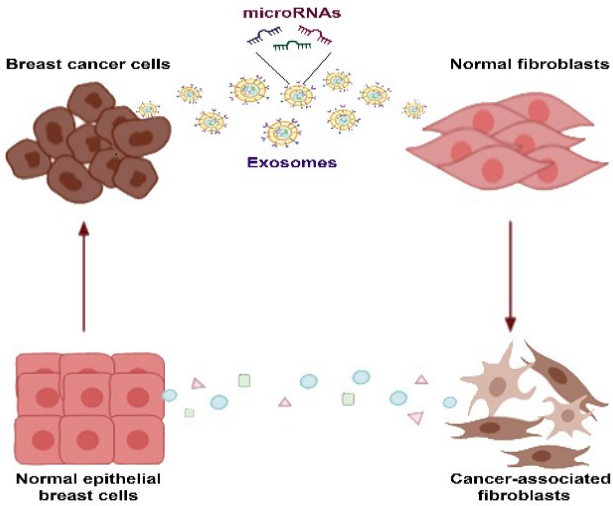
UNIVERSITY OF NAPLES FEDERICO II

DOCTORATE IN
MOLECULAR MEDICINE AND MEDICAL BIOTECHNOLOGY

XXXIV CYCLE

Iolanda Scognamiglio

"The relevance of exosomal microRNAs in the re-education of stromal fibroblasts: a framework for the comprehension of triple negative breast cancer"



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Iolanda Scognamiglio

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LIST OF ABBREVIATIONS

ATRA	All- trans retinoic acid
BC	Breast cancer
BRCA1-2	Breast Related Cancer Antigen 1-2
CAF	Cancer-Associated Fibroblast
CD	Cluster of differentiation
CDK4/6	Cyclin Dependent Kinase 4/6
circRNA	Circular RNA
CM	Conditioned Media
Combo miRs	Combined microRNAs
CTGF	Connective tissue growth factor
CXCR4	CXC- chemokine receptor 4
DMEM	Dulbecco's Modified Eagle's Medium
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
ER	Estrogen receptor
ESCRT	Endosomal sorting complexes required for transport
FAK	Focal adhesion kinase
FAP	Fibroblast activation protein
FBS	Fetal Bovine Serum
FDR	False Discovery Rate
FGFR	Fibroblast growth factor receptor
HER-2	Human epidermal growth factor-2
HSP70	Heat shock protein 70
IHC	Immunohistochemistry
IL-2,	Interleukin-2
ITG	Integrin
lncRNA	Long non coding RNA
LOXL2	Lysyl oxidase- like 2
MCT4	Monocarboxylate transporter 4
miRISC	MiRNA induced silencing complex
miRNA/miR	MicroRNA
MMP	Matrix metalloproteinase
MRX34	MicroRNA miR-RX34
MTs	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NF	Normal Fibroblast
nSMase2	Neutral sphingomyelinase 2
NT	Not treated
O.N.	Over night

PAM50	Prediction Analysis of Microarray 50
PARP	Poli ADP-ribose polymerase
PBS	Phosphate Buffered Saline
PD-L1	Programmed death-ligand 1
PDTO	Patient-derived tumor organoids
PDX	Patient-derived xenograft
PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
PR	Progesterone receptor
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
R.T.	Room Temperature
ROCK	RHO kinase
rpm	Round per minute
Scra	scrambled
TBS	Tris buffered saline
TEAD	Transcriptional enhanced associate domain
TEM	Transmission electron microscopy
TGFβ	Transforming growth factor- β
TME	Tumor microenvironment
TNBC	Triple negative breast cancer
TSG101	Tumor susceptibility gene 101
UTR	Untranslated region
YAP1	Yes-associated protein 1

ABSTRACT

Triple negative breast cancer (TNBC) is the most aggressive subtype of breast cancer, characterized by elevated metastatic potential. TNBC progression is strongly sustained by the recruitment of the tumor microenvironment (TME), mainly composed of Cancer-Associated Fibroblasts (CAFs) able to endorse tumor hallmarks. Increasing evidence demonstrated that exosomes mediate the crosstalk between cancer cells and TME through delivering their molecular cargo. In this thesis, we examined the role of TNBC cell-derived exosomes and their microRNAs (miRNAs) cargo in the activation of stromal fibroblasts towards CAFs. We demonstrated that TNBC cell-derived exosomes increased fibroblast-mediated collagen contraction ability and migration potential alongside CAF-related molecular markers. Furthermore, fibroblasts activated by exosomes promoted the invasion potential of normal breast epithelial cells as assessed by the three-dimensional organotypic co-culture assay which resembled the *in vivo* context. We further investigated the role of TNBC cell-derived exosomes cargo in activating normal fibroblasts, by performing a small RNA-sequencing on fibroblasts incubated with exosomes. The findings revealed multiple upregulated miRNAs in fibroblasts upon exosome incubation. Among these, miRNAs-185-5p, -652-5p, and -1246 (combo miRs) were found to synergistically boost fibroblast migration, invasion, and contraction abilities together with the related molecular markers, thus promoting a specific CAF sub-specialization towards a pro-migratory functional state rather than a proliferative phenotype. Furthermore, conditioned medium from stromal fibroblasts transfected with combo miRs (CM_combo miRs) induced the migration of non-tumorigenic epithelial breast MCF10A cells, as assessed by transwell assay and increased the proliferation of breast cancer MCF7 cells, and MCF10A cells, as tested by MTS assay. Moreover, we investigated the invasion ability of patient-derived breast cancer organoids when incubated with CM_combo miRs through the *in vitro* collagen invasion assay. We found wider invasive protrusions in organoids cultured with CM_combo miRs compared to control (CM_Scra) indicating an increased invading trend of cancer cells mediated by fibroblasts activated by exosomal miRs. All together these data highlighted the role of exosomes and their miRNA cargo in the re-education of fibroblasts within the TME, shedding light on processes related to triple negative breast cancer evolution.

1. BACKGROUND

1.1 Breast cancer: general features

Currently breast cancer (BC) is the most diagnosed malignancy among women accounting for 30% of female cancers (Siegel 2020). BC is very heterogeneous disease that comprises different subtypes according to the molecular, histochemical, and histological classification. From the histological point of view, breast cancer is primarily divided into *in situ* and invasive carcinomas, based on the ability of infiltrate the stroma. In situ carcinoma is further subclassified into ductal in situ carcinoma, more common, and lobular in situ carcinoma, depending on the architectural features. Similarly, invasive carcinoma comprises different subtypes, the most invasive are infiltrating ductal, invasive lobular, ductal/lobular, mucinous (colloid), tubular, medullary and papillary carcinomas (Malhotra 2010). Based on the presence/absence of histochemical markers such oestrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor-2 (HER-2) and proliferation markers (e.g., Ki67), BC is classified into: Luminal A (ER^+/PR^+, HER-2^-, $Ki67_{low}$), Luminal B (ER^+/PR^-, HER-$2^{+ \text{ or } -}$, $Ki67_{high}$), HER-2 overexpressing (ER/PR^-, HER-2^+, $Ki67_{high}$), and triple negative breast cancer (TNBC- ER/PR^-, HER-2^-, $Ki67_{high}$). In recent years, BC molecular characterization has expanded including genomic markers (e.g., BRCA1, BRCA2, and PIK3CA), and immunomarkers (e.g., tumour-infiltrating lymphocytes and PD-L1) with new biomarker combinations emerging for a more accurate diagnosis (Loibl 2021). Among these various group of malignancies, triple negative breast cancer (TNBC) represents the most aggressive subtype constituting 15% to 20% of all BCs. Since it is primarily characterized for the absence of ER, PR, and HER-2 expression, TNBC is ineligible for hormone-based and receptor-targeted therapies (mainly trastuzumab and pertuzumab). Despite that, receptor expression in TNBC is more tangled than it seemed. In fact, TNBC is highly heterogeneous, since itself includes six subtypes in total (basal-like1, basal-like2, mesenchymal, luminal androgen receptor type, immune modulatory, and mesenchymal stem–like) based on gene expression profiles (Lehmann 2011, Prat 2013, Lehmann 2016, Garrido-Castro 2019) (**Figure1.1**). Consequently, this subtyping dictates a diverse response to chemotherapies that further fuels disease-related heterogeneity (Santonja 2018). Wherefore, TNBC is associated to a poor clinical prognosis with a significant mortality rate at 5 years from the diagnosis. The aggressiveness of TNBC is further related to its high metastasis occurrence, strictly associated to tumor plasticity and heterogeneity (Kvokackova 2021) (Karaayvaz 2018). Furthermore, TNBC metastatic potential has been increasingly related to the presence of activated tumor microenvironment (TME) able to sustain diverse functions during cancer initiation and evolution (Keren 2018, Janiszewska 2019). This item will be further discussed in the next sections.

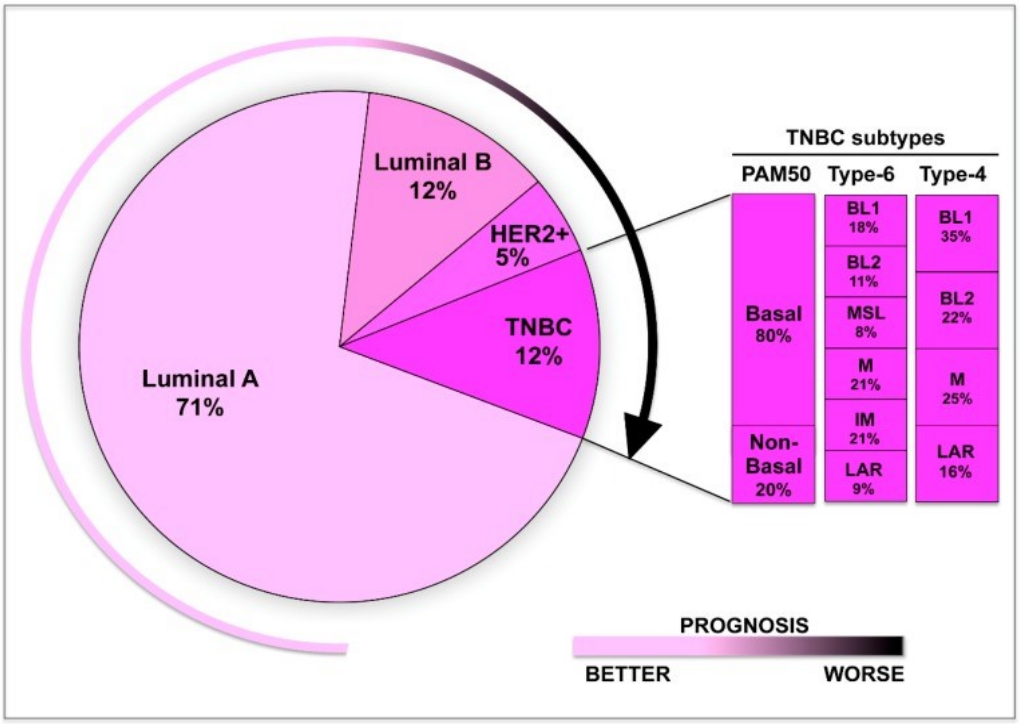

Figure1.1. Schematic representation of TNBC heterogeneity.
Pie chart on the left represents the recurrence of different BC subtypes expressed in percentage. The circled arrow indicates patients' prognosis (better for Luminal A and worse for TNBC). Right panel schematizes TNBC heterogeneity according to PAM50 (Prediction Analysis of Microarray 50), Type-6, and type-4 subtyping. (From:(Gatti 2019)).

1.2 Breast cancer management: standard vs precision medicine.

Breast cancer is usually diagnosed with mammography screening or for the appearance of symptoms represented by pain or palpable nodule. However, mammography presents several disadvantages, including radiation exposure, pain, negative psychological effects, and the presence of false positive, especially in women between 40-50y (Pace and Keating 2014). To reduce false positive results, several imaging techniques have been employed in combination with mammography, including digital breast tomosynthesis, ultrasounds and magnetic resonance imaging, that seem to better detect occult cancers (Friedewald 2014) (Lehman 2012). After clinic imaging screening, the pathological evaluation of breast tissue specimens derived from surgical excision, needle aspiration, or core biopsy is performed by IHC or molecular tests to detect tumor morphology and perform marker characterization. Indeed, clinical treatment decision mainly depends on the expression of ER, PR, HER-2, and proliferation markers, in combination with the detection of multiple genes predictive of breast cancer prognosis (Perou 2000). According to standard medicine, primary breast cancer treatment includes tumor removal by surgery, with different innovative approaches developed during the years. As an instance, surgical breast-conserving procedures have been established in modern years thanks to the improved imaging techniques, that are able to detect also non-palpable tumors. For early breast cancer treatment, they comprise lumpectomy and radiation with survival rates similar to total mastectomy (Fischer 2014) (Veronesi 1981). After surgical removal, adjuvant chemotherapy is recommended for cancers with high risk of recurrence, such as TNBC, that is predicted by marker expression (ER-, PR-, and HER2-negative; HER2-positive), tumor size, and positive lymph nodes. In particular, for HER-2 overexpressing tumors, trastuzumab, a HER2-specific monoclonal antibody, is administered in addition to chemotherapy to improve the survival of patients with early-stage breast cancer. Instead, ER- or PR-positive breast cancer is treated with endocrine therapy, such as aromatase inhibitors. However, for high metastatic breast cancer at advanced stages, chemotherapy remains the only therapeutic chance (McDonald 2016). Nonetheless, the standard therapeutical approaches remain unsuccessful for some patients, dictating high variability in therapeutic efficacy. Starting from these issues, the main goal for the future medical oncology has been representing by the "precision medicine" approach, that considers the inter-individual heterogeneity, accountable for variable response to therapy and delay in precise diagnosis. According to this innovative procedure, breast cancer management should comprise the combination of structural imaging, immunohistochemical and genetic information to recreate an integrative stratification of patients (**Figure 1.2**).

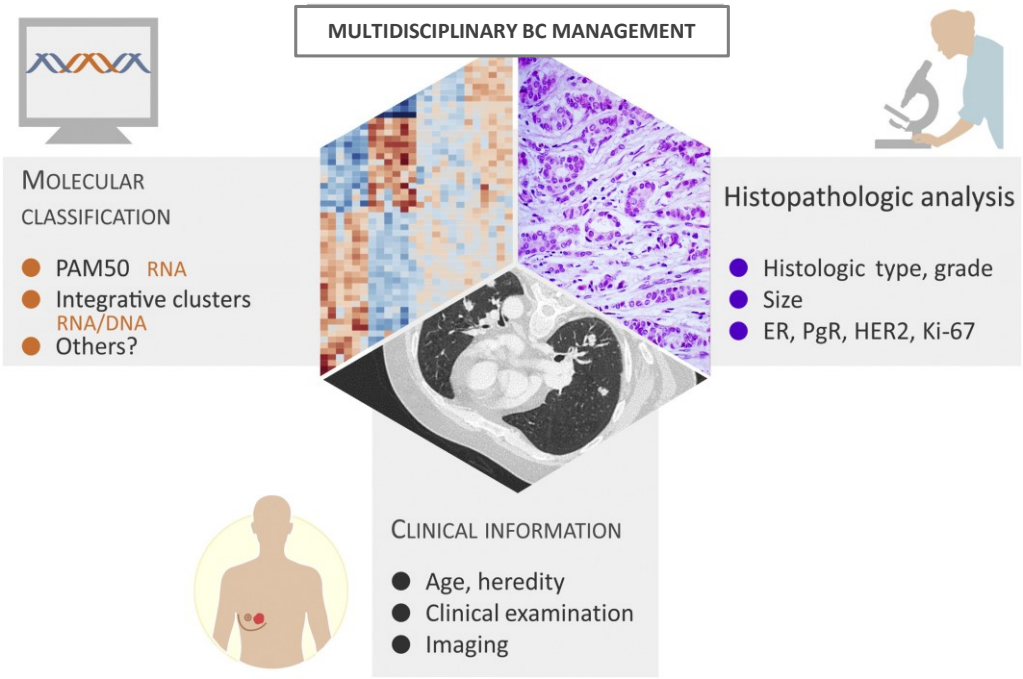

Figure 1.2. Representation of breast cancer management according to a multidisciplinary approach. Clinical information, histopathologic analysis, and molecular-based classification concur to provide an integrated patient stratification, necessary for a precise cancer management. (Adapted from: (Russnes 2017).

Furthermore, the development of high-throughput molecular screenings and the advancement in big data managing have enabled to enlarge the molecular scenario of BC, that is informative both for cancer development and treatment. For example, the individuation of novel tumor-associated genes via high next-generation sequencing have been helping to develop new drugs or to elucidate some of the molecular mechanisms underlying the resistance to therapy. This allows to predict and follow patients' response to therapies at different stages and to promptly change the therapeutic strategy if resistance occurs (Sachdev 2019) . Furthermore, thanks to this novel procedure, new molecules and new combinatorial therapies have been approved for breast cancer treatment, for example Abemaciclib (CDK4/6 inhibitor) for hormone-receptor-positive metastatic breast cancer (Dickler 2017), or trastuzumab emtansine combination for the treatment of metastatic HER2+ breast cancer (Verma 2012). Definitely, precision medicine represents a malleable strategy for cancer management, exhibiting the advantages to adapt to tumor evolution and to preserve patient individuality.

1.3 Tumor microenvironment: a novel cancer hallmark.

As defined by Hanahan in 2011, TME has been officially recognized as a cancer hallmark effectively participating in "sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis" (Hanahan and Weinberg 2011). TME comprises not only cancerous cells, but also not-transformed cells and other molecular components including extracellular matrix (ECM) elements. Fibroblasts, adipocytes, immune cells (including dendritic cells, M1- and M2- macrophages, natural killer, T-cells) inflammatory cells, endothelial cells represent the main cellular constituents distinctive of TME and are usually defined as "tumor stroma". The result is a complex interaction machinery that allows a specific communication between cancer cells and tumor stroma by the exchange of cytokines, growth factors, ECM proteins and other signal molecules (Mittal 2018) (**Figure1.3**).
This peculiar crosstalk can also mediate systematic effects during tumor development promoting as an instance the formation of long-distance metastasis. In breast cancer, metastatization process often occurs at bone level with a strong influence of the TME. Indeed, it is known that BC-secreted chemokines and cytokines recruit bone marrow-derived cells that are released into the blood circulation and mediate the formation of pre-metastatic niche even before cancer cell harnessing (Kakonen and Mundy 2003) (Place 2011). More interestingly, during metastasis formation, fibroblasts from the tumor stroma migrate together with cancer cells to recreate a permissive microenvironment for their growth and sustainment in that new colonized organ (Duda 2010) (Malanchi 2011) (Oskarsson 2011).
Given the importance of TME in breast cancer progression, several therapeutic strategies have been addressing towards different TME components with the scope to restore a "normal stroma" that restrains cancer cell expansion and/or promote immune-mediate antitumor effects (Criscitiello 2014). Unfortunately, these alternative combinatorial approaches are not full effective because lots of other molecular mechanisms remain unclear and need to be further elucidated.
Despite all these malignant features, the real concern is that mechanisms underlying TNBC initiation and evolution are still unknown, remaining a challenge for all physician and scientists.

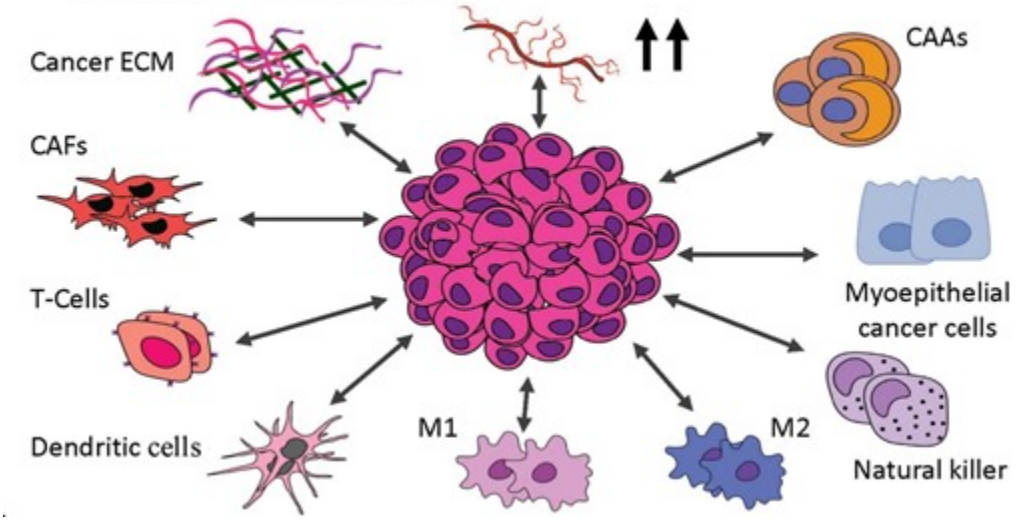

Figure 1.3. Graphic illustration of tumor microenvironment components.
Different cellular and non-cellular elements composing the TME. Double-pointed arrows indicate the reciprocal crosstalk between cancer cells (ate the centre) and TME constituents. (Adapted from: (Moccia and Haase 2021)).

1.3.1 A focus on cancer-associated fibroblasts

Cancer-associated fibroblasts (CAFs) are the most abundant cell type within the TME. Basically, they are activated not-transformed cells of the tumor stroma with the ability to exert pro-tumorigenic functions, sustaining cancer cell growth, invasion, metastasis, and inducing therapy resistance (Olumi 1999) (Paulsson and Micke 2014). Currently, many hypotheses for CAF origin exist. Generally, the majority of CAFs originates from local pre-existing fibroblasts; otherwise, CAFs can arise from the transformation of adipocytes, pericytes, endothelial cells and bone marrow- derived mesenchymal stem cells (Raz 2018) (Zhang 2012) (Dirat 2011).

Several scientific evidence demonstrated that CAF activation occurs through the contact between fibroblasts and epithelial cancer cells through inducing pro-inflammatory and proliferative signalling, and/or epigenetic switch (Strell 2019) (Erez 2010, Procopio 2015). Moreover, fibroblast transformation to CAFs can be promoted by changes in ECM rigidity and properties inducing a contractile phenotype in CAFs through the activation of specific transcriptional programs (i.e., Yes- associated protein 1 (YAP1)–TEAD). As a consequence, the improved contraction abilities together with the release of matricellular molecules by fibroblasts themselves can in turn provoke tissue stiffness by establishing a positive feedback loop that self-sustains CAFs within the TME (Calvo 2013) (Foster 2017). The ECM remodelling process mediated by CAFs can lead to a variety of pro-tumoral consequences. Predominantly, it could promote local and systemic invasion, pro-survival and pro-proliferative signaling together with angiogenic-related disfunctions and drug delivery impairment in cancer cells (Kaur 2019) (Diop-Frimpong 2011) (Chauhan 2013)

(Jain 2014). Importantly, the ECM reshaping orchestrated by CAFs is both force-based and enzyme-mediated, occurring through the activation of mechano-transduction signaling including integrin-mediated adhesion, and the secretion of matrix metalloproteinases (MMPs) that degrade different components of ECM. This allows the creation of easy-going tracks for local and systemic invasion of cancer cells (Gaggioli 2007, Hooper 2010, Goetz 2011) (Kechagia 2019) (Zeltz 2020). Among this wide range of pro-tumoral effect, increasing evidence demonstrated that CAFs can acquire peculiar phenotypes with specialized functions (**Figure1.3.1**) and gene expression depending on tumoral spatiotemporal factors (Ohlund 2017, Philippeos 2018) (Bartoschek 2018). However, it is still unknow whether these specific functions acquired by CAFs are interchangeable or stably driven by cancer cells and thus permanent.

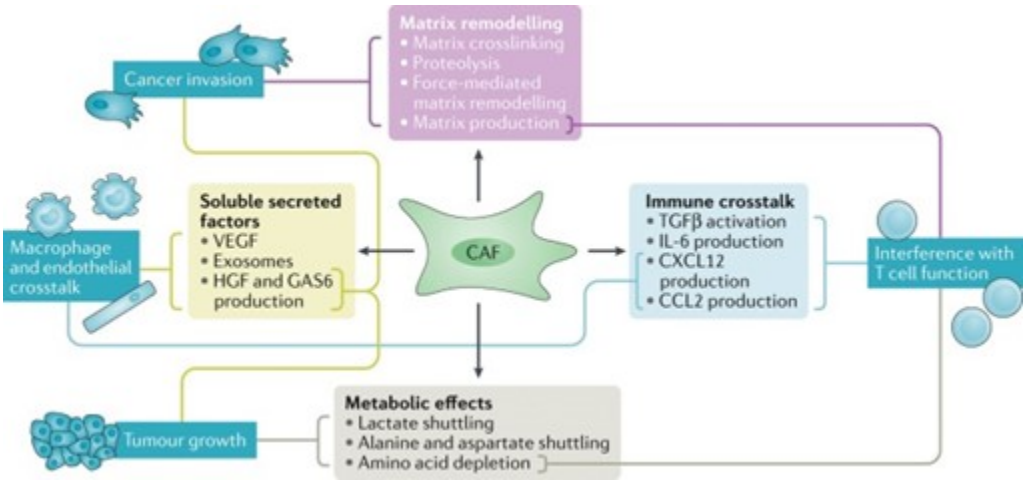

Figure 1.3.1 Schematic organization of functions exerted by cancer-associated fibroblasts within the tumor microenvironment. Blue text boxes indicate biological functions regulated by CAFs in the tumor context. Light blue, green, purple, and grey text boxes connected by lines show the mechanisms underlying the regulated functions. (From: (Sahai 2020).

In breast cancer, the majority of stromal fibroblasts are present in an active form and are associated to poor clinical outcome (Sappino 1988) (Hu 2018, Piersma 2020). It is also known that TNBC is particularly affected by the presence of heterogeneous CAF populations that contribute to its aggressiveness (Bianchini 2016) (Yu and Di 2017). Given the influence of CAFs in cancer evolution, several therapeutical approaches have been developed to interfere with CAF activation and functionality. Some examples of clinical trials involving CAFs are listed in **Table1**.

Table1. Current clinical trials targeting cancer-associated fibroblasts (Adapted from (Sahai 2020)

Drug target	Drug name	Drug type	Mechanism	Status
Interference with CAF activation and action				
FGFR	JNJ-42756493	Small- molecule inhibitor	Prevents CAF activation	Phase I and phase II trials under way [1]
Hedgehog	IPI-926 (saridegib), vismodegib	Small- molecule inhibitor	Reduces CAF activation	Clinical trials ongoing [2,3]
TGFβ	Various, including galunisertib	Blocking Abs, small-molecule inhibitor	Prevents CAF activation and immunosuppression	Phase I, phase II and phase III trials under way [4,5]
Angiotensin receptor	Losartan	Small- molecule inhibitor	Reduces collagen and hyaluronan levels	Phase II trial completed; randomized trial ongoing [6,7]
CXCR4	AMD3100	Small- molecule inhibitor	Prevents signalling from CAFs to immune cells	Clinical trials ongoing [8]
ROCK	AT13148	Small- molecule inhibitor	Reduces contractility	Phase I trial completed [9]
FAK	Defactinib (VS-6063, PF-04554878)	Small- molecule inhibitor	Reduces signalling downstream of integrins	Clinical trials ongoing [10]
LOXL2	Simtuzumab (GS 6624)	Blocking Ab	Anticrosslinking	Preclinical and fibrosis trials [11]
CTGF	FG-3019	Blocking Ab	Blocks binding to receptors	Early- phase clinical trials ongoing
Hyaluronic acid	PEGPH20 (PVHA)	Pegylated enzyme	ECM degradation, improvement in immuno- and cytotoxic therapies	Phase III trial complete, awaiting final analysis [12, 13]
FAP- expressing cells	Various, including PT630, RO6874281	Blocking Abs, molecular radiotherapy, inhibitors, Ab–IL-2 fusion	Blocks FAP^+ CAFs, promotes T cell function	Phase I and phase II trials under way [14]
CAF normalization				
Vitamin A metabolism	ATRA	Vitamin A metabolite	'Normalizes' stellate cells	Clinical trials ongoing [15,16]
Vitamin D receptor	Paricalcitol	Small- molecule agonist	'Normalizes' stellate cells	Clinical trial started [17]

Ab, antibody; ATRA, all- trans retinoic acid; CAF, cancer- associated fibroblast; CTGF, connective tissue growth factor; CXCR4, CXC- chemokine receptor 4; ECM, extracellular matrix; FAK, focal adhesion kinase; FAP, fibroblast activation protein; FGFR, fibroblast growth factor receptor; IL-2, interleukin-2; LOXL2, lysyl oxidase-like 2; ROCK, RHO kinase; TGFβ, transforming growth factor- β.

Nonetheless, CAFs still represent a challenge for their enormous heterogeneity. Furthermore, lack of precise markers univocally associated to CAFs slow the way for their total integration in the clinic. Indeed, the absence of epithelial and endothelial marker expression remains the main way to distinguish fibroblasts within the tissue. Further studies are needed to better characterize CAF subtypes with the scope to use them as prognostic and therapeutic tools.

1.4 MicroRNAs in cancer regulation

MicroRNAs (miRNAs or miRs) are a group of small non-coding functional RNAs of approximately 18–22 nucleotides in length widespread in plants and animals (Bartel 2004). MiRNAs are transcribed by RNA polymerase II/III from independent genes or intronic sequences as long primary transcripts (pri-

miRNAs). Their maturation occurs through sequential events, starting from the nuclear processing of the pri-miRNA into stem-loop precursors of approximately 70 nucleotides (pre-miRNAs), and the cytoplasmic processing of pre-miRNAs into mature miRNAs. Finally, mature miRNAs are incorporated into the miRNA induced silencing complex (miRISC) which allows miRNAs to target mRNAs through binding their 3' UTR and induce a rapid silencing of the mRNA transcripts via translation repression or RNA degradation (Lee 2003, Han 2004, Chendrimada 2005, Winter 2009) (**Figure1.4**). Given the prominent role in gene expression regulation, miRNAs are involved in multiple physiological and pathological events, including embryogenesis, developmental stages, cell differentiation, metabolism, organogenesis, and apoptosis (Ha and Kim 2014). Nonetheless, miRNAs are not required for the delineation of individual tissues, meaning that the total absence of a miRNA does not provoke organ miss-development. However, tissue-distinctive miRNAs are necessary for the maintenance of tissue homeostasis and differentiation state. Indeed, several miRNAs are dysregulated in various diseases and for this reason are still widely investigated (Svoronos 2016, Saliminejad 2019). In particular, miRNAs constitute key regulator messengers in cancer. As a matter of fact, miRNAs implicated in cancer regulation have been grouped in the class of oncomiRs and can act in two different manners. In most studies, "OncomiR" term often refers to miRNAs upregulated in cancer, with oncogenic potential that results in the downregulation of their target tumor suppressor genes. Instead, miRNAs with tumor suppression functions are usually downregulated in cancer, leading to the overexpression of their oncogene targets. Some oncomiRs may act as tumor suppressor or oncogenic miRNAs depending on the tumor scenario they live. Probably, this phenomenon occurs because an individual miRNA can regulate the expression of a multiplicity of target genes (Svoronos 2016, Saliminejad 2019). What is important is that microRNA dysregulation persist during all stages of tumor progression, influencing cancer cell proliferation, metastasis, angiogenesis, stem phenotype, and resistance to therapies. Thereby miRNA profiling has been employed for cancer diagnosis, prognosis, and drug response prediction in patients (Lee and Dutta 2009, Iorio and Croce 2012). Beside their role as cancer biomarkers, miRNAs have also been investigated as alternative and/or combinatorial therapeutic tools and targets (Rupaimoole and Slack 2017). Interestingly, MRX34 is the first-in-human phase 1 study of a microRNA-based cancer therapy. This clinical trial (NCT01829971) has been based on the administration of liposomal miR-34 mimic in adult patients with different solid tumors refractory to standard treatments (Hong 2020). Even though the clinical trial prematurely terminated, it constituted the first concrete application of miRNAs in the clinics and a solid proof-of-concept for miRNA-based cancer therapy.

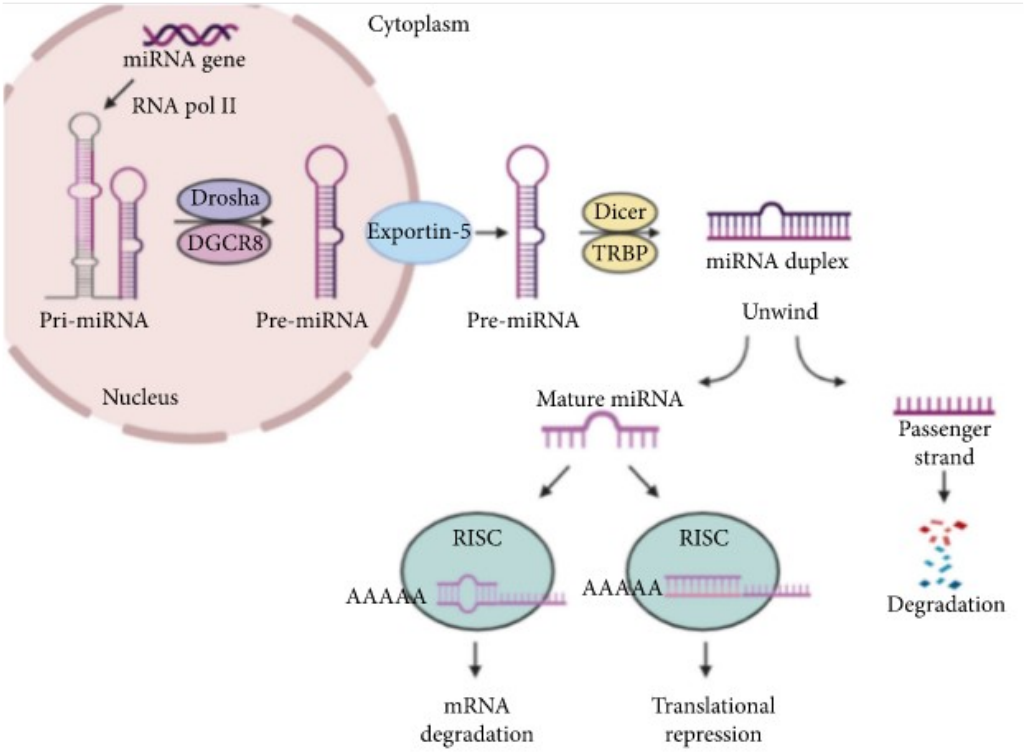

Figure 1.4. Representation of microRNA biogenesis pathway. After the maturation, functional strand of miRNA is uploaded onto the RNA-induced silencing complex (RISC) and negatively regulates gene expression through mRNA degradation or translational repression, while the passenger strand is degraded (Abd-Aziz 2020).

1.5 The emerging role of exosomes in cancer

Exosomes are nano-sized extracellular vesicles originated by the endocytic pathway through the inward budding of the plasma membrane followed by multivesicular body formation. Thanks to their origin, they represent the "mirror of the cells" since can hold the same molecular content, except organelles. Exosome main function is to mediate inter-cellular communication through conveying their cargo, that is composed of RNA, DNA, proteins, lipids, and other molecules (Valadi 2007, Colombo 2014) (**Figure1.5**). Given the importance of inter-cellular crosstalk, they are involved in a plethora of physiological and pathological processes. Initially, exosomes were thought to be garbage disposals employed by the cells for the elimination of misfolded proteins and other molecular wastes. Subsequently, several studies associated exosomes to further important physiological functions, including cell-to-cell communication, immune response modulation, tissue homeostasis regulation, stem cell maintenance and tissue repair (Simons and Raposo 2009) (Lai 2011) (Cossetti 2012). Potentially, exosomes can be released by all types of cells and found in several body fluids, including serum, plasma, saliva, liquor, amniotic liquid, breast milk, and seminal fluid (Keller 2006). Beside the physiological

role, exosomes are key players also in different pathologies. They are involved in infections, neurodegenerative diseases, and cancer since they are able to deliver any kind of molecular messages, spreading pathogens and activating pro-tumorigenic pathways (Iraci 2016). Interestingly, cancer cells release a higher number of exosomes compared to normal ones due to favorable microenvironmental conditions (Riches 2014). Indeed, well-known tumor-associated aspects, such as hypoxia and acidic pH promote the efficiency of exosome up-take and release (Parolini 2009). Not only the number, but also the molecular cargo of exosomes is different in cancer context compared to healthy conditions. Indeed, exosome molecular content directly participate to different stages of tumor formation and evolution, by interfering with immune system, supporting cancer cell proliferation, metastasis spreading, and drug resistance (Liu 2021). Moreover, with the same mechanisms, exosomes mediate the transformation of tumor microenvironment, already described to be fundamental for tumor progression. In TNBC, it is widely demonstrated that exosomes mediate cancer invasion and metastasis through the delivery of signaling molecules from cancer cells to TME and vice versa (Galindo-Hernandez 2014) (Campos 2018) (Kia 2019) (Singh 2014) (Li 2017) (Santos 2018) (Baroni 2016) (Luga 2012) (Wang 2020).
Considering the relevance of exosomes and their molecular cargo during the development and treatment of cancer, it made sense to use them in diagnosis, patient monitoring and therapy. Indeed, exosomes show high stability in serum and other biological fluids, making them better candidates compared to "classical" biomarkers. In particular, exosomal microRNAs have shown a high diagnostic value in different types of cancer (Su 2019) (Jiao 2017, He 2018).

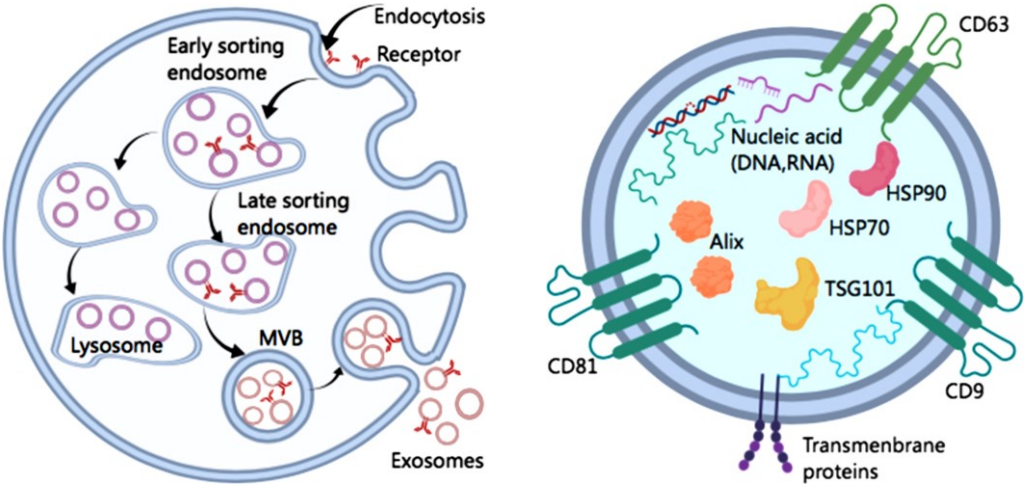

Figure1.5. Graphic representation of exosome biogenesis and cargo.
On the left, schematization of exosomes biogenesis, occurring in the endocytic pathway. At the final step of maturation, exosomes are released from multivesicular bodies in the extracellular space. On the right, a representation of exosome structure and molecular content that comprise structural and functional proteins, different kinds of nucleic acids

(DNA, miRNAs, lncRNAs, circRNAs) that collectively play important role in tumor development and progression. ((Dai 2020))

1.5.1 Exosomal microRNAs in cancer communication

As previously described, intercellular communication at long and short distances is fundamental in cancer progression and abundantly mediated by exosomes. Cancer cells take advantages of exosomes to modify the surrounding environment for their self-sustainment through carrying molecular messages. Furthermore, it is known that the RNA content of exosomes is mainly constituted by miRNAs (Wu and Shen 2020) that can be passively or actively sorted into them. In the first case, miRNAs are just released into exosomes in a way that is directly proportional to their intracellular concentration. However, miRNAs can be actively loaded into exosomes in an ATP-dependent manner, independently from their intracellular levels, probably for specific cellular requirements. Different mechanisms have been described for the active sorting, including neutral sphingomyelinase 2 (nSMase2)-dependent pathway (Mittelbrunn 2011) (Kosaka 2010), or sumoylated chaperone protein hnRNPA2B1. The latter works by binding to specific nucleotide motifs contained in miRNA sequences named "EXOmotifs" that allow them to be loaded into exosomes (Villarroya-Beltri 2013).
In mammary gland, non-malignant cells tend to retain miRNAs in the intracellular space, whereas cancer cells seem to selectively load miRNAs into exosomes for a specific extracellular communication (Pigati 2010), that can intervene in the transformation of TME components (short-distance effect) and in the formation of pre-metastatic niches (long-distance effect). Indeed, numerous miRNAs from BC cells can be transferred through exosomes to the surrounding stroma for their own advantage. As an instance, BC cell-derived exosomal miR-210 can activate endothelial cells to promote angiogenesis and metastatic initiation through targeting Ephrin-A3 protein (Kosaka 2013). Furthermore, miR-1246 delivered through exosomes from BC cells to non-tumorigenic epithelial breast cells foster proliferation, invasion, and drug resistance thus regulating different aspects of BC progression (Li 2017). In other cases, cancer cells can communicate each other's through the release of specific exosomal miRNAs to amplify tamoxifen resistance (Wei 2014). However, it is equally proven that TME component themselves promote and sustain cancer progression once recruited and activated by the tumor. As an instance, Donnarumma (2017) demonstrated that exosomes derived from breast CAFs increased breast cancer cell aggressiveness through the horizontal transfer of microRNAs that promoted stem phenotype, epithelial-mesenchymal transition (EMT), and anchorage-independent cancer cell growth (Donnarumma 2017). Furthermore, exosomal miRNAs mediate extracellular communication also at long distance, with the participation in pre-metastatic niche formation, that represent the preliminary step for the colonization of a new organ during metastatization process. Indeed, exosomes from cancer cells can enter the blood

stream through specific molecular signals and reach the target organ recreating a permissive microenvironment that facilitates cancer cell engraftment. Interestingly, Yuan (2021) demonstrated that high metastatic breast cancer cells transfer exosomal miR-21 to osteoclasts to promote pre-metastatic niche that serve for the facilitation of bone metastasis (Yuan 2021).
Given the relevance of exosomal miRNAs to cancer regulation, their use as diagnostic and prognostic biomarkers, along with targets for molecular therapy, may be consistent. Indeed, exosomal miRNAs present higher stability in biological fluids than miRNAs alone, thanks to the double lipidic layer that protect against RNase degrading activity (Nedaeinia 2017). Consequently, they could demonstrate high applicability as non-invasive biomarkers to be measured regularly for early cancer identification or patient follow-up.

1.6 *In vitro* and *in vivo* models for studying breast cancer

The translational research has done enormous progresses in the study of BC mechanisms and in the discovery of innovative therapies, thanks to the development of *in vitro* and *in vivo* models that resembled the human BC disease. The predominant BC *in vitro* model has been represented by primary or immortalized cell line cultures, with the advantages to be easily manageable and cryoconserved in biobanks, but moderately genome editable (Holliday and Speirs 2011). However, they do not represent the organ physiology and the tumor heterogeneity and could not be optimal for large-scale drug screening. Regarding *in vivo* models, patient-derived xenografts (PDXs) are the most employed animal models in the preclinical research (Pompili 2016). They are generated by inoculating human tumor specimens in immunodeficient mice to enable tumor engraftment and propagation in a living organism. Given to this, they highly depict the physiological conditions, with the presence of vascularization and organ interaction. Because the tumor mass is allowed to grow and develop in a murine organism, some environmental component of tumor tissues, such as the stroma, could be replaced by those of mice and for these reasons they not-fully reflect the genetic and the heterogeneity of the primary human tumor. Furthermore, as *in vivo* models, they and are not easily handling for high-throughput drug screening and the genome editing manipulation could be difficult for the complexity of tissue environment (Yang 2020) (Ben-David 2017).
In recent years, patient-derived tumor organoids (PDTOs) have been representing the turning point for the study of human cancers. The growing knowledges in stem cell biology and the need of a real and accurate models of human tumor disease have opened the way for the development of *in vitro* three-dimensional models directly derived from human tissues. In fact, mammary PDTOs are constituted by epithelial stem cells embedded in basement membrane-rich matrix, recreating the three-dimensional ECM, where can self-organize in hormone-sensitive gland structures, thus conserving cellular

polarization and architecture (Dontu 2003, Lee 2007) (Parry 1987). Thus, PDTOs recreate semi-physiologic conditions, reflecting the primary tumor heterogeneity that allows the study of cell-cell communication and morphogenesis. Furthermore, PDTOs contemplate the whole BC spectrum and can be employed for drug resistance testing. Indeed, BC organoids were sensitive to HER2-targeted therapies only when HER2 receptor was overexpressed. Similarly, organoids with a high BRCA1/2 signature were sensitive to PARP inhibitors, while organoids from low BRCA1/2 signature tumors were not sensitive (Walsh 2014) (Sachs 2018). Furthermore, they can be employed in individualized treatments and facilitate the high throughput drug screenings.

Definitely, PDTOs represent promising manageable tools for the study of breast cancer. However, their relatively recent development did not allow to establish a standard protocol for their propagation and stabilization over time, therefore, the results obtained in different laboratories are not yet perfectly comparable.

2. AIM OF THE STUDY

Triple negative breast cancer (TNBC) remains the most aggressive and heterogeneous subtype of breast cancer, even characterized by high recurrence and metastatic potential. However, the molecular mechanisms at the basis of TNBC development are still incompletely known, reflecting the lack of efficacious targeted therapies for patient treatment. Nonetheless, the interaction between cancer cells and tumor microenvironment (TME) has been increasingly associated to TNBC aggressiveness and heterogeneity. In this scenario, exosomes have held key role since they mediate the extracellular communication potentially among all types of cells by delivering their molecular content at short and long distances. Moreover, exosomal miRNA cargo participates in the crosstalk between cancer cells and the TME, that sustains tumor progression.
To shed light on mechanisms responsible of TNBC evolution, the aim of this study was to evaluate the relevance of TNBC cell- derived exosomes and the miRNA cargo they hold in the activation of stromal fibroblasts to cancer-associated fibroblasts (CAFs), that represent the most abundant cell population of the TME.
To this purpose, we investigated:

- The effect of exosomes derived from TNBC cells on stromal fibroblast activation, by analyzing functional and molecular aspects of fibroblast conversion to CAF.
- The role of TNBC cell-derived exosomal miRNA cargo in fibroblast transformation, by studying the differential expression of miRNAs in stromal fibroblasts after TNBC-exosome exposure.
- The specific impact of overexpressed exosomal miRNAs in mediating fibroblast activation, by exploring different functions acquired by transformed fibroblasts.

We further aimed to examine the consequence of fibroblast conversion to CAF on breast cell behaviour, by evaluating:

- The strengthen of tumorigenic functions of breast cancer cells, through analyzing the proliferation of luminal A-subtype breast cancer cell and the invasion potential of patient-derived breast cancer organoids after the influence of activated fibroblasts.
- The fostering of pro-tumorigenic effects on non-tumorigenic epithelial breast cells, by assessing the proliferation and migration abilities impacted by activated fibroblasts.

3. MATERIALS AND METHODS

3.1 Primary and continuous cells cultures.

Primary cultures of fibroblasts (NFs) were obtained from patients undergoing breast reduction surgery from Clinica Mediterranea (Naples, Italy). Patients' informed consent was obtained before samples collection. Briefly, human breast specimens were cut by mechanical fragmentation with sterile blades and tongs. Extracellular matrix was digested with collagenase (Sigma-Aldrich) for 2h under continuous agitation (200 rpm) at 37°C. Then, the cellular suspension was differentially centrifuged to separate epithelial cells from fibroblasts population (200 rpm for 2 minutes to obtain a pellet of epithelial cells and 1300 rpm for 5 minutes to obtain fibroblast population). Fibroblasts were grown in Dulbecco's Modified Eagle's Medium/Nutrient F12-Ham (DMEMF12 – Sigma Aldrich, Cod. D8437, lot. #RNBG9065) supplemented with 10% heat-inactivated foetal bovine serum (FBS, Sigma-Aldrich, Cod. F7524, Lot. #BCBW0228), 1% penicillin/streptomycin (A/A, Gibco, Cod. 15240-062, Lot. #2321085), 1% amphotericin B (Gibco, NY-USA, Cod. 15290-026, Lot #2244434) at 37°C with 5% CO2. Breast cancer continuous cell line MDA-MB-231(ATCC) was grown in RPMI-1640 medium (Sigma-Aldrich, Cod. R8758, Lot. #RNBF0094) supplemented with 10% heat inactivated FBS, 1% A/A. Normal Breast Epithelial cell line MCF-10A (ATCC) was cultured in DMEM-F12 supplemented with 5% heat-inactivated foetal horse serum, 1% of A/A, 1% amphotericin B and with all hormones and factors needed for their growth: EGF (1µg/µl), hydrocortisone (1µg/µl), cholera toxin (100µg/µl), insulin (20µg/µl). All media and supplements were from Sigma-Aldrich (Milan, Italy).

3.2 Exosome Isolation

Exosomes were isolated from cell culture media of MDA-MB-231 cells. In detail, $4x10^6$ cells were pleated in 150mmx25mm cell culture dishes (Corning, #430599) with their usual growth medium (described in the previous section) to allow plate attachment. After 24h, cells were washed twice with PBS (Dulbecco's Phosphate Buffered Saline, Sigma-Aldrich, Cod. D8537, Lot. #RNBH3372) and 12 ml of RPMI medium (Sigma-Aldrich) supplemented with 10% FBS depleted of exosomes (Exo-FBS, SBI, System Biosciences, Cod. EXO-FBS-250A-1, Lot. #161004-002), 1% of A/A and 1% amphotericin B was added. After 48h, culture media were collected and centrifuged at 3000 g for 15 minutes RT to remove cellular debris. The supernatants were transferred into new sterile tubes and the appropriate volume of the ExoQuick-TC™ Exosome Isolation Reagent (SBI, System Biosciences, Cod. EXOTC50-A) was added according to manufacturers' instructions. Then, tubes were gently mixed until the separation between the two phases was no longer visible. Tubes were kept standing at 4°C O/N. The following day, tubes were centrifuged first at 1500 g for 30 minutes, and then at 1500 g for 5 minutes to ensure the complete removal of the ExoQuick-TC™ solution. Lastly, exosome pellets were resuspended in 300 µl of PBS solution.

3.3 Nanoparticle tracking analysis (NTA)

Exosome size and particle number were analyzed using the NS300 nanoparticle characterization system (NanoSight, Alfatest – Rome, Italy) equipped with a blue laser (405 nm). In brief, 40 µl of exosome isolation (see "Exosome Isolation" section) was diluted with PBS to a final volume of 400µl and loaded into the instrument. For the measurement, the instrument software (NTA 3.1 Build 3.1.54) was used under the following settings. Capture settings: Camera type: sCMOS; Camera level: 15; Slider shutter: 1206; Slider gain: 366; FPS: 25.0; Number of frames: 1498; Temperature: 24.6-24.7°C; Viscosity (water): 0.895-0.897 cP; Dilution factor: 2 x 10e2; Syringe pump speed: 20. Analysis settings: Detect threshold: 5; Blur size: auto; Max jump distance: auto (12.3-12.9 pix). Sample measurement was performed in triplicate.

3.4 Transmission electron microscopy (TEM)

TEM imaging was carried out at the Department of Radiology of Leiden University Medical Center (Leiden, The Netherlands). Carbon-coated grids (Formvar/Carbon on 200 Mesh Copper; AGS162; Van Loenen Instruments; Zaandam, the Netherlands) were glow-discharged for 1 minute at 2x10-1 mbar and 20 mA using the Emitech K950X Turbo Evaporator (Quorum Technologies; Ashford, UK). Afterwards, 3 µl of sample solution was transferred to the glow-discharged grid and left for 1 min to adhere. Excess liquid was blotted onto filter paper, and 3 µl of 2% uranyl acetate in distilled water was applied to the grid for negative staining. Excess uranyl acetate was removed by blotting after 1 min, and the sample was air-dried for 10 min. Grids were placed on a room temperature holder and observe at a voltage of 120 kV with a Tecnai 12 Twin (FEI Company; Oregon, USA) fitted with a OneView Camera Model 1095 (Gatan; Pleasanton, USA). DigitalMicrograph 3.4 was used to capture and save digital images (Gatan).

3.5 Exosome labelling and immunofluorescence assay

Exosomes isolated from MDA-MB-231cells (see "Exosome isolation and characterization" section) were labelled with the red fluorescent cell membrane linker PKH26 (Sigma-Aldrich, #SLBT6344). Briefly, exosomes (40 µg) were stained with PKH26 (0,33 µl) for 5 minutes in dark at RT in a final reaction volume of 2ml. Then, the same amount of 1% BSA (2 ml) was added to stop the labelling reaction. Finally, samples were ultracentrifuged (Beckman coulter, Optima MAX) twice at 100,000 g for 70 minutes at 4°C, and pellets were resuspended in 500µl of PBS. For the immunofluorescence assay, 5x 105 NFs were plated on glass coverslips in a 24 multiwell plate. The following day, NFs were treated with PKH26-labelled exosomes for 12h, then washed three times with PBS and finally fixed with Methanol/Acetone 1:1 for 10 minutes at -20°. After 3 washes in PBS, cells were blocked in 1% PBS-BSA with 0.3% Triton X-100 (SIGMA-ALDRICH, cat. # 9002-93-1) solution at RT for 15 minutes. Subsequently, cells were stained with anti- β-Actin primary antibody (1:1000) diluted in blocking solution for 1 hour at RT for cytoskeleton detection. After 3 washes in PBS, the secondary antibody Goat Anti-Mouse IgG-FITC (Santa Cruz Biotechnology, #F0211-1:300 in PBS) was added for 30 minutes at RT. Lastly, cells were incubated with DAPI (BD Pharmingen™, Cat.564907 1:1000 in PBS) for 10 minutes at RT in dark for nuclei visualization. Coverslips were washed and mounted with 2µl of 1:1 Glycerol (Sigma-Aldrich, #114K0183V) in PBS on a microscope slide and images from confocal microscopy (Leica LSM700) were taken and analyzed to check exosome uptake.

3.6 Cell treatment with exosomes

Exosomes isolated from MDA-MB-231 (see “exosome isolation” section) were quantized using Bradford reagent (Protein assay dye-Bio- Rad, Cod. 5000006, Lot. # 64254929) and a total amount of 40ug was used for NF treatments. Briefly, NFs (2,5x105) were seeded in p60mm dishes in 10% FBS-DMEM-F12 as long as they get attached, then washed twice with PBS solution and kept in DMEM-F12 medium supplemented with 10% Exo-FBS for the exosome treatment. Lastly, NFs were collected after 24h and/or 48h for downstream analysis.

3.7 Protein isolation and Western Blotting

Cells were washed twice in ice-cold PBS and exosomes previously isolated were lysed in JS buffer (50 mM HEPES pH 7.5 containing 150mM NaCl, 1% Glycerol, 1% Triton X100, 1.5mM MgCl2, 5mM EGTA, 1 mM Na3VO4, and 1X protease inhibitor cocktail). Protein concentration was determined by the Bradford reagent (Protein assay dye-BioRad, Cod. 5000006, Lot. # 64254929) using bovine serum albumin as the standard, and equal amounts of proteins were analyzed by SDS-PAGE (12.5% acrylamide Bio-Rad, Cod. 1610158, Lot. #64269544). First, gels were electroblotted into nitrocellulose membranes (GE Healthcare Life Science, cat. #10600002); then, membranes were blocked for 1h with 5% blotting-grade blocker (Bio-Rad, #1706404) in Tris Buffered Saline (TBS- Bio-Rad, #1706435) containing 0.1% Tween-20 (SIGMA-ALDRICH, Co., #P1379-1L), and finally incubated at 4°Cover night with the primary antibodies. Signal detection was performed by peroxidase-conjugated secondary antibodies using the enhanced chemiluminescence system (Thermofisher, Milan Italy). Primary antibodies used were: anti-FAP (Abcam, UK, ab53066- 1:1000 in 5% TBS-milk), anti-Caveolin-1 (Santa Cruz Biotechnologies, MA, USA,sc-53564 -1:500 in 5% TBS-BSA), anti-MCT4 (Santa Cruz Biotechnologies, MA, USA, sc-376140-1:500 in 5% TBS-BSA), anti-β-ACTIN (Signa, USA, A5441-1:15000 in 5% TBS-milk), anti-phospho-FAK(Y576/577) (Cell signaling technology, #3281- 1:500 in 5% TBS-BSA), anti-FAK(Cell signaling technology, #71433-1:1000 in 5% TBS-milk), anti-CD63 (Santa Cruz Biotechnologies, MA, USA,sc-15363-1:500 in TBS-milk), anti-TAPA1(Abcam, UK, ab35026- 1:1000 in 5% TBS-milk), anti-CD9 (Abcam, UK, ab92726-1:1000 in 5% TBS-milk), anti-Tsg101(Abcam, UK,ab83- 1:1000 in 5% TBS-milk), anti-Calnexin (Abcam, UK, ab10286-1:1000 in 5% TBS-milk), anti MMP-1 (Santa Cruz Biotechnologies, MA, USA, sc-21731, 1:500 in 5% TBS-milk), MMP-2 (Santa Cruz Biotechnologies, MA, USA, sc-21731, 1:250 in 5% TBS-milk), MMP-3 (Santa Cruz Biotechnologies, MA, USA, sc-21731, 1:500 in 5% TBS-milk), anti-Integrin α5 (Santa Cruz Biotechnologies, MA, USA, sc-13547, 1:500 in 5% TBS-milk), and anti-Integrin β1 (Santa Cruz Biotechnologies, MA, USA, sc-13547, 1:1000 in 5% TBS-milk).

3.8 RNA extraction and Real Time PCR

Total RNA (miRNAs and mRNA) was extracted using TRIZOL reagent (Life technologies, #15596018, Milan, Italy). Reverse transcription was performed starting from equal volume of total RNA/sample (150-300 ng) using miScript reverse Transcription Kit (QIAGEN, cat #218161) for total miRNAs, and SuperScript® III First-Strand (Invitrogen, cat. #18080051) for mRNAs. Quantitative analysis of miR-185-5p, miR-652-5p, miR-1246 e and RNU6A (as an internal reference) was performed by Real Time PCR using miScript SYBR Green PCR Kit (QIAGEN, cat. #218075) and miScript Primer Assays (QIAGEN, cat. #3406126). The reaction for detection of miRs was performed as follows: 95°C for 15, 40 cycles of 94°C for 15'', 55°C for 30'', and 70°C for 30''. For the mRNA amplification of FAP, Caveolin-1, SLC16A3, SLC2A1, and β-Actin as internal normalizer gene we performed a Real Time PCR with iTaq™ Universal SYBR® Green Supermix (Bio-Rad, cat. #1725124) and custom-made primers for mRNAs (IDT, Milan, Italy) and. The reaction for the detection of mRNAs was performed as follows: 95°C for 15', 40 cycles of 94°C for 15'', 58°C for 30'', and 72°C for 30'. All reactions were run in triplicate.

3.9 3D Organotypic coculture assay

NFs were starved in DMEM F12 media without FBS for 24 h and then seeded in 35mmx 10mm cell culture dishes (Corning Incorporated, #430165) in a neutralized matrix made of type- 1 collagen either treated with MDA-MB-231-derived exosomes (or PBS as control) to ensure their activation (the same procedure described in "Contraction assay" section). Additionally, 1 x 10^5 MCF10A cells were seeded on the top of the collagen plug for 48h. Then, plugs were transferred to an invasion grid (Screens for CD-1 size 40 mesh, SIGMA-ALDRICH) in a 60 mm plate and complete growth medium was added underneath in order to create an air/liquid interface to trigger epithelial cell invasion. After 14 days, matrices were fixed, paraffin embedded and cut into 10 μm sections. Organotypic matrices were stained with anti-pan cytokeratin (Santa Cruz Biotechnologies, MA, USA, sc-8018- 1:400 in blocking solution) O.N. at 4°C and then with the secondary antibody ALEXA594-conjugated goat anti-mouse (Abcam, ab150116- 1:400 in blocking solution) for 1h at RT in dark. Images were taken either with an inverted microscope and with a fluorescent one. Number of Pan-cytokeratin positive cells was counted by ImageJ software in different fields of the images to quantify the number of invading cells.

3.10 RNA Sequencing

NFs (5x105) were pleated in 100mm dishes with DMEM-F12 culture media supplemented with 10% Exo-FBS (SBI, System Biosciences) and 1% A/A and

treated with 120 µg of MDA-MB-231 derived exosomes and the same volume of PBS as control. After 24 hours, cells were collected and RNA was extracted using TRIZOL reagent (Life technologies, #15596018, Milan, Italy). Samples were sent to Genomix4Life S.r.l (Baronissi, Salerno, Italy) that performed Small-RNA-sequencing by using Illumina HiSeq2500 (SmallRNA 1X20M Cod. G4L1630 – iMir, Cod. G4L15055) and the bioinformatics analysis (PCA component and differentially expression analysis). Two biological replicates for each experimental point were analyzed. For the statistical analysis p. value < 0.05 alone was considered for the experimental significance; no p value adjustment was performed because of the small sample size analyzed (NFs pt.#1).

3.11 Fibroblast transfection

NFs (2,5 x10^5) were seeded in 60mm x 15mm cell culture dishes (Corning Incorporated, #353004) and the combination of pre-miR miRNA precursor-185-5p, pre-miR-652-5p, and pre-miR-1246 (combo miRs), as well as pre-mir-Scramble (Ambion, Life technologies Milan Italy) were transiently transfected at the final concentration of 150µM for each transfection point by using Oligofectamine™ Reagent (Invitrogen, Thermo Fisher Scientific – Milan, Italy- Cod. 12252-011, Lot. #2030861) in a reduced serum condition (Opti-MEM, Gibco, Cod. 31985-047, Lot. #2091581). After 4h, cell medium was supplemented with a final concentration of 10%FBS to restore optimal cell growth conditions. Cells were collected at 24h, 48h, and/or 72h of transfection for downstream analysis or applications.

3.12 Collagen contraction assay

Collagen contraction assay was performed with NFs in 35mmx 10mm cell culture dishes (Corning Incorporated, #430165) for exosome treatment and in 12-well plates (Corning Incorporated, #3513) for combo miRs transfection. Type-1 collagen (Corning, #354236-33ng/µl) was resuspended in acidic environment composed of Acetic Acid (5mM) and Minimum Essential Medium Eagle (Sigma, MO275, 10X); then NaOH (1M) was added drop by drop to restore the neutral pH for cell resuspension. For contraction assay performed with combo miRs, NFs were previously transfected with combo miRs (scrambled for control) for 48h and then used for contraction assay; whereas for exosomes, NFs were first pleated in the collagen plug and then treated with exosomes. In both cases, 1,5x105 NFs resuspended in 250µl of FBS were added to type-1 collagen mix prepared before. All steps during collagen handling must be performed on ice to avoid early collagen solidification. After that, plates containing collagen plugs and cells were taken at 37°C with 5%CO2 for 3h to allow collagen solidification. Subsequently, collagen plugs were detached from plate walls to allow cell contraction and DMEM-F12 FBS- free medium (with or without exosomes, depending on the experiment) was added. Images of entire

collagen plugs were taken after 24h with the camera tool of a mobile phone held in a fixed position. Then, plug areas were calculated with ImageJ software and analyzed to check NFs contraction ability after exosomes treatment or combo miRs transfection**.**

3.13 Transwell - Migration assay.

Migration assay was carried out with 8.0 μm polycarbonate membrane permeable 6.5 mm Transwell® inserts (Corning Incorporated, NY, USA – Cod. 3422, Lot. #11619021). NFs pretreated with exosomes (40 μg) or transfected with miRNAs (as described in "Cell transfection and exosome treatment" section) were harvested with a trypsin-EDTA solution (Sigma Aldrich, USA – Cod. T4D49 – Lot. #SLCH3365) and counted with Neubauer's chamber. Then, 1,0 × 105 cells were washed with PBS to remove any FBS residues, resuspended in DMEM-F12 FBS free medium, and seeded in the upper part of the Transwell® chamber. The lower part of the chamber was filled with 600 μL of DMEM-F12 medium supplemented with 10% FBS, 1% of A/A and 1% amphotericin B to create the chemical gradient needed for cell migration. Cells were incubated at 37 °C with 5% CO2 for 24 h. Subsequently, the Transwell® chambers were stained and fixed with 0.1% Crystal Violet in 25% methanol for 20 minutes at RT in dark. The reaction was stopped with water and non-migrated cells were scraped off the top of the chamber with a cotton swab. Representative images were taken with the contrast phase microscopy (Leica DMI3000 B). The percentage of migrated cells was calculated by eluting Crystal Violet with 600 μl of 1% SDS for each well and measuring the respective absorbance at 490 nm with MULTISKAN FC plate reader (Thermo Scientific).

3.13 Invasion assay

In vitro invasion assay was performed in 24-well Corning Multiwell, with 8.0 μm polycarbonate membrane permeable 6.5 mm Transwell inserts (Corning Incorporated, NY, USA – Cod. 3422, Lot. #11619021). NFs (1.2 x 105). NFs previously transfected with combo miRs for 24h were resuspended in a mix containing Matrigel Matrix Basement Membrane (Corning, NY – USA, Cod. 354230, Lot. #6207017) diluted 1:5 in DMEM-F12 FBS free. The lower part of the chamber was filled with 600μl of DMEM-F12 medium supplemented with 10% FBS, % antibiotic-antimycotics and 1% of amphotericin B to create the chemical gradient for cell movement. Cells were incubated at 37°C with 5% CO2 for 72h. The Transwell supports were stained and fixed with 0.1% Crystal Violet in 25% methanol for 20 minutes at RT in dark. The reaction was stopped with water, and non-migrated cells together with residual Matrigel solution were scraped off the top of the Transwell with a cotton swab. Representative images were taken with a contrast phase microscopy (Leica DMI3000 B). The percentage of migrated cells was evaluated by eluting Crystal Violet with 600μl of 1% SDS for each well and measuring the respective absorbance at 490 nm with MULTISKAN FC plate reader (Thermo Scientific).

3.14 Scratch assay

NFs ($5x10^4$) were seeded in a 12-well plate (Corning Incorporated, #3513) and the following day transfected with miR 185-5p, 652-5p, 1246 or with scrambled sequence as control. After 48h, cells were starved for 3h in DMEM F12 FBS-free culture media. Next, a scratched wound was scraped with a 200-μL tip in each well and then cells were continuously grown in DMEM-F12 culture media complemented with 10% FBS and 1% A/A for 24h. Microscopy images were taken in different fields of the wound at the scratch moment (t0) and after 24h (t24) using a 5x objective of an inverted microscope (Leica DMI3000 B, Leica, Milan, Italy). Scratch area was calculated with ImageJ software and analyzed to measure the wound healing ability of NFs after combo miRs transfection.

3.15 *In vitro* cell proliferation assay

Cell proliferation was evaluated with the CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS assay, Promega, Milan, Italy. REF: G3581), according to the manufacturer's protocol. After 1h of incubation, the plates were analyzed on a Multilabel Counter (Bio-Rad, Milan, Italy) to measure the absorbance values used for the analysis.

3.16 Primary breast cancer organoid culture

Breast cancer organoids were obtained by patients' biopsies. Briefly, biopsies were mechanically disrupted and then digested in 10 mL of AdDF (Advanced DMEM/F12, 1× Glutamax, 10 mM HEPES, and 1% antibiotics/antimycotic) containing 2 mg/mL−1 collagenase (Sigma, C9407) on a shaker (200rpm) at 37 °C for 3 h. Then, the digested suspension was shared using plastic with a small diameter and finally a glass Pasteur pipette in a sequential manner. After that, the suspension was centrifuged at 400rcf. If erythrocytes were present, the pellet was suspended in a red blood cell lysis buffer (Roche 11814389001) for 5 min at 37°C. The pellet was washed in PBS and centrifuged again at 400 rcf. Finally, the pellet was resuspended in cold Cultex growth factor reduced BME type 2, and 40 µL drops (= 1 dome) were allowed to solidify in Not Treated Multiple Well Plates (Corning ®Costar®, CLS3738) for 20 min at 37 °C. Breast cancer organoids were then covered with 400 µL of organoid medium as described by the Hans Clevers protocol.

3.17 Immunofluorescence assay of breast cancer organoids.

Two domes of breast cancer organoids (see: "Primary organoids cultures." Section) with at least 60 % confluence were resuspended in 20 µl Cultex growth factor reduced BME type 2 over a coverslip glass plated in Not Treated Multiple Well Plates (Corning ®Costar®, CLS3738). The day after, culture medium was removed, and organoids were fixed and permeabilized in Acetone-Methanol (1:2) for 1 h at R.T. Then, organoids were washed in a washing solution (1% FBS,0,1% Triton in PBS) for 2 h at R.T., by replacing the solution every 30 min. Then, blocking solution (10% FBS,0,1% Triton in PBS) was added and let 1h at R.T. Primary antibodies were incubated overnight at 4°C and diluted in washing solution as follows: Anti-Cytokeratin14 (Santa Cruz Biotechnologies, MA, USA, sc-53253 lot.# B2217, 1:50), Anti-Cytokeratin8 (Santa Cruz Biotechnologies, MA, USA, sc-130312 lot# C1009, 1:50), Anti- HCAM (Santa Cruz Biotechnologies, MA, USA, 1:200, sc-7297, lot#D1619), Anti-β-Catenin (Cell signaling, cat. 95825, 1:50). Subsequently, washed solution was added for 2h and replaced every 30 min. Secondary antibodies conjugated with fluorophores were incubated for 2h at R.T. and diluted in washing solution as follows: FITC-conjugated-Goat anti Rabbit IgG (BD Pharmigen, cat 554020, 1:200), FITC-conjugated-Goat anti Mouse IgG (Santa Cruz Biotechnologies, MA, USA, sc-2079, cat#F0211, 1:200), and Alexa647-conjugated Goat anti-Mouse IgG (Thermo Fisher, Cat # A32728). After a wash of 2h as previously described, organoids were stained with DAPI (2µg/µl) for nuclei detection for 10 min at R.T. and then washed for 40 min. Finally, glass coverslips containing organoids were mounted on a slide with 2 µl of 90% glycerol and observed with a confocal microscopy to check fluorescent signals.

3.18 Organoid invasion assay.

Type-1 collagen (Corning, #354236-33ng/μl) was resuspended in acidic environment composed of Acetic Acid (5mM) and Minimum Essential Medium Eagle (Sigma, MO275, 10X); then NaOH (1M) was added drop by drop to restore the neutral pH. Organoids were resuspended in B-27 supplement (10x) and then combined with the collagen mix previously obtained. Then, 100μl of organoid-containing collagen mix was plated in 96-Multiple Well Plate (Corning ®Costar®, CLS3738) and let solidify for 1h at 37°C. Subsequently, conditioned medium collected from fibroblasts transfected for 72h with combo miRs and Scra was mixed with organoid culture medium (1:2) and added in each well at 0h and 48h. Images were captured every 24 hours with optical microscope (Leica, DMI3000 B) in different fields of the wells and used for the analysis.

4. RESULTS

4.1 Isolation and characterization of exosomes from MDA-MB-231 cells.

To study whether exosomes were involved in the crosstalk between breast cancer cells and surrounding stromal fibroblasts occurring in the TME transformation, we isolated exosomes from MDA-MB-231, a TNBC immortalized cell line. To this aim, MDA-MB-231 cells were grown in a culture medium complemented with exosome-depleted foetal bovine serum (Exo-FBS) for 48h to avoid contamination of external exosomes. Then, for exosome isolation we adopted a polymeric precipitation method (ExoQuickTC, SBI) that allows the sequestration of exosomes based on the diameter size. Isolated exosomes were then characterized by Nanoparticle Tracking Analysis (NTA), transmission electron microscopy (TEM) and western blotting analyses. By performing NTA, we assessed particle size and distribution through NanoSight LM10 instrument, that is based on optical microscope equipped with a laser light source (405nm) to illuminate nanoparticles moving under Brownian motion. Results are shown as the ratio between particle size and concentration with a major size peak at approximately 105nm, corresponding to mean diameter of exosomes (**Figure 4.1A**). To confirm the exosomal origin of the isolated vesicles, we looked for the expression of protein markers canonically associated to exosomes. Western blot analysis revealed the expression of transmembrane CD63, CD81, and CD9 proteins belonging to tetraspanin family, that demonstrated the lipid-bilayer structure specific of extracellular vesicles. Furthermore, the presence of cytosolic Tsg101 protein belonging to the ESCRT complex confirmed the endocytic derivation of our vesicles; whereas the absence of the endoplasmic reticular protein Calnexin, indicated there was no cellular contamination in the exosome isolation (**Figure 4.1B)**. In addition, morphologic analysis performed by TEM showed that our particles were surrounded by a lipidic bilayer and confirmed exosome-corresponding diameter (**Figure 4.1C**). Altogether, these data confirmed that the extracellular vesicles isolated from MDA-MB-231 cells were enriched in exosomes.

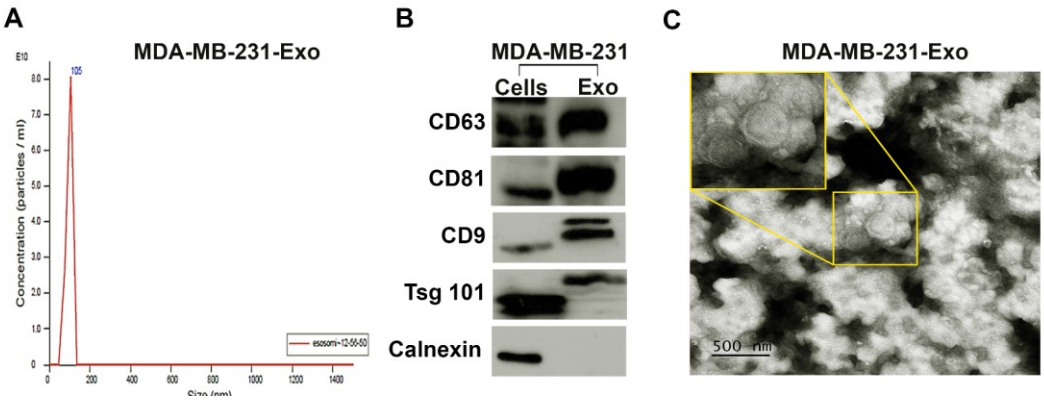

Figure 4.1. Characterization of exosomes from MDA-MB-231 cells.
A) Graph of results from the Nanoparticle tracking analysis performed on MDA-MB-231 cell-derived exosomes. The peak (at 105nm) indicates the mean ratio of vesicle size and concentration derived from three measurements. **B)** Western blot showing the expression of exosome-specific markers (CD63, CD81, CD9, and Tsg101) in MDA-MB-231 cells and exosomes, and the absence of the endoplasmic reticulum protein Calnexin. **C)** Representative TEM images of exosomes from MDA-MB-231 cells showing morphology and size typical of exosomes (scale bar: 500nm). Yellow square indicates a larger magnification (Scale bar: 100nm).

4.2 Exosomes from MDA-MB-231 cells are transferred to stromal fibroblasts.

To determine whether exosomes from TNBC cells were transferred to fibroblasts, we took advantage of primary stromal fibroblasts derived from patients undergoing to surgical resection of normal breast tissues and/or fibroadenomas. Due to the benign origin of fibroadenomas that usually show no stromal activation, we chose to define fibroblasts isolated from this tumor by convention as normal fibroblasts (NFs). More in detail, primary fibroblasts were derived from three female patients (pt. #1, #2, and #3) between 21 and 44 years of age presenting with various clinicopathological features, including two fibroadenomas (pt. #1 and #2) and one mammary reduction without any pathological signs (pt. #3). Once set up, the primary fibroblast cell lines were employed to assess the up-take of MDA-MB-231-derived exosomes. To this aim, exosomes isolated from MDA-MB-231 cells were labeled with PKH26 lipophilic red fluorescent dye, that marks exosomes through binding to their lipidic bilayer. Then, labeled exosomes were incubated for 12h with NFs (pt.#1) and subjected to immunofluorescence analysis to verify their transfer. We observed that PKH26-labeled exosomes were distinctly taken up by NFs. Indeed, confocal microscopy indicated co-localization of PKH26 derived from exosomes and β-Actin from NF cytoskeleton, confirming that fibroblasts actively internalize MDA-MB-231-derived exosomes (**Figure 4.2**).

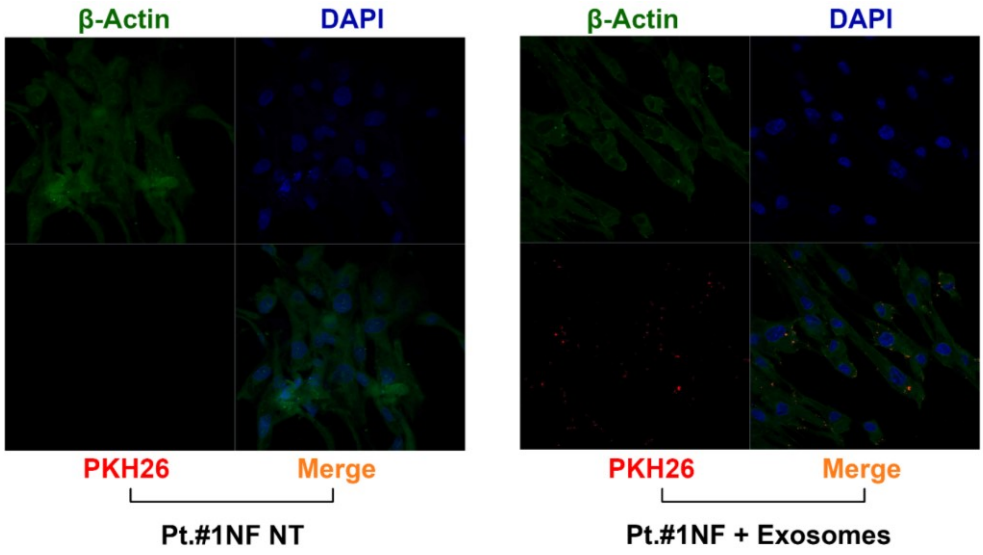

Figure 4.2. The up-take of MDA-MB-231-derived exosomes by stromal fibroblasts. Immunofluorescence assay performed on NFs (pt. #1) incubated with MDA-MB231 cell-derived exosomes labeled with PKH26 dye (+Exosomes) and with PBS (NT). The images from confocal microscopy show co-localization of the red signal derived from PKH26-labeled exosomes and the green signal from FITC-conjugated anti-β-Actin (Merged), indicative of exosome uptake by NFs. Magnification 63x.

4.3 Exosomes from MDA-MB-231 cells induce a cancer-associated-like phenotype in normal fibroblasts.

After assessed the transfer of exosomes from TNBC cells to stromal fibroblasts, we wanted to evaluate the effect mediated by exosomes in the activation of NFs to CAFs. To this aim we incubated MDA-MB-231-derived exosomes with NFs (pt.#1, #2, and #3) and examined the progression of NFs towards a CAF-related phenotype in different molecular and functional aspects. Foremost, we investigated the expression of some genes canonically associated to CAF. Indeed, we performed real time-PCR to measure the levels of FAP, Caveolin-1, SLC16A3, and SLC2A1 mRNAs in NFs cultured with MDA-MB-231 cell-derived exosomes. The presence of exosomes was associated with upregulation of these genes (**Figure 4.3.1 A, B**), that belong to panel of genes canonically up regulated in CAFs (Goetz 2011, Roy and Bera 2016, Sahai 2020) .

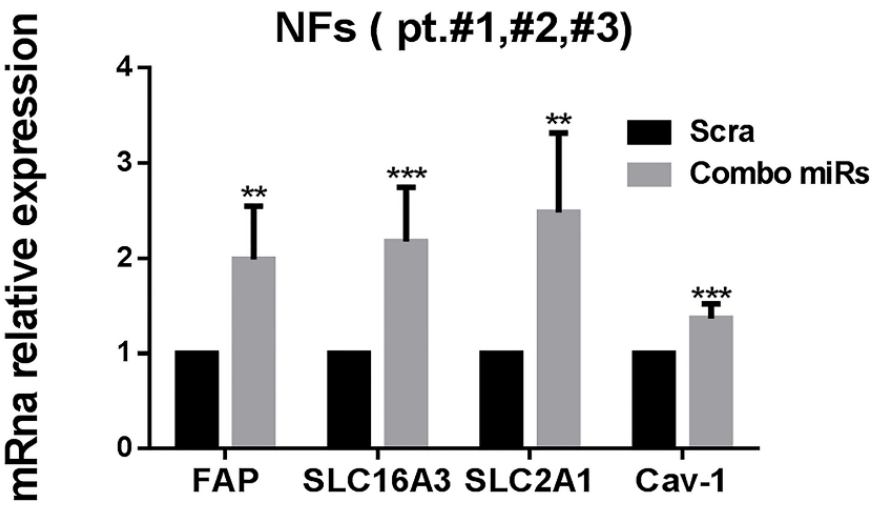

Figure 4.3.1. Upregulation of CAF-related gene in normal fibroblasts by MDA-MB-231 exosomes. Histogram of quantitative real time-PCR showing FAP, Caveolin-1, SLC16A3, and SLC2A1 mRNAs relative expression in NFs +Exosomes over NT. Standard deviations were calculated on replicates from two independent experiments performed on three different NF cell lines (pt. #1, #2, #3). P-value was calculated using the multiple t-test with FDR adjustment (FAP and SLC2A1** p=0.0014; SLC16A3 ***=0.00064; Caveolin-1*** p=0.00014).

Among several functions known to be enhanced in CAFs, cell contractility and motility are regarded as major hallmarks of activated fibroblasts within the TME to foster cancer cell invasion. Therefore, we assessed the effect of TNBC cell-derived exosomes on fibroblast-mediated collagen contraction. To this end, NFs (pt. #1, #2, #3) were plated inside a type 1-collagen matrix and then incubated with MDA-MB-231 cell-derived exosomes or PBS (control) to examine their contractile ability. After 24h, we observed that NFs cultured in the presence of exosomes significantly reduced the collagen plug area compared to control, reflecting increased cell contractility mediated by exosomes (**Figure 4.3.2 A, B**).

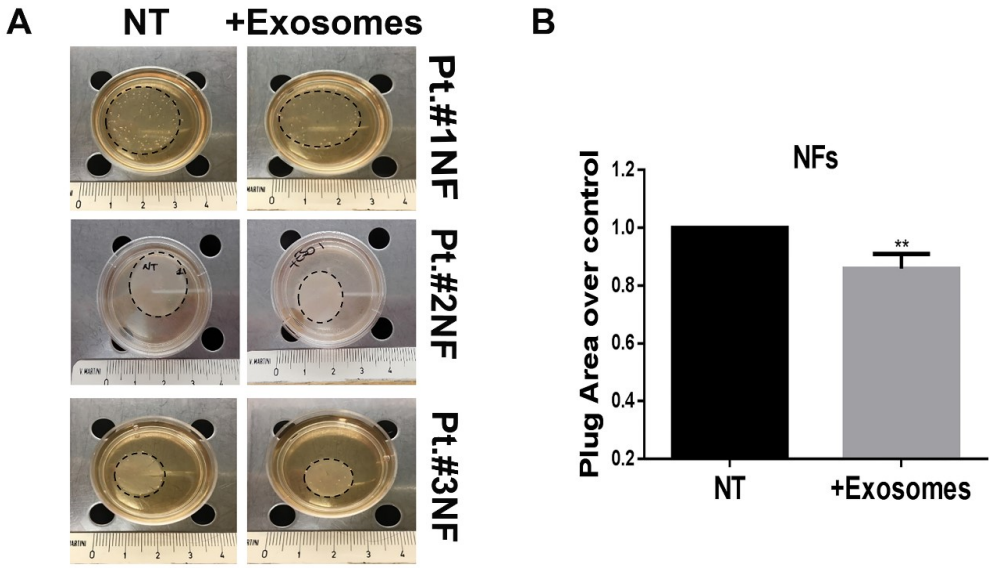

Figure 4.3.2. MDA-MB-231-derived exosomes increase contraction ability of normal fibroblast. A) Collagen contraction assay. Representative pictures of collagen plugs containing NFs (pt.#1, #2, and #3) cultured with MB-231 cell-derived exosomes (+Exosomes) or PBS (not treated, NT) for 24h. **B)** Histogram of mean collagen plug areas for NFs +Exosomes over NT measured with ImageJ. Standard deviations were calculated on replicates from two independent experiments performed with three different NF cell lines (pt. #1, #2, #3). P-value was calculated using the two-tailed unpaired t-test, ** p=0.0028

Likewise, to verify whether breast cancer-derived exosomes affected the migratory potential of the fibroblasts, we pre-incubated NFs (pt. #1, #2, #3) with MDA-MB-231 cell-derived exosomes or PBS for 48h and then performed a Transwell® migration assay in presence of a chemical gradient. We found improved migration when NFs were cultured in the presence of exosomes, as shown in **Figure 4.3.3 A, B** by the higher value of crystal violet absorbance that reflects a larger number of migrated cells. These results supported our initial hypothesis of fibroblasts being converted to a CAF-like phenotype under cancer exosome influence.

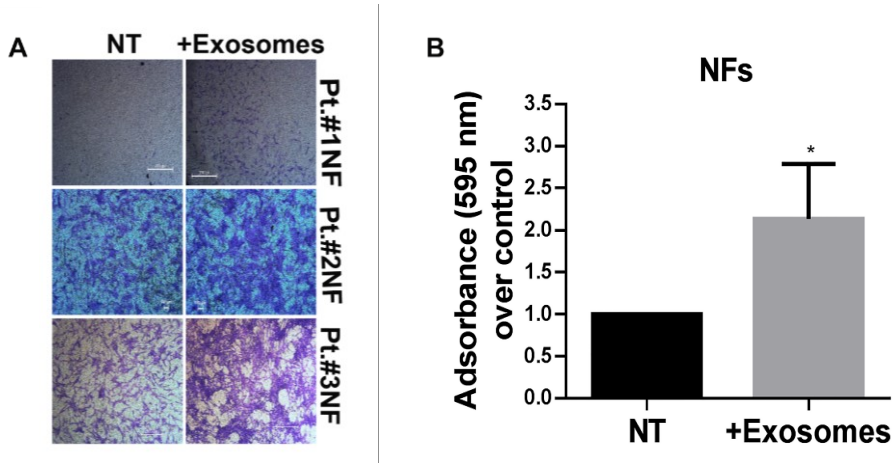

Figure 4.3.3 MDA-MB-231-derived exosomes improve fibroblast migration ability. **A)** Migration assay performed with NFs (pt. #1, #2, #3) cultured in the presence of MDA-MB-231 cell-derived exosomes for 48h (+Exosomes) or PBS (NT). Representative bright-field images of NFs migrated through the Transwell® chamber and colored with crystal violet. Bars indicate size, expressed in µm (pt. #2, #3) or pixels (pt. #1). Magnification 5x. **B**) Histogram of mean absorbance of crystal violet eluted from NFs +Exosomes over NT. Standard deviations were calculated on replicates from two independent experiments performed on three different NF cell lines (pt.#1, #2, #3). P-value was calculated using the two-tailed unpaired t-test, * p=0.039.

4.4 Exosome-activated fibroblasts induce invasion of non-tumorigenic breast epithelial cells.

To investigate the effects of exosome-mediated fibroblast activation within the TME, we set up a three-dimensional co-culture model to better study cell dynamics. As already reported by Timpson (2011), we set up an in vitro invasion organotypic assay in presence of collagen I (Timpson 2011). In detail, NFs (pt. #3) were seeded on a type 1 collagen matrix, then cultured in the presence of MDA-MB-231 cell-derived exosomes or PBS for 48h to ensure fibroblast activation. Subsequently, non-tumorigenic breast epithelial MCF10A cells were seeded on the top of the fibroblast-containing collagen matrix for 14 days. Then, collagen matrices were transferred to an invasion grid and finally paraffin-embedded for the analysis. Results displayed that the activation of NFs induced by the presence of the exosomes increased the number of MCF10A cells invading the matrix, as demonstrated by the positive Pan-cytokeratin signal from cells stained in the collagen section (**Figure 4.4**). These data suggested that MDA-MB-231-derived exosomes have a pro-tumor stromal function triggering the activation of NFs to a CAF-like phenotype. Moreover, our organotypic assay outlined how the impact of cancer-derived exosomes on fibroblasts contribute to

malignant transformation promoting the invasive capacity of non-tumorigenic breast epithelial cells.

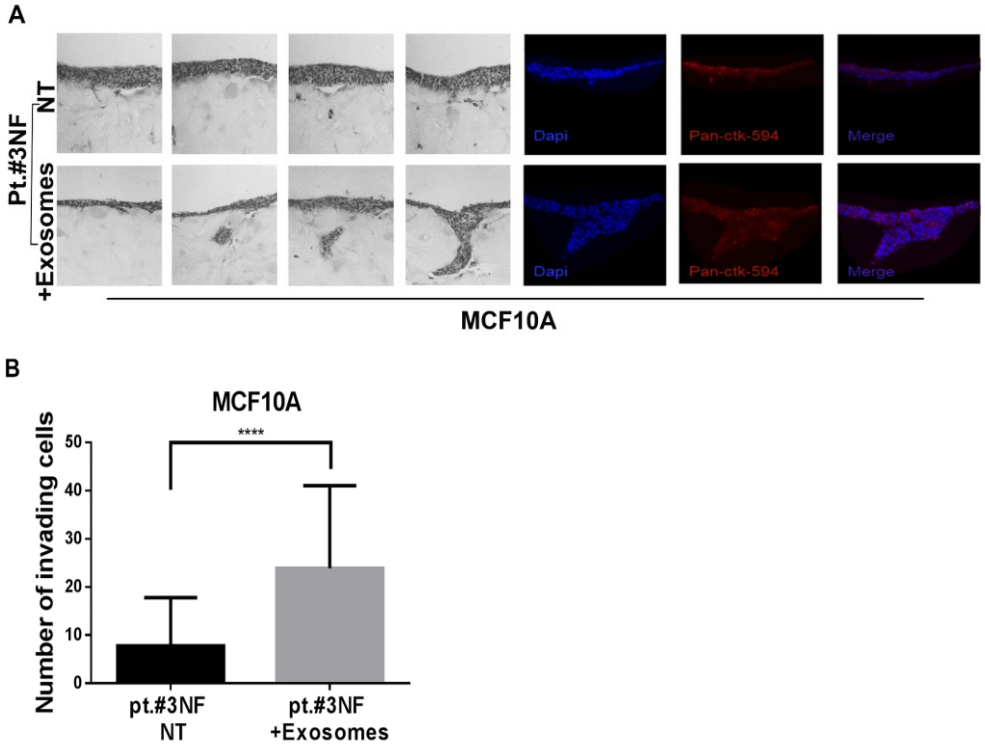

Figure 4.4. 3D Organotypic invasion assay of breast epithelial cells co-cultured with exosome-activated fibroblasts. A) Left panel: representative phase contrast images of collagen matrix embedded section (10µm slides) showing MCF10A cell invading the matrix when three-dimensional co-cultured with NFs (pt.#3) incubated with MDA-MB-231-derived exosomes (+Exosomes) or PBS (NT). Right panel: confocal microscopy images of collagen matrix sections showing MCF10A cells stained with DAPI for nuclei (blue signal) and ALEXA594-conjugated anti-Pan-cytokeratin antibody (red signal) as epithelial marker. B) Histogram of the number of invading MCF10A cells (NF + Exosomes and NF_NT) calculated in different fields of confocal microscopy images. Standard deviations were calculated on technical replicates. P-values were calculated using two-tailed unpaired t test; ****$p<0.0001$.

4.5 MiRNA expression profile of normal fibroblasts exposed to MDA-MB-231 cell-derived exosomes.

Given the importance of exosomal miRNAs in cancer progression, we investigate whether the miRNA cargo carried by TNBC cell-derived exosomes to fibroblasts could participate in fibroblast activation within the TME. To do this, we performed small-RNA sequencing to identify miRNAs differentially expressed in NFs cultured with MDA-MB-231 cell-derived exosomes or PBS (NT, control) for 24h. We found that 14 miRNAs were significantly upregulated in NFs (pt. #1) when exposed to the exosomes (**Figure 4.5.1**).

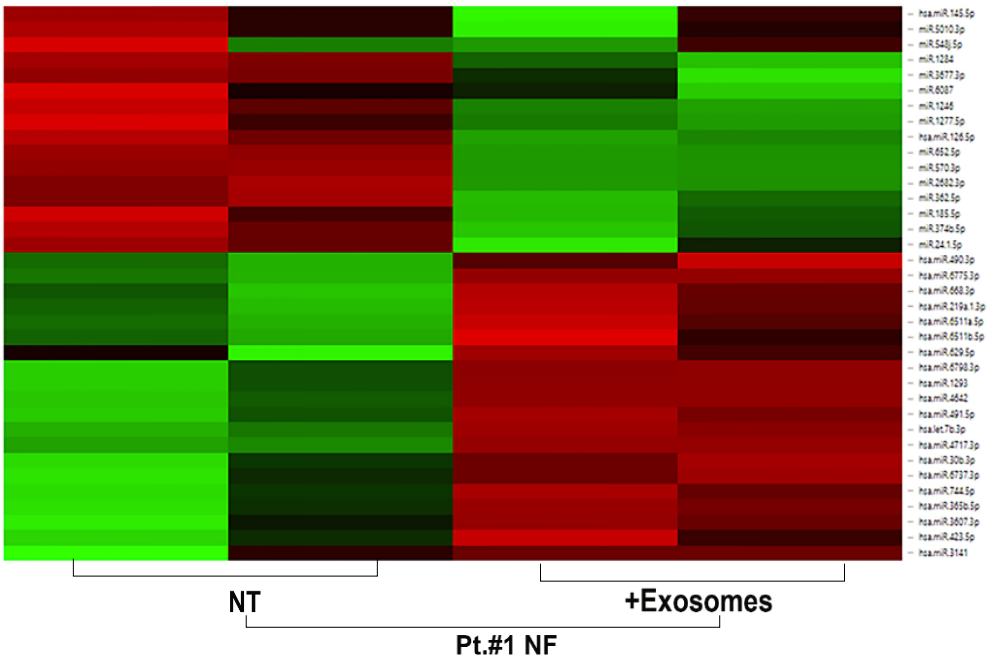

Figure 4.5.1. MiRNA differentially expressed in fibroblasts upon exosome exposure. Heatmap of data from small-RNA sequencing analysis showing miRNAs differentially expressed in NFs (pt. #1) incubated with MDA-MB-231 cell-derived exosomes (+Exosomes) and PBS (NT). Upregulated miRNAs represented in green, downregulated miRNAs in red. The results are based on technical duplicates (p-value < 0.05).

Among miRNAs up-regulated in fibroblasts by exosomes, we focused our attention on miR-185-5p, -652-5p, and -1246, since they were predicted to be actively sorted into exosomes by the MDS2 bioinformatics tool (http://cse-jcui-08.unl.edu:7000/input), previously described (Villarroya-Beltri 2013). We found short-sequence motifs (EXOmotifs) in the three miRNAs that predicted their loading into exosomes (**Figure 4.5.2**). This finding directed us to study these three miRNAs among the other upregulated miRNAs identified by RNA-sequencing.

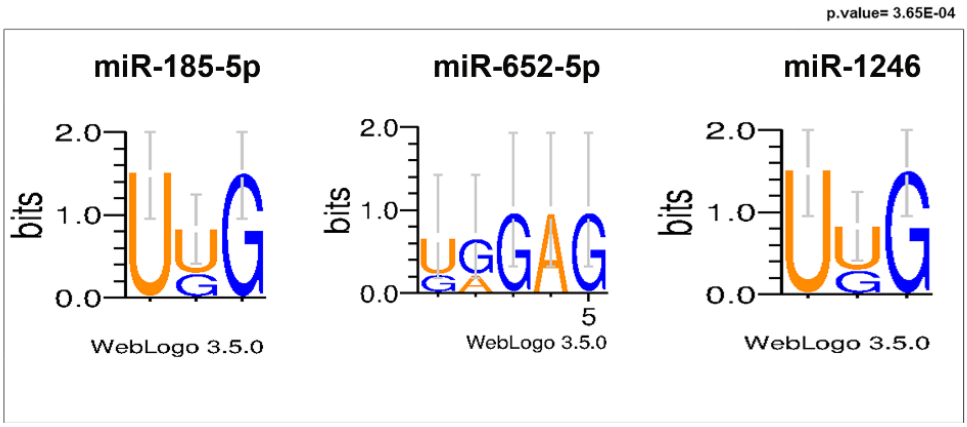

Figure 4.5.2. Bioinformatic prediction of miRNA loading into exosomes. Representative images of the Exomotif analysis with MDS2 software showing short motif (3 to 5 nucleotides) contained in miR-185-5p, -652-5p, and miR-1246 sequences and predictive of their active loading into exosomes.

4.6 Exosomal miR-185-5p, -652-5p, and-1246 operate in combination.

We then investigated whether the miR-185-5p, -652-5p, and 1246 shuttled via breast cancer-derived exosomes triggered fibroblast activation. We found that transfection of the individual miRNAs had no significant biological effects on fibroblast conversion, as shown by western blot for FAP, MCT4, and Caveolin1, known markers associated to CAF phenotype (**Figure 4.6.1**).

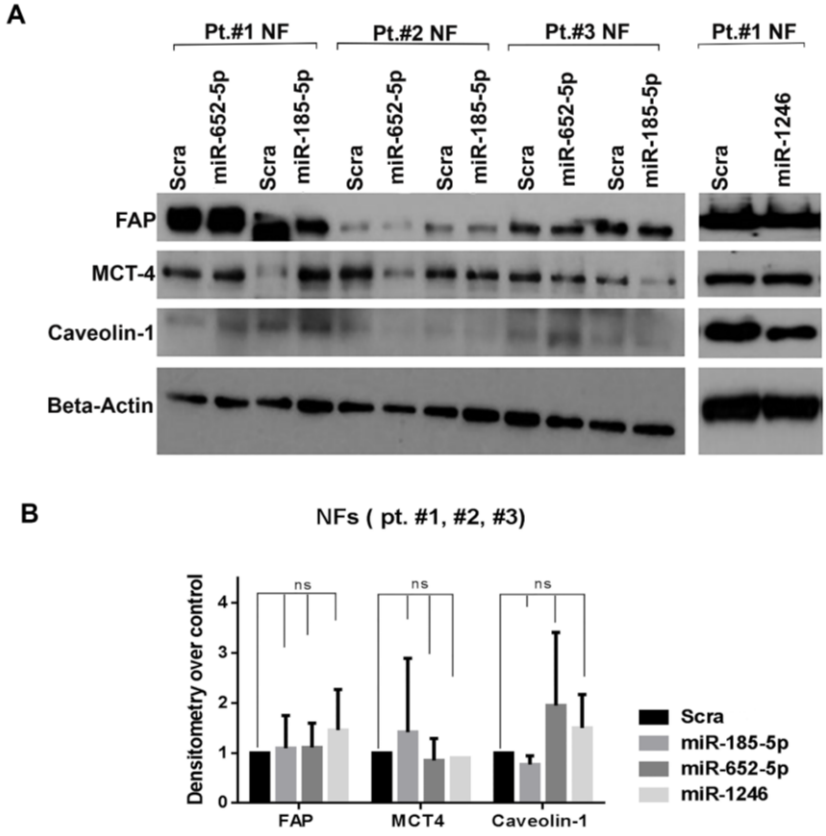

Figure 4.6.1. Transfection of individual miRNAs have no significant effect on fibroblast activation. A) Representative western blot images showing no significant variation in the expression of FAP, MCT-4, and Caveolin-1 proteins in NFs (pt. #1, #2, #3) transfected with miR-185-5p, -652-5p, and -1246 after 72h**. B)** Histogram of mean densitometric measurement of bands (ImageJ) for NFs transfected with miR-185-5p, -652-5p, and -1246 over Scra. Standard deviations were calculated on replicates from two independent experiments performed on three different NF cell lines (pt. #1, #2, and#3 for miR-185-5p, and -652-5p/ pt.#1 for miR-1246). P-value was calculated using 2way ANOVA-multiple comparisons (p=0.37).

Therefore, we hypothesized that miR-185-5p, -652-5p, and -1246 might work synergistically, as already reported for miR-185-5p in severe alopecia areata

(Sheng 2019). Indeed, we found that combined transfection of miR-185-5p, -652-5p, and -1246 (combo miRs) was primarily associated with the upregulation of CAF markers (FAP, MCT4, and Caveolin1) in all three patient-derived NFs (**Figure 4.6.2**)

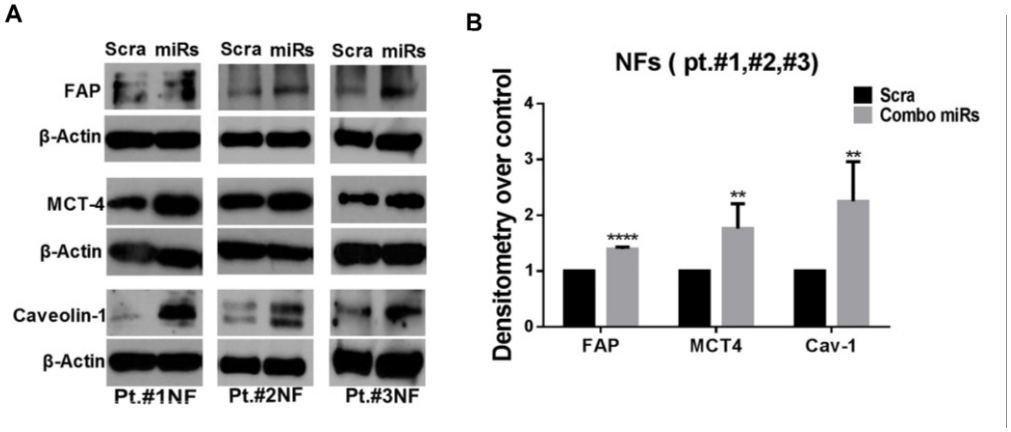

Figure 4.6.2. Exosomal miRNAs synergistically up-regulate CAF markers in normal fibroblasts. **A**) Western blot showing the overexpression of FAP, Caveolin-1, and MCT4 proteins in NFs (pt. #1, #2, #3) transfected with combo miRs as compared to control (Scra) after 72h. **B**) Histogram of densitometric measurement of bands, obtained with ImageJ. Quantification of protein expression is represented as the mean of folded densitometry from NFs transfected with combo miRs over Scra. Standard deviations were calculated on replicates from two independent experiments performed on three different NF cell lines (pt. #1, #2, #3). P-value was calculated using the multiple t-test with FDR adjustment (FAP ****$p<0.0001$; MCT4 ** $p=0.0019$; Caveolin-1**$p=0.0016$).

4.7 Exosomal combo miRs activate stromal fibroblasts towards a pro-migratory functional phenotype.

It is known that fibroblasts are recruited by cancer cells within the TME to support different tumor traits (Erdogan and Webb 2017, Joshi and Kanugula 2021). Therefore, we investigated different aspects of fibroblast activation mediated by exosomal combo miRs. First, we evaluated the cell proliferation with MTS assay in NFs transfected with combo miRs after 24h and 48h, but surprisingly, we did not find any significant effect (**Figure 4.7.1, A**). We also found that the expression levels of known markers of cell proliferation, self-renewal, and survival in cancer (Duchartre 2016) (Yoshida 2018, Ediriweera 2019) (Chen 2018) did not change in NFs transfected with combo miRs compared to control. Indeed, western blot for the phosphorylated form of AKT (S473) and phosphorylated β-catenin (S33/37/T41) showed no significant alteration in protein levels mediated by combo miRs compared to control (**Figure 4.7.1, B**).

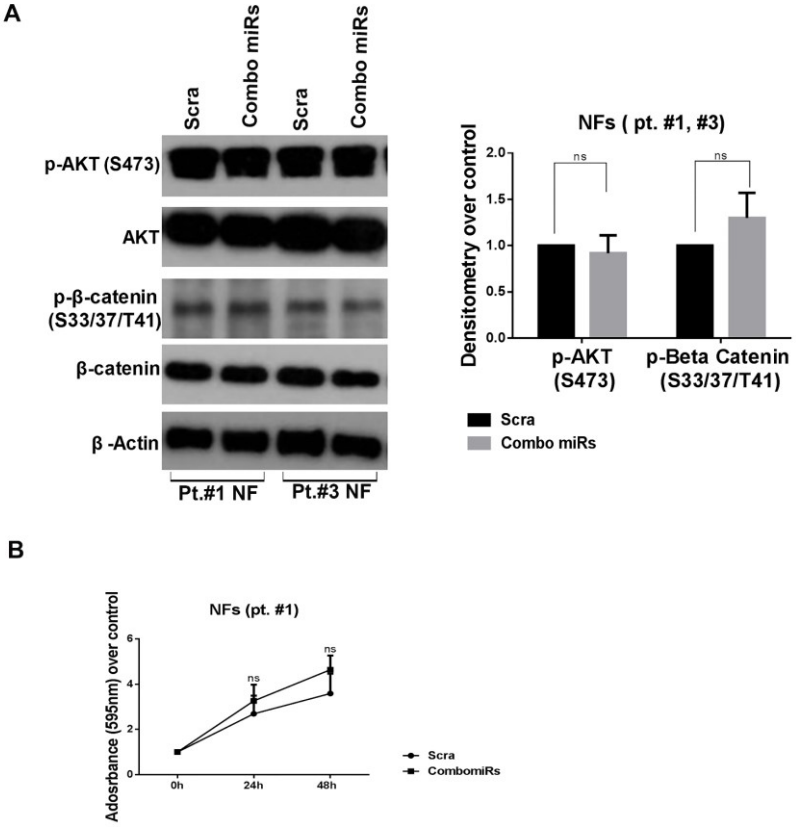

Figure 4.7.1. Combo miRs do not promote pro-proliferative and pro-survival phenotype. A) On the left, representative image of western blot showing no significant variation in the expression of phosphorylated AKT (p-AKT-S473) and phosphorylated β-catenin (p- β-catenin- S33/37/T41) in NFs transfected with combo miRs compared to Scra after 48h. On the right, mean densitometric measurement of bands (ImageJ) for NFs transfected with combo mRs over Scra. Standard deviations were calculated on replicates from a single experiment performed on two different NF cell lines (pt. #1, and#3). P-value was calculated using 2way ANOVA-multiple comparisons (ns= p-value >0,05). **B**) Cell proliferation analyzed by MTS assay at 24 and 48h normalized on 0h in NFs transfected with combo miRs and Scra. The graph shows no significant variation in cell proliferation and survival of NFs transfected with combo miRs and Scra after 24 or 48h. Standard deviations were calculated on replicates from two independent experiments performed on NF cell line (pt. #1). p-value was calculated using multiple t-test with FDR adjustment (24h p=0.329; 48h p=0.099).

Otherwise, we observed that combo miRs exert significant effects in promoting invasive, contractile, and migratory phenotype in fibroblasts. We first found that NF migration ability was increased by the synergistic action of the three miRs

as shown by wound healing assay (**Figure 4.7.2 A, B**) and transwell migration assay (**Figure 4.7.2 C, D**). To corroborate these results, we investigated expression of FAK, the main regulator of focal adhesion turn-over, responsible for cell movement. We found upregulation of the activated form of FAK protein (phosphorylated at Y576/577) using western blot analysis (**Figure 4.7.2 E, F**) in NFs (pt.#1, #2, #3) transfected with combo miRs, indicating an activation of the molecular pathway involved in cell migration.

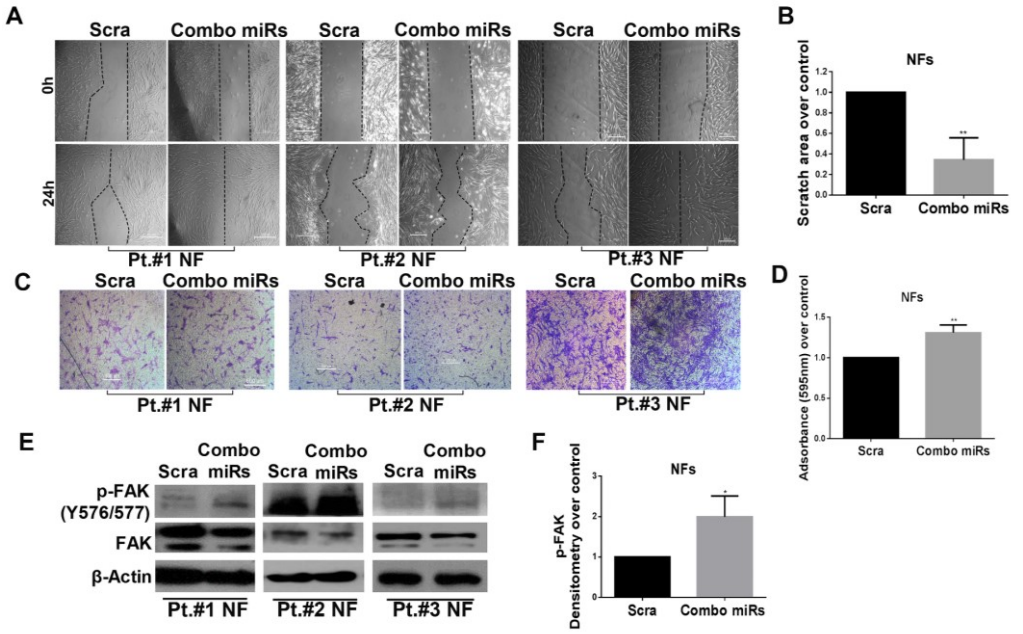

Figure 4.7.2. Combo miRs promote migration of stromal fibroblasts. A) Wound healing assay performed with NFs transfected with combo miRs or Scra. Representative images in bright field of NFs at 0h and 24h from the scratch. Bars indicate size, expressed in pixels (px). **B**) Histogram of mean scratch area at 24h, normalized on 0h of NFs transfected with combo miRs over Scra. Standard deviations were calculated on replicates from two independent experiments performed on three different NF cell lines (pt. #1, #2, #3). P-value was calculated using the two-tailed unpaired t-test, ** p=0.0061. **C**) Transwell migration assay performed with NFs (pt.#1, #2, #3) transfected with combo miRs and Scra (control). Representative images in bright field of NFs migrating through the Transwell chamber and colored with crystal violet. Bars on the images indicate size, expressed in µm (pt. #1, #2) or pixels (pt. #3). **D**) Histogram of mean absorbance values of crystal violet eluted from migrated cells. Standard deviations were calculated on replicates from two independent experiments performed on three different NF cell lines (pt. #1, #2, #3). P-value was calculated using the two-tailed unpaired t-test, ** p=0.0049. **E**) Western blot showing overexpression of phosphorylated FAK (Y576/577) protein in NFs transfected with combo miRs compared to Scra after 72h. **F**) Histogram of mean densitometric measurement of bands (ImageJ) for NFs transfected with combo miRs over Scra. Standard deviations were calculated on replicates from two independent experiments performed on three different NF cell lines (pt. #1, #2, #3). P-value was calculated using the two-tailed unpaired t-test, * p=0.029.

Furthermore, we investigated the invasion potential induced by combo miRs in NFs. Coherently, the above results demonstrated that the combo miRs mediated the activation of NFs by primarily boosting their motility in the ECM. To explore this behavior, we set up a collagen contraction assay, plating NFs pre-transfected with combo miRs or scrambled sequence as control onto type 1 collagen matrices. We found that the synergistic action of the three miRNAs promoted fibroblast collagen contraction, as shown by reduction of collagen plug area in combo miRs-transfected NFs (**Figure 4.7.2 A,B**).

Another way to study the invasion potential of fibroblasts is represented by their ability to move within the ECM as a result of its degradation. To this end, we transfected NFs (pt. #1, #2, #3) with combo miRs and performed a Transwell invasion assay, seeding the cells in a Matrigel solution plated on top of the migration chamber, thus simulating three-dimensional ECM. We observed increased invasion by NFs transfected with combo miRs, as demonstrated by the higher absorbance values of crystal violet eluted from migrated cells (**Figure 4.7.2 5 C, D**).

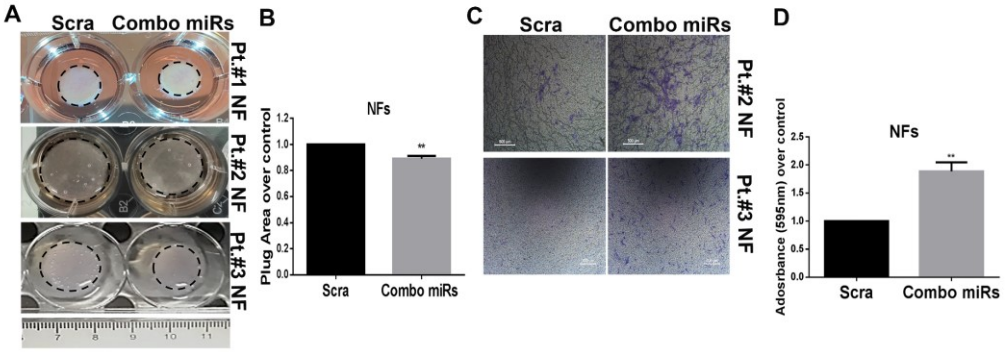

Figure 4.7.2. Combo miRs promote fibroblast-mediated ECM remodeling. A) Representative pictures of collagen plugs containing NFs transfected with combo miRs or Scra taken after 24h. B) Histogram of mean collagen plug area for NFs transfected with combo miRs, fold over Scra at 24h (ImageJ). Standard deviations were calculated on replicates from two independent experiments performed with three different NF cell lines (pt. #1, #2, #3). P-value was calculated using the two-tailed unpaired t-test; ** p=0.0011. **C**) Transwell invasion assay with NFs (pt. #2, #3) transfected with combo miRs or Scra (control). Representative bright field images of NFs that invaded the matrix and migrated through the Transwell chamber fixed and colored with crystal violet. Bars indicate size, expressed in μm or px. Magnification 5x. **D**) Histogram of mean absorbance values for crystal violet eluted from NFs transfected with combo miRs folded on Scra. Standard deviations were calculated on replicates from two independent experiments performed on two different NF cell lines (pt. #2, #3). P-value was calculated using the two-tailed unpaired t-test, ** p=0.0018.

Considering that ECM remodeling depends on force-mediated and protease-dependent mechanisms, we investigated integrin (ITG) and matrix metalloprotease (MMP) expression in NFs after combo miRs overexpression. For this, we conducted western blot analysis for the expression of ITG β1 and α5 proteins, major players in mechanical force-mediated cell invasion and extracellular matrix modeling, especially during CAF transformation. We found that ITG β1 and ITG α5 were increased in NFs transfected with combo miRs after 72h (**Figure 4.7.3 A, B upper**), suggesting there was a higher driving force exerted by fibroblasts to contract the collagen matrix. However, the protease activity involved in matrix degradation is arbitrated by different members of the MMP protein family. In particular MMP1, 2, 3, and 9 are mainly upregulated in breast cancer stroma under the influence of cancer cells (Ito 1995, Eck 2009, Eiro 2015). We found that after 48h of transfection, combo miRs led to increased levels of MMP 1, 2, and 3 in NFs. These results justified the boost in fibroblast invasive potential by combo miRs from the moleculr standpoint (**Figure 4.7.3 A, B lower**).

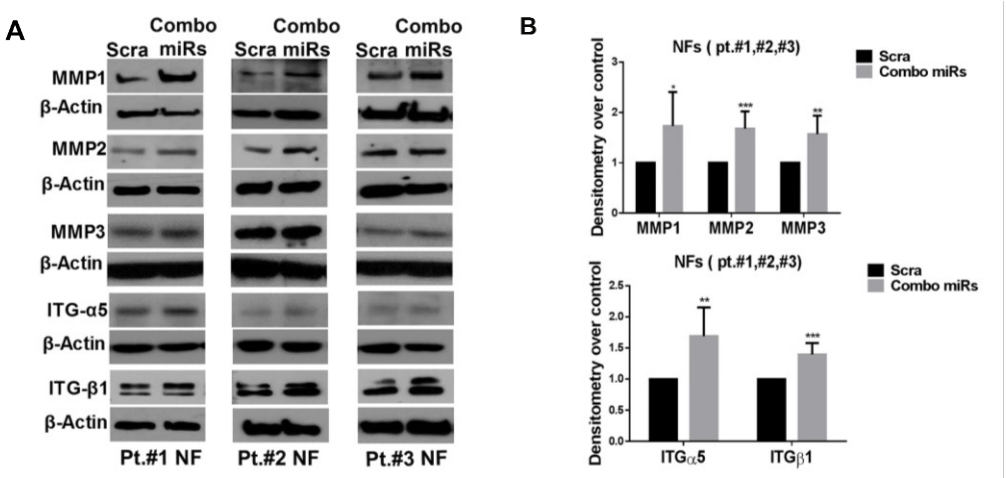

Figure 4.7.3. Combo miRs upregulate invasion markers in stromal fibroblasts. A) representative western blot images showing overexpression of MMP1, MMP2, and MMP3 proteins together with ITGβ1 and ITGα5 in NFs (pt. #1, #2, #3) transfected with combo miRs and Scra after 48h and 72h. **B**) Histograms of mean densitometric measurement of bands (ImageJ) for NFs transfected with combo miRs over Scra. Standard deviations were calculated on replicates from two independent experiments performed on three different NF cell lines (pt. #1, #2, #3). P-value was calculated using multiple t-test with FDR adjustment (MMP1* p=0.023; MMP2*** p=0.00087; MMP3**p=0.0035; ITGβ1***p=0.00014; ITGα5 **p=0.0054).

Given these consistent results, we can assume that exosomal combo miRs derived from TNBC cells activate stromal fibroblasts to obtain a specific pro-migratory and pro-invasive functional phenotype. Furthermore, the proprerty of ECM remodeling improved in fibroblasts by combo miRs could led to the

formation of permissive tracks, exploited by cancer cells to invade and metastatize.

4.8 Fibroblasts activated by exosomal combo miRs exert downstream pro-tumorigenic effects on breast cells.

We aimed to better characterize the impact of stromal fibroblast activation mediated by cancer-derived exosomal miRNAs on other breast cells populating the TME context. Assuming that once activated, fibroblasts could secret pro-tumorigenic factor in the extracellular space to promote tumor functions, we collected the conditioned medium (CM) from the transfection of NFs with combo miRs after 72h. Thus, we used CM as chemoattractant for a migration assay involving non tumorigenic breast epithelial MCF10A cells. Results shown in **Figure 4.8.1** revealed that the treatment with CM from the transfection of NFs with combo miRs (+CM_combo miRs) increased MCF10A cell migration compared to control (+CM_Scra).

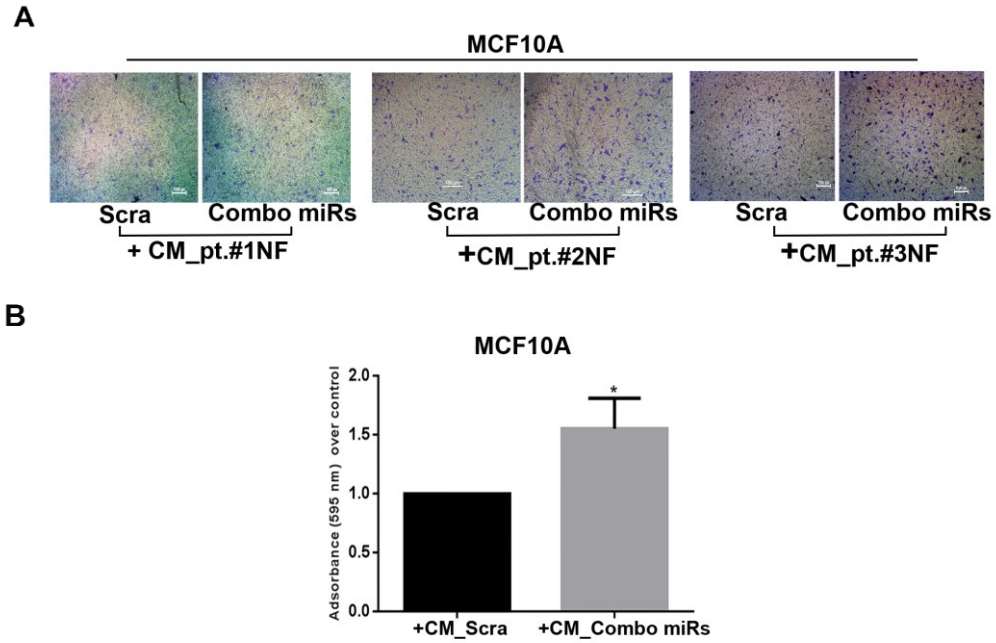

Figure 4.8.1. Conditioned medium from combo miRs-transfected fibroblasts increases MCF10A cell migration. **C**) Transwell migration assay with MCF10A cells. CM from NFs (pt.#1, #2, and#3) transfected with combo miRs (+CM_combo miRs) and Scramble (+CM_Scra) was used as chemoattractant. Representative images in bright field of MCF10A migrating through the Transwell chamber and colored with crystal violet. Bars on the images indicate size, expressed in μm (+CM_pt.#2NF) or pixels (+CM_pt. #2NF, #3NF). **D**) Histogram of mean absorbance values of crystal violet

eluted from migrated cells. Standard deviations were calculated on replicates from two independent experiments. P-value was calculated using the two-tailed unpaired t-test, * $p<0.05$.

Subsequently, we tested the effect of combo miRs-activated fibroblasts on epithelial breast cell proliferation. Hence, by using MTS assay we measured proliferation ability of MCF10A and breast cancer cells MCF7 (luminal A subtype) incubated with CM from combo miRs transfection of NFs. After 48h, we observed increased cell proliferation of MCF7 and MCF10A cells treated with CM from combo miRs transfection compared to control (**Figure 4.8.2**).

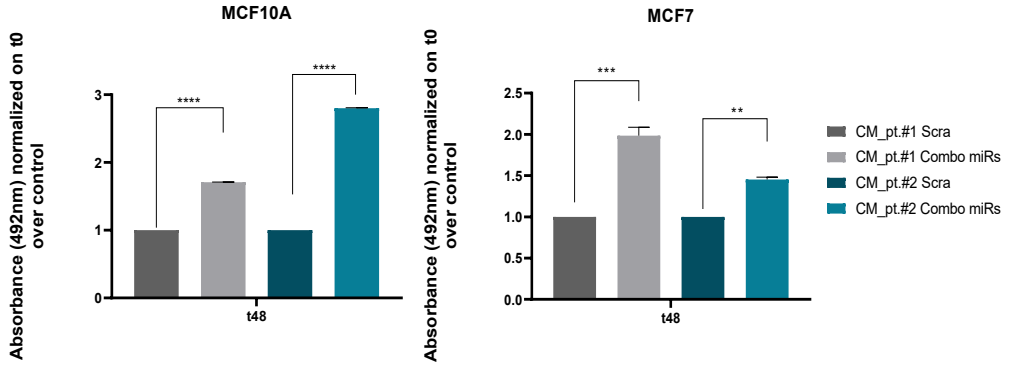

Figure 4.8.2. Conditioned medium from combo miRs-transfected fibroblasts increases MCF10A and MCF7 cell proliferation. Graphs of cell proliferation analyzed by MTS colorimetric assay performed on MCF10A cells (upper) and MCF7 cells (lower) treated with CM of NFs (pt.#1, #2) transfected with combo miRs and Scra (control). Absorbance (492nm) measured at 0h (t0) and after 48h (t48) was normalized at t0 and folded over control. Standard deviations were calculated on technical replicates. Adjusted p-value was obtained using ordinary one-way ANOVA-Multiple comparison (**** $p<0.0001$; *** $p=0.0002$; ** $p=0.0035$).

Furthermore, to recreate a scenario that better resembled the *in vivo* conditions, we set up primary breast organoid cultures from patients' biopsy specimens. For this study, we cultured organoids from two different patients affected by breast cancer luminal A subtype (pt.#530 and pt.#535). Once obtained, patient-derived breast organoids (pt.#530) were characterized through immunofluorescence assay to confirm the polarization and the epithelial stem origin of the three-dimensional cultured cells (**Figure 4.8.3**). Coherently, we found a positive expression for cytokeratin 14, a known marker of basal multi-layered epithelia, that localizes in direct contact with ECM and usually correlates with tumor invasiveness (Cheung 2013). We also observed a positivity for cytokeratin 18 glandular epithelial marker, also correlated to breast adenocarcinomas (Yang 2018) (Cîmpean 2008). Furthermore, we observed the expression of CD44 and beta-catenin proteins, associated to cancer cell stem-phenotype, that is typical of

cells growing in three-dimensional state (Murat Dogan 2015) (Morin 1999) (Sokol 2011).

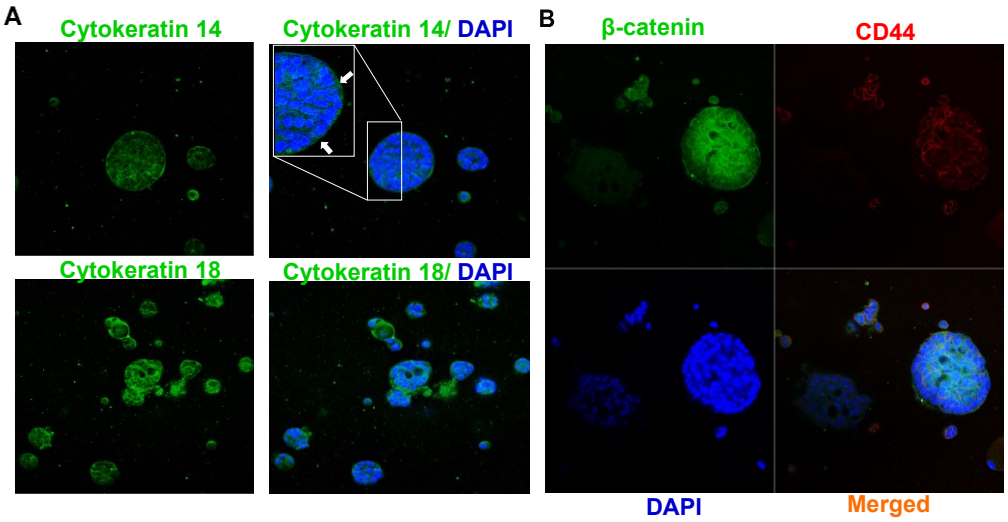

Figure 4.8.3. Breast cancer organoid characterization by immunofluorescence assay. A) Confocal microscopy images of breast cancer organoids (pt.#530) stained for DAPI (nuclei) and for cytokeratin14 (upper panel) and cytokeratin 18 (lower panel) epithelial markers. White arrows in the expanded white square indicates the basal localization of cytokeratin14 signal. Magnification 63x **B)** Confocal microscopy images of breast cancer organoids (pt.#530) stained for DAPI (nuclei), and CD44 and β-catenin stem markers. Magnification 63x.

Subsequently, breast cancer organoids were employed as *in vivo*-like model to test the effect of activated fibroblast on cancer cell invasion. More in detail, we performed a collagen invasion assay by plating breast cancer organoids (luminal A-subtype, pt.#535) in type 1 collagen matrix in presence of conditioned medium from NFs (pt.#1) transfected with combo miRs (CM_pt#1 Combo miRs) and Scra (CM_pt#1 Scra, control) for 96 h. As already described by Nguyen-Ngoc (2015), collagen 1-enriched matrix induces mammary organoid invasion, revealed by the formation of multicellular invasive protrusions (Nguyen-Ngoc 2015). According to the authors, invasive protrusions need to be clearly distinguishable from single cells escaped away from organoids or from groups of cells that have lost their three-dimensionality. Coherently, our results shown that organoids were able to produce invasive multicellular protrusion tips in presence of collagen, that are clearly detectable after 72h, with a trend to form wider protrusions when incubated with CM_pt#1 Combo miRs compared to control. In particular, this tendency was highlighted after 96 h of incubation with CM_pt#1 Combo miRs, whereas at 72h the differences were not yet significant (**Figure 4.8.4 A, B**). Further experiments are necessary to confirm the epithelial

origin of invading cells, that should be positive to cytokeratin 14 expression. As a conclusion, our preliminary data suggest that fibroblasts activated by combo miRs could enforce invasion ability of breast cancer cells, thus promoting cancer aggressiveness as already assumed by our previous observations.

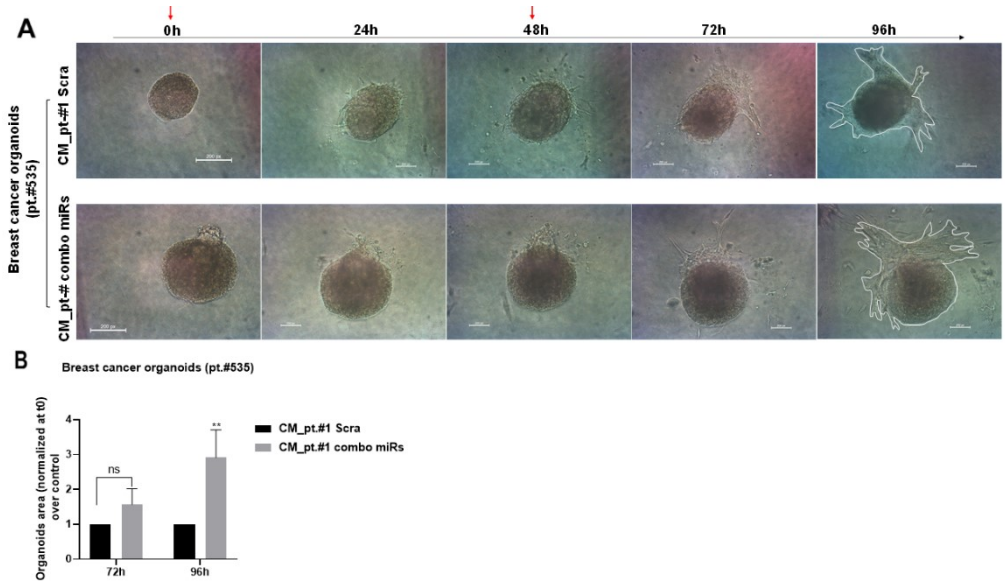

Figure 4.8.4. Invasion assay with breast cancer organoids in presence of conditioned medium from combo miRs transfection. A) Representative images from optical microscope taken at 0h, 24h, 48h, 72h, and 96h of collagen-embedded breast cancer organoids (pt.#535) incubated with CM_pt#1 Combo miRs and CM_pt#1 Scra (control). Red arrows indicate the addition of the CM. White lines confine the invading protrusions around the organoids. Scale bars are expressed in pixel. Magnification 20x. **B)** Graph indicating the quantification of organoid invasion obtained by measuring the area of organoids at 72h and 96h normalized on the respective areas at 0h and folded on the control. Area measurements were performed with Image J. Standard deviation were calculated on technical replicates. Adjusted P value was obtained with 2-way ANOVA (**p=0.0018).

5. DISCUSSION

The data presented here shed light on the role of exosomes in the crosstalk between the TME and breast cancer cells. Our results strengthen knowledge on the mechanisms adopted by breast cancer to potentiate the oncogenic phenotype of neighboring cells within the TME. Indeed, we demonstrate that TNBC cell-derived exosomes and the miRNA cargo they hold activate stromal fibroblasts to obtain a specific pro-migratory functional phenotype, so potentially enabling tumor invasion and metastasis.

It is known that fibroblasts are recruited by cancer cells within the TME to support different tumor traits (Erdogan and Webb 2017, Joshi 2021). However, the major obstacle in studying CAFs is high functional heterogeneity and lack of specific molecular markers defining their status, even considering occasional antitumorigenic roles (Sahai 2020). However, some researchers have tried to associate precise gene signatures to CAF subtypes (Busch 2017). In our case, fibroblast activation mediated by cancer cell exosomal miRNAs was associated with upregulation of MCT-4, FAP, and Caveolin-1, among other canonical CAF markers. The role of Caveolin-1 in CAF activation is controversial. In fact, some studies have demonstrated that loss of Caveolin-1 expression is a trait associated with CAF transformation (Martinez-Outschoorn 2014) (Mercier 2008, Sotgia 2011). In contrast, our data are in line with those of Goetz (2011) showing that the presence of Caveolin-1-enriched CAFs correlates with tumor invasion and metastasis by promoting the biomechanical remodeling of tumoral stroma (Goetz 2011).

In the context of breast cancer TME, exosomal miRNAs have been widely reported to mediate the crosstalk between CAFs and cancer cells (Kim 2020) (Wu 2020) (Baroni 2016) (Chen 2021) (Dou 2020) (Donnarumma 2017). In this manuscript, we provided evidence supporting the notion that breast cancer cell exosomal miRNAs induce a CAF-related pro-migratory phenotype, rather than a proliferative pro-survival one. Indeed, any significant effect on the activation of proliferative and survival pathways mediated by combo miRs was observed, as shown by cell viability assay and western blotting for phosphorylated AKT (S473) and phosphorylated β-catenin (S33/37/T41), known markers of cell proliferation, self-renewal, and survival in cancer (Duchartre 2016, Chen 2018, Yoshida 2018, Ediriweera 2019). In contrast, delivery to fibroblasts of the breast-cancer cell exosomal combo miRs induced the former to increase migration, contraction, and invasion, all characteristics acquired by specific CAF subtypes. Moreover, the overexpression in fibroblasts of combo miRs induced the expression of MMPs (type 1, 2, and 3) and ITGs (α5 and β1 subunits) and increased FAK phosphorylation (Y576/577), components of major pathways involved in cancer cell invasion and motility, since they operate in protease-

dependent ECM remodeling and cellular movement (Jang and Beningo 2019) (Wu 2020) (Nagase 2006).
Although breast cancer prognosis has been improved by the development of molecular targeted therapies, the treatment of TNBC is still a challenge due to its highly invasive nature and relatively low response rate. These adverse clinicopathological aspects are often caused by CAFs populating the activated tumoral stroma and the exosomal cargo shed by cancer cells into the surrounding TME (Bianchini 2016) (Yu and Di 2017) (Dong 2020). Thus, discovering new molecular targets for TNBC prognosis and drug response prediction has become fundamental. Regarding this point, our study shows that three novel exosomal miRNAs (miR-185-5p, miR-652-5p, and miR-1246) act synergistically to promote fibroblast transformation in the context of TNBC.
miR-185-5p has already been reported in patients with severe active alopecia to work in synergy with other miRNAs rather than alone (Sheng 2019), a finding coherent with our initial hypothesis. Furthermore, upregulation of this miRNA has been observed specifically in lymph nodes with metastases from breast cancer; similarly, it has emerged as a prognostic factor of radiation-related toxicity in the serum of patients with oropharyngeal cancer(Tomasik 2021), and as a predictive biomarker of chemotherapy response and metastasis formation in colorectal and gastric cancer (Sur 2021) (Sun 2021).
Likewise, miR-652-5p has been reported upregulated in breast malignancies. However, decreased expression of this miRNA has been correlated with esophageal carcinoma progression and recurrence (Matsui 2016) (Gao 2020). This difference could be explained by the fact that miRNAs can operate in different ways depending on the biological system they are acting in.
Regarding miR-1246, this is a well know master regulator in cancer. In particular, its upregulation has been associated with tumor growth, metastasis, and drug resistance in different types of cancer(Chen 2021, Torii 2021, Ueta 2021). In breast cancer, exosomal miR-1246 has been used as diagnostic biomarker due to its high expression specificity.
Given the considerable role of these miRNAs in cancer, the novelty of our study lies in the combined effect exerted by these exosomal miRNAs on fibroblast activation within the TME. This could reflect the existence of a specific miRNA profile in the tumor cells' exosomal cargos, with a well-defined scope of action (Kruger 2014).
Furthermore, our results demonstrated that fibroblasts activated by TNBC-derived exosomal miRNAs have subsequent pro-tumorigenic impact on breast cells. In fact, we found a cancer cell-fibroblast-cancer cells loop triggered by exosomal miRNAs, that resulted not only in the strengthening of tumoral function, but also in the fostering of pro-tumorigenic effects on non-transformed cells. As a matter of fact, conditioned medium collected from the transfection of fibroblasts with exosomal miR-185-5p, miR-652-5p, and miR-1246 improved the proliferation of luminal A MCF7 cells and induced the proliferation and the migration of normal epithelial breast MCF10A cells.

Patient-derived tumor organoids are emerging as promising tools for studying breast cancer. Thanks to the preservation of cell-cell-interaction and organ-like architecture, they have been employing for dissecting the mechanisms of cancer development and drug resistance (Yang 2020). Hence, by taking advantage of patient-derived breast cancer organoids, we investigated the effect of the activated fibroblast in a way that better resembled the tumor context compared to two-dimensional cell cultures. Indeed, our preliminary results showed that the conditioned medium from combo miRs transfection enforced the invasion potential of primary breast cancer organoids (luminal A subtype), by provoking the formation of wider invasive protrusions after 96h, but not at 72h of incubation. Even though these findings need to be confirmed, we could assume a pro-tumorigenic effect for fibroblasts activated by combo miRs. Hypothetically, this means that miR-185-5p, miR-652-5p, and miR-1246 are transferred from TNBC cells through exosomes and activate stromal fibroblasts within the TME. Furthermore, the activated fibroblasts are able in their turn to secrete factors in the extracellular space that positively influence breast cancer cell growth and invasion, as well as non- tumorigenic breast cell migration and proliferation, indicating their active involvement in the TME. Further studies are needed to better characterize tumorigenic functions for activated fibroblasts and to find specific determinants responsible of their pro-tumorigenic role within the TME.

Altogether, our results demonstrate that exosomes encourage breast cancer development by delivering specific miRNAs that stimulate the formation of a singular and aggressive TME. These findings may potentially aid the development of novel alternative strategies for TNBC management, since exosomal miRNAs have already shown promising application in cancer diagnosis and prognosis. Better comprehension of the mechanisms underlying the behavior of CAFs within the context of a tumor may help to adapt them for specific clinical benefits.

6. CONCLUSIONS

In this study we examined the relevance of triple negative breast cancer exosomes and the miRNA cargo they hold in the re-education stromal fibroblasts within the tumor microenvironment context. We found that miR-185-5p, -652-5p, and -1246, found upregulated in fibroblasts upon breast cancer exosome exposure, synergistically induced a pro-migratory functional state in stromal fibroblasts, that in turn promote breast cell growth, migration, and invasion. Our findings supported the notion that exosomes mediate the construction of a forceful TME that promote cancer evolution, by providing the discovery of novel miRNAs involved in these processes. Further studies are needed to elucidate the mechanisms of action of these miRNAs, including their specific target genes. Finally, our results contributed to the comprehension of the molecular mechanisms underlying TNBC progression, paving the way for the discovery of novel alternative strategies for triple breast cancer management.

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10.LIST OF PUBLICATIONS

- Exosomal microRNAs synergistically trigger stromal fibroblasts in breast cancer. Iolanda Scognamiglio, Lorenza Cocca, Ilaria Puoti, Francesco Palma, Francesco Ingenito, Cristina Quintavalle, Alessandra Affinito, Giuseppina Roscigno, Silvia Nuzzo, Rosario Vincenzo Chianese, Stefania Belli, Guglielmo Thomas Timo Schomann, Alan Chan, Maria Patrizia Stoppelli, and Gerolama Condorelli. Molecular Therapy and Nucleic Acid. 2022 June 14. doi: 10.1016/j.omtn.2022.02.013

- Modulating the Crosstalk between the Tumor and the Microenvironment Using SiRNA: A Flexible Strategy for Breast Cancer Treatment. Giuseppina Roscigno, Iolanda Scognamiglio, Francesco Ingenito, Rosario Vincenzo Chianese, Francesco Palma, Alan Chan, Gerolama Condorelli. Cancers (Basel). 2020 Dec 13;12(12):3744. doi: 10.3390/cancers12123744.

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