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# REPORT DOCUMENTATION PAGE

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<b>14. ABSTRACT</b> The overall objective of this work is to create novel "chromatin engines", named <u>Designed Epigenetic Remodeling Factors</u> (DERFs). These factors will be designed to recognize 18-base pairs (bp) specific sequences in self-renewal gene promoters (found up-regulated in CSCs). These proteins will then incorporate specific silencing marks, which will trigger endogenous self-renewal gene silencing. Thus, the IDEA is to direct the "chromatin editing" of the breast cancer stem genome, by altering the collection of the epigenetic marks at specific histone tails ("histone code" or "histone grammar") in CSCs. As a proof of principle, we will focus on the gene promoter SOX2, which plays a critical role controlling self-renewal of CSCs (10). Our specific hypothesis is that the delivery of sequence-specific ZF domains (engineered to bind the SOX2 promoter) tethered to specific silencing enzymes will result on forced epigenetic silencing of SOX2, inhibition of CSC self-renewal and inhibition of tumorigenicity in a mouse model of breast cancer.					
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## **Strahl, Brian – Final Progress Report**

### **I. PROPOSAL INTRODUCTION**

A significant amount of research has shown that the majority of cells within the bulk of a breast tumor are differentiated cells that lack tumorigenic potential. As it turns out, the tumor initiating cells comprise a small population that can generate tumors with very small amounts (for example, as little as 100 cells) once injected in immunodeficient mice. 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. Like their normal stem cell counterparts, 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. 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. *Thus, there is 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 **Idea Award** objective was 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, are up-regulated in basal-like breast cancer patients, and that their overexpression is associated with poor prognosis. 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. Strikingly, histone proteins are highly modified by an array of diverse post-translational modifications, including acetylation and methylation. The large number of modifications and the ability of individual histones to be combinatorially modified has given rise to the idea that a “histone code” exists that functions in a combinatorial manner to elicit distinct biological effects. 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. Several histone modifications have been linked to oncogenesis and cancer stem cell biology. 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. 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.

Collective studies like the ones mention above highlight that the chromatin landscape itself, and diversity of chromatin “editing” enzymes that regulate chromatin structure are behind the control of gene expression. Thus, 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. This **Idea Award** proposal aimed 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.

## **II. BRIEF SUMMARY HIGHLIGHTING THE SUCCESSES FROM THIS AWARD**

As highlighted below, and with the end of this award, we have successfully generated a variety of epigenetic remodeling factors. Importantly, we have used these factors to shut-down breast cancer cell growth both in culture and in mouse models! We have published two research articles of this work, presented our data at several meetings, and are excited over the success of our proposed studies. Our Progress and success has prompted Dr. Blancafert and I to apply for an Idea Extension Award, to take these findings further. We have submitted a new proposal aimed at a high-through approach to targeting many potential drives of breast cancer, as a combinatorial means to therapeutically treat cancers that historically have no cures. We are very excited to continue this line of work.

## **III. DETAILED FINAL PROGRESS REPORT FOR THE PROJECT PERIOD**

In this section we will describe the main results and conclusions for the tasks outlined in the statement of work for **years 1, 2** and the **awarded extension** 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 Blancafert 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 (Blancafert) (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 Blancafert 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 (Blancafert):** At the end of the first 6 months the Blancafert 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 (Blancafert) and to trigger specific epigenetic silencing by ChIP assay (Strahl). Months 6-9*

**Task 2. To analyze the phenotype of DERF-transduced cells (Blancafert).**

- a) *To analyze the proliferative capabilities of DERF-transduced cells by self-renewal assays (tumorsphere) assays on DERF-transduced cells: Months 9-10*  
b) *To analyze the proliferative capabilities of DERF-transduced cells by soft-agar anchorage-independent growth assays: Months 10-11*  
c) *To study the capability of DERF-transduced cells to sensitize SUM cell lines to chemotherapy agents (taxol, doxorubicin and cis-platin) by cell viability assays: Months 11-12*

**Final product after month number 12:** The Blancafert lab will have characterized the phenotype of the DERF-transduced cells. At the end of the first year, Dr. Strahl's lab will have completed the ChIP assays to determine the changes in the SOX2 epigenetic landscape. For preparation of task 3, we will focus our efforts on those 6ZF-chromatin remodeling fusions that show the greatest effects on epigenetic changes and SOX2 gene expression.

**Task 3. To investigate if the DERFs characterized in Aims 1 and 2 are able to modulate tumor formation in a xenograft/nude mouse model (Blancafert, Strahl) (Months 12-24). The post-doctoral fellows of Drs. Blancafert and Strahl will work on the injection and monitoring of tumor modulation in SCID mice.**

In this task, we will retrovirally transduce SUM159 or SUM102 cells (depending on activity measurements finalized in year 1) with the most effective SOX2 silencer among the different 6ZF-chromatin remodeling constructs generated in Aim1. We will test a total of five groups: one control (cells retrovirally transduced with an empty retroviral vector) and four DERFs (one DERF for each enzyme subtype: PLU-1, SUV39H1, Set2 and EZH2; Table 1). The DERF clones will be chosen with the highest biological activity as addressed in Task 2. Transduced cells will be injected in the flank of nude mice (N=9 mice/group, 5x9=45 mice). These animals will be induced with doxycycline when the tumors reach approximately 100 mm<sup>3</sup> in volume. The tumors will be monitored every week during a minimum period of a month (depending on the tumor growth). Tumors will be dissected from animals and recovered for real-time and protein detection analyses.

- a) *To retrovirally transduce the SUM lines with DERFs and controls: Months 12-13*  
b) *To Inject these cells if nude mice: Months 13-14*  
c) *To induce the animals with doxycycline, monitor tumor growth (BLI/caliper): Months 14-16*  
d) *To extract the tumors, analyze them by real-time, western, immunohistochemistry: Months 16-17*

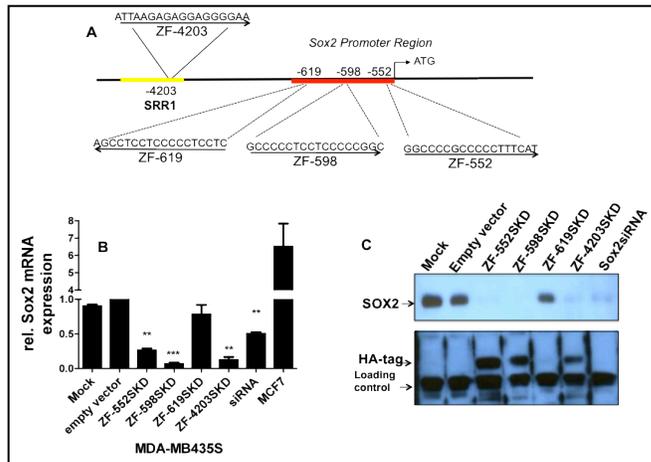
f-i) To perform the same study, but injecting the cells tail vein for experimental metastasis formation. The same groups as described above (45 mice; the same time-frames): Months 17-24

**Final product months 12-24:** At the end of the second year of funding, we will have finished evaluation of the effect of the DERFs on tumor burden and lung colonization, and will have established which constructs are able to modulate SOX2 function *in vivo*.

## OBTAINED RESULTS AND DISCUSSION:

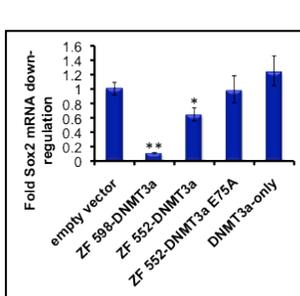
### KEY RESEARCH ACCOMPLISHMENTS FOR YEAR 1

We 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].



**Fig. 1. A:** 18-bp targeted duplexes in the Sox2 promoter / enhancer SRR1 region. **B:** Sox2 mRNA levels by real-time quantification of MDA-MB-435s-transduced cells. Data was normalized to mock-transduced cells. **C:** western blot analysis of nuclear extracts of transduced cells, analyzing Sox2 levels, ZF construct expression (HA). Loading control is total Histone 3. \*\*p<0.05; \*\*\*p<0.001 relative to vector control.

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-

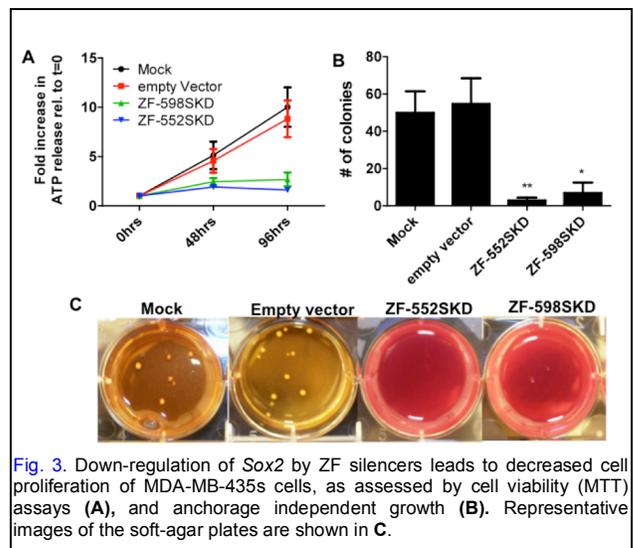


**Fig. 2.** Down-regulation of Sox2 by ZF-DNMT3a silencers in MDA-MB-435s cells, as assessed by real-time PCR. \*p<0.05; \*\*p<0.001 relative to empty vector.

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 inhibition of MDA-MB-435s cells (Fig 3). To verify that the DNMT3a constructs are catalytically active we measured DNA<sub>me</sub> 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

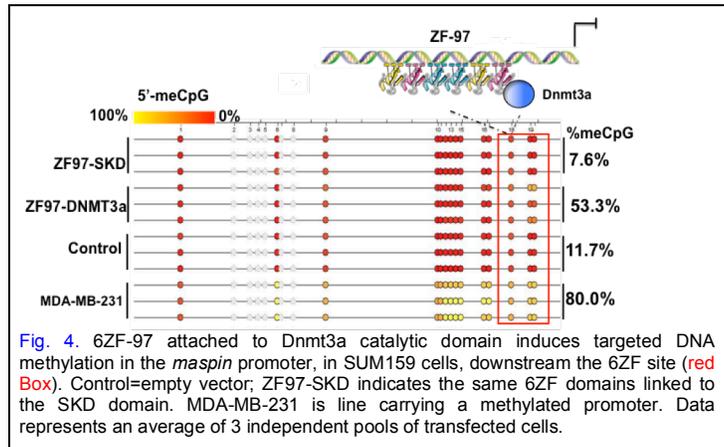


**Fig. 3.** Down-regulation of Sox2 by ZF silencers leads to decreased cell proliferation of MDA-MB-435s cells, as assessed by cell viability (MTT) assays (A), and anchorage independent growth (B). Representative images of the soft-agar plates are shown in C.

SKD or controls, induced DNA<sub>me</sub> in the CpG islands adjacent to the 6ZF-binding site. MDA-MB-231 cells were processed as positive control for DNA<sub>me</sub> [36]. Interestingly, recent data shows that individual ZF-97Dnmt3a transduced clones (but not catalytic death mutants or untargeted Dnmt3a) retained DNA<sub>me</sub> 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, **6ZFDnmt3a constructs were able to stably reprogram the promoter.**

### Summary of 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 DNA<sub>me</sub> in the CpG islands adjacent to the 6ZF-binding site.



### REPORTABLE OUTCOMES

#### ABSTRACTS AND MANUSCRIPTS:

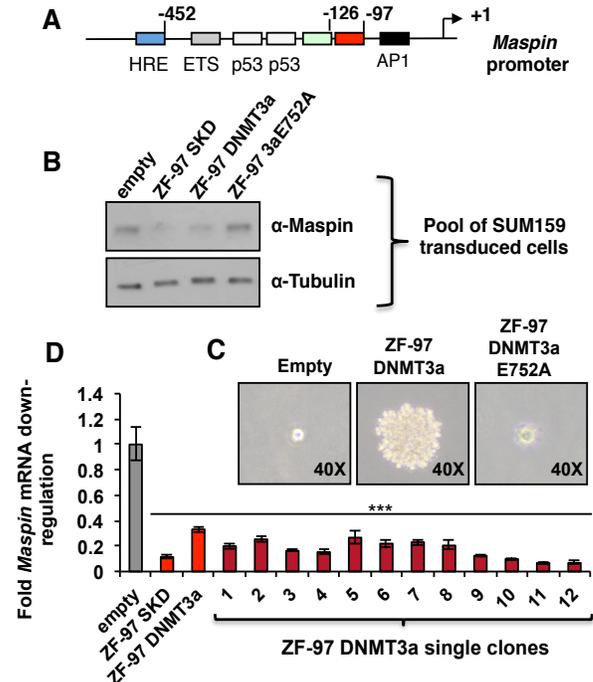
Stolzenburg, S., Yuan, X., Rots, M.G., Strahl, B.D., Blancafort, P. Epigenetic silencing of the Sox2 Oncogene using Artificial Transcription Factors in Breast Cancer Stem Cells. *Era of Hope 2011 sponsored by Department of Defence Breast Cancer Research Program.*

#### CONCLUSIONS FROM YEAR 1

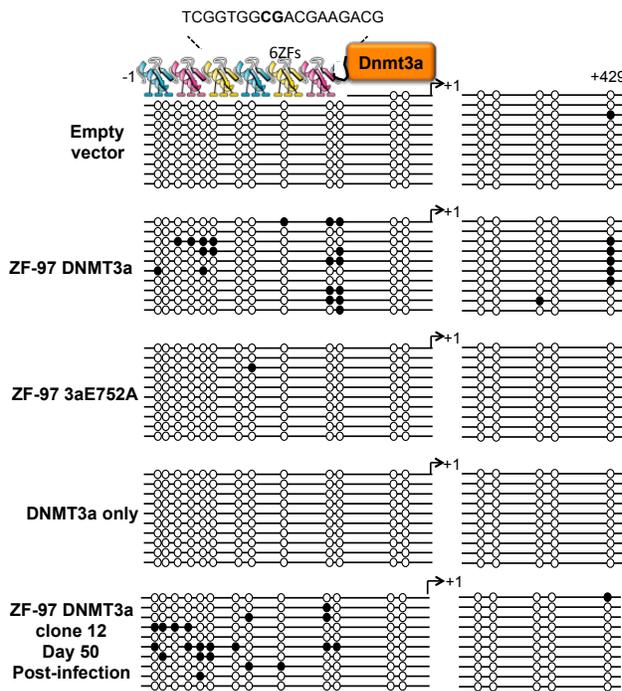
We generated arrays of Artificial Transcription Factors (ATFs) made of specific six-zinc finger domains (ZF) targeting unique 18-base pair sites in the SOX2 promoter. The 6ZFs were linked to the repressor domain Krüppel Associated Box (KRAB) domain or DNA methyltransferase 3a (DNMT3a) catalytic domain 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.

## KEY RESEARCH ACCOMPLISHMENTS FOR YEAR 2 AND EXTENSION

**1. Success in targeting Dnmt3a-directed DNA methylation.** To develop “epigenetic OFF switches”, we first took advantage of the 6ZF proteins previously constructed in the lab, targeting the tumor suppressor *mammary serine protease inhibitor (maspin)*. We have characterized the construction of 3 Artificial Transcription Factors (ATFs) in which the 6ZFs were linked to the transactivator domain VP64. The 6ZFs from ATF-97, -126 and -452 were C-terminally linked to the human catalytic domain of Dnmt3a (residues 614-914), an enzyme that catalyzes *de novo* DNA<sub>me</sub>. We also generated the E752 => A mutant, known to abolish catalytic activity. As control, we attached a Kruppel Associated Box domain (SKD; see Fig. 1 above), which has been shown to promote HDAC-dependent repression when linked to ZFs. These constructs were retrovirally transduced in breast SUM159 cancer cells, which express high levels of *maspin*. We found that the most potent repressive effect was mediated by the proximal protein -97, in which SKD and



**Fig. 1.** Retrovirally delivered 6ZFs linked to silencing domains promote *maspin* down-regulation in SUM159 cells. **A:** 18-bp target sites (colored boxes) of the engineered 6ZFs in the *maspin* promoter. **B:** Down-regulation of Maspin by 6ZF-97 linked to silencing domains, assessed by western blot. SKD=Kruppel Associated Box silencing domain. DNMT3a and catalytically inactive mutant (E752A) are indicated. **C:** Morphology of single clones of transduced cells isolated from soft agar. **D:** Down-regulation of *maspin* in single clones from ZF-97 DNMT3a transductions isolated from soft-agar. Real-time PCR was used to quantify *maspin* mRNA levels relative to empty vector (\*\*\*) $p < 0.0001$ .



**Fig. 2.** 6ZFs linked to Dnmt3a promote targeted DNA<sub>me</sub> in the *maspin* promoter. Methylated (C<sub>me</sub>pG) islands are represented with solid circles.

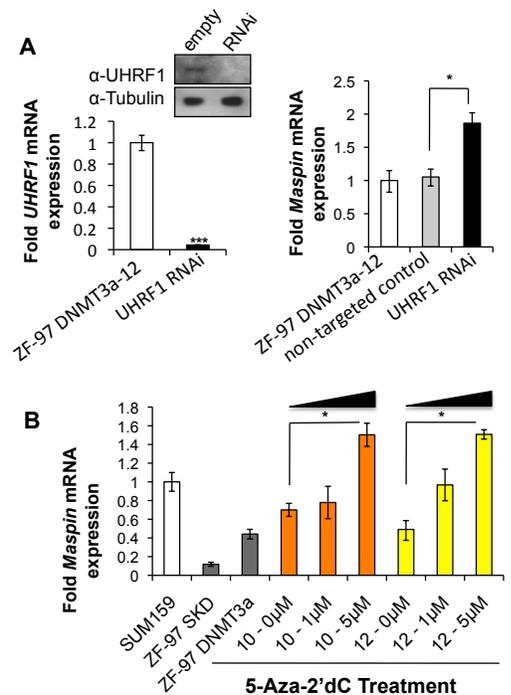
Dnmt3a, but not catalytic mutants, induced transcriptional silencing relative to control (Fig. 1B,D). Further, when single cells from the pool of ZF-97Dnmt3a-transduced cells were seeded in soft agar, colony formation was highly induced relative to the cell line or mutant constructs, which were not tumorigenic in soft agar (Fig. 1C). This phenotype was expected from *maspin* as being a tumor suppressor [8-10]. qRT-PCR analysis of these single clones recovered from the agar demonstrated high levels of repression potentiated by Dnmt3a (Fig. 1D). Retroviral integrated DNA was detected in genomic preparations by PCR, demonstrating that these clones were infected. Importantly, after 50 days post-isolation, these clones began the silencing of the ATF-expressing retrovirus; however, they stably maintained

*maspin* silencing, suggesting that the ATF was able to epigenetically reprogram the promoter.

To verify that Dnmt3a was catalytically active and that targeted DNA<sub>me</sub> was dependent on the specific 6ZF DNA-binding domains, SUM159 cells were transduced with the following constructs: ZF-97Dnmt3a, ZF-97 linked to a catalytically dead Dnmt3a, untargeted Dnmt3a, and empty vector samples. Genomic preparations of infected cells (72hrs post-transduction) were processed by sodium bisulfate conversion, followed by sequencing of single clones, to analyze the % of DNA<sub>me</sub> in the *maspin* promoter (**Fig. 2**). These results demonstrated that the 6ZF domains promoted targeted DNA<sub>me</sub> into the *maspin* promoter, with the highest density of methylated CpG islands immediately adjacent to the 6ZF-targeted site. Some CpG islands were found methylated downstream the 6ZF-binding site (+429; **Fig. 2**), which could reflect the folding and higher accessibility of these di-nucleotides in chromatin. Catalytically dead mutants and untargeted Dnmt3a did not induce DNA<sub>me</sub>, indicating that both, a functional 6ZF-binding domain and an active enzyme were required for targeted methylation *in vivo*. In contrast with our Dnmt3a enzymes, the ZF-97SKD constructs did not result in targeted DNA<sub>me</sub>.

**2. Demonstration that targeted methylation by 6ZF-Dnmt3a is stably transmitted through cell generations.** To address whether the initial methylation pattern was faithfully transmitted through cell generations, we analyzed DNA<sub>me</sub> patterns of single-cell clones of SUM159 cells previously transduced with ZF-97Dnmt3a, maintained in culture for more than 50 passages. These clones had lost the expression of the ZFs-Dnmt3a constructs yet integrated retrovirus was still detected in genomic preparations by qRT-PCR. The transcriptional silencing of retroviral constructs has been described after long-term culture of infected cells, for example with reprogramming TFs. As shown in **Fig. 1**, clone 12 maintained *maspin* silencing with DNA<sub>me</sub> patterns closely resembling those of the initial pool of infected cells at 72 hrs post-infection (ZF-97-Dnmt3a). These data suggested that DNA<sub>me</sub> patterns originally nucleated by the 6ZFs were stably maintained over cell generations.

To further verify the stable transmission of DNA<sub>me</sub> we challenged clone 12 with siRNAs targeting the endogenous protein UHRF1, a factor necessary for the transmission of DNA<sub>me</sub> during replication. UHRF1 recruits endogenous Dnmt1 to faithfully maintain DNA<sub>me</sub> over cell generations. We found that knock-down of UHRF1 lead to a re-activation of *maspin* relative to mismatch-treated cells (**Fig. 3A**). Similarly, treatment of ZF-97Dnmt3a-silenced clones with 5-Aza-2'-dC, an inhibitor of DNA-methyltransferases, also resulted in *maspin* reactivation (**Fig. 3B**). Collectively, these data show that ZFs-Dnmt3a fusions nucleated an initial DNA<sub>me</sub> pattern, which was further "read" and "transmitted" by endogenous proteins, and that 6ZF-Dnmt3a expression was not longer required to maintain the silencing.



**Fig. 3.** Inhibition of endogenous epigenetic silencing proteins in clones previously silenced by ZFs-Dnmt3a fusions resulted in promoter re-activation. **A:** siRNA-mediated knock-down of UHRF1. **B:** 5-Aza-2'dC-inhibition of Dnmts. Transcript level was assessed by qRT-PCR, (\*P<0.05, \*\*\*P< 0.0001).

## **REPORTABLE OUTCOMES**

### **ABSTRACTS AND MANUSCRIPTS:**

Rivenbark, A.G., Stolzenburg, S., Beltran, A.S., Yuan, X., Rots, M.G., **Strahl, B.D.**, and Blancafort, P. (2012) Epigenetic reprogramming of cancer cells via targeted DNA methylation. *Epigenetics* 7:1-11.

Rivenbark, A.G., Stolzenburg, S., Yuan, X., **Strahl, B.D.**, and Blancafort, P. (2012) A designer artificial transcription factor stably reprograms cancer cells by targeted DNA methylation. *FASEB J.*, **26**:142.2. (Meeting Abstract)

Stolzenburg, S., Rots, M. G., Beltran, A. S., Rivenbark, A. G., Yuan, X., **Strahl, B. D.** & Blancafort, P. (2012) Targeted silencing of the oncogene transcription factor SOX2 in breast cancer. *Nucleic Acid Res.* **40**:6725-6740.

Stolzenburg, S., M. G., Beltran, A. S., Rivenbark, A. G., Swift-Scanlan, T., Yuan, X., Rots, **Strahl, B. D.** & Blancafort, P. (2012) Epigenetic reprogramming of breast tumors by engineered Zinc Finger DNA methyltransferases. Meeting Abstract for the Max Planck Freiburg Epigenetics Meeting, December 2012

### **CONCLUSIONS FOR YEAR 2 AND EXTENSION**

We have successfully employed a new technology using artificial transcription factors (ATFs) to control gene expression in cancer cells through targeting and changing the epigenetic landscape. We showed that site-specific DNA methylation and long-term stable repression of the tumor suppressor *Maspin* and the oncogene *SOX2* (in collaboration with Dr. Blancafort) can be achieved in breast cancer cells via zinc-finger ATFs targeting DNA methyltransferase 3a (DNMT3a) to the promoters of these genes. Using this approach, we showed *Maspin* and *SOX2* down-regulation is more significant as compared to transient knockdown, which is also accompanied by stable phenotypic reprogramming of the cancer cell. These findings indicate that multimodular Zinc Finger Proteins linked to epigenetic editing domains can be used as novel cell resources to selectively and heritably alter gene expression patterns to stably reprogram cell fate. We are happy to report that year 2, and the time given by the extension was very successful, and we have achieved our objective. To continue this line of work, we have submitted a new proposal aimed at a high-through approach, involving a new DNA-targeting technology (TALE), to target many potential oncogenic drivers of breast cancer, especially those for which there are no therapeutically means for treatment.