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Engineering Transcription Factors in Breast Cancer Stem Cells

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1. Introduction

Breast cancers are classified in at least six different subtypes (normal-like, luminal A, luminal B, Her2, basal-like, claudin-low), which are characterized by distinct genome-wide transcriptional profiles and response to therapy [1]. Recently, it has been shown that these intrinsic types of breast cancers are associated with unique DNA-methylation patterns [2,3,4]. In 2009, a large-scale genomic analysis of breast cancer cohorts has identified a novel subtype of breast cancer enriched in putative cancer stem-cell (CSC) markers, named claudin-low [5]. In addition to cancer or stem cell signatures, claudin-low tumors are enriched in Epithelial-to-Mesenchymal transition (EMT) markers, such as high expression of the Transcription Factors (TFs) Twist and Snail, and loss of epithelial junction proteins, such as cadherins, claudins and ocludins. Together with basal-like breast cancers, claudin-low carcinomas are mostly triple negative, hence their lack of expression of the Estrogen Receptor (ER), Progesterone Receptor (PR) and Her2. Consequently, these carcinomas are refractory to regimens to treat breast cancers, such as anti-estrogens and conventional chemotherapy. Similarly to these breast cancers, a subtype of serious epithelial ovarian cancers also appear to be poorly differentiated, high grade, and associated with poor clinical outcome. These serous epithelial ovarian tumors, named type II, are often associated with p53 and BRCA mutations [6]. Thus, there is a need to develop novel and more effective strategies to target poorly differentiated carcinomas. This will begin with a better understanding of molecular pathways that are activated in these tumors, which maintain aberrant proliferation and potentially, tumor initiation.

Little is known regarding the molecular determinants of tumor initiation and progression in poorly differentiated cancers. it has been proposed that claudin-low and basal-like breast tumors are originated by oncogenic transformation of bipotent stem and progenitor cells, respectively. Consistent with this idea, we found that many Transcription Factors (TFs) normally expressed in both, adult and embryonic stem cells (hESCs), are also over-expressed in poorly differentiated breast and ovarian carcinomas. In the first part of this chapter we will overview oncogenic TFs and TF networks that could play a role in maintaining aberrant self-renewal, with special focus on the OCT4-SOX2-NANOG embryonic TF network. In addition to abnormal reactivation of oncogenic TFs, tumor suppressor genes undergo epigenetic silencing

processes during tumor initiation, such as p16^{INK4A} a critical factor involved in immortalization of normal epithelial cells. *Mammary serine protease inhibitor* (*maspin*) is a tumor suppressor epigenetically silenced during metastatic progression of several epithelial cancers, including breast carcinomas. In the second part of the chapter we will describe the design of Artificial Transcription Factors (ATFs) and their potential applications to redirect or reprogram the epigenetic and transcriptional state of aggressive carcinomas towards a more benign or less aggressive phenotype. ATFs are typically composed of arrays of specific six Zinc Finger (ZF) domains, and are designed to bind, unique eighteen base pairs (bps) in targeted promoters. These ZFs are linked to several effector domain functions, which mediate activation, repression or epigenetic regulation of the gene of interest [7]. We will review ATFs generated in our laboratory and others, able to reprogram the epigenetic status of endogenous promoters, oncogenes and tumor suppressors, to revert some of the phenotypic hallmarks of aggressive cancer cells.

1.1 Overview of molecular subtypes of breast cancers

Large-scale genomic analyses of breast cancer patients have revealed that breast cancer is, clinically and biologically, a heterogeneous disease. Moreover, gene expression microarray technology has stratified breast cancer patients in distinct subtypes: normal-like, luminal A, luminal B, Her2, basal-like, claudin-low [1,8,9]. Recently, a novel subtype of breast cancer has been discovered, named claudin-low [5]. Although this subtype affects 5-10 % of all breast cancers, is associated with poor response to conventional therapy and with tumor relapse. Together with the basal-like subtype, the majority of claudin-low carcinomas are triple negative, (Estrogen Receptor (ER), Progesterone Receptor (PR) and Her2 negative [8,10].

One of the molecular hallmarks of claudin-low tumors is the down-regulation of tight junction proteins, such as claudin 3, 4, 7 and E-cadherin (CDH1), and an over-representation of mesenchymal proteins characteristic of stromal cells, such as vimentin. Claudin-low carcinomas express TFs associated with epithelial-to-mesenchymal transition (EMT), including Snail1/2, Twist1/2 and ZEB1/2. Additional characteristics of claudin-low tumors are the enrichment in putative cancer stem-cell signatures, such as high CD44 and low CD24 levels, high degree of lymphocyte infiltration, and resistance to chemotherapy [8,10,11,12].

The cell of origin of breast tumors. Although genome-wide expression analyses have clearly defined subtypes of breast cancer patients, the cell of origin that is target of transformation is still subject of intense investigation and debate. To isolate and characterize the target cell giving rise the distinct subtypes of breast cancer is crucial in order to develop effective, targeted, and tailored therapies. It is generally accepted that human mammary epithelial cell preparations are composed of a hierarchic organization of cell types, where more undifferentiated, multipotent stem cells, can either remain quiescent, self-renew (generating progeny of undifferentiated cells identical to the mother cells) or differentiate towards specific, defined lineages (Figure 1). In addition to specific transcriptional profiles, the subtypes of cells within the breast hierarchy are characterized by distinct epigenetic landscapes, including DNA-methylation [13], and possibly, histone modifications. In addition, stromal cells surrounding the epithelial ductal structures of the mammary gland highly influence the degree of self-renewal or differentiation of stem and progenitor cells. Stromal-tumor cell interactions are of critical importance during tumor formation and progression [14]. For example, Mesenchymal stem cells (MSCs) originated in the bone-

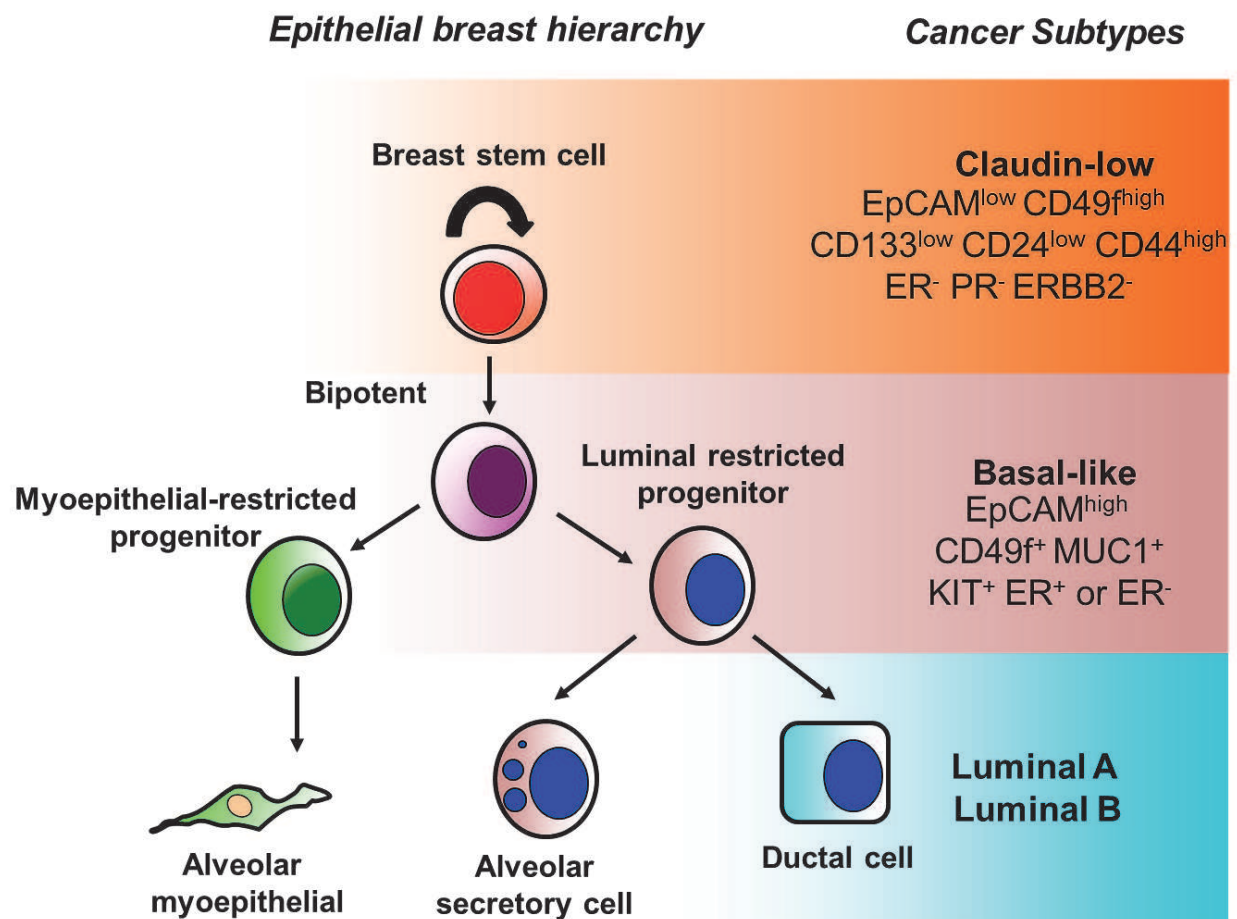## Epithelial breast hierarchy   Cancer Subtypes

Fig. 1. Schematic representation of the human breast epithelial cell hierarchy and its association with the main breast cancer subtypes. Each subpopulation of breast cells is defined by the expression of selected cell surface markers. Gene expression profiles of these cell populations upon fractionation of the mammary gland and cell sorting, revealed similarities to specific subtypes of breast cancer. Modified from [25].

marrow can home to breast tumors and promote metastasis [15,16]. Fibroblasts or "tumor-associated fibroblasts" can similarly influence the growth and motility of breast epithelial cells [14,17].

Mammary stem cells (MSCs). The mammary epithelium is composed of two main lineages of epithelial cells: an inner layer of luminal cells and an outer sheath of myoepithelial cells (Figure 2). These cells compose the basic architecture of the human mammary gland, named terminal duct lobular units (TDLUs) [18,19]. MSCs are defined as cells having self-renewal ability and able to generate all the cell types of the mammary epithelium [20]. Progenitor cells are more restricted or committed cells having proliferative capabilities. Both stem and primitive progenitors are believed to reside within the ducts of the mammary gland, rather than in the terminal ductal lobular units. Bipotent epithelial cells are capable to fully differentiate in luminal and myoepithelial cells. These cells retain the ability to generate entire TDLUs both *in vitro* and also upon transplantation in nude mice [18,19,20,21]. These primitive cells occupy a suprabasal position *in vivo* and co-express the luminal keratin K19 and the basal-specific keratin K14. The most likely candidates for MSCs are basal positioned small electro-lucent (light) cells (SLCs), occurring at a frequency of 1-3% within the epithelium.
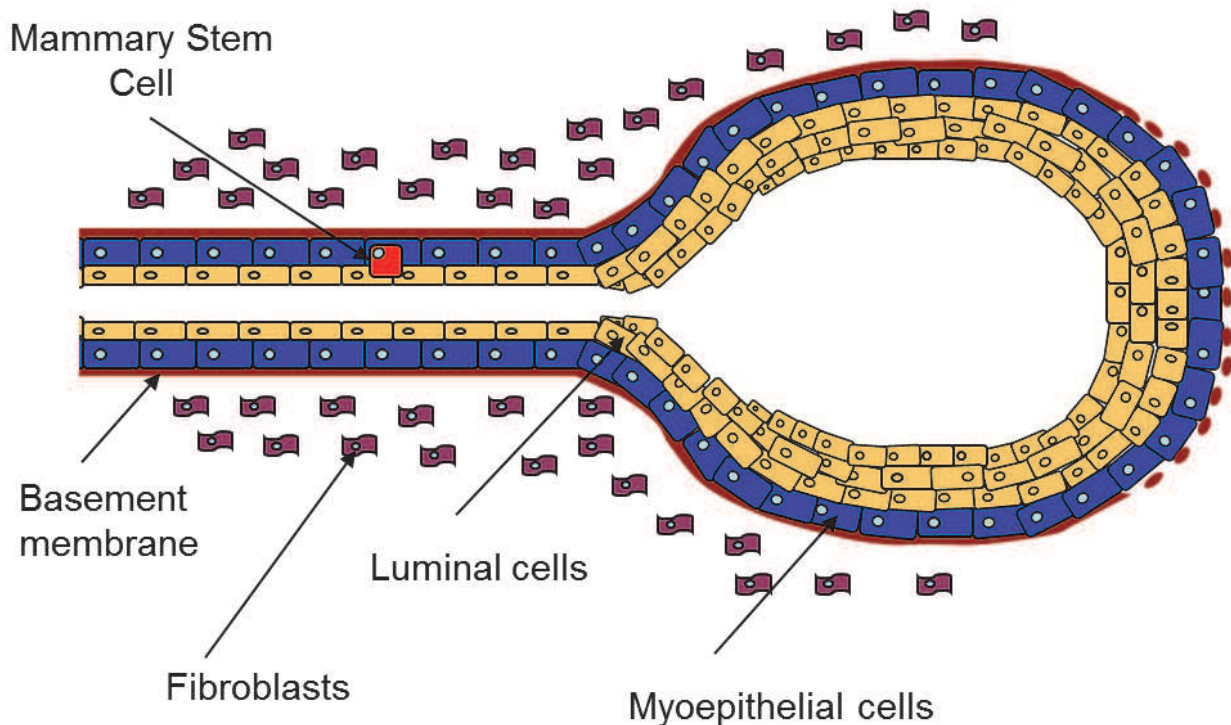
Fig. 2. Schematic representation of a terminal end duct in the mammary gland. Expansion (shown in red) occurs during puberty and pregnancy.

Dontu and colleagues have developed a culture system to isolate and propagate *in vitro* human breast multipotent stem/progenitor cells in a non-differentiated state. They have generated mammospheres (MMS), or non-adherent spherical cell clusters, clonally derived from single cells possessing self-renewal ability. Primary MMS contain eight-times more of bi-potent progenitors than freshly cultured human mammary cells [22,23]. The majority of bi-potent progenitors are able to generate colonies in 3D matrigel cultures containing all the main lineages of the mammary gland [24].

To date, combination of markers for the isolation of human stem/progenitor cells have been proposed based on the ability of the resulting populations to regenerate TDLUs by *in vivo* transplantation experiments. Based on prospective cell surface fractionation markers the current model of breast hierarchy is shown in Figure 1. In this model, the putative human MSC is a CD49f^{hi} EpCAM$^{-/low}$ CD24$^-$ CD133$^{-/low}$ ER$^-$ PR$^-$ ERBB2$^-$ and the luminal progenitor is a CD49f$^+$EpCAM$^+$ cell [25,26].

In a seminal experiment by Lim *et al.*, gene expression microarray studies of specific (sorted) populations of human breast revealed that the "prospective stem cell population" closely resembles claudin-low tumors (Figure 1). This has intrigued investigators and indicated that the cell of origin target of transformation in claudin-low carcinomas is possibly an early, undifferentiated EpCAM$^-$ stem cell. Basal-like breast cancers, which are highly proliferative, mostly triple negative and associated with BRCA mutation carriers, are possibly target of transformation of a more downstream, luminal-restricted, EpCAM+ progenitor. Finally, it has been proposed that both, luminal A and luminal B breast carcinomas are originated from "more" differentiated luminal cells [25].

Stem/progenitor cells as potential targets of transformation. Stem and primitive progenitor cells exhibit the ubiquitous feature of either remaining quiescent or sustaining self-renewal

in response to their microenvironment. These cells also undergo an asymmetric cell division and differentiate towards more committed cell lineages [18,21,27,28]. The identification of factors regulating self-renewal and differentiation in the mammary gland is of primary importance, as these factors could be potentially involved in oncogenesis. Unlike somatic cells, which undergo senescence, adult stem cells exist in the period of a life-time of an individual. These "long lived" cells have the potential to accumulate genetic and epigenetic aberrations contributing to oncogenesis [19]. The BRCA1 gene, a major tumor suppressor associated with basal breast cancer, has been shown to regulate human mammary stem/progenitor cell fate. Knock-down of BRCA in primary breast cells leads to an increase in cells displaying the stem/progenitor cell marker ALDH1 and a decrease in cells expressing luminal epithelial markers and estrogen receptor [29]. Similarly, the protein Musashi (Msi1) has been shown to modulate mammary progenitor cell expansion by activation of Wnt and Notch pathways, which are often dys-regulated in breast cancers [30].

Breast Cancer Stem Cells (breast CSCs). It has been demonstrated that only a small population of cancer cells (named "Tumor Initiating Cells, TICs, or Cancer Stem Cells, CSCs) retains the ability to form new tumors after transplantation in immunodeficient mice [31,32,33]. TICs display stem/progenitor cells properties, namely competence for self-renewal and capacity to re-establish tumor heterogeneicity [18]. Like normal stem/progenitor cells, CSCs can be propagated as spheroids cultures, named tumorspheres, when grown in low adherence conditions. Tumorspheres isolated from human cancer cell lines and patients are resistant to chemotherapy agents, such as paclitaxel and 5-fluorouracil [24,34]. Given their capability to promote tumor formation and the fact that these cells are refractory to chemotherapy, CSCs are primordial targets in breast cancer therapeutics. Very active research aims to the identification of prospective CSC markers for the isolation and study of these tumor-initiating cells. In a seminal study reported by Al-Hajj and colleagues, CSCs from breast cancer patients were identified by isolating the Lin⁻CD44⁺CD24⁻/low population (where Lin- refers the lineage negative population) [35]. As little as 100 cells of the sorted population formed palpable tumors when injected in immunodeficient animals. Recently, the same CD44⁺CD24⁻/low signature was identified in a panel of eight different human cancer cell lines. Importantly, the highest percentage of CD44⁺CD24⁻/low positive cells was found in basal-like breast cancer lines, whereas that more differentiated breast cancer cell lines, such as luminal and luminal-mix lines, showed significantly less percentage of cells expressing this signature. [34]. Reciprocally, TICs have also been isolated from three breast cancer lesions using the tumorsphere method. The resulting breast carcinoma lines exhibited the CD44⁺CD24⁻/low signature, over-expressed cytoprotective factors (including Survivin) and were able to self-renew and differentiate into both, luminal and basal lineages, indicating that these cells could arise from a bi-potent progenitor. Importantly, these cells expressed the OCT4 stem cell marker, and as few as 1000 cells generated tumors in nude mice [36]. In addition to the CD44⁺CD24⁻/low signature, another potential marker for CSCs is the aldefluor (ALDH1). ALDH1 is a detoxifying enzyme responsible for the oxidation of intracellular aldehydes and thought to play a role in stem cell differentiation through metabolism of retinal to retinoic acid. Two recent publications have demonstrated that the ALDH1 positive population (which is typically 1% of the total tumor cell lines) displayed CSC characteristics and were able to promote tumor formation in immunodeficient mice [31,37].

In summary, the above results suggest that cancer cell lines contain a hierarchical organization of cell populations, and that rare CSCs exhibiting tumor-initiating capabilities can be isolated with at least two defined molecular signatures.

Basal-like (BL) and claudin-low (CL) breast cancers are enriched in putative Cancer Stem Cell (CSC) signatures. Recent studies suggest that basal-like and claudin-low breast cancers are enriched in gene signatures associated with Cancer Stem Cells (CSCs). First, Honeth et al. performed immunohistochemistry with the CD44 and CD24 antibodies in 240 human breast tumors, and demonstrated that the CD44+CD24- phenotype was enriched in the basal-like subtype. From all BRCA- breast cancers analyzed, 94% contained the CD44+CD24- signature. However, this phenotype was detected in 31% of the all breast tumors scored, hence not all the basal-like tumors, and very few Her2+ tumors, contained this signature. Thus, is likely than other molecular signatures remain to be discovered for these tumors [38]. Second, characterization of mammary TICs revealed expression of many proteins that are up-regulated in basal-like and claudin-low tumors: CK5, CK14, α6 integrin, and αB-crystallin in the case of basal-like tumors [1], and ALDH1 in the case of claudin-low tumors [5]. Third, immunohistochemistry analyses indicate that basal-like tumors express both luminal and basal CKs, suggesting that the cell type of origin of these tumors is bi-potent [39]. Finally, histo-pathological analysis of basal-like tumors reveals a poorly differentiated phenotype, nuclear atypia and mitotic index that are reminiscent to stem cells [40]. The recently identified claudin-low breast cancer subtype is characterized by a high enrichment of epithelial-mesenchymal transition (EMT) markers, such as high TWIST1, SNAI1, ZEB2 and Vimentin. More importantly, a hallmark of these tumors is the presence of CD44+/CD24-/low CSC signature and also poor response to chemotherapy or hormonotherapy [41]. Overall, these findings suggest that this novel mesenchymal subtype is potentially enriched with CSCs that might be resistant to standard therapy.

Targeting claudin-low and basal-like breast cancers. As expressed above, both claudin-low and basal-like breast cancers are triple negative and associated with poor prognosis. Basal breast cancers are currently treated with a combination of chemotherapeutic and anti-angiogenic regimens [12]. However, because claudin-low tumors are refractory to chemotherapy and biologic therapy, there is an urgency to develop novel strategies to target these patients.

The first targeting genetic approach consists in down-regulating potential oncogenes that are over-expressed in CSCs. This can be achieved by knock-down strategies, including siRNA, and repressive Artificial Transcription Factors, which will be discussed more in detail in the following sections. The second strategy is the forced up-regulation of tumor suppressors, which are often silenced in tumor cells. This can be achieved by over-expression of tumor suppressors, by epigenetic remodeling drugs, which target repressive chromatin, and Artificial Transcription Factors (Figure 3).

In the following sections we will overview first, embryonic TFs and their regulatory networks in stem and CSCs, and second, we will focus on the biology of selected tumor suppressor genes. Finally we will review recent investigations aiming to characterize ATFs targeting the tumor suppressor gene *mammary serine protease inhibitor* (*maspin*), which is down-regulated in claudin-low carcinomas.

2. Targeting self-renewal transcription factors, TFs

Because claudin-low tumors are possibly originated from primitive stem cells, dys-regulation of self-renewal and differentiation gene pathways might be at the basis of their oncogenic potential. In that regard, it is possible that claudin-low/basal breast cancers use an operational machinery of transcription factors (TFs), which regulates self-renewal in stem
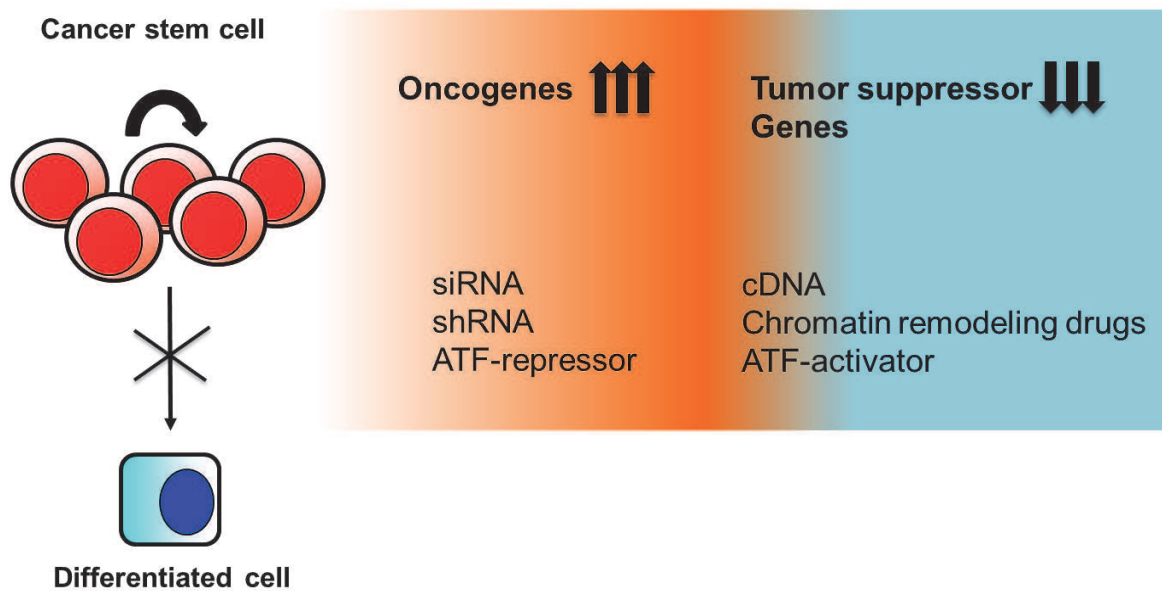
Fig. 3. Current approaches to target tumor initiating cells by down-regulation of potential oncogenes and up-regulation of tumor suppressors.

cells. The improper function of this circuitry could unbalance a delicate equilibrium between self-renewal and differentiation, thereby locking stem/early progenitor cells in an aberrant, excessive self-renewal stage. Aberrant self-renewal could prevent or limit stem cells from undertaking downstream differentiation gene programs. The initial pool of self-renewal cells could accumulate subsequent genetic and epigenetic aberrations, particularly in patients associated with mutations in the BRCA tumor suppressor gene (basal and claudin-low breast cancer). How is self-renewal dys-regulated in cancer stem cells? Can we learn some lessons from stem cell biology? Is there a network of TFs over-activated/operative in cancer stem cells?

The self-renewal TF network of human embryonic stem cells (hESCs). The self-renewal TF network has been characterized in hESCs by ChIP-chip and Chip-seq. This network involves the transcriptional activation as well as the physical association of multiple TFs. The "heart" or "core" of this network is composed of three master regulators TFs: OCT4, SOX2 and NANOG. Interactions between these TFs results on an autoregulatory feed-forward feedback loop [42]. The OCT4-SOX2-NANOG circuit functions as an epigenetic switch, which controls the maintenance of the self-renewal state. In hESCs DNA-methylation of the OCT4, SOX2 and NANOG promoters acts as an "irreversible switch" promoting differentiation gene programs [43]. Are OCT4, SOX2 and NANOG, and downstream self-renewal TFs activated in breast cancers? If so, a very attractive approach would consist in promoting persistent, imprinted, and inherited silencing on these upstream master regulators to force tumor cells to switch from a "proliferative" towards a "differentiation" stage. OCT4 positive cells have been reported in breast cancer cell lines derived from tumors [36,44]. The SOX2 gene is over-expressed in basal breast cancer specimens and cell lines, and thus, represents a potential cancer stem cell/progenitor marker [44]. In the following sections we will describe the OCT4 and SOX2 TF targets and their functional importance in hESCs and potentially in CSCs.

OCT4/POU5F1 (Octamer binding transcription factor 4). As a member of the POU transcription factor family (Pit, Oct, Unc), OCT4 contains a bipartite DNA-binding domain,

which consists of two sub-domains, namely the POU-specific and the POU-homeodomain. These domains are connected by a flexible linker of variable length. The POU proteins have an intrinsic ability to bind DNA with sequence specificity. They interact with different binding partners as well as transcriptional co-activators or co-repressors, which mediate the activation or repression of their targets [45], [46]. This has a fundamental importance as different constellations of co-activator, co-repressors and chromatin remodeling complexes exist in different cell types. Thereby, depending on the chromatin microenvironment OCT4 could behave either as an activator or a repressor.

OCT4 was the first gene to be identified as a master regulator of pluripotency [47]. OCT4 is essential for the maintenance of pluripotency and this is mediated by up-regulation of a self-renewal TF network. These downstream TF targets of OCT4 coordinately activate self-renewal gene programs, while preventing the expression of genes that are activated during stem cell differentiation [48],[42,49].

The regulatory core network in embryonic cells comprises OCT4, SOX2, and NANOG transcription factors. The transcription factors OCT4, SOX2 and NANOG form the basic core of the self-renewal TF network. OCT4, SOX2 and NANOG are able to physically associate with different TFs as well as with large co-activators/co-repressor complexes, and co-occupy a set of 179 target promoters, namely the "NOS" gene set. This mediates the activation of self-renewal transcription factors, while it represses targets associated with differentiation gene programs [42] [50]. The results reported by Boyer *et al*. [42] suggested a model of ESCs transcriptional regulation. Some of the OCT4, SOX2, and NANOG targets include genes involved maintenance of self-renewal (e.g. *OCT4, SOX2, NANOG, STAT3* and *ZIC3*), members of the Wnt (*DKK1, FRAT1/2*) and TGFβ *(TDGF1, LEFTY2/EBAF)* signaling pathways, and histone-modifying and chromatin remodelers, such as *SMARCAD1*, *MYST3* and *SET*. Genes that encoded for homeodomain proteins, such as *DLX5, HOXB1, LHX5, TITF1, LBX1* and *HOP* implicated in developmental gene programs were repressed [42]. Importantly, genome-wide studies have shown that NOS targets are over-represented in poorly differentiated carcinomas, such as breast carcinomas and gliomas, which suggest that embryonic TFs could similarly play an important role in maintaining aberrant self-renewal in these tumors [51].

OCT4 in cancer stem cells (CSCs). In humans, CSCs have been associated with multiple malignancies, including breast [35], ovarian [52] and prostate cancer [53]. The role of OCT4 in CSCs has been a subject of intense investigation. Several studies suggest that OCT4 is critical for tumor cell survival and for the formation of tumor-initiation units *in vitro*, or tumor-spheroids. High expression of OCT4 has been reported *in vitro* in Lewis lung carcinoma 3LL and human breast cancer MCF7 cells. Silencing of Oct4 by siRNA resulted in cell apoptosis, suggesting that OCT4 is essential for cell survival [54]. Furthermore, human breast cancer cell lines have been isolated that exhibit the CSC signature CD44+CD24- and these cells express high levels of OCT4 [36]. In the same line, another group has shown a 60% reduction in cell viability in epithelial ovarian cancer PA-1 cells transfected with siRNA specific for OCT4, and the reduction in cell viability was in part due to an increase of apoptosis [55].

SRY (Sex determining Region Y) Box 2 (SOX2). SOX2 is a member of the SRY-related High Mobility Group (HMG) box transcription factor. SOX2 is key regulator of cell fate and is necessary for the maintenance of self-renewal in embryonic stem cells. SOX2 has been proposed to be a marker of CSCs (together with other genes, e.g. OCT4, CD133, CD44) in several tumor types, including glioblastoma, prostate, lung, liver and breast carcinomas [51], [56], [57], [58], [59], [60]. Gene-expression microarrays revealed that SOX2 up-regulated

Cyclin D1, which contributes to cell cycle progression. Consistently, Cyclin D1 over-expression is observed in a variety of tumors [61]. However, the functional role of SOX2 in breast organogenesis remains still unexplored. SOX2 is over-expressed in breast cancer cells with increasing levels of expression in poorly differentiated cancer cells [62],[44]. Loss of function studies using SOX2-specific shRNA resulted in an inhibition of tumorigenic phenotype of MCF-7 breast cancer cells in xenograft studies in nude mice ([63]). These results give evidence that down-regulation of SOX2 in breast cancer cells can be used for clinical applications for the treatment of aggressive breast cancers.

3. Targeting tumor suppressors in breast cancers

In addition to activation of oncogenes, aggressive carcinomas inactivate multiple panels of tumor suppressor genes. The p16^{INK4A}, p53, and BRCA tumor suppressor genes are commonly inactivated in breast cancers. As expressed earlier, BRCA mutation carriers are associated with risk to develop basal-like breast cancers [26]. Loss of p53 and p16^{INK4A} tumor suppressor genes, which are also a main blockade for reprogramming of somatic cells, occurs in the earliest steps of mammary carcinogenesis [64,65,66]. Together with up-regulation of telomerase reverse transcriptase (hTERT), loss of p16^{INK4A} is involved in immortalization of normal epithelial cells [64]. Loss of p16^{INK4A} gene function could thus be a critical initial stem for prospective cancers stem cells to bypass the senescence pathway. In addition to the above described tumor suppressor genes, DKK1 inactivation has been associated with claudin-low tumors [67,68]. DKK1 functions as an antagonist of the Wnt signaling pathway, which plays an essential role in maintaining self-renewal and in stem cells, and possibly, in cancer stem cells.

The loss of tumor suppressive functions associated with self-renewal of stem cells could result in further genetic and epigenetic instability leading to tumor initiation. In addition to mutations and/or re-arrangements of tumor suppressors, epigenetic silencing mechanisms, for example DNA- and histone-methylation, and histone deacetylation, are very often observed in tumors. Some tumor suppressor genes named Class II tumor suppressors, such as the gene *maspin*, are ideal epigenetic targets because are solely down-regulated by epigenetic mechanisms in aggressive cancer cells.

The maspin tumor suppressor. *Mammary serine protease inhibitor (maspin or SERPIN B5)* is a multifaced protein able to induce apoptosis and suppress cell motility and metastasis [69,70,71,72,73,74]. The mechanisms by which *maspin* exert their functions are still under investigation but recent evidence suggest that the tumor suppressive and possibly the anti-metastatic responses are associated with the nuclear localization of this protein [75]. It has been demonstrated that *maspin* physically associates with chromatin and functions as a histone-deacetylase (HDAC) inhibitor [75,76]. The function of *maspin* as metastasis suppressor has been associated with the regulation of the urokinase-type plasminogen activator (uPA) and receptor (uPAR) protein system [77,78]. In addition to enhanced tumor cell apoptosis and inhibition of tumor cell motility, ectopic expression of *maspin* results in decreased angiogenesis, which as been attributed to regulation of endothelial cell migration and adhesion [79], as well as induction of endothelial apoptosis [80].

Maspin expression is regulated at many levels, including TFs (p53, p63, AP1/2) [81], microRNAs (mirRNA-21; [82]) and lastly, by promoter epigenetic regulation [83]. Epigenetic inhibitors, affecting both DNA- and histone methylation (for example 5-Aza-2'dC) and histone deacetylation (for example, SAHA) are commonly used to re-activate silenced tumor

suppressors, including *maspin*. These inhibitors are presently in clinical trials particularly for non-solid malignancies. However, the problem of using these inhibitors in a clinical setting is their high toxicity due to their lack of targeted specificity. Thus, novel strategies are required to target more specifically tumor suppressor genes.

Our laboratory has developed Artificial Transcription Factors (ATFs), able to specifically bind the regulatory regions of both, oncogenes and tumor suppressors, to repress or activate gene expression. A hallmark or unique property of ATFs is their ability to revert the epigenetic state of the targeted genes, thereby reprogramming the phenotype of the tumor cell. This is particularly important for silencing oncogenes; the induction of epigenetic silencing by ATF has the unique capacity to promote inherited and stable changes in the tumor cell, which entails epigenetic, transcriptional and phenotypic memory. In the following sections we will describe the anatomical constituents of ATFs, the recent advances in the technology, and their potential to modulate gene expression in the breast cancer field.

4. Artificial transcription factors for targeting cancer-associated genes

Designer zinc finger transcription factors or Artificial Transcription factors (ATFs) are engineered proteins composed of a DNA-binding-domain (DBD) and an effector domain (ED). The DBD is designated to recognize specific DNA sequences, while the ED enables to edit or modify DNA. EDs comprise transcriptional activators or repressors, as well as enzymatic domains, such as methyltransferases, recombinases, and site-specific nucleases. Thus, unique features of ATFs comprise their capability to bind specific regions of the genome, as well as to edit, modify and sculpt the chromatin landscape of the cell. These technologies facilitate the directed and specific modification of the genome for gene therapy purposes.

Design of the DNA binding domain (DBD). The DBD is designed to bind specific DNA sequences, typically in the promoter region of the gene of interest. Most of the DBDs used to engineer ATFs are based on zinc finger (ZF) scaffolds, because of their modular nature and the simplicity of their DNA–protein interactions. Cys2-His2 ZF domains are compact, 30 amino acids units composed of a recognition α-helix and two antiparallel β-strands stabilized by a zinc ion [84,85]. Each ZF α-helix recognizes 3 base-pairs (bps) of DNA in an antiparallel manner [84]. Importantly, each ZF recognize the DNA in a quasi-independent manner, which has facilitated the selection and rational design of multimodular ("polydactyl" ZF proteins). Novel DNA-binding specificities are generated by mutagenesis of the recognition α-helix, without altering the rest of the scaffold. This has facilitated the construction of arrays of ZFs to recognize large DNA sequences using simple and relatively quick molecular biology approaches. Nevertheless, proteins of improved DNA-binding specificity can be further optimized by randomization and subsequent selection of the ZF backbone, using both *in vitro* and *in vivo* approaches (for an overview of these, see references [7,86,87]). Our laboratory has focused on six-ZF arrays recognizing 18-bp sequences because of the high affinity of these proteins (with K_ds in the picomolar range). In addition, 6ZF proteins are capable of binding and regulating single genes in complex genomes and thus, possess higher selectivity than three ZF proteins [88].

Effector domains linked to the DNA binding domain (DBD). The effector domain (ED) mediates the ATF function by either modulating transcription or by modifying or editing the chromatin. The VP16 [89], VP64 [90] and p65 [91] are common transcriptional activators that interact with the Mediator protein recruiting the polymerase-II transcriptional complex and associated enzymes to facilitate transcription. Additional activator domains used to
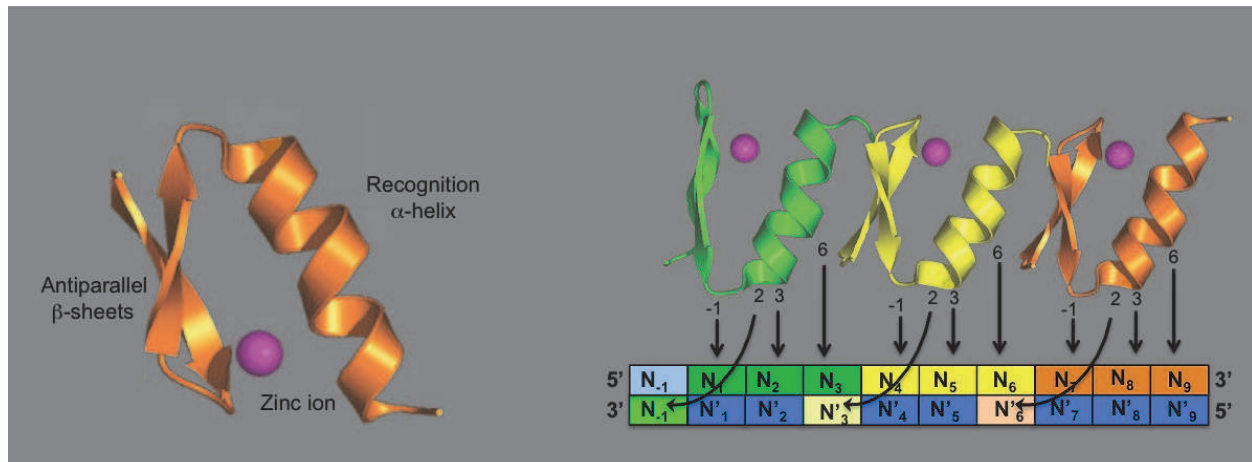
Fig. 4. Structure of Cys2Hys2 ZF proteins and their DNA-recognition mode. (a) Crystal structure of a ZF protein illustrating the recognition α-helix, the two antiparallel β-strands, and the zinc ion. (b) ZF-DNA interactions of a prototype 3ZF protein binding a 9-bp target.

construct ATFs comprise the S3H domain [92] and the $(FDTDL)_{11}$ domain derived from the C-terminal transcriptional activator domain of β-catenin [93].

Commonly used repressor domains include the Krüppel-associated box domain of KOX1 (KRAB) [94] and the SID domain (derived from the amino acid residues 1- 36 of the Mad mSIN3 interaction domain [95]). These EDs repress transcription by interacting with transcriptional co-repressors and chromatin condensing enzymes, resulting in a decreased accessibility of the promoter, which impedes the access of transcription factors and the polymerase II complex. Additional repressor domains include the ERD domain (comprising the residues 473-530 of the est2 repressor factor) [96], the vErbA (the ligand-binding domain of the thyroid hormone receptor v-erbA) [97], and the SRDX domain, derived from the transcription factor SUPERMAN of *Arabidopsis thaliana* [93].

Recently, DBDs have been linked to methyltransferase domains to silence promoter activity [98]; this fusion induced an epigenetic modification conferring stably and inherited gene silencing. DNA-methyltransferases catalyze the incorporation of methyl groups in position 5 of a cytosine base in CpG dinucleotides. In the cell, DNA methylation marks are "read" by methyl-binding-proteins (MBPs) proteins, which recruit large repressive complexes resulting in chromatin compaction and gene silencing. ZFs have been also linked to the Histone methyltransferase G9a to promote H3K9 methylation in the VEGF-A promoter [98]. Our laboratory has recently found that the VP64 activator domain can directionally de-methylate the *maspin* promoter [99]. Overall, these results hold great promise to develop novel ATFs able to alter the epigenetic code at specific promoter contexts, thereby promoting stable and inherited phenotypic changes in target cells.

Construction of Artificial Transcription Factors. Two main strategies have been developed to engineer ZF proteins, namely the modular assembly and the combinatorial-selection. The modular strategy involves the assembly of a basic repertoire of ZF building blocks of pre-characterized DNA-binding specificities. The modular approach is commonly performed by the "helix grafting" method ([90,100,101,102,103]). The combinatorial method produces ZFs with high specificity and binding affinity after the interrogation of randomized libraries and selection techniques. Here again, several strategies are available for investigators to select for ZF DBDs, such us as Phage display [104,105,106,107,108], ribosome display [109], two

hybrids [110,111], and more recently the OPEN [112] and CODA systems [113]. The selection of the specific ZF units is a first critical step, and researchers can take advantage of several web-based tools available for designing their suitable array of ZFs, such as Zinc Finger Tools, (http://www.scripps.edu/mb/barbas/zfdesign/zfdesignhome.php) [114]; Zinc Finger Targeter, ZiFiT (http://bindr.gdcb.iastate.edu:8080/ZiFDB/) [115]; and ZFNGenome (http://bindr.gdcb.iastate.edu/ZFNGenome) [116]. These web-based tools are user-friendly interfaces that identify potential ZF binding sites on the target gene and provide scores for each ZF protein.

Endogenous gene regulation by Artificial Transcription Factors. In 1997 at the Barbas' lab demonstrated that 6ZF ATFs coupled either with the VP16 or KRAB-A box EDs were able to regulate the expression of an ErbB2-driven reporter gene [117]. The same group later demonstrated that an ATF was able to target the endogenous ErbB2 promoter [90]. For the purpose of modulating gene expression in mammalian cells, ATFs are cloned either in transient expression vectors (pcDNA), or retroviral expression vectors. These constructs facilitate the expression of ATFs in primary and transformed mammalian cells. Some of the genes successfully regulated by ATFs are listed on **Table I**. Recently regulated genes include: *mammary serine protease inhibitor* (*maspin*) [103,118], dystrophin-related gene *utrophin* [119], *vascular endothelial growth factor A* (*VEGF-A*) [120,121], *gamma-globin* [122], *heme oxygenase-1 gene* (*HMOX1*) [123], *glial cell line-derived neurotrophic factor gene* (*GDNF*) [124], derived tyrosine kinase receptors *ErbB2* [90,125], and *ErbB3* [100,125]. Genes that were successfully down-regulated by ATFs comprise: *VEGF-A* [120,121], *ErbB2* [90,125], *ErbB3* [100], the human telomerase reverse transcriptase (*hTERT*) [126], the nuclear hormone receptor PPARγ gene (*PPARγ*) [127], and the repression of the checkpoint kinase 2 (*CHK2*) gene [88].

The most recent application of engineered ZF DBDs embrace the ability to specifically modify the genome using zinc finger nucleases (ZFNs). These ZFNs are composed of 2-4 ZF arrays linked to DNA-cleavage domain of Fok-I. Gene correction has been successfully demonstrated in human embryonic and hematopoietic cells [128].

Regulation of cancer associated genes by ATFs. ATFs are versatile tools, which offer a unique therapeutic strategy to modulate the expression of oncogenes and tumor suppressors. Currently, several ATFs have been constructed to regulate cancer genes. Falke *et al*. reported an ATF up-regulating the pro-apoptotic *Bax* gene. Upon transfection in Saos-2 cells this ATF promoted a 40% reduction in cell viability and apoptosis induction [130].

High levels of VEGF-A protein expression have been associated with tumor vascularization. In 2003 Snowden et al. [97] generated an ATF coupled with the repressor domain v-ErbA targeting the *VEGF-A* gene. Transfection of this ATF into tumorigenic HEK293 cells resulted in a 50% reduction of protein expression.

Several cancers are associated with the over-expression of the ErbB-family of receptors (e.g. breast, prostate, colon, pancreas and ovary). The ErbB2 receptor has an important role in tumor cell proliferation and metastasis, particularly in breast cancer cell models. ATFs designed to up- and down-regulate this gene in A431 cells, derived from an epidermoid squamous cell carcinoma line, caused an increase in cell migration when ErbB2 was over-expressed, while its down-regulation led to a significant reduction in cell migration [125]. Recently Lund CV. *et al*. described the generation of a single twelve-ZF construct designed to down-regulate the ErbB2 and ErbB3 genes. This ZF protein was properly expressed in the cells, co-regulated both targets, and was able decrease breast tumor cell proliferation *in vitro* [125].

Endogenous gene	Regulation	Target site length (bp)	Effector Domain	Reference
CCK2R	↑	9	VP16	Liu et al. 2004 [129]
Utrophin	↑	9	VP16	Corbi et al. 2000 [119]
Bax	↑	15	VP16	Falke et al. 2003 [130]
Human erythropoietin	↑	9	VP16	Zhang et al. 2000 [131]
EPO-1	↑	9	VP64	Zhang et al. 2000 [131]
γ-Globin	↑	18	VP64	Blau et al. 2005 [122]
Maspin	↑	18	VP64	Beltran et al. 2007 [103]
HMOX1	↑	18	p65	Guo et al. 2010 [123]
GDNF	↑	18	p65	Laganiere et al. 2010 [124]
PEDF	↑	18	p65	Yokoi et al. 2007 [132]
PTHR1	↑	9	VP16/p65	Liu et al 2005 [133]
VEGF-A	↑	9	VP16/p65	Liu et al. 2001, Mori et al. 2008 [121,134]
IGF2/H19	↑↓	9	VP16/p65/v-ErbA	Jouvenot et al. 2003 [132]
ErbB2/ErbB3	↑↓	9	VP16, VP64 KRAB, ERD, SID	Beerli et al, 1998, 2000 [90,100]
ErbB2/ErbB3	↑↓	18	VP64/KRAB	Lund et al. 2005 [125]
ErbB2	↑↓	18	VP64/KRAB	Beerli et al. 2000 [100]
OCT-4	↑↓	18	VP16/KRAB	Bartsevich et al. 2003 [135]
MDR1	↑↓	15	VP16/KRAB	Bartsevich et al. 2000 [136]
hTERT	↓	12	KRAB	Sohn et al. 2010 [126]
PPARγ	↓	18	KRAB	Ren et al. 2002 [127]
CHK2	↓	18	KRAB	Tan et al. 2003 [88]

Table 1. Genes regulated by Artificial Transcription Factors.

Targeting the maspin tumor suppressor gene. Recently, our laboratory has targeted the gene *maspin*, a tumor suppressor silenced by epigenetic mechanisms in aggressive cancer cells. We have constructed three six-ZF ATFs designed to bind 18-pbs sites in the *maspin* proximal promoter. These ATFs were fused to the VP64 transactivator domain and designed to awake the silenced gene. These ATFs offer a versatile strategy to study the phenotypic consequences of re-expressing the endogenous gene in different cancer cell models. ATFs -97 and -126 strongly reactivated *maspin* in highly metastatic breast and lung cancer cell lines [99,103,118]. The up-regulation of *maspin* decreased cell motility and induced tumor-cell apoptosis in approximately 60% of the transfected cells; the remaining cell population restored cell junction proteins, such as E-cadherin, and other normal-like features. When ATF-126 was expressed in MDA-MB-231 cells using inducible retroviral vectors, ATF induction resulted in 50% of tumor burden reduction and totally abolished metastatic colonization in nude mice. Furthermore, gene expression microarrays demonstrated that the ATF-responsive genes predicted normal-like cell behavior, better prognosis and therapeutic response of breast cancer patients. These data point to the future clinical application of ATFs targeting *maspin* in breast cancers.

Subsequent studies with *maspin*-specific ATFs in several cancer cell backgrounds revealed that the re-activation of the gene was partially compromised by the epigenetic status of its promoter. Maspin is aberrantly silenced in metastatic tumors by epigenetic mechanisms including DNA methylation, H3K9 methylation, and histone de-acetylation. Co-treatment of low-maspin expressing breast and lung cancer cell lines with ATF-126 with either methyltransferase or HDAC inhibitors resulted in a synergistic interaction in re-activating *maspin* expression, and cell death induction. Furthermore, the triple combination (ATF-126 + methyltransferase + HDAC inhibitor) was far more potent in re-activating *maspin* and in inducing cell death as compared to single or double treatments, even at low concentration of inhibitors. These observations demonstrated the importance of the promoter context, particularly promoter methylation, in the reactivation of *maspin* by ATFs.

Later experiments have demonstrated that both ATFs, ATF-126 and ATF-97, were able to re-activate *maspin* in non-small cell lung carcinoma (NSCLC) cancer cells carrying a hypermethylated *maspin* promoter. Sodium bisulfate methylation studies in ATF-97 and ATF-126 transfected cells demonstrated substantial DNA demethylation upstream the ATF-binding sites (~70% reduction relative to control cells). This unidirectional, site-specific, de-methylation effect was dependent on the positioning of VP64 along the promoter. The VP64 transactivator domain interacts with the mediator protein to recruit RNA polymerase complex and chromatin remodeling enzymes in the *maspin* promoter, which acts to relax the chromatin and to ignite transcription. Although the mechanism by which ATFs de-methylate the *maspin* promoter remains elusive, these results demonstrate that ATFs can be target DNA demethylation in specific promoters.

ATFs designed against the *maspin* promoter resulted in successful target gene regulation. The next step involves the delivery of the ATFs into tumors and metastasis in a pre-clinical setting. To this end, our laboratory and others are developing targeted nanoparticles and adenoviral delivery systems. Although these delivery systems are at this time under development, an ATF made to target the Vascular Endothelial Growth Factor (VEGF-A) gene is currently in clinical trials. Nevertheless, the forthcoming engineering of ATFs will benefit from EDs that actively shape the chromatin to either compact or relax its native architecture. In addition, combinations of ATFs to target both, oncogenes and tumor suppressors, are anticipated to achieve more potent therapeutic outcomes. The development of ATFs able to alter the chromatin landscape of multiple loci represents a powerful novel genetic tool to target malignant breast cancer.

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6. References

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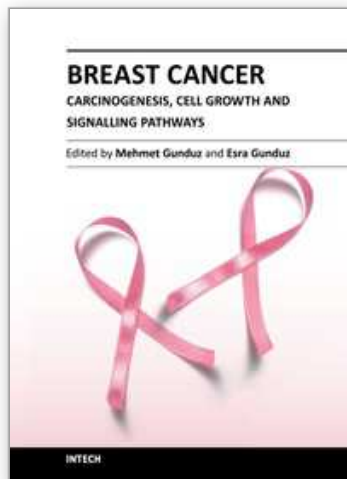
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Cancer is the leading cause of death in most countries and its consequences result in huge economic, social and psychological burden. Breast cancer is the most frequently diagnosed cancer type and the leading cause of cancer death among females. In this book, we discussed various aspects of breast cancer carcinogenesis from clinics to its hormone-based as well as genetic-based etiologies for this deadly cancer. We hope that this book will contribute to the development of novel diagnostic as well as therapeutic approaches.

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