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# An *in vitro* model of EMT/MET using human near-normal and cancer-derived breast cell lines

Modelo *in vitro* de EMT/MET em linhas celulares de mama normal e de cancro de mama

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Dissertação de Candidatura ao grau de Mestre em Oncologia (especialidade: Oncologia Molecular) submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.


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**ABSTRACT**

Epithelial-Mesenchymal Transition (EMT) is a biological process involving the loss of epithelial characteristics and acquisition of mesenchymal features. Non-pathological EMT occurs both during development (embryogenesis) as well as in adult individuals in situations of wound healing and tissue remodeling. Pathological EMT has been associated with cancer, in cells with *de novo* oncogenic ability and with genetic/epigenetic alterations underlying metastatic dissemination. While literature accumulates concerning EMT, few studies have focused on EMT's reverse process, the Mesenchymal-Epithelial Transition (MET). This process is characterized by the down-regulation of mesenchymal markers and up-regulation of epithelial markers as well as of cell-cell adhesion. EMT and MET have relevant roles during cancer progression, as proposed by several authors. It has been suggested that cancer cells, derived from normal epithelial cells that have suffered a given set of genetic/epigenetic alterations, upon certain chemical cues from the microenvironment, could undergo EMT thus gaining mobility and detaching from the primary tumor. In addition, these now invasive cancer cells could then intravasate to the blood stream and reach a secondary site in the organism. The reversible nature of EMT would at this point be activated: cancer cells at the secondary site, could then undergo MET, regaining enough adhesion thus forming secondary tumors.

Several molecules are able to induce EMT *in vitro* such as bone morphogenic proteins, platelet-derived growth factor and transforming growth factor beta. These molecules could be the chemical triggers to induce/allow EMT and consequently cancer progression *in vivo*: for example, cancer cells may secrete non-physiological levels of TGF-β receptor ligands that both in a paracrine/autocrine manner may lead to tumor progression and metastasis.

EMT/MET occurrence during cancer progression has been supported by several facts such as: presence of the same (epithelial) markers both in primary tumors and metastases from clinical samples; overexpression of mesenchymal markers in the invasive front of tumors. However, given that histological samples are a static scenario and EMT/MET is characterized by its dynamicity, *in vitro* models that

mimic these processes are key for understanding the biological alterations associated with EMT/MET. *In vitro* EMT/MET models can be the solution to uncover novel biological markers and regulatory mechanisms and hence novel therapeutic approaches. **Therefore our aim was to establish novel EMT/MET *in vitro* models, using the cytokine TGF-β1 as an inducer, in near-normal and cancer-derived cell lines.**

In **Chapter One** of this Thesis we have tested several mammary cell lines aiming at selecting the best suited for an EMT induction. The expression of several epithelial and mesenchymal markers was assessed and MCF10A was the selected cell line. In **Chapter Two** of this Thesis we demonstrate the induction of EMT and MET in this cell line, thus creating a novel model that faithfully recreates the dynamic process of gain (EMT) and loss (MET) of mesenchymal markers and concomitant opposite variation of epithelial markers. These variations were confirmed by analyzing the RNA and protein expression of several markers. In **Chapter Three** of this Thesis, we have provided preliminary data on the comparison between the MCF10A EMT/MET model and another in house established EMT/MET model. We have compared the expression and regulation of the recently annotated gene *Dies1* and observed that although with similar patterns of expression variation, the inherent regulatory mechanism is different in both models. This observation enhances the relevance of creating novel EMT/MET *in vitro* models and stresses the importance of the work described in this Thesis.

**RESUMO**

A transição Epitelial-Mesenquimal (EMT) é um processo biológico que envolve a perda de características epiteliais e aquisição de características mesenquimais. EMT não patológica, ocorre tanto durante o desenvolvimento (embriogénese), assim como em indivíduos adultos em situações de cicatrização de feridas e remodelação do tecido. EMT patológica tem sido associada com cancro, tendo sido observada em células com capacidade oncogénica e com alterações genéticas/epigenética subjacentes disseminação metastática. Enquanto que vários estudos abarcam a temática da EMT, poucos se concentraram no processo inverso à EMT, a transição Mesenquimal-Epitelial (MET). Este processo é caracterizado pela diminuição na expressão de marcadores mesenquimais e aumento de marcadores epiteliais, bem como aumento da adesão entre células. EMT e MET têm papéis relevantes durante a progressão do cancro, como proposto por diversos autores. Tem sido sugerido que células cancerosas, derivadas de células epiteliais normais que tenham sofrido um conjunto de alterações genéticas/epigenéticas, mediante sinais químicos determinados a partir do microambiente, podem submeter-se a EMT ganhando assim mobilidade e destacando-se do tumor primário. Estas células cancerosas invasivas poderiam então intravasar para a corrente sanguínea e chegar a um local secundário no organismo. A natureza reversível da EMT seria neste momento ser activada: células cancerosas no local secundário, poderiam submeter-se a MET, recuperando suficiente adesão entre células que leve à formação de tumores secundários. Várias moléculas são capazes de induzir EMT *in vitro*, tais como "bone morphogenic proteins" (BMP), "platelet-derived growth factor" (PDGF) e "transforming growth factor beta 1" (TGF-β1). Estas moléculas podem ser responsáveis por induzir EMT e, consequentemente, permitir a progressão de cancro *in vivo*: por exemplo, as células cancerígenas podem segregar níveis não fisiológicos de ligandos de receptores TGF-β que, tanto de uma forma parácrina como autócrina, podem levar à progressão do tumor e formação de metástases.

A ocorrência de EMT/MET durante a progressão do cancro tem sido apoiada por diversos factos, tais como a presença dos mesmos marcadores (epiteliais), tanto em tumores primários como em metástases e a sobre-expressão de marcadores

mesenquimais na frente invasiva de tumores. No entanto, dado que as amostras histológicas são um cenário estático e EMT/MET é caracterizada pelo seu dinamismo, modelos *in vitro* que imitam a esses processos são essenciais para a compreensão das alterações biológicas associadas com EMT/MET. Modelos *in vitro* de EMT/MET podem ser a solução para descobrir novos marcadores biológicos e mecanismos de regulação e, assim, novas abordagens terapêuticas. Portanto **o nosso objectivo foi estabelecer novos modelos *in vitro* de EMT/MET, utilizando a citoquina TGF-β1 como um indutor, em linhas de células "normais" e de cancro.** No **Capítulo Um** desta Tese testámos várias linhas celulares mamárias visando seleccionar a mais adequada para uma indução de EMT. A expressão de vários marcadores epiteliais e mesenquimais foi avaliada e MCF10A foi a linha celular seleccionada. No **Capítulo Dois** desta Tese, demonstrámos a indução de EMT e MET nesta linha celular, criando assim um novo modelo que recria fielmente o processo dinâmico de ganho (EMT) e perdas (MET) de marcadores mesenquimais e variação oposta concomitante de marcadores epiteliais. Estas variações foram confirmadas por análise da expressão de proteínas e de ARN de vários marcadores. No **Capítulo Três** desta Tese, apresentámos dados preliminares sobre a comparação entre o modelo EMT/MET em MCF10A e um outro estabelecido pelo nosso grupo. Comparámos a expressão e regulação de um gene recentemente anotado, *Dies1*, e observámos que, embora com padrões semelhantes de variação de expressão, o mecanismo regulador inerente é diferente em ambos os modelos. Esta observação reforça a relevância da criação de novos modelos *in vitro* de EMT / MET e salienta a importância do trabalho descrito nesta Tese.

# GENERAL INTRODUCTION

## GENERAL INTRODUCTION

### NOVEL AVENUES FOR CANCER RESEARCH

In 2000, Hanahan and Weinberg published a review where they defined invasion and metastasis as hallmarks of cancer[1]. Although there was little knowledge on the mechanisms involved in these processes, the authors pointed that the activation of extracellular proteases and the alteration in the expression and function of cell-cell adhesion proteins, such as cadherins, and integrins were crucial for invasion and metastasis [1]. Later in 2011 another review from the same authors came out where they established a succession of cell-biologic changes: local invasion, intravasation into blood or lymphatic vessels, circulation of cancer cells through blood or lymph, extravasation, formation of micrometastases and finally, colonization [2]. The occurrence of metastatic spread of primary tumors is responsible for about 90% of cancer-related deaths[3]. For this reason during the past years, research concerning novel biological pathways/targets (in)activated during metastatic spread has dramatically increased, with several studies focused on the key biological process of Epithelial-Mesenchymal Transition (EMT).

Epithelial and mesenchymal cells are the two major types of cells present in every animal tissue [4]. These two types of cells are the most common however their phenotypes are not static. Under certain conditions these cells convert between each other by two processes: the Epithelial-Mesenchymal Transition (EMT) and the reverse Mesenchymal-Epithelial Transition (MET) [5]. Without these two processes the development of multicellular organisms would be impossible since they would not be able to evolve from the blastula stage of embryogenesis [6].

### EMT AS A CANCER-RELEVANT BIOLOGICAL PATHWAY

What is EMT and why has it been the focus of so many cancer research studies? The Epithelial-Mesenchymal transition is a biological process involving the loss of some characteristics of epithelial cells, marked by loss of “cobblestone-like” phenotype due to rearrangement of the cytoskeleton that leads to changes in cell shape and organization, loss of cell polarity and cell-cell adhesion, and the gain of mesenchymal features, marked by the “spindle-shaped” phenotype leading to increased invasion ability, migratory capacity, resistance to apoptosis and increased production of ECM components[7]. The activation of transcription factors and the altered expression of specific cell-surface proteins also happen when epithelial cells undergo EMT: loss or decrease of expression of epithelial markers, such as *adherens* junction proteins (E-cadherin, β-catenin), tight junction proteins (occludin, claudin) and cytokeratins[8]. Concomitantly, EMT also leads to

the gain or increase of the expression of mesenchymal markers, such as Fibronectin, Vimentin and N-cadherin [7,8,9,10].

EMT can be divided in three subtypes according to its function: type 1 EMT occurs during embryogenesis, for example during gastrulation leading to the formation of the ectoderm, mesoderm and endoderm; type 2 EMT is associated with wound healing and tissue remodeling after a noxious event or injury in adult individuals; and type 3 EMT is directly associated with cancer and occurs in cells with *de novo* oncogenic ability and with several genetic and epigenetic alterations leading to metastatic dissemination [10].

The dynamic characteristic of the Epithelial-Mesenchymal Transition makes it impossible to be identified according to the parameters defined by pathologists [6,11]. Due to this, it took a long time to be accepted as important for tumor progression and both EMT and MET are now closely associated with invasion and metastasis [6]. In fact, *in vitro* and *in vivo* studies have revealed several common features between EMT and cancer progression: 1) invading carcinoma cells, thought to be responsible for invasion and metastasis, can display a mesenchymal phenotype and express mesenchymal markers such as Vimentin[12]; 2) tumor budding in colorectal cancer, a phenomenon by which tumor cells in the invasion front become detached and move towards the stroma in a EMT-like fashion[7]; 3) in some types of colon cancer, single migratory cells have been observed detaching from the primary tumor with concomitant loss of E-cadherin expression [7,13]; 4) in cervical cancer, tumor cells located in the invasive front have been shown to, besides displaying loss of E-cadherin, possesses increased Vimentin expression; 5) expression of many EMT drivers, such as Snail and Slug, in breast, colorectal and ovarian cancer, have been shown to be significantly correlated with poor clinical outcome [14].

However, David Tarin (2005) claims that EMT experiments *in vitro* are extremely artificial because cells cultured both in a two or three dimensional environments are not representative of vascular, endocrine or neurologic dynamism. The author also refers that the classification of cell lines as epithelial or mesenchymal is commonly based on cell shape and that the evaluation of one or a few markers is not enough to assume the occurrence of EMT, *i.e.*, to conclude that a cell population has completely changed its whole-scale gene expression programming [11].

Even so, to extend the knowledge on these processes, there has been an attempt to establish several *in vitro* models from different origins (normal and cancer cell lines) that undergo partial or complete EMT in order to follow the dynamism of the transition [6]. In an *in vitro* condition, EMT can be induced using several extracellular factors such as: transforming growth factor-β (TGF-β), bone morphogenetic proteins (BMPs), Wnt, Notch

ligands, hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) [15].

### EMT, TGF-β PATHWAY AND CANCER

TGF-β1is a cytokine that is produced in our organism regulating proliferation and differentiation, involved in immune suppression, apoptosis, angiogenesis and also related to EMT during carcinogenesis [16,17,18].

TGF-β1 binds to membrane serine-threonine kinase receptor type 2 that recruit the type 1 receptor (TGFβR2 and TGFβR1, respectively) and activates its kinase activity by phosphorylation. The intracellular signaling is mainly transduced through Smad proteins; however it can also be a non-Smad signaling. After Smad2 or Smad3 are phosphorylated by TβRI, they associate with Smad4 and are translocated to the nucleus to modulate gene transcription. The receptors of TGF-β1 can also activate Erk, JNK, p38 MAP kinases, PI3 kinase and small GTPases like Cdc42 and Rac[15,16,18,19,20].

There has been much interest in studying the dual behavior of TGF-β during cancer progression, also known as “TGF-β Paradox”. In an earlier stage, Smads regulate the expression of many genes including *c-myc*, which is repressed and *p21* and *p15* that are induced. These genes promote the arrest of cell cycle in G1 phase preventing cells to divide [16]. However, it is very common the loss of this antiproliferative effect of TGF-β in cancer, *e.g.* due to mutations in components of the signaling cascade [18]. It has been observed resistance to the antiproliferative effect of TGF-β in head and neck squamous cell carcinomas, lung cancer, gastric cancer, colon cancer, pancreatic cancer, ovarian cancer and in some recurrent breast cancers [19].

With the aim to better understand the response to TGF-β1 and to determine the frequency of success in inducing EMT *in vitro* using TGF-β1, Brown and colleagues (2003) used an extended panel of 18 established normal and tumorigenic cell lines of human (epidermoid, breast, lung, pancreas and prostate) and mouse (epidermoid, mammary gland and kidney) origin and primary cultures of two human mammary epithelial cells. All cells were treated with the same amount of TGF-β1 (5ng/ml) for 48h. At the end of the experiments only NMuMG and MCT cell lines (a normal murine mammary gland epithelial and a murine proximal tubular cell line, respectively) underwent TGF-β1 mediated EMT, which was confirmed by the expression of E-cadherin, *Zona Occludens* 1 (ZO-1) and the formation of actin stress fibers. The authors have also seen formation of actin stress fibers in MCF10A cells (a normal human mammary gland epithelial cell line) but only after 6 days of culturing cells with TGF-β1. With this study, Brown *et al.* (2003) refuse the hypothesis that cells need to gain resistance to the antiproliferative effects caused by TGF-β since it was observed that these cells exhibited decreased S-phase of cell cycle

and underwent EMT [19]. Unlike these results, there are some reports of TGF-β induced EMT *in vitro* with different cell lines, *e.g.*, established from prostate tumors [21] or non-tumorigenic mouse mammary cells [22].

The possible origin of EMT-inducing TGF-β pathway ligands has been recently explored by several groups. Moustakas *et al* (2002), suggest that cancer cells can start secreting non-physiological levels of TGF-β receptor ligands affecting not only their differentiation in an autocrine manner, but also the surrounding cellular environment in a paracrine manner, which leads to tumor progression and consequent metastasis [16].Another study, by Labelle *et al*(2001) has shown that contact between tumor cells and platelets can be sufficient to induce an EMT-like transition and invasive behavior *in vitro*, and to stimulate metastatic seeding in the lung *in vivo*. If we take into account that cancer cells, which are able to intravasate,can/will be in contact with platelets, the results of Labelle *et al*(2011) suggest a later acquisition of the mesenchymal phenotype, *i.e.*, after leaving the primary tumor microenvironment. Therefore, cancer cells may/had to intravasate as epithelial cells via leaky blood vessels or via collective invasion mechanisms [23]. To confirm this hypothesis, the authors found expression of epithelial markers such as epithelial cell adhesion molecules and cytokeratins in circulating tumor cells, which suggest that EMT may be not crucial to access the blood flow. Labelle *et al*(2011)also collected results showing that the observed prometastatic effects were largely mediated via the activation of the TGF-β pathway. In addition, upon either TGF-β pathway abrogation in tumor cells or TGF-β expression inhibition in platelets there was no induction of EMT. This clearly indicates that platelets are an important source of this growth factor for tumor cells in circulation [23].

### MET AND CANCER

Unlike EMT that has been extensively studied, the interest of the scientific community in understanding the reverse process, MET, is relatively recent. The Mesenchymal-Epithelial Transition is characterized by the down-regulation of mesenchymal markers and transcriptional factors such as N-cadherin, Zeb1 and Snail and also by the up-regulation of epithelial markers such as E-cadherin and Occludin. During MET, cells re-gain the polarity and establish more cell-cell adhesion contacts [4,6]. In 2010, Li *et al* found that it was possible to transform fibroblasts, which are a product of EMT, into induced pluripotent stem cells (iPSCs), via MET by suppressing pro-EMT factors, *e.g.*, blocking TGF-β signaling. This was particularly interesting because it enabled the authors to unravel a key cellular mechanism to induce pluripotency that can be useful to produce pluripotent stem cells from an individual that are compatible with his/her own immune system [24].

Korpal *et al* (2011) have shown that the overexpression of miR200 family is associated with high risk of metastasis in breast cancer due to the ability to regulate MET and metastatic colonization in mouse models [25]. The findings of the authors indicate that both EMT and MET have an important role in different stages of metastatic colonization: while EMT is accompanied by low levels of miR200s, promote invasion and intravasation; MET and high levels of mirR200s are needed for successful colonization of distant organs [25]. These results are in line with Thiery's hypothesis suggesting MET as a necessary event that allows the establishment of metastases [6].

***In conclusion, and to dissect whether these mechanisms have a parallel in clinical samples of cancer patients, it is mandatory to confirm the occurrence of EMT and/or MET in primary cancer and metastases samples. If that is the case, the identification of in vivo EMT/MET may provide the grounds to develop novel therapeutic approaches: on one hand by trapping disseminated cancer cells in a latent state of micrometastases, thus inhibiting the development of solid tumor metastases, and; on the other hand by targeting and preventing the re-epithelialization process which appears to be relevant for metastases establishment [26].***

***The creation of in vitro models that recreate EMT/MET are powerful tools as a first step to study these processes and this has been the major goal of this Thesis.***

# AIMS AND THESIS OUTLINE

## AIMS AND THESIS OUTLINE

The Epithelial to Mesenchymal Transition (EMT) hypothesis suggests that cancer epithelial cells, at the periphery of the tumor, receive signals from the surrounding stroma, thus acquiring properties that enable them to become motile and invade. Some recent reports also suggest that although EMT may drive the initial steps of the metastasis process, a reversion of the mesenchymal phenotype back to epithelia (MET) of malignant cells is needed to facilitate the establishment of macroscopic metastases. This has been proven essentially by using clinical samples of primary tumors and metastases from several cancer types, however the dynamic transition processes are difficult to assess. Therefore, **the global aim of this study was to establish a dynamic *in vitro* model of EMT/MET using human near-normal and cancer-derived breast cell lines, in order to better understand the alterations that occur during EMT and MET.** Several molecules exist that have been shown to be able to induce EMT *in vitro*. Many studies have been published which have used the cytokine TGF-β1 as an *in vitro* EMT inducer. Therefore, **in order to accomplish our global aim, we have elected this cytokine to establish our EMT/MET model.**

The work enclosed in this Thesis has been divided in three distinct chapters. Each chapter has been subdivided into three sections: aims, results and discussion. Given that similar techniques were applied in all three chapters, this Thesis also includes a general **MATERIAL AND METHODS** section. Concerning the work described in each chapter:

- In **CHAPTER ONE**, we characterized several breast cell lines (near-normal and cancer-derived) aiming at selecting those with endogenous epithelial features as models for *in vitro* TGF-β1-induction of EMT/MET.

- In **CHAPTER TWO**, the major focus was the generation of an EMT/MET *in vitro* model using the cell lines selected in Chapter One and TGF-β1 treatment. This Chapter enclosed two tasks: 1) finding the best suited concentration of TGF-β1 to induce EMT *in vitro* in each selected cell line and; 2) reversing EMT by removal of TGF-β1, to obtain a near-epithelial phenotype through MET. To confirm the occurrence of both EMT and MET, we characterized a panel of epithelial and mesenchymal markers at RNA and protein levels.

- In **CHAPTER THREE**, we aimed at demonstrating the relevance of TGF-β1-induced EMT/MET models in the study of cellular regulatory functions. We herein present interesting results concerning the EMT/MET model established in the present work and a previous similar model (EpH4 EMT/MET *in vitro* model).

Integrated data from all chapters was summarized in the **SUMMARY AND CONCLUSIONS** section of this Thesis. Planned/ongoing applications of the established *in vitro* EMT/MET model were described in the **FUTURE PERSPECTIVES** section of this Thesis.

# MATERIAL AND METHODS

## MATERIAL AND METHODS

### NEAR-NORMAL AND CANCER-DERIVED BREAST CELL CULTURE

HB4a cells was derived from normal breast luminal cells and were cultured in RPMI (Invitrogen, Oregon, USA) culture medium supplemented with fetal bovine serum (10%, Lonza, Switzerland), penicillin-streptomycin (1%, Invitrogen, Oregon, USA),hydrocortisone (5µg/ml, Sigma-Aldrich, Missouri, USA), recombinant human insulin (5 µg/ml, Invitrogen, Oregon, USA), Cholera toxin (25 ng/ml, Sigma-Aldrich, Missouri, USA).

226L cell line, a normal epithelial mammary cell line was cultured in DMEM and F12 (1:1, Invitrogen, Oregon, USA) supplemented with fetal bovine serum (10%, Lonza, Switzerland), penicillin-streptomycin (1%, Invitrogen, Oregon, USA), hydrocortisone (1 µg/ml, Sigma-Aldrich, Missouri, USA), recombinant human insulin (5 µg/ml, Invitrogen, Oregon, USA), Cholera toxin (20 ng/ml, Sigma-Aldrich, Missouri, USA) and recombinant human epidermal growth factor (20 ng/ml, Sigma-Aldrich, Missouri, USA).

*MCF10A* cells derived from mammary gland were cultured in DMEM and F12 (1:1, Invitrogen, Oregon, USA) supplemented with horse serum (5%, Lonza, Switzerland), penicillin-streptomycin (1%, Invitrogen, Oregon, USA), hydrocortisone (500 ng/ml, Sigma-Aldrich, Missouri, USA), recombinant human insulin (40 µg/ml, Invitrogen, Oregon, USA), Cholera toxin (100ng/ml, Sigma-Aldrich, Missouri, USA) and recombinant human epidermal growth factor (20 ng/ml, Sigma-Aldrich, Missouri, USA).

MCF 7AZ breast cancer cell line was cultured in DMEM and F12 (1:1, Invitrogen, Oregon, USA) supplemented with fetal bovine serum (10%, Lonza, Switzerland) and penicillin-streptomycin (1%, Invitrogen, Oregon, USA).

All cell lines were cultured at 37ºC and 5% $CO_2$.All cell lines were kindly provided by Joana Paredes (Cancer Genetics Group, IPATIMUP).

When referred, mesenchymal cells were obtained by supplementing the normal culture medium with Transforming Growth Factor β1 (TGF-β1, Sigma-Aldrich, Missouri, USA). Transient state cells were obtained by replacing the TGF-β1 enriched medium by normal culture medium.

### RNA ISOLATION AND RT-PCR

RNA was isolated from the four cell lines using the *mirVana miRNA Isolation Kit* (Invitrogen, Oregon, USA), following the kit's instruction manual. Complementary DNA (cDNA) was obtained from 1 µg of RNA of each cell line and random primers, for 10 minutes at 70ºC and 2 minutes at 4ºC. The enzyme SuperScript Reverse Transcriptase II (Invitrogen, Oregon, USA) was used for cDNA synthesis. The reaction occurred for 60 minutes at 37ºC. PCR reaction was performed using 50 ng of cDNA (forward and reverse

primers described in **Table 1**) and Taq Polymerase (GE Healthcare, USA). PCR was done using the following conditions: 5 minutes at 94ºC, 30 cycles of 30 seconds at 94ºC, 30 seconds at 60/56ºC (depends on the pair of primers used), 2 minutes at 72ºC and for last 10 minutes at 72ºC.

**Table 1 – Primers sequence of TGF-β receptors 1 and 2 and of GAPDH.**

| | |
|---|---|
| TGFβR1 | |
| Forward | GCAAAGGTCGATTTGGAGAAGT |
| Reverse | CTGCCAGTCCTAAGTCTGCAAT |
| TGFβR2 | |
| Forward | GGAGTACCTGACGCGGCATG |
| Reverse | GGTGGTTGAGCCAGAAGCTG |
| GAPDH | |
| Forward | TCAAGGCTGAGAACGCGAAG |
| Reverse | AGAGGGGGCAGAGATGATGA |

#### WESTERN BLOT FOR E-CADHERIN, β-CATENIN AND FIBRONECTIN

HB4a, 226L, MCF10A and MCF7AZ cells were washed with PBS and then lysed in cold PBS containing 1% Triton X-100 (Sigma-Aldrich, Missouri, USA), 1% NP40 (Sigma-Aldrich, Missouri, USA), protease inhibitor cocktail (Roche, USA) and phosphatase inhibitor cocktail (Sigma-Aldrich, 1:100 dilution). Total protein was quantified using Bradford Protein Assay (Bio-Rad).

Equal amounts of total cell protein lysates from each cell line were subjected to 30% SDS-PAGE electrophoresis and then transferred to nitrocellulose membranes. After blocking with 5% nonfat milk, the membranes were incubated with primary antibodies against: E-cadherin (24E10, Cell Signalling, MA, USA); β-catenin (purified mouse anti-β-catenin, 610153, BD Transduction Laboratories) and Fibronectin (sc-69681, Santa Cruz Biotechnology). Next, menbranes were washed four times with PBS Tween solution, followed by incubation with horseradish peroxidase-linked secondary antibody for blot analysis. Blots were also probed with anti-actin antibody (Santa Cruz Biotechnology) or anti-tubulin antibody (Sigma-Aldrich) for loading control analysis.

### IMMUNOFLUORESCENCE FOR E-CADHERIN, β-CATENIN AND VIMENTIN

HB4a, 226L, MCF10A and MCF 7AZ cells were grown in normal medium in 9mm Petri plates with coverslips (Becton Dickinson Labware, Franklin Lakes, NJ, USA) inserted. After adequate cell growth, the culture medium was removed and the cells were washed twice with PBS supplemented with calcium (1:100). Cells were fixed in ice-cold methanol for 10 minutes. Prior to incubation during 1 hour with a mixture of primary antibodies the cells were blocked with 5% bovine serum albumin for 30 minutes. The primary antibodies used were: anti-E-cadherin (24E10, Cell Signalling, MA, USA); anti-β-catenin (purified mouse anti-β-catenin, 610153, BD Transduction Laboratories); anti-Vimentin (Monoclonal mouse anti-Vimentin, MO725, Clone V9, DakoCytomation). After four washes with PBS, secondary antibody anti-rabbit or anti-mouse Alexa 488 or Alexa 594 (Invitrogen, Oregon, USA), were incubated for 1 hour at room temperature in the dark. Coverslips with cells were washed six times with PBS and mounted using Vectashield mounting medium containing DAPI (Vector Laboratories Inc, CA, USA). Images were taken with a Zeiss Imager.Z1, AxioCamMRm.

### RNA EXPRESSION QUANTIFICATION

RNA was isolated from the four cell lines using the *mirVana miRNA Isolation Kit* (Invitrogen, Oregon, USA), following the kit's instruction manual. Approximately 1000 ng of total RNA were reverse transcribed to single stranded cDNA using Superscript II Reverse Transcriptase and random hexamer primers (Invitrogen, Oregon, USA). Quantitative Real-Time-PCR (qRT-PCR, using a ABI Prism 7000 Sequence Detection System) was carried out in triplicates using source RNA, using *TaqMan Gene Expression Assays* (Applied Biosystems, California, USA) or *PrimeTime qPCR Assays* (Integrated DNA Technologies, Iowa, USA) for the following target genes: human *CDH1*(Hs.PT.49a.3324071), *Ocln* (Hs.PT.49.14927371), *CDH3*(Hs00999918_m1), *FN1* (Hs.PT.47.1565512), *Vim* (Hs.PT.47.14705389), *CDH2* (Hs.PT.49.15618412),*Zeb2* (Hs.PT.47.1778946), *Dies1* (Hs00735289_m1), *Id2* (Hs00747379_m1) and *Id3* (Hs00171409_m1).. The endogenous control was human *GAPDH* (Hs02758991_g1). Data was analyzed by the comparative $2^{(-\Delta\Delta CT)}$ method. We performed t-test to evaluate the significance of the data obtained from the quantification of mRNA expression.

### *DIES1* METHYLATION STATUS ANALYSIS

DNA from a MCF10A E and TS cells was extracted using the kit *Invisorb Spin Tissue Mini Kit* following the manufacturer's instructions (STRATEC Molecular, Berlin, Germany). Approximately 300 ng of DNA from each cell type was then subjected to complete bisulfite conversion and subsequent clean-up using the *Epitect Bissulfite Kit* following

manufacturer's instructions (Qiagen, Hilden, Germany). Bisulfite treated DNA was amplified using primers flanking the predicted *Dies1* promoter. This prediction was based on the classical approach of analyzing the bioinformatically predicted CpG islands within the gene's locus, given the known association between CpG islands and promoter regions. The criteria used for CpG island prediction was as follows: 1) genomic area with >500 bp; 2) a percentage of CG>55 and; 3) the observed/expected CpG dinucleotides>0,65 (CpG Island Searcher Website). Concerning human *Dies1* the predicted CpG island was located at chromosome 10:73533894-73534693 and the CpG sites analyzed were located at chromosome 10:73534292-73534425. The bisulfite PCR products were sequenced for methylation status determination.

# CHAPTER ONE

## Characterization of near-normal and cancer-derived cell lines

## AIM

The objective of this part of the work was to characterize several breast cell lines(near-normal and cancer-derived) aiming at choosing those with the most adequate features to be used as an *in vitro* TGF-β1-induced EMT/MET model. We have assessed the expression (RNA and/or protein) of a set of epithelial and mesenchymal markers to characterize a series of cell lines using several techniques such as quantitative real-time-PCR (qRT-PCR), immunofluorescence, western blot (WB) and reverse transcriptase-PCR (RT-PCR). The selected cell line(s) would have to display key epithelial features: 1) classical cobble-stone morphology; 2) expression of epithelial markers; 3) absent/diminished expression of mesenchymal markers;4) expression of TGF-β receptors and; 5) literature supporting the feasibility of EMT induction.

## RESULTS

### 1. PHENOTYPICAL FEATURES AND PROTEIN LOCALIZATION OF EPITHELIAL AND MESENCHYMAL MARKERS IN SEVERAL HUMAN BREAST CELL LINES

We started by characterizing three different near-normal cell lines of human mammary origin. HB4a cell line derives from normal breast luminal cells[27].**Figure 1**, in bright field microscopy images, displays the general morphology of a sub-confluent culture of the human HB4a cell line. As morphology is not enough to characterize a cell line as epithelial, we proceeded by characterizing the protein expression/localization by immunofluorescence of two known epithelial markers, E-cadherin and β-catenin, as well as of a mesenchymal marker, Vimentin. In **Figure 1**, in the column correspondent to HB4a cells, it is possible to observe that both E-cadherin and β-catenin localized at the cell membrane while no expression of Vimentin was detected.

The normal human epithelial mammary 226L cell line [28] morphology can be also observed in **Figure 1**, in bright field microscopy images. These cells exhibited an epithelial phenotype as well as E-cadherin and β-catenin localization at the cell membrane, and residual expression of Vimentin.

The third of the near-normal human cell lines chosen was MCF10A cell line, which derived from human fibrocystic mammary tissue [29]. Its epithelial morphology is present in the bright field microscopy images (**Figure 1**) and both E-cadherin and β-catenin were expressed at the cell membrane. These cells displayed considerable expression of Vimentin when compared to other cell lines, an observation confirmed in multiple biological replicas.

We also used a cancer-derived cell line, MCF7AZ, which is a variant of MCF7 human mammary carcinoma cell line[30]. These cells displayed an epithelial morphology as observed in the bright field microscopy images (**Figure 1**). MCF7AZ presented E-cadherin and β-catenin expression located at the cell membrane and the expression of Vimentin was absent, as it is possible to observe in the immunofluorescence images (**Figure1**).

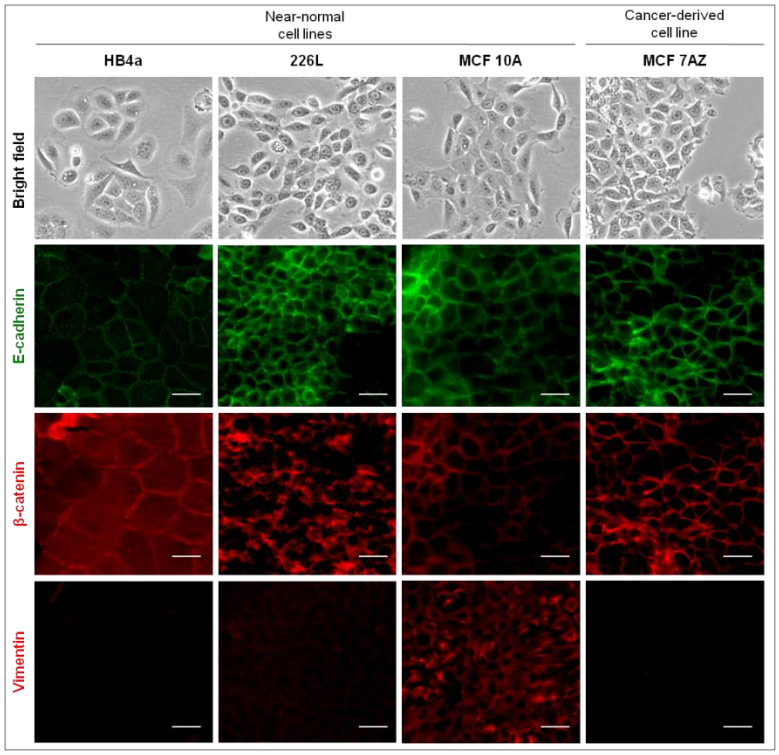



**Figure 1 –Characterization of three near-normal and one cancer-derived breast cell lines.** Bright field microscopy images exhibit the epithelial phenotype of the parental cell lines (100x). All cell lines displayed an epithelial morphology with a typical cobble-stone phenotype. Immunofluorescence was performed for the four cell lines using antibodies for epithelial markers (E-cadherin (green), β-catenin (red)) and mesenchymal markers (Vimentin (red)). The white scale bar corresponds to 50 μm. All cell lines displayed E-cadherin and β-catenin expression at the cell membrane. Vimentin expression was detected in 226L and MCF10A.

Using total protein lysates extracted from all four cell lines we assessed the expression profile of the abovementioned epithelial markers, as well as of Fibronectin, a mesenchymal marker (by WB).We did not assess Vimentin by WB and Fibronectin by immunofluorescence due to technical difficulties. All cell lines expressed E-cadherin and β-catenin as it was expected and only 226L expressed Fibronectin (**Figure 2A-C**). After adequate normalization to actin (endogenous control) we observed that MCF7AZ displayed the highest levels of total E-cadherin, while all other cell lines displayed similar levels (**Figure 2D**). Concerning β-catenin, all cell lines displayed similar levels (**Figure 2D**).

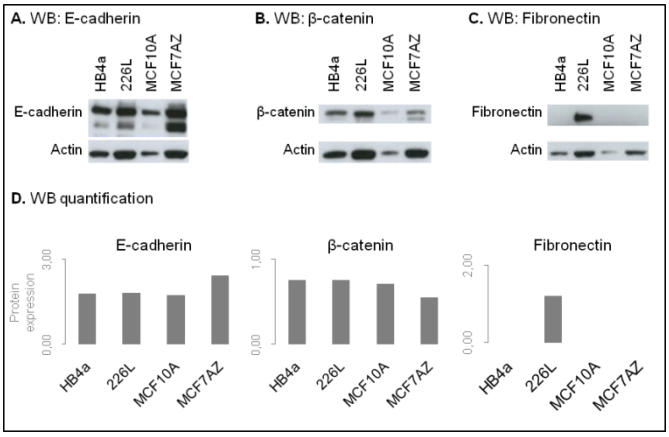



**Figure 2 – Protein expression of epithelial and mesenchymal markers across all selected cell lines (one biological replicate).** Results of the Western Blot performed for E-cadherin (**A**), β-catenin (**B**), Fibronectin (**C**) using total protein lysates extracted from HB4a, 226L, MCF10A and MCF7AZ cell lines. **D.** Quantification of the bands present in A, B and C. All cell lines expressed both epithelial markers and only 226Lcell line expressed Fibronectin.

## 2. QUANTIFICATION OF GENE EXPRESSION OF SEVERAL EPITHELIAL AND MESENCHYMAL MARKERS

For each cell line we collected total RNA to evaluate and quantify the expression of several genes known to be markers of epithelial or mesenchymal cells. For this, we used the quantitative real-time-PCR (qRT-PCR) technique, not aiming at comparing the expression levels between samples, rather observing whether each gene was expressed or not.

The epithelial markers evaluated were: 1)*CDH1*, which encodes for E-cadherin and was detected in all cell lines(**Figure3A**); 2) *Ocln*, which encodes for Occludin, a tight-junction component, which was also detected in all cell lines (**Figure3B**) and; 3) *CDH3*, which encodes for P-cadherin, again expressed in the four cell lines (**Figure 3C**).

Next, we evaluated the RNA expression of three mesenchymal markers: 1) *FN1*, which encodes for Fibronectin and was expressed in 3 out of 4 cell lines, being MCF10A the only cell line that was negative for the expression of this gene (**Figure 3D**); 2) *Vim*, which encodes for Vimentin, was only expressed in 226L and MCF10A cell lines (**Figure 3E**) and; 3) *CDH2*, which encodes for N-cadherin, was expressed in all cell lines (**Figure 3F**). In addition, we evaluated the RNA expression of the EMT inducer *Zeb2*, which displayed a residual expression in the MCF10A cell line.

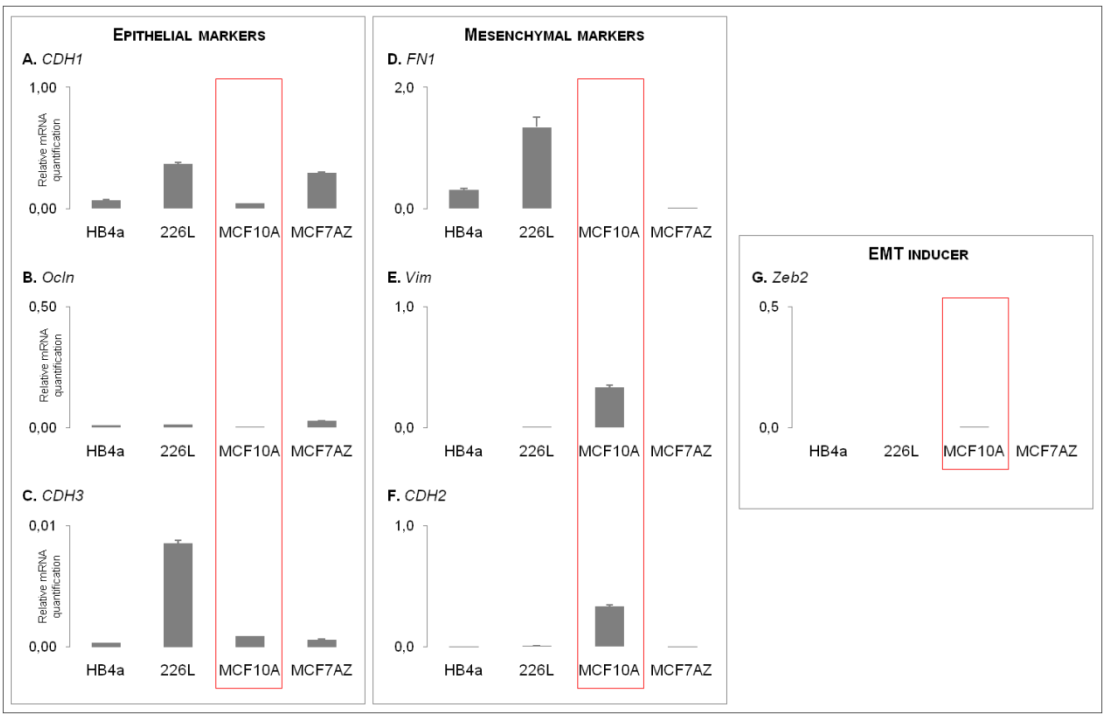



**Figure 3 – mRNA expression of epithelial and mesenchymal markers across all selected cell lines (one biological replicate).** Expression levels of epithelial markers: *CDH1* (**A**), *Ocln* (**B**) and *CDH3* (**C**); Expression levels of mesenchymal markers: *FN1* (**D**), *Vim* (**E**) and *CDH2* (**F**). Expression levels of the EMT inducer *Zeb2* (**G**). Expression pattern of the assessed markers led to the choice of MCF10A for EMT/MET induction (highlighted by a red box).

### 3. EXPRESSION OF TGF-β RECEPTORS 1 AND 2

One of the goals of this part of the work was also to choose the cell line(s) with operational TGF-β receptors 1 and 2 (TGFβR1, TGFβR2), crucial for TGF-β pathway activation. The absent expression of one of the TGF-β receptors would be an exclusion criterion. Therefore, we performed a RT-PCR to confirm the expression of TGFβR1 and TGFβR2. In **Figure 4**we can observe that all four cell lines expressed both receptors. All cell lines studied had the phenotypical features and E-cadherin/β-catenin expression/localization expected for epithelial cell lines (**Table 1**). In addition, similar results were observed concerning epithelial markers' gene expression. Concerning the mesenchymal marker Fibronectin, we observed: 1) protein expression was only detected in 226L although its RNA was detected also in HB4a and MCF7AZ; 2) neither protein nor RNA expression was detected in MCF10A cell line, an observation crucial for the cell line selection process (**Table 1**).

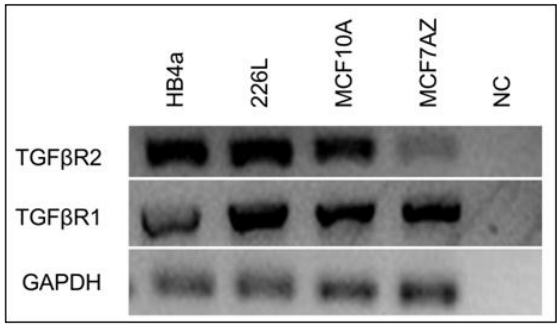



**Figure 4 –*TGFβR1* and *TGFβR2* expression in HB4a, 226L, MCF10A and MCF7AZ cell lines.** NC refers to the negative control. All cell lines expressed both receptors.

In fact, we selected the MCF10A cell line for our next studies given that: 1) previous studies in our group have already emphasized the relevance of *FN1* as a key mesenchymal marker in an established in house mouse EMT/MET *in vitro* model [22]; 2) past studies have shown that it is possible to induce EMT in MCF10A, unlike HB4a and 226L for which no studies have been published. In addition, we have also selected the MCF7AZ cell line for our next studies given that it is cancer-derived and could be viewed as a pathological counterpart of the MCF10A cell line.

**Table 1** – Summary of the characterization performed on the four selected cell lines.

| *Criteria assessed* | | | **HB4a** | **226L** | **MCF10A** | **MCF7AZ** |
|---|---|---|---|---|---|---|
| Morphology | | | Cobble-stone | Cobble-stone | Cobble-stone | Cobble-stone |
| Protein Cellular Localization | Epithelial Marker | E-cadherin | Cell Membrane | Cell Membrane | Cell Membrane | Cell Membrane |
| | | β-catenin | Cell Membrane | Cell Membrane | Cell Membrane | Cell Membrane |
| | Mesenchymal Marker | Vimentin | Absent | Residual, Cytoplasm | Cytoplasm | Absent |
| Protein Expression | Epithelial Marker | E-cadherin | + | + | + | + |
| | | β-catenin | + | + | + | + |
| | Mesenchymal Marker | Fibronectin | - | + | - | - |
| Gene Expression | Epithelial Marker | *CDH1* | + | + | + | + |
| | | *Ocln* | + | + | + | + |
| | | *CDH3* | + | + | + | + |
| | Mesenchymal Marker | *FN1* | + | + | - | + |
| | | *Vim* | - | + | + | - |
| | | *CDH2* | + | + | + | + |
| | EMT inducer | *Zeb2* | - | - | Residual | - |
| TGFβ Receptors | | *TGFβR1* | Expressed | Expressed | Expressed | Expressed |
| | | *TGFβR2* | Expressed | Expressed | Expressed | Expressed |

## DISCUSSION

In this part of the study we characterized a series of cell lines with the goal of choosing at least one with the adequate features to be used in an *in vitro* human TGF-β1-induced EMT/MET model.

All cell lines that were studied had the classical features of epithelial cells: classical cobble-stone morphology; expression of E-cadherin and β-catenin (RNA and/or protein level)at the cell membrane; expression of TGF-β receptors, and; expression, at the RNA level, of a panel of epithelial markers (**Table 1**).

Despite the presence of epithelial markers, all cell lines expressed at least one mesenchymal marker (**Table 1**): HB4a presented expression of *FN1* and *CDH2*; 226L displayed *FN1*, *Vim* and *CDH2* expression; MCF7AZ expressed *FN1* and *CDH2* and; MCF10A exhibited *Vim* and *CDH2*. Concerning *Vim*/Vimentin expression in MCF10A cell line, this result was confirmed in distinct biological replicas and it has also been reported in the literature[31]. This cell line also displayed residual levels of *Zeb2*, again in agreement with current literature [32]. Nevertheless, MCF10A cell line is currently considered a *bonafide* model of an epithelial cell line, thus fulfilling the requisites to be used as a model for TGF-β1-induced EMT/MET model. Therefore, we decided to choose MCF10A cell line to establish our *in vitro* model of EMT/MET. In addition, there have been studies reporting that EMT can be efficiently induced *in vitro* using MCF10A cells cultured with TGF-β1-enriched-medium [19,33,34]. Concerning HB4a and 226L cell lines there are, currently no studies that have used these cell lines for EMT induction. In addition, both cell lines display *FN1* expression, a key mesenchymal marker [22]. Therefore, we did not select any of these cell lines for the next steps of this study.

Due to the connection of EMT with tumor progression, as discussed in **General Introduction**, it would be interesting to have a pathological counterpart for the induction of EMT. As the cancer-derived cell line MCF7AZ fulfills the criteria established for the definition of an epithelial cell line, we decided to include this cell line in the experiments of Chapter Two.

# CHAPTER TWO

## Induction of mesenchymal phenotype

## Recovery of epithelial phenotype

## AIM

The major focus of this part of the work was to induce EMT using TGF-β1and to be able to reverse the process, thus creating a dynamic EMT/MET *in vitro* model. To accomplish this aim, we have used the cell lines selected in the previous Chapter, MCF10A and MCF7AZ. The first task was to find the best-suited concentration of TGF-β1 to induce EMT *in vitro* in both cell lines. The second task was to reverse EMT, thus inducing MET by allowing cells that have undergone EMT to re-acquire an epithelial phenotype.

To confirm the occurrence of both EMT and MET, we have characterized a panel of epithelial and mesenchymal markers (RNA and/or protein) in each step of these transitions using techniques, such as qRT-PCR, immunofluorescence and WB.

## RESULTS

### 1. OPTIMIZATION OF TGF-β1CONCENTRATION ABLE TO INDUCE EMT IN MCF10A AND MCF7AZ CELL LINES

To test the best concentration of TGF-β1 to induce EMT we have cultured both cell lines with normal culture medium enriched with 10 or 20 ng/ml of TGF-β1. With this experiment we aimed at observing alterations on: 1)the morphology of cells; 2) protein expression/localization by immunofluorescence and; 3) RNA expression by qRT-PCR.

### 1.1 Cancer-derived cell line MCF7AZ

When cultured in normal medium enriched with 10 or 20 ng/ml of TGF-β1**,** MCF7AZ cells did not lose the epithelial morphology, represented in the bright field microscopy images of **Figure 1**. In the co-immunofluorescence images of **Figure 1** it is possible to observe that the cell membrane expression of E-cadherin was not lost and that Vimentin did not form fibers, as it was expected if these cells had undergone classical EMT.

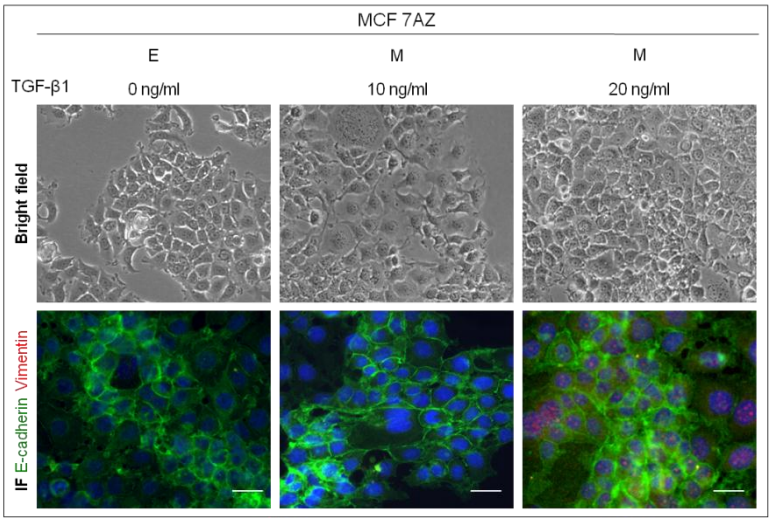



**Figure 1 – Effect of distinct TGF-β1 concentrations on EMT induction in MCF7AZ cell line.** The cells were treated with different concentrations of TGF-β1 (0, 10 and 20 ng/ml) in order to induce EMT. For each condition is represented a bright field microscopy image (100x) and a co-immunofluorescence for E-cadherin (green) and Vimentin (red). No alterations were observed with any of the distinct TGF-β1 concentrations tested. The white scale bar corresponds to 50 µm.

Next, we evaluated the expression at theRNA level of key epithelial and mesenchymal markers by qRT-PCR on MCF7AZ cells cultured under varying TGF-β1 concentrations. We observed that the expression of *CDH1* significantly decreased when MCF7AZ cells were cultured with both 10 and 20 ng/ml of TGF-β1 (*p*-value=6,30E-06 and 4,16E-05 in comparison with no TGF-β1 added, **Figure 2A**). However, the mesenchymal markers did not indicate the occurrence of EMT: 1) *CDH2* expression was not detected in any condition (**Figure2B**); 2) *Vim* significantly decreased even when the highest concentration of TGF-β1was added (*p*-value=2,02E-05 and 2,58E-05 in comparison with no TGF-β1 added, **Figure2C**) and; 3)*FN1* also had a significant decrease in expression however only when the 20 ng/ml of TGF-β1 were added (*p*-value=2,59E-02 in comparison with no TGF-β1 added, **Figure2D**). Interestingly, both in the case of *CDH1* and *Vim* expression, MCF7AZ cells subjected to TGF-β1 treatment displayed significant differences when comparing the two concentrations tested: with 20ng/ml of TGF-β1, the levels of *CDH1* and *Vim* where superior to those detected with 10 ng/ml of TGF-β1, although still significantly lower than those observed in the absence of this cytokine (*p*-value=1,25E-02 for *CDH1* and 1,54E-02 for *Vim* when comparing TGF-β1 concentrations of 10ng/ml *vs.* 20ng/ml, **Figure 2A** and **2C**).

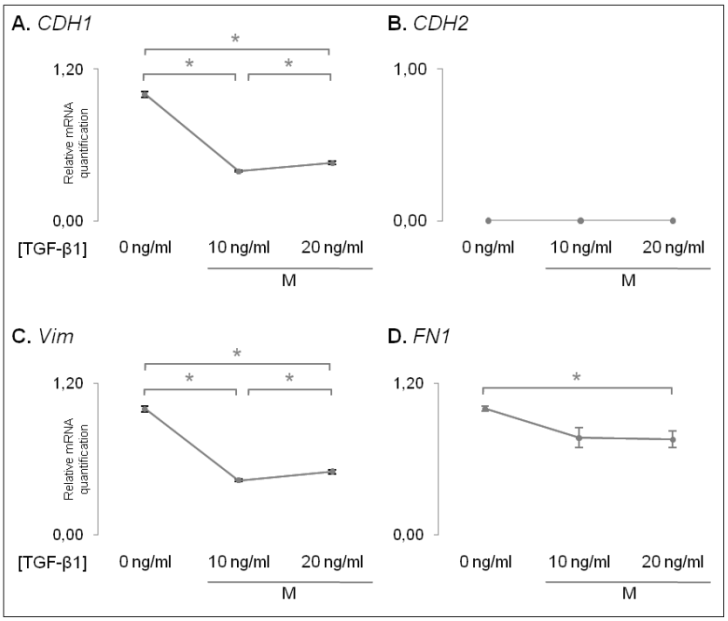



**Figure 2 – Results of relative mRNA quantification obtained using qRT-PCR technique of epithelial and mesenchymal markers of MCF 7AZ cells treated with different concentrations of TGF-β1.** A. *CDH1* gene (epithelial marker) exhibit a decreased expression in cells treated with TGF-β1 independently of the concentration used. *CDH2* (B), *Vim* (C) and *FN1* (D) genes (mesenchymal markers) also display decreased expression with the increase of TGF-β1 concentration. Asterisk stands for significant comparisons ($p$<0.05).

Several attempts were made to induce EMT on MCF7AZ cells, varying different culture conditions, such as the confluence state of cells (data not shown). The results obtained were similar to those presented previously.

The results described lead us to believe that MCF7AZ was not an EMT inducible cell line, at least not with TGF-β1 as an EMT driver. For this reason we did not perform any other experiments with this cell line.

### 1.2 Near-normal cell line MCF10A

We followed the same strategy described previously to assess the best adequate TGF-β1 concentration to induce EMT in the MCF10A cell line. Unlike MCF7AZ cells, we were able to induce EMT in MCF10A cells, as indicated by bright field microscopy (Figure 3). The morphological alterations upon TGF-β1 treatment (both 10 and 20 ng/ml) point to the successful EMT occurrence.

Alterations of protein expression/localization were also detected by co-immunofluorescence for E-cadherin and Vimentin (**Figure 3**). Cells cultured withoutTGF-β1 added, displayed expression of E-cadherin at the cell membrane. However, this pattern of expression was lost

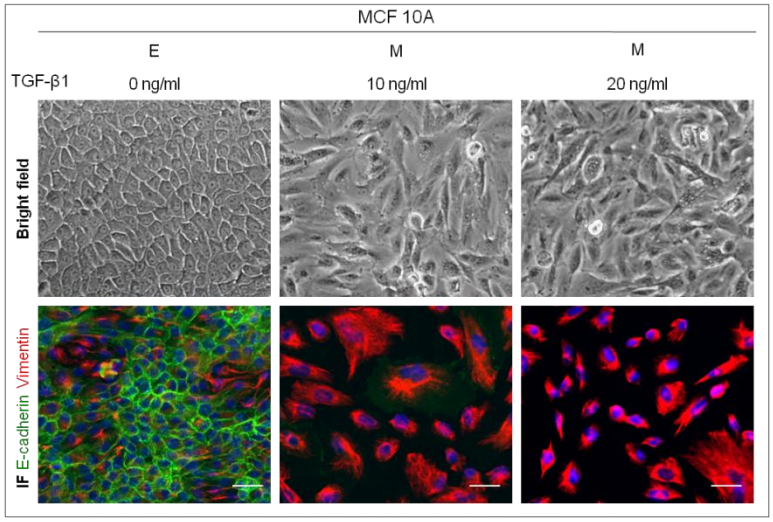



**Figure 3 – Effect of distinct TGF-β1 concentrations on EMT induction in MCF10A cell line.** The cells were treated with different concentrations of TGF-β1 (0, 10 and 20 ng/ml) in order to induce EMT. For each condition is represented a bright field microscopy image (100x) and a co-immunofluorescence for E-cadherin (green) and Vimentin (red). The white scale bar corresponds to 50 µm. When cells were treated with 10 and 20 ng/ml of TGF-β1, E-cadherin lost its typical expression and fibers of Vimentin were formed. The white scale bar corresponds to 50 µm.

in cells cultured with both 10 and 20 ng/ml of TGF-β1 and was replaced by a strong expression of Vimentin forming well defined fibers.

Next, we evaluated the expression of epithelial and mesenchymal markers by qRT-PCR on MCF10A cells cultured with distinct concentrations of TGF-β1.

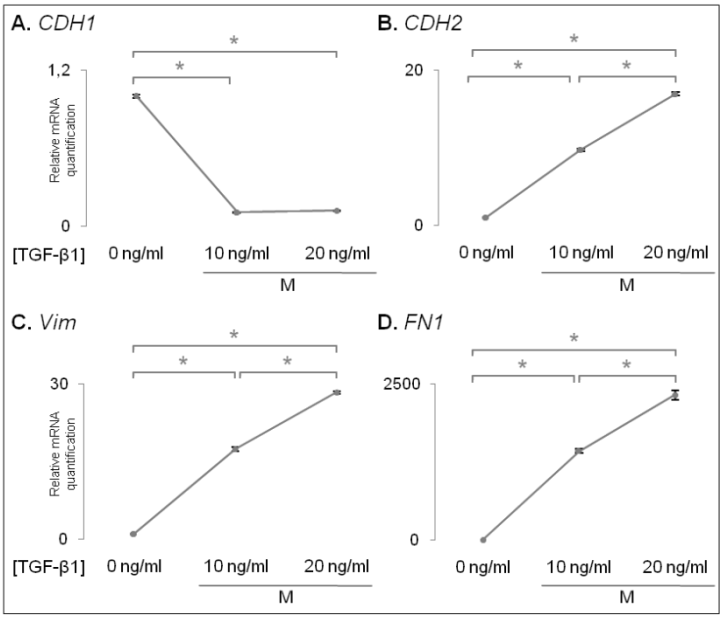



**Figure 4 –Relative mRNA quantification of epithelial and mesenchymal markers in MCF10A cells treated with different concentrations of TGF-β1.**A.*CDH1* gene (epithelial marker) exhibit a decreased expression in cells treated with TGF-β1 independently of the concentration used.

*CDH2*(B), *Vim*(C) and *FN1*(D) genes (mesenchymal markers) display a trend to increase its expression with the increase of TGF-β1 concentration. Asterisk stands for significant comparisons ($p$<0.05).

The results confirmed that MCF10A effectively underwent EMT. We observed that *CDH1*significantly decreased when MCF10A cells were cultured with 10 and 20 ng/ml of TGF-β1 ($p$-value=4,52E-10 and $p$-value=5,02E-07 in comparison with no TGF-β1 added, **Figure 4A**). Concerning the mesenchymal markers: 1) *CDH2* significantly increased when cells were cultured with 10 and 20 ng/ml of TGF-β1 ($p$-value=1,73E-07 and $p$-value=1,69E-08, in comparison with no TGF-β1 added, **Figure 4B**); 2) *Vim* also had a significant increase when cells were cultured in the presence of 10 and 20 ng/ml of TGF-β1 ($p$-value=7,63E-09 and $p$-value=3,83E-09, in comparison with no TGF-β1 added, **Figure 4C**)and; 3) similar to *CDH2* and *Vim*, *FN1* also significantly increased with 10 and 20 ng/ml of TGF-β1 being added to the culture medium ($p$-value=7,71E-10 and $p$-value=3,19E-09, in comparison with no TGF-β1 added, **Figure 4D)**.The three mesenchymal markers tested also showed a significant increase when comparing the results of culture with 10 ng/ml with 20 ng/ml, indicating a dose-dependent expression ($p$-value=7,30E-05, 6,74E-06, 6,62E-05 for *CDH2*,*Vim* and*FN1* when comparing TGF-β1 concentrations of 10ng/ml *vs.* 20ng/ml, **Figure 4B-D**). No dose-dependent expression was observed for *CDH1* expression.

Given that both TGF-β1 concentrations efficiently induced EMT on MCF10A cells, as confirmed by the significant alterations detected for all markers tested, we selected the lowest concentration (10 ng/ml) for the subsequent experiments. We have also renamed MCF10A cells after TGF-β1 treatment as Mesenchymal MCF10A cells (M cells).Since our results supported the occurrence of EMT in MCF10A cells, we used only this cell lineto perform the EMT reversion.

### 2. MET, THE RECOVERY OF THE EPITHELIAL PHENOTYPE?

We reproduced again the EMT process using only the chosen concentration of TGF-β1 (10ng/ml) in order to obtain mesenchymal MCF10A cells (M cells).As expected, we observed an acquisition of a spindle-shaped morphology (bright field microscopy images), the loss of the typical expression of E-cadherin (IF) and the formation of Vimentin fibers in M cells indicating that EMT had occurred. Next, we continued to grow M cells in culture medium, deprived of TGF-β1. The culture of M cells was interrupted when bright field microscopy images pointed towards a recovery of an epithelial phenotype. These post-EMT epithelial-like MCF10A cells were renamed as transient state cells (TS cells, **Figure 6**). In fact, as displayed in **Figure 5**, bright field microscopy images of TS cells showed a

partial recovery of epithelial features. In addition, by immunofluorescence, we observed that E-cadherin expression was recovered to some extent at the cell membrane (**Figure 3**). Regarding the expression of Vimentin (**Figure 5)**, by immunofluorescence it was possible to observe that the fibers formed in M cells were lost in TS cells: in fact, the pattern of Vimentin expression observed in E cells was recovered in TS cells.

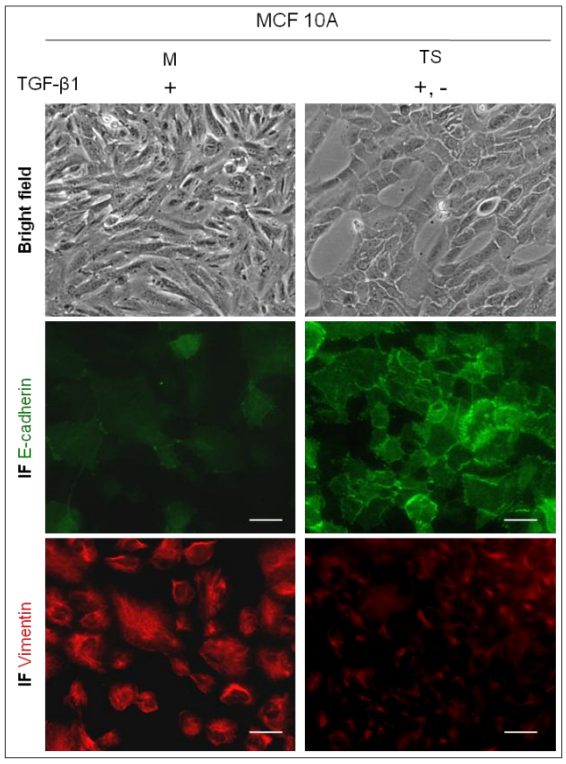



**Figure 5 –MET, the recovery of the epithelial phenotype after the removal of TGF-β1 from the culture medium.** M stands for mesenchymal cells and TS stands for transient state cells. Bright field microscopy images showing the recovery of the cobble-shaped phenotype of cells (100x). The re-expression of E-cadherin at the cell membrane can be observed by immunofluorescence (IF). The loss of Vimentin expression is evident when comparing M with TS cells by immunofluorescence. The white scale bar represents 50 µm.

Facing these results it was our conviction that our *in vitro* model successfully mimicked EMT which was then successfully reverted indicating the occurrence of MET.

### 3. THE ESTABLISHMENT OF A HUMAN *IN VITRO* EMT/MET MODEL

We have successfully created an *in vitro* model of EMT and MET, using the human epithelial cell line MCF10A (**Figure 6**). **Figure 6** summarizes the alterations in morphology and in protein expression/localization of MCF10A cells through the three steps of EMT/MET: E cells, the original cell line; M cells, upon EMT induction and; TS cells, generated after MET (or partial MET) occurrence.

By immunofluorescence, we observed a significant loss of E-cadherin expression in M cells, later recovered in TS cells (**Figure 6**). Concerning Vimentin expression, immunofluorescence revealed an altered pattern of expression, exclusively in M cells which displayed well defined fibers, in line with EMT occurrence (**Figure 6**).

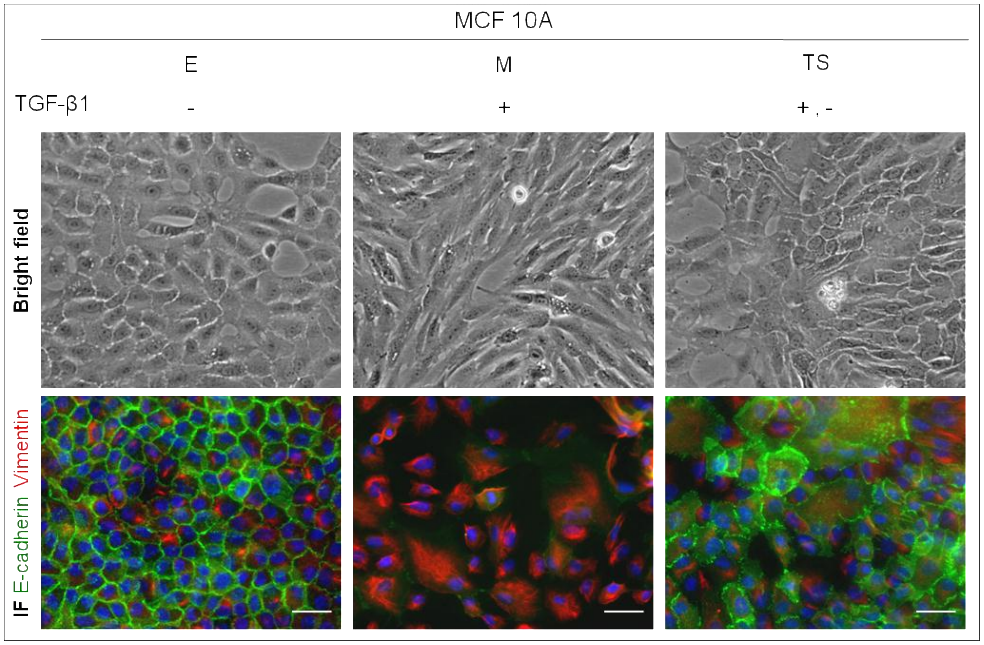



**Figure 6 – EMT/MET model validation.** Bright field microscopy images showing the phenotypical alterations and co-immunofluorescence for E-cadherin and Vimentin during EMT/MET induction (100x). E stands for epithelial cells, M stands for mesenchymal cells and TS stands for transient-state cells. E cells lose the cobble-shaped phenotype to become M cells and a partial recovery of the cuboid phenotype occur in TS cells. In the co-immunofluorescence images M cells lose the E-cadherin expression at the cell membrane and acquire a filamentous expression of Vimentin. TS cells recover some expression of E-cadherin and lose Vimentin expression. The white scale bar represents 50 µm.

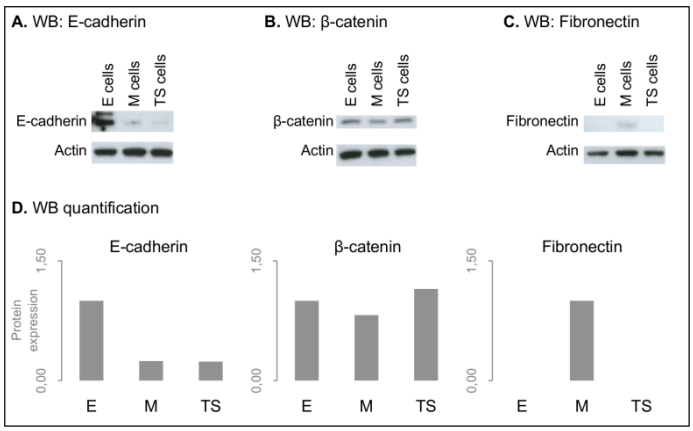



**Figure 7 – Protein expression of epithelial and mesenchymal markers during EMT and MET (one biological replicate).** Results of the Western Blot performed for E-cadherin (**A**), β-catenin (**B**), Fibronectin (**C**) using total protein lysates extracted from E, M and TS cells from the *in vitro* model of EMT/MET using MCF10A cells. **D.** Quantification of the bands present in A, B and C.E-

cadherin expression decreased during EMT; β-catenin displayed similar levels in all cells and only M cells expressed Fibronectin.

We next ran a preliminary assay to evaluate the protein expression of epithelial and mesenchymal markers in the EMT/MET model (**Figure 7**). Concerning the epithelial markers: 1) E-cadherin had a decreased expression during EMT and no recovery of its expression was observed by Western Blot (**Figure 7A and 7D**) and; 2) the expression of β-catenin was constant across EMT and MET (**Figure 7B and 7D**). Regarding the mesenchymal marker Fibronectin, its expression was absent in E and TS cells, being only expressed in M cells (**Figure 7C and 7D**).

We also evaluated the mRNA expression of a panel of epithelial (*CDH1* and *Ocln*) and mesenchymal (*FN1* and *Vim*) markers, as well as an EMT inducer (*Zeb2*). Regarding the epithelial markers: 1) *CDH1* had a significant decrease of expression in M cells (*p*-value=3,66E-04, in comparison with E cells, **Figure 8A**) and a significant increase in TS cells (*p*-value=1,73E-04, in comparison with M cells, **Figure 8A**), recovering almost the same expression levels of E cells and; 2) *Ocln* had the same behavior as *CDH1*, with significant decrease of expression during EMT(*p*-value=4,74E-06, in comparison with E cells, **Figure 8B**) and a recovery of expression during MET(*p*-value=4,97E-06, in comparison with M cells, **Figure 8B**). Concerning the mesenchymal markers/EMT inducers: 1) *FN1* gene had an evident and significant increase of expression during EMT (*p*-value=5,23E-07, comparing E with M cells, **Figure 8C**) and significantly decreased almost recovering to its basal levels of expression during MET (*p*-value=1,44E-04, comparing M with TS cells, **Figure 8C**);2) interestingly, *Vim* expression did not vary with EMT and significantly increased with MET (*p*-value=8,66E-03, comparing M with TS cells, **Figure 8D**), unlike expected of a mesenchymal marker. In addition, TS cells displayed increased levels of Vim in comparison with E cells (*p*-value=7,60E-03, **Figure 8D**) and; 3)the EMT inducer *Zeb2*expression significantly decreased during EMT (*p*-value=7,35E-05, comparing E with M cells, **Figure 8E**) while during MET its expression significantly increased (*p*-value=3,68E-04, comparing M with TS cells, **Figure 8E**).

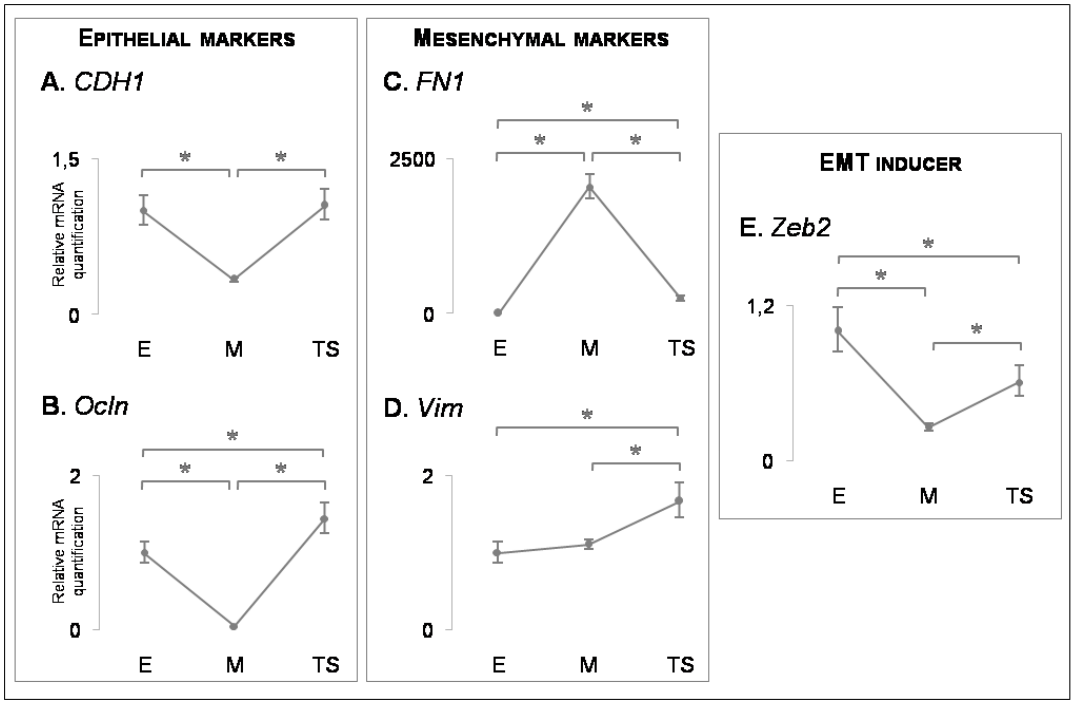



**Figure 8 – Relative mRNA quantification of Epithelial and Mesenchymal markers and an EMT inducer (one biological replicate)**.A.*CDH1*revealed a trend to decrease with EMT and to increase with MET. B. *Ocln* exhibited a decreased expression with EMT and increased with MET. C. *Vim* had a slight increase with EMT and also increased with MET. D.*FN1* massively increased with EMT and decreased with MET. E.*Zeb2* decreased with EMT and increased with MET. Asterisk stands for significantly comparisons ($p$<0.05).

The cell lines, MCF10A and MCF7AZ, used to induce EMT and MET with TGF-β1, did not present the same results. Although several attempts at EMT were made, MCF7AZ cell line was excluded because it did not exhibit any of the features that were expected after TGF-β1 treatment. Nevertheless, MCF10A cells cultured with TGF-β1,displayed classical phenotypical alterations, decreased expression of epithelial markers and concomitant increase in the expression of mesenchymal markers, clearly pointing towards the occurrence of EMT and a subsequent reversion of this process, MET.

## DISCUSSION

The aim of this part of the work was to establish an *in vitro* EMT/MET model using the cell lines selected in Chapter One, MCF10A and MCF7AZ, using TGF-β1 as an EMT inducer. The first step to accomplish this aim was to define the best suited concentration of TGF-β1 needed to successfully induce EMT. Both cell lines were treated with 10 or 20 ng/ml of TGF-β1. In literature many different concentrations of TGF-β1 have been described, ranging from 2 to 20 ng/ml [35]. We decided to use 20 ng/ml to assure that cells were given enough TGF-β1 to undergo EMT (top limit in several published studies). The second concentration of TGF-β1 used (10 ng/ml) was selected given that it is one of the most common concentrations used to induce EMT according to several studies [36]. MCF7AZ cell line treated with both concentrations of TGF-β1 did not present alterations in its morphology and in the expression/localization of E-cadherin and Vimentin. Regarding the RNA expression levels of epithelial and mesenchymal markers tested, the only marker that could indicate the occurrence of EMT was *CDH1*:*CDH1*displayed a significant expression decrease when MCF7AZ cells were cultured with both concentrations of TGF-β1 in comparison with no TGF-β1 added to the culture medium. Despite the decrease of expression of *CDH1*, the expression of E-cadherin at the cell membrane did not suffer any alteration. We performed several other attempts, varying different culture conditions; however we were never able to successfully induce EMT in MCF7AZ cells (data not shown). Facing these results we decided to exclude this cell line from our study.
MCF10A cell line displayed several alterations that were expected in the case of successful TGF-β1-induced EMT: 1) acquisition of a spindle-shaped morphology; 2) loss of the typical membrane expression of E-cadherin; 3)formation of well-defined Vimentin fibers, a classical mesenchymal feature [31]; 4)decreased *CDH1* expression and; 5)increased *CDH2*, *Vim* and *FN1* expression. In addition, mesenchymal markers showed a dose-dependent expression since the expression of *CDH2*, *Vim* and *FN1* was significantly higher when cells were cultured with 20 ng/ml of TGF-β1(in comparison with 10 ng/ml).Our results combined with the fact that most TGF-β1-induced-EMT studies use 10 ng/ml of TGF-β1, lead us to select this concentration for the subsequent inductions with the MCF10A cell line.

As a second task of this part, we wanted to reverse the EMT process in MCF10A cells. We first reproduced the EMT step thus creating 2 individual cell populations: E cells, corresponding to epithelial cells (the parental MCF10A cell line), and M cells, corresponding to mesenchymal cells (obtained after EMT had occurred). Next, we removed TGF-β1 from the culture medium and M cells recovered a near-epithelial

morphology, thus creating a third cell population, which we denominated as transient-state (TS) cells. When TGF-β1 was removed from the culture medium, TS cells displayed: 1) a phenotype similar to that of E cells, recovering the cobble-shaped morphology; 2) expression of E-cadherin at the cell membrane, again as in E cells; 3) less presence of Vimentin fibers, most visible in M cells; 4) recovery of *CDH1* and *Ocln* expression and; 4) loss of *FN1*expression. Concerning *Vim*, and in this particular biological replicate, RNA expression did not reproduce the behavior of a classical mesenchymal marker. Despite this unexpected finding, the immunofluorescence images clearly showed that M cells displayed Vimentin protein expression organized in well-defined fibers, unlike E or TS cells. Such fiber-like pattern is typical of mesenchymal-like cells and demonstrate partial recovery of an epithelial-like phenotype in TS cells.

The results concerning *Zeb2*, are very preliminary and were not clear: unlike most *in vitro* EMT models, our MCF10A model exhibited decreased expression in M cells. However, this observation was only made at the RNA level, given that we did not evaluate Zeb2 protein expression. In fact, Zeb2 protein is increased upon EMT due to the existence of a post-transcriptional mechanism via an Internal Ribosome Entry Site (IRES) necessary for its translation. Therefore, Zeb2 RNA levels are not necessarily related to Zeb2 protein expression [37].

As final conclusion of this chapter, it is reasonable to state that we successfully created a human *in vitro* EMT/MET model that can be explored to evaluate regulatory pathways.

# CHAPTER THREE

## How to explore regulatory functions with this EMT/MET model

## AIM

The aim of this Chapter was to demonstrate some applications of the previously described TGF-β1-induced EMT/MET model using the MCF10A cell line. Here we present some interesting parallels between the MCF10A EMT/MET *in vitro* model and another similar model previously established by our group (EpH4 EMT/MET *in vitro* model) [22]. The results herein presented are part of ongoing work.

## RESULTS

### 1. *DIES1*, A KEY GENE FOR MOUSE EMBRYONIC STEM CELL DIFFERENTIATION VARY ALONG EMT/MET

A recent study by Aloia *et al*2010 has pinpointed *Dies1* (Differentiation of Embryonic Stem Cells 1) as a relevant gene for the differentiation process of mouse embryonic stem cells (mESCs)[38].Given that EMT is a dedifferentiation phenomenon [39] – we have hypothesized that*Dies1*could have a role in EMT/MET. Preliminary studies on an *in vitro* model, established by our group, ofTGF-β1-induced EMT/MET using the near-normal murine EpH4 cell line[22] has strengthen our hypothesis: *Dies1* expression was significantly decreased in EMT and recovered upon MET to levels above those observed in the parental cell line, in concomitance with *Dies1* promoter demethylation. Therefore, we analyzed*Dies1* expression and promoter methylation in MCF10A E, M and TS cells. We observed that*Dies1* had a significant decreased expression during EMT (*p*-value=7,46E-06, comparing E and M cells, **Figure 1A**) and was significantly increased during MET (*p*-value=3,56E-06, comparing M and TS cells, **Figure 1A**). Interestingly, *Dies1* expression also displayed a significant increase when comparing E with TS (*p*-value=9,17E-04, **Figure 1A**). These results reproduced completely the ones previously observed for EpH4 cells.

Aloia *et al* (2010) have also observed an involvement of *Dies1* in the BMP signaling pathway. Therefore, we next analyzed the expression of effectors of the BMP pathway,*Id2* and *Id3*. We observed that*Id2*expression was significantly lower in M cells (*p*-value=1,02E-06, comparing with E cells, **Figure 1B**) and had a significant increase in TS cells (*p*-value=1,49E-05, comparing with M cells, **Figure 1B**). Concerning *Id3*, we observed a significant decrease during EMT (p-value=1,21E-03, comparing E with M cells, **Figure 1C**) and in MET (p-value=2,89E-02, comparing M with TS, **Figure 1C**).

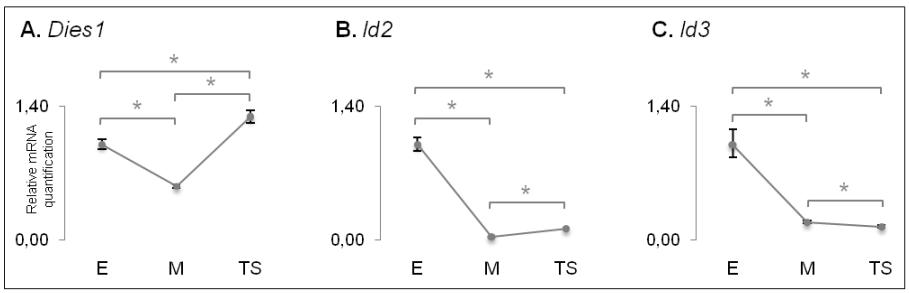



**Figure 1 - Relative mRNA quantification of *Dies1* (A), *Id2* (B) and *Id3* (C) during EMT and MET.** Significant variations were detected for all genes (asterisk stands for $p$<0,05).

As *Dies1* mRNA expression followed, in MCF10A, the same trend we observed in EpH4, we reasoned that the mRNA expression decreased in the M state could also be explained by promoter methylation. Then, we proceeded by evaluating the methylation status of *Dies1* promoter in MCF10A EMT/MET model. Previous studies by our group predicted the presence of a *CpG* island in the vicinity of the human*Dies1*gene (chromosome 10: 73533894-73534693) and 40 *CpG* sites were evaluated (chromosome 10: 73534292-73534425). Despite all efforts, we were able to observe that *Dies1* promoter was demethylated both in E and TS cells, but the Dies1 methylation status in M state, was impossible to assess due to technical difficulties.

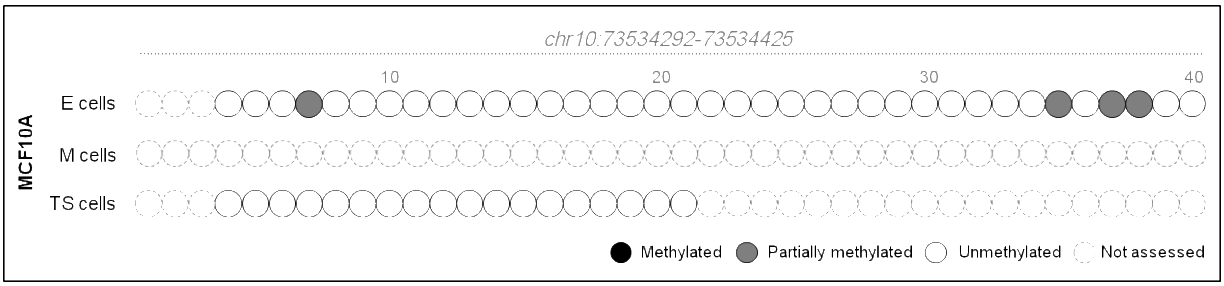



**Figure 2 – Result of the evaluation of the methylation status of the *Dies1* promoter of MCF10A E and TS cells (one biological replicate).** Circles represent individual *CpG* sites assessed. Black circles stand for methylated *CpG* sites; gray circles for partially methylated *CpG* sites; white circles for unmethylated *CpG* sites and; dashed white circles for *CpG* sites not assessed.

## DISCUSSION AND UNANSWERED QUESTIONS

The creation and validation of the MCF10A EMT/MET model, extensively described in the previous Chapters, enabled us to go one step beyond and establish parallels with an already published EMT/MET model by our group. Using the near-normal murine EpH4 cell line and TGF-β1 as inducer, we were earlier able to create three distinct cell populations, similarly to the MCF10A *in vitro* EMT/MET model: EpH4-E, EpH4-M and EpH4-TS cells [22]. The later three cell populations were subjected to whole transcriptome sequencing (Illumina Genome Analyzer, RNAseq) and more than 4000 differentially-expressed genes were detected. At the same time the study by Aloia *et al* (2010) was published, revealing an intriguing relationship between the recently annotated gene*Dies1* and mESCs differentiation. Knowing that EMT is a dedifferentiation phenomenon [39], we hypothesized whether*Dies1* could have a relevant role in EMT/MET. Using the RNAseq data produced, we observed a significant variation of *Dies1* expression with EMT/MET in our previous model: *Dies1* was significantly downregulated with EMT and greatly increased upon MET. In fact, EpH4-TS cells, generated after the differentiation process of MET, displayed massive amounts of *Dies1*. These results, validated by qRT-PCR in distinct biological replicas, strengthened our hypothesis of *Dies1* involvement with EMT/MET. In light of the aim of this part of the study, we assessed *Dies1* expression in MCF10A E, M and TS cells: results similar to those described for EpH4 E, M and TS cells were observed, which may indicate a consistent behavior of *Dies1* in both EMT/MET models. However, *Dies1* promoter methylation status was different when comparing the MCF10A EMT/MET with EpH4 EMT/MET model: while *Dies1* promoter methylation status varied during Eph4 EMT/MET, no variation was observed in the MCF10A EMT/MET. In addition, we could not assess the *Dies1* promoter methylation status of MCF10A M cells.

The study by Aloia *et al* had also shown an involvement of *Dies1* with the BMP signaling pathway. In fact, the authors had shown that upon *Dies1*downregulation in mESCs, the BMP signaling cascade was greatly decreased, an observation supported by: 1) decreased expression of *Id1*, *Id2* and *Id3*, downstream effectors of the BMP pathway; 2) decreased activity of *Id1* gene promoter and; 3) reduced phospho-Smad1 levels. This study also showed that*Dies1* knock-down (and the consequent BMP pathway downregulation), led to maintenance of mESCs in an undifferentiated state [38]. Therefore, we evaluated the expression of *Id1* (by RNAseq), *Id2* and *Id3*(by RNAseq and qRT-PCR) in the EpH4 EMT/MET model: EpH4-TS cells displayed increased *Id2* and *Id3* expression, both in comparison to EpH4-E and EpH4-M cells. No expression variation was detected for *Id1*.These results pointed towards an over-activation of the BMP

pathway in EpH4-TS cells in concomitance with *Dies1* expression. Concerning the MCF10A EMT/MET model, we have only evaluated *Id2* and *Id3*expression by qRT-PCR. We have observed that both *Id2* and *Id3* expression significantly decreased upon EMT and only *Id2* revealed a small recovery upon MET. This expression variation was distinct to that observed in the EpH4 EMT/MET model.

Although both EpH4 and MCF10A were EMT/MET induced using a similar strategy, it is necessary to take into account that the two models differ in several aspects: 1) EpH4 cell line derives from mouse mammary gland while MCF10A cell line has a human origin; 2) EpH4 TS cells displayed an heterogeneous pattern of epithelial and mesenchymal markers unlike the extremely homogenous population of MCF10A TS cells and;3) unlike MCF10A M cells, EpH4 M cells did not display a decreased *CDH1*/E-cadherin expression. Concerning this last observation, our study has shown that in EpH4 M cells, E-cadherin, although present, exhibited loss of functionality due to incorrect post-transcriptional modifications, in particular, incorrect glycosylation [22]. The differences observed in both EMT/MET models only highlighted the relevance of the similarities found: common events will be of relevance for the study of EMT/MET, regardless of the context, or in this case, of the cell line used. This fact only stresses the importance of *Dies1* expression variation during EMT/MET, found to be common between both models. Further studies are currently ongoing, such as *Dies1* expression inhibition and its consequences on EMT/MET occurrence.

# SUMMARY AND CONCLUSIONS

## SUMMARY AND CONCLUSIONS

In this Thesis, we generated data that match, in its majority, our general aim: we established and characterized a human *in vitro* model of EMT and MET. We have started by characterizing a series of human near-normal mammary cell lines: HB4a, 226Land MCF10A. This characterization, described in **Chapter One** of this Thesis, aimed at selecting the most adequate cell line to induce EMT and MET, two normal occurring biological processes of significance in cancer initiation/progression. This characterization encompassed RNA and protein expression analysis using qRT-PCR, immunofluorescence and western-blot of both epithelial and mesenchymal markers. The combined analysis of this characterization, together with existing literature data, led to the choice of MCF10A as a suited model for EMT/MET induction. Given the link between EMT and cancer progression, we have also characterized and used a human cancer-derived mammary cell line, MCF7AZ, for EMT/MET induction as a pathological counterpart of MCF10A.

After the appropriate cell line selection, we proceeded by testing several conditions to induce EMT in both MCF10A and MCF7AZ cell lines. We were able to successfully induce EMT in MCF10A cells, a fact confirmed by the loss of expression of several epithelial markers and *de novo* expression of mesenchymal markers. The same was not valid for MCF7AZ cells, a task that was later on abandoned, despite several attempts as described in **Chapter Two** of this Thesis. After adequate confirmation of EMT occurrence in the newly created MCF10A M cells, we successfully accomplished the second aim of our work: MET induction. The MCF10A TS cell population created was also extensively characterized and confirmed the occurrence of MET, with expression recovery of several epithelial markers and concomitant loss of mesenchymal markers.

The relevance of this newly created MCF10A EMT/MET model was also assessed by comparison with another model, previously created by our group, using a distinct near-normal cell line (EpH4 of murine origin).**Chapter Three** presented some preliminary results concerning a recently annotated gene (*Dies1*), which revealed both similarities and differences among the two EMT/MET models. While the differences may be due to distinct features of each cell line, the similarities emphasized EMT/MET general characteristics and strengthen the general aim of this Thesis, which was to create novel EMT/MET *in vitro* models.

# FUTURE PERSPECTIVES

## FUTURE PERSPECTIVES

Although many studies have been published concerning EMT and cancer progression, few focus on MET and its relevance, particularly in metastization. The extensive characterization described in this Thesis has provided the backbone for future studies, such as:

- Expression analysis of key elements of several biological pathways such as PI3K/mTOR/AKT signaling pathway and Wnt signaling pathway. Data from the RNAseq performed on the EpH4 EMT/MET model pointed towards a differential activation of these (and other) pathways.

- Expression analysis of microRNAs in MCF10A E, M and TS cells as well as their corresponding conditioned mediums. Preliminary data pointed towards a differential expression of several microRNAs, which could impact the expression of several other target molecules.

- Evaluation of the differential tumorigenic/metastization potential of MCF10A E, M and TS cells using *in vivo* assays. EpH4 E, M and TS cells have been previously injected into the mammary fat pad of nude mice and preliminary results have pointed towards an increased tumorigenic ability of EpH4 TS cells. In addition, all three types of cells were injected into the tail vein of nude mice (a classical metastization assay) and increased lethality has been associated specifically with EpH4 TS cells.

These and other studies are currently ongoing and highlight the relevance of the work described in this Thesis.

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