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Re-Writing the Histone Code of Breast Cancer Stem Cells

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The transcription factor SOX2 is a Cancer Stem Cell (CSC) marker that plays an pivotal role maintaining self-renewal in both embryonic and adult breast stem cells. In human embryonic stem cells SOX2 expression is down regulated by means of epigenetic mechanisms, including DNA methylation. SOX2 is overexpressed in many breast cancer cell lines as well as in poorly differentiated triple negative breast tumor specimens. Thus, enforced down-regulation of SOX2 in tumor cells offers a unique therapeutic opportunity to suppress breast cancer cell self-renewal and tumor initiation. Epigenetic mechanisms, such as DNA methylation, are inherited during cell division and result in permanent and stable silencing. We have generated arrays of Artificial Transcription Factors (ATFs) made of specific six-zinc finger domains (ZF) targeting unique 18-base pair sites in the SOX2 promoter. The 6ZFs were linked to the repressor domain Krüppel Associated Box (KRAB) domain or DNA methyltransferase 3a (DNMT3a) catalytic domain and expressed in aggressive MDA-MB-435s breast cancer cells using retroviral vectors. Our results show that two ATFs were able to silence SOX2 mRNA and protein levels with virtually 100% down-regulation. This was accompanied with a potent suppression of both tumor cell proliferation and anchorage independent growth. These epigenetic switches represent a promising therapeutic strategy to effectively target breast cancer stem cells.
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INTRODUCTION

It is perhaps surprising that most of the cells within the bulk of a breast tumor are differentiated cells, which indeed do not have tumorigenic potential at all. The Tumor Initiating Cells (TIC) comprise a small population that can generate tumors with very small amounts (for example, as little as 100 cells) once injected in immunodeficient mice (1-4). These cells, named Cancer Stem Cells (CSCs), are able to sustain self-renewal ability in vitro and have tumorigenic capabilities. CSCs could be visualized as “corrupted” versions of normal stem cells. Because of their ability to self-renew during a lifetime of an individual, these cells could be primary targets of transformation, by acquisition of genetic defects (for example, mutations in tumor suppressor genes and oncogenes) but also by acquiring transcriptional and epigenetic aberrations (3-4). Like their normal stem cell counterparts (5), CSCs are believed to naturally overexpress proteins in the surface that extrude DNA-damaging agents (like the ones used in chemo-therapy for breast cancer) possibly as natural mechanism of stem cells to protect the integrity of their long-life genomes (6-7). Because of their ability to initiate a tumor, their potential to migrate, disseminate and differentiate, and their intrinsic resistance to chemotherapeutic agents, CSCs are primordial targets in breast cancer therapeutics. Recently, the triple negative breast cancer subgroup or basal-like breast cancer, associated with the poorest prognosis in breast cancer patients, has been found enriched in CSCs (8,9) Thus, we are in a critical need for the development of novel technologies to detect and specifically target CSCs, in order to suppress the intrinsic growth capabilities of the tumor.

Our objective is to develop novel technologies to target genes differentially expressed in CSCs, which play a role in maintaining self-renewal and tumorigenicity. We have recently found that the primordial embryonic transcription factor genes, such as SOX2, responsible for self-renewal of embryonic stem cells (10), are up-regulated in basal-like breast cancer patients, and that their overexpression is associated with poor prognosis (11). The structure of the chromatin in these self-renewal gene promoters is a major determinant associated with transcriptional dysregulation and oncogenesis. Chromatin structure and function is controlled in large part by the post-translational modification of histones and the incorporation of specialized histone variants into nucleosomes (12). Strikingly, histone proteins are highly modified by an array of diverse post-translational modifications, including acetylation and methylation (12). The large number of modifications and the ability of individual histones to be multiply modified has given rise to the idea that a “histone code” may exist that acts in a combinatorial manner to elicit distinct biological effects (13-14). This code is thought to function through the physical alteration of chromatin structure and/or through the recruitment of effector proteins to the sites of histone modification (12). Several histone modifications have been linked to oncogenesis and cancer stem cell biology (15-18). Histone H3 lysine 9 (H3K9) methylation, for example, is critical for gene repression and heterochromatin formation through the recruitment of heterochromatin protein 1, which binds the methyllysine residue (19-21). In contrast, the methylation of H3 lysine 4 (H3K4) is linked to transcriptional activation via the recruitment of TFIID and several chromatin-remodeling and modifying enzymes (22-24). Histone methylation was once considered to be irreversible, however, recent identification of lysine-specific histone demethylases (KDMs) has revealed histone methylation to be a dynamically regulated process (25-26). KDMs contain a JmjC domain, a signature motif conserved from yeast to humans (27). To date, there have been a number of H3K4 demethylases identified, including PLU-1 (JARID1B) (25-26). H3K4 demethylation can help maintaining a repressed chromatin state (28). The diversity of chromatin “editing” enzymes underscores the importance of the epigenetic landscape in controlling gene expression. Further, our ability to stably alter gene expression states via epigenetic reprogramming is likely to have far reaching implications for controlling human diseases including breast cancer (15, 29). This Idea Award proposal aims to specifically test the utility of targeting the enzymes that
methylate or demethylate these lysine residues in order to shut down the expression of genes that promote breast cancer stem cell renewal. We propose the construction of novel factors named Designed Epigenetic Remodeling Factors (DERFs). DERFs will be targeted to specific self-renewal promoters using engineered arrays of six-Zinc Finger (ZF) domains, which target 18-bp sites and potentially have unique specificity in the human genome. The result of this work should lead to the generation of novel chromatin remodeling factors targeting CSC self-renewal and tumorigenicity.

BODY

In this section we will describe the main results and conclusions for the tasks outlined in the statement of work for year 1 (months 1-12) of this proposal.

List of Tasks and expected outcomes:

Task 1. The generation of Designed Epigenetic Remodeling Factors (DERFs) able to bind and regulate the endogenous SOX2 promoter in basal breast cancer cell lines enriched in cancer stem cells (SUM102 and SUM159 cell line) (months 1-12)

During months 1-6 the Blancafort lab will construct the DNA-binding domains of five Zinc Finger (ZF) proteins, whereas Dr. Strahl’s laboratory will construct the chromatin remodeling domains. The Strahl lab will then clone their inserts into the final retroviral vector for the expression of the resulting DERFs into SUM cell lines.

a) To generate highly specific 6ZF domains able to bind different 18-bp sequences in the SOX2 human proximal promoter (Blancafort) (months 1-6)

a.1. To generate the 6ZF DNA-binding domains. We have budgeted the generation of five 6ZF proteins binding five different sites in the proximal SOX2 1kb promoter region. We have chosen five 6ZF proteins since in our hands, approximately half of 6ZF proteins built against a given gene promoter show endogenous activity in vivo. Thus, by making five proteins we have a very good confidence to produce at least two proteins able to effectively bind the SOX2 promoter in vivo. We will use an overlapping PCR procedure to generate multimodular 6ZFs. We expect to complete the construction, cloning and sequencing of the five 6ZF proteins in six month, with one protein being made and characterized per month.

a.2. To perform DNA-Binding analyses (ELISA). To verify that the proteins bind DNA well, we will perform routine DNA-binding analyses in vitro (for example, to calculate the dissociation constant, Kd, of these proteins for their cognate substrates by ELISA). These protocols and methods are well established in the Blancafort lab and using previously published conditions. These analyses will be run every month for each 6ZF DNA-binding protein constructed.

Final product after month number 6 (Blancafort): At the end of the first 6 months the Blancafort laboratory will have completed the construction, cloning and in vitro characterization of five different 6ZF DNA-binding domains.

b) To generate fusions of the engineered 6ZFs with specific panel of Histone modifying domains (Strahl). (months 1-6). In these experiments, DNA binding domains (made of ZF domains) will be linked to a specific set of modular histone modifiers. The construction of these fusions should be completed by the end of month 6.
Final product after month number 6 (Strahl): At the end of the first 6 months the Strahl laboratory will have completed the construction and cloning (in the retroviral expressing vector) of the chromatin-modifying domains. These constructs will be all verified by DNA sequencing.

c) To assess the capability of these fusions to regulate endogenous SOX2 expression by real-time/western analysis (Blancafort) and to trigger specific epigenetic silencing by ChIP assay (Strahl). Months 6-9

Obtained Results and Discussion:

Previous work and follow-up work from year 1.
We have constructed four 6ZF proteins recognizing highly specific sequences in the Sox2 human promoter: three close to the ATG start site, and one additional ZF protein binding the SRR1 enhancer, which controls Sox2 expression and is critically modified by DNA methylation [30]. The 6ZF domains were first linked to the Kruppel Associated Box (SKD) repressor domain, which has been shown to promote HDAC-dependent repression when linked to ZFs [31,32,33]. These constructs were expressed using retroviral vectors in breast cell lines carrying high Sox2 expression [34]. Repression of Sox2 was measured by qRT-PCR and western blot, and normalized to mock-transduced cells. As shown in Fig 1A, all constructs except ZF-619 were properly expressed in MDA-MB-435s cells. ZFs -552 and -598 knock-down Sox2 mRNA and protein expression levels, even with higher potency than siRNAs (Fig 1B-C). Further, when the proximal 6ZF domains were linked to DNA methyltransferase 3a (DNMT3a), but not with a catalytic mutant, a repression of Sox2 was observed (Fig 2). Silencing of Sox2 led to a strong suppression of cell proliferation and anchorage independent growth (Fig 3). To verify that the DNMT3a constructs are catalytically active we measured DNAme frequencies using the Sequenom EpiTyper platform (Yale). We have begun preliminary data with previously characterized 6ZF domains targeting the maspin promoter (ZF-97; [35]) in the SUM159 breast cell line. As shown in Fig 4, when transduced in SUM159 cells, the ZF-97DNMT3a but not SKD or controls, induced DNAme in the CpG islands adjacent to the 6ZF-binding site. MDA-MB-231 cells were processed as positive control for DNAme [36]. Interestingly, recent data shows that individual ZF-97Dnmt3a transduced clones (but not catalytic death mutants or untargeted Dnmt3a) retained DNAme frequencies for more than 50 days post-transduction, even though the retroviral constructs were not longer expressed. In conclusion, our data suggest that our Dnmt3a constructs induced
directional, targeted methylation transmitted over cell generations and thus, **6ZF**Dnmt3a constructs were able to stably reprogram the promoter.

![Fig. 4](image)

**Fig. 4.** 6ZF-97 attached to Dnmt3a catalytic domain induces targeted DNA methylation in the maspin promoter, in SUM159 cells, downstream the 6ZF site (red Box). Control=empty vector; ZF97-SKD indicates the same 6ZF domains linked to the SKD domain. MDA-MB-231 is line carrying a methylated promoter. Data represents an average of 3 independent pools of transfected cells.

**KEY RESEARCH ACCOMPLISHMENTS**

- Constructed four 6ZF proteins recognizing highly specific sequences in the Sox2 human promoter.

- Two 6ZF proteins knock-down Sox2 mRNA and protein expression levels, even with higher potency than siRNAs.

- When the proximal 6ZF domains were linked to DNA methyltransferase 3a (DNMT3a), but not with a catalytic mutant, a repression of Sox2 was observed.

- Silencing of Sox2 led to a strong suppression of cell proliferation and anchorage independent growth inhibition of MDA-MB-435s cells.

- When transduced in SUM159 cells, the ZF-97DNMT3a but not SKD or controls, induced DNAme in the CpG islands adjacent to the 6ZF-binding site.

**REPORTABLE OUTCOMES**

**ABSTRACTS AND MANUSCRIPTS:**


**AWARDS**

- V-Foundation award for breast cancer research to Pilar Blancafort, May 2005
- SPORE breast cancer award UNC-Chapel Hill
CELL LINES AND CLONES

- Retrovirally-transduced breast cancer cells able to invade in vitro
- ATF-sequences and clones able to down-regulate gene expression in breast cancer cell lines

CONCLUSION

We have generated arrays of Artificial Transcription Factors (ATFs) made of specific six-zinc finger domains (ZF) targeting unique 18-base pair sites in the SOX2 promoter. The 6ZFs were linked to the repressor domain Krüppel Associated Box (KRAB) domain or DNA methyltransferase 3a (DNMT3a) catalytic domain and expressed in aggressive MDA-MB-435s breast cancer cells using retroviral vectors. Our results show that two ATFs were able to silence SOX2 mRNA and protein levels with virtually 100% down-regulation. This was accompanied with a potent suppression of both tumor cell proliferation and anchorage independent growth. These epigenetic switches represent a promising therapeutic strategy to effectively target breast cancer stem cells.

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