JOINT PHD PROGRAMME

IN MOLECULAR BIOLOGY

PhD Thesis

Mutant p53 alters tumor microenvironment by reprogramming the cancer cell secretome *via* miR-30d

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To my parents,

Rudy and Rita,

for their constant support

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ABSTRACT

Solid tumors can be considered as complex organs, in which neoplastic cells are surrounded by a tumor microenvironment (TME) that consists of an extracellular matrix (ECM) and many non-transformed cell populations (e.g. fibroblasts, endothelial cells). Cancer cells shape their TME through secretion of soluble and insoluble factors, including proteins, metabolites, extracellular vesicles (EVs), and circulating nucleic acids, among which miRNAs. The cancer secretome has both local and systemic effects on tumor growth, dissemination and metastatic colonization. Several cancer-related pathways concur to reprogram the secretome, and thereby alter the TME to sustain cancer development.

In human cancers, one of the most frequently mutated gene encodes for the transcription factor and tumor suppressor TP53. The majority of these mutations occur within the DNA binding domain of TP53, leading to expression of mutant p53 (mut-p53) proteins that not only lose the oncosuppressive features of the wild-type form, but may also acquire novel oncogenic properties, generally referred to as gain of function (GOF). Indeed, mut-p53 proteins are capable of promoting cancer progression, metastasis and chemoresistance. mut-p53 can exert its oncogenic functions by different mechanisms, a major one being the alteration of coding and non-coding (including miRNAs) gene expression profiles.

In the laboratory where this PhD project has been developed, miR-30d was recently identified as a novel target of mut-p53 and an effector of its oncogenic functions in breast cancer cells. In an attempt to dissect the molecular mechanisms underlying the effects exerted by the mut-p53/miR-30d axis, we have demonstrated that miR-30d impacts the cancer secretome, suggesting an effect on TME remodeling.

Starting from these observations, here I provided evidence that the mut-p53/miR-30d-dependent secretome is able to promote *in vitro* paracrine effects towards different components of TME, such as ECM, endothelial cells, fibroblasts, as well as other cancer cells. Moreover, the mut-p53/miR-30d axis also contributes to the loading of proteins within EVs. In addition, I showed that miR-30d secreted by cancer cells can exert a pro-oncogenic effect in non-transformed receiving cells and may represent a promising secreted biomarker associated with p53 status *in vivo*.

This study provides new insights into the mechanisms by which mut-p53, through miR-30d induction, can exert pro-tumorigenic functions in a paracrine fashion, and highlights potential non-invasive biomarkers and therapeutic targets to treat tumors harboring mut-p53.

List of abbreviations

α-SMA	alpha smooth muscle actin
AFM	atomic force microscopy
BMDC	bone-marrow-derived cell
CAF	cancer-associated fibroblast
CCL	CC-chemokine ligand
CM	conditioned medium
CXCL	CXC-chemokine ligand
DC	dendritic cell
dy	decoy
ECM	extracellular matrix
EMT	epithelial-to-mesenchymal transition
EV	extracellular vesicle
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GO	gene ontology
GOF	gain of function
GSEA	gene set enrichment analysis
HIF	hypoxia-inducible factor
HSP	heat-shock protein
HUVEC	human umbilical vein endothelial cell
IL	interleukin
LOX	lysyl oxidase
MDSC	myeloid-derived suppressor cell
miRNA	microRNA
MMP	matrix metalloproteinase
mut-p53	mutant p53
NK	natural killer cell
PDGF	platelet-derived growth factor
PMN	pre-metastatic niche
SASP	senescence-associated secretory phenotype
TAM	tumor-associated macrophage
TAZ	transcriptional coactivator with a PDZ-binding domain
TCA	trichloroacetic acid
TGF	transforming growth factor
TME	tumor microenvironment
TNFα	tumor necrosis factor alpha
Treg	T regulatory cells
VEGF	vascular endothelial growth factor
YAP	yes-associated protein

1. Introduction

Cancer is nowadays the second leading cause of death worldwide, following cardiovascular disease, with 9.6 million deaths estimated in 2018[1]. Tumor development involves a multistep process of genetic mutations and epigenetic changes that incites normal cells to progressively evolve towards a neoplastic state. Along this process, cancer cells acquire common features, known as the "Hallmarks of Cancer", including sustained proliferative signaling, evasion of growth suppressive checkpoints, resistance to cell death, replicative immortality, induction of angiogenesis, activation of invasion and metastasis, deregulation of cellular metabolism and evasion of the immune system[2].
The biology of tumors involves complex interactions that are established between cancer cells and the surrounding environment and are essential for cancer progression. The underlying mechanisms and involved cell types are not yet fully known and have to be explored. The characterization of these processes promises to shed new insights for therapeutic treatments against tumor development.

1.1 The cancer ecosystem

Cancers are complex organ-like systems, where cancer cells undergo heterotypic and dynamic interactions with other tumor and non-tumor cells both contributing to the acquisition of oncogenic traits, similarly to natural ecosystems[3–5]. Studying cancer from an ecological perspective implies that tumor phenotypes are not fully tumor cell-autonomous, but rather that functional cooperative networks exist among multiple subpopulations[4], including crosstalk between tumor cells inside the tumor mass and between tumor cells and stromal cells, which cooperate to shaping the tumor microenvironment (TME).
Importantly, the relationship between tumors and their surrounding microenvironment has a dual nature. In fact, microenvironment can both support cancer growth and survival, and, vice versa, limit it, competing for the availability of vital resources (e.g. oxygen, nutrients, metabolites) or triggering an immune response. Furthermore, non-cell-autonomous interactions between cancer cells and TME regulate tumor evolution. As the cancer progresses, leading to dissemination and colonization of secondary sites, the surrounding microenvironment co-evolves through continuous paracrine communication, creating dynamic and heterotypic signaling entities[6].

1.2 The tumor microenvironment (TME)

1.2.1 Components of the primary tumor microenvironment

The TME is composed by a set of various stromal cells, such as fibroblasts, immune cells, blood and lymphatic vessels, pericytes and eventually adipocytes (Figure 1), enclosed in a heterogeneous extracellular matrix (ECM)[7].

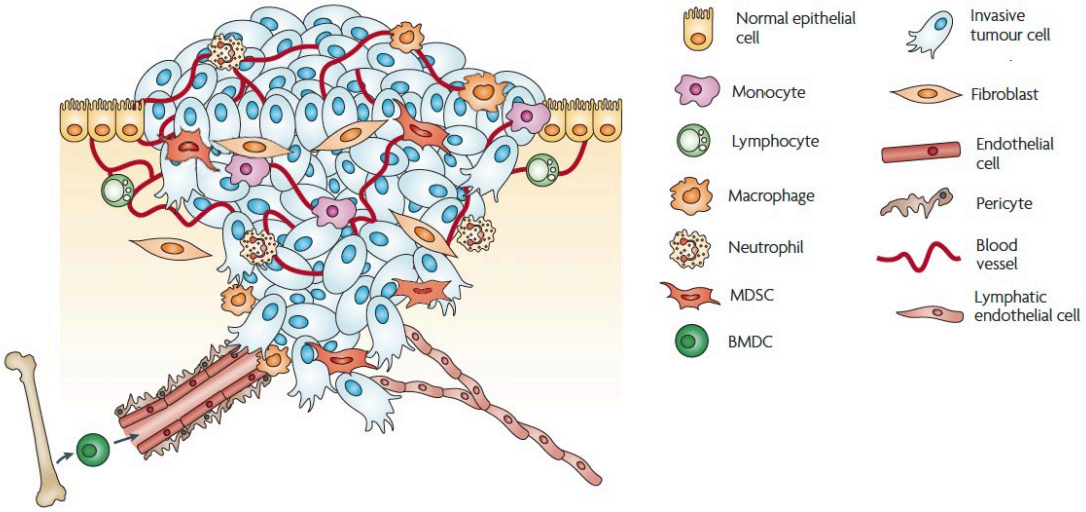

Figure 1. The primary tumor microenvironment. Cancer cells in primary tumors are surrounded by a complex microenvironment comprising numerous cells including a variety of immune and inflammatory cells, endothelial cells of the blood and lymphatic circulation and stromal fibroblasts. Moreover, bone-marrow-derived cells (BMDCs) can move from bone marrow to primary tumor site. (modified from [8])

The type, activity and evolution of the cells in the TME resemble the processes of wound healing and inflammation. In fact, during classic wound healing response in a tissue, fibroblasts, inflammatory and immune cells are recruited, promoting angiogenesis and deposition of ECM to repair the wound. Likewise, accumulation of cancer cells in a given tissue causes a persistent tissue injury, thus initiating a chronic wound healing response, known as cancer fibrosis. Indeed, tumors are often described as "wounds that do not heal"[9]. In addition, cancer resembles a site of chronic inflammation, since neoplastic tissues are infiltrated by immune and inflammatory cells that can enhance tumor progression, by releasing bioactive molecules and ECM-modifying enzymes. Therefore, inflammation is recognized as an enabling characteristic of cancer[2].

The origin of cancer-associated stromal cells is still controversial. They predominantly derive by proliferation of existing cells from the normal tissue adjacent to the tumor, or by differentiation of local stem/progenitor cells. In recent years, it has been found that bone marrow-derived stem/progenitor cells move to the tumor, where they can differentiate into stromal cell types or persist in an undifferentiated or partially differentiated state[2,8].

The following paragraphs will present in detail those components of TME which will be further discussed in this thesis.

1.2.1.1 The extracellular matrix (ECM)

The ECM is composed of two main classes of macromolecules: proteoglycans and fibrous proteins, among which the most important are collagens, elastins, fibronectins and laminins[10]. Collagen and elastin fibers are reoriented and cross-linked by the lysyl oxidase (LOX) enzyme family (including LOX and LOXL) and transglutaminase, forming larger and more rigid fibrils. Cell adhesion to the ECM is mediated by ECM receptors, such as integrins, discoidin domain receptors and syndecans. A bidirectional interdependence exists between cells and the surrounding ECM: cells constantly secrete and remodel ECM, whereas the latter in turn signals back to the cells and influences their features and activities[11].

ECM components confer unique physical, biochemical and biomechanical properties that are essential for regulating cell behaviour[12]. Physical properties include rigidity, porosity, insolubility, spatial arrangement, orientation and other features that together define the scaffold supporting tissue architecture and integrity. Biochemical properties include ECM-derived chemical stimuli that initiate signal transduction cascades. Biomechanical properties related to elasticity of the ECM, ranging from soft to stiff, represent a cue that is perceived by the cells. The ECM also regulates the availability of growth factors and cytokines and maintains the hydration and pH of the local microenvironment.

The ECM is a highly dynamic structure that is constantly being remodeled and its molecular components are subjected to a myriad of post-translational modifications[10]. Tumors often display desmoplasia, characterized by increased deposition, altered organization and enhanced post-translational modifications of ECM proteins[12] (Figure 2). In particular, accumulation of collagens I, II and III, fibronectin, elastin, and changes in the levels and composition of proteoglycans have been observed in tumors[13]. Moreover, collagens are organized into thick linearized bundles. Abnormal changes in the amount and composition of the ECM can largely alter both its biochemical and physical properties, through reorientation of collagen fibers, ultimately potentiating the oncogenic effects of various signaling pathways. Tumors are usually stiffer than the surrounding normal tissues[14–16]. For example, breast cancer tissue can be up to 10-fold stiffer than normal breast tissue, on the order of 5 kPa and 0.4 kPa, respectively, as measured by Atomic Force Microscopy (AFM). Part of the increased stiffness is due to elevated activity of LOX, LOXL1 and LOXL2, that create the thick linearized collagen fibrils[13,14].

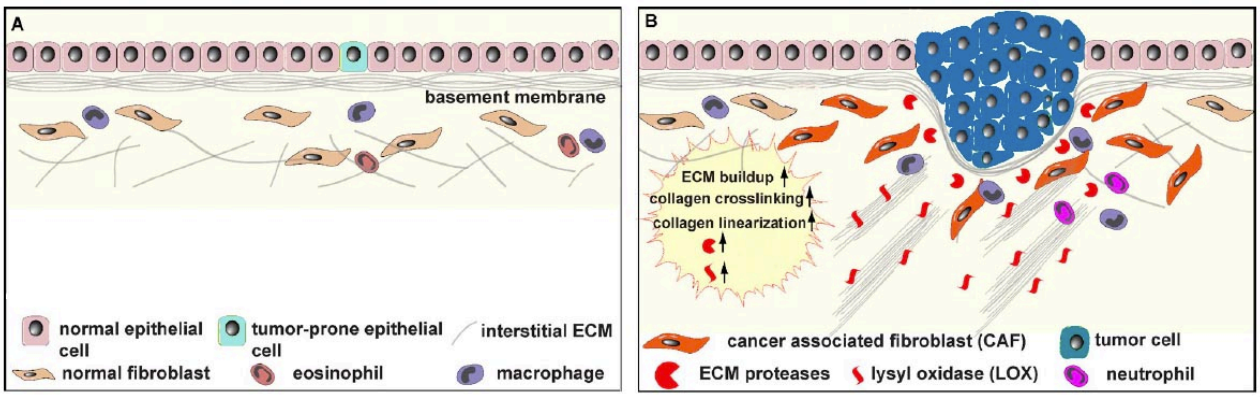

Figure 2. Differences between normal and cancer-associated ECM. Normal ECM (in A) is essential for maintaining tissue integrity and an overall healthy microenvironment. During tumorigenesis, the ECM undergoes important changes (in B). In particular, there is an increased ECM deposition, accumulation and reorganization of collagens fibers, deregulated expression of ECM remodeling enzymes (such as proteases or LOX). This aberrant ECM leads also to increased stiffness of the tissue. (modified from [12])

1.2.1.2 Cancer-associated fibroblasts (CAFs)

Fibroblasts are the major cell type in connective tissue, whose functions include deposition of ECM and basement membrane components, regulation of epithelial differentiation and modulation of immune response[17]. In normal tissue, fibroblasts are quiescent and become activated as part of wound healing responses to support the repair of injured tissue[18]. Activated fibroblasts acquire a stellate or cruciform shape, migratory and proliferative abilities, and the capacity to produce an active secretome (Figure 3). Due to a chronic wound healing response, TME is characterized by an aberrant number of activated fibroblasts that are called cancer-associated fibroblasts (CAFs) and are clearly distinct from normal fibroblasts from a molecular and phenotypic viewpoint.

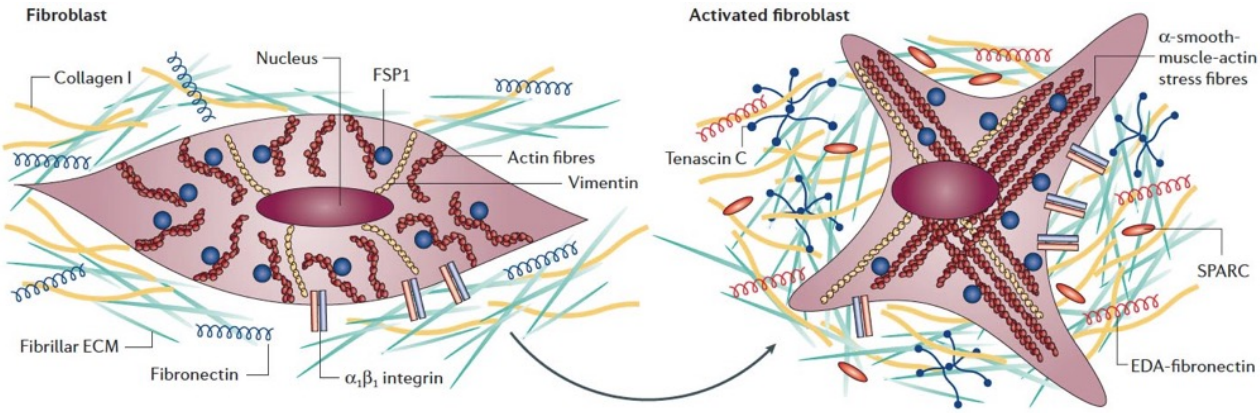

Figure 3. **Activation of fibroblasts.** Normal quiescent fibroblasts are embedded within the ECM of stromal tissue, interacting with their surrounding microenvironment through integrins. They typically appear as fusiform cells with a prominent actin cytoskeleton and vimentin intermediate filaments. Upon different stimuli, fibroblasts can acquire an activated phenotype, which is associated with an increased proliferative activity and enhanced secretion of ECM proteins such as collagens, fibronectin (EDA-fibronectin, that contains the extra domain A) and matricellular proteins (tenascin C and SPARC). Phenotypically, activated fibroblasts are commonly characterized by the expression of α-smooth-muscle actin. (adapted from [17])

Activated fibroblasts are commonly identified by their expression of α-smooth muscle actin (α-SMA), a cytoskeletal protein associated with smooth muscle cells[19]. In addition to α-SMA, other important, but not specific, markers of activation are fibroblast-specific protein 1 (FSP1; also known as S100A4), vimentin, fibroblast activation protein (FAP), platelet-derived growth factor receptor α (PDGFRα), PDGFRβ, desmin and discoidin domain-containing receptor 2 (DDR2)[18]. Once activated, CAFs express distinct arrays of markers, creating a large degree of heterogeneity. Nevertheless, a common feature of activated fibroblasts is an increased production of constituents of the ECM and basement membranes, such as collagens (type I, III, IV and V), many different laminins, fibronectin and matricellular proteins, contributing to abnormal ECM stiffening[18]. CAFs participate also to ECM remodeling by producing ECM-degrading proteases such as the matrix metalloproteinases (MMPs) MMP3 and MMP1. After activation, CAFs actively secrete pro-tumorigenic growth factors, including VEGF-A, cytokines and chemokines, which affect immune cells' recruitment, activation and polarization, thereby promoting an immunosuppressive TME[18,20].
CAF activation requires also a transcriptional reprogramming, leading to deregulation of Notch and p53 signaling pathways[21] and induction of the transcription cofactors Yes-associated protein (YAP)/transcriptional coactivator with a PDZ-binding domain (TAZ)[22]. Notably, YAP/TAZ, downstream effectors of the Hippo signaling pathway, are key sensors as well as mediators of mechanical signals exerted by the tissue environment, including ECM[23]. Activated YAP/TAZ are required for CAFs to promote ECM remodeling, supporting invasion and angiogenesis of cancer cells[22]. Moreover, YAP regulates multiple factors that modulate both matrix stiffness and cell mechanoresponsiveness, which further enhance YAP protein levels, establishing a feed-forward loop that maintains the CAF phenotype.
Activation of fibroblasts involves also metabolic rewiring, with an increased reliance on aerobic glycolysis instead of oxidative phosphorylation (Warburg effect), coupled with enhanced catabolic activity and autophagy. This metabolic adaptation may have a crucial role in redistributing nutrients for other cells of the TME and cancer cells. The drivers of this metabolic shifts are transforming growth factor β (TGF-β), PDGF, hypoxia-inducible factor 1α (HIF-1α) and reactive oxygen species (ROS)-mediated suppression of caveolin 1 (CAV1)[24–26].

1.2.1.3 The blood vascular network

Tumor vascularization requires the cooperation of vascular endothelial cells and pericytes[27]. The former create tight adhesions to guarantee vessel integrity, the latter provide vessel coverage and paracrine support signals.

Angiogenesis is a well-known hallmark of cancer[2], responding to the increased requirement of oxygen and nutrients of a growing tumor. Angiogenic signals due to hypoxia or deriving from malignant or inflammatory cells, trigger an "angiogenic switch" in quiescent endothelial cells[27,28]. In this way, endothelial cells detach their junctional adhesions from neighbor cells, proliferate to form temporary tubes, recruit pericytes to provide stability and, finally, remodel to create a new network. Remarkably, the structure of tumor vasculature is abnormal[29]. Blood vessels are heterogeneous and leaky, with chaotic branching structures and a rough lumen. Moreover, a reduced coverage of tumor vessels by pericytes destabilizes vascular integrity and function. Vascular leakiness increases the interstitial fluid pressure altering blood flow, oxygenation, nutrient and drug distribution in the TME[7].

1.2.1.4 Other stromal cells

Immune cells present in tumors belong to both adaptive and innate systems[7,8]. Some cell types exert an immunosuppressive function, such as $CD8^+$ and $CD4^+$ T cells, natural killer (NK) and dendritic cells (DC). Conversely, other immune cells are able to promote tumor development, as in the case of T helper, T regulatory cells (Tregs), and B lymphocytes. Tumor-associated macrophages (TAMs) are crucial for tumorigenesis[30]. Indeed, macrophages are functionally plastic, altering their polarization status from M1 to M2. While M1 macrophages produce type I proinflammatory cytokines with a tumoricidal role, on the contrary M2 macrophages produce type II cytokines and are anti-inflammatory and pro-tumorigenic[31]. TAMs resemble M2 macrophages and they influence tumor biology by producing growth-inducing molecules, regulating inflammatory response and adaptive immunity, enhancing angiogenesis and promoting ECM deposition and remodeling. Furthermore, myeloid-derived suppressor cells (MDSCs) also play a key protumorigenic role[32]. They are functionally defined as immature immunosuppressive myeloid cells that maintain normal tissue homeostasis. During tumorigenesis, MDSCs are mobilized to primary tumor sites, where they promote vascularization and block the principal mechanisms of immune suppression.

In addition to blood vascular network, functional lymphatic vessels can be found at the tumor margin[33]. Indeed, tumors can drive peritumoral lymphangiogenesis to facilitate the spreading of metastases in the draining lymph nodes.

A growing body of evidence demonstrates that the adipose tissue has an important role in boosting cancer development, particularly establishing a highly proinflammatory environment and secreting many different cytokines, chemokines and hormone-like factors[34]. Intriguingly, several reports highlighted a role in tumorigenesis for neuroendocrine cells, which are able to influence the function of the immune system[34].

1.2.2 The TME at metastatic sites

The great majority of cancer-associated deaths (about 90%) are caused by metastatic disease rather than by the primary tumor. The dissemination of cancer cells from primary tumors and their subsequent seeding in distant tissues involves a multi-step process known as the invasion-metastasis cascade[35,36]. One of the initiating steps of this cascade is the epithelial-to-mesenchymal transition (EMT), during which cancer cells lose epithelial markers and gain mesenchymal traits that confer stem-like and migratory properties[37]. After invasion of the surrounding stromal tissue cancer cells intravasate into the circulatory system as circulating tumor cells (CTCs) and finally colonize a foreign microenvironment in a distant tissue[36].

The microenvironment plays a central role in all the steps of the metastatic cascade[38]. Particularly, entry and colonization of CTCs into secondary and/or distant organ sites are key steps in the metastatic cascade and are critically affected by the local microenvironment, which determines whether or not tumor cell colonization will occur. The "seed and soil" hypothesis proposed by Stephen Paget more than a century ago tried to explain the organ tropism of metastasis[39]. Pro-metastatic cancer cells (the "seed") colonize specific organ sites (the "soil") bearing a favorable microenvironment. Some reports have in fact demonstrated that primary tumors can release factors and microvesicles in the bloodstream, able to modify the microenvironment in a secondary site in a way that it will be supportive for survival and growth of tumor cells before their arrival. This predetermined microenvironment is defined the "pre-metastatic niche" (PMN)[40,41]. Similarly to the primary tumor microenvironment, the PMN is characterized by an alteration of locally resident cells, such as fibroblast activation, and recruitment of BMDCs, including VEGFR-1$^+$ hematopoietic progenitor cells, CD11b$^+$ myeloid cells, and regulatory and suppressive immune cells (i.e. MDSCs, TAMs, Tregs)[40]. ECM remodeling as well is pivotal for establishing the PMN. It is achieved by two mechanisms: deposition of new ECM components (e.g. periostin, versican, tenascin C) and alteration of the physical properties of pre-existing ECM at PMN (e.g. collagen I crosslinking by LOX results in increased stiffness[42]).

1.3 Cancer-derived mediators of communication within the TME

Within solid tumors, communication between cancer cells and TME is extremely important for tumor progression and establishment of TME itself. Although heterotypic communication can occur in a contact-dependent manner by cell-cell interactions mediated by membrane receptor proteins or through gap junctions, it is mainly realized in a paracrine fashion, through secretion of soluble and insoluble factors, collectively called secretome[43]. The secretome consists mainly of proteins, but includes also lipids, metabolites, extracellular vesicles (EVs) with their cargo of proteins,

microRNA and mRNA. The secreted proteins in humans account for 13-20% of the entire cellular proteome and include all the proteins secreted either with the classical secretory pathway or unconventional mechanisms (e.g. proteins present within EVs accounts for up to 42% of the secretome[44]).

The secretome is involved in several physiological processes and is tightly regulated. Each cell type displays a specific secretome signature, which can change in response to pathological conditions, including cancer, which is characterized by an aberrant pattern of secretion[45]. The cancer secretomes have been shown to have both short-range and systemic effects, allowing the formation of a supportive and reactive microenvironment around the primary tumor and of a PMN, respectively[46].

1.3.1 Tumor-derived secreted molecules

Cancer cells secrete a plethora of both protein (i.e. cytokines, chemokines, growth factors, proteases and ECM components) and non-protein molecules (e.g. metabolites and lipids), overall able to shape the surrounding primary TME[43,44].

Cytokines, low-molecular-weight proteins, are well-known mediators of inflammation and are also involved in tumor growth, EMT, angiogenesis, and metastasis[47]. They can modulate both a pro- and anti-tumor response, depending on the balance of pro- and anti-inflammatory cytokines, expression of their receptors and the activation state of surrounding cells. The most important cytokines involved in tumor development are tumor necrosis factor alpha (TNFα), transforming growth factor β (TGF-β), interleukin 1 (IL-1), IL-6, and IL-10.

Chemokines are small secreted peptides that mediate the chemotactic migration of cells expressing appropriate chemokine receptors[48]. Among the most common, there are CC-chemokine ligand 2 (CCL2, also known as MCP1), CCL5, CCL21, CXC-chemokine ligand 12 (CXCL12, known also as SDF-1), CXCL1, CXCL2, CXCL8. Their principal function is to mediate immune cells and BMDCs recruitment within TME.

The main growth factors present in the extracellular milieu are epidermal growth factor (EGF), transforming growth factor α (TGF-α), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and vascular endothelial growth factor A (VEGF-A)[49]. VEGF-A is the major angiogenetic growth factor in the TME and together with EGF, PDGF, TGF-β and chemokines, stimulate endothelial cells and pericytes during neoangiogenesis[28].

Proteases can be present in the extracellular space either associated to membranes or secreted. The most relevant within the TME are matrix metalloproteinases (MMPs) and ADAMs (A Disintegrin And Metalloproteinase), cathepsins and urokinase plasminogen activator (uPA)[50]. They are

responsible for pericellular proteolysis, leading to ECM degradation and cleavage of cell adhesion molecules, growth factors, cytokines and kinases. In this way, they allow cancer cells to proliferate, survive, locally invade, colonize distant organs and, in addition, they can regulate angiogenesis and immune response.

Besides structural ECM components and enzymes (e.g. LOX family), cancer cells secrete also matricellular proteins, not playing a primary role in ECM structure but able to modulate cell interactions and functions[51]. In cancer, matricellular proteins are involved in different steps of tumorigenesis due to their ability to bind different cell receptors[52]. The matricellular protein family includes thrombospondins, tenascins, SPARC (Secreted Protein Acidic And Cysteine Rich), periostin, osteopontin and CCN proteins (among which CYR61 and CTGF)[53].

Cancer cells undergo global metabolic reprogramming and, as a result of an accelerated and imbalanced metabolism, tumor-derived metabolites accumulate in the TME. Strikingly, recent evidence suggests that such metabolites can act as signaling molecules, impairing antitumor immunity[54,55]. In fact, increased secretion of lactate by cancer cells can repress monocytes and T cells[56,57], promote M2 macrophages polarization[58] and furthermore modulate angiogenesis in endothelial cells[59,60]. An immunosuppressive function has been showed also for extracellular adenosine and its associated nucleotides (i.e. ATP, ADP, AMP), which can impinge on DCs and T cells activity[61]. In addition, cancer cells secrete in the extracellular milieu bioactive lipid mediators capable of mediating tumor-stroma communication[62]. The most characterized are prostaglandin E2 (PGE_2), which stimulate angiogenesis and immunosuppression, and sphingosine-1-phosphate (S1P), which is involved in angiogenesis and lymphangiogenesis.

All these factors secreted by the primary tumor can play critical roles in preparing the PMN, exerting a systemic effect[46]. This was firstly shown by Kaplan and colleagues, by injecting a Lewis lung carcinoma (LLC) cell line into the flank of syngeneic mice pretreated with melanoma conditioned medium (CM)[63]. LLC cells normally metastatize only to the lung, but in melanoma CM-treated mice, their metastatization was redirected to organs typically associated with melanoma metastasis, demonstrating the essential role of tumor-secreted factors in metastatic organotropism. One of the major functions of secreted factors is to recruit BMDCs in host tissue in order to form the PMN and favor the homing of cancer cells[41,64]. For example it has been reported that secretion of VEGF-A, TNFα and TGF-β by the primary tumor induces expression of the inflammatory chemoattractants S100A8 and S100A9, which in turn recruit $CD11b^+$ myeloid cells to premetastatic lung[65]. Once secreted into circulation from primary breast tumors, LOX colocalized with fibronectin at sites of future metastasis where it served to crosslink collagen IV in the lung basement membrane, increasing the presence of $CD11b^+$ myeloid cells[66]. Noteworthy, ATP or UTP

released from highly metastatic breast cancer cells activate the $P2Y_2$ receptor to facilitate PMN formation by mediating LOX secretion, collagen crosslinking and monocytes recruitment[67].

1.3.2 Extracellular vesicles (EVs)

EVs are membrane-encapsulated particles released by any type of cell into the extracellular space and are detectable in various body fluids, such as blood, urine and milk[68]. EVs are involved in intercellular communication at both the local and the systemic level. They participate in a plethora of physiological processes (e.g. coagulation, immunity, stem cell differentiation, tissue regeneration, autophagy, pregnancy and embryology) and also pathological conditions, such as neurodegenerative diseases and cancer[69,70]. In the latter context, tumor-secreted EVs are involved in a wide range of oncogenic processes, including inflammatory response, angiogenesis, cell migration and proliferation, invasion and EMT, metastasis. Indeed it has been demonstrated that cancer cells secrete more EVs than non-transformed cells[71].

Biogenesis and characteristics of EVs

Based on the current knowledge of their biogenesis, EVs can be divided into two main categories: exosomes and microvesicles (also commonly called ectosomes or microparticles or oncosomes)[72]. Exosomes are small vesicles, ranging from 30 to 150-200 nm in diameter, with a lipid bilayer membrane. They derive from intraluminal vesicles (ILVs) formed by the inward budding of endosomal membrane during maturation of multivesicular bodies (MVBs), which are intermediates of the endosomal system, and secreted upon fusion of MVBs with the cell surface (Figure 4.A). Their biogenesis and release are modulated by the endosomal sorting complex required for transport (ESCRT) machinery and the ceramide-dependent pathway[73,74]. Instead, microvesicles range in size from 100 nm to 1000 nm in diameter, but can be even larger (up to 10 μm) in the case of oncosomes[75]. They are generated by the outer budding and fission of the plasma membrane and the subsequent release into the extracellular space. Their biogenesis involves plasma membrane reorganization, redistribution of phospholipids, disassembly of the cytoskeleton network, and contraction of the actin-myosin machinery (Figure 4A).

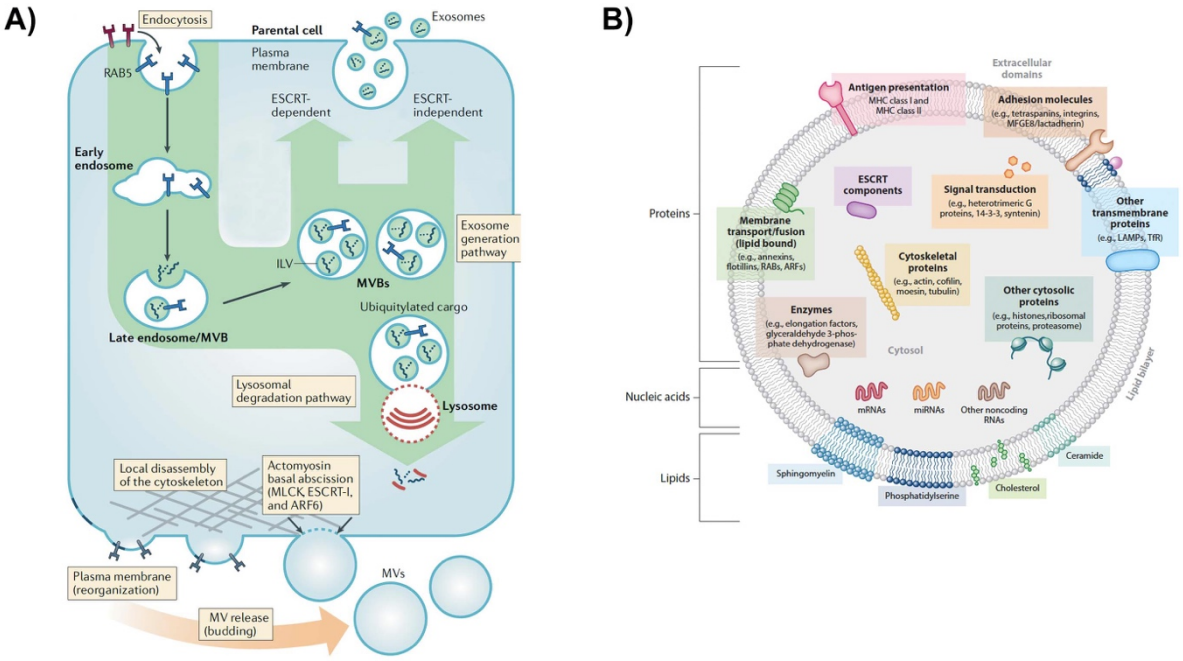

Figure 4. EVs biogenesis and cargo contents.
A) Schematic overview of the biogenesis pathways of EVs in a cell. Exosome biogenesis, in the upper part, starts from late endosome/MVBs (derived from the endocytic process), in which the inward budding of the membrane forms intraluminal vesicles (ILVs). MVBs traffic to and fuse with the plasma membrane to release ILVs into the extracellular space, where the ILVs are recognized as exosomes. Two pathways, ESCRT-independent or ESCRT-dependent, operate in parallel for exosomes generation. Alternatively, MVBs can fuse with lysosomes, resulting in degradation. The biogenesis and release of microvesicles, in the lower part, includes the reorganization of plasma membrane, with the repositioning of phosphatidylserine to its outer layer. These particular domains are then released from cell as microvesicles via direct budding through ARF6 and rearrangement of the actin cytoskeleton. (modified from [70])
B) A schematic representation of the overall content of both EVs. In addition to their different sizes, EVs differ only partially for their compositions in terms of proteins families, lipids and nucleic acids. Note that each listed component may be present only in one of the two subtypes of EVs. (adapted from [76])

EVs are carriers of lipids and proteins[76] (Figure 4B). This content can vary widely between cells and conditions, directly affecting their fate and functions. However, the two EV types share some properties and differ in many others. Both EVs have high levels of cholesterol, sphingomyelin, ceramide and phosphatidylserine. Instead, exosome membranes are enriched in tetraspanins (such as CD63, CD9, CD81), flotillin, adhesion molecules (e.g. ICAM-1), integrins, proteoglycans (e.g. Glypican-1), while that of microvesicles abound with some receptors, glycoproteins and metalloproteinases. The cargoes enclosed within the lumina of all EV types contain many cytosolic proteins, most of which have cytoskeletal function (e.g. actin, tubulin, annexin). Classical exosome cargoes include heat shock proteins (HSP70 and HSP90), synthesis proteins (Alix and TSG101) and enzymes (pyruvate kinase, enolase, GAPDH). It has been demonstrated that EVs carry also nucleic acids; their function and role in cell-to-cell communication will be further analyzed in the next paragraph.

Roles of EVs in cancer progression

EVs can fuse with recipient cells in the TME, both in the vicinity (horizontal transfer) and in a distant tissue. As a consequence, their molecular cargoes are transferred inside the receiving cells, eliciting changes in function and gene expression. For these reasons, EVs acquire a pivotal role in all the steps of cancer progression, from primary tumor development to PMN formation and metastasis.

Tumor EVs exert complex effects on neighboring cells. Tumor-secreted EVs participate in the horizontal propagation of oncogenic molecules (e.g. EGFRvIII and mutant KRAS) between cancer cells within the primary tumor[77,78]. Noteworthy, in breast cancer and hepatocellular carcinoma, cancer-derived EVs might induce transforming effects when taken up by normal cells of the same tissue[79,80].

Tumor EVs influence also surrounding stromal cells. Exosomes from prostate cancer and mesothelioma cell lines contain TGF-β that activate normal fibroblasts into CAFs[81,82]. Several studies reported the ability of EVs to promote both proliferation and proangiogenic activity in endothelial cells[83,84]. In various cancer contexts EVs modulate evasion from immune surveillance, mainly through the loading of several immunoregulatory molecules which can induce modifications in the maturation and activity of different cells of the immune systems[83,84]. On the other hand, EVs can stimulate immunity against cancer because they express MHC class I molecules and/or tumor antigens which can be transferred to DCs for antigen presentation and induction of specific anti-tumor immune responses[83,85].

Furthermore, EVs can promote local tumor invasion through ECM remodeling achieved with bioactive cargoes, such as Annexins, α3 integrins, ADAM10, ARF6, Cav-1, MMP9, MMP2[86,87].

Over the last 10 years, great importance has been given to circulating EVs that can mediate the reprogramming or education of multiple cell types, such as fibroblasts, endothelial cells, macrophages and BMDCs at distant sites, creating the PMN. For example, melanoma-exosomes containing the receptor tyrosine kinase MET foster PMN formation in the lung through the reprograming of BMDCs[88]. These exosomes also promote vascular leakiness in the lung, dysregulated ECM remodeling and increased expression of inflammatory proteins, which resulted in the recruitment of reprogrammed BMDCs at lung PMN. In pancreatic tumors, exosomes loaded with macrophage migration inhibitory factor MIF promote TGF-β expression and secretion in Kupffer cells, the resident macrophages in the liver, stimulating neighboring hepatic stellate cells to secrete fibronectin[89]. These cells subsequently facilitate the recruitment of BMDCs and ultimately complete PMN formation. Remarkably, exosomes can dictate metastasis organotropism. Hoshino *et al.* demonstrated that exosomes from breast cancer express a unique integrin profile that addresses

them to specific target organs, where they prepare the PMN by fusing with resident cells and upregulating pro-inflammatory S100 molecules[90].

There is also evidence about the role of tumor-derived microvesicles in PMN formation, for example contributing to BMDCs mobilization through osteopontin[91] or promoting endothelial cell activation mediated by AKT phosphorylation[92]. Tumor-derived large oncosomes containing metalloproteinases, CAV-1 and ARF6, are biologically active toward tumor cells, endothelial cells as well as fibroblasts at distant sites[87].

1.3.3 Cancer-secreted nucleic acids and microRNAs

Circulating nucleic acids are known to be present in human blood, reflecting physiological and pathological conditions, including cancer[93]. They have become increasingly popular due to their potential use as a "liquid biopsy", which is a tool for non-invasive diagnosis, prognosis and monitoring of diseases, in particular of cancer. In addition, recent evidence indicates active trafficking of nucleic acids between cells, highlighting their involvement in cell-to-cell communication[94].

In order to be protected from nuclease-mediated degradation in the extracellular space and in body fluids, most of circulating nucleic acids (including DNA fragments, mRNAs and noncoding RNAs (ncRNAs)), are loaded into EVs[95,96] and, in this way, can shuttle between cells[97].

Tumor exosomal DNA may reflect the oncogenic mutational status of parental tumor cells and affect the metastatic behaviors of recipient cancer cells once transferred. For example, medulloblastoma-derived microvesicles contain high levels of single-stranded DNA fragments, which amplify the oncogene c-Myc[98]. Full length or sometimes fragmented mRNA are carried by EVs to recipient cells, in which they can be actively translated[97]. mRNAs in cancer-derived EVs can promote angiogenesis and PMN formation[99–101].

Among secreted ncRNAs, microRNAs (miRNAs), long ncRNAs (lncRNAs) and circular RNAs (circRNAs) have been demonstrated to be involved in cell-to-cell communication[95], the most extensively studied class being that of miRNAs. Secreted miRNAs were found in several body fluids, in which they operate as hormone-like molecules to influence the behaviors of different cells in a paracrine or endocrine manner[102]. The most well-studied way of extracellular miRNAs secretion is the packaging within exosomes, but they are detected also in complexes with the proteins Argonaute 2 (AGO2)[103,104] or nucleophosmin 1 (NPM1)[105] or with high density lipoproteins (HDLs)[106]. Owing to their high abundance and stability, circulating miRNAs carry potential utility as non-invasive, blood-based biomarkers that can provide information on tumor biology and the effects of treatments. Cancer-secreted miRNAs can be delivered to other cells in the

TME where they can finely modulate target genes expression, thus reprogramming the TME and favoring cancer progression. Further, tumor-derived exosomes, but not those released by normal cells, contain key enzymes involved in miRNA biogenesis, which enables cell-independent miRNA biogenesis within exosomes[107].

Secreted miRNAs at primary tumor sites have been shown to affect several processes, among which invasion (miR-10b), activation of fibroblasts (miR-155), angiogenesis (miR-21, miR-494, miR-9, miR-210), and modulation of immune response (miR-150, miR-203, miR-23 and others)[84,108]. Interestingly, tumor-secreted miR-21 and miR-29a in exosomes bind to the highly conserved pathogen recognition Toll-like receptor 8 (TLR8) in macrophages, stimulating downstream activation of the NF-κB pathway and secretion of inflammatory cytokines[109]. Recently, it has been demonstrated that MYC activity in breast cancer cells leads to the secretion of miR-105-containing exosomes, which, taken up by surrounding CAFs, activates in turn a MYC-driven metabolic program that fuels cancer cell growth by releasing important metabolites in the TME[110].

In the last years, great attention was given to the contribution of miRNAs packaged inside EVs on metastasis dissemination, seeding and outgrowth. Metastatic breast cancer cells that secrete miR-105-containing exosomes cause vascular permeability by destroying tight junction protein ZO1 in recipient endothelial cells[111]. Instead, miR-181c from breast cancer-derived EVs is able to disrupt the permeability of endothelial cells of the blood-brain barrier[112]. Several secreted miRNAs, such as miR-494, miR-542p, miR-1247-3p, can participate in the formation of a reactive PMN[113,114]. In particular, breast cancer-derived microvesicles transfer miR-122 to stromal cells (i.e. lung fibroblasts, brain astrocytes and neurons) where, by inhibiting pyruvate kinase and thus preventing glucose uptake from the PMN, they increase glucose availability for the metastatic cancer cells[115]. Moreover, large oncosomes can mediate intercellular transfer of miR-1227 from tumorigenic prostate cells to CAFs, enhancing their migration to secondary sites for PMN formation[116].

1.4 Regulation of cancer secretome reprogramming

The cancer secretome is finely tuned and actively regulated by cancer cells, in order to change their interactions with the TME for the ultimate purpose of enhancing tumor growth and progression. This reprogramming of the secretome is modulated by cancer cells in response to various stimuli and/or alterations that arise from both extracellular and intracellular sources.

For instance, the extracellular environment is characterized by the presence of biomechanical forces, such as solid stresses, increased matrix stiffness and abnormal interstitial fluid pressure and flow[117]. These biomechanical forces can impinge on the behavior of cancer cells and on their interactions with the TME. In fact, solid stress and ECM stiffness activate the cellular production

and secretion of WNT family proteins[13]. Moreover, ECM stiffness can release latent TGF-β from the ECM, increasing its availability within TME. Tumors use the abnormal interstitial fluid flow to generate autologous gradients of soluble factors (such as cytokines) responsible for both cancer and stromal cells' migration through chemotaxis[118].

Furthermore, cancer cells have been shown to modify the surrounding microenvironment during the adaptive response to proteotoxic stress at the endoplasmic reticulum (ER). ER stress is the result of both a strong environmental pressure (such as hypoxia, glucose deprivation, oxidative stress and low pH) and a high demand in protein synthesis and secretion due to hyperproliferation[45]. To resolve this proteotoxic stress and maintain protein homeostasis, cells activate the unfolded protein response (UPR). In addition to its intrinsic effect on tumor biology, activation of UPR can modulate the expression and secretion of ECM components, MMPs, pro-angiogenic factors and pro-inflammatory as well as immunogenic molecules[119,120].

Instead, the hypoxic stress is a well-characterized extrinsic factor, which causes an adaptive response in cancer cells, with a complex and significant impact on TME that will be discuss below.

Finally, cancer cells present deregulated expression of miRNAs, which, acting as pleiotropic modulators of gene expression, allow cancer cells to transform the TME. Moreover, genetic alterations in signaling pathways presented by cancer cells exert not only a cell-autonomous effect initiating and/or promoting malignant transformation, but can also have a non-cell-autonomous effect, fostering tumorigenesis. These aspects will be addressed in detail in the following paragraphs.

1.4.1 Hypoxia

Due to rapid cancer cell proliferation and aberrant tumor vascularization, solid tumors present regions with reduced oxygen availability, namely hypoxia. Cancer cells adapt to hypoxic environment through the transcriptional activity of hypoxia-inducible factors (HIFs), that function as heterodimers: an oxygen-regulated HIF-1α (or HIF-2α) subunit and a constitutively expressed HIF-1β subunit[121]. HIFs' transcriptional activity in cancer cells mediates angiogenesis, metabolic regulation, invasion, metastasis and it has also an influence on the TME, in terms of recruitment of stromal components and regulating intercellular communication[122,123].

The principal outcome of HIFs' response is vascularization. In both tumor and stromal cells, HIF-1α promotes the secretion of angiogenetic factors, such as VEGF-A, that influences endothelial cells, pericytes and BMDCs to promote the growth of new vessels. In addition, HIF-1α regulates lymphangiogenesis by secretion of VEGF-C and PDGF-B[124].

HIF1 controls secretion of signaling molecules, such as TGF-β, bFGF, PDGF-B, to prompt activation of fibroblasts in the TME. Under hypoxic conditions, cancer cells synthesize chemokines and cytokines to recruit immunosuppressive Tregs and MDSCs, and secrete chemoattractants (e.g. SEM3A, EMAPII, ET-1 and -2) to promote the chemotaxis of macrophages from the blood[123].

Importantly, hypoxia affects ECM deposition and remodeling by inducing expression of collagene genes and regulating that of intracellular collagen-modifying enzymes and extracellular crosslinking enzymes[125]. Furthermore, hypoxia contributes to ECM degradation, upregulating the expression of MMP2, MMP9, MMP15, and of urokinase receptor (uPAR), while downregulating the tissue inhibitors of MMPs TIMP2 and TIMP3.

Hypoxic signaling may exert a long-distance effect to promote PMN formation[126]. Breast cancer cells exposed to hypoxia secrete LOX, LOXL2 and LOXL4 causing collagen remodeling in lung and, consequently, allowing the recruitment of BMDCs to this primed area[66]. Hypoxic breast tumors favor metastasis to the bone through secretion of parathyroid hormone-related protein (PTHrP) and LOX into the circulation, where they precondition the bone marrow for metastatic colonization[127,128].

Hypoxic microenvironment can control quantity and quality of EVs release. Indeed, increased quantities of exosomes are released under hypoxic conditions compared with normoxic ones[129]. Breast cancer cell lines exposed to hypoxia boost microvesicles shedding mediated by the HIF-dependent expression of the small GTPase RAB22A[130]. Hypoxia can alter the miRNAs profile within exosomes, for example promoting the release of miR-135, miR-210, miR-103a[84,131].

1.4.2 Aberrant miRNA expression

miRNAs, major players of post-transcriptional gene regulation, exert pleiotropic effects in multiple steps of cancer progression[132]. Cancer cells present aberrant miRNAs expression, due to several mechanisms, such as chromosomal deletion or amplification, mutations, epigenetic silencing and transcriptional dysregulation. Another level of regulation affects the enzymes involved in miRNAs biogenesis, namely Drosha and Dicer.

Increasing evidence show that miRNAs can have a non-cell-autonomous role in cancer cells[133,134]. In addition to their direct role in intercellular communication within TME through their secretion from cancer cells (as described in paragraph 1.3.3), altered miRNAs expression in cancer cells promotes tumorigenesis by transforming the TME.

Endothelial cells function is regulated by several miRNAs. In breast cancer cells, MYC/MYCN-induced miR-9 targets E-cadherin, which in turn activates β-catenin signaling increasing VEGF-A expression and angiogenesis[135]. miR-126 is frequently suppressed in various types of cancer and

thus it promotes endothelial cells recruitment by deregulating the signaling cascades in which its three main target genes are involved, namely *MERTK*, *IGFBP2* and *PITPNC1*[136]. The downregulation of miR-29b by the loss of GATA3 enhances angiogenesis and ECM remodeling due to the deregulation of its target (VEGF-A, ANGPTL4, PDGF, LOX, MMP9)[137].

miRNAs are important regulators of ECM formation and remodeling[138]. In nasopharyngeal carcinomas, miR-29c downregulation induces the expression of ECM proteins, including COL1A2, COL3A1, COL4A1 and laminin-γ1[139]. Furthermore, several miRNAs have been found to regulate, directly or indirectly, the expression of MMPs.

Multiple miRNAs are involved in regulation of immune and inflammatory response, mainly affecting, directly or indirectly, cytokines secretion. Downregulation of miR-126/126* levels in breast tumors lead to increased levels of both CXCL12 and CCL2 thereby promoting recruitment of inflammatory monocytes[140]. In hepatocellular carcinoma, miR-34a, suppressed by TGF-β, causes an increased production of CCL22 important for Tregs recruitment[141]. Among miRNAs which indirectly regulate cytokines release, miR-135 is worth mentioning in anaplastic large cell lymphomas[142], miR-30b and miR-30d in melanoma[143], and miR-145 in head and neck cancer[144].

Surprisingly, the miR-200 family promotes metastatic colonization of breast cancer altering the cancer cell-derived secretome by targeting *SEC23A*, which mediates secretion of metastasis-suppressive proteins, including IGFBP4 and TINAGL1[145].

1.4.3 Genetic alterations in signaling pathways

Cancer cells are characterized by genetic alterations in proto-oncogenes and tumor suppressors and epigenetic changes that confer the ability to divide and grow in an unscheduled way[2]. Genetic alterations in signaling pathways that control cell-cycle progression, apoptosis, and cell growth are common hallmarks of cancer[146]. Besides cell-intrinsic functions, these signaling pathways can affect also the TME through non-cell-autonomous effects, modulating the secretion of soluble factors and altering secretion and cargo assembly of EVs[147].

For instance, constitutively activating mutations in the tyrosine kinase domain of HER2 upregulate the expression of multiple growth factors including TGF-β and -α, amphiregulin and VEGF, supporting both an autocrine and paracrine signalling[148]. Several reports have implicated the oncogenic Ras pathway in TME modulation at least in part due to production of cytokines and chemokines, such as IL-8, IL-6, TGF-β, GM-CSF (Granulocyte-Macrophage Colony-Stimulating Factor)[148–151]. Moreover, in colorectal cancer cells mutant KRAS affects proteins and miRNAs content within exosomes, altering also the metabolic state of recipient epithelial cells[78,152,153]. Also

relevant to cite here is endogenous MYC that in tumor cells regulates changes in the TME, sustaining angiogenesis by VEGF signaling and allowing a complex inflammatory response[154].
Increased amounts of proteins loaded into EVs and enhanced membrane budding were observed in brain tumor cells harboring oncogenic EGFRvIII[77,155]. Overexpression of a constitutively active form of AKT1 triggers formation of large oncosomes by prostate cancer cells[75].
YAP, the transcriptional regulator of the Hippo pathway, favors the secretion of the growth factor amphiregulin and of matricellular proteins (such as CYR61 and CTGF)[156,157]. YAP has been demonstrated as a critical regulator of immunosuppressive microenvironment: in prostate cancer it regulates the secretion of CXCL5 to recruit MDSCs[158]; in pancreatic ductal adenocarcinoma YAP drives the expression and secretion of multiple cytokines and chemokines, promoting differentiation and accumulation of MDSCs and macrophage reprogramming[159].
The p53 signaling pathway, which is one of the most commonly altered pathways in cancer, will be deeply described in a next section, being the focus of this thesis.

1.5 The p53 pathway in cancer

One of the most important signaling pathways involved in cancer is the p53 pathway. The p53 protein is primarily involved in restraining tumor onset and outgrowth. Not surprisingly, inactivating mutations in *TP53* are most frequent in human tumors and associated with adverse prognosis in various cancer types[160]. Germline *TP53* mutations cause the rare Li-Fraumeni cancer predisposition syndrome[161] and mice lacking one or both *Trp53* alleles are prone to develop spontaneous tumors[162].

1.5.1 Wild-type p53

The human *TP53* gene encodes for a sequence-specific DNA binding protein that functions primarily as a tetrameric transcription factor. p53 belongs to a family of related proteins, including also p63 and p73, which exert important roles in organism development and both possess tumor suppressive activities. The p53 protein consists of two N-terminal transactivation domains followed by a conserved proline-rich domain, a central DNA binding domain, and a C-terminal region encoding its nuclear localization signals, and an oligomerization domain required for transcriptional activity.
p53 responds to diverse stresses, including hyperproliferative signals, DNA damage, ribonucleotide depletion, oxidative stress, hypoxia, and nutrient starvation, which may compromise genomic stability promoting neoplastic transformation[163] (Figure 5). Indeed, p53 acts as the “guardian of the genome”, because it prevents the accumulation of oncogenic mutations and limits their

consequences[164,165]. In unstressed cells, p53 is maintained at low levels through degradation, mainly mediated by the MDM2 E3-ubiquitin ligase in complex with the related protein MDM4 (known as MDMX in humans). Stress-induced post-translational modifications of both p53 and MDM2/MDM4 abolish their interaction, leading to p53 stabilization, accumulation and induction of its transcriptional activity. The best characterized p53 responses are cell cycle arrest either transient or permanent (senescence), and programmed cell death (apoptosis).

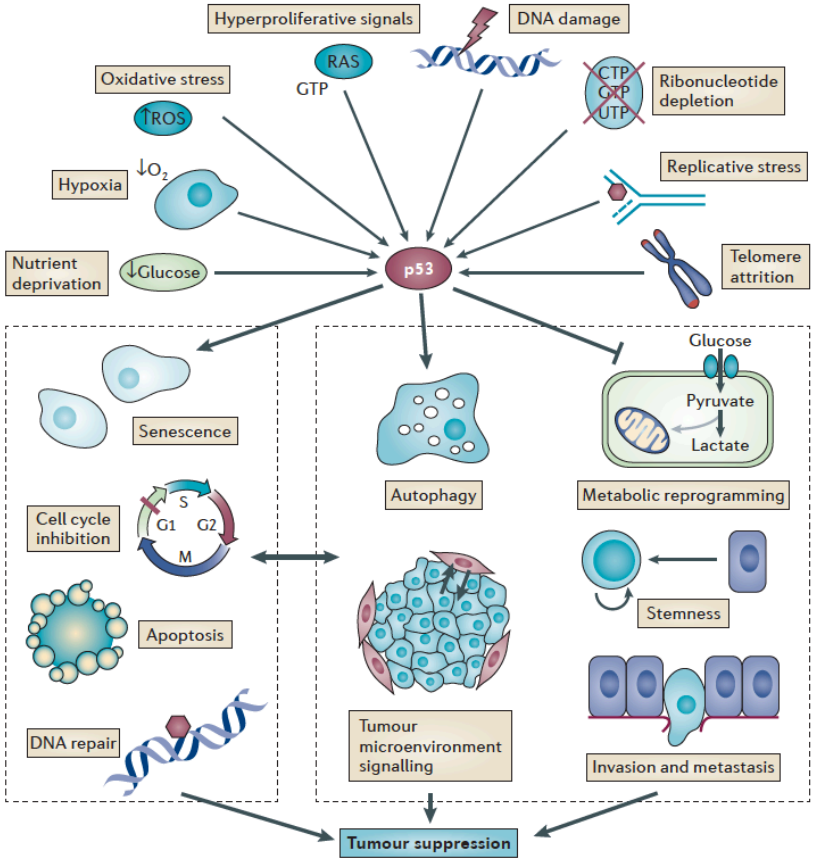

Figure 5. p53-activating signals and responses that are important for tumor suppression. In tumors, a host of different stresses can activate p53, including nutrient deprivation, hypoxia, oxidative stress, hyperproliferative signals, DNA damage and ribonucleotide depletion. Activation of p53 can consequently promote diverse responses that ultimately lead to tumor suppression. The canonical responses (on the left side) consist in cell cycle arrest, senescence, apoptosis and DNA repair. However, beyond these classical responses, activated p53 can modulate several additional cellular processes (on the right side), including opposing oncogenic metabolic reprogramming, activating autophagy, inhibiting stem cell self-renewal and reprogramming of differentiated cells into stem cells, restraining invasion and metastasis, and promoting communication within the tumor microenvironment. (adapted from [163])

The p53 pathway controls the G1/S and G2/M checkpoint mechanisms to block cell-cycle progression, thus preventing propagation of DNA damage while cells attempt to repair it. p53 can induce G1 arrest through transcriptional induction of the cyclin dependent kinase inhibitor p21 (*CDKN1A*)[166] and G2 arrest by inducing *GADD45* and *SFN* (14-3-3σ)[167,168]. However, if the damage is too severe, p53 promotes apoptotic cell death to avoid the division of cells with unrepaired DNA. To this aim, p53 induces the expression of pro-apoptotic genes (*BAX*, *BID*,

PUMA, *NOXA*, *TP53AIP1*) while repressing anti-apoptotic genes (*BCL2*, *BCLXL*)[163], or it promotes apoptosis in transcriptional-independent ways[169,170]. Interestingly, in response to oxidative stress, p53 can induce necrosis instead of apoptosis[171]. Upon DNA damage and oncogenic stress, p53 activation can also lead to senescence, a permanent cell-cycle arrest and relevant onco-suppressor mechanism. Indeed, restoration of p53 in various p53-deficient tumors causes complete tumor regression through induction of senescence[163].

Besides these antiproliferative responses, p53 can regulate several cellular processes such as metabolic reprogramming, accumulation of reactive oxygen species, autophagy, stem cell maintenance, invasion and metastasis[163] (Figure 5). The cellular context, including cell type, epigenetic state, stress and environmental signals, contributes to modulate p53 activity and to direct the biological outcome of p53 response[165].

In addition to transcriptional regulation of protein-coding genes, p53 transcriptionally induces expression of miRNAs to carry out its tumor suppressive functions[172]. The most studied p53-regulated miRNAs belong to miR-34 family, which promote p53-mediated apoptosis, cell cycle arrest and senescence. Furthermore, p53 is able to modulate the transcription of miRNAs, their maturation and accessibility to their corresponding targets.

1.5.1.1 Non-cell-autonomous functions of p53

Although the function of p53 as a brake against cancer development has been extensively studied at the cell-autonomous level, more recent data point also to non-cell-autonomous effects of p53 in stimulating an anti-tumorigenic microenvironment[163]. Indeed, once cells enter senescence, they develop a senescence-associated secretory phenotype (SASP), characterized by high secretion of pro-inflammatory cytokines and immune modulators, which provoke immune surveillance to eliminate senescent cells. During senescence, p53 can modulate the SASP in non-malignant and tumor cell lines, thus promoting an antitumor microenvironment to restrain cancer in a non-cell-autonomous way. In fact, after liver damage, hepatic stellate cells expressing p53 undergo senescence and their SASP includes factors that shift macrophages toward the anti-tumorigenic M1 phenotype and activate NK cells, ultimately leading to clearance of senescent cells[173]. Instead, loss of p53 in hepatic stellate cells promotes an M2 phenotype in macrophages, increasing liver fibrosis and enhancing transformation of adjacent epithelial cells into hepatocellular carcinoma. p53 reactivation in liver tumors drives senescence and tumor regression through upregulation of inflammatory cytokines thereby triggering an innate immune response[174]. Moreover, gene expression analyses prove that p53 directly induces numerous genes involved in the recruitment of immune cells and immune surveillance[175].

Recently it has been found that p53 is able to alter the liver secretome differently under various conditions[176]. In the physiological state, p53 controls secreted factors that are related mainly to lipid metabolism and injury response. Conversely, upon exposure to various chemotherapeutic drugs, activated p53 rewires the secretome profile, inducing the secretion of proteins related to insulin, glucocorticoids, ECM modulators, cell adhesion and immune response.

Other evidence involves the role of p53 in the regulation of exosome release from cells *in vitro*. Upon activation by DNA damage, p53 transcriptionally upregulates the expression of *TSAP6* and *CHMP4C*, which enhance production of exosomes from lung cancer cells[177,178]. Lately, it has been shown that p53 regulates *HGS* expression, which is necessary for the maintenance of larger-sized exosomes in colorectal cancer[179]. Even more intriguing is the demonstration that endogenous p53 protein has been found released within exosomes *in vitro* and *in vivo*, and that suppressed growth and proliferation after being transferred to p53-deficient cells[180].

1.5.2 Mutant p53 proteins

Extensive characterization of somatic mutations in human cancers highlighted *TP53* as the most commonly altered gene, with variable frequencies between tumor types[160]. Unlike most tumor suppressor genes, which are predominantly inactivated by deletion or truncation, *TP53* is frequently inactivated by missense mutations (73.15% according to current release of the IARC TP53 database[181], R19, August 2018) (Figure 6A). As a consequence, in the vast majority of tumors, cells express a full-length mutant form of p53, which differs from the wild-type counterpart in a single amino acid substitution. Most of these missense mutations occur within the DNA binding domain, with six residues mutated at high frequency, called “hot spot” mutations (R248, R273, R175, G245, R249, and R282)[182] (Figure 6B). These missense mutations are classified in two main categories according to the structural alteration and stability on p53 protein: DNA-contact mutations, that change amino acids critical for DNA binding, and conformational mutations, that cause local or global conformational alteration on the protein structure[183].

The functional impact of *TP53* mutations can be classified into three non-mutually exclusive groups[184] (Figure 6C). First, mutations can abrogate or attenuate the tumor suppressor function of wild-type p53 (wt-p53), resulting in absent activation of downstream effectors. This phenomenon is called “Loss Of Function” (LOF) and it is a common characteristic across all cancer-associated p53 mutants. Second, most mutant p53 (mut-p53) proteins are able to oligomerize with the wild-type protein encoded by the second allele, forming a heterotetramer unable to bind DNA (Dominant Negative effect). Finally, several mutations are able to confer truly neomorphic or “Gain Of Function” (GOF) activities to mut-p53, independent on wt-p53 and actively promoting tumor

growth. Thus, mutations in *TP53* are not equivalent to simple loss of the p53 tumor suppressor pathway, but rather can render it oncogenic.

Mut-p53 has been involved in several aspects of tumorigenesis, including proliferation and survival, chemoresistance, genomic instability, somatic cell reprogramming and stem cell characteristics, disruption of tissue architecture, angiogenesis, migration, invasion and metastasis, deregulation of cellular energy metabolism[182,185].

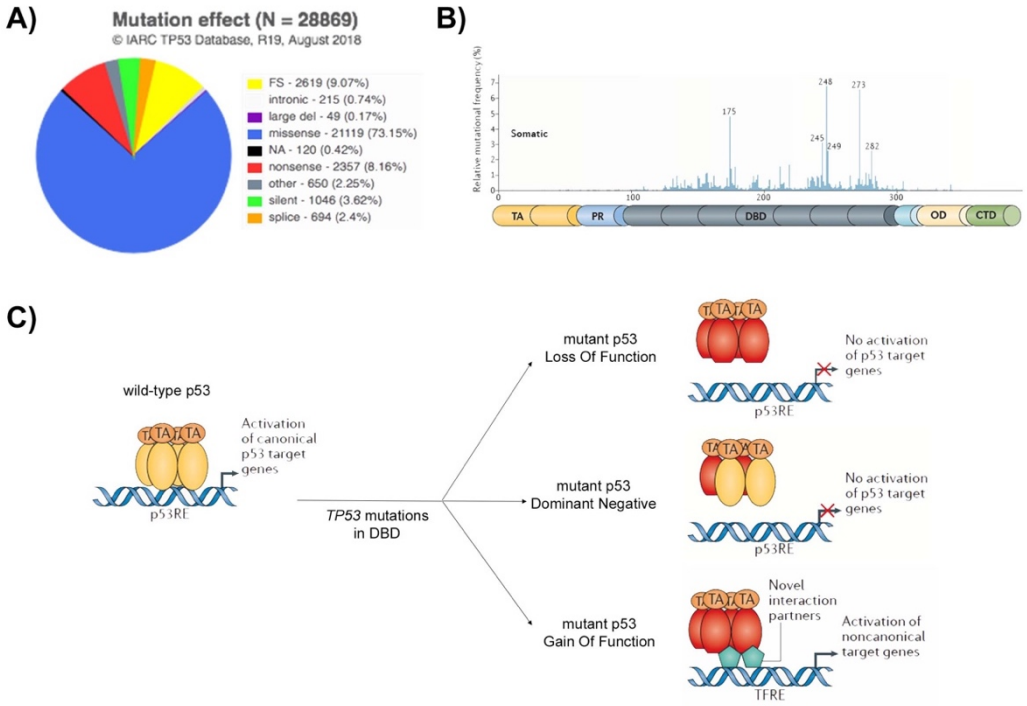

Figure 6. Mutations in *TP53* in cancer. A) Pie chart representing the different cancer-derived mutation types reported in the IARC TP53 Mutation Database, version R19, August 2018 (http://p53.iarc.fr)[181]. **B)** The distribution of reported missense mutations along the 393 amino-acid sequence of p53. The domain architecture of p53 is aligned below. The six most common hotspot mutations are highlighted. Abbreviations: CTD, carboxyl-terminal domain; DBD, DNA-binding domain; OD, oligomerization domain; PR, proline-rich domain; TA, transactivation domain. (adapted from [186]). **C)** Schematic cartoon representing the functional impacts of *TP53* mutations, as described in the main text. Wild-type and mutant p53 monomers are represented in yellow and red, respectively. (modified from [186])

1.5.2.1 Mechanisms of mut-p53 gain of function

Mutant p53 drives the acquisition of many tumor phenotypes both through transcriptional and non-transcriptional processes[187]. Despite mutant p53 proteins are unable to recognize wt-p53 consensus on DNA, they typically retain an intact transactivation domain and have been reported to regulate gene expression. However, although mut-p53 ChIP-sequencing data are available, a mut-p53-specific DNA site has not yet been characterized[188], rather it has been proposed to bind in a DNA-structure selective mode[189].

In the vast majority of cases, mutant p53 exerts its transcriptional activity by interaction with other transcription factors and cofactors to enhance or prevent their activities. Several factors, among which NF-Y, YAP, SREBP, ETS2, E2F1, VDR, SP1, NRF2 and PGC1α, have been reported as

transcriptional partners of mut-p53[190]. Some of these factors are partners of wild-type p53 and usually mediate its oncosuppressor functions, but their interaction with mut-p53 blunts or diverts them to a cancer-promoting outcome[191]. Among the interactors of mut-p53 there are the p53 homologs p63 and p73[192,193]. In that way, mut-p53 inhibits p73-dependent apoptosis and chemosensitivity[194] and suppresses TA-p63 anti-metastatic target genes[195,196].

Mut-p53 GOF is exerted also through alterations in gene expression that involve chromatin remodeling. Indeed, mut-p53 cooperates with the SWI/SNF complex to remodel the chromatin architecture of the *VEGFR2* promoter, increasing its expression[197]. Interacting with ETS2, mut-p53 upregulates some epigenetic regulators (i.e. MLL1, MLL2 and MOZ), leading to a genome-wide increase of histone methylation and acetylation, that promotes cancer cell proliferation[198].

In addition to the effects on the transcriptional machinery, mut-p53 can interact with other proteins to directly change their function. For example, interaction with MRE11 or Topoisomerase 1 results in enhanced genomic instability[199,200].

Although less investigated, cytoplasmic activities of mut-p53 are relevant for its oncogenic potential. Mut-p53-dependent alteration of cancer cell metabolism requires direct inhibition of AMPK in the cytoplasm[201]. Cytoplasmic activities of mut-p53 supports tumor cells survival by suppressing autophagy and inhibiting apoptosis[202–204]. Recently, it has been proved that mut-p53 activates small GTPase Rac1 through SUMOylation, promoting tumor progression[205]. Moreover, mut-p53 binds and inhibits the tumor suppressor DAB2IP in the cytoplasm of cancer cell lines, modulating the response to multiple oncogenic extracellular signals, including TNFα[206] and insulin[207].

Similarly to its wild-type counterpart, mut-p53 is able to regulate miRNAs both transcriptionally and post-transcriptionally[172]. The transcriptional regulation is achieved mainly by activating transcription factors or by inhibiting others, such as p63. Mut-p53-regulated miRNAs are involved in different GOF activities, such as chemoresistance (miR-128 and miR-223), proliferation (miR-27a), invasion and migration (miR-155, miR-130b, miR-205 and let7i)[172]. Finally, mut-p53 affects the processing of miRNAs by interfering with the Drosha/Microprocessor complex[208,209] and by downregulating Dicer[210] and KSRP[211].

Numerous studies have clearly established that mutant p53 protein stabilization is a major mechanism for its GOF[191] and can be achieved in different ways. Interestingly, mutant p53 protein levels are not uniform in all cells of a tumor, but immunohistochemical analyses have often unveiled a rather heterogeneous staining of mut-p53, being absent in some while accumulated in other cells within the same tumor, implying that, even though the mutation is present in all tumor cells, only in some of them there could be functional consequences of the missense mutation. A

notable recent discovery of our group demonstrated that mut-p53 stability can be controlled by mechanical cues from the extracellular environment, such as matrix stiffness, through the mevalonate/RhoA/HDAC6 molecular axis[212]. These results suggest that conditions of tumor fibrosis, ECM stiffening (fibrosis) and cell contractility could induce mutant p53 stabilization and stimulate its oncogenic properties.

1.5.2.2 Interplay between mut-p53 and TME

The ability of cancer cells to undertake advantageous interactions with the tumor stroma and to actively create a permissive microenvironment is pivotal for cancer progression. Indeed, increasing evidence highlights the impact of mut-p53 in modulating the TME and altering the cancer cell secretome[213] (Figure 7). The vast majority of these reports concern the transcriptional regulation by mut-p53 of genes encoding secreted factors playing a role in the TME.

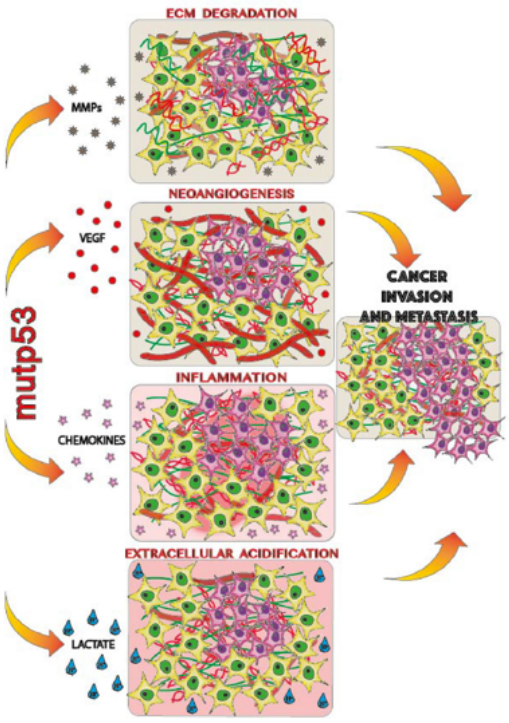

Figure 7. Impact of mutant p53 on tumor microenvironment. Cartoon depicting some of the effects of mut-p53 in modulating the TME, in order to promote cancer cell invasion and metastasis. (adapted from [213])

Mut-p53 contributes to ECM remodeling through the transcriptional repression of TIMP-3[214]. Moreover, periostin cooperates with mut-p53 to mediate invasion by inducing STAT1 signaling in the esophageal TME[215]. Recently, it has been discovered that mice carrying mutant *Trp53* influence COL6A1 expression through a GOF mechanism in part dependent on TGF-β signalling[216].

The expression of the pro-angiogenetic factor VEGF-A is promoted by mut-p53. Indeed, a significant direct correlation between mut-p53 proteins and VEGF expression has been identified in

breast cancer[217]. In addition, mut-p53 can induce *ID4*, a member of ID family proteins involved in neoangiogenesis through post-transcriptional stabilization of the proangiogenic cytokines IL8 and CXCL1[218].

Mut-p53 can impact on inflammatory microenvironment largely depending on both activation and amplification of NF-κB pathway upon TNFα stimulation and induction of *NFKB2* gene expression[219–221]. It has been shown that mut-p53 upregulates the secretion of CXCL5, CXCL8 and CXCL12 chemokines through the NF-κB pathway, thus enhancing cell migration[222]. Moreover, thanks to a NF-κB-alternative mechanism, mut-p53 supports a proinflammatory environment, through binding and activating the CXCL1 promoter in colon cancer cells[223] or suppressing the expression of the secreted interleukin-1 receptor antagonist (sIL-1Ra), a specific antagonist of the IL-1 pro-inflammatory cytokine[224].

Mut-p53 can impinge on the crosstalk between cancer cells and the surrounding stroma. Based on *in vitro* co-culture studies, it has been proved that mut-p53 is able to protect lung carcinoma cells from the anti-tumor effect of IFN-β secreted by CAFs[225]. Finally, stimulating the Warburg effect, mut-p53 favors extracellular acidification and enhances the secretion of lactate, that can act as both a metabolic and a signaling molecule[226].

Recent reports have highlighted additional cell-extrinsic effects of mut-p53 in sustaining invasive cancer phenotypes. Using p63 as a chaperone to bind its target promoters, mut-p53 induces the expression of a proinvasive cluster of secreted factors[227]. In addition, analyzing transcriptome and secretome profiles from mut-p53-inducible non-small lung cancer cell lines, it has been demonstrated that mut-p53 modulates the extracellular environment mainly through changes in the gene expression of numerous targets that are subsequently secreted from cells[228]. Among these genes, *SERPINA1*, encoding for A1AT (alpha-1 antitrypsin, a secreted serine protease inhibitor), is a critical effector of mut-p53 to drive invasion and alter EMT markers expression.

Recently, a new non-cell-autonomous GOF mechanism of mut-p53 has been discovered. In colon cancer cells, mut-p53 can reprogram macrophages to M2 status by shedding miR-1246-enriched exosomes[229]. When such exosomes are internalized, macrophages undergo reprogramming and produce anti-inflammatory and tumor supportive factors (e.g. IL-10, TGF-β, MMPs).

2. Preliminary data

In the laboratory in which my PhD project was developed, we focused on miRNAs, broad modulators of gene expression, as oncogenic effectors of the mut-p53 pathway in tumor cells. We chose to concentrate our studies on triple negative breast cancer (TNBC) which presents high prevalence of missense mutations in the *TP53* gene, around 54%[230]. Of note, TNBC (lacking expression of oestrogen receptor, progesterone receptor and HER2[231]) is a breast cancer subtype characterized by a highly aggressive phenotype, unfavorable prognosis and absence of specific therapy.

We knocked down mut-p53 expression in a TNBC cell line harboring the p53 R280K mutation (MDA-MB-231 cells) by RNAi, and analyzed the expression levels of a panel of miRNAs previously reported to be overexpressed in solid tumors by qPCR[232]. This analysis highlighted miR-30d as the strongest hit positively regulated by mut-p53. miR-30d is an intergenic miRNA located in chromosome 8q24 and it has been already reported as an oncogenic miRNA. Indeed, its locus is frequently amplified in multiple types of human solid tumors[233]. In different contexts (e.g. hepatocellular carcinoma, melanoma, breast, ovarian and prostate cancer) miR-30d has been demonstrated to mediate several processes related to cancer development and progression, including migration, invasion, EMT, autophagy, angiogenesis and metastasis[143,233–237].

We recapitulated mut-p53-dependent miR-30d expression in other TNBC cell lines endogenously expressing different p53 missense mutants, in particular MDA-MB-468 (mut-p53 R273H) and SK-BR-3 (mut-p53 R175H) (Figure 8A). Moreover, we found that endogenous wtp53 had no effect on miR30d in normal-like breast epithelial cells, whereas ectopic expression of mut-p53 variants R175H, R273H and R280K in this context upregulated miR-30d levels (Figure 8B).

Preliminary experiments have also suggested that the mut-p53/miR-30d axis exerts aggressive cancer phenotypes, such as migration, invasion, EMT and epithelial cell polarity disruption (data not shown).

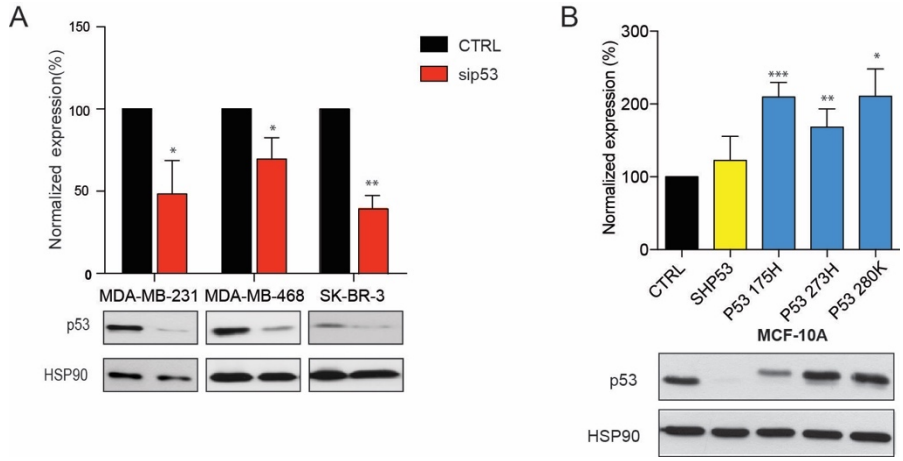

Figure 8. mut-p53 promotes miR-30d expression. A. Bar graph showing expression of miR-30d in MDA-MB-231, MDA-MB-468, SK-BR-3 cells upon mut-p53 knockdown. Mut-p53 levels are shown by western blot analysis below. Cells were transfected with either p53-specific or control siRNA, and collected 48h after transfection. The RNA was extracted and q-RT-PCR was performed. (*: $p<0.05$, **: $p<0.01$; t test) **B**. Bar graph showing expression of miR-30d in MCF10A stably silenced for wt-p53 and overexpressing three different hotspot mutants (mut-p53 R175H, R273H and R280K). p53 levels following silencing of wt-p53 (shp53) and mut-p53 overexpression are shown by the western blot analysis in the lower panels. (**: $p<0.01$, ****: $p<0.0001$; t test)

To identify the biological processes regulated by miR-30d, we stably inhibited miR-30d expression and function by engineering MDA-MB-231 cells to stably express a miR-30d GFP-decoy vector (MDA-MB-231 dy-30d) (as described by Bonci *et al.*[238]). Transcriptomic profiles of MDA-MB-231 dy-30d and control cells were obtained through microarray technology. Genes that display a statistically significant change in expression were subjected to unbiased analysis using Gene Set Enrichment Analysis (GSEA), which highlighted a strong correlation between the presence of miR-30d and protein secretion (Figure 9A). To investigate whether miR-30d has an impact on protein secretion, we performed radioactive metabolic labeling of MDA-MB-231 dy-30d and control cells. This assay proved that depletion of endogenous miR-30d did not impinge on neosynthesis of intracellular proteins (data not shown), but significantly reduced total protein secretion, similarly to Brefeldin A (BFA), a well-known inhibitor of protein transport (Figure 9B). Given that mut-p53 regulates miR-30d expression, we asked if mut-p53 is also able to affect protein secretion. Figure 9C shows that mut-p53 knockdown is able to reduce the amount of secreted proteins by MDA-MB-231 cells. Importantly, overexpression of miR-30d in this context rescued protein secretion, without promoting comparable alterations in global expression of intracellular proteins (data not shown), thus supporting the notion that this miRNA could directly mediate the impact of mut-p53 on protein secretion. Furthermore, overexpression of miR-30d in MCF-10A cells is able to enhance total protein secretion (data not shown). All together, these data indicate the presence of an axis by which mut-p53, through miR-30d, promotes protein secretion in cancer cells.

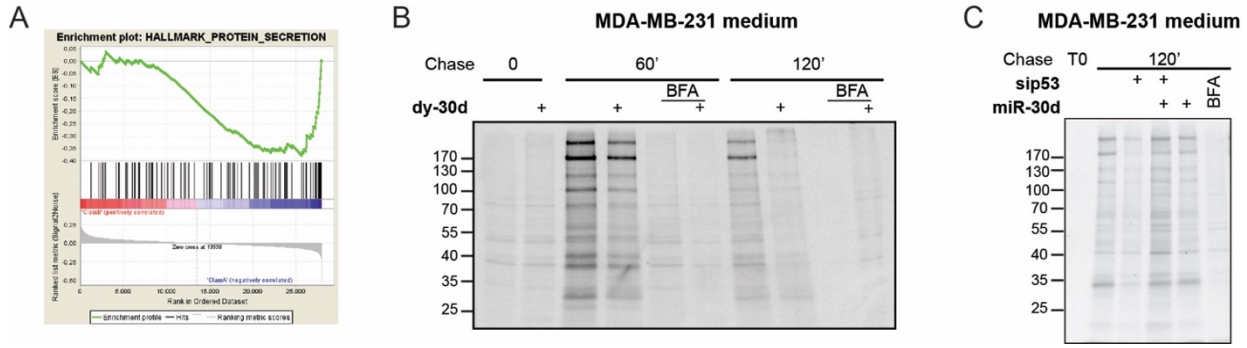

Figure 9. miR-30d-depedent regulation of protein secretion is a novel process regulated by mut-p53. A. Gene set enrichment analysis of microarray data upon stable inhibition of miR-30d function in MDA-MB-231 dy-30d cells compared to control cells. GSEA enrichment plot for the cluster related to protein secretion. The control condition is indicated as class A, while the condition characterized by stable inhibition of miR-30d function is named class B. **B.** MDA-MB-231 dy-30d cells and control cells were labeled with [^{35}S]Met/Cys aminoacids for 1h. Then newly synthesized proteins were chased in medium containing unlabeled Met/Cys for the indicated times. BFA was used as positive control. At each time point, the medium was collected, and secreted proteins recovered by Trichloroacetic/acetone (TCA) precipitation. Radiolabeled secreted proteins were resolved using a 12.5% SDS-PAGE gel and visualized by autoradiography. Note that dy-30d cells display a reduction on total protein secretion at both collection time points. **C.** Autoradiography of secreted proteins from MDA-MB-231 cells silenced with either p53-specific or control siRNA and transfected with miR-30d mimic. After 48h of transfection were subjected to pulse/chase experiment as previously described. Autoradiography shows a reduction in protein secretion after mut-p53 silencing, that is restored overexpressing in this context miR-30d. However, ectopically overexpression of miR-30d in MDA-MB-231 cells seems to not impinge on protein secretion. BFA was used as positive control.

To better understand the mechanisms regulated by the mut-p53/miR-30d axis and to characterize the increasingly secreted proteins, we collected conditioned medium (CM) from MDA-MB-231 control cells, mut-p53-depleted MDA-MB-231 cells, or mut-p53-depleted MDA-MB-231 cells overexpressing miR-30d, and performed mass spectrometry analysis of secreted proteins by means of LC MS/MS technology (Figure 10A). In order to reduce contaminants and/or background proteins in the proteomic analysis, all statistically significant protein hits were filtered using a previously described bioinformatic pipeline[239], which considers proteins released via the classical ER-Golgi secretory pathways, through unconventional pathways (e.g. exosomal secretion) or generated by proteolytic cleavage of extracellular domains of plasma membrane proteins. This analysis identified 1039 differentially secreted proteins. Whole secretome analysis highlighted that, upon mutp53 depletion, the repertoire of proteins secreted by MDA-MB-231 cells changed significantly, with both up- and down-regulation of different hits. Of note, we found that only about 30% of the differentially secreted proteins are regulated by mut-p53 at the transcriptional and/or protein level[211] (data not shown), which suggests that mut-p53 may globally impact the secretion process. Restoring miR-30d levels in mut-p53 depleted cells massively recovered the observed mut-p53-dependent secretome regulation (Figure 10B). These data suggest that mut-p53 reprograms the cancer cell secretome through miR-30d.

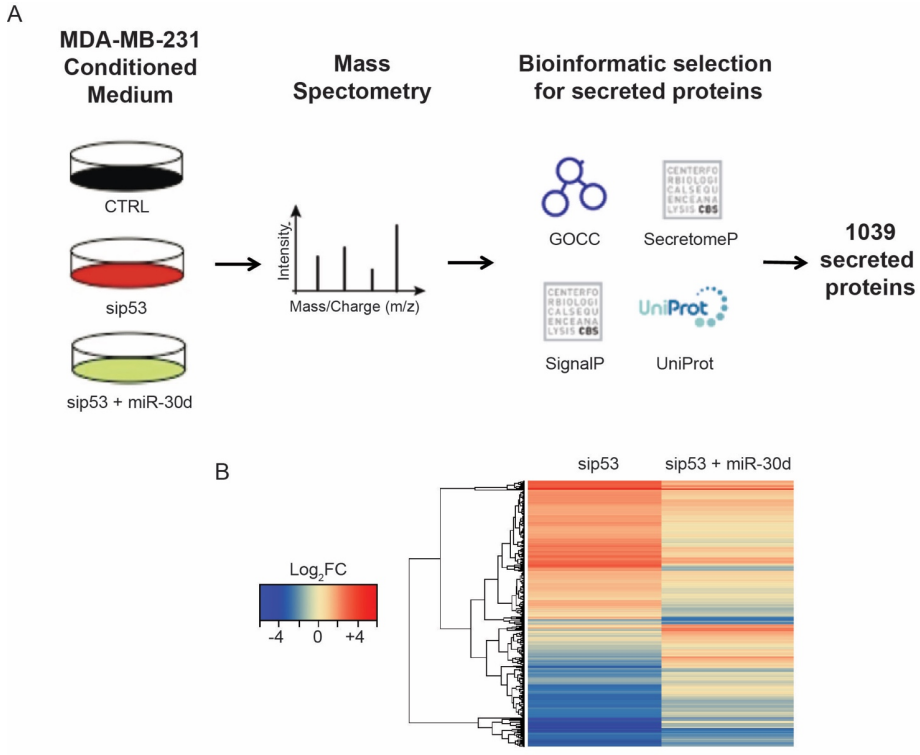

Figure 10. Mass spectrometry analysis of secreted proteins affected by mut-p53/miR-30d axis. A. Schematic overview of the experimental workflow used to obtain information about the mut-p53/miR-30d-dependent secretome in MDA-MB-231 cells. Conditioned medium from control, mut-p53-depleted or mut-p53-depleted with ectopically overexpressed miR-30d cells was collected after 72 hours and filtered through a 0.22 mm filter. Mass spectrometry analysis of secreted proteins through LC-MS/MS technology was performed (n=2). The predicted secreted proteins were identified through a bioinformatic pipeline previously described in Deshumukh *et al.*[239], which employed the indicated bioinformatic tools. **B.** Heat map with horizontal dendrogram clustering showing log2(FoldChange) distribution of secreted proteins identified. This global secretome analysis revealed both significantly upregulated proteins (red scale) and downregulated ones (blue scale) upon mut-p53 depletion. Note that restoring miR-30d expression massively rescued the mut-p53-dependent regulation, as visible from the color shift towards the yellow scale (intermediate values).

Intriguingly, miR-30d has been reported as a miRNA secreted in both physiological[240] and pathological conditions, among which cardiovascular disfunctions[241–244], systemic inflammatory response[245], type 2 diabetes mellitus[246] and cancer. Indeed, higher serum levels of miR-30d have been associated with poor prognosis in lung cancer patients[247,248]. Moreover, circulating miR-30d has been proposed to have a diagnostic role in multiple myeloma[249] and a prognostic role for melanoma recurrence[250]. The modality of miR-30d secretion is not clear and unique. According to the existing literature and to a database of extracellular miRNAs (miRandola), secreted miR-30d has been found in exosomes or associated with proteins, both in normal and cancer contexts[104,105,251]. Moreover, it has been demonstrated that miR-30d can be shuttled between normal cells[240,252]. All these data suggest that secreted miR-30d is involved in intercellular communication and that it could represent a non-invasive biomarker.

3. Aim of the thesis

In established tumors, cancer cells are surrounded by a supportive and reactive environment called tumor microenvironment (TME), which includes non-transformed cells (e.g. fibroblasts, endothelial cells), and the extracellular matrix (ECM). Cancer cells modify the TME by secreting an array of heterogeneous molecules collectively called secretome, which has been shown to have both local and systemic effects. Several cancer-related pathways concur to reprogram the cancer cells' secretome, impinging on communication with the TME and thus promoting tumorigenesis. Vesicles and factors secreted by tumor cells have been demonstrated to strongly contribute to these dynamic short- and long-range interactions, however little is known about how this secretion becomes deregulated in cancer. A better understanding of the crosstalk between cancer cells and microenvironment will allow identifying novel potential therapeutic targets and non-invasive biomarkers in tumors.

In the laboratory in which I carried out my PhD project, miR-30d was identified as a novel target of mut-p53 and an effector of a novel process regulated by mut-p53 in breast cancer cells. Indeed, we have demonstrated that in cancer cells the mut-p53/miR-30d axis is able to promote total protein secretion, which was further characterized by performing mass spectrometry analysis of secreted proteins. In addition, miR-30d has been reported to be secreted from cancer cells, exerting a diagnostic or prognostic role in some cancer types[247–250].

Starting from these data, I hypothesized that the mut-p53/miR-30d molecular axis could alter the TME through modulation of the secretome. Therefore in this thesis I pursued two aims:

1. to characterize the impact of mut-p53/miR-30d-dependent secretome on different TME constituents and to understand how it can reshape the local microenvironment;
2. to verify whether miR-30d secretion by cancer cells is mut-p53-dependent and to understand what role it could exert in receiving cells.

The results of this research were expected to shed light on how mut-p53, through miR-30d induction, could reprogram the cancer secretome and to understand the impact of this process on specific compartments of the TME, favoring the identification of potential therapeutic targets and non-invasive biomarkers in mut-p53 expressing tumors.

4. Results

4.1 Functional characterization of the mut-p53/miR-30d-dependent secretome

In order to uncover the functional significance of the mut-p53/miR-30d-dependent secretome, a Gene Ontology enrichment analysis using the Database for Annotation, Visualization and Integrated Discovery[253,254] (DAVID, https://david.ncifcrf.gov/), was performed on the secreted proteins identified in the mass spectrometry analysis (see preliminary data, Figure 10). I first focused the analysis on the GO term Biological Process, in order to gain insights in the pathways and processes affected by the secreted proteins. As shown in Figure 11A, this unbiased analysis revealed an enrichment of proteins involved in cell-ECM and cell-cell adhesion, ECM composition, proteolysis, angiogenesis and regulation of cell migration. Afterwards, I performed the GO term analysis for Cellular Components, to explore the subcellular localization of the proteins identified in the secretome. This analysis highlighted that a great amount of differentially secreted proteins has been reported to be present in the extracellular exosome compartment (Figure 11B), and further confirmed the presence of proteins that are secreted in the extracellular space and that compose the ECM.

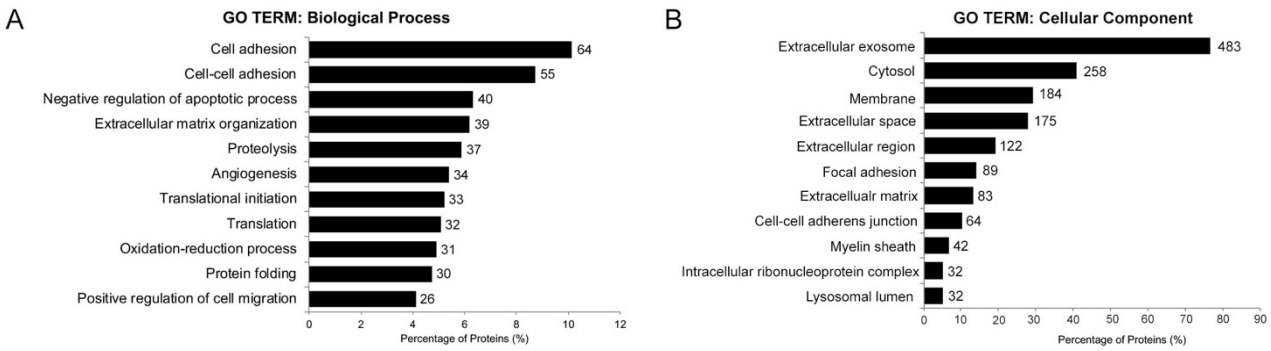

Figure 11. Gene ontology term enrichment analysis of mut-p53/miR-30d secreted proteins. Proteins identified in the mut-p53/miR-30d-dependent secretome were analyzed by enrichment analyses using DAVID database. The bar graph shows the top results of the analysis for the GO term Biological Process in **A** and the GO term Cellular Component in **B**. The amount of proteins found in the secretome compared to the total gene list included in DAVID clusters is indicated next to the bars.

The predicted processes emerged by the first GO term analysis indicate that proteins secreted in a mut-p53/miR-30d-dependent manner are able to modulate cancer progression, invasion and metastasis, thus suggesting a protumorigenic role of the investigated secretome. These findings prompted to deeply explore the effect of the mut-p53/miR-30d-dependent cancer secretome towards various components of the tumor microenvironment, such as the ECM and stromal cells, as well as paracrine stimulation towards cancer cells within the heterogenous tumor mass. In addition, the

predicted localization suggested to analyze the contribution of mut-p53/miR-30d axis in the release of extracellular vesicles (EVs).

4.2 mut-p53/miR-30d axis in cancer cells alters the composition and properties of the ECM

Cancer cells are characterized by an increased ECM deposition, with the accumulation of specific components (e.g. collagens and fibronectin), and by an altered organization and remodeling.

As mentioned in the preliminary data, miR-30d inhibition in MDA-MB-231 cells reduced total protein secretion. To verify if miR-30d could impinge on secretion of ECM components, I collected conditioned medium (CM) from control and MDA-MB-231 dy-30d cells. Secreted proteins were recovered after precipitation of CM with trichloroacetic acid (TCA) and analyzed by immunoblotting. As shown in Figure 12A, miR-30d inhibition was able to reduce the secretion of some ECM components, such as Fibronectin, Laminin-β1 and Laminin-γ2, validating in part the results of the GO analysis.

Next, to test whether mut-p53 could alter secretion of ECM components through miR-30d, I performed immunoblot analysis of the above reported secreted ECM proteins recovered from MDA-MB-231 control cells, MDA-MB-231 cells silenced for mut-p53 and MDA-MB-231 cells silenced for mut-p53 (a condition in which miR-30d levels are reduced) overexpressing miR-30d precursor hairpin mimic to restore its levels. As reported in Figure 12B, mut-p53 silencing strongly inhibited the secretion of the three ECM components that however was rescued by miR-30d overexpression.

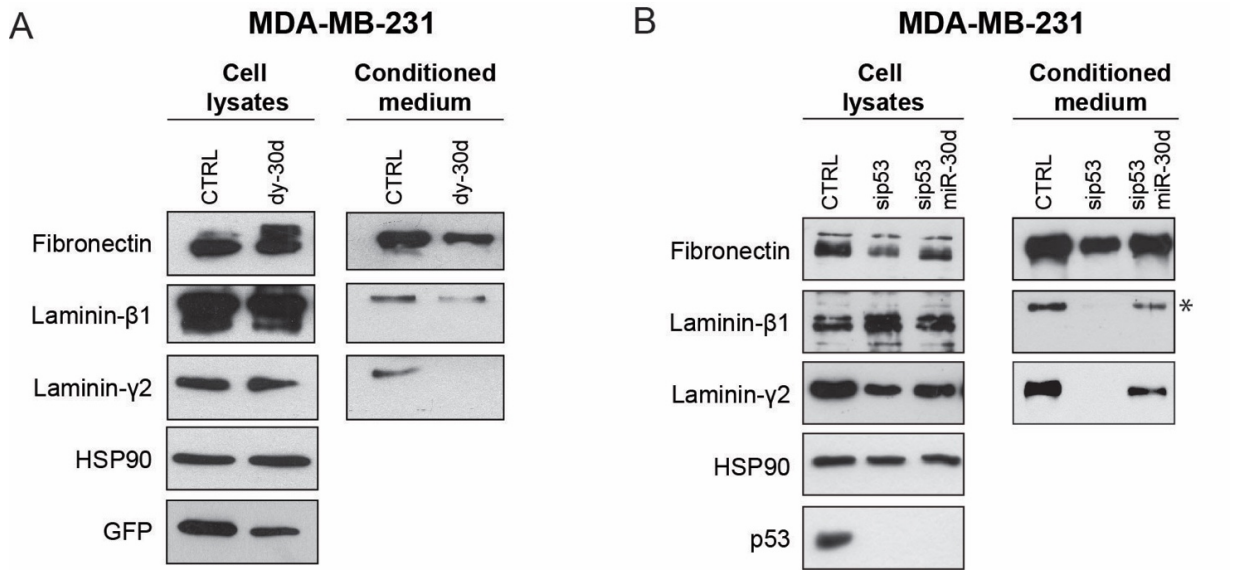

Figure 12. mut-p53/miR-30d axis alters the secretion of ECM components. A. Western blot analysis of ECM components from cellular lysates (left) or CM (right) from MDA-MB-231 control and dy-30d cells after 60 hours of starvation. HSP90 was used as loading control. GFP protein levels confirmed the functionality of miR-30d decoy vector. **B.** Western blot analysis of cell lysates

(left) or CM (right) from control, mut-p53-depleted or mut-p53-depleted with overexpression of miR-30d mimic MDA-MB-231 cells was collected after 60 hours of serum starvation. HSP90 was used as loading control. p53 was blotted to control the efficiency of silencing. Asterisk indicates a shift in band due to post-translational modifications in secreted Laminin-β1.

Alterations in ECM composition and assembly have a marked impact on the biomechanical properties of the ECM. In particular, this can cause an alteration of ECM stiffness. Therefore, I asked if the mut-p53/miR-30d axis in cancer cells could impact on increased matrix stiffness. For this aim, we employed atomic force microscopy (AFM). The basic principle of this method is to indent cells or ECM with an AFM tip, measuring the applied force from the deflection of the AFM cantilever, which will result in a quantitative measurement of the stiffness. AFM measurement highlighted that mut-p53 depletion in MDA-MB-231 cells caused a reduction in ECM stiffness compared to control cells (on average of 0.15 kPa and 0.25 kPa, respectively) (Figure 13), whereas miR-30d overexpression in mut-p53 depleted cells significantly recovered ECM stiffness.
All together these results demonstrate that the mut-p53/miR-30d axis in cancer cells impacts the secretion of single ECM components and increases ECM stiffness.

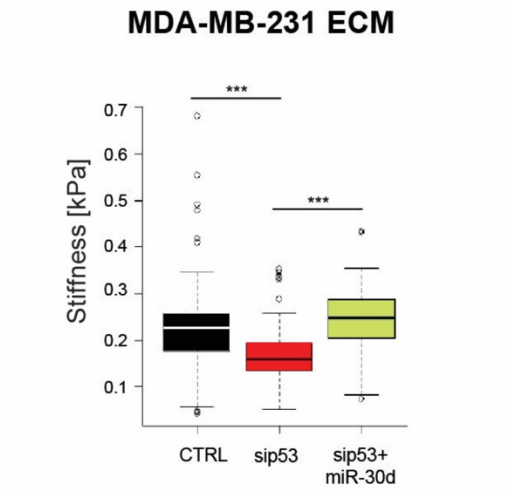

Figure 13. mut-p53/miR-30d axis alters the stiffness of ECM. MDA-MB-231 cells were seeded on coverslips and silenced with either control or p53-specific siRNA and transfected with miR-30d mimic. After 60 hours of serum starvation, cells were fixed in PFA and ECM was visualized using Picro Sirius Red Stain. Samples were then analysed by means of AFM to measure ECM stiffness. Box plots indicate the stiffness values. (***: $p<0.001$, Kolmogorov-Smirnov test)

4.3 mut-p53/miR-30d-dependent secretome contributes to angiogenesis and vascular permeability

Cancer cells secrete a plethora of proangiogenic signals into the TME, which induces an angiogenic program in recipient endothelial cells. VEGF-A is the principal secreted growth factor that regulates this process. In addition, tumors display an aberrant network of blood vessels characterized by vascular leakiness and permeability, due to the loss of cellular adhesions. Since the secretome GO

term annotation unveiled proteins involved in angiogenesis and cell-cell adhesion, I investigated the role of mut-p53/miR-30d axis in promoting angiogenesis and vascular permeability *in vitro*.

Firstly, by immunoblot analysis, I unveiled that mut-p53/miR-30d axis sustained a differential secretion of VEGF-A in MDA-MB-231 cells (Figure 14A), suggesting a role in angiogenesis for the mut-p53/miR-30d-dependent secretome. In order to verify this hypothesis, human umbilical vein endothelial cells (HUVECs) were used to perform tube formation assays, thus evaluating their capacity to form capillary-like structures. As shown in Figure 14B, upon VEGF treatment HUVECs organize themselves to create tubes mimicking the generation of new vessels. Treatment of HUVECs with MDA-MB-231 control CM increased the number of closed tube loops of more than double with respect to HUVECs grown in their own medium. This angiogenic increase was impaired after treatment with CM obtained from MDA-MB-231 cells after silencing of mut-p53, but recovered using CM from cells in which miR-30d levels were restored.

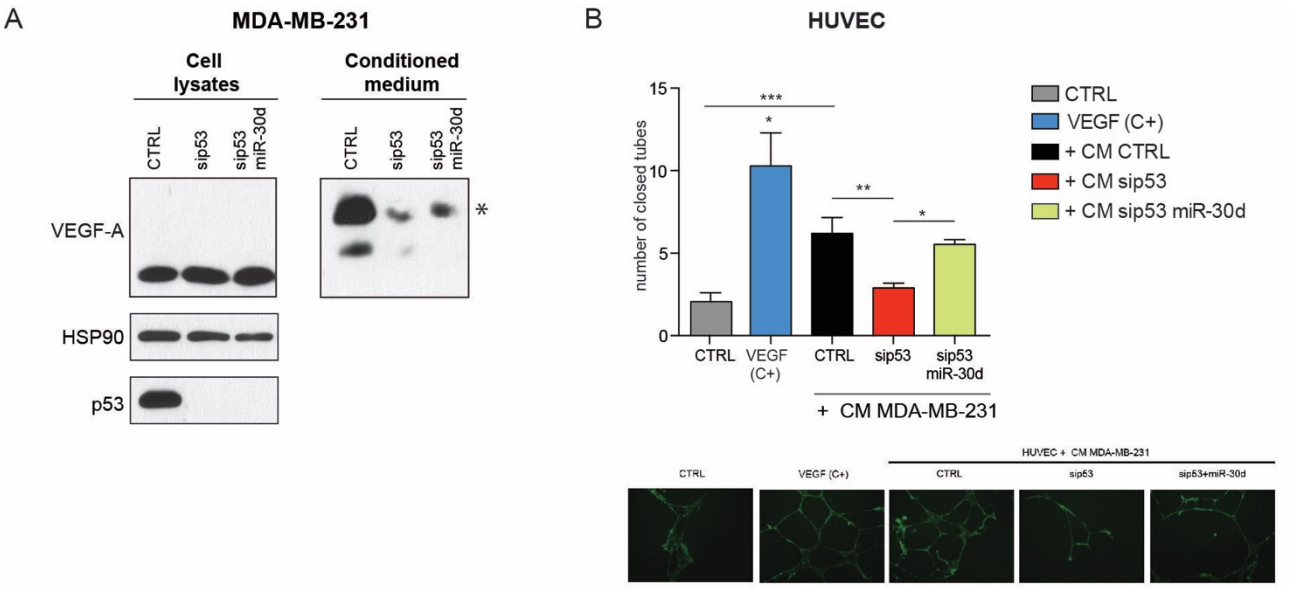

Figure 14. mut-p53/miR-30d-dependent secretome promotes neoangiogenesis *in vitro*. A. Conditioned medium from control, mut-p53-depleted or mut-p53-depleted plus overexpressed miR-30d MDA-MB-231 cells was collected after 60 hours of serum starvation. Cells were lysed and secreted proteins were recovered after precipitation with TCA. Western blot analysis of VEGF-A; HSP90 was used as loading control; immunoblot of p53 confirms the silencing. Asterisk indicates the presence of different isoforms in secreted VEGF-A. **B.** HUVECs were treated for 24 hours with CM recovered from MDA-MB-231 cells as described above. The tubes were visualized after staining with Phalloidin and counted. Recombinant VEGF was used as positive control. The bar graph indicates the number of closed tubes after each treatment. Values are mean ± SEM from n=3 independent experiments. (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; t test). The bottom panel shows representative pictures of Phalloidin staining in HUVEC.

Subsequently, to evaluate the properties of the vessels induced by the mut-p53/miR-30d axis, I performed an *in vitro* permeability assay by measuring the crossing of FITC-dextran through HUVEC monolayers grown on 0.4 μm filters. Since the relative molecular mass of FITC-dextran is 70 kDa, only in the presence of a discontinuous monolayer the passage of the fluorescent probes can occur from the top to the bottom of the wells. Addition of MDA-MB-231 control CM allowed

the passage of dextran through the endothelial monolayer similarly to the effect induced by TNFα treatment known to induce vessel permeabilization (Figure 15). However, CM from mut-p53-depleted cells abrogated this effect, which in turn was rescued when miR-30d levels were restored in mut-p53-depleted cells. Overall, these results confirm that the mut-p53/miR-30d-dependent secretome is able to promote angiogenesis and vascular permeability in receiving endothelial cells.

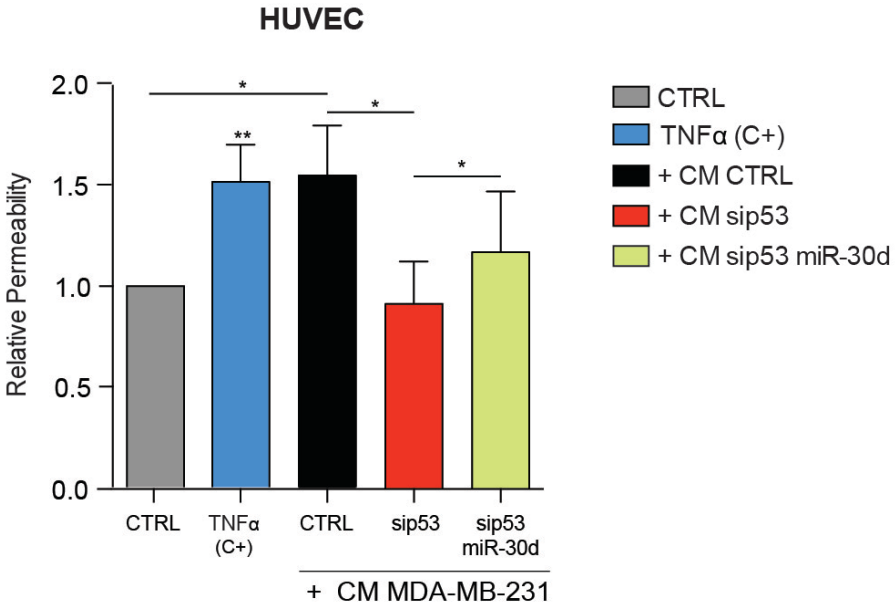

Figure 15. mut-p53/miR-30d-dependent secretome promotes vascular permeability *in vitro*. HUVEC monolayers were grown on 0.4 µm filters. CM recovered from MDA-MB-231 cells as described in Figure 14 was added in the upper chamber for 24 hours. Then, FITC-dextran was added to the top well for 30 minutes. The permeability of treated HUVEC was determined by the appearance of FITC-dextran in the lower chamber, measuring the absorbance at 495 nm. TNFα treatment was used as positive control. The absorbance was compared and normalized with control condition (no treatment with CM). Values reported in the graph are the mean ± SEM of n=3 independent experiments. (*: $p<0.05$; **: $p<0.01$; t test)

4.4 mut-p53/miR-30d-dependent secretome causes activation of fibroblasts

Within the TME, CAFs (cancer-associated fibroblasts), which derive from activation of stromal fibroblasts, represent a key cellular component. Activated fibroblasts are characterized by morphological changes, acquisition of migratory capabilities, increased production of ECM components, transcriptional and metabolic reprogramming. The cancer cells' secretome can mediate fibroblasts activation, which in turn fosters growth and invasion of cancer cells. Given the protumorigenic role of the secretome under investigation, I assessed its contribution to fibroblasts activation. To this aim, two different fibroblast cell lines were employed: BJ-EHT immortalized human skin-derived fibroblasts, and WI-38 primary human lung fibroblasts.

Firstly, prompted by the cluster of secreted proteins involved in cell migration highlighted by the GO analysis, I performed wound-healing assays to test fibroblast migratory capabilities, an index of fibroblast activation. I hypothesized that inhibition of miR-30d, reducing protein secretion, was also able to regulate fibroblast activation. In fact, CM derived from MDA-MB-231 control cells led to a

2-fold increase in migratory ability of BJ cells, which was dampened using CM from MDA-MB-231 dy-30d cells, thus confirming the hypothesis (Figure 16).

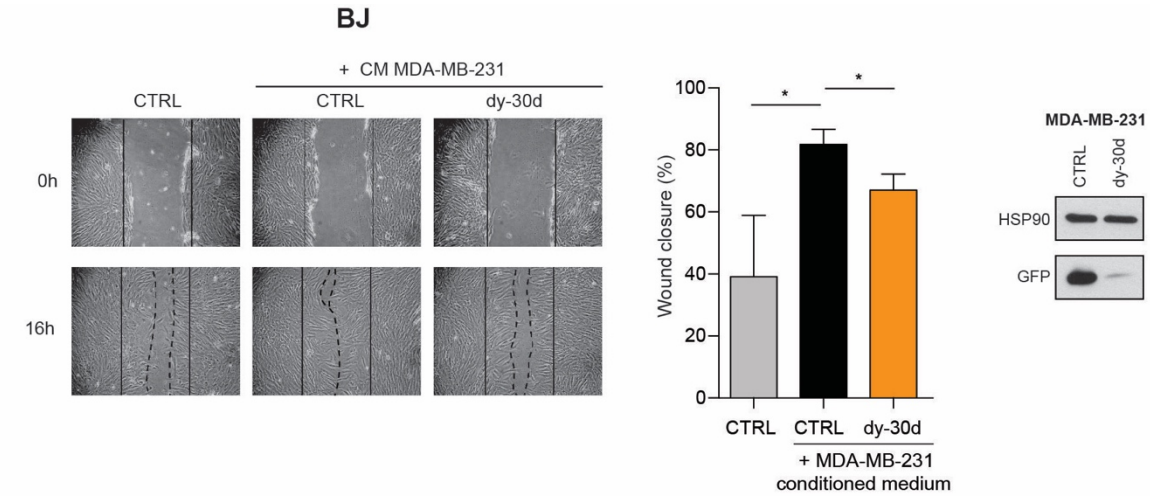

Figure 16. miR-30d-dependent secretome triggers paracrine migration of fibroblasts. BJ fibroblasts were cultured until they created a monolayer. Subsequently, wound closure assay was performed in presence of CM from control or dy-30d MDA-MB-231 cells. Representative pictures were taken at the beginning of the experiment and after 16 hours of migration. Bar graph shows the quantification of wound closure area. Values are mean ± SEM from n=3 independent experiments with 3 replicates. (*: $p<0.05$; t test). The efficiency of miR-30d inhibition in MDA-MB-231 cells used to recover CM was checked by western blot.

Subsequently, I tested the ability of mut-p53/miR-30d-dependent CM in promoting migration of BJ and WI-38 fibroblasts in a paracrine fashion. Same experiments as above were performed using CM derived from control and MDA-MB-231 mut-p53-depleted cells: while the CM of control cells enhanced fibroblast migration, CM derived from MDA-MB-231 cells with mut-p53 knockdown caused a 25-35% reduction (Figure 17). Recovering miR-30d levels in mut-p53-depleted cells by overexpression restored the ability of CM to promote migration in both types of fibroblasts. Similar results were obtained in BJ cells exposed to CM derived from MDA-MB-468 and SK-BR-3 cells, harboring different mut-p53 variants, following mut-p53 knockdown and miR-30d rescue experiments (Figure 18). All these results strongly suggest that mut-p53 proteins, through the expression of miR-30d, can promote fibroblasts recruitment, enhancing their migration in a paracrine manner.

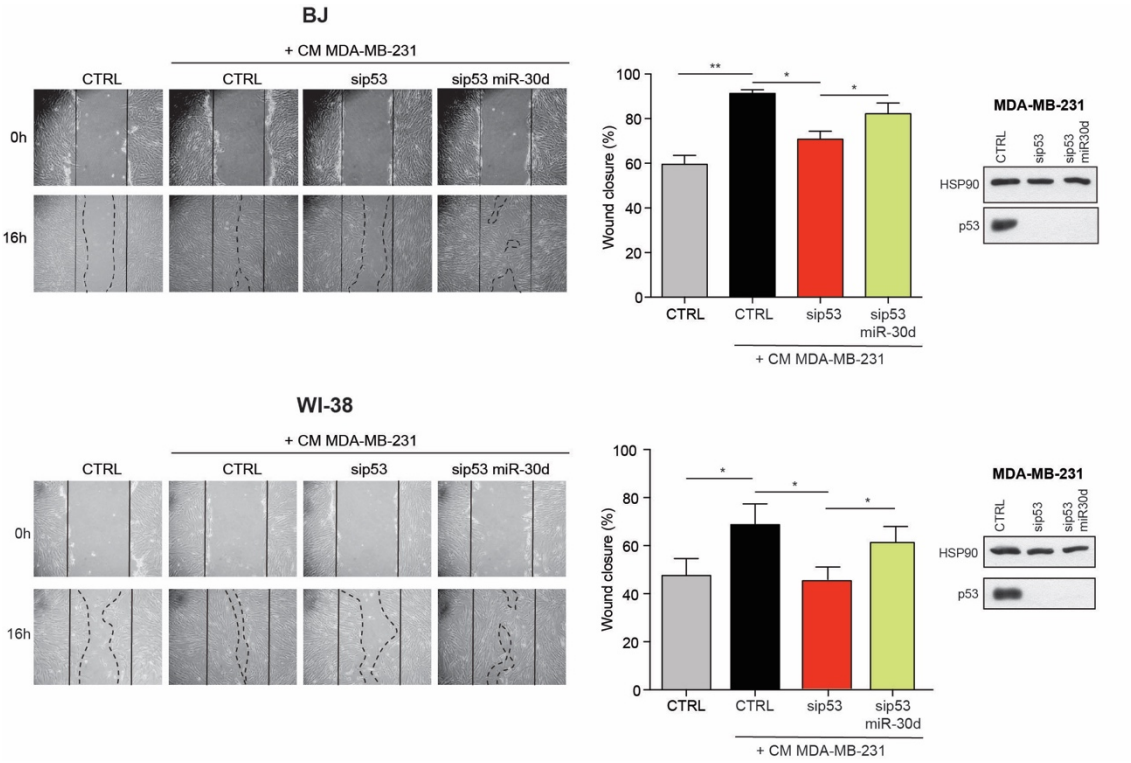

Figure 17. mut-p53/miR-30d-depedent secretome activates migration of fibroblasts. CM was recovered from control, mut-p53-depleted or mut-p53-depleted overexpressed miR-30d MDA-MB-231 cells after 60 hours of serum starvation and added to confluent BJ and WI-38 fibroblasts to perform wound healing assay. Representative pictures were taken at the beginning of the experiment and after 16 hours of migration. Bar graph shows the quantification of wound closure area. Values are mean ± SEM from n=3 (for BJ) or n=4 (for WI-38) independent experiments with 3 replicates. (*: $p<0.05$; **: $p<0.01$; t test). Silencing of p53 was checked by western blot.

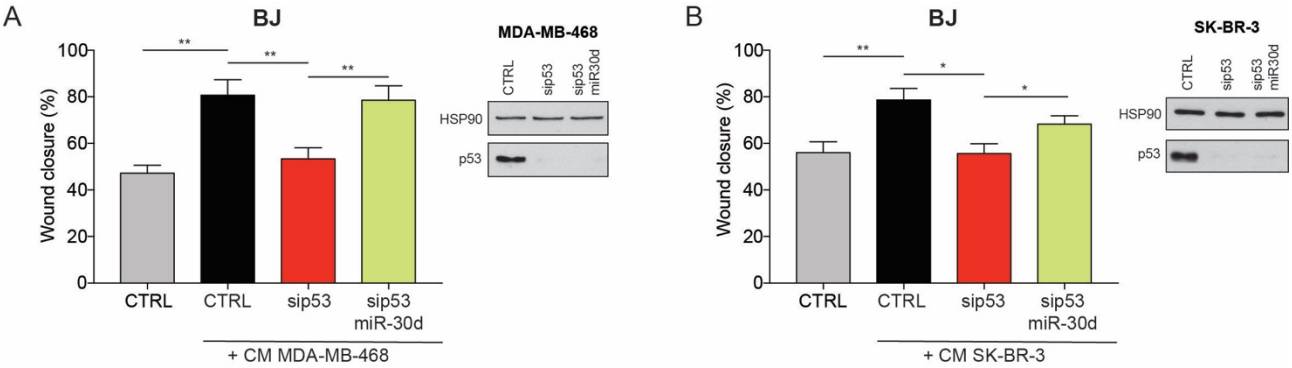

Figure 18. Fibroblasts migration is influenced by a mut-p53/miR-30d-dependent secretome from different TNBC cell lines. MDA-MB-468 (mut-p53 R273H) and SK-BR-3 (mut-p53 R175H) cells were silenced with either control or p53-specific siRNA and transfected with miR-30d mimic. After 48 hours of serum starvation, CM was recovered, added to confluent BJ cells, and wound healing assays were performed. Migration was evaluated for 16 hours. Bar graph shows the quantification of wound closure area. Values are mean ± SEM from n=3 independent experiments with 3 replicates. (*: $p<0.05$; **: $p<0.01$; t test). Silencing of p53 in both cell lines was checked by western blot.

Afterwards, I functionally characterized fibroblasts' activation by performing immunoblot analysis of their activation markers. In particular, I observed a regulation of fibronectin and HIF-1α in BJ cells and of α-SMA, fibronectin, YAP, TAZ and HIF-1α in WI-38 cells (Figure 19A). The expression of these markers was induced adding CM from MDA-MB-231 control cells, but blunted

after exposure to CM from MDA-MB-231 mut-p53-depleted cells. However, their expression was rescued adding CM after restoration of miR-30d levels in MDA-MB-231 mut-p53-depleted cells. Moreover, similar results for α-SMA, fibronectin and YAP regulation were observed using CM derived from SK-BR-3 cells in a mut-p53/miR-30d-depedent manner (Figure 19B). These data suggest that mut-p53/miR-30d-dependent secretome is able to trigger fibroblast activation affecting cytoskeleton rearrangement (α-SMA), ECM production (fibronectin), transcriptional reprogramming (YAP, TAZ) and metabolic rewiring (HIF-1α).

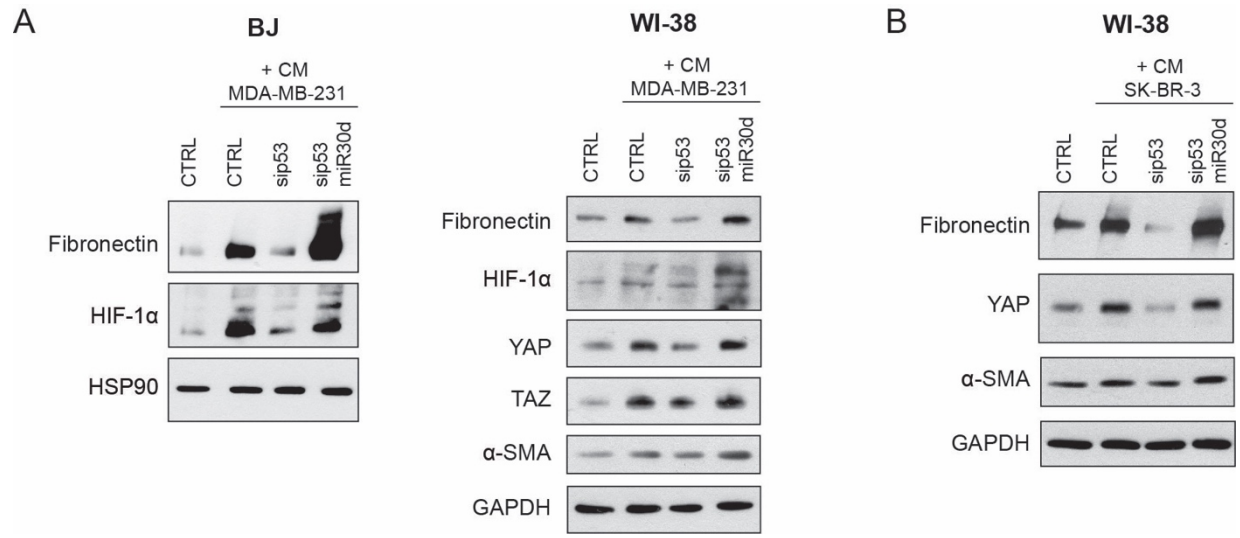

Figure 19. mut-p53/miR-30d-depedent secretome induces expression of CAFs markers in receiving fibroblasts. A. BJ and WI-38 fibroblasts were treated for 16 hours with CM from control, mut-p53-depleted or mut-p53-depleted plus overexpressed miR-30d MDA-MB-231 cells. Then, cells were washed and lysed, and western blot analysis of the indicated proteins was performed. HIF-1α blot was taken from a different gel, loaded in parallel with the same amount of lysate. HSP90 and GAPDH were used as loading control. **B.** CM was recovered from SK-BR-3 cells silenced with either control or p53-specific siRNA and transfected with miR-30d mimic. CM was added to WI-38 fibroblasts for 16 hours and then cells were lysed. Immunoblot analysis of the indicated proteins was shown. GAPDH was used as loading control.

4.5 The mut-p53/miR-30d-dependent secretome promotes paracrine cancer cells migration

The GO term functional annotation suggests that the mut-p53/miR-30d-dependent secretome can exert protumorigenic effects towards both extrinsic (as described so far) and intrinsic components of the tumor mass. Indeed, cancer cells within the same tumor can influence each other in a paracrine fashion and this is believed to underlie intratumor heterogeneity, characterized by the presence of cells with distinct morphological and phenotypic features, such as motility, proliferation and metastatic potential, within the same tumor. Thus, I next asked whether the mut-p53/miR-30d-dependent secretome could influence surrounding cancer cells in a paracrine fashion. In particular, since the GO term analysis of the secretome highlighted the annotation “positive regulation of cell

migration", I assessed the potential of the mut-p53/miR-30d dependent secretome to influence cancer cell migration.

As a proof of principle, I tested the migration of p53-null lung cancer cells (H1299) exposed to different CM obtained from MDA-MB-231 cells for 48 hours in a transwelling assay. As expected, CM obtained from MDA-MB-231 control cells causes a strong increase in migration of receiving H1299 cells, whereas CM derived from MDA-MB-231 dy-30d cells displayed a significantly reduced ability to stimulate cancer cell migration (Figure 20).

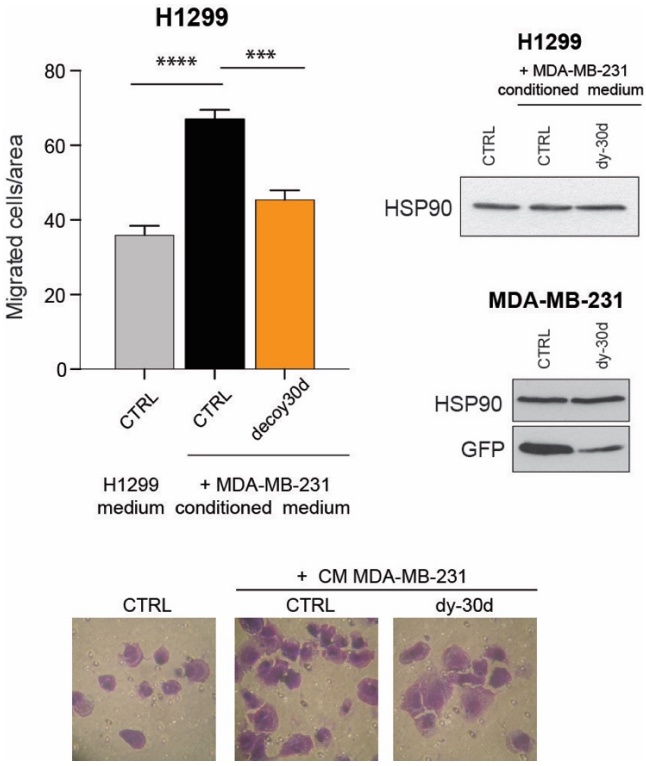

Figure 20. miR-30d-dependent secretome influences paracrine migration of cancer cells. H1299 cells were exposed for 48 hours to CM derived from control or MDA-MB-231 dy-30d cells. Afterwards, migration was evaluated for 16 hours using a transwelling assay. Upper left: the bar graph summarizes the migrated cells per area. Values are mean ± SEM from n=3 independent experiments with 2 replicates. (***: $p<0.001$; ****: $p<0.0001$; t test). In the bottom panel a representative picture of migrated cells is reported. Upper right: HSP90 western blot analysis was added as a control for equal amounts of migrated cells. The efficiency of miR-30d inhibition in MDA-MB-231 cells used to recover CM was checked by western blot.

As stated in the Introduction (paragraph 1.3), the cancer secretome is not solely composed by proteins, but includes also lipids and metabolites. Since the conditioned medium recovered from MDA-MD-231 cells is heterogeneous in composition, I verified if the migration ability induced by miR-30d-dependent secretome is mainly due to secreted proteins. To this purpose, I assessed the effect of heat on the properties of the conditioned medium. In fact, boiling causes most of the proteins to be denatured without affecting heat-stable factors, such as lipids and small metabolites[255–257]. Figure 21 shows that after boiling, the CM lost its ability to drive migration of receiving H1299 cells, suggesting that this property mainly relies on secreted proteins.

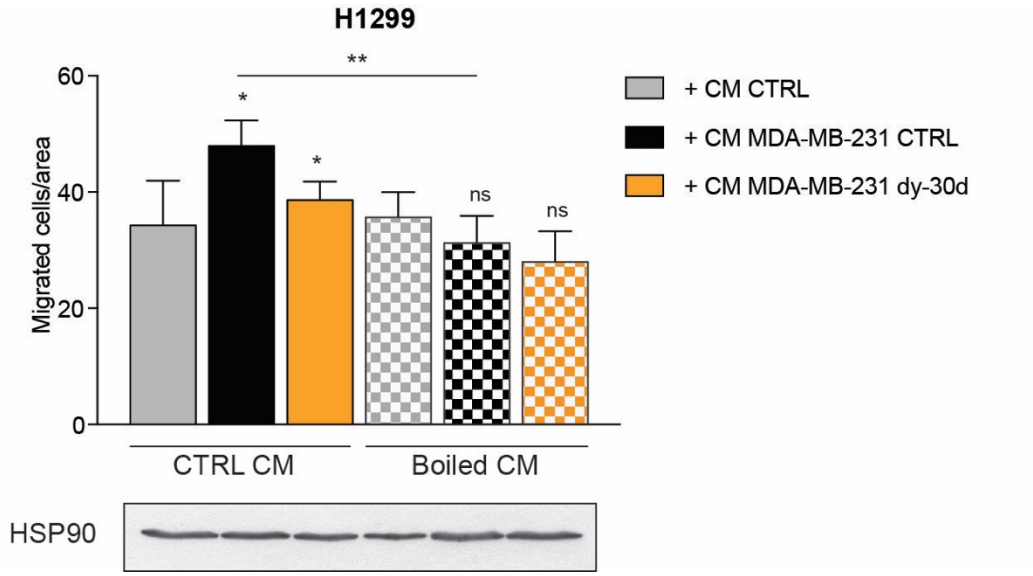

Figure 21. Paracrine effects of the miR-30d-dependent secretome mainly rely on secreted proteins. Conditioned medium was recovered from MDA-MB-231 control or dy-30d starved cells and divided in two aliquots. One of them was boiled for 10 minutes. Next, CM was added to H1299 cells for 48 hours, and then transwell migration assay was performed for 16 hours. The bar graph summarizes the migrated cells per area. Values are mean ± SEM from n=4 independent experiments with 2 replicates. (*: $p<0.05$; **: $p<0.01$; t test). HSP90 western blot analysis was added as a control for equal amounts of migrated cells.

Finally, I assessed also the contribution of mut-p53 and miR-30d towards paracrine stimulation of cancer cell migration. H1299 cells were exposed to their own CM or to CM from MDA-MB-231 cells for 48h: compared to H1299 CM, that of MDA-MB-231 induced a 2-fold increase of the migratory capabilities of H1299 cells (Figure 22). Conversely, exposing H1299 cells to CM from mut-p53-depleted cells dampened the induction of migration, which was rescued with CM from mut-p53-depleted cells with overexpression of miR-30d to restore its levels. Overall, these results support the notion that mut-p53, by altering tumor cells' secretome via miR-30d exert a pro-migratory paracrine effect on neighboring cancer cells.

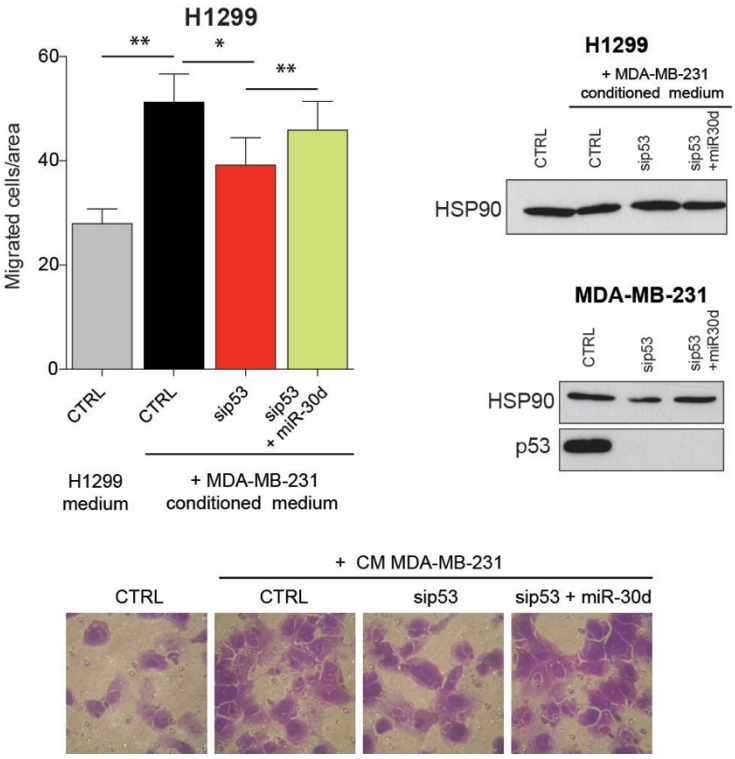

Figure 22. mut-p53/miR-30d-dependent secretome promotes paracrine migration of cancer cells. H1299 cells were exposed for 48 hours to CM derived from control, mut-p53-depleted or mut-p53-depleted overexpressing the miR-30d mimic MDA-MB-231 cells. Next, migration was evaluated for 16 hours using a transwell system. The bar graph summarizes the migrated cells per area. Values are mean ± SEM from n=3 independent experiments with 2 replicates. (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; t test). In the bottom panel a representative picture of migrated cells is reported. HSP90 western blot analysis was added as a control for equal amounts of migrated cells. Silencing of p53 in MDA-MB-231 cells used to recover CM was checked by western blot.

4.6 miR-30d expression activates a pro-migratory secretome in a non-transformed context

As mentioned in the preliminary data, overexpression of miR-30d in normal-like breast epithelial MCF-10A cells is capable of promoting enhanced protein secretion. Therefore, I asked whether and how this enhanced protein secretion in a normal context is able to influence the surrounding microenvironment.

I transiently overexpressed miR-30d precursor hairpin mimic in MCF-10A cells and collected the CM. Immunoblot analysis of secreted proteins precipitated from CM revealed an increased production and secretion of fibronectin after miR-30d overexpression (Figure 23A). Next, I evaluated the effect of CM on paracrine migration of fibroblasts. As shown in Figure 23B, BJ fibroblasts receiving CM from MCF-10A control cells caused a mild change (6%) in their migratory abilities, while adding CM from MCF-10A overexpressing miR-30d led to a statistically significant increase of about 20% in their migration. Finally, I assessed also the contribution of miR-30d-dependent secretome on paracrine migration of cancer cells. After 48 h of exposure to CM, H1299 cells were subjected to a transwelling migration assay. The CM from control MCF-10A

cells caused a significant increase in migration that is further boosted after exposure to CM from MCF-10A cells overexpressing miR-30d (Figure 23C). Overall these results indicate that the increased total protein secretion caused by overexpressing miR-30d in a non-transformed context produces a secretome with a pro-migratory effect on the surrounding microenvironment.

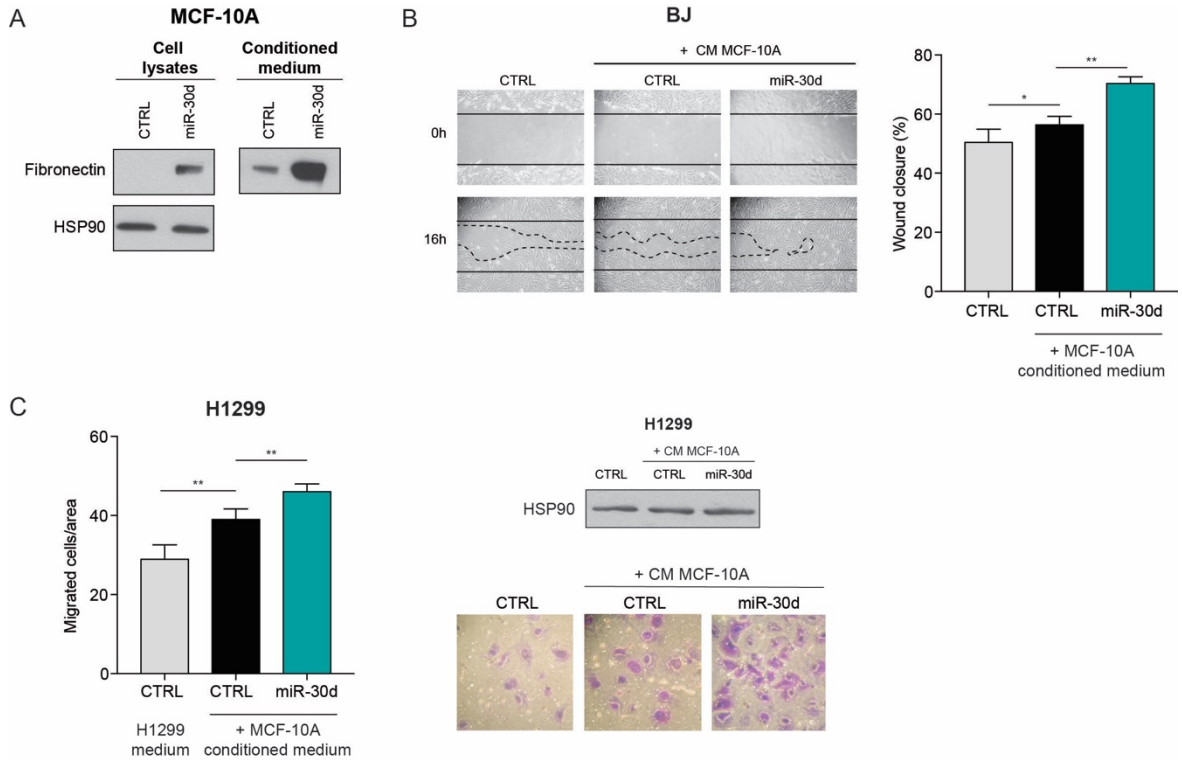

Figure 23. Overexpression of miR-30d in MCF-10A produces a pro-migratory secretome. A. MCF-10A cells were transiently transfected with miR-30d precursor hairpin mimic. Then, cells were lysed and conditioned medium was collected to recover secreted proteins. Immunoblot analysis of fibronectin was shown. HSP90 was used as loading control. **B.** CM collected as previously described was added to BJ cells grown in monolayer and wound healing assays were performed. Representative pictures were taken at the beginning of the experiment and after 16 hours of migration. Bar graph shows the quantification of wound closure area; values are mean ± SEM from n=3 independent experiments with 2 replicates. (*: $p<0.05$; **: $p<0.01$; t test). **C.** CM obtained as described above was added to H1299 cells for 48 hours. Next, migration was evaluated for 16 hours using a transwelling system. The bar graph summarizes the migrated cells per area. Values are mean ± SEM from n=4 independent experiments with 2 replicates. (**: $p<0.01$; t test). HSP90 western blot analysis was added as a control for equal amounts of migrated cells. A panel of representative pictures of migrated cells is reported below.

4.7 mut-p53/miR-30d axis affects the release of proteins within EVs

All the results presented so far suggest that the mut-p53/miR-30d-dependent secretome could alter the local microenvironment by altering ECM deposition and by acting on surrounding cells, both tumor and stromal, through secreted factors, thus supporting protumorigenic phenotypes. Nevertheless, it is known that the cancer secretome can impinge on TME through the release of extracellular vesicles (EVs), which can act both at the short and distant range. Thus, prompted by the GO term analysis of Cellular Component showed in Figure 11, I asked whether the mut-p53/miR-30d-dependent axis affects the release of EVs from cancer cells.

The most frequently used method to isolate EVs involves a series of ultracentrifugations, which requires a specific equipment not available in our laboratory. Therefore, I employed polymer-based precipitation using a commercial kit, a method that ensures a high recovery yield compared to ultracentrifugation, although at slightly lower purity[258]. I precipitated secreted EVs from CM of control, mut-p53-depleted or mut-p53-depleted overexpressing miR-30d MDA-MB-231 cells cultured for 60 h in serum-free medium. Firstly, I provided a molecular characterization of EVs by western blot analysis. Immunoblotting of precipitated EVs uncovered that mut-p53 silencing did not affect exosomal membrane markers, such as CD63 and CD9, but reduced the levels of well-known cargo proteins such as HSP70, HSP90, β-actin, and Glypican-1 (Figure 24). In this condition, miR-30d overexpression totally recovered secretion of these proteins within EVs. These data suggest that mut-p53/miR-30d axis does not impinge on the amount of secreted EVs but rather could reduce their protein cargo.

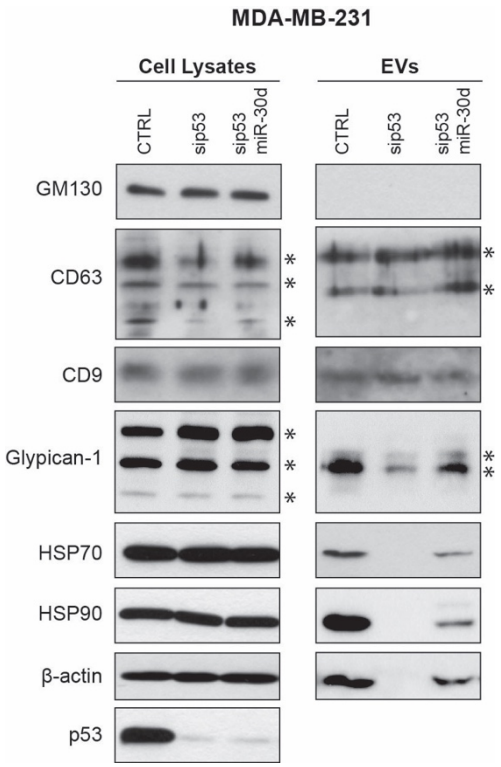

Figure 24. mut-p53/miR-30d axis influences secretion of proteins within EVs. Conditioned medium from control, mut-p53-depleted or mut-p53-depleted overexpressed miR-30d MDA-MB-231 cells was collected after 60 hours of serum starvation; while cells were lysed. The same amount of CM volume was precipitated through Total Exosome Isolation Reagent (Invitrogen). Precipitated EVs were lysed and, together with cell lysates, were resolved using SDS-PAGE. The indicated exosome markers were revealed by western blot. Immunoblot of GM130, a resident Golgi protein, confirms the absence of intracellular membranes contaminants in precipitated EVs. Silencing of p53 was checked by western blot. Asterisks indicate multiple bands of the indicated proteins migration at different heights due to post-translational modifications, e.g. mainly glycosylation.

Next, we characterized the physical properties of precipitated EVs employing AFM, which has been recently considered as a very powerful instrument for the detection and characterization of nanovesicles[259]. AFM analysis determined that all the three conditions under investigation

displayed a similar surface density, indicating a comparable total amount of secreted EVs (Figure 25A). Representative images presented in Figure 25B show the established spherical morphology of the precipitated particles. In addition, diameter measurement by AFM revealed a heterogeneous population of EVs, including vesicles in the size range of exosomes. Interestingly, observing the size frequency distribution, I noted that mut-p53-silencing causes an increased presence of EVs with smaller diameter (below 100 nm), a condition that is partly rescued in a miR-30d-dependent manner (Figure 25C). These data lead to the hypothesis that the reduced size of EVs isolated from mut-p53-depleted cells might correlate with the observed reduction of the cargo. Nevertheless, overall these results suggest an impact of mut-p53/miR-30d axis on EVs proteins cargo rather than on EVs release.

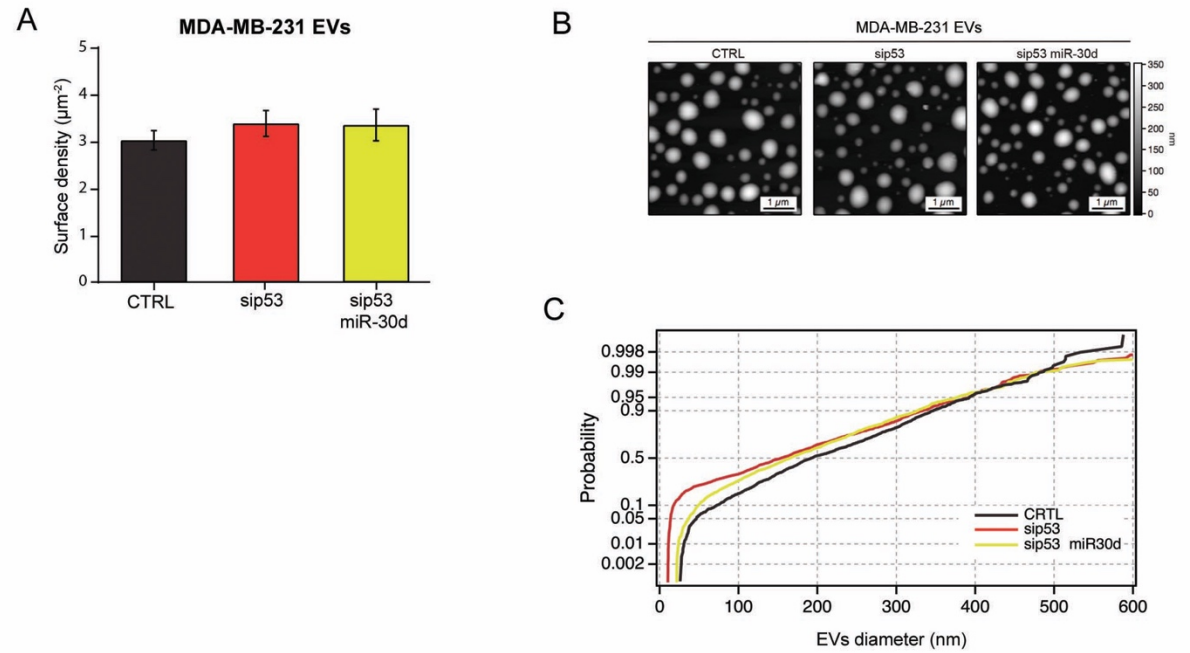

Figure 25. Atomic force microscopy (AFM) characterization of EVs. EVs were isolated as previously described from MDA-MB-231 cells in the indicated conditions; then were fixed in paraformaldehyde and subjected to AFM measurement as described in the Materials and methods section. **A.** AFM density (particles concentration) of EVs expressed as particles/µm^2. **B.** Representative AFM images of precipitated EVs. 5x5 µm^2 images. Scale bar: 1 µm. **C.** Probability size distribution of precipitated EVs. Note that in the size range below 100 nm, the red curve (EVs from mut-p53 depleted MDA-MB-231 cells) follows a different trend compared to the other two conditions.

To further demonstrate an effect of mut-p53 on the cargo of EVs, I precipitated EVs from CM of MCF-10A cells stably depleted of endogenous wtp53 and overexpressing the mut-p53 variant R175H, after 24 h of culture in serum-free medium. As reported in Figure 26, the overexpression of mut-p53 R175H did not affect the level of CD63, but increased the levels of some cargo proteins (Glypican-1, HSP90, HSP70, and β-actin) compared to both control cells and wt-p53-depleted cells. These data suggest that mut-p53 could promote the loading of proteins within EVs.

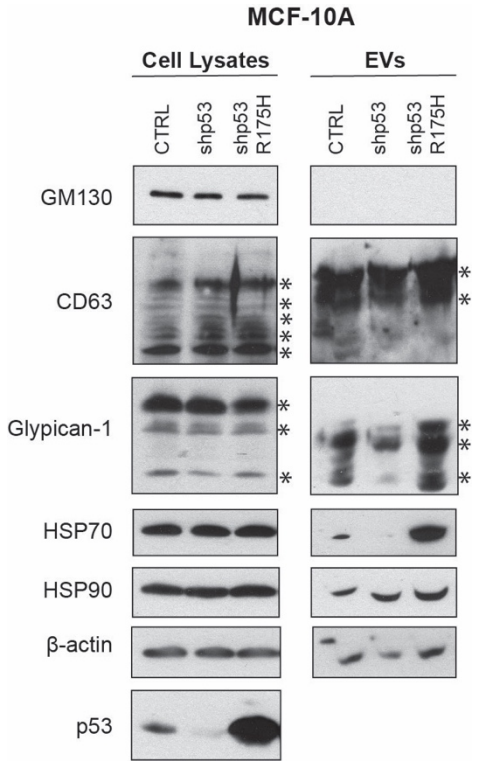

Figure 26. Overexpression of mut-p53 R175H in MCF-10A promotes the release of proteins within EVs. MCF10A control cells, MCF-10A stably silenced for wt-p53 and MCF-10A stably silenced for wt-p53 and overexpressing the hotspot mutant p53 variant R175H were plated and serum-starved for 24 hours. CM was collected and the same amount of volume was precipitated through Total Exosome Isolation Reagent (Invitrogen). Precipitated EVs were then lysed and resolved using SDS-PAGE together with cell lysates. The indicated exosome markers were revealed by western blot. Immunoblot of GM130, a resident Golgi protein, confirms the absence of intracellular membranes contaminants in precipitated EVs. Silencing of p53 and overexpression of mutant p53 variant were checked by western blot. Asterisks indicate multiple bands of the indicated proteins migration at different heights due to post-translational modifications, e.g. mainly glycosylation.

Finally, I wanted to assess whether EVs could contribute to the observed paracrine phenotypes of mut-p53/miR-30d-dependent secretome. To do this aim, I precipitated EVs from mut-p53/miR-30d-dependent CM of MDA-MD-231 cells as described above. Isolated EVs were then resuspended in serum-free medium and added to confluent BJ cells and wound healing assays were performed for 16 hours. Figure 27 shows that EVs isolated from MDA-MB-231 control cells were able to promote fibroblasts migration, which conversely was abrogated using EVs from MDA-MB-231 mut-p53-depleted cells. The enhanced migration was rescued adding EVs from MDA-MB-231 mut-p53-depleted cells with recovered expression of miR-30d. Thus, EVs secreted by mut-p53/miR-30d axis may contribute at least to paracrine fibroblasts migration.

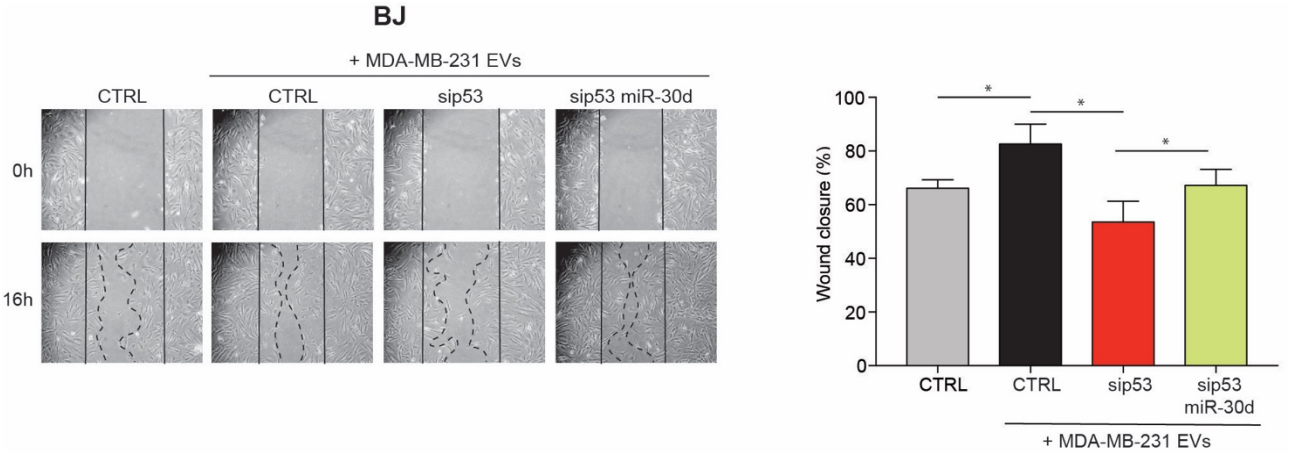

Figure 27. EVs regulated by the mut-p53/miR-30d axis are able to trigger paracrine migration of fibroblasts. MDA-MB-231 EVs were precipitated from mut-p53/miR-30d-dependent CM as described above. EVs were then resuspended in serum-free medium and added to BJ cells grown in monolayer and wound healing assay were performed. Representative pictures were taken at the beginning of the experiment and after 16 hours of migration. Bar graph shows the quantification of wound closure area; values are mean ± SEM from n=3 independent experiments with 2 replicates. (*: $p<0.05$; t test).

4.8 Cancer-secreted miR-30d influences receiving cells

The previous results suggest that the mut-p53/miR-30d axis may regulate the differential loading of cargo proteins within EVs. Of note, EVs are one of the means by which miRNAs are released from cells to mediate intercellular communication. miR-30d has been reported to be released in the extracellular environment, both in exosomes and associated with proteins, and to be transferred between normal cells[240,252]. I therefore asked if secreted miR-30d from cancer cells can contribute to communication in TME.

Firstly, in order to potentiate the levels of secreted miR-30d to better study its function, I generated clones of MDA-MB-231 cells stably overexpressing a miR-30d construct (miRVec30d). I measured by qPCR the levels of miR-30d from these cells and their media and compared them to those derived from parental cells. Extracellular levels of miR-30d were normalized using an exogenous spike-in control (cel-miR-39)[260]. MDA-MB-231 miRVec30d cells presented high miR-30d intracellular expression and an increase of miR-30d release in the extracellular space (Figure 28).

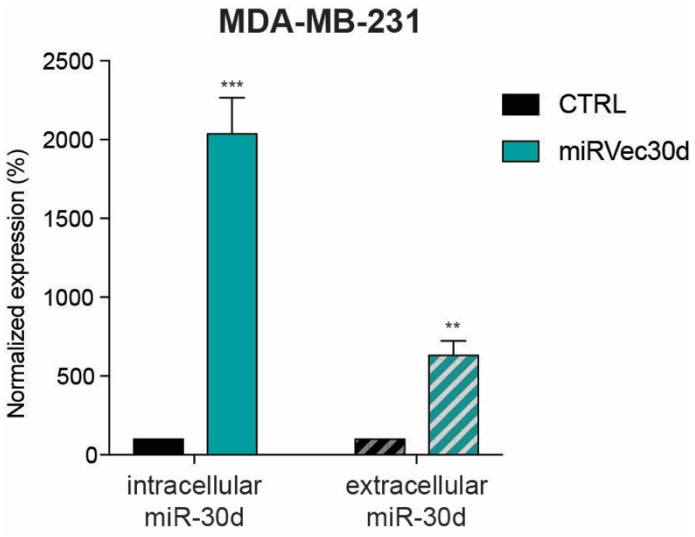

Figure 28. Intracellular and extracellular levels of miR-30d in MDA-MB-231 stable clones. MDA-MB-231 control cells and miRVec30d cells were grown for 48 hours and then both cells and medium were collected. The RNA was extracted from cells and medium as described in the Materials and methods section. Extracellular levels were normalized to a standard amount of cel-miR-39 added prior to RNA extraction. Bar graphs shows the results of q-RT-PCR for miR-30d expression; values are mean ± SEM from n=5 independent experiments (**: $p<0.01$; ***: $p<0.001$; t test).

Subsequently, I performed co-culture experiments of either control or miR-30d overexpressing MDA-MB-231 cells with normal-like breast epithelial MCF-10A cells (as receiving cells), using a transwelling filter system. In this previously described system[261], the two cell types cells shared the same medium for 16 h, being separated by a 8.0 μm porous membrane. I did not observe significant changes in the extracellular miR-30d levels of MCF-10A/MDA-MB-231 control co-culture as compared to MCF10A alone, with a slight but not significant increase in the intracellular miR-30d levels in MCF-10A (Figure 29A). As expected, in the co-culture of MCF-10A and MDA-MB-231 miRVec30d cells there was a significant enrichment of extracellular miR-30d levels compared to the previous experiment; strikingly, this was associated with a concomitant increase of intracellular miR-30d within recipient MCF-10A cells, suggesting that miR-30d secreted by tumor cells is taken up by normal-like cells.

Next, I verified whether miR-30d, after its uptake, can perform its post-transcriptional functions in receiving cells. Indeed, MCF-10A cells co-cultured with MDA-MB-231 miRVec30d cells displayed the downregulation both at mRNA and protein level of two miR-30d targets, namely DLG5 and DGKZ that we have previously identified in our laboratory (Figure 29B-C, and data not shown). Furthermore, since miR-30d promoted EMT in epithelial cells, as mentioned in the preliminary results, I investigated if the absorbed miR-30d might exert a transforming effect on normal receiving MCF-10A cells. Interestingly, upon co-culture with MDA-MB-231 miRVec30d cells, MCF-10A cells showed a reduction in the protein levels of E-cadherin and an increase in the protein level of vimentin, two classical EMT markers, compared to co-culture with MDA-MB-231

control cells (Figure 29C). This suggested that secreted miR-30d is able to exert its oncogenic function in receiving cells, for example triggering the EMT process.

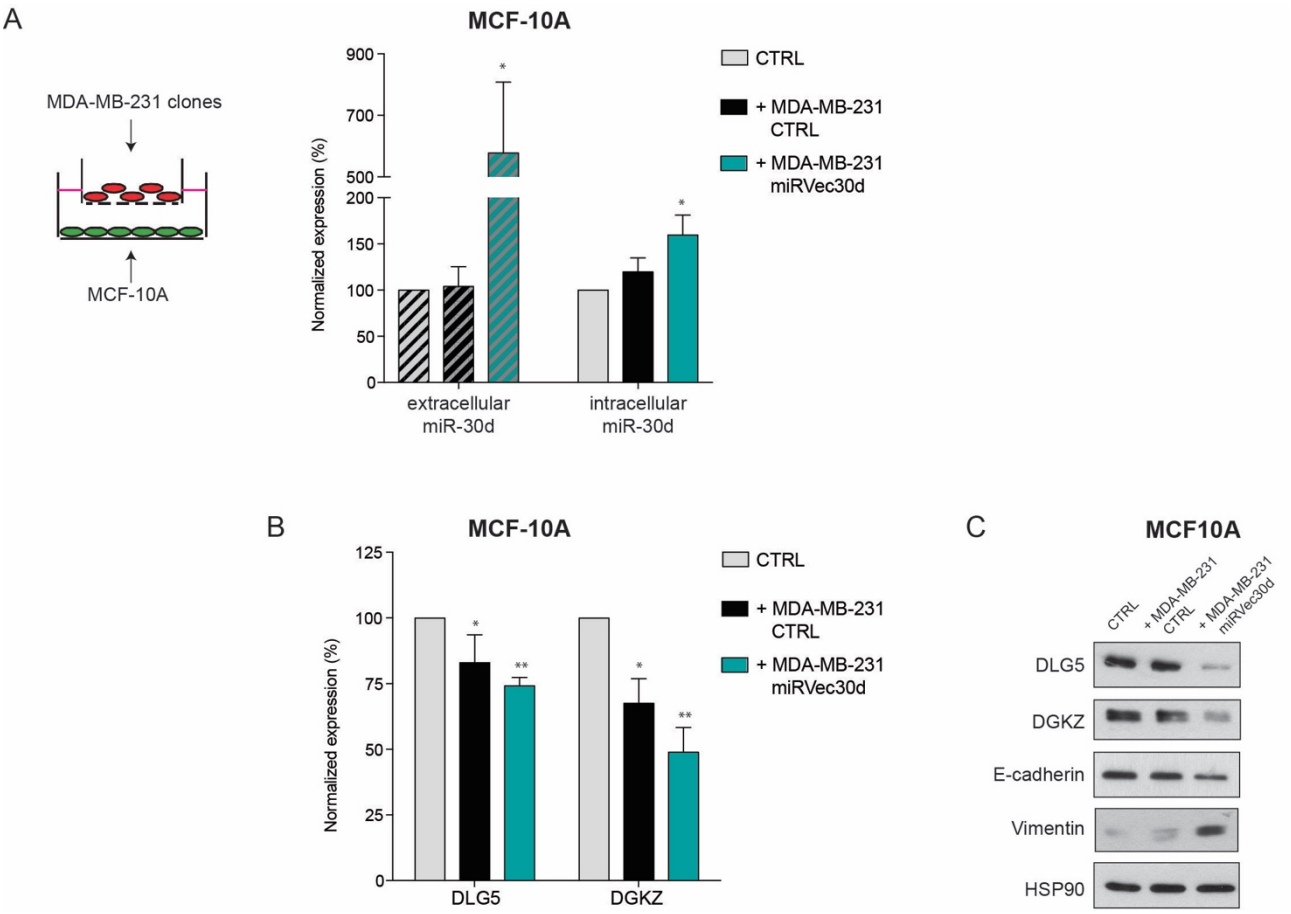

Figure 29. **Effect of cancer-secreted miR-30d in co-colture experiments.** MDA-MB-231 control cells or stably overexpressed miR-30d were cocultured with MCF-10A using a transwelling system as schematically depicted in the cartoon in **A**. After 16 hours of coculture, the shared medium was recovered, and RNA was extracted, adding a standard amount of cel-miR-39 to allow normalization. Cells were collected and subjected to RNA or protein extraction. **A.** Bar graphs show the extracellular levels of miR-30d in the coculture medium and the intracellular miR-30d expression in the indicated coculture conditions. Values are mean ± SEM from n=5 independent experiments (*: $p<0.05$; t test). **B.** Bar graphs with the q-RT-PCR analysis of two target genes (DLG5 and DGKZ) previously identified in our laboratory as direct target of miR-30d. Values are mean ± SEM from n=3 independent experiments (*: $p<0.05$; **: $p<0.01$; t test). **C.** Western blot analysis of the protein levels of the two miR-30d target genes (DLG5 and DGKZ) and of the well-known EMT markers E-cadherin and vimentin. HSP90 was used as loading control.

4.9 miR-30d as a potential biomarker of tumors expressing mut-p53

We have demonstrated that mut-p53 regulates miR-30d expression in cancer cells (see preliminary data). Since, as mentioned above, miR-30d is also secreted by cancer cells, I decided to investigate whether its secretion parallels its expression regulated by mut-p53.

I silenced mut-p53 by RNAi in MDA-MB-231 cells and then extracted total RNA both from cells and the recovered culture medium. qPCR analysis confirms the intracellular reduction of miR-30d expression after mut-p53 depletion and revealed a 35% reduction of secreted miR-30d levels in the same context (Figure 30A), indicating that mut-p53 sustains both expression of miR-30d and its secretion in the extracellular compartment. This result prompted to uncover a role of miR-30d as a

putative biomarker for mut-p53 expressing cancers. I thus analyzed miR-30d levels from sera of a cohort of 43 mice with different p53 status. In particular, the available cohort consisted of wild-type p53 mice ($p53^{+/+}$, n=15), homozygous mut-p53 R172H knock-in mice ($p53^{M/M}$, n=16) and homozygous p53 knock-out mice ($p53^{-/-}$, n=12). Knock-in and knock-out mice spontaneously develop lymphomas and sarcomas, with a median latency of 8-9 months[196,262]. We sacrificed $p53^{M/M}$ and $p53^{-/-}$ mice when tumor growth occurred together with age- and sex matched wild-type controls ($p53^{+/+}$) and collected their total peripheral blood. After sera purification, RNA was extracted adding exogenous cel-miR-39 to allow normalization of circulating miRNAs. Figure 30B shows the results of qPCR analysis for miR-30d from the sera of the mice cohorts. I unveiled that mice harboring p53 mutation and developing cancer presented a significant increase of miR-30d levels in the sera compared to healthy mice. Conversely p53 knock-out mice, even if they had developed a tumor, did not display significant changes in miR-30d sera levels compared to control mice. Thus, miR-30d levels in the blood correlated with p53 status, supporting its role as possible cancer biomarker for tumors with p53 GOF mutations.

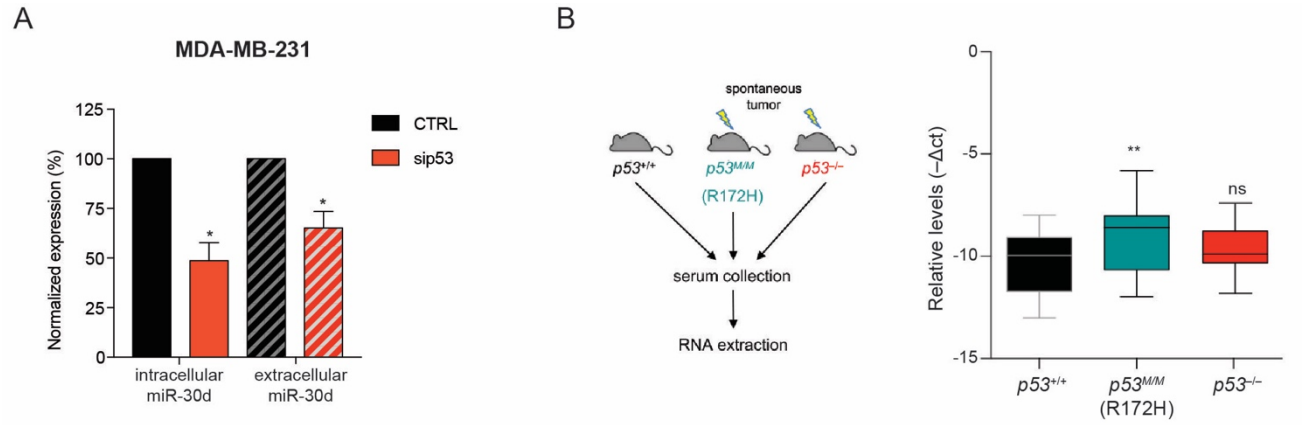

Figure 30. **miR-30d secretion depends on mut-p53 *in vitro* and *in vivo*. A.** MDA-MB-231 cells were silenced with either control or p53 specific siRNA. After 48 hours, both cells and medium were collected and RNA was extracted, adding a standard amount of cel-miR-39 to allow normalization. Bar graph shows the results of q-RT-PCR analysis for miR-30d expression. Values are mean ± SEM from n=3 independent experiments (*: $p<0.05$; t test). **B.** Schematic overview of the in *vivo* experimental workflow. The mice cohort consists of n=15 wild-type p53 mice ($p53^{+/+}$), n=16 homozygous mut-p53 R172H knock-in mice ($p53^{M/M}$) and n=12 homozygous p53 knock-out mice ($p53^{-/-}$). When tumor occurred, $p53^{M/M}$ and $p53^{-/-}$ mice were sacrificed together with matched control $p53^{+/+}$ mice and sera purified from peripheral blood. Then, RNA was extracted from the same amount of clarified sera (200 µl), adding a standard amount of cel-miR-39 to allow normalization. Box plot shows the levels of miR-30d relative to cel-miR-39 presented as −Δct. Mann Whitney test was used for statistical analysis (**: $p<0.01$)

5. Discussion

During tumor progression, cancer cells communicate with each other and the surrounding tissue to shape a permissive tumor microenvironment. The main mechanism of paracrine communication includes the secretion by cancer cells of soluble and insoluble factors, such as proteins, metabolites, extracellular vesicles (EVs), and also circulating nucleic acids, among which miRNAs[43,70,95]. Overall, these components constitute the cancer secretome, which can be finely reprogrammed by cancer cells to sustain tumor growth and progression. Thus, a growing interest is addressed to the study of the mechanisms by which cancer cells are able to reprogram their secretome and consequently alter the TME. Indeed, a better knowledge of these mechanisms will be instrumental to provide the basis for new effective therapeutic interventions against tumors, as well as for novel potential biomarkers for clinical use.

Preliminary data, produced by the laboratory where this PhD project has been developed, proved that the mut-p53/miR-30d axis promotes total protein secretion in breast cancer cells, highlighting a novel biochemical process instigated by mut-p53. This aberrant secretion was further characterized by means of mass spectrometry, unveiling that the mut-p53/miR-30d axis can modulate the cancer cell secretome and thus suggesting an impact of the axis on TME.

The work presented in this thesis pointed out that mut-p53, through the regulation of miR-30d expression, is able to alter the tumor microenvironment with a dual mechanism: on one hand, by promoting total protein secretion, which results in a secretome with protumorigenic effects on different components of the TME, and, on the other hand, by sustaining secretion of miR-30d itself, that exerts oncogenic functions in receiving cells.

miR-30d belongs to the miR-30 family, which in humans consists of five members (miR-30a, -30b, -30c, -30d and -30e), which share a common seed sequence, but present different compensatory sequences that allow them to target different genes. In cancer, miR-30 family members were described as both tumor suppressor and oncogenic miRNAs, and these differential behaviors may be due to the different targeting ends or different tumor contexts[263]. Nevertheless, an oncogenic role for miR-30d was reported in a wide range of tumors[143,233–237].

The role of miR-30d in influencing TME is still elusive. In 2011 Gaziel-Sovran *et al.* have demonstrated that miR-30d, in cluster with miR-30b, targets and downregulates the GalNAc transferase GALNT7 in human melanoma, thus indirectly promoting the secretion of the immunosuppressive IL-10[143]. Strikingly, secretion of miR-30d has been reported for both normal

and cancer cells, exerting a role in intercellular communication between normal cells[240,252] and as putative diagnostic or prognostic biomarker for some human tumors[247–250].

Several studies have underlined cell-extrinsic effects for mut-p53 in modulating the interplay with TME[213]. This is achieved mainly through transcriptional regulation of genes that encode secreted proteins[228]. Recently, it has been reported that mut-p53 can reprogram macrophages towards a tumor supportive and anti-inflammatory state through the secretion of miR-1246 within exosomes[229]. Interestingly, our preliminary data demonstrated that the mut-p53/miR-30d axis fosters total protein secretion by cancer cells. Furthermore, comparing the results of the mut-p53-dependent secretome in MDA-MB-231 cells to our previously published data on mut-p53-regulated transcriptomic and proteomic profile in the same cell line[211], we unveiled that about the 70% of differentially secreted proteins did not appear to be affected by mut-p53 neither at the mRNA nor at the protein level. This result indicates that the effect of mut-p53 on secretome is mainly a consequence of a global impact on the process of secretion.

Interestingly, GSEA analysis of the transcriptomic profile regulated by miR-30d indicated protein secretion as a process strongly affected by miR-30d, impacting on the expression of key factors of ER-Golgi vesicular trafficking. Moreover, identifying the miR-30d targets is mandatory to understand how the deregulated expression of this miRNA subvert the secretory pathway. Of note, preliminary data produced in the laboratory identified DGKZ as mut-p53/miR-30d target gene. DGKZ is an enzyme that acts as a negative regulator of diacylglycerol in membrane, affecting membrane trafficking and exocytosis[264,265]. It would be interesting to investigate if the mut-p53/miR-30d axis enhances protein secretion by downregulating DGKZ.

In this thesis, I provided evidence that the enhanced protein secretion prompted by the mut-p53/miR-30d axis alters the TME *in vitro* by producing a protumorigenic secretome.

First, I dissected the effects of the mut-p53/miR-30d-dependent secretome on TME constituents, such as ECM, stromal cells (endothelial cells and fibroblasts) as well as other cancer cells. I unveiled that in cancer cells the mut-p53/miR-30d axis promotes the secretion of some ECM components and increases the matrix stiffness. This result is interesting considering that increased ECM stiffness can in turn influence the behavior of cancer cells, for instance by activating mechanotransduction pathways which trigger cytoskeleton reorganization and regulate the levels and activity of mechanosensitive transcription factors to drive wide gene expression programs[13]. Among these are YAP/TAZ transcription cofactors, which play essential roles in organ growth and tissue homeostasis[266]. Consistently, YAP/TAZ are aberrantly activated in many human tumors, where they have been shown to be essential for cancer progression, metastasis and

chemoresistance[156]. YAP/TAZ are mechanosensors, regulated by ECM stiffness, cell shape and cytoskeletal tension, and also mechanotransducers, integrating mechanical cues with cell-specific transcriptional programs[267]. For instance, cells cultured on stiff ECM displayed increased nuclear localization and consequently transcriptional function of YAP/TAZ[23]. In addition, very recently our group has demonstrated that mechanical cues from ECM are able to control mut-p53 stability and thus its function through a mevalonate/RhoA/HDAC6 axis[212]. Thus the results provided in this thesis suggest the existence of a pro-tumorigenic feedback-loop: mut-p53 promotes ECM stiffness, which in turn sustains its own stability and GOF activity, along with YAP/TAZ oncogenic function. Furthermore, increased ECM stiffness in cancer cells could influence non-transformed cells in TME, in particular promoting CAFs migration and contractility, endothelial cells function and angiogenesis, TAMs migration, as well as T cells activation and maturation[117]. The mut-p53/miR-30d-dependent secretome induces the formation of new vessels, with a concomitant increase of vascular permeability. These results corroborate the results of a previous report by Fontemaggi *et al.*[218], where an angiogenic potential of mut-p53 was reported, and my data add on to this evidence, showing that mut-p53 contributes also to vascular leakiness. This latter aspect might be relevant considering that leaky vessels in tumors can lead to hypoxia, facilitate immune cells infiltration, metastatic spreading and impair chemotherapy delivery[268]. Interestingly, I first provided evidence about the ability of mut-p53/miR-30d axis to induce paracrine activation of fibroblasts. In fact, conditioned media from mut-p53/miR-30d axis derived from TNBC cells harboring different mut-p53 variants were able to confer CAF-like features to normal fibroblasts. In particular, I observed enhanced migration and induction of some markers of activation (e.g. fibronectin, α-SMA, YAP/TAZ, HIF-1α). The induction of YAP/TAZ might promote ECM remodeling and stiffness, as described by Calvo *et al.*[22], whereas HIF-1α induction and stabilization under normoxic conditions suggested that activated fibroblasts may undergo metabolic reprogramming[26]. Recruitment and activation of fibroblasts by cancer cells is extremely important for cancer development and dissemination[18]. Indeed, CAFs in turn influence proliferation, invasion and survival of cancer cells. Furthermore, CAFs exert a strong and broad influence on TME, promoting ECM deposition and remodeling, angiogenesis, metabolic and immune reprogramming. Beyond the aforementioned protumorigenic effects on stromal components, the mut-p53/miR-30d-dependent secretome can also influence other malignant cells to gain aggressive features, such as enhanced migration, contributing to cancer heterogeneity.

Notably, I demonstrated a critical role for miR-30d expression in a non-transformed context. Indeed, our preliminary data unveiled that overexpression of miR-30d in normal breast immortalized MCF-10A cells was able to sustain total protein secretion. In this thesis I proved that

this enhanced protein secretion in a non-tumor context was able to produce a secretome with promigratory effects in the surrounding environment. Importantly, it has been demonstrated that under hypoxic conditions HIF transcription family factors induce miR-30d levels in the breast cancer cell line MCF-7[269]. Since hypoxia is a master regulator of secretome reprogramming[122,125,270], it would be interesting to understand whether miR-30d could mediate the impact of the hypoxic response in TME. It has been reported that tumors bearing *TP53* mutations are generally characterized by higher levels of hypoxia[271]: mut-p53 appears to stimulate HIF-1α stabilization in hypoxic conditions, consistently with its reported ability to repress the ability of TAp63 to mediate Sharp-1-dependent HIF degradation, resulting in enhanced metastatic potential[272]. Thus, it would be interesting to characterize a possible interplay between mutant p53 and HIFs in regulation of miR-30d expression and its downstream phenotypes.

Subsequently, given that the GO enrichment analysis of the secretome highlighted a role of secreted EVs, I assessed the contribution of the mut-p53-miR-30d axis in the secretion of EVs. I found that mut-p53/miR-30d axis did not impinge on the amount of secreted EVs, but can affect the release of proteins within EVs, accordingly to its role in regulating total protein secretion by cancer cells. The role of mut-p53 in exosome secretion is still controversial. A recent report showed that dysfunction in *TP53* (knock-out or presence of a mutant p53 variant) led to a reduced size of exosomes compared to wildtype cells in the colorectal cancer cell line HCT116[179]. However, this result can be tumor-context specific and also mut-p53 variant specific (R273H).

The mut-p53/miR-30d axis, through a differential loading of proteins within EVs, may modulate its interactions with TME, exerting both local and systemic effects. Indeed, EVs have been reported to promote both primary tumor growth and "education" of a supportive pre-metastatic niche (PMN)[69,70]. Specifically, EVs released by primary tumor may alter specific distant tissue sites favoring the colonization and outgrowth of disseminated cancer cells. Proteins loaded within EVs lumina and surface can modulate their effect in receiving cells[72]. For instance, in a subset of breast cancer cell lines a different pattern of surface integrins perturbs organotropism of EVs conferring tissue-specificity to colonization sites[90]. Moreover, once docked to recipient cells, EVs can deliver their cargo, activating various responses and processes inside cells. Thus, regulating protein cargo within EVs, the mut-p53/miR-30d axis could strongly regulate its impact on TME.

Further experiments are required to demonstrate the contribution of mut-p53/miR-30d-dependent secretome *in vivo*, for instance through systemic administration of conditioned medium or EVs regulated by this axis in nude mice to evaluate its ability to foster PMN formation and/or metastasis. Moreover, it would be interesting to deeply investigate the molecular mediators, either free or carried by EVs, that are responsible for conditioning both local and distal microenvironment.

Among the molecules secreted by cancer cells, an interesting role is played by secreted miRNAs, which can be internalized by cells of the surrounding microenvironment, carrying out their multiple effects. Moreover, cancer-secreted miRNAs acquired great importance also for their putative role as non-invasive cancer biomarkers[95]. In fact, they have been found released in the blood and other biological fluids, and they can correlate with tumor onset, evolution and response to treatment.
In this thesis, I showed that miR-30d secreted by cancer cells can be taken up by receiving non-transformed cells, where it can downregulate its target genes and trigger an EMT process, according to its proposed oncogenic role. These results sustain the role of miR-30d in mediating intercellular communication, as previously reported in non-tumor contexts[240,252]. However, my results suggest the possibility that in a tumor context miR-30d, secreted by cancer cells, can mediate its oncogenic effects in a paracrine way, promoting the transformation of normal cells.
Moreover, I demonstrated that miR-30d secretion is mut-p53-dependent both *in vitro* and *in vivo*. Strikingly, in this latter context I proved that miR-30d secretion in the sera of mice was associated with tumors bearing mutation in *Trp53*. This result leads us to consider miR-30d as a candidate biomarker associated with p53 status in tumors. A diagnostic or prognostic role for secreted miR-30d has been already reported in patients with lung cancer[247,248], melanoma[250] and multiple myeloma[249], but this is the first evidence of its association with p53 mutations. To date, only higher levels of miR-1246 in exosomes have been associated with colorectal cancer patients carrying mut-p53[229].

The mut-p53/miR-30d-dependent regulation of secretome unveiled in this thesis can allow the identification of non-invasive biomarkers and potential therapeutic targets associated with mutations in *TP53* gene in cancer.
Indeed, secreted molecules, including proteins and miRNAs, can represent promising cancer biomarkers, that could be associated with mut-p53 presence. To date, only two reports attribute a role for mut-p53 in regulating putative secreted biomarkers. The first evidence regards the increased expression and secretion of prostate-specific antigen (PSA) by prostate cancer cells *in vivo* and *in vitro*[273]; the second one is the abovementioned exosomal miR-1246 levels increased in the plasma of colorectal cancer patients[229]. The demonstration of the *in vivo* association of increased sera levels of miR-30d in tumors harboring p53 mutations can be added to the list of possible mut-p53-dependent secreted biomarkers. The discovery of additional mut-p53-related secreted biomarkers may provide new tools for diagnostic and prognostic evaluation, as well as new effective therapeutic strategies for cancer patients harboring mutations in *TP53.*

Targeting the secreted mediators of the communication between cancer cells and the surrounding TME is considered a promising strategy to counteract tumor progression. Indeed, secreted factors can be expected to be more susceptible to inhibition than the paracrine or juxtacrine signaling mediators that act within solid tumors[46]. Examples from available literature include the use of neutralizing antibodies against specific secreted molecules such as LOX[128] or angiogenesis inhibitors[28]. Recently, our group has demonstrated that administration of statin or zoledronic acid, two inhibitors of the mevalonate pathway, was able to reduce stroma-tumor mechanosignaling and mut-p53 destabilization in cancer cells[212]. Starting from our mass spectrometry data, I can expect to identify a subset of secreted proteins able to mediate the protumorigenic effects on the TME and then pursue their inhibition through antibodies or small molecule inhibitors.

In sum, since I demonstrated that miR-30d in cancer cells is a critical mediator of TME alteration, both promoting a protumorigenic secretome, and mediating intercellular communication as secreted miRNA, I believe that its inhibition may represent a promising anticancer therapeutic strategy. In fact, several systems for miRNA inhibition exist that are able to target *in vitro* and *in vivo* both intracellular and extracellular miRNAs[115,238,274], and it would be intriguing to testing them in the near future.

6. Materials and methods

Cell cultures

Cell lines

MDA-MB-231, MDA-MB-468, SK-BR-3 and BJ cells (immortalized and engineered as previously described in Voorhoeve and Agami[275]) were cultured in DMEM medium (Lonza) supplemented with 10% FBS (EuroClone) and Penicillin-Streptomycin Mixtures (100 IU/mL, Lonza). H1299 cells were cultured in RPMI medium (Lonza) supplemented with 10% FBS and antibiotics. WI-38 cells were cultured in EMEM medium (Lonza) supplemented with 10% FBS, Penicillin-Streptomycin Mixtures (100 IU/mL, Lonza) and 1% non essential aminoacids (Lonza); these cells were used within the 38th passage of culture to avoid senescence. MCF-10A cells have been maintained in culture in a medium made by DMEM and Ham's F12 (Lonza) mixed in a ratio 1:1, and supplemented with 5% horse serum (LifeTechnologies), Penicillin-Streptomycin Mixtures (100 IU/mL, Lonza), insulin (10 µg/mL, Sigma-Aldrich), hydrocortisone (0.5 µg/mL, Sigma-Aldrich) and epidermal growth factor (EGF, 20 µg/mL, Peprotech).

HUVECs (Human umbilical vein endothelial cells) were kindly provided by Dr. Chiara Agostinis (IRCCS "Burlo Garofolo", Trieste) and isolated as previously described[276]. The cells were seeded in plates precoated with fibronectin (Roche) and maintained in endothelial serum-free basal medium (Life Technologies) supplemented with 20 ng/ml bFGF (basic Fibroblast Growth Factor), 10ng/ml EGF (Epidermal Growth Factor) (Life Technologies) and 10% of FBS (EuroClone). All the experiments with HUVECs were carried out within the fourth passage of cell culture.

All cells were maintained in an incubator set at 37°C and 5% pCO2.

Stably transduced cell lines

MDA-MB-231 cells with stable inhibition of miR-30d were obtained by lentiviral transduction with TWEEN 3'UTR EGFP empty, that was used as a negative control, and with the miR-30d decoy construct. The TWEEN 3'UTR EGFP empty was kindly provided by R. De Maria, and the miR-30d decoy was cloned as described by Bonci et *al.*[238].

MCF-10A cells silenced for endogenous wtp53, overexpressing the mut-p53 variant R175H were obtained by retroviral transduction with pRS-shp53, kindly provided by R. Agami, and pMSCV-p53 R175H vector. This latter vector was obtained by cloning p53 R175H coding region into a pMSCV empty vector, after having introduced silent mutations in the region targeted by p53 siRNA by site-directed mutagenesis in pLPC p53 R175H.

MDA-MB-231 cells overexpressing miR-30d were obtained by retroviral transduction with pRS-Blast and miR-Vec-30d, kindly provided by R. Agami.
Infected cell populations were selected using puromycin (Sigma-Aldrich) and/or blasticidin (InvivoGen), 2µg/mL each, for at least one week.

Transfection

For siRNA and miRNA transfections, cells were plated 24 hours before the transfection experiment. Lipofectamine RNAiMax (Invitrogen) was used to transfect 40 nM siRNAs or 3 nM miRNAs following the manufacturer's instructions, using a control siRNA at the same molar concentration as a control.
siRNAs and miRNAs used in this thesis are listed in the table below:

siRNA/miRNA	**Sequence**	**Manufacturer**
Control siRNA	Unknown	All Star Negative Control (1027281,Qiagen)
sip53 ORF	GACUCCAGUGGUAAUCUAC	Eurofins MWG
miR-30d mature miRNA	UGUAAACAUCCCCGACUGGAAG	Pre-miR™ miRNA Precursors hsa-miR-30d (PM10756, Ambion)
miR-Negative Control	Unknown	Pre-miR™ miRNA Precursor Negative Control #1 (AM17110, Ambion)

Conditioned medium

After 6 hours from transfection or 24 hours from seeding, cells were washed twice with PBS and replaced with fresh serum free medium (supplemented with antibiotics). After a certain time interval (24 hours for MCF-10A cells, 48 hours for MDA-MB-46 and SK-BR-3 cells, 60 hours for MDA-MB-231 cells), the conditioned medium (CM) was collected, filtered through a 0.22 µm syringe filter (EuroClone) and centrifuged 2,000 xg for 5 min to remove cells and debris. Cell-free CM was transferred to a new tube for subsequent use. CM was used usually immediately or kept a 4°C for up to 2 days or aliquots were stored at -80°C for up to 1 week.

Extracellular vesicles (EVs) isolation

EVs were collected from equivalent amounts of conditioned medium using Total Exosome Isolation reagent from cell culture media (4478359, Invitrogen) according to the manufacturer protocol. Briefly, 0.5 volumes of the reagent were added to CM; then the mixture was mixed well by vortex and the samples were incubated at 4°C overnight. After, samples were centrifuged at 10,000 xg for

1 hour at 4°C. The supernatant was carefully removed and the pellet was proper used according to the subsequent procedures.

Protein expression analysis

Protein extraction

Total cell extracts were lysed with Lysis Buffer (50 mM Tris-HCl pH 7,5, 300 mM NaCl, 1% NP40, 1 mM EDTA) supplemented with 1 mM PMSF, 5 mM NaF, 1 mM Na_3VO_4, 10 μg/ml CLAP. Cells were then centrifuged at 10,000 rpm for 10 minutes at 4°C. Protein concentration was determined with Bio-Rad Protein Assay Reagent (#500-0006, Bio-Rad). Samples obtained were denatured in Laemmli Sample Buffer 2X or 6X.

For the recovery of secreted proteins, 1 ml of CM was precipitated by addition of 100% Trichloroacetic acid (TCA) (Sigma) to a final concentration of 10% and incubation at 4°C overnight. The following day, the samples were centrifuged at 13,200 rpm for 15 min at 4°C. Pellets were resuspended in 0.4 ml 100% chilled acetone by vortexing, and protein precipitates were recovered by centrifugation at 13,200 rpm for 10 min at 4°C and air-dried at room temperature for approximately 30 min. Finally, pellets are dissolved in Laemmli Sample Buffer 2x.

EVs pellets were resuspended in lysis buffer supplemented with with protease and phosphatases inhibitors, and then denatured in Laemmli Sample Buffer 6X.

All the samples were denatured by heating at 95°C for 5 min.

Western blot

Lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Amersham). Blocking was performed in Blotto-tween (PBS, 0.2% Tween-20, not fat dry milk 5%) or with TBST-milk (0.2% Tween-20, Tris/HCl 25mM pH 7.5, plus 5% not fat dry milk) depending on the antibody. Anti-mouse and anti-rabbit HRPO-conjugated (Sigma) were used as secondary antibodies. Membranes were analyzed by chemiluminescence using ECL™ Western Blotting Reagents (Amersham) or LiteAblot Extend Long Lasting Chemiluminescent Substrate (EuroClone). The following primary antibody were used.

Target	**Antibody**
β-actin	a9718 (Sigma-Aldrich)
α-SMA	ab5694 (Abcam)
CD63 (H-193)	sc-15363 (Santa Cruz)
CD9 (C-4)	sc-13118 (Santa Cruz)
DGKZ	HPA051336 (Sigma-Aldrich)

DLG5	HPA000555 (Sigma-Aldrich)
E-cadherin	610182 (BD)
Fibronectin (N1N2)	GTX112794 (GeneTex)
GAPDH (6C5)	sc-32233 (Santa Cruz)
GFP	Home-made produced rabbit polyclonal
Glypican-1 (N3C3)	GTX104557 (GeneTex)
GM130	610822 (BD)
HIF-1α (D2U3T)	#14179 (Cell Signaling)
HSP70 (C92F3A-5)	sc-66048 (Santa Cruz)
HSP90 (HSP90 α/β, F-8)	sc-13119 (Santa Cruz)
Laminin 5 (γ_2 chain)	MAB19562 (Millipore)
Laminin-β1	ab69633 (Abcam)
p53 (DO-1)	sc-129 (Santa Cruz)
VEGF	GTX102643 (GeneTex)
Vimentin (V9)	ab8069 (Abcam)
WWTR1 (TAZ)	HPA007415 (Sigma-Aldrich)
YAP (63.7)	sc-101199 (Santa Cruz)

Atomic force microscopy (AFM) analysis

AFM was used to asses ECM mechanical properties and investigate extracellular vesicles (EVs) dimensions and distribution.

Stiffness measurement

Elastic assessment of ECM stiffness was performed as previously described[212]. MDA-MB-231 cells were seeded on coverslips and transfected as described above. Cells were washed with 1X PBS and fixed with 4% paraformaldehyde for 20 min. Nuclei were marked via haematoxylin staining, while ECM was visualized using the collagen- and amyloid-specific dye Picro Sirius Red Stain (Abcam, ab 150681). AFM imaging was performed at room temperature on a Smena AFM (NT-MDT Co., Russia) mounted on an inverted fluorescence microscope (Nikon Eclipse Ti-U). For each sample, 60 randomly chosen areas were measured and analysed. The cantilever used was a tip-less probe characterized by a spring constant of about 0.03 $nN \cdot nm^{-1}$ (HQ:CSC38 cantilevers from MikroMasch Co), at the end of which a 18 μm diameter silica bead (Thermo Fisher Scientific) was glued using UV curable glue (Norland Products Inc.). Force spectroscopy measurements were performed at constant speed (2.5 $\mu m \cdot s^{-1}$) and triggered to a maximum force applied to the sample of 5 nN. Elastic modulus values, in kPa, were determined by fitting obtained force/displacement

curves with a Hertzian model for the tip used taking advantage of the NOVA (NT-MDT Co., Russia) control and analysis software. Statistics and data processing were performed using Igor Pro software (www.wavemetrics.com) and R statistical computing software (www.R-project.org). The significance of the differences in the data was established as equality of probability distributions via the Kolmogorov-Smirnov test.

Characterization of EVs

Precipitated EVs were resuspended and fixed in 30 μL of 4% paraformaldehyde for 20 min, and then diluted to 100 μL in H_20. EVs were subsequently suspended in a 1.0% solution of osmium tetroxide in 0.1 M Na-Cacodylate buffer, pH 7.4. After being rinsed with 0.1 M Na-Cacodylate buffer alone samples were carefully washed with mQ water and processed following the procedure described in Junker *et al.*[277] and Rauti *et al.*[278]. Briefly, a 15 μL drop of sample solution was placed and left to adsorb (15 min) onto a freshly peeled mica substrate, thereafter rinsed with mQ water. EVs were then dried under a gentle stream of nitrogen. AFM was used in semicontact mode at RT in air using a commercial instrument (Solver Pro, NTMDT, RU). Silicon tips (NSC36/CR-AU, MikroMash, USA) with a typical force constant of 0.6 nN/nm and a resonance frequency of about 65 kHz were employed. Topographic height and phase images were recorded at 512 × 512 pixels at a scan rate of 0.5 Hz. Image processing was performed using Gwyddion freeware AFM analysis software, version 2.40.66. For statistical analysis, 10 5x5 μm^2 fields were acquired for each sample and EVs' dimensions and densities were evaluated. In particular, the height of each vesicle was evaluated from cross-line profiles associated to an average diameter.

Tube formation assay

Tube formation assay was performed as previously described[276]. HUVECs were placed on wells coated with Matrigel (Becton Dickinson) and incubated for 24 hours with VEGF (20 ng/mL) or CM from different conditions to allow tube formation. After fixation with 4% paraformaldehyde and staining with Phalloidin-Alexa Fluor 488 (Invitrogen), the number of tubules was counted under a Leica AF6500 microscope using LAS software (Leica).

Permeability assay

HUVECs were seeded onto fibronectin-coated transwell inserts (0.4 μm pore size; Corning). When cells are confluent, they were pre-treated for 24 hours with CM from different conditions, or for 30 minutes with TNFα (100 ng/ml, Invitrogen). FITC-dextran (1 mg/ml, 70 kDa, Sigma) was added to monolayer (upper chamber) for 30 minutes. The presence of FITC-dextran in the lower chamber

was assayed at 495 nm by using Enspire multimode plate reader (PerkinElmer). Fluorescence intensity measurements were expressed as relative permeability by calculating the fold increase over the basal permeability of untreated monolayer (control).

Wound healing assay

Equal numbers of BJ-EHT or WI-38 cells were grown in 6-well or 12-well plates until they reached confluence. Then, cells were scraped with a pipette tip and, after washing with PBS, were incubated with CM from different conditions or serum-free medium alone as a control. For each experimental point, two or three scratch were performed, and one image of the wounding area were acquired immediately after scratching and then in the same field after 16 hours of migration. The relative wound closure was quantified by measuring the wound area at the time of scratching and the end point of the experiment using ImageJ (NIH Image).

For the experiments performed with WI-38, CM or control medium (DMEM) was diluted 1:1 in the basal medium for culture of WI-38 cells. Instead, EVs pellets were resuspended in 1 ml of serum-free DMEM before using them for wound healing assay.

Migration assay

H1299 cells were grown in 6-well in the presence of CM (supplemented with 10% FBS) for 48-72 h. Then $5x10^4$ cells were seeded in 24 well PET inserts (8.0 μm pore size, Falcon) in the presence of CM (supplemented with 1% FBS) in both compartments. After 16 hours, cells on the upper part of the membrane were removed with a cotton swab and cells that passed through the filter were fixed in 4% PFA, stained with 0.05% crystal violet and counted.

For the migration assay performed with boiled conditioned medium, CM was first heat-inactivated at 100°C for 10 min as previously described[257], and then used according to the procedure above described.

Co-culture experiments

$2x10^5$ MCF-10A cells were seeded in 24-well plates suitable for transwell insert (Falcon), and allowed to adhere for 4 hours. Then, $2x10^5$ MDA-MB-231 control or miRVec30d cells were seeded in 24 well PET inserts (8.0 μm pore size, Falcon) placed above the wells with MCF-10A. All the cells were seeded in the same medium, that is the complete basal medium for culture of MCF-10A cells. After 16 hours, the inserts were removed, and the medium and cells collected for the subsequent analysis.

Collection of sera from mice

The mice cohort was generated, monitored and genotyped as previously described[196]. All experimental protocols were approved by the Animal Ethics Committee of the University of Trieste. When tumor occurred, $p53^{M/M}$ and $p53^{-/-}$ mice were sacrificed together with age- and sex matched wild-type controls ($p53^{+/+}$). Total peripheral blood was collected and immediately processed to recover the sera. After an overnight incubation at 4°C to allow coagulation, serum is recovered after two subsequent centrifugations at 4,000 rpm for 10 minutes at 4°C. The sera thus collected were stored at -80°C until their use.

RNA extraction and RT-qPCR

Total RNA was extracted with QIAzol (Qiagen) following manufacturer's instructions. For analysis of miRNA expression in culture medium and mice sera, cel-39 miRNA mimic (0.1 nM) was previously added to QIAzol. 1 ml of culture medium or 200 µl or mice sera were diluted 1:5 in QIAzol according to manufacturer's instruction. For RNA extraction from mice sera, RNeasy MinElute Cleanup Kit (Qiagen) was used.

For microRNAs expression analysis, 1µg of cellular RNA or 20µl of RNA extracted from culture medium/mice sera was retro-transcribed with miScript PCR System (Qiagen). miR-30d and the control housekeeping U6B and SNORD25 small nuclear RNAs were amplified with miScript SYBR Green PCR kits (Qiagen), following manufacturer's instructions on a CFX96™ Real-Time PCR System (Biorad).

For mRNA expression analysis, 1µg of total RNA was reverse-transcribed with QuantiTect Reverse Transcription (Qiagen). Analyzed genes were amplified using SsoAdvanced™SYBR® Green Master Mix (Biorad) on a CFX96™ Real-Time PCR System (Biorad).

List of primers used:

miScript Primer Assay Name	**Official Symbol**	**Catalog Number**
Hs_SNORD25_11	SNORD25	MS00014007 (Qiagen)
Hs_RNU6B_13	RNU6B	MS00014000 (Qiagen)
Hs_miR-30d_2	MIR30D	MS00009387 (Qiagen)
Cel_miR-39_1	MIR39	MS00019789 (Qiagen)

Target	**Sequence**
H3	Fw: 5'-GAAGAAACCTCATCGTTACAGGCCTGGT-3' Rev: 5'-CTGCAAAGCACCAATAGCTGCACTCTGGAA-3'

DLG5	Fw: 5'-CGGCCGAAGCTTGCTCCAG-3' Rev: 5'-TCCGGGGAACAGTGCCCACA-3'
DGKZ	Fw: 5'-AGCAGTACTGTGTAGCCAGGAT-3' Rev: 5'-CACGGAAGGACGGCTTACAG-3'

GO term analysis

The significant and positive results of the secretome were entered in the DAVID tool (http://david.abcc.ncifcrf.gov/) using their official gene symbols. The default options were selected and the functional annotation clustering combined view was generated. A maximum p-value of 0.05 was chosen to select only significant categories.

Statistical analysis

Differences between values reported in the figures were analyzed by Student's t test using Prism 7 (GraphPad), except when otherwise indicated. P-values < 0.05 were considered significant.

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