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Regulation of cellular plasticity and extracellular vesicle secretion in breast cancer

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Abstract

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Epithelial-to-mesenchymal transition (EMT) is a dynamic process controlling the transition of cells between epithelial and mesenchymal states in various physiological or pathological conditions. In cancer, EMT promotes cell dissemination and metastatic colonization, enriches tumors with stem cell populations and confers resistance to anticancer therapy. Instigators of EMT activate a cohort of transcription factors (EMT-TF) which regulate the expression of each other and confer dynamic chromatin modifications to transcriptionally repress epithelial and induce mesenchymal genes. In this respect, transforming growth factor-β (TGF-β) is a potent inducer of EMT in different types of cancer.

In this study we first identified a link between the EMT-TF SNAI1 that is highly expressed in aggressive triple-negative breast cancers (TNBC), with the establishment of an intermediate epithelial-mesenchymal phenotype and the dual transcriptional induction of FOXA1 and androgen receptor which define mammary epithelial cell differentiation towards the luminal subtype. Studying additional phenotypes of *SNAI1* mutant TNBCs, we showed that SNAI1 through TGF-β/SMAD signaling and repression of FOXA1, induces the guanine exchange factor PSD4/EFA6B, driving a vesicular trafficking program that promotes cell-matrix interactions and invasiveness.

Tumor-derived extracellular vesicles (EV) are important mediators of intercellular communication and of microenvironment formation where tumors develop. In this study we showed that MEK/ERK signaling, drives TGF-β promoting EV secretion by regulating cholesterol homeostasis in TNBC cells. Additionally, TGF-β ligands and matrix metalloproteases identified as EV protein contents, conferred pro-invasive attributes and resistance to chemotherapeutic drugs in recipient cells.

Metabolism has a well-documented role in tumor progression and EMT maintenance and here we propose that a hybrid epithelial-mesenchymal state upon knockout of the EMT-TF *SNAI2* in TNBC cells, associated with altered expression of genes involved in metabolic pathways. This perturbed cell cycle progression in the mutant cells presumably via the transcription and stem cell factor SOX4.

In conclusion, this study provides insights into the contribution of the SNAIL family EMT-TFs, in the dynamic EMT process and the mechanisms by which this manifests the development of aggressive breast carcinomas. Furthermore, it provides means on the way TGF-β impacts on the biogenesis, secretion, and functional transfer of EV cargo molecules in the context of cancer.

Keywords: Cellular plasticity, extracellular vesicles, breast cancer, EMT

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To those who stand by my dreams and find charm in my intriguing spirit

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. **Tsirigoti, C.**, Ali, M.M., Maturi, V., Heldin, C.-H. and Moustakas, A. (2022) Loss of SNAI1 induces cellular plasticity in invasive triple-negative breast cancer cells. *Cell Death Dis.*, 13, 832.

II. **Tsirigoti, C.**, Ali, M.M., Rodrigues-Junior, D.M., Johansson, S., Heldin, C.-H. and Moustakas, A. SNAI1 and SMAD/TGF-β signals antagonize FOXA1 to control PSD4/EFA6B expression and couple integrin-mediated adhesion to endocytic fate in triple-negative breast cancer cells (2023). *Manuscript*.

III. Rodrigues-Junior, D.M. **Tsirigoti, C**. Psatha, K., Kletsas, D., Aivaliotis, M., Heldin, C.-H. and Moustakas, A. TGF-β induces cholesterol accumulation to regulate the fate of tumor-derived extracellular vesicles (2023). Submitted *manuscript*.

IV. **Tsirigoti, C.**, Ali, M.M., Heldin, C.-H. and Moustakas, A. SNAI2 knockout induces metabolic and cell cycle changes in breast cancer cells (2023). *Manuscript*.

Related publications

I. Rodrigues-Junior, D.M., **Tsirigoti, C.**, Lim, S.K., Heldin, C.-H. and Moustakas, A. (2022) Extracellular vesicles and transforming growth factor β signaling in cancer. *Front. Cell Dev. Biol.*, 10, 849938.

Contents

Abbreviations

AKT: AKR mouse thymoma AKT-8 retroviral oncogene (v-AKT), a serine/threonine protein kinase (also known as PKB, protein kinase B or RAC-alpha (related to A and C) kinase
ALDH1A: Aldehyde dehydrogenase 1 alpha
ALIX: ALG2-interacting protein X/Programmed cell death 6-interacting protein
AP2: Adaptor protein 2
AR: Androgen receptor
ARF1: ADP-ribosylation factor 1
ARF6: ADP-ribosylation factor 6
CDH1: E-cadherin
E/M: Epithelial/Mesenchymal
ECM: Extracellular matrix
EGFR: Epidermal growth factor receptor
EMT: Epithelial to mesenchymal transition
EMT-TF: EMT-transcription factor
ERK: Extracellular signal regulated kinases
ESCRT: Endosomal sorting complexes required for transport
ESR: Estrogen receptor
EV: Extracellular vesicle
FAK: Focal adhesion protein
FN1: Fibronectin
FOXA1: Forkhead box A1
H3K27: Histone 3, lysine 27
H3K4: Histone 3, lysine 4
ILV: Intraluminal vesicle
lncRNA: long non-coding RNA
MAPK: Mitogen-activated protein kinase
MET: Mesenchymal to epithelial transition
miRNA: micro-RNA
MVB: Multivesicular body
PDGFR: Platelet-derived growth factor receptor
PI3K: Phosphatidylinositol 3-kinase
PPFIA1: PTPRF interacting protein alpha 1
SMAD: Small mothers against decapentaplegic

SNARE: Soluble NSF attachment protein receptor
SRC: Sarcoma kinase
TGF-β: Transforming growth factor β
TGFβR: Transforming growth factor β receptor
TGN: Trans-Golgi network
TME: Tumor microenvironment
TNBC: Triple negative breast cancer
TSG101: Tumor susceptibility gene 101
VIM: Vimentin
ZEB1: Zinc finger E-box-binding homeobox 1
ZO-1: Zonula occludens-1
α-SMA: alpha-Smooth muscle actin

Introduction

"What causes cancer?" stands as the central question in cancer biology and molecular oncology and many years of research are devoted in exploring cancer causality "how does a tumor grow and spread within an organism?" and etiology "what are the factors that initiate and promote cancer development?". As recent as in mid-seventies, Harold Varmus and J. Michael Bishop set the ground in understanding the molecular mechanisms underlying cancer formation, by identifying a family of genes, the so-called oncogenes, which upon alterations can result in cancer development. This milestone discovery has since then broadened our insight into the molecular and signalling systems that control the transformation of normal into tumor cells and into how tuned functions and behaviors of the latter form life-threatening tumors and metastases.

As such, the complexities of human cancer pathogenesis are nowadays being conceptualized as the products of multistep processes that allow the development of premalignant cells which acquire multiple phenotypic and functional attributes during tumorigenesis and malignant progression. In an effort to provide a common principle that justifies the complex phenotypes of human tumors, a set of shared traits among all types of cancer cells, called hallmarks of cancer, was generated. These comprise the acquired abilities of cancer cells for genome instability, sustaining proliferative signalling, evading growth suppressors, promoting inflammation, enabling replicative immortality, resisting cell death, activating invasion and metastasis, inducing or accessing the vasculature, avoiding immune destruction and reprogramming cellular metabolism (Hanahan, 2022).

Taking a step further, increasing evidence demonstrate the existence of heterogeneous cancer cell populations in a given tumor and appreciate their integral role in malignant cancer progression together with the stromal cells of the tumor microenvironment. A potent mechanism in this respect that provides the tumor with its remarkable adaptive abilities allowing metastasis, immune evasion and drug resistance is plasticity in terms of differentiation. Cellular plasticity does not represent a novel invention of cancer cells, but rather an appropriation of fundamental properties of multicellular life that enables coordination during organogenesis in early development, repair and regeneration. One illuminating case for the convergence of developmental and regenerative processes as distinct events of metastatic progression, shows that the

metastatic program of lung adenocarcinoma cells, recapitulates key stages of lung morphogenesis as if the tumor tries to "grow a lung tissue" at the wrong time and place (Laughney et al., 2020).

As is evident, our understanding about cancer cells and thus disease progression is ultimately connected to the fundamental and evolutionarily conserved rules of life. By providing context and inventive perspective to these principles, we can build comprehensive scaffolds that will delineate the origins of cancer and lead to advancements in the prevention and cure of the disease. In this respect, cancer research has made great discoveries of individual molecules that govern diverse traits of cancer cells; however, we still lack a clear understanding of how the instigation and transmission of complex signals, formed by these molecules, decide on the fate of individual cells in our bodies.

On this ground, the present thesis work aims to contribute to our knowledge on how transcription factors initiating epithelial to mesenchymal transition, evidenced to take place both in embryonic and cancer development, regulate cellular plasticity favoring the development of highly aggressive and heterogeneous breast cancer subtypes. During the course of this study, we additionally investigated alternative sources of cancer cell heterogeneity, emphasizing in the induction of differentiation reprogramming by intercellular communication via extracellular vesicles.

1. Epithelial to mesenchymal transition – EMT

1.1 EMT in development and cancer

EMT is a cellular process that takes place during embryonic development, wound healing, fibrosis and cancer development, and governs cell transition between epithelial and mesenchymal states. Betty Hay was the first to introduce the tern "epithelial to mesenchymal transformation" in embryos, while later on she pointed out the plastic and dynamic nature of this process (Hay, 1990, 1991). EMT causes epithelial cells to express a mixture of molecular constituents, resulting in the adoption of a mesenchymal phenotype presenting migratory behavior. Cells with the latter phenotype have the ability to undergo the inverse process called mesenchymal-to-epithelial transition (MET). During EMT, epithelial cells create looser cell-cell adhesions (i.e., replacement of E-Cadherin by N-Cadherin and low expression of tight junctional components), modify their interactions with the ECM (based on high expression of specific and new sets of integrin receptors), reorganize their cytoskeleton (i.e., lower numbers of cytokeratins and higher numbers of mesenchymal vimentin) and remodel the secreted extracellular proteins. As such cells acquire a highly motile phenotype, and, development of an altered ECM, will enforce additional phenotypic changes (Derynck and Weinberg, 2019; Huang et al., 2013; Moustakas and Heldin, 2012; Nieto et al., 2016).

EMT has key roles during embryogenesis. One of the first studies revealing the implication of EMT during embryonic development, showed that cells of the primitive ectoderm in rabbit embryos delaminated the epiblast and migrated in order to give rise to mesodermal tissues during gastrulation (Greenburg and Hay, 1982). Plethora of following developmental studies established the role of EMT in the neural crest and mesectoderm formation as well as heart development. EMT is also associated with wound healing and fibrosis, by providing epithelial cells with motile characteristics to re-establish the damaged tissue and transforming epithelial cells into myofibroblasts which accumulate in the ECM, respectively. However, these processes will not be further described since this report focuses on the role of EMT in cancer development. Involvement of the EMT process in cancer progression was pointed out more than a hundred years ago when Santiago Ramon y Cajal described undifferentiated breast tumors as follows: "*The epithelial islands are not surrounded by a basement membrane… We shall mention the fusiform, pear-like and star-like forms… The cells are not attached to each other… This*

explains their invasive ability, since free of intercellular cement, they can migrate through the connective tissue" (Ramon y Cajal, 1890). In this context, EMT is linked with the reformation of the primary tumor microenvironment in order to facilitate its outgrowth, whilst important is its contribution in cancer cell dissociation from the primary tumor and dissemination in distant organs via the bloodstream to form metastases (Massague and Obenauf, 2016). However, lack of morphological evidence in clinical samples and of specific markers as well as oversimplification of the addressed process, has created confusion and strongly debated the role of EMT during cancer progression. Currently, the EMT status in a primary tumor is being validated by the expression of a combination of epithelial and mesenchymal genes, as was established in a breast cancer microarray (Sarrio et al., 2008). In addition, the EMT status of a given tumor type predicts the overall- and disease free-survival of patients (Tan et al., 2014), suggesting that EMT most likely occurs *in vivo* and impacts on cancer progression.

1.2 Epithelial mesenchymal plasticity – Intermediate EMT phenotypes

As mentioned earlier, the simplified model of EMT describing the transition of cells between two states (epithelial, mesenchymal) generated ambiguities and thus in an effort to better understand this concept, experimental cancer and mathematical models of EMT dynamic equilibria, proposed the existence of partial EMT or hybrid epithelial/mesenchymal (E/M) cancer cell phenotypes (Bierie et al., 2017; Jolly et al., 2019; Kröger et al., 2019; McFaline-Figueroa et al., 2019; Pastushenko et al., 2018). In this context, epithelial cells that undergo EMT generate derivative phenotypes of mixed molecular identity, whereby epithelial and mesenchymal proteins are co-expressed (Tsubakihara and Moustakas, 2018). This model supports the phenotypic heterogeneity and plasticity present within a tumorigenic tissue and reflects the different biological properties (i.e., migration, survival and proliferation) of its constituent cancer cells (Huang et al., 2013; Williams et al., 2019).

1.3 Regulation of EMT

The phenotypic and functional changes observed in cancer cells have been traditionally explained by the differential activities of signalling and transcriptional programs, stimulated by secreted factors from the tumor microenvironment. Such a factor, capable of inducing EMT is the transforming growth factor β (TGF-β) (Derynck and Weinberg, 2019; Moustakas and Heldin, 2012). TGF-β signaling is directly coupled to the transcriptional induction of a cohort of transcription factors that initiate the EMT (EMT-TFs), such as SNAI1, SNAI2, ZEB1, ZEB2 and TWIST1, TWIST2, many of which (SNAI1, ZEB1,

ZEB2) also receive signals from TGF-β that control their activity (de Herreros et al., 2010; Derynck and Weinberg, 2019; Moustakas and Heldin, 2012; Nieto et al., 2016). Shortly, TGF-β ligands deposited on the ECM upon their activation, bind to transmembrane receptors known as TGF-β receptor type II and type I with a serine/threonine kinase activity and form a hetero-hexameric complex. This complex further phosphorylates the receptor-activated SMAD proteins SMAD2/3, which oligomerize with SMAD4 and translocate to the nucleus in order to bind to DNA and regulate the expression of target genes, in this case to induce the EMT-TF expression (Moustakas and Heldin, 2009). These transcription factors even though they have distinct and characteristic DNA-binding and transactivation domains, they regulate the expression of a similar cohort of genes. An overview of the SNAIL EMT-TF family protein structures is provided in Figure 1. EMT-TFs function by conferring dynamic chromatin modifications in the promoters of target genes and thus affecting their expression. In turn, EMT-TF activities, can be modulated by the expression of other EMT-TFs, post-translational modifications and miRNAs. For a detailed overview of EMT-TF function and regulation, see section 1.4. These events, create feed-forward and negative feed-back loops in EMT regulation, contributing to the diverse phenotypic spectrum of the hybrid E/M states (Meyer-Schaller et al., 2019; Zhang et al., 2014).

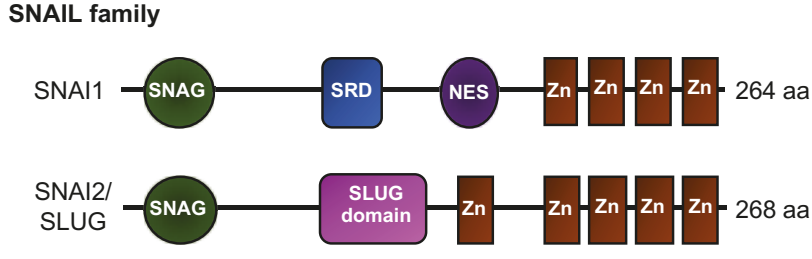

Figure 1. Overview of the SNAIL family protein structures. The Snail family proteins have one DNA-binding domain, represented in brown. aa, amino acids; SNAG, Snail corepressor binding domain; SRD, serine-rich domain; NES, nuclear export sequence.

1.4 Regulation of EMT-TF function and activity

As mentioned above, the extent to which epithelial cancer cells will transit towards a more mesenchymal state via EMT, highly depends on the activation of EMT-TFs and their post- transcriptional and post-translational processing. Such procedures may be context-dependent and not be relevant for all cancer types, thus a complete overview will not be presented in this report; besides examples of well established activity regulation mechanisms of the EMT-TFs SNAI1 and SNAI2/SLUG will be discussed.

SNAI1 is responsible for the repression of the expression of epithelial genes, including E-cadherin (Batlle et al., 2000; Cano et al., 2000). Its function

is mostly mediated by transient binding to promoters of target genes together with SMAD proteins, or by recruiting chromatin modifiers i.e. histone deacetylase 1 (HDAC1), HDAC2, lysine-specific demethylase 1 (LSD1) and components of the polycomb repressor-2 (PRC2) complex (Herranz et al., 2008; Lin et al., 2010; Peinado et al., 2004; Vincent et al., 2009). The transcriptional activity of SNAI1 is regulated by diverse signalling pathways, including the glycogen synthase kinase-3β, which phosphorylates nuclear SNAI1 at two distinct domains, one inducing SNAI1's export to the cytoplasm and the other recruiting ubiquitin ligases that promote cytoplasmic SNAI1 proteasomal degradation (Zhou et al., 2004). In an inverse signalling scenario, the collagen receptor discoidin domain receptor 2, upon adhesion to collagen matrix, activates the LATS2 protein kinase, which phosphorylates nuclear SNAI1, thus retaining SNAI1 to the nucleus and prolonging its stability and transcriptional activity (Zhang et al., 2013; Zhang et al., 2012). Recently, it was demonstrated that SNAI1 also presents non-canonical, EMT-independent functions, which are not based on E-cadherin repression, but rather on the inactivation of the Retinoblastoma (Rb)-restriction checkpoint of senescence allowing cell cycle progression (Paul et al., 2023).

Repression of the expression of epithelial genes is a function conserved in SLUG as well, however it differs from SNAI1 in terms of specificity and mode of binding to specific DNA motifs known as 5′-CANNTG-3′ or E-boxes (Villarejo et al., 2014). The transcriptional activity of SLUG is positively regulated by epigenetic modifications mediated by a histone H3K27 demethylase (the Jumonji domain-containing protein 3 (JMJD3)) while its phosphorylation by the glycogen synthase kinase-3β promotes its proteasomal degradation (Kim et al., 2012a; Tang et al., 2016).

Additionally, EMT-TFs can also form negative-feedback loops with epithelial associated miRNAs i.e. miR-34 and miR-200 and control the EMT plasticity (Diaz-Lopez et al., 2014). As such, miR-34 downregulates the expression of SNAI1, while SNAI1 can bind to the promoters of the miRNAs and repress their expression (Diaz-Lopez et al., 2015; Siemens et al., 2011).

1.5 EMT in tumor metastasis and metastatic colonization

Outgrowth of the primary tumor drives intravasation of cancer cells into the bloodstream or the lymphatic vessels, extravasation and metastatic colonization to another tissue (Massague and Obenauf, 2016). This process is associated with circulating tumor cells (CTCs) that exhibit EMT in order to degrade their surrounding basal membrane and intravasate, and with MET induction that favors metastatic colonization (Khoo et al., 2015; Ocana et al., 2012). The role of EMT in tumor growth and invasion has been indicated by EMT-TF loss of function studies. For example, silencing of *SNAI1* in the TNBC model

MDA-MB-231 revealed loss of mesenchymal protein expression and decreased invasiveness *in vitro*, accompanied by reduced tumor growth and metastatic potential in xenografted mice (Olmeda et al., 2007a; Olmeda et al., 2007b). Additional CRISPR/Cas9-mediated *SNAI1* knockouts in TNBC cells, indicated the generation of intermediate E/M phenotypes with decreased proliferation and migration capacity (Maturi et al., 2018; Yamamoto et al., 2017). Whether, EMT is required for metastatic colonization, has been controversial. Reports utilized breast and pancreatic cancer lineage-tracing studies in mice, using a Cre recombinase activated by mesenchymal-related specific transcriptional promoters, demonstrated that EMT is not required for metastatic colonization (Fischer et al., 2015; Zheng et al., 2015). In opposition to this, another study utilizing the same pancreatic cancer model, revealed the role of ZEB1 in cell plasticity and metastasis induction (Krebs et al., 2017). In line with this, a lineage-tracing study based on N-cadherin expression in mice, indicated that an active EMT programme takes place prior to breast cancer metastasis to the lung (Li et al., 2020). As mentioned earlier, the oversimplification of the process of EMT, accompanied by the generation of experimental models considering changes in the expression of end-stage (epithelial or mesenchymal) markers, most probably accounts for such controversies. As such, the notion that EMT is a continuum of molecular changes, accompanying plasticity in epithelial cancer cell differentiation (Jolly et al., 2019; McFaline-Figueroa et al., 2019), may better reflect the "reality". Research focused on the establishment of E/M markers, depicts the heterogeneity of tumor cell populations in regard to the EMT transition stages and their tumor associated functions (Pastushenko et al., 2018). At least in experimental mice, the various subpopulations of cancer cells that exhibited the plasticity of the E/M phenotype, presented similar tumor initiation potential with yet diverse metastasis rate and distinct localization within the tissue (Pastushenko et al., 2018). In addition, a study in a prostate cancer mouse model revealed that mesenchymal cells, even though presenting similar tumor initiation potential with cells of a hybrid E/M phenotype, could not generate metastases (Ruscetti et al., 2015). The latter supports the notion that CTCs need to acquire a more epithelial phenotype, permissive of effective proliferation in a normal tissue, whereas prevention from such process leads to dormancy (Castano et al., 2018; Harper et al., 2016).

1.6 EMT and cancer stem cells

As pointed out earlier, tumors consist of highly heterogeneous cell populations with diverse tumorigenic and metastatic potential. Among these, cancer cells with high tumor initiating potential are referred as cancer stem cells (CSCs) due to their ability to self-renew and give rise to more differentiated cell populations, similar to their normal counterparts. The first indication that EMT is

involved in the generation of CSCs, was when transiently expressed EMT-TFs in human breast cancer cells, enhanced the frequency with which these cells formed tumors in appropriate host mouse models (Mani et al., 2008; Morel et al., 2008). An overview of EMT implication in stem cell formation in normal and neoplastic mammary tissues, is presented in sections 2.1 and 2.2. Interestingly, accumulating evidence suggests that CSCs reside within a hybrid E/M state (Jolly et al., 2019; Schmidt et al., 2015). However, our understanding on the molecular mechanisms that associate EMT with the stem cell state, remains poor (Lambert and Weinberg, 2021). Efforts to unravel these mechanisms, have shown that EMT-TFs can interact with stem cell self-renewal regulators (i.e. the proto-oncogene and Polycomb ring finger BMI1 and the protein kinase A (PKA)), control the balance of symmetric (two stem cells) and asymmetric (one stem cell, one differentiating cell) cell divisions and facilitate DNA repair preventing stem cell ageing (Gross et al., 2019; Hwang et al., 2014; Pattabiraman et al., 2016; Yang et al., 2010). Early observations have also implicated the role of EMT and tumor microenvironment in the transduction of signals, important for stem cell maintenance, whilst interesting is the notion that certain E/M states may provide advantages by supporting the metabolic demands of stem cells (Gupta et al., 2019; Intlekofer and Finley, 2019; Shibue et al., 2013).

1.7 EMT and therapeutic interventions

Development of strategies aiming in the specific inhibition and/or reversion of EMT has been proven quite challenging over the years due to the plastic nature of the process, giving rise to cancer cells with distinct tumorigenic phenotypes, and its interconnection with multiple signalling pathways.

Inhibitors of signalling pathway components (i.e. TGFβR, EGFR, PDGFR, ERK/MAPK) are currently investigated as anti-EMT drugs, however, proof of concept of such chemical compounds in regard to EMT specificity is difficult to be assessed (Lamouille et al., 2014). Of the most widely studied anti-EMT drugs are TGFβR inhibitors (i.e., LY2157299), which have been shown to effectively inhibit EMT and metastasis in a breast cancer mouse model, when combined with the cytotoxic drug paclitaxel (Park et al., 2015). In addition, inhibitors targeting the focal-adhesion kinase (FAK), which mediates integrin-ECM interactions, have been shown promising in their ability to reverse EMT (Infante et al., 2012).

Efforts are currently focused on the development of EMT reversal agents which specifically target EMT-TFs. For example, HDAC inhibitors reversed ZEB1-mediated EMT in pancreatic cancer, while conditional knockout of ZEB1, but not of SNAI1 or TWIST, in a mouse model of pancreatic adenocarcinoma, suppressed metastatic progression (Krebs et al., 2017; Meidhof et

al., 2015; Zheng et al., 2015). Contradictory to the above were the observations of two additional studies, which showed that partial EMT reversion in mouse models, generates cancer cells with increased metastatic colonization capacity (Korpal et al., 2011; Ocana et al., 2012). Thus, better understanding of the regulation of the metastable EMT states and further characterization of the phenotypic aspects of the hybrid cells, will contribute to the development of promising and specific anti-EMT therapies. In such a concept, however, it is of crucial importance to determine to what extend the cells can be reversed and which phenotype - if any - is compatible with eliminating metastatic potential and re-sensitization to anti-cancer drugs without enhanced, MET-associated, metastatic colony formation ability.

2. Mammary gland development and carcinogenesis

The mammary gland is a specialized organ which undergoes multiple dynamic morphogenetic and differentiation changes throughout the lifespan of a mammal. Such changes are triggered by the actions of the ovarian steroid hormones estrogen and progesterone, during puberty, the reproductive (estrus) cycle and pregnancy (Arendt and Kuperwasser, 2015; Fu et al., 2020). Thus, understanding the mammary gland physiology and mammary cell lineage differentiation plasticity with concomitant decoding of molecular regulatory mechanisms, in health and disease, will open further opportunities for advances in breast cancer management.

2.1 Mammary gland development and regulation of its differentiation program

The mammary gland is formed by bilayered epithelia consisting of basal and luminal cell lineages. The inner layer of luminal cells surrounds the ducts and alveoli, while the outer layer of basal cells lies together with the basement membrane and adjacent to the vasculature (Fu et al., 2020). Mammary gland differentiation initiates from mammary stem cells (MaSCs) that generate unipotent luminal and basal mammary progenitor cells, which eventually differentiate into ductal luminal, alveolar and myoepithelial cells, all necessary for the proper lactating function of the gland (Fu et al., 2020). Recent single cell transcriptomic and chromatin accessibility screens in human and mouse mammary glands have elucidated new intermediate mammary progenitor cells and transcriptional networks that guide the mammary differentiation program (Nguyen et al., 2018; Pervolarakis et al., 2020). An overview of the transcriptional regulators determining the stem cell state, basal and luminal identity are presented in Figure 2, but will not be described further in this report. A special emphasis in the role of FOXA1 in lineage plasticity regulation is given in section 2.2.

One of the best characterized processes of de- or trans-differentiation that generates plasticity of phenotypic alteration within the mammary tissue is EMT (Lambert and Weinberg, 2021; Nieto et al., 2016). One of the first inci-

dents demonstrating the above, was when basal cells derived from the mammary gland of a mouse, displayed a mesenchymal phenotype when cultured *in vitro* with concomitant downregulated CDH1 and upregulated FN1 and VIM levels (Batlle et al., 2000; Cano et al., 2000; Mani et al., 2008). Such cell populations, could successfully reconstitute the mammary epithelium in cleared mammary fat pads of mice, demonstrating the existence of basal cells with mesenchymal and MaSC properties (Prater et al., 2014). Involvement of the EMT-TFs SNAI1 and SNAI2/SLUG in the maintenance of the basal phenotype and MaSC activity of mammary cells, further supports the activation of EMT programs in normal epithelial stem cells (Guo et al., 2012; Nassour et al., 2012). Interestingly, a homeostatic mechanism present in the mammary gland, provides luminal cells, via activity of the tumor necrosis factor (TNF), with the ability to abolish the stem cell potential of their neighboring basal cells driven by the Notch, Wnt and EGFR signalling pathways (Centonze et al., 2020).

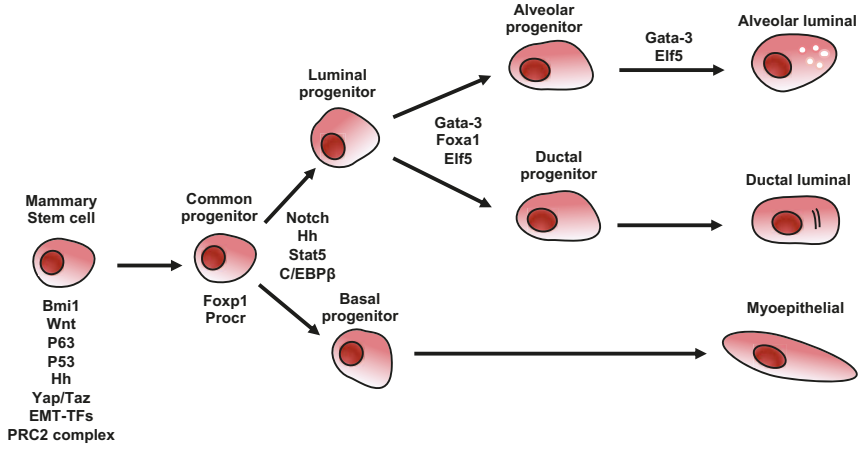

Figure 2. Molecular regulators acting along the mammary epithelial hierarchy according to Fu et al., 2020. These have been demonstrated to affect mammary stem cell self-renewal and activation, lineage commitment and luminal differentiation. Bmi1, Polycomb complex protein BMI1; Hh, hedgehog; PRC2 complex, polycomb repressive complex 2; Foxp1, Forkhead box P1; Procr, Protein C receptor; Stat5, Signal transducer and activator of transcription 5; C/EBPβ, CCAAT/enhancer binding protein beta; Foxa1, Forkhead box protein A1; Elf5, E74 like ETS transcription factor 5.

2.2 Breast cancer subtypes and cell of origin

Breast cancer classification divides the spectrum of phenotypes into tumors expressing estrogen receptor-α (ERα/ESR1), progesterone receptor (PGR) and/or human epidermal growth factor (EGF) family receptor HER2/ERBB2, which emanate from luminal epithelial progenitors and are classified as luminal-A or -B; the TNBCs do not express the above three receptors and are

further subdivided into basal-like 1 and -2, mesenchymal (or claudin-low), mesenchymal stem-like, luminal androgen receptor and immunomodulatory tumor cells (Beca and Polyak, 2016; Lehmann et al., 2011; Nagarajan and McArdle, 2018; Pommier et al., 2020; Shipitsin et al., 2007). The above classification is very complex and does not always link the observed tumor to a specific developmental event of the mammary tissue hierarchy, yet it emphasizes the variety of perturbations normal development can suffer leading to the heterogeneous, plastic tumor phenotype.

Several TNBCs, especially the most aggressive, metastatic and chemoresistant breast tumors, exhibit metaplasia, including mesenchymal differentiation, and present the molecular features of the EMT (Hennessy et al., 2009). Such tumors have accordingly been classified as mesenchymal or claudin-low tumors (Prat et al., 2010), a feature reproduced well in many established breast cancer cell lines (Blick et al., 2008; Neve et al., 2006), such as the mesenchymal and metastatic MDA-MB-231 cell that was derived from the pleural effusion of a patient (Cailleau et al., 1974). As such, a given breast tumor is normally composed of mixtures of phenotypes, some of which (in certain cases the majority), exhibit the mesenchymal phenotype and the rest exhibit epithelial features, with evidence of the inverse to EMT process, MET (Liu et al., 2014; Rios et al., 2019). This heterogeneity can explain why "mesenchymal", claudin-low breast tumors are sub-classified based on their content of luminal, EMT-exhibiting basal, and even cells that resemble best mammary stem cells (Lehmann et al., 2011; Pommier et al., 2020). This model of breast cancer diversification proposes that cellular phenotypes related to MaSCs carry the tumor-initiating potential under experimental conditions, whereas phenotypes related to an ability to invade and metastasize exist in both luminal and basal or mesenchymal, essentially deeply heterogeneous, breast cancers (Kim et al., 2012b; Pommier et al., 2020).

A pioneering factor with a pivotal role in directing the mammary luminal lineage is FOXA1. FOXA1 is a member of the winged-helix transcription factors, and its expression has been established not only in the development and differentiation of the mammary gland, but also in ERα- positive breast tumors (Friedman and Kaestner, 2006; Lacroix and Leclercq, 2004). FOXA1 acts as a pioneering factor and its recruitment to chromatin structures correlates with the regulation of transcriptional programs specific to ERα- positive breast tumors (Eeckhoute et al., 2006). Additionally, FOXA1 regulates the expression of androgen receptor target genes in both prostate and breast cancer, and TNBCs that co-express FOXA1 and AR have been shown to lose metastatic potential and present favorable survival outcomes (Jin et al., 2014; Lehmann et al., 2016; Maeda et al., 2016; Tang et al., 2012). Regulation of these transcriptional networks by FOXA1 is being achieved after its recruitment to condensed chromatin structures, in promoter or enhancer regions, increase of local chromatin accessibility further allowing binding of other transcription fac-

tors including nuclear receptors (Jozwik and Carroll, 2012). In such cases, expression of FOXA1 has been associated with DNA demethylation and *de novo* gain of active histone modifications, including H3K4 mono and/or di-methylation as well as H3K27 acetylation at lineage-specific enhancers (Lupien et al., 2008; Serandour et al., 2011; Yang et al., 2016).

2.3 Determinants of breast cancer heterogeneity

The hierarchy of mammary gland differentiation is of primary importance for the understanding of breast cancer development (Arendt and Kuperwasser, 2015; Fu et al., 2020). This is because the cell of origin that initiates breast tumorigenesis, eventually defines the type and fate of the developing tumor; oncogenic processes initiate in the archetypal stem cell of the tissue or in luminal or basal mammary progenitor cells (Fu et al., 2020). Alternatively, mechanisms of de-differentiation of mammary cells into progenitor-like phenotypes eventually cause phenotypic and molecular heterogeneity in breast tumors among patients (Shipitsin et al., 2007; Yuan et al., 2019). Another concept that adds to the complexity of breast cancer heterogeneity, is the existence of the CSCs that maintain tumor initiating properties, seed metastases and resist to anti-cancer treatment. EMT has been linked with the formation of breast CSCs (BCSCs) with either epithelial or mesenchymal phenotypes (Liu et al., 2014; Mani et al., 2008; Morel et al., 2008). $CD44^{+}CD24^{-}$ BCSCs associate with mesenchymal gene expression, invasive properties and maintenance in a quiescent state, whilst $ALDH1^{+}$ BCSCs associate with epithelial gene expression and a profound proliferative capacity (Liu et al., 2014).

Interestingly, oncogenic mutations and signals of the tumor microenvironment (i.e. TGF-β, WNT) may regulate the BCSC transition between the different states of the EMT continuum and the residence of the new cell types in a specific phenotypic state or locale (Liu et al., 2014; Shibue et al., 2013; Van Keymeulen et al., 2011). Giving emphasis on the last possibility, it has been shown that TGF-β signaling is a strong regulator of the lineage determinant FOXA1 in prostate cancer, while re-expression of the last in nasopharyngeal carcinoma cells, reprogrammed the TGF-β transcriptional program from a metastasis promoter to a tumor suppressor, generating cancer cell populations with less aggressive characteristics (Li et al., 2019; Song et al., 2019). Another interesting concept that adds to cellular plasticity as a mechanism of cancer heterogeneity is metabolism. In this respect, metabolic regulation during cancer progression and EMT is the main focus of numerous recent reports, which attempt to demonstrate the importance of such an interconnection not only in the survival and expansion of cancer cells, but also in the transition of one state of the EMT spectrum to another (Bergers and Fendt, 2021; LeBleu et al., 2014; Luo et al., 2018). Interestingly many signals that activate the EMT pro-

gram have been shown to reprogram metabolism. TGF-β for example, promotes glycolysis in glioblastoma cells, by inducing the expression of the glucose transporter 1 (GLUT1), the glycolytic hexokinase 2 (HK2) and of lactate dehydrogenase A (LDHA) to provide cells undergoing EMT the energetic demands for migration and invasion (Rodriguez-Garcia et al., 2017). Also, EMT-TFs have been involved in metabolic rewiring in connection with EMT status of a given cell type. These include alterations in the gene expression of enzymes involved in glycolysis and glucose-based oxidative phosphorylation (OXPHOS) (Dong et al., 2013; Krebs et al., 2017; Masin et al., 2014; Rosland et al., 2019; Yang et al., 2015). Overall, these observations reflect upon molecular and cellular mechanisms by which cancer cells can adopt to the dynamic alterations occurring during neoplasia.

3. Cell adhesion molecules: function and role in cancer progression

Cell-to-cell and cell-to-ECM adhesions, both consisting of transmembrane proteins interacting with intracellular scaffolds or signalling proteins and the cytoskeleton, establish connections in between the cells and anchorage to the substratum, respectively. Beside their role in the organization of tissues, cell adhesions are involved in the cytoskeleton rearrangement, cell migration and ECM remodelling. Thus, cell adhesions constitute dynamic structures that can be rearranged not only in response to physiological cellular processes, but also during pathophysiological conditions i.e., cancer, while important is their contribution to signal transduction regulation.

3.1 Cell-to-cell adhesion molecules

Cell-to-cell adhesions are comprised of adherens junctions (AJ), tight junctions (TJ), desmosomes and gap junctions. Adherens junctions mediate a calcium-dependent adhesion between the cells and are characterized by the presence of cadherin-catenin complexes in the junctions (Meng and Takeichi, 2009). Of the cadherin family members, E-cadherin is the most commonly expressed in epithelial tissues. The latter consists of five repetitive subdomains, each containing a calcium-binding sequence, which control the conformation of the extracellular domain and allow for trans-homophilic binding to the same type cadherins of the neighboring cell (Meng and Takeichi, 2009). The intracellular domain of E-cadherin interacts with β-catenin and p120-catenin which form a complex with α-catenin and get associated with the actin cytoskeleton (Drees et al., 2005; Yamada et al., 2005).

Tight junctions composed of claudin and occludin proteins intracellularly bound to ZO family adaptors, such as ZO-1, are located at the apical membrane parts of epithelial cells and either restrict the diffusion of substances outside the cell or drive the diffusion of proteins to the specific membrane domain (Tsukita et al., 2001; Tsukita et al., 2008). Desmosomes are intercellular junctions comprised of transmembrane desmocollin and desmoglein associated intracellularly with desmoplakin, plakoglobin (γ-catenin) and intermediate filaments (Delva et al., 2009). Gap junctions formed by connexin hexamers, form intercellular tunnels that allow the diffusion of small molecules between the cells (Laird, 2010).

3.2 Cell-to-ECM adhesion receptors

The major cell surface receptors mediating cell-to-ECM interactions are integrins. Integrins belong to an evolutionary conserved family of type I transmembrane receptors constituted by α and β subunits. In humans, integrins form 24 heterodimers, consisting of 18 α and 8 β subunits (Hynes, 2002). The integrin heterodimers are present in a closed, inactive conformation at focal adhesion sites or hemidesmosomes, where they can get activated with subsequent alteration of their conformation and transition to an active state. Hemidesmosomes are specialized desmosome-like adhesions located at the basal side of epithelial cells and interconnect two adjacent epithelial cells via the ECM that penetrates the lateral inter-epithelial space (Walko et al., 2015). Interestingly, integrins can be activated either by an inside-out or an outside-in signalling mechanism referred to as bidirectional signalling. In the first case, adaptor proteins (i.e., talin and kindlin) bind to the cytoplasmic region of integrin β subunits, change their conformation and increase their affinity for ECM ligands (i.e., fibronectin, laminin, collagen and vitronectin). In the second case, ECM proteins bind to integrin heterodimers, change their conformation and induce their clustering across the cell membrane with subsequent binding in their cytoplasmic tail and activation of adaptor and signalling molecules (i.e. vinculin, FAK, paxillin) (Sun et al., 2019). Activated integrins not only can mediate mechanical signalling to the actin or intermediate filament cytoskeleton via proteins including vinculin, paxillin, α-actinin and tensin, but also activate signalling pathways (i.e., PI3K/AKT, RAS/MAPK) essential for cell survival, proliferation and migration via autophosphorylation of FAK and activation of the Src family of tyrosine kinases (SFK) (Katoh, 2020). In addition, integrins can interact with leucocyte common antigen-related (LAR) in focal adhesions, the sites where the cell-to-ECM interactions occur. Liprin (LAR-interacting protein) scaffolds are linked with integrin-bound talin via CLIP-associating (CLASP) proteins and in cooperation with LAR, dephosphorylate integrin associated proteins, enhancing the turnover of focal adhesions for efficient protrusion of the front of the migrating cell (de Curtis, 2011; Stehbens et al., 2014).

3.3 Cell adhesion molecules and cancer progression

As explained in earlier sections, cell invasion and migration concur with cancer progression and metastasis. Signals from the tumor microenvironment initiate such processes by regulating the reorganization of the cytoskeleton and the turnover of the cell-to-cell and cell-to-ECM interactions.

During migration, the cell morphology is altered, and actin is organized in linear microfilaments (lamellipodia) in the leading edge, which form complexes with surface receptors and adhesion molecules, structures called filo-

podia (Friedl et al., 1997; Kaverina et al., 2002). In addition, cell surface proteases bind to and degrade locally, components of the ECM (Friedl and Wolf, 2009), while the tension generated by actomyosin contractions is released by the turnover of the focal adhesions at the rear of the cell, allowing for forward movement and further generation of protrusions in the leading edge (Friedl and Alexander, 2011). The modulatory factors of the tumor microenvironment (i.e., growth factors, ECM components) determine the rate and mode of cancer cell migration. As such, cancer cells can predominantly follow single-cell, multicellular or collective cell migration. In the first case, cell-to-cell adhesions are lost or replaced by looser ones (e.g. E-cadherin is replaced by N-cadherin during EMT), cells adopt an elongated morphology and develop strong adhesive and proteolytic properties toward the ECM substrata (Friedl and Alexander, 2011; Friedl and Wolf, 2003). During multicellular migration, cells maintain loose cell-to-cell adhesions and migrate one after the other along the same direction (Friedl and Alexander, 2011; Friedl and Wolf, 2003). Epithelial cells undergoing partial EMT and CSCs often adopt this type of migration, which protects them, while present in the circulation, against mechanical forces and the immune system (Aceto et al., 2014; Cayrefourcq et al., 2015). Cells with a collective migration pattern, retain their cell-to-cell junctions and move in a coordinated manner. In collective cancer cell invasion, leader cells with mesenchymal characteristics are often required, in order to orchestrate the ECM remodelling and movement of the bulk of the cells that follow (Friedl and Alexander, 2011; Friedl and Wolf, 2003).

The individual steps of the migration process are tightly regulated by integrins and their effector SFK proteins. Defective endocytosis/exocytosis of the first and/or altered expression of the second, can lead to increased cell growth and migration with clinical repercussions (Katoh, 2020; Kawauchi, 2012). Another example that points to the role of integrins in ECM assembly and cancer progression, shows that Kank proteins bound to liprin scaffolds direct fibronectin secretion at the focal adhesions to mediate fibrillogenesis and induce cancer cell migration (Chiaretti et al., 2016; Mana et al., 2016). Comprehensive reviews on integrins and cell adhesion can be found in: (Bachmann et al., 2019; Cooper and Giancotti, 2019; Yousefi et al., 2021).

Interestingly, integrins and their associated proteins, beside cancer cell invasion and migration, and among other functions, have also been implicated in CSC formation and colonization and development of drug resistance, molecular processes discussed extensively in this report (Cooper and Giancotti, 2019; Yousefi et al., 2021). An overview of the integrins involved in such programmes that mediate breast cancer progression is provided in Table 1.

Table 1. Integrins and integrin-associated proteins involved in breast cancer progression

Integrins	Integrin-associated protein	Main function	Breast cancer-related function
α3β1 **α6β1** **α6β4**	Laminin	Component of the basal lamina	Migration of metastatic breast cancer cells (Desai et al., 2018) Modulate stem cell population (Yousefi et al., 2021)
α2β1 **α1β1** **αvβ3**	Collagen	ECM structural protein	Chemoresistance (Baltes et al., 2020) Survival of human breast cancer cells (Badaoui et al., 2018)
α5β1	Fibronectin	cell adhesion, growth, migration	Breast cancer cell spreading and progression (Park and Helfman, 2019)
β1, β2, β3	α-actinin	Link between actin microfilaments and focal adhesions	Resistance to radiotherapy and breast cancer invasiveness (Desai et al., 2018; Yousefi et al., 2021)
α5β1 **α4β1** **α6β1** **α5β3**	Uroplasminogen activator receptor (uPAR)	ECM degradation, local fibrillogenesis, cell migration, inflammation	Breast tumor invasion and metastasis (Annis et al., 2018)

αvβ3	Matrix metalloproteinases (MMPs)	ECM degradation	Breast cancer cell proliferation, migration, angiogenesis (Radisky and Radisky, 2015)
	Insulin receptor substrate-1	Insulin and insulin-like growth factor signaling pathways	Breast cancer cell survival and invasion (Mardilovich and Shaw, 2009)

4. Vesicular trafficking

4.1 The biological basis of vesicular trafficking and regulation

Vesicular trafficking refers to the biological process in which vesicles transfer material either between a cell and its ECM or between cells of the tissue microenvironment. This process can be divided in three phases including endocytosis, vesicle intracellular movement and exocytosis.

Endocytosis (derived by the complex Greek word endokyttárosi) defines the process of internalization of molecules and surface proteins into the cell. Three modes of endocytosis have been described consisting of a) the non-selective molecule and nutrient internalization, b) phagocytosis and pinocytosis, and c) the molecule-specific receptor-mediated endocytosis (RME) (Haigler et al., 1979; Underhill and Ozinsky, 2002). Endocytosis via specific receptors can take place via clathrin-mediated (CME), caveolae-mediated (CavME) and non-clathrin-, non-caveolae-mediated endocytosis. CME was initially found to be involved in iron transport via transferrin uptake, while its significance in the internalization and recycling of multiple signal transduction receptors i.e. EGFR, TGFβR, was later pointed out (Ciechanover et al., 1983; Takei and Haucke, 2001). Briefly, AP2 present in the clathrin-coated pits (CCP) recognizes internalized receptors and together with other adaptor proteins recruits and concentrates them in the CCPs. Mature clathrin-coated vesicles are disassembled from the plasma membrane by the GTPase dynamin and the clathrin coating is removed (Schmid and Frolov, 2011; Traub and Bonifacino, 2013). CavME whose involvement is mainly in the transcellular transport of macromolecules and mechanosensing, arises by the binding of a ligand to its receptor, concentrated in cholesterol-enriched plasma membrane invaginations called caveolae. Mature caveolae are released by the plasma membrane by the action of the small GTPase dynamin (Parton and Simons, 2007).

The released vesicles derived by each of the endocytic pathways, are released in the intracellular space and can fuse with their target endosome during the vesicle intracellular movement phase. The first sorting of the endocytosed cargo takes place in the early endosomes. Then the internalised receptors of the early endosomes, can either follow a recycling pathway back to the cell surface or mature to late endosomes by receptor sorting into intraluminal vesicles (ILVs), ILV accumulation and multivesicular body (MVB) formation.

The endosomal sorting complexes required for transport (ESCRT) is a multi-protein machinery that plays crucial roles during this process. For detailed description, see section 5.2. Some of the cargo of the early endosomes can also be sorted to the trans-Golgi network (TGN) via the retromer complex. Late endosomes can either fuse with the cell membrane or move to lysosomes, where degradation of the endocytosed cargo takes place (Huotari and Helenius, 2011). In the first case, late endosomes can release their cargo (ILVs) extracellularly, via exocytosis. This aims either in the regulation of the levels of certain components intracellularly or to intercellular communication (Blanc and Vidal, 2018). In addition, lysosomal exocytosis which involves the fusion of lysosomes with the plasma membrane and subsequent release of their content, is a mechanism important for the disposal of lysosomal undigested material (Buratta et al., 2020). Alternatively, cellular contents or defective organelles can be delivered to lysosomes via a self-eating process called autophagy. During this process, a newly formed membrane arising by a phagophore assembly site (PAS), elongates and engulfs the material to be degraded in autophagosomes which are then driven to and fused with the lysosomes (Mizushima et al., 2011; Tong et al., 2010). Interestingly, autophagosomes can interact with late endosomes and form hybrid domains called amphisomes, which can either fuse with the plasma membrane and release their content or move to lysosomes for subsequent degradation (Buratta et al., 2020).

Vesicular trafficking is controlled by RAB GTPases of the RAS superfamily, which can cycle between an inactive GDP-bound and an active GTP-bound state via GTPase-activating proteins (GAP) and guanine nucleotide exchange factors (GEF) (Wandinger-Ness and Zerial, 2014). Each endosomal compartment is enriched in certain GTP-bound RAB proteins, which interact with specific effector proteins and catalyse multiple steps of the membrane trafficking, including cargo selection and vesicle formation, vesicle uncoating, movement and tethering as well as vesicle fusion with the plasma membrane (Hutagalung and Novick, 2011). The RAB proteins have been implicated in the regulation of the autophagic process and its interconnection with the endosomal pathway as well (Szatmari and Sass, 2014). A schematic description of vesicular trafficking and its regulation by well-characterized RAB proteins is depicted in Figure 3.

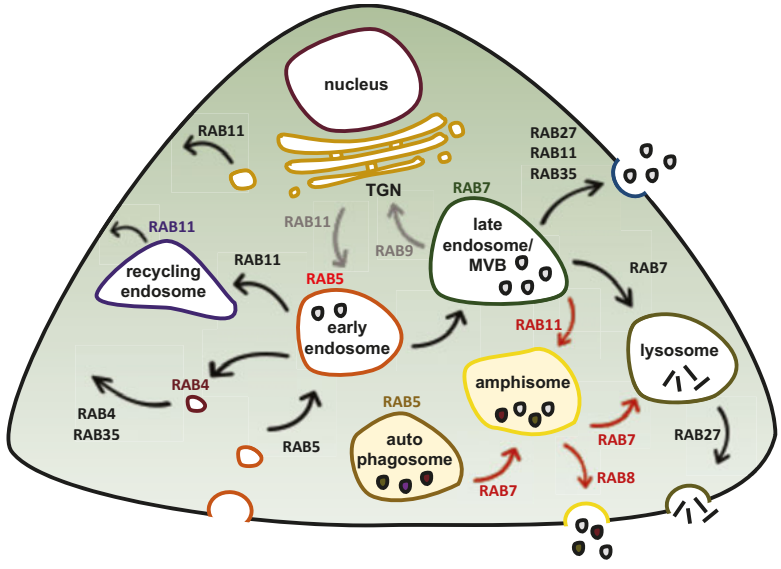

Figure 3. RAB GTPases orchestrate vesicular trafficking. The illustration depicts the different endosomal maturation routes after molecule-specific receptor-mediated endocytosis (black arrows) leading to either recycling or degradation of the receptor. Alternatively, early endosomes can mature to late endosomes, which recycle by fusing with the plasma membrane and releasing their content to the extracellular milieu. Autophagy and its association with the endosomal pathway are shown (red arrows). Retrograde transport of vesicles from endosomes is also depicted (grey arrows). RAB proteins not only mark each of the endosomal and autophagocytotic compartments, but also control every step of the process and thus the fate of the endocytosed molecules.

The vesicular trafficking system also depends on the function of the SNARE protein family, which ensures that vesicles fuse with the right compartment. During this process, a specific vesicular v-SNARE binds to its corresponding t-SNARE in the target membrane (Hutagalung and Novick, 2011).

4.2 Vesicular trafficking, EMT and metastasis

The dynamic and reversible transitions between epithelial and mesenchymal states of the EMT spectrum in addition to transcriptional reprogramming of certain gene networks appear to be dependent on the rewiring of cellular circuitries, including vesicular trafficking networks. These determine the signal output by controlling the spatial and temporal protein transport through endocytic and membrane trafficking pathways (Di Fiore and von Zastrow, 2014). An interesting example in this respect emerges from the endocytic regulation of TGF-β, which affects the duration and the extent to which TGF-β signaling is being activated and its ability to induce EMT (Balogh et al., 2013). Thus, ligand bound TGF-β receptors are known to be internalized via CME into

early endosomes leading to recycling of the receptor back to the plasma membrane for a new round of signalling (Di Guglielmo et al., 2003). Internalization of ligand bound TGF-β receptors is also known to incur in cholesterol-rich membrane micro-domains which instead promote the binding of the receptor to SMAD7-SMURF2 complexes that ubiquitinate the receptor and target it into MVBs for lysosomal degradation and subsequent signal termination (Di Guglielmo et al., 2003; Zhang et al., 2001).

Endocytic vesicular trafficking is also important for cell-cell junction stability, cell polarity and motility regulation. In this respect, the dynamics of adherens junction formation and the plasticity of epithelial cells and tissues can be regulated by endocytic trafficking of E-cadherin. The latter upon CME internalization can either be targeted to lysosomal degradation or endosomal recycling back to the adherens junctions, based on the function of divergent molecular factors (Baum and Georgiou, 2011). An important instigator of E-cadherin internalization and adherens junction dyamics is NUMB that upon binding to E-cadherin cytoplasmic motives allows for p120CTN and/or endocytic adaptor, e.g., AP-2 and EPS15, recruitment (Sato et al., 2011; Wang et al., 2009). TGF-β signaling and other EMT inducing factors including EGF or SRC activation have been shown to induce destabilization of adherens junctions while activation of their downstream effector ARF6 leads to E-cadherin internalization and cell migration (Janda et al., 2006; Palacios and D'Souza-Schorey, 2003). Interestingly, it has been found that ARF6 activation ensures the absence of adherens junction formation by blocking E-cadherin recycling, while consistent with its EMT promoting role, ARF6 overexpression disrupted the formation of renal and mammary epithelial spheres by accumulation of signalling endosomes leading to hyperactive ERK signalling (Frasa et al., 2010; Tushir et al., 2010). In a similar scenario, a recent report has demonstrated that NUMB negatively regulates cell membrane protrusions and HGF-induced cell migration and invasion by modulating ARF6 and EFA6B (exchange factor for ARF6) activity (Zobel et al., 2018). Moreover, it has been reported that mammary cells undergo collective invasion upon loss of EFA6B that is dependent on CDC42 activation (Fayad et al., 2021). Finally, regulation of integrin endocytosis by NUMB and aPKC, appears be important in the regulation of directional cell migration, while redistribution of integrin β1 to the migration front has been shown to be dependent on ARF6 and RAB11 mediated recycling endocytosis (Nishimura and Kaibuchi, 2007; Powelka et al., 2004).

Despite the advancements in our understanding of the critical role of vesicular trafficking in EMT and metastasis regulation, the role EMT-TFs play in the regulation of endocytosis, remains unclear. So far, in an effort to pinpoint this action of EMT-TFs, a recent report has demonstrated the contribution of ZEB1 in the induction of endosomal trafficking as a mechanism of cell polarity and motility establishment in lung adenocarcinoma (Banerjee et al., 2021).

5. Extracellular vesicles

5.1 Types of extracellular vesicles

Cells of multiple origins secrete different types of membranous vesicles, known as extracellular vesicles (EVs), which mediate cellular homeostasis maintenance and intercellular communication. EVs were first identified as carriers of cellular debris to the extracellular space. Their significance in signal transmission to the cell microenvironment and their role in determining cellular processes as well as the fate of other neighboring or distant cells, was later on pointed out (Johnstone et al., 1987; Lo Cicero et al., 2015). EVs constitute a highly heterogenous population of membrane vesicles, which until recently were classified based on the cell of origin, size, content and morphology. Such a definition however, brought confusion to the field, and thus, now EVs can be classified into two distinct categories, based mainly on their biogenesis mode and size; these of the exosomes and of the microvesicles. Exosomes (30-150 nm in diameter) are of endosomal origin since they arise by the formation of ILVs and MVB maturation followed by fusion to the plasma membrane and release to the extracellular milieu. Microvesicles (50-1,000 nm in diameter) arise after outward budding of the cell membrane, fission and the subsequent release to the extracellular space (Raposo and Stoorvogel, 2013). The two EV populations are composed by different molecules present on their surface and in their lumen, determined mainly by the type and maturation status of their cell of origin, biogenesis and environmental conditions, such as stress. The EV cargo involves proteins, lipids, enzymes and nucleic acids which will directly affect the function and fate of the vesicles (van Niel et al., 2018). A number of proteins are currently used in order to identify EVs, including members of the ESCRT machinery (ALIX, TSG101) and tetraspanins (CD81, CD9, CD63), all involved in the EV biogenesis machineries (Thery et al., 2018). Additional factors aiming in the distinction between extracellular and non-extracellular matter and categorization of the different vesicle subpopulations, have been proposed in constant attempts of a clearer understanding of the vesicular heterogeneity (Jeppesen et al., 2019).

Numerous techniques have been developed in order to isolate and analyse EVs. Widely used and accepted by the International Society of Extracellular Vesicles (ISEV), EV isolation procedures include differential ultracentrifugation, size exclusion chromatography, centrifugation after floating on density

gradients, immunoprecipitation and affinity-based immunoselection. Assessment of the purity, integrity and concentration of the isolated EVs is achieved by nanoparticle tracking analysis (NTA), immunoblotting, transmission electron microscopy (TEM) and flow cytometry. Such analyses can be complemented by proteomic and/or transcriptomic profiling and functional assays (Coumans et al., 2017; Thery et al., 2006).

Despite the great advances being achieved in regard to EV isolation and characterization, the overlapping size range, morphology similarities and variable composition of the different vesicular subpopulations, challenge modern attempts that aim at the investigation and validation of their functional capabilities. Furthermore, it remains mysterious why some EV subclasses might have unique biological properties, when most of them are generated by parallel or even the same process of vesicular secretion. Nevertheless, the contribution of EVs in several physiological and pathophysiological states i.e., neuronal communication, inflammation and tumor progression, is well characterized (Yanez-Mo et al., 2015). The present report focuses on the role of EVs in the context of cancer progression and metastasis, a topic which will be further analysed in section 5.3.

5.2 Extracellular vesicle biogenesis and secretion mechanisms

As previously mentioned, exosomes and microvesicles have distinct biogenesis routes, which however, share similar intracellular mechanisms and cargo sorting machineries. The two main steps of EV biogenesis involve the recognition and targeting of the cargoes to be secreted and their subsequent clustering to either the plasma membrane (microvesicles) or the MVBs (exosomes), budding, fission and vesicle release.

Biogenesis of exosomes

The internalised material of the early endosomes that is targeted for subsequent secretion matures to late endosomes by cargo sorting into ILVs, ILV accumulation and MVB formation, processes that are orchestrated by the ESCRT machinery. ESCRT complexes, their effector proteins and functions are listed on Table 2. ESCRT-0 recognises ubiquitinated cargo in the membrane of the early endosomes and recruits ESCRT-1 which participates in the cargo clustering and the initiation of the inward budding of the late endosome membrane. ESCRT-I binds ESCRT-II and form the ESCRT-cargo-enriched zone, where accumulation of cargo takes place. ESCRT-III is then recruited and drives the deubiquitination and packaging of the cargoes into the mature vesicle and implements vesicle budding. VPS4 is then responsible for ESCRT-III disassembly and the subsequent cargo sorting and vesicle scission (Frankel and Audhya, 2018; Henne et al., 2011). An alternative biogenesis

pathway controlled by ARF6 involves the direct interaction of the ESCRT accessory protein ALIX with ESCRT-III and subsequent recruitment of syntenin and syndecan, a complex that bridges cargoes and promotes membrane remodelling favouring ILV formation (Ghossoub et al., 2014). Exosomes can be generated via an ESCRT-independent manner as well, based either on ceramide or proteins of the tetraspanin family. In the first case, ceramide can generate membranous curves and its metabolite, sphingosine, can activate the G_i-protein coupled sphingosine 1-phosphate receptor, which is needed for the cargo sorting into the ILVs (Trajkovic et al., 2008). Clustering of tetraspanins including CD63, CD81 and CD9 can anchor different transmembrane and cytosolic proteins and mediate the formation of microdomains that will finally bud and form vesicles (Chairoungdua et al., 2010; van Niel et al., 2011; Zimmerman et al., 2016). Lipid raft domains rich in cholesterol that can interact with CD81 have also been implicated in the formation of ILVs (de Gassart et al., 2003).

Table 2. List of the ESCRT complexes and their function in human cells according to Henne et al., 2011

ESCRT complex	Effector proteins	Function
ESCRT-0	HRS STAM1/2	Engage and cluster ubiquitinated cargo Recruit ESCRT-I
ESCRT-I	TSG101 hVPS28 VPS37A, B, C hMVB12A, B	Bind and cluster ubiquitinated cargo Recruit ESCRT-II
ESCRT-II	EAP45 EAP30 EAP20	Bind and cluster ubiquitinated cargo Recruit ESCRT-III
ESCRT-III	CHMP6 CHMP4A, B, C CHMP3	Vesicle maturation and budding Recruit deubiquitination machinery

	CHMP2A, B	
VPS4	SKD1 CHMP5 LIP5	ESCRT-III disassembly Vesicle scission

Biogenesis of microvesicles

The biogenesis of microvesicles requires the reorganization of the plasma membrane and the cytoskeleton. This is achieved by the activation of Ca^{2+}-dependent enzymatic machineries that change the lipid asymmetry of the plasma membrane leading to a curvature formation. This is accompanied by the reconstruction of the underlying actin cytoskeleton and thus rendering cytoskeletal elements and their regulators i.e., the RHO family of small GTPases and the RHO-associated protein kinase (ROCK) important coordinators of the process (Li et al., 2012; Piccin et al., 2007).

Once formed, EVs get released to the extracellular space either by pinching off the plasma membrane (microvesicles) or by docking of the MVBs to the plasma membrane (exosomes). Interestingly, MVBs upon formation, are mainly targeted for lysosomal degradation. The mechanisms through which the fate of MVBs is determined are poorly understood, however recent evidence suggests that post-translational modifications of the EV cargo and involvement of the different sorting machineries at MVBs, might regulate such processes. As such, the ESCRT machinery seems to be associated with degradative MVBs, while the syndecan-syntenin-ALIX pathway with vesicle secretion. Also, ubiquitinated MHC class II seems to be targeted to MVBs for degradation, whilst ubiquitin-independent mechanisms target MHC class II to MVBs directed towards secretion (Baietti et al., 2012; Buschow et al., 2009; Palmulli and van Niel, 2018). Maintenance of a balance between lysosomal degradation and EV secretion is important for the normal function and survival of the cell. Interestingly, the lysosomal protein LAMP2 has been found to transcriptionally repress *SNAI1*, suggesting a regulatory connection between SNAI1 and lysosomal function, while impaired lysosomal function promotes secretion of EVs rich in defective proteins, as a mechanism aiming at the conservation of cellular homeostasis (Gauthier et al., 2017; Zheng et al., 2018). A similar balance also exists between autophagy and EV secretion. MVBs can fuse with autophagosomes (amphisome) which can either degrade in lysosomes or fuse with the plasma membrane and release their content (Figure 3). Autophagy inhibition, similar to lysosomal dysfunction, increases the autophagosome-related proteins in the secreted EVs, however it does not affect the EV biogenesis (Hessvik et al., 2016; Papandreou and Tavernarakis, 2017).

MVB intracellular movement to either lysosomes or the plasma membrane depends on the tuned function of several trafficking pathways, involving the cytoskeleton (actin microfilaments, microtubules), associated molecular motors (dynein, myosin, kinesin) and RAB GTPases (van Niel et al., 2018). Accordingly, MVB fusion with the cell membrane and the subsequent release of EVs is dependent on acto-myosin interactions mediated by the small GTP-binding proteins ARF6 and ARF1 and the engagement of proteins of the RAB and SNARE families. A schematic description of the interdependency of the intracellular trafficking pathways in the biogenesis and secretion of EVs is depicted in Figure 4.

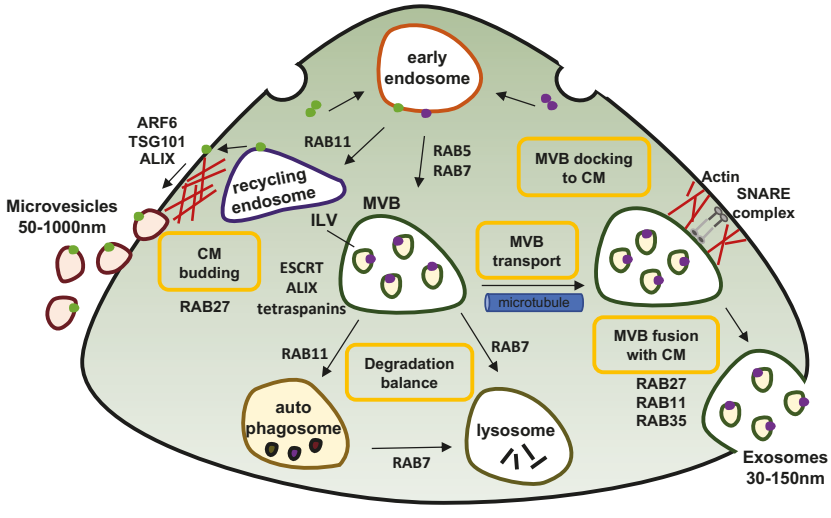

Figure 4. Trafficking pathways involved in EV biogenesis. Exosomes: endocytosed cargoes are directed to the early endosomes, where they are sorted in ILVs and mature into MVBs. These processes are orchestrated by proteins of the RAB GTPases family, the ESCRT-0-III machineries, VPS4 and the ESCRT accessory protein ALIX (ESCRT-dependent biogenesis), or ceramide, ALIX and tetraspanins (ESCRT-independent biogenesis). MVBs that are not targeted for degradation are transported along microtubules to the cell membrane (CM). MVBs dock and finally fuse with the cell membrane in order to release exosomes, processes mediated by actin rearrangement and proteins of the RAB and SNARE families. Microvesicles: membrane or endocytosed-recycled cargoes are sorted into membrane curvatures which finally pinch off to the extracellular space. These processes are dependent on RAB GTPases, cytoskeleton rearrangement via ARF6 and CM budding via the ESCRT-I factor TSG101 and the ESCRT accessory protein ALIX.

5.3 Extracellular vesicles and their cargo molecules as mediators of intercellular communication

Upon their release in the extracellular space, EVs interact with target cells and initiate functional responses. This can be mediated by different interaction trajectories including binding of ligands present on the surface of EVs (including TGF-β ligands) with cell surface receptors or fusion of EVs with the cell membrane of the recipient cells and subsequent release of the EV content in the cytoplasm, affecting downstream signalling pathways (Del Conde et al., 2005; Valadi et al., 2007). Alternatively, EVs can be uptaken by receptor-mediated endocytosis during which specific cargo molecules initiate signalling events from the endocytic or phagocytic compartments of the recipient cells (Svensson et al., 2013). As such, the molecular constituents of EVs not only determine the EV-cellular interaction routes, but also direct the functional response of the recipient cells. Multiple proteins, metabolites and lipids as well as nucleic acids, including mRNAs and non-protein coding RNAs, have been reported to make up EV cargo (http://www.exocarta.org). In the context of cancer, these EV contents can regulate tumor initiation and progression, metastasis, angiogenesis, immune escape and response to anticancer therapy, while they can also serve as prognostic and disease monitoring markers. A comprehensive list of the EV cargo and EV source, with the corresponding function and cancer process involvement can be found in Rodrigues-Junior, D.M. et al., 2022. Among these molecular constituents, the role of proteins rather than RNAs is much better established in the delivery of tumorigenic attributes to target cells (Toh et al., 2018) and this is mainly due to some contradictions associated with the format and abundance of functional RNAs in the EVs (Albanese et al., 2021; Chen et al., 2010; Enderle et al., 2015; Garcia-Martin et al., 2022; Jenjaroenpun et al., 2013; Kesimer et al., 2009). Nevertheless, most of the studies delineating the role of EV carried proteins and/or RNAs are based on gain or loss of function of a given bioactive molecule in the secreting cells, which raises the question on whether such analyses of the cargo represent the reality or are indicative of an adaptive cell condition derived by the genetic perturbation occurred. In this respect, it has been demonstrated that transcriptional repression of *TGFBR2*, affects the protein and miRNA content of colorectal cancer cell-derived EVs, indicative of a TGF-β dependent regulation of EV biogenesis or secretion (Fricke et al., 2019a; Fricke et al., 2019b).

In any case the molecular constituents of EVs are highly dependent on the cell type they derive from. As such, cells with cancer properties secrete EVs carrying oncoproteins and/or inhibitors of tumor suppressor genes, which can transform surrounding and distant normal or pre-malignant epithelial cells in multiple experimental systems, allowing cancer growth and spreading. A schematic representation of the tumor derived EV content and their biological functions in cancer via TGF-β and RAS signalling, is depicted in Figure 5. In

addition, a more detailed overview of the role of extracellular vesicles in tumorigenesis, EMT and metastasis is provided in the following section.

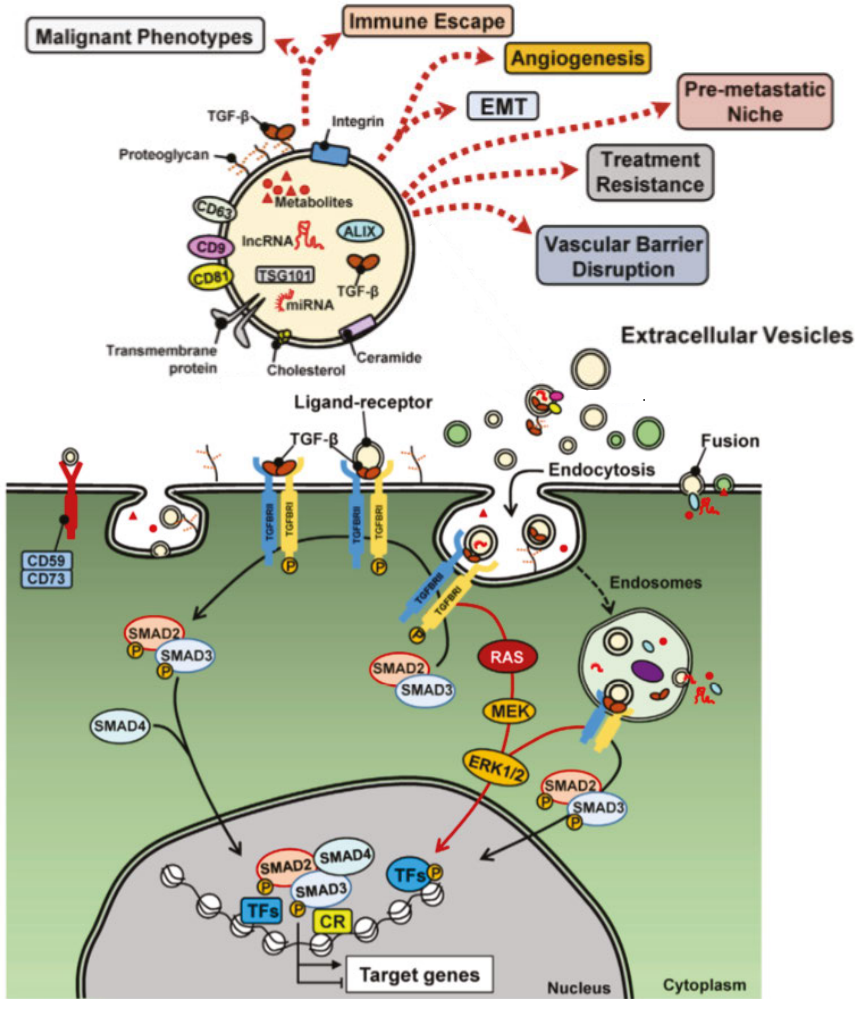

Figure 5. Biological functions of EVs in cancer and TGF-β signaling. A single EV is magnified in order to highlight its various cargo molecules and dotted arrows indicate diverse cell biological functions of EVs that relate to the hallmarks of cancer. Extracellular TGF-β binds to the type II and type I cell surface receptors. The type I receptor phosphorylates the R-SMADs (SMAD2 and SMAD3), oligomerize with SMAD4 and translocate to the nucleus. The ligand-bound receptors also activate RAS, MEK, ERK among other protein kinases. EV-associated TGF-β signals in the same manner, yet the ligand is presented from the surface of EVs, as endocytosis of these EVs is in progress. The signaling proteins, SMADs and MAPKs regulate gene transcription via direct binding to DNA (SMADs) and via phosphorylation of transcription factors (TF) and association with chromatin regulatory protein (CR). Obtained and modified from Rodrigues-Junior, D.M. et al., 2022.

5.4 Role of extracellular vesicles in cancer progression, EMT and metastasis

The pro-tumorigenic and pro-metastatic role of cancer associated EVs has been reported in numerous studies. For instance, tumor-derived EVs have been shown to induce neo-angiogenesis and vascular leakiness promoting fibroblast differentiation into cancer associated fibroblasts that further suppress the activity of immune cells (Liu et al., 2016; Nabet et al., 2017; Nazarenko et al., 2010; Webber et al., 2010). Another example derives from a study showing that cancer-derived EVs carry fibronectin, which once bound to integrin receptors of normal fibroblasts, promotes their anchorage-independent growth (Antonyak et al., 2011). Similarly, prostate cancer cell-derived EVs enriched in TGF-β, induced SMAD3 and α-SMA expression in normal fibroblasts, leading to their myofibroblast differentiation (Webber et al., 2010). Additional studies have indicated the role miRNAs and lncRNAs released by tumor derived EVs play in the regulation of gene expression and transformation of non-cancer recipient cells, favoring primary tumor growth and drug resistance (Hsu et al., 2017; Qu et al., 2016; Wei et al., 2014). However, as explained earlier, these observations should be considered with caution.

EMT as explained in earlier sections, has an inevitable role in cancer progression and metastasis formation and TGF-β signaling is an integral part of this process. In this respect, multiple studies report that mesenchymal cells secrete elevated levels of EVs, capable of transitioning epithelial cells to a mesenchymal state by inducing TGF-β signaling and transcriptionally activating EMT-TFs (Gopal et al., 2017). An example of such function emanates from a study showing that EVs derived from mesenchymal prostate cancer cells inhibited androgen receptor signalling in recipient androgen responsive epithelial prostate cancer cells by parallel induction of TGF-β signaling (El-Sayed et al., 2017). On top of this, specific miRNAs (*miR-21*, *miR-31* and *miR-145*) that are known to regulate androgen receptor, were found increased in both mesenchymal-derived EVs and in recipient cancer cells, indicating a horizontal transfer of EV cargo that induced EMT and allowed the generation of an aggressive cancer cell population (El-Sayed et al., 2017).

Significant appears to be the role of cancer associated EVs in the metastatic outgrowth of the primary tumor to distant organs, which is mainly achieved by modifying the migratory and invasive properties of cancer cells. In this respect it has been demonstrated that EV secretion promotes and stabilizes the leading-edge adhesive protrusions of migrating cancer cells (Sung et al., 2015) while the last create exosome trails that can be followed by other cancer cells fuelling the transmission of migratory properties (Sung et al., 2020). At the same time EVs also carry ECM related molecules promoting ECM remodelling and altered cell-ECM interactions. For example, fibronectin carried by EVs induces cell motility upon binding to integrin receptors (Sung et al.,

2015), while MVB-dependent transfer of metalloproteinases can either lead to invadopodial formation or ECM degradation and acquisition of an amoeboid mode of migration (Clancy et al., 2015; Hoshino et al., 2013). Alternatively, $\alpha_v\beta_6$ integrins delivered by prostate cancer cell-derived EVs, bound to and activated the TGF-β latency-associated peptide (LAP) in epithelial prostate cancer cells leading to the release of mature TGF-β and cell migration induction (Fedele et al., 2015).

5.5 Role of extracellular vesicles in the metastatic niche formation

Several studies have pointed out the contribution of EVs in the formation of pre-metastatic niches which can be correlated with the high occurrence of tumor derived EVs in the blood circulation of cancer patients (Baglio et al., 2017; Baran et al., 2010; Costa-Silva et al., 2015; Galindo-Hernandez et al., 2013; Hoshino et al., 2015; Hyenne et al., 2019; Logozzi et al., 2009). During this process, tumor-derived EVs in combination with other tumor-secreted soluble factors, enter the circulation in order to reach distant secondary organs and create a tumor supportive microenvironment, mainly by induction of inflammation, immune suppression, vascular leakiness and stromal cell activation (Liu et al., 2016; Nabet et al., 2017; Nazarenko et al., 2010). For instance, TGF-β present in metastatic osteosarcoma cell-derived EVs has been shown to interact with its receptor on the surface of mesenchymal stem cells and induce pro-inflammatory signals, including IL-6 production, as well as fibroblast to myofibroblast differentiation (Baglio et al., 2017; Webber et al., 2015). Interestingly, assessment of the circulation of exogenous melanoma labelled EVs in a zebrafish model, revealed their internalization by primarily endothelial cells and macrophages during the pre-metastatic niche formation (Hyenne et al., 2019).

Cancer associated EVs have also been implicated in organ-specific metastasis. In such cases, EVs, based on their cargo molecules, mediate non-random metastatic patterns by displaying preference for target organs where they specifically prepare the microenvironment for metastatic seeding. An example of such a process derives from an experimental mouse model of pancreatic cancer metastasis to the liver, which demonstrated that pancreatic cancer cell-derived EVs, promoted the formation of a pre-metastatic niche in the liver, where they induced TGF-β expression and release upon their internalization by Kupffer cells (Costa-Silva et al., 2015). The observed EV mediated organotropism is proposed to be dependent on ECM-related molecular constituents that promote EV adhesion to specific organs. In this respect, studies have demonstrated that EVs expressing $\alpha_v\beta_5$ bind to Kupffer cells in the liver, while EVs expressing $\alpha_6\beta_4$ and $\alpha_6\beta_1$ bind to fibroblasts and epithelial cells in the lung (Hoshino et al., 2015). In a similar scenario colorectal cancer cell-derived EVs

enriched in integrin β-like 1, activate stellate cells and fibroblasts specifically in the lung and the liver inducing the secretion of TGF-β, IL-6 and IL-8 to promote metastatic growth and invasiveness (Ji et al., 2020).

5.6 Role of extracellular vesicles in drug resistance

As mentioned in previous sections, TGF-β signaling and EMT are important mediators of conventional radiotherapy, chemotherapy and immunotherapy resistance in cancer patients, mostly by fueling tumors with heterogeneous cancer cell populations, including dormant stem-like cell populations, and modulation of the tumor microenvironment. An additional mechanism conferring drug resistance involves the function of EVs by transferring bioactive molecules from resistant donor cells to sensitive recipient cells and eliminating drugs from their target sites, in a way that cytotoxicity cannot be reached (Samuel et al., 2017). Alternatively, EVs have been shown to carry surface proteins that bind therapeutic monoclonal antibodies targeting tumor cell surface receptors (Whiteside, 2016). In this respect, it has been demonstrated that hepatocellular carcinoma cells resistant to cisplatin, released EVs carrying TGF-β3 that activated TGF-β signaling in recipient cells which subsequently acquired a drug-resistant phenotype (Rodrigues-Junior et al., 2019). Another study emphasized the role of TGF-β1-enriched breast cancer-cell derived EVs, in EMT induction and a parallel increase in adriamycin resistance (Tan et al., 2021). Important appears to be the role of TGF-β in the secretion of cancer associated EVs that carry immunosuppressive molecules, including programmed death-ligand 1 (PD-L1) which facilitate tumor immune evasion (Chatterjee et al., 2021; Kang et al., 2020; Mathew et al., 2020). Alternatively, EV cargoes, including miRNAs and arginase-1, have been shown to impact on macrophage differentiation or T cell activation respectively (Marar et al., 2021; Zhou et al., 2020).

Based on the above references it is obvious that special attention has recently been given on the EV-mediated communication of tumor and immune cells in an effort to understand immune evasion mechanisms and improve anticancer immunotherapy. However, it is not yet clear whether EV-mediated processes may be advantageous in the treatment of cancer patients. As such, recent studies focus on the identification of EVs and cargo molecules that can stand as markers not only for prognosis but also for cancer cell detection and subclassification of tumors (Hoshino et al., 2020; Javadi et al., 2021).

6. Present investigations

Breast cancer is a complex disease, where genetic and phenotypic heterogeneity contribute to the generation of distinct tumor cell populations that greatly impacts on cancer progression, metastasis formation and therapy resistance (Dagogo-Jack and Shaw, 2018; Luond et al., 2021). In contrast to genetic heterogeneity that arises from the accumulation of oncogenic mutations in the diverse mammary epithelial cell types, phenotypic heterogeneity originates from the dynamic and reversible cell state transitions in response to a combination of extrinsic and intrinsic cues. Such plastic features enable cancer cells to adapt to external stressors, evolve and ultimately expand, having a strong impact on cancer progression and disease outcome (Hanahan, 2022). Signals arising from the TME are important instigators of such differentiation plasticity, and thus intercellular communication stands as an integral part of the evolution of the proper tissue microenvironment both for primary tumor formation and metastatic colonization. In this respect, EVs constitute vehicles that based on their molecular cargo can alter the physiology of surrounding and distant cells to promote cancer growth and metastasis. Among such cargo, TGF-β and other members of the TGF-β family with well-known tumor and metastasis-promoting functions, have been reported as constituents of EVs (Rodrigues-Junior et al., 2022).

A fundamental process that causes cellular plasticity during cancer progression is the EMT and its reverse process, MET, that allow bidirectional transitions among epithelial, mesenchymal and multiple hybrid E/M phenotypes (Nieto et al., 2016). This plasticity confers changes in cellular responses including cell proliferation, tumor-initiation potential, drug resistance, metabolic reprogramming and immune response (Ye and Weinberg, 2015). TGF-β drives the EMT in every cell type examined so far (Moustakas and Heldin, 2012) and TGF-β signalling is directly coupled to the transcriptional induction of the EMT-TFs. Thus, the aim of our study was to investigate the mechanisms through which the SNAIL family EMT-TFs, SNAI1 and SNAI2, contribute to the generation and maintenance of the dynamic EMT spectrum and how this is linked to the development of aggressive and heterogenous breast carcinomas which are naturally characterized by enhanced manifestation of the EMT process. Such tumors highly express SNAI1 and SNAI2 and present increased metastasis formation, resistance to anti-cancer therapy and high disease relapse rate, all leading to poor prognosis and unfavorable clinical outcomes. In

addition, we attempted to unravel the mechanism through which TGF-β regulates the biogenesis of tumor-derived EVs and the way TGF-β molecules, present as EV cargoes, activate TGF-β signaling in recipient cells to promote invasion and resistance to chemotherapeutic drugs. In Paper I, we focus on the function of SNAI1 in the regulation of mammary epithelial cell differentiation and maintenance of heterogeneous phenotypes of TNBCs and we provide means to separate cell invasiveness from progenitor cell de-differentiation as independent cellular programs. In Paper II, we show that SNAI1 regulates a gene network that connects cell-matrix adhesion to endocytosis and vesicular trafficking, including EV secretion, that is mutually connected with the differentiation plasticity observed in TNBCs upon *SNAI1* knockout. In Paper III, we uncover signalling mechanisms through which TGF-β regulates the biogenesis and determines the cargo of EVs as means to promote TNBC cell invasion and resistance to chemotherapeutic drugs. Finally, in paper IV we attempt to identify downstream molecular targets of SNAI2 that contribute to the aggressive phenotype of TNBCs, and we demonstrate the establishment of an EMT-metabolism regulatory network that is critical for TNBC cell migration and survival.

6.1 Paper I. Loss of SNAI1 induces cellular plasticity in invasive triple-negative breast cancer cells

Breast cancer is a heterogeneous disease composed of several subtypes with diverse pathological features, molecular signatures and clinical outcomes. Based on gene expression profiling, breast cancers are classified as tumors expressing ERa and/or PGR as well as epidermal growth factor family receptor HER2 that derive from luminal epithelial progenitors and are classified as luminal-A or –B (Beca and Polyak, 2016; Shipitsin et al., 2007). Tumors that do not express the above receptors comprise the TNBC subtype that derive from luminal progenitors and mammary stem cells and are subdivided into basal-like-A and -B, mesenchymal (claudin low), mesenchymal-like, luminal androgen receptor and immunomodulatory (Lehmann et al., 2011; Nagarajan and McArdle, 2018; Pommier et al., 2020). The observed heterogeneity can be explained by the differential activities of signaling and transcriptional programs that generate tumors with a mixture of epithelial, mesenchymal and intermediate phenotypes of the EMT spectrum.

In this study we focused on the EMT-TF SNAI1, an evolutionary conserved zinc finger transcription factor, that together with SNAI2 forms a small family of EMT inducers during embryogenesis and cancer (de Herreros et al., 2010; Manzanares et al., 2001). Main function of SNAI1 is the transcriptional repression of epithelial and induction of mesenchymal genes that further determine the tumor-initiating, invasive, and metastatic potential of cancer cells as shown by many SNAI1 gain- and loss-of function studies (Batlle et al., 2000;

Beyes et al., 2019; Cano et al., 2000; Dong et al., 2013; Haraguchi et al., 2015; Maturi et al., 2018; Ohkubo and Ozawa, 2004; Olmeda et al., 2007a; Olmeda et al., 2007b; Yamamoto et al., 2017). However, these studies could not identify possible links between SNAI1 and mammary cell differentiation programs that define certain phenotypes within the heterogenous TNBCs.

To this end, we stably ablated *SNAI1* by CRISPR/Cas9 in metastatic TNBC cells and we demonstrated that its loss was associated with the establishment of an intermediate epithelial-mesenchymal phenotype of the EMT spectrum, and this was further accompanied by decreased invasion, TGF-β signaling and chemotherapeutic resistance. Transcriptomic profiling of the knockout cells revealed a remarkable shift of their differentiation towards the luminal epithelial lineage that was functionally associated with increased proliferation and mammosphere formation capacity in 3D cultures. Mechanistically and based on chromatin binding assays, we proved that loss of *SNAI1* transcriptionally derepressed the pioneering transcription factor of the luminal cell lineage, FOXA1, that in turn induced the expression of androgen receptor (AR). Importantly AR expression has been reported in the luminal-AR subset of TNBC patients and it is correlated with a favourable outcome of breast cancer patients (Tang et al., 2012). Thus, we hypothesize that the *FOXA1*-driven AR expression in TNBC *SNAI1*-mutant cells triggers a transcriptional program reminiscent of ER-mediated transcription as in luminal breast cancer.

In summary, we propose that *SNAI1* mutant TNBC cells are reprogrammed to the hybrid epithelio-mesenchymal phenotype of the EMT spectrum, while dual transcriptional induction of FOXA1 and AR generate basal-luminal plasticity that shifts the differentiation balance towards a more proliferative and less invasive breast cancer cell population.

6.2 Paper II. SNAI1 and SMAD/TGF-β signals antagonize FOXA1 to control PSD4/EFA6B expression and couple integrin-mediated adhesion to endocytic fate in triple-negative breast cancer cells

Motivated by the differentiation plasticity causing the emergence of the luminal epithelial lineage upon *SNAI1* deletion in the metastatic TNBC MDA-MB-231 cells, we studied additional phenotypes of these cells related to the observed reprogramming.

Based on RNA-sequencing data and functional assays we demonstrated the extensive dysregulation of cell-matrix adhesion pathways and endocytosis, including EV secretion, in *SNAI1* knockout cells. Adhesion of cancer cells to the ECM, formation of invadopodia and ECM degradation, as well as secretion of EVs that mediate intercellular communication to shape the TME and

contribute to the pre-metastatic niche formation are integral aspects of the invasive and pro-metastatic program of EMT (Lambert and Weinberg, 2021; Nieto et al., 2016; Rodrigues-Junior et al., 2022). Further analysis provided evidence of a common gene network acting downstream of SNAI1 regulating the interrelated pathways of integrin-depended adhesion, invadopodial/metalloprotease-mediated invasion and endocytic vesicular trafficking. Of critical importance was the role of the guanine exchange factor PSD4/EFA6B as regulator of cell adhesion, invasion and endocytic dynamics in TNBC cells. PSD4/EFA6B activates the small GTPase ARF6, whose role in promoting cell motility and EV biogenesis and secretion is well documented (van Niel et al., 2018; Zobel et al., 2018). Mechanistically, we showed that SNAI1 through TGF-β/SMAD signaling and repression of FOXA1, induces PSD4/EFA6B expression driving a vesicular trafficking program that promotes cell-matrix interactions and invasiveness and correlates with unfavourable clinical outcomes of TNBC patients.

In conclusion, this study addresses key components of the gene network operating downstream of SNAI1 that control endocytic vesicular trafficking and EV biogenesis. In addition, we provide evidence of the importance of vesicular trafficking programs in regulating cell polarity and cell-matrix interactions during EMT and metastasis.

6.3 Paper III. TGF-β induces cholesterol accumulation to regulate the fate of tumor-derived extracellular vesicles

EVs are secreted vesicles that consist of a lipid bilayer and are important mediators of cellular communication. In the context of cancer, EVs can influence tumor progression, metastatic spread, immune evasion and response to anticancer treatment, due to transfer of bioactive molecules (Rodrigues-Junior et al., 2022). Such molecules include TGF-β and TGF-β signaling related constituents, however, whether TGF-β regulates the biogenesis, secretion or functional transfer of EV cargoes in the context of cancer is poorly understood.

In this study we demonstrated the mechanism through which TGF-β regulates the biogenesis of tumor-derived EVs and the way TGF-β related molecules, present as EV cargoes, activate TGF-β signaling in recipient cells to promote invasion and resistance to chemotherapeutic drugs. To this end, we provided evidence that MEK/ERK signaling, independent of SMAD activation, is key mediator of TGF-β, promoting EV secretion by regulating cholesterol homeostasis in TNBC cells. Cholesterol signaling has an established role in the regulation of EV biogenesis in the context of cancer (Baek et al., 2021), and here we show that *7-dehydrocholesterol reductase* (*DHCR7*) is transcriptionally regulated by TGF-β. DHCR7 is an enzyme that synthesizes choles-

terol in a single step from its substrate 7-dehydrocholesterol. Proteomic characterization of the content of the EVs identified many proteins, including TGF-β ligands and matrix metalloproteases, that when transferred to either normal or malignant cells, conferred increased pro-invasive attributes and resistance to conventional chemotherapeutic drugs. Interestingly, we demonstrated that TGF-β carried by EVs, signals mostly via cell surface receptors and does not require EV uptake to guide the molecular responses of the recipient cells.

Altogether, these data uncover signalling mechanisms that regulate the biogenesis and determine the cargo of EVs and propose a multitargeted signalling inhibition approach for better disease outcomes in TNBC patients.

6.4 Paper IV. SNAI2 knockout induces metabolic and cell cycle changes in breast cancer cells

In this study we focused on the other member of the SNAIL family EMT-TF, SNAI2, whose expression is also linked with heterogenous and drug-resistant breast cancer subtypes with high tumor-initiating and metastatic potential (Alves et al., 2018; Fan et al., 2020; Fenouille et al., 2012; Idoux-Gillet et al., 2018). As with SNAI1, SNAI2 transcriptionally represses epithelial genes that are mainly involved in cytoskeletal and intermediate filament rearrangement procedures, cell polarity and motility regulation, as well as apoptosis and stem/progenitor cell dynamics (Hajra et al., 2002; Idoux-Gillet et al., 2018). Yet, in many cases, SNAI2 appears to have distinct roles during breast cancer progression indicative of alternative regulatory gene networks downstream of the two EMT-TFs (Come et al., 2006).

To test this hypothesis, we generated TNBC cells carrying a stable *SNAI2* knockout by utilizing CRISPR/Cas9, and we confirmed a partial reversion of the mesenchymal phenotype of the parental cells, indicative of a hybrid epithelial-mesenchymal state of the EMT spectrum, that was associated with decreased migration and stem-like potential. RNA-sequencing analysis of the mutated cells identified significant alterations in the expression of genes involved in metabolic pathways with a well-documented role in tumor progression and EMT maintenance (Masin et al., 2014; Rosland et al., 2019). In this respect, we found that *SNAI2* knockout cells produced increased levels of mitochondrial superoxide while surprisingly maintained glycolysis and fatty acid oxidation. We further propose that the observed metabolic reprogramming upon *SNAI2* deletion caused significant perturbations in the cell cycle progression as depicted by reduced proliferation and apoptosis induction. Interestingly, the transcription and stem cell factor SOX4 that positively correlates with SNAI2 expression, EMT and TGF-β signaling, was the top significantly downregulated gene in *SNAI2* knockout cells. Its role however in regulation

of metabolism and its mechanism of action in the TNBC cells remains to be validated.

In summary, this study can help us distinguish the distinct roles that SNAI1 and SNAI2 play during breast cancer progression, as well as understand the impact that SNAI2 has on heterogeneity generation via regulation of metabolism and cell cycle progression.

7. Future perspectives

The overall work presented in this thesis highlights the molecular mechanisms through which the EMT-TFs, SNAI1 and SNAI2, contribute to cellular plasticity within breast cancer and on the way TGF-β impacts on EV secretion and content, processes that govern cancer cell aggressiveness and disease outcomes in breast cancer patients. Although significant progress has been made in this respect, further questions have arisen through this work that can stand as the starting point for future investigations.

In **paper I** we showed that upon loss of SNAI1, MDA-MB-231 TNBC cells acquired an intermediate epithelio-mesenchymal phenotype. This was accompanied by the expression of the transcription factor FOXA1, allowing for upregulation of factors of the AR pathway which suppressed the basal phenotype of these cells and allowed the transition to a luminal-like state. Repression of FOXA1 by SNAI1 has been reported in colorectal carcinoma (Jägle et al., 2017), while here we established its regulation in breast cancer as shown by regression and correlation analysis of FOXA1 and SNAI1 expression in TCGA breast cancer samples and rescue experiments upon SNAI1 overexpression in the mutant MDA-MB-231 cells. Even though our results were convincing we never tested if this is a direct or indirect effect of SNAI1 on FOXA1 transcription in our cell system. Following this, we proved that FOXA1 binds to AR gene regulatory sequences in our SNAI1-KO model and regulates its expression. Upon enzalutamide treatment, an AR antagonist, we observed that the mutant cells acquired an elongated morphology and generated mammospheres with deformed lumen and decreased cross-section area, while the proliferative and invasive capacities of the cells remained unaffected. This confirmed our hypothesis that FOXA1-driven AR expression is important for the observed basal-luminal plasticity, based also on our knowledge that AR binds and regulates ESR1 cis-regulatory elements and its expression in TNBC patients is associated with the LAR subgroup that presents favorable outcome in terms of disease-free and overall survival (Lehmann et al., 2016; Maeda et al., 2016; Tang et al., 2012). However further work is needed to define the exact mechanism of action of AR in the absence of SNAI1. In this respect, it would be interesting to identify additional downstream targets not only of FOXA1 but also of AR in the SNAI1-KO cells in order to acquire a more comprehensive view of the molecular events taking

place during the observed cellular differentiation program. Additional to this, we could determine the deposition of repressive and/or active histone marks e.g., of H3K27me3 or H3K4me3, in the genome of the SNAI1 mutant cells before and after treatment with the AR antagonist enzalutamide. Such analysis would allow for identification of gene expression changes related to the histone modification in question, upon AR inhibition.

In a broader spectrum and to better understand the transcriptional pathways determining the differentiation status of our cell model, it would be ideal to find the differentially expressed genes across the SNAI1 WT and mutant cells, the TNBC cells of the LAR subtype and of the SNAI1-KO co-residing breast cancer basal/luminal cell lines as illustrated by our hierarchical clustering analysis. Moreover, phosphoproteomic analysis could help us understand the dependencies on signalling pathways that determine the differentiation status of the aforementioned tumor cells. This would allow a better stratification of the mutant cells regarding their differentiation status and could potentially lead to the application of more robust and targeted therapeutic interventions. In this respect, it has been shown for example that the LAR subtype of the TNBCs displays dependencies in AR and PI3K/mTOR signalling, as well as on CCND1 and CDK4. As such these tumors are uniquely sensitive to AR antagonists, PI3K/mTOR and CDK4/6 inhibitors. In comparison, TNBCs belonging to the basal-like-A and mesenchymal subtype can be mainly benefited by cell cycle and DNA repair inhibitors, since they display elevated phosphorylation of proteins involved in these pathways (Lehmann et al., 2021). From this point of view, it would be interesting to test the tumorigenic and metastatic capacity of the cells not only in vitro but also in vivo after combinatorial treatment with inhibitors of the aforementioned pathways.

In **paper II** we showed that upon SNAI1 deletion, MDA-MB-231 TNBC cells present defective cell-matrix adhesive and endocytic dynamics as opposed by RNA sequencing analysis and functional assays. Mechanistically, we provided evidence that key mediator of this action of SNAI1 is the exchange factor for the small GTPase ARF6, EFA6B, whose dual regulation by TGFβ/SMAD and FOXA1 signals provides a mechanism for coupling the endocytic dynamics to the transcriptional circuit of the EMT. In a more detailed approach, we observed that decreased receptor-mediated endocytosis was also followed by a significant reduction in EV secretion and a parallel decrease in the expression of the late endosomal proteins RAB27A and RAB27B in the mutant cells. Contradictory to this, we observed an upregulation in the expression of the early endosomal markers RAB4 and RAB5 in the SNAI1-KO cells which awaits further investigation. RAB4 is involved in fast recycling and its silencing has been reported to increase EV release due to reduction of recycling and accumulation of late endosomes (Zhang et al., 2022). In this respect, it would be interesting to test if the high RAB4 levels in our cell model are associated with increased recycling of cell surface proteins after induction of

endocytosis. In any case, we never examined the catalytic activity of these RAB GTPases, an investigation that will potentially shed some light in the complex roles of endocytic proteins in the regulation of vesicular trafficking and EV release. In addition to this, we provide evidence that the mutant cells present a significant downregulation of late endosomal to lysosomal maturation. Interestingly a regulatory connection between SNAI1 and lysosomal function has been reported in hepatocellular carcinoma that further affects EV secretion as a cellular homeostatic mechanism (Alvarez-Erviti et al., 2011; Tancini et al., 2019; Vingtdeux et al., 2007; Zheng et al., 2018). Inspired by this observation, it would be worth to deeper investigate this network of vesicular transport mechanisms and demonstrate whether the *PSD4* gene that encodes for EFA6B, is critical for such pathways. Involvement of EFA6B/ARF6 in the establishment of cell-ECM interactions is another aspect that remains to be critically evaluated in our cell model. So far, we have good indications that EFA6B/ARF6 function is important for promoting cell motility and this is based on reports showing that delivery of paxillin from the perinuclear space to focal adhesions depends on ARF6 (Kondo et al., 2000), observation that is in accordance with our data demonstrating regression of paxillin into the perinuclear space upon loss of SNAI1. Whether dysregulation of the adhesive and invasive properties of the mutant cells is interconnected with the endocytic dynamics and EV release, with a possible implication of EFA6B/ARF6 is a question we have started addressing but requires further research in order to draw comprehensive conclusions.

Driven by the indications we have so far on the central role PSD4 plays in the regulation of all the above, we investigated the molecular mechanisms defining its expression and we demonstrated a positive correlation with TGFB1 and binding of SMAD2/3 upon TGF-β stimulation to its transcriptional starting site. At the same time significant was the enrichment of FOXA1 binding in the *PSD4* promoter in the absence of TGF-β, while FOXA1 silencing efficiently rescued PSD4 expression and EV secretion. The exact mechanism through which SMAD2/3 and FOXA1 regulate PSD4 expression however remains to be elucidated. In this respect, we would like to evaluate whether binding of SMAD2/3 and FOXA1 could rather be linked to specific epigenetic modifications on the *PSD4* promoter and/or enhancer regions. For this we believe that ChIP-qPCR analysis of either repressive e.g., H3K27me3 or active histone marks e.g., H3K4me3 in WT and SNAI1-KO cells, in the absence or presence of TGF-β, will highlight the interplay between the pioneering factors of interest and the chromatin modifications they conduct for efficient gene regulation. Whether these factors are important for the induction of these chromatin modifications or just contribute to the recruitment of additional transcriptional regulators in genomic regions marked by these specific histone marks is another interesting aspect that could be addressed by performing ChIP-qPCR of e.g., H3K27me3 and/or H3K4me3 after FOXA1 and/or SMAD2/3 silencing in the mutant and WT cells respectively. Such studies

could help us understand how vesicular trafficking programs affect cell polarity and cell-matrix interactions during cell differentiation coupled with the EMT program and potentially provide means to interfere with the progression of TNBCs towards the highly invasive and metastatic form of the disease.

In **paper III** we provided evidence that the signalling mechanism initiated by TGF-β receptors, engages MEK/ERK kinase activity to regulate the biogenesis of EVs. The exact signalling module that drives the MEK/ERK-dependent pathway operating in the absence of active SMAD signalling is however something we need to investigate further. Examining signalling mediators acting downstream of TGF-β via MEK/ERK signalling we found that DHCR7 expression is important for cholesterol homeostasis and biogenesis of EVs, even though genetic silencing or chemical inhibition of DHCR7 has not yet provided clear evidence of regulation of TGF-β in our hands. This observation is of great interest, since the use of statins, the most commonly prescribed cholesterol-reducing drugs, has been shown to reduce cellular plasticity of solid tumors by locking the cells in a partial EMT state and whose survival is now dependent on ERK activation for counteracting statin-induced apoptosis (Dorsch et al., 2021). Thus, it would be worth to examine such hypothesis in our cell model and demonstrate a possible mechanism through which intracellular cholesterol biosynthesis regulates EV secretion via MEK/ERK signalling as an aftereffect of cellular plasticity driven by EMT and TGF-β signalling. In addition, proteomic analysis showed unique proteins carried by EVs after TGF-β stimulation of TNBC cells. However, we never tested if this enrichment could be due to enhanced activity of the TGF-β signalling itself or whether it is a consequence of the adaptation of the cancer cells in a differentiation state which further affects the content of the secreted EVs upon TGF-β stimulation. Relative to the invasive properties that exogenous TGF-β confers to TNBC cells, we demonstrated that EVs derived by TNBCs upon TGF-β stimulation could transfer pro-invasive properties and confer resistance to chemotherapeutic drugs of either cancer cells or normal fibroblasts. For this, we proposed that TGF-β and MMP9 protein specific cargoes of TGF-β-stimulated TNBC-derived EVs, are important mediators of the observed phenotypes. However more experiments need to be done that not only will address the mechanisms through which these EV cargoes interact with the recipient cells to promote further signalling activation, but also will ensure their contribution in the acquisition of aggressive phenotypes by the recipient cells. Some preliminary data in this direction, point out that TGF-β carried by EVs signals mostly via plasma membrane receptors and does not require EV uptake, while the use of MMP inhibitors blocked the pro-invasive function of TNBC derived EVs. In addition, it would be interesting to analyse whether TGF-β ligands associate with specific MMP family members in different EV populations generated by breast cancer cells with divergent tumor-initiating and metastatic

potential. Overall, this study can potentially reveal a strong advantage of combinatorial inhibition of cholesterol biosynthesis with established cytotoxic drugs in the deterioration of the tumorigenicity and/or metastatic seeding and growth of breast cancer cells, aspect that needs further assessment by mechanistic in vitro and functional in vivo analyses.

In **paper IV** we generated SNAI2-KO MDA-MB-231 TNBC cells and we verified the acquisition of an intermediate epithelio-mesenchymal phenotype, with a parallel decrease in cell migration, stem-like potential and TGF-β signalling. As revealed by RNA sequencing analysis, this transition was accompanied by altered gene profiles that mediate central metabolic requirements of cancer cells and perturbations in cell cycle progression leading to apoptosis. SNAI1 and SNAI2 belong to the same family of zinc finger transcription factors, and both share structural and functional similarities (de Herreros et al., 2010; Villarejo et al., 2014), however comparison of the transcriptomic profiles of the cells that have suffered knockout of each gene showed noteworthy differences that awaits further investigation. In any case, this observation suggests that SNAI1 and SNAI2 apart from regulating common gene regulatory networks mainly involved in metabolism, cell migration and ECM interactions, they also present unique downstream molecular targets responsible for distinct phenotypic and functional attributes acquired by the mutant cells. As such, it would be of importance to address the role of the stem cell and transcription factor SOX4, which appeared to be the top significantly downregulated gene only upon loss of SNAI2 in MDA-MB-231 cells. Early evidence demonstrated a positive correlation of SNAI2 with SOX4 expression in breast cancer, however we need to verify its direct regulation by SNAI2 and its contribution, if any, in cell proliferation, migration, and stem cell maintenance. Another finding that caught our attention but requires further validation was the metabolic dependency of the SNAI2-KO cells on both oxidative phosphorylation and glycolysis as well as fatty acid oxidation. Interestingly, there are reports supporting the notion that cells residing in intermediate states of the EMT spectrum rely on a hybrid metabolism in order to fulfil their bioenergetic needs based on their proliferative, stem-like and migratory properties (LeBleu et al., 2014; Luo et al., 2018). For this reason, performing a comprehensive metabolic analysis of MDA-MB-231 WT and SNAI2-KO cells is necessary and will provide clear evidence of the exact metabolic programs modulated upon loss of SNAI2 in these cells. Mechanistically, differential and compensatory activity of numerous signalling pathways can result in such hybrid metabolism. In this respect we provide early evidence of dysregulated TGF-β and upregulated mTOR signalling pathways in the mutant cells, while we have also started investigating the hypoxic and autophagic response of these cells in normal and serum or amino acid-deprived conditions. Such analyses can

potentially unravel the molecular mechanisms that connect certain environmental cues upon loss of SNAI2 to the observed metabolic rearrangement in MDA-MB-231 TNBC cells, and the way they affect cellular homeostasis.

An obvious observation emanating from the research work of **paper I**, **II** and **IV** is that breast cancer cells which are naturally characterized by enhanced manifestation of the EMT process, activate alternative molecular programs that allow for cellular plasticity in terms of differentiation as a survival mechanism upon loss of function mutations of single SNAI1 and SNAI2 EMT-TFs. In this respect a great continuation of this work would be to define the pathways and genes that are essential for cellular viability in the absence of SNAI1 and SNAI2 and additionally determine if such pathways/genes can be targeted by anticancer therapy. This scientific question could be addressed by performing a synthetic lethality screen. Synthetic lethality occurs when the concomitant inhibition of two genes leads to cellular death while separate inhibition of each of the genes is not lethal. An experimental approach towards this direction would be to perform a genome-wide CRISPR-Cas9 loss of function screen in single SNAI1, SNAI2, double SNAI1-SNAI2 KO and WT MDA-MB-231 TNBC cells and identify the genes that are systemically depleted in the single or double KOs as compared to the WT cells as well as the enriched pathways corresponding to the top hits. By this way we could determine gene networks that are essential for breast cancer cell survival in the SNAI1 and SNAI2 KO cells. We then could proceed with additional genetic mutations or pharmacological inhibition targeted to the identified pathways/genes and assess attributes including cell morphology, proliferation and/or cell death, stem-cell and metastatic potential. Furthermore, these studies, though very extensive, and although supported by unbiased gene expression analyses in multiple breast cancer cell types linked to TCGA database, they will benefit for additional targeted experiments in one or two complementary cell models of TNBCs.

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Last but not least, my little **Rick**! My life has changed so much since the day we started living together. People say dogs are a man's best friend, but I say you are a family to me. We are living together for eight years now, and I cannot find a moment that you did not show your affection, dedication and loyalty to me. Even when sometimes I am coming back home later than expected, you are always there waiting for me, wagging your tail, showing me your joy, and getting ready for our beloved walk that relieves the stress of the day! I even have a picture of you sitting close to me looking with absorbed attention at a figure of a scientific article I was reading at home, while I cannot count how many Monday group meetings and virtual conferences you have attended via zoom during the COVID-19 times. For me you deserve the title "doctor" as well after all the support and unconditional love you are showing to me every single day!

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