# From Cancer Cell Plasticity to Differentiation Therapy

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# Summary

Cancer is a systemic heterogeneous disease that can undergo several rounds of latency and activation. Malignant tumours evolve by increasing diversity and in progressive response to microenvironment signals and resistance development after therapeutic interventions. Thus, adaptation is required for cancer cell survival during tumour dissemination and metastatic outgrowth.

An epithelial-mesenchymal transition (EMT) plays a major role in facilitating cell plasticity in cancer and allows cancer cells to escape chemotherapy and targeted therapies by dedifferentiation and signalling adaption processes. EMT commonly describes a process in which differentiated epithelial cells lose their epithelial characteristics such as cell-cell adhesions and apical-basal polarity and gain migratory properties. While an EMT is mainly responsible for primary tumour cell invasion, its reversal mesenchymal-epithelial plasticity (MET) has been shown to contribute to the metastatic outgrowth of disseminated cancer cells in distant organs. Hence, the therapeutic reversion of an EMT in cancer could be counterproductive. However, it has also been noted that cells undergoing an EMT and/or an MET are in a state of high cell plasticity and thus, may offer a window of opportunity for therapeutic targeting.

Here, I have aimed at utilizing breast cancer cell plasticity by inducing terminal differentiation into postmitotic adipocytes. Giving the inherent growth arrest of adipocytes they are unlikely to adapt and dedifferentiate, and therefore lose cellular plasticity. I report the efficient conversion of breast cancer cells, which have undergone an EMT, into functional post-mitotic adipocytes. By combining the diabetic drug Rosiglitazone and bone morphogenetic protein 2 (BMP2) I have been able to achieve almost 100% adipogenesis efficiency in mesenchymal breast cancer cells *in vitro*. Delineation of the molecular pathways underlying such trans-differentiation has motivated a combination therapy with a MEK inhibitor and Rosiglitazone to demonstrate the conversion of invasive cancer cells into adipocytes and the repression of primary tumour invasion and metastasis formation in mouse

models of breast cancer. The results indicate the high potential of utilizing the increased cell plasticity inherent to invasive cancer cells for differentiation therapy. They consequently raise the possibility of employing pharmacological treatments to interfere with tumour invasion and metastasis.

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# 1 Introduction

## 1.1 Plasticity as a General Survival Mechanism

Plasticity refers to the adaptability of a system and its capacity to undergo changes. Applying this rough definition to any aspect of the world around us stresses the true essence of plasticity. Because when do we adapt? The answer is quite simple: when we are forced to. A solid piece of metal will bend if heated or enforced to by any other outside energy. Even an elastic rubber band will change its shape, only when stretched by external force. This means that plasticity is relevant or acknowledgeable only in the context of a stressful moment. The degree of plasticity of a system is known only when plasticity is about to be lost. When we stretch the rubber band too much it will stay in the stretched form or be torn – thus losing its plasticity. If the plasticity of an organism has reached its limit and cannot adapt to a new condition, the organism will die. Plasticity in biology should hence be viewed as the capacity to adapt and survive under changes.

Plasticity in biology is used in various research fields such as neural plasticity and synaptic plasticity, cancer plasticity, plasticity in cell reprogramming, but also phenotypic plasticity and developmental plasticity. Indeed plasticity appears to be a term which explains many phenomena in biology. It is required for the survival of a tissue under inflammatory stress, as well as the adaption of a flower to the change of season and of a population undergoing global warming.

In this first chapter I would like to present a “zoom-in” picture of plasticity in order to stress its common and essential nature. I will start from a general overview, proceed to describe some general mechanisms in biology and reach eventually the kind of cell plasticity my research is concentrated on.

### *1.1.1 Evolution –Plasticity in Species*

Evolution is the natural selection over generations, whereas adaptation is the movement towards a phenotype that better fits the current environment (Orr, 2005). Thus, adaptation is often reversible unless an environmental change endures or risks the population's survival, in which case natural selection may occur (Dobzhansky, 1956).

Referring to evolution as the plasticity of species, involves the survival capacity of the population in novel environments (Hollander et al., 2015) and its inherent (Corbett-Detig et al., 2013) or emerging epistasis (Orr, 1996).
To understand epistasis contribution to plasticity one should first refer to the Darwin paradox, here explained by A. Orr (Orr, 1996):

> How could something as patently maladaptive as the evolution of sterility or inviability be allowed by natural selection? ...
>
> To see DARWIN paradox, consider the simplest possible scenario: a single gene causes hybrid sterility. One species has genotype AA and the other aa. While each species is fertile, Aa hybrids are sterile. Now consider how these species could evolve from a common ancestor, say, AA. They can't. Starting with two allopatric AA populations, one simply remains AA while the other must become Aa. But how can it? The a mutation, like any mutation, has the unfortunate property of arising in the heterozygous state. But the resulting Aa individual is the sterile hybrid genotype, and the line comes crashing to an end.

The solution to the paradox emerges when rethinking the problem in the context of two genes and the interaction between them, namely epistasis. The epistasis theory of incompatibility, also known as Bateson-Dobzhansky-Muller incompatibility, shows that emergence of a new mutation in population A may lead to hybrid sterilization, if this is incompatible with mutation of a gene in population B. This means that populations A (AA) and B (BB) are compatible, and can generate fertile hybrids of AB. Aa mutation is still fertile and so is

mutation Bb. However aB and Ab may cause hybrid sterility, and thus, lead to speciation (Dobzhansky, 1936).

In short, enduring changes leading to favourable mutations may produce incompatibilities that are eventually responsible for speciation. Speciation increases the diversity of the total population favouring population survival.

### *1.1.2 Polyphenism – Organism Plasticity*

Phenotypic plasticity is the capacity of an organism to produce different phenotypes in response to environmental variation (Forsman, 2015; Ghalambor et al., 2015). Aphids (plant lice) become winged or wingless, for example, depending on food availability. Tomato hornworm changes its colour depending on environmental temperature variations. In the cooler northern United States, the caterpillars that emerge in the autumn are black in order to absorb more sunlight. However, in the south, where camouflage is more important than heat conservation, caterpillars are green. In contrast, tobacco hornworms are typically green, regardless of temperature (Pennisi, 2006).

Suzuki and Nijhout demonstrated how species can mask the effects of genetic mutations until that moment when an environmental change reveals them (Suzuki and Nijhout, 2006). The latency amounts to an adaptive mechanism that helps organisms survive under changing conditions. The study focuses on the tobacco hornworm mutant coloured black rather than the normal green. This mutation reduces secretion of juvenile hormone, which regulates skin colouring. This mutant strain, however, generates caterpillars with varying degrees of green when heat-shocked—that is, briefly exposed to a very high temperature—at an early stage of development. The authors suggest that polyphenism, depends on mutations in the mechanism that control hormone titre. This can shift the phenotypic threshold and reveal previously covert genetic variation. Subsequent small-scale changes in hormone titre, or in the timing of hormone secretion, can progressively reveal further genetic variation leading to selection.

The role that external stimuli variations can have on the plasticity of a cell population will be further discussed throughout this introduction.

### *1.1.3 Epithelial-Mesenchymal Transition (EMT) – Cellular Plasticity*

In the context of species or organisms, a reciprocal connection exists between diversity and plasticity (Forsman, 2015). What however is plasticity in a single cell? A cell would markedly respond to an external signal in one way or another, but when can we ascribe its response to plasticity and distinguish it from its general cellular functioning? A possible definition is that plasticity in a cell equals its escape mechanism, when the cell changes to adapt itself to fluctuating conditions. This capacity of a cell to adapt is determined by cellular plasticity; therefore plasticity can be viewed as a quality of the cell. Escape requires the involvement of all cellular components: of cytoskeleton rearrangements, transcriptional and post-transcriptional changes, and even of altered cellular function. Such global changes take place under differentiation, differentiation being one domain in which plasticity is demonstrated. A stem cell can always differentiate, which means that it always has a powerful escape mechanism at hand. Stem cells and differentiation will be further discussed in the next chapter.

Another example of cellular plasticity is EMT. EMT is a process in which differentiated epithelial cells lose their epithelial characteristics such as cell-cell adhesions and apical-basal polarity and gain migratory properties (**Box1**) (Tiwari et al., 2012).

**Box 1 – Hallmarks of EMT:** EMT can be induced by various extracellular stimuli such as cytokines belonging to transformation growth factor β (TGFβ) family, hypoxic conditions or matrix stiffness. These activate signalling cascades that regulate structural and functional changes in epithelial cells. (Lamouille et al., 2014; Nieto, 2013)

Epithelial cells are constituted of sheets of cells that are tightly packed via specialized cell-cell junctions. One of which are the cell-cell adhesion junctions that require epithelial cadherin (E-cadherin). Upon EMT cells undergo a "Cadherin-switch", whereby E-cadherin is downregulated and replaced by neural-cadherin (N-cadherin) (Christofori, 2006). This switch is directly linked to the loss of cell-cell adhesions, activation of EMT regulatory pathways (e.g. Wnt signalling) and rearrangement of the cytoskeleton. The cortical actin is typical to epithelial cells and is reorganized to form stress fibres. The epithelial apical-basal polarity is essential to their function, and is lost during EMT, resulting in front-rear polarity and fibroblast-like morphology (Yilmaz and Christofori, 2009).

These major morphological changes are the result but also the cause of transcription factor activation and EMT-associated signalling regulation. Transcription factors regulating EMT, such as Zeb, Snail and Twist, are tightly controlled at the post-transcriptional level by various micro-RNAs (miRNAs) (Lamouille et al., 2014).

Members of the miR-200 family are associated with epithelial cell morphology and their expression is decreased upon the induction of an EMT. Zeb1 and Zeb2 directly bind to miR-200 promoters and repress their expression, in turn miR-200 repress Zeb1/2. A number of such double-negative feedback loops between miRNAs and key EMT TFs have been described. These negative feedback loops function as molecular switches and are important mechanisms underpinning the fine-tuning and reversibility of an EMT and, thus, epithelial/mesenchymal cell plasticity (Brabletz, 2012b; Diepenbruck and Christofori, 2016).

The process of EMT is considered reversible. It is not a process of trans-differentiation of epithelial into a mesenchymal cell or a fibroblast; unlike trans-differentiation, EMT is considered a process of dedifferentiation (Berx et al., 2007). The full process of EMT is complex and prolonged in time. At any point during the process, if the signal is removed, the cells will revert to the

epithelial state through a mesenchymal-to-epithelial-transition (MET) (Lamouille et al., 2014). However, the plasticity acquired during this process seems to be reduced again if the signal remains in force, bringing about a stabilization of mesenchymal state (Zhang et al., 2014). When stabilized in the mesenchymal state it is hard to distinguish between EMT-derived cells and a fibroblast. Yet, a fibroblast is less likely to undergo MET or any other transition. Partial EMT refers to the different states from EMT induction to full mesenchymal state and can include markers of both epithelial and mesenchymal cells at varying levels (Nieto, 2013). These cells are in an unstable state and will quickly revert to the epithelial state once the external stimulus is removed (**Figure 1**).

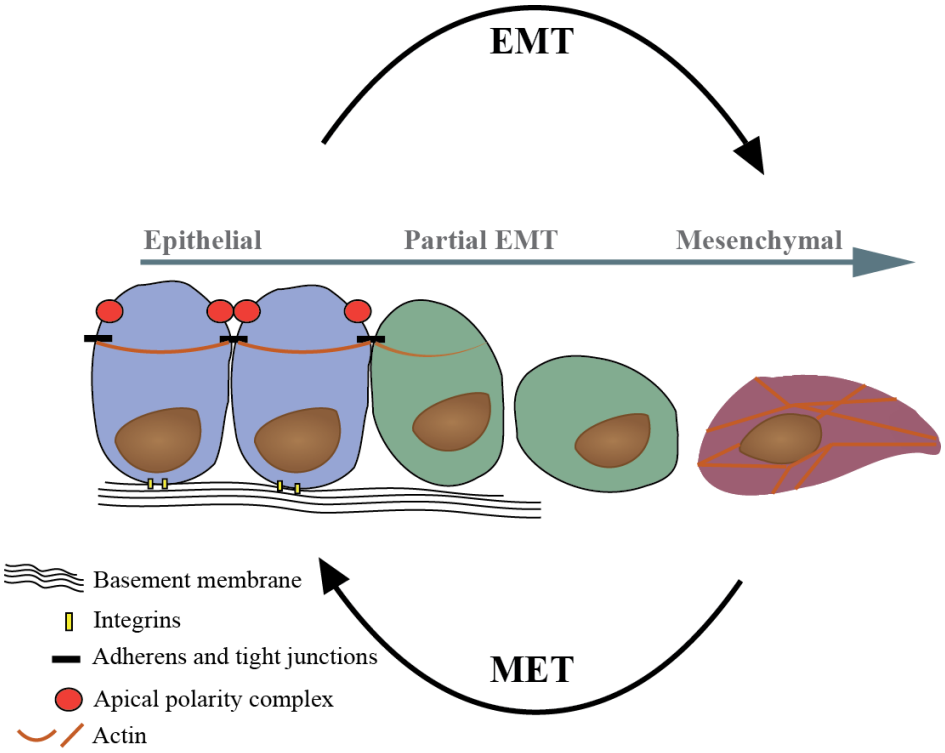


**Figure 1: Representation of structural and cellular changes during EMT/MET**

Epithelial cells (blue) exhibit apical-basal polarity and cortical actin organization. Epithelium comprises tightly packed and functionally synchronized epithelial cells connected to each other via cell-cell junctions and are anchored to the basement membrane via integrins. Upon EMT, cells lose epithelial characteristics and become dedifferentiated single cells (partial EMT – green). The full conversion gives rise to mesenchymal-like cells (purple) with front-rear polarity and actin stress fibres formation.

While the removal of stimulus induces MET, it is not yet known whether MET-derived cells are identical to their epithelial ancestors. In embryogenesis, EMT and MET are of central importance as they evidently generate cells different from their cell of origin and are therefore crucial for normal development (Nieto, 2013) (See chapter: EMT and MET in Development).

EMT and MET play a role also in wound healing processes and are activated in pathological contexts of organ fibrosis and cancer (Nieto, 2013). It is possible that *in vivo* partial EMT is a much more frequent event than a full transition (Berx et al., 2007). However, due to the unstable state of partial EMT and its dynamic nature, studying these events has been technically challenging. Lineage tracing studies concentrating on the different aspects of this transition can contribute to our understanding of partial EMT's frequency and effects (Beck and Blanpain, 2013).

EMT is further described and discussed throughout this work but I would like to give one example at this point that emphasizes the plasticity cells gain during an EMT. EMT is in fact an embryological, tightly regulated process. From the early stages of gastrulation and throughout organogenesis, EMT and MET facilitate embryonic development (Nieto, 2013) (See chapter: EMT and MET in Development). The reversibility of these processes hints that certain plasticity endures in cells undergoing EMT or MET (Berx et al., 2007). These processes can be reactivated in pathological conditions such as chronic inflammation and cancer. This correlation between developmental EMT and pathological EMT can be well demonstrated in the case of kidney fibrosis:

The generation of the kidney epithelium is the result of several rounds of EMT and MET. The entire epithelium of the kidney is derived from the intermediate mesoderm during the urogenital development. The mesodermal cells undergo a mesenchymal-epithelial transition to form the kidney's epithelium (Kalluri and Weinberg, 2009). Snai1 is an important transcription factor regulating EMT by repressing E-cadherin expression or cadherin-16 in the case of kidney epithelium (Grande et al., 2015). Snai1 is expressed in the precursors of the renal epithelial cells and is downregulated upon epithelial differentiation. Thus, Snai1 is maintained in a silent state during adulthood. The reactivation

of Snai1 in renal epithelial cells leads to renal fibrosis and renal failure in an inducible transgenic mouse model (Boutet et al., 2006). Snai1 expression is induced in chronic inflammation by a persisting inflammatory stress mediated by cytokines, such as TGFβ. EMT in organ fibrosis is a complex process that might have its origin in kidney development. By retaining some imprint of their mesenchymal origins, kidney epithelial cells may be particularly prone to undergo EMT. In agreement with the concept of high plasticity in kidney epithelium, it has been observed that EMT in kidney fibrosis is highly heterogeneous; the cells undergo EMT to a different extent omitting an invasive phenotype. Therefore, EMT in kidney fibrosis is considered partial EMT. (Grande et al., 2015; Nieto et al., 2016)

The dynamic nature of EMT and MET leads to increased plasticity. The end points of these transitions, namely full epithelial or full mesenchymal states, are usually relatively stable (Greenburg and Hay, 1982; Nieto et al., 2016). Nevertheless, one can legitimately hypothesize that every epithelial cell carries a corresponding ability to undergo EMT.

## 1.2 Stem Cells and Plasticity

### *1.2.1 Stem Cells*

Stem cells are classically defined by their ability to self-renew and to differentiate. Long-term self-renewal is characterized by the ability to produce at least one identical daughter cell. The ability to differentiate refers to the potential of the cell to give rise to more than one type of differentiated cell. If a cell can differentiate only within one specific lineage, it is considered a progenitor and not a stem cell. Totipotent stem cells can differentiate into all cell types including extraembryonic tissues (placenta, yolk sac and supporting tissues). Pluripotent stem cells can generate all cell types of the organism, and multipotent stem cells produce cells of a specific tissue. Unipotent stem cells, such as spermatogonial stem cells, share the capacity for self-renewal, yet exhibit limited developmental potential giving rise to only a single cell type, such as sperm. (Laplane, 2016)

Most adult stem cells, however, are considered multipotent stem cells. They are residents of a specific tissue, responsible for the tissue's homeostatic self-renewal and can be rapidly recruited in case the need of regeneration arises (Reya and Clevers, 2005). Adult stem cells are maintained in specialized niches in a quiescent state. The stem cell niche, or in other words the stem cell microenvironment has crucial role in maintaining the stem cells in their multipotent state. This is not a trivial quest, since maintaining the stem cell potential means stem cells are kept away from signals that will induce their differentiation. Differentiation can be induced not only through differentiation signals but it also requires the cells entering into cell cycle. Indeed, keeping the cells in a quiescent state is one of the major roles of the niche to avoid stem cells exhaustion (Cheng et al., 2000; Orford and Scadden, 2008; Porlan et al., 2013).

One can argue that a stable stem cell state is a challenge confronted only by adult stem cells. In fact, the pluripotent stem cells of the developing embryo keep their state only transiently. It is already during the transition from

8- to 16-cell stage in early mammalian development that different cell lineages arise for the first time. A recent study demonstrates that differences in contractility and polarity confer different fates in the 16-cell stage (Maitre et al., 2016). The daughter cells congregating at this stage have differential levels of contractility due to asymmetric segregation of a polarized apical domain at cell division. In the less-contractile polar cells, the transcriptional activator protein YAP enters the nucleus and activates a gene-expression program giving rise to the trophecdoterm. The highly contractile cells at this early stage do not have nuclear YAP, and localize to the centre of the cell aggregate to eventually become the inner cell mass, from which the embryo will form (Maitre et al., 2016).

The broadly studied embryonic stem cells (ESC) are in fact pluripotent stem cells expanded *in vitro* from the inner cell mass of the embryo (Weinberger et al., 2016). They are pluripotent because they can give rise to cells from the three germ layers *in vitro* and *in vivo*. During gastrulation they rapidly proliferate and differentiate. Maintaining self-renewal *in vivo* or *ex vivo* requires transformation, explaining why ESC give rise to tumours called teratomas or teratocarcinomas upon re-transplantation or reactivation (Burdon et al., 1999). Another interesting point is that teratomas are highly differentiated tumours. The cells that composite the teratomas are cells of the three germ layers but are usually of terminal differentiated phenotype (hair and teeth- like structures are frequently found in these tumours) (Pierce and Dixon, 1959). Indeed these tumours are usually considered benign. When ESC are expanded *in vitro* they are maintained in a special medium containing leukaemia inhibitory factor (LIF), which activates the JAK–STAT3 (Janus kinase–signal transducer and activator of transcription 3) pathway. LIF is a key ingredient that supports ESC propagation in the undifferentiated state (Weinberger et al., 2016). Once LIF is removed, the cells rapidly commit to a specific lineage. When these cells are re-transplanted or re-activated *in vivo* they undergo differentiation in the form of a teratoma (Weinberger et al., 2016).

These features of embryonic stem cells suggest the necessity of stem cell niches in adult tissue. Stem cell function in the niche is tightly regulated by

morphogens (including Notch, Wnt and Hedgehog), cell–cell and cell–extracellular matrix (ECM) adhesion molecules (cadherins and integrins) as well as hypoxic conditions (Laplane, 2016). Self-renewal requires the exact positioning of the mitotic spindle to allow the generation of two different daughter cells (Clevers, 2005; Maitre et al., 2016). Thus, any change in the microenvironment may lead to differentiation.

Plasticity seems inherent to the understanding of stem cell function. Stem cells are considered plastic cells, since they can easily change their phenotype. They are so prone to change and adaptation that every slight change will lead to their differentiation.

### *1.2.2 EMT and MET in Development*

EMT plays an essential role in implantation, embryogenesis and organ development. Both embryo implantation and the initiation of the placenta are associated with EMT involving the parietal endoderm. The trophectoderm cells undergo an EMT in order to facilitate invasion into the endometrium and the following anchoring of the placenta (Kalluri and Weinberg, 2009). During gastrulation the primitive epithelium (epiblast cells) migrate toward the centre of the embryo to form the mesoderm. This ingression of mesendodermal precursors at the primitive streak requires EMT both for the cell migration and for the generation of primary mesoderm. A MET that follows generates the endodermal layer. EMT also enables delamination of the neural crest (Nieto, 2013).

Mesodermal cells then migrate to occupy different positions along the medio-lateral axis of the embryo. Upon MET these cells generate notochord, somites and the urogenital system. Organogenesis then often requires further rounds of EMT and MET (Locascio and Nieto, 2001; Nieto, 2013).

It is intriguing that EMT and MET in early development involve different types of stem cells. In fact, EMT directly induces primitive epithelium stem cells to become pluripotent, by allowing their differentiation into cells of both the endodermal and mesodermal layer. As mentioned above, at least some epithelial cells, though differentiated, maintain the ability to undergo EMT.

This possibly suggests that cellular plasticity is a quality contemporaneous with the differentiation state of a cell and not a property enhanced by stemness (**Figure 2**). Of course not all cells can easily change their phenotype. As discussed earlier, plasticity potentially increases the survival of a population. Perhaps it is due to the high plasticity potential of epithelial cells (Donati and Watt, 2015) that they are more prone to undergo malignant transformation. This hypothesis is further supported by the observation that skin keratinocytes, an epithelial cell type, give rise to induced pluripotent stem cells (iPSCs) more efficiently and faster than fibroblasts (Aasen et al., 2008; Maherali et al., 2008).

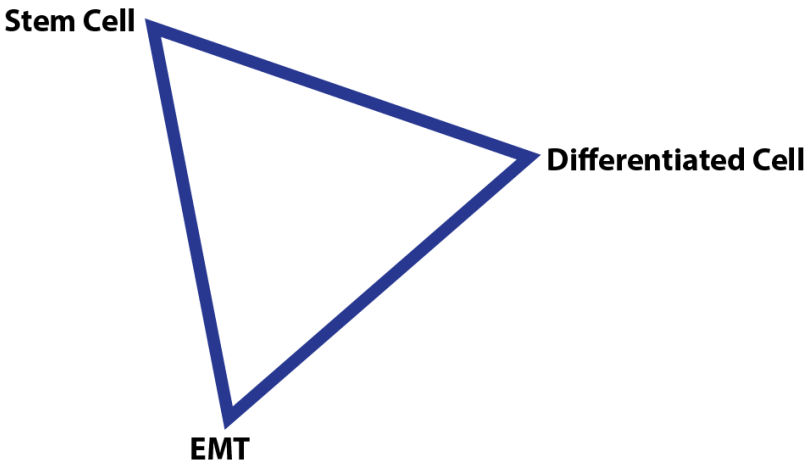


**Figure 2: EMT enhances cellular plasticity regardless of differentiation state**

A stem cell can undergo differentiation to a specific cell type. However, both the differentiated cell as well as the stem cell can undergo EMT-like processes that will increase cell plasticity. Suggesting that cell plasticity is contemporaneous with cell differentiation state.

### *1.2.3 Induced Pluripotent Stem Cells (iPSC)*

Overexpression of the transcription factors Oct4 (Pou5f1), Klf4, Sox2, and c-Myc (OKSM) in somatic cells gives rise to induced pluripotent stem cells (iPSCs) in a process named cell reprogramming. IPSC exhibit molecular and functional similarities to ESC (Takahashi and Yamanaka, 2006). The generation of a pluripotent stem cell from a somatic- embryonic or adult cell is

a powerful tool for studying numerous degenerative diseases with a clear therapeutic potential.

Reprogramming is considered efficient when at least the following functional assays deliver results comparable with those of ESCs. 1. *In vitro* differentiation to derivatives of all three embryonic germ layers—ectoderm, mesoderm and endoderm. 2. The teratoma formation assay assesses the spontaneous generation of differentiated tissues from the three germ layers following the injection of cells into immune-compromised mice. 3. The blastocyst chimaera formation determines whether test cells can re-enter development when introduced into host embryos at either of two pre-implantation stages: by aggregation with cleavage-stage morulas or by injection into blastocysts. Pluripotent stem cells should support normal development and generate high-grade chimaeras with extensive colonization of all embryonic tissues including the germ line (De Los Angeles et al., 2015). These assays test the pluripotency of stem cells generated and expanded *in vitro*. The initial identification of iPSC following the conversion is usually done on hand of Nanog expression (Stadtfeld et al., 2008). Reprogramming has also proven possible *in vivo*, whereby a transitory induction of OSKM factors in mice generated teratomas originating from various epithelial structures (Abad et al., 2013).

Reprogramming is usually slow and inefficient (1-3%) (Apostolou and Hochedlinger, 2013). Various approaches emerged over the few years since this popular field opened its gates to overcome the parsimonious efficiency of this process. In 2013, small interfering RNA (siRNA) screen of epigenetic factors, revealed Mbd3, a component of the nucleosome remodelling and histone deacetylase (NuRD) complex, as a clear repressor of reprogramming. By downregulating Mbd3 together with OSKM transduction nearly 100% of mouse embryonic fibroblasts (MEF) were reprogrammed into iPSC within 6-8 days. The authors suggested that Mbd3 is recruited once the factors are introduced and represses their function. Similar efficiency was achieved in different cell lines, including in terminally differentiated cells such as mature B- and T-cells. Mbd3 was shown to inhibit reprograming until the late stages of the process. Nevertheless, once reprograming was achieved, Mbd3

expression had no effect on the maintenance of pluripotency (Lujan et al., 2015; Rais et al., 2013).

By transiently overexpressing CCAAT/enhancer binding protein-α (C/EBPα) prior to the introduction of the OSKM factors B-cells were efficiently (~95%) reprogrammed within 12 days. Importantly, the enhanced efficiency was achieved only when using B-cell precursors and not by using terminally differentiated mature B-cells or MEF. The transient expression of C/EBPα correlated with the gain of mesenchymal markers. The authors suggested that the expression of mesenchymal markers induced with C/EBPα enabled the activation of MET and consequently of cell reprograming. C/EBPα was shown to induce reprograming by activating Tet2, which enables Oct4 binding to pluripotency inducing genes (Di Stefano et al., 2014).

### *1.2.4 EMT and MET in Cell Reprogramming*

Reprogramming is a process in which somatic cells gradually lose their differentiated identity and assume embryonic gene expression pattern and growth behaviour. It is a multi-stage process involving early stochastic and late deterministic phases. The stochastic model describes the unpredictability of reprogramming in a cell population, demonstrating that reprogramming depends on the number of cell divisions. A sequence of probabilistic events eventually lead to the small and unpredictable fraction of iPSC (Hanna et al., 2009). The deterministic late phase refers to hierarchical gene activation circuitry towards pluripotency after the initial gene expression changes (Buganim et al., 2012).

The inefficient and stochastic nature of reprogramming left the mechanisms governing reprogramming largely obscure in the first years. However, the emergence of single cell technologies on one hand and achieving 95-100% efficient reprogramming on the other hand enabled the deciphering of this process to some extent. This conversion is accompanied by drastic morphological changes generating multilayered epithelial-like cells. However, the different approaches and juvenility of the field lead to a quite confusing picture of the mechanisms underlying reprogramming.

Nevertheless, some interesting insights regarding the process have been described and demonstrated.

Two major waves of gene expression changes are typical for the early extinction of somatic genes and the late activation of core pluripotency genes (Apostolou and Hochedlinger, 2013; Buganim et al., 2012). The intermediate phase of reprogramming is poor in major transcriptional changes, suggesting that cells undergo gradual epigenetic alterations to prime the genome for transcriptional activation of pluripotency genes. In agreement with this is the observation that histone marks associated with pluripotency enhancers are established at early and intermediate stages of reprogramming. The establishment of pluripotency-specific long-range chromatin interactions and Tet-mediated conversion of 5mC into 5hmC at pluripotency promoters also characterize the intermediate period. DNA methylation is considered to be the most stable epigenetic modification occurs late in the process (Apostolou and Hochedlinger, 2013).

This suggests that plasticity of reprogrammed cells is enhanced in the early and intermediate phases of reprogramming, whereas the late phase requires the stabilization of the cells in a stem cell state. In agreement with this data, EMT and MET like processes seem to facilitate cell reprogramming mostly in the early and intermediate phases. Although reprogramming is frequently induced in fibroblasts or B-cells, it has been shown that EMT-related transcription factors are suppressed to allow MET activation (Di Stefano et al., 2014; Liu et al., 2013; Samavarchi-Tehrani et al., 2010). The data demonstrate that the induction of reprogramming includes the suppression of epithelial genes as well as the upregulation of mesenchymal genes (Di Stefano et al., 2014), even in the conversion of fibroblasts to iPSC (Liu et al., 2013). The OSKM factors can directly induce or suppress EMT related transcription factors, such as Snail1 (Li et al., 2010; Nieto et al., 2016). In turn MET is actively induced by the regulation of Klf4 (Li et al., 2010) or external MET inducers such as bone morphogenetic proteins (BMPs) (Samavarchi-Tehrani et al., 2010).

One can rightfully argue that the MET-like changes are required merely for the upregulation of the epithelial protein E-cadherin (encoded by the gene

Cdh1) which is also expressed in ESC and iPSC. However, hints to a prior EMT-like process as well as the timing of these transitions during the time course of reprogramming from different cell types suggest that EMT and MET coincide with increased plasticity required for reprogramming.

## 1.3 Cancer Plasticity

As discussed in the previous chapters, the survival of a population is achieved through its inherent variations (epigenetic modifications, mutations, epistasis), and its capacity to undergo adaptation induced by dynamic changes. On the cellular level, adaptation is possible via EMT and MET which enhance cell plasticity.

Diversity in a tumour cell population increases its survival potential. External signals from the tumour microenvironment trigger cancer cell plasticity via processes like EMT/MET. Plasticity is exhibited in the varying responses of cells depending on cell state and location, thus creating a variety of phenotypic changes (**Figure 3**).

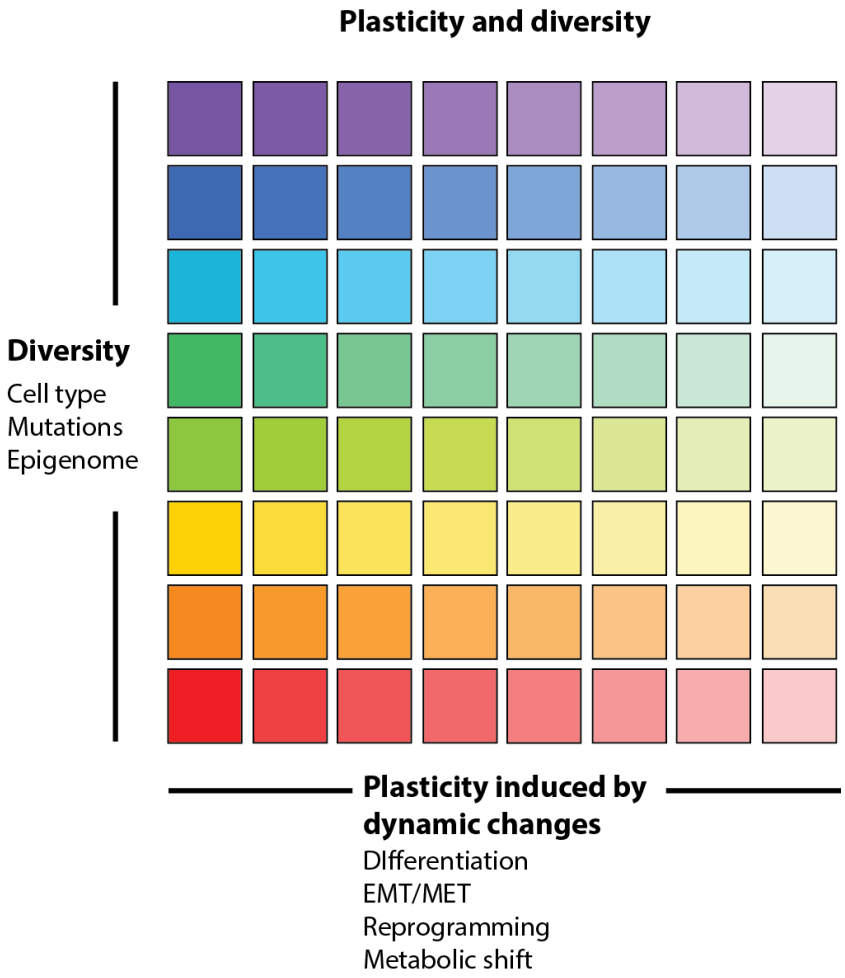

**Figure 3: Plasticity and diversity in a cell population**

This figure represents the inherent plasticity of every differentiated state and of every cell type on the vertical axis. Differentiated states are on the left where different colours are marked, and dedifferentiated and stem cell-like states are on the right where colours are light. Cell diversity includes cell identity, mutations and epigenetic landscape of cells creating cellular heterogeneity. Plasticity induced by dynamic changes is represented on the horizontal axis. Plasticity is triggered by external signals from the tumour microenvironment and is exhibited in the varying response of cells depending on cell state and location, thus creating a variety of phenotypic changes.

In this chapter I would like to emphasize the different aspects of plasticity in breast cancer and its contribution to disease progression (**Figure 4**).

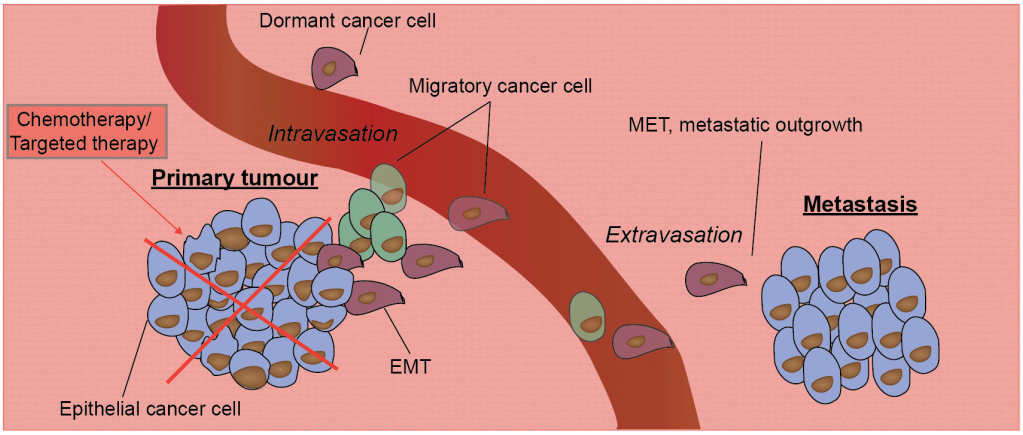


**Figure 4: Cancer plasticity and the metastatic cascade of breast cancer.** The primary tumour site is highly heterogeneous and comprises epithelial cell populations with different mutations, proliferation states and differentiation phenotype. At the invasive tumour front, epithelial cells respond to microenvironment signals such as TGFβ by undergoing EMT (purple cells) to varying extent. Cell clusters can detach from the primary tumour exhibiting mixed expression of epithelial and mesenchymal genes as demonstrated in partial EMT (green cells). Migratory tumour cells disseminate through blood vessels and extravasate at distant organs. The primary tumour is surgically removed and systemic therapy is usually applied triggering drug resistance and dormant cancer cell formation. These cells are reactivated at distant site and revert to their former differentiated state to allow metastatic outgrowth via MET.

### *1.3.1 Heterogeneity*

Tumour heterogeneity coincides with major challenges to cancer diagnosis and treatment success. The primary tumour site comprises cell populations with different mutations, proliferation states and differentiation phenotype. For example in breast cancer, the same tumour can combine cells extensively expressing oestrogen receptor (ER) and cells negative for ER. Intriguingly, these two populations can arise from the same cell of origin (Keller et al., 2012). Thus the histopathological analysis of the tumour has to base on different sites. The treatment is usually decided according to the more malignant counterparts of the tumour and its receptor status (Koren and Bentires-Alj, 2015).

A further aspect of heterogeneity concerns the tumour's cell of origin. The mammary gland contains two types of epithelial cells. The luminal layer lining the ducts and alveoli is characterized by the expression of oestrogen and/or progesterone receptors and apical-basal polarity. The myoepithelial cells are typically of a basal location and function as the contractile apparatus of the gland with specialized cytoskeleton arrangement and the expression of its contractile protein smooth muscle actin (SMA) (Weigelt and Bissell, 2008). This basal layer has been a source of confusion due to its communalities with basal or triple-negative (negative for ER, progesterone receptor (PR) and HER2 receptors) breast cancer cells. However, studies demonstrate that the myoepithelial cells are usually less likely to undergo malignant transformation, suggesting that the highly invasive basal-like cells do not originate from the myoepithelial lineage (Gudjonsson et al., 2005).

Two recent studies support this notion by underlining the multipotency role of $PIK3CA^{H1047R}$ oncogene in breast cancer (Koren et al., 2015; Van Keymeulen et al., 2015). The authors demonstrate how the expression of the frequent breast cancer mutation $PIK3CA^{H1047R}$ in basal or luminal cells gives rise to tumours with different degrees of malignancy. The mutation induces mammary cell plasticity and enhances tumour heterogeneity when expressed in either of the cells of origin. However, heterogeneous tumours arising from

luminal mammary cells show gene expression similarities to more aggressive breast cancer subtypes and more frequent malignant lesions than tumours derived from basal cells, which show mostly a benign phenotype (Koren et al., 2015).

Analysing the genetic diversity evolution and driver mutations in tumours demonstrates the development and proliferation of robust clones (Koren and Bentires-Alj, 2015). Single cell analysis from a primary tumour and its liver metastasis indicated that a single clonal expansion formed the primary tumour and seeded the metastases (Navin et al., 2011).

Taken together, tumour heterogeneity refers to different parameters of the tumour such as genetic diversity, cell type and receptor status variability as well as lineage plasticity, all of which contribute to the survival of the tumour.

### *1.3.2 Cancer Stem Cells (CSC)*

The concept of CSC is disputable which generates diverse definitions, making the discourse on CSC even more complex (Clevers, 2011; Koren and Bentires-Alj, 2015; Pattabiraman and Weinberg, 2014). CSC are also referred to as cancer-initiating cells or cancer-propagating cells, whereas each term actually stands for a different function of these cells (Laplane, 2016). This moot question regarding stem cells possibly has its roots in cancer plasticity, but to address the question of cancer plasticity we will have to come to terms with the lack of clarity as described above.

A major question in the CSC field refers to the cell-of-origin of a tumour (Beck and Blanpain, 2013). Is cancer a disease originating in a transformed stem cell (hierarchical model) (Pardal et al., 2003) or do CSC have their ancestors in non-stem cancer cells (Koren and Bentires-Alj, 2015)? A further possible variation implies that oncogene activation can directly induce stemness in non-stem cells (Koren et al., 2015). Cancer types, like teratomas, or some types of leukaemia, like Acute promyelocytic leukemia (APL), seem to fit the hierarchical model (Clevers, 2011). However, tumours originating from epithelium (carcinomas) are able to undergo dedifferentiation

processes like EMT, demonstrating that CSC could originate from non-stem cells (Lamouille et al., 2014).

A recently published book by Lucie Laplane, entitled “cancer stem cells: philosophy and therapy” represents a deconvolution of the concept of CSC (Laplane, 2016). Laplane demonstrates through a comprehensive analysis that the complexity of the field has its roots in vague definitions of basic terms such as stem cells and dedifferentiation. These unclarities originate in our incomplete understanding of the nature of stemness (Zipori, 2004). To overcome these challenges Laplane suggests a new definition to the concept of stemness divided into four versions:

1. Categorical: Stemness is an intrinsic and essential property of a stem cell, independent of its environment.
2. Dispositional: Stemness is essential and specific to stem cells but the expression of stemness depends on extrinsic factors. Thus, stemness emerges only in the right environment.
3. Relational: Stemness is an extrinsic property induced in a cell that would otherwise be a non-stem cell by its microenvironment.
4. Systemic: Stemness is an extrinsic property of a system such as tissue, rather than an individual cell. Thus, stemness is regulated by factors maintaining tissue homeostasis.

Taken together, cancer stem cells, although broadly studied are ill defined. This has to do with the evasive definition of stemness and possibly with the transient dynamic nature of CSC. Considering cellular plasticity as a quality contemporaneous to differentiation state, it is possible that targeting CSC alone will not inhibit cancer plasticity.

### *1.3.3 Microenvironment, EMT and MET*

Cancer is a systemic disease that can frequently result in metastatic dissemination, which is the main cause of cancer-associated death. The formation of distant metastasis requires the invasion of primary tumour cells, dissemination through blood vessels, seeding at the distant site, and

colonization of the host organ to form macrometastases (Brabletz, 2012b) (**Figure 4**). Metastatic cells need to survive blood circulation, evade immune defences, and adapt to a constantly changing microenvironment: as a consequence, metastasis is a highly inefficient process (Diepenbruck and Christofori, 2016; Massague and Obenauf, 2016).

How do tumour cells overcome microenvironmental changes? It has been demonstrated that a small population of cells via direct non-cell-autonomous stimulation can support tumour growth. A sub-population of cells in the tumour can stimulate proliferation of all tumour cells by inducing tumour-promoting microenvironmental changes. Hence, intra-tumoural sub-clonal interactions can facilitate metastatic outgrowth (Marusyk et al., 2014).

Tumour cell dissemination is an early event in cancer progression also in cancer types that are considered curable (Husemann et al., 2008). Clinically, cancer relapses within months or decades after initial tumour removal and treatment in the form of a metastatic disease is a frequent outcome. This latency between primary cell dissemination and metastatic outgrowth remains largely elusive due to technical challenges in studying molecular mechanisms over a long period of time (Massague and Obenauf, 2016). Yet, clinical studies investigating blood circulating tumour cells (CTC) and tumour cells in bone marrow of early stage cancer patients provided first insights into the molecular state of these cells, demonstrating their quiescent state (dormancy) (Pantel et al., 2008). Moreover, the incidence of disseminated tumour cells in the bone marrow is a predictor of metastatic disease with a higher prognostic value than CTC (Massague and Obenauf, 2016; Pantel et al., 2008). Nevertheless, given the necessary step of disseminating tumour cells to intravasate and survive in the circulation, studies focusing on CTC are of highly clinical relevance. Notably, CTC can be detected both as single cells and as CTC clusters. The latter exhibit gene expression of both epithelial and mesenchymal genes (partial EMT) and demonstrate a higher probability to survive the circulation and to form metastasis (Aceto et al., 2014; Diepenbruck and Christofori, 2016). Micrometastasis in other organs than bone marrow also shows features of dormancy induced by immune response and microenvironmental signals.

These together with an insufficient vascularization at the distant metastatic site create a stem cell-like niche that supresses proliferation (Gao et al., 2012b; Massague and Obenauf, 2016).

Various *in vivo* models were able to shed light on the mechanisms underlying tumour cell dormancy and survival. Disseminating cancer cells reside in specialized niches resembling adult stem cell niches (Massague and Obenauf, 2016). Existing stem-cell niches are rich in extracellular signalling molecules, such as members of the TGFβ family, Hedgehog, Wnt and the chemokine CXCL12 (Gao et al., 2012b; Massague and Obenauf, 2016; Zhang et al., 2013). These signals supress re-differentiation and keep cells in a quiescent state, which in turn leads to therapy resistance (Brabletz, 2012b; Giancotti, 2013).

EMT and MET are induced by external stimulation as the result of a tumour cell's interaction with the microenvironment at different sites and stages in tumour progression. A lineage-tracing model to study the effect of TGFβ-responsive cells in squamous cell carcinoma revealed its role in malignant progression. The authors demonstrate the direct effect of TGFβ on tumour cells localized in the perivascular niche as an underlying mechanism for non-genetic tumour heterogeneity. TGFβ-responsive cells show reduced proliferation, aberrant differentiation along with EMT changes and an invasive phenotype with enhanced drug resistance. The data indicates that TGFβ in the tumour microenvironment generates dedifferentiated resistant cells that eventually lead to tumour recurrence (Oshimori et al., 2015).

Further evidence for a TGFβ response was found in tumour cells' passage in the circulation. Intravascular tumour-platelet interaction promoted metastasis via platelet-derived TGFβ. TGFβ and platelet elements directly induced EMT and the upregulated expresssion of prometastatic genes in tumour cells (Labelle et al., 2011).

Another environmental contribution to metastasis formation is the recruitment of non-tumour cells to the metastatic site to facilitate metastatic outgrowth. It has been demonstrated in mouse models of breast cancer that a bone marrow-specific population of myeloid progenitor cells recruit almost exclusively to the metastatic lung. The recruited myeloid cells showed

increased expression of Versican in the lung with direct correlation to metastatic outgrowth. Further analysis revealed that Versican expressed by myeloid cells directly induced MET in metastatic cells, enabling metastasis outgrowth (Gao et al., 2012a).

Further data support the notion that upon metastatic colonization cancer cells need to undergo MET in order to overcome the EMT-induced growth arrest (Ocana et al., 2012; Tsai et al., 2012). However, highly aggressive carcinomas, such as triple negative breast cancer sub-types, often maintain a claudin-low and inherent EMT-like phenotype at the metastatic sites. This phenotype is also observed in short- term relapsed patients with an originally differentiated sub-type. This type of metastatic disease is puzzling in view of our current understanding of tumour dormancy and metastatic colonization, suggesting two distinct metastasis pathways (Brabletz, 2012b; Diepenbruck and Christofori, 2016).

Yet, some studies focusing on mesenchymal, basal-like breast cancer models contribute to our understanding of the possible mechanisms responsible for cancer progression in these aggressive tumours. Single cell analysis of a metastatic signature in triple-negative (ER−/PR−/HER2−), basal-like patient-derived xenograft (PDX) models was preformed using a microfluidics-based platform (Fluidigm). This technique allowed a multiplex gene expression analysis in individual cells. Early-stage metastatic cells (low burden) expressed a distinct basal/stem-cell signature with upregulation of pluripotency genes as well as EMT markers (Lawson et al., 2015). Furthermore, the mesenchymal invasive human cell line MDA-MB-231 showed an increase in EMT markers upon chemotherapeutic drug treatment. EMT was directly responsible for the drug resistance in these cells (Saxena et al., 2011).

These results raise the possibility that in the context of dedifferentiated invasive cancer cells, a cancer cell can still be induced to undergo EMT or MET that will allow further adaptation. The consequence of this transition is not a full phenotypic change, but rather an enhancement of plasticity. This hypothesis is in agreement with the concept of plasticity as discussed in the previous chapters:

- Cellular plasticity is a quality contemporaneous to the differentiation state of a cell and not a property enhanced by stemness (**Figure 2**).
- EMT and MET-like changes are required for cellular reprogramming regardless of the cell type. Also MEF and B-cells can undergo initial EMT inducing their conversion into iPSC, suggesting that EMT and MET coincide with increased plasticity required for cellular reprogramming (See Chapter: EMT and MET in Cell Reprogramming).

### *1.3.4 Therapy Resistance*

Breast cancer therapy typically starts with the surgical removal of the primary tumour. In some cases of more advanced disease neoadjuvant therapy to shrink the tumour is given prior to surgery. Treatment decisions are not trivial in breast cancer therapy and are the result of interdisciplinary discussion between surgeons, oncologists and pathologists. Generally, hormone-positive cancer types (ER+/PR+) will be treated with specific inhibitors, whereas triple-negative or highly proliferative, aggressive subtypes will be treated with chemotherapy. Tumour resistance to chemotherapy and targeted therapies is one of the main reasons for cancer recurrence and mortality.

Drug resistance can be intrinsic (present before treatment) or acquired during treatment by various therapy-induced adaptive responses. Indeed cancer therapy resistance is a paradigm of cancer plasticity; diverse molecular mechanisms have been implicated in tumour cell adaption to therapy (Holohan et al., 2013). These include increased rates of drug efflux mediated by ABC-transmembrane protein family members, activation of survival signalling pathways and the inactivation of apoptosis signalling pathways can also lead to drug resistance. Tumour heterogeneity, epigenetic changes, microenvironment influence and CSC have also been identified as important contributors to chemoresistance. Dormant cancer cells are intrinsically resistant to many therapeutic approaches, which typically target dividing cells. As mentioned above many of these mechanisms leading to

resistance have been directly linked to tumour-dedifferentiation and EMT (Holohan et al., 2013; Ma et al., 2015).

A further interesting example comes from HER2 expression patterns in resistant tumours. HER2+ breast cancer cells exhibit aggressive phenotype, with loss of epithelial characteristics and invasiveness (Moasser, 2007). The development of anti-HER2 targeted therapy (Trastuzumab) has significantly improved the survival of HER2+ breast cancer patients. However drug resistance is a common event in this type of tumours, and correlates with PTEN loss. Resistant cells often lose HER2 expression and express EMT-related markers (Burnett et al., 2015). This suggests that EMT program correlates with malignancy regardless of cell phenotype.

A recent study investigated the observed clinical transition in HER2 expression patterns after chemotherapy (Jordan et al., 2016). After multiple courses of therapy, women with advanced ER+/HER2- breast cancer acquire a HER2-positive subpopulation of CTC. HER2+ CTC counterparts are more proliferative but not addicted to HER2 due to the activation of multiple signalling pathways. However the HER2− subpopulation of circulating tumour cells show activation of Notch and DNA damage pathways, exhibiting resistance to cytotoxic chemotherapy, but sensitivity to Notch inhibition. This is in agreement with the drug resistance premise, since these tumours originate from well-differentiated ER+ breast cancer cells, but they activate cancer stem cell-associated pathways, such as Notch signalling, to survive treatment. The authors demonstrate that the HER2+ subpopulation spontaneously arises from therapy-resistant HER2- cells. The more rapidly proliferating single HER2+ CTCs also generated HER2− progeny. Single cell RNA sequencing revealed the activation of a MET program in the newly generated HER2+ cells.

Taken together, the data demonstrate the high plasticity acquired by chemotherapy-resistant cancer cells allows a rapid inter-conversion between phenotypes, which involves EMT and MET-like processes.

## 1.4 Plasticity as a Challenge in Cancer Therapy

Cancer cell plasticity plays a critical role in cancer survival, invasion and metastasis formation (Massague and Obenauf, 2016), as well as in tumour heterogeneity (Koren et al., 2015; Van Keymeulen et al., 2015) and in the development of therapy resistance (Gupta et al., 2009; Jordan et al., 2016). Cancer cell plasticity is of a dynamic nature and can be the result of changing cues in the microenvironment (Oshimori et al., 2015). An epithelial-mesenchymal transition (EMT) seems to play a major role in facilitating cell plasticity in cancer and allows cancer cells to escape chemotherapy and targeted therapies by dedifferentiation and signalling adaption processes (Gao et al., 2012b; Labelle et al., 2011; Nieto, 2013; Valiente et al., 2014). Targeting EMT-derived cells by specific cytotoxic compounds, such as Salinomycin (Gupta et al., 2009), or by inducing their re-differentiation via MET (Pattabiraman et al., 2016) has been proposed as a possibility to overcome metastasis.

While an EMT is mainly responsible for primary tumour cell invasion, its reversal MET, has been shown to contribute to the metastatic outgrowth of disseminated cancer cells in distant organs (Ocana et al., 2012; Tsai et al., 2012). Hence, the therapeutic reversion of an EMT in cancer could be counterproductive (Nieto, 2013). However, it has also been noted that cells undergoing an EMT and/or an MET are in a state of high cell plasticity and thus may offer a window of opportunity for therapeutic targeting (Brabletz, 2012b; Laplane, 2016; Nieto, 2013; Nieto et al., 2016).

## 1.5 Differentiation Therapies

### *1.5.1 Differentiation Therapy in Solid Tumours by G.B. Pierce*

The notion that cancer stem cells can be induced to undergo differentiation has been suggested by G.B. Pierce in his study of teratomas in 1959 (Pierce and Dixon, 1959). This initial study was followed by a life-time work of Pierce on the concept of cancer stem cells and differentiation potential, establishing a crucial milestone in the field of stem cell biology (Pierce, 1993). Supported by his revolutionary studies on CSC and hierarchical tumour development Pierce was resistant to the concept of tumour dedifferentiation (Pierce, 1993; Pierce et al., 1977). His results rather demonstrate as he describes "cancer cells as a caricature of the normal process of tissue renewal" (Pierce, 1993). This notion implies that all tumours originate from tissue stem cells and that tumours differ only in the potential for differentiation of their stem cells: embryonal carcinomas form the three germ layers, breast cancer stem cells form only glandular epithelium, and stem cells of squamous cell carcinoma of the skin differentiate into well-differentiated squamous cells (Pierce and Dixon, 1959; Pierce et al., 1977; Pierce and Wallace, 1971). Indeed in this latter report, Wallace and Pierce demonstrate that the progeny of malignant stem cells can differentiate into benign cells incapable of forming a tumour (Pierce and Wallace, 1971).

### *1.5.2 Differentiation Therapy in Leukaemia*

APL is a distinct highly malignant subtype of acute myeloid leukaemia. It is characterized by a chromosomal translocation, which results in the fusion between the promyelocytic leukaemia (PML) gene and the retinoic acid receptor (RAR) gene. Early treatment with chemotherapy was the front-line treatment of APL with limited remission success and low long- term survival rate (Wang and Chen, 2008).

A new era in the treatment of this disease began with a differentiation therapy approach initially developed in China. As the authors describe, this new direction in cancer treatment has its origins in disease control models employed in China that had been influenced by the Chinese ancient philosophy on the management of society. These are best illustrated by Confucius' famous saying: "If you use laws to direct the people, and punishments to control them, they will merely try to evade the laws, and will have no sense of shame. But if by virtue you guide them, and by the rites you control them, there will be a sense of shame and of right." The translation of this philosophy into cancer therapy in their research was described as "educating" cancer cells rather than killing them (Wang and Chen, 2008). This philosophy led to the introduction of all-trans retinoic acid (ATRA) in APL patients to induce terminal differentiation of the leukemic promyelocytes into mature granulocyte. Further development in this therapeutic strategy by applying arsenic trioxide (ATO) improved the clinical outcome of refractory or relapsed as well as newly diagnosed APL patients. The combination of ATRA and ATO demonstrated synergism in inducing differentiation and apoptosis turning this disease from highly fatal to highly curable (Coombs et al., 2015; Wang and Chen, 2008).

### *1.5.3 PPARγ Ligands to Induce Benign Differentiation*

The success of ATRA inspired various attempts to induce differentiation in less differentiated solid tumours. The group of Professor Bruce Spiegelman demonstrated exciting results in various cancer types by manipulating the nuclear receptor peroxisome proliferator-activated receptor-*γ* (*PPARγ)*. PPARγ is a ligand-activated transcription factor that plays an important role in a variety of physiological processes. PPARγ was initially characterized as the master regulator for adipogenesis but PPARγ signalling has also been implicated in the control of cell proliferation and metabolism. Ligands for PPARγ include naturally occurring fatty acids and a class of anti-diabetic drugs, the thiazolidinediones (TZD). The clinical benefit of these drugs in the

treatment of diabetes is mostly owing to the improvement of insulin sensitivity in these patients (Tontonoz and Spiegelman, 2008).

PPARγ as a crucial transcription factor in adipocytes is also extensively present in malignancies of adipose tissue, liposarcoma (Tontonoz et al., 1997). This observation led the authors to the hypothesis that treating transformed dedifferentiated cells of this tumour with TZD would induce a terminal differentiation into benign adipocytes and inhibit tumour progression, thanks to the post-mitotic characteristic of mature adipocytes. Preclinical and clinical experiments demonstrated a gain of adipocyte markers, reduced proliferation and typical adipocyte morphology in treated liposarcoma tumours (Demetri et al., 1999; Tontonoz et al., 1997).

Surprisingly, Spiegelman and colleagues also observed high PPARγ levels in colon tumours, a cancer type originating from transformed epithelial cells. Thus, they tested the effect of TZD in colon cancer cells demonstrating here as well reduced cancer cell proliferation and increased differentiation. However, in this case the induced differentiation did not result in adipogenesis, but in a re-differentiation into colonic epithelial cells with decreased malignancy (Sarraf et al., 1998).

Relatively high levels of PPARγ were also found in breast cancer metastasis. Here the combination of PPARγ agonists with a MEK inhibitor (PD98059) resulted in decreased proliferation and upregulation of epithelial markers (Mueller et al., 1998).

Since this pioneer work on the effects of PPARγ ligands in cancer, multiple clinical studies in various cancer types were conducted. The majority of these studies were done in advanced stage disease and mostly as monotherapy using TZD. Yet, most of these studies did not show a significant clinical benefit (Hatton and Yee, 2008).

Interestingly, the combination of PPARγ agonists with tyrosine kinase inhibitors in chronic myeloid leukaemia (CML) resulted in the erosion of the CSC pool by suppression of quiescence and stemness leading to long- term cancer cell eradication (Prost et al., 2015).

### *1.5.4 Inducing MET*

The concept of CSC in solid tumours and the relevance of EMT during malignant tumour progression evolved the induction of epithelial re-differentiation as a possible therapeutic approach targeting specifically those cells. It is important to point out that such re-differentiation approaches require combination with chemotherapy or targeted therapies to achieve long-term clinical benefit (Pattabiraman and Weinberg, 2014).

Mesenchymal invasive cells from mammary gland carcinomas were induced to undergo differentiation into epithelial cells by treatment with a histone deacetylase (HDAC) inhibitor called SAHA. Treatment with SAHA reduced proliferation and induced differentiation in these cells (Munster et al., 2001). A different HDAC inhibitor was shown to induce differentiation and to increase sensitivity to chemotherapy in EMT-derived pancreatic cancer cells (Meidhof et al., 2015). Indeed, during the last years numerous clinical trials with HDAC inhibitors have been performed for the treatment of different cancer types. However, HDAC inhibitors seem to have conflicting effects on regulating cell-state transitions, and the clinical results did not yet meet the expectations from this class of drugs (Tam and Weinberg, 2013).

A recent study aimed at specifically inducing MET in breast cancer cells in order to overcome invasiveness and drug resistance correlating with EMT. Screening for compounds that induce the upregulation of CDH1 (the gene encoding for E-cadherin) revealed that the activation of adenylate cyclase (cAMP) could induce the acquisition of epithelial properties. The study further demonstrated a role for the cAMP-downstream effector protein kinase A (PKA) in inducing MET and maintaining an epithelial state (Pattabiraman et al., 2016).

However, as emphasized above, cancer cell plasticity and the dynamic nature of EMT and MET during tumour dissemination and metastatic outgrowth underscore the possible weakness of epithelial differentiation. Although these processes are not fully understood, there is growing evidence that inducing MET may enhance metastatic outgrowth (Brabletz, 2012b; Pattabiraman and Weinberg, 2014). A further open question concerns the

ability of re-differentiated cells to undergo another round of EMT, a possible outcome that would endure plasticity response.

### *1.5.5 Conclusion*

Forced differentiation as a therapeutic approach to specifically target EMT-derived cells seems like addressing cancer cell plasticity in the same terms. However, a dedifferentiation potential remains in cells of epithelial origin and the rather plastic epithelial state could further allow tumorigenic phenotype. As discussed in the chapters about stem cells and iPSC, cell cycle plays an important role in the processes of differentiation and dedifferentiation. Cell cycle arrest keeps stem cells in their undifferentiated state (Orford and Scadden, 2008) as observed also in the case of dormant cancer stem cells (Massague and Obenauf, 2016). Moreover, cell cycle progression seems to play an important role in cellular reprogramming and a crucial step in generating iPS cells (Hanna et al., 2009). Hence, cell cycle arrest retains cells in their differentiated state and post-mitotic states could potentially inhibit processes of dedifferentiation.

## 1.6 Adipogenesis

In the past, adipose tissue was considered a simple structure of connective tissue containing lipid droplets. Only recently the complexity of adipose tissue and its central role as an endocrine organ at the centre of energy homeostasis have been fully appreciated. The profound effect on pathophysiology of major metabolic disorders such as diabetes mellitus and obesity had placed adipose function and development in the focus of research and clinical interest (Rosen and Spiegelman, 2014).

The epidemic disease of the modern society namely obesity and related metabolic disorders drew the attention to adult white adipose tissue (WAT). WAT has an important role in buffering nutrient availability and demand by storing excess calories and preventing the toxic accumulation of excess nutrients in non-adipose tissues. However, WAT is by no means a passive organ. WAT is able to communicate with metabolically relevant organs by secreting so-called adipokines as part of a dynamic endocrine system that regulates nutrient partitioning into peripheral tissues. Adipocyte-secreted adipokines, such as leptin and adiponectin, have a central role in energy homeostasis and adipose tissue communication with other organs and the immune system (Peirce et al., 2014; Rosen and Spiegelman, 2014).

However, the adult adipose organ also contains brown adipose tissue (BAT) whose main function is to maintain the core body temperature in response to cold stress by generating heat, a process known as thermogenesis. Like the uni-locular (single lipid droplet) white adipocytes in WAT, the multi-locular brown adipocytes in BAT also accumulate and store lipids. However, the highly specialized brown adipocytes are distinct from white adipocytes by more abundant mitochondria, which are enriched for the expression of uncoupling protein 1 (UCP1), enabling chemical energy storage in the form of heat (Peirce et al., 2014).

Exposure to cold or activation of β-adrenergic receptor (β3) agonists that enhance lipolysis in adipocytes triggers the appearance of WAT browning, due to the generation of 'brown-like' fat cells, termed beige

adipocytes. Unlike classic brown adipocytes these beige adipocytes, do not derive from precursors that are positive for myogenic factor 5 (Myf5) (Peirce et al., 2014). Instead beige adipocytes seem to arise from *de novo* adipogenesis in WAT (Wang et al., 2013).

In this chapter I will focus only on few aspects of this broad field of research, mainly on the most important issues regarding the process of adipogenesis and the general concepts of adipocyte characteristics.

### *1.6.1 The Process of Adipogenesis*

During adipogenesis cells accumulate triglyceride, acquire the signet ring appearance of adipose cells, and ultimately lose their ability to revert to the growing state (Green and Kehinde, 1975). At the cellular level adipogenesis is defined as a two-phase process with an initial determination phase followed by the terminal differentiation to mature adipocyte (Farmer, 2006; Rosen and MacDougald, 2006; Rosen and Spiegelman, 2014). Determination requires commitment to the progressive adipogenesis transcriptional program in parallel to the suppression of alternate differentiation pathways, together resulting in the conversion of a stem cell to a pre-adipocyte (Rosen and MacDougald, 2006; Rosen and Spiegelman, 2014). A pre-adipocyte cannot be distinguished morphologically from its precursor cell, usually of mesenchymal origin, but has lost the potential to differentiate into other cell types (Rosen and MacDougald, 2006; Tontonoz and Spiegelman, 2008). Once committed to the adipogenic fate, a pre-adipocyte undergoes terminal differentiation and acquires mature adipocyte characteristics - including the machinery that is necessary for lipid transport and synthesis, insulin sensitivity and the secretion of adipocyte-specific proteins (Rosen and MacDougald, 2006). Adipogenesis research developed mainly on the basis of *in vitro* cellular models that are already committed to the adipose lineage (e.g., 3T3-L1, 3T3-F442A cells). Hence, much more is known about the later phases of adipogenesis and adipocyte terminal differentiation than about the cell fate decisions of a multipotent cell (Rosen and Spiegelman, 2014).

Most of the studies on pathways regulating cell fate between adipogenic and non-adipogenic commitment have been performed using bone-marrow-derived mesenchymal cells, and thus the “bone-fat switch” is the most commonly described fate choice. The activation of canonical and alternative Wnt signalling as well as of Hedgehog signalling tends to promote osteogenesis and to inhibit adipogenesis in both committed and uncommitted precursor cells (Park et al., 2015; Rosen and Spiegelman, 2014; Tang and Lane, 2012). The fate decisions via these pathways and others were partially linked to the regulation of YAP/TAZ transcriptional activity. YAP/TAZ activity is regulated by mechanical signals and have been shown to be the downstream effectors of, for example, the Hippo pathway (Halder et al., 2012) and alternative Wnt signalling (Park et al., 2015). Apart from YAP/TAZ transcriptional regulation of the osteogenic lineage, which in turn suppresses adipogenesis (Park et al., 2015), TAZ was shown to directly repress PPARγ-dependent adipogenic gene transcription (Hong et al., 2005). The TGFβ family has also a profound effect on adipogenic commitment and progression and will be discussed in the next chapter on TGFβ signalling.

The principle pathway of adipogeneis involves the activation of CCAAT-enhancer binding protein β and δ (C/EBPβ, C/EBPδ) which facilitate the upregulation of PPARγ and C/EBPα, the master regulators of adipogenesis (Farmer, 2006). Different mechanisms have been suggested for the initial activation of C/EBPβ (Gubelmann et al., 2014). Nuclear receptor corepressor 2 (NCoR2), also known as silencing mediator of retinoic acid and thyroid hormone receptor (SMRT), is an interesting example acting as a gate-keeper of adipognesis by masking C/EBPβ enhancers (Raghav et al., 2012). Krüppel-like factor 4 (Klf4) was shown to be necessary for adipogenesis by directly activating C/EBPβ, which in turn supresses Klf4 via a tightly controlled negative feedback loop (Birsoy et al., 2008). As described in the latter study, C/EBPβ has a regulatory effect on adipogenesis activating factors. A recent study identified Zeb1, an EMT-transcription factor (see **Box1**), as a central regulator of adipogenesis acting in conjunction with C/EBPβ. Zeb1 is bound to open/activate gene regulatory regions before the onset of adipogenesis in committed pre-adipocytes, many of which are targeted by first-wave

adipogenic transcriptions factors, especially by C/EBPβ (Gubelmann et al., 2014).

This transcriptional initiation of adipogenesis results in the expression of the downstream targets and adipogenesis master regulators PPARγ and C/EBPα. PPARγ is both necessary and sufficient for adipogenesis. Hence, PPARγ is such a potent adipogenic factor that its ectopic expression can drive non-adipogenic fibroblasts to become adipocytes (Rosen and Spiegelman, 2014). Murine studies demonstrated that PPARγ is essential for adipose tissue formation (Farmer, 2006). In agreement with these results, humans with rare loss-of-function mutations in PPARγ exhibit lipodystrophy and severe insulin resistance (Rosen and Spiegelman, 2014). C/EBPα is also required for adipogenesis, and its absence impairs adipose tissue formation. However, C/EBPα is not sufficient to induce adipogenesis, if PPARγ is absent, whereas PPARγ can activate adipogenesis even in C/EBPα-deficient cells (Farmer, 2006). Once activated, adipogenesis is then “locked in” by a positive feedback loop between PPARγ and C/EBPα; a second positive feedback loop between PPARγ and C/EBPβ reinforces the decision to differentiate (Rosen and Spiegelman, 2014).

Alternative splicing is responsible for the generation of two protein isoforms of PPARγ. PPARγ2 has an additional 30 amino acids at its N-terminus as compared to PPARγ1 and is more specific for adipose tissue. Genetic mouse models demonstrate that PPARγ1 has adipogenic action, although PPARγ2 appears more potent in this function (Tontonoz and Spiegelman, 2008).

PPARγ is a nuclear receptor that acts as ligand-gated transcription factor (Tontonoz and Spiegelman, 2008). Fatty acids and their derivatives can bind and activate PPARγ, however the identification of specific endogenous PPARγ ligands has been widely unsuccessful. In contrast, synthetic ligands, such as TZDs have been identified as specific PPARγ ligands with a well-studied mechanism of activation. TZDs, such as rosiglitazone and pioglitazone, are potent activators of PPARγ with robust insulin-sensitizing activities and are highly effective oral medications used in the treatment of difficult-to-manage type 2 diabetes (Ahmadian et al., 2013). TZD regulation of

PPARγ is two-fold, since these agonists are capable not only to activate PPARγ and initiate adipogenesis, but also to protect PPARγ against phosphorylation. The second mechanism is directly responsible for the insulin sensitizing effect of these compounds (Banks et al., 2015).

PPARγ and C/EBPα act as transcription factors of a large number of genes responsible for adipocytes formation and function including fatty acid binding protein 4 (FABP4), the adipokine adiponectin and the insulin-sensitive glucose transporter 4 (GLUT4). Indeed, PPARγ is also necessary for adipocyte maintenance as demonstrated *in vitro* and *in vivo* (Tontonoz and Spiegelman, 2008).

As mentioned above, ectopic expression of PPARγ in non-adipogenic cells, such as fibroblasts, is sufficient to induce adipogenesis (Rosen and Spiegelman, 2014). However, the activation of adipogenesis with TZD via endogenous PPARγ alone is not enough for effective adipogenesis even in pre-adipocytes (Hong et al., 2005). Moreover, PPARγ activation in other cell types expressing this receptor, such as colon epithelial cells, does not result in adipogenesis (Rosen and MacDougald, 2006; Sarraf et al., 1998). These results demonstrate the complex signalling networks orchestrating adipogenesis progression even in adipocyte-primed cellular states.

### *1.6.2 Growth Arrest*

Adipogenesis is classically induced in a confluent population of preadipocytes by exposure to insulin, inducers of cAMP signalling and glucocorticoids in serum-complemented medium. The cells are then induced to re-enter cell cycle and undergo at least one round of clonal expansion before proceeding into terminal differentiation (Farmer, 2006).

In pre-adipocytes, growth arrest and not cell confluence or cell-cell contact per se appears to be required for adipocyte differentiation as demonstrated by the differentiation of re-seeded confluent 3T3-F442A cells in suspension. Furthermore, primary rat pre-adipocytes plated at low density in serum-free medium were also able to differentiate in the absence of cell-cell contact (Gregoire et al., 1998).

Both adipocyte regulators C/EBPα and PPARγ appear to be critical for the growth arrest that is required for adipocyte differentiation. Both transcription factors' activation and expression mediate the upregulation of cyclin-dependent kinase inhibitors, thereby providing a molecular mechanism coupling growth arrest and adipocyte differentiation (Gregoire et al., 1998; Morrison and Farmer, 1999). One study indicated that the cell cycle block in 3T3-L1 cell adipogenic differentiation occurs despite high levels of c-Myc and hyperphosphorylated Retinoblastoma (Rb) (Reichert and Eick, 1999). Cell-cycle proteins of the E2F family of transcription factors and associated pocket proteins seem to have a regulatory role in adipogenesis. E2F transcription factors and pocket proteins seem to regulate two separate but parallel pathways that result in the activation of PPARγ1 and PPARγ2 expression. Factors such as Rb which activates C/EBPβ promotes PPARγ2 production, whereas factors that induce E2F1 activity support PPARγ1 expression. Since PPARγ2 and not PPARγ1 is considered to be the predominant regulator of adipogenesis, factors such as E2F may lead to C/EBPα expression induced by PPARγ1 which in turn enhances PPARγ2 expression (Farmer, 2006).

The simultaneous regulation of cell cycle and adipogenic regulators eventually results in cell cycle exit and the generation of terminally differentiated-postmitotic adipocytes (Wang et al., 2013).

## 1.7 TGFβ-Signalling in Plasticity and Differentiation

Polyphenism, as described for the black tobacco hornworm in the first part of the introduction (See chapter: Polyphenism – Organism Plasticity) implies the central role of signal variations in plasticity induction. The contribution of microenvironmental stimuli to stem cell function and cancer plasticity was discussed thereafter. Different signalling pathways were briefly mentioned in this context, including TGFβ signalling. TGFβ signalling has been proven central in various processes such as proliferation, differentiation, and cancer invasion, specifically in EMT. The thought that plasticity and perhaps cancer malignancy per se may be substantially influenced by cell extrinsic factors is intriguing. In this part, I specifically discuss TGFβ signalling as a paradigm to the above-mentioned premise.

TGFβ's effect on cellular response has been described as context-dependent (Massague, 2012). Intriguingly, TGFβ family members are able to target just a few genes in pluripotent ES cells but hundreds of genes in differentiated cells. Hence, TGFβ can activate or inhibit transcription depending on the targeted gene and the cellular context. For example, in mammary epithelial cells TGFβ represses inhibitor of differentiation 1 (ID1) but induces this gene in metastatic breast cancer cells.

Three types of contextual determinants are defined to shape the TGFβ transcriptional response. First, the epigenetic landscape of the cell modifies the chromatin and dictates which genes are open for expression and thus susceptible to regulation. For example, ESC maintain pluripotency genes in an open chromatin conformation that permits transcriptional activation downstream of TGFβ signals, whereas genes involved in differentiation remain repressed and refractory to these inputs. When conditions are permissive for ESC differentiation, specific chromatin marks open master differentiation genes to their activation by the TGFβ family member NODAL, which regulates pluripotency and differentiation.

A second determinant of TGFβ target gene regulation is the availability of SMAD- TGFβ signalling effectors or other transcription co-factors. The third

determinant described refers to extracellular and intracellular composition of the TGFβ signal transduction system. The intensity of the TGFβ signal is the result of this system and determines the response by regulating SMAD function or activating non-canonical TGFβ pathways. Inputs that affect the intensity of the TGFβ signal may qualitatively influence the cellular response as observed in cell fate decisions. These emerge from fine-tuned gradients of BMP acting alongside WNT and Hedgehog signals during development (Massague, 2012).

The TGFβ pathway is indeed essential for developmental processes, including mesoderm specification and dorsal–ventral axis formation, and is dysregulated in a variety of cancers. The TGFβ superfamily acts via canonical (SMAD- mediated) and non-canonical signal transductions that orchestrate the function and balance of this pathway.

One example for the contextual role of the TGFβ superfamily refers to the function of the secreted BMP inhibitor COCO in development and disease. Deglincerti and co-workers focused on the canonical TGFβ pathway, which consists of two branches (Deglincerti et al., 2015). TGFβ/activin/nodal ligands signal through type1 receptors Alk4/5/7 to induce the phosphorylation of Smad2/3, BMPs activate Smad1/5/8 via Alk1/2/3/6 receptors. Phosphorylation of the receptor-activated Smads (R-Smads) from either branch of the pathway results in complex formation with Smad4 and its nuclear translocation. Smad4 localization upon TGFβ pathway activation was used as a reporter system to monitor TGFβ1 signalling dynamics and response. The Smad4 localization reporter was previously published by the same group (Warmflash et al., 2012) and was used in this paper to study the molecular mechanisms regulating the rapid TGFβ1 response by combining it with a genome-wide RNAi screen. 48 hours after RNAi library transfection, mouse myoblast C2C12 GFP-Smad4 reporter cells were stimulated with TGFβ1. Cells were fixed and analysed at the peak Smad4 nuclear translocation time point (1 hour after stimulation) to study genes that prevent GFP-Smad4 nuclear localization as well as 6 hours after stimulation to study genes that prevent its return to the cytoplasm. 321 genes were shown to significantly affect GFP-Smad4 localization.

Among these genes the authors identified the secreted protein Coco (Dand5), which is known to inhibit BMP4 and Nodal. Surprisingly, the authors observed that the RNAi-mediated ablation of Coco expression impaired GFP-Smad4 nuclear localization. Further investigation of Coco's effect on TGFβ1 signalling by various knockdown and overexpression experiments demonstrated an enhancing effect of Coco on TGFβ1 signalling accompanied by the known inhibitory effect of Coco on BMP4. The authors further demonstrate that Coco directly enhances the interactions of TGFβ1 and its receptor Alk5.

The implication of Coco as both an inhibitor and enhancer of TGFβ pathway depending on the ligand it interacts with may explain its critical function in development but also in disease as a possible enhancer of cancer metastasis. Coco was also identified as an activator of lung metastasis outgrowth in mouse breast cancer model (Gao et al., 2012b). These authors demonstrated that Coco promoted the exit from dormancy by alleviating the ability of lung- stromal BMP to enforce a dormant state.

Taken together these studies suggest that Coco can have one effect on microenvironmental signals and an opposing effect on invasive cancer cells during metastasis.

### *1.7.1 TGFβ-Signalling in EMT and MET*

TGFβ induces EMT during heart development, palate fusion and renal fibrosis, as well as in breast and hepatic epithelial cells (Massague, 2012). TGFβ directly regulates EMT-related transcription factors, such as Snail, Twist and Zeb (Xu et al., 2009). BMPs, which are known to induce miR-200 and miR-205 are considered inhibitors of an EMT and activators of a MET process (Massague, 2012). However, BMP4 together with Wnt1 induces neural crest delamination during development, a process that shares transcriptional and molecular similarities with EMT and also resulting into a migratory phenotype (Theveneau and Mayor, 2012).

TGFβ induces EMT by canonical SMAD activation but also elicits signalling responses through pathways that are generally considered as

important effector pathways for tyrosine kinase receptors in response to ligands that do not belong to the TGFβ family. These non-canonical signalling pathways are rapidly activated by TGFβ and often follow similar kinetics as SMAD signalling. Attenuation of SMAD signalling does not generally affect the activation of these pathways. Non-canonical signalling responses to TGFβ can also occur with delayed kinetics and are then often indirect, presumably as a result of SMAD-mediated changes in gene expression. The direct activation of non-SMAD signaling pathways by TGFβ occurs through interactions of signalling mediators either directly with the TβRII and/or TβRI receptors or through adaptor proteins. Among the non-canonical signalling responses, activation of MAP kinases, MEK and ERK kinases, Rho GTPases and the PI3 kinase/Akt pathway have been linked to TGFβ-induced EMT through their regulation of distinct processes, such as cytoskeleton re-organization, cell growth, survival, migration and invasion (Xu et al., 2009).

### *1.7.2 TGFβ Family Members in Adipogenesis*

BMPs usually show a minor effect on pre-adipocyte differentiation. However, exposure of multipotent mesenchymal cells to BMP4 commits these cells to the adipocyte lineage, allowing them to undergo adipogenesis (Rosen and MacDougald, 2006). BMP2 seems to promote osteogenesis by upregulating TAZ expression (Hong et al., 2005). However, when combined with adipogenic inducers, BMP2 activates adipogenesis in multipotent cells (Gubelmann et al., 2014). While BMP2 and BMP4 enhance white adipogenesis, BMP7 is a potent inducer of brown adipocyte differentiation (Tseng et al., 2008).

Indeed, TGFβ signalling is found activated in mature adipocytes and adipose tissue. However, TGFβ inhibits adipogenesis and impairs the development of adipose tissue. Inhibiting endogenous TGFβ signalling by either expressing dominant-negative TGFβ receptor or by direct inhibition of SMAD3 lead to increased adipogenesis. SMAD3 was found to bind to C/EBPs

and to inhibit their transcriptional activity, including their ability to activate PPPARγ2 expression (Rosen and MacDougald, 2006).

TGFβ-mediated inhibition of adipogenesis is usually linked to its canonical signalling. However, ERK has been previously reported to interfere with PPARγ function leading to obesity-linked insulin resistance (Rosen and MacDougald, 2006). ERK directly phosphorylates PPARγ at serine 273 in a robust manner and induces diabetogenic gene expression in adipose tissues (Banks et al., 2015). Moreover, in the terminal differentiation phase, ERK1 phosphorylation of PPARγ inhibits differentiation (Rosen and MacDougald, 2006). Surprisingly, TGFβ activation of ERK as a possible mechanism for the blockade of adipogenesis by TGFβ has never been demonstrated.

# 2 Aim of the study

Cancer is a systemic heterogeneous disease that can undergo several rounds of latency and activation. Malignant tumours evolve by increasing diversity and in progressive response to microenvironment signals and therapeutic interventions. Hence, both cancer diversity and cellular plasticity contribute to cancer progression and thus are an obstacle for cancer therapy (**Figure 3 in the introduction**).

New therapeutic approaches and drugs targeting cancer cells are constantly developed; some of which target cancer cell hyperproliferation, others target specific signalling pathways mutated and/or hyperactivated in cancer cells, and still others aim to specifically eliminate or inhibit cancer stem cells. Yet, in many cancer types, including breast cancer, the therapeutic success remains limited making cancer recurrence and metastatic outgrowth a frequent clinical event.

Cancer plasticity may possibly explain cancer progression regardless of the therapeutic approach. Processes like EMT and MET enable tumour cells to elude a hostile microenvironment and to develop drug resistance. Such plasticity induced dynamic changes are contemporaneous to the differentiation state of the cell and are plausible in both a well-differentiated cancer cell and in a cancer stem cell (**See Figure 2 in Introduction**).

In my studies, I have aimed at utilizing cancer cell plasticity by inducing their terminal differentiation into postmitotic adipocytes. Adipocytes are specialized robust cells; given their inherent growth arrest they are unlikely to adapt and dedifferentiate. Thus these are cells whose intra-cellular plasticity is inhibited. Since they are non-proliferative, many of the typical cancer mutations may become irrelevant in adipocytes.

By targeting cancer cell plasticity to force trans-differentiation into adipocytes we may be able to disarm malignant tumours both from their diversity and from their cellular plasticity. If such a conversion will be proved possible, we can potentially transform malignant tumours into a benign phenotype.

# 3 Results:

# Gain fat – lose metastasis:

## Converting invasive breast cancer cells into adipocytes

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## 3.1 Abstract

**A high degree of cell plasticity seems to promote malignant tumour progression, and an epithelial-mesenchymal transition (EMT) is suspected to provide cancer cells with increased cell plasticity for the development of metastasis and therapy resistance. Here, we have tested whether the EMT-induced cancer cell plasticity can be therapeutically exploited and we report the efficient conversion of breast cancer cells, which have undergone an EMT, into post-mitotic adipocytes. Delineation of the molecular pathways underlying such transdifferentiation has motivated a combination therapy with a MEK inhibitor and Rosiglitazone to demonstrate the conversion of invasive cancer cells into adipocytes and the repression of primary tumour invasion and metastasis formation in mouse models of breast cancer. The results indicate the high potential that lies in the increased cell plasticity of invasive cancer cells for differentiation therapy and they raise the possibility of pharmacological interference with tumour invasion and metastasis.**

## 3.2 Introduction

Cancer cell plasticity plays a critical role in cancer cell survival, invasion and metastasis formation (Massague and Obenauf, 2016), as well as in tumour heterogeneity (Koren et al., 2015; Van Keymeulen et al., 2015) and in the development of therapy resistance (Holohan et al., 2013; Jordan et al., 2016). Cancer cell plasticity is of a dynamic nature and can be the result of changing cues in the microenvironment (Oshimori et al., 2015). An epithelial-mesenchymal transition (EMT) seems to play a major role in facilitating cell plasticity in cancer and allows cancer cells to escape chemotherapies and targeted therapies by dedifferentiation and signalling adaption processes (Fischer et al., 2015; Gao et al., 2012b; Labelle et al., 2011; Valiente et al., 2014; Zheng et al., 2015). While an EMT is mainly responsible for primary tumour cell invasion, its reversal, a mesenchymal-epithelial transition (MET), has been shown to contribute to the metastatic outgrowth of disseminated

cancer cells in distant organs (Ocana et al., 2012; Tsai et al., 2012). Hence, the therapeutic reversion of an EMT could be counterproductive (Nieto, 2013). However, it has also been noted that cells undergoing an EMT and/or an MET are in a state of high cell plasticity and thus may offer a window of opportunity for therapeutic targeting (Brabletz, 2012a; Gupta et al., 2009; Nieto, 2013; Nieto et al., 2016).

The observation that cell dedifferentiation processes in cancer are often correlated with increased malignancy and cancer-initiating potential (Lawson et al., 2015; Oshimori et al., 2015; Tam and Weinberg, 2013) has readily lead to the appealing strategy of treating malignancies by inducing cancer cell differentiation. While attempted already in 1959 as a possible treatment for teratoma (Pierce and Dixon, 1959), a prominent example of a highly successful differentiation therapy has been the introduction of all-trans retinoic acid (ATRA) as a therapy for Acute Promyelocytic Leukemia (APL) (Wang and Chen, 2008). In carcinomas, therapeutic approaches to revert dedifferentiated cancer cells into normal epithelial cells have reduced cell proliferation and increased the sensitivity to chemotherapy (Girnun et al., 2007; Mueller et al., 1998; Wielenga et al., 2015). However, the dynamic nature of the processes underlying cell plasticity may explain the limited clinical benefit of epithelial re-differentiation therapy.

## 3.3 Results

### *3.3.1 EMT-Breast Cancer Cells Convert into Adipocytes*

We set out to assess whether the cell plasticity of cancer cells acquired by an EMT can be exploited to force their transdifferentiation into cells with benign traits, such as adipocytes. Adipocytes represent an ideal cell type for a benign trans-differentiation of malignant entities; they are usually benign and they present features of terminal differentiation and post-mitotic cell cycle arrest.

As cellular models of EMT-induced cancer cell plasticity we used Py2T murine epithelial breast cancer cells that undergo a reversible EMT upon

induction with transforming growth factor β (TGFβ) *in vitro* and gain mesenchymal characteristics when implanted into mice *in vivo* (Waldmeier et al., 2012). Py2T-LT cells are Py2T cells that have been treated with TGFβ for at least 20 days to induce a complete EMT program. As a second experimental system, we have used epithelial MTflECad murine breast cancer cells which carry floxed alleles of the E-cadherin gene (*Cdh1*) and undergo an EMT upon Cre-mediated genetic ablation of the E-cadherin gene (MTΔECad cells). They represent an irreversible EMT model of murine breast cancer cells with demonstrated cancer stem cell characteristics (Fantozzi et al., 2014).

We first optimized an adipogenesis protocol in 3T3-L1 pre-adipocytes which are well-known to differentiate into adipocytes when induced with insulin, Dexamethasone and Rosiglitazone (a potent ligand of peroxisome proliferator-activated receptor γ (PPARγ) (Gubelmann et al., 2014; Hong et al., 2005) **(Fig. 1a)**. For the induction of adipogenesis in breast cancer cells, this protocol was combined with bone morphogenetic protein-2 (BMP2), as suggested for multipotent mesenchymal stem cell differentiation (Gubelmann et al., 2014; Tseng et al., 2008). The generation of bona fide adipocytes was first assessed by staining with fluorescent Nile Red to visualize lipid droplets. The accumulation of lipid droplets was readily detected in both mesenchymal breast cancer cells but not in their epithelial counterparts (**Fig. 1a**). C/EBPα is a master regulator of adipogenesis and is expressed in the pre-adipocytic cell line 3T3-L1 when undergoing adipogenesis. Immunofluorescence analysis showed the presence of C/EBPα in mesenchymal Py2T-LT and MTΔECad cells. The expression of C/EBPα could not be detected in epithelial Py2T or MTflECad cells treated with the identical differentiation protocol (**Fig. 1a**). C/EBPβ, an early regulator of adipogenesis and gatekeeper of the adipogenic lineage determination (Raghav et al., 2012), was found absent in untreated and treated epithelial MTflECad cells, yet already expressed in untreated as well as in treated MTΔECad mesenchymal cells (**Extended Data Fig. 1a**).

Our main goal was to use adipogenesis of malignant cancer cells as a therapeutic option. Thus, we aimed at reducing the treatment protocol to a

minimal number of pharmaceutical compounds. In a minimal factor scenario, we were able to induce adipogenesis in mesenchymal breast cancer cells with Rosiglitazone alone at a concentration of 2µM. The efficiency of adipogenesis was further enhanced when combining Rosaglitazone with BMP2. Thus, all *in vitro* experiments described hereafter were performed using these two compounds, unless stated otherwise.

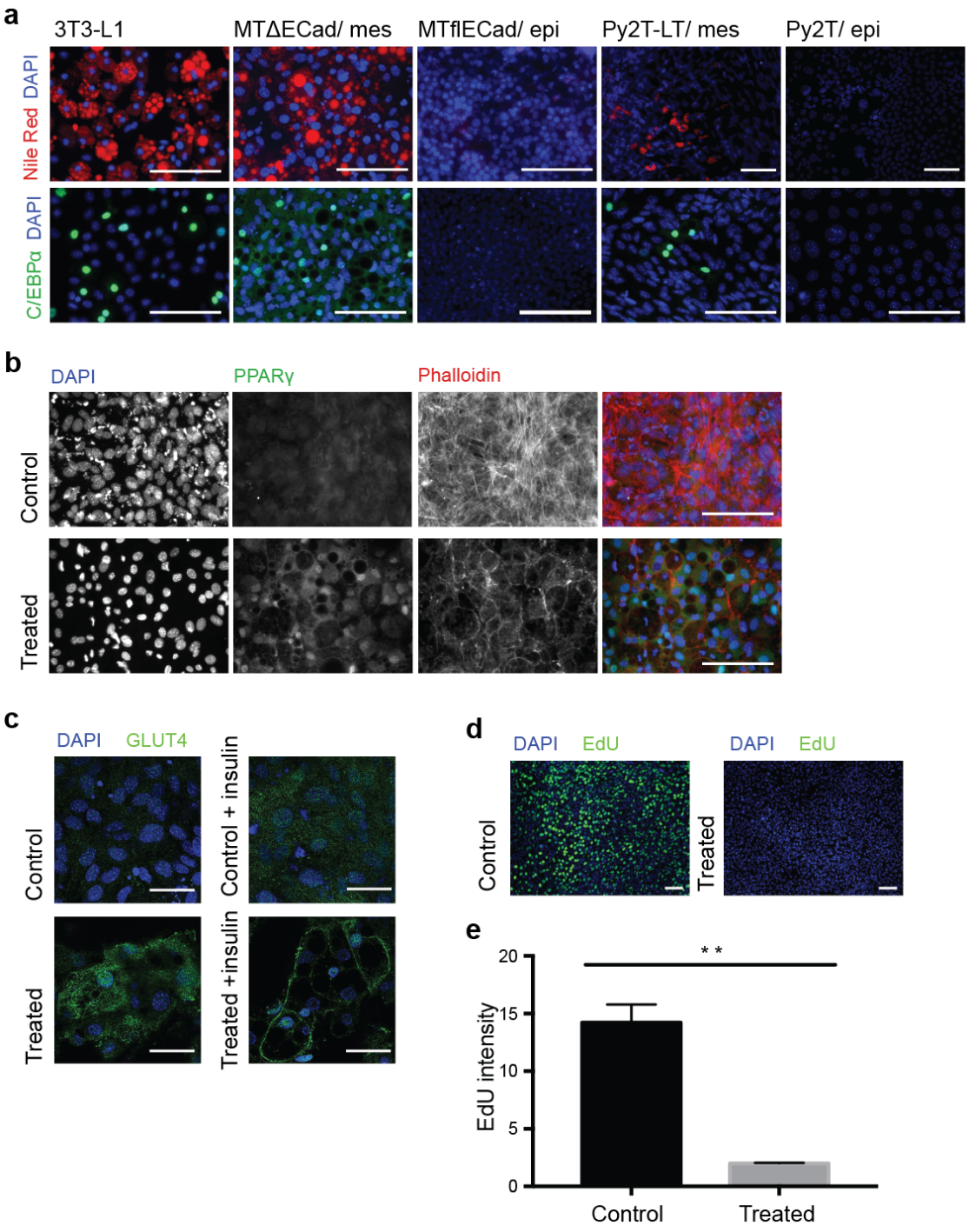



**Figure 1. Mesenchymal murine breast cancer cells can be induced to differentiate into functional adipocytes and to lose malignant characteristics.**

**(a)** Fibroblastic pre-adipocytes (3T3-L1) were treated with insulin, Dexamethasone and Rosiglitazone to induce adipogenesis. Mesenchymal (MTΔEcad and Py2T-LT; mes) and epithelial (MTflEcad and Py2T; epi) cancer cells were treated with insulin, Dexamethasone, Rosiglitazone and BMP2 to induce adipogenesis. Lipid droplets

were visualized by immunofluorescence microscopy with Nile Red (red), the adipogenesis regulator C/EBPα by a specific antibody (green), and nuclei by DAPI (blue).

**(b)** MTΔEcad cells were treated with Rosiglitazone and BMP2 (Treated) or DMSO solvent (Control) to induce adipogenesis. The adipogenesis regulator PPARγ (green), Phalloidin (F-actin, red) and nuclei (DAPI) were visualized by immunofluorescence staining.

(**c)** Control and Rosiglitazone/BMP2-treated MTΔECad cells were stimulated with insulin for 25 minutes to induce GLUT4 translocation to the plasma membrane. GLUT4 (green) and DAPI (blue) were visualized by immunofluorescence staining and confocal microscopy.

**(d, e)** Control and Rosiglitazone/BMP2-treated MTΔECad cells were incubated with EdU for 72 hours to quantify proliferating cells. Representative images of EdU staining (green) and DAPI staining are shown (blue) (d). EdU intensities were quantified using Image J. The graph shows means ± SEM; unpaired Student's t-test; **P =0.001 (e).

Scale bar, 100μm.

To further characterise the cancer cells undergoing adipogenesis we analysed their expression of mesenchymal and adipocyte-specific markers. A continuous increase in the expression of the adipogenesis master regulator PPARγ and of the adipokine produced by mature adipocytes, Adipoq, were detected by quantitative RT-PCR (**Extended Data Fig. 1b**) and by immunofluorescence microscopy analysis of differentiated cells (**Extended Data Fig. 2a,b**). Immunofluorescence microscopy analysis further showed the down-regulation of alpha-smooth muscle actin (αSMA) expression in differentiated adipocytes, a further sign for the immobilization and differentiation status of the cells (**Extended Fig. 2a**). Visualisation of the actin cytoskeleton by Phalloidin staining revealed a reorganization of mesenchymal stress fibres into cortical actin, a hallmark of cell immobilization, in both mesenchymal cell lines, even in the absence of E-cadherin expression in MTΔECad cells (**Fig. 1b and Extended Data Fig. 2b**). We further tested the functionality of the transdifferentiated adipocytes by inducing translocation of

the insulin-sensitive glucose transporter GLUT4 which is usually expressed in adipocytes, muscle cells and cardiomyocytes. MTΔECad cells were induced to undergo adipogenesis and then stimulated with insulin to activate GLUT4 translocation to the plasma membrane. Indeed, GLUT4 expression was increased in differentiated cells and differentiated cells responded to insulin stimulation by GLUT4 translocation to the plasma membrane (**Fig. 1c**).

As mentioned above, when considering cancer therapy, one of the most appealing characteristics of adipocytes is their cell-cycle arrest. We thus assessed the cell cycle status of transdifferentiated mesenchymal breast cancer cells. Adipogenesis-induced MTΔECad were incubated with EdU for 72 hours to quantify proliferating cells. The results revealed a significant decrease in proliferation of transdifferentiated adipocytes (**Fig. 1d,e**). Immunoblotting analysis also demonstrated the downregulation of phosphorylated histone 3 (pH3) and of cyclin D1, additional markers of cell proliferation (**Extended Data Fig. 2c**).

YAP and TAZ play a central role in tissue homeostasis, organ size regulation and tumourigenesis (Halder et al., 2012; Moroishi et al., 2015). YAP/TAZ transcriptional activation upon translocation to the nucleus induces cell proliferation and EMT (Diepenbruck et al., 2014; Moroishi et al., 2015). YAP/TAZ regulate also stem cell maintenance and differentiation and were shown to induce osteogenesis and inhibit adipogenesis in multipotent mesenchymal stem cells (Halder et al., 2012; Hong et al., 2005). YAP/TAZ cellular localization was visualized during MTΔECad- adipogenesis time course. During the same time course, cells were incubated with Edu for 24 hours to detect proliferating cells. The data demonstrates a progressive translocation and downregulation of YAP/TAZ expression during adipogenesis. As expected, YAP/TAZ translocation to the cytoplasm correlated with proliferation decrease (**Supplementary Data Fig. 1**).

Replacing the differentiation medium by normal culture medium for 7 days after differentiation treatment and subsequent Nile Red staining showed that differentiated cells did not revert to an epithelial or mesenchymal state and maintained the typical adipocyte morphology (**Supplementary Data Fig. 2**). These data demonstrate that breast cancer cells undergoing an EMT gain

the potential to irreversibly differentiate into *bona fide* adipocytes with post-mitotic cell cycle arrest. Epithelial cancer cells lack this transdifferentiation potential, supporting the notion that an EMT coincides with increased cell plasticity.

### *3.3.2 Multi-Differentiation Potential*

We next assessed whether the potency of EMT-derived breast cancer cells includes the ability to differentiate into further cell types of the mesenchymal lineage, such as osteoblasts and chondrocytes. MTΔECad cells were treated with differentiation protocols adapted from previous reports about multipotent mesenchymal cell differentiation to induce osteogenesis and chondrogenesis (Karamboulas et al., 2010; Mackay et al., 1998; Pittenger et al., 1999). Immunofluorescence microscopy analysis showed the presence of the osteoblast regulator, Osterix in osteogenesis-induced cells (**Fig. 2a**). Chondrogenesis-induced cells expressed Sox9, a master transcription factor of chondrogenesis, and chondrocyte-secreted collagen type 2 (Col2) (**Fig. 2b**). However, we did not optimize the protocols used in these experiments to achieve higher efficiency, since the therapeutic potential seemed less promising compared to adipogenesis of mesenchymal breast cancer cells. Nevertheless, the results underscore the high cell plasticity and transdifferentiation potential of breast cancer cells that have undergone an EMT.

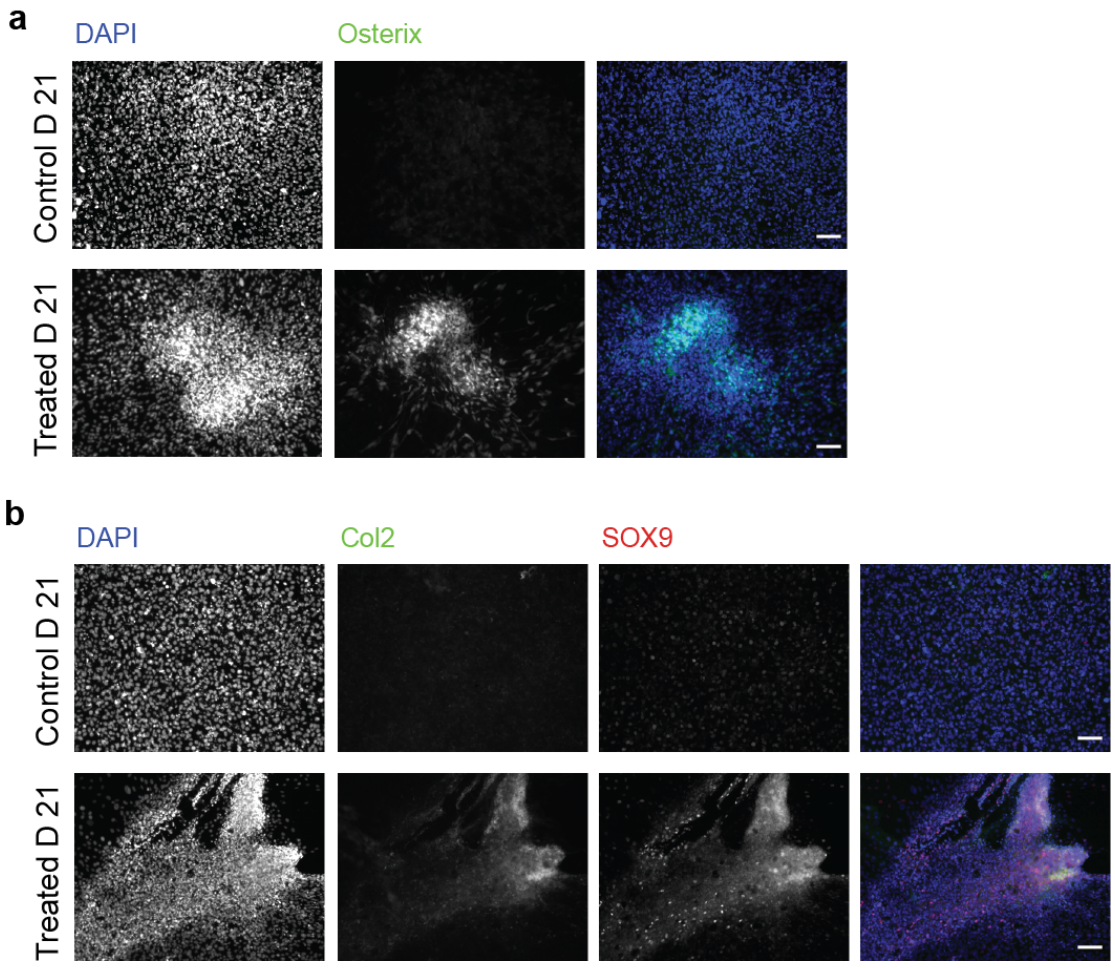



**Figure 2: EMT-derived murine breast cancer cells show multi-cell type differentiation potential.**

**(a)** Osteogenesis was induced in MTΔECad cells for 21 days (Treated D 21). The osteoblast-specific transcription factor Osterix (green) was visualized by immunofluorescence staining.

**(b)** Chondrogenesis was induced in MTΔECad cells for 21 days (Treated D 21). The chondrocyte transcription factor SOX9 (red) and cartilage-characteristic type II collagen (Col2, green) were visualized by immunofluorescence staining. Scale bars, 100µm.

### *3.3.3 MEK Inhibition Promotes Adipogenesis*

As previously reported, BMPs play a major role in inducing adipogenesis and are inducers of a MET (Samavarchi-Tehrani et al., 2010; Suenaga et al., 2013). On the other hand, TGFβ acts as an inhibitor of this differentiation

process, yet is one of the most potent inducers of an EMT, and TGFβ-signalling pathways are activated in metastatic cancer cells (Bruna et al., 2012; Oshimori et al., 2015). Indeed, the presence of TGFβ during the induction of adipogenesis repressed efficient adipogenesis of MTΔECad cells, while the inhibition of TGFβ signalling with a pharmacological inhibitor (SB431542) promoted adipogenesis (**Fig. 3; Extended Data Fig. 2b,c**). To overcome TGFβ-mediated inhibition of adipogenesis, we inquired which of the TGFβ-induced pathways was responsible for this inhibition. Immunoblotting analysis identified the non-canonical MEK/ERK pathway as the major inhibitor of adipogenesis in cancer cells (**Fig. 3a**). Other canonical (pSMAD2, pSMAD3) and non-canonical (pTAK1, pP38) TGFβ signalling pathways did not show a direct correlation with adipogenesis of mesenchymal breast cancer cells (data not shown). ERK has been previously reported to interfere with PPARγ function leading to obesity-linked insulin resistance (Banks et al., 2015). We thus tested whether inhibition of the MEK/ERK non-canonical TGFβ signalling pathway was able to overcome TGFβ-mediated inhibition of adipogenesis. Indeed, pharmacological inhibition of MEK activation with the MEK inhibitor (MEKi) PD98059 allowed the differentiation of mesenchymal cancer cells even in the presence of TGFβ as demonstrated by immunoblotting of adipocyte marker expression and Nile Red staining analysis (**Fig. 3a-c**).

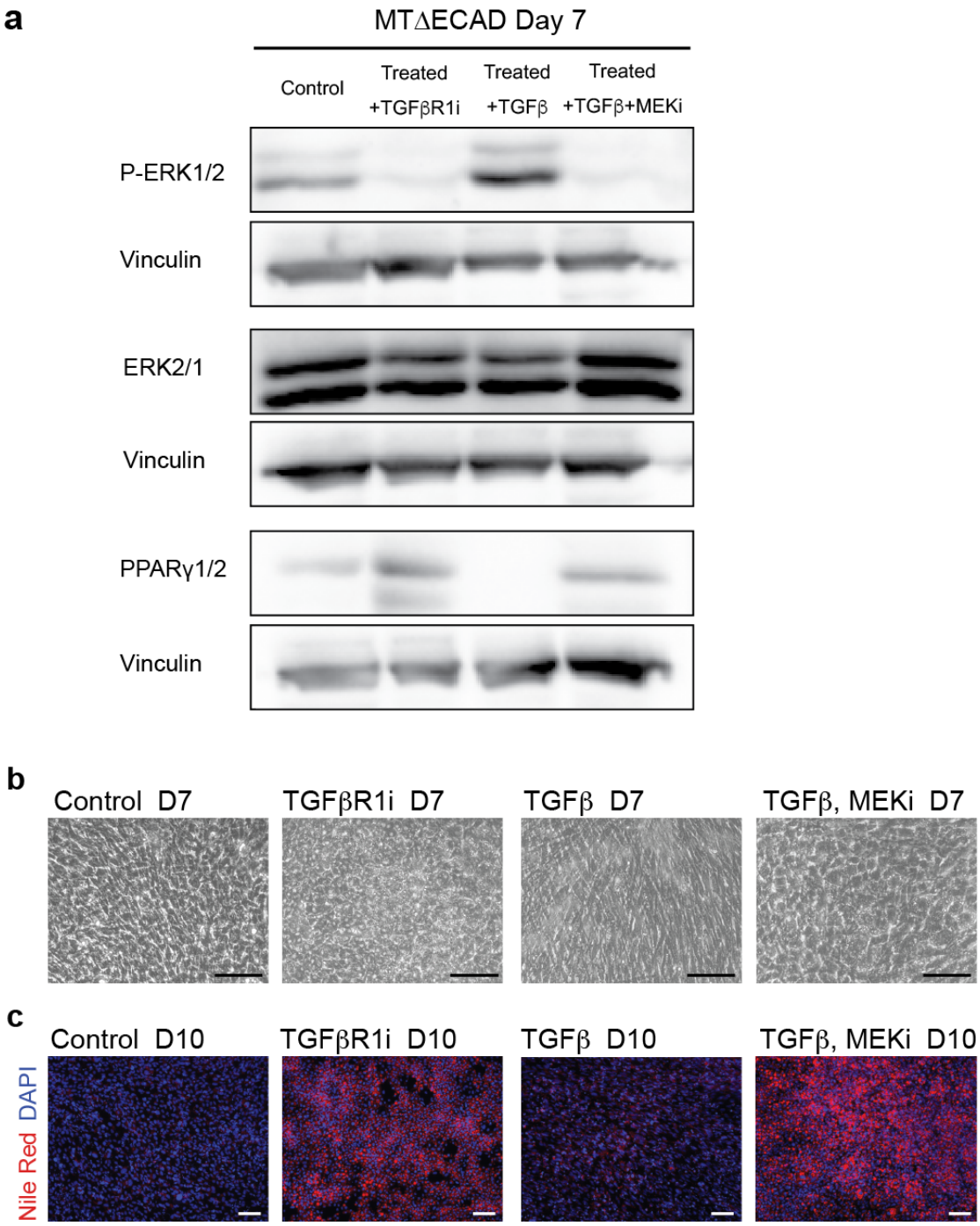



**Figure 3. Inhibition of MEK facilitates adipogenesis under TGFβ treatment.**

**(a, b, c)** MTΔECad cells were treated with Rosaglitazone and BMP2 (Control) and in addition with TGFβ, the TGFβ receptor inhibitor SB431542, and the MEK inhibitor (MEKi) PD98059 as indicated. After 7 days, the levels of phosphorylated ERK1/2 (P-ERK1/2) and total ERK (ERK1/2) as well as PPARγ1/2 were determined by immunoblotting analysis (a). After 7 days, phase contrast microscopy images were taken (b) and, after ten days, lipid droplets and nuclei were visualized by immunofluorescence Nile Red and DAPI stainings (c), Scale bars, 100µm.

### *3.3.4 Forced Adipogenesis Inhibits Tumour Invasion and Metastasis*

Overcoming the known TGFβ-mediated inhibition of adipogenesis motivated us to test our hypothesis *in vivo*. In a first proof-of-concept study, we transplanted GFP-expressing Py2T murine breast cancer cells, known to undergo EMT *in vivo* (Waldmeier et al., 2012), into the mammary fat pad of female RAG2-/-;common γ receptor -/- (RSG) mice. After an initial growth of the primary tumours, mice were divided into three groups with the following treatment protocols of two weeks: a control group treated with vehicle alone, a low treatment group treated with 16mg/kg Rosiglitazone and 2mg/kg MEKi PD98059, and a high treatment group treated with 16mg/kg Rosiglitazone and 5mg/kg MEKi PD98059. After termination of the treatment, the expression of the adipocyte markers Adipoq and Fatty acid binding protein 4 (FABP4) was found significantly increased in tumours of Rosaglitazone/MEKi-treated mice (**Extended Data Fig. 3a**). Interestingly, there was no significant change in the tumour mass between the treatment groups (**Extended Data Fig. 3b**), although MEK inhibition was anticipated to exert an inhibitory effect on cell proliferation.

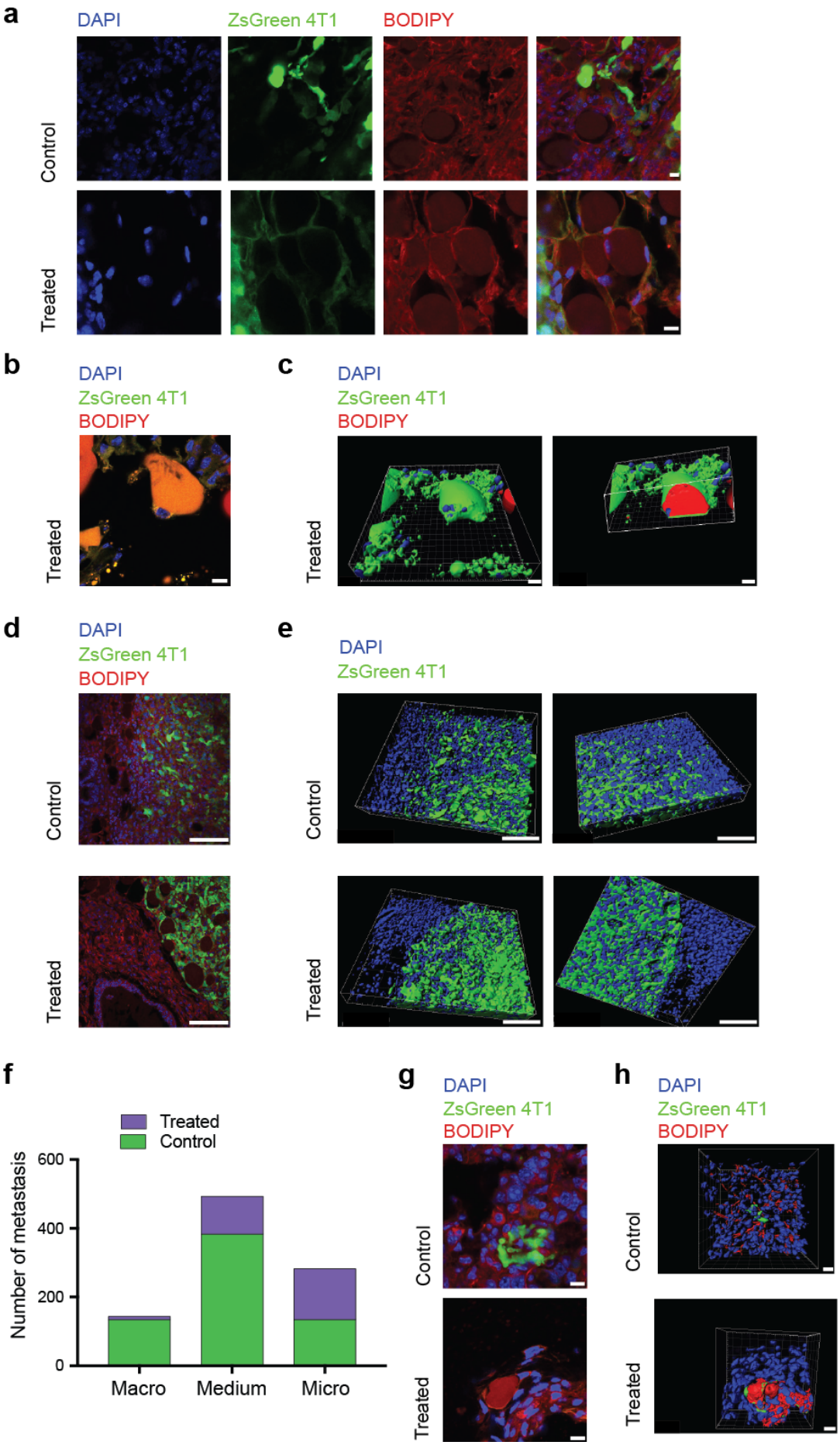
We next assessed whether the up-regulation of adipocyte markers is due to generally increased adipogenesis in the tumour stroma or due to the cancer cells' direct transdifferentiation into adipocytes. Lipid droplets accumulating within cells of tumour sections were visualized with red BODIPY dye and tumour cells were identified by GFP expression. Strikingly, many of the lipid droplets-containing cells in the tumours of Rosaglitazone/MEKi-treated mice expressed GFP and, thus originated from transplanted cancer cells. As a control for this co-localization between GFP and BODIPY we specifically searched for adipocytes in control-treated tumours. Here, the adipocytes of the mammary fat pad were negative for GFP (**Extended Data Fig. 3c-e**).

We next asked whether the direct differentiation of breast cancer cells into adipocytes might inhibit the metastatic dissemination or metastatic outgrowth of invasive cancer cells. ZsGreen-expressing 4T1 murine metastatic breast cancer cells were injected into the mammary fat pad of

immune-deficient nu/nu mice. After initial primary tumour growth, mice were divided into two treatment groups. The control group was treated with vehicle alone, and the experimental group was treated with 16mg/kg Rosiglitazone and 2mg/kg MEKi PD98059 for 17 days. Monitoring primary tumour growth in the individual mice showed that the primary tumours in the treatment group grew slower than in the control group, and primary tumour mass was significantly lower in treated mice upon termination of the experiment (**Extended Data Fig. 4a,b**).

To identify labelled tumour cells that had differentiated into adipocytes, tumour sections were stained with red BODIPY to visualize lipid droplets. Confocal microscopy revealed that tumours of mice treated with MEKi and Rosiglitazone, but not of control-treated mice, exhibited adipocytes expressing ZsGreen (**Fig. 4a-c)**. We further observed that these adipocytes predominantly localized at the rim of the tumour next to the normal mammary fat pad. Confocal microscopy and 3D reconstruction analysis of the tumour rims on thick histological tumour sections (80µm) revealed that the invasive fronts observed in the control-treated tumours were completely impeded in tumours of Rosaglitazone/MEKi-treated mice (**Fig. 4d,e**). An appealing explanation for this finding is that tumour cells at the invasive front undergo an EMT or EMT-like changes allowing their transdifferentiation into adipocytes, and that this forced differentiation of invasive tumour cells keeps the tumour in a non-invasive, benign state. Delayed primary tumour growth in treated mice may thus be explained by their less aggressive growth pattern and by an inhibitory effect of the MEKi on proliferation.

We then examined the effect of forced adipogenesis on metastasis formation in the two different treatment cohorts. For quantification of lung metastasis, metastatic nodules were classified in three groups: Macrometastases were defined as macroscopically identifiable metastases; medium-size metastases referred to microscopically detectable metastatic nodules, and micrometastases were individual cancer cells or cell clusters without clear metastatic outgrowth (**Extended Data Fig. 4c**). The number and size of metastases was found significantly reduced in the Rosiglitazone/MEKi-treated mice as compared to control-treated mice (**Fig. 4f)**.


a
DAPI
ZsGreen 4T1
BODIPY
Control
Treated
b
DAPI
ZsGreen 4T1
BODIPY
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c
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d
DAPI
ZsGreen 4T1
BODIPY
Control
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Control
Treated
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Number of metastasis
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Control
Treated

**Figure 4. *In vivo* adipogenesis of metastatic 4T1 murine breast cancer cells correlates with decreased invasion and metastatic colonization.**
ZsGreen-expressing 4T1 cells were injected into the mammary fat pad of female NMRI nu/nu mice. After initial primary tumour growth, mice were treated daily with vehicle (Control) or 2mg/kg MEKi PD98059 and 16mg/kg Rosiglitazone (Treated). After 17 days of treatment, mice were euthanized, and tumours and lungs were processed for histopathological analysis. n=6 mice per group.
**(a)** 558/568 BODIPY (red) was used to visualize lipid droplets on 80µm thick frozen sections. Tumour cells are visualized by ZsGreen and nuclei by DAPI staining. Shown are representative images of tumour cells containing lipid droplets in tumours of treated mice (Treated) but not in tumours of control-treated mice (Control).
**(b,c)** 7µm thick tumour sections were stained with BODIPY to identify adipocyte structures formed by differentiated ZsGreen-expressing 4T1 cells (green) using confocal microscopy. DAPI staining visualizes nuclei (b). 3-dimensional reconstructions of the confocal microscopy images using Imaris software (c). Scale bars, 10µm.
**(d,e)** Representative confocal microscopy images of an invasive (Control) and a non-invasive, smooth (Treated) tumour rim bordering to the mammary fat pad on 80µm thick sections stained with BODIPY (red). Tumour cells were visualized by ZsGreen and nuclei by DAPI staining (d). 3-dimensional reconstructions of (d) excluding BODIPY staining. Nuclei are visualized by DAPI staining. Scale bars, 100µm.
**(f)** Quantification of lung metastasis in treated and control mice. The graph shows the number of metastases in each group (Control = green, Treated = purple) classified by size in macro, medium or micrometastases. Chi-square test, P-value < $2^{-16}$.
**(g,h)** 80µm thick frozen sections of lungs were stained with BODIPY (red) to visualize adipocytes in the lungs using confocal microscopy (g) and 3-dimensional reconstruction (h). Tumour cells were visualized by ZsGreen and nuclei by DAPI staining. Scale bars, 10µm.

We then assessed whether the treatment was able to induce adipogenesis in tumour cells invading the lungs. Thick histological lung sections (80µM) were stained with BODIPY to visualize lipid droplets. Here, BODIPY staining led to an extensive background staining of all cells. Nevertheless, individual ZsGreen and BODIPY double-positive tumour cells

were detected in the lungs of treated mice but not in control-treated mice (**Fig. 4g,h**). Together, the data indicate that the forced adipogenesis of invasive tumour cells results in a reduction of primary tumour growth and invasion and of metastasis formation.

### *3.3.5 Cancer Cell Plasticity Facilitates Adipogenesis*

The fact that we achieved a nearly 100% efficiency in converting murine breast cancer cells into adipocytes *in vitro* allowed a whole transcriptomic survey of changes in gene expression during a time course of mesenchymal breast cancer cells (MTΔECad) undergoing transdifferentiation into terminally differentiated adipocytes. Cells were harvested at six different time points during the adipogenesis time course and subjected to RNA sequencing (**Fig. 5a**). Gene expression analysis documented a full transcriptomic conversion of malignant to benign differentiation. A clustered heat map of most variable genes in the time-course of differentiation demonstrated the extensive molecular changes cancer cells undergo when forced into adipogenesis (**Fig. 5b**). Notably, among the increased expression of adipocyte-specific genes, this analysis also identified the differentiated cells as white fat adipocytes expressing Resistin (Retn) and no UCP1, a marker for brown fat adipocytes **(Fig. 5c)**.

Analysis of cell cycle-related genes showed the down-regulation of proliferation-related genes alongside with the up-regulation of cell cycle arrest-related genes (**Fig. 5d,e**). This data further supports our findings that cancer cells undergoing adipogenesis exit the proliferative state and undergo cell-cycle arrest (see also **Fig. 1d,e and Extended Data Fig. 2c**).

The gene expression data also provided insights into the possible mechanisms of how an EMT/MET interconversion may manifest cell plasticity and allow the transdifferentiation of breast cancer cells into adipocytes. For example, we noted that BMP2 had a crucial effect on the efficiency of adipogenesis in MTΔECad cells, yet not in Py2T-LT cells as shown by immunoblotting analysis and by Nile Red staining (**Extended Data Fig. 5**).

When inducing adipogenesis in Py2T-LT cells, TGFβ signalling had to be repressed to overcome its inhibitory effect on adipogenesis. However, the cells then slowly revert to their epithelial state by a MET. In contrast, MTΔECad cells lack E-cadherin and cannot revert back to the epithelial state, and we speculate that BMP2 initiates a MET-like process in MTΔECad cells *in vitro* resulting in the enhancement of cell plasticity. This notion is supported by the loss of mesenchymal marker gene expression and by the up-regulation of MET inducers, such as KLF4 (Tiwari et al., 2013), at the beginning of the transdifferentiation process (**Fig. 5f**). Notably, the transcription factors Zeb1 and Zeb2, well-known inducers of EMT (Chaffer et al., 2013) as well as of adipogenesis (Gubelmann et al., 2014), are upregulated by BMP2 (**Fig. 5f**).

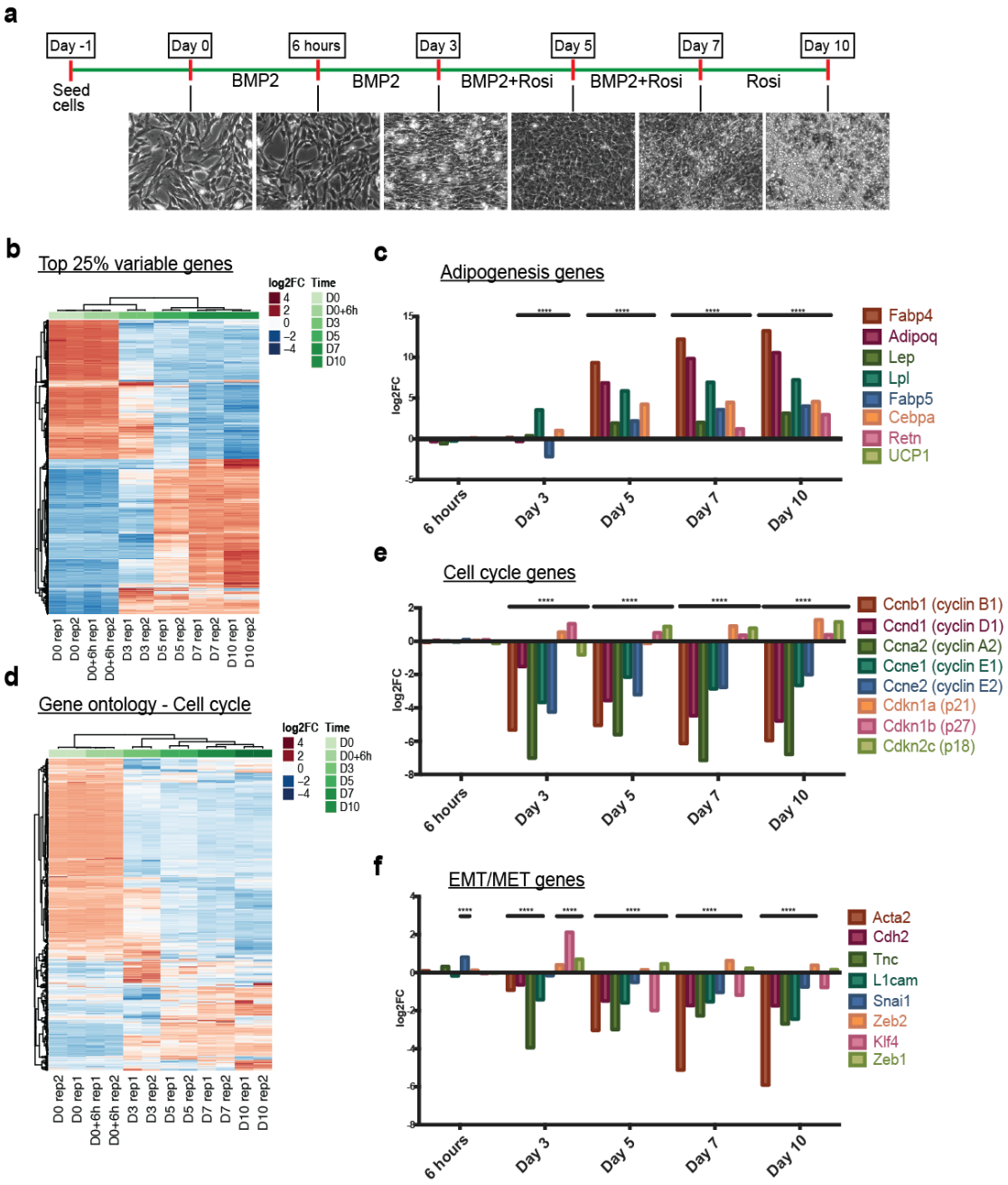



**Figure 5. The transcriptome of cancer cell adipogenesis.**

**(a)** Schematic representation of the treatment protocol and timed microphotographs of MTΔECad cells undergoing forced adipogenesis *in vitro*. RNA was extracted for RNA sequencing at the days indicated.

**(b)** Heat map of the top 25% most variably expressed genes during MTΔECad cell adipogenesis.

**(c)** RNA sequencing analysis of selected adipogenesis-related genes at different time points of MTΔECad cell adipogenesis.

**(d)** Heat map of cell cycle-related genes genes annotated in the GO database (GO:0007049) during MTΔECad cell adipogenesis.
**(e)** RNA sequencing analysis of cell cycle-related genes at different time points of MTΔECad cell adipogenesis.
**(f)** RNA sequencing analysis of selected EMT/MET-related genes at different time points of MTΔECad cell adipogenesis.

## 3.4 Discussion

Taken together, our data demonstrate that EMT in cancer correlates not only with malignant progression, but also with increased cell plasticity. This status of high cell plasticity in cancer cells can be exploited *in vitro* and *in vivo* to force differentiation into terminally differentiated and post-mitotic adipocytes with benign traits. Our proof-of-concept experiments in mouse models highlight the relevance of cancer plasticity not only as a means of cancer dissemination and survival but also as a possibility for novel therapeutic approaches. We note that the tested approaches have targeted specifically invasive, malignant cancer cells, thus efficiently repressing primary tumour invasion and metastasis, yet leaving highly proliferating epithelial tumour cells untouched. Therefore, further studies will be required to target tumour invasion and metastasis by the adipocyte transdifferentiation therapy reported here in combination with repressing tumour cell proliferation by cytostatic therapy. Since EMT has been demonstrated to increase resistance to chemotherapy and targeted therapy, we envision that the ablation of invasive mesenchymal cancer cells by transdifferentiation therapy will overcome therapy resistance and cancer relapse. Of course, improving and optimizing cancer cell transdifferentiation/adipogenesis with newer and more potent pharmacologicals will be the next step before assessing the clinical benefit of this approach.

## 3.6 Material and Methods

*Cell lines and cell culture*

3T3-L1 cell line was previously described (Green and Kehinde, 1975) and was a kind gift from Professor B Deplancke (EPFL, Lausanne). Py2T, MTflECad, MTΔECad were previously described (Fantozzi et al., 2014; Waldmeier et al., 2012). Cells were routinely tested for Mycoplasma contamination.

All cells were cultured in Dulbecco's modified eagle medium (DMEM; Sigma-Aldrich) supplemented with fetal calf serum (FCS, 10 %; Sigma-Aldrich), glutamine (2 mM; Sigma-Aldrich), penicillin (100 U; Sigma-Aldrich) and streptomycin (0.2 mg/l; Sigma-Aldrich). All cell lines were grown at 37 °C, 5 % $CO_2$, 95 % humidity.

All *in vitro* experiments were independently repeated at least 3 times. No samples have been excluded from the analysis.

*Antibodies and fluorescent dyes*

C/EBPβ (E299, Abcam), PPARγ (81B8, Cell Signaling), Adiponectin (C45B10, Cell Signalling), ERK Diphospho (M8159, Sigma), ERK total (M7927, Sigma), Vinculin (S19, Santa Cruz), GLUT4 (ab65267, Abcam), PH3 (Upstate), Smad3 (9523, Cell Signaling), Smad3-Phospho (9520, Cell Signaling), α-SMA (F3777, Sigma), Nile Red (Sigma), BODIPY 558/568 (D3835, Thermo Fisher), BODIPY 493/503 (D3922, Thermo Fisher), Yap/Taz (sc-101199, Santa Cruz).

*In vitro adipogenesis*

Cells were seeded at density of 20,000 cells/$cm^2$ and incubated overnight at 37°C in 5% $CO_2$. To induce differentiation of 3T3-L1, the growth medium was replaced with medium containing 5µg/ml insulin, 1µM Dexamethasone (dissolved in ethanol), 2µM Rosiglitazone (dissolved in DMSO) for 48 hours, then in medium containing 5µg/ml insulin for 48h, then in medium containing 1µM Rosiglitazone. To induce differentiation in cancer cells, cells were seeded and incubated overnight, incubated with 200ng/ml human recombinant BMP2 (Sigma) for 3 days with 200ng/ml BMP2 and 2µM Rosiglitazone (Rosi) for 4 days, and with medium containing 2µM

Rosiglitazone for another 3 days. The addition of other growth factors or inhibitors is indicated in the figures, usually from day 0 to day 7 such as human recombinant TGFβ1 at 2ng/ml, the MEK inhibitor PD98059 at 40μM and the TGFβR1 inhibitor SB431542 at 10μM. Control cells were treated with medium containing DMSO.

*In vitro chondrogenesis*

Cells were seeded at a density of 60,000 cells/$cm^2$ in normal growth medium (DMEM supplemented with glutamine, penicillin, streptomycin and 10% FBS) and incubated at 37°C in 5% $CO_2$ over night. Medium was removed and cells were washed with serum free Opti-MEM media (to remove traces of serum). Differentiation medium: Opti-MEM serum free media (Gibco) containing ITS 1% (BD), Dexamethasone 10uM (Sigma Aldrich), Ascorbate 100 uM (Sigma Aldrich) and 10ng/ml human recombinant TGFβ1. One day later, medium was removed, and cells were washed. From day 2 to day 21, differentiation medium was replaced every 48 hours containing 10ng/ml human recombinant BMP2 and BMP4. For control cells normal culture medium was used.

*In vitro osteogenesis*

Cells were seeded at density of 20,000 cells/1$cm^2$ in normal growth medium (DMEM supplemented with glutamine, penicillin, streptomycin and 10% FBS) and incubated at 37°C in 5% $CO_2$ for 48 hours. From day 2 until day 21 differentiation medium was used: Supplemented DMEM containing 1mg/ml β-Glycerophosphate (Calbiochem), 10-7M Dexamethasone and 100μM L-Ascorbate, 1mM Sodium Pyruvate (Sigma) and 10 ng/ml human recombinant BMP4 was added each time. Control cells were incubated with culture medium.

*In vivo adipogenesis*

For primary tumour growth experiments, $0.5x10^6$ GFP-expressing Py2T cells were injected into the mammary fat pad of 8-10 weeks old female RAG2-/-;common γ receptor -/- (RSG) mice. After 2 weeks of initial tumour growth,

tumour volume was measured, and mice were divided into a control group (n=7), low treatment group (n=7) and high treatment group (n=7).
Test on volume in the first day: Equal variances assumption does not hold, there for Wilcoxon test was used on each pair of groups, none of the three comparisons was significant:

| **Comparison (tumour volume)** | **Result** |
|---|---|
| Control vs low treatment | W=24, P-value=0.731 |
| Control vs high treatment | W=15, P-value=0.61 |
| Low treatment vs high treatment | W=12, P-value=0.79 |

Mice were treated daily with i.p injections of drugs or vehicle for two weeks. High dose group, PD98059 5mg/kg and Rosiglitazone 16mg/kg dissolved in vehicle; low dose group, PD98059 2mg/kg and Rosiglitazone 16mg/kg dissolved in vehicle; control group, vehicle alone (4%DMSO, 30% PEG300, 5% Tween 80). Not included animals were euthanized during the experiment. Final group size: Control group (n=6), low treatment (n=7) and high treatment group (n=4).

For tumour invasion and metastasis experiments, 8 weeks old female NMRI nu/nu mice were injected into the mammary fat pad with $0.5x10^6$ ZsGreen-expressing 4T1 cells. After 5 days of initial tumour growth, tumour volume was measured, and mice were divided into a control group (n=6) and a low dose group (n=6) treated as above. Test on volume in the first day: Equal variances assumption does not hold, there for Wilcoxon test was used. The groups' means are not different W=14.5, P-value=0.624. Mice were treated daily with i.p. injections of drugs or vehicle for 17 days.

Animals were sacrificed, tumour volumes and weights were measured, and tumours and lungs were embedded in paraffin for H&E staining and OCT for immunofluorescence staining and frozen for RNA isolation.

All studies involving mice have been approved by the Swiss Federal Veterinary Office (SFVO) and the Cantonal Veterinary Office of Basel Stadt (licenses 1878, 1907, and 1908).

*Lung metastasis quantification and statistical analysis*

The dataset consists of 12 mice, for each mouse the number of metastases was counted and classified by size types: “Macro”, “Medium” or “Micro” across 9 slides per size type. The test aims to detect dependency between the metastases’ size type and group, using chi-square test on the frequency table below:

| | **Macro** | **Medium** | **Micro** | **Total** |
|---|---|---|---|---|
| Control | 134 | 383 | 134 | 651 |
| Treatment | 9 | 110 | 148 | 267 |
| Total | 143 | 493 | 282 | N=918 |

*RNA isolation and RT-PCR*

Total RNA was prepared using TriReagent (Sigma-Aldrich) for cells or RNeasy mini kit (74104, Qiagen) for tissues. For RNA sequencing total RNA was isolated using miRNeasy mini kit (217004, Qiagen). RNA was reverse transcribed with M-MLV reverse transcriptase (Promega), and transcripts were quantified by PCR using SYBR-green PCR MasterMix (Invitrogen). Riboprotein L19 primers were used for normalization. PCR assays were performed in triplicate, and fold induction was calculated using the comparative Ct method (ΔΔ Ct). Primers used for quantitative RT-PCR are listed in table 1

*Immunofluorescence microscopy*

Cells were fixed with 4%PFA and permeabilized with 0.5% NP40. Cells were blocked with 3%BSA and incubated with primary antibody for 1.5h at room temperature or overnight at 4°C. After washing, cells were incubated with

secondary antibodies for 1h at room temperature, washed and incubated with DAPI for 10 minutes, and washed and mounted with fluorescent mounting medium (Dako). Samples were imaged with a confocal microscope (LSM 510 Meta, Zeiss or Leica SP5) or with a fluorescence microscope (Leica DMI4000).

*GLUT4 translocation*

Before and after the full ten-days adipogenesis protocol, cells were incubated for 25 minutes with 100nM insulin or normal growth medium and subsequently analysed for the expression and localization of GLUT4 by immunofluorescence microscopy.

*EdU incorporation to detect proliferating cells*

Cells before and after a full ten-day adipogenesis protocol were incubated for 24 hours with 1µM EdU (Invitrogen) or 72 hours with 0.1µM EdU. Cells were fixed with 4% PFA for 20 minutes, washed with PBS, permeabilized with 0.5% NP40 and washed with PBS-T (PBS + 0,01% TX-100). ClickIT reaction was performed according to the manufacturer's protocol (BaseClick), cells were washed with PBS-T and used for immunostaining.

*BODIPY staining on frozen sections*

Cryosections (7µM or 80µM) were rehydrated in PBS and incubated with BODIPY in the dark at room temperature for 30 minutes. Cells were washed and incubated with DAPI for 10 minutes, then washed and mounted with fluorescent mounting medium (Dako).

*Immunoblotting*

Cells were lysed in boiling lysis buffer (0.29M Tris-HCl PH6.8, 4.7% SDS, 23% glycerol). Protein concentration was determined using the BCA assay kit (Pierce). Equal amounts of protein were diluted in SDS-PAGE loading buffer (10% glycerol, 2% SDS, 65 mM Tris, 1 mg/100 ml bromophenol blue, 1% β-mercaptoethanol) and resolved by SDS-PAGE. Proteins were transferred to nitrocellulose, 0.45µM pore size membranes by wet transfer, blocked with 5%

skim milk powder in TBS/0.05% Tween 20 and incubated with the antibodies indicated. HRP-conjugated secondary antibodies were detected by chemiluminescence using a Fusion Fx7 chemiluminescence reader (Vilber Lourmat, France).

*RNA sequencing analysis*

Total RNA was isolated from cells of 2 independent experiments using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instruction. RNA quality control was performed with a fragment analyser using the standard or high sensitivity RNA analysis kit (DNF-471-0500 or DNF-472-0500) from Labgene and RNA concentration was measured by using the Quanti-iT$^{TM}$ RiboGreen RNA assay kit (Life Technologies/Thermo Fisher Scientific). 200 ng of RNA was utilized for library preparation with the TruSeq Stranded Total RNA LT Sample Prep Kit (Illumina). Poly-A+ RNA was sequenced by HiSeq SBS kit v4 (Illumina) on an Illumina HiSeq 2500 using protocols defined by the manufacturer.

Obtained single-end RNA-seq reads (63-mers) were mapped to the mouse genome assembly, version mm10, with RNA-STAR (Dobin et al., 2013), with default parameters except for allowing only unique hits to genome (outFilterMultimapNmax=1) and filtering reads without evidence in spliced junction table (outFilterType="BySJout"). Using RefSeq mRNA coordinates from UCSC (genome.ucsc.edu, downloaded in December 2015) and the qCount function from QuasR package (version 3.12.1), we quantified gene expression as the number of reads that started within any annotated exon of a gene. The differentially expressed genes were identified using the edgeR package (version 1.10.1). Genes with FDR smaller than 0.05 and minimum log2 fold change of +/-1 were used for downstream analysis.

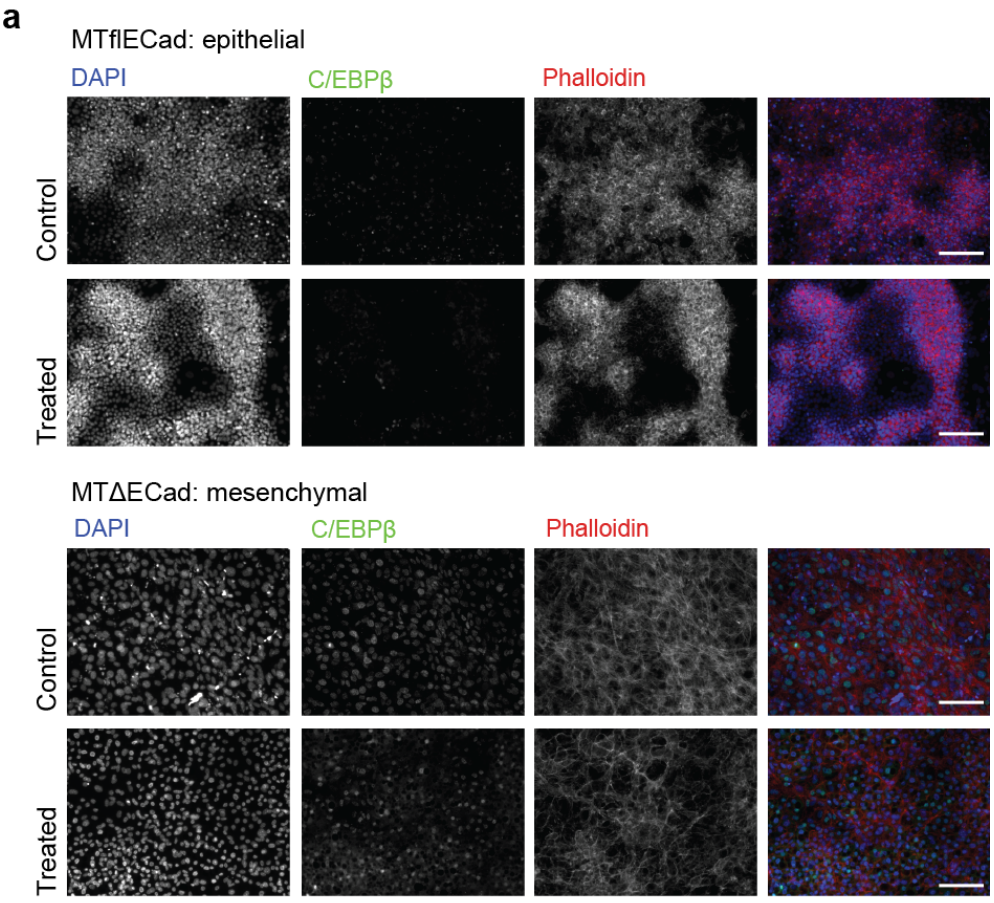
*Statistical analysis*

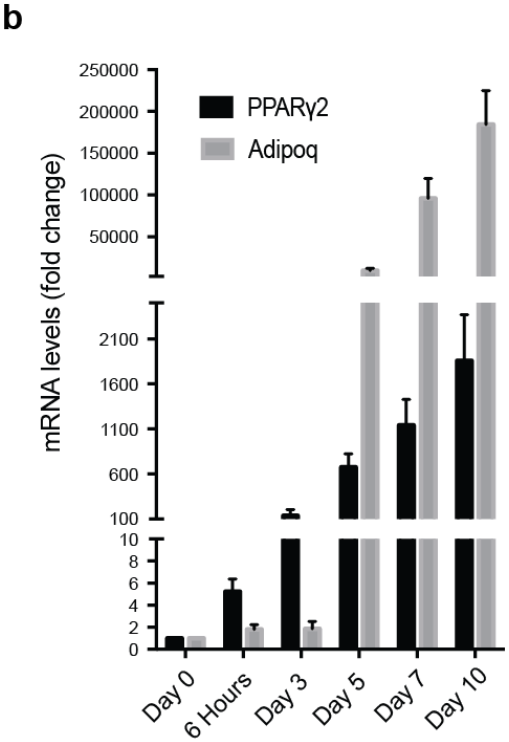
Statistical analysis and graphs were generated using the R software and GraphPad Prism software. Statistical analysis was preformed as indicated in the figure legends.

**Table 1. RT-PCR primers**

| Gene (mouse) | Primers | |
|---|---|---|
| Pparγ2 | GCTGTGAAGTTCAATGCACTGG | GCAGTAGCTGCACGTGCTCTG |
| Adipoq | TGTTCCTCTTAATCCTGCCCA | CCAACCTGCACAAGTTCCCTT |
| FABP4 | GATGCCTTTGTGGGAACCT | CTGTCGTCTGCGGTGATTT |

## 3.7 Extended Data Figures

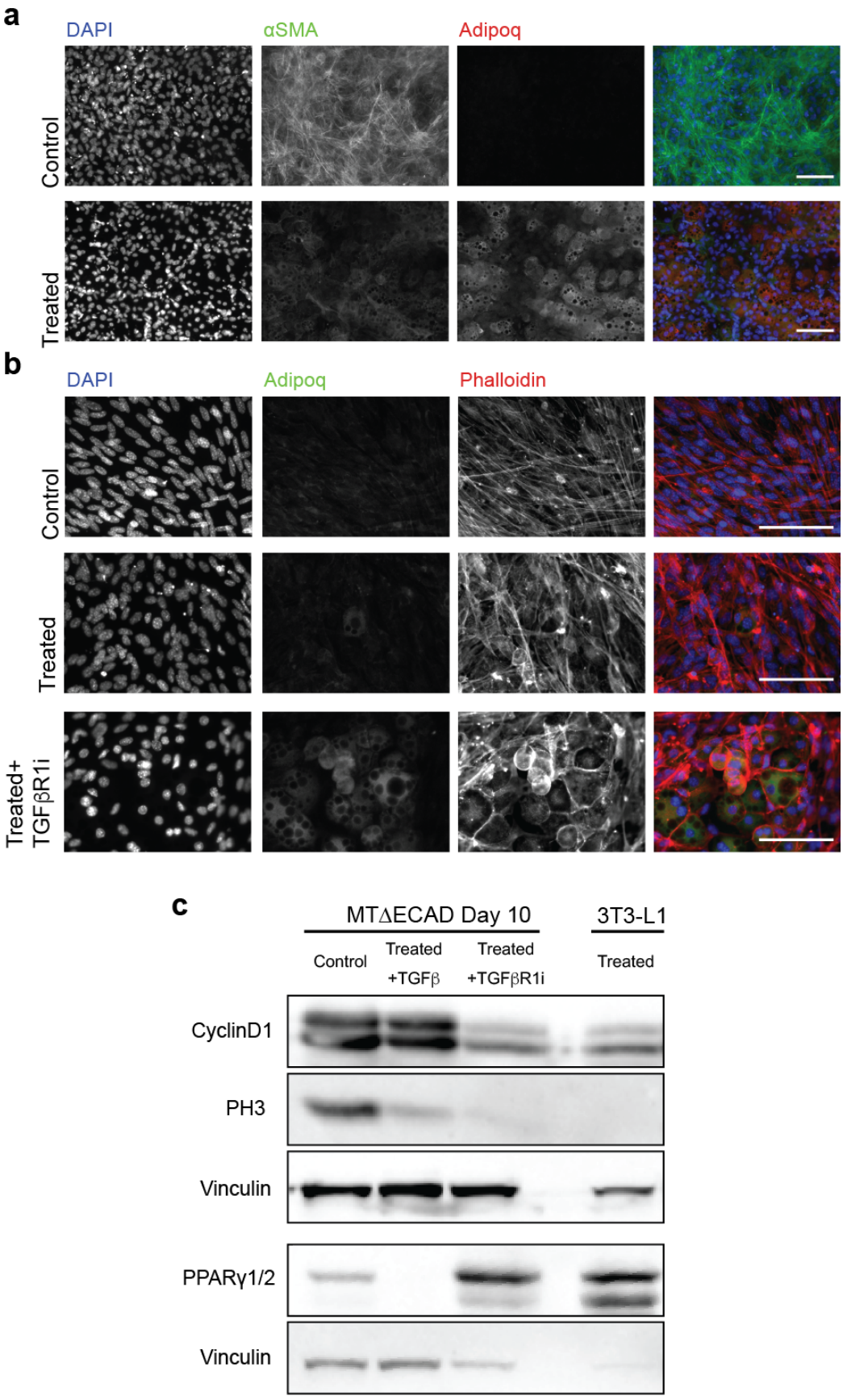


**Extended Data Figure 1. Adipogenesis can be induced in EMT-derived cancer cells but not in their epithelial ancestors.**

**(a)** Epithelial MTflEcad cells (upper panels) and mesenchymal MTΔECad cells (lower panels) were treated to induce adipogenesis. The early adipogenesis regulator C/EBPβ and the actin cytoskeleton (Phalloidin) were visualized by immunofluorescence stainings. Scale bars, 100µm.

**(b)** Adipogenesis was induced in MTΔECad cells for 10 days, and mRNA levels of PPARγ2 (early adipogenesis) and Adipoq (mature adipocytes) were quantified by quantitative RT-PCR at the time points indicated. The graph shows means ± standard deviation (s.d.).

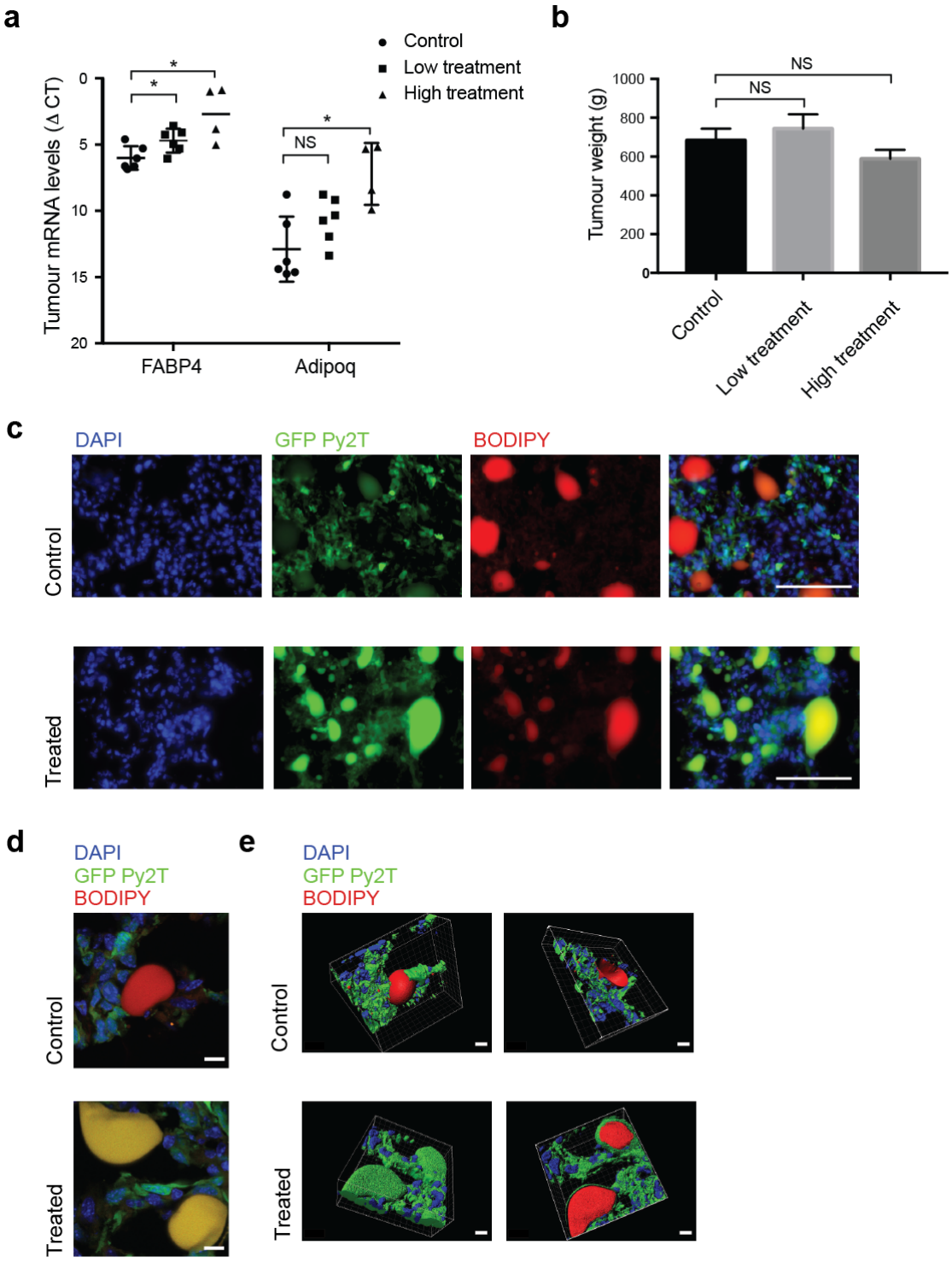

a
DAPI
αSMA
Adipoq
Control
Treated
b
DAPI
Adipoq
Phalloidin
Control
Treated
Treated+
TGFβR1i
c
MTΔECAD Day 10
3T3-L1
Control
Treated
+TGFβ
Treated
+TGFβR1i
Treated
CyclinD1
PH3
Vinculin
PPARγ1/2
Vinculin

**Extended Data Figure 2. Cancer cells undergoing post-mitotic differentiation into adipocytes lose their invasive, mesenchymal traits.**

**(a)** Immunostaining of adipogenesis-induced MTΔECad cells (Treated) or control-treated cells (Control) for the mesenchymal marker alpha smooth muscle actin (α-SMA, green) and for the adipocyte marker Adipoq (red). Scale bars, 100μm.

**(b)** Immunostaining of adipogenesis-induced or control-treated Py2T-LT cells for Adipoq (green) and the actin cytoskeleton (Phalloidin, red). The cells were in addition treated with TGFβR inhibitor (TGFβRi; SB431542) as indicated. Scale bars, 100μm.

**(c)** MTΔECad and in 3T3-L1 were treated to undergo adipogenesis for 10 days (Treated) or treated with solvent (Control). MTΔECad cells were in addition treated with TGFβ or TGFβR1 inhibitor (TGFβR1i; SB431542) as indicated. Levels of proliferation-related phospho-histone 3 (PH3) and CyclinD1 as well as of the adipogenesis regulator PPARγ were determined by immunoblotting analysis.




**Extended Data Figure 3. GFP-labelled Py2T breast cancer cells undergo differentiation into adipocytes *in vivo*.**

GFP-expressing Py2T cells were injected into the mammary fat pad of female RAG2 -/-;common γ receptor -/- (RSG) mice. After initial tumour growth, mice were treated daily with vehicle (Control) or 2mg/kg MEK inhibitor (MEKi, PD98059) and 16mg/kg Rosiglitazone (Low treatment) or 5mg/kg MEKi (PD98059) and 16mg/kg Rosiglitazone (High treatment). After 14 days of treatment mice were sacrificed and tumours sectioned and stained.

**(a)** mRNA levels of Adipoq and FABP4 in tumours of control, low treatment and high treatment groups were detected by quantitative RT-PCR. The graph shows means ± standard deviation (s.d.). Student's t-test; P-value: * < 0.05; NS, not significant.

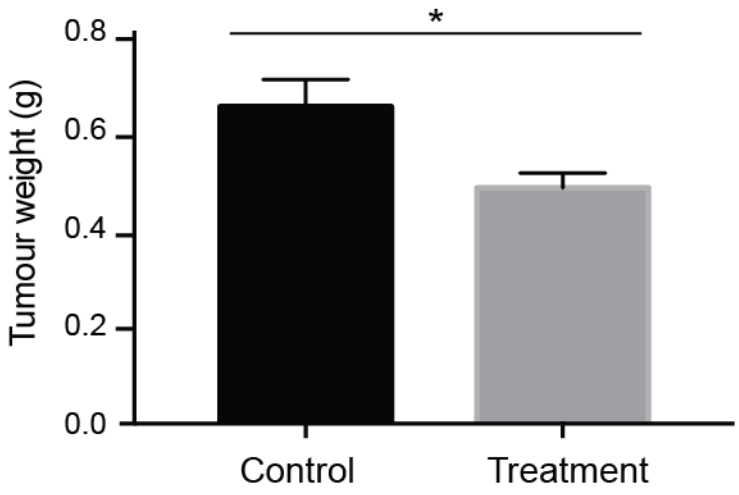
**(b)** Tumour weights in mice of the control, low treatment and high treatment groups. The graph shows means ± SEM. Student's t-test; NS, not significant.

**(c)** Representative images of adipocytes inside tumours in both control and treated mice. Lipid droplets were visualized with BODIPY (red) and fluorescence microscopy; Scale bars, 100μm.

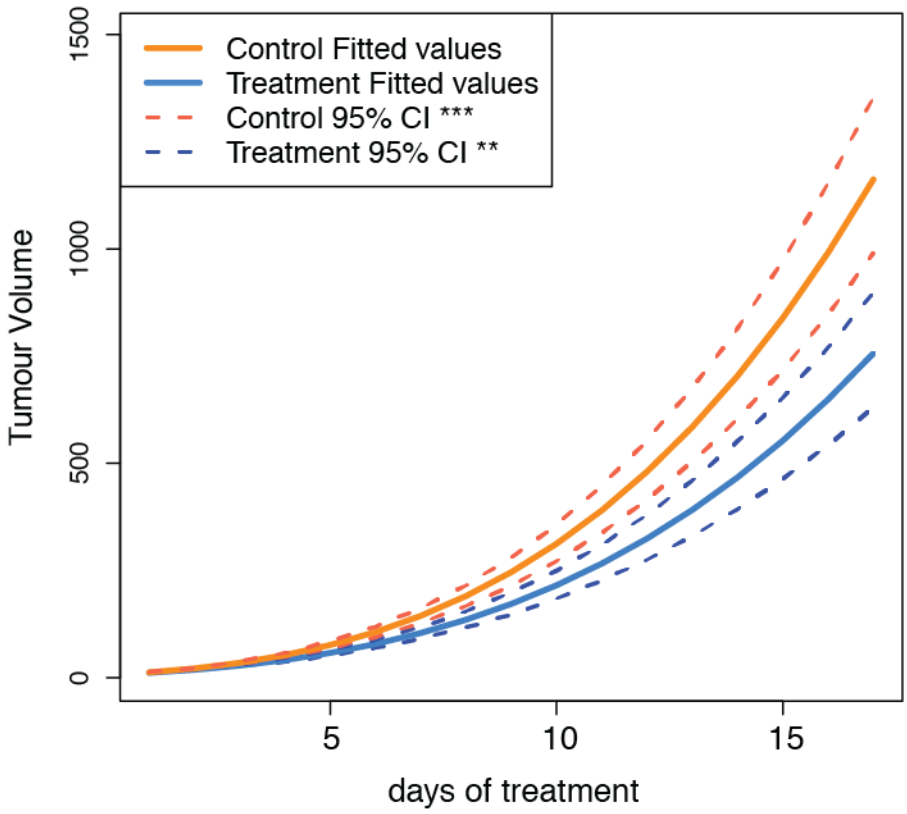
**(d)** Co-localization of GFP (labelled Py2T cells) and BODIPY was visualized by confocal microscopy in control and treated tumours. Scale bars, 10μm.

**(e)** The adipocyte cellular structure of (d) was visualized by 3-dimensional reconstruction using Imaris software. Scale bars, 10μm.

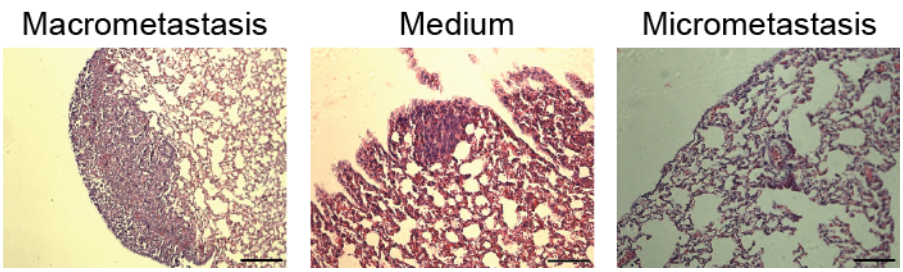
**a**


*
0.8
0.6
0.4
0.2
0.0
Tumour weight (g)
Control
Treatment

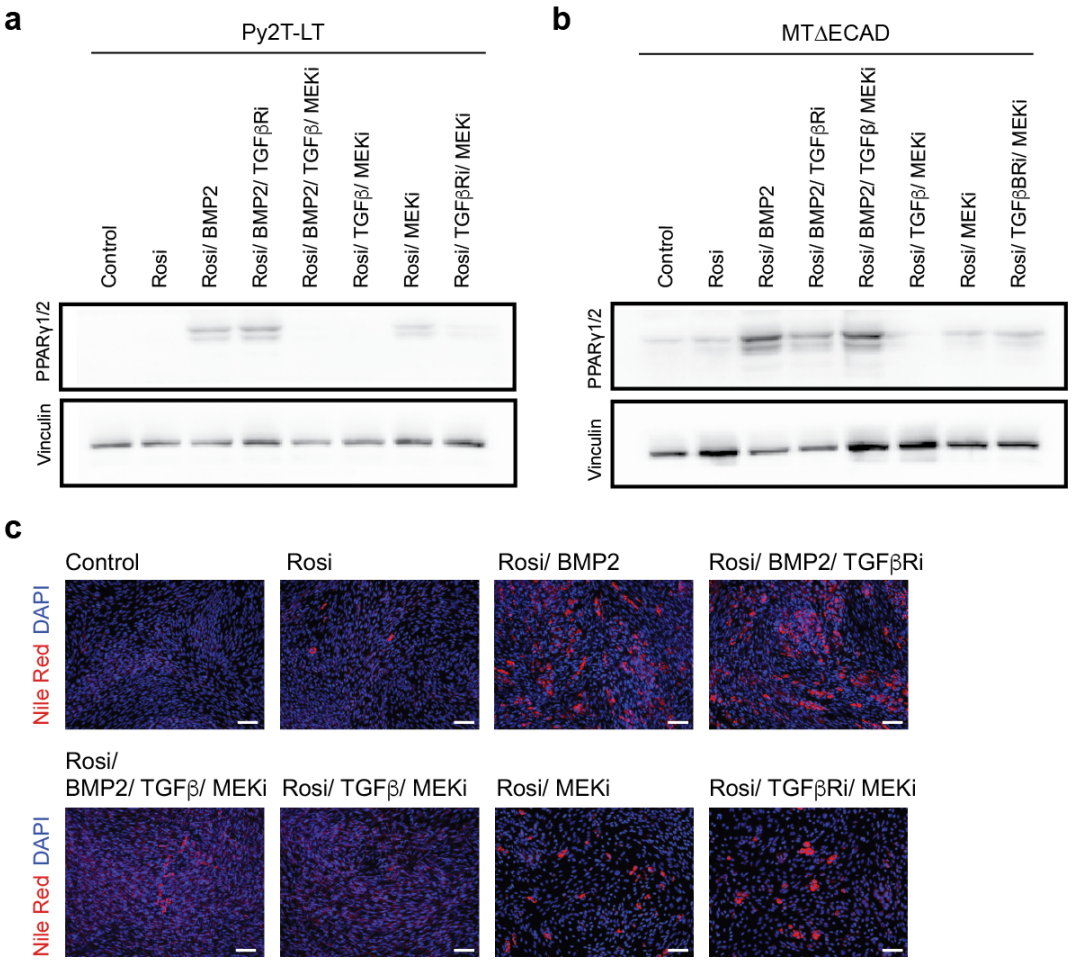
**b**


Control Fitted values
Treatment Fitted values
Control 95% CI ***
Treatment 95% CI **
1500
1000
500
0
Tumour Volume
5
10
15
days of treatment

**c**


Macrometastasis
Medium
Micrometastasis

**Extended Data Figure 4 *In vivo* adipogenesis of metastatic 4T1 murine breast cancer cells correlates with decreased invasion and metastatic colonization.**
Further analysis of the experiment described in Figure 4.
**(a)** Tumour weights of control or treated animals were determined after euthanasia. The graph shows means ± SEM. Student's t-test; P-value < 0.05.
**(b)** Tumours size was measured at least once per week over time and after euthanasia (day 17). A random intercept regression model with an interaction term between the group and day of measurement was fitted after performing box-cox transformation on the volume with $\lambda = 0.303$. Graph shows transformed volume for fitted values as well as the 95% confidence interval (CI) in each group, control (orange) and treatment (blue). Significant interaction term between the group factor and number of days (P-value: * = 0.0011)
**(c)** Lungs were sectioned and stained by hematoxilin and eosin (H&E). Representative images of macroscopically detectable lung metastases (Macrometastasis), medium-sized metastases (Medium), and microscopically visible, infiltrating tumour cells (Micrometastasis) are shown.

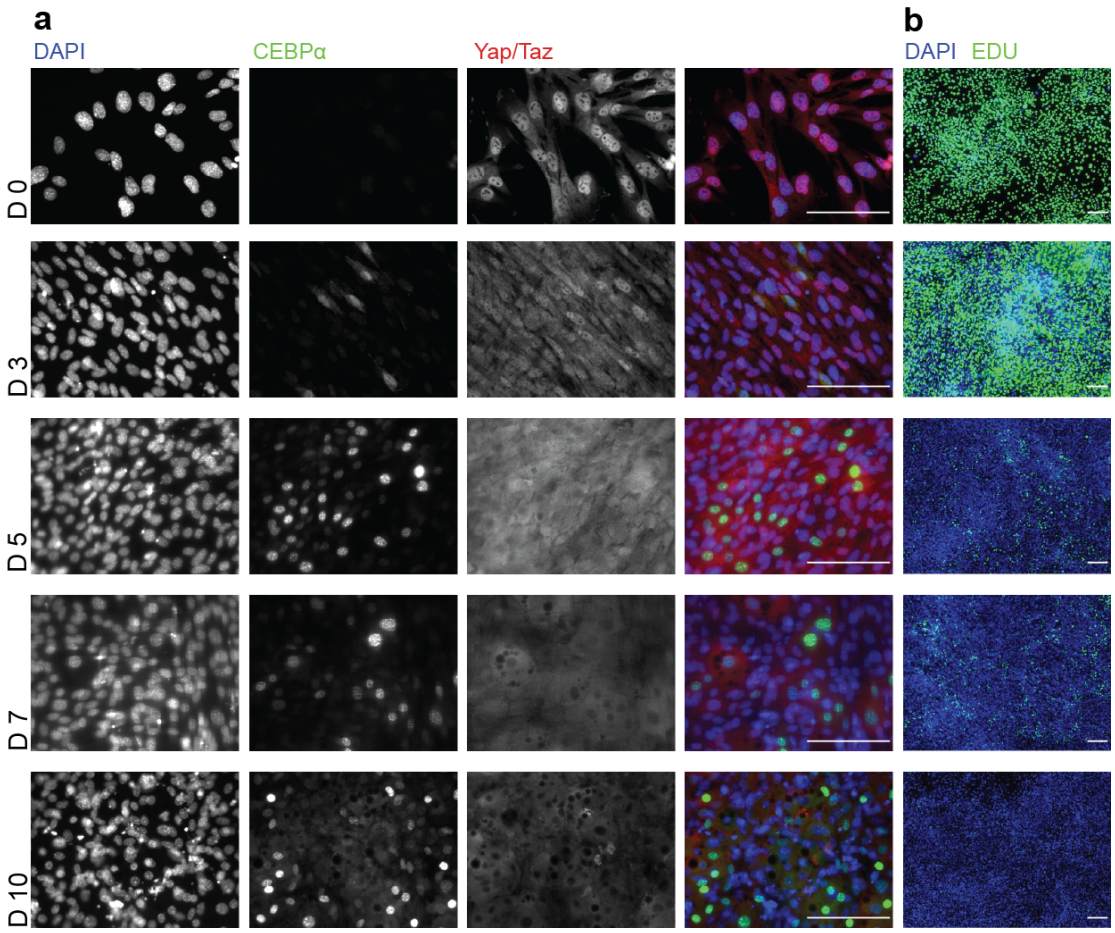



**Extended Data Figure 5. Role of BMP2 in adipogenesis of EMT-derived cancer cells.**

**(a,b)** Mesenchymal Py2T-LT cells (a) and MTΔECad cells (b) were treated as indicated for 7 days and the expression of the adipogenesis regulator PPARγ was analysed by immunoblotting. Detection of vinculin was used as loading control.

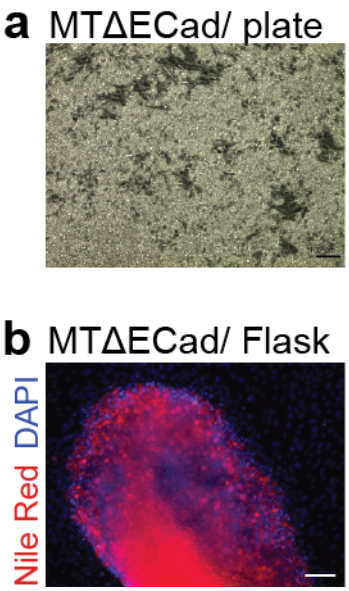
**(c)** Py2T-LT cells were treated as indicated for 10 days and then stained with Nile Red (red) to visualize lipid droplets. (TGFβR1i, SB431542; MEKi, PD98059; Rosi, Rosiglitazone). Scale bars, 100μm.

## 3.8 Supplementary Data Figures




**Supplementary Figure 1: Yap/Taz translocation during adipogenesis correlates with decreased proliferation**

**a,** During adipogenesis induction in MTΔECad cells, Yap and Taz (red) localization and Cebpα (green) expression were visualized by immunofluorescence staining. **b,** The same cells were incubated with EdU for 24 hours to detect proliferating cells. Representative images show EdU (green) and DAPI (blue) stainings. Scale bars: 100µm




**Supplementary Figure 2: Adipocyte- derived breast cancer cells revert neither to mesenchymal nor to epithelial cells**

**a, b,** MTΔECad cells were treated to induce adipogenesis. Cells were incubated for further 7 days with normal culture medium and representative images were taken (**a**). Cells were trypsinized and replated in a collagen I pre-coated- flask. The flask was filled completely with culture medium and placed up side down to allow adipocytes to adhere to the flask's bottom. After 7 days of incubation medium was removed and cells stained with Nile Red (Red) to visualize lipid droplets (**b**). Scale bars: 100µm.

# 4 Outlook and Future Plans

The introduction section defines cellular plasticity as a quality of the cell that determines the cell's capacity to adapt to environmental cues. EMT and MET represent a paradigm to plasticity, they are evident in various types of cells and have been demonstrated essential for many biological processes. I argue that EMT and MET can be viewed as processes contemporaneous to the cell differentiation state and are therefore activated during cellular adaptation and modification processes, including cellular reprogramming to iPSC. Given the enhanced plasticity acquired by EMT/MET in different cell types, it will be necessary to reveal the molecular essence of these processes. Distinguishing cell plasticity events from cell identity-related processes in EMT could substantiate our understanding of cell plasticity.

In order to identify the molecular plasticity mechanisms underlying EMT/MET, we have recently initiated a study comparing the transcriptomic changes during EMT/MET and cell reprogramming in iPSC. We ask whether a plasticity signature can be revealed when finding the similarities between these processes. Such a signature can potentially be composite of specific gene expression or favourable reciprocal connection between different recognized cellular mechanisms. We speculate that understanding the molecular mechanisms underlying cell plasticity will enable a better understanding of cell fate decisions and cancer plasticity.

It is likely that the EMT/MET processes observed *in vivo* are only partial and/or transient. To evaluate the role of EMT/MET in cancer progression and validate the potential of adipogenesis therapy, lineage-tracing studies are required. The monitoring of cancer cells that have initiated an EMT program, even if not to its full extent, will allow an assessment whether adipogenesis utilizes the acquired plasticity in all cells. Zeb1 has been suggested as a crucial EMT transcription factor associated with plasticity and important also for adipogenesis. Therefore, a possible approach is to generate 4T1 breast cancer cells carrying a Zeb1 promoter-reporter and

transplant them into mice for adipogenesis treatment. This could be used to detect tumour cells that had initiated an EMT program. Correlating Zeb1 expression with the expression of adipogenesis genes may provide additional insights into the molecular mechanisms underlying cell plasticity and into the efficiency of differentiation therapy to target cancer cell plasticity.

Cancer cell dormancy seems to facilitate cancer cell survival and is an underlying mechanism for cancer latency. Adipogenesis, on the other hand, requires cell cycle arrest to allow terminal differentiation, and adipocytes represent postmitotic cells. It is therefore possible that adipogenesis therapy can target and force differentiation also of dormant cancer cells. This may allow a therapeutic approach to inhibit breast cancer recurrence. However, further studies on the effect of adipogenesis treatment on dormant bone marrow-infiltrating tumour cells will be required.

As described in the discussion section of this work, adipogenesis therapy is aimed to target specifically cancer cell plasticity. Non-invasive tumour cells and re-differentiated tumour cells at the metastatic organ will require classical treatment with targeted therapies or chemotherapy. It is intriguing to test the benefit of combining these approaches in mouse models of breast cancer. Notably, it is plausible that adipogenesis will inhibit the development of drug resistant cells.

Finally, MEK inhibition, though clinically used for cancer treatment, exhibits high toxicity. Delineating the role of BMP2 in breast cancer cell adipogenesis *in vitro* and the transcriptomic analysis of this process will be instrumental for identifying new specific targets to induce adipogenesis. To reveal the role of BMP2 derived from MTΔEcad and Py2T-LT cells treated for seven days with the adipogenesis differentiation protocol with and without BMP2. The analysis of these data will provide further insights into the pathways that can induce adipogenesis in malignant cancer cells.

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