Published online 4 May 2012 Nucleic Acids Research, 2012, Vol. 40, No. 14 6725–6740
doi:10.1093/nar/gks360

Targeted silencing of the oncogenic transcription factor *SOX2* in breast cancer

Sabine Stolzenburg[1,2], Marianne G. Rots[1], Adriana S. Beltran[2], Ashley G. Rivenbark[2,3], Xinni Yuan[2], Haili Qian[4], Brian D. Strahl[3,5] and Pilar Blancafort[2,5,*]

[1]Epigenetic Editing, Department of Pathology and Medical Biology, University Medical Center Groningen, University of Groningen, Hanzeplein 1, 9713 GZ Groningen, The Netherlands, [2]Department of Pharmacology, [3]Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, NC 27599, USA, [4]State Key Laboratory of Molecular Oncology, Cancer Hospital/Institute, Chinese Academy of Medical Sciences, Pan Jia Yuan Nan Li 17, Chaoyang District, Beijing 100021, P.R. China and [5]UNC Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC 27599, USA

Received January 17, 2012; Revised March 22, 2012; Accepted April 11, 2012

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).

*To whom correspondence should be addressed. Tel: +1 919 966 1615; Fax: +1 919 966 5640; Email: pilar_blancafort@med.unc.edu

© The Author(s) 2012. Published by Oxford University Press.
This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/3.0), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

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 293 T-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, 00.1 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. MDA-MB-453s 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% CO_2.

ATF construction

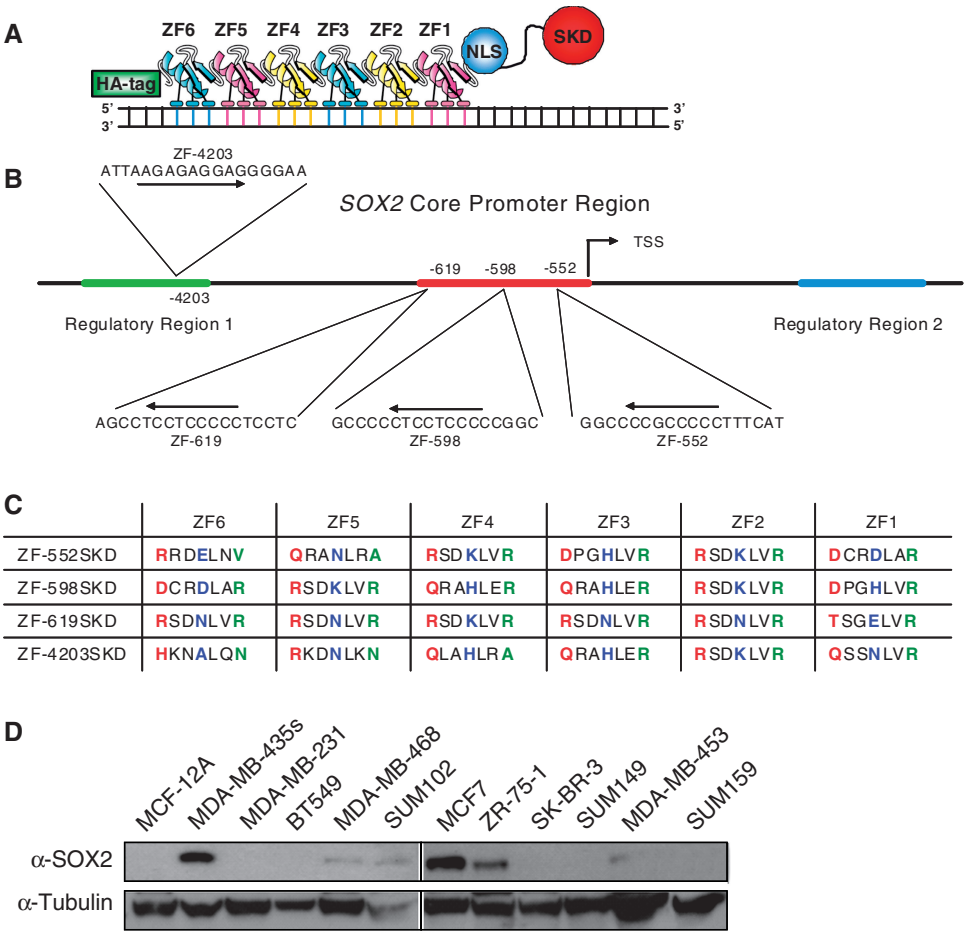
The ZF target sites within the *SOX2* promoter were selected using the website www.zincfingertools.org (42). The selection of three 18-bp target sites was based on the close proximity to the transcriptional start site and the high content of GNN-triplets in the target sequence. One ATF was designed to 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) decapeptide 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



	ZF6	ZF5	ZF4	ZF3	ZF2	ZF1
ZF-552SKD	RRDELNV	QRANLRA	RSDKLVR	DPGHLVR	RSDKLVR	DCRDLAR
ZF-598SKD	DCRDLAR	RSDKLVR	QRAHLER	QRAHLER	RSDKLVR	DPGHLVR
ZF-619SKD	RSDNLVR	RSDNLVR	RSDKLVR	RSDNLVR	RSDNLVR	TSGELVR
ZF-4203SKD	HKNALQN	RKDNLKN	QLAHLRA	QRAHLER	RSDKLVR	QSSNLVR

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 supernantant was harvested 48 h post-transfection, filtered, and concentrated using Amicon Ultra centrifugal filter Units (Millipore, Billerica, MA). MCF7 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 μg/ml geneticin (Gibco/Invitrogen) and 5 μg/ml puromycin (InvivoGen, San Diego, CA) for 10 days. ATF expression was induced using Doxycycline (Dox, 100 μg/ml) for 72 h.

Lentiviral transduction of SOX2 cDNA

HEK 293T cells were transfected with a lentiviral vector encoding the *SOX2* cDNA (pSin*SOX2*, Addgene, Cambridge, MA), together with the accessory plasmids Gagpol, VSVG and RSV-REV as described (19). As control, parallel transfections were performed with empty plasmid. Viral supernatants from either *SOX2* cDNA or control transfections were used to infect the MCF7 cell lines stably transduced with either empty vector, ZF-552SKD, or ZF-598SKD, with a density of 5×10^5 cells in 10 cm plates. These cells were cultured for 48 h and cells were un-induced or induced with Dox for 72 h and then analyzed with a cell culture microscope.

Treatment with 5-aza-2′-deoxycytidine (5-Aza)

MCF7 cells stably transduced with empty vector or ZF-552SKD were plated in a density of 5×10^5 cells in 10 cm plates. Cells were un-induced or induced with Dox (+Dox) and simultaneously treated with either vehicle or 5 μM 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 RNeasy Kit (Qiagen; Valencia, CA) and 3 μg 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 #4333764F). Detection of *Cyclin D1* mRNA was carried out using Absolute Blue QPCR SYBR Green Low ROX Mix (Thermo Scientific, Rockford, IL) with the following *Cyclin D1* detection primers (Applied Biosystems, Foster City, CA): forward 5′-GCTCCTGGTGAACAAGCT CAA-3′ and reverse 5′-TTGGAGAGGAAGTGTTCAA TGAAA-3′. For detection of human *GAPDH* as endogenous control the primers forward 5′-CCATGTTCGTCAT GGGTGTGA-3′ and reverse 5′-CATGGACTGTGGTC ATGAGT-3′ were used. Data were analyzed using the comparative ΔCt 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 μg 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 μg/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 1:10 000 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′-AGTGGAAATTTTTGTTGGAG-3′ and 5′-ATATACTT ATCCTTCTTCATAA-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% CO_2 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*-specific 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 α-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

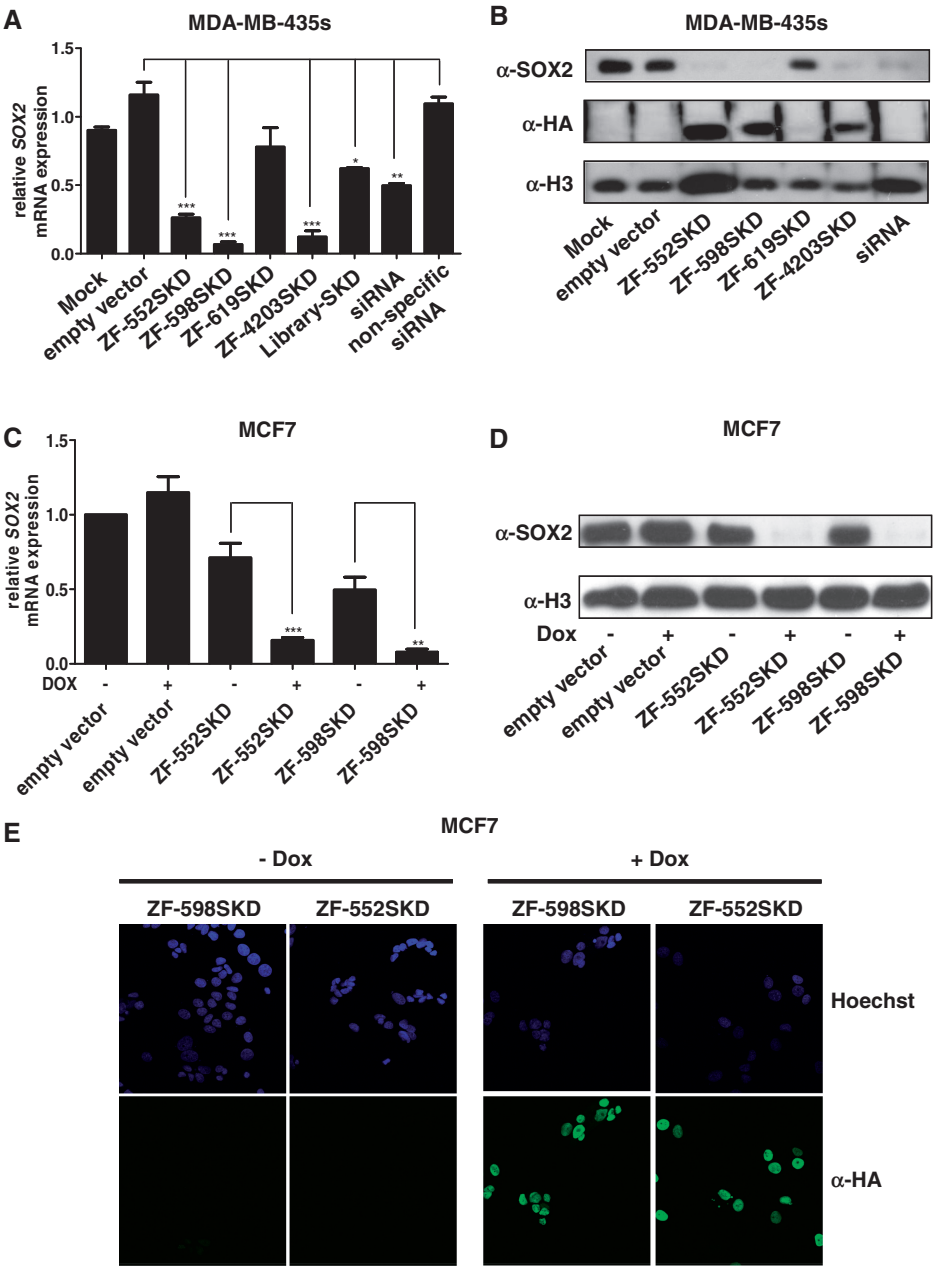


Figure 2. ATFs down-regulate *SOX2* expression in MDA-MB-435s and MCF7 breast cancer cells. (**A**) Quantification of *SOX2* mRNA expression by qRT-PCR in MDA-MB-435s cells. PMX-IRES-GFP (empty vector), ZF proteins –552SKD, –598SKD, –619SKD, –4203SKD, or a pool of 10^7 ZF domains [Library-SKD (48)] were retrovirally delivered in the cells and total mRNA was extracted. Mock-treated cells (Mock) are also indicated as control. Real-time quantification of gene expression was normalized to empty vector control samples. As positive controls for knock-down, cells were transfected with an anti-*SOX2* siRNA. A non-specific siRNA targeting another TF (*PATZ1*) was used as a negative control for siRNA transfection. Error bars represent the standard deviation of three independent experiments. Statistical significance was analyzed using *t*-test (***$P < 0.001$, *$P < 0.05$) (**B**) Detection of *SOX2* protein levels by western blot in MDA-MB-435s cells transduced with the same constructs as in (A). An anti-Histone H3 antibody was used as a loading control. ATFs were designed with a C-terminal Hemagglutinin (HA) tag, used for ATF detection. (**C**) Quantification of *SOX2* mRNA by qRT-PCR in MCF7 breast cancer cells. MCF7 cells were stably transfected with either empty vector control, ZF-552SKD, or ZF-598SKD. The ATF expression was induced by Doxycyclin as indicated in the *x*-axis (−/+ Dox). Error bars show SD of three independent experiments and statistical significance was analyzed using Student's *t*-test (***$P < 0.001$, **$P < 0.01$). (**D**) Detection of *SOX2* protein by western blot in MCF7 cells. An anti-Histone H3 antibody was used as a loading control. Samples are the same as in (C). (**E**) Immunofluorescence analysis of MCF7 cells transduced with ZF-552SKD and ZF-598SKD. Detection of ZF-552SKD and ZF-598SKD is indicated in green (α HA-tag) and nuclear staining in blue (Hoechst). The left panel shows un-induced (–Dox) and the right panel induced (+Dox) MCF7 cells. Images are taken at 40×.

by flow cytometry (data not shown). Quantitative changes in *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 *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). These 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

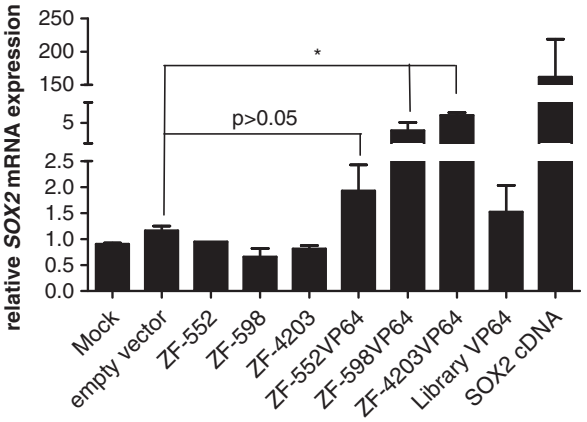


Figure 3. 6ZF domains linked to transcriptional activators enhance *SOX2* mRNA expression in MDA-MB-435s cells. Cells were retrovirally transduced with either ZF-552, ZF-598 or ZF-4203 (retroviral constructs expressing the specific DNA-binding domains but lacking the SKD effector domain or with the same ZFs linked to the VP64 transactivator domain (ZF-552VP64, ZF-598VP64, ZF-4203VP64). Library-VP64 sample refers to a pool of $\sim 10^6$ different 6ZF domains (48). Quantification of *SOX2* mRNA cells was analyzed by qRT-PCR and normalized to empty vector control. ($*P < 0.05$).

(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 engineered 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

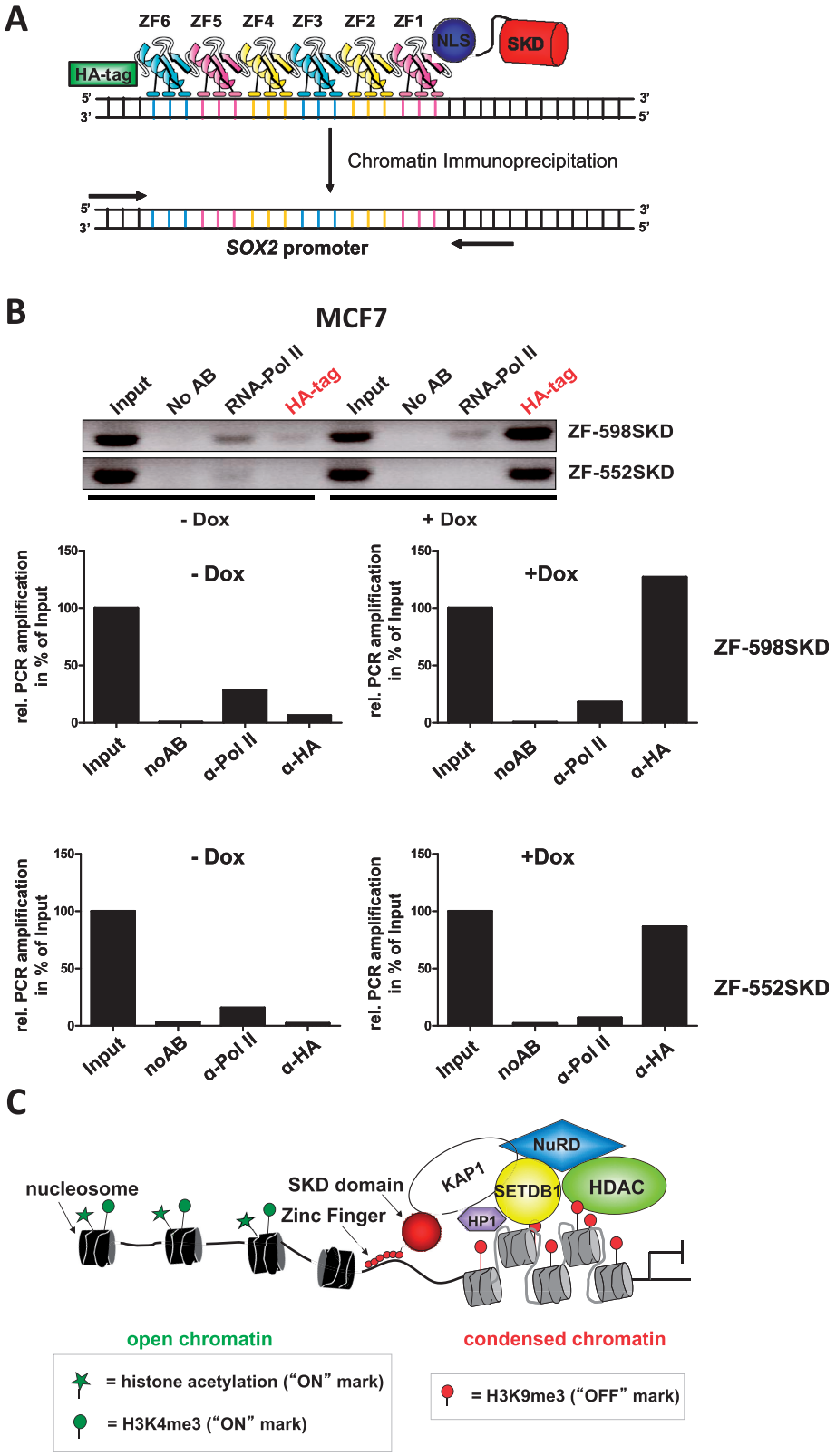


Figure 4. ATFs bind their targeted site in the endogenous *SOX2* promoter. (**A**) Schematic illustration of the chromatin Immunoprecipitation (ChIP) assay. (**B**) ZF-598SKD (upper panel) and ZF-552SKD (lower panel) are binding their target sites, as assessed by ChIP using an anti-HA antibody. Genomic DNA bound by the corresponding ATF was amplified using *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 *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.

not induced by DNA methylation, as subsequent treatment of the ATF-transduced cells with the methyltransferase inhibitor 5-Aza-2′deoxycytidine (5-Aza) did not result in a reactivation of *SOX2* (Supplementary Figure S2).

These findings are in agreement with the molecular mechanism of SKD-mediated repression (Figure 4C). In this model, SKD interacts with the co-repressor KAP1 (53). Subsequent recruitment of the nucleosome remodeling and histone deacetylase (NuRD) complex, histone deacetylases (HDACs), histone methyltrasferase (SETDB1) and heterochromatin protein 1 (HP1) catalyzes the formation of condensed chromatin, which is inaccessible for the binding of RNA Pol II.

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-345s 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 (pSin*SOX2*) 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 xenogaft 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 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 2×10^6 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 $\sim 50\,mm^3$ (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

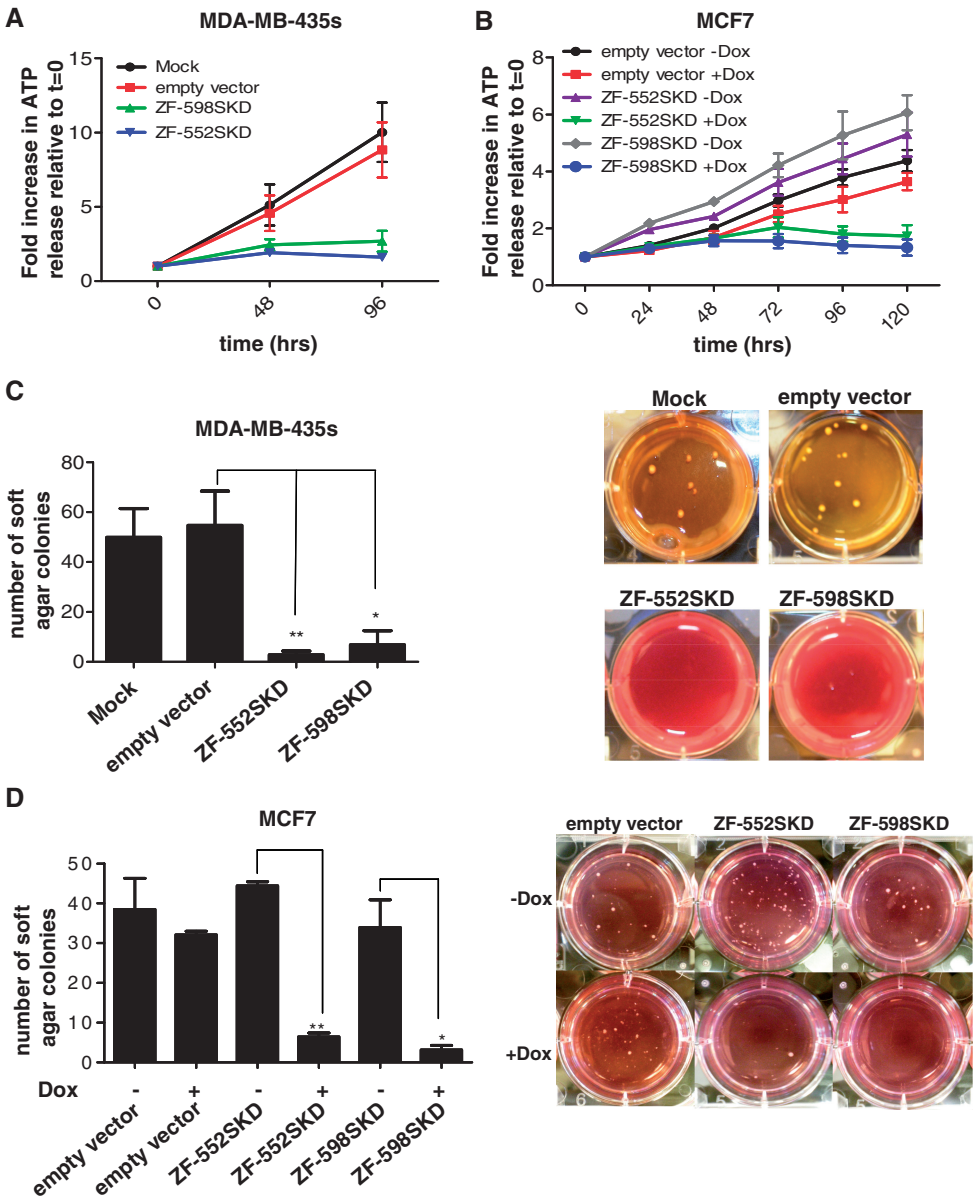


Figure 5. Repression of *SOX2* decreases cell viability and anchorage-independent growth. (**A**) Cell viability analysis of MDA-MB-435s cells transduced with either empty vector, ZF-598SKD or ZF-552SKD. Mock-transfected cells (Mock) were used to assess background. Cell viability over time was monitored over a period of 96 h after the initial seeding of the infected cells. Cell viability was monitored using a CellTiter Glo Assay (19). (**B**) Cell viability assays in MCF7 cell cells. Empty vector, ZF-552SKD- or ZF-598SKD-transduced cells were induced with Doxycyclin every 48 h. Un-induced (–Dox) cells were used as controls. The *y*-axis indicates fold increase in ATP release relative to time point 0 measured by luminescence. Statistical significance was analyzed using two-way ANOVA. The *P*-values for both, ZF-552SKD and ZF-598SKD +Dox samples versus the same samples in –Dox conditions were $P < 0.001$ at the last time point (120 h). (**C** and **D**) Anchorage-independent growth of MDA-MB-435s and MCF7 cells. (C) Quantification of the number of soft-agar colonies from un-transduced MDA-MB435s cells (Mock), MDA-MB435s cells transduced with empty vector, ZF-552SKD and ZF-598SKD. (D) Quantification of the number of soft-agar colonies from MCF7 cells transduced with either empty vector, ZF-552SKD or ZF-598SKD. –Dox and +Dox indicate un-induced and induced cells, respectively. Left panels show the quantification of colony numbers. Right panels show representative pictures of the soft-agar plates. Error bars represent SDs of three independent experiments. Statistical significance was analyzed by Student's *t*-test (**$P < 0.01$, *$P < 0.05$).

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

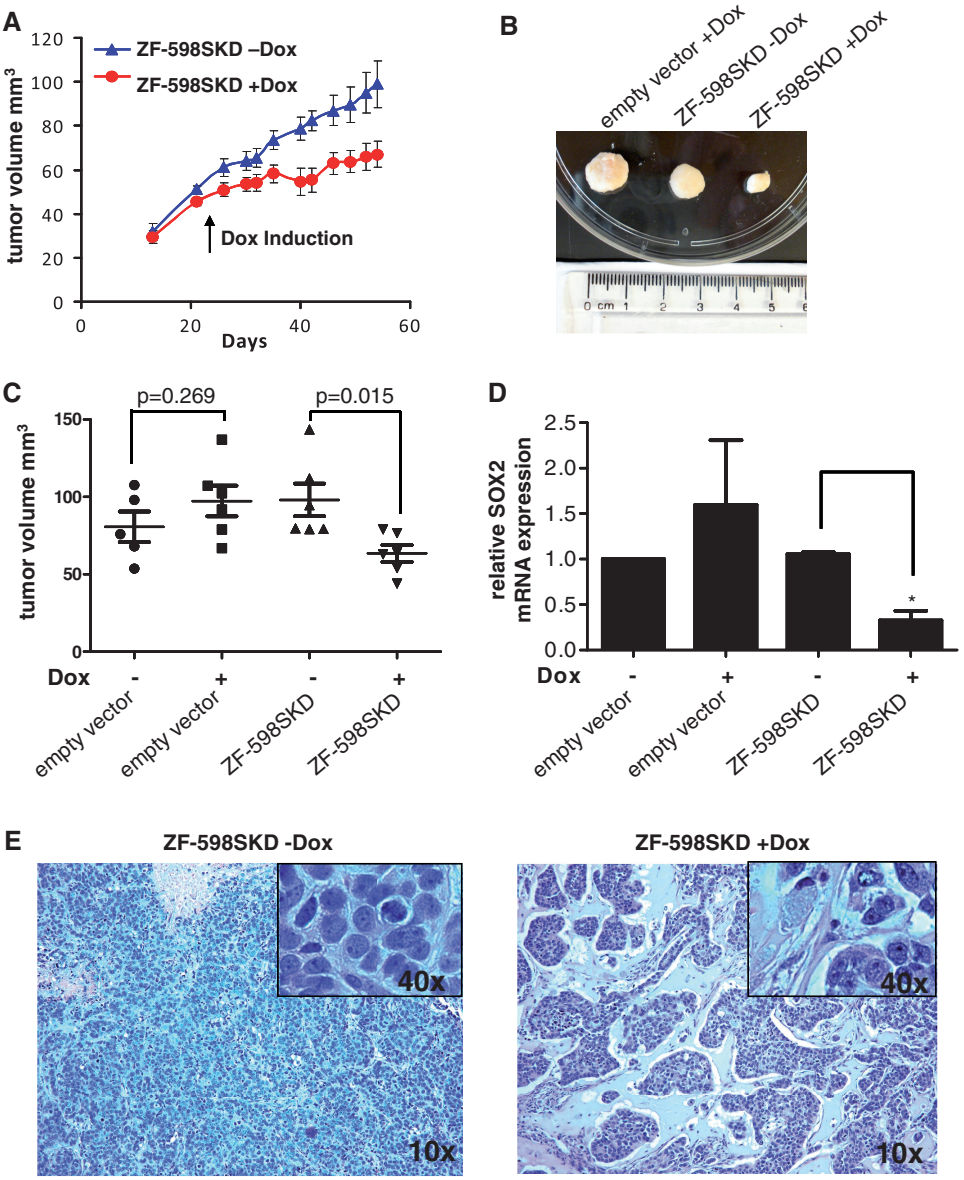


Figure 6. A *SOX2*-specific ATF inhibits the growth of pre-existing s.c xenografts of MCF7 cells. (**A**) Time course plot of tumor volume monitored by caliper measurements. Animals ($N = 6$) were either maintained in a Dox-free diet (-Dox) or induced with Dox diet (arrow) at day 21 post-injection. (**B**) Picture of representative tumors collected at day 28 post-induction from induced empty vector, un-induced ZF-598SKD, and induced ZF-598SKD animals. (**C**) Tumor volume measurements at day 21 post-induction from empty vector and ZF-598SKD groups ($N = 6$ animals per group). Differences between groups were assessed by a Wilconxon rank sum test. (**D**) Quantification of *SOX2* mRNA expression by qRT-PCR in tumor samples from a representative tumor xenograft. Bar graphs represent the mean and SD of three tumor samples. Differences in gene expression were calculated with a Student's *t*-test, $*P = 0.01$ (**E**) Hematoxylin-Eosin staining of representative ZF-598SKD –Dox and +Dox tumor sections. Un-induced (–Dox) animals revealed highly compact tumors. Induced (+Dox) ZF-598SKD sections comprised discrete islands of tumor cells, separated by intervening stroma. Pictures were taken at 10× and a detail of a 40× magnification is shown.

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.

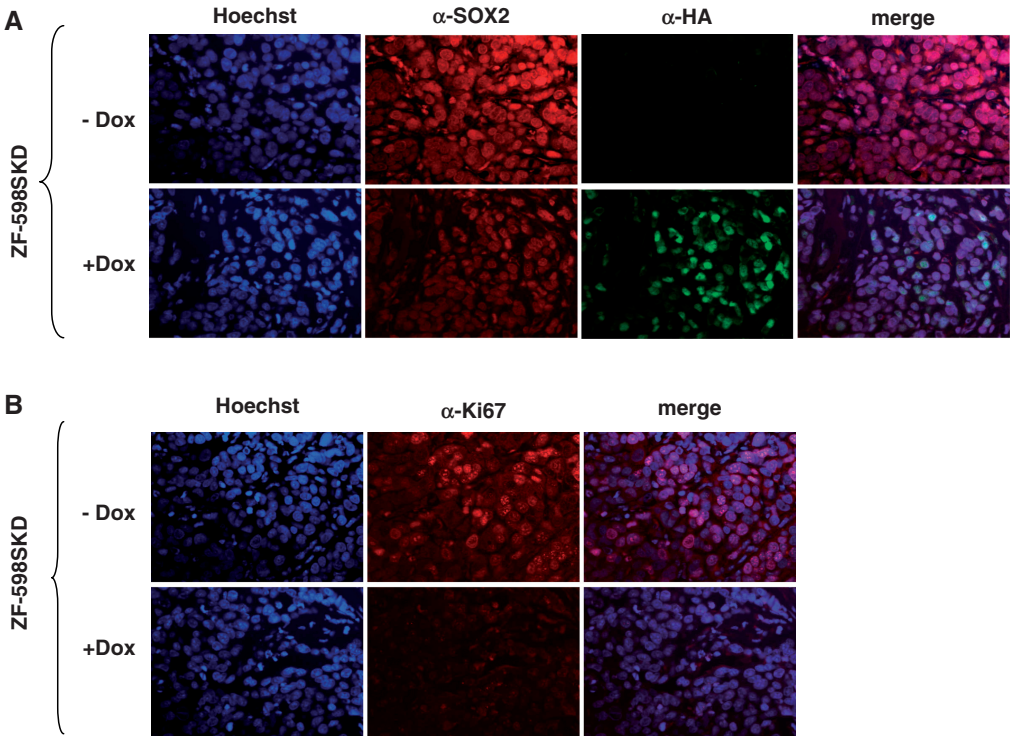


Figure 7. Induction of ZF-598 SKD reduces *SOX2* expression and tumor cell proliferation *in vivo*. (**A**) *SOX2* (red) and ZF-598SKD (α-HA, green) detection by immunofluorescence (IF) analyses of representative sections from ZF-598SKD un-induced (–Dox) and induced (+Dox) animals. (**B**) Ki67 expression (red) analyzed by IF in the same samples. Nuclei were labelled with Hoechst (blue). Images were taken at 40×.

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 mammoplastic 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 would prevent gene expression by silencing the promoter with no opportunity for 'residual' oncogenic transcriptional activity. In addition, genomic 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 6ZF 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 (KAP1). By assembling a complex with heterochromatin protein 1 (HP1), the histone methyltransferase SETDB1, nucleosome-remodeling (NuRD) and histone deacetylases (HDAC), KAP1 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.

Metastatic 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 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 *MASPIN* 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 epigenetic 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.

ACKNOWLEDGEMENTS

The authors thank Prof. David Alan Eberhard (Department of Pathology and Laboratory Medicine, UNC, Chapel Hill) for pathological evaluation of xenograft tumor samples.

FUNDING

NWO/VIDI/91786373 (to M.R.); National Cancer Institute/National Institutes of Health (NCI/NIH) [1R01CA125273, 3R01CA125273-03S1]; DoD awards [W81XWH-10-1-0265 to P.B.; W81XWH-10-1-0266 to P.B. and B.D.S.]. Funding for open access charge: NCI/NIH [1R01CA125273].

Conflict of interest statement. None declared.

REFERENCES

1. Darnell,J.E. Jr (2002) Transcription factors as targets for cancer therapy. *Nat. Rev. Cancer*, **2**, 740–749.
2. Ben-Porath,I., Thomson,M.W., Carey,V.J., Ge,R., Bell,G.W., Regev,A. and Weinberg,R.A. (2008) An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. *Nat. Genet.*, **40**, 499–507.
3. Perou,C.M. (2010) Molecular stratification of triple-negative breast cancers. *Oncologist*, **15(Suppl. 5)**, 39–48.
4. Perou,C.M., Sorlie,T., Eisen,M.B., van de Rijn,M., Jeffrey,S.S., Rees,C.A., Pollack,J.R., Ross,D.T., Johnsen,H., Akslen,L.A. *et al.* (2000) Molecular portraits of human breast tumours. *Nature*, **406**, 747–752.
5. Prat,A., Parker,J.S., Karginova,O., Fan,C., Livasy,C., Herschkowitz,J.I., He,X. and Perou,C.M. (2010) Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. *Breast Cancer Res., BCR*, **12**, R68.
6. Dontu,G. (2008) Breast cancer stem cell markers - the rocky road to clinical applications. *Breast Cancer Res.*, **10**, 110.
7. Honeth,G., Bendahl,P.O., Ringner,M., Saal,L.H., Gruvberger-Saal,S.K., Lovgren,K., Grabau,D., Ferno,M., Borg,A. and Hegardt,C. (2008) The CD44+/CD24- phenotype is enriched in basal-like breast tumors. *Breast Cancer Res.*, **10**, R53.
8. Park,S.Y., Lee,H.E., Li,H., Shipitsin,M., Gelman,R. and Polyak,K. Heterogeneity for stem cell-related markers according to tumor subtype and histologic stage in breast cancer. *Clin. Cancer Res.*, **16**, 876–887.
9. Tysnes,B.B. and Bjerkvig,R. (2007) Cancer initiation and progression: involvement of stem cells and the microenvironment. *Biochim. Biophys. Acta*, **1775**, 283–297.
10. Marchini,C., Montani,M., Konstantinidou,G., Orru,R., Mannucci,S., Ramadori,G., Gabrielli,F., Baruzzi,A., Berton,G., Merigo,F. *et al.* Mesenchymal/stromal gene expression signature relates to basal-like breast cancers, identifies bone metastasis and predicts resistance to therapies. *PLoS One*, **5**, e14131.
11. Fillmore,C.M. and Kuperwasser,C. (2008) Human breast cancer cell lines contain stem-like cells that self-renew, give rise to phenotypically diverse progeny and survive chemotherapy. *Breast Cancer Res.*, **10**, R25.
12. Wegner,M. (1999) From head to toes: the multiple facets of Sox proteins. *Nucleic Acids Res.*, **27**, 1409–1420.
13. Rao,R.R., Calhoun,J.D., Qin,X., Rekaya,R., Clark,J.K. and Stice,S.L. (2004) Comparative transcriptional profiling of two human embryonic stem cell lines. *Biotechnol. Bioeng.*, **88**, 273–286.
14. Wang,J., Rao,S., Chu,J., Shen,X., Levasseur,D.N., Theunissen,T.W. and Orkin,S.H. (2006) A protein interaction network for pluripotency of embryonic stem cells. *Nature*, **444**, 364–368.
15. Adachi,K., Suemori,H., Yasuda,S.Y., Nakatsuji,N. and Kawase,E. (2010) Role of SOX2 in maintaining pluripotency of human embryonic stem cells. *Genes Cells*, **15**, 455–470.
16. Boyer,L.A., Lee,T.I., Cole,M.F., Johnstone,S.E., Levine,S.S., Zucker,J.P., Guenther,M.G., Kumar,R.M., Murray,H.L., Jenner,R.G. *et al.* (2005) Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell*, **122**, 947–956.
17. Hochedlinger,K. and Plath,K. (2009) Epigenetic reprogramming and induced pluripotency. *Development*, **136**, 509–523.
18. Sikorska,M., Sandhu,J.K., Deb-Rinker,P., Jezierski,A., Leblanc,J., Charlebois,C., Ribecco-Lutkiewicz,M., Bani-Yaghoub,M. and

Walker,P.R. (2008) Epigenetic modifications of SOX2 enhancers, SRR1 and SRR2, correlate with in vitro neural differentiation. *J. Neurosci. Res.*, **86**, 1680–1693.
19. Beltran,A.S., Rivenbark,A.G., Richardson,B.T., Yuan,X., Quian,H., Hunt,J.P., Zimmerman,E., Graves,L.M. and Blancafort,P. (2011) Generation of tumor initiating cells by exogenous delivery of OCT4 Transcription Factor. *Breast Cancer Research: BCR*, **13**, R94.
20. Otsubo,T., Akiyama,Y., Yanagihara,K. and Yuasa,Y. (2008) SOX2 is frequently downregulated in gastric cancers and inhibits cell growth through cell-cycle arrest and apoptosis. *Br. J. Cancer*, **98**, 824–831.
21. Li,X.L., Eishi,Y., Bai,Y.Q., Sakai,H., Akiyama,Y., Tani,M., Takizawa,T., Koike,M. and Yuasa,Y. (2004) Expression of the SRY-related HMG box protein SOX2 in human gastric carcinoma. *Int. J. Oncol.*, **24**, 257–263.
22. Wilbertz,T., Wagner,P., Petersen,K., Stiedl,A.C., Scheble,V.J., Maier,S., Reischl,M., Mikut,R., Altorki,N.K., Moch,H. *et al.* (2011) SOX2 gene amplification and protein overexpression are associated with better outcome in squamous cell lung cancer. *Mod. Pathol.*, **24**, 944–953.
23. Gangemi,R.M., Griffero,F., Marubbi,D., Perera,M., Capra,M.C., Malatesta,P., Ravetti,G.L., Zona,G.L., Daga,A. and Corte,G. (2009) SOX2 silencing in glioblastoma tumor-initiating cells causes stop of proliferation and loss of tumorigenicity. *Stem Cells*, **27**, 40–48.
24. Lu,Y., Futtner,C., Rock,J.R., Xu,X., Whitworth,W., Hogan,B.L. and Onaitis,M.W. (2010) Evidence that SOX2 overexpression is oncogenic in the lung. *PLoS One*, **5**, e11022.
25. Leung,E.L., Fiscus,R.R., Tung,J.W., Tin,V.P., Cheng,L.C., Sihoe,A.D., Fink,L.M., Ma,Y. and Wong,M.P. (2010) Non-small cell lung cancer cells expressing CD44 are enriched for stem cell-like properties. *PLoS One*, **5**, e14062.
26. Bae,K.M., Su,Z., Frye,C., McClellan,S., Allan,R.W., Andrejewski,J.T., Kelley,V., Jorgensen,M., Steindler,D.A., Vieweg,J. *et al.* (2010) Expression of pluripotent stem cell reprogramming factors by prostate tumor initiating cells. *J. Urol.*, **183**, 2045–2053.
27. Xu,X.L., Xing,B.C., Han,H.B., Zhao,W., Hu,M.H., Xu,Z.L., Li,J.Y., Xie,Y., Gu,J., Wang,Y. *et al.* (2010) The properties of tumor-initiating cells from a hepatocellular carcinoma patient's primary and recurrent tumor. *Carcinogenesis*, **31**, 167–174.
28. Lengerke,C., Fehm,T., Kurth,R., Neubauer,H., Scheble,V., Muller,F., Schneider,F., Petersen,K., Wallwiener,D., Kanz,L. *et al.* (2011) Expression of the embryonic stem cell marker SOX2 in early-stage breast carcinoma. *BMC Cancer*, **11**, 42.
29. Rodriguez-Pinilla,S.M., Sarrio,D., Moreno-Bueno,G., Rodriguez-Gil,Y., Martinez,M.A., Hernandez,L., Hardisson,D., Reis-Filho,J.S. and Palacios,J. (2007) Sox2: a possible driver of the basal-like phenotype in sporadic breast cancer. *Mod. Pathol.*, **20**, 474–481.
30. Chen,Y., Shi,L., Zhang,L., Li,R., Liang,J., Yu,W., Sun,L., Yang,X., Wang,Y., Zhang,Y. *et al.* (2008) The molecular mechanism governing the oncogenic potential of SOX2 in breast cancer. *J. Biol. Chem.*, **283**, 17969–17978.
31. Sera,T. (2009) Zinc-finger-based artificial transcription factors and their applications. *Adv. Drug Deliv. Rev.*, **61**, 513–526.
32. Tan,S., Guschin,D., Davalos,A., Lee,Y.L., Snowden,A.W., Jouvenot,Y., Zhang,H.S., Howes,K., McNamara,A.R., Lai,A. *et al.* (2003) Zinc-finger protein-targeted gene regulation: genomewide single-gene specificity. *Proc. Natl Acad. Sci. USA*, **100**, 11997–12002.
33. Bartsevich,V.V. and Juliano,R.L. (2000) Regulation of the MDR1 gene by transcriptional repressors selected using peptide combinatorial libraries. *Mol. Pharmacol.*, **58**, 1–10.
34. Beerli,R.R., Segal,D.J., Dreier,B. and Barbas,C.F. 3rd (1998) Toward controlling gene expression at will: specific regulation of the erbB-2/HER-2 promoter by using polydactyl zinc finger proteins constructed from modular building blocks. *Proc. Natl Acad. Sci. USA*, **95**, 14628–14633.
35. Laganiere,J., Kells,A.P., Lai,J.T., Guschin,D., Paschon,D.E., Meng,X., Fong,L.K., Yu,Q., Rebar,E.J., Gregory,P.D. *et al.* (2010) An engineered zinc finger protein activator of the endogenous glial cell line-derived neurotrophic factor gene provides functional neuroprotection in a rat model of Parkinson's disease. *J. Neurosci.*, **30**, 16469–16474.
36. Segal,D.J., Goncalves,J., Eberhardy,S., Swan,C.H., Torbett,B.E., Li,X. and Barbas,C.F. 3rd (2004) Attenuation of HIV-1 replication in primary human cells with a designed zinc finger transcription factor. *J. Biol. Chem.*, **279**, 14509–14519.
37. Beltran,A.S., Russo,A., Lara,H., Fan,C., Lizardi,P.M. and Blancafort,P. (2011) Suppression of breast tumor growth and metastasis by an engineered transcription factor. *PLoS One*, **6**, e24595.
38. Beltran,A.S. and Blancafort,P. (2011) Reactivation of MASPIN in non-small cell lung carcinoma (NSCLC) cells by artificial transcription factors (ATFs). *Epigenetics*, **6**, 224–235.
39. Gommans,W.M., McLaughlin,P.M., Lindhout,B.I., Segal,D.J., Wiegman,D.J., Haisma,H.J., van der Zaal,B.J. and Rots,M.G. (2007) Engineering zinc finger protein transcription factors to downregulate the epithelial glycoprotein-2 promoter as a novel anti-cancer treatment. *Mol. Carcinog.*, **46**, 391–401.
40. Sohn,J.H., Yeh,B.I., Choi,J.W., Yoon,J., Namkung,J., Park,K.K. and Kim,H.W. (2010) Repression of human telomerase reverse transcriptase using artificial zinc finger transcription factors. *Mol. Cancer Res.*, **8**, 246–253.
41. Lund,C.V., Popkov,M., Magnenat,L. and Barbas,C.F. 3rd (2005) Zinc finger transcription factors designed for bispecific coregulation of ErbB2 and ErbB3 receptors: insights into ErbB receptor biology. *Mol. Cell. Biol.*, **25**, 9082–9091.
42. Mandell,J.G. and Barbas,C.F. 3rd (2006) Zinc finger tools: custom DNA-binding domains for transcription factors and nucleases. *Nucl. Acids Res.*, **34**, W516–523.
43. Beltran,A., Parikh,S., Liu,Y., Cuevas,B.D., Johnson,G.L., Futscher,B.W. and Blancafort,P. (2007) Re-activation of a dormant tumor suppressor gene maspin by designed transcription factors. *Oncogene*, **26**, 2791–2798.
44. Liu,Q., Segal,D.J., Ghiara,J.B. and Barbas,C.F. 3rd (1997) Design of polydactyl zinc-finger proteins for unique addressing within complex genomes. *Proc. Natl Acad. Sci. USA*, **94**, 5525–5530.
45. Rae,J.M., Creighton,C.J., Meck,J.M., Haddad,B.R. and Johnson,M.D. (2007) MDA-MB-435 cells are derived from M14 melanoma cells–a loss for breast cancer, but a boon for melanoma research. *Breast Cancer Res. Treatment*, **104**, 13–19.
46. Chambers,A.F. (2009) MDA-MB-435 and M14 cell lines: identical but not M14 melanoma? *Cancer Res.*, **69**, 5292–5293.
47. Hennessy,B.T., Gonzalez-Angulo,A.M., Stemke-Hale,K., Gilcrease,M.Z., Krishnamurthy,S., Lee,J.S., Fridlyand,J., Sahin,A., Agarwal,R., Joy,C. *et al.* (2009) Characterization of a naturally occurring breast cancer subset enriched in epithelial-to-mesenchymal transition and stem cell characteristics. *Cancer Res.*, **69**, 4116–4124.
48. Blancafort,P., Magnenat,L. and Barbas,C.F. 3rd (2003) Scanning the human genome with combinatorial transcription factor libraries. *Nat. Biotechnol.*, **21**, 269–274.
49. Magnenat,L., Blancafort,P. and Barbas,C.F. 3rd (2004) In vivo selection of combinatorial libraries and designed affinity maturation of polydactyl zinc finger transcription factors for ICAM-1 provides new insights into gene regulation. *J. Mol. Biol.*, **341**, 635–649.
50. Beltran,A., Liu,Y., Parikh,S., Temple,B. and Blancafort,P. (2006) Interrogating genomes with combinatorial artificial transcription factor libraries: asking zinc finger questions. *Assay Drug Dev. Technol.*, **4**, 317–331.
51. Blancafort,P., Tschan,M.P., Bergquist,S., Guthy,D., Brachat,A., Sheeter,D.A., Torbett,B.E., Erdmann,D. and Barbas,C.F. 3rd (2008) Modulation of drug resistance by artificial transcription factors. *Mol. Cancer Ther.*, **7**, 688–697.
52. Blancafort,P., Chen,E.I., Gonzalez,B., Bergquist,S., Zijlstra,A., Guthy,D., Brachat,A., Brakenhoff,R.H., Quigley,J.P., Erdmann,D. *et al.* (2005) Genetic reprogramming of tumor cells by zinc finger transcription factors. *Proc. Natl Acad. Sci. USA*, **102**, 11716–11721.
53. Groner,A.C., Meylan,S., Ciuffi,A., Zangger,N., Ambrosini,G., Denervaud,N., Bucher,P. and Trono,D. (2010) KRAB-zinc finger proteins and KAP1 can mediate long-range transcriptional repression through heterochromatin spreading. *PLoS Genet*, **6**, e1000869.

54. Leis,O., Eguiara,A., Lopez-Arribillaga,E., Alberdi,M.J., Hernandez-Garcia,S., Elorriaga,K., Pandiella,A., Rezola,R. and Martin,A.G. Sox2 expression in breast tumours and activation in breast cancer stem cells. *Oncogene*, **31**, 1354–1365.
55. Bass,A.J., Watanabe,H., Mermel,C.H., Yu,S., Perner,S., Verhaak,R.G., Kim,S.Y., Wardwell,L., Tamayo,P., Gat-Viks,I. *et al*. (2009) SOX2 is an amplified lineage-survival oncogene in lung and esophageal squamous cell carcinomas. *Nat. Genet*., **41**, 1238–1242.
56. Hussenet,T., Dali,S., Exinger,J., Monga,B., Jost,B., Dembele,D., Martinet,N., Thibault,C., Huelsken,J., Brambilla,E. *et al*. (2010) SOX2 is an oncogene activated by recurrent 3q26.3 amplifications in human lung squamous cell carcinomas. *PLoS One*, **5**, e8960.
57. Challen,G.A., Sun,D., Jeong,M., Luo,M., Jelinek,J., Berg,J.S., Bock,C., Vasanthakumar,A., Gu,H., Xi,Y. *et al*. (2011) Dnmt3a is essential for hematopoietic stem cell differentiation. *Nat. Genet*., **44**, 23–31.
58. Epsztejn-Litman,S., Feldman,N., Abu-Remaileh,M., Shufaro,Y., Gerson,A., Ueda,J., Deplus,R., Fuks,F., Shinkai,Y., Cedar,H. *et al*. (2008) De novo DNA methylation promoted by G9a prevents reprogramming of embryonically silenced genes. *Nat. Struct. Mol. Biol*., **15**, 1176–1183.
59. Christophersen,N.S. and Helin,K. (2010) Epigenetic control of embryonic stem cell fate. *J. Exp. Med*., **207**, 2287–2295.
60. Schoenhals,M., Kassambara,A., De Vos,J., Hose,D., Moreaux,J. and Klein,B. (2009) Embryonic stem cell markers expression in cancers. *Biochem. Biophys. Res. Commun*., **383**, 157–162.
61. Oppel,F., Muller,N., Schackert,G., Hendruschk,S., Martin,D., Geiger,K.D. and Temme,A. (2011) SOX2-RNAi attenuates S-phase entry and induces RhoA-dependent switch to protease-independent amoeboid migration in human glioma cells. *Mol. Cancer*, **10**, 137.
62. 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**, 350–360.
63. Margolin,J.F., Friedman,J.R., Meyer,W.K., Vissing,H., Thiesen,H.J. and Rauscher,F.J. 3rd (1994) Kruppel-associated boxes are potent transcriptional repression domains. *Proc. Natl Acad. Sci. USA*, **91**, 4509–4513.
64. Alonso,M.M., Diez-Valle,R., Manterola,L., Rubio,A., Liu,D., Cortes-Santiago,N., Urquiza,L., Jauregi,P., de Munain,A.L., Sampron,N. *et al*. (2011) Genetic and epigenetic modifications of Sox2 contribute to the invasive phenotype of malignant gliomas. *PLoS One*, **6**, e26740.
65. Cancer Genome Atlas Research Network (2008) Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature*, **455**, 1061–1068.
66. Mikkelsen,T.S., Ku,M., Jaffe,D.B., Issac,B., Lieberman,E., Giannoukos,G., Alvarez,P., Brockman,W., Kim,T.K., Koche,R.P. *et al*. (2007) Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. *Nature*, **448**, 553–560.
67. Chen,Y., Bathula,S.R., Li,J. and Huang,L. (2010) Multifunctional nanoparticles delivering small interfering RNA and doxorubicin overcome drug resistance in cancer. *J. Biol. Chem*., **285**, 22639–22650.
68. van der Gun,B.T., Wasserkort,R., Monami,A., Jeltsch,A., Rasko,T., Slaska-Kiss,K., Cortese,R., Rots,M.G., de Leij,L.F., Ruiters,M.H. *et al*. (2008) Persistent downregulation of the pancarcinoma-associated epithelial cell adhesion molecule via active intranuclear methylation. *Int. J. Cancer*, **123**, 484–489.
69. Beltran,A.S., Sun,X., Lizardi,P.M. and Blancafort,P. (2008) Reprogramming epigenetic silencing: artificial transcription factors synergize with chromatin remodeling drugs to reactivate the tumor suppressor mammary serine protease inhibitor. *Mol. Cancer Ther*., **7**, 1080–1090.