Targeted silencing of the oncogenic transcription factor SOX2 in breast cancer

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ABSTRACT

The transcription factor (TF) SOX2 is essential for the maintenance of pluripotency and self-renewal in embryonic stem cells. In addition to its normal stem cell function, SOX2 over-expression is associated with cancer development. The ability to selectively target this and other oncogenic TFs in cells, however, remains a significant challenge due to the ‘undruggable’ characteristics of these molecules. Here, we employ a zinc finger (ZF)-based artificial TF (ATF) approach to selectively suppress SOX2 gene expression in cancer cells. We engineered four different proteins each composed of 6ZF arrays designed to bind 18 bp sites in the SOX2 promoter and enhancer region, which controls SOX2 methylation. The 6ZF domains were linked to the Kruppel Associated Box (SKD) repressor domain. Three engineered proteins were able to bind their endogenous target sites and effectively suppress SOX2 expression (up to 95% repression efficiencies) in breast cancer cells. Targeted down-regulation of SOX2 expression resulted in decreased tumor cell proliferation and colony formation in these cells. Furthermore, induced expression of an ATF in a mouse model inhibited breast cancer cell growth. Collectively, these findings demonstrate the effectiveness and therapeutic potential of engineered ATFs to mediate potent and long-lasting down-regulation of oncogenic TF expression in cancer cells.

INTRODUCTION

Transcription factors (TFs) are crucial molecules orchestrating gene programs involved in self-renewal, differentiation and organism’s developmental patterning. Maintaining the proper threshold of expression of TFs is critical for the normal homeostatic function of cells and tissues. Aberrant regulation of TF expression is frequently found in human malignancies and associated with specific tumor subtypes (1). Over-expression of oncogenic TFs is well documented in the mammary gland, particularly in poorly differentiated, triple negative breast cancers (TNBCs) (2). TNBCs are characterized by the lack of expression of Estrogen Receptor (ER), Progesterone Receptor (PR) and Epidermal Growth Factor Receptor 2 (Her2). Recent progress revealed that some TNBCs belonging to the basal-like and claudin-low intrinsic subtypes of breast cancers are highly aggressive and resistant to treatment (3–5). It has been proposed that these breast cancers are enriched in stem cells, which might be critical for tumor initiation, progression and resistance to chemotherapy and radiation (6–11). Albeit their fundamental role in tumor etiology and progression, TFs are currently refractory to target-based drug discovery approaches due to their lack of small molecule binding pockets. Thus, novel strategies are required to efficiently silence the aberrant expression of oncogenic TFs in cancer cells. Ideally these novel approaches should restore and stably maintain the expression pattern of these TFs, like it is observed in normal epithelial cells.

The SOX2 gene encodes a TF belonging to the high-mobility group (HMG) family (12). SOX2 expression is critical for the maintenance of self-renewal in embryonic stem cells (ESCs) and neural progenitor cells (13–15).

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While SOX2 is highly transcribed in self-renewal conditions, its promoter undergoes epigenetic silencing during the onset of differentiation of stem cells (16,17). In neural stem cells epigenetic modifications in two SOX2 enhancer elements, SRR1 and SRR2, control the onset of differentiation gene programs (18). Thus, in the majority of differentiated cells, including mammary epithelial cells, the SOX2 promoter is silenced (19). However, SOX2 has been detected in normal gastric mucosae and promoter silencing by DNA methylation has been reported in some human gastric carcinomas (20,21). In contrast to gastric cancers, SOX2 has been found over-expressed in multiple malignancies. The SOX2 gene was found amplified in a subset of squamous cell lung and esophageal cancers in which the amplification/upregulation of SOX2 was associated with improved clinical outcome (22). Several publications report over-expression of SOX2 in glioblastomas (23), non-small cell lung cancer (24,25), prostate cancer (26), hepatocellular carcinomas (27) and breast carcinomas (28), supporting a role of SOX2 as an oncogene in these tissues. SOX2 was found over-expressed in 28% of all invasive breast carcinomas and in 43% of basal-like TNBCs (29). These reports suggest that SOX2 could activate important gene cascades involved in tumor initiation and progression and in the maintenance of a poorly differentiated state.

Previous studies targeting SOX2 in breast cancer cell lines have shown that shRNA-mediated knock-down of SOX2 resulted in cell cycle arrest by down-regulation of Cyclin D1 (30). This arrest in the cell cycle was accompanied by an inhibition of tumor cell proliferation in xenograft models (30). Although shRNA or siRNA approaches are widely used to silence gene expression, there are potential limitations associated with inhibitory RNA (RNAi). First, oncogenes are expressed at very high levels in the mammary tissue, and thereby these targets are difficult to knock-down completely by RNAi. Second, siRNAs have a transient effect in tumor cells due to the short half-life of the small RNAs, which limits the long-term effect of RNAi in tumor cells. We reasoned that molecules able to directly silence the promoter and DNA regulatory regions necessary for oncogenic transcription would result in potent transcriptional down-regulation of the targeted gene.

Direct alteration of endogenous gene expression at DNA level requires a sequence-specific DNA-recognition module and an effector domain, which modulates transcriptional activity. Zinc-finger (ZF)-based artificial transcription factors (ATFs) are currently the state-of-the-art molecules able to bind genomic sequences with potentially single locus specificity (31,32). Because ZFs bind endogenous DNA sequences with high selectivity, they provide an opportunity to modify, edit, and sculpt the epigenetic and transcriptional state of endogenous promoters. In the past, several genes have been targeted with ZF-based ATFs for transcriptional up- and down-regulation of targeted promoters (33–36). Recently, our laboratory has reported ATFs able to reactivate the expression of the tumor-suppressor gene MASPIN, which is silenced by epigenetic mechanisms in metastatic tumor cells. Expression of our ATFs in breast cancer cells decreased tumor growth and metastasis in vivo (37,38). Likewise, ATFs have been designed to repress potential oncogenes, such as Epithelial Cell Adhesion Molecule (EpCAM), human Telomerase Reverse Transcriptase (hTERT) and ErbB2/ErbB3 (34,39–41). In this article, we investigated the capability of ATFs to down-regulate the oncogenic TF SOX2 in breast cancer cell lines. Retroviral delivery of three out of four designed ATFs led to a potent (~95%) down-regulation of endogenous SOX2 mRNA and protein expression in two breast cancer cell lines. This strong suppression of the endogenous SOX2 promoter activity was accompanied by a long-term inhibition of tumor cell proliferation and anchorage-independent growth. Furthermore, one of our ATFs was able to efficiently inhibit tumor growth in a xenograft model of breast cancer. Importantly, repression of SOX2 was still maintained in the tumors in vivo even 48 days post-injection of the tumor cells. Overall, our data outline the therapeutic potential of ATFs to effectively repress oncogenic TFs that are highly expressed in cancer cells.

MATERIALS AND METHODS

Cell lines and cell culture

The packaging cell line 293T-GagPol cells and the human breast cancer cell lines MDA-MB-231, MDA-MB-435s were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS, BenchMark, Gemini Bio Products) and 1% Penicillin streptomycin (Pen/Strep, Invitrogen, Carlsbad, CA). Culture media of MDA-MB-435s cells contained additionally 0.01 mg/ml Bovine Insulin (Invitrogen). MCF7 breast cancer cells were cultured in Minimum Essential Media (MEM) supplemented with 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids (NEAAs), 1 mM sodium pyruvate, 0.01 mg/ml Bovine Insulin, 10% FBS and 1% Pen/Strep. MCF7 and MCF-12A cells were cultured in DMEM containing 20 ng/ml Epithelial Growth Factor (EGF), 100 ng/ml cholera toxin, 0.01 mg/ml Bovine Insulin, 500 ng/ml hydrocortisone, 5% Horse serum and 1% Pen/Strep. MDA-MB-468 breast cancer cells were cultured in L15 media supplemented with 10% FBS and 1% Pen/Strep. ZR-75-1 and BT549 cells were cultured in RPMI 1640 media supplemented with 10% FBS and 1% Pen/Strep. SUM102 and SUM149 cells were cultured in human mammary epithelial cell (HuMEC) media containing HuMEC supplemental bullet kit (Gibco/Invitrogen), bovine pituitary extract (Gibco/Invitrogen) and 1% Pen/Strep. For SUM149 cells media contained additionally 5% FBS. SUM159 breast cancer cells were cultured in Ham's F12 media containing 5 μg/ml Bovine Insulin, 1 μg/ml hydrocortisone, 10 mM Hepes, 5% FBS and 1% Pen/Strep. SK-Br-3 cells were cultured in McCoy’s 5a Medium, supplemented with 10% FBS and 1% Pen/Strep. For SUM149 cells media contained additionally 5% FBS. SUM159 breast cancer cells were cultured in Leibovitz’s L15 Medium, supplemented with 10% FBS and 1% Pen/Strep. All cell lines were purchased...
from ATCC (American Type Culture Collection, Manassas, VA, USA) maintained at 37°C and 5% CO₂.

**ATF construction**

The ZF target sites within the SOX2 promoter were selected using the website www.zincfingertools.org (42). The selection of three 18-bp target sites was based on the close proximity to the transcriptional start site and the high content of GNN-triplets in the target sequence. One ATF was designed to target an 18-bp sequence in the SOX2 enhancer region 1, ~4-kb upstream of the TSS (Figure 1B). Specific primers were designed coding for the amino acids in the recognition helix of the ZFs responsible for the binding to the target sequence (Figure 1C). The ZF proteins were generated by overlapping PCR as described (43), SfiI-digested fragments were subcloned into the retroviral vector pMX-IRES-GFP-SKD, generating pMX-ZF552SKD, pMX-ZF598SKD, pMX-ZF619SKD and pMX-ZF4203SKD. Each ATF contains an internal SV40 nuclear localization signal (NLS) and a terminal hemagglutinin (HA) decapptide tag. The correct ZF-sequence of the obtained product was confirmed by plasmid sequencing.

**Retrovirus infection of MDA-MB-435s**

The pMX retroviral vectors containing the SOX2-ATFs were first co-transfected with the plasmid (pMDG.1) expressing the vesicular stomatitis virus envelope protein into 293TGagPol cells to produce retroviral particles. Transfection was performed using Lipofectamine™ (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. For cell proliferation and soft agar assays, cells were harvested 24 h after the last infection. For flow cytometry analysis, protein, and mRNA extraction, transduced cells were harvested 48 h post-infection.

**siRNA transfection**

MDA-MB-435s breast cancer cells were transfected with either a SOX2-specific siRNA pool (siGENOME D-011778-01-04), an irrelevant (non-specific) siRNA

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Figure 1. Design of ATFs to down-regulate SOX2 expression. (A) Schematic representation of a 6 ZF ATF bound to DNA with the orientation of the domains depicted. (B) Schematic illustration of the SOX2 promoter outlining the ZF-552SKD, ZF-598SKD, ZF-619SKD and ZF-4203SKD targeted sequences and their location relative to the transcription start site (TSS). Highlighted are the core promoter (red), regulatory region 1 (green), and regulatory region 2 (blue). Arrows show the orientation of the 18-bp binding site in the promoter (from 5' to 3'). (C) Alpha-helical ZF amino acid sequences chosen to construct the ATFs. Residues at position –1, +3 and +6 making specific contacts with the recognition triplets are indicated in color (red refers to position –1, blue to position +3 and green to +6 of the ZF recognition helix). (D) Quantification of SOX2 expression in 12 breast cancer cell lines by western blot.
pool targeting the TF *PATZ1* (siGENOME M-013539-00) or human a positive control for transfection, a cytotoxic siRNA pool *UBB* (siGENOME MU-013382-01-0002). The siRNAs were transfected using DharmaFECT (Dharmacon, Lafayette, CA) according to manufacturer's protocol. Cells were collected 72 h after transduction for RNA or protein preparations.

**Generation of MCF7 stable cell lines**

The coding region of the ATFs ZF-552SKD and ZF-598SKD was subcloned using the *BamHI/EcoRI* restriction sites into the expression vector pRetroX-Tight-Pur (CloneTech, Mountain View, CA). Retroviral particles from pRetroX-Tight-ZF-552SKD (ZF-552SKD), pRetroX-Tight-ZF-598SKD (ZF-598SKD), pRetroX-Tight-empty vector (empty vector), and pRetroX-Tet-On-Advanced (pTet-On) were generated by Lipofectamin transfection (Invitrogen, Carlsbad, CA) of 293T-Gagpol cells according to manufacturer's recommendation. Virus-containing supernatant was harvested 48 h post-transfection, filtered, and concentrated using Amicon Ultra centrifugal filter Units (Millipore, Billerica, MA). MCF7 cells were selected with 2\( \mu \text{g/mL} \) puromycin (InvivoGen, San Diego, CA) and 3\( \mu \text{g/mL} \) geneticin (Gibco/Life Technologies, Grand Island, NY). For staining of tumor sections we used the following antibodies: rabbit anti-fibronectin (Sigma-Aldrich, St Louis, MO). Immunofluorescence was performed using an anti-HA antibody 1:500 (Covance, Princeton, NJ) 1:2500 or anti-H3 (Active Motif, Carlsbad, CA) diluted 1:1000, monoclonal mouse anti-HA-tag 1 \( \mu \text{g/mL} \) (Covance, Princeton, NJ) 1:2500 or anti-H3 (Active Motif, Carlsbad, CA) diluted 1:10,000. The horseradish peroxidase-conjugated secondary mouse anti-rabbit and rabbit anti-mouse antibodies were used for detection (Jackson Immunoresearch, West Grove, PA) diluted 1:10000 and visualized using ECL plus kit (Amersham, Piscataway, NJ).

**Lentiviral transduction of SOX2 cDNA**

HEK 293T cells were co-transduced with both supernatants, one containing empty or ATF-expressing retroviral particles, and the second containing the transactivator pTet-On particles (CloneTech, Mountain View, CA) in a ratio of 1:1. Double stable MCF7 cells were selected with 2\( \mu \text{g/mL} \) genetin (Gibco/Invitrogen) and 5\( \mu \text{g/mL} \) puromycin (InvivoGen, San Diego, CA) for 10 days. ATF expression was induced using Doxycycline (Dox, 100 \( \mu \text{g/mL} \)) for 72 h.

**Treatment with 5-aza-2'-deoxycytidine (5-Aza)**

MCF7 cells stably transduced with empty vector or ZF-552SKD were plated in a density of \( 5 \times 10^5 \) cells in 10 cm plates. Cells were un-induced or induced with Dox (+Dox) and simultaneously treated with either vehicle or 5 \( \mu \text{M} \) 5-Aza (+5-Aza, Sigma Aldrich, Milwaukee, WI). Cells were cultured for 48 h and then processed for quantitative real-time PCR (qRT-PCR).

**qRT-PCR**

Total RNA was extracted using RNaseasy Kit (Qiagen; Valencia, CA) and 3 \( \mu \text{g} \) of RNA was converted into cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). qRT-PCR was carried out as described (38). *SOX2* mRNA expression levels were detected using Taqman primer and probes (Applied Biosystems, Foster City, CA). *SOX2* (Hs01053049s1) and *GAPDH* (FAM/MGB #433764F). Detection of *Cyclin D1* mRNA was carried out using Absolute Blue PCR SYBR Green Low ROX Mix (Thermo Scientific, Rockford, IL) with the following primers forward 5'-GCCCTGTGAAACAAGCTCAA-3' and reverse 5'-TGGAGAGGAGGTGCCATGACCA-3'. For detection of human *GAPDH* as endogenous control the primers forward 5'-CCATGTTCGTCATGGGTGTA-3' and reverse 5'-CATGGACTGTTGGTCATGAGT-3' were used. Data were analyzed using the comparative \( \Delta \text{Ct} \) method (ABPrism software, Applied Biosystems, Foster City, CA) using *GAPDH* as an internal normalization control. Data represented an average of at least three independent experiments and statistical analysis was performed using Student’s \( t \)-test.

**Nuclear extract preparation and western blotting**

MDA-MB-435s cells were harvested 48 h post-transduction and MCF7 cells were harvested 72 h after Dox-induction. Nuclear protein was extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Thermo Scientific, Rockford, IL) according to manufacture’s instruction. For western blot 25 \( \mu \text{g} \) of nuclear protein per lane was loaded and resolved on 10% pre-cast NuPAGE Bis-Tris Mini Gels (Invitrogen, Carlsbad, CA). Proteins were transferred from the gel on a Sequi-Blot PVDF membrane (BioRad, Hercules, CA). Membranes were blocked with 5% non-fat dry milk/TBST for at least 1 h, and then probed with the following antibodies: rabbit anti-SOX2 polyclonal antibody (Cell Signalling Technology, Danvers, MA) diluted 1:1000, monoclonal mouse anti-HA-tag 1 \( \mu \text{g/mL} \) (Covance, Princeton, NJ) 1:2500 or anti-H3 (Active Motif, Carlsbad, CA) diluted 1:10,000. The horseradish peroxidase-conjugated secondary mouse anti-rabbit and rabbit anti-mouse antibodies were used for detection (Jackson Immunoresearch, West Grove, PA) diluted 1:10000 and visualized using ECL plus kit (Amersham, Piscataway, NJ).

**Immunofluorescence and Immunohistochemistry**

MCF7 cells were plated in 24-well plates coated with fibronectin (Sigma-Aldrich, St Louis, MO). Immunofluorescence was performed using an anti-SOX2 antibody (AB 5603, Millipore, Billerica, MA) diluted 1:200 and an anti-HA antibody 1:500 (Covance, Princeton, NJ). For staining of tumor sections we used the following antibodies: anti-SOX2 (AB 5603, Millipore, Billerica, MA) 1:500, an anti-Ki67 antibody (ab833, Abcam, Cambridge, MA) 1:100, and an anti-HA antibody (Covance, Princeton, NJ) 1:1000. SOX2 was detected using an Alexa-Fluor555 anti-rabbit IgG (Invitrogen, Carlsbad, CA) 1:1000 dilution in MCF7 cells and 1:750 on tumor sections. Detection of the HA epitope tag was performed with an Alexa-Fluor488
anti-mouse IgG (Invitrogen, Carlsbad, CA) 1:1000 dilution in MCF7 cells and 1:500 on tumor sections. Images were taken using a confocal Leica microscope at 40× magnification.

**Chromatin immunoprecipitation (ChIP) assay**

Doxycycline-induced and un-induced MCF7 cells were fixed, sonicated, and incubated with either an anti-HA (Covance, Princeton, NJ) antibody or anti-RNA Polymerase II (8GW16; Covance, Princeton, NJ) antibody, respectively (1 μg/reaction). DNA complexes were immunoprecipitated using Protein A Sepharose 4 Fast Flow beads (GE Healthcare, Pittsburgh, PA). DNA was amplified by PCR using the SOX2-specific primers: 5’-AGTGGAAATTTTGTGGAG-3’ and 5’-ATATACCTATCCTTCTCATAA-3’, with the following conditions: cycle 1, 5 min at 95°C; cycle 2, 1 min at 95°C; cycle 3, 1.30 min at 53°C; cycle 4, 1 min at 72°C; repeat cycle 2 to 4, 35 times followed by a final step of 10 min at 72°C. PCR-products were visualized on a 1.8% agarose gel.

**Cell proliferation assays**

Six replicates of MDA-MB-435s/MCF7 cells were plated in 96-well flat bottom plates in a density of 1000 cells per well. Cell proliferation was assessed every 24 h using a CellTiter Glo assay (Promega; Madison, WI) according to the manufacturer’s instructions. Emitted luminescence was detected in a PHERAstar plate reader (BMG LABTECH, Durham, NC) and analyzed using PHERAstar software. Results were normalized to readings obtained immediately after seeding of the cells (day = 0). Statistical analyses were performed by 2-way analysis of variance (ANOVA).

**Anchorage independent colony formation assays**

For colony formation assays, 1.8% Agarose/PBS was diluted with cell culture media to a final concentration of 0.6%, and 2 ml/well media/agar solution was plated in the bottom layer of a 6 well plate. For the top layer 5000 cells were re-suspended in 0.3% media/agar solution and plated in a volume of 2 ml/well on the solidified bottom layer. The soft agar was covered with 0.5 ml culture media and cultured in 5% CO2 humidified incubator at 37°C for 50 days. Experiments were performed in three replicates. Plates were counted visually for the presence of colonies that were greater than 2 mm in diameter. Statistical analysis was performed with a Student’s t-test with level of significance \( P < 0.05 \).

**Subcutaneous injections**

Female NUDE mice (age 4 weeks) were purchased from Taconic Farms and housed under pathogen-free conditions. The Institutional Animal Care and Use Committee (IACUC) at the University of North Carolina at Chapel Hill approved all experiments described herein. Estrogen pellets containing 2 mg 17β-Estradiol (Sigma-Aldrich Corp. St. Louis, MO) and 8 mg Cellulose (Sigma-Aldrich Corp. St. Louis, MO), were subcutaneously implanted in the animals 7 days prior of the injection of the cells. MCF7 cells (2 × 10^6) were collected and re-suspended with matrigel (BD Bioscience, San Diego, CA) 1:1 volume ratio in a total volume of 100 μl. The cell–matrigel mixture was injected into the mouse flank. Tumor growth was monitored by caliper twice a week. When the tumor reached a size of approximately 50–100 mm^3, Doxycycline (+Dox) was administered to the mice in the form of green food pellets (200 mg/kg of mice chow) for a period of 28 days. During the entire experiment the mice weight was monitored to ensure absence of toxicity. Animals were euthanized 28 days after Dox induction. Statistical differences between control and ATF animals were assessed by Wilcoxon Ranks Sum Test analysis.

**RESULTS**

**Delivery of SOX2-specifc ATFs in breast cancer cells suppresses SOX2 expression**

In order to down-regulate SOX2 expression in tumor cells, we designed ATFs consisting of arrays of 6ZF domains linked to a potent repressor domain, the Kruppel Associated Box (SKD) domain. Each ZF domain recognizes 3 bp of genomic DNA, and arrays of 6ZF domains will read an 18-bp stretch of endogenous DNA (Figure 1A). Using the helix grafting or modular approach, initially developed by the Barbas group (44), we engineered four distinct ATFs. Three ATFs were designed to bind the proximal SOX2 promoter (ZF-552SKD, ZF-598SKD, and ZF-619SKD) and one ATF (ZF-4203SKD) was directed against the SOX2 regulatory region I (SRR1), which controls SOX2 silencing in stem cells (Figure 1B) (18). ZF-552SKD was engineered to recognize a sequence that was perfectly conserved between the murine and the human promoters. The ZF proteins were constructed by PCR using the helix grafting approach as we have previously described (43). The specific z-helical sequences used for the assembly of the proteins are shown in Figure 1C.

We first investigated SOX2 expression levels in a panel of 12 breast cancer cell lines by western blot (Figure 1D). We found that SOX2 was over-expressed in several breast cancer lines relative to non-transformed breast epithelial cells, such as MCF-12 A. The highest expression of SOX2 was detected in the ER+ luminal MCF7 cell line, followed by the claudin-low triple negative MDA-MB-435s cell line. SOX2 was also found up-regulated in the ER+ luminal line ZR-75-1 (Figure 1D). Gene expression microarrays have recently questioned whether the cell of origin of the MDA-MB-435 line is melanoma or basal breast cancer (45,46). More recently, with the discovery of the new mesenchymal intrinsic subtype of breast cancer (47), MDA-MB-435s cells have been clustered within the claudin-low subtype of breast cancer (5). To examine if the ATFs were able to silence the endogenous SOX2 promoter, we chose the highest SOX2 expressing lines MCF7 and MDA-MB-435s as model cell lines.

For transduction of MDA-MB-435s cells, the retroviral vector pMX-IRES-GFP was used. These cells were transduced with up to 80–90% efficiency, as measured
by flow cytometry (data not shown). Quantitative changes in \( \text{SOX2} \) mRNA expression upon transduction of MDA-MB-435s cells were assessed by real-time expression analyses (qRT-PCR; Figure 2A). As shown in Figure 2A, a significant down-regulation of \( \text{SOX2} \) mRNA expression was achieved with ZF-552SKD, ZF-598SKD but not with ZF-619SKD, relative to empty vector control. Furthermore, the targeting of the regulatory region I of
SOX2 by ZF-4203SKD led to a potent down-regulation of SOX2 mRNA (Figure 2A). When siRNA was used to knock-down the SOX2 mRNA, only 50% SOX2 mRNA down-regulation was achieved by the SOX2-specific siRNA relative to control cells transduced with a non-specific siRNA. Importantly, ZF-552SKD, ZF-598SKD and ZF-4203SKD resulted in 74, 94 and 88% down-regulation of SOX2 mRNA levels relative to empty vector control. Consistent with the results in Figure 2A, reduction of SOX2 mRNA expression resulted in strong suppression of SOX2 protein expression by ZF-552SKD, ZF-598SKD and ZF-4203SKD, but not with ZF-619SKD (Figure 2B; Supplementary Figure S1). The ZF-619SKD construct was not properly expressed in the tumor cells, as assessed by western blotting using an anti-HA antibody to detect the terminal HA-tag in the ZF protein (Figure 2B). Improper translation of designed proteins could be due to instability of the protein or ineffective codon usage. Thus, this construct had no significant effect on SOX2 mRNA expression. As an unspecific SKD control we used a diversity library of 6ZF domains comprising more than 10^7 different ZFs capable of targeting any 5’-(GNN)6-3’ sequence in the genome (48). These 6ZF-library members were linked to the SKD repressor domain (library-SKD). Some down-regulation of SOX2 (38%) was observed upon transduction of this library in the cells, which was expected based on the potential of multiple library constituents to regulate not only the SOX2 cis-regulatory regions but also other regulatory sequences, which could indirectly affect SOX2 expression. The effect of library members on gene expression has been well documented (48–52). However, the repressive effect of the 6ZF library was significantly lower than the effect of the proteins −552, −598 and −4203, demonstrating the sequence selectivity of the engineered ZF arrays. Similarly, the retroviral delivery of the SOX2-specific 6ZFs in absence of effector domain had no impact in SOX2 transcriptional regulation. In addition, when the same 6ZFs were linked to the transcriptional activator VP64, a significant up-regulation of SOX2 mRNA expression was achieved with ZF-598VP64 and ZF-4203VP64 in MDA-MB-435s cells (Figure 3). This data indicated that the regulatory effect of the engineered proteins required both, a sequence specific DNA binding domain and a functional effector domain. The down-regulation of SOX2 by the ZF proteins was validated at protein level by western blot (Figure 2B and D) and is quantitated in Supplementary Figure S1.

We next focused on the two most potent proximal proteins, ZF-552SKD and ZF-598SKD, to assess their capability to suppress SOX2 expression in a second cell line, MCF7. Since MCF7 cells have lower transduction efficiencies than MDA-MB-435s, we generated stable cell lines using the pRetroX-tight retroviral vector system, by which the expression of the ZF protein is controlled by Doxycyclin (Dox). The induction of ZF-552SKD and ZF-598SKD in MCF7 cells (+Dox) resulted in potent down-regulation of both SOX2 mRNA and protein expression, compared with un-induced control (−Dox). In contrast, no change of SOX2 expression levels were detected in −Dox cells transduced with empty vector (Figure 2C and D; Supplementary Figure S1). The expression of the ZF proteins in MDA-MB-435s and MCF7 cells was validated by western blotting and immunofluorescence (IF), respectively, using an anti-HA antibody to detect the C-terminal tag of the ZF constructs (Figure 2B and E). Collectively these results demonstrated that the ZF silencers resulted in strong suppression of SOX2 expression in MDA-MB-435s and MCF7 cells.

The engineered ATFs ZF-552SKD and ZF-598SKD bound their targeted DNA in vivo

To verify the binding of our engineered proteins to their target sites in the SOX2 promoter in vivo, ChIP assays were performed. MCF7 cells stably transduced with either ZF-552SKD or ZF-598SKD were induced with Dox (+Dox) or maintained in −Dox media. Cells were fixed, cross-linked, and chromatin was extracted. First, ZF–DNA complexes were immunoprecipitated with an anti-HA antibody, which detects the C-terminal tag of the ZF constructs. The ChIP products were next amplified by PCR using specific primers flanking the 18-bp ZF binding sites (Figure 4A). As shown in Figure 4B induction of ZF-552SKD and ZF-598SKD by Dox led to a strong enrichment of the HA-immunoprecipitated products, indicating that the ZF constructs were binding to their target sites in the context of the endogenous SOX2 promoter. In addition, when the ChIP experiments were performed with an anti-RNA Polymerase II (RNA Pol II) antibody, a decrease of RNA Pol II-immunoprecipitated products was detected in +Dox cells relative to the un-induced cells (Figure 4B). These experiments indicate that the engineered ZF proteins were physically associated with the SOX2 promoter and directed potent transcriptional repression. This silencing of SOX2 expression was
Figure 4. ATFs bind their targeted site in the endogenous \textit{SOX2} promoter. (A) Schematic illustration of the chromatin Immunoprecipitation (ChIP) assay. (B) ZF-598SKD (upper panel) and ZF-552SKD (lower panel) are binding their target sites, as assessed by ChIP using an anti-HA antibody. Genomic DNA bound by the corresponding ATF was amplified using \textit{SOX2}-specific primers. An anti RNA-polymerase II (RNA-Pol II) antibody and no antibody (No AB) samples were used in the same assay, as positive and negative controls, respectively. A quantification of the ChIP assay by densitometry analyses of the bands from the same gels is outlined below. (C) A schematic illustration of the proposed repressive mechanism induced by ZF silencers in the \textit{SOX2} promoter. Upon recruitment of the co-repressor KAP1 (KRAB-associated protein 1) and NuRD (nucleosome remodeling and deacetylase) by SKD in the targeted site, a repressive complex including HDACs (histone deacetylases), SETDB1 (histone methyltransferase), and HP1 (heterochromatin protein 1) is assembled. This repressive complex catalyzes the formation of condensed chromatin by de-acetylation of histones, demethylation of H3K4me3, and incorporation of H3K9me3.
ATF-mediated down-regulation of SOX2 expression decreased cell proliferation and anchorage-independent growth of MDA-MB-435s and MCF7 cells

Ectopic expression of the SOX2 cDNA has been associated with an induction of oncogenic properties in different cancer cell types, including breast cancer. Reciprocally, shRNA-mediated knock-down of SOX2 in breast (30,54) and lung (55,56) cancer cell lines resulted in decreased tumor cell growth both in vitro and in vivo. Consequently, we investigated whether the down-regulation of SOX2 expression mediated by our SOX2-specific ATFs would also entail a decreased tumorigenic phenotype of breast cancer cells. To this end, MDA-MB-435s and MCF7 cells transduced with either ZF-552SKD or ZF-598SKD were first subjected to cell viability assays. We monitored cell viability of MDA-MB-435s cells transduced with either empty vector, ZF-552SKD or ZF-598SKD over time for a total period of 96 h (Figure 5A). We found that cells expressing ZF-552SKD and ZF-598SKD exhibited a significant reduction in tumor cell growth relative to un-transduced mock cells or empty vector-transduced cells (both ATFs P < 0.001). In MCF7 cells stably transduced with the same constructs, Dox treatment of the ZF-transduced cells resulted in decreased cell proliferation relative to controls, even at 120 h after seeding of the cells (Figure 5B).

To further validate that the down-regulation of SOX2 expression by the ZF silencers resulted in a decreased tumorigenic phenotype, we performed colony formation assays, which monitor anchorage-independent growth (Figure 5C and D). MDA-MB-435s un-transduced mock cells, empty vector, ZF-552SKD- and ZF-598SKD-transduced cells were seeded in soft agar and the number of colonies was quantified. While mock treated and empty vector transduced cells formed abundant foci in soft-agar, down-regulation of SOX2 by either ZF-552SKD or ZF-598SKD abolished colony formation (Figure 5C). These results were also validated in the MCF7 cell line stably transduced with ZF-552SKD and ZF-598SKD, where induction of the ATFs by Dox effectively suppressed colony formation (Figure 5D).

The oncogenic properties of SOX2 have been associated with activation of Cyclin D1 promoter, by direct binding and trans-activation of the SOX2 TF. Reciprocally, down-regulation of SOX2 was shown to arrest the proliferation of the breast cancer cells by down-regulation of Cyclin D1 (30). We therefore analyzed Cyclin D1 mRNA levels in MCF7 cells stably transduced with either ZF-552SKD, ZF-598SKD or controls. As shown in Supplementary Figure S3, induction of the ZF silencers resulted in a significant down-regulation of Cyclin D1 mRNA relative to control cells. Overall, these data demonstrate that our engineered proteins promote a down-regulation of tumor cell proliferation and anchorage-independent growth.

To confirm that the phenotype of the ZF silencers in inhibiting tumor cell proliferation was dependent on the SOX2 target, rescue experiments with the SOX2 cDNA were performed (Supplementary Figure S4). MCF7 cells stably transduced with either ZF-552SKD or ZF-598SKD were challenged with either a SOX2 cDNA-expressing lentiviral vector (pSinSOX2) or with an empty vector control. 48 h after adding the lentiviral supernatants, the cells were either maintained in a -Dox medium or switched to a Dox-containing medium to activate the expression of the ZF proteins. As shown in Supplementary Figure S4, the delivery of SOX2 cDNA in -Dox cells resulted in enhanced cell proliferation relative to control, consistent with the oncogenic function of SOX2 cDNA in breast cancer. The delivery of the SOX2 cDNA in +Dox cells rescued the cell proliferation phenotype of the ZF proteins. These functional assays demonstrate that the observed phenotype can be directly attributed to SOX2 expression.

Down-regulation of SOX2 using ZF-598SKD inhibited tumor growth in a breast cancer xenograft model in immunodeficient mice

To analyze the effect of the SOX2 ZF silencers in vivo, we focused on ZF-598SKD since this protein mediated potent repression of breast tumor proliferation in vitro. We took advantage of the Tet-ON inducible ZF-598SKD and empty vector control transfected MCF7 cell lines to analyze whether Dox induction of the ZF repressor resulted in long-term repression of SOX2 and decreased tumor cell growth in a mouse model. Unlike constitutive viral vectors, inducible systems have the unique capability to interrogate the role of the therapeutic agent when tumors are already established (37). A total of 2 × 10⁶ MCF7 cells stably transduced with either ZF-598SKD or empty vector control were implanted into the flank of nude mice. Tumor growth was monitored every other day using a digital caliper. Tumor volume was determined by measurement of length (L) and the width (W) as described (37). When the tumors reached ~50 mm³ (at day 21 post-injection) half of the animals for each group (N = 6) were switched to a Dox-containing diet (+Dox), whereas the other half (N = 6) was maintained in Dox-free diet (–Dox). As shown in Figure 6A, ZF-598SKD animals induced with Dox underwent a significant inhibition of tumor growth relative to the dox-free diet (–Dox) animals. In contrast, control tumors maintained an exponential growth during the entire experiment. Moreover, the ZF-mediated inhibition of tumor
growth was evident in most of the animals even 27 days post-induction (Figure 6B). A significant ($P = 0.015$) reduction of tumor growth was observed in ZF-598SKD induced animals relative to the ZF-598SKD –Dox animals. In contrast, empty vector animals did not exhibit significant reduction on tumor volume upon induction with Dox ($P = 0.269$) (Figure 6C). Examination of the tumors by qRT-PCR demonstrated that repression of $SOX2$ was maintained in ZF-598SKD induced animals relative to un-induced ZF-598SKD and controls (Figure 6D). Pathological analysis of ZF-598SKD –Dox tumors by hematoxylin-eosin staining revealed an amorphous tissue with higher density of closely packed tumor cells (Figure 6E, left panel). The same morphology was found
in tumors derived from empty vector control (data not shown). In contrast, the ZF-598SKD +Dox tumors exhibited a more organized tissue with increased amount of intervening stroma separating small islands of tumor cells (Figure 6E, right panel). In addition, immunofluorescence analyses of the tumor sections demonstrated that the ZF proteins were expressed in the nucleus of the majority of tumor cells in ZF-598SKD +Dox animals, but not in un-induced animals (Figure 7A) or controls (data not shown). This induction of ZF expression was accompanied by a decreased nuclear SOX2 staining (Figure 7A), and by a decreased proliferation of the tumor cells, as indicated by a Ki67 staining of the tumor sections (Figure 7B). In summary, our in vivo analyses indicated that the tumor suppressive functions of the engineered silencers were maintained after long-term inoculation of the tumor cells, resulting in the maintenance of the SOX2 down-regulation and decreased tumor cell proliferation in animal models of breast cancer.
DISCUSSION

In this study, we investigated the capability of ATFs to promote sequence-specific silencing of the oncogenic transcription factor (TF) SOX2. SOX2 is a self-renewal TF crucial to maintain pluripotency in embryonic stem cells (ESCs) (13,14). During differentiation of ESCs, self-renewal gene promoters undergo several layers of epigenetic silencing by means of DNA, H3K4, H3K9 and H3K27 methylation (57–59). Although the function of SOX2 in the normal mammary gland hierarchy has not been well explored, our lab has found that the gene is silenced in human mammary epithelial cells (HUMECs) derived from mammoplastical reductions (19). In contrast, over-expression of SOX2 is frequently associated with the development of many malignancies, including breast cancer (30,54). Over-expression of SOX2 in breast carcinomas has been associated with disease progression and poor clinical outcome (28). It has been proposed that SOX2 is expressed in a subpopulation of cells within the tumor with tumor-initiating characteristics (2). This subpopulation of cells shares remarkable similarities in their overall gene expression profiles with stem cells and exhibit important phenotypic characteristics, such as sustained proliferation and resistance to apoptotic insults (60). Therefore, being able to target SOX2 and other TFs involved in tumor initiation and maintenance would provide a unique opportunity for anti-cancer intervention. However, because of their lack of small molecule binding pockets, TFs are currently an example of ‘undruggable targets’. Thus, novel strategies to effectively down-regulate these targets are required; these agents are anticipated to block specific gene programs involved in the maintenance of proliferation of the bulk of the tumor, stably abolishing tumor growth.

Previously, knock-down experiments using shRNAs targeting SOX2 demonstrated that down-regulation of SOX2 in cancer cells resulted in decreased tumor cell proliferation by down-regulation of Cyclin D1 and induction of cell cycle arrest (30,61). Although RNAi is widely used to induce specific gene silencing, one potential limitation of interference approaches has been the achievement of complete knock-down of highly expressed gene transcripts, such as oncogenic TFs. In contrast with post-transcriptional approaches, transcriptional and epigenetic silencing of targeted genes provides additional advantage since only two genomic copies of the target promoter need to be silenced. Such genome-based approaches have the unique property to impact the epigenetic state of the targeted promoter, which have the potential to enhance the longevity of the silencing and the therapeutic effect in vivo. Indeed, we have recently demonstrated that 6ZFs proteins can target DNMT3a into specific promoter sites in vivo, resulting in stable, phenotypic reprogramming of the tumor cell (62).

In order to down-regulate SOX2 expression directly at DNA level, we generated four sequence-specific ZF DNA-binding domains (31). Three ATFs were designed...
to bind the core promoter of SOX2 in close proximity to the transcriptional start site (TSS) and one ATF was designed to bind in the regulatory region of SOX2 (4200 bp upstream the TSS). These multi-modular ZF genomic ‘readers’ were linked to the transcriptional repressor domain Kruppel-Associated box (SKD domain) (63). SKD recruits the co-repressor KRAB-associated protein 1 (KAPI). By assembling a complex with heterochromatin protein 1 (HP1), the histone methyltransferase SETDB1, nucleosome-remodeling (NuRD) and histone deacetylases (HDAC), KAPI facilitates heterochromatin formation through methylation of H3K9 (53). In this manuscript the SKD domain was recruited to the SOX2 promoter via the 6ZF proteins to promote gene silencing and chromatin condensation in breast cancer cells lines expressing high levels of SOX2. Our ChIP analyses demonstrated that retroviral delivery of our ZF proteins results in decreased RNA-Pol II recruitment to the SOX2 promoter. These results support the notion that the ATFs were able to impact the epigenetic state of SOX2 by preventing the binding of the transcription complex. The ATF-induced condensation of active chromatin is most likely not based on DNA-methylation, since co-treatment of ATF-transduced cells with the DNA methyltransferase inhibitor 5-aza-2’-deoxycytidine (5-Aza) failed to rescue SOX2 expression (Supplementary Figure S2). Instead, the SKD domain could induce specific histone deacetylation and/or histone methylation resulting in potent SOX2 silencing and chromatin condensation.

The importance of the epigenetic modifications in the regulation of SOX2 and in the phenotype of tumor cells has been well documented in other reports. The SOX2 promoter has been found hypomethylated in glioblastoma tumor specimens as compared with normal cell lines or normal adjacent tissue (64,65). Treatment of SOX2-negative glioma cell lines with 5-Aza resulted in re-activation of the endogenous gene thereby supporting the role of DNA methylation as a critical regulator of SOX2 silencing in glioblastoma (64). In addition to DNA methylation maps, genome-wide high-throughput profiling of histone modifications of embryonic, pluripotent and lineage-committed cells demonstrated that specific histone modifications, such as H3K4me3, H3K27me3, could play a role in determining the transcriptional state of SOX2. In embryonic stem cells, the SOX2 locus presented a high abundance of H3K4me3 marks, together with an enrichment of H3K36me3 in the 3' of the gene. Moreover, SOX2 was found flanked by two bivalent CpG islands, which could poise the gene for repression (66). In this regard, more analyses need to be performed in the breast cancer cells to uncover the role of specific histone combinations in the transcriptional status of SOX2 and the resulting phenotypic outcomes.

Three out of four ATFs mediated strong silencing of SOX2 mRNA expression, even with higher potency than siRNA. The ATFs ZF-552SKD and ZF-598SKD, designed to bind the core promoter region, down-regulated SOX2 mRNA expression by 74 and 94%, respectively, and thus, nearly abolished expression of SOX2 in MDA-MB-435s cells. ZF-4203SKD, which was designed to bind the enhancer regulatory region I (18), resulted in 88% repression of SOX2 expression. This finding demonstrated that ATFs targeting regulatory regions in chromatin promote potent down-regulation of endogenous promoter activity. In our hands, the modular approach for engineering of ZF proteins yielded 75% success rate; hence three out of four ZF proteins were able to silence a highly expressed oncogene in breast cancer cells. When ZF-552SKD and ZF-598SKD were expressed in MCF7 cells by means of inducible retroviral vectors, an arrest in tumor cell proliferation was observed. Our xenograft experiments demonstrated that ZF-598SKD inhibited tumor growth of breast cancer cells in vivo, and this inhibitory phenotype was maintained long-term, even 48 days post-injection. Pathological examination of the tumors revealed that ZF-598SKD induced animals exhibited decreased proliferation, as demonstrated by Ki67 staining, relative to un-induced or control tumors. In addition, expression of the ZF proteins and stable down-regulation of SOX2 in the tumors was validated by qRT-PCR and immunofluorescence. Interestingly, the hematoxylin-eosin staining of the ZF-598SKD induced tumors revealed small structured islands of tumor cells separated by large areas of intervening stroma, free of tumor cells. This phenotype was in contrast with the highly dense and compact growth of the tumor cells in un-induced and control tumors. The significance of this distinct phenotype induced by the ZF proteins is not known. However, it is reminiscent with the notion that transcriptional and/or epigenetic silencing of SOX2 could induce cell arrest resulting in a more structured or normal-like growth of the tumor cells in vivo.

To date, multimodular proteins composed of 6ZF domains represent the state of the art molecules for the engineering of designer transcription factors since they are potentially capable of regulating single genes (32). The specificity of our 6ZF silencers for SOX2 was further evaluated by SOX2 cDNA rescue experiments, which suggested that the cell proliferation defect mediated by the ATFs was dependent on the down-regulation of SOX2. Nevertheless, we are currently performing the genome-wide mapping of 6ZF binding sites by ChIP-seq in our MCF7 cell lines stably expressing the 6ZFs. These experiments will provide important insights regarding the endogenous specificity of our proteins in the breast cancer genome.

Previously our group has reported the ATF-mediated re-activation of the tumor suppressor gene Mammary Serine Protease Inhibitor (MASPIN) (43). We demonstrated that MASPIN reactivation in breast cancer cells resulted in tumor and metastasis suppression in breast cancer and non-small cell lung carcinoma cell lines (37,38). The reactivation of MASPIN using the VP64 activator domain was mediated at least partially by DNA demethylation (38). Herein we have reported the capability of the SKD domain to down-regulate highly expressed oncogenic TFs in breast cancer cells. Overall these results indicate that ATFs can modify the transcriptional landscape of tumor cells to direct cell fate. Thereby, this work opens the door to design an ‘alphabet’ of chromatin ‘editors’, with the ultimate goal to stabilize
the longevity of the epigenetic, transcriptional, and phenotypic state. Ideally, such ‘ZF editors’ will be able to reprogram the tumor cells epigenetic landscape like it is observed in normal epithelial cells.

Metastasis resistance and disease recurrence, which ultimately affect multiple pathways, including activation of ‘undruggable’ oncogenic TFs, are presently the main causes of death of cancer patients. Novel treatments able to suppress disease recurrence pathways will provide great hope for targeting this disease, potentially in combination with small molecules. Moreover, the delivery in vivo of ATFs into the tumor cells has historically been a major challenge and limitation for clinical applications. To this aim, we are developing targeted nanoparticles encapsulating chemically modified ATF-encoded mRNA. RNA-based delivery of nanoparticles circumvents several problems associated with plasmid-based DNA delivery. RNA has a negligible chance of integration in the chromosome, it is less toxic, and less immunogenic than DNA. The in vitro synthesis of RNA incorporating ribonucleotide analogues enhances the stability of the in vitro some, it is less toxic, and less immunogenic than RNA has a negligible chance of integration in the chromosome.

RNA-based delivery of nanoparticles circumvents several problems associated with plasmid-based DNA delivery. RNA has a negligible chance of integration in the chromosome, it is less toxic, and less immunogenic than DNA. The in vitro synthesis of RNA incorporating ribonucleotide analogues enhances the stability of the RNA and the half-life inside the cells (Wang et al. submitted for publication). Systemic delivery of nanoparticles encapsulating an ATF-mRNA designed to up-regulate the MASP IN promoter in ovarian cancer cell lines demonstrated potent regulation of the endogenous gene and robust therapeutic effect in vivo (Lara et al., submitted for publication). These data confirm that ATFs can be delivered into the tumors in vivo and achieve targeted and potent anti-tumor effects. In the future, encapsulation of multiple agents, for example small molecule inhibitors in combination with chemically modified RNA, which has been successfully performed with siRNA and doxorubicin in prostate cancer cells (67), could improve therapeutic outcome. Delivery of multiple agents together with ZF-encoded mRNA or even protein (68), is particularly interesting given the inherent plasticity of ZF domains to be designed for oncogenes and tumor suppressor genes, the availability of epi-genetic editors, which could stabilize the longevity of the therapeutic effect in vivo, and the reported synergisms of ATFs with chromatin remodeling drugs (69). In summary, our data suggest that the targeted down-regulation of highly expressed oncogenes using ATF-based technologies can be used as a powerful tool for the long-term targeting of oncogenic TFs with potential application in cancer biology and other human diseases.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online: Supplementary Figures 1–4.

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