Universidade de Lisboa
Faculdade de Farmácia

Dissecting miRNA-194-5p's and MEF2C's roles in breast cancer brain metastases

Sara Cristina Barbosa Caetano

Dissertação orientada pela Professora Doutora Maria Alexandra Brito e co-orientada pela Doutora Inês Figueira

Dissertação de Mestrado para obtenção do grau de Mestre em Ciências Biofarmacêuticas

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“Do not follow where the path may lead.
Go, instead, where there is no path and leave a trail.”
Ralph Waldo Emerson

The studies presented in this master thesis were performed in the research group "Neurovascular Lab", from the Research Institute for Medicines (imed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa, under the supervision of Professor Doctor Maria Alexandra Brito, and co-supervision of Doctor Inês Figueira. All the presented images of immunofluorescence were acquired at the Faculty of Sciences of the University of Lisbon's Microscopy Facility, a node of the Portuguese Platform for BioImaging, reference PPBI-POCI-01-0145-FEDER-022122, under collaboration with Professor Rui Malhó. The figures of this work were created with aid of Servier medical art and BioRender software. This study was supported by Fundação para a Ciência e Tecnologia (FCT – PTDC/MED-ONC/29402/2017, UIDB/04138/2020 and UIPD/04138/2020)

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Abstract

Breast cancer (BC) is the most commonly diagnosed tumor in women worldwide. Among BC subtypes, triple negative BC is the most aggressive, with proneness to metastasize to the brain. In fact, BC brain metastases are a major contributor to mortality in BC patients, owing to lack of targeted therapies. During the metastatic cascade, BC cells undergo phenotypic changes, known as epithelial-mesenchymal transition, which endow motility properties essential for metastases occurrence. Nevertheless, the intricated complexity that characterizes BC brain metastases formation, specially from triple negative BC, is still poorly understood. Hence, discovery of new BC metastases players involved in metastatic BC is urgently needed.

In previous studies, we found that microRNA (miR)-194-5p is downregulated in blood circulation at early stages of BC brain metastases development, pointing to its role as tumor suppressor. Through bioinformatical analysis, we uncovered the transcription factor myocyte enhancer factor 2C (MEF2C) as a miR-194-5p target. Moreover, analysis of MEF2C expression in the brain parenchyma revealed that MEF2C is increasingly expressed along BC brain metastases development, in a mouse model of the pathology, and along disease severity in resected brain metastases from BC patients. Therefore, both miR-194-5p and MEF2C may constitute new players in BC brain metastases. However, the causality between miR-194-5p's downregulation and MEF2C's upregulation and their contribution to malignant cells features remain undetermined. Hence, this study aims at disclosing the role of miR-194-5p and MEF2C in tumorigenesis and establishing whether miR-194-5p's and MEF2C's deregulation are linked.

A triple negative BC cell line (4T1 cells) was used for mechanistic studies via transfections with a siRNA or a plasmid for MEF2C, or with a pre-miR-194-5p. Modulation efficiencies were ascertained by real-time quantitative polymerase chain reaction (RT-qPCR), as well as by immunofluorescence analysis of MEF2C, or *in situ* hybridization of miR-194-5p. In both cases, cells' viability was evaluated by MTT assay, while phenotypic alterations were inspected by immunofluorescence analysis of cytokeratin and vimentin, as epithelial and mesenchymal markers, respectively. Additionally, migration of 4T1 cells was assessed via wound healing assay. To analyze the reflex of miR-194-5p's modulation on MEF2C's expression, 4T1 cells were transfected with pre-miR-194-5p and MEF2C's mRNA and protein levels were

determined by RT-qPCR and immunofluorescence anaçysis, respectively.

MEF2C's silencing was confirmed by reduced mRNA and protein expression levels, whereas the augment seen in MEF2C's levels with the plasmid did not reflect an acceptable transfection efficiency regarding protein levels, which led to the exclusion of this procedure from further experiments. Interestingly, MEF2C-silenced cells presented a decline in both vimentin and cytokeratin expression, in addition to a loss of migratory capability. Pre-miR-194-5p treatment induced a dose-dependent augment of the miRNA's expression in 4T1 cells, albeit with increasing toxicity. The non-toxic concentration of pre-miR-194-5p was selected and its cellular expression was validated via *in situ* hybridization. Overexpression of miR-194-5p promoted an increase of cytokeratin and a reduction of vimentin, concomitant with a decrease of migratory capability. Furthermore, MEF2C's mRNA and protein levels at three different timepoints (24, 48 and 72 h) did not suffer alterations upon transfection with pre-miR-194-5p.

Together, the effects of silencing MEF2C on the epithelial and mesenchymal markers expression, alongside with the reduction of 4T1 cells' migratory capability, suggest that MEF2C determines BC cells invasive properties by partially determining the occurrence of epithelial-mesenchymal transition. Overexpression of miR-194-5p also reduced 4T1 cells' motility, which coupled with the observed shift from mesenchymal to epithelial phenotype, reflect a decline in BC cells aggressive behavior and reinforce the miRNA role as tumor suppressor in triple negative BC. Even though bioinformatically MEF2C emerged as a target of miR-194-5p, the *in vitro* results did not demonstrate the link between these two players, which may be explained by the fact that the altered levels of miR-194-5p and MEF2C were detected in different samples (plasma and malignant cells, respectively). Additionally, not only *in vitro* studies cannot fully mimic the temporal sequence of events occurring *in vivo*, but also activation of alternative and/or compensatory pathways may have taken place, preventing the establishment of a direct relationship between miR-194-5p and MEF2C.

By revealing miR-194-5p and MEF2C's contributions to tumorigenesis, this study discloses novel modulation targets in BC patients to improve disease-free survival.

Keywords: Epithelial-mesenchymal transition; invasiveness; MEF2C; miR-194-5p; triple negative breast cancer.

Resumo Alargado

A nível global, o cancro mais diagnosticado em mulheres é o cancro da mama, tendo uma incidência de 2,3 milhões de novos casos por ano, o que levou à sua classificação como uma das principais causas de morte relacionada com o cancro. De entre os diversos subtipos de cancro da mama, o triplo negativo é considerado o subtipo mais agressivo, caracterizado por uma elevada propensão para desenvolver metástases, especialmente no parênquima encefálico. De facto, as metástases encefálicas de cancro da mama são um dos fatores que mais contribui para os elevados números de mortalidade em pacientes de cancro da mama, em muito devido à falta de terapias direcionadas e específicas para este subtipo.

Durante o desenvolvimento de metástases, por um processo conhecido como a cascata metastática, as células tumorais sofrem alterações fenotípicas e morfológicas associadas sobretudo à ocorrência de um processo designado por transição epitelial-mesenquimal. Neste sentido, durante a transição epitelial-mesenquimal, as células tumorais perdem algumas das suas características epiteliais e adquirem um fenótipo parcial ou completamente mesenquimal, o que lhes confere propriedades de motilidade essenciais para processos invasivos que podem culminar na formação de metástases. No entanto, a complexidade que caracteriza a formação de metástases encefálicas de cancro da mama, especialmente derivadas do subtipo de cancro da mama triplo negativo, ainda é pouco compreendida. Por conseguinte, a descoberta de novas estratégias terapêuticas que consigam atuar na prevenção e mitigação das metástases de cancro da mama é necessária. Para tal, é imperativa a utilização de novos alvos passíveis de serem modulados e que visem prevenir ou atenuar o prognóstico associado à formação das metástases.

Apesar das descobertas relativamente recentes no âmbito dos miRNAs e de proteínas oncogénicas, ainda há um longo caminho a percorrer para aumentar a sobrevivência global em pacientes com cancro da mama, especialmente aqueles que sofrem com a forma metastática de cancro da mama do subtipo triplo negativo. Estudos prévios realizados no nosso laboratório revelaram que o microRNA (miRNA ou miR)-194-5p está sub-expresso na corrente sanguínea numa fase inicial do desenvolvimento de metástases encefálicas de cancro da mama, o que aponta para que este miRNA possa ter um papel supressor no desenvolvimento de tumores. Através de uma análise bioinformática, foi também

revelado que o fator de transcrição, conhecido como fator ativador do miócito 2C (MEF2C, do inglês *myocyte enhancer factor 2C*) é um alvo do miR-194-5p. Para além disso, a análise da expressão do MEF2C no parênquima encefálico, utilizando um modelo de ratinho da doença, demonstrou que este fator de transcrição tem uma expressão crescente ao longo do desenvolvimento de metástases encefálicas de cancro da mama. Adicionalmente, em amostras de pacientes com metástases encefálicas de cancro da mama, foi possível observar que com o aumento da severidade da doença, os níveis de MEF2C também incrementavam. Por estes motivos, tanto o miR-194-5p como o MEF2C surgem como novos intervenientes a serem estudados, com grande potencial para serem modulados e, desta forma, conseguir melhorar o prognóstico dos pacientes que sofrem de cancro da mama triplo negativo.

Apesar da previsão *in silico* apontar para o MEF2C como um alvo do miR-194-5p, a causalidade entre a desregulação dos dois ainda não foi analisada até à data. Assim, este estudo pretende esclarecer o envolvimento tanto do MEF2C, tal como do miR-194-5p na tumorigénese, tentando estabelecer uma relação direta entre a sub-expressão deste miRNA e a sobre-expressão desta proteína. Adicionalmente, será determinante também perceber o papel do MEF2C e do miR-194-5p isoladamente nas propriedades metastáticas das células tumorais de cancro da mama.

Uma linha celular de ratinho de cancro da mama triplo negativo (células 4T1) foi usada para as transfeções com um siRNA e um plasmídeo específicos para o MEF2C, ou com o pre-miR-194-5p, com o intuito de modular a expressão da proteína e do miRNA, respetivamente. As eficiências de modulação foram determinadas por *real-time quantitative polymerase chain reaction* (RT-qPCR) e por análise dos níveis de expressão do MEF2C e do miR-194-5p por imunofluorescência e por hibridização *in situ*, respetivamente. Em ambos os casos e após as modulações dos respetivos alvos, a viabilidade das células tumorais foi determinada pelo ensaio colorimétrico de MTT, enquanto as alterações fenotípicas das células foram avaliadas por imunofluorescência, analisando proteínas como a citoqueratina e a vimentina, marcadores epitelial e mesenquimal, respetivamente. Além disso, o comportamento migratório das células tumorais 4T1 foi seguido através da técnica de *wound healing*. Por último, o reflexo direto da modulação do miR-194-5p na expressão do MEF2C foi estudado recorrendo à análise dos níveis de mRNA e expressão proteica do MEF2C, através de RT-qPCR e análise de imunofluorescência respetivamente, ambos em células tumorais 4T1 a sobre-expressar o miR-194-5p.

A modulação dos níveis de expressão do MEF2C foi conseguida com a transfeção de um siRNA e um plasmídeo, de forma a silenciar e sobre-expressar o MEF2C, respetivamente. O resultado do silenciamento do MEF2C foi confirmado pela redução significativa dos seus níveis de mRNA e da proteína. Apesar do aumento significativo dos níveis de mRNA do MEF2C, causado pelo plasmídeo, o mesmo não aconteceu com os seus níveis proteicos, uma vez que a eficiência de transfeção demonstrou ser consideravelmente baixa. Por esta razão, o plasmídeo usado para induzir a sobre-expressão do MEF2C foi excluído das restantes experiências em que se estudou o efeito desta proteína na tumorigénese das células de cancro da mama triplo negativo. Curiosamente, as células tumorais 4T1 que foram sujeitas ao silenciamento do MEF2C apresentaram uma diminuição na expressão tanto de vimentina como de citoqueratina, promovendo ainda uma perda significativa de capacidade migratória destas células. Por outro lado, o tratamento com o pre-miR-194-5p induziu um aumento dependente da dose na expressão do miRNA em células tumorais 4T1, embora com crescente toxicidade. Apesar da redução de viabilidade observada nas células tumorais tratadas com o pre-miR-194-5p a uma concentração de 30 nM, esta condição deverá ser estudada mais a fundo no futuro como uma possível opção terapêutica direcionada para as células tumorais de cancro da mama triplo negativo. A concentração não tóxica mais eficaz de pre-miR-194-5p (10 nM) foi selecionada e a sua expressão celular foi validada através de hibridização *in situ*, que mostrou novamente o aumento dos níveis celulares deste miR. Para além disso, a sobre-expressão do miR-194-5p promoveu ainda um aumento de citoqueratina e uma redução de vimentina, juntamente com uma diminuição significativa da capacidade migratória das células tumorais 4T1. Por fim, não foi possível estabelecer uma relação de causalidade entre o miR-194-5p e o MEF2C, uma vez que o aumento da expressão deste miR não induziu um efeito percetível nos níveis de mRNA nem proteicos do MEF2C. É importante realçar que com o intuito de perceber se o resultado anterior foi influenciado por algum mecanismo compensatório, foi feito um estudo adicional, a dois tempos diferentes (24 e 72 h). Ainda assim, não foi possível verificar a relação de causalidade entre o aumento da expressão do miR e o MEF2C.

Coletivamente, os efeitos do silenciamento do MEF2C na expressão dos marcadores epitelial (citoqueratina) e mesenquimal (vimentina), juntamente com a redução da capacidade migratória das células tumorais 4T1, sugerem que o MEF2C tem um papel determinante nas propriedades invasoras das células tumorais, determinando a ocorrência parcial de transição epitelial-mesenquimal. De forma semelhante, a sobre-expressão do

miR-194-5p também levou a uma redução da mobilidade das células tumorais 4T1, que, em conjunto com a mudança observada do fenótipo mesenquimal para o fenótipo epitelial, reflete um declínio no comportamento agressivo das células tumorais e reforça o papel do miR como supressor de tumores no cancro da mama triplo negativo.

Em suma, ao perceber o contributo tanto do MEF2C como do miR-194-5p em cancro da mama triplo negativo, este estudo revela novos alvos passíveis de serem modulados em pacientes que sofrem deste subtipo de cancro da mama, de forma a tentar melhorar o seu prognóstico e, se possível, torná-lo livre de metástases.

Palavras-chave: Cancro da mama triplo negativo; capacidade invasiva; MEF2C; miR-194-5p; transição epitelial-mesenquimal

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Abbreviations

AKT2	RAC-beta serine/threonine-protein kinase 2
AP	Alkaline phosphatase
BBB	Blood-brain barrier
BC	Breast cancer
BCCs	Breast cancer cells
BCBM	Breast cancer brain metastases
BMECs	Brain microvascular endothelial cells
bp	Base pairs
BSA	Bovine serum albumin
CHD2	Chromodomain helicase DNA binding protein 2
DIG	Digoxigenin/Digoxin
DPX	Dibutylphthalate polystyrene xylene
ECs	Endothelial cells
EMT	Epithelial-mesenchymal transition
F-actin	Filamentous actin
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HBEGF	Heparin-binding EGF-like growth factor
HBSS	Hank's Balanced Salt Solution
HER	Human epidermal growth factor receptor

HR	Hormone receptor
IF	Immunofluorescence
ISH	*In situ* hybridization
MAP	Mitogen-activated protein
MEF2C	Myocyte enhancer factor 2C
MET	Mesenchymal-epithelial transition
MicroRNAs	MiRNAs or miR-
MLCK	Myosin light-chain kinase
MTT	Thiazolyl blue tetrazolium bromide
NBT/BCIP	Nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate
PanCK	Pan-cytokeratin
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol 3-kinase
RAP2B	RAS-related protein 2B
RPMI	Roswell Park Memorial Institute
RT-qPCR	Real time quantitative polymerase chain reaction
STAT1	Signal transducer and activator of transcription 1
TNBC	Triple negative breast cancer
VE	Vascular endothelial
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor 2

Chapter I – Introduction and Aims

1. Breast cancer – A brief history

In 2018, breast cancer (BC) was ranked second, after lung cancer, among the most common diagnosed tumors worldwide with an incidence of 2.1 million new cases per year, which represents approximately 11.6% of all new cases, being directly and indirectly responsible for 626,679 deaths (6.6%)[1]. Only two years later, in 2020, BC surpassed lung cancer, becoming the type of malignancy with the most diagnosed new cases per year and ranking in fifth as a leading cause of cancer related death worldwide[2]. It was estimated that in 2020 around 2.3 million people developed BC, which corresponds to 11.7% of new cancer cases globally – an increase of 0.1% and an alarming high mortality of around 6.9% per year[2].

Incidence rates of BC in women vary, with Western Europe and the United States presenting the highest ones, whereas Africa and Asia present the lowest ones[3]. Undoubtedly, BC continues to be the most common malignant tumor diagnosed in women and, regrettably, the implementation of primary prevention programs remains a challenge for BC[2]. To our knowledge, there are several contributing factors for BC development that range from reproductive and hormonal factors, such as oral contraceptives or a reduction in breastfeeding, to lifestyle risk factors, like elevated body weight or alcohol consumption[2–4].

Physiologically, BC occurs when there is an up-regulation in cell growth in any part of the breast, even so, the most predominantly histological form is the invasive ductal carcinoma, representing about 50-75% of all BC cases[5–7]. Regardless of the origin, BC tumors, and therefore BC patients, can be categorized into three groups, depending on the type of expression of the receptors in BC cells (BCCs): hormone receptor (HR)-positive, when the estrogen and/or progesterone receptors are positive; human epidermal growth factor receptor 2 (HER2)-positive; and triple negative, when neither the HRs nor HER2 are present[5] (Figure 1).

With an incidence rate of approximately 60% of all BC cases, HR-positive BC is the least aggressive type of BC, and it is more likely to affect older women[7]. HER2-positive BC affects approximately 20% of BC patients and, without systemic therapy, can be associated with a poor prognosis[7]. On the other hand, due to its high metastatic potential within the first 3 to 5 years, triple negative BC (TNBC) is the most aggressive and has the worst survival rate of all three BC subtypes, with an incidence of around

20%[5,7–9]. Younger women are more likely to develop TNBC, but even so, chances are that it will be diagnosed at a later stage (stage III or IV), where the overall survival is reduced to 1 year[7,10].

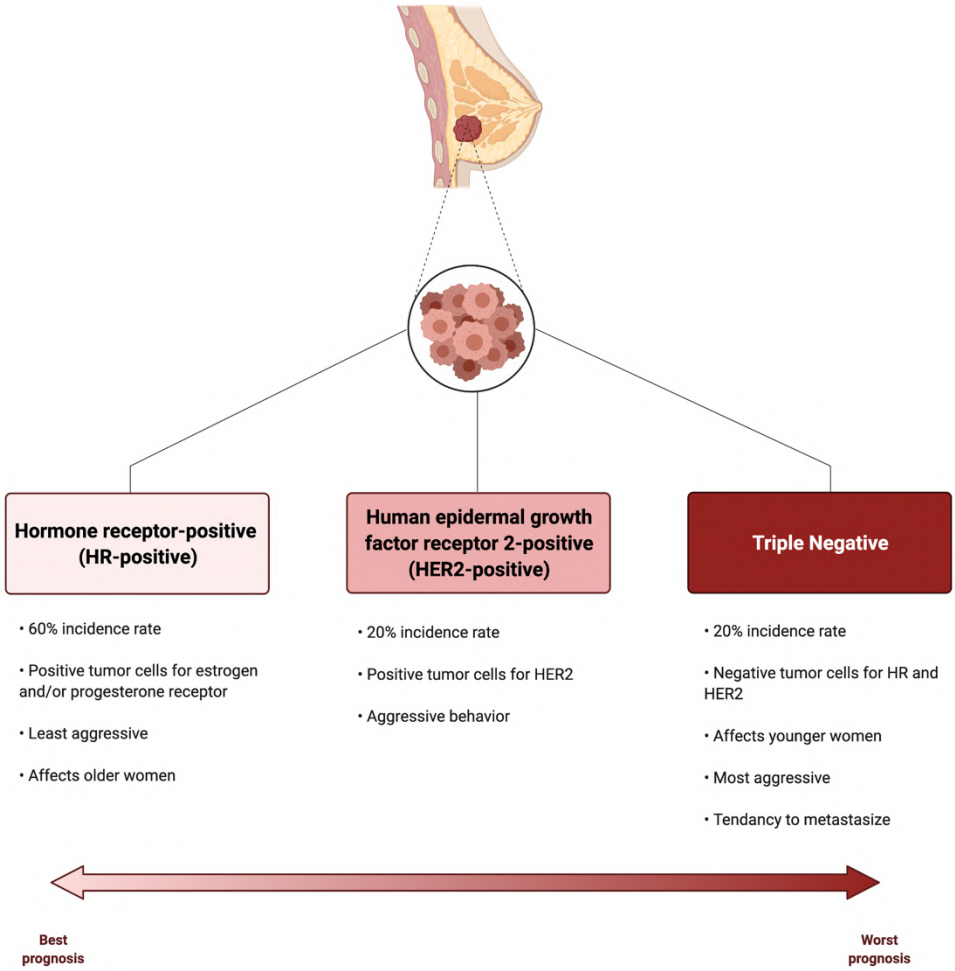

Figure 1. Major breast cancer (BC) subtypes. Three major BC subtypes classified according to cell receptor expression. Hormone receptor (HR)-positive tumors present estrogen and/or progesterone receptor expressing cells and accounts for 60% of BC incidence. This subtype mostly affects older women and is the least aggressive of the three. The second subtype represents around 20% of BC cases and expresses human epidermal growth receptor 2 (HER2) at the cell surface, which defines it as HER2-positive. Lastly, the triple negative has a 20% incidence rate and is characterized by the lack of HR and HER2 receptors. This subtype is most commonly diagnosed in younger women, and due to its tendency to metastasize, is the most aggressive and presents the worst prognosis.

Even though clinical treatment for BC patients varies tremendously depending on several factors (such as the subtype and clinical history), the most commonly used treatment options include surgery, chemotherapy, radiation and hormone therapy[5,8].

These systemic adjuvant treatments are intended to reduce the recurrence rate, by eliminating any remnant of the disease, and consequently improve long-term survival[11]. However, the risk of distant recurrence in BC patients is higher in the first decade after diagnosis, coupled with the fact that these therapies have low rate responses in patients with the metastatic form of BC[9], highlight the need for new, effective and long term therapeutic approaches.

2. Metastatic Breast Cancer: A Focus on Triple Negative Breast Cancer Subtype

BC subtypes are characterized not only by the presence or lack of membrane receptors, but also by the different signaling pathways affected[12]. In most cases, the affected pathways lead to known hallmarks of tumorigenesis, such as tumor cell proliferation and migration, and impact metastases formation as well as its site[12,13]. Alarmingly, metastases are responsible for 90% of all BC deaths[14]. Different BC subtypes have distinct site-specific metastasis formation, a non-random process known as organotropism, or metastatic organotropism, in which the arrest and growth of tumor cells is dependent on several factors, such as the cancer's subtype, genetic and molecular characteristics of the malignant cells, the host microenvironment, and the crosstalk between tumor and local cells[8,14]. From the different BC subtypes, HR-positive is more likely to metastasize to the bone and distant lymph-nodes, whereas HER2-positive tends to spread to the liver and brain, while lastly, TNBC mainly metastasize in the brain and lungs[14].

Overall, the brain is the second organ more likely to sustain metastasization from BC and, even when BC is diagnosed at early stages, around 30% of BC patients will develop metastasis, mainly derived from TNBC, which is responsible for about 77% of deaths[15,16]. This evident aggressive behavior makes metastatic BC, in particularly metastatic TNBC, undeniably, the leading cause of death in the majority of BC patients[14,17]. Moreover and in line with brain metastasization, TNBC patients usually have an upregulation of the Wnt/β-catenin signaling pathway, which, in non-metastatic conditions, is involved with regulation of tumorigenesis[18].

3. The Metastatic Cascade

Metastasization from any primary tumor to the 'target organ' is a complex multistep process, known as the metastatic cascade, that comprises local invasion of the neighboring normal tissue; intravasation into circulation, which can happen through the bloodstream or the lymphatic system; survival in circulation and arrest in a distant organ site (homing); extravasation to the new organ; and colonization[8,19] (Figure 2). The metastatic cascade starts with BCCs undergoing morphological alterations, known as the epithelial-mesenchymal transition (EMT)[8]. This reversible process increases cell invasiveness, since tumor cells acquire a more migratory, mesenchymal phenotype, with the increase in markers like vimentin, concomitant with the loss of cell-cell junctions, and a reduction of specific epithelial cell markers, such as the intermediate filament cytokeratin[20–22].

Although BCCs that suffer EMT gain mesenchymal characteristics, not all epithelial trades are lost, which gives tumor cells the ability to form clusters and consequently, invade and migrate together[8,19]. Following EMT, and in order to invade and spread within the mammary tissue, malignant cells detach from the original site of the tumor and migrate towards the surrounding tissue (stroma), until they reach the proximity of a blood vessel[20]. Upon contact with the endothelial barrier of capillaries, BCCs can enter the circulation, a process known as intravasation, mainly by paracellular but also via transcellular transendothelial migration, though less commonly[19].

When in circulation, tumor cells need to survive in a different microenvironment, in order to be able to arrest at the secondary organ, as the brain. Due to the numerous challenges that BCCs have to endure while in the bloodstream, such as shear stress effect and presence of immune cells, it is not surprising that this step is the limiting one of the metastatic cascade[23]. To increase chances of survival, BCCs developed a strategy where they interact with platelets forming shield-coated platelets, which grants them protection from the hostile environment that is the blood circulation[24]. Additionally, this cooperation between BCCs and platelets can also be found in several important steps of the metastatic cascade, like migration, cell adhesion and extravasation[23].

During rolling, even under the influence of shear stress, tumor cells can adhere to specific sites of the vessels at the target organ[8]. In the case of the brain, BCCs establish interactions with brain microvascular endothelial cells (BMECs), which form the

anatomic basis of the BBB[25]. Afterwards, through transendothelial migration, BCCs are able to cross the BBB and enter into the brain microenvironment. This can happen when intercellular junctions and the actin cytoskeleton of the endothelial cells (ECs) are disrupted and reorganized around the invasion site (paracellular migration), or by encapsulating BCCs in a transitory pore-like structure, maintaining barrier integrity (transcellular migration)[8]. Similarly to melanoma cells, it has been shown that one way BCCs can cross the BBB is also through the transcellular pathway[26].

Once in the target organ, tumor cells undergo the reverse process of EMT which allows them to infiltrate the blood vessels, known as mesenchymal-epithelial transition (MET)[8]. At this stage, BCCs lose the previously obtained mesenchymal markers, like vimentin, and acquire their normal epithelial markers. Despite such phenotypical alterations, a partial MET in BCCs after reaching the brain has been observed, suggesting that these cells retain some migratory properties which allows them to further disseminate to other areas[27].

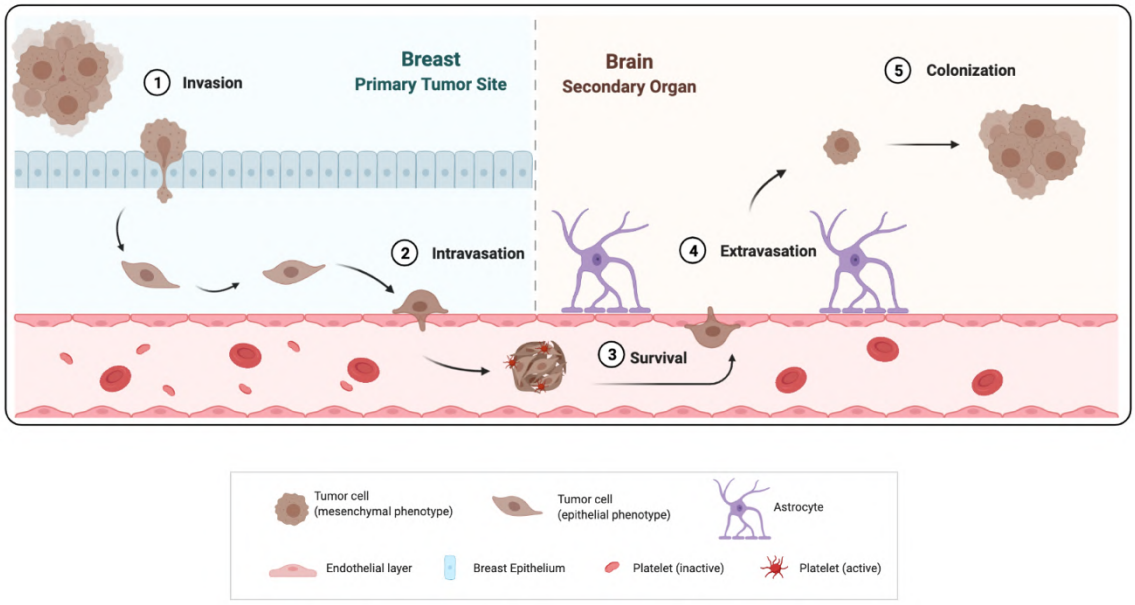

Figure 2. Metastatic cascade in breast cancer, focusing on brain metastasis. The metastatic cascade starts with malignant cells undergoing a phenotypic alteration known as the epithelial-mesenchymal transition (EMT). This modification enhances tumor cells' migratory capacity, allowing them to move from the primary tumor site and invade the normal surrounding tissue. Through intravasation, malignant cells reach the blood circulation where they are subjected to shear stress, which is responsible for the death of most tumor cells in circulation. To bypass this problem, malignant cells form clusters with platelets until they arrive at the secondary organ. There, tumor cells need to transpose the BBB, a process known as extravasation, which involves rolling, adhesion and transendothelial migration, suffering the reverse

phenotypic modification, the mesenchymal-epithelial transition (MET). The metastatic cascade ends with tumor cells colonization in the secondary organ (i.e., the brain).

4. Myocyte Enhancer Factor 2C (MEF2C)

Myocyte enhancer factor 2 (MEF2) is a family of transcription factors, which includes several proteins, such as MEF2A, MEF2B, MEF2C and MEF2D[28]. These four transcription factors play central functions in vital processes, such as differentiation and adaptive responses[29]. MEF2 transcription factors can interact not only with co-activators, but also with co-repressors, enabling them to be involved in both normal and pathological regulation of several signaling pathways[30]. This makes consensus regarding transcription factors' role as oncogenes or tumor suppressors a rather unclear field[29].

MEF2 transcription factors can interact not only with numerous miRNAs, but also several signaling pathways, such as the Wnt/β-catenin, the phosphatidylinositol 3-kinase (PI3K)/AKT, and the mitogen-activated protein (MAP) kinase, which can happen during normal or pathological processes, including cancer[31]. A common receptor activated in these pathways is the vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2), known to play a vital role during metastasis development and, in turn, to activate MEF2C[32,33]. This transcription factor is known for its key role in many development processes, such as the regulation of muscle, neural crest, ECs, chondrocyte, neuron and lymphocyte development, regulating tissue-specific gene expression in embryonic period and, in adulthood, it is expressed in neuronal, muscle and hematopoietic lineages[34,35]. Additionally, a deregulation of MEF2C's levels can affect the differentiation process, ultimately leading to an increase in cellular proliferation, which is of particular interest when it comes to cancer[36].

MEF2C's role in tumor development presents some duality accordingly with its subcellular location, for instance in hepatocellular carcinoma, when in the nucleus, MEF2C induces VEGF signaling, which increases malignancy through angiogenesis and tumor invasion; in contrast, by blocking Wnt/β-catenin signaling pathway MEF2C inhibits malignant cell proliferation[37]. Accordingly, in BCBM, membrane VEGFR2 has been shown to decrease along with disease severity, whereas nuclear β-catenin expression tends to increase contrarily to membrane β-catenin, which decreases[30]. Interestingly,

MEF2C has been shown to have mainly an oncogenic role in several tumors, such as in colorectal cancer, where its upregulation was associated with disease progression[38], and in cooperation with Sox4 gene, MEF2C promoted myeloid leukemia[39]. Additionally, in pancreatic ductal adenocarcinoma by inducing the transcription of the metalloproteinase (MMP) 10, MEF2C appears to be a player in a dependent mechanism that promoted metastases development[40]. However, the biological role played by MEF2C in BC is still not fully understood.

Previous studies from our team[41], showed that MEF2C is expressed in perivascular cells, in early stages of metastatic development, as well as in well-established metastases, in a mouse model of BC brain metastases (BCBM) formation (Figure 3). This study further revealed MEF2C's increasing tendency to translocate from the cytoplasm to the nucleus along BCBM development[41], consistent with transcription factor activation. Our additional studies.[30] further corroborated these findings. Different MEF2C's cellular localization was observed, which led to the classification of patients in three different phenotypes (P): P1; P2 and P3. In P1, MEF2C was mainly found extranuclear; in P2 around 50% of the cells presented extranuclear staining and 50% presented extranuclear and nuclear MEF2C expression; in P3, nearly all BCCs exhibited an all-over the cell staining[30]. The majority of patients presented either P2 or P3 phenotypes, with P3 being associated with an increased number of metastases, as well as an increased tumor size, suggesting a link between MEF2C's translocation and disease severity[30]. Additionally, MEF2C was found to be expressed in primary BCCs in both the ducts and the parenchyma of P3 patients with the proliferative marker Ki-67 increasingly expressed along the three phenotypes[30].

By directly interacting with effector proteins, MEF2C plays a crucial role as an essential regulator in several signaling pathways, such as the Wnt/β-catenin and the VEGF signaling pathways[35]. Once these pathways have also been reported to be altered in BC tumorigenesis[42–46], including in BCBM formation[30], MEF2C appears as a new player in BC, and possibly in the development of BCBM, remaining undetermined its regulation and effects in tumorigenesis.

5. MicroRNAs

MicroRNAs (miRNAs or miR-) are small and highly conserved non-coding RNA molecules, with 18 to 24 nucleotides in length. MiRNAs have essential roles in many biological processes[47–49], including cell development and differentiation, proliferation and apoptosis[50]. Recently, miRNAs have been highlighted as possible biomarkers in cancer, as evidenced by the identification of several different miRNA expression patterns present in human cancers, including brain cancer and BC[51]. In fact, in 2007 one of the first studied correlations between altered miRNA levels and metastasis formation was established, where miR-10b was found to be overexpressed in metastatic BCCs, which led to a positive regulation of cell migration and invasion[52].

In several types of cancer, miRNAs have been reported to function both as tumor suppressors or as oncogenes, depending on their expression levels, since they can impact different pathways which are important for tumor development[50]. In fact, the same miRNA can also present such duality of effects depending on the cell and cancer types[53]. miR-194-5p is a perfect example of this duality, not only it has been reported to act as an oncogene in BC tissues[54] and in prostate cancer[55], but also to function as a tumor suppressor in glioblastoma tissues[56] and in non-small cell lung cancer[57].

On one hand, miR-194-5p was shown to work as a potential promoter of BC growth by interfering in the Wnt signaling pathway through degradation of β-catenin, which increases the chances of metastasis formation and, therefore, decreases the survival rates for BC patients[58–60]. Likewise, in prostate cancer, miR-194 overexpression was shown to be positively related with metastatic development and, consequently, with a poor prognosis[55]. On the other hand, miR-194-5p was found to function as a tumor suppressor in glioblastoma tissues and in non-small cell lung cancer, where its downregulation increased the proliferative profile of cancer cells and was indicative of a poorer survival rate[56,57]. This seeming duality of the overexpression of miR-194-5p is therefore an incredibly rich and deep topic worth investigating further as it may hold the key to the diagnosis, prevention, and treatment of many different types of cancer.

Data concerning miRNAs' expression pattern in BCBM is limited[41]. Nonetheless, published data from our team[41] established a link between circulating miRNAs and the occurrence of BCBM *in vivo*, by Next-Generation Sequencing analysis. The aberrant expression of miRNAs in the plasma of mice injected with TNBC cells (4T1) increased

as metastases developed: the number of downregulated miRNAs was shown to decrease, while the number of the upregulated ones increased[41]. Among the several dysregulated miRNAs, miR-194-5p appeared to be downregulated prior to metastases development (Figure 3). Bearing in mind that miRNAs with decreased expression in cancers can act as tumor suppressor genes and negatively regulate oncogenes[61], miR-194-5p can potentially be related with the predicted upregulation of oncogenic proteins, such as the signal transducer and activator of transcription 1 (STAT1), the RAS- related protein 2B (RAP2B), the heparin-binding EGF-like growth factor (HBEGF), the RAC-beta serine/threonine-protein kinase 2 (AKT2), the MEF2C, and the chromodomain helicase DNA binding protein 2 (CHD2)[41].

We previously tested by which means miR-194-5p dysregulation is related with specific phases of BCBM formation[62]. In this sense, miR-194-5p downregulation prior to metastatic development was further validated in brain sections and by the number of miR-194-5p-positive cells[62]. These findings highlighted this miRNA as a possible early biomarker of the upcoming BCBM formation. Moreover, miR-194-5p levels secreted by BMECs (b.End5) and BCCs (4T1 cells), both separately or in mixed cultures, revealed that 4T1 cultures secrete high quantities of this miRNA, unlike b.End5 cultures, which present the lowest release values[62]. Interestingly, miR-194-5p levels decreased throughout time in b.End5 cells, while 4T1 cells clearly secrete this miRNA, not only by themselves, but also as 'metastasis-like' clusters[62]. This indicates that b.End5 cells low release of miR-194-5p may be the cause of its observed downregulation, both in plasma and in the brain, and that the interaction between BMECs and BCCs is somehow involved in miR-194-5p's regulation, in addition to the individual regulation of both cell types[62].

Downregulation of miR-194-5p seen both *in vivo* and *in vitro* suggests that this miRNA can comprise an efficient predictor of the upcoming occurrence of brain metastasis in BC, making it a putative biomarker of BC and a possible target to be explored. Interestingly, a bioinformatics analysis revealed MEF2C as a target of miR-194-5p, with no causality nexus ascertained between miR-194-5p downregulation and MEF2C upregulation[41] (Figure 3).

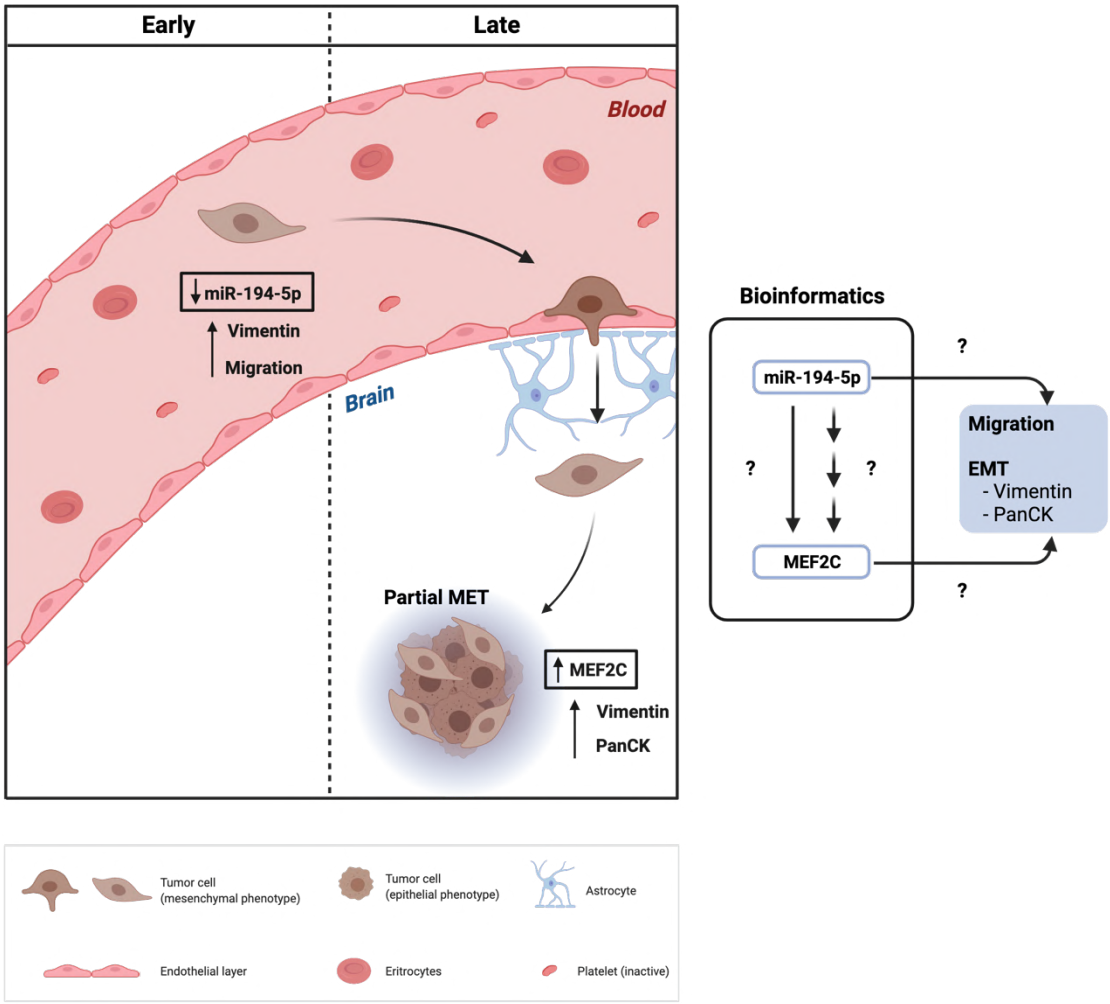

Figure 3. Gaps in knowledge in early and late events of breast cancer (BC) brain metastases formation involving miR-194-5p and MEF2C. In early stages, before BC brain metastases detection, microRNA (miR)-194-5p presented decreased levels in circulation in a mouse model of the disease[41,62]. Additionally, BC cells entering the brain parenchyma were already positive for the mesenchymal marker vimentin, reflecting high migratory cell capacity[63]. Later, when well-established metastases are already patent in the brain, myocyte enhancer factor 2C (MEF2C) levels were up-regulated[41], alongside with an increase in vimentin and the epithelial marker pan-cytokeratin (PanCK)[63]. Together, elevated levels of vimentin and PanCK suggest that triple negative BC (TNBC) cells undergone a partial mesenchymal-epithelial transition (MET) within metastatic brain lesions. Importantly, MEF2C is a bioinformatically predicted target of miR-194-5p, but a direct or indirect relation between the two of them has yet not been established, neither their contribution for BC phenotype nor migratory capacity.

Aims

There is a clear and urgent need for novel approaches to tackle TNBC. Despite the relatively recent discoveries in the realm of miRNAs and oncogenic proteins, there is still a long way to go to increase overall survival of BC patients, especially those suffering with the metastatic form of TNBC.

The hypothesis of this work is that the downregulation of miR-194-5p is linked to an increase in the expression levels of MEF2C, being both alterations directly related with the metastatic properties of BCCs. To this end, the main goal of this thesis is to disclose the role of miR-194-5p and of MEF2C expression in BC aggressiveness, focusing on hallmarks of tumorigenesis such as EMT and migration. To achieve this goal, three distinct objectives were set up:

1. Evaluate the putative effect of miR-194-5p on MEF2C expression;
2. Assess the metastatic properties of BCCs after modulation of MEF2C;
3. Study the metastatic profile of BCCs upon miR-194-5p modulation.

By understanding the role of miR-194-5p and MEF2C in tumorigenesis, this work will reveal new players and pinpoint novel modulation targets in TNBC patients, this way paving the way for a new therapeutic option to improve metastasis-free survival.

Chapter II – Materials and Methods

1. Cell Culture Conditions

A murine mammary carcinoma triple-negative 4T1 cell line (ATCC, Middlesex, UK), was used. 4T1 cells were cultured and maintained in Roswell Park Memorial Institute (RPMI) 1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2 mM mM L-glutamine (Sigma-Aldrich) and 5% (v/v) fetal bovine serum (FBS, Sigma-Aldrich), at 37 °C in humid atmosphere enriched with 5% CO_2.

2. Transient Transfection

In vitro transfection studies in 4T1 cells used a siRNA specific for MEF2C and its respective control, purchased from Ambion (Life Technologies, Carlsbad, CA, USA). Additionally, a plasmid pcDNA3.1-MEF2C (pMEF2C), which encodes for MEF2C, and empty plasmid pcDNA3.1, used as negative control were used. Both plasmids (backbone structures in Chapter VII – Supplementary Figures 1 and 2) were kindly donated by Dr. Eros DiGiorgio, University of Udine, Italy. Pre-miR-194-5p and miRNA negative control (Scramble) were purchased from Ambion (Life Technologies).

In all experiments, 4T1 cells were seeded, and after 24 h they were transiently transfected with the siRNA (10 nM), plasmid (2 µg/mL) or pre-miR (10, 30 and 50 nM), using Lipofectamine™ 3000 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and Opti-MEM™ Reduced Serum Medium (Gibco, Thermo Fisher Scientific). Transfection mixes were diluted in complete cell medium, and transfections were stopped after 24 h of siRNA/plasmid incubation, and 48 h of pre-miR-194-5p incubation.

3. Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

Total RNA was isolated from cells using TrilpeXtractor reagent (Grisp, Porto, Portugal), according to the manufacturer's instructions. For gene expression, RNA was

then transcribed into cDNA, using the Xpert cDNA Synthesis Kit (Grisp), while for miRNA expression the miRCURY LNA RT Kit (Qiagen, Hilden, Germany) was used, according to manufacturer's instructions. In the later, prior to the reverse transcription reaction, the synthetic RNA UniSp6 RNA spike-in (Qiagen) was added to the mixture. The reactions were performed on a Biometra T-Combi thermocycler (Analytic Jena, Jena, Germany), using the following conditions for gene expression: 65 °C for 5 min; 50 °C for 15 min, 85 °C for 5 min to heat-inactivate the reverse transcriptase, and cooling down and storage at 4 °C; whereas for miRNA expression the following conditions were used: 42 °C for 60 min; 95 °C for 5 min to heat-inactivate the reverse transcriptase, and cooling down and storage at 4 °C.

RT-qPCR was performed using QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific)[62]. The Xpert Fast SYBR Green (Grisp) was used for gene expression, according to the manufacturer's instructions, diluted at 1:2 and using the following conditions: 1 cycle of 95 °C for 2 min, 40 cycles of 95 °C for 5 s, 60-65 °C for 20-30 s, followed by a dissociation/melting curve analysis. In the case of miRNAs expression, miRCURY LNA SYBR Green PCR Kit (Qiagen) was used according to the manufacturer's instructions using cDNA diluted at 1:6. The following conditions were used: 50 cycles of 95 °C for 15 s, 56 °C for 30 s, 72 °C for 30 s, and a ramp rate of 1.6 °C/s, followed by a melting curve analysis.

Forward and reverse primer pairs for MEF2C and housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Table 1) were purchased from Stab Vida (Caparica, Portugal), while specific predesigned LNA primer pairs for miR-194-5p (mmu-miR-194-5p) and endogenous control miR-16-5p (mmu-miR-16-5p) were purchased from Qiagen.

RT-qPCR was performed in 384-well plates, with each sample performed in triplicate, and a no-template control was included for each sample amplification. Determination of the threshold cycle was performed using the QuantStudioTM Real-time PCR software (Applied Biosystems), and the quantifications performed by the ΔΔC method. The results are presented as fold-change.

Table 1. Real-time quantitative PCR primers.

Target	Primer Sequence	Length (bp)
GAPDH	Forward: 5- GTG GCA AAG TGG AGA TTG TTG CC -3	23
	Reverse: 5- GAT GAT GAC CCG TTT GGC TCC -3	21
MEF2C	Forward: 5- AGA TCT GAC ATC CGG TGC AG -3	20
	Reverse: 5- TCT TGT TCA GGT TAC CAG GT -3	20

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MEF2C, myocyte enhancer factor 2C.

4. Cell Viability Assay

Upon transfection and to assess the effect of this procedure in TNBC cells, 4T1 cells' viability was evaluated using Thiazolyl Blue Tetrazolium (MTT) assay. 4T1 cells were seeded into 96-well plates at a density of $2.5x10^4$ cells/mL and after the transfection period, medium was discarded and 0.5 mg/mL of MTT (Alfa Aesar, Haverhill, MA, USA) diluted in RPMI was added to each well, and cells were incubated for 1 h at 37 °C and 5% CO_2. Supernatants were carefully removed and the formazan crystals, developed in viable cells, were solubilized with a solution of 0.04 N HCl in isopropanol. The absorbance values were obtained using a microplate reader (Zenyth 3100, Anthos Labtec Instruments, Salzburg, Austria) at 595 nm. Cell viability was calculated as a percentage of control (untreated 4T1 cells). All experiments were performed in triplicate for three independent experiments.

5. *In situ* Hybridization (ISH)

Upon transfection, ISH[62] was performed to assess the expression of miR-194-5p. 4T1 cells were seeded onto coverslips in 24-well plates at a density of $5x10^4$ cells/mL and fixed post-transfections with 4% PFA (Sigma-Aldrich). Fixed cells were permeabilized with 0.3% of Triton X-100 (VWR International, Radnor, PA, USA) for 15 min on ice and

hybridized with correspondent probe (50 nM) at the hybridization temperature of miRNA-194-5p (54 ºC) for 1 h. The hybridization signal was detected by adding alkaline phosphatase (AP)-labelled anti-Digoxigenin/Digoxin (DIG, 1:1500, Roche, Basel, CH) for 1 h at room temperature. Nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP, Roche, Basel, CH, 1:50) was used as a chromogenic substrate for AP. Nuclei counterstaining was performed with Hoechst 33342 dye (Thermo Fisher Scientific, 1:1000 in PBS), for 10 min at room temperature. Negative control assays were performed without probe.

6. Immunofluorescence (IF)

MEF2C's levels, as well as proliferative and phenotypic alterations in tumor cells upon selected transfections were evaluated by immunofluorescence (IF)[64] analysis of the transcription factor MEF2C and the epithelial and mesenchymal markers (pan-cytokeratin – PanCK - and vimentin, respectively).

4T1 cells were seeded onto coverslips in 24-well plates at a density of $5x10^4$ cells/mL and fixed post-transfections with freshly prepared 4% (v/v) paraformaldehyde (PFA, Sigma-Aldrich) in phosphate-buffered saline (PBS) for 20 minutes at room temperature. Following fixation, cells were washed with PBS and permeabilized for 5 min, blocked for 60 min at room temperature and incubated overnight at 4 °C with the primary antibodies, followed by incubation with the corresponding secondary antibodies for 60 min at room temperature, as specified in Table 2. Both primary and secondary antibodies were diluted in blocking solutions. Nuclei were counterstained with Hoechst 33342 dye (Thermo Fisher Scientific, 1:1000 in PBS), for 10 min at room temperature. Between incubations cells were washed three times with PBS. Methanol (Honeywell) dehydrated cells were then mounted in microscopy slides with dibutylphthalate polystyrene xylene [DPX (Merck Millipore, Burlington, MA, EUA)], properly dried and stored at 4 °C until image acquisition. Negative control assays were performed without primary antibody.

Table 2. Summary of the experimental conditions for immunofluorescence analysis.

Target Protein	Permeabilization	Blocking	Primary Antibody	Secondary Antibody
MEF2C	0.3% Triton X-100	3% BSA	MEF2C (1:100) Santa Cruz Biotechnology #sc-518152, Mouse	Alexa Fluor® 555 (1:500) Thermo Fisher Scientific, #A21428, Goat Anti-Mouse
PanCK			Pan-Cytokeratin (1:50) Thermo Fisher Scientific #MA512231, Mouse	Alexa Fluor® 488 (1:500) Thermo Fisher Scientific, #A11001, Goat Anti-Mouse
Vimentin			Vimentin (1:100) Thermo Fisher Scientific #MA3745, Mouse	

BSA, bovine serum albumin; MEF2C, myocyte enhancer factor 2C; PanCK, pan-cytokeratin.

7. Wound Healing Assay

Migratory behavior of tumor cells overexpressing miR-194-5p or silenced for MEF2C was evaluated through wound healing assay. Briefly, 4T1 cells were seeded in 96-well plates at a density of $5x10^4$ cells/mL (siRNA/plasmid) or $4x10^4$ cells/mL (pre-miRNA) and after transfection period, a sterile 10 µl pipet tip was used to scrape a longitudinal wound through the cell monolayer, with an even diameter. Medium was removed and the cells were gently washed with Hank's Balanced Salt Solution (HBSS, Gibco), followed by a 24 h incubation in RPMI, after which BCC's migration and wound closure was monitored along time.

8. Image Acquisition

Images from 10 fields per coverslip (n=3) were acquired at the Faculty of Sciences, University of Lisbon, BioIsi Facility. For IF, images were obtained using a 40x objective with immersion oil in an Olympus BX60 microscope equipped with Olympus U-RFL-T Mercury lamp and Hamamatsu Orca R2 cooled monochromatic CCD camera. For ISH images were acquired using a 40x objective of a bright field microscope (Olympus, model BX51) with an integrated Olympus DP50 digital camera with mercury fluorescence illuminator, and Nomarski/DIC Prism for Transmitted Light.

For the wound-healing assay images were acquired at 0, 3 and 24 h after the wound was inflicted (n=3, performed in triplicate, one image per well), using a 10x objective with a phase contrast microscope (Carl Zeiss, Primovert, NY, USA).

9. Image Analysis

IF images were analyzed using ImageJ (National Institutes of Health, Bethesda, MD, USA) software. In the wound healing assay, wound closure was quantified using the freehand selections tool in ImageJ software to measure the area between the wound's ends. The results are presented as wound closure percentage of control (0 h), using the following equation:

$$\%\ closure = 100 - \frac{Distance\ at\ chosen\ timepoint \times 100}{Distance\ at\ 0h} \quad (1)$$

10. Statistical Analysis

Results were analyzed with GraphPad Prism® 8.4.3 (GraphPad Software, San Diego, CA, USA) and are expressed as mean ± SEM, representing the average from three independent experiments (n=3).

Results' normality were tested with D'Agostino Pearson test. When normality was verified, significance of differences between treatments and controls were evaluated by analysis of the variance with one-way ANOVA or two-way ANOVA multiple comparison test, in case of grouped data, such as in wound healing assay. When no normality between data was observed, significance between differences was tested with Kruskal-Wallis test. Differences were considered statistically significant when $p < 0.05$.

Chapter III – Results

1. Modulation of miR-194-5p's levels does not affect MEF2C's expression

1.1. Pre-miR-194-5p effect in TNBC cells expression and viability

To assess the transfection efficiency and to ensure its safety, miRNA-194-5p's expression levels and 4T1 cells' viability after the transfection were evaluated via RT-qPCR and ISH, and MTT assay, respectively (Figure 4).

In an initial screening, different concentrations (10, 30 and 50 nM) of scramble and pre-miR-194-5p were tested to evaluate the effect that each concentration would have on miR-194-5p's levels. Notably, all the tested concentrations significantly increased this miRNA's expression, with the highest concentration (50 nM) inducing the greatest upregulation (Figure 4A).

Once all the concentrations tested significantly increased miR-194-5p's levels, the safety of these concentrations on 4T1 cells' viability was ensured. At 50 nM, both the scramble and the pre-miR-194-5p significantly compromised 4T1 cells' viability (Figure 4B), which resulted in the exclusion of this concentration for further experiments. Similarly, the 30 nM concentration was also excluded since it also induced a significant reduction in 4T1 cells' viability. Then, the 10 nM concentration was chosen for the subsequent studies, since it did not significantly affect the viability of these cells, presenting a similar profile to the one seen in the untreated and scramble groups.

To further validate the increased expression of miR-194-5p, an ISH was performed (Figure 4C) using the previous selected concentration (10 nM). A clear increase in miR-194-5p's cellular expression was observed, reflected by the upsurge in signal intensity (blue) both in the cytoplasm and nuclei in 4T1 cells transfected with the pre-miR, by opposition to untreated or scramble transfected cells, where only a faint blue signal was observed along cell cytoplasm.

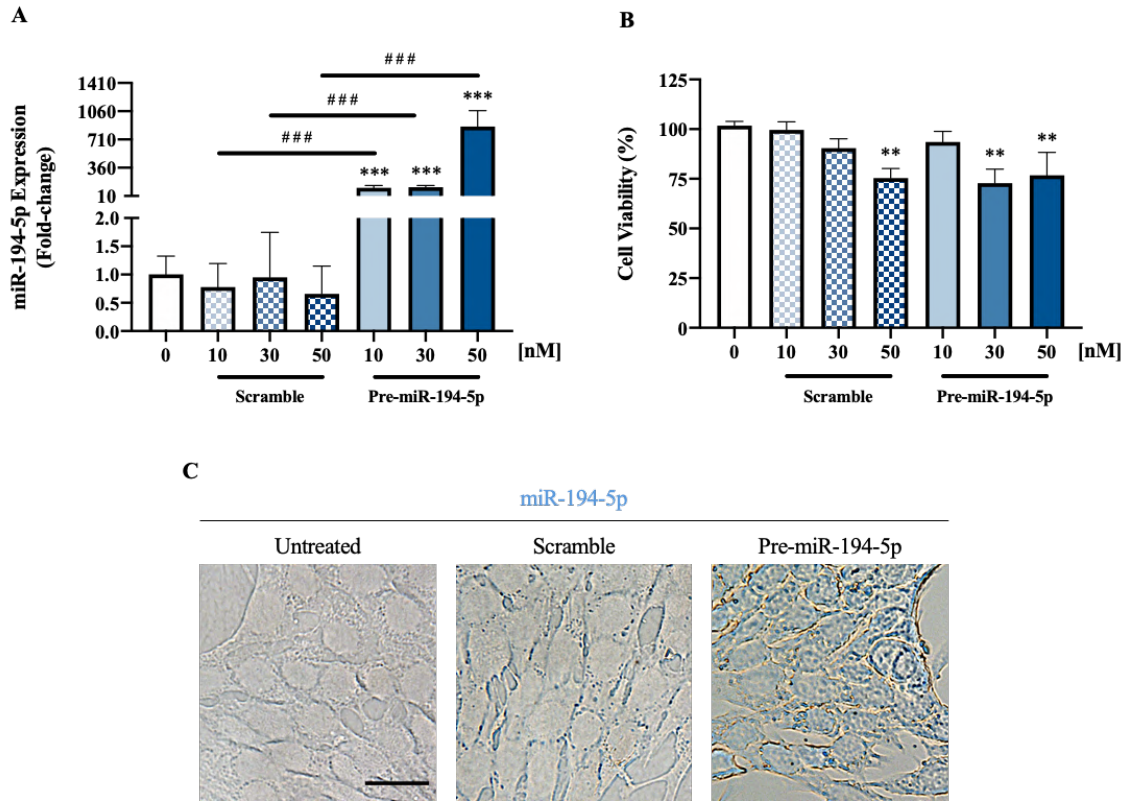

Figure 4. Pre-miR-194-5p at 10 nM increases miR-194-5p expression without compromising triple negative breast cancer (TNBC) cells' viability. TNBC cells were incubated with pre-miR-194-5p or scramble at increasing concentrations of 10, 30 and 50 nM, for 48 h, after which miR-194-5p expression levels and cells' viability were analyzed. **(A)** Quantitative analysis by real-time quantitative polymerase chain reaction (RT-qPCR) revealed that miR-194-5p expression is significantly increased after 48 h of pre-miR-194-5p exposure, with all the tested concentrations. **(B)** The effect of pre-miR-194-5p on TNBC cells' viability was assessed by MTT assay, showing toxicity in TNBC cells for 30 and 50 nM, but not at 10 nM. This concentration (10 nM) was then used to further corroborate miRNA increased expression in TNBC cells by **(C)** *in situ* hybridization of miR-194-5p (blue). Scale bar: 30 µm. Results are presented as fold-change and percentage vs. untreated cells (RT-qPCR and MTT assay, respectively) as mean ± SEM (n=3, performed in triplicate). Statistical differences are denoted as ** $p < 0.01$, *** $p < 0.001$ vs. untreated, and by $^{###}$ $p < 0.001$ between indicated conditions, determined by one-way ANOVA.

1.2. Increased miR-194-5p's levels do not translate in a reduction of MEF2C's expression

In previous work from our lab, miR-194-5p was found to be downregulated prior to metastases development and MEF2C, which emerged as bioinformatically predicted target, was upregulated in early stages of metastatic development and also in well-

established metastases[41]. Nonetheless, no causality nexus between miR-194-5p downregulation and MEF2C upregulation was evaluated. To assess this possible relation, MEF2C's levels in 4T1 cells upon 48 h of pre-miR-194-5p incubation were analyzed via RT-qPCR and IF (Figure 5).

Unexpectedly, the increase in miR-194-5p's levels did not significantly affect MEF2C's mRNA levels (Figure 5A). This result was also observed regarding MEF2C's protein intensity, in which no alterations are observed among untreated cells and pre-miR-194-5p treated ones (Figure 5B). Likewise, the semi-quantitative analysis of MEF2C's mean intensity did not reflect any significant changes between conditions and between the different time points (24, 48 and 72 h, Figure 5C).

Together, these results suggest that miR-194-5p may be a too far upstream target of MEF2C to have a direct effect on this protein.

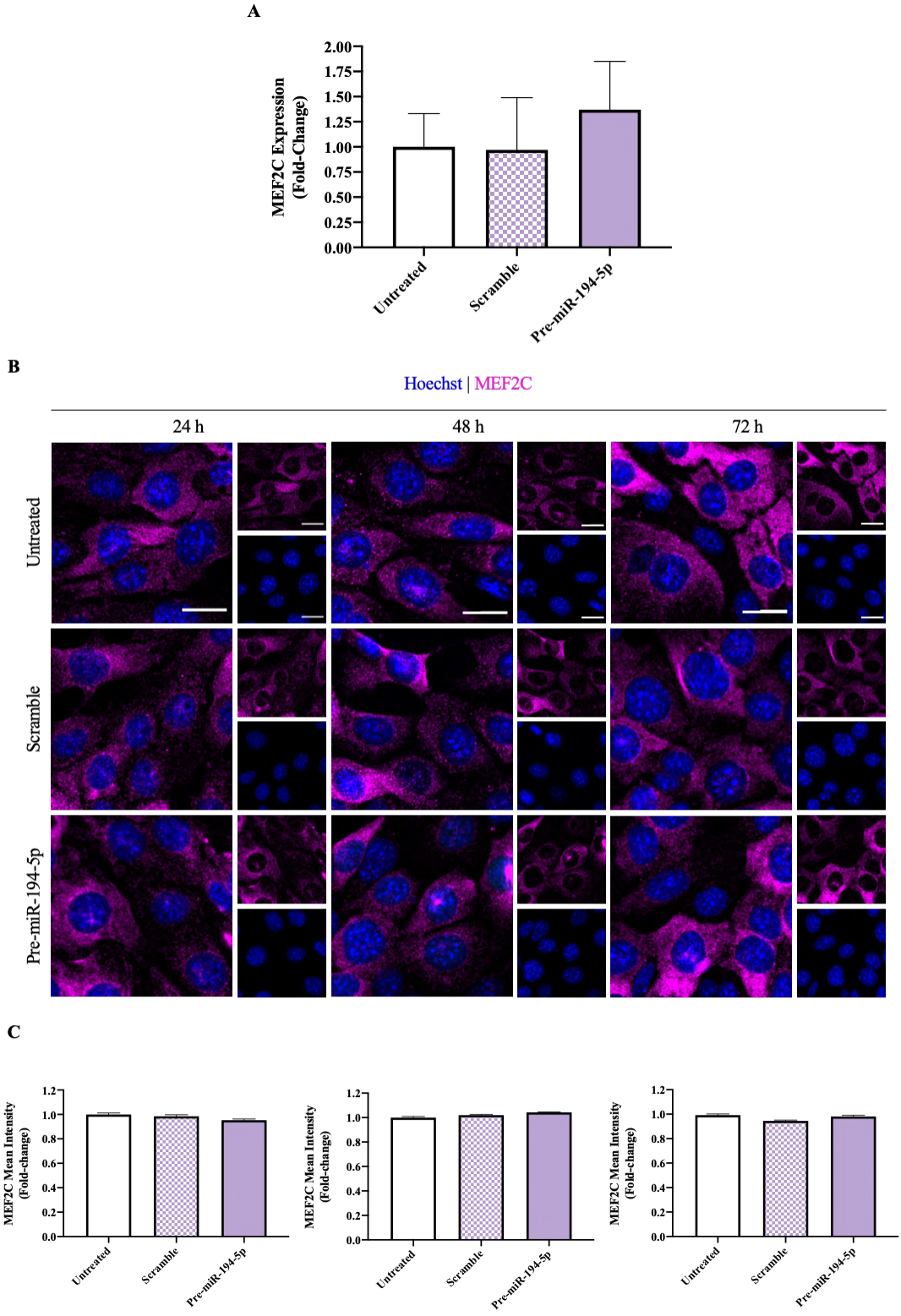

Figure 5. Increase in miR-194-5p does not alter MEF2C's expression in triple negative breast cancer cells. 4T1 cells were incubated with pre-miR-194-5p or scramble at 10 nM, for 48 h, after which MEF2C's expression was assessed by real-time quantitative polymerase chain reaction (RT-qPCR) and immunofluorescence (IF). **(A)** Quantitative analysis revealed that MEF2C's gene expression does not seem to suffer significant alterations after 48 h of pre-miR-194-5p exposure. These results were further

corroborated by **(B)** IF at 24, 48 and 72 h and **(C)** semi-quantitative analysis of MEF2C's intensity in the same time points. Scale bar: 20 µm. Results are presented as fold-change vs. untreated cells (RT-qPCR and IF) as mean ± SEM (n=4 for RT-qPCR and n=3 for IF, performed in triplicate).

Despite the lack of causality between miR-194-5p and MEF2C in the presently used experimental setting, MEF2C's role along BCBM formation from TNBC has been described by our team as a putative determinant and a key player in the a more aggressive phenotype in brain metastasis[30,41]. In that regard, the dissection of its mechanisms towards tumorigenesis is of utmost importance.

2. Metastatic properties of TNBC cells after MEF2C's modulation

2.1. MEF2C's effect in TNBC cells expression and viability

To disclose the role of MEF2C in TNBC, a mechanistic study using siRNA targeted to MEF2C (siMEF2C), as well as DNA plasmid in order to promote MEF2C overexpression (pMEF2C), was conducted in 4T1 cells. The transfection efficiency was assessed by RT-qPCR and IF of MEF2C, ensuring maintenance of cell viability, via MTT assay (Figure 6).

MEF2C's mRNA levels, appraised by RT-qPCR, were significantly reduced using the siMEF2C (10 nM), as expected, in comparison to siControl-treated (10 nM) cells. Moreover, an increased expression of MEF2C's transcripts was detected with the pMEF2C (2 µg/mL), when compared with untreated and pEmpty-transfected (2 µg/mL) cells (Figure 6A). The safety of this transfection in 4T1 cells was then assessed, showing that neither the siRNAs, nor the plasmids affected TNBC cells' integrity (Figure 6B). Additionally, the previously seen alterations in MEF2C's mRNA levels were validated regarding protein expression within the cells, where MEF2C-silenced cells presented a notorious reduction in this protein's intensity, which contrasts with the upregulation observed in pMEF2C-transfected cells (Figure 6C). However, MEF2C overexpression was not homogeneous, with some cells exhibiting a notorious expression of the protein, whereas others did not reveal MEF2C protein overexpression (Figure 6C), despite the upregulation of the corresponding mRNA (Figure 6A).

In line with the previous results, semi-quantitative analysis of IF data showed that the silencing significantly reduced MEF2C's mean intensity per cell (Figure 6D), while the transfection with the MEF2C plasmid led to a significant increase in fluorescence intensity.

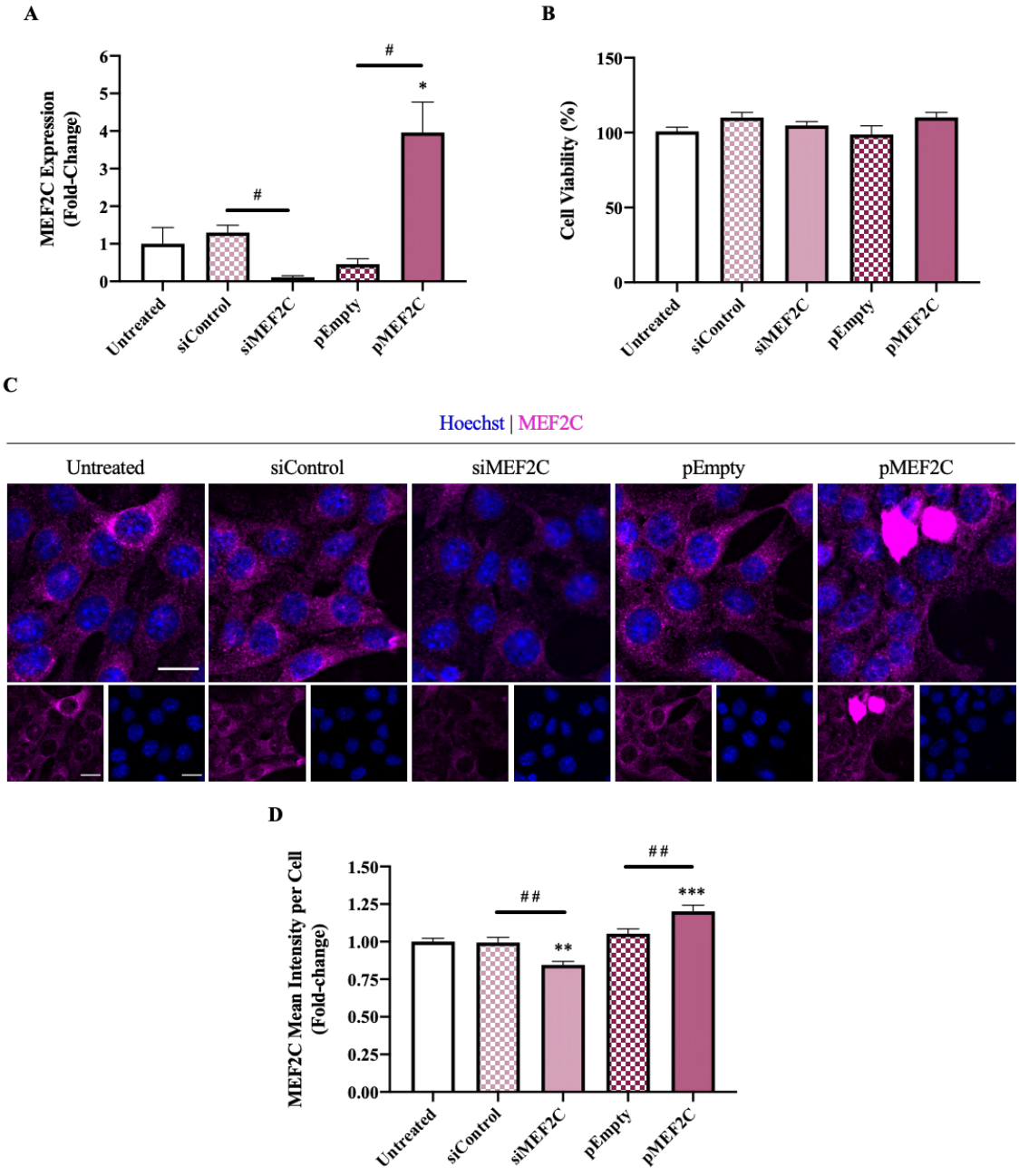

Figure 6. Efficient modulation of myocyte enhancer factor 2C (MEF2C) does not compromise triple negative breast cancer (TNBC) cells' viability. TNBC cells were incubated with siRNAs (siControl and siMEF2C) or plasmids (pEmpty and pMEF2C) for 24 h, after which MEF2C's expression and TNBC cells' viability were assessed, by real-time quantitative polymerase chain reaction (RT-qPCR) and immunofluorescence (IF), and by MTT, respectively. **(A)** Analysis of RT-qPCR revealed a decrease promoted by siMEF2C and an increase induced by pMEF2C, compared to the respective controls (siControl

and pEmpty, respectively). Transfections effects on TNBC cells viability was assessed with **(B)** MTT assay, showing no toxicity in TNBC cells. MEF2C's expression in TNBC cells was further validated by **(C)** immunofluorescence analysis of MEF2C (magenta). Nuclei were counterstained with Hoescht 33342 (blue). Scale bar: 20 μm. Alterations on MEF2C's expression were further corroborated by **(D)** semi-quantitative analyses of MEF2C's mean intensity per cell. Results are presented as fold-change and percentage vs. untreated cells (RT-qPCR and MTT assay, respectively) as mean ± SEM (n=3, performed in triplicate). Statistical differences are denoted as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. untreated, and by $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ between indicated conditions, determined by one-way ANOVA.

Even though pMEF2C transfection led to an increased intensity of the protein, the transfection efficiency was low (i.e., maximum 4-6 overexpressing cells/field). Several attempts of optimization of the transfection efficiency were performed with no clear improvement on the increase in MEF2C's protein expression itself (more information in Chapter VII - Supplementary Figure 3). Due to this insurmountable obstacle, further experiments were only carried out using the siRNAs.

2.2. Reduced levels of MEF2C affect EMT in TNBC cells

Once increased MEF2C's expression has been correlated with an aggressive phenotype in brain metastasis from TNBC patients[30], we aimed to dissect its role towards EMT. As a way to understand the effects of MEF2C in mesenchymal and epithelial features of TNBC cells, vimentin and PanCK expression were assessed by immunofluorescence analysis (Figure 7).

It was possible to see that when MEF2C was silenced there was a visible decrease in the mesenchymal marker vimentin, when compared to both controls (Figure 7A). This loss of vimentin intensity was aligned with an apparent alteration in the morphology of 4T1 cells, that appeared less elongated and more polygonal. Regarding PanCK, its expression was also present in all conditions, appearing to also be decreased in MEF2C-silenced cells (Figure 7B). Moreover, cells low on the PanCK marker gain an elongated and fusiform phenotype.

Semi-quantitative analysis of immunofluorescence data showed that cells treated with siMEF2C indeed presented a significant reduction not only in vimentin mean intensity per cell (Figure 7C), but also in cytokeratin mean intensity per cell (Figure 7D).

Although TNBC cells silenced for MEF2C present a decline in both the mesenchymal marker vimentin and in the epithelial marker PanCK, these data point to a partial EMT modulation, possibly reducing BCCs invasiveness.

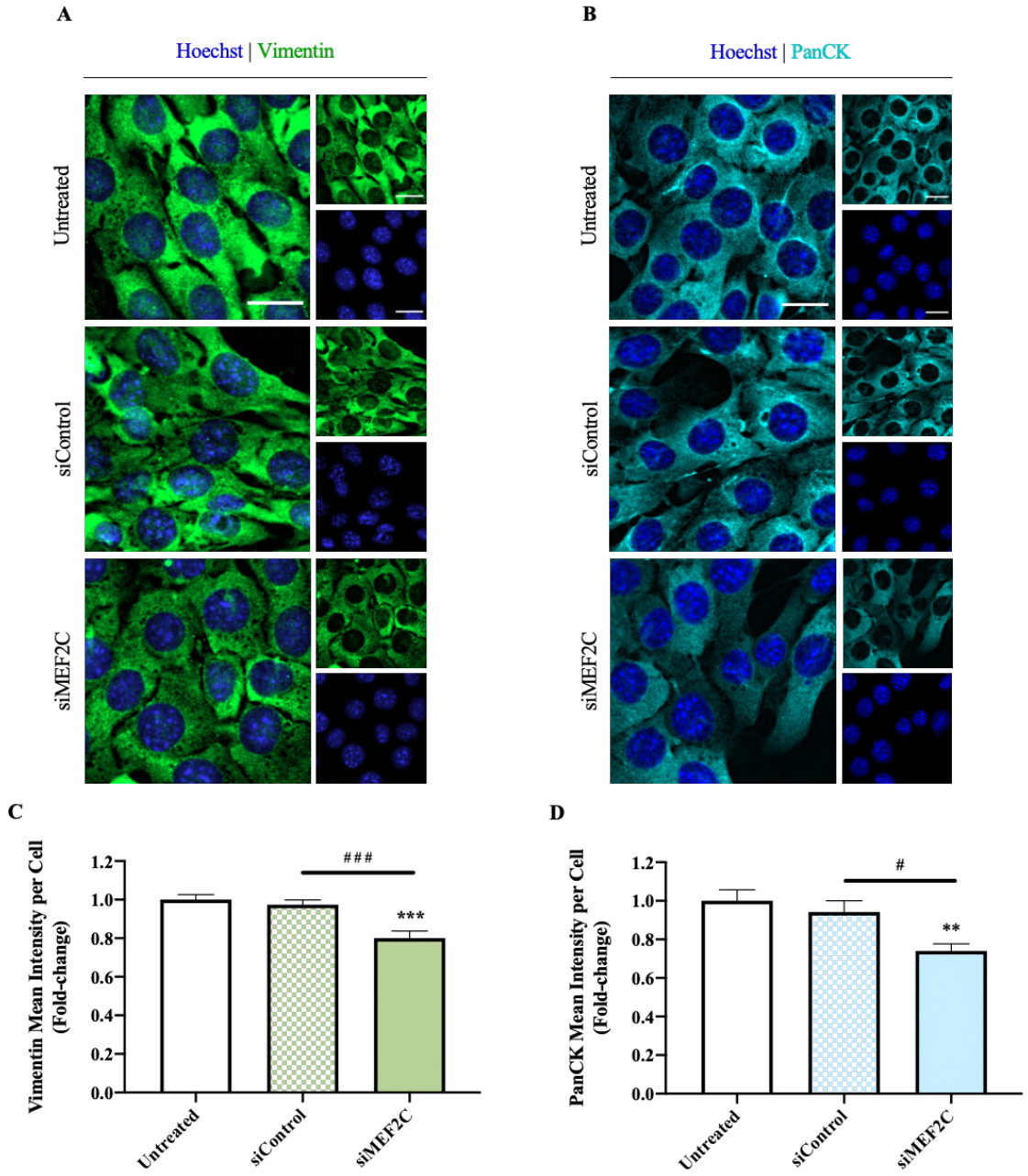

Figure 7. MEF2C-silencing leads to loss of mesenchymal and epithelial properties of triple negative breast cancer (TNBC) cells. TNBC cells were incubated with siMEF2C (10 nM), siControl (10 nM) or with medium supplemented with Opti-MEM (untreated), for 24 h. Expression of **(A)** the mesenchymal marker vimentin (green) and of **(B)** the epithelial marker pan-cytokeratin (PanCK, cian) were evaluated by immunofluorescence, which revealed a decrease of both vimentin and PanCK in MEF2C-silenced cells. Hoechst 33342 was used as counterstaining for nuclei (blue). Scale bar: 20 µm. Semi-quantitative analysis

further corroborates **(C)** the significant decrease in vimentin's intensity, as well as in **(D)** PanCK's intensity upon siMEF2C treatment. Data are given as mean ± SEM (n = 3, performed in triplicate, 10 fields/condition). Significant differences were represented by ** $p < 0.01$ and *** $p < 0.001$ vs. untreated, $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ between the indicated conditions, determined by one-way ANOVA.

2.3. Loss of MEF2C promotes a reduction in TNBC cells' migration

In order to perceive the effect of silencing MEF2C in TNBC cells invasiveness, their migratory capability was determined with a wound-healing assay (Figure 8), where transfection was stopped after 24 h of exposure to the transfection agents (siControl and siMEF2C). Even though the effects of the transfection after 3 h are still not very pronounced, it is visible that BCCs slowly began to migrate (Figure 8A). Moreover, after 24 h the silencing of MEF2C led to a clear reduction in the migratory behavior of 4T1 cells (Figure 8A). In fact, at this timepoint, both controls (untreated 4T1 cells and siControl) showed an evident increase in wound closure, seen by the complete closure of some wound sites.

Likewise, the semi-quantitative analysis of the percentage of wound closure (Figure 8B) showed no significant differences between conditions at 3 h. Additionally, the clear alterations in BCCs migration seen at 24 h were further corroborated, with the untreated 4T1 cells presenting a percentage of wound closure of around 59%, similar to cells transfected with the siControl (59%), whereas MEF2C-silenced cells had the lowest closure percentage of approximately 37%. This translates in a reduction of migration around 20% between 4T1 untreated and transfected, pointing to a reduction in the migratory phenotype in MEF2C-silenced 4T1 cells.

In sum, 4T1 cells migratory capability is reduced by downregulation of MEF2C, in line with the reduced mesenchymal phenotype. Additionally, TNBC cells silenced for MEF2C also presented a reduced polyhedral morphology. These results, suggest an overall contribution of MEF2C to a decrease in BCCs invasiveness/aggressiveness.

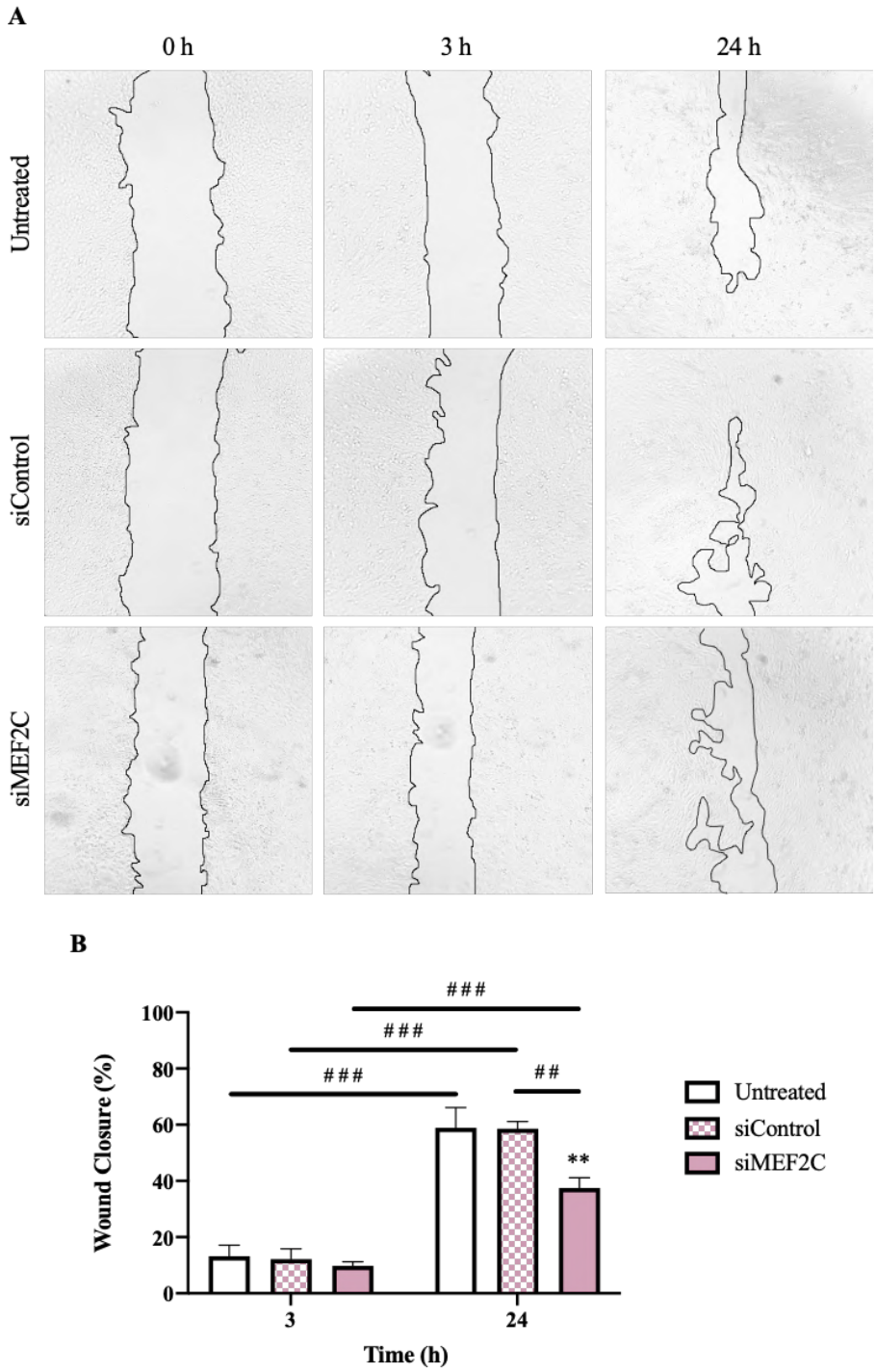

Figure 8. Triple negative breast cancer (TNBC) cells' migration is reduced in MEF2C-silenced cells. TNBC cells were incubated with siMEF2C (10 nM), siControl (10 nM) or with medium supplemented with Opti-MEM (untreated), for 24 h, at which point a wound was inflicted. **(A)** Images of wound closure throughout time (0, 3 and 24 h) were acquired with a phase contrast microscope (100x magnification), showing a notorious inhibitory effect on cell migration in cells silenced for MEF2C. This was further confirmed with the semi-quantitative analysis of the **(B)** percentage of wound closure compared to untreated cells. Data are given as mean ± SEM (n=3, performed in triplicates). A two-way ANOVA test was used to evaluate the significant differences, represented by **$p < 0.01$ vs. untreated at 24 h, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ for differences between the indicated conditions.

3. Metastatic profile of BCCs upon miR-194-5p modulation

Previous studies from our team showed a downregulation of miR-194-5p prior to metastatic development, suggesting that this miRNA can work as a tumor suppressor and could constitute a possible early biomarker for the occurrence of BCBM formation[41,62]. This raised the interest in better comprehending the role of miR-194-5p in TNBC tumorigenesis. To this end, and to increase miR-194-5p's expression, 4T1 cells were transfected with a pre-miR specific for miR-194-5p or a negative control (scramble).

3.1. EMT in TNBC cells is affected by increased levels of miR-194-5p

BCCs undergo phenotypic and morphological alterations associated with the occurrence of EMT, in which they acquire a mesenchymal phenotype, becoming more elongated, with the increase in mesenchymal markers like vimentin, and loss of specific epithelial cell markers, such as the intermediate filament PanCK[8,49]. These alterations have been associated with increased invasiveness of tumor cells, which in turn is associated with an aggressive behavior.

To perceive the putative effects of miR-194-5p in mesenchymal and epithelial features of TNBC cells, vimentin and PanCK expression were assessed by IF (Figure 9). All 4T1 cells expressed the mesenchymal marker vimentin, both in untreated and scramble-transfected cells, with a notorious reduction in pre-miR-194-5p-transfected cells (Figure 9A). In opposite, PanCK expression, which was also patent in all conditions, was increased when miR-194-5p expression was augmented (Figure 9B). Semi-quantitative analysis of IF data corroborated the qualitative observations, since 4T1 cells treated with pre-miR-194-5p presented a significant reduction in vimentin intensity (Figure 9C), concomitant with a significant increase in PanCK intensity (Figure 9D).

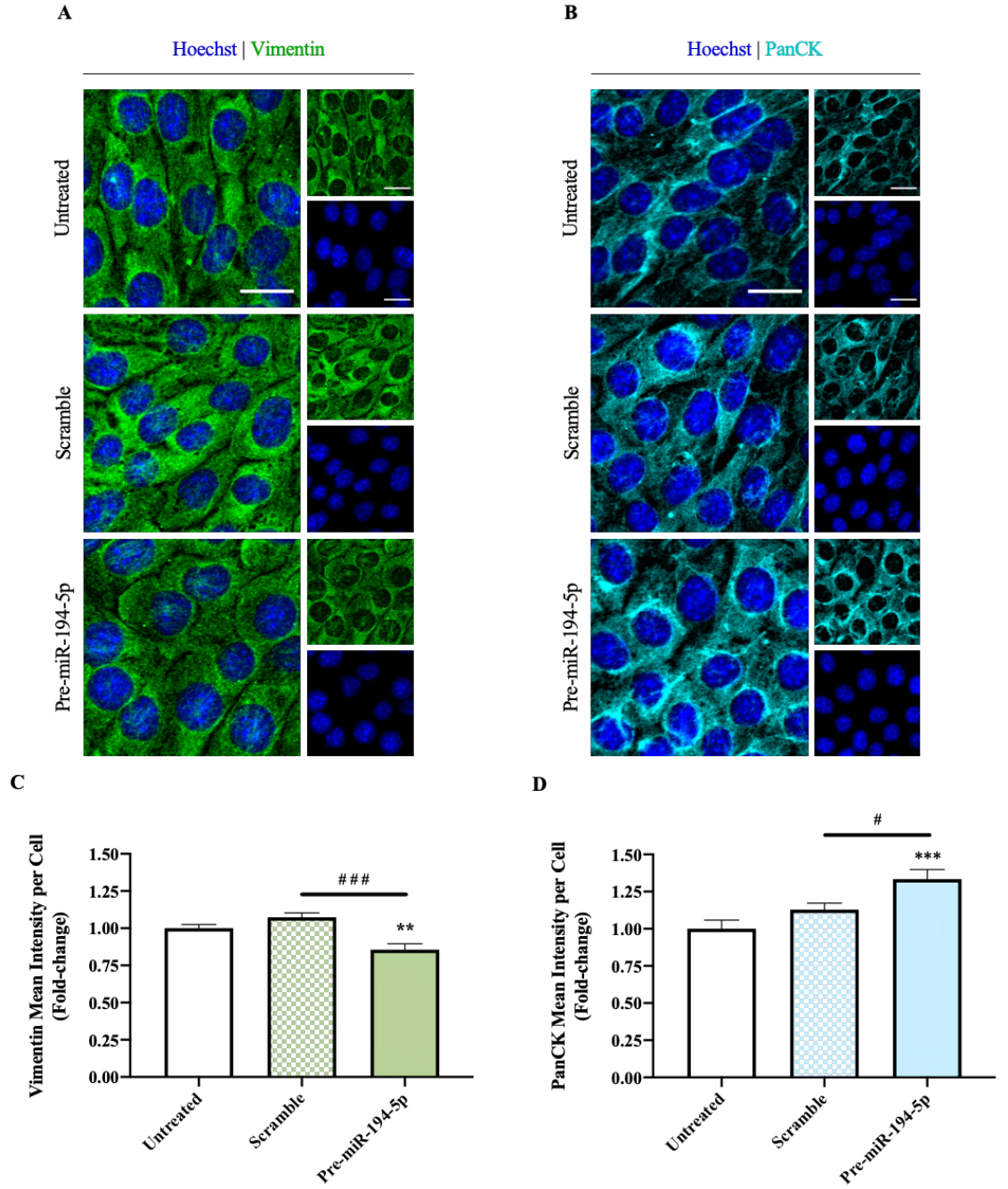

Figure 9. Increase of miR-194-5p levels leads to loss of mesenchymal properties and gain of epithelial features in triple negative breast cancer (TNBC) cells. TNBC cells were incubated with pre-miR-194-5p (10 nM), scramble (10 nM) or with medium supplemented with Opti-MEM (untreated), for 48 h. Expression of **(A)** the mesenchymal marker vimentin (green) and of **(B)** the epithelial marker pan-cytokeratin (PanCK, cian) were evaluated by immunofluorescence, which revealed a decrease of vimentin and an increase of PanCK in cells overexpressing miR-194-5p. Hoechst 33342 was used as counterstaining for nuclei (blue). Scale bar: 20 µm. Semi-quantitative analyses further corroborate **(C)** the significant decrease in vimentin's intensity and **(D)** the increase of PanCK's intensity upon pre-miR-194-5p treatment. Data are given as mean ± SEM (n = 3, performed in triplicate, 10 fields/condition). Significant differences were represented by ** $p < 0.01$ and *** $p < 0.001$ vs. untreated, $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ between the indicated conditions, determined by one-way ANOVA.

Collectively, these data point to a modulation of EMT in TNBC cells by pre-miR-194-5p, once the increase in miR-194-5p's levels not only promoted a reduction in the mesenchymal marker vimentin, but also induced an increase in the epithelial one PanCK, possibly reducing BCCs invasiveness.

3.2. Increased levels of miR-194-5p reduce TNBC cells migration

It is known that when cancer cells undergo morphological alterations that lead to EMT, they become more migratory. This ability to invade the surrounding tissue and enter into circulation, through cell migration, is a hallmark of metastases formation[65]. Hence, migratory studies comprise a way to appraise cancer cells' aggressiveness, mirroring a loss of invasive capability.

To assess if the loss of mesenchymal features and gain of endothelial ones is reducing BCCs' migratory capability, a wound-healing assay was performed (Figure 10). Upon 48 h of transfection (with the previously selected 10 nM concentration), the effect of miR-194-5p's overexpression on 4T1 cells' migration was assessed. The results showed that BCCs slowly began to migrate at 3 h, slightly reducing the wound size (Figure 10A). However, after 24 h, migration of 4T1 cells overexpressing miR-194-5p was notoriously reduced (Figure 10A). Indeed, at 24 h, both controls (untreated 4T1 cells and scramble) showed an evident increase in wound closure, whereas cells treated with pre-miR-194-5p remained essentially unclosed.

Semi-quantitative analysis of the percentage of wound closure (Figure 10B) showed that 3 h after the end of the transfection period, BCCs began to migrate, with no significant differences between conditions. Additionally, the perceivable visual effect seen at 24 h is further corroborated, with the untreated 4T1 cells presenting a percentage of wound closure (52%) similar to cells transfected with the scramble (47%), whereas cells overexpressing miR-194-5p had the lowest closure percentage (22%). This translates in a reduction of migration around 30% between 4T1 untreated and transfected cells. Interestingly, the differences between 3 and 24 h migration in cells overexpressing miR-194-5p increases (15% to 21%), albeit with no significant difference. This, once again, highlights the reduction in wound closure, promoted by increased levels of miR-194-5p.

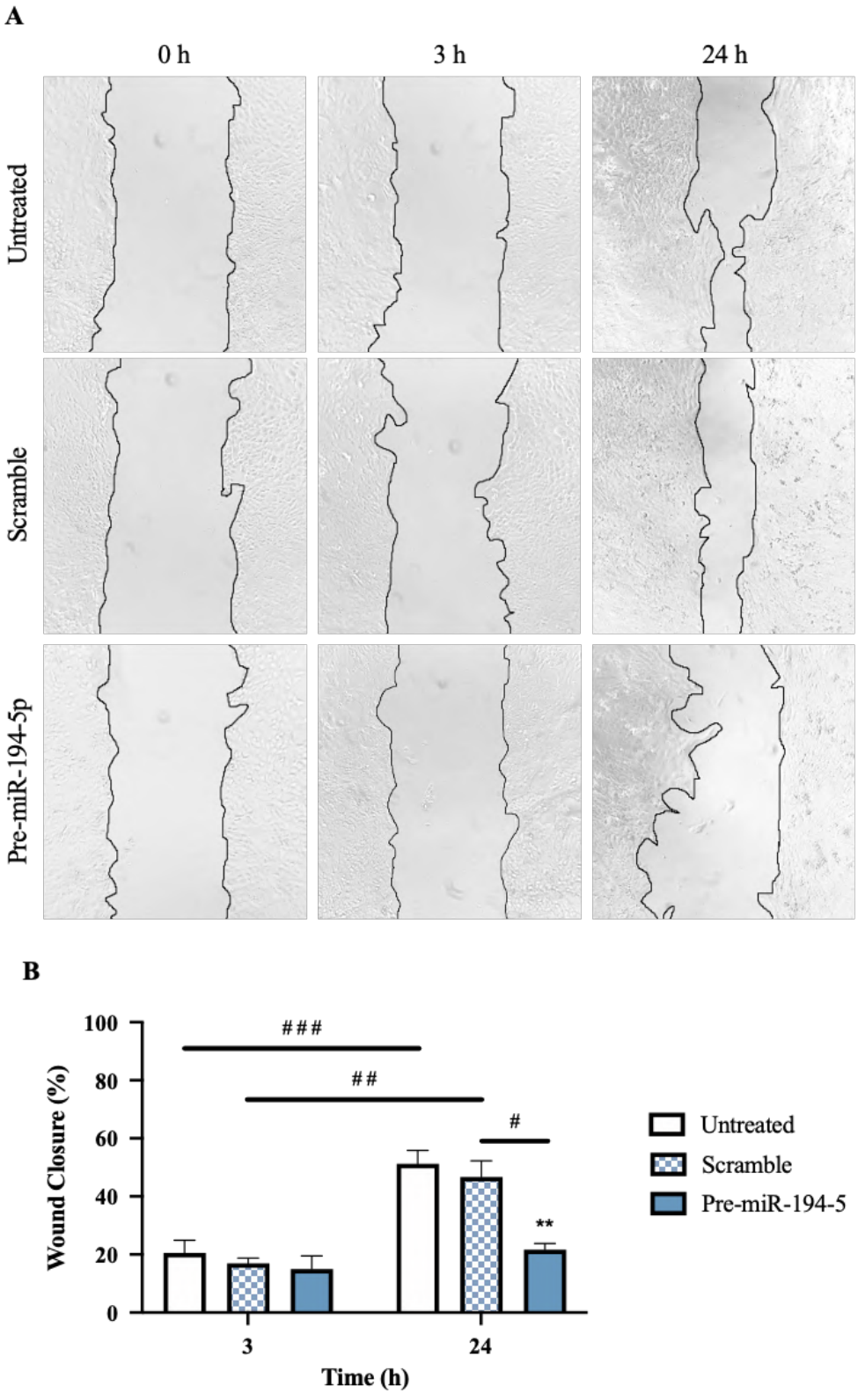

Figure 10. Increase of miR-194-5p levels significantly inhibits triple negative breast cancer (TNBC) cells' migration. TNBC cells were incubated with pre-miR-194-5p (10 nM), scramble (10 nM) or with medium supplemented with Opti-MEM (untreated), for 48 h, at which point 4T1 cells were wounded. **(A)** Images of wound closure throughout time (0, 3 and 24 h) were acquired with a phase contrast microscope (100x magnification) and revealed a notorious inhibitory effect on cell migration in cells overexpressing

miR-194-5p, validated by the semi-quantitative analysis of the **(B)** percentage of wound closure compared to untreated cells. Data are given as mean ± SEM (n=3, performed in triplicate). A two-way ANOVA test was used to evaluate the significant differences, represented by **$p < 0.01$ vs. untreated at 24 h, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ between the indicated conditions.

All in all, an increase in miR-194-5p's levels is able not only to reverse EMT in 4T1 cells, but also to reduce their migratory capacity, overall contributing to a decrease in BCCs invasiveness/aggressiveness.

Chapter IV – Discussion

Among BC subtypes, TNBC is widely known for its invasive and aggressive behavior[66]. Still lacking effective treatments, due to absence of disease biomarkers and prone to metastasize, TNBC is often associated with a poor prognosis[67]. Since most BC related deaths arise from its metastatic form, and due to its heterogeneity, recent research is focusing on prognostic factors that can be used to identify not only at-risk patients, but also the most effective treatments for each patient[68,69]. Recently, in published work from our team, miR-194-5p's plasma levels were found to be decreased prior to metastasis development, in a mouse model of BCBM, while MEF2C was increasingly expressed along brain metastasis development[41]. Moreover, MEF2C was bioinformatically identified as a target of miR-194-5p, but no relation between these two players was investigated. In addition to analyzing the possible link between MEF2C and miR-194-5p, this study also brings an understanding of the role of MEF2C and of miR-194-5p in BC tumorigenesis.

A limitation of this study relies in the fact that the initially planned assessment of the reflex of modulating the miRNA's and the transcription factor's levels in BCBM formation was not undertaken. To this fact accounted the confinement periods and experimental work restrictions imposed by the Covid-19 pandemics, which limited the foreseen experimental work that could be developed. Moreover, considerable efforts towards MEF2C overexpression were undertaken, which consumed a significant fraction of the time dedicated to the experimental development. Nevertheless, this thesis unravels new contributions to BC tumorigenesis, paving the way for future studies particularly directed to BCBM.

In an initial screening for the optimal pre-miR-194-5p concentration, the ones that induced any toxicity were excluded, with only the 10 nM concentration meeting the non-toxic criteria. One way of studying a miRNA's impact in pathological processes of any cancer is by assessing its role on major protein targets and signaling pathways, affected by the miRNA dysregulation[70]. Baring this in mind, the selected non-toxic concentration of pre-miR-194-5p was used to appraise the supposed relation between miR-194-5p and MEF2C by mRNA and protein expression analysis. In previous *in vivo* studies, an early downregulation of miR-194-5p and a late upregulation of MEF2C were observed[41]. In fact, miR-194-5p early downregulation was further validated both *in vivo* and *in vitro* by ISH and RT-qPCR[62]. Hence, by inducing miR-194-5p upregulation it was expected that MEF2C's expression would decrease, which would be indicative of a direct effect between these two players. When no effect was observed at 48 h, it raised the question if

at that time any compensatory mechanism had already been put to action. So, to enlighten if this could be the case, two additional timepoints (24 and 72 h) were tested by IF, which, similarly to the 48 h, did not show any effect of overexpressing miR-194-5p in MEF2C's expression. Even though two bioinformatic tools predicted that MEF2C is a target of miR-194-5p, these *in vitro* results did not demonstrate the link between the miRNA and the transcription factor in TNBC cells. This may be explained by the fact that the decreased miR-194-5p expression was observed in plasma, whereas the increased MEF2C expression was observed in malignant cells. On the other hand, the temporal sequence of events occurring *in vivo* cannot be fully mimicked *in vitro*. Finally, it cannot be neglected the possibility that activation of alternative and/or compensatory pathways took place, precluding the establishment of a direct relationship between the miRNA and the transcription factor. Therefore, the role of these players should be further dissected, namely in more complex systems, as the BCBM *in vitro* model established by our team[64].

EMT is one of the most studied tumorigenic processes and yet, in some cases like TNBC, the mechanisms preceding this phenomenon are still poorly understood[69]. During EMT, tumor cells undergo a series of intricate modifications both in shape and in cytoskeletal organization with loss of cell-to-cell connections, in which they partially or completely lose their epithelial phenotype and acquire mesenchymal characteristics[65,71]. Our results revealed that BCCs silenced for MEF2C present a reduction not only in the mesenchymal marker vimentin, but also in the epithelial marker panCK, suggesting that this procedure induces a partial EMT in TNBC cells (Figure 11). In the literature, a partial EMT is considered a "metastable" intermediate transition, characterized by weak cell-cell junctions, in addition to increased motility and invasive properties[72]. This process has been observed in different cancer types, ranging from head and neck[72], lung[73], renal[74] and BC[75,76]. However, our results regarding TNBC cells' migration showed a reduced migratory capability in MEF2C-silenced cells, when compared to the controls. These observations are particularly visible at 24 h, where not even 50% of the wound was closed, suggesting that MEF2C downregulation significantly limits TNBC cells' motility and invasive phenotype. It is known that one key step in the metastatic development, is the ability of tumor cells to invade the surrounding tissue[77], and, to our knowledge, MEF2C has not been directly related with the migratory capability of TNBC cells. In fact, MEF2C's dysregulation has been correlated with different diseases, including several types of cancer. In accordance with our data, MEF2C's silencing was shown to promote a reduction in glioma cells' migration and proliferation, in addition to an increase in cell

apoptosis[28]. Similarly, in hepatocellular carcinoma, MEF2C-silenced cells appeared to counteract the migratory effect caused by VEGF-overexpression, by maintaining the percentage of wound area[37]. On the other hand, in cervical cancer, several tumorigenic processes, like proliferation, migration and invasion, were inhibited when MEF2C's expression levels were up-regulated[78]. Indeed, in gastric cancer cells, the opposite effects were observed with the knockdown of MEF2C, which lead to an enhanced migration[79]. Even though MEF2C's appears to have a dual effect on cancer cells' migration, our results put on evidence, for the first time, that MEF2C silencing leads to a significant reduction on TNBC cells' migration, corroboration its role as tumorigenic (Figure 11). However, further studies focusing on cell-cell adhesion are needed to better understand whether MEF2C's silencing is truly inducing a "metastable" state, in addition to analyze the effects that MEF2C's overexpression may induce in TNBC cells and how it affects their tumorigenesis.

In the case of miRNAs, their dysregulation can affect EMT through interaction with a variety of specific pathways and protein targets, which, in turn, will affect pathogenic processes, like cell growth and differentiation, metastasis and apoptosis[70,80]. In line with this, the effects of overexpression of miR-194-5p levels on EMT features were assessed by IF analysis of a mesenchymal (vimentin) and an epithelial (PanCK) marker. Our results showed a substantial reduction in vimentin, in opposition to an increase in PanCK, disclosing a modulation of EMT in TNBC cells through augmented levels of this miRNA, which can reflect a reduction on BCCs' invasiveness and point to the tumor suppressor role of miR-194-5p. A tumor suppressor role of this miRNA has also been observed in several types of cancer, such as Wilms tumor[81], colorectal adenocarcinoma[82,83], glioma[84] and gastric cancer[85]. In these studies, and similarly to our results, an induced overexpression of miR-194-5p led to a decline in mesenchymal markers, like N-cadherin, vimentin and Twist1/2, in opposite to an increase in epithelial markers, such as E-cadherin, ultimately suggesting a total inhibition of EMT[81–85]. Interestingly, in BC, miR-194-5p knockdown was shown to suppress tumor growth, through inhibition of cell proliferation, migration and invasion, yet the effect of this dysregulation on EMT has not been established[58]. Even so, and since the knockdown of this miRNA leads to a decrease in tumorigenic processes of BC, it would be expected that this procedure would translate in an inhibition of EMT. Nonetheless, the inhibitory effect on EMT caused by the overexpression of miR-194-5p in TNBC cells, not only seems to bear great potential

towards inhibition of invasive properties, but also appears to be described for the first time in our study, worthwhile to be explored in deeper extent.

To our knowledge, a direct relation between miR-194-5p's dysregulation and TNBC cells' migratory capability has not yet been established. Baring this in mind, and to better understand the impact of overexpressing miR-194-5p, a wound healing assay was performed, where it was possible to observe that cells overexpressing miR-194-5p appear to have a slower closure than the respective controls, particularly at 24 h. At this time point, BCCs' migration was notoriously reduced with the pre-miR-194-5p at 10 nM, indicating that increased levels of this miR significantly limit TNBC cells' motility and invasive phenotype. Depending on the cancer type or subtype, miR-194-5p can have a dual behavior, acting as tumor suppressor or oncogene. A tumor suppressor role has been seen in several types of cancer, as in non-small cell lung cancer[57], osteosarcoma[86], gastric cancer[87] and in colorectal cancer[88], where miR-194 had an inhibitory effect on tumorigenic processes such as migration, proliferation and invasion. In contrast, miR-194-5p was found to be upregulated in BC tissues and its knockdown led to an inhibitory effect in the same tumorigenic processes of BCCs[58]. These data are in line with another study, in which miR-194-5p's overexpression in BCCs was associated with an aggressive behavior, by counteracting a specific long non-coding RNA – LNC00641 – and increasing tumorigenic parameters, such as migration and invasion[54]. Hence, the different roles of miR-194-5p emphasizes the dual effect that this miRNA may have, depending on the cell and cancer types. In our study, this miRNA appears to have a tumor suppressor role in TNBC, since its overexpression is aligned with a decrease migratory capability of BCCs and, thus, with a putative reduction in aggressiveness, as corroborated by a reduction in BCC's mesenchymal phenotype reduction (Figure 11).

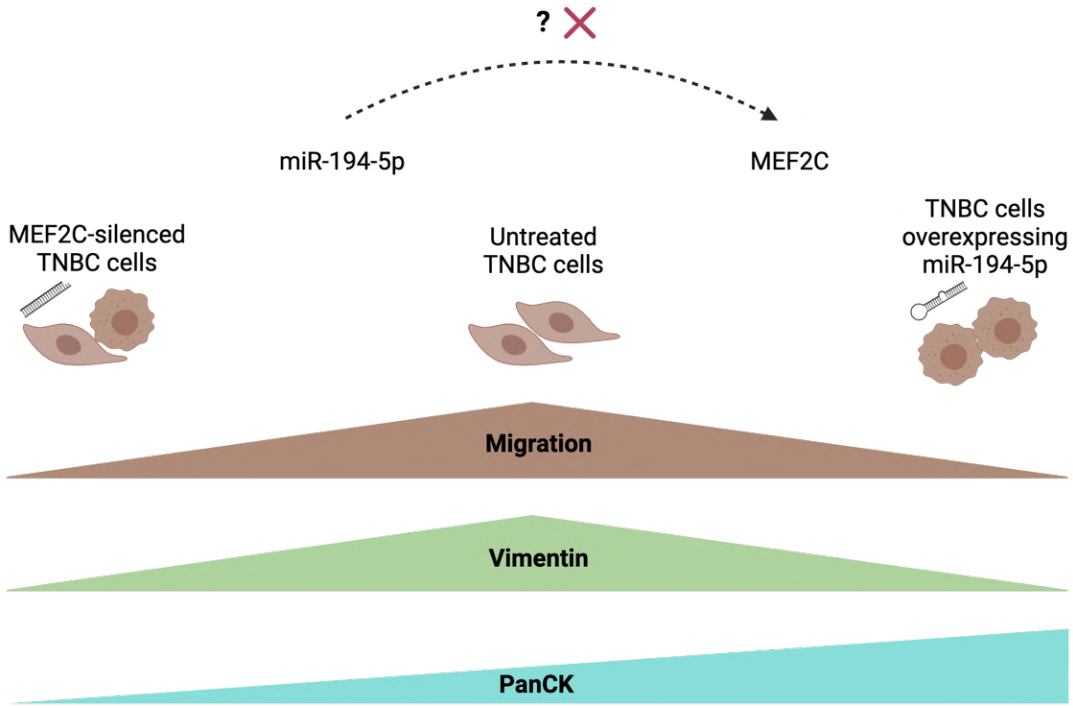

Figure 11. Myocyte enhancer factor 2C (MEF2C) and microRNA (miR)-194-5p modulate TNBC cells phenotype and migratory capacity. Despite the known alterations regarding MEF2C and miR-194-5p along BCBM formation, as well as their bioinformatically predicted downstream regulation, in the tested conditions, it was not possible to discern a relation between miR-194-5p overexpression and MEF2C's levels. TNBC cells silenced for MEF2C presented a decrease in migratory capability, along with reduced vimentin and PanCK levels, suggestive of a partial epithelial-mesenchymal transition (EMT). TNBC cells overexpressing miR-194-5p also presented a decline in migration and vimentin expression, whereas PanCK expression was boosted, concomitant with a reduction in TNBC cells aggressiveness. By understanding the role of miR-194-5p and MEF2C in tumorigenesis, this work revealed them as new players and putative modulation targets in TNBC patients, paving the way for new therapeutic options to improve metastasis-free survival.

To the best of our knowledge, together with the mesenchymal phenotype reduction observed, this study reports for the first time, an inhibitory effect of not only MEF2C but also miR-194-5p in TNBC cells' migratory and invasive capacity, bringing a new understanding of the potential of these two players in the pathogenesis of BC.

Notwithstanding, the preliminary safety screening also provided a basis for a possible therapeutic approach to tackle TNBC, since only pre-miR-194-5p at 30 nM and not the scramble showed some toxicity against TNBC cells. In this sense, this concentration of pre-miR-194-5p could be further studied in a delivery system to specifically target BCCs. In fact, this procedure has already been performed in TNBC using nanoparticle deliveries

of different miRNAs. For instance, suppression of TNBC migration was successfully achieved with an encapsulation of miR-203, whereas tumor growth and metastasis formation were inhibited and delayed using a nanoparticle delivery of both miR-34a and miR-10b[89,90]. Additionally, a combined therapy consisting of an encapsulation of both orlistat and antisense-miR-21 lead to a better cellular therapeutic response[91]. Together with CD133, a highly expressed marker in TNBC, miR-21 was also used, in a mouse model, showing a significant tumor growth inhibition, in addition to a highly specific to target TNBC tumors[92]. These promising studies emphasize the unexplored interest in understanding the effect of a specific targeted delivery of pre-miR-194-5p at a concentration of 30 nM to TNBC cells, as a way to limit BCCs survival.

Chapter V – Concluding Remarks

Due to a general lack of knowledge in what are the complex and dynamic processes underlying BC tumorigenesis, particularly regarding formation of metastases in the brain, it becomes imperative to explore new players in order to develop novel therapeutic approaches. In the present study, a miRNA and a transcription factor have been highlighted to have a role in key mechanisms involved in TNBC cells aggressiveness, such as their invasion and migration, owing to morphological and phenotypic alterations. Importantly, both MEF2C silencing and miR-194-5p upregulation showed an inhibitory role in BCCs migration and invasion. These effects were observed for the first time in TNBC, pointing to the modulation effect of these two players in the tumorigenic processes.

Despite these promising achievements, further studies are still needed to have a deeper knowledge of the modulation effect of MEF2C and miR-194-5p not only on morphological alterations, focusing on elongation and cytoskeleton markers such as the filamentous actin (F-actin) and the myosin light-chain kinase (MLCK), but also by assessing intervenients of the MEF2C's signaling pathway, such as the β-catenin and VEGF. These studies will allow to better understand MEF2C's and miR-194-5p's mechanisms of action. Furthermore, the assessment of the toxicity effects of pre-miR-194-5p at 30 nM in TNBC cells could also be relevant as a therapeutic alternative, aimed to directly target invasive tumor cells. Moreover, it will also be important to further consolidate these findings in mixed cultures of ECs and BCCs, as an *in vitro* model of BC brain metastasization established in our lab[64], which will allow the study of tumor cells extravasation, cluster formation, BBB impairment and migration through this barrier. Additionally, this microenvironment may be helpful to remove any lingering questions regarding the relationship between miR-194-5p dysregulation and MEF2C's expression, since it is a model that better mimics the *in vivo* conditions.

To add to the reported results of this study, it would be of interest to improve the transfection efficiency of the MEF2C plasmid to understand the effects of its overexpression on TNBC cells tumorigenesis. This could be achieved using alternative approaches to the lipid-based transfections, such as physical transfection methods like electroporation. Similarly, additional studies using an antagomiR to diminish miR-194-5p's expression would be relevant to assess the impact of low levels of this miR in TNBC pathogenesis. As a result, TNBC cell's regulation confirmation will be possible through cytoskeleton/morphological alterations and extravasation processes. Elucidation of these

processes will, hopefully, allow to develop a therapeutic strategy to, not only, timely avoid the dissemination of TNBC cells, but also the occurrence of BCBM itself.

In sum, this study offers a new understanding of the roles and contributes of MEF2C and miR-194-5p in tumorigenesis, by providing new information beyond the current state of art in BC and, consequently, disclosing novel modulation targets aimed to improve TNBC patients' metastases-free survival, particularly those presenting brain metastases.

Chapter VI – References

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Chapter VII – Supplementary Material

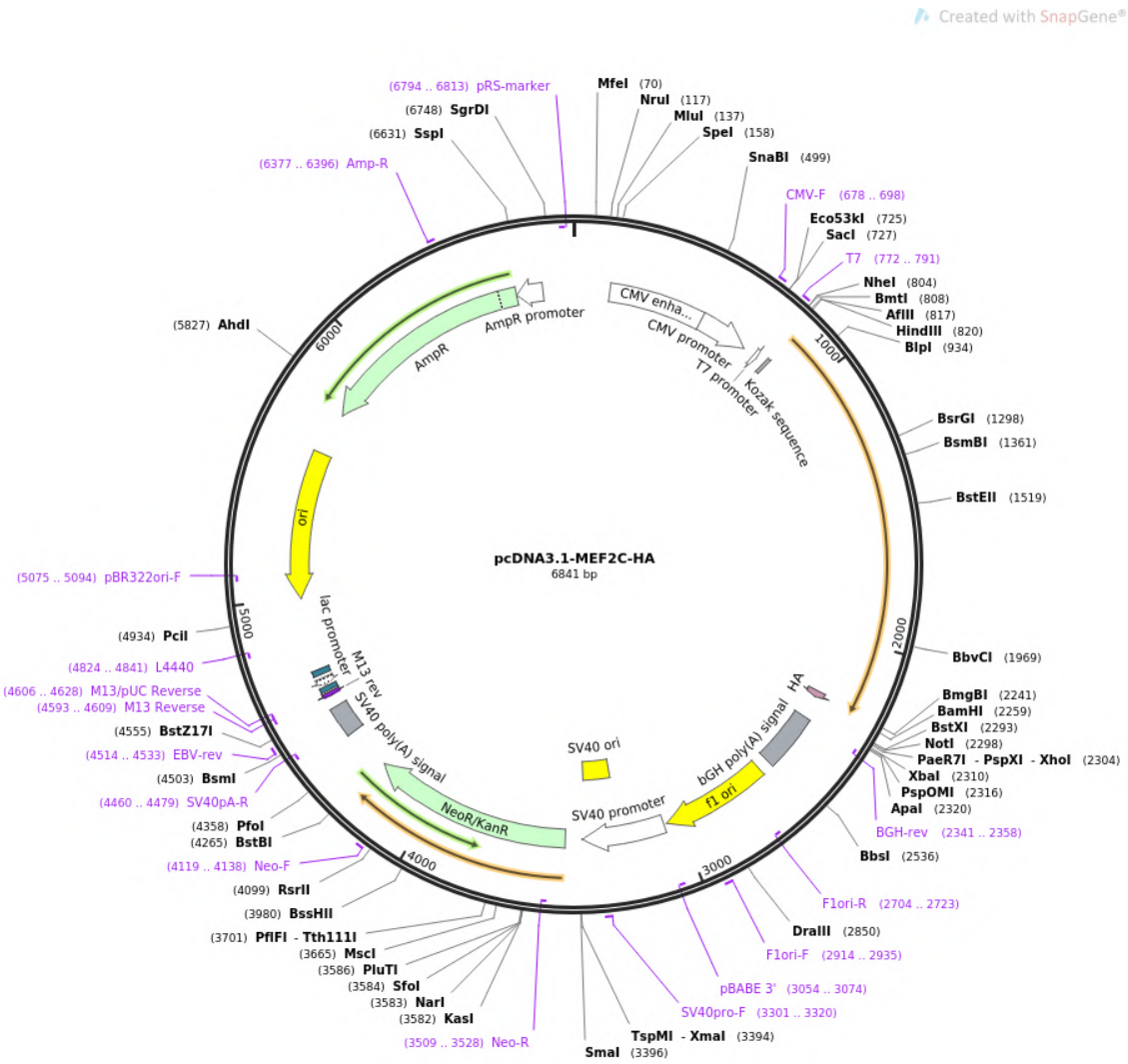


Figure 1. Schematic and structural representation of MEF2C's plasmid. Backbone of the pcDNA3.1-MEF2C-HA vector backbone, containing its size (6841bp), bacterial resistance to Ampicillin and promoter (CMV). This plasmid was created by Andrew Lassar and it was published form the first time in 2009[93]. Additionally, MEF2C plasmid can be found in addgene.org with the catalog number of #32515.

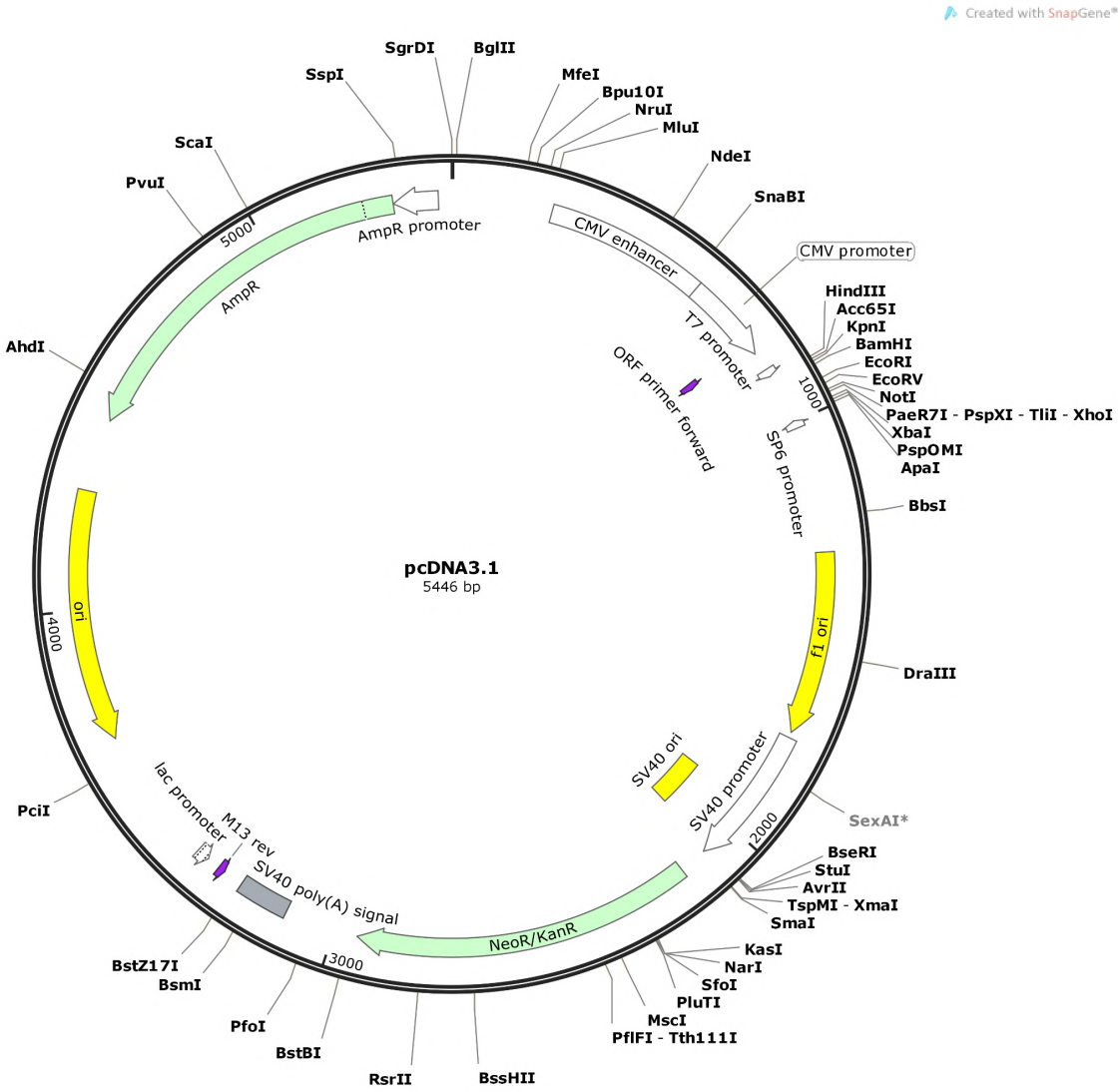
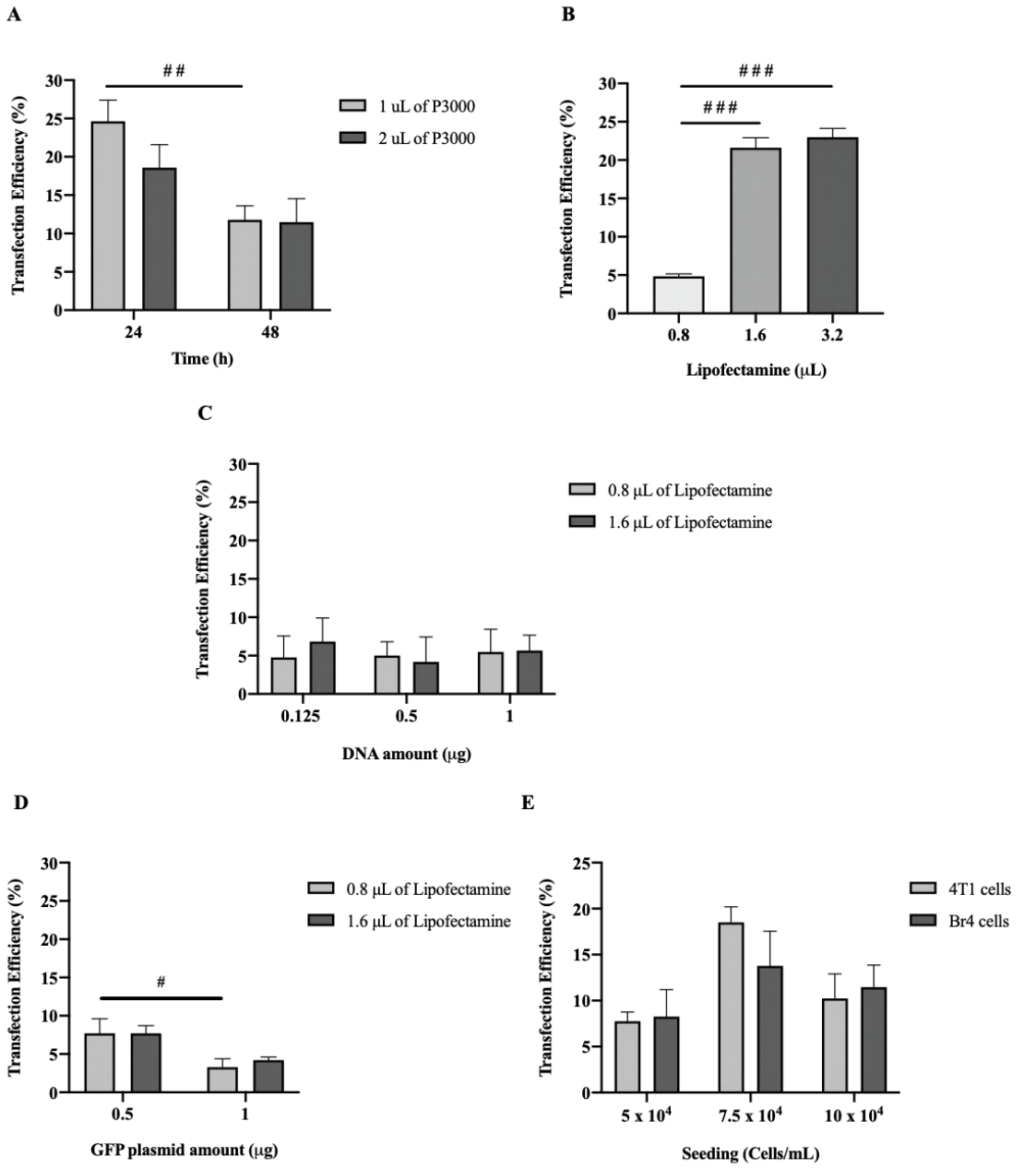


Figure 2. Schematic and structural representation of the empty plasmid. Backbone of the pcDNA3.1 vector backbone, containing its size (5446 bp), bacterial resistance to Ampicillin and promoter (CMV). This plasmid can be found in genomics-online.



a

Figure 3. Optimization to increase the transfection efficiency of MEF2C plasmid, in 4T1 cells. Several efforts to improve modulation efficiency of MEF2C, using a plasmid specific for this protein (pMEF2C) were carried out. **(A)** Optimization of P3000 volume to perform the transfections with pMEF2C. **(B)** Evaluation of Lipofectamine 3000 volume necessary to increase the transfection efficiency, showing that both 1.6 µL and 3.2 µL significantly increase the number of pMEF2C-transfected cells. **(C)** Optimization of the amount of DNA (0.125, 0.5 and 1 µg of DNA), showing no significant differences between not only the two different volumes of Lipofectamine 3000, but also among DNA quantities. **(D)** To understand if the low transfection efficiencies were due to any defect of the MEF2C plasmid, 4T1 cells were transfected a green fluorescent protein (GFP) plasmid, using two DNA amounts (0.5 and 1 µg of DNA), showing a slightly better efficiency with the 0.5 µg of GFP plasmid. **(E)** Three different seeding concentrations of 4T1 and the human TNBC cell line MDA-MB-231Br4 cells (in short TNBC cells) were used, and, in both cases,

the intermediate concentration (7.5 x 10^4 cells/mL) appears to have a higher number of cells transfected. Data are given as mean ± SEM (n=1). A two-way ANOVA test was used to evaluate the significant differences, apart from graph **(B)**, where a one-way ANOVA test was performed. Significant differences are represented by $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ and $^{\#\#\#}$ $p < 0.001$ between the indicated conditions.