1	-0.31	-0.33	0.58	0.938366	BMPR2	-0.09	-0.16	0.36	0.800475
ZFP36L2	-0.3	-0.3	0.59	0.810248	PATZ1	-0.09	-0.09	ORF	0.855072
GRIN2B	-0.25	-0.25	0.36	0.888724	ZFHX4	-0.09	-0.09	0.58	0.88181
PPP2R2A	-0.25	-0.25	< 0.1	0.930702	CREBZF	-0.08	-0.2	0.39	0.754205
UBE2E2	-0.24	-0.34	0.36	0.871245	DIP2C	-0.08	-0.1	0.38	0.987795
XPR1	-0.23	-0.24	0.57	0.946169	EPHA4	-0.07	-0.08	< 0.1	0.918228
OTP	-0.22	-0.22	0.41	0.844445	SGMS1	-0.07	-0.07	0.34	0.750379
MOB1A	-0.22	-0.23	0.34	0.812535	CORO2A	-0.07	-0.27	0.56	0.822486
CTBP2	-0.22	-0.22	0.36	0.834557	ATG2B	-0.07	-0.07	0.36	0.793108
ARL4C	-0.21	-0.21	< 0.1	0.843944	TEAD1	-0.07	-0.07	0.35	0.824171
CEPT1	-0.21	-0.21	0.35	0.921258	PRPF4B	-0.07	-0.07	0.35	0.858397
ZFPM2	-0.21	-0.21	0.35	0.95941	ARHGAP35	-0.07	-0.07	0.35	0.732051
ATP1B1	-0.2	-0.2	0.36	0.923197	GREM2	-0.07	-0.22	0.36	0.741489
MSL2	-0.19	-0.19	0.38	0.940437	TMEM65	-0.07	-0.17	0.36	0.905668
RS1	-0.19	-0.19	0.36	0.874091	TSC1	-0.06	-0.07	0.36	0.929274
TSC22D2	-0.19	-0.2	0.36	0.845449	MIER3	-0.06	-0.08	0.34	0.721805
ELAVL2	-0.19	-0.2	0.58	0.95043	NLK	-0.06	-0.18	0.35	0.786771
PRKD1	-0.18	-0.18	0.36	0.804505	CNIH4	-0.06	-0.15	0.36	0.877934
USP1	-0.17	-0.22	0.36	0.806931	MBNL1	-0.06	-0.06	0.36	0.786062
AKIRIN1	-0.17	-0.19	0.35	0.718441	SFXN1	-0.06	-0.17	0.36	0.818939
EIF4G3	-0.17	-0.29	0.58	0.840273	SLC4A4	-0.05	-0.06	0.36	0.915747
CSNK2A1	-0.17	-0.19	0.35	0.718696	UHRF1BP1L	-0.05	-0.09	0.36	0.781742
UBE3A	-0.16	-0.16	0.36	0.902209	GRM5	-0.05	-0.05	0.36	0.894382
RBPJ	-0.16	-0.25	0.51	0.817749	ATXN1	-0.05	-0.07	0.36	0.956079
PAX6	-0.16	-0.23	0.37	0.949229	PLXNA4	-0.05	-0.09	0.38	0.755992
HOXA3	-0.16	-0.16	0.36	0.839103	SETX	-0.05	-0.15	0.34	0.903034

NR5A2	-0.16	-0.16	0.36	0.708876	CASZ1	-0.05	-0.05	0.36	0.789926
PDE4D	-0.15	-0.17	0.58	0.753964	ENAH	-0.05	-0.05	0.36	0.873663
KLF4	-0.15	-0.15	0.36	0.896054	RASD1	-0.04	-0.21	0.36	0.774396
SPAG9	-0.14	-0.16	0.36	0.885706	ZRANB1	-0.04	-0.1	0.36	0.707826
RORB	-0.14	-0.14	0.58	0.92313	PURB	-0.03	-0.05	0.37	0.943915
UST	-0.14	-0.15	0.36	0.850621	ARHGEF12	-0.02	-0.04	0.37	0.959875
SERBP1	-0.14	-0.14	0.36	0.843258	SLC7A11	-0.02	-0.09	0.36	0.789644
PITPNA	-0.14	-0.14	0.35	0.729815	PDE5A	-0.02	-0.07	0.36	0.864356
MATR3	-0.14	-0.14	0.36	0.82016	SWAP70	-0.02	-0.15	0.35	0.725131
GRIK2	-0.14	-0.14	0.35	0.891284	SOX6	-0.02	-0.06	0.58	0.776056
HAS2	-0.13	-0.13	0.36	0.85278	NFIX	-0.01	-0.04	< 0.1	0.99207
PDPK1	-0.13	-0.14	0.36	0.868238	NUFIP2	-0.01	-0.07	0.36	0.899627
EVI5	-0.13	-0.13	0.36	0.813342	MAP3K1	-0.01	-0.19	0.35	0.922994
CCDC176	-0.12	-0.12	0.34	0.742126	MED13	0	-0.04	0.35	0.976558
LSM12	-0.12	-0.17	0.34	0.846146	CYB5B	0	-0.29	0.5	0.811311
EHMT1	-0.12	-0.17	0.36	0.841575	IGSF10	0	-0.06	0.35	0.729088
DNAJC16	-0.12	-0.14	0.36	0.819471	CADM1	0	-0.03	0.36	0.886206
SCN2B	-0.12	-0.12	0.39	0.724991	TBC1D9	0	-0.14	0.36	0.749672
MAPRE2	-0.12	-0.12	0.35	0.723485					

Supplementary Table 6 – Results of the target prediction. using TargetScan v.7.2. and DIANA Tools MicroT-CDS v.5.0.. for miR-205-5p

Target Gene	Cumulative weighted context++ score	Total context++ score	Aggregate PCT	MiTG	Target Gene	Cumulative weighted context++ score	Total context++ score	Aggregate PCT	MiTG
CHN1	-0.92	-0.92	0.97	0.999993252	RBFOX3	-0.15	-0.15	0.17	0.73603808
MTFR1L	-0.73	-0.76	< 0.1	0.999525229	ERBB4	-0.15	-0.19	0.84	0.98597218
RBMX	-0.67	-0.68	< 0.1	0.952901649	LPCAT1	-0.15	-0.23	0.6	0.975751437
SH3GL3	-0.63	-0.63	ORF	0.733970037	SLC24A2	-0.15	-0.15	0.17	0.778414112
CDH11	-0.6	-0.66	0.25	0.981799127	MAGI2	-0.15	-0.15	0.55	0.920241676
SLC35B3	-0.58	-0.66	0.87	0.938354779	RUNX2	-0.15	-0.15	0.51	0.848211814
RNF146	-0.58	-0.58	ORF	0.72278313	FAM104A	-0.14	-0.27	< 0.1	0.754973635
COL16A1	-0.56	-0.56	0.6	0.926933374	CPEB2	-0.14	-0.14	0.56	0.934905009
HS3ST1	-0.54	-0.55	0.89	0.99546152	PPP3R1	-0.14	-0.14	0.15	0.914369043
SEPT4	-0.49	-0.49	ORF	0.99127931	MED1	-0.14	-0.15	0.12	0.979514007
SLC35A1	-0.49	-0.49	0.38	0.991811429	ARFGEF1	-0.14	-0.18	0.16	0.84797578
GXYLT1	-0.47	-0.47	0.45	0.914210714	USP7	-0.14	-0.15	0.2	0.857230071
RCBTB1	-0.47	-0.51	0.18	0.997861681	STK3	-0.14	-0.14	0.15	0.717986453
SSBP3	-0.46	-0.46	0.8	0.919610945	CDC27	-0.13	-0.2	0.55	0.961094045
SELT	-0.45	-0.45	0.74	0.919103634	CREBRF	-0.13	-0.13	0.34	0.825448902
BICC1	-0.43	-0.43	0.58	0.984277347	AMOT	-0.13	-0.18	0.25	0.87796691
MARCKS	-0.43	-0.45	0.94	0.992188679	DOK4	-0.13	-0.24	0.4	0.99787169
NDUFA4	-0.43	-0.44	0.63	0.885879847	TNRC6C	-0.12	-0.13	0.43	0.704901862
ACSL1	-0.43	-0.49	0.54	0.999853733	RAB11FIP1	-0.12	-0.33	0.77	0.87174434
LENG9	-0.43	-0.43	< 0.1	0.738991679	GRB10	-0.12	-0.15	0.15	0.734196062
ARRB2	-0.42	-0.42	ORF	0.720210716	UBIAD1	-0.12	-0.25	0.23	0.905361141
RAP2B	-0.41	-0.6	0.72	0.751604115	ZCCHC14	-0.11	-0.12	0.82	0.999888663
CASC4	-0.39	-0.39	< 0.1	0.999057233	YES1	-0.11	-0.12	0.19	0.776862968
CTPS2	-0.38	-0.54	0.27	0.966235919	BCL6	-0.11	-0.11	0.15	0.801774655
RTN3	-0.34	-0.34	ORF	0.979534977	PJA2	-0.11	-0.24	< 0.1	0.706322003
DDX5	-0.34	-0.34	0.36	0.990647314	NAA10	-0.11	-0.11	ORF	0.712775437
PTPRM	-0.34	-0.34	0.48	0.980408592	FAM84B	-0.11	-0.22	0.64	0.963696843
LHFPL2	-0.33	-0.33	0.13	0.934212645	MICAL2	-0.11	-0.11	0.41	0.992118624
FAM126A	-0.33	-0.43	0.32	0.97998278	LCOR	-0.1	-0.1	0.49	0.938548892
SGMS1	-0.32	-0.33	0.28	0.88687212	GTF3C2	-0.1	-0.16	0.14	0.832298378
UBE2N	-0.32	-0.35	0.55	0.916768162	LYSMD4	-0.1	-0.1	0.16	0.880004607
NAA11	-0.32	-0.32	< 0.1	0.802985979	SMIM14	-0.1	-0.1	0.16	0.803350247
CRMP1	-0.32	-0.32	0.15	0.845437419	UBE2E3	-0.1	-0.12	0.15	0.900154621
EZR	-0.31	-0.4	0.18	0.877699421	ITGA5	-0.09	-0.09	0.15	0.870049795
TMEM255A	-0.31	-0.31	0.37	0.966052396	DNM3	-0.09	-0.18	0.15	0.941321691
SBF2	-0.31	-0.34	0.74	0.998177791	AP1G1	-0.09	-0.09	0.14	0.896858213
LSAMP	-0.31	-0.52	0.24	0.970999734	PPP1R3A	-0.09	-0.09	0.15	0.885846296
ABHD17B	-0.3	-0.3	0.2	0.987641095	UBFD1	-0.09	-0.09	0.15	0.847038958
ADAMTS9	-0.3	-0.3	0.82	0.999292073	STRBP	-0.09	-0.09	0.15	0.94855762
NTNG1	-0.29	-0.29	< 0.1	0.985800072	CSF1	-0.09	-0.09	0.52	0.86669592
AAK1	-0.29	-0.3	0.79	0.929579165	KLHL31	-0.09	-0.14	< 0.1	0.733184382
MFNG	-0.28	-0.28	0.13	0.888952325	KLF7	-0.09	-0.13	0.24	0.870669959
ZEB2	-0.27	-0.31	0.2	0.949596923	ACBD5	-0.09	-0.09	0.15	0.740118816
CALU	-0.27	-0.43	0.48	0.926005913	RBPMS2	-0.09	-0.41	0.27	0.847065105
PHC2	-0.27	-0.27	0.59	0.999909005	AXIN2	-0.09	-0.09	0.15	0.811660502
LIMS2	-0.26	-0.26	0.21	0.882267543	HMGB1	-0.08	-0.23	0.16	0.766985227
GATA3	-0.26	-0.36	0.44	0.8607067	STXBP6	-0.08	-0.26	< 0.1	0.768967766
RAB11FIP2	-0.26	-0.27	0.17	0.779180309	SCMH1	-0.08	-0.08	0.46	0.722155904
ELF1	-0.26	-0.27	0.16	0.932311147	ETF1	-0.08	-0.09	0.19	0.892559419
FBXO24	-0.26	-0.26	< 0.1	0.963043192	LRCH3	-0.08	-0.34	0.65	0.999225786
RBM47	-0.25	-0.25	0.37	0.96075938	GALNT3	-0.08	-0.08	< 0.1	0.797236432
ENC1	-0.25	-0.26	0.54	0.933483908	PAFAH1B1	-0.08	-0.13	< 0.1	0.709441751
DGCR8	-0.25	-0.25	0.15	0.792630328	ZEB1	-0.08	-0.08	0.59	0.95658008
PHB	-0.24	-0.24	0.27	0.942033265	KLHL15	-0.08	-0.08	0.34	0.919104238
ESRRG	-0.24	-0.24	0.6	0.944665844	KAZN	-0.07	-0.21	0.16	0.704917971
MGRN1	-0.24	-0.24	0.91	0.960333878	PTPRJ	-0.07	-0.08	0.52	0.864906774
CBX1	-0.23	-0.23	0.3	0.865406798	SRGAP1	-0.07	-0.1	0.13	0.910910396
DUSP7	-0.23	-0.39	0.84	0.990130059	LUC7L3	-0.07	-0.25	< 0.1	0.773759121
CNIH1	-0.23	-0.24	< 0.1	0.727955829	KSR1	-0.07	-0.07	0.6	0.754976653
IRF2BPL	-0.23	-0.23	0.15	0.847668051	NFAT5	-0.07	-0.09	0.79	0.888654354
FNBP1L	-0.23	-0.23	0.15	0.857705505	DSC1	-0.07	-0.07	ORF	0.816335742
TBCEL	-0.23	-0.4	0.22	0.882258541	POU2F1	-0.06	-0.07	0.45	0.988942977
MLLT4	-0.23	-0.23	0.74	0.994133999	WWC2	-0.06	-0.07	0.39	0.746953504
EAF1	-0.23	-0.24	< 0.1	0.810976238	CCDC93	-0.06	-0.21	0.16	0.750168736

SLC19A2	-0.23	-0.23	0.18	0.862316937	HNRNPK	-0.06	-0.06	0.16	0.780812016
VEGFA	-0.22	-0.23	0.53	0.964016185	TNRC6B	-0.05	-0.07	0.15	0.793505058
MIER3	-0.22	-0.25	0.15	0.794972754	NHS	-0.05	-0.05	< 0.1	0.792377623
MMD	-0.22	-0.22	< 0.1	0.866612867	PRKCA	-0.05	-0.09	< 0.1	0.872703581
DLG2	-0.22	-0.22	0.6	0.983278957	DDX52	-0.05	-0.09	0.16	0.864676578
FA2H	-0.22	-0.22	0.29	0.803072096	TRPS1	-0.05	-0.06	0.18	0.724851769
ERRFI1	-0.22	-0.24	0.2	0.965187168	LIN9	-0.04	-0.23	0.53	0.955846215
HIATL1	-0.22	-0.22	< 0.1	0.751577033	ACTB	-0.04	-0.16	< 0.1	0.727822925
E2F1	-0.21	-0.21	0.19	0.772707982	MTRF1L	-0.04	-0.04	ORF	0.72165992
CCDC43	-0.21	-0.21	0.17	0.892674628	KMT2A	-0.04	-0.04	0.39	0.988582169
IL1R1	-0.21	-0.21	0.13	0.888622728	CMTM4	-0.04	-0.12	0.15	0.796109514
RBMS1	-0.21	-0.21	0.59	0.922485683	MAGI1	-0.03	-0.08	0.2	0.715465315
RNF157	-0.21	-0.23	0.46	0.871539997	PSD3	-0.03	-0.1	0.16	0.949168421
CCDC176	-0.21	-0.21	< 0.1	0.838593893	TIAL1	-0.03	-0.03	0.16	0.802738327
NACC2	-0.21	-0.3	0.97	0.998641047	CDC42BPB	-0.03	-0.03	< 0.1	0.826726593
CLK3	-0.2	-0.2	0.13	0.819476251	SEPT3	-0.03	-0.03	< 0.1	0.742390638
PLCB1	-0.2	-0.2	0.15	0.999110121	RARA	-0.03	-0.1	0.15	0.726973094
FAM155A	-0.2	-0.2	0.32	0.954006614	MTMR4	-0.03	-0.03	< 0.1	0.861373076
FAM168A	-0.2	-0.29	0.31	0.835083967	MAP3K9	-0.03	-0.03	0.15	0.801937473
RORA	-0.19	-0.23	0.57	0.993529416	STK10	-0.03	-0.29	0.49	0.846486873
TNR	-0.19	-0.19	0.35	0.831282215	RPS6KA3	-0.03	-0.17	0.61	0.906383808
NCOA1	-0.19	-0.2	0.29	0.804713828	NSUN5	-0.03	-0.15	0.16	0.939357455
CALCRL	-0.19	-0.51	0.86	0.837944438	HOXD13	-0.02	-0.21	0.23	0.96584158
GLIS3	-0.18	-0.18	0.45	0.920216282	PDE7A	-0.02	-0.02	0.16	0.940721633
TRAK2	-0.18	-0.18	0.26	0.972603855	ARMC8	-0.02	-0.12	< 0.1	0.914997176
BAMBI	-0.18	-0.18	0.16	0.944987005	MPRIP	-0.02	-0.02	< 0.1	0.920842137
AFF1	-0.18	-0.3	0.15	0.763660049	NR3C2	-0.01	-0.01	0.15	0.812906919
UBE2G1	-0.18	-0.21	0.12	0.711123012	IPPK	-0.01	-0.19	0.39	0.893529093
FRK	-0.18	-0.43	0.98	0.992187598	ANK2	-0.01	-0.01	ORF	0.858822353
PAX9	-0.18	-0.21	0.38	0.959988208	LRP6	-0.01	-0.01	0.16	0.77409024
TMEM236	-0.18	-0.18	0.33	0.8062065	ARID1A	-0.01	-0.01	0.15	0.72157762
NFIB	-0.18	-0.2	0.56	0.963019587	TMEM136	0	-0.14	0.17	0.766204693
TMEM236	-0.17	-0.17	0.34	0.8062065	PAPD5	0	-0.09	0.84	0.987748336
HERC3	-0.17	-0.21	0.23	0.871832487	SIPA1L1	0	-0.15	0.74	0.939530097
VTI1B	-0.17	-0.2	0.15	0.760560931	EIF4E	0	-0.12	0.16	0.745426026
TXNRD1	-0.16	-0.16	0.16	0.714739043	TM9SF2	0	-0.28	0.29	0.975710769
TLK1	-0.16	-0.2	0.29	0.762889503	CADM1	0	-0.23	0.8	0.998396394
TM9SF3	-0.16	-0.2	0.36	0.877885712	ZBTB20	0	-0.38	0.9	0.999816235
RAB9B	-0.15	-0.15	0.32	0.772182941	EPS8	0	-0.1	0.18	0.814913423
TTI1	-0.15	-0.16	0.15	0.762503424					

Supplementary Table 7 - Results of the target prediction. using TargetScan v.7.2 and Diana MicroT-CDS v.5.0. for miR-92a-1-5p

Target Gene	Cumulative weighted context++ score	Total context++ score	Aggregate PCT	MiTG	Target Gene	Cumulative weighted context++ score	Total context++ score	Aggregate PCT	MiTG
SLC25A23	-1.27	-1.27	N/A	0.724338153	RPH3AL	-0.19	-0.28	N/A	0.719469008
ANAPC15	-0.75	-0.75	N/A	0.740113401	ENG	-0.18	-0.18	N/A	0.711306376
SF3A3	-0.67	-0.67	N/A	0.765194043	ZBTB16	-0.18	-0.2	N/A	0.704966765
YBX3	-0.58	-0.59	N/A	0.91528158	NFIC	-0.17	-0.17	N/A	0.897584357
PAX5	-0.55	-0.55	N/A	0.73296523	PLEC	-0.17	-0.17	N/A	0.805138175
FOXP1	-0.54	-0.54	N/A	0.74974079	DUSP13	-0.17	-0.17	N/A	0.725291403
UBQLN4	-0.54	-0.54	N/A	0.73940766	PVR	-0.16	-0.16	N/A	0.804009942
PPME1	-0.5	-0.5	N/A	0.993161162	MATN1	-0.16	-0.16	N/A	0.702411274
CEP128	-0.47	-0.47	N/A	0.710240527	ANO5	-0.15	-0.2	N/A	0.901922797
MSI1	-0.46	-0.46	N/A	0.883522177	CNTN4	-0.15	-0.16	N/A	0.793381793
NANP	-0.45	-0.45	N/A	0.765256616	QSER1	-0.15	-0.18	N/A	0.725760411
WNK3	-0.44	-0.44	N/A	0.906745437	SEMA5A	-0.14	-0.14	N/A	0.917690472
FBXL16	-0.41	-0.41	N/A	0.897573645	PLEKHO2	-0.14	-0.14	N/A	0.819790157
NECAB1	-0.4	-0.4	N/A	0.867738479	WASF2	-0.14	-0.14	N/A	0.723182919
NECAB1	-0.4	-0.4	N/A	0.797531498	SEPT11	-0.13	-0.13	N/A	0.822719738
PRPF3	-0.39	-0.39	N/A	0.741453403	UGGT1	-0.11	-0.32	N/A	0.731965789
KCNC1	-0.39	-0.39	N/A	0.704196136	CLSTN2	-0.1	-0.18	N/A	0.934204647
ENSA	-0.34	-0.43	N/A	0.771236868	TNPO1	-0.1	-0.1	N/A	0.96483842
AAK1	-0.31	-0.39	N/A	0.732052895	DNAJC5	-0.1	-0.12	N/A	0.847838553
DOC2A	-0.3	-0.3	N/A	0.828597686	SLC6A17	-0.1	-0.1	N/A	0.725119025
TRPS1	-0.3	-0.3	N/A	0.709692094	PAPPA	-0.09	-0.09	N/A	0.735435294
FBXW7	-0.29	-0.29	N/A	0.839990156	ANGEL1	-0.07	-0.07	N/A	0.844329569
DCLK2	-0.29	-0.29	N/A	0.744061585	MLX	-0.07	-0.26	N/A	0.742852151
ELK1	-0.28	-0.28	N/A	0.871306395	ARPP19	-0.06	-0.18	N/A	0.886682773
SERPINA6	-0.28	-0.28	N/A	0.716462407	EIF4E3	-0.05	-0.18	N/A	0.810498788
TMEM167B	-0.26	-0.26	N/A	0.784646285	TNRC6B	-0.04	-0.04	N/A	0.744628843
LRRN2	-0.26	-0.26	N/A	0.774779473	NOS1	-0.03	-0.03	N/A	0.816510378
BACH2	-0.26	-0.27	N/A	0.75160741	WIPF2	-0.03	-0.16	N/A	0.810571013
SV2B	-0.25	-0.35	N/A	0.822705169	LPHN1	-0.03	-0.05	N/A	0.735511748
EPB42	-0.25	-0.25	N/A	0.746322714	HNF1A	-0.02	-0.02	N/A	0.915450583
JPH4	-0.24	-0.24	N/A	0.759273395	GAS7	-0.01	-0.16	N/A	0.830489739
NAV1	-0.23	-0.29	N/A	0.807522062	LIG3	-0.01	-0.17	N/A	0.713390023
ZBTB7A	-0.22	-0.22	N/A	0.727907619	SLC22A1	0	-0.14	N/A	0.902476233
ATP2B4	-0.22	-0.22	N/A	0.709972891	SLC16A3	0	-0.18	N/A	0.891252288
BSDC1	-0.21	-0.21	N/A	0.794279074	ZBTB20	0	-0.05	N/A	0.868198765
MMP9	-0.21	-0.42	N/A	0.706774012	ERCC6	0	-0.01	N/A	0.78574321
NTNG1	-0.2	-0.2	N/A	0.812611243	SRPK2	0	-0.41	N/A	0.778547884
POP5	-0.2	-0.2	N/A	0.758119971					