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Epithelial-Mesenchymal transitions and cell behaviour

DOCTORAL THESIS

REBECA CÓRCOLES CÓRCOLES

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CERTIFICA que la tesis doctoral titulada *"Epithelial-Mesenchymal transitions and cell behaviour"*, ha sido realizada por Dña. Rebeca Córcoles Córcoles, bajo la dirección de la Dra. M. Ángela Nieto Toledano y da su conformidad para que sea presentada a la Comisión de Doctorado de la Universidad Miguel Hernández.

Para que así conste a los efectos oportunos, firmo el presente certificado en San Juan de Alicante a 11 de Junio de 2015.

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La **Dra. M.Ángela Nieto Toledano**, Profesor de Investigación del Consejo Superior de Investigaciones Científicas en el Instituto de Neurociencias de Alicante, centro mixto CSIC-UMH,

DA SU CONFORMIDAD a la lectura de la tesis doctoral titulada "Epithelial-Mesenchymal transitions and cell behaviour", realizada por Dña. Rebeca Córcoles Córcoles, bajo mi inmediata dirección y supervisión en el Instituto de Neurociencias (CSIC-UMH) y que presenta para la obtención del grado de Doctor por la Universidad Miguel Hernández.

Para que así conste a los efectos oportunos, firmo el presente certificado en San Juan de Alicante a 11 de Junio de 2015.

Fdo.: Dra. M.Ángela Nieto Toledano

A mis padres Mario y Basi

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Abbreviations

- α –SMA Smooth muscle α-actin
- BSA Bovine serum albumin
- CSC Cancer stem cell
- CTC Circulating tumour cell
- CoRe Contact repulsion
- DAB 3,3-Diaminobenzidine
- DAPI Diamidinophenylindole
- EMT Epithelial to mesenchymal transition
- EMT-TF Epithelial to mesenchymal transition-Transcription factor
- FBS Fetal bovine serum
- HEK-293T Human embryonic kidney 293T
- MET Mesenchymal to epithelial transition
- SCC Squamous cell carcinoma
- PBS Phosphate-buffered saline
- PH3 Phospho-histone-3
- PR1 Prrx1 (Paired-related homeobox 1)
- TdT Terminal desoxinucleotidil transferase
- TIC Tumour initiating cell
- TW1 Twist1

Abstract

The epithelial to mesenchymal transition (EMT) is required in the embryo for the formation of tissues which cells originate far for their final destination. This programme endows cells with migratory and invasive properties. Interestingly, the reverse process, known as mesenchymal to epithelial transition (MET), is also essential in embryogenesis to allow the differentiation of tissues and organs once the embryonic migratory cells reach their final destination. The pathological activation of the EMT programme in the adult promotes tumour progression and organ fibrosis and recent findings indicate that the EMT can also confer stem cell properties. Similar to the situation in embryos, it has been suggested that a reversion of the EMT (MET) might also be necessary for metastatic colonization once malignant cells extravasate and find their niche in distant organs.

The main inducers of the EMT are transcription factors of the Snail, Zeb and Twist families (EMT-TFs). Recently in our lab it has identified Prrx1, another transcription factor that can trigger EMT in embryos and in cancer cells. Importantly, the loss of Prrx1 in mesenchymal cancer cells induces a complete reversion to the epithelial phenotype. Moreover, Prrx1 loss in cancer cells is accompanied by the acquisition of stem cell-like properties, indicating that, unlike the classical EMT transcription factors, Prrx1 uncouples EMT and stemness.

One question that emerges is why the organisms need so many EMT inducers and whether there are differences in the EMT triggered by each of them. Therefore, this thesis focuses on the characterization of the cells that have undergone EMT triggered by each individual factor or by a combination of them, as different developing tissues and human tumours usually express several inducers. Specifically, we have examined cell behaviour in 2D and 3D cultures and parameters related to cell movement, invasion, proliferation and stemness as well as cell morphology and molecular markers. As the reverse process, MET, is important for organ differentiation and metastasis formation, we examine how the regulation of these factors impact on epithelial plasticity

and the acquisition or maintenance of stem cell properties. The latter has obvious implications in the colonization of migratory embryonic or malignant tumour cells to form organs or metastasis, respectively.

Published paper related to this thesis:

Ocaña, O. H., Corcoles, R., Fabra, A., Moreno-Bueno, G., Acloque, H., Vega, S., Barrallo-Gimeno, A., Cano, A., and Nieto, M. A. (2012). Metastatic colonization requires the repression of the epithelial-mesenchymal transition inducer Prrx1. Cancer Cell *22*, 709-724.

Resumen

En el embrión la transición epitelio-mesénquima (TEM) es necesaria para que se puedan formar los tejidos cuyas células se originan lejos del que será su destino final. Este programa proporciona propiedades migratorias e invasivas a las células. De manera interesante, el proceso contrario, conocido como transición mesénquima-epitelio (TME), es también esencial en embriogénesis ya que permite la diferenciación de los tejidos y los órganos una vez que las células migratorias embrionarias han llegado a su destino. La activación patológica del programa de TEM en el adulto causa progresión tumoral y fibrosis de los órganos y un descubrimiento reciente indica que la TEM puede conferir también propiedades de células madre. De manera similar a lo que ocurre en embriones, se ha sugerido que una reversión de la TEM (TME) podría ser necesaria para la colonización metastásica una vez que la célula maligna extravasa y encuentra su nicho en un órgano lejano.

Los principales inductores de la TEM son factores de transcripción pertenecientes a las familias de Snail, Zeb y Twist. En nuestro laboratorio se ha identificado recientemente Prrx1, otro factor de transcripción que promueve TEM en embriones y células tumorales. De manera importante, la pérdida de Prrx1 en células tumorales mesenquimáticas induce una reversión completa al fenotipo epitelial. Además, la pérdida de Prrx1 en células tumorales va acompañada de la adquisición de propiedades de células madre, lo que indica que, a diferencia de los factores de transcripción de TEM clásicos, Prrx1 separa la TEM de las propiedades de células madre.

Una pregunta que surge es por qué los organismos necesitan tantos inductores de TEM y si hay diferencias en la TEM promovida por cada uno de ellos. Por ello, esta tesis está enfocada en la caracterización de células que han experimentado una TEM promovida por cada factor individual o por una combinación de ellos, ya que diferentes tejidos durante el desarrollo embrionario y tumores humanos expresan varios inductores. Hemos examinado de manera específica el comportamiento celular en cultivos en 2 y 3

dimensiones y parámetros relacionados con los movimientos celulares, invasión, proliferación y propiedades de células madre así como morfología celular y marcadores moleculares. Como el proceso contrario (TME) es importante para la diferenciación de los órganos y la formación de la metástasis, también hemos examinado como impacta la regulación de estos factores en la plasticidad epitelial y en la adquisición o mantenimiento de propiedades de células madre. Esto último, tiene implicaciones obvias en la colonización de células migratorias embrionarias o tumorales para formar órganos o tumores, respectivamente.

Publicaciones relacionadas con la tesis:

Ocaña, O. H., Corcoles, R., Fabra, A., Moreno-Bueno, G., Acloque, H., Vega, S., Barrallo-Gimeno, A., Cano, A., and Nieto, M. A. (2012). Metastatic colonization requires the repression of the epithelial-mesenchymal transition inducer Prrx1. Cancer Cell *22*, 709-724.

I. CONTENTS

II. INTRODUCTION

II.1. The concept of Epithelial Plasticity: EMT and MET

Metazoans consist mainly of two cell types, epithelial and mesenchymal, which differ both morphologically and functionally (Acloque et al., 2009). Epithelial cells are adherent cells that form coherent layers as they are closely attached by intercellular adhesion complexes. These cell junctions consist of multiprotein complexes that provide contacts between neighbouring cells and between cells and the extracellular matrix (Thiery and Sleeman, 2006). In vertebrates, there are four major types of cell-cell junctions -tight junctions, adherens junctions, desmosomes and gap junctions- and two types of cell-extracellular matrix junctions, namely focal adhesion and hemidesmosomes. They differ from one another in the cytoskeletal protein anchor as well as in the transmembrane linker protein that extends through the membrane (Figure 1).
Epithelial cells also display apico-basal polarity, with a characteristic basally located basement membrane that separates the epithelium from other tissues. These cells do not detach or move away from the epithelial layer under normal conditions (Thiery and Sleeman, 2006).

Mesenchymal cells, on the other hand, do not form an organized layer. They lack intercellular junctions, and as such they can move as individual cells or organized in mesenchymal networks throughout the extracellular matrix. They do not display apicobasal polarity and are not typically associated with a basal lamina. In culture, mesenchymal cells have a spindle shape, fibroblast-like morphology and tend to be highly mobile, whereas epithelial cells grow as clusters of cells that maintain cell-cell adhesion with their neighbours. Moreover, a number of molecular differences are observed between these two phenotypes. For example, mesenchymal cells do not express epithelial (E)-cadherin, whereas epithelial cells do (Peinado et al., 2004). The composition of the intermediate filament is also different, with vimentin being typical of mesenchymal cells.

Interestingly, there is plasticity between these two phenotypes. Epithelial cells can turn into mesenchymal cells and vice versa so that both these

phenotypes are not irreversible and cells frequently cycle between epithelial and mesenchymal state. The events that convert adherent epithelial cells into mesenchymal migratory cells are known collectively as epithelial to mesenchymal transition (EMT). Elizabeth Hay coined this term in 1968 when she highlighted the importance of the EMT process for cell movements in the embryo (Hay, 1968). In the reverse process, the mesenchymal to epithelial transition (MET), mesenchymal cells recover intercellular adhesion complex and the apico-basal polarity.

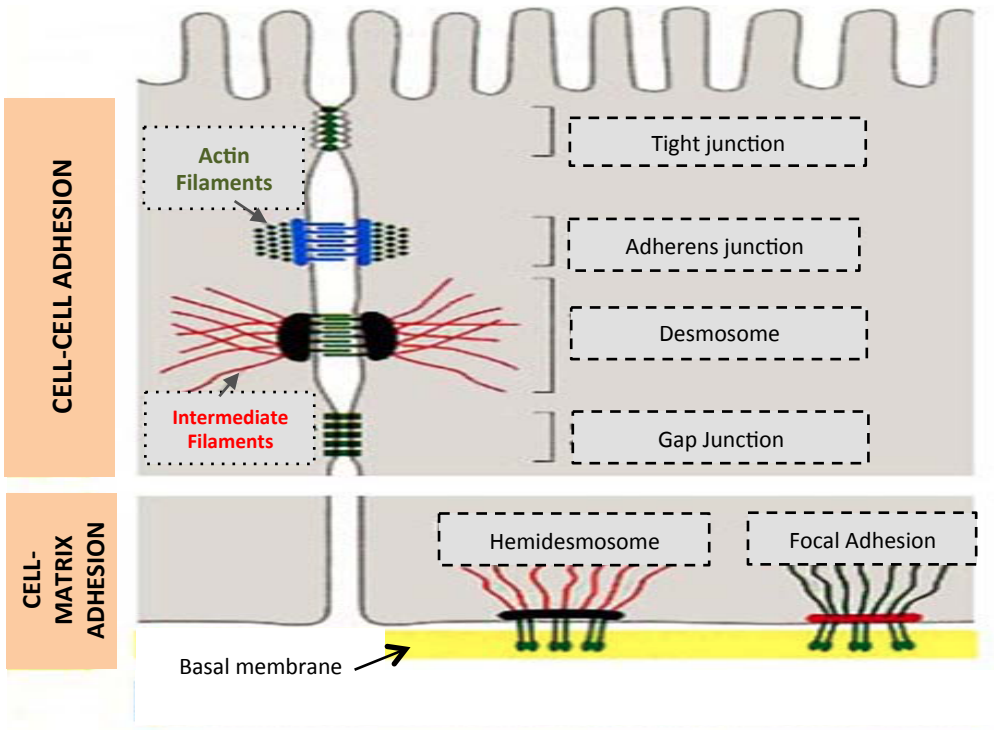


Figure 1. Types of cell junctions. Tight junction seals neighbouring cells together in an epithelial sheet to prevent leakage of molecules between them. Adherens junction joins an actin bundle in one cell to a similar bundle in a neighbouring cell. Desmosome joins the intermediate filaments in one cell to those in a neighbour. Gap junction allows the passage of small water-soluble ions and molecules. Hemidesmosome and focal adhesion anchor intermediate filaments or actin filaments respectively, to the basal membrane.

Adapted from Molecular Biology of the Cell, Bruce Alberts (Fourth edition, 2004)

II.1.1. Cellular aspects of the EMT process

The transition from epithelial to mesenchymal characteristics encompasses a spectrum of inter- and intracellular changes, which are determined by the integration of extracellular signals the cell receives. The EMT process can be delineated into several steps (Figure 2; Acloque et al., 2009), some of which can occur simultaneously:

1. Disruption of the intercellular adhesion complexes and loss of apico-basal polarity (Barrallo-Gimeno and Nieto, 2005; Peinado et al., 2007; Moreno-Bueno et al., 2008).
2. Profound cytoskeletal remodelling, which favours:
    - Cell delamination after apical constrictions and disorganization of the basal membrane (Nakaya et al., 2008; Martin et al., 2009).
    - Acquisition of motility properties
3. Activation of protease activity leading to:
    - Breakdown of the basement membrane
    - Acquisition of invasive properties (Haraguchi et al., 2008).

Thus, upon undergoing EMT, the cells acquire migratory and invasive properties that allow them to migrate through the extracellular matrix (Thiery et al., 2009; Lamouille et al., 2014).

II.1.2. Epithelial Plasticity

Plasticity between epithelial and mesenchymal cells is crucial during embryonic development as several rounds of EMT and MET are necessary for the generation of tissues and organs whose precursors originate far for their final destination. EMT is necessary for the embryo to allow epithelial cells to migrate over what may be long distances. Once cells have reached their destination, they differentiate into different cell types, a process that very often involves the reverse process, MET (Thiery et al., 2009; Lamouille et al., 2014).

The reactivation of the EMT programme can be highly deleterious in the adult as epithelial homeostasis is disturbed. As such, pathological activation of the EMT programme promotes tumour progression and organ fibrosis (Nieto, 2011). Similar to the situation in embryos, a reversion of the EMT (MET) was proposed to be necessary for metastatic colonization once malignant cells extravasate and find their niche in distant organs (Thiery et al., 2002; Thiery et al., 2009). Depending on the signalling context and the tissue, some cells may show some epithelial and mesenchymal characteristics, which is considered as a partial EMT. Therefore, epithelial plasticity holds a range of changes in morphological phenotypes and cell behaviour.

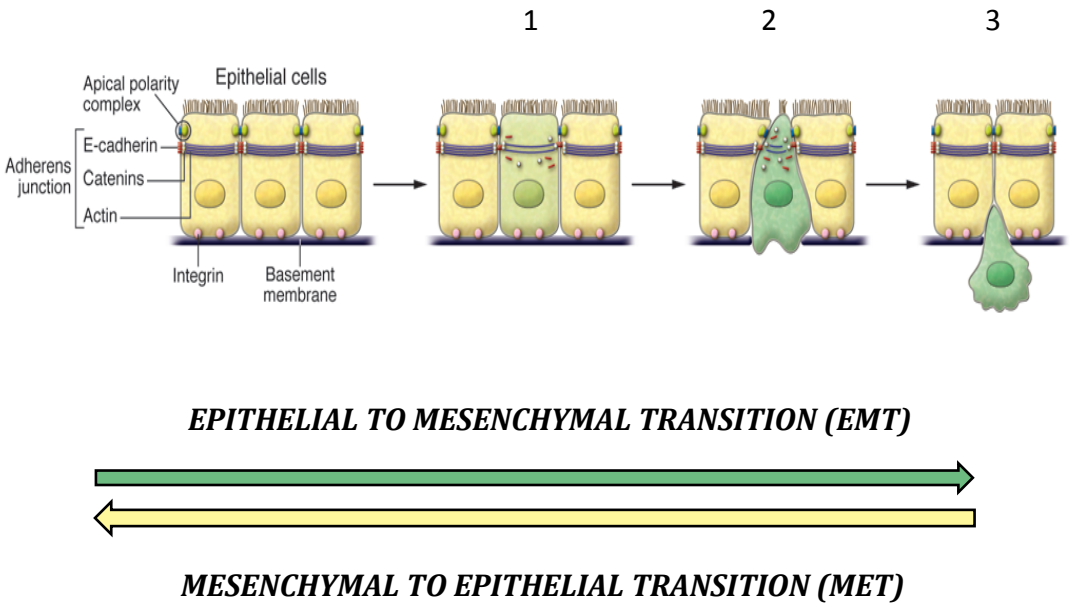


Figure 2. Cellular aspects of EMT. Adherens junctions in epithelial cells contain E-Cadherin, catenins and actin rings. Tight junctions are associated with apical polarity complexes. Integrins interact with components of the basement membrane. (1) Loss of cell-cell adhesion. E-cadherin is internalized and targeted for degradation. (2) Profound cytoskeletal remodelling and apical constriction. Breakdown of the basement membrane. (3) Cell delamination and invasion of adjacent tissues.

Adapted from Acloque et al., 2009

II.1.3. Types of EMT

EMT and MET processes are induced in different physiological and pathological contexts, although exhibit some peculiarities depending on whether it occurs during embryonic development, tumour progression or organ fibrosis. For instance, in fibrotic processes there is an excessive deposition of extracellular matrix and inflammation, which does not occur in embryonic development (Kalluri and Weinberg, 2009; López-Novoa and Nieto, 2009). Inflammation is also present in tumour microenvironment. In contrast to fibrotic cells, embryonic or tumour cells have the ability to disseminate, although only tumour cells can do it through both lymphatic and blood vessels (Nieto, 2011).

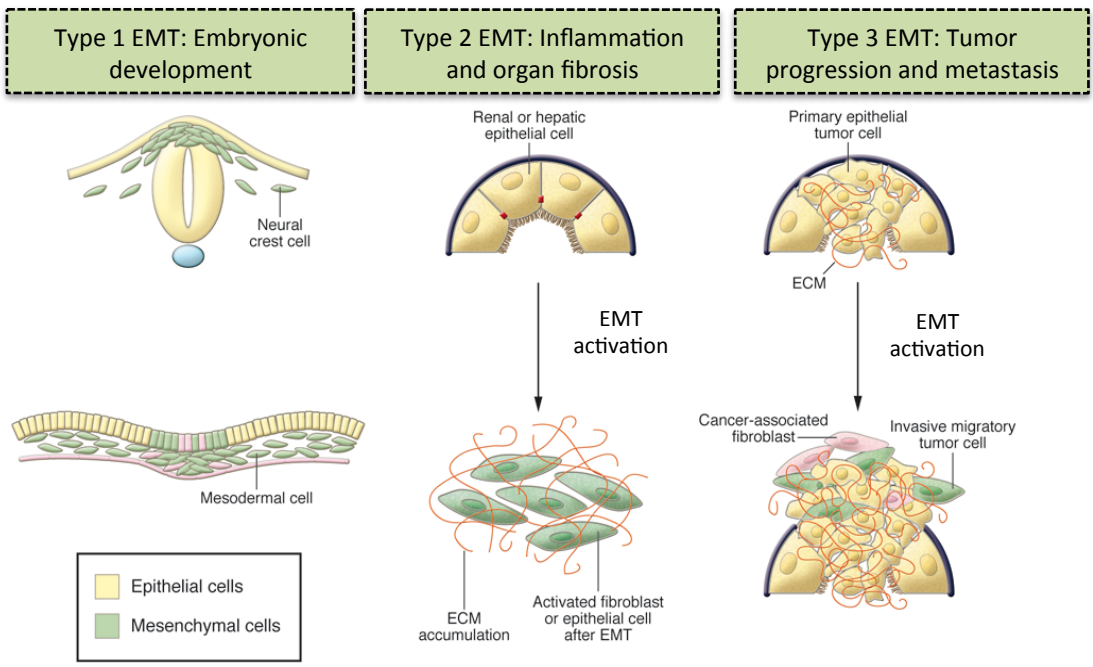


Figure 3. Different types of EMT. Type 1 EMT is associated with neural crest cells delamination and embryonic gastrulation. Type 2 EMT is related to the context of inflammation and fibrosis. Unlike type 1 EMT, type 2 EMT is expressed over extended periods of time and can destroy an affected organ if the primary inflammatory insult is not removed. Type 3 EMT is associated with cancer cells.

From Acloque et al., 2009 and adapted from Kalluri and Weinberg, 2009

Based on these differences, the EMT has recently been classified into three different groups, namely types 1, 2 and 3, referring to what occurs during development, organ fibrosis and cancer progression respectively (Figure 3; Kalluri and Weinberg, 2009).

Epithelial plasticity occurs both during embryonic development and cancer. However, the EMT associated with organ fibrosis is not a plastic process. The acquisition of a mesenchymal state in fibrosis can be considered an end stage, which leads to organ degeneration and failure (Nieto, 2013).

II.2. EMT and MET during embryonic development

Throughout evolution, the capacity of a cell to transition from an epithelial to a mesenchymal state has been fundamental in the generation of complex body patterns (Acloque et al., 2009). Several rounds of the EMT and MET are necessary in the embryo for the final differentiation of specialized cell types and the acquisition of the complex three-dimensional structure of internal organs (Thiery et al., 2009). Indeed, with the exception of the epidermis and part of the central nervous system, all adult tissues and organs are the result of one or several rounds of EMT and MET (Figure 4).

Primary EMT occurs at gastrulation and during neural crest cell emigration, both fundamental processes during embryonic development. This primary EMT is followed by differentiation events that generate different cell types, which often involve the MET process. Although many signalling pathways have been identified for EMT (Thiery et al., 2009; De Craene and Berx, 2013), the signals involved in the induction of MET have not been well characterized in embryonic development.

II.2.1. EMT in gastrulation

The first EMT in the embryo occurs at gastrulation, an ancient process

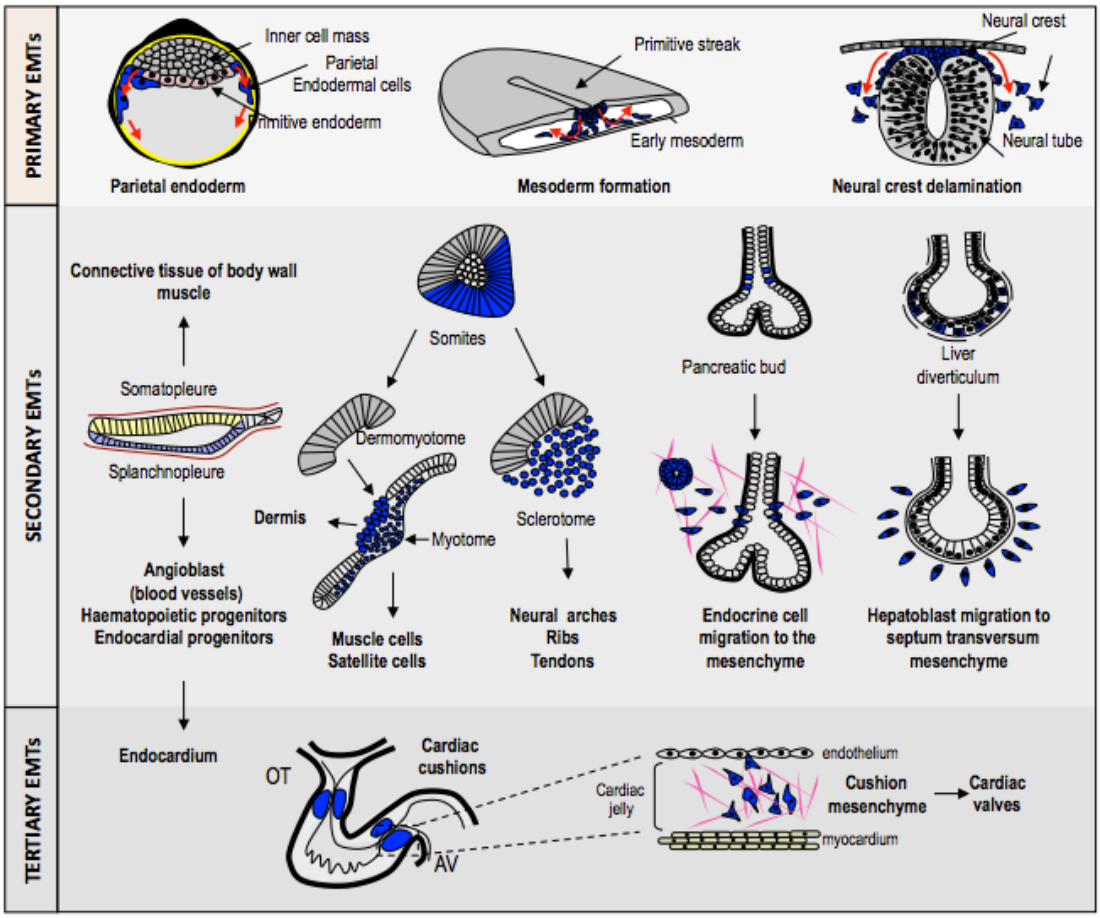


Figure 4. Reversibility of EMT during embryonic development. EMTs during embryonic development are reversible. Primary EMT occurs very early in the embryo, even before implantation, such as during the formation of the parietal endoderm in mice. The first EMT after implantation is that undergone by the mesendodermal progenitors during gastrulation, whereas the delamination of neural crest cells from the dorsal neural tube is a later event. Thereafter, the mesodermal precursors migrate to occupy different positions along the mediolateral axis of the embryo and upon undergoing MET generate the notochord, the somites and the somatopleure and splenchnopleure. The majority of these epithelial derivatives undergo a second round of EMT as occurs in pancreas and liver to induce the dissociation of endocrine cells and hepatoblasts from their respective epithelial primordia. A tertiary EMT arises during the formation of the cushion mesenchyme, which is the precursor of the cardiac valves

Adapted from Thiery et al., 2009

fundamental for the formation of the different embryonic layers. At first, the embryo is formed by a single epithelial cell layer known as epiblast. After receiving specific signals, a subset of cells from the epiblast move to the midline

to form the primitive streak, a linear structure that bisects the embryo along the anterior-posterior axis. These cells undergo EMT and internalize to generate mesoderm and endoderm, while those remaining in the epiblast become ectoderm. Thus, the embryo is transformed from a single layer, to three germ layers (Acloque et al., 2009).

The mesoderm initially comprises mesenchymal cells and rapidly becomes subdivided into axial, paraxial, intermediate and lateral plate mesoderm. After migration and distribution along the entire mediolateral axis of the embryo, mesodermal cells undergo MET and give rise to various derivatives. Therefore, the axial mesoderm gives rise to the notochord, paraxial mesoderm undergoes MET to form the somites, intermediate mesoderm will later form the urogenital system and the lateral mesoderm condenses to form somatopleure and splanchnopleure (Figure 5A). Except for the notochord, all the other embryonic structures derived from the early mesoderm are later remodelled by successive EMT and MET events to define the architecture of the organism (Perez-Pomares et al., 2002). For the formation of the heart, three successive cycles of EMT and MET are necessary.

Endodermal derivatives can also undergo several rounds of EMT and MET to generate internal organs such as the pancreas and liver (Johansson and Grapin-Botton, 2002; Tanimizu and Miyajima, 2007).

II.2.2. EMT during neural crest cells emigration

Primary EMT also occurs during vertebrate nervous system development to generate migrating neural crest cells. Epithelial cells from the dorsal midline of the neural tube undergo EMT and migrate throughout the embryo. Following their migration, they differentiate into many diverse derivatives including ganglia of the peripheral nervous system, glial cells, melanocytes and cranial bones. Formation of these derivatives again often involves a MET step (Figure 5B; Le Douarin and Kalcheim, 1999).

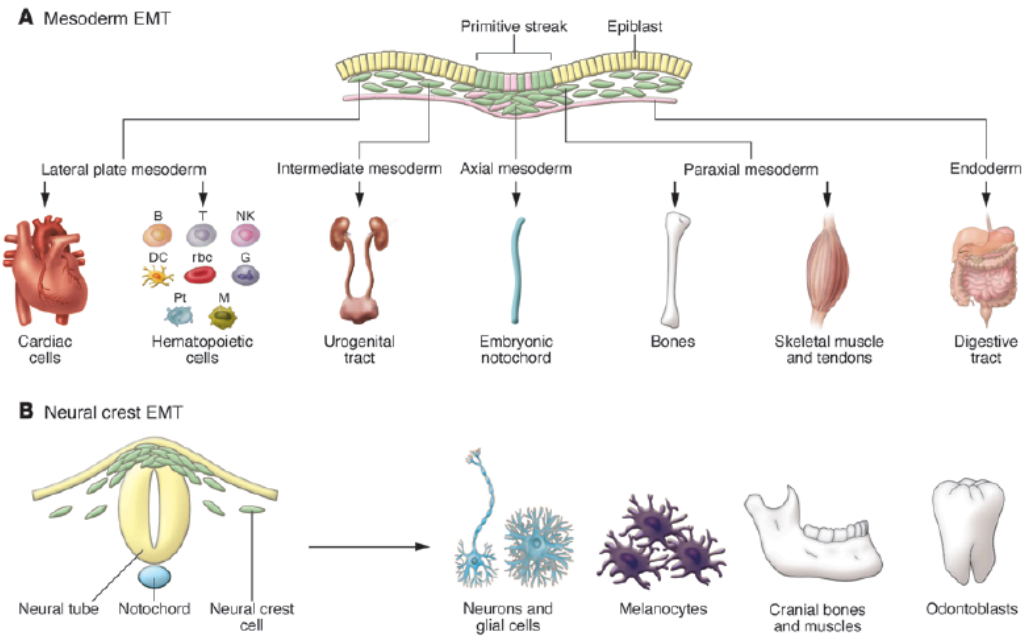


Figure 5. Primary EMTs give rise to progenitors of many organs and tissues. A. Cells from the primitive streak undergo EMT (green cells) and internalize at gastrulation giving rise to different mesodermal and endodermal population**.** Pt, platelets; B, T, and NK, lymphocytes; G, granulocytes; M, macrophages **B.** Neural crest cells delaminate from the dorsal neural tube and will generate ganglia of the peripheral nervous system, glial cells, melanocytes and cranial bones, which involve the MET process.

From Acloque et al., 2009

II.3. EMT as a physiological response to injury

EMT also occurs as a physiological response to injury. During wound healing, cells at the border of the wound recapitulate part of the EMT process and they acquire an intermediate phenotype known as the “metastable” state. This “metastable” state has been used to indicate intermediate states, which allow cells to move while maintaining loose contacts rather than migrating as individual cells. Due to this partial EMT, cells can still revert to the epithelial phenotype once the wound is healed.

II.4. EMT and MET in cancer progression

EMT not only occurs during embryonic development or as a physiological response to injury, but is also an important element in cancer progression and other pathologies such as organ degeneration.

At the cellular level, pathological EMTs are similar to physiological EMTs in that similar signalling pathways, regulators and effector molecules, govern them. However, as already mentioned, some peculiarities exist depending on whether it occurs during embryonic development, tumour progression or organ fibrosis.

EMT has been recognized as a crucial step in the metastatic cascade (Polyak and Weinberg, 2009; Thiery et al., 2009). The best evidence of EMT in cancer comes from the recent observation *in vivo* of single cells delaminating from primary tumours (Entenberg et al., 2013) and from the finding that circulating tumour cells (cancer cells traveling through the circulation, CTCs) from patients show EMT features and a high degree of epithelial plasticity (Raimondi et al., 2011; Yu et al., 2013).

Excessive epithelial cell proliferation and angiogenesis are crucial steps in the initiation and early growth of primary epithelial cancers (Hanahan and Weinberg, 2000). Afterwards, activation of the EMT program allows cells to delaminate from the primary tumour and invade surrounding tissues, initiating the metastatic dissemination. Therefore, the invasion-metastatic cascade can be delineated into several steps (Figure 6; Thiery, 2002):

1. *Invasion*. Individual cells in the invasive front of the tumour undergo EMT to delaminate. Mesenchymal cancer cells can breakdown the basement membrane invading the surrounding tissues.

2. *Intravasation*. Mesenchymal cancer cells enter the microvasculature of the lymph and blood systems (intravasation), allowing their passive transport to distant organs. CTCs represent carcinoma cells that are en route between primary tumours and sites of dissemination.

3. *Extravasation*. Cancer cells leave the blood stream.

4. *Colonization* (“*homing*”). Cells undergo MET, adapt to the foreign microenvironment and they can either remain solitary (micrometastasis) or proliferate forming a secondary tumour (macrometastasis).

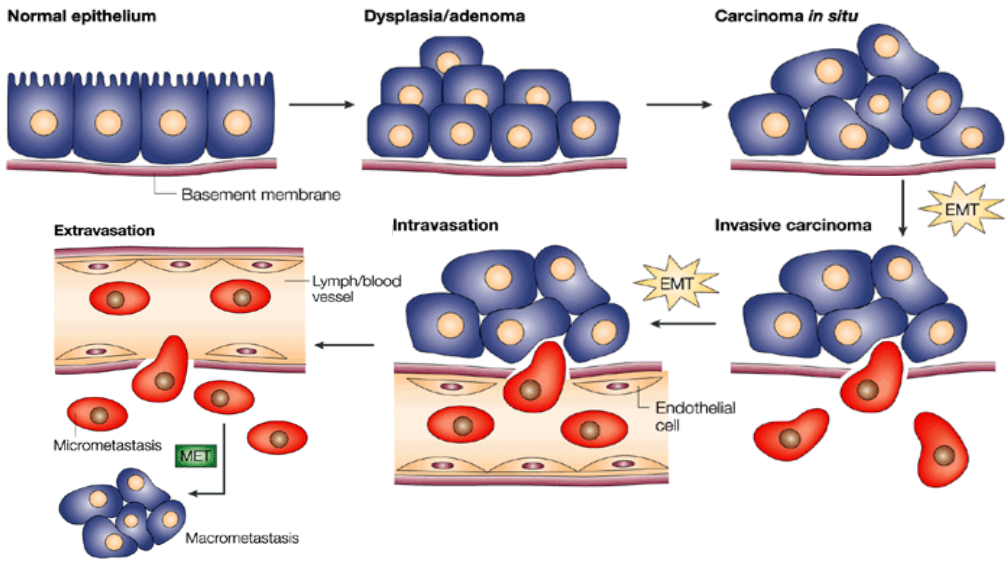


Figure 6. Stages of the invasion-metastasis cascade. Normal epithelia, separated from other tissues by a basement membrane, can proliferate locally to give rise to an adenoma. Further transformations caused by genetic and epigenetic alterations lead to a carcinoma *in situ,* still outlined by an intact basement membrane. Activation of the EMT program induces a fragmentation of the basement membrane and local dissemination of carcinoma cells. These cells can intravasate into lymph or blood vessels, allowing their passive transport to distant organs. At secondary sites, cells leave the blood stream and they can remain solitary (micrometastases) or form a new carcinoma through a MET.

From Thiery, 2002

As in the embryos, a MET step is also necessary for metastatic colonization once malignant cells extravasate and find their niche in distant organs. MET not only implies a reversion to the epithelial phenotype but also an increase in cell proliferation, important for the growth of the secondary tumour (ñet al., 2012). The description of signals that induce MET at the metastatic site

remain unknown, but it is clear that interactions between cancer cells and the tumour microenvironment play an important role (Karnoub et al., 2007; Malanchi et al., 2012). In the same way, activation of the EMT program during tumourigenesis often requires signalling between cancer cells and neighbouring stromal cells (Yang and Weinberg, 2008). Island of cancer cells in advanced primary carcinomas recruit a variety of cell types into the surrounding stroma, such as fibroblast, myofibroblast, granulocytes, macrophages and lymphocytes. These recruited cells create the "reactive" stroma, an inflammatory microenvironment that can activate the EMT program enabling cell dissemination (Chaffer et al., 2011). Therefore, the stroma influences the primary tumour and the metastatic site (Gao et al., 2012; Calon et al., 2012).

The EMT program also seems to help recently extravasated cells to extend filopodium-like protrusions and interact productively with the niche, promoting homing and proliferation (Shibue et al., 2013). In summary, EMT is associated with metastasis and it is thought to confer invasive properties that enable cancer cells to disseminate whereas the reverse process, MET, is thought to favour the outgrowth of these cells at distant sites.

II.5. EMT inducers

All EMTs have in common the loss of adherent junctions and apico-basal cell polarity. This loss occurs upon activation of transcription factors known as EMT inducers (EMT-TFs) and with subsequent *E-cadherin* (encoded by CDH1) transcriptional repression (Peinado et al., 2007). This is the reason why the EMT-TFs are also known as *E-cadherin* transcriptional repressors. Multiple transcription factors are able to directly trigger EMT when expressed in epithelial cells. They belong to different gene families that can be divided into two groups depending on whether they can repress *E-cadherin* transcription directly or indirectly (Nieto, 2011; Puisieux et al., 2014). As such, Snail1, Snail2 (previously known as Slug), Zeb1, Zeb2 (also known as Sip1), E47 and Klf8 (Kruppel-like factor 8) can bind to the *E-cadherin* promoter and repress its

transcription, so they belong to the *E-cadherin* direct transcriptional repressor class. However, factors such as Goosecoid, E2.2 (also known as TCF4), and Foxc2 (fork-head box protein C2) repress *E-cadherin* transcription indirectly (Peinado et al., 2007; Yang and Weinberg, 2008). Twist, another EMT-TF can act as an indirect repressor through Slug or as a direct repressor when complexed with the polycomb-group protein BMI-1 (Casas et al., 2011, Yang et al., 2010). Recently in our lab a new EMT-TF has been identified (Ocaña et al., 2012). It is known as Prrx1, and belongs to the class of indirect repressors of *E-cadherin* transcription (Figure 7).

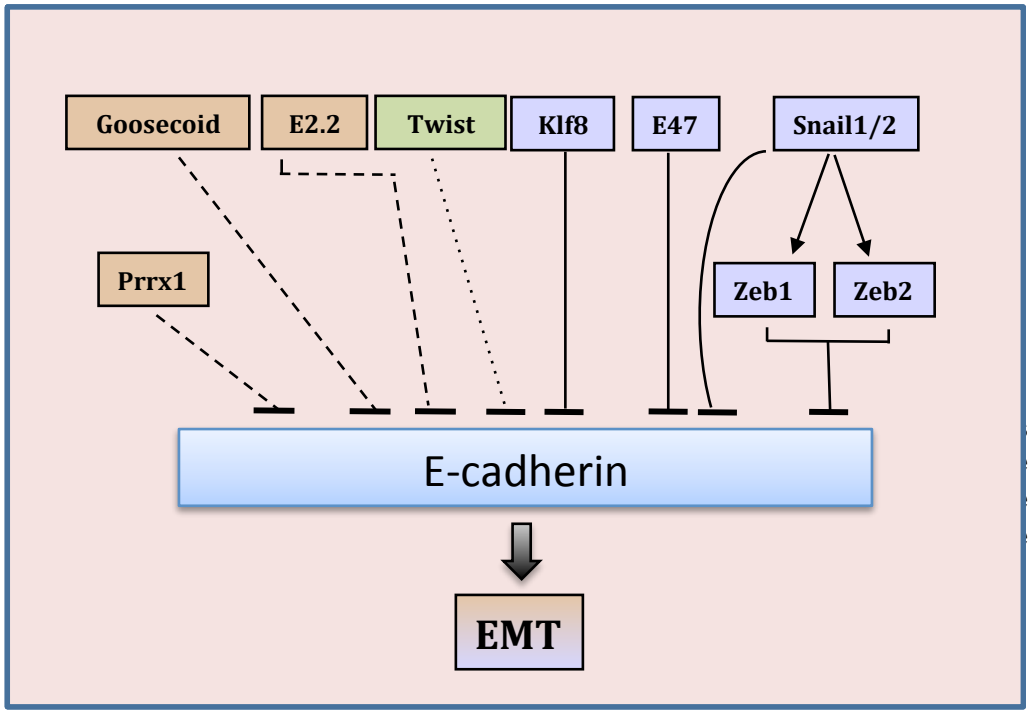


Figure 7. E-cadherin transcriptional repressors. Snail, Zeb, E47 and KLF8 factors directly repress *E-cadherin* transcription, whereas Goosecoid, E2.2 and Prrx1 are indirect E-cadherin repressors. Snail1 activates the expression of the *Zeb* genes. The EMT inducers that indirectly repress *E-cadherin* transcription frequently activate some of the direct repressors and they also have multiple specific targets.

Adapted from Thiery et al., 2009

II.5.1. Snail factors

The Snail superfamily consists of two related, yet independent, families: Snail and Scratch, with three members of the Snail superfamily described in vertebrates: Snail1, Snail2 y Snail3 (Barrallo-Gimeno and Nieto, 2005). Members of the Snail family are zinc-finger transcription factors expressed during gastrulation and neural crest cell delamination, as well as in most developmental process where EMT is required. Members of the Snail family share a highly conserved C-terminal region containing from four to six zinc fingers (C_2H_2 type) that function as the sequence-specific-DNA-binding domains, which recognize consensus E-box type elements in the promoter of target genes. This region also contains the nuclear localization domain (NLD) recognized by importins, which translocate the Snail protein from the cytoplasm to the nucleus (Mingot et al, 2009). The N-terminal part of the protein is more divergent and usually contains the SNAG domain, which consists of 7 to 9 amino acids that harbour the repressor capacity. Moreover, this domain also works as a nuclear export signal (NES) (Mingot et al., 2013). The central region of the Snail proteins has a serine-proline rich region that is highly divergent between the family members. Snail2 contains a so called Slug domain whose function remains elusive.

Snail1 and *Snail2* expression has been reported in several types of carcinomas. The expression of *Snail1* in breast carcinomas is associated with invasion, metastasis, tumour recurrence and poor prognosis (Blanco et al., 2002; Come et al., 2006; Kudo-Saito et al., 2009; Tran et al., 2014) and similarly, the expression of *Snail2* has been associated with poor clinical outcome in breast and ovarian tumours (Martin et al., 2005). *Snail1* expression has also been found in colorectal cancer, squamous cell carcinomas (SCC) and hepatocarcinomas (Palmer et al., 2004; Takeno et al., 2004; Miyoshi et al., 2005; Olmeda et al., 2008). The expression of *Snail2* has also been found in colorectal cancer, oesophageal SCC, in lung adenocarcinomas and in gastric carcinomas (Shioiri et al., 2006; Uchicado et al., 2005; Shih et al., 2005; Castro-Alves et al., 2007).

II.5.2. Twist factors

Twist1 and Twist2 transcription factors belong to the family of basic helix-loop-helix factors (bHLH), and contain two parallel amphipatic α-helices joined by a loop required for dimerization. Twist, but not other bHLH proteins, recognizes a unique double E-box motif with two boxes separated by 5 nucleotides. This double E-box motif aligns two Twist-E47 dimers on the same face of DNA, providing a high affinity side for a highly stable tetramer (Chang et al., 2015). Moreover, Twist also displays robust interactions with Snail1 and Snail2 (Lander et al., 2013). Twist factors are involved in gastrulation and in dorso-ventral symmetry as well as in neurogenesis and muscle development in invertebrates (Castanon et al., 2002). In vertebrates, it is important for head mesenchyme, neural crest migration and limb bud formation among other processes (Chen and Behringer, 1995; Bildsoe et al., 2013; Lander et al., 2013). Moreover Twist is a key factor during invasion and intravasation and induces invadopodia-mediated extracellular matrix degradation (Yang et al., 2004; Eckert et al., 2011).

Increased *Twist1* expression has been associated with disease aggressiveness and poor survival in high-grade invasive ductal carcinomas (Mironchik et al., 2005, Watson et al., 2007; Eckert et al., 2011). Moreover, association of *Twist1* expression with solid metastasis has been reported in prostate cancer, SCC and hepatocarcinomas (Kwok et al., 2005; Yuen et al., 2007; Lee et al., 2006; Kallergi et al., 2011; Tsai et al., 2012) and is linked to poor survival in patients with endometrial tumours or melanomas.

II.5.3. Zeb family factors

The Zeb family of transcription factors contains two members, Zeb1 and Zeb2 and are characterized by the presence of three-four zinc fingers (C_2H_2 and C_3H type) at each end and a central homeodomain (Comijin et al., 2001; Eger et al., 2005). Zeb factors interact with DNA through the simultaneous binding of the two zinc finger domains to high affinity binding sites composed of bipartite

E-boxes. They are expressed in the embryo in the central nervous system, heart, skeletal muscle and haematopoietic cells.

The expression of these factors has also been reported in different tumour series. *Zeb2* expression has been found in ovarian, gastric and pancreatic tumours (Elloul et al., 2006; Rosivatz et al., 2002; Imamichi et al., 2007), while *Zeb1* expression has been studied in prostate, colorectal, breast and pancreatic tumours (Graham et al., 2008; Pena et al., 2006; Spaderna et al., 2006; Wellner et al., 2009) and recently also in glioblastomas (Siebzehnrubl et al., 2013).

II.5.4. Paired-related homeobox factors: Prrx1

Paired-type homeobox transcription factors are important regulators of morphogenetic processes and have been conserved in several species. The *Prrx1* gene is a member of this class and encodes a protein that contains a paired-type DNA binding homeodomain but lacks a second DNA binding domain present in other paired-related homeoproteins (Galliot et al., 1999). *Prrx1* is expressed in mesoderm during embryogenesis, particularly in craniofacial regions and branchial arches, as well as mesenchymal cells of the developing limbs and cranial cushions (Cserjesi et al., 1992; Norris et al., 2000). Recently in our lab Prrx1 has been identified as a novel EMT inducer both in embryos and in cancer cells (Ocaña et al., 2012).

PRRX1 expression has been reported in breast and lung carcinomas and in colorectal cancer patients (Ocaña et al., 2012; Takahashi et al., 2013) and recent studies have also shown that it is involved in pancreatic regeneration and carcinogenesis (Reichert et al., 2013).

II.6. Functions of the EMT inducers

EMT-TFs not only repress the transcription of the *E-cadherin* gene, but also repress the expression of other junction proteins genes, including claudins

and occludins, thus facilitating EMT. Therefore, EMT inducers act regulating the expression of a variety of genes repressing the epithelial character and promoting the mesenchymal state. In addition, they repress epithelial cell polarity through regulation of protein complexes involved in apicobasal polarity such as Par, Crumbs and Scribble (Moreno-Bueno, 2008).

The dramatic cytoskeletal changes that take place during EMT are probably incompatible with cell division (Barrallo-Gimeno and Nieto, 2005). Indeed, cells transiently stop dividing before undergoing migration during embryonic development. Similarly, although unregulated proliferation is fundamental for tumours to form and grow, this is not the case during their malignant phase. Interestingly, Snail1 and other EMT-TFs, block cell cycle progression by repressing the expression of the *Cyclin D* gene and increasing that of the cell cycle inhibitor p21 (Vega et al., 2004, Mejlvang et al.; 2007; Evdokimova et al., 2009; Tsai et al., 2012; Beck et al., 2015). Therefore, EMT-TFs attenuate cell proliferation, which favour invasion versus proliferation. Another property associated with Snail1 and other EMT-TFs is the resistance of cells to cell death. They confer a selective advantage to embryonic cells migrating towards their final destination and to invasive malignant cells in their attempts to disseminate and form metastasis (Vega et al, 2004; Leroy and Mostov, 2007; Valdes et al., 2002; Beck et al., 2015).

In conclusion, Snail1 together with other EMT-TFs not only affect the expression of epithelial and mesenchymal markers together with that of cytoskeletal proteins, but also they attenuate cell proliferation and confer resistance to cell death (Figure 8).

II.7. Hierarchy and cooperation among EMT inducers

Cooperation between different EMT-TFs is a hallmark of EMT induction (Thiery et al, cell 2009; Peinado et al., 2007). The existence of many EMT-TFs and their cooperation provides robustness to the system, ensuring the

implementation of the EMT program during embryonic development (Ocaña et al., 2012; Nieto and Cano, 2012; Nieto, 2013).

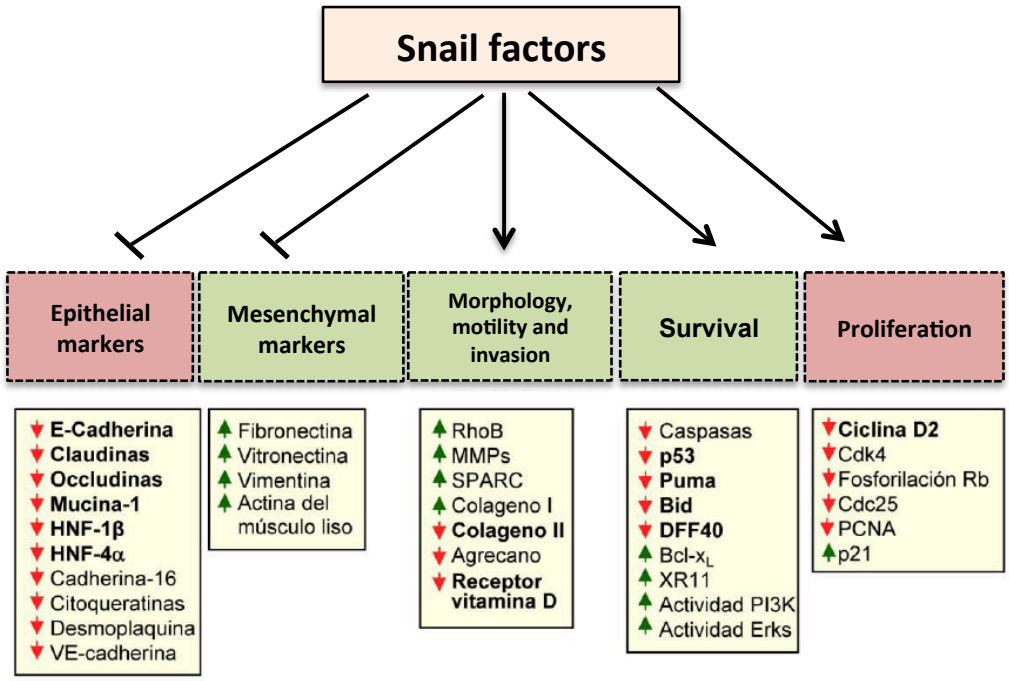


Figure 8. Functions and downstream targets of Snail. Snail factors induce the loss of epithelial markers and the gain of mesenchymal markers, as well as inducing changes in cell morphology and the acquisition of mobility and invasive properties. The *Snail* genes also regulate cell proliferation and cell death. Not all of these targets are directly regulated by Snail. The molecules and processes shown in red are downregulated or impaired by Snail, and those in green are upregulated or promoted by Snail.

From Barrallo-Gimeno and Nieto, 2005

There is increasing evidence of a hierarchy that controls the expression of these EMT-TFs. During embryonic development, *Snail* expression normally precedes the expression of other factors, which are more important to maintain the mesenchymal phenotype (Thiery et al., 2009; Nieto, 2013). This temporal hierarchy of EMT-TFs activation and cooperation also seems to operate during carcinoma progression, where *Snail1* is expressed at the onset of the transition, whereas *Snail2*, *Twist*, and *Zeb* are subsequently expressed to maintain the

migratory mesenchymal state (Peinado et al., 2007). As such, Snail1 and Snail2 cooperate both in the primary tumour and in metastatic cascade (Olmeda et al., 2008). Snail1 also cooperates with Twist1 during cancer progression and both transcription factors can regulate *Zeb1* expression in breast epithelial cells (Tran et al., 2011; Dave et al., 2011). Moreover, Snail1 upregulates the expression of Zeb proteins in cancer cells, although Zeb is active in some tumours that lack *Snail* expression (Peña et al., 2006). Thus, the contribution of different EMT inducers is also dependent on the cellular context. For instance, *Zeb1* expression is important during colon cancer progression, whereas *Zeb2* has been studied in ovarian, gastric and pancreatic tumours (Peinado et al., 2007).

Together, all these data indicate that in general terms, Snail1 is implicated in the initial migratory phenotype of primary tumours and can be considered as an early marker of EMT. In contrast, Snail2, Twist, Zeb1 and Zeb2 are subsequently activated and could be responsible for the maintenance of the mesenchymal phenotype. Moreover, once activated, EMT-TFs can cooperate and activate the same signalling pathways to generate positive regulatory autocrine and paracrine loops that help to maintain the migratory state (Scheel et al., 2011; Nieto 2013). In addition to that, these repressors could be differentially or sequentially expressed at distinct anatomical sites during tumour progression. For instance, Snail is important in local invasion while Twist1 plays an essential role in intravasation (Yang et al., 2004).

II.8. Regulation of EMT and stemnes

The induction of the EMT program has also been associated with the acquisition of stem cell-like properties. Upon expression of *Snail1* or *Twist1*, untransformed immortalized human mammary epithelial cells undergo EMT concomitant with the acquisition of stem cell properties (Mani et al., 2008: Morel et al., 2008). Interestingly *Zeb1* expression is also able to induce stem cell properties (Wellner et al., 2009). This is consistent with the concept of

"migratory cancer stem cells" (Brabletz et al., 2005), which combines stem cell properties and the mesenchymal phenotype necessary for efficient dissemination in tumour progression.

II.8.1. Intratumoral heterogeneity

Cells within individual tumours are not homogeneous and they display a great deal of intratumoral heterogeneity. Therefore, tumours consist mainly of two different cell types, cancer stem cells (CSCs) and non-cancer stem cells. CSCs maintain typical stemness properties, including the capacity to self-renew (ability to divide while maintaining their undifferentiated state), proliferate and differentiate. However, contrary to normal stem cells, CSCs do not need full pluripotency as during metastasis formation, cancer cells do not generate multiple cell types but instead revert to the phenotypes of the primary carcinoma (Polyak and Weinberg 2009). CSCs are also known as tumour initiating cells (TICs) as they are thought to be crucial for the initiation and maintenance of tumours as well as their metastasis. In many tumours they may represent a tiny fraction of the total cellularity of individual tumours. However, TICs may be the critical drivers of their malignant progression. Of particular relevance to metastatic colonization, the CSC hypothesis asserts that one or more self-renewing TICs must disseminate during the course of disease progression in order for macroscopic metastases to develop. Conversely, the limited self-renewal capacity of disseminated non-CSCs may preclude them from spawning macroscopic metastases (Brabletz et al., 2005).

II.8.2. EMT and Cancer Stem Cells (CSCs)

As described above, recent studies have demonstrated that the EMT can induce non-CSCs to enter into a CSC-like state (Mani et al., 2008: Morel et al., 2008, Wellner et al., 2009). However, these induced CSCs cannot represent the only source of cells in the CSC pool within a tumour and intrinsic CSCs are likely to exist within tumours (Figure 9; Chaffer and Weinberg, 2009).

This unexpected convergence between a molecular pathway that promotes both invasiveness and self-renewal is of particular note, as these transcription factors appear to concomitantly facilitate physical dissemination of carcinoma cells and, following dissemination, the proliferation (self-renewal) of these cells at distant organ sites (Valastyan and Weinberg, 2011). However, recent studies including those to be presented below, indicate that the mesenchymal phenotype is not always associated with stem cell-like properties and suggest that phenotype and "stemness" can be independently regulated (Celià-Terrassa et al., 2012; Ocaña et al., 2012; Schmidt et al., 2015).

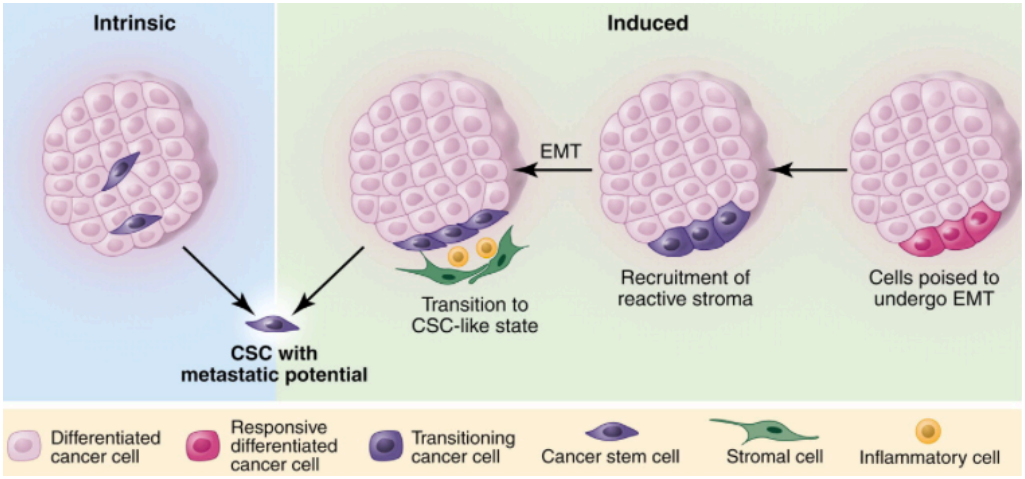


Figure 9. Mechanisms proposed for the generation of CSC. Tumours contain heterogeneous populations of cells. Intrinsic CSCs are thought to exist in primary tumours from the very early stages of tumorigenesis and may be the oncogenic derivatives of normal-tissue stem or progenitor cells. Induced CSC can arise as a consequence of the EMT. In this case, carcinoma cells initially recruit a variety of stromal cells, such as fibroblast, myofibroblast, macrophages, mesenchymal stem cells and lymphocytes. Together, these cells create a reactive microenvironment that causes the neighbouring cancer cells to undergo EMT and acquire CSC-like properties.

From Chaffer and Weinberg, 2011

II.9. Cell Motility

As it has been described above, in multicellular organisms cell migration is essential during normal development in the embryo for the formation of tissues, which cells originate far for their final destination (Acloque et al., 2009; Thiery et al., 2009; Nieto, 2011). Cell migration is also required throughout life for numerous processes, including wound healing and macrophage dispersion. However, deregulation in the control of cell migration can lead to cancer metastasis, as tumour cells can invade adjacent tissues and enter into the blood or lymphatic vessels.

II.9.1. Types of cell motility

Migratory cells can be classified into two groups: epithelial-like cells, which migrate collectively maintaining contacts and epithelial polarity, and mesenchymal cells, which are produced by an EMT and migrate solitary. In some cases, mesenchymal cells also can travel in large and dense groups exhibiting key features of collective migrating cells such as coordination and cooperation. This intermediate state corresponds to partial EMT (Figure 10).

II.9.1.1. Collective cell migration

Collective cell migration is a migratory process by which cells maintain close contact and E-cadherin expression as well as epithelial polarity during the whole process. Effective migration is likely to require coordinated regulation of the cytoskeleton of cells moving in the group. Examples of collective migration include the migration of the lateral line primordium in the zebrafish and the migration of the border cells cluster in the *Drosophila* ovary (Arboleda-Estudillo et al., 2010; López-Schier, 2010). Moreover, this kind of movement is often observed at the edge of SCC (Wicki et al., 2006) and during lymph node colonization from breast tumours (Giampieri et al., 2009).

II.9.1.2. Individual cell migration

Individual cells can migrate long distances to their final destination. Examples of full EMT are found in amniote embryos (avian and mammals) during the migration of mesendodermal precursors from the primitive streak and during the migration of neural crest cells from the neural tube. Individual cancer cells have also been seen to detach from the tumour mass in mouse models of breast carcinoma (Wang et al. 2002) and in human colorectal carcinomas (Brabletz et al., 2001). Although the cells migrate individually they do in a coordinated manner and with transient contacts with other cells.

II.9.1.3. Collective mesenchymal migration and partial EMT

Within the EMT the epithelial and mesenchymal cells can be considered as the two extremes of the process. As it has been mentioned above, an intermediate phenotype corresponds to partial EMTs when a full transition to the mesenchymal phenotype is not completed. Therefore, cells maintain loose cell-cell contacts while they are migrating. This migration has been described during wound healing and during the early migration of cranial neural crest in *Xenopus* and zebrafish (Theneveau et al, 2010). A partial EMT has also been observed in carcinosarcomas, claudin-low breast carcinomas and during renal or liver fibrosis.

II.9.2. Contact inhibition of Locomotion or Contact Repulsion

To generate productive movements, all migratory cells must show directionality, which is imposed by different mechanisms. One of these mechanisms that can direct cell migration during embryonic development is contact inhibition of locomotion (CIL), also known as contact repulsion (CoRe). For instance, neural crest cells from *Xenopus* exhibit CoRe on their way towards the branchial arches (Theveneau el at. 2010). This mechanism was described more than 50 years ago as the process by which cultured cells retract their protrusions and change their direction of migration upon contact with

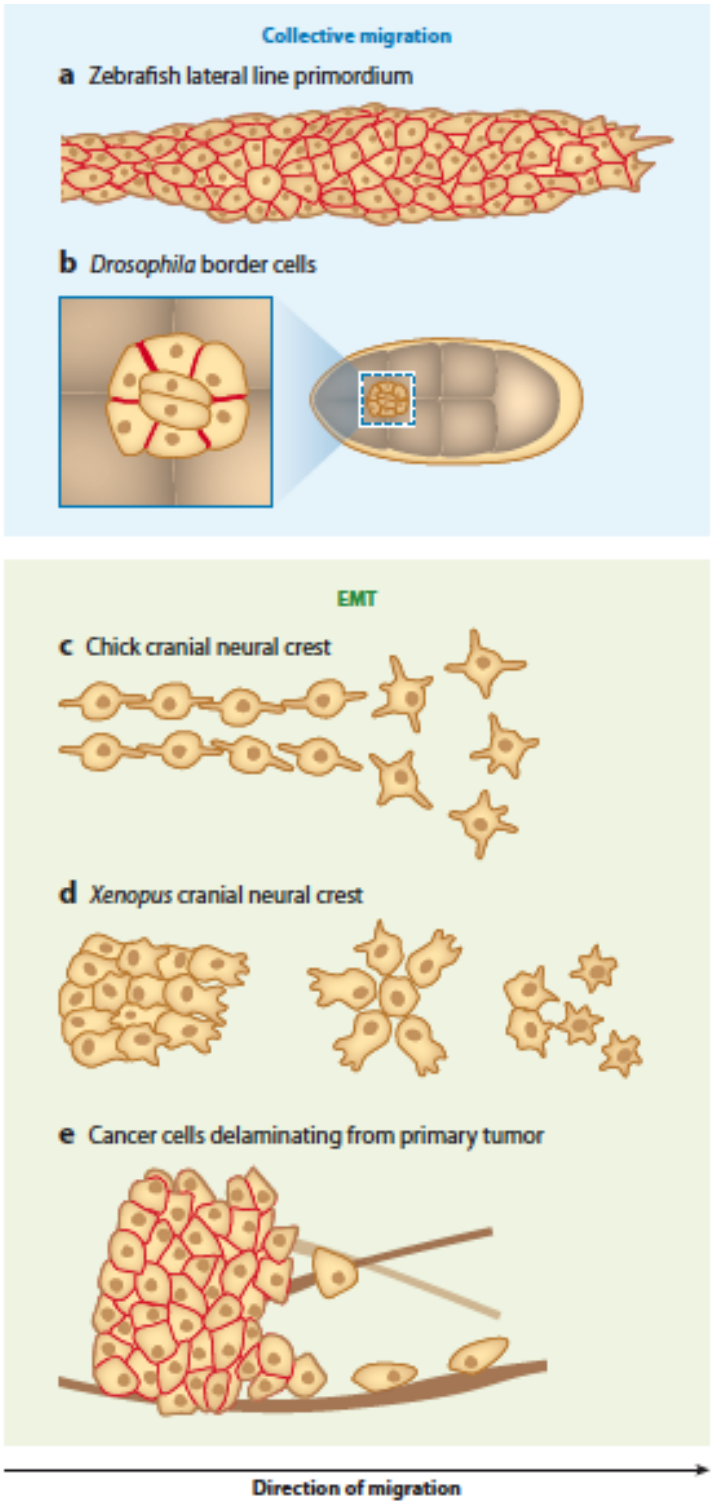


Figure 10. Types of cell motility. *(a,b)* Examples of collective cell migration are the primordium of the lateral line in zebrafish and the migration of the border cell cluster in the *Drosophila* ovary. In both cases, cells maintain some degree of epithelial polarity and E-cadherin at the cell junctions (*red*). (*c,d)* Migration of cranial neural crest cells of chick and *Xenopus* embryos. Cells migrating in a coordinated manner, either as loose groups or as individual cells with transient contacts. Cells lose *E-cadherin* expression and apicobasal polarity. (*e*) Individual cancer cells delaminating from the primary tumour in a mouse model of breast cancer. Cells can activate EMT programs, lose E-cadherin, and migrate along extracellular matrix fibers to form chains of coordinated individual cells.

Adapted from Nieto, 2011

another cell (Abercrombie, 1953). The behaviour of cells undergoing CoRe promotes dispersion, helping cell movements (Carmona-Fontaine et al. 2008; Nieto, 2011).

In summary, in all types of migration contacts between migrating cells help interpret directionally cues. Indeed, in all processes of cell migration, contacts exist between neighbouring cells. These contacts can favour cell directionality, which is crucial for cells spread out when approaching their target tissues

III. AIMS OF STUDY

The epithelial to mesenchymal transition (EMT) is a process that converts adherent and polarized epithelial cells into mesenchymal cells with migratory and invasive properties. During embryonic development, rounds of EMT and the reverse process, mesenchymal to epithelial transition (MET) are crucial for the formation of many tissues and organs (*Nieto, 2013*). The reactivation of the EMT programme in the adult promotes tumour progression and organ fibrosis, and can also confer stem cell properties (*Nieto, 2013; Mani et al., 2009; Morel et al., 2008*). Similar to the situation in embryos, a reversion of the EMT (MET) seems to be necessary for metastatic colonization once malignant cells extravasate and find their niche in distant organs.

The main inducers of the EMT (EMT-TFs) are transcription factors of the Snail, Zeb and Twist families. Different EMT-TFs are expressed during embryonic development as well as in tumour tissues. Therefore, one question that emerges is ***why the organism needs so many EMT inducers and whether there are differences in the EMT triggered by each of them***.

Main aim

To compare the EMT process mediated by each individual factor or by a combination of them, as different developing tissues and human tumours usually express several inducers.

This comparative analysis was carried out through:

- Gain of function analysis
  - In an epithelial cell line upon ectopic expression of different EMT-TFs
  - In a highly metastatic human cancer cell line, which expresses a combination of EMT-TFs.
- Loss of function analysis
  - Down-regulation of these factors in mesenchymal cancer cells.

As a new EMT-TF had been recently identified in the lab able to induce a full EMT in embryos, Prrx1, before addressing the main aim mentioned above, this work included the characterization of the EMT triggered by Prrx1 in cancer cells.

IV. MATERIALS AND METHODS

IV.1. Cell culture

IV.1.1. Culture media

The MCF7, MDA-MB-453, MDA-MB-468, MDA-MB-231, HBL100, A375P, MDA-MB-435, BT-549, Hs578T and MDA-MB-436 human tumour cell lines were cultured in DMEM: F12 HAM media (1:1) supplemented with 10% FBS (fetal bovine serum), 10 µg/ml insulin (Roche), 1% penicillin-streptomycin (Sigma) and 1% amphotericin (Sigma). MDCK cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin (Sigma). Three-dimensional cultures in Matrigel™ (BD Biosciences) were performed as described in Lee et al., 2007). Briefly, $3x10^4$ cells from a monolayer culture were trypsinized and disaggregated into single cells suspension, and then resuspended into 150 µl of Matrigel™, previously diluted with media 1:4. The mixture was seeded in a 48-well plate which had previously been covered with a thin layer of Matrigel™: Media (1:1). The cells were incubated for 6 days at 37°C and 5% CO_2. The media was replaced every two days. The names of MDA cell lines have been reduced in the text for convenience.

IV.1.2. Cell lines

Cell lines were purchased from the ATCC (Virginia, USA). Derivatives from cell lines where obtained as follows:

- BT-549 Control, BT-549 shPRRX1, BT-549 shTWIST1, BT-549 shPRRX1+TWIST1, MDA-231 Control, MDA-231 HsPRRX1, MDA-231 HsTWIST1, MDA-231 HsPRRX1+TWIST1, MDCKmock and MDCK-Prrx1 were generated in the lab by Oscar Ocaña and Herve Acloque, as described in Ocaña et al., 2012. Hs578T Control, Hs578T shPRRX1, MDA-436 Control, MDA-436 shPRRX1 were generated following the same protocol as BT-549 Control and BT-549 shPRRX1 cells.
- MDCK-Snail1 and MDCK-Snail2 were kindly provided by Amparo Cano and were generated as described in Cano et al., 2000 and Bolos et al., 2003, respectively.

- MDCK-Twist1. Stable transfectants were generated in MDCK cells and selected for 3 weeks with 400 mg/ml G418. Afterwards, 100 cells were seeded in culture 100 mm Petri dishes (Falcon) to isolate clones. Once clones had reached enough density, they were carefully trypsinized to avoid mixing with other clones and re-seeded in a 12-well plate (Falcon). The expression of *Twist1* was analysed by RT-PCR in five independent clones. The mouse *Twist1* coding sequence was amplified by RT-PCR from total mRNA obtained from a MEF (mouse embryonic fibroblast) cell line. The amplified fragment was subcloned into the pcDNA3-vector (Invitrogen) and transfected into cells with Lipofectamine 2000 (Life Technologies).

- MCDK cells expressing Prrx1 deletions constructs. Different Prrx1 deletion constructs generated in the lab by J.M. Mingot were subcloned in the pBABE-neo retroviral vector. The corresponding constructs were transfected into HEK-293T cells (Human Embryonic Kidney 293T) with Lipofectamine 2000 (Life Technologies). Two days after transfection, the viral supernatants were collected and used to infect MDCK cells. Two days after infections, MDCK cells expressing pBABE-neo with the corresponding constructs were selected with 400 mg/ml G418 for 2 weeks. Retroviral supernatants were produced using a viral packaging system that includes the psPAX2 and pM2DG plasmids (purchased from Open Biosystems). At least two independent pools were generated in each case and the cultured cells were initially tested for changes in morphology and expression. Different pools behaved similarly, and one representative pool per condition was selected for further analyses.

IV.2. Total RNA Extraction, cDNAs Isolation and RT-qPCR Analysis

Total mRNA was extracted from the cells using the Illustra RNAspin Mini isolation kit (GE Healthcare 25-0500-70). Reverse transcription was performed using oligo (dT), and random hexamer primers with the Maxima First Strand cDNAs synthesis kit (Fermentas). RT-qPCR analysis was performed using Fast SYBR Green Mastermix (Applied Biosystems) in a Step One Plus PCR machine

(Applied Biosystems). The primer sequences (listed below) were designed with the Primer3 programme (http://frodo.wi.mit.edu/primer3/).

List of oligonucleotides used

(*Cf*, dog; *Hs*, human; *Mm*, mouse)

Primer name	Forward	Reverse
QPCR *Cf*RS17	CAAGATCGCAGGCTATGTGA	CCTCGATGATCTCCTGATCC
QPCR *Cf*Snail1	TGGAAAGGCCTTCTCTAGGC	CAAAGGCCACCAAGAGAGC
QPCR *Cf*Snail2	CGTTTTCCAGACCCTGGTTA	TGACCTGTCTGCAAATGCTC
QPCR *Cf*Twist1	GCCGGAGACCTAGATGTCATT	CACGCCCTGTTTCTTTGAAT
QPCR *Cf*Prrx1	TCCTACTCAGGGGACGTGAC	GGCACATGTGGCAGAATAAG
QPCR *Cf*Zeb1	GAAGGTGATCCAGCAAATG	CTTCCGCATTTTCTTTTTGG
QPCR *Cf*Zeb2	GCCATTATTTACCCCGAAGC	AGTAGGGGCAGGTCAGCAGT
QPCR *Hs*36B4	GTGATGTGCAGCTGATCAAGACT	GAAGACCAGCCCAAAGGAGA
QPCR *Hs*Snail1	GGTTCTTCTGCGCTACTGCT	TAGGGCTGCTGGAAGGTAAA
QPCR *Hs*Snail2	TTCGGACCCACACATTACCT	TCTCCCCCGTGTGAGTTCTA
QPCR *Hs*Twist1	CCGGAGACCTAGATGTCATTG	CACGCCCTGTTTCTTTGAAT
QPCR *Hs*Prrx1	CTGATGCTTTTGTGCGAGAA	ACTTGGCTCTTCGGTTCTGA
QPCR *Hs*Zeb1	AGGATGACCTGCCAACAGAC	TGCCCTTCCTTTCTGTCATC
QPCR *Hs*Zeb2	GTACCTTCAGCGCAGTGACA	CAGGTGGCAGGTCATTTTCT
QPCR *Hs*CD24	GAGACTCAGGCCAAGAAACG	ACCTGTTTTTCCTTGCCACA
QPCR *Mm*Snail2	CGAACCCACACATTGCCTTG	TGATCTGTCTGCAAAAGCCCT
QPCR *Mm*Twist1	CTCGGACAAGCTGAGCAAG	CAGCTTGCCATCTTGGAGTC
QPCR *Mm*Prrx1	TTACCCGGATGCTTTTGTTC	ACTTGGCTCTTCGGTTCTGA

The levels of expression were calculated using the comparative Ct method normalized to the internal control (housekeeping) genes *RS17* and *36B4* in dog and human cells, respectively. Final results were expressed as the relative mRNA levels, calculated with the ΔΔCt method:

$$\Delta\Delta CT = (2^{-\Delta CT(test)}) / (2^{-\Delta CT(control)}), \text{ where}$$

$$\Delta CT = CT(target) - CT\ mean\ (housekeeping)$$

As relative quantification of EMT-TFs expression levels have been performed using MDCK and MCF7 cells as a reference and these epithelial cells do not express EMT-TFs, a list of Ct (Cycle threshold) values are shown in Figures 26 and 27 to help in the interpretation of these data.

IV.3. Invasion Assays

Invasion assays on collagen type-IV gels were performed as described previously (Cano et al., 2000). Briefly, 35000 cells were seeded onto the upper surface of the filters in modified Boyden chambers. After 7 hours of incubation, cells attached to the lower part of the filters were fixed in methanol, stained with 4,6-diamidinophenylindole (DAPI) and counted using a Leica DMR microscope.

IV.4. Immunofluorescence microscopy studies

Cells grown in two-dimensional cultures were seeded on coverslips for a better visualization in immunofluorescence analyses. After 72 hours, cells were washed with phosphate-buffered saline (PBS) twice and fixed in 4% paraformaldehyde for 15 minutes at room temperature. Cells were then washed 3 times with PBS and incubated with the blocking solution (1% BSA -albumin from bovine serum- in PBS) for 90 minutes. Primary antibodies were diluted in 1% BSA and incubated at 4^{o}C overnight. After several washes with PBS, the samples were incubated with fluorescent-conjugated secondary antibodies and DAPI for 2 hours at room temperature. The slides were washed extensively with PBS and mounted with mowiol. The antibodies used are listed in the next page.

To analyse three-dimensional cultures (3D-cultures), cells were fixed directly with 4% paraformaldehyde for 2 hours. The cultures were then

permeabilized and blocked in 0,3% Tritón-X-100 and 10% BSA in PBS for 3,5 hours. After several washes in PBS, the cells were incubated with the primary antibodies at 4°C overnight, washed eight times with PBS and incubated with the secondary antibodies. The cultures were subsequently washed eight times with PBS and counterstained with DAPI.

For F-actin staining, cells were fixed with 4% paraformaldehyde in PBS for 15 minutes and incubated with Rhodamine-conjugated phalloidin (50μgr/ml) for 45 minutes. Images were acquired using a Leica TCS SP2 inverted confocal microscope.

Primary Antibodies	**Source**	**Catalogue Number**	**Dilution**
Phalloidin-TRITC	Sigma	P1951	1/20
Vimentin	Santa Cruz	SC7557	1/200
Fribronectin	Dako	A0245	1/100
Alpha-smooth muscle actin (α-SMA)	Sigma	A2547	1/100
E-Cadherin (used in BT-549 cells)	Transduction Laboratories	610181	1/200
β-Catenin	Transduction Laboratories	C19220	1/500
Laminin	Sigma	L9393	1/25
E-Cadherin (used in MDCK cells)	Takara	M108	1/100
Secondary Antibodies	**Source**	**Catalogue Number**	**Dilution**
Alexa Fluor 568 goat anti-mouse	Invitrogen	A11004	1/3000
Alexa Fluor 488 goat anti-mouse	Invitrogen	A11001	1/3000
Alexa Fluor 568 goat anti-rabbit	Invitrogen	A11011	1/3000
Biotinylated anti-mouse	Vector	BA9200	1/100
Alexa Fluor 568 Streptavidin	Invitrogen	S-11226	1/3000
FICT goat anti-rat IgG (used in MDCK cells)	Jackson	112 095 167	1/100

IV.5. Soft Agar Assays and Mammosphere Cultures

Approximately $3,5x10^3$ cells for BT-549 and $5x10^3$ cells for MDA-231 were resuspended in 2ml DMEM: F12 HAM containing 0.35% agar and seeded in six-well plates previously covered with a thin layer of DMEM:F12 HAM medium (1:1) containing 0.5% agar. BT-549 and MDA-231 cells were incubated for 15 or 30 days, respectively, at 37ºC and 5% CO_2, and they were fed twice weekly. At the end of the incubation time, cell cultures were photographed and the colonies larger than 50 µm in diameter were counted and measured using the ImageJ software.

Mammosphere cultures were performed as described in Dontu et al., 2003. Single cells were plated in six-well ultralow attachment plates (Corning) at a density of $2x10^4$ cells/well. Cells were grown in DMEM:F12 HAM media (1:1) and supplemented with 5 µg/mL insulin, 20 ng/mL hEGF, 4 µg/mL heparin (Sigma) and 0.5 µg/mL hydrocortisone. Mammospheres were collected after 10 days and primary mammospheres with diameters larger than 75 µm were enzymatically dissociated into single cells with trypsin. After dissociation, the cells were sieved through a 40µm pore sieve, analysed microscopically for single-cellularity and replated. After a further 10 days, BT-549 secondary mammospheres larger than 75 µm in diameter were photographed and counted. This procedure was repeated up to six times. For MDA-231 cells, tertiary mammospheres were analysed.

IV.6. Analysis of CD24/CD44 by flow cytometry

For cell sorting analysis, cells were cultured in DMEM: F12 HAM media (1:1) supplemented with 10% FBS (fetal bovine serum), 10 µg/ml insulin (Roche), 1% penicillin-streptomycin (Sigma) and 1% amphotericin (Sigma). After 2 days in culture, 10^6 cells were trypsinized and washed with ice-cold PBS, 1% FBS and 0.1% NaN_3. Anti-CD44-PE (BD Pharmingen) and anti-CD24-A647 (Biolegend) antibodies were diluted 1/10 and 1/5 respectively, in 3% BSA and incubated in the dark at 4ºC. After 30 minutes, cells were fixed in

paraformaldehyde 1% during 15 minutes and then washed three times and resuspended in 400 µl ice-cold PBS, 1% FBS and 0.1% NaN_3. Cells were analysed on a flow cytometer (FACSAria ^{TM} III, BD).

IV.7. Analysis of cell Proliferation

To follow cell growth, $1,5x10^5$ cells were seeded in a six-well culture dish per condition. After two, three and four days from seeding, cells were trypsinized and counted in a Neubauer chamber.

Cells undergoing mitosis were identified by the presence of phospho-histone-3 (Prigent and Dimitrov, 2003). Cells were seeded on coverslips in DMEM: F12 HAM media (1:1). After 16 hours, the cells were fixed with methanol for 5 minutes. The cells were then blocked with 10% FBS and 1% BSA in PBS for 1 hour at room temperature. Phospho-histone-3 (PH3) antibody (Uptase) was diluted 1/200 and incubated for 30 minutes at 37^{o}C, and the secondary antibody (Alexa Fluor 568 goat anti-rabbit, Invitrogen) for 2 hours at room temperature. PH3 positive cells were counted using a Leica DMR microscope.

IV.8. *TUNEL* Assay

To detect DNA fragmentation, cells were analysed by the TUNEL method using the *In Situ* Cell Death Detection kit (Roche). This kit is based on the detection of single- and double-stranded DNA breaks that occur at the early stages of apoptosis. Cells were cultured in DMEM: F12 HAM media (1:1) supplemented with 10% FBS (fetal bovine serum), 10 mg/ml insulin (Roche), 1% penicillin-streptomycin (Sigma) and 1% amphotericin (Sigma). After two days, cells were fixed with paraformaldehyde at 4% and permeabilized. Subsequently, the cells were incubated with the TUNEL (TdT mediated dUTP nick end labelling) reaction mixture that contains TdT (terminal desoxinucleotidil transferase) and fluorescein-dUTP. During this incubation period, TdT catalyses the addition of fluorescein-dUTP at free 3'-OH groups in single- and double-

stranded DNA. After washing, the label incorporated in the damaged sites of the DNA was marked with an anti-fluorescein antibody conjugated with the reporter enzyme peroxidase. After washing to remove unbound enzyme conjugates, the peroxidase retained in the immune complex was visualized by a substrate reaction. Cells were developed in the dark with DAB (3,3-Diaminobenzidine, Sigma). A Leica DMR microscope was used to take the pictures. Positive control was obtained treating the cells with 200U DNAse I during 15 minutes.

IV.9. Cell cycle analysis

Cells were detached from dishes with trypsin, fixed in 70% ethanol (−20°C) for 1 minute, and treated with RNAse (1mg/ml) for 15 minutes at 37°C. After propidium iodide staining (0.05mg/ml in PBS for 15 minutes at room temperature in the dark), the cellular DNA content was evaluated in a FACS flow cytometer (FACSAria^{TM} III, BD). For computer analysis, only signals from single cells were considered (10,000 cells/assay).

IV.10. Time-Lapse Confocal Imaging and Cell Tracking analysis

For wound healing assays (movies 1 to 4 corresponding to Figure 13 and movies 9 to 13 corresponding to Figure 23), $1x10^6$ cells were seeded on glass-bottom culture Petri dishes (35mm, MatTek). A wound was made in the centre of the culture 24 hours later and cells were further incubated in a chamber at 37°C and 5% CO_2 (Life Imaging Services, Basel, Switzerland) surrounding an inverted confocal Laser Scanning Spectral Confocal Microscope TCS SP2 AOBS (Leica Microsystems, Heidelberg GmbH). For time-lapse analysis one image was captured every 10 minutes for a total of 15 or 17 hours in BT-549 and MDCK cells cultures, respectively. Movies were assembled using the ImageJ software (http://rsbweb.nih.gov/ij/). Individual cells were tracked using the ‘‘Manual Tracking’’ plug-in described by F. Cordelieres (http://rsbweb.nih.gov/ij/plugins/track/track.html) from ImageJ (movies 5 to 8 corresponding to Figure 13 and movies 14 to 18 corresponding to Figure 23). Velocity, net displacement and directionality index was quantified after tracking

22-25 cells per condition. Net displacement was measure as the distance of a straight line from the beginning point of the track to the end point, whereas the directionality index was calculated as the net displacement of a cell divided by the total path length followed by the cell. All quantifications represent the mean ± SD of 22-25 cells depending on the cell line (see Figure legends).

For the extracellular matrix degradation assays (movies 19 to 23 corresponding to Figure 24), $1x10^6$ cells were resuspended in Matrigel™: Media (1:1) and a drop of the mixture was placed onto a glass-bottom Petri dish (MatTek). After 20 minutes, the drop is covered with culture medium and two hours later the dish was placed into an incubation chamber surrounding an inverted confocal as described above. For time-lapse analysis, one image was captured every 10 minutes for a total of 17 hours. Movies were assembled using the ImageJ software (http://rsbweb.nih.gov/ij/) as described above.

For the analysis of cell behaviour upon cell-cell contact (movies 24 to 51 corresponding to Figures 25, 28, 29 and 30), $5x10^3$-$1x10^4$ cells were seeded onto glass-bottom 35 mm Petri dishes (MatTek) and 24 hours later the dish was placed into the incubation chamber surrounding an inverted confocal as described above. For time-lapse analysis, one image was captured every 5 minutes for a total of 3 or 4 hours depending on the cell line (see supplementary movie legends). Control cells in Figure 25 (movies 24 and 25) were recorded for 16 hours to assess the formation of an epithelial layer. To analyse cell behaviour after collision, three outcomes were considered: adhesion, repulsion or no-response. When cells remained together after contact for more than 15 frames (75 minutes) they were considered as adhesive, while when cells repelled each other and changed direction of movement after contact in less than 15 frames, behaviour was considered as repulsion (15 was the mean of frames in contact of MDCK cells expressing either of the EMT-TFs). When one cell contacted another cell and walked past each other and their trajectory was not affected after their physical interaction, this interaction was considered as no-response. One hundred and 65 cells were studied for Figures 25 and 28

respectively. For Figures 29 and 30, 30 cells were analysed. Only cells that did not divide during the analysis were considered.

IV.11. Statistical Analysis

Results were expressed as mean ± SD (standard deviation). Differences between two groups were compared using Student's t-test (two-tailed, unequal variances) in Excel. Differences among several groups were tested after checking the normality of data sets with a Kolmogorov_Smirnov's test, using one-way analysis of variance (ANOVA) with a Turkey´s multiple comparisons post-test in Prism6 (Graph Pad).

V. RESULTS

V.1. Prrx1 confers invasive and migratory properties to cancer cells

The transcription factor Prrx1 had been implicated in several developmental processes and in the regulation of fibroblast behaviour (Cserjesi et al., 1992; McKean et al., 2003). In addition, our lab had identified Prrx1 as a novel EMT-TF as it induces a massive invasive phenotype when is ectopically expressed in zebrafish embryos (Ocaña et al., 2012). Given the relationship between EMT and Prrx1 during embryonic development, we wondered whether Prrx1, as other EMT-TFs, could also have a role in tumour progression. Therefore, we firstly analysed the expression of this factor in a panel of human cancer cell lines. We found that a high level of *PRRX1* expression was associated with the mesenchymal phenotype and with invasive properties (Figure 11A and 11B). In addition, when we compared the expression of other EMT-TFs in these cell lines, we noted that *PRRX1* expression was frequently associated with that of *TWIST1*, indicating that both factors could cooperate in the induction of EMT (Figure 11C).

To further investigate the cooperation between Prrx1 and Twist1 in conferring invasive properties, we examined the effect of their down-regulation in the invasive BT-549 breast human cancer cell line, which expresses significant levels of both factors (Figure 11) and lacks *SNAIL1* expression (Moreno-Bueno et al., 2011). We stably silenced *PRRX1* or *TWIST1* expression by short-hairpin interference mediated by lentiviral infection as described in Ocaña et al, 2012. The levels of expression of *PRRX1* and *TWIST1* were strongly reduced**,** as *PRRX1* expression decreased 95% and that of *TWIST1* decreased 75% (Figure 12A). In cells where PRRX1 was downregulated the expression of *TWIST1* also decreased 20% while in cells where TWIST1 was downregulated, the expression of *PRRX1* increased 30%. We found that the down-regulation of PRRX1 or both PRRX1 and TWIST1, strongly reduced the ability of BT-549 cells to invade collagen-type-IV gels. However, down-regulation of TWIST1 alone was not sufficient to significantly reduce collagen invasive properties (Figure 12B and 12C).

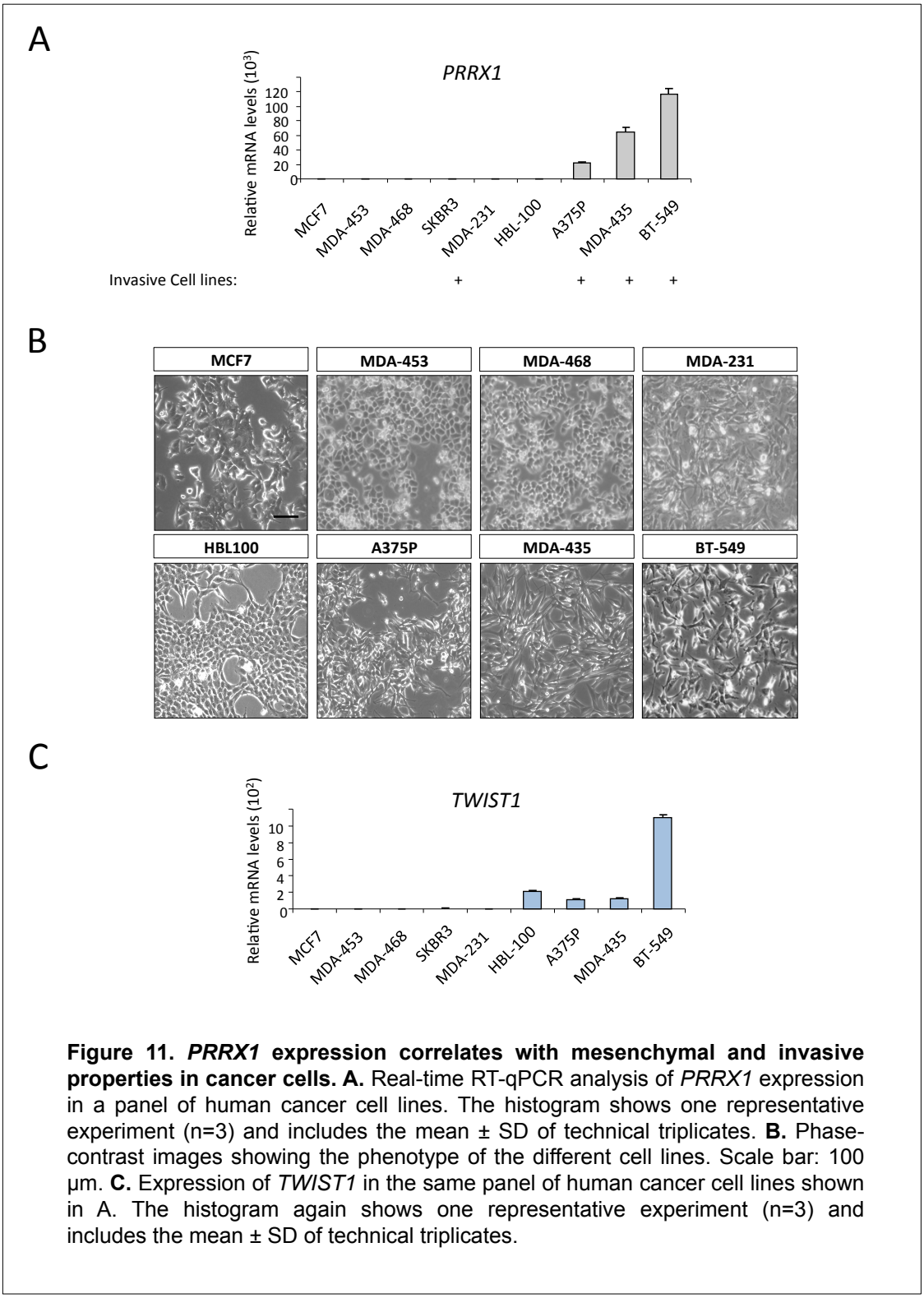


Figure 11. *PRRX1* expression correlates with mesenchymal and invasive properties in cancer cells. A. Real-time RT-qPCR analysis of *PRRX1* expression in a panel of human cancer cell lines. The histogram shows one representative experiment (n=3) and includes the mean ± SD of technical triplicates. **B.** Phase-contrast images showing the phenotype of the different cell lines. Scale bar: 100 µm. **C.** Expression of *TWIST1* in the same panel of human cancer cell lines shown in A. The histogram again shows one representative experiment (n=3) and includes the mean ± SD of technical triplicates.

As the invasive properties of the cells were altered when PRRX1 was downregulated, we then wanted to know whether this downregulation also

affected the migratory properties, as they are also closely related during cancer progression. Therefore, we performed wound-healing assays with the different BT-549 cells and we observed that both PRRX1-deficient cells and PRRX1+TWIST1-deficient cells were unable to heal the wound, as opposed to the control cells, which healed the wound after 16 hours in culture.

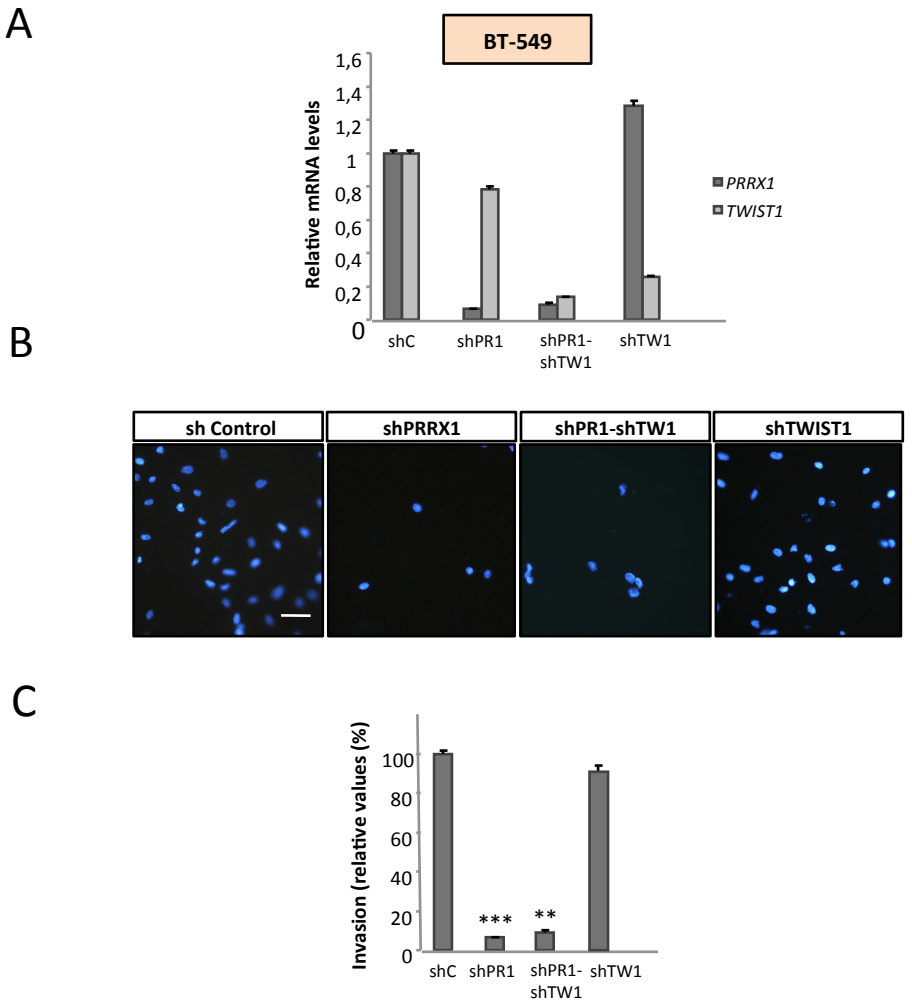


Figure 12. PRRX1 confers invasive properties to cancer cells. A. Relative expression of *PRRX1* and *TWIST1* in BT-549 control cells and in those transduced with shRNA to down-regulate either PRRX1, TWIST1 or PRRX1 plus TWIST1. One representative of three independent experiments is shown and includes the mean ± SD of technical triplicates. **B.** Invasive behaviour of the different BT-549 cells in collagen-typeIV-gels, as observed in Boyden chamber invasion assays. Nuclei of invasive cells are visualized with DAPI. Scale bar: 100 µm. **C.** Invasion represented relative to that observed in control (shC) BT-549 cells. The histogram shows one representative of three independent experiments and includes the mean ± SD (**<0,01, ***<0,001 compared to the control condition). PR, PRRX; TW, TWIST.

TWIST1-deficient cells were able to heal the wound almost completely (Figure 13A and see also Supplementary movies 1 to 4). To better analyse cell migration, we tracked the different cells over time (Figure 13B and see also Supplementary movies 5 to 8) and analysed their overall behaviour, including velocity and net displacement. The latter indicates the distance of a straight line from the starting to the end point. We found that both the velocity and the net displacement of the cells were reduced in those cells where PRRX1 o PRRX1+TWIST1 were downregulated (Figure 13C). When we downregulated TWIST1 alone, cells diminish their velocity and net displacement, but not as much as when PRRX1 was also downregulated (Figure 13C). Therefore, these data indicate that PRRX1 confers, not only invasive properties, but also migratory properties to cancer cells.

V.2. The loss of PRRX1 reverts EMT

Having shown that the loss of PRRX1 was sufficient to diminish invasive and migratory properties, we examined the impact of its down-regulation on the phenotype of cancer cells. Firstly, we found that the loss of RRRX1 was sufficient for BT-549 cells to revert EMT, undergoing a mesenchymal to epithelial transition (MET) in culture, proved by the changes in morphology and actin filament reorganization (Figure 14). When cells were grown in 3D Matrigel™ cultures, PRRX1-deficient BT-549 cells did not form mesenchymal networks like control cells, but rather they formed spheroids. PRRX1 plus TWIST1-deficient cells also formed spheroids. However, the loss of TWIST1 alone did not induce significant changes in morphology with respect to control cells (Figure 14).

In addition to the changes in morphology, using immunohistochemical analysis we could see that those spheroids had lost the expression of the mesenchymal markers Vimentin, Fibronectin and Smooth muscle α-actin (α-SMA), and expressed significant levels of the epithelial markers E-cadherin and β-catenin at the cell membrane. Moreover, spheroids secreted Laminin I,

indicative of the formation of a basement membrane-like structure (Figure 15). A similar phenotype was observed in cells in which both PRRX1 and TWIST1 were downregulated (Figure 15). However, TWIST1 down-regulation in BT-549 cells was unable to revert EMT. It is worth noting here that these cells still express high levels of *PRRX1*. In summary, it appears that the loss of PRRX1 is sufficient to revert the EMT.

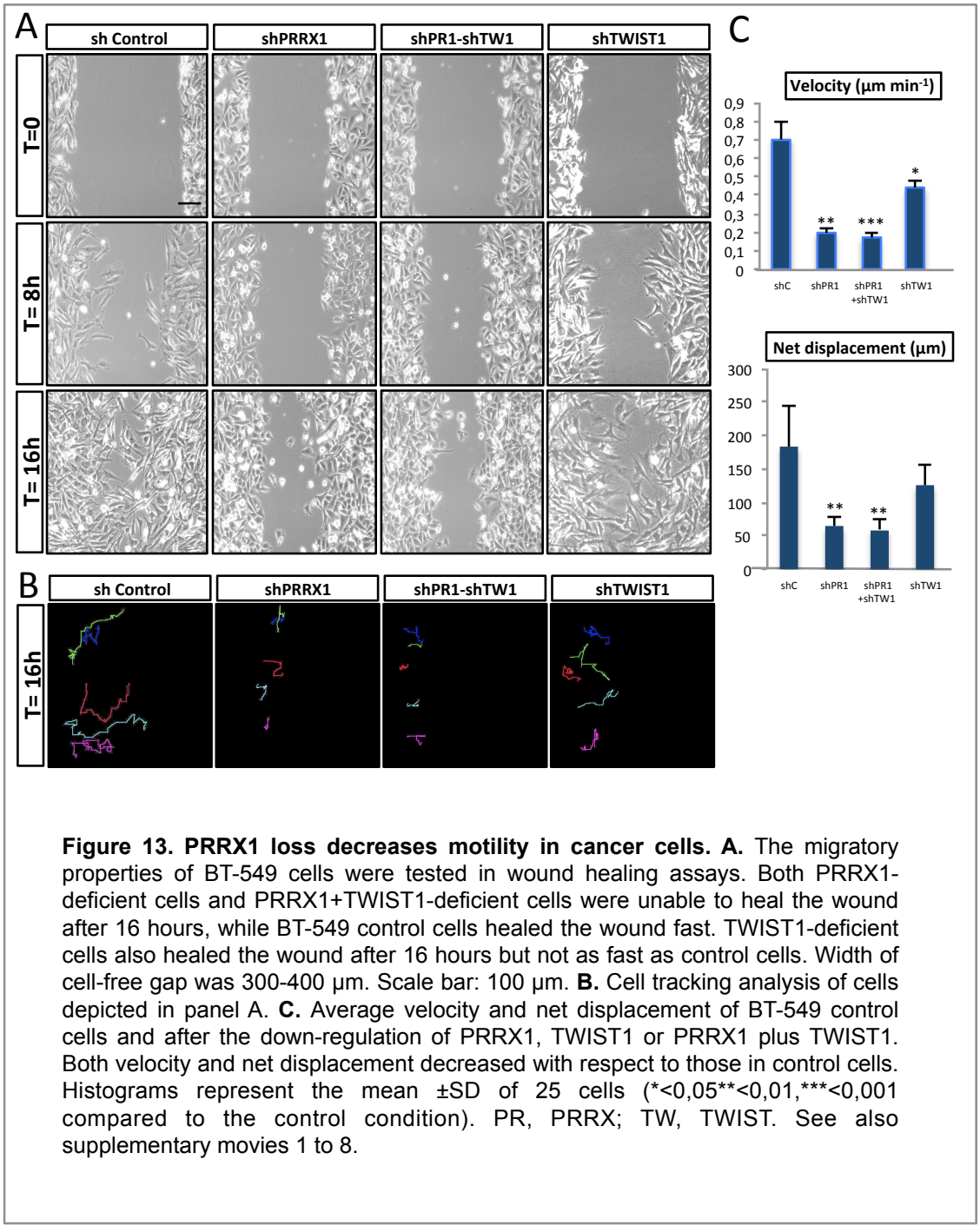


Figure 13. PRRX1 loss decreases motility in cancer cells. A. The migratory properties of BT-549 cells were tested in wound healing assays. Both PRRX1-deficient cells and PRRX1+TWIST1-deficient cells were unable to heal the wound after 16 hours, while BT-549 control cells healed the wound fast. TWIST1-deficient cells also healed the wound after 16 hours but not as fast as control cells. Width of cell-free gap was 300-400 µm. Scale bar: 100 µm. **B.** Cell tracking analysis of cells depicted in panel A. **C.** Average velocity and net displacement of BT-549 control cells and after the down-regulation of PRRX1, TWIST1 or PRRX1 plus TWIST1. Both velocity and net displacement decreased with respect to those in control cells. Histograms represent the mean ±SD of 25 cells (*<0,05**<0,01,***<0,001 compared to the control condition). PR, PRRX; TW, TWIST. See also supplementary movies 1 to 8.

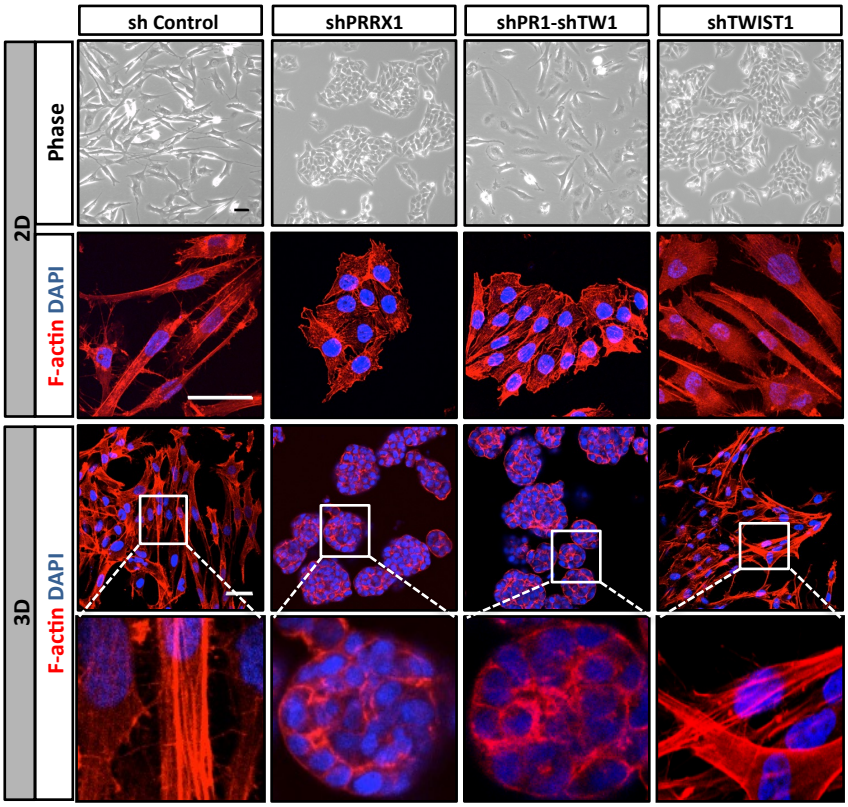


Figure 14. PRRX1 knockdown in BT-549 cells induce a Mesenchymal to epithelial transition (MET) and spheroid formation. Phase contrast and Phalloidin staining (red) showing the phenotype of BT-549 cells transfected with the indicated shRNAs grown in 2D and 3D cultures. Pictures in lower panel of 3D cultures were taken at a higher magnification. DAPI staining (blue) was used to visualize the nuclei. Scale bars 50 μm. PR, PRRX; TW, TWIST.

In addition to the effect of PRRX1 down-regulation in BT-549 cells, we tested other cell lines to make sure that the observed effects were due to a decrease in the expression levels of *PRRX1* and not to a peculiarity of this cell line. Therefore, we stably downregulated *PRRX1* expression by short-hairpin interference in two other cell lines: Hs578T and MDA-436, both also human breast cancer cell lines. Upon down-regulation, *PRRX1* expression levels decreased 80% with respect to the control-Hs578T line and 90% when compared with the control-MDA-436 cells (Figure 16A). When cells were grown in 3D Matrigel™ cultures, PRRX1-deficient Hs578T or MDA-436 cells did not form mesenchymal networks like control cells, but rather they formed spheroids,

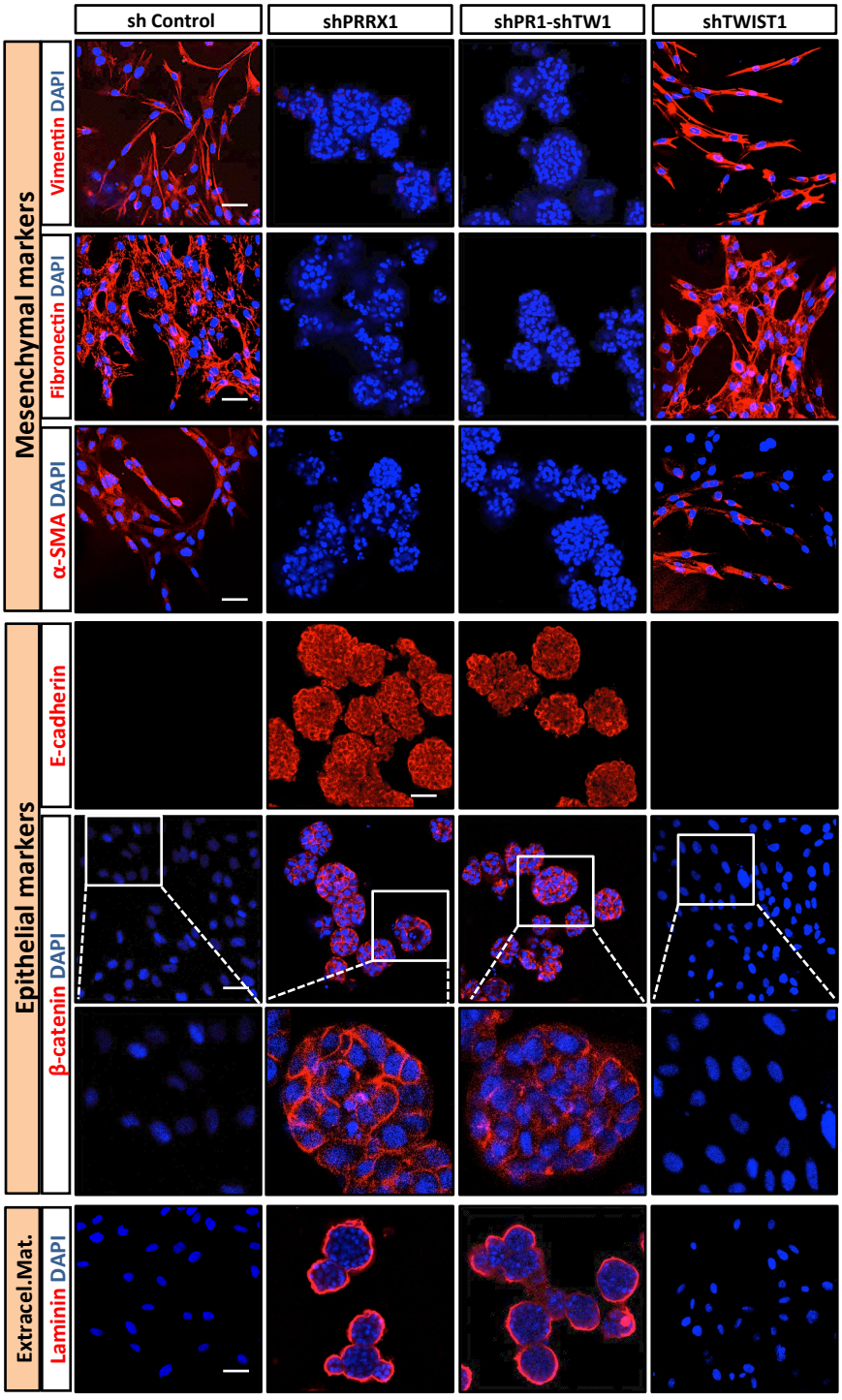


Figure 15. PRRX1 knockdown in BT-549 cells induces loss of mesenchymal markers and gain of epithelial markers, both characteristic of a Mesenchymal to epithelial transition (MET). Confocal images depicting the phenotype of the different BT-549 cells transfected with indicated shRNAs grown in 3D matrigel cultures and assessed for the presence of mesenchymal (Vimentin, Fibronectin and α-SMA), epithelial (E-Cadherin and β-catenin) and extracellular matrix (Laminin I) markers. Pictures in lower panel of β-catenin were taken at a higher magnification. DAPI staining (blue) was used to visualize the nuclei. Scale bar: 50 μm. PR, PRRX; TW, TWIST.

similarly to those observed in PRRX1-deficient BT-549 cells. By immunohistochemical analysis we could see that in those spheroids, the mesenchymal marker Vimentin did not totally disappear. However, cells re-expressed the epithelial markers E-Cadherin and β-catenin, as opposed to control cells. Moreover, these cells also secreted Laminin I, indicative of the formation of a basement membrane-like structure (Figure 16B). In summary, the down-regulation of PRRX1 in Hs578T and MDA-436 induces similar effects to those observed in BT-549 cells. Therefore, these data indicate that the loss of PRRX1 is sufficient to revert the EMT and that this is independent of the cell line.

V.3. The loss of PRRX1 induces stem cell properties

While we had shown that *PRRX1* expression was important for invasion and migration and that its loss was sufficient for the reversion to the epithelial phenotype, parallel experiments in the lab also showed that PRRX1 loss was necessary for metastatic colonization. The latter was puzzling at the beginning, because the expression of EMT-TFs had been for years associated with tumour progression towards the metastatic state. In fact, it was shown that EMT generates cells with properties of stem cells (Mani et al., 2008; Morel et al., 2008; Wellner et al., 2009). Thus, we asked whether the altered phenotype of BT-549 cells following PRRX1 loss could have an impact on the tumour-initiating properties of the non-tumourigenic BT-549 cells. Soft agar cultures and mammosphere formation assays in ultra low attachment plates were used to examine the ability of the cells to support attachment-independent growth and to self-renew, respectively, taken as indicators of tumour-initiating capacity.

We found that, while BT-549 control cells were unable to grow in soft-agar, silencing of PRRX or of both PRRX1 and TWIST1 enabled these cells to form soft agar colonies (Figures 17A and 17B), and facilitated the formation of secondary mammospheres (Figures 17C and 17D). The number of soft agar colonies and mammospheres increased when TWIST1 was additionally

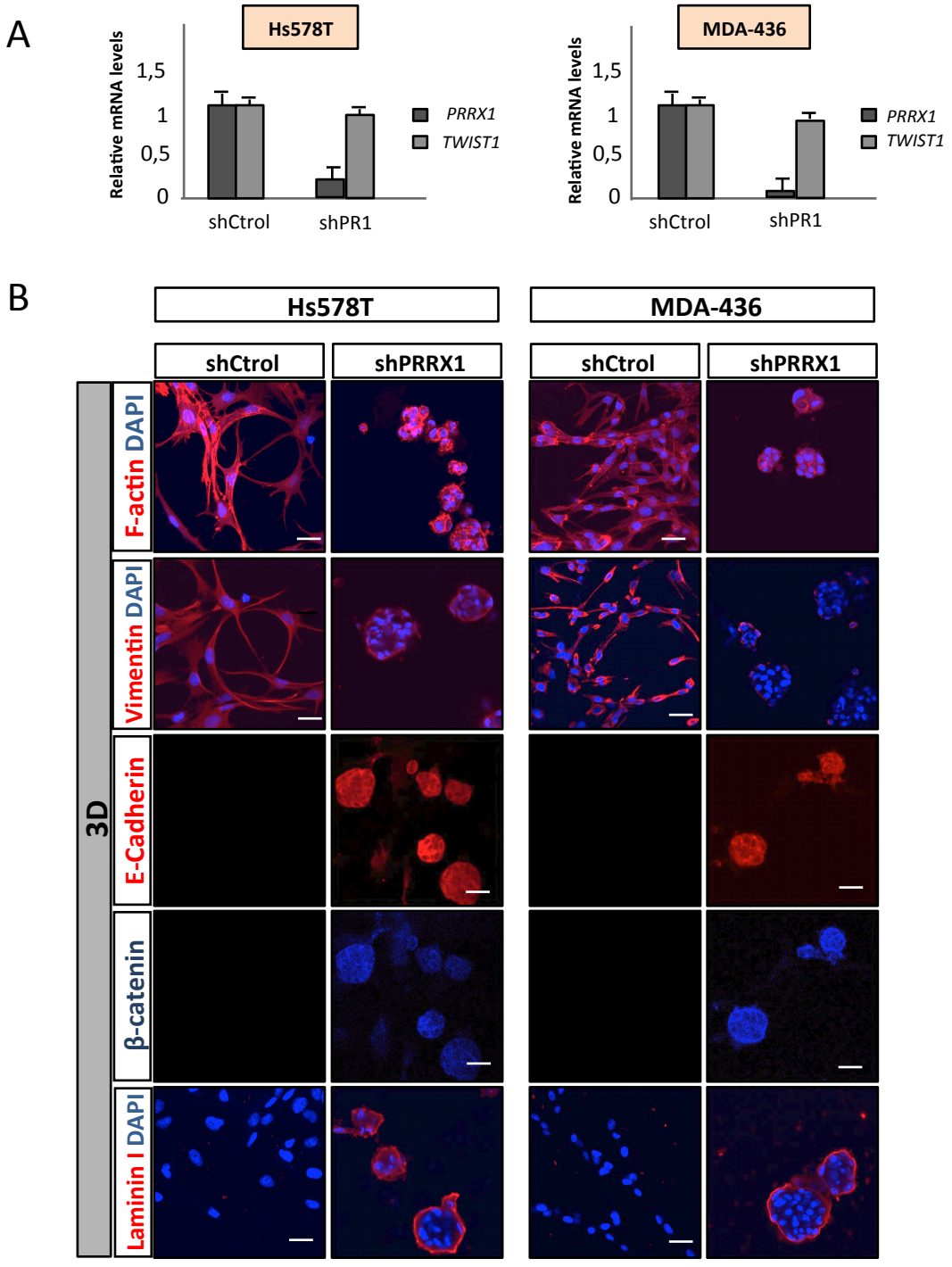


Figure 16. PRRX1 knockdown induces a Mesenchymal to epithelial transition (MET) and spheroid formation in Hs578T and MDA-436 cells. A. Relative expression of *PRRX1* and *TWIST1* in Hs578T and MDA-436 cells. One representative experiment (n=3) is shown and includes the mean ± SD of technical triplicates. **B.** Confocal images depicting the phenotype of the different cells transfected with indicated shRNAs grown in 3D cultures and assessed for the presence of mesenchymal (Vimentin), epithelial (E-Cadherin and β-catenin) and extracellular matrix (Laminin I) markers. DAPI staining (blue) was used to visualize nuclei. Scale bar: 50 µm. PR, PRRX; TW, TWIST.

silenced, compatible with cooperation between these two factors. However, TWIST1 down-regulation alone was not able to induce BT-549 cells to grow in soft agar or to form mammospheres (Figures 17A-17D). This is compatible with the previous finding that *Twist1* expression can endow cells with stem cell properties (Mani et al., 2008; Morel et al., 2008). Importantly, PRRX1 loss was sufficient to sustain mammosphere growth in up to at least six consecutive passages.

To further characterize the impact of PRRX1 loss, we examined the expression of the CD44 and CD24 cell surface markers, given that their relative expression and the $CD44^{high}/CD24^{low}$ profile in particular, has been associated with stemness in both normal mammary and breast cancer cells (Sleeman et al., 2006). Moreover, $CD44^{high}CD24^{low}$ is the described profile for breast cancer stem cells associated with the most aggressive and metastatic types (Marotta et al., 2011). Silencing PRRX1 or PRRX1 and TWIST1 converted BT-549 cells (mostly CD44/CD24 double-positive) into $CD44^{high}$ single positive cells (Figure 17E), indicative of a stem cell phenotype (Marotta et al., 2011). Therefore, PRRX1 down-regulation was sufficient to shift cells towards a population of CD44 single positive cells. In contrast, TWIST1 down-regulation decreased CD44 expression again compatible with the previous finding that *Twist1* expression can endow cells with stem cell properties (Mani et al., 2008; Morel et al., 2008). As CD24 was almost abrogated from the cell surface after PRRX1 silencing, we wondered whether PRRX1 could regulate its expression. We found that PRRX1 loss leads to an almost complete down-regulation of *CD24* transcription. In contrast, TWIST1 down-regulation increases the levels of *CD24* transcripts, pointing again to an antagonistic role between PRRX1 and TWIST1 in relation to the acquisition of stem cell properties (Figure 17F). Interestingly, all these data indicate that the EMT is not always associated with the acquisition of cancer stem cell properties, or better, with tumour initiating capacity. These results also indicate that the cell phenotype and “stemness” are two cell properties that can be independently regulated. Finally, they also point to the fact that in order for cells to make metastasis, while maintaining tumour-

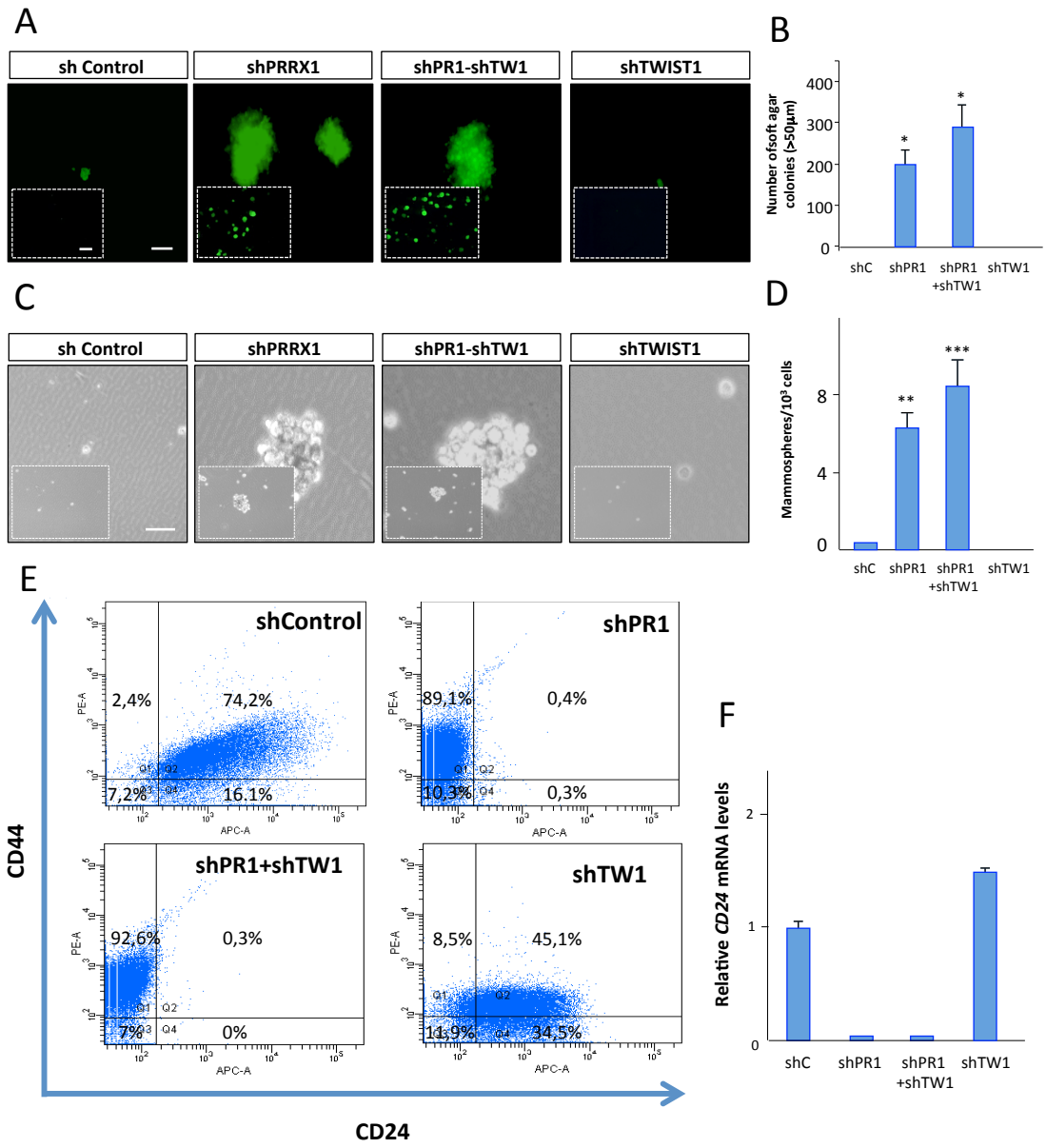


Figure 17. PRRX1 knockdown Induces the acquisition of Stem Cell Properties in Cancer Cells. A. Representative fluorescence images showing the ability of the cells to induce colony formation in soft agar 15 days after seeding. Inserts are low magnification images. Cells are expressing GFP. Scale bars: 100 µm (main pictures) and 500 µm (inset). **B.** Quantification of soft agar colonies above 50 µm in diameter. Histograms represent the mean ± SD of three independent experiments (*<0,05 compared to the control condition). **C.** Phase-contrast images showing the mammosphere-forming capacity of different BT-549 cells. Scale bars: 50 µm (main pictures) and 200 µm (inset). **D**. Quantification of secondary mammospheres as the number formed/10^3 cells seeded. Histograms represent the mean ± SD of three independent experiments (**<0,01, ***<0,001 compared to the control condition). **E.** BT-549 control cells and those in which PRRX1,TWIST1 or both had been downregulated were evaluated for their surface expression of CD44 and CD24 by FACS. **F.** *CD24* transcript levels observed in BT-549 control cells or in those where PRRX1, TWIST1 or both were downregulated. Histograms show one representative experiment (n=3) and include the mean ± SD of technical triplicates. PR, PRRX; TW, TWIST.

initiating capacities, they need to revert to the epithelial state. All this is compatible with (i) the fact that metastasis in distant organs are epithelial in nature and (ii) data in the lab showing that the reversion to the epithelial phenotype supports metastatic colonization *in vivo* (see Ocaña et al., 2012 and the discussion section).

V.4. The loss of PRRX1 enhances cell proliferation and cell division

It has been shown that EMT inducers attenuate proliferation, which may also compromise tumour growth but favour migration (Vega et al., 2004; Mejlvang et al., 2007; Evdokimova et al., 2009; Tsai et al., 2012; Beck et al., 2015). To assess whether PRRX1 has the same effect on cell growth, we performed several experiments with the different BT-549 cells. Firstly, in a growth curve, we observed that PRRX1-deficient cells showed an increase in proliferation. A similar effect was observed when both PRRX1 and TWIST1 were silenced. In the presence of PRRX1, TWIST1 down-regulation was not able to significantly alter cell growth (Figure 18A). We also quantified the cells undergoing mitosis by the expression of the mitotic marker phospho-histone 3 (PH3; Prigent and Dimitrov, 2003). We observed that the percentage of PH3 positive cells was higher in those cells transduced with sh lentiviruses to down regulate PRRX1, TWIST1 or both than in control cells, as we expected (Figure 18B). Then, we confirm that the increase in cell number was not also due to a higher rate of death upon PRRX1 downregulation, as EMT-TFs have also been associated with cell survival (Vega et al., 2004; Leroy and Mostov, 2007, Cheng et al., 2008; Browne et al., 2010). Therefore, we performed TUNEL staining, a method that detects DNA fragmentation by labelling the terminal end of nucleic acids. We found that none of the cell lines underwent cell death (Figure 18C), indicating that Prrx1 can attenuate cell proliferation and that its downregulation was not associated with an increase in cell death.

Finally, we analysed the cell cycle through a flow-cytometry-driven assay that distinguishes cells in different phases of the cycle. To carry out this

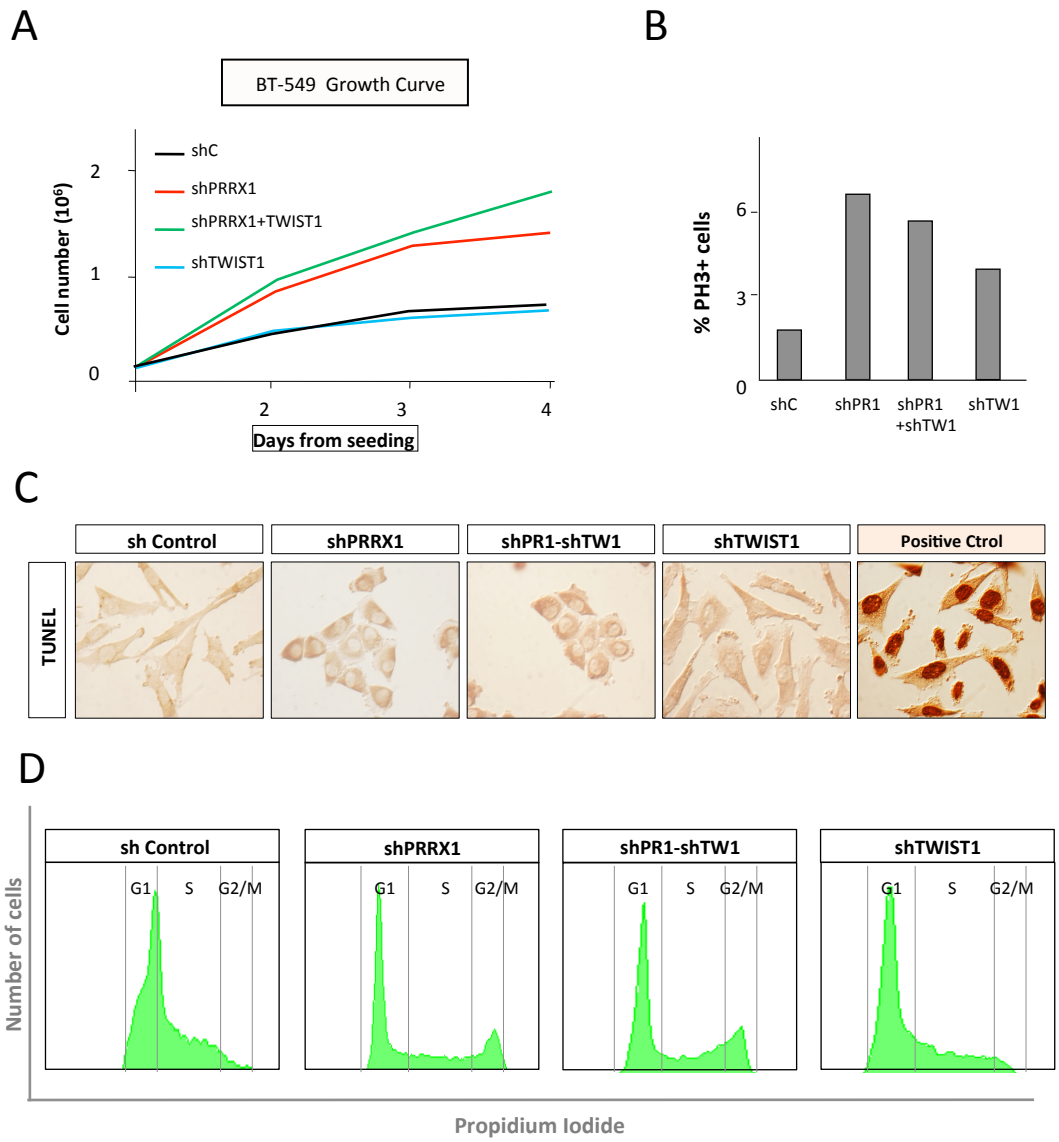


Figure 18. PRRX1 loss enhances cell proliferation. A. Growth curve of different BT-549 cells, either control or after being transduced with sh lentiviruses to downregulate PRRX1,TWIST1 or both. PRRX1-deficient cells show an increase in cell number when compared to control BT-549 cells. A similar effect is observed when both PRRX1 and TWIST1 were downregulated. In the presence of PRRX1, TWIST1 downregulation was not able to significantly alter cell growth. The graphic shows a representative experiment (n=3). **B.** Quantification of cells undergoing mitosis as assessed by PH3 staining 16 hours after seeding the same cell populations as in A. Histogram shows a representative experiment (n=3). **C.** Cell death was analysed by TUNEL staining in the same cell populations. A positive control for TUNEL is also included (see Materials and methods). **D.** FACS analysis of the cell cycle in BT-549 control cells and upon down-regulation of PRRX1, TWIST1 or PRRX1 plus TWIST1. PR, PRRX; TW, TWIST.

analysis, the cells were permeabilized and treated with the DNA intercalation agent propidium iodide. The fluorescence intensity of the stained cells can therefore be correlated with the amount of DNA that they contain. As the DNA content of cells doubles during the S phase of the cell cycle, the relative amount of cells in G_0/G_1, S, G_2 and M phases can be determined and the fluorescence of cells in the G_2/M phase will be twice as high as that of cells in the G_0/G_1 phase. Sixteen hours after cell seeding, there were many more cells in G_2/M phase when PRRX1 was downregulated than in control cells, indicating again that the loss of PRRX1 enhances cell proliferation. A similar effect was observed when both PRRX1 and TWIST1 were silenced. Again, in the presence of PRRX1, TWIST1 down-regulation was not able to significantly alter cell growth (Figure 18D). Altogether, these data suggests that PRRX1 loss enhanced cell division.

V.5. PRRX1 Ectopic Expression Impairs Stem Cell Properties in Cancer Cells

If PRRX1 loss was sufficient to induce stem cell properties in a non-tumourigenic cell line, ectopic expression of PRRX1 in tumourigenic PRRX1-negative cells may have a negative impact on their stem cell properties. To test it, we chose the tumourigenic and highly metastatic MDA-MB-231 cell line (from now on MDA-231), which expresses EMT-TFs but is devoid of PRRX1 and TWIST1 (Figure 11A). As expected, stable PRRX1 or TWIST1 ectopic expression in MDA-231 cells did not have significant effects on the morphology of these already mesenchymal cells (Figures 19A and 19B).

However, both the number of colonies formed in soft agar cultures and particularly, the number of secondary and tertiary mammospheres significantly decreased upon PRRX1 ectopic expression (Figure 20A-20D). Interestingly, all these effects were reinforced by the additional ectopic expression of TWIST1. Surprisingly, after ectopic expression of TWIST1 alone, the number of soft agar colonies and the number of secondary and tertiary mammospheres also

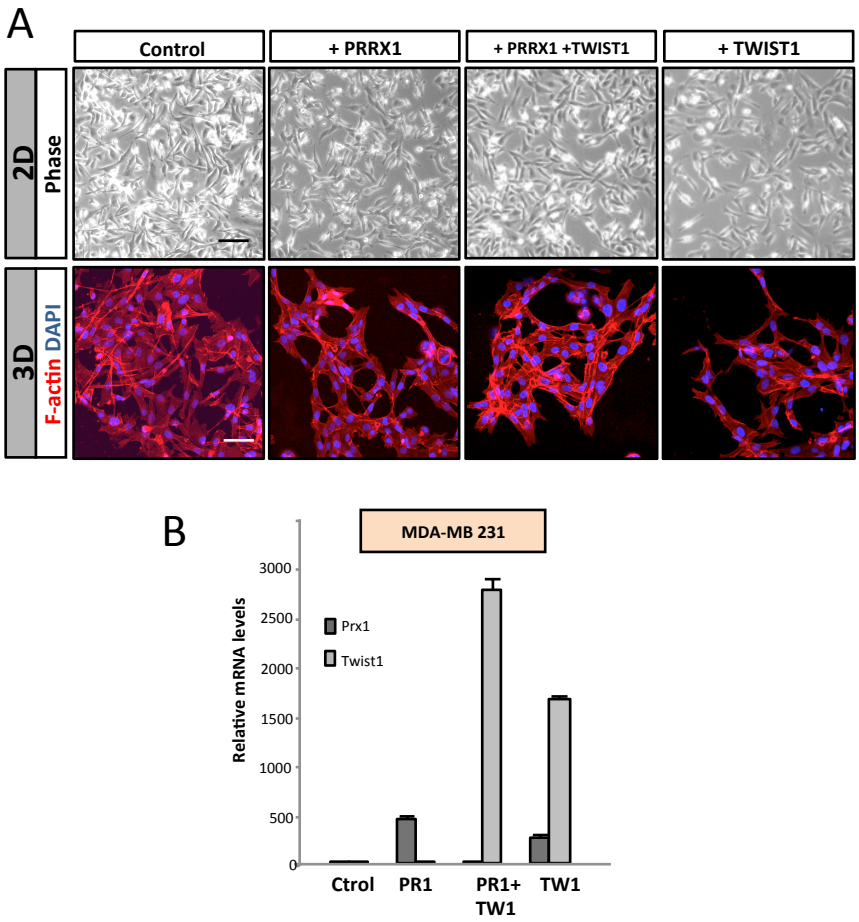


Figure 19. PRRX1 ectopic expression in MDA-231 cells does not significantly affect morphology. A. Phase contrast and phalloidin staining images showing the phenotype of MDA-231 control cells or upon overexpression of PRRX1, PRRX1 plus TWIST1 or only TWIST1. Cell morphology is not significantly affected, as MDA-231 cells are mesenchymal cells. Scale bar: 100 µm (upper panel) and 50 µm (lower panel) **B**. Relative expression of PRRX1 and TWIST1 in MDA-231 control cells and in those transduced with viruses to ectopically express PRRX1, TWIST1 or PRRX1 plus TWIST1. One representative experiment (n=3) is shown and includes the mean ± SD of technical triplicates. PR, PRRX; TW, TWIST.

significantly decreased with respect to the control cells. This was contrary to the previous finding that *Twist1* expression endows cells with stem cell properties (see discussion section). Furthermore, MDA-231 cells (mostly CD44 single positive) lose CD44 expression and shift toward $CD44^{-}/CD24^{-}$ double-negative population after ectopic expression of PRRX1, TWIST1 or both genes (Figure 20E). Hence, as hypothesized, the effect of ectopically expressing PRRX1 in

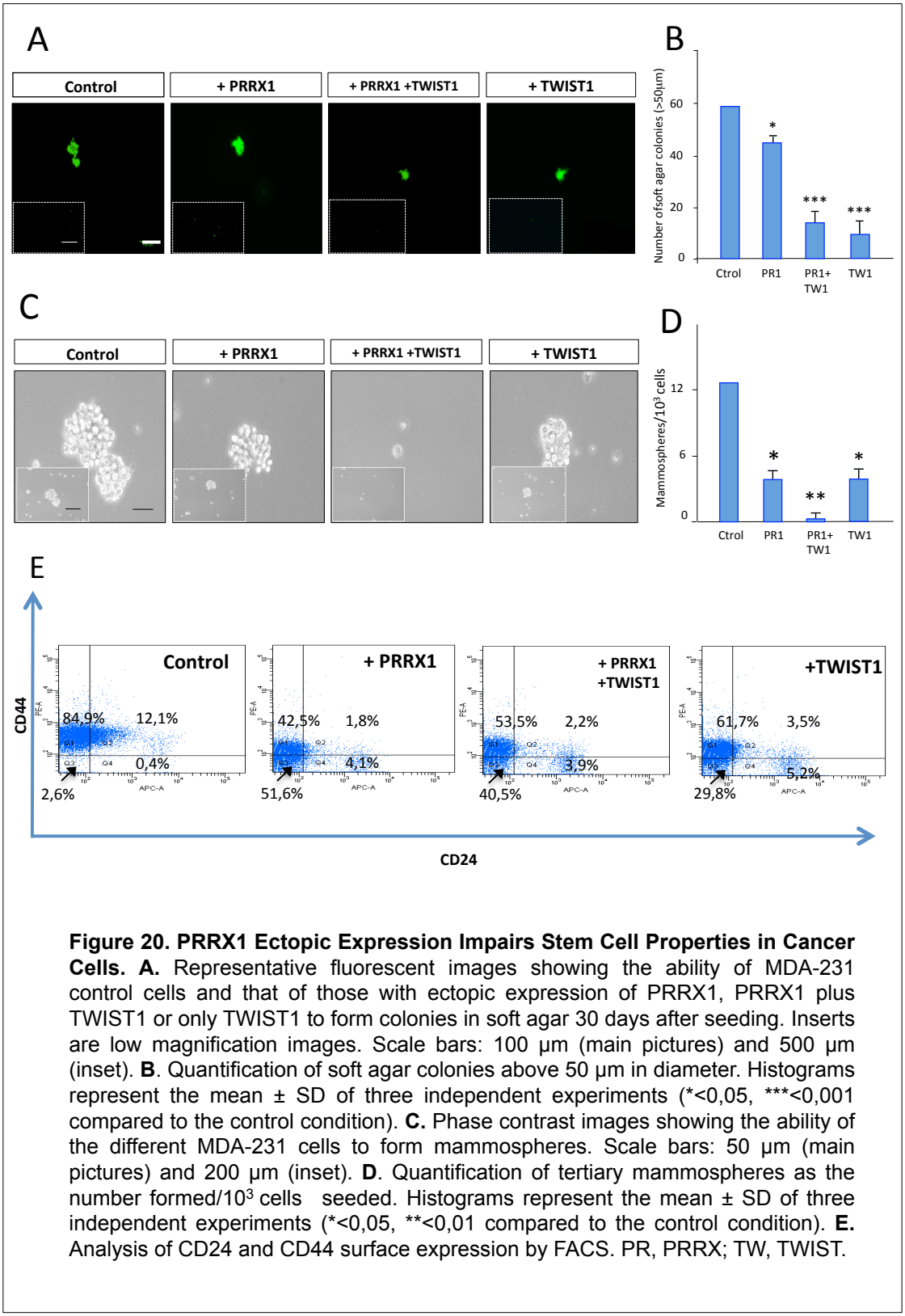


Figure 20. PRRX1 Ectopic Expression Impairs Stem Cell Properties in Cancer Cells. A. Representative fluorescent images showing the ability of MDA-231 control cells and that of those with ectopic expression of PRRX1, PRRX1 plus TWIST1 or only TWIST1 to form colonies in soft agar 30 days after seeding. Inserts are low magnification images. Scale bars: 100 µm (main pictures) and 500 µm (inset). **B**. Quantification of soft agar colonies above 50 µm in diameter. Histograms represent the mean ± SD of three independent experiments (*<0,05, ***<0,001 compared to the control condition). **C.** Phase contrast images showing the ability of the different MDA-231 cells to form mammospheres. Scale bars: 50 µm (main pictures) and 200 µm (inset). **D**. Quantification of tertiary mammospheres as the number formed/10^3 cells seeded. Histograms represent the mean ± SD of three independent experiments (*<0,05, **<0,01 compared to the control condition). **E.** Analysis of CD24 and CD44 surface expression by FACS. PR, PRRX; TW, TWIST.

MDA-231 cells was contrary to that found after PRRX1 down-regulation in BT-549 cells.

Collectively, our data indicate that the loss of Prrx1 alone is sufficient to revert EMT, inducing a MET accompanied by the acquisition of stem cell properties and increased proliferation, all important events for metastatic colonization.

V.6. Characterization of EMT processes triggered by different EMT-TFs: Analysis of cell behaviour

The results shown above in cells in which we downregulated PRRX1 expression indicate that PRRX1 is a new EMT-TF that endows cancer cells with invasive properties and that impinges into tumour-initiating capacities. In contrast to SNAIL and other EMT-TFs such as TWIST, PRRX1 attenuated tumour-initiating capacities. This important difference prompted us to analyse in detail the EMT processes triggered by different EMT-TFs.

V.6.1. Ectopic expression of Snail1, Snail2, Twist1 and Prrx1 induce a mesenchymal phenotype in MDCK cells

Previous studies have indicated that the EMT triggered by each factor has many similarities but also important differences. These analyses have characterized cells mainly at the morphological and molecular level, and pioneer and subsequent works characterized the transcriptomes of cells expressing individual EMT-TFs (Moreno-Bueno et al., 2006, Taube et al., 2010; Díaz-Martin et al., 2014; Díaz-Lopez et al., 2015). In addition to confirm the changes described in cells that underwent EMT triggered by the ectopic expression of Snail1, Snail2 and Twist1, we also characterized those that occur upon Prrx1 ectopic expression. We examined morphology and molecular markers in MDCK (*Madin-Darby canine kidney*) cells, an epithelial cell line that does not express any EMT-TFs.

As expected, while epithelial cells established close contacts with their neighbours and an apico-basal axis of polarity, cells expressing any of the

EMT-TF were loosely organized and adopted a mesenchymal morphology, including those expressing Prrx1 (Figure 21A). When cells were grown in 3D Matrigel™ cultures, they all formed cellular networks, as opposed to epithelial tubular structures formed by epithelial cells (Figure 21B). In addition to the change in morphology, using immunohistochemical analysis we observed that cells expressing EMT-TFs had lost the expression of the epithelial marker E-Cadherin (Figure 22A) and expressed high levels of the mesenchymal marker Vimentin (Figure 22B). Therefore, as previously shown, MDCK cells expressing Snail1, Snail2 and Twist, but now also those expressing Prrx1, adopted a mesenchymal morphology concomitant with loss of E-cadherin expression and gain of Vimentin.

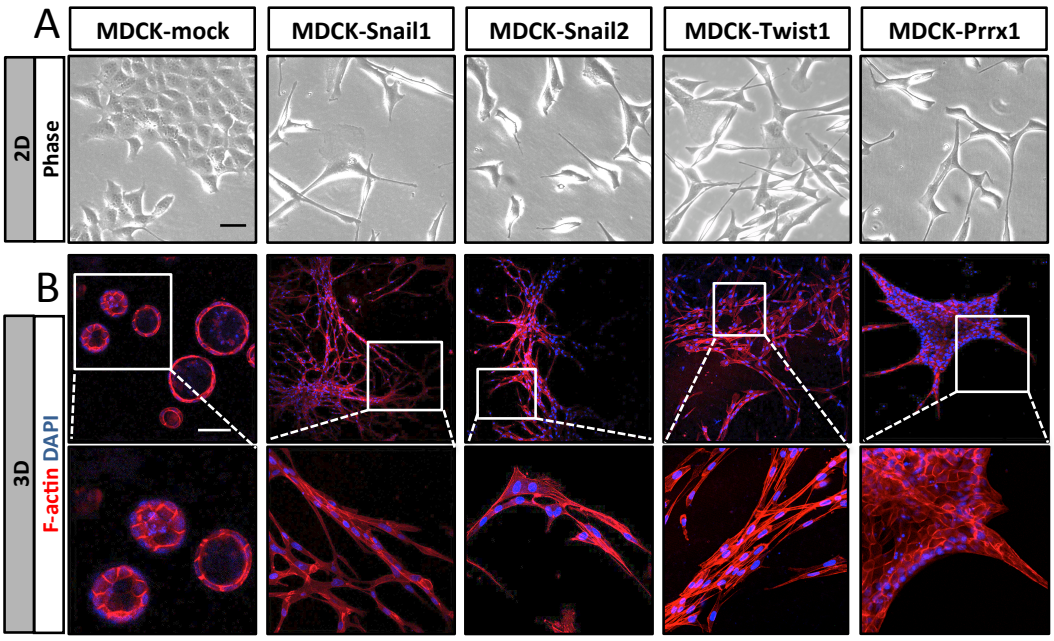


Figure 21. Ectopic expression of Snail1, Snail2, Twist1 or Prrx1 in MDCK cells induce Epithelial to Mesenchymal Transition (EMT). **A.** Phase contrast images in 2D cultures showing the phenotype of MDCK cells transfected with the indicated EMT-TFs. **B**. Phalloidin staining (red) images of MDCK cells grown in 3D cultures. DAPI staining (blue) reveals the nuclei. Cells expressing any of the EMT-TFs can form cellular networks in 3D cultures, as opposed to epithelial tubular structures formed by epithelial cells. Pictures in lower panel of 3D cultures were taken at a higher magnification. Scale bar: 50 µm.

V.6.2. Analysis of cell motility: Snail2 induces directional migration, while Snail1, Twist1 and Prrx1 promote a more random migration

After setting the system in which we could compare cells stably transfected with one of the four EMT-TFs (Snail1, Snail2, Twist1 or Prrx1), we decided to further analyse these cells and address the putative differences in cell behaviour. We first analysed their migratory properties and performed wound-healing assays. We observed that while control MDCK cells were unable to heal the wound after 16 hours in culture, MDCK cells expressing any of the EMT-TFs completely healed the wound (Figure 23A and see also Supplementary movies 9 to 13).

We next followed the different cells over time (Figure 23B and see also Supplementary movies from 14 to 18) and analysed their overall behaviour. We examined velocity, net displacement and the directionality index. Net displacement, as previously described, indicates the distance of a straight line from the starting to the end point while the directionality index was calculated as the net displacement of a cell divided by the total path length followed by the cell. We found that both velocity and displacement were increased in cells expressing any of the EMT-TFs when compared to control cells (Figure 23C). Velocity was higher in MDCK-Snail1, MDCK-Twist1 and MDCK-Prrx1 than in MDCK-Snail2. However, MDCK-Snail2 cells showed a greater displacement than the others. Therefore, although MDCK-Snail2 cells move slower than their counterparts (excluding the control cells), their net displacement is bigger, indicating that MDCK-Snail2 follow a more directional migration than the others, which seem to change the direction of migration frequently. This is compatible with the observed directionality indexes (displacement/path length), which is higher in MDCK-Snail2 (Figure 23C).

In summary, these results indicate that different EMT-TFs confer not only a mesenchymal phenotype, but also different motility properties to epithelial cells. Snail2 ectopic expression induces a more directional migration, while Snail1, Twist1 and Prrx1 ectopic expression seem to promote a more random

migration.

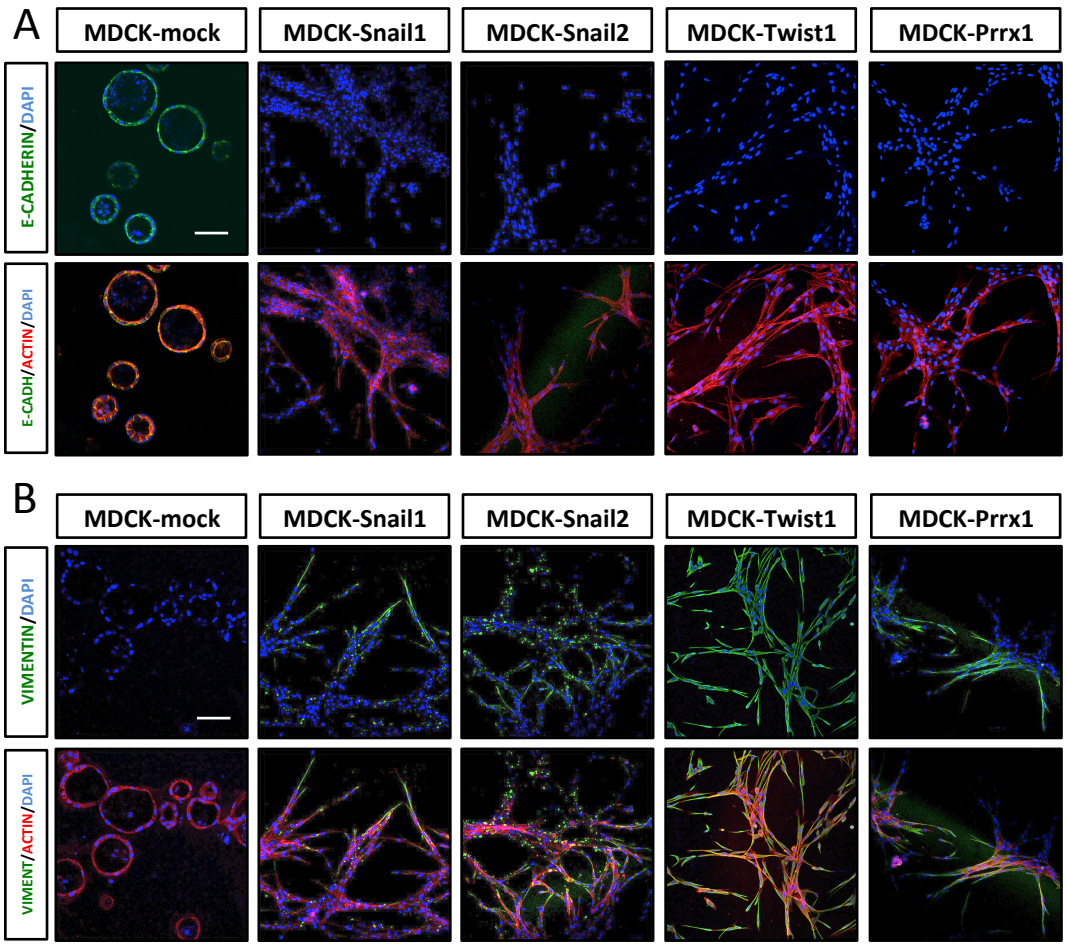


Figure 22. Ectopic expression of EMT-TFs in MDCK cells induces changes in molecular markers, characteristic of the Epithelial to Mesenchymal Transition (EMT). A. Confocal images showing the expression of the epithelial marker E-Cadherin (green) in MDCK cells transfected with indicated genes and grown in 3D matrigel cultures. After ectopic expression of any of the EMT-TF in epithelial cells, the expression of E-Cadherin was lost. Lower panel, E-cadherin immunofluorescence (green) is combined with Phalloidin staining (red) to reveal actin fibers, and with DAPI staining to visualize the nuclei (blue) **B.** Confocal images depicting the expression of the mesenchymal marker Vimentin (green). Control epithelial cells do not express Vimentin. By contrast, MDCK cells transfected with EMT-TFs express the mesenchymal marker. The lower panel combines Vimentin immunofluorescence with phalloidin and DAPI stainings. Scale bar: 50µm.

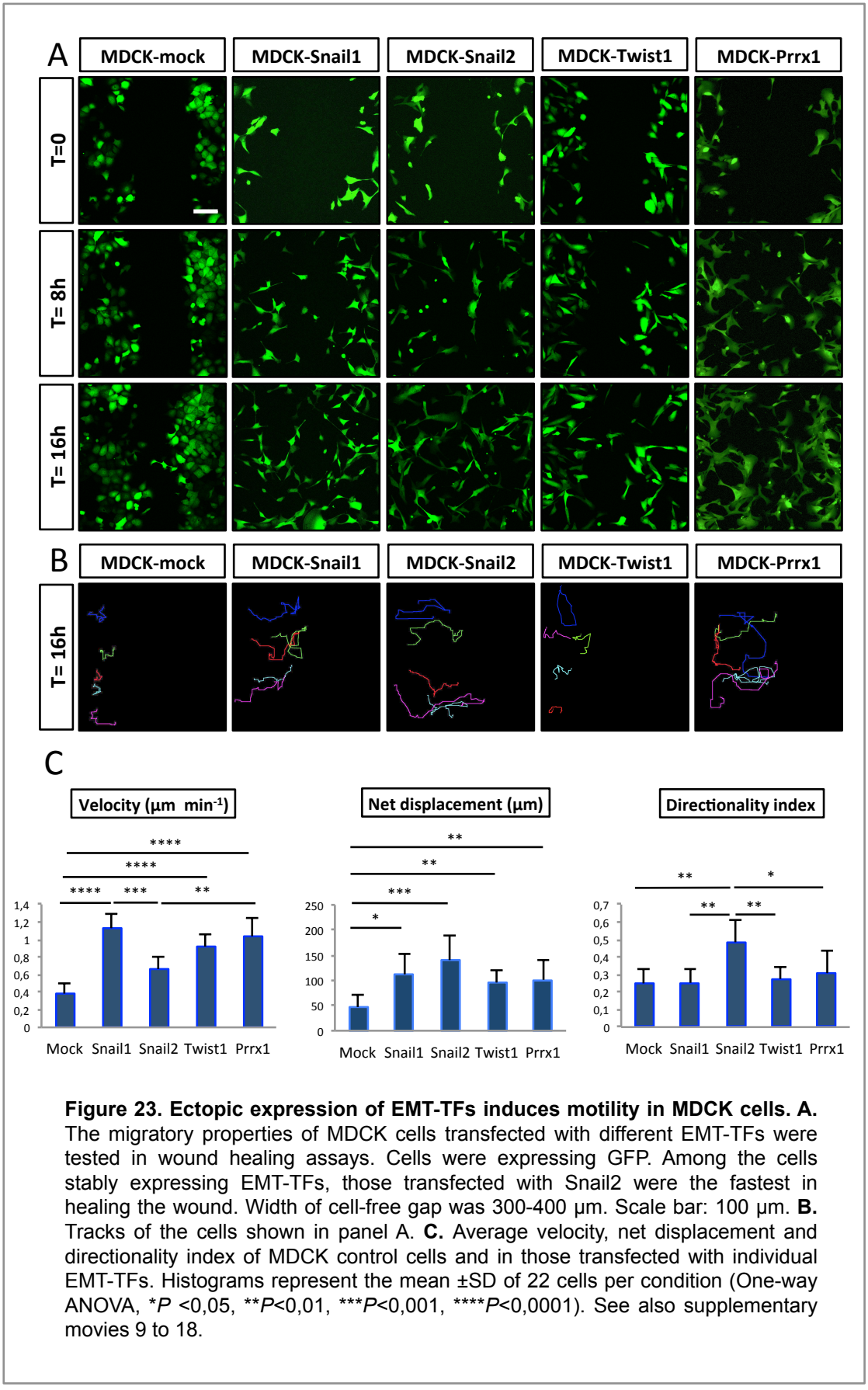


Figure 23. Ectopic expression of EMT-TFs induces motility in MDCK cells. A. The migratory properties of MDCK cells transfected with different EMT-TFs were tested in wound healing assays. Cells were expressing GFP. Among the cells stably expressing EMT-TFs, those transfected with Snail2 were the fastest in healing the wound. Width of cell-free gap was 300-400 µm. Scale bar: 100 µm. **B.** Tracks of the cells shown in panel A. **C.** Average velocity, net displacement and directionality index of MDCK control cells and in those transfected with individual EMT-TFs. Histograms represent the mean ±SD of 22 cells per condition (One-way ANOVA, **P* <0,05, ***P*<0,01, ****P*<0,001, *****P*<0,0001). See also supplementary movies 9 to 18.

V.6.3. Analysis of matrix degradation: Snail2 and Prrx1 are more efficient than other EMT-TFs

We next wanted to test the ability to degrade extracellular matrices, as this is one of the hallmarks of the EMT and the base for the invasive behaviour of both embryonic and cancer cells. To test this property, MDCK control cells and those transfected with EMT-TFs were included in a drop of Matrigel™ and the cultures followed by time-lapse imaging. After 8 hours, Snail2-, Twist- and Prrx1-expressing cells had degraded the extracellular matrix and started to migrate. After 16 hours in culture all MDCK cells transfected with individual EMT-TFs were able to degrade the matrix and start migrating as opposed to MDCK control cells, which were unable to invade the extracellular matrix and migrate (Figure 24 and Supplementary movies 19 to 23).

Collectively, our data indicate and confirm that Snail1, Snail2, Twist1 and Prrx1 induce a full epithelial to mesenchymal transition in MDCK cells concomitant with the acquisition of migratory and invasive properties.

V.6.4. Cell behaviour upon cell-cell contact: different EMT-TFs induce different responses

The extent to which EMT produces fully mesenchymal cells or cells with an intermediate phenotype *in vivo* varies with the species and the cell context. For example, as it was mentioned in the introduction, cranial neural crest cells in mouse and chick embryos migrate in a coordinated manner as individual cells, sometimes forming chains (Kulesa and Gammill, 2010) whereas in amphibian and fish embryos cells maintain contacts while migrating (Theveneau el at. 2010; Nieto, 2011). A similar degree of heterogeneity, with the generation of full or intermediate EMT phenotypes, can also be observed during the delamination of cells from the primary tumours. But, even for individual mesenchymal cells, contacts with their neighbours help them interpret directional cues (Oda et al., 1998, Peinado et al., 2004). Therefore, we next decided to study the behaviour of these cells when they contacted one another.

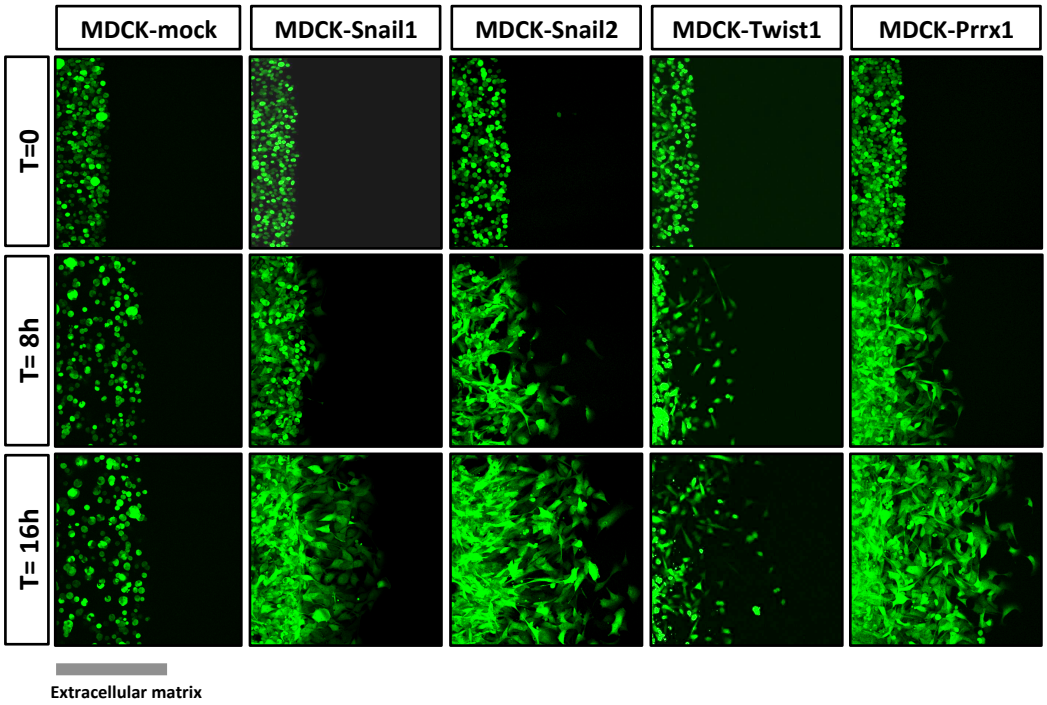


Figure 24. Snail2 and Prrx1 degrade Matrigel™ matrices more efficiently than other EMT-TFs. The invasive properties of MDCK control cells and those transfected with EMT-TFs were tested in Matrigel assays. Cells were resuspended in a drop of Matrigel and the culture was followed for 16 hours. After 8 hours in culture, cells expressing Snail2, Twist1 or Prrx1 had degraded the extracellular matrix and started to migrate on the culture dish. After 16 hours in culture, cells expressing any of the EMT-TFs were able to degrade the Matrigel™ and migrate, but Snail2- and Prrx1-expressing cells displaced more than the others. In contrast, MDCK control cells are unable to invade the extracellular matrix and migrate after 16 hours (as expected). See also supplementary movies 19 to 23.

Essentially, as a first approach, we decided to examine the behaviour of pairs of individually migrating cells after they contacted. We analysed MDCK control cells and those expressing Snail2, Twist1 and Prrx1. As Snail1-expressing cells displayed a mixed and complex behaviour due to its high position in the hierarchy of EMT-TFs and its ability to activate other EMT-TFs, we did not study MDCK-Snail1 cells in this preliminary analysis. Three different outcomes were observed after collision: (1) Adhesion, when cells stayed together upon collision (Figure 25 A1); (2) Repulsion, when upon cell-cell contact the two cells migrated away from each other (Figure 25 A2) or (3) no-response, when cell migration

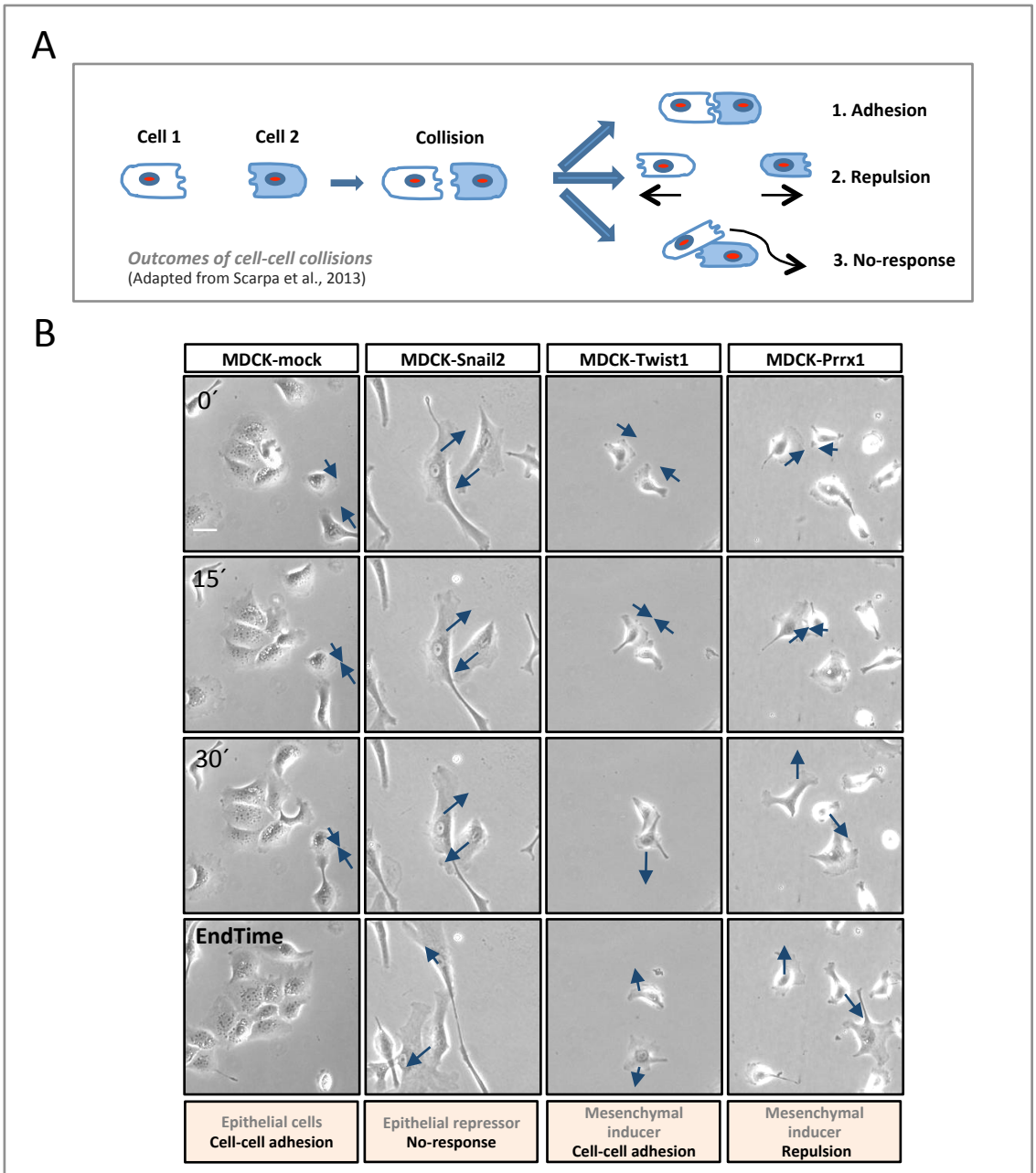


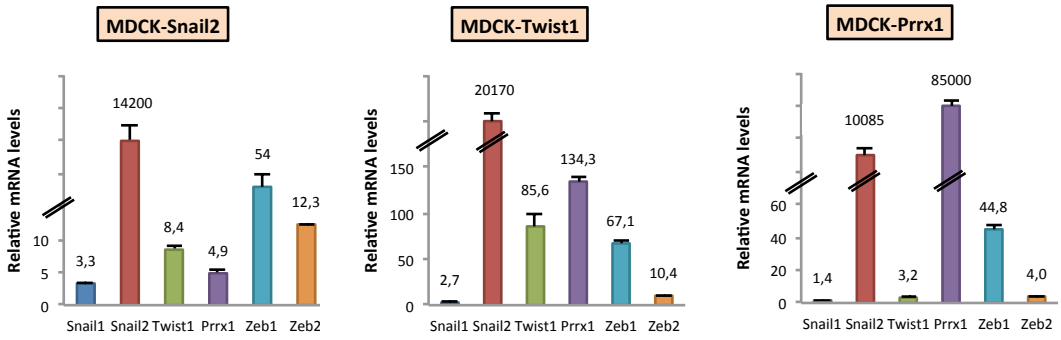
Figure 25. Prrx1 ectopic expression induces contact repulsion. A. Diagram showing the possible outcomes of cell-cell collisions in 2D cultures. **B.** Pictures showing the position of pairs of the indicated cells in culture at different times (before, during and after collision). Time is shown in minutes. The arrows show the direction of migration. Scale bar: 50 μm. 100 cells of each condition were analyzed. After contact Twist1- and Prrx1-expressing cells change the direction of migration. Nevertheless, Prrx1-expressing cells remain significant less time in contact, that may contribute to a better dispersion and distribution of these cells. MDCK-Snail2 cells were unaffected by the physical interaction and walked pass each other. See also supplementary movies 24 to 31.

were not significantly affected by their physical interaction and cells walked past each other (Figure 25 A3).

We found that MDCK control cells move when they are isolated in culture but upon contact they attach to each other and form a pavement, typical of epithelial cells (Figure 25B and Supplementary movies 24 and 25). However, Snail2-expressing cells showed individual cell migration and walked past each other when they met, migrating always in the same direction and ignoring the presence of other cells (Figure 25B and Supplementary movies 26 and 27). In contrast, Twist1-expressing cells migrated together upon contact. They showed certain degree of cell-cell adhesion but after several hours migrating together, cells migrated away from each other, indicating their mesenchymal condition, in contrast to epithelial cells (Figure 25B and Supplementary movies 28 and 29). By contrast, Prrx1-expressing cells displayed repulsion after collision as cells migrated away from each other (Figure 25B and Supplementary movies 30 and 31). This repulsion can play an important role in the dispersion of migratory cells, helping cell movements.

Even though the overall behaviour of the different MDCK derivatives was very different, when analysed on an individual basis, cells showed complex or mixed behaviours. As EMT-TFs can activate the expression of other EMT-TFs, we decided to study the expression of the different factors upon transfection of only one EMT-TF. This analysis can help in the understanding of the corresponding behaviours. Through RT-qPCR analysis we found that, as expected, stable expression of one factor can trigger the expression of other members (Figure 26). As such, ectopic expression of Snail2 strongly induced the activation of Twist1, Zeb1 and Zeb2. Ectopic expression of Twist1 upregulated the expression of all EMT-TFs except Snail1. In contrast, Prrx1 ectopic expression induced strong activation of Snail2 and Zeb1 (Figure 26). Interestingly, none of these cells expressed high levels of Snail1 indicating that cells may be protected from highly expressing Snail1. In contrast, Snail2 is expressed at very high levels and seemed to be induced by all the other EMT-TFs. Similarly, Zeb1 is also activated upon ectopic expression of Snail2, Twist1

and Prrx1, although the levels are not as high as in the case of Snail2.



Ct Values	RS17	Snail1	Snail2	Twist1	Prrx1	Zeb1	Zeb2
MDCK-mock	18,4	29,9	34,6	29,5	31,6	30,6	31,9
MDCK-Snail2	18,6	28,3	21	26,6	29	25,1	28,4
MDCK-Twist1	19,9	30	21,8	25,8	25,9	26	30
MDCK-Prrx1	18,5	29,5	21,4	27,8	15,4	25,1	30

Figure 26. The expression of one EMT-TFs induces the expression of other EMT-TFs. Relative expression of EMT-TFs in MDCK epithelial cells transfected with the factors indicated, compared to mock cells. Each cell line expresses a combination of different EMT-TFs. Histograms show one representative experiment (n=3) and include the mean ± SD of technical triplicates. The table showed below indicate the Ct (cycle threshold) values. RS17 was used as an endogenous control.

Cells ectopically expressing Zeb1 and Zeb2, similarly to Twist1-expressing cells, showed adhesion after collision (data not shown) although again, upon several hours in contact, they migrated away each other. Preliminary results also showed that these cells did not express either Prrx1 or Snail1, and only upregulated the expression of Snail2 and Twist1.

All together these data indicate that EMT-TFs can differentially activate the expression of other members and that cell behaviour should reflect the combination of factors they express (see discussion section).

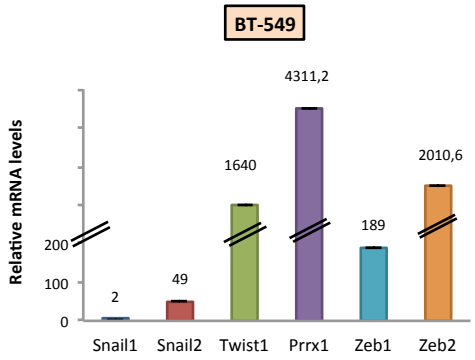
V.7. PRRX1 induces contact repulsion

Once we analysed the behaviour of epithelial cells upon transfection of a particular EMT-TF in an epithelial cell line, we decided to examine the behaviour of human cancer cells, which as embryonic cells, naturally express particular combinations of EMT-TFs. We chose cells that express high levels of PRRX1, as we were interested in characterizing this novel EMT-TF and also because data in the lab indicated that the expression or downregulation of this factor has a big impact on the ability of cells to form distant metastasis (Ocaña et al., 2012). In addition, MDCK-Prrx1 cells, in contrast to the other cells analysed, showed clear contact repulsion, which may significantly impinge into their movements and behaviour *in vivo*, both in embryonic development and tumour progression.

We selected BT-549 cancer cells for our subsequent analysis, which express significant levels of SNAIL2, ZEB1, particularly high levels of TWIST1, ZEB2 and PRRX1 (the latter similar to those observed in the MDCK-Prrx1 cells) and they lack SNAIL1 expression (Figure 27). This is compatible with the fact that Snail1 and Prrx1 do not seem to be co-expressed in embryonic cells or in cancer patients (Ocaña et al., 2012), indicating that these cells are an appropriate model to study the impact of PRRX1 in cell behaviour.

Our analysis in time-lapse experiments of BT-549 cells in 2D cultures, clearly showed contact repulsion behaviour (Figure 28A control cells), again suggesting that this behaviour is associated with *PRRX1* expression. Therefore, in order to confirm it we examined the impact of PRRX1 downregulation on the behaviour of these cells. We analysed BT-549 cells in which we had stably downregulated the expression of *PRRX1*, *TWIST1* or both by short-hairpin interference, as previously described. We found that in contrast to BT-549 control cells, in which cells mostly showed repulsion when they collided (Figures 28A and Supplementary movies 32 and 33), PRRX1-deficient BT-549 cells acquired cell-cell adhesion and attached to each other when they met. Furthermore, they did revert to an epithelial phenotype and also increased

proliferation (as shown above and can be observed in Figures 28A and Supplementary movies 34 and 35). Therefore, PRRX1 loss abolished cell dispersion and induced cell division. When TWIST1 was downregulated, contact repulsion was still present, as PRRX1 was still active, and the time in contact decreased due to the loss of TWIST1 (Figures 28A and Supplementary movies 36 and 37). Similar behaviour to that showed by PRRX1-deficient cells was observed when both genes were downregulated (Figures 28A and Supplementary movies 38 and 39).



Ct Values	36B4	Snail1	Snail2	Twist1	Prrx1	Zeb1	Zeb2
MCF7	19,1	28,7	28,5	33,8	35,6	31,5	35,6
BT549	19,6	28	23,8	23,8	24,1	24,8	25,4

Figure 27. PRRX1 and TWIST1 are highly expressed in BT-549 cells. Expression of different EMT-TFs in BT-549 cells compared to MCF7 epithelial cell line. Histogram shows one representative experiment and include the mean ± SD of technical triplicates. The table showed below indicate the Ct (cycle threshold) values. 36B4 was used as an endogenous control.

To better understand the behaviour of these cells, we wanted to examine the combination of factors that these cells expressed upon downregulation of PRRX1 or TWIST1. Through RT-qPCR analysis we found that upon downregulation of PRRX1 almost all the EMT-TFs, except SNAIL1 and ZEB2, were downregulated. Similar levels were observed upon downregulation of both, PRRX1 and TWIST1, again showing the epithelial phenotype. Upon downregulation of TWIST1, cells still presented several EMT inducers including

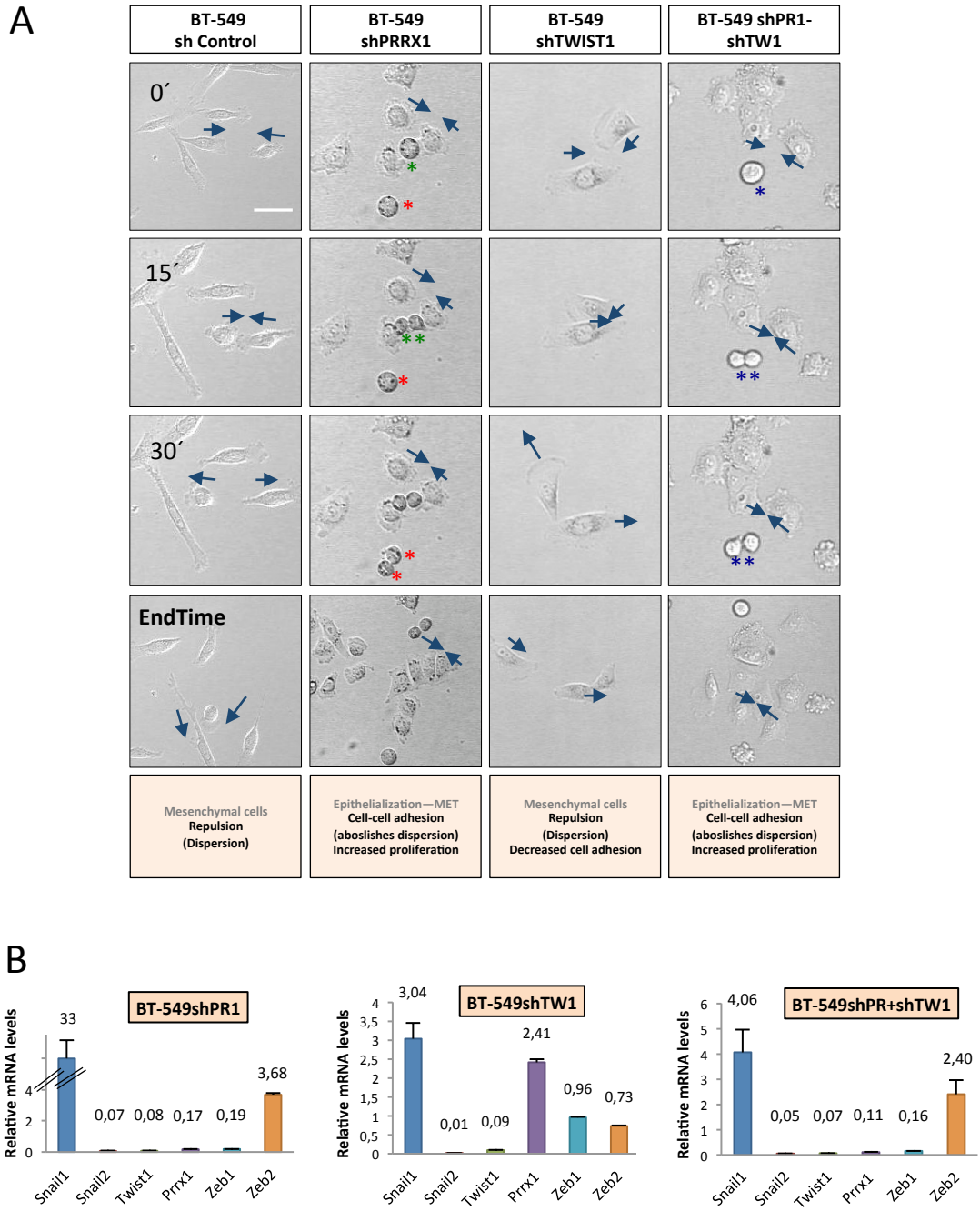


Figure 28. PRRX1 downregulation abolishes cell dispersion and promotes high proliferation. A. Pictures showing the position of pairs of BT-549 cells transfected with the indicated shRNAs in culture at different times (before, during and after collision). Time is shown in minutes. The arrows show the direction of migration. Scale bar: 50 µm. 65 cells of each condition were analyzed. Asterisks indicate proliferating cells. **B.** Relative expression of different EMT-TFs in different BT-549 cells shown in A compared to BT-549sh Control cells. One representative experiment (n=3) is shown and includes the mean ± SD of technical triplicates. PR, PRRX; TW, TWIST. See also supplementary movies 32 to 39.

PRRX1, which was reflected in their mesenchymal phenotype and the contact repulsion behaviour upon cell-cell contact (Figure 28B).

In conclusion, these data indicate that the presence of PRRX1 is required for cells to show contact repulsion. In addition, PRRX1 loss induces MET, abolishes cell dispersion and promotes high proliferation, all favouring metastatic colonization (see discussion section).

V.8. The OAR and AD3 domains in Prrx1 are required for the induction of EMT and contact repulsion

Alternative splicing of Prrx1 results in two isoforms, Prrx1 Long and Prrx1 Short isoforms. Very little is known about the expression patterns of these two isoforms. Although both appear to be coexpressed in all samples of mouse and human tissues examined, there are dramatic tissue-specific differences in the ratio of these two mRNAs in different species and human tissues (Kern et al., 1992; Norris et al., 2000; Ocaña et al., unpublished).

The Prrx1 Long isoform (L) contains 245 amino acids, and the short isoform (S), 217 amino acids. Both proteins are identical from amino acids 1 to 199, but their carboxy termini are completely different (Figure 29A; Norris et al., 2001). The carboxy tail of the long isoform (amino acids 200 to 245) contains an evolutionary conserved domain (OAR) that it is not encoded in the carboxyl terminus of the short isoform. This 14-amino acid motif has been identified within the C-terminal region of several members of the paired-homeobox transcription factors. Although it has been proposed that this domain could be important for transactivation and for protein-protein interactions or DNA binding, its function is not yet known. In vertebrates, the carboxy terminus of the long isoform also contains an activation domain (AD3: amino acids 199-221; Norris et al., 2001) that is not present in the short isoform (Figure 29A).

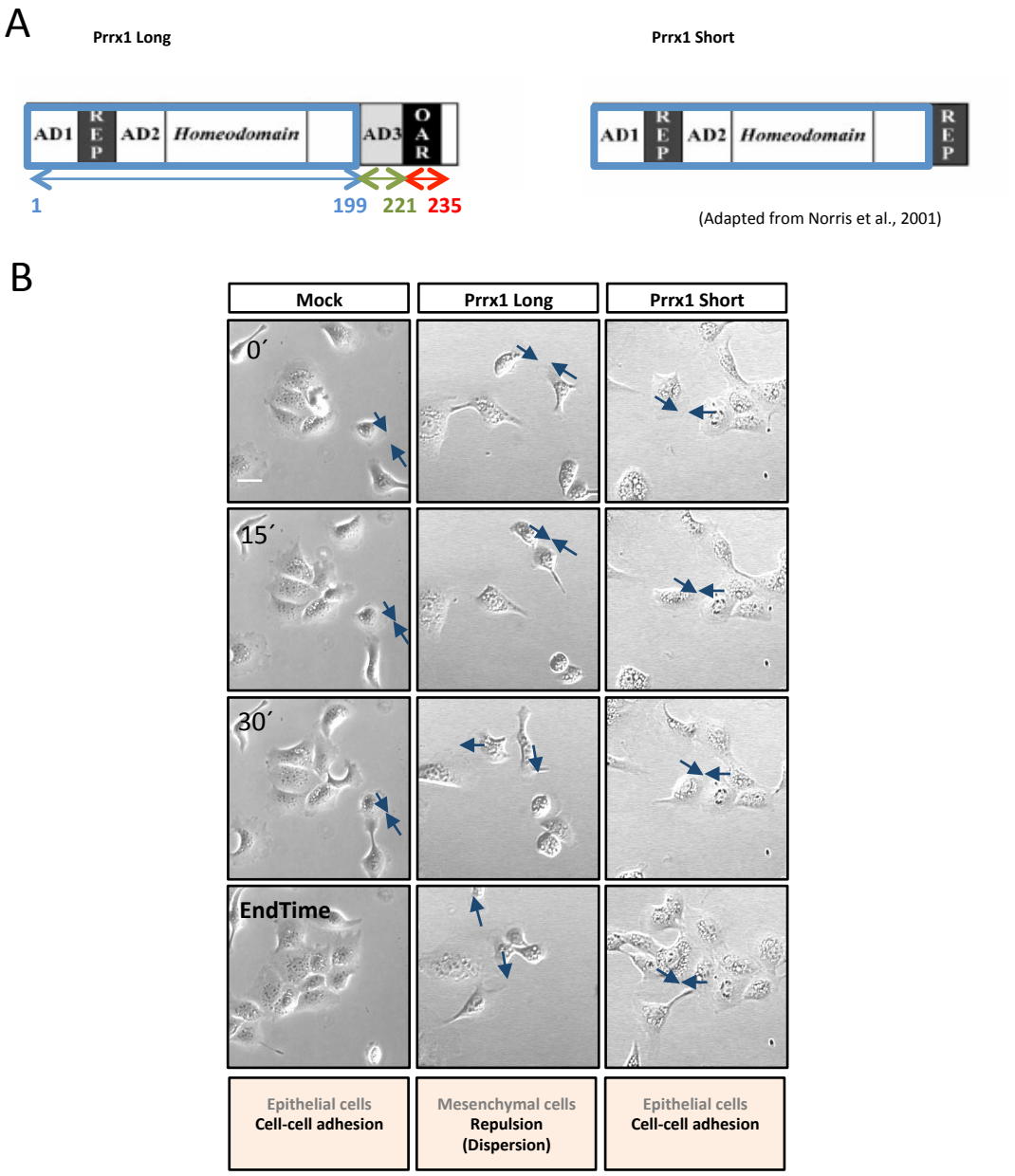


Figure 29. Ectopic expression of the PRRX1 short isoform does not induce EMT in MDCK epithelial cells. A. Domains of the Prrx1 Long and Prrx1 Short proteins. Alternative splicing of *Prrx1* transcripts results in two isoforms: Prrx1 Long, containing a carboxyl activation domain (amino acids from 199 to 220) and an OAR domain (aminoacids 221 to 235), and the Prrx1 Short isoform containing a repression domain. The blue square indicates the common region (aminoacids 1-199). AD, activation domain; REP, repressor domain. **B.** Panels showing MDCK control cells or those expressing either the Prrx1 long or short isoforms in culture at different times (before, during and after collision). Time is shown in minutes. The arrows show the direction of migration. Scale bar: 50 µm. 30 cells of each condition were analyzed. See also supplementary movies 24, 25 and 40 to 43.

Having shown that Prrx1 can induce EMT and contact repulsion, we wanted to examine the capabilities of each isoform. The behaviour previously observed in MDCK-Prrx1 cells shown above (Figures 21-26) corresponded to that induced by the long isoform, as we transfected a construct that only encoded this variant. Thus, we also needed to transfect MDCK cells with the short isoform. When we analysed the cells transfected with the short isoform in 2D cultures we observed that, in contrast to MDCK cells expressing the long isoform of Prrx1, cells failed to undergo EMT. As such, cells attached to each other as control cells (Figure 29B and Supplementary movies 40 to 43). Therefore, the short isoform does not behave as an EMT-TFs.

We next wanted to address the impact that the different domains of the Prrx1 long protein has on the behaviour of cells. We firstly examined the behaviour of MDCK cells expressing a Prrx1 version containing amino acids 1 to 199, the common part between Prrx1 S and L, and found that cells behave like MDCK control cells (Figure 30 and Supplementary movies 44 and 45). We then included both the AD3 and the OAR domains (1-235 amino acids) and found that cells showed signs of EMT and of contact repulsion (Figure 30 and Supplementary movies 46 and 47). We next deleted the OAR domain and found that both the mesenchymal features of cells and contact repulsion were highly attenuated (Figure 30 and Supplementary movies 48 and 49). This was also the case when we tested a construct that lacked the AD3 domain (Figure 30 and Supplementary movies 50 and 51).

All together, these data indicate that contact repulsion is linked to the EMT induced by the long isoform of Prrx1 and that both the OAR and the AD3 domains seem to play a crucial role in the implementation of this behaviour.

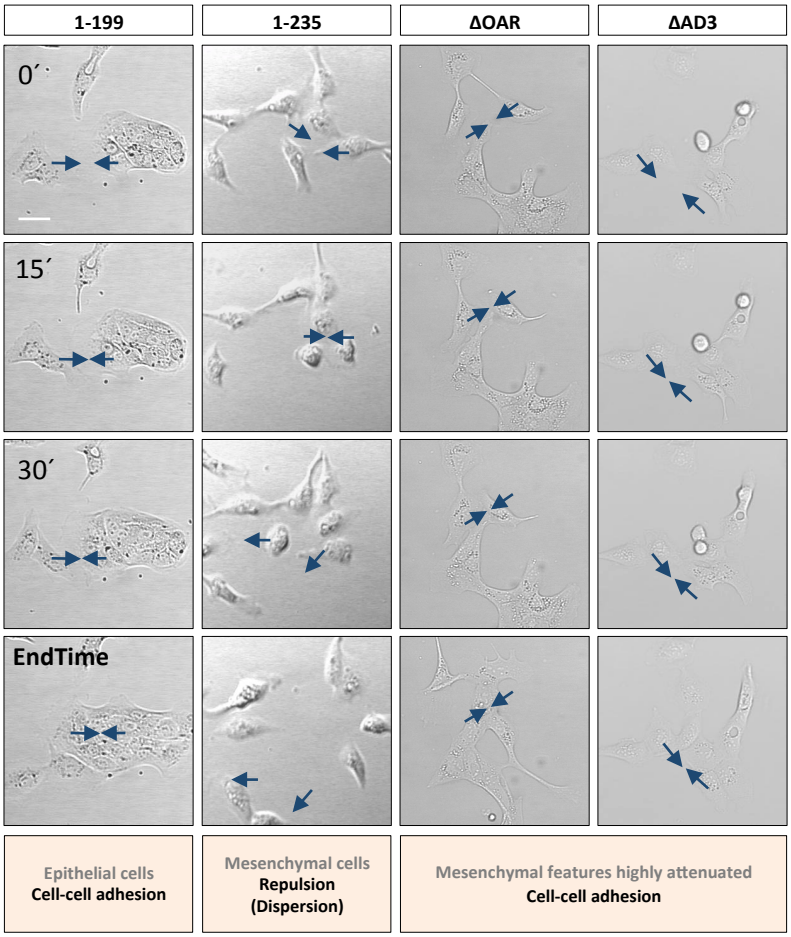


Figure 30. The OAR and AD3 domains in Prrx regulate EMT and contact repulsion. Pictures showing the position of MDCK cells expressing the indicated deletion constructs of Prrx1 in culture at different times (before, during and after collision). Time is shown in minutes. The arrows show the direction of migration. Scale bar: 50 μm. 30 cells of each condition were analyzed. See also supplementary movies 44 to 51.

VI. DISCUSSION

The EMT endows epithelial cells with migratory and invasive properties, allowing them to disperse and colonize distant territories both during embryonic development and cancer progression. As different developing tissues and human tumours usually express several EMT-TFs, one question that emerges is ***why the organism needs so many EMT inducers and whether there are differences in the EMT triggered by each of them***. Therefore, the main aim of this work was to compare the EMT process mediated by each individual factor or by a combination of them. The main inducers of the EMT are transcription factors of the Snail, Twist and Zeb families, but we had identified a novel EMT-TF, called Prrx1, that could induce invasion when ectopically expressed in zebrafish embryos. Thus, we set out to analyse the behaviour of epithelial non-transformed or cancer cells expressing different EMT-TFs, including Prrx1.

VI.1. Prrx1 is a novel EMT inducer in cancer cells

In this work we show that Prrx1 behaves as a novel EMT inducer in cancer cells. Prrx1 expression correlates with invasive and migratory properties and its loss reverts those cells to the epithelial phenotype. As such, mesenchymal cancer cells undergo MET when Prrx1 is downregulated. Importantly, this occurs even in the presence of other EMT inducers such as Twist. This indicates that there is a hierarchy among EMT-TFs and that, although Prrx and Twist are co-expressed in many tissues and in cancer cells from patients and in fact cooperate in the induction of invasive properties, Prrx1 function as an EMT-TF dominates over Twist.

Altogether, these results extend our previous data showing that Prrx1 can induce a dramatic migratory and invasive behaviour when overexpressed in chicken and zebrafish embryos (Ocaña et al., 2012). In addition, this adds a new type of transcription factor to the list of EMT-TFs, as Prrx1 belongs to a different TF group, the paired-like and homeobox family (Galliot et al., 1999; Norris et al., 2000).

As mentioned above, the organisms express many different EMT-TFs and one question that emerges is whether the EMTs induced by each factor are somehow different. With respect to Prrx1 in comparison with the other previously characterized, we find some similarities—i.e. all of them convert epithelial cells into mesenchymal cells with migratory and invasive properties. Another similarity is that Prrx1, as Snail, Twist or Zeb1, attenuates cell division (Vega et al., 2004; Mejlvang et al., 2007; Evdokimova et al., 2009; Tsai et al., 2012; Beck et al., 2015). Indeed, when Prrx1 is downregulated, the cells show an increase in proliferation. This is consistent with the EMT favouring migration versus proliferation both in embryos and in cancer cells, providing a selective advantage to reach distant destinations (Vega et al., 2004). As such, invasive, de-differentiated cancer cells are growth arrested, and proliferation is again detected in re-differentiated metastasis (Brabletz et al., 2001; Brabletz, 2012).

Importantly, in addition to the above-mentioned similarities, we have found substantial differences with respect to the type of cell migration, the response after cell-cell contacts and last, but not least important, in the induction of cancer stem cell-like properties.

VI.2. Prrx1 uncouples EMT and stemness

The induction of the EMT program has been associated with the acquisition of stem cell-like properties. As such, upon expression of Snail1 or Twist1, untransformed immortalized human mammary epithelial cells undergo EMT concomitant with the acquisition of stem cell properties as assessed by the formation of soft agar colonies and mammospheres in ultralow attachment plates (Mani et al., 2008: Morel et al., 2008). Similarly, the EMT inducer Zeb1 can also contribute to stemness maintenance (Wellner et al., 2009), indicating that the EMT generates cells with properties of stem cells.

The importance of the acquisition of cancer stem cell properties by cancer cells was already proposed in the context of EMT with the concept of

"migrating cancer stem cells" (Brabletz et al., 2005), which combines stem cell properties and the mesenchymal phenotype necessary for efficient dissemination in tumour progression. The presence of stem cell properties is associated with the ability of those cells to seed new tumours and this is why cancer stem cells are also referred to as cells with tumour initiating capacity (TIC). It is worth noting that Snail2 (Slug) and Sox9 can cooperate in the acquisition of the mesenchymal mammary stem cell state, with Snail2 essentially inducing the EMT and Sox9 facilitating entry into the stem cell state (Guo et al., 2012). Thus, the mesenchymal phenotype and stemness co-exist. However, the coexistence of these combined traits also implies phenotypic plasticity, because mesenchymal cells must revert to the epithelial phenotype to form the typical well-differentiated metastases while maintaining stemness (Chaffer et al., 2006; Dykxhoorn et al., 2009; Cèlia-Terrassa et al., 2012; Beck et al., 2015).

Considering the discussion above, in addition to the complete reversion to the epithelial phenotype (MET), we unexpectedly found that the loss of PRRX1 in mesenchymal human cancer cells was concomitant with the acquisition of the ability to (i) sustain an anchorage-independent growth, (ii) form mammospheres and (iii) convert from a mostly double positive CD44/CD24 population to $CD44^{high}$ single-positive cells, all associated with stemness in breast normal and cancer cells. Thus, our results indicate that it is Prrx1 loss rather than gain in cancer cells what it is accompanied by the acquisition of stemness related capabilities. Nevertheless, this observation is compatible with the finding that a MET process is essential for fibroblasts to acquire stem cells properties during cellular reprogramming (Li et al., 2010, Samavarchi-Tehrani et al., 2010), and is also compatible with the epithelial-like phenotype of the embryonic stem cells. These data link MET and stemness rather than EMT and stemness. Therefore, our findings showing that PRRX1 loss induces both MET and stemness, indicate that all will be favouring tumour-initiating capacities when PRRX1 is downregulated after extravasation at the secondary organ. By contrast, *PRRX1* expression can preclude progression to

the metastatic state due to both deficiency in tumour-initiating capacity and the persistence of an EMT state.

As MET is required for metastatic colonization in vivo (Ocaña et al., 2012; Tsai et al., 2012; Tran et al., 2014) and implies the downregulation of EMT-TFs in general, for EMTs induced by the classical factors, their loss would also convey the loss of stem cell properties associated with tumour-initiating capacity, thereby impairing metastatic colonization. However, it has been shown that while transient Twist1 activation primes cells for stem-cell-like traits, a continued expression impairs the stem cell properties (Schmidt et al., 2015). This is also compatible with our data showing that ectopic expression of TWIST1 in the tumour cell line MDA-231 did not concur with the aforementioned stem cell properties traits. In our experiments TWIST1 was stably expressed and thus it is likely that was indeed unable to convey cancer stem cell properties, and therefore not promoting the formation of colonies in soft agar and mammospheres, similarly to the situation after ectopic expression of PRRX1. This reinforces the concept that EMT and stemness are independently regulated as we found for Prrx and now has also been described for Twist (Beck et al., 2015). This also indicates that stable stem-cell-like traits may arise after Twist1 deactivation. Likewise, transient but not continuous *Snail1* expression in primary breast tumours increases breast cancer metastasis (Tran et al., 2014).

All these data allow us to classify the EMT processes into two groups: Snail1-type (EMT triggered by the classical EMT-TFs), which is accompanied by the acquisition of stem cells properties, and Prrx1-type, that is not linked to stem related capabilities. Two different kinds of CSCs can arise within tumours: migratory cancer stem cells and stationary cancer stem cells. Migratory CSCs are those cells expressing classical EMT-TFs and they are mobile but non proliferative. Stationary CSCs are cells that have lost Prrx1 expression; they are not mobile cells and are embedded in the epithelial tumour mass both in the primary tumour and metastasis. They are also highly proliferative (Brabletz, 2012).

In summary, our data indicate that the loss of Prrx1 alone is sufficient to induce a MET accompanied by increased proliferation and the acquisition of stem cell properties, all favouring metastatic colonization. As such, this is supported by *in vivo* experiments in mice (Ocaña et al., 2012). Therefore, unlike the classical EMT-TFs, Prrx1 uncouples EMT and stemness (Figure 31).

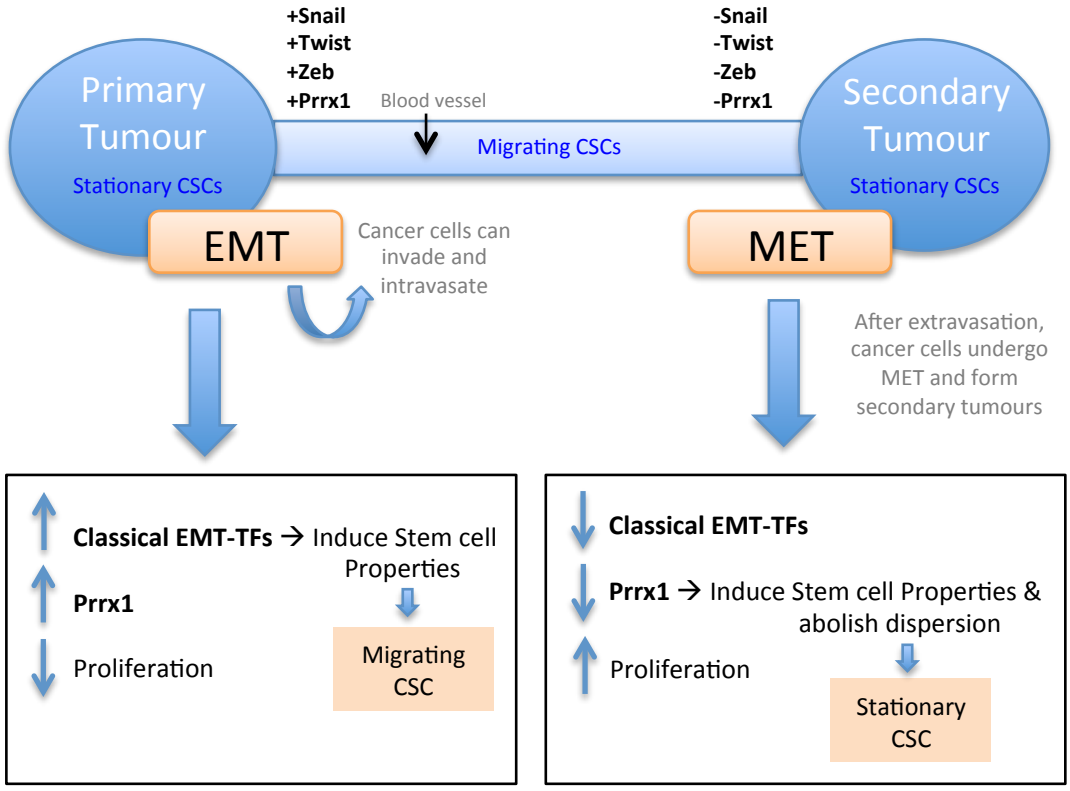


Figure 31: EMT-TFs are developmental factors that, when aberrantly activated in tumors, initiate the invasion-metastatic cascade. Thus, cancer cells expressing EMT-TFs delaminate from primary tumour, migrate through the extracellular matrix and enter the lymphatic and blood vessels. Migratory cancer cells expressing classical EMT-TFs (Snail, Twist and Zeb factors) present stem cell properties, whereas those expressing Prrx1 do not. After extravasation, tumour cells undergo MET upon downregulation of Prrx1 and other EMT-TFs. This MET is accompanied by an increase in proliferation, which favour the formation of the secondary tumour. The loss of Prrx1 in addition to revert the EMT, induces stem cell properties giving rise to stationary cancer stem cells, which favours tumour-initiating capacities and therefore allowing the metastatic colonization. Moreover, Prrx1 loss abolishes contact repulsion and therefore cells dispersion, favouring the coalescence of the cells and the formation of the macrometastasis. How Prrx1 and the other EMT-TFs are downregulated once cancer cells exit from the blood stream is still unknown.

VI.3. EMT triggered by Snail1, Snail2, Twist1 or Prrx1 induce different cell behaviours

Having shown that Prrx1 behaves differently with respect to other EMT-TFs as far as the acquisition of stem cell properties is concerned, we decided to compare the EMT processes triggered by different EMT-TFs in detail. After expressing individual EMT-TFs in MDCK cells, a prototypical epithelial model for EMT, we confirmed previous studies showing that ectopic expression of Snail1, Snail2, Twist1 and Prrx1 induce a full EMT in MDCK cells, characterized by the loss of E-Cadherin and the increased expression of the mesenchymal marker vimentin (Moreno-Bueno et al., 2006; Díaz-López et al., 2015).

We were particularly interested in analysing the motility properties, modes of migration and responses after cell-cell interactions, as the movements of migratory embryonic and cancer cells are complex and seem to be tightly regulated. One plausible explanation for their different motility behaviour could be a differential expression of the factors that endow them with the ability to migrate. When we compared the behaviour of MDCK cells transfected with each of the EMT-TFs we found that MDCK-Snail2 cells depict directional migration, while cells transfected with either Snail1, Twist1 or Prrx1 seem to promote a more random migration. In our experiments, cells were grown on plastic but in view of these differences, it would be interesting to examine how these behaviours are modified depending on the substrate. In addition, Snail2 and Prrx1 are more efficient in the degradation of extracellular matrix, indicating that they have stronger invasive properties. These data already indicate that different EMT-TFs impinge different motility properties.

As migratory cells are usually surrounded by their peers in the migratory pathway, we next analysed the cell responses after they contacted one another. For this still preliminary analysis, we examined MDCK cells transfected with either Snail2, Twist1 and Prrx1. All cells show individual cell migration but the outcomes upon contact were very different. While control cells attach to each other to form the typical pavement of epithelial cells, cell expressing Snail2 walk

past each other, and continue migrating in the same direction. In contrast, cells expressing Twist1 migrate together upon contact before finally migrating away from each other. Interestingly, when two Prrx1-expressing cells make contact, they rapidly change the direction of migration and migrate away from each other. This behaviour has been described as contact repulsion, first described in cancer cells by Abercrombie (named contact inhibition of locomotion or CIL; Abercrombie et al., 1953) and observed as well during the migration of the neural crest cells in vertebrate embryos (Carmona-Fontaine et al., 2008) and that of Cajal-Retzius cells in the developing cortex (Villar-Cerviño et al., 2013). These results immediately suggest that while cells expressing Twist would be prone to migrate together at least for a period of time, those expressing Prrx1 would rather separate from each other, favouring cell dispersion. Both types of migration are important in embryos and during tumour progression at different stages. One can speculate that Twist can impinge a mode of migration similar to that observed at the very early stages of neural crest migration in anamniotes (fish and *Xenopus*) and Prrx1 may help at later stages when crest cells need to disseminate and populate the branchial arches. Similarly, Twist may be highly expressed in cells that delaminate from tumours as groups and Prrx1 would help in the dissemination process of cancer cells. Although the expression patterns of both Twist and Prrx1 are compatible with this in embryos, a deeper analysis after the isolation of specific migratory cancer cells in animal models is necessary to better understand this process.

The significant differences in the responses of the MDCK transfected with different EMT-TFs suggests that the final behaviour observed *in vivo* is very likely the result of the “code” of EMT-TFs that they may express, as it is known that different embryonic and cancer cells express combinations of these factors (Thiery et al, 2009; Peinado et al., 2007; Tran et al., 2011). In relation to this, we have found that even the ectopic expression of one individual EMT-TF in MDCK cells induces the expression of others. As such, we observe a strong induction of *Snail2* and *Zeb1* expression after the expression of any of the three EMT-TFs tested. The upregulation of Zeb1 expression after ectopic expression of other EMT-TFs has also been found in other models (Taube et al., 2010;

Tran et al., 2011; Díaz-López et al., 2015). Specifically, ectopic expression of Snail2 induces the activation of significant levels of Twist1, Zeb1 and Zeb2. In addition to induce the expression of Snail2, ectopic expression of Twist1 also strongly induces the expression of Prrx1, compatible with the finding that both factors are usually co-expressed in embryos and cancer cells and can cooperate in inducing invasiveness (Ocaña et al., 2012). Furthermore, the activation of Prrx1 in these cells also explains the observed contact repulsion, only manifested after some time of collective migration, likely favoured by the presence of high levels of Twist1, which is a very potent mesenchymal/motility factor but a weak epithelial repressor. In contrast, Prrx1 ectopic expression does induce *Twist1* expression. Interestingly, none of the EMT-TFs are able to significantly activate *Snail1* expression, suggesting that cells may be protected from expressing high levels of Snail1. Indeed, Snail1 is a very potent EMT inducer and it is able to function at low expression levels. Moreover, during embryonic development, *Snail1* expression normally precedes the expression of the other factors such as Snail2, Twist1, Zeb1 and Zeb2, which are subsequently activated and could be responsible for the maintenance of the mesenchymal phenotype (Thiery et al., 2009; Nieto, 2013). A recent study suggests that not only Snail1 but also Zeb1 is required for EMT induction (Díaz-López et al., 2015). This study also shows that *Snail1* and *Snail2* expression are needed for the maintenance of the mesenchymal phenotype. These observations are in agreement with our results, which show a strong upregulation of Zeb1 and Snail2 upon ectopic expression of any of the EMT-TFs tested. On the other hand, most of Snail2-expressing cells walked past each other when they met indicating that in general terms, Snail2 does not endow cells with an instructive signal to modify their motility mode upon cell-cell interaction. This suggests that although Snail2 is important to maintain the mesenchymal phenotype, may be not so relevant in terms of impinging behaviour whenever other EMT-TFs are present. The relationship between Snail1 and Prrx1 is also worth noting here. Ectopic expression of Prrx1 in MDCK cells leads to the activation of the EMT-TFs except for Snail1. This is in agreement with the finding that Prrx1 and Snail1 show mutually exclusive expression patterns in embryo and do not seem to be co-expressed in cancer

patients (Ocaña et al., 2012). Thus, in contrast to the situation between Prrx1 and Twist1, Snail1 and Prrx1 do not seem act together, but they seem to be mutually excluded.

In summary, the responses upon cell-cell contact depends on the combination of EMT-TFs that cells express, with Twist1 behaving as a poor epithelial repressor, with mesenchymal cells maintaining some degree of cell-cell adhesion and Prrx1 acting as a potent mesenchymal inducer endowing cells with the ability to repel each other upon contact.

VI.4. PRRX1 mediates contact repulsion

After analysing the behaviour of epithelial cells forced to undergo EMT by the ectopic expression of a particular EMT-TF that induces the expression of a specific subset of the other factors, we next were interested in studying cell behaviour in cancer cells as they naturally express several inducers. We chose one human breast cancer cell line that expresses high levels of PRRX1 and in agreement with the correlations we have discussed above, also expresses TWIST1 and does not express SNAIL1.

As expected from our previous analysis, BT-549 cells display contact repulsion. We later confirmed that this behaviour was due to the expression of PRRX1, as upon its downregulation, cells underwent a MET, depicted high cell-cell adhesion properties and lost contact repulsion. The loss of contact repulsion upon PRRX1 downregulation can favour the coalescence of disseminated cancer cells and therefore, favour metastatic colonization. In addition, the concomitant downregulation of other EMT-TFs upon PRRX1 loss explains the reversion to the epithelial phenotype. Moreover, as mentioned above, these cells have an increased proliferation rate, which will also favour the growth of the secondary tumour. Therefore, PRRX1 loss induces MET, abolishes cell dispersion and increases proliferation, all favouring metastatic colonization.

We have previously referred to the observation that TWIST1 downregulation is unable to revert cells to the epithelial phenotype in the presence of PRRX1, suggesting that Prrx1 function dominates over that of Twist1. Compatible with this, we also find that TWIST1 downregulation does not induce MET, does not increase proliferation or abolishes contact repulsion in the presence of PRRX1. Nevertheless, *Twist1* expression impinges into cell-cell adhesion attenuating the potent Prrx1-mediated contact repulsion response. Therefore, even though both factors cooperate in inducing invasiveness, they can antagonize in terms of the cell repulsion response.

VI.5. The Prrx1 AD3 and OAR domains are required for the induction of EMT and contact repulsion

Prrx1 exists in two different isoforms, the long isoform, composed by four exons and the short isoform formed by five exons. The short isoform appears later in the evolution and contains one extra exon which is inserted between the third and fourth exons. This new exon encodes for a stop codon generating a protein shorter than the ancestral form. Therefore, both isoforms have identical N-termini whereas their C-termini are completely different (Norris et al., 2001). Previous studies have suggested that both isoforms might have opposite roles in proliferation and cell survival (Jones et al., 2001; Peterson et al., 2005). Moreover, biochemical approaches found that the long isoform act as a transcriptional activator whereas the short one is a transcriptional repressor (Norris and Kernt, 2001). Our results show that with respect to its function as an EMT-TF, only the long isoform can induce EMT and contact repulsion. It is interesting to note that both in embryos and cancer cells both isoforms are expressed, although the relative proportion varies depending on the context (Oscar Ocaña et al., unpublished). Therefore, it will be interesting to check how different levels of the short isoform can impinge into de function of the long. In any case, BT-549 cells express both isoforms and we show here that cells are mesenchymal and depict contact repulsion. More importantly, we also show that downregulating both isoforms (our silencing construct affects both) BT-549

cells revert to the epithelial phenotype and lose contact repulsion.

As contact repulsion is fundamental to promote cell dispersion and our data indicate that this function can be associated to Prrx1, we also wanted to assess whether this function is regulated by a particular protein domain and found that the AD3 and OAR domains present only in the long isoform are important both for the induction of EMT and with that, for the contact repulsion response. This explains why the short isoform, although still contains transactivation domains plus the DNA binding domains including the homeobox, is unable to induce EMT.

A summary of the plasticity undergone by cancer cells from the activation of the EMT program in primary tumours to the formation of secondary tumours at the metastatic site together with the regulation of the different EMT-TFs and the properties that they impinge into cancer cells is shown in Figure 31.

VII. CONCLUSIONS

1. Prrx1 is a novel EMT inducer in cancer cells. *The comparative analysis with other well-known EMT-TFs unveils commonalities and differences as summarized below.*

2. Prrx1 attenuates cell proliferation. *Similarly to other EMT-TFs, PRRX1 favours invasion versus proliferation.*

3. Prrx1-induced EMT does not concur with the acquisition of cancer stem cell properties. *Therefore, unlike the classical EMT transcription factors, Prrx1 uncouples EMT and stemness.*

4. Different EMT-TFs induce different behaviours upon cell-cell contact. Prrx1 induces contact repulsion, *which favours cell dispersion. The AD3 and OAR domains seem to be responsible for this behaviour.*

5. The Prrx1 short isoform cannot induce EMT or contact repulsion and lacks both the AD3 and OAR domains. *This isoform, present in all vertebrates, may attenuate the function of the long isoform.*

6. Prrx1 impinges its EMT mode in cancer cells even in the presence of other EMT-TFs.

7. PRRX1 loss is sufficient to:

- **revert cells to the epithelial phenotype**
- **abolish cell dispersion**
- **increase proliferation, and**
- **maintain stem cell properties**

All these features are required for metastatic colonization. *These results are supported by in vivo experiments in mice, confirming the role of Prrx1 in the metastatic cascade.*

VII. CONCLUSIONES

1. Prrx1 es un nuevo inductor de TEM en células tumorales. *Los análisis comparativos con otros inductores de TEM ya conocidos desvelan semejanzas y diferencias que se resumen a continuación.*

2. Prrx1 disminuye la proliferación celular. *De manera similar a otros inductores de TEM, PRRX1 favorece la invasión versus la proliferación.*

3. La TEM inducida por Prrx1 no coincide con la adquisición de propiedades de células madre. *Por tanto, a diferencia de los inductores clásicos de TEM, Prrx1 separa la TEM de las propiedades de células madre.*

4. Distintos inductores de TEM inducen comportamientos diferentes tras el contacto célula-célula. Prrx1 induce repulsión por contacto, *lo cuál favorece la dispersión celular. Los dominios AD3 y OAR parecen ser los responsables de este comportamiento.*

5. La isoforma corta de Prrx1 no puede inducir TEM ni repulsión por contacto y carece de dominios AD3 y OAR. *Esta isoforma, presente en todos los vertebrados, puede atenuar la función de la isoforma larga.*

6. Prrx1 afecta al modo de TEM en células tumorales incluso en presencia de otros inductores de TEM.

7. La pérdida de Prrx1 es suficiente para:

- **revertir las células al fenotipo epitelial**
- **abolir la dispersión celular**
- **aumentar la proliferación, y**
- **mantener las propiedades de células madre**

Todas estas características son necesarias para la colonización metastásica. *Estos resultados están apoyados por experimentos in vivo en ratones, confirmando el papel de Prrx1 en la cascada metastásica.*

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Supplementary Movies legends

Movie 1. Wound healing assay of BT-549 control cells. After 15 hours in culture, BT-549 mesenchymal cancer cells completely heal a wound of 400 µm. They are highly motile. Time-lapse movies were recorded 24 hours after cell seeding for 15 hours, taking one picture every 10 minutes.

Movie 2. Wound healing assay of BT-549 shPRRX1. Prrx1-deficient cells are unable to heal the wound after 15 hours in culture, as opposed to control cells. Time-lapse movies were recorded 24 hours after cell seeding for 15 hours, taking one picture every 10 min.

Movie 3. Wound healing assay of BT-549 shPR1+ shTW1. Prrx1- and Twist1-deficient cells do not heal the wound after 15 hours in culture. Time-lapse movies were recorded 24 hours after cell seeding for 15 hours, taking one picture every 10 minutes.

Movie 4. Wound healing assay of BT-549 shTWIST1. After 15 hours in culture, Twist1-deficient cells heal the wound of 400 µm, although the velocity is lower than BT-549 control cells. Time-lapse movies were recorded 24 hours after cell seeding for 15 hours, taking one picture every 10 minutes.

Movie 5. Tracking of Individual BT-549 control cells showed in Movie 1. Some individual cells from Movie 1 were tracked using the “Manual Tracking” plug-in by F. Cordelières (http://rsb.info.nih.gov/ij/plugins/track/track.html).

Movie 6. Tracking of Individual BT-549 shPRRX1cells. Some individual cells from Movie 2 were tracked using the “Manual Tracking” plug-in by F. Cordelières (http://rsb.info.nih.gov/ij/plugins/track/track.html).

Movie 7. Tracking of Individual BT-549 shPR1+ shTW1 cells. Some individual cells from Movie 3 were tracked using the “Manual Tracking” plug-in by F. Cordelières (http://rsb.info.nih.gov/ij/plugins/track/track.html).

Movie 8. Tracking of Individual BT-549 shTWIST1 cells. Some individual cells from Movie 4 were tracked using the "Manual Tracking" plug-in by F. Cordelières (http://rsb.info.nih.gov/ij/plugins/track/track.html).

Movie 9. Wound healing assay of MDCK-mock cells. Epithelial MDCK cells are unable to heal the wound after 17 hours in culture. Time-lapse movies were recorded 24 hours after cell seeding for 17 hours, taking one picture every 10 minutes. Cells were expressing GFP.

Movie 10. Wound healing assay of MDCK-Snail1 cells. Ectopic expression of Snail1 in MDCK cells induces EMT and migration. Cells heal completely the wound after 17 hours in culture. Time-lapse movies were recorded 24 hours after cell seeding for 17 hours, taking one picture every 10 minutes. Cells were expressing GFP.

Movie 11. Wound healing assay of MDCK-Snail2 cells. Ectopic expression of Snail2 in MDCK cells induces EMT and migration. Cells heal completely the wound after 17 hours in culture but velocity is lower than MDCK-Snail1 cells. Time-lapse movies were recorded 24 hours after cell seeding for 17 hours, taking one picture every 10 minutes. Cells were expressing GFP.

Movie 12. Wound healing assay of MDCK-Twist1. Ectopic expression of Twist1 in MDCK cells induces EMT and migration. Cells heal completely the wound after 17 hours in culture. Time-lapse movies were recorded 24 hours after cell seeding for 17 hours, taking one picture every 10 minutes. Cells were expressing GFP.

Movie 13. Wound healing assay of MDCK-Prrx1. Ectopic expression of Prrx1 in MDCK cells induces EMT and migration. Cells heal completely the wound after 17 hours in culture. Time-lapse movies were recorded 24 hours after cell seeding for 17 hours, taking one picture every 10 minutes. Cells were expressing GFP.

Movie 14. Tracking of Individual MDCK-mock. Some individual cells from Movie 9 were tracked using the "Manual Tracking" plug-in by F. Cordelières (http://rsb.info.nih.gov/ij/plugins/track/track.html).

Movie 15. Tracking of Individual MDCK-Snail1. Some individual cells from Movie 10 were tracked using the "Manual Tracking" plug-in by F. Cordelières (http://rsb.info.nih.gov/ij/plugins/track/track.html).

Movie 16. Tracking of Individual MDCK-Snail2 cells. Some individual cells from Movie 11 were tracked using the "Manual Tracking" plug-in by F. Cordelières (http://rsb.info.nih.gov/ij/plugins/track/track.html).

Movie 17. Tracking of Individual MDCK-Twist1 cells. Some individual cells from Movie 12 were tracked using the "Manual Tracking" plug-in by F. Cordelières (http://rsb.info.nih.gov/ij/plugins/track/track.html).

Movie 18. Tracking of Individual MDCK-Prrx1 cells. Some individual cells from Movie 13 were tracked using the "Manual Tracking" plug-in by F. Cordelières (http://rsb.info.nih.gov/ij/plugins/track/track.html).

Movie 19. Epithelial MDCK cells are unable to degrade the extracellular matrix and migrate. Cells were resuspended in Matrigel (BD Biosciences): Media (1:1) and a drop of the mixture was placed onto a glass-bottom culture Petri dish (MatTek). The drop was covered with culture medium. For time-lapse movies, one image was captured every 10 minutes for a total of 17 hours. Movies were assembled using the ImageJ software (http://rsbweb.nih.gov/ij/). Cells were expressing GFP.

Movie 20. Epithelial cells stably transfected with Snail1 undergo a full EMT, degrade extracellular matrix and migrate. For time-lapse movies, one image was captured every 10 minutes for a total of 17 hours. MDCK-Snail1 cells were cultured and images processed as control cells (See Movie 19 legend). Cells were expressing GFP.

Movie 21. Epithelial cells stably transfected with Snail2 undergo a full EMT, degrade extracellular matrix and migrate. For time-lapse movies, one image was captured every 10 minutes for a total of 17 hours. MDCK-Snail2 cells were cultured and images processed as control cells (See Movie 19 legend). Cells were expressing GFP.

Movie 22. Epithelial cells stably transfected with Twist1 undergo a full EMT, degrade extracellular matrix and migrate. For time-lapse movies, one image was captured every 10 minutes for a total of 17 hours. MDCK-Twist1 cells were cultured and images processed as control cells (See Movie 19 legend). Cells were expressing GFP.

Movie 23. Epithelial cells stably transfected with Prrx1 undergo a full EMT, degrade extracellular matrix and migrate. For time-lapse movies, one image was captured every 10 min for a total of 17 hours. MDCK-Prrx1 cells were cultured and images processed as control cells (see Movie 19 legend). Cells were expressing GFP.

Movies 24 and 25. MDCK control cells move when they are isolated in culture but when they meet they attach to each other in order to form the typical pavement of epithelial cells. Time-lapse movies were recorded 24 hours after cell seeding for 16 hours, taking one picture every 5 minutes.

Movie 26 and 27. Snail2-expressing cells show individual cell migration and walk past each other when they meet, ignoring the presence of other cells Time-lapse movies were recorded 24 hours after cell seeding for 3 hours, taking one picture every 5 minutes.

Movies 28 and 29. Twist1-expressing cells migrate together upon contact. They show certain degree of cell-cell adhesion but after several hours migrating together, cells migrate away from each other, very likely reflecting the expression of Prrx1. Time-lapse movies were recorded 24 hours after cell seeding for 3 hours, taking one picture every 5 minutes.

Movies 30 and 31. Prrx1-expressing cells displayed repulsion after collision as cells migrated away from each other. This repulsion can play an important role in the dispersion of cells that have migrated. Time-lapse movies were recorded 24 hours after cell seeding for 3 hours, taking one picture every 5 minutes.

Movies 32 and 33. BT-549 shControl cells show repulsion when they collide, favouring dispersion. Time-lapse movies were recorded 24 hours after cell seeding for 4 hours, taking one picture every 5 minutes.

Movies 34 and 35. BT-549 shPRRX1 cells, in addition to revert to the epithelial phenotype and increase proliferation, they show cell-cell adhesion and attach to each other when they meet. Therefore, PRRX1 loss abolishes cell dispersion and induces proliferation. Time-lapse movies were recorded 24 hours after cell seeding for 4 hours, taking one picture every 5 minutes

Movies 36 and 37. Similarly to BT-549 shControl cells, BT-549 shTWIST1 cells show repulsion when they meet. Time-lapse movies were recorded 24 hours after cell seeding for 4 hours, taking one picture every 5 minutes.

Movies 38 and 39. Similarly to BT-549 shPRRX1 cells, BT-549 shPR1+shTW1 cells show adhesion when they meet and also increased proliferation. Time-lapse movies were recorded 24 hours after cell seeding for 4 hours, taking one picture every 5 minutes.

Movies 40 and 41. MDCK cells expressing the long isoform of Prrx1 display repulsion after collision. Time-lapse movies were recorded 24 hours after cell seeding for 4 hours, taking one picture every 5 minutes.

Movies 42 and 43. In contrast to the long isoform of Prrx1, cells expressing the short isoform do not show a repulsion response after collision, but rather cell-cell adhesion. Time-lapse movies were recorded 24 hours after cell seeding for 4 hours, taking one picture every 5 minutes.

Movies 44 and 45. MDCK cells expressing a construct that encodes amino acids from 1 to 199 of the Prrx1 protein behave as MDCK control cells (movies 24 and 25). Time-lapse movies were recorded 24 hours after cell seeding for 4 hours, taking one picture every 5 minutes.

Movies 46 and 47. MDCK cells expressing a construct that encodes amino acids from 1 to 235 of the Prrx1 protein depict contact repulsion. These constructs encode a protein that contains the AD3 and the OAR domains of Prrx1. Cells showed signs of EMT and of contact repulsion Time-lapse movies were recorded 24 hours after cell seeding for 4 hours, taking one picture every 5 minutes.

Movies 48 and 49. MDCK cells expressing a Prrx1 protein in which the OAR domain is deleted are not converted to mesenchyme and contact repulsion is not apparent. Time-lapse movies were recorded 24 hours after cell seeding for 4 hours, taking one picture every 5 minutes.

Movies 50 and 51. MDCK cells expressing Prrx1 protein lacking the AD3 domain, similarly to those expressing the protein lacking the OAR domain, loose contact repulsion and adhere to each other. Time-lapse movies were recorded 24 hours after cell seeding for 4 hours, taking one picture every 5 minutes.