Carcinogenesis vol.35 no.4 pp.942–950, 2014
doi:10.1093/carcin/bgt410
Advance Access publication December 9, 2013

The stem cell factor SOX2 regulates the tumorigenic potential in human gastric cancer cells

Katharina Hütz[1,8,†], Raquel Mejías-Luque[1,†], Katarina Farsakova[2], Manfred Ogris[2], Stefan Krebs[3], Martina Anton[4], Michael Vieth[5], Ulrich Schüller[6], Marlon R.Schneider[3], Helmut Blum[3], Ernst Wagner[2], Andreas Jung[7] and Markus Gerhard[1,*]

[1]Institut für Medizinische Mikrobiologie, Immunologie und Hygiene, Technische Universität München, 81675 Munich, Germany, [2]Department Pharmazie, Pharmaceutical Biotechnology and [3]Gene Center, Ludwig-Maximilians-Universität München, 81377 Munich, Germany, [4]Institut für Experimentelle Onkologie und Therapieforschung, Klinikum rechts der Isar der Technischen Universität München, 81675 Munich, Germany, [5]Institut für Pathologie, Klinikum Bayreuth GmbH, 95445 Bayreuth, Germany, [6]Zentrum für Neuropathologie und Prionforschung, Medizinische Fakultät and [7]Pathologisches Institut, Medizinische Fakultät, Ludwig-Maximilians-Universität München, 81377 Munich, Germany
[8]Present address: Institute of Anatomy and Cell Biology, Ludwig-Maximilians, University Munich, 80336 Munich, Germany

*To whom correspondence should be addressed. Institut für Medizinische Mikrobiologie, Immunologie und Hygiene, Technische Universität München Trogerstrasse 30, 81675 Munich, Germany. Tel: +49 89 4140 2477; Fax: +49 89 4140 4139;
Email: markus.gerhard@tum.de

Gastric cancer (GC) is still one of the most common causes of cancer-related death worldwide, which is mainly attributable to late diagnosis and poor treatment options. Infection with *Helicobacter pylori*, different environmental factors and genetic alterations are known to influence the risk of developing gastric tumors. However, the molecular mechanisms involved in gastric carcinogenesis are still not fully understood, making it difficult to design targeted therapeutic approaches. Aberrant expression of the specific gastric differentiation marker SOX2 has been observed in stomach cancer. However, the role of SOX2 in gastric tumors has not been well established to date. To elucidate the role of SOX2 in gastric tumorigenesis, SOX2 transcriptional activity was blocked in AZ-521 cells. Interestingly, inhibition of SOX2 reduced cell proliferation and migration, increased apoptosis and induced changes in cell cycle. Blocking of SOX2 also reduced the tumorigenic potential of AZ-521 cells *in vivo*. In addition, correlation of SOX2 expression and proliferation was observed in a subset of human gastric tumors. Finally, target genes of SOX2 were for the first time identified by RNA microarray in GC cells. Taken together, the results presented here indicate that SOX2 controls several aspects related to GC development and progression by regulating the expression of members of important signaling pathways. These findings could provide new therapeutic options for a subset of GCs exhibiting SOX2 deregulation.

Introduction

Gastric cancer (GC) is the second most common cause of cancer-related death worldwide with mortality rates still over 70% (1). *Helicobacter pylori* infection, diet and life style have been linked to the development of intestinal-type stomach tumors, whereas diffuse-type tumors have been more related to genetic alterations. Nevertheless, accumulating genetic and molecular changes, including activation of oncogenes, inactivation of tumor suppressor and DNA repair genes as well as overexpression of growth factors have been extensively described to occur during the gastric carcinogenetic process (2).

Recently, the gastric differentiation marker SOX2 (sry-related HMG box 2) has been identified as an adult stem cell marker in mice (3). SOX2 is an intronless, highly conserved gene known to be important in organogenesis, development and maintenance of 'stemness' of cells (4). Moreover, it is essential to maintain pluripotency in embryonic stem cells (5) and it is important for reprogramming fibroblasts into induced pluripotent cells (6). SOX2 has been described to be involved in a number of cancers including prostate cancer (7), pancreatic cancer (8), breast cancer (9), glioblastoma (10) and colorectal cancer (11). Interestingly, in several endoderm-derived SOX2-proficient tissues, SOX2 acts as an oncogene. For example, in prostate cancer, SOX2 promotes tumorigenesis and decreases apoptosis (12) and plays a critical role in epidermal growth factor receptor-mediated self-renewal of human prostate cancer stem-like cells (13). In squamous cell carcinomas of esophagus and lung, SOX2 is highly upregulated and it promotes cell migration and proliferation, acting as a lineage survival oncogene and driving cells toward squamous differentiation and pluripotency (14,15). Regarding the role of SOX2 in GC progression, there is still some controversy, basically due to the fact that it is only expressed in a subset of GCs. This observation even led to the assumption of a tumor suppressor function of SOX2 (16). On the other hand, more recent studies showed that SOX2 enhances the tumorigenicity and chemoresistance of cancer stem-like cells derived from GC (17), suggesting an oncogenic effect of SOX2 in the stomach.

In this study, we investigated the role of SOX2 in gastric carcinogenesis by abrogating its transcriptional activity in the stomach carcinoma cell line AZ-521. The loss of SOX2 resulted in reduced cell proliferation and tumorigenicity, whereas in human gastric tumor samples, SOX2 expression was found at high proliferation rate sites. Furthermore, we could identify novel target genes of SOX2 in GC.

Abbreviations: BSA, bovine serum albumin; DEG, differentially expressed genes; dnSOX2, dominant-negative SOX2; FCS, fetal calf serum; GC, gastric cancer; HA, hemagglutinin epitope; LEF, lymphoid-enhancer-factor; mRNA, messenger RNA; PBS, phosphate-buffered saline; TCF, T-cell factor.

[†]These authors contributed equally to this work.

Materials and methods

Cell culture

AZ-521 (Japanese Collection of Research Biosources, Osaka, Japan) were cultured in MEM-GlutaMAX™-I (Gibco, Darmstadt, Germany) supplemented with 10% tetracycline-negative fetal calf serum (FCS; PAA, Pasching, Austria). AGS, Kato III, NCl-N87 and SNU-1 (American Type Culture Collection), MKN7, MKN45 (18), NUGC4 (19), St3051, St23132 and St2957 (20) were grown in 10% FCS-supplemented Dulbecco's modified Eagle's medium (Gibco). All cells were maintained at 37°C in CO_2 atmosphere.

Cloning of a dominant-negative SOX2 construct

Dominant-negative SOX2 (dnSOX2) was cloned into a tet-responsive pcDNA4TO-vector (Promega, Mannheim, Germany) and amplified via PCR using a sense primer with a BamHI restriction site and an antisense primer with a NotI restriction site and an hemagglutinin epitope (HA)-tag. dnSOX2 se: 3′ CCA GGA TCC ATG TAC AAC ATG ATG GAG ACG GAG 5′, dnSOX2 as: 3′ GCA GCG GCC GCT CAA GCG TAA TCT GGA ACA TCG TAT GGG TAA CCC CCG CCG GGC AGC GTG TAC 5′.

Stable transfection of AZ-521 cells

AZ-521 cells were transfected with Lipofectamine 2000 (Invitrogen, Darmstadt, Germany) according to manufacturer's recommendations. Stable AZ-521 clones constitutively expressing a tetracycline repressor were generated using 20 µg/ml blasticidin (Invitrogen) and tested for activity of the repressor system by reporter assay. Tetracycline-repressor-positive cells were stably transfected with the HA-tagged dnSOX2 construct, which was under the control of the tetracycline repressor. Colonies resistant to 400 µg/ml of Zeocin (Invitrogen) were tested for induction of dnSOX2 expression by western blot and SOX2 reporter assays using 1 µg/ml of doxycycline (Fluka, St Louis, MO).

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Luciferase reporter gene assays

Transient transfections were carried out by using 100 ng of the SOPFlash/NOPFlash or TOPFlash/FOPFlash constructs. These reporter constructs contain six copies of the SOX2 consensus sequence (SOPFlash) or mutated copies (NOPFlash) upstream of a minimal thymidine kinase promoter directing transcription of a luciferase gene. TOPFlash/NOPFlash contains three copies of the T-cell factor (TCF)/lymphoid-enhancer-factor (LEF)-binding sites (TOPFlash) or three mutated versions (FOPFlash) (21). Cells were co-transfected with 10 ng of simian virus 40-Renilla luciferase plasmid (Promega) as control for transfection. The expression of firefly and renilla luciferases was performed 48 h postinfection, according to the manufacturer's instructions (Promega).

Cell proliferation analysis

Measurement of cell viability was performed by adenosine triphosphate quantification using CellTiter-Glo kit (Promega) according to the manufacturers' protocol. Briefly, 5×10^3 cells were seeded in 96-well plates and serum starved for 24 h. Ten percent FCS was added and adenosine triphosphate yield was measured in a Mithras 96-well reader LB 940 at 24, 48 and 72 h after treatment of cells with 1 µg/ml of doxycycline.

For cell proliferation analysis, 10^5 cells were seeded into 10 cm dishes and serum starved for synchronization. After 24 h, cells were stained with 2.5 µM of eFluor proliferation dye (eBioscience, San Diego, CA) according to supplier's recommendations and seeded into 10 cm dishes with 10% FCS. Fluorescence-activated cell sorting analysis was done after 24, 48 and 72 h of doxycycline stimulation (1 µg/ml) in a CyAn ADP analyzer (Beckman Coulter, Brea, CA). Data were evaluated using FloJo software (Tree Star, Ashland, OR).

Cell cycle analysis

Cells were serum starved for synchronization. After 24 h, medium with 10% FCS was added and dnSOX2 expression was induced by adding 1 µg/ml of doxycycline. After 48 h, cells were harvested, washed with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and fixed with 70% ethanol for 24 h at 4°C. Cells were washed twice in PBS containing 1% BSA, treated with 2 µg/ml ribonuclease A (Sigma–Aldrich, St Louis, MO) and stained with 10 µg/ml of propidium iodide (Sigma–Aldrich). Cell cycle profile was analyzed by flow cytometry with a CyAn ADP analyzer and the FloJo software.

Caspase 3/7 assay

About 5×10^5 cells were seeded in triplicates in a 96-well plate and serum starved overnight for synchronization. Medium containing 10% FCS was added and cells were treated with 1 µg/ml of doxycycline for 8, 24 and 48 h. Caspase 3/7 assay (Promega) was performed according to manufacturer's protocol. About 1 µM of staurosporine (Sigma–Aldrich) was used as a positive control.

Analysis of senescence-associated β-galactosidase activity

Fifty percent confluent cells were fixed in 5 mM of potassium ferricyanide crystalline, 5 mM of potassium hexacaynoferrate (II) trihydrate, 2 mM of $MgCl_2$ and 1 mg/ml of X-Gal in PBS for 5 min at room temperature. Subsequently, cells were washed and staining solution was added. Cells were incubated at 37°C without CO_2 overnight and microscopically analyzed and photographed in a Leica DMB microscope.

Real-time–PCR assays

Total RNA was extracted using GenElute Mammalian Total RNA Miniprep kit (Sigma–Aldrich). After deoxyribonuclease treatment (Ambion, Austin, TX), complementary DNA was prepared using M-MLV reverse transcriptase, ribonuclease H(-) (Promega). Quantitative real-time–PCR gene expression analyses were carried out using Maxima SYBR Green/ROX (Fermentas, St Leon-Rot, Germany) on a Light Cycler 480 System (Roche, Penzberg, Germany) for 40 cycles (95°C/15 s and 60°C/1 min). Results were normalized to glyceraldehyde 3-phosphate dehydrogenase. The following primers were used: hGAPDH fwd - GAAGGTGAAGGTCGGAGT, hGAPDH rev - GAAGATGGTGATGGGATTTC; hSOX2 fwd - CCCT GTGGTTACCTTTTCCT, hSOX2 rev - AGTGCTGGGACATGTGAAGT; hDKK4 fwd - GGAGGTGCCAGCGAGATG, hDKK4 rev - GGTGCCCA GTTGTTCCTTC; hp21 fwd - CAAAGGCCCGCTCTACATCTT, hp21 rev - AGGAACCTCTCATTCAACCGC; hLEF1 fwd - TCCAGCTCCTGATATCCC TACTTT, hLEF1 rev - CTGACCTTGCCAGCCAAGAG; hCycB1 fwd - GGAAA CATGAGAGCCATCCT, hCycB1 rev - TTCTGCATGAACCGATCAAT.

Wound healing

About 70 µl of cell suspension containing 3×10^6 cells/ml were seeded into each well of a silicone culture insert (ibidi, Munich, Germany). Cells were grown to 100% confluence and the culture insert was removed, leaving a 500 µm gap. About 1 µg/ml of doxycycline was added, and cell growth and migration were documented microscopically after 0, 12 and 24 h of treatment. Cell wound closure evaluation was done by WimScratch Wimasis Image Analysis (www.wimasis.com).

Western blot

Cells were lysed with sodium dodecyl sulfate lysis buffer (Tris–HCl pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate, 0.01% bromophenol blue and 50 mM dithiothreitol). Lysates were sonicated and boiled at 95°C. An equal volume of lysate was electrophoresed and transferred to a nitrocellulose membrane. Membranes were blocked in 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 for 1 h at room temperature. Subsequently, membranes were incubated overnight with anti-SOX2, anti-cyclin B1 (Cell Signaling, Danvers, MA) or anti-p21 (BD Biosciences, San Jose, CA) anti-HA antibodies (Sigma–Aldrich) according to manufacturers' instructions. After washing, membranes were incubated with the corresponding secondary antibody and proteins were detected with SuperSignal West Pico Chemiluminescence Substrate (Thermo Scientific, Waltham, MA).

RNA microarray

Total RNA from 10^6 dnSOX2-transfected AZ-521 cells harvested at different time points after/without doxycycline induction was isolated using Trizol (Invitrogen). Experiments were performed with three independent biological replicates. Labeled complementary DNA probes generated from total RNA were hybridized to an Affimetrix Gene Chip human gene 1.0 ST array (Affimetrix, Santa Clara, CA). Robust multiarray average normalized data were analyzed using LIMMA (22). Genes were scored as differentially expressed at a false discovery rate ≤0.05 and alog2-fold-change ≥1.

Raw data can be accessed at GEO, accession number GSE42937.

Immunofluorescence

About 60% confluent cells grown on cover slips were fixed in methanol/acetone for 15 min. After washing, samples were permeabilized and blocked with PBS containing 3% BSA, 0.1% Triton-X 100 and 1% saponin for 15 min. To detect dnSOX2, mouse anti-HA antibody (Sigma–Aldrich) was incubated in PBS–3% BSA–1% saponin overnight at 4°C in a humidified chamber. After washing, anti-mouse Alexafluor 488 (Invitrogen) was incubated for 1 h. Cell membranes were stained using 5 µg/ml of Cell Mask Deep Red Plasma Membrane Stain (Invitrogen). Cover slips were mounted with VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA), microscopically analyzed and photographed using a confocal microscope (Leica SP5; Leica, Solms, Germany).

Immunohistochemistry

Gastric tumor samples (n = 31) were obtained from the paraffin-embedded tissue bank of the Institut für Pathologie, Klinikum Bayreuth (Bayreuth, Germany), after approval of the local ethics committee. Four micrometer sections were incubated with anti-SOX2 or Ki67 antibodies (Cell Signaling) after antigen retrieval in epitope retrieval solution pH 6 (Novocastra; Leica Biosystems, Bucks, UK). Slides were then incubated with ImmPRESS (peroxidase) Polymer Detection kit (Vector Laboratories, Peterborough, UK) for 30 min and developed using AEC single solution (Invitrogen, Carlsbad, CA) following manufacturer's instructions. Sections were counterstained with hematoxylin (Vector Laboratories). For automated image acquisition, Olympus Virtual Slide System VS120 (Olympus, Hamburg, Germany) was used.

In vivo *analysis of dnSOX2*

For *in vivo* experiments, 6- to 10-week-old female NMRI nude mice (Janvier, Le Genest-Saint-Isle, France) were used (n = 5 per group). AZ-521 dnSOX2 cells were lentivirally transduced with an enhanced green fluorescent protein-Luc construct. Production of VSV.G pseudotyped third-generation lentiviral vectors was as described in ref. 23. AZ-521 dnSOX2 cells were seeded to culture plates and infected with 1 ml of virus supernatant in presence of 8 µg/ml of polybrene (Sigma–Aldrich Taufkirchen, Germany). Cells were passaged and absence of lentiviral particles was confirmed by p24 enzyme-linked immunosorbent assay (Quick Titer Lentivirus Quantification kit; BIOCAT GmbH, Heidelberg, Germany). Cells were sorted for enhanced green fluorescent protein by fluorescence-activated cell sorting. After washing, 10^6 cells were diluted in 150 µl of cold PBS and injected subcutaneously. Mice received doxycycline via food uptake from day 7 after cell injection to induce expression of dnSOX2 in injected AZ-521 cells. About 100 µl of luciferin solution (60 mg/ml) was injected intraperitoneally 15 min prior to bioluminescent measurement. Bioluminescence signal was measured 4 and 24 h after tumor cell injection and then every 2–4 days using the IVIS Lumina system (Caliper Life Science, Hopkinton, MA).

Results

SOX2 is expressed in a subset of GC cell lines

In order to investigate the frequency of SOX2 expression in cells derived from gastric tumors, 11 GC cell lines were screened for SOX2 expression and transcriptional activity. AZ-521 cells expressed high SOX2 messenger

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RNA (mRNA) levels (Figure 1A). Lower levels were observed in AGS and Kato III cells, whereas most other cell lines screened showed no or marginal expression of SOX2 mRNA. Similar results were observed at the protein level (Figure 1B). We then analyzed SOX2 transcriptional activity using a SOX2 reporter containing six SOX2 binding sites (termed SOPFlash). Highest SOX2 transcriptional activity was observed in AZ-521 cells (Figure 1C), correlating with the expression of SOX2 mRNA and protein. However, some of the cell lines exhibiting high SOX2 mRNA or protein expression, such as AGS or MKN45 cells, only presented moderate or low transcriptional activity. Interestingly, similar proportion of SOX2-positive human gastric tumors was observed, as described as described in the SOX2 expression in GC tissue samples.

Role of SOX2 in proliferation, apoptosis, cell cycle and migration

To examine the role of SOX2 in GC cells in detail, SOX2 was functionally inhibited in AZ-521 cells. For this purpose, cells were stably transfected with a tetracycline-inducible C-terminally truncated version of SOX2 (dnSOX2), which is able to bind SOX2 recognition sites on DNA, thus competing with SOX2 wild-type, but devoid of a transactivation domain. Expression of dnSOX2 was detected as early as 4 h after induction with doxycycline and increased over time (Supplementary Figure 1A, available at *Carcinogenesis* Online). Nuclear expression of dnSOX2 was visible after 8 h (data not shown), and after 12 h, approximately 80% of cells expressed dnSOX2 (Supplementary Figure 1B, available at *Carcinogenesis* Online). In order to analyze if the dnSOX2 construct, activated in AZ-521 cells, successfully inhibited SOX2 transcriptional activity, SOX2 reporter assays were performed. A time dependent decrease of SOX2 activity in inducible AZ-521 dnSOX2 stable cell clones was observed with highest reduction of SOX2 activity achieved 24 h after induction (Supplementary Figure 1C, available at *Carcinogenesis* Online). Thus, stable dnSOX2 cell clones showed significantly downregulated SOX2 transcriptional activity after treatment with doxycycline and represented a functional tool to investigate the role of SOX2 in GC cells.

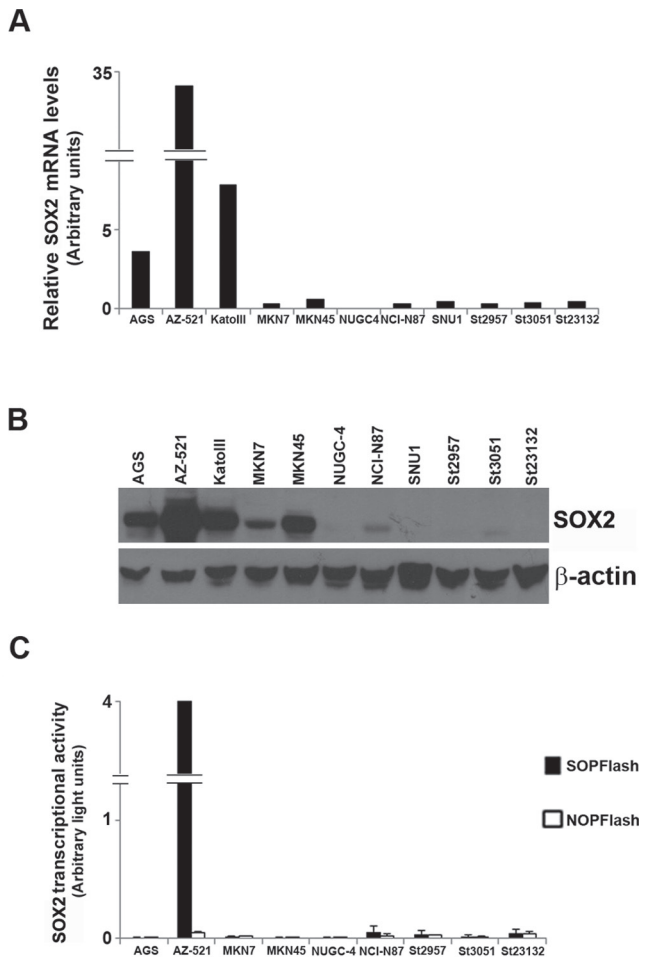

Fig. 1. Expression and transcriptional activity of SOX2 in a panel of gastric cancer cell lines. (**A**) SOX2 mRNA expression levels in gastric cancer cells, analyzed by quantitative real-time–PCR. (**B**) SOX2 (35 kDa) protein expression in gastric cancer cell lines was determined by western blot. β-actin (45 kDa) was used as a loading control. (**C**) SOX2 basal transcriptional activity in gastric cancer cells (SOPFlash and control NOPFlash). Results were normalized to renilla luciferase, used as a control for transfection efficiency.

SOX2 has been described to be a key regulatory gene during early stages of embryonic development and stemness, suggesting that it plays an important role in cell differentiation, growth and proliferation. We first analyzed the effect of SOX2 on cell proliferation by inducing dnSOX2 in synchronized AZ-521 cells. After 24 h, no differences in proliferation of stable AZ-521 dnSOX2 cell clones were observed compared with parental control cell lines (data not shown). At 48 h after induction, cell proliferation was significantly reduced to 50%, and a reduction of 70% was detected 72 h after induction (Figure 2A). Similar results were detected in AGS cells (Supplementary Figure 2A, available at *Carcinogenesis* Online) and were confirmed by using small hairpin RNA for SOX2 in AZ-521 and AGS cells (Supplementary Figure 2B, available at *Carcinogenesis* Online). These results suggest an important role of SOX2 in regulating cell proliferation of GC cells.

To explore whether the inhibition in cell proliferation observed after blocking SOX2 transcriptional activity was related to an induction of apoptosis, caspase 3/7 assays were performed in AZ-521 cells. After 8 and 24 h of doxycycline induction, no significant differences were observed compared with the non-induced cell clones or the parental cell line (data not shown). After 48 h of doxycycline treatment, almost 60% of induced AZ-521 dnSOX2 cell clones were apoptotic compared with only 20–40% of the control cells (Figure 2B), indicating that SOX2 inhibits cellular apoptosis in AZ-521 cells, thus promoting uncontrolled cell growth. Comparable results were observed in dnSOX2-expressing AGS cells (Supplementary Figure 2C, available at *Carcinogenesis* Online). Effects on apoptosis were also analyzed after expression of shSOX2 in AZ-521 and AGS. No differences in apoptosis could be detected in AZ-521 cells probably due to the fact that no complete abrogation of SOX2 expression was achieved after transducing cells with SOX2 small hairpin RNA (Supplementary Figure 2B, available at *Carcinogenesis* Online), whereas increased number of apoptotic cells was observed in AGS cells (Supplementary Figure 2C, available at *Carcinogenesis* Online).

Moreover, the inhibition of proliferation in AZ-521 dnSOX2 cells could be also linked to senescence because >30% of cells with inhibited SOX2 activity underwent senescence compared with only 5% of control cells (Supplementary Figure 2D, available at *Carcinogenesis* Online).

To depict the mechanism underlying growth inhibition, we also investigated changes in cell cycle after blocking SOX2 in AZ-521 GC cells. Although distribution of the different cell cycle phases in non-induced AZ-521 dnSOX2 stable clones was comparable with the parental cell line, a cell cycle arrest was observed in cells with blocked SOX2 activity (Figure 2C and Supplementary Figure 2E, available at *Carcinogenesis* Online). Cells were accumulating in G_2/M phase of the cell cycle and a concomitant reduction of cell numbers in the S-phase was observed, correlating with the results observed in proliferation analysis.

Further, we examined possible changes in the expression levels of cell cycle regulators that could explain the cell cycle arrest observed after blocking SOX2 activity. No significant changes were observed in cyclin D1, cyclin D3, cyclin E and c-myc (data not shown) mRNA levels, whereas a significant decrease in cyclin B1 levels was observed when inhibiting SOX2 (Figure 2D). Taken together, these results suggest that loss of SOX2 transcriptional activity induces a cell cycle arrest that can be partly be mediated by downregulation of cyclin B1

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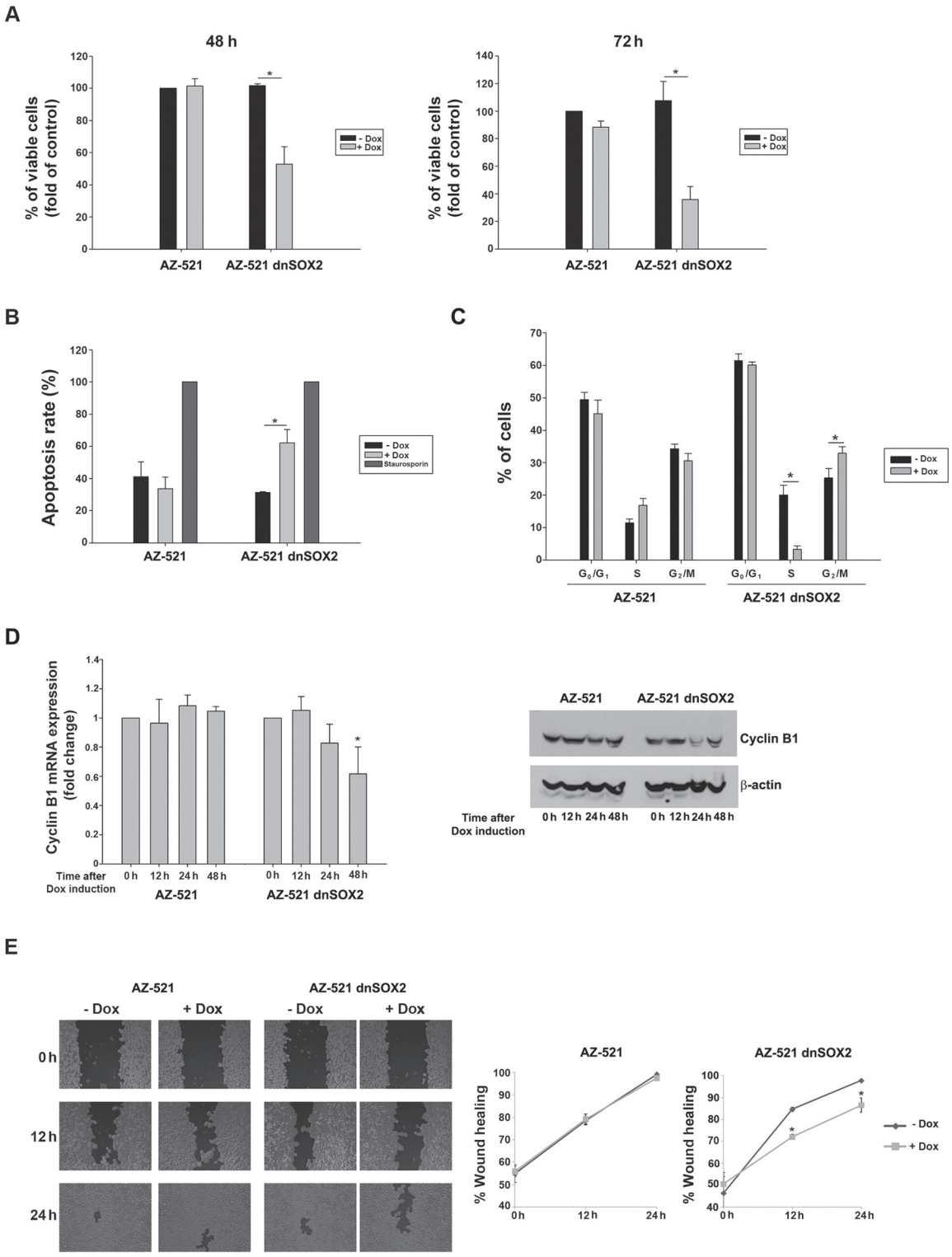

Fig. 2. Inhibition of SOX2 transcriptional activity influences cell proliferation, apoptosis, cell cycle and migration in AZ-521 cells. (**A**) Cell proliferation after inhibition of SOX2 in AZ-521 cells was measured after 48 and 72 h after doxycycline induction. AZ-521 cells were used as control. Results expressed as mean ± SD (error bars) from three independent experiments are shown. (*$P < 0.05$). (**B**) Apoptosis rate analyzed by caspase 3/7 assay after 48 h doxycycline induction in AZ-521 and AZ-521 dnSOX2 cells. Staurosporine (1 μM) was used as a positive control. Data are presented as mean ± SD (error bars) of three independent experiments (*$P < 0.05$). (**C**) Cell cycle analysis 24 h after induction with doxycycline in AZ-521 and AZ-521 dnSOX2 cells. Data are presented as mean ± SD (error bars) of three independent experiments (*$P < 0.05$). (**D**) Cyclin B1 mRNA and protein expression 12, 24 and 48 h after doxycycline induction in control AZ-521 and AZ-521 dnSOX2 cells. Results are expressed as mean of three independent experiments ± SD (error bars; *$P < 0.05$). (**E**) Wound healing experiments were performed in AZ-521 and AZ-521 dnSOX2 cells. Wound closure was monitored microscopically (left panel) 12 and 24 h after doxycycline induction. Percentage of wound healing was evaluated using WimScratch Wimasis Image Analysis (right). Results of three independent experiments are shown (*$P < 0.05$).

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expression in AZ-521 cells, which is then followed by increased apoptosis of GC cells.

Recently, SOX2 has been described to correlate with lymph node metastases and distant spread in right-sided colon cancer (11). To determine if SOX2 could be also involved in GC cell migration, wound healing assays were performed in AZ-521 cells. After inhibition of SOX2 transcriptional activity, AZ-521 cells showed a significant decrease in migration (Figure 2E). Compared with cells with high SOX2 activity, which achieved 98% wound closure after 24 h, cells with inhibited SOX2 activity showed a wound closure of only 85%, suggesting a role of SOX2 expression in migration of gastric tumor cells. Similar results were observed after using shSOX2 (Supplementary Figure 2F, available at *Carcinogenesis* Online). We also performed matrigel invasion assays, however, no changes in the (very low) invasive capacity of AZ-521 cells was detected after induction of dnSOX2 (data not shown).

Tumorigenic capacity of SOX2 in vivo

Having observed a functional role for SOX2 in regulating proliferation, apoptosis, cell cycle and migration in GC cells, we next sought to investigate the tumorigenic potential of SOX2 expression *in vivo*. Therefore, AZ-521 dnSOX2 cells were stably transduced with an enhanced green fluorescent protein-Luc lentiviral vector, which allowed monitoring cell growth via bioluminescence live imaging. Athymic nu/nu mice (n = 10) were injected subcutaneously with the stably luciferase-expressing clone of AZ-521 dnSOX2 cells. Bioluminescence was measured in an *in vivo* imaging system to assess tumor development for 24 days after injection. A significant reduction in tumor growth was observed in mice in which dnSOX2 expression was induced via doxycycline food uptake. Differences in tumor growth were visible already 3 days after induction (Figure 3), strongly emphasizing the fundamental role of SOX2 in gastric tumor development.

SOX2 expression in GC tissue samples

Downregulation of SOX2 expression has been previously observed in GC, and SOX2 levels are reported to be lower in intestinal-type compared with diffuse-type tumors (24). To analyze whether SOX2 was related to proliferation in GC, the expression pattern of SOX2 in relation to the proliferation marker Ki67 was evaluated in 31 GC tissue samples. From those, 8 cases were classified as diffuse-type tumors (25.8%) and 23 (74.2%) as intestinal-type tumors. Unexpectedly, in only two of the diffuse tumors SOX2 was detected, whereas nine (45.0%) of the intestinal-type cancers presented SOX2 nuclear expression. The percentage of SOX2-positive cells detected in the tumors was heterogeneous, ranging from 1% to 70%. Importantly, SOX2 expression correlated with Ki67 (Figure 4), indicating that SOX2 is mainly expressed at high proliferation rate sites within the tumors. Correlation of SOX2 expression and different clinicopathological characteristics of the tumors was also analyzed (Supplementary Table I, available at *Carcinogenesis* Online). Although SOX2 expression positively correlated with higher tumor grade, this was not significant due to the limited number of cases that were available for analysis. Also, no correlation was observed between *H.pylori* infection and SOX2 positivity. Here, it is important to note that around 25% of the patients with GC had already lost active *H.pylori* infection, though some of these still showed signs of past *H.pylori* gastritis.

Identification of SOX2 target genes in GC

Although many reports deciphered SOX2 targets in embryonic stem cells, to date, not many genes have been described to be regulated by SOX2 particularly in the stomach. To identify target genes of SOX2 that could explain its tumorigenic potential, the gene expression profile in the stable dnSOX2 cell clones was analyzed by RNA microarray 8, 12, 18 and 24 h after doxycycline induction, together with the corresponding un-induced controls. As there were no genes differentially expressed (with significance levels set at false discovery rate < 0.05) in the early time points (8 and 12 h), the further analyses were done with the values obtained at later time points. Quality control of RNA did not show any outliers in the expression data when normalized with robust multiarray average algorithm. Hierarchical clustering of the array data showed that induction with dnSOX2 is strongly influencing the expression profile of the cells, but there are only small differences between the two different time points of induction (Supplementary Figure 3A, available at *Carcinogenesis* Online). Results were analyzed with LIMMA (25). When setting the false discovery rate-adjusted P-value to 5%, 578 differentially expressed genes (DEG) could be identified after 24 h of induction with doxycycline.

Cluster analyses were done for the 578 DEGs using the SOTA algorithm as implemented in the program MeV4.5 (www.tm4.org/mev), which assigned the genes correlating to the progression of their expression at the different time points. The time points of 8 h and the 12 h after induction were included in this analysis as it was assumed that genes regulated at later time points would be already regulated during these earlier time points. DEGs were grouped into six different clusters of upregulated and downregulated genes. Cluster 1 shows DEGs, which increased their expression continuously during expression of dnSOX2, whereas clusters 4, 5 and 6 show all DEGs, which decreased during induction with doxycycline. Cluster 2 includes DEGs with a peak at 18 h. DEGs in cluster 3 also showed a peak at 18 h but almost no regulation after 24 h (Supplementary Figure 3B, available at *Carcinogenesis* Online).

For further analyses, we focused on DEGs represented in either cluster 1 or cluster 6 with a change of 2-fold or more (n = 33), as these genes were most significantly and continuously upregulated or downregulated after inhibiting SOX2 activity. Among those 33 genes, 19 genes were downregulated after inhibition of SOX2, and 14 genes were found to be upregulated (Table I).

Interestingly, *SOX2* was found to be downregulated, although the fold change observed was lower than 2. The decrease of SOX2 expression was confirmed by real-time mRNA expression analysis (Figure 5A). A constant reduction of SOX2 mRNA levels over time was observed, indicating that dnSOX2 is competing with SOX2 to

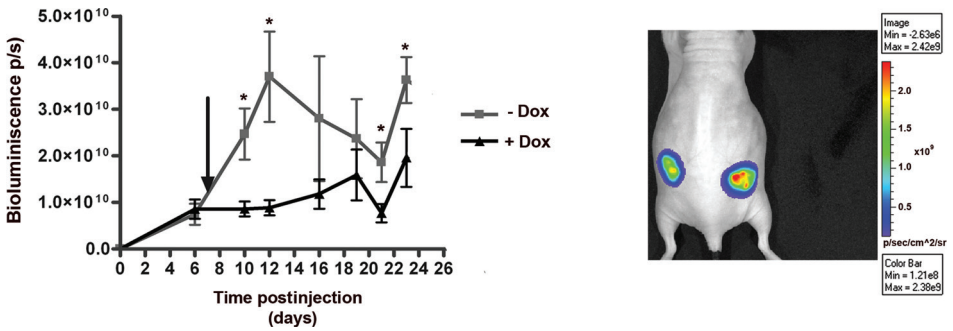

Fig. 3. Cellular tumorigenicity of AZ-521 dnSOX2 cells in nude mice. 10^6 cells were injected subcutaneously. The arrow indicates when mice (n = 5) started to receive doxycycline via food uptake to induce dnSOX2 expression in the injected cells. Bioluminescence signal was measured every 2–4 days after injection using IVIS Lumina system (*P < 0.05). A representative image of tumor growth in a control mouse after 26 days of injection is shown.

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bind its own promoter, disrupting the autoregulation loop that has been well documented for SOX2 in embryonic stem cells (26).

Remarkably, some genes related to the Wnt/β-catenin signaling pathway such as *LEF1*, *DKK4* and *FGF10* were found to be differentially regulated when SOX2 was inhibited in AZ-521 cells. To verify these results from the microarray data, gene expression at the mRNA level was analyzed. A significant increase in the mRNA levels of the downstream effector of the canonical Wnt/β-catenin pathway LEF1 and the Wnt target gene DKK4 was observed after dnSOX2 induction (Figure 5B and 5C). In contrast, mRNA expression of the Wnt target gene, FGF10, which is essential in lung and foregut patterning and development (27), decreased over time (Figure 5D), confirming the results obtained in the array.

Because SOX2 has been described previously to antagonize Wnt/β-catenin pathway (28–30) and our data suggested that this antagonism could be also occurring in GC cells, TOPFlash assays were performed to measure TCF/LEF transcriptional activity after blocking SOX2. AZ-521 dnSOX2 cells treated with doxycycline showed a significant upregulation of TCF4-activity when SOX2 was downregulated (Figure 5E), indicating antagonism between Wnt signaling and SOX2 in SOX2-positive GC cells.

Finally, several genes were identified to be differentially expressed, which did not show a 2-fold or higher change in the RNA microarray. For instance, a significant upregulation of p21 (Cip1) was confirmed after dnSOX2 expression in AZ-521 cells (Figure 5F) and after knocking down SOX2 expression in AGS cells by shSOX2 (Supplementary Figure 4A, available at *Carcinogenesis* Online). p21 prevents cell proliferation and reduction of cell growth promoted by p21 expression can lead to cell differentiation. Indeed, we observed that p21 knock down in cells expressing dnSOX2 induced a decrease in cellular apoptosis, whereas no significant effects were detected in cell proliferation (Supplementary Figure 4B and C, available at *Carcinogenesis* Online). These results indicate that p21 repression by SOX2 is involved in blocking apoptosis in gastric cancer cells.

Lastly, downregulation of the ΔN variant of p63, known to be involved in adult stem cell and progenitor regulation, was detected (Figure 5G).

Thus, we have for the first time identified and confirmed novel target genes of SOX2 in GC cells.

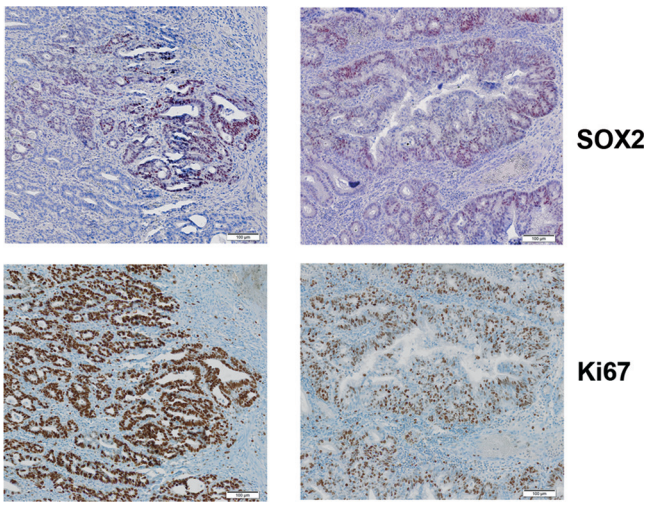

Fig. 4. SOX2 and Ki67 expression in human gastric tumors detected by immunohistochemistry.

Discussion

Aberrant expression of the transcription factor SOX2 has been observed in several different types of tumors, including stomach cancer. Interestingly, although stomach cells, and particularly gastric stem cells, are described to be SOX2 proficient, the role of SOX2 in gastric tumors has not been well established to date. Rather, conflicting roles of SOX2 in gastric carcinogenesis have been postulated. Although some studies suggested that loss of SOX2 might be related to gastric carcinogenesis and poor prognosis (16,31,32), other reports showed correlation between SOX2 expression and tumor invasion and lymph node metastasis (33). Thus, the function of SOX2 in gastric tumor development remains unclear.

To elucidate the role of SOX2 in GC, we first screened a panel of gastric tumor cell lines for the expression and transcriptional activity of SOX2. Unexpectedly, expression levels of SOX2 did not correlate with its transcriptional activity in some of the cell lines analyzed. Although AZ-521 cells showed high SOX2 expression and transcriptional activity, AGS and Kato III cells clearly expressed SOX2 but hardly showed any transcriptional activity. SOX2 can undergo different post-translational modifications, such as sumoylation (34), phosphorylation (35) and acetylation (36), which can explain the lack of correlation between

Table I. DEG after Sox2 inhibition

Downregulated genes		Upregulated genes	
BIRC3	Baculoviral IAP repeat-containing 3	*ZNF114*	Zinc finger protein 114
XRCC4	X-ray repair complementing defective repair in Chinese hamster cells 4	*APOE*	Apolipoprotein E
PCDH18	Protocadherin 18	*NELF*	Nasal embryonic LHRH factor
FGF10	Fibroblast growth factor 10	*SNAPC1*	Small nuclear RNA activating complex, polypeptide 1, 43 kDa
P2RY5	Purinergic receptor P2Y, G-protein coupled, 5	*C18orf19*	Chromosome 18 open reading frame 19
COQ3	Coenzyme Q3 homolog, methyltransferase (*Saccharomyces cerevisiae*)	*SFN*	Stratifin
MMP10	Matrix metallopeptidase 10 (stromelysin 2)	*CD68*	CD68 molecule
TFDP2	Transcription factor Dp-2 (E2F dimerization partner 2)	*GDF15*	Growth differentiation factor 15
EGR1	Early growth response 1	*LEF1*	Lymphoid-enhancer-binding factor 1
GOLPH3L	Golgi phosphoprotein 3-like	*IFI30*	Interferon, gamma-inducible protein 30
ARHGAP24	Rho GTPase activating protein 24	*ELAVL3*	ELAV (embryonic lethal, abnormal vision, *Drosophila*)-like 3
CYB5R1	Cytochrome b5 reductase 1	*DKK4*	Dickkopf homolog 4 (*Xenopus laevis*)
COL3A1	Collagen, type III, alpha 1	*IFITM1*	Interferon-induced transmembrane protein 1 (9–27)
MAMDC2	MAM domain containing 2	*CRABP2*	Cellular retinoic acid binding protein 2
COL5A2	Collagen, type V, alpha 2		
RBMS3	RNA binding motif, single-stranded interacting protein		
CHRDL1	Chordin-like 1		
PCOTH	Prostate collagen triple helix		
FRMD5	FERM domain containing 5		

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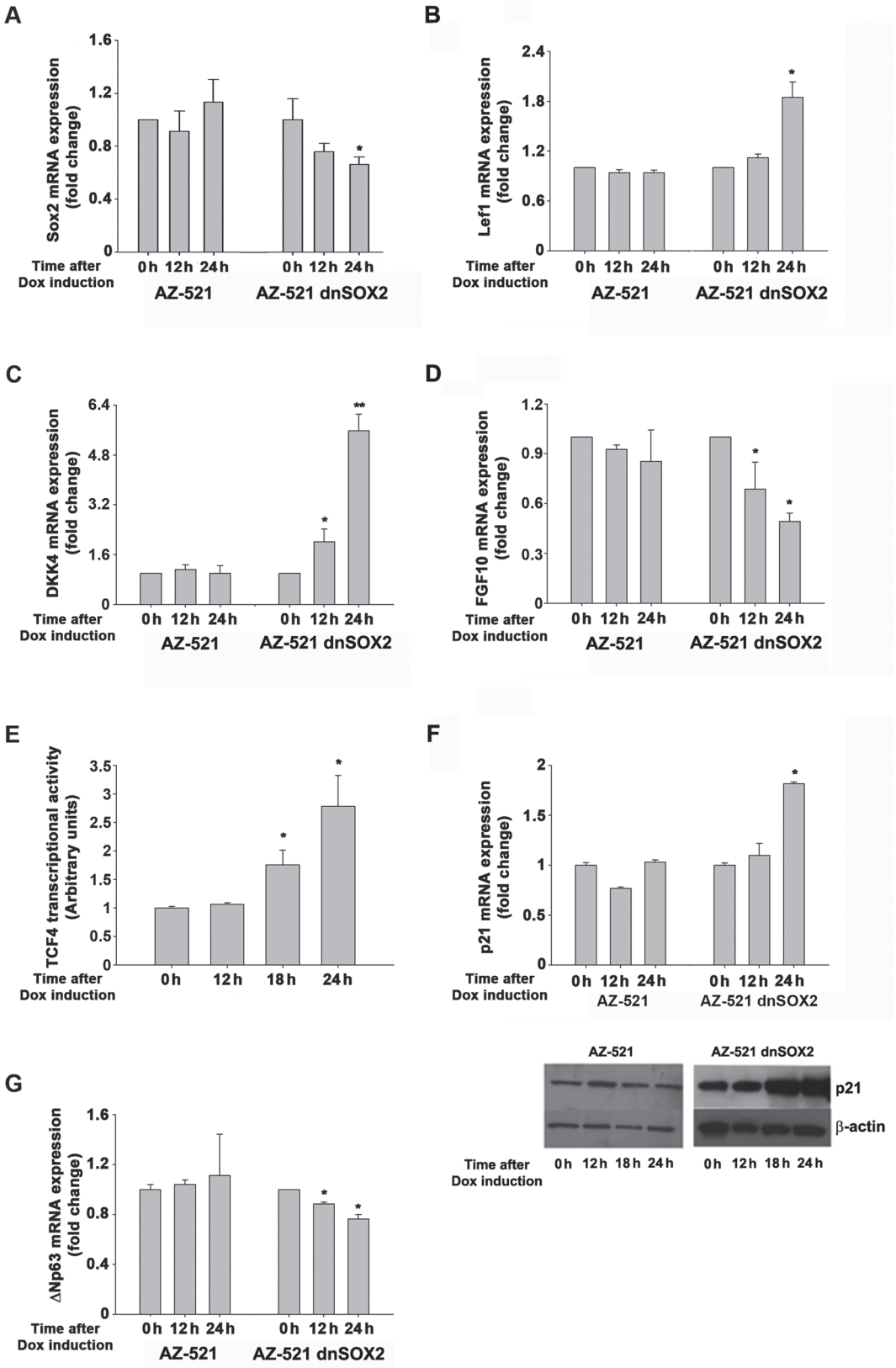

Fig. 5. Analysis of DEG after SOX2 inhibition. mRNA levels of SOX2 (**A**), Lef1 (**B**), DKK4 (**C**), FGF10 (**D**), p21 (**F**) and ΔNp63 (**G**) were analyzed after 12 and 24 h of doxycycline induction in control AZ-521 and AZ-521 dnSOX2 cells. The protein levels of p21 were also analyzed by western blot (F). TCF4 transcriptional activity (**E**) was measured by TOPFlash/FOPFlash reporter assays. Renilla luciferase was used as a control for transfection efficiency. TOP values normalized to FOP are shown. Results are presented as mean ± SD (error bars) from five independent experiments (*$P < 0.05$, **$P < 0.01$).

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protein levels and transcriptional activity because all such modifications affect SOX2 binding to DNA. Furthermore, this validates our strategy of inhibiting SOX2 transcriptional activity to study the role of SOX2 in GC cells, given that its capacity to bind DNA (and not merely its expression) is essential to regulate target genes that might be in turn involved in gastric tumorigenesis.

We observed that suppression of SOX2 in AZ-521 cells induced a significant decrease in cell proliferation concomitant to increased apoptosis. *In vivo* blocking of SOX2 in a xenograft mouse model resulted in reduced tumor growth. In addition, expression of SOX2 in human gastric tumor samples was observed at high proliferation rate sites. The inhibition of cell growth was found to be due to changes in cell cycle because inhibition of SOX2 led to cell cycle arrest in G_2/M phase and translated into a lower cell migration rate. In line with our findings, SOX2 has been reported to be essential for proliferation of glioma and breast cancer cells (10,37), whereas downregulation of SOX2 inhibited proliferation and induced apoptosis in human lung cancer cells (14,15,38). Moreover, attenuated S-phase entry was observed in human glioma cells upon inhibition of SOX2 (39), whereas inhibition of SOX2 led to cell cycle arrest in prostate cancer cells (12). In the latter report, SOX2 was shown to influence cell cycle through inhibition of G_1 to S-phase transition by targeting cyclin E. However, our results indicated that cell cycle arrest in GC cells with abolished SOX2 activity occurred at least partly through a reduction in cyclin B1 levels, whereas altered expression of cyclin E was not detected. This could be due to the different cellular context, or a differential dependency of the cell lines employed on cell cycle regulators. On the other hand, our data are in contrast to the observations by Otsubo *et al.* (16), describing inhibition of proliferation and apoptosis upon over expression of SOX2 in GC cell lines. The use of cells with distinct tumor origin could explain these discrepancies.

To identify potential SOX2 downstream target genes, we performed comparative RNA gene expression microarrays after inhibition of SOX2. We could observe differential expression of several genes. For instance, upregulation of p21 mRNA was detected after downregulating SOX2. Deregulation of p21 has been observed in a number of human cancers (40), and specifically in GC, expression of p21 was associated with a favorable prognosis, whereas absence of p21 correlated with poor survival and advanced stage and lymph node metastasis (41,42). Functionally, p21 could be linked to the effects of SOX2 on cellular apoptosis, but not on proliferation, indicating that rather a concert of different factors regulated by SOX2 are involved in the cellular effects mediated by SOX2. Furthermore, we could observe a downregulation of ΔNp63, a splice variant of p63. In squamous cell carcinomas of the gastrointestinal tract and of the lung, SOX2 was found to be co-expressed with p63 (43,44), and more recently, ΔNp63 has been described to mediate proliferation and apoptosis in human GC cells (45). Interestingly, we observed a cluster of genes related to the Wnt pathway being regulated by SOX2. In different studies, SOX2 has been observed to antagonize TCF/β-catenin activity, repressing the Wnt pathway by promoting the transcription of its negative regulators (28–30). We observed an upregulation of the Wnt effector gene *LEF1* after blocking SOX2 activity, which was concomitant to an increase in TCF/LEF transcriptional activity, indicating that SOX2 is indeed antagonizing Wnt signaling in GC. Moreover, changes in the expression of other Wnt target genes, namely DKK4 and FGF10, were detected. DKK4 is induced by the canonical Wnt pathway (46), and very recently, its over expression in hepatocellular carcinoma cells inhibited cell proliferation, reduced colony formation and retarded cell migration (47). Thus, DKK4 over expression upon SOX2 inhibition could be also contributing to the changes in proliferation and invasion observed in our dnSOX2-expressing cells. Finally, we could confirm the downregulation of FGF10 after blocking SOX2. Although FGF10 has been described to antagonize SOX2 during stomach development (27,48), we observed that inhibition of SOX2 induced also downregulation of FGF10 in GC cells. On the other hand, Wnt signaling has been reported to suppress FGF10 activity during branching morphogenesis of lung and the lacrimal glands leading to a decrease in proliferation (49). Here, further experiments would be necessary to clarify if FGF10 downregulation is an indirect effect of Wnt upregulation upon loss of SOX2 or if SOX2 can directly influence FGF10 expression in GC cells. Taken together, our array data indicate a dual role for SOX2 as transcriptional activator and transcriptional repressor. This is not surprising because by using a number of different domains, SOX2 was shown to associate with diverse co-activators and co-repressors (50). Therefore, depending on the SOX2–protein complexes engaged, SOX2 will preferentially activate or repress the transcription of specific target genes.

In summary, our results demonstrate that SOX2 is involved in several aspects of gastric carcinogenesis *in vitro* and *in vivo* by regulating the expression of genes implicated in cell proliferation, apoptosis and cell cycle regulation. Importantly, our data support a model of gastric carcinogenesis that involves the gastric stem cell and differentiation marker SOX2, indicating a novel pathway apart from activating mutations in the Wnt signaling pathway or E-cadherin.

Supplementary material

Supplementary Table I and Figures 1–4 can be found at http://carcin.oxfordjournals.org/

Funding

Schering Stiftung Graduate Fellowship to K.H.; Deutsche Forschungsgemeinschaft OG 63/4-1 and the Nanosystems Initiative Munich to O.G.; Deutsche Forschungsgemeinschaft GE 2042/2-1 to M.G.

Acknowledgements

We thank E.Wolf for helpful contributions to this work.

Conflict of Interest Statement: None declared.

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Received June 11, 2013; revised November 10, 2013; accepted November 29, 2013

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