Published OnlineFirst December 3, 2013; DOI: 10.1158/1541-7786.MCR-13-0351

Molecular Cancer Research

Genomics

Hypoxia-Independent Gene Expression Mediated by SOX9 Promotes Aggressive Pancreatic Tumor Biology

Peter Camaj[1], Carsten Jäckel[3], Stefan Krebs[4], Enrico N. DeToni[2], Helmut Blum[4], Karl-Walter Jauch[1], Peter J. Nelson[3], and Christiane J. Bruns[1]

Abstract

Pancreatic cancer aggressiveness is characterized by its high capacity for local invasion, ability to promote angiogenesis, and potential to metastasize. Hypoxia is known to represent a crucial step in the development of aggressive malignant features of many human cancers. However, micrometastatic tumors are not typically subjected to hypoxic events during early stages of dissemination; therefore, it is unclear how these tumors are able to maintain their aggressive phenotype. Thus, the identification of regulators of hypoxia-related genes in aggressive/metastatic tumors represents a fundamental step for the design of future therapies to treat pancreatic cancer. To this end, transcriptomic profiles were compared between the nonmetastatic pancreatic cancer cell line FG (LMET) and its angiogenic/metastatic derivate L3.6pl (HMET) under normoxic or hypoxic conditions. Cluster analysis revealed a number of transcripts that were induced by hypoxia in nonmetastatic cancer cells. Strikingly, this cluster was determined to be constitutively activated under normoxia in the metastatic cancer cells and could not be further induced by hypoxia. A subset of these transcripts were regulated by the transcription factor SOX9 in the aggressive-metastatic cells, but driven by hypoxia-inducible factor-1α (HIF-1α) in the parental nonmetastatic cell line. Moreover, these transcripts were enriched in cancer-related networks including: *WNT*, *CXCR4*, retinoic acid, and (FAK) focal adhesion kinase, gene PTK2 signaling pathways. In functional assays, inhibition of *SOX9* expression in HMET cells led to increased apoptosis and reduced migration *in vitro* and a significant reduction in primary tumor growth, angiogenesis, and metastasis following orthotopic tumor cell injection. At the molecular level, the control of *SOX9* expression was associated with changes in the methylation status of the *SOX9* promoter. Finally, *SOX9* upregulation was verified in a series of tumor specimens of patients with pancreatic carcinoma.

Implications: SOX9 represents a novel target for pancreatic cancer therapy. *Mol Cancer Res; 12(3); 421–32. ©2013 AACR.*

Introduction

Pancreatic cancer is a leading cause of cancer-related deaths in western countries (1). The biologic aggressiveness of pancreatic cancer is defined by local invasion, tumor angiogenesis, and its potential to metastasize. Hypoxia plays a significant role in these processes by readjustment of gene expression to support tumor cell survival. Here, we demonstrate that the transcription factor SOX9 also plays an important role in the oxygen independent regulation of hypoxia-associated gene expression responsible for tumor progression and metastases in pancreatic cancer.

SOX9 [SRY (sex determining region Y)-box 9] was originally identified as a factor necessary for development of the testis (2). SOX9 acts as a transcription factor, binding to a defined consensus sequence (3, 4). Phosphorylation of Sox9 on 2 S64 and S181 protein kinase A sites is cAMP-dependent and associated with activation of the transcription factor (5).

During cartilage development, hypoxia helps promote the differentiation of mesenchymal cells into chondrocytes in part through the activation of the transcription factor Sox9 via a hypoxia-inducible factor-1α (HIF-1α)–dependent mechanism. This expression of *SOX9* is abolished by deletion of hypoxia responsive element sites in the *SOX9* promoter (6).

SOX9 has also been described as a specific marker and maintenance factor for multipotential progenitors during pancreas organogenesis. In the developing pancreas, *SOX9* expression is restricted to a mitotically active, Notch-responsive subset of PDX1(+) pluripotent progenitors, and is absent from committed endocrine precursors or differentiated cells.

Authors' Affiliations: Departments of [1]Surgery and [2]Gastroenterology, Munich University Medical Center; [3]Medizinische Klinik und Poliklinik IV AG Klinische Biochemie; and [4]Gene Center Munich, LMU Munich, Munich, Germany

Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

Corresponding Author: Peter Camaj, Klinikum Großhadern, VH02 426, Marchioninistr. 15, 81377 München, Germany. Phone: 49-89-7095-6443; Fax: 49-89-7095-6433; E-mail: peter.camaj@med.uni-muenchen.de

doi: 10.1158/1541-7786.MCR-13-0351

©2013 American Association for Cancer Research.

Downloaded from mcr.aacrjournals.org on October 24, 2021. © 2014 American Association for Cancer Research.

Published OnlineFirst December 3, 2013; DOI: 10.1158/1541-7786.MCR-13-0351

Seymour and colleagues demonstrated that SOX9 maintains pancreatic progenitors by stimulating their proliferation, survival, and persistence in an undifferentiated state (7).

The pancreatic carcinoma cell line L3.6pl (ref. 8; HMET) contains typical genetic alterations that are associated with an early developmental phase of human pancreatic cancer including somatic mutation of the *KRAS* gene ($KRAS^{mut}$) as well as an amplification of the *ERB2* gene (9). L3.6pl (HMET) contains $KRAS^{G12D}$, the clinically most relevant activating point mutation found in Codon 12 of the *KRAS* gene (10–12). This mutation is not present in the parental cell line and thought to have arising during *in vitro* selection of the tumor line (10, 12).

Typical hypoxic gene regulation would bring advantage of more flexible rearrangement of metabolism and regulations toward hypoxic functions already for micrometastasis. Nevertheless, small cellular aggregates typical for micrometastasis do not yet suffer on hypoxia and therefore HIF-1α cannot be the main regulator causing the regulatory and metabolic rearrangement. In this study, we demonstrate that overexpression of *SOX9* is associated with the unusual regulation of hypoxia-inducible genes such as *VEGF* with consecutive activation of primary tumor growth and metastasis in clinically relevant human pancreatic cancer.

Materials and Methods

Cell culture

The following human pancreatic carcinoma cell lines were used in this study: FG (low-metastatic, e.g. LMET) and L3.6pl (high-metastatic, e.g. HMET; ref. 8). Both cell lines were maintained in Dulbecco's Modified Eagle Medium under culture conditions and with supplements as described previously (8). The last 24 hours cells were incubated either in hypoxic (1% O_2, 5% CO_2, 94% N_2) or remained at atmospheric conditions.

VEGF ELISA

To analyze the protein concentration of VEGF in the conditioned media, the human VEGF ELISA Kit (BenderMedsystems) was used according to the manufacturer's protocol.

Microarray analysis

Total RNA was isolated from the cultured cells by the TRizol method assayed for integrity and purity by agarose gel electrophoresis. Synthesis of cDNA, transcription labeling, and purification was performed with Affymetrix Kits according to the manufacturer's protocol. Affymetrix GeneChips HGU 133 plus 2.0 were hybridized, stained, and scanned. The full dataset is available at the GEO database (http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE9350. Differentially expressed genes were identified by fitting a linear model for a 2 × 2 factorial design with the factors culture conditions (hypoxia vs. atmospheric oxygen pressure) and cell line with the R-package LIMMA (13). Three independent biologic replicates were subjected to analysis. The threshold of significance in the moderated *t* test was set to a P value < 0.01 after false discovery rate adjustment and to a fold change >2.0. The expression values for the genes significant for a specific pair-wise comparison extracted from the linear model were converted to log fold change relative to the mean of all samples and clustered using the SOTA algorithm with a Pearson uncentered metric within the program suite MeV 4.0 (http://www.tm4.org/mev.html). The genes constituting a cluster were analyzed for enrichment of transcription factor binding sites with the program oPOSSUM 2.0 using the recommended significance threshold of Z-score > 10.0 and Fisher P-value < 0.01 (14).

Methylation assay

Methylation assay was performed using the MetylCollector Kit (Active Motif) with *Mse*I digestion according to the manufacturer's protocol. The presence and quantity of purified methylated-Sox9 fragments were detected via PCR using the primers (Supplementary Materials). Methylated *BRCA*-DNA delivered with the kit was used as a positive control. Products of purification in absence of methyl-CpG-binding domain protein were used as a negative control.

Construction of plasmids for regulated inhibition of Sox9 expression

pENTRSox9shRNA and control pENTRLacZshRNA based on the plasmid vector pENTR/H1/TO were constructed using BLOCK-iT Inducible H1 RNAi Entry Vector Kit (Invitrogen) with oligonucleotides Sox9shRNAforw, Sox9shRNArev (see Supplementary Materials) and verified by DNA sequencing. The negative control construct expresses shRNA with no target in human cells (*Escherichia coli LacZ* gene) was constructed using oligonucleotides LacZshRNAforw and LacZshRNArev (see Supplementary Materials).

Inhibition of Sox9 expression in cell culture

HMET cells were stably transfected with the plasmid pcDNA6TR (Invitrogen) and evaluated for overproduction of *E. coli* tetracycline repressor via Western blotting with anti-tetracycline repressor (MoBiTech). The overproducers were then stably transfected with Sox9shRNA or LacZshRNA constructs and selected. Expression of the respective shRNAs was induced by treatment with 10 μg/mL tetracycline for 48 hours.

Determination of apoptosis using FACS

To evaluate the fraction of apoptotic cells, cell-cycle analyses were performed according to methods described elsewhere (15). Tumor cells transfected with shRNA against Sox9 were cultivated in 6-well plates with or without tetracycline treatment.

Orthotopic tumor injection

For analysis of orthotopic tumor growth, HMET Sox9sh and HMET LacZsh cells, respectively, were injected into the pancreas of 20 male athymic nu/nu BALB/c mice (Charles River WIGA), as described previously (8). Expression of Sox9 was inhibited by induction of transcription of Sox9-specific shRNA with tetracycline. Therefore, the animals were divided into 2 experimental groups (10 animals each): one group

Downloaded from mcr.aacrjournals.org on October 24, 2021. © 2014 American Association for Cancer Research.

Published OnlineFirst December 3, 2013; DOI: 10.1158/1541-7786.MCR-13-0351

received 1 g/L tetracycline with glucose in the drinking water, the other group received isotonic solution of glucose (Sigma) in tetracycline-free MilliQ-water. Orthotopic tumor growth was monitored every second day by measuring the tumor volume using a caliper. On day 37 after tumor cell injection, animals were sacrificed and examined for primary tumor growth, lymph node, and liver metastases.

RT-PCR

Reverse transcription (RT)-PCR or quantitative reverse transcription PCR (qRT-PCR) was performed using One-Step RT-PCR Kits with Platinum Taq High Fidelity (Invitrogen) and Light Cycler with total RNA isolated from cell culture or tissue samples using RNeasy Kit (Qiagen) according appropriate manufacturer's protocol. The following primers were designed to span 2 exons if it sequence allowed. Primer sequences: see Supplementary Materials.

Chromatin immunoprecipitation

Chromosomal immunoprecipitation (ChIP) of the promoters binding Sox9 was performed using ChIP-IT Express Magnetic Chromatin Immunoprecipitation Kit (Active Motif). Sox9 complexes were immunoprecipitated using anti-Sox9 antibody (Tebu-bio). Immunoprecipitated promoter sequences were identified using PCR with the following primers (see Supplementary Materials). The primers recognize the sequence either within published promoter sequence or 1 kb upstream of transcription start. One twentieth of the immunoprecipitated sample and one twentieth of the sample precipitated using the negative control antibody, or sample containing no antibody or beads were subjected to PCR using promoter-specific primers and analyzed by agarose gel electrophoresis.

Western blotting

Proteins from cell lysates or from immunoprecipitation were subjected to Western blotting using the appropriate antibody according to method described elsewhere (15). Bands were visualized using WesternDot 625 Western Blot Kit (Invitrogen) and documented using gel documentation system.

Investigation of HIF-1α stability

HMET Sox9shRNA was cultivated under hypoxia or normoxia in presence or absence of tetracycline, respectively. Nuclear and cytoplasmatic extracts were prepared using Nuclear Extract Kit (Active Motif) according manufacturer's manual. Both extracts were subjected to Western blotting using anti-HIF-1α antibody (Abcam), the membrane was stripped and reprobed with anti-β-actin (Sigma) or anti-H4 histone antibody (Abcam), respectively. Data show that reduced amount of Sox9 does not influence hypoxic stabilization of HIF-1α.

Examination of *SOX9*, *VEGF* expression, and *SOX9* methylation in patient samples

The collection of human tumor tissue was approved by the local ethics committee. Operative specimens of tumor and corresponding normal tissues were obtained from 15 patients with histologically confirmed pancreatic ductal adenocarcinoma. The tissues were snap frozen and stored in liquid nitrogen. RNA or chromosomal DNA extraction were done using RNeasy Mini Kit or DNeasy Blood & Tissue Kit, respectively (both Qiagen). Quantitative results were normalized for glyceraldehyde-3-phosphate dehydrogenase.

Boyden chamber assay

Cell migration activity was determined using a colorimetric QCM Haptotaxis Cell Migration Assay—Fibronectin (Millipore). Complete growth medium [supplemented with 10% fetal calf serum (FCS)] was used as chemo-attractant. Untreated cells as well as membrane coated with arbitrary protein (bovine serum albumin, BSA) were used as a negative control.

Statistical analysis

Data are given as the mean ± SE in quantitative experiments. For statistical analysis of differences between the groups, an unpaired Student *t* test was done. Resulting *P* values were utilized for testing of the significance of certain event. As the significant are qualified only events that difference shows P value < 0.05.

Results

VEGF expression is increased in metastatic HMET cells independent of hypoxia

The LMET and HMET pancreatic cell lines derive from the same parental cell line, but HMET shows a more "aggressive" metastatic phenotype. The biology of these cell lines was examined with regards to their response to hypoxic stress and related processes associated with the development of a metastatic phenotype.

Increased expression of *VEGF* is an important indicator of hypoxic stress. VEGF protein and RNA expression in LMET and HMET cells was evaluated under normoxic and hypoxic conditions. Interleukin (IL)-6 mRNA expression was used as an additional control of hypoxic inducibility (16, 17). Results presented in Fig. 1A and B show inducible upregulation of *VEGF* RNA and protein expression in LMET cells ($P < 0.0001$), whereas metastatic HMET cells constitutively exhibited increased VEGF expression under normoxic conditions ($P < 0.0001$), which was not further inducible by hypoxia (Fig. 1A and B). In these cells, constitutive oxygen-independent upregulation of *VEGF* was detected on transcriptional (Fig. 1A) and protein levels (Fig. 1B).

Microarray analysis revealed an enrichment of genes with binding sites for SOX9 under hypoxia in LMET cells

Microarray analysis was used to profile the transcriptome of the cell lines cultivated under normoxic or hypoxic conditions. RNA was isolated and hybridized to Affymetrix human U133 Plus 2.0 GeneChip microarrays. Of the 24,734 probe sets, analysis yielded 2,986 differentially expressed genes at a significance threshold of $P < 0.01$ and a fold change higher than 2. A total of 742 genes were found to respond (either up or down) to hypoxic changes in LMET

Downloaded from mcr.aacrjournals.org on October 24, 2021. © 2014 American Association for Cancer Research.

Published OnlineFirst December 3, 2013; DOI: 10.1158/1541-7786.MCR-13-0351

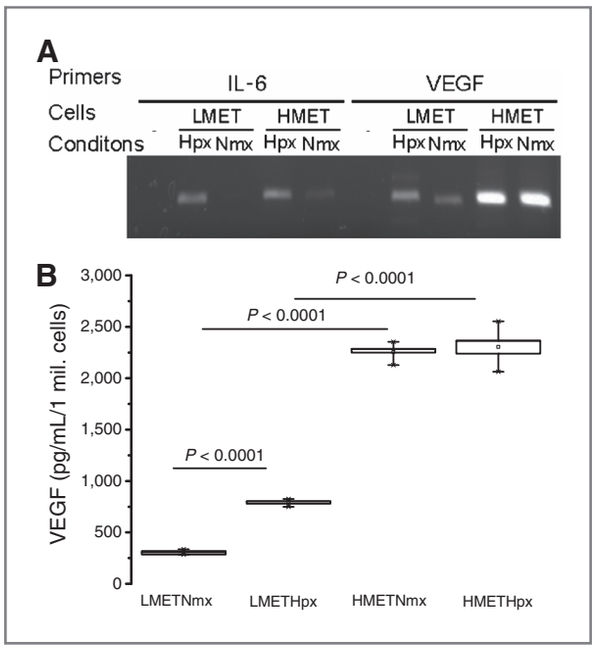

Figure 1. Expression of *VEGF* in HMET and LMET under hypoxia or normoxia. A, RT-PCR. Used primes and total RNAs are indicated. Hpx, hypoxic cultivation; Nmx, normoxic cultivation of indicated cell culture. *IL-6* expression was used as a control of hypoxia-controlled gene. B, ELISA. Concentration of VEGF in conditioned media of LMET and HMET cells under normoxia (Nmx) or hypoxia (Hpx) were measured by ELISA. Results are normalized to the cell number and statistical significance is indicated.

cells (Supplementary Fig. S1A; patterns 1 and 2), whereas these genes showed only slight expression changes (Supplementary Fig. S1A; pattern 1), or were not regulated (Supplementary Fig. S1A; pattern 2) in the HMET cells where 280 genes were found to be oxygen-responsive (Supplementary Fig. S1A; pattern 3).

We hypothesized that the regulatory activity and gene expression seen may result from the actions of a small set of specific transcription factors linked to the constitutive upregulation of "hypoxia" associated genes in the HMET line. In this regard, transcriptomic profiling identified hypoxia-induced expression of *SOX9* in the LMET cells, whereas it was found to be constitutively expressed in HMET cells (3.5-fold; $P = 0.014$; normoxia vs. hypoxia; GEO database: accession number GSE9350).

Sox9 is constitutively phosphorylated in HMET cells independent of hypoxia

The mRNA and protein expression of *SOX9* was investigated in more detail in the LMET and HMET cells under hypoxic and normoxic conditions. The results showed increased *SOX9* expression in HMET at the protein level (Fig. 2A). *SOX9* and *VEGF* transcription was constitutively upregulated in HMET cells and was not further inducible under hypoxic culturing conditions (Fig. 2B). The oxygen-dependent regulation of *VEGF* exhibited a similar transcriptional pattern to that seen for *SOX9* (Fig. 2B). The results showed no significant difference in protein activation (phosphorylation of Ser-181; ref. 5) when normalized to total amount of SOX9 in both cell lines (Fig. 2C).

Pathway analysis and expression of SOX9 target genes

The synchronization of gene expression linked to regulatory pathways can be associated with the activity of transcription factors such as SOX9. To better understand the functional consequences of the gene regulation seen in

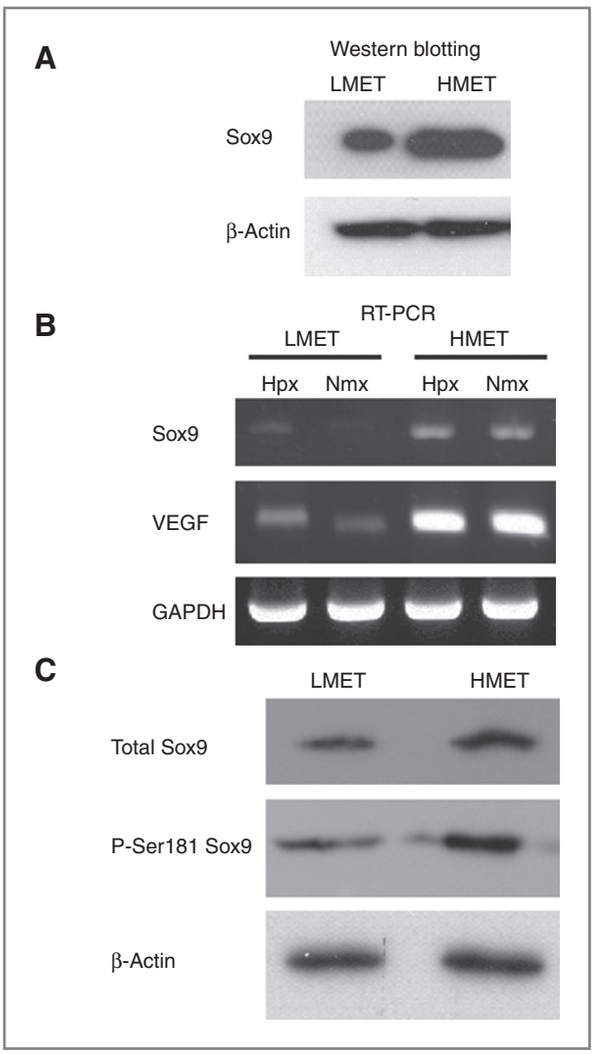

Figure 2. Expression and activation of *SOX9*. A, expression of SOX9 on protein level. Result of Western blotting using anti-Sox9 antibody is demonstrated. Cells were cultivated under normoxia. Detection of β-actin was used as a loading control. Cell lines used for protein extraction are indicated. B, transcription of *SOX9* and *VEGF* under normoxia and hypoxia. RT-PCR results are demonstrated. Primers are indicated on the left side. Cell lines used for total RNA isolation and their cultivation conditions are indicated. RT-PCR patterns show similarity of *SOX9*- and *VEGF*-transcription patterns in LMET and HMET cells with respect to hypoxia and normoxia. C, phosphorylation of SOX9. Western blotting result demonstrates the level of phosphorylation of SOX9 in position Ser-181 in HMET and LMET cells. β-Actin and total SOX9 controls are provided.

Downloaded from mcr.aacrjournals.org on October 24, 2021. © 2014 American Association for Cancer Research.

Published OnlineFirst December 3, 2013; DOI: 10.1158/1541-7786.MCR-13-0351

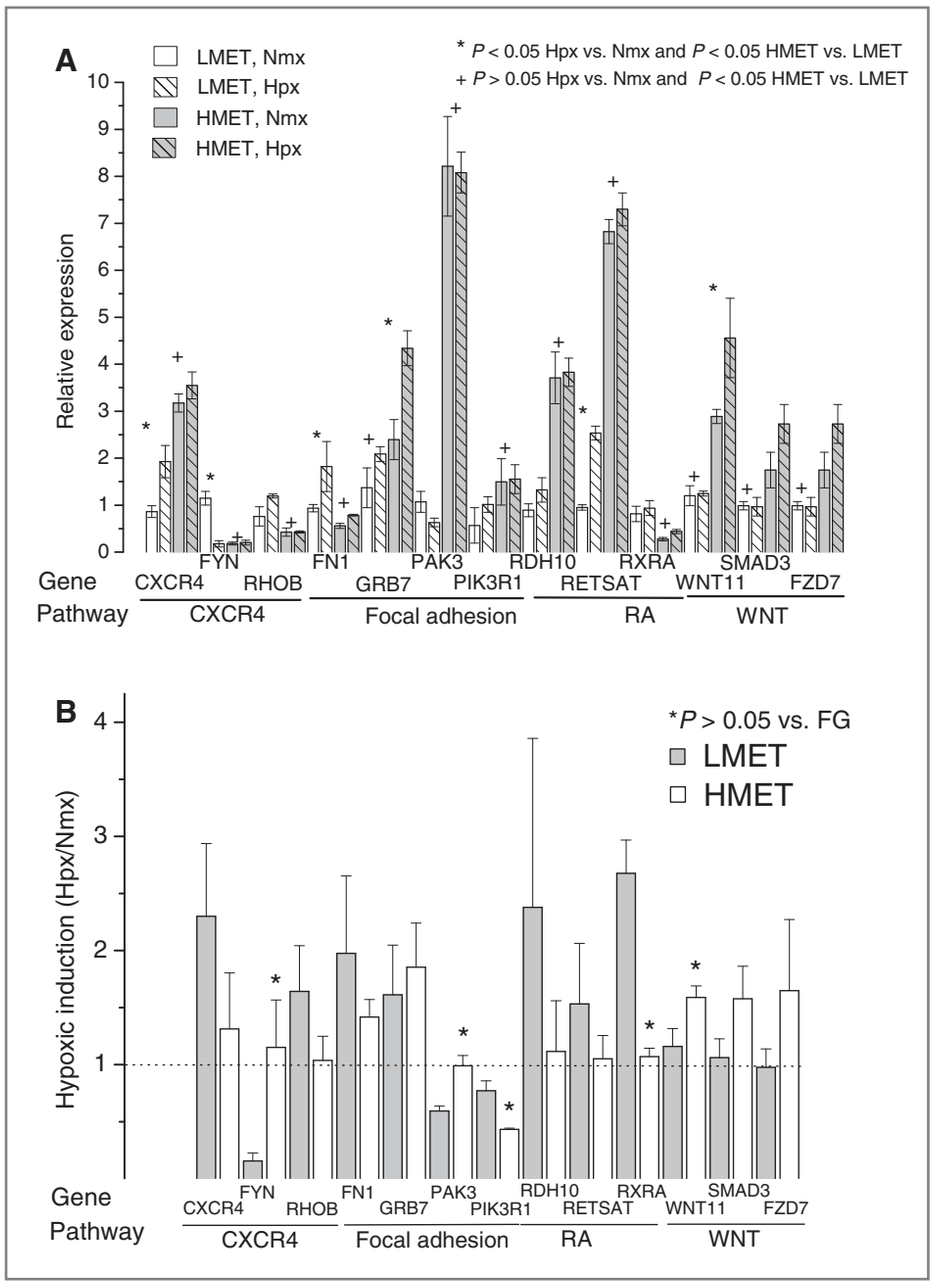

Figure 3. Hypoxic induction of expression of Sox9-driven genes. A, expression of Sox target genes in LMET and HMET cells under hypoxia or normoxia, respectively. White bars represent gene expression in LMET cells and the gray bars expression in HMET cells. Hatched bars depict the hypoxic (Hpx) gene expression and empty bars depict normoxic (Nmx) gene expression. B, relative changes of gene expression shown fold change of hypoxic expression induction. Dashed line indicates fold change 1 where hypoxia has no effect on gene expression. The values above this boarder indicate increased expression of certain gene under hypoxic conditions. Cell lines as well as statistical significance of the changes in hypoxic inducibility of certain gene in HMET compared with inducibility of the same gene in LMET are indicated. Sox9 target genes showing altered sensitivity to hypoxia in HMET as compared with LMET cells, as well as the pathways where these genes belong are indicated.

HMET, the genes were grouped according their differential reactivity to hypoxia in LMET versus HMET cells. The genes were also analyzed for presence of Sox9-binding site in their promoter region using the GeneQuest program from Lasergene package from DNASTAR. Genes from this subgroup were ordered into known pathways and analyzed using Pathvisio software (18). These data show a high prevalence of potential SOX9 target genes in cancer relevant pathways including the CXCR4, focal adhesion, retinoic acid, and WNT pathways. The expression of key genes in these pathways was then verified by qRT-PCR. The results as depicted in Fig. 3A shows the expression of Sox9 target genes and Fig. 3B describes the fold change of transcription of the investigated gene under hypoxia compared with normoxia (values greater than 1 show upregulation whereas less than 1 define downregulation under hypoxia). A significant difference between hypoxia-induced regulation of expression in LMET versus HMET cells of the gene *FYN* belonging to CXCR4 pathway, *PAK3* linked to the focal adhesion pathway, Retinoic acid receptor (*RXRA*) associated with the retinoic acid pathway, and the *WNT11* gene belonging to WNT pathway was observed in LMET versus HMET cells.

ChIP verified SOX9 binding to target gene promoters

ChIP was used to verify binding of SOX9 protein to promoter sequences of the investigated genes. Cross-linked

Downloaded from mcr.aacrjournals.org on October 24, 2021. © 2014 American Association for Cancer Research.

Published OnlineFirst December 3, 2013; DOI: 10.1158/1541-7786.MCR-13-0351

chromosomal fragments were immunoprecipitated using an anti-SOX9 antibody. The results showed SOX9 binding to promoters of the genes from investigated pathways as follows (Fig. 4): *CXCR4*, *FYN*, and *RHOB* from CXCR4 pathway, FN1 and PIK3R1 from focal adhesion pathway, *RDH10*, *RETSAT*, and *RXRA* from the retinoic acid pathway, as well as *WNT11* and *FZD7* from the canonical WNT pathway. No binding of Sox9 to *GRB7*, *PAK3*, and *SMAD3* promoters could be demonstrated (data not shown). Sox9 binding was further verified on the promoters of other cancer relevant genes, which contain conserved Sox9 binding sites including; *VEGF*, *mTOR*, β-*catenin*, and *IFIT3* (Supplementary Fig. S1B).

Epigenetic changes are linked to Sox9 induction

To investigate whether differential methylation of the SOX9-promoter could underlie the changes in SOX9 expression seen in LMET versus HMET cells, the methylation analysis was performed. Chromosomal DNA was isolated from both cell lines grown under hypoxia or normoxia and digested with the methylation-sensitive *Mse*I restriction endonuclease. Under normoxic conditions, the Sox9-promoter in LMET cells was hypermethylated as compared with HMET cells (Supplementary Fig. S2A). However, under hypoxic conditions methylation of the *SOX9*-promoter was substantially reduced in the LMET cells, in contrast, methylation of the *SOX9*-promoter in HMET cells was constitutively low suggesting reduced potential responsiveness to hypoxia. Methylation of the *SOX9*-promoter in LMET and HMET cells seems to be inversely related to the expression of VEGF and SOX9 at the protein and RNA level under normoxia and hypoxia.

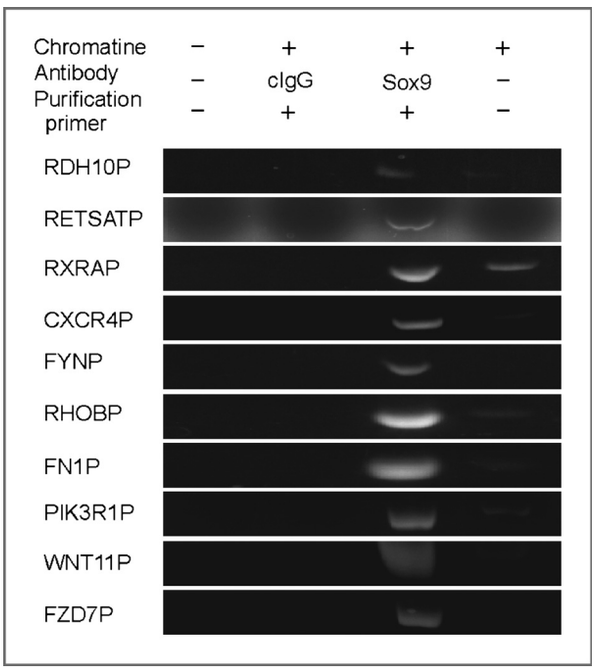

Figure 4. Binding of Sox9 on different promoters. Chromatin-immunoprecipitation. Cross-linked fragments of chromosomal DNA were immunoprecipitated with anti-Sox9 antibody. Immunoprecipitate was used as a template for PCR. Primers specific for the different promoters are indicated. PCR reaction without template (immunoprecipitate) was used as negative control. Treatment with control unrelated antibody (cIgG) demonstrates specificity of immunoprecipitation. PCR product of unprecipitated chromosomal DNA was used as positive PCR control.

Inhibition of *SOX9* expression impairs the aggressive behavior of HMET cells

We then asked whether inhibition of *SOX9* expression could revert the biologic aggressive phenotype of HMET cells toward a less aggressive phenotype as in LMET cells. To this end, HMET cells were stably transfected with an inducible vector expressing *SOX9* shRNA. Treatment with 10 μg/mL tetracycline for 48 hours induced shRNA expression. A reduction of *SOX9* expression was confirmed by Western blotting (Supplementary Fig. S1D).

Induced expression of Sox9shRNA lead to changes in the expression of all investigated downstream genes. *CXCR4* (CXCR4 pathway), *FN1*, *GRB7*, *PAK3*, and *PIK3R1* (focal adhesion pathway), *RDH10*, *RETSAT*, and *RXRA* (retinoic acid pathway), *SMAD3* and *FZD7* (*WNT* pathway) also showed reduced expression after inhibition of Sox9 expression. Expression of *FYN* and *RHOB* from CXCR4 pathway as well as *WNT11* from WNT pathway showed a significant increase in expression following knockdown of Sox9 expression (Fig. 5A). The results also showed (Supplementary Fig. S4) that reduction of *SOX9* expression did not lead to alterations in the steady-state concentration of HIF-1α in the nucleus or in cytoplasm suggesting that *SOX9*-dependent regulation of hypoxic transcription represents an alternative to HIF-1α–mediated gene transcription.

Resistance to apoptosis is associated with a malignant tumor phenotype. An increased fraction of apoptotic cells under both serum depletion as well as normal culturing conditions was also detected in the Sox9shRNA transfectants [Fig. 5B: typical fluorescence-activated cell sorting (FACS) scans]. The induced inhibition of Sox9 expression also led to a significant reduction in VEGF production (Fig. 5C, $P < 0.0001$, Sox9 shRNA transfectants: normoxia: + Tet vs. −Tet, hypoxia: + Tet vs. −Tet), and importantly, restored the hypoxia-associated inducibility of VEGF expression (Fig. 5C, $P < 0.0001$, Sox9 shRNA transfectants: + Tet: hypoxia vs. normoxia) in this cell line. Migration and invasion is an important component in tumor aggressiveness. A significant reduction in cell migration was observed after inhibiting *SOX9* expression (Fig. 5D, $P < 0.0005$).

These phenomenon were then evaluated in an *in vivo* setting. The cell lines were implanted orthotopically in nude mice. Tetracycline-induced inhibition of *SOX9* expression in HMET cells *in vivo* led to a significant reduction in primary tumor growth and metastasis as compared with control tumors (Fig. 5E and Table 1). Following implantation of HMET, LacZshRNA cells developed large tumors [average volumes 2.1 (±0.3) cm^3 −tet, 2.2 (±0.4) cm^3 +tet] and high metastatic activity (table 1). Animals with implanted HMET *Sox9*sh cells exhibited substantially [0.8

Downloaded from mcr.aacrjournals.org on October 24, 2021. © 2014 American Association for Cancer Research.

Published OnlineFirst December 3, 2013; DOI: 10.1158/1541-7786.MCR-13-0351

Table 1. Animal experiments. Balb/c$^{nu/nu}$ mice were orthotopically injected with 1×10^6 cells

			Metastasis		
	Tetracycline	*n*	Liver	Lymph nodes	Death
LacZ shRNA	−	10	9	10	2
	+	10	9	10	1
Sox9 shRNA	−	10	7	9	2
	+	10	0	2	0

NOTE: HMET stable transfectants expressing Sox9 shRNA or *E. coli* LacZ shRNA in the presence of tetracycline. Tetracycline treatment, total number of mice, and the incidence of liver or lymph node metastases are indicated.

($\pm$0.4) cm^3] and statistically significant ($P < 0.01$) reduction of primary tumor volume and incidence of liver metastasis (Table 1).

Histologic sections prepared from the HMET tumors were stained for antigen Ki-67 specific for proliferating cells and counterstained using hematoxylin and eosin. A significant reduction in proliferation activity of the HMET cells *in vivo* was seen after inhibition of *SOX9* expression (proliferative index: 0.73 vs. 0.27). Expression of unspecific shRNA showed no significant effect on proliferation of the tumor cells *in vivo* (proliferative index: 0.82 vs. 0.85; Fig. 5F). The tissue sections were then fluorescent stained using Terminal deoxynucleotidyl transferase dUTP nick end labeling. An increased apoptosis was seen in tumors expressing *Sox9*shRNA construct after induction by tetracycline. Negative control cells expressing non-relevant shRNA showed no significant effect on apoptosis (Supplementary Fig. S1F).

Clinical significance of *SOX9* expression

To examine the potential role of Sox9 in clinical settings, paired tumor and normal tissue samples obtained from the same human pancreas were analyzed for expression of *VEGF* and *SOX9* genes. In 22 of 28 analyzed tissue samples, significant upregulation of *SOX9* expression was observed in pancreatic cancer in comparison to normal tissue. Analysis of *VEGF* transcription in the same samples revealed the significant upregulation in 23 of 28 tumor compared with normal samples (Fig. 6A).

To further validate the clinical role of *SOX9* at the level of promoter methylation, chromosomal DNA was isolated from the same tissue samples used for investigation of *Sox9* and *VEGF* transcription described earlier, and analyzed for *SOX9*-promoter methylation. *SOX9*-promoter methylation was increased in normal tissue as compared that seen in corresponding tumor tissues in 9 of the 15 investigated paired samples (Supplementary Fig. S5A and S5B). Three samples exhibited increased *SOX9*-promoter methylation in cancer tissues in comparison to normal tissue. Interestingly, *SOX9* or *VEGF* expression is in these samples stronger in normal tissue or they show no significant difference in both paired samples (Supplementary Fig. S5B). Elevation of *VEGF* expression (tumor vs. normal tissue) in patient samples strongly correlates with the elevation of *SOX9* expression in tumor samples (Fig. 6B). We have observed less significant (correlation coefficient: 0.7) negative correlation between *SOX9* promoter methylation and *SOX9* expression (Fig. 6C).

Discussion

SOX9 expression was linked to aggressive pancreatic tumor phenotypes. *SOX9* expression was found to cluster with constitutively upregulated genes in the HMET cells under normoxic conditions (but not hypoxia). Genes within the clusters were demonstrated to be differentially responsive to hypoxia and to be targets of SOX9 regulation. Four regulatory pathways associated with tumorigenesis: the CXCR4 pathway, focal adhesion pathway, retinoic acid pathway, and WNT pathways were found to be overrepresented among SOX9 target genes. Sox9shRNA inducible transfectants in the HMET cell line showed reduced expression of genes identified in the HMET cluster suggesting a central role for SOX9 in pancreatic tumor progression (Fig. 5 and Supplementary Fig. S1E; refs. 19–21).

Tumor progression is associated with hypoxia. HIF helps to mediate responses to hypoxia (22). VEGF family members are commonly overexpressed in human pancreatic cancer and contribute to tumor growth and metastasis (23). We recently demonstrated that the metastatic potential of a human pancreatic cancer cell line HMET as compared with its nonmetastatic derivative LMET that was associated with increased expression of proangiogenic molecules, in particular, VEGF. As expected, *VEGF* RNA expression and protein production was inducible by hypoxia in LMET cells (reviewed in ref. 24). Interestingly, in HMET cells *VEGF* expression was constitutively expressed under normoxia and not inducible by hypoxia. An increase in the biological aggressiveness of HMET cells *in vivo* was previously reported as compared with LMET cells based on the development of an angiogenic phenotype and an increase of their metastatic potential (8).

Transcriptomic profiling defined genes that coclustered in HMET cells with VEGF. These genes were found to be inducible by hypoxia in LMET cells, whereas in HMET cells the same genes were constitutively upregulated under normoxia and not inducible by hypoxia (Supplementary Fig. S1A). Presumably, the aggressive HMET pancreatic tumor cells gained via *in vivo* selection the capability to overexpress hypoxia inducible genes under normoxic conditions.

Downloaded from mcr.aacrjournals.org on October 24, 2021. © 2014 American Association for Cancer Research.

Published OnlineFirst December 3, 2013; DOI: 10.1158/1541-7786.MCR-13-0351

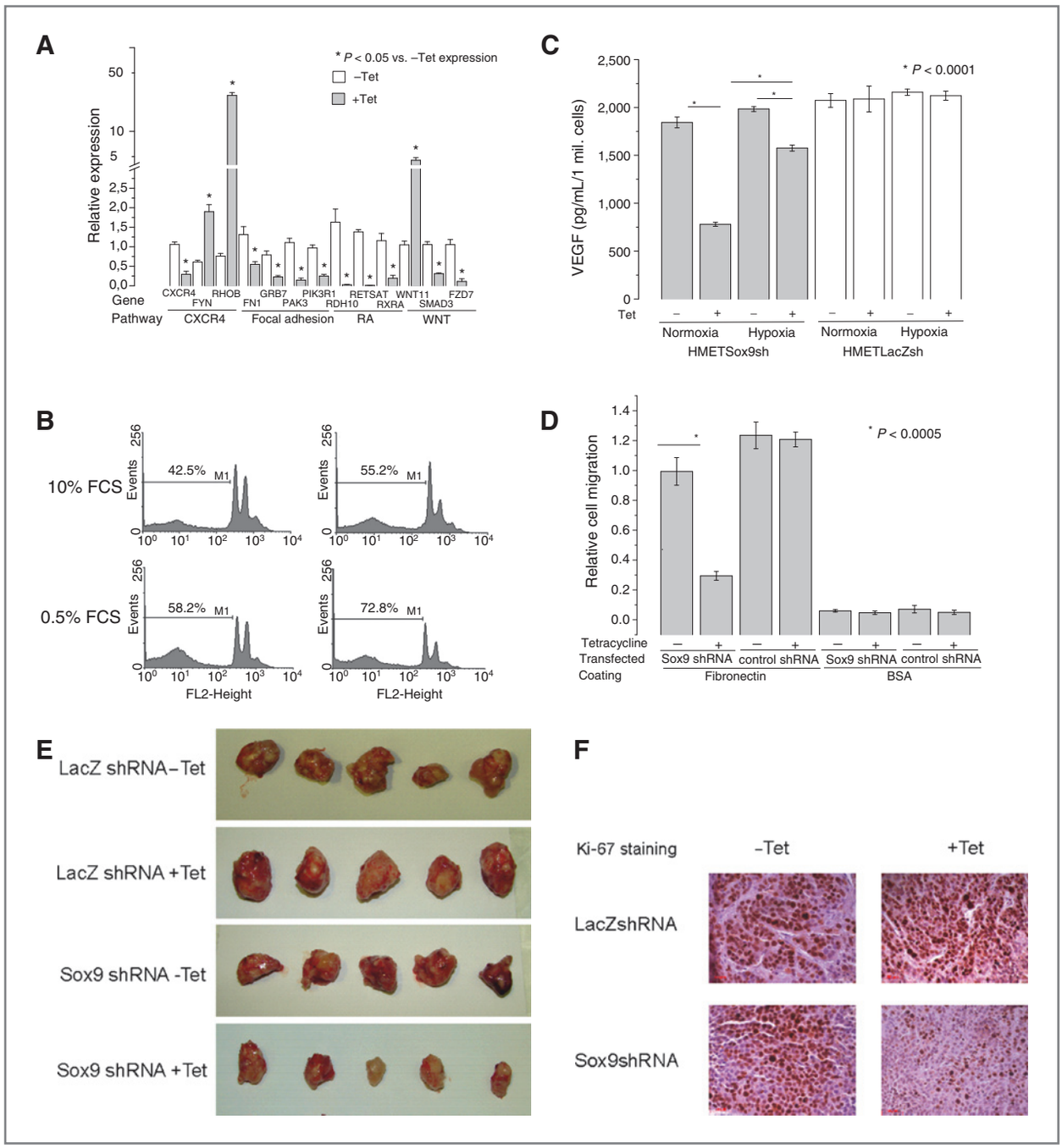

Figure 5. Effect of tetracycline-induced expression of Sox9 shRNA *in vitro* and *in vivo*. HMET cells stable transfected with the plasmid system leading to inhibition of *SOX9* expression via activity of appropriate shRNA after treatment with tetracycline were used to investigate: A, expression of SOX9-driven genes. Expression of SOX9-driven genes was investigated via qRT-PCR with (gray bars) or without (white bars) added tetracycline. Genes and their pathway are indicated. Statistical significance ($P < 0.05$) of the expression changes induced by downregulation of Sox9 expression is indicated. B, cell cycle and apoptosis. Representative FACS scans of propidium iodide-stained HMET Sox9sh cells treated with or without tetracycline are presented. Cells were cultivated in normal medium or in starvation medium containing 0.5% FCS, respectively. Cultivation conditions as well as percentage of apoptotic cells are indicated. C, production of VEGF. HMET Sox9sh cells treated with or without tetracycline were cultivated under normoxic or hypoxic conditions, respectively. Cells expressing unspecific shRNA, lacking its target in the human cells (white bars), were used as negative control. Statistical significances ($P < 0.0001$) are indicated. D, cell migration assay. HMET cells stably transfected with Sox9shRNA or LacZshRNA (negative shRNA control) were treated with 10 μg/mL tetracycline for 48 hours and subjected to cell migration assay using Boyden chambers. FCS was used as a chemoattractant. Data are demonstrated relative to untreated control cells. Cell lines and tetracycline treatment are demonstrated. Chambers coated with BSA was used as the control for unspecific cell migration. Statistical significance ($P < 0.0005$) is indicated. E, animal experiment. (*Continued on the following page.*)

Downloaded from mcr.aacrjournals.org on October 24, 2021. © 2014 American Association for Cancer Research.

Published OnlineFirst December 3, 2013; DOI: 10.1158/1541-7786.MCR-13-0351

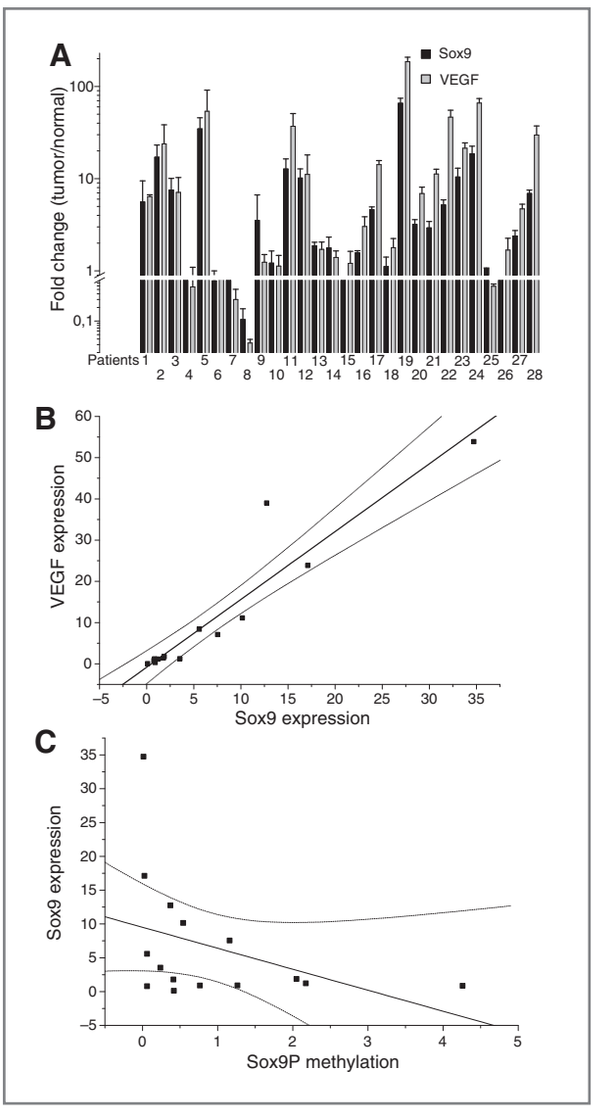

Figure 6. A, transcription of Sox9 in patient samples. Total RNA isolated from tumor as well as healthy tissue from the same pancreas were isolated and subjected to real time qRT-PCR. Results are demonstrated as a ratio between *SOX9* and *VEGF* expression in tumor tissue compared with normal tissue. Fold change of the expression and numbers of patients are indicated. B, correlation between fold change of *VEGF* expression (tumor/normal tissue) and fold change of *SOX9* expression similarly expressed as the ratio between the expression in the tumor and normal cells. 95% confidence intervals (CI) are indicated. C, correlation between fold change of *SOX9* promoter methylation (tumor/normal tissue) and fold change of *SOX9* expression similarly expressed as the ratio between the expression in the tumor and normal cells. 95% CIs are indicated.

The promoter sequence analysis of the gene cluster revealed a significant enrichment for the binding site of the transcription factor Sox9 (14). We then hypothesized that SOX9-dependent transcriptional regulation of hypoxia-associated genes under normoxic conditions could be responsible for the aggressive biology of HMET-induced pancreatic cancer, and may therefore contribute to aggressive tumor stages of pancreatic cancer. A series of survival and migration-related genes are found in the gene clusters, exhibiting the differential hypoxic response in LMET versus HMET cells, suggesting a potential role for these gene products in the observed phenotype (Table 2). Although HIF-1α is a major factor driving hypoxia-inducible genes in pancreatic tumors under oxygen deprivation (25), SOX9-based transcription of hypoxia-associated genes may represent an alternative pathway for the induction of these tumor-critical genes in the absence of hypoxia. Knockdown of *SOX9* led to restoration of HIF-1α–driven hypoxic inducibility of the downstream target genes (Fig. 5C for *VEGF*), suggesting overlap of activity between these transcription factors.

The concept of "stemness" has been linked to the regulation of tumor aggressiveness, including metastasis, chemoresistance, and adaptation to hypoxia. To date, the transcription factor SOX9 has not been associated with pancreatic cancer development and progression. Galmiche and colleagues described cytoplasmatic expression of *SOX9* and *PDX1* associated with β-*catenin* mutations in pediatric solid pseudopapillary tumors (26). The authors suggested that these tumors may originate from the transformation of normally quiescent pancreatic stem cells (26). Results from the ChIP analysis presented here showed *in vivo* binding of SOX9 to the β-catenin gene promoter (Supplementary Fig. S1B). Seymour and colleagues described SOX9 as a specific marker and maintenance factor for multipotential progenitors during pancreas organogenesis (7). Genes identified in their study represent downstream targets of SOX9 regulation and may therefore be active in the establishment of a tumor progenitor phenotype. In this study, we identified SOX9 target genes exhibiting differential hypoxic responsiveness to hypoxia in HMET and specifically belonging to the WNT and CXCR4 pathways. The constitutive normoxic upregulation of *SOX9* may thus have a direct effect on tumor growth, invasiveness, and metastasis.

The knockdown of *SOX9* led to restoration of oxygen sensitivity presumably because of a reactivated HIF-1α pathway. Our data also show that SOX9 expression does not seem to interfere with *HIF-1α* gene regulating machinery (Supplementary Fig. S4). The stability of HIF-1α protein is regulated the same regardless of the intracellular concentration of SOX9. *SOX9* activation seems to have a stronger effect on the transcriptome and therefore can overcome hypoxic HIF-1α–based hypoxic regulation.

Four pathways associated with tumorigenesis were linked to *SOX9* expression: the CXCR4 pathway, focal

(*Continued.*) Male athymic nu/nu BALB/c mice were subjected to orthotopic implantation of 1×10^6 HMET cells stably transfected with Sox9shRNA or LacZshRNA (negative control, plasmid construct), respectively. Tetracycline was administered to 50% of all animals in each group with the drinking water. Animals were sacrificed 45 days after tumor cell injection. Tumors have been photographed. shRNAs and tetracycline treatments are indicated. F, Ki-67 immunohistochemistry staining. Tumors depicted in E were fixed, sectioned, and stained for proliferation-specific nuclear protein Ki-67. Expressed shRNA as well as presence of inducer are indicated. The size bar indicates length 50 μm.

Downloaded from mcr.aacrjournals.org on October 24, 2021. © 2014 American Association for Cancer Research.

Published OnlineFirst December 3, 2013; DOI: 10.1158/1541-7786.MCR-13-0351

Table 2. Overview of the genes containing in their promoter, the SOX9-recognition sequence, and at the same time belong to the cluster of the genes which in LMET cells respond to hypoxia differently than in HMET cells

Gene	Migration	Survival	HMET/LMET (Hpx)	HMET/LMET (Nmx)
MAP2	−	−	0.395	0.082
SYNJ2	+		2.6	3.18
ETV1	+	+	4.29	5.7
SEMA3A		+	2.2	7.16
SEMA3C	+		3.6	6.87
WNT11	−	+	4.85	5.43
FOXC1	+	+	2.04	8.39
ITGA6	+	+	6.36	10.63
HAS3	+		4.66	3,691
ERF	+	−	0.28	0.17
SMAD7	−	−	0.19	0.0011
ITGB8	+		2.97	9.78
FOXP1		−	0.44	0.46
LAMA3	+		2.3	2.38
ARHGAP21	−		0.3	0.22
HBEGF	+	+	16.22	95.01
RASSF8	+		10.34	16.34
CPEB4	−		0.22	0.24
PCDH17		−	0.017	0.087
TBCCD1	+		2.71	6.62
CALD1	−		0.31	0.22
SGPP1		−	0.29	0.28
EREG	+	+	26.72	26.17
NOV	+		19.56	6.49
SSH2	+		2.3	2.53
THBS1	−	−	0.37	0.31
GRB7	+	+	2.38	3.46
EHF		+	3.68	9.38
TPM1	−		0.12	0.0063
DUSP6	+	+	2179.23	51,418
CAST		+	2.53	4.08
TIAM1	+	+	4.79	4.82
PIK3R1	+	+	3.43	5.66
PTPRR	−		0.000006	0.0013
LATS2		−	0.4	0.36
PPP2R2B		−	0.28	0.13
SSBP3		−	0.45	0.37
HHEX		+	5.62	9.32
DEDD2		−	0.23	0.21
DOCK1	+		2.23	2.75
TNIK		+	3.43	3.14
ATF3		−	0.44	0.0093
SH3RF1		−	0.36	0.19
DLC1	−	−	0.34	0.4
SMAD3	+	+	4.23	5.94

(Continued on the following column)

Table 2. Overview of the genes containing in their promoter, the SOX9-recognition sequence, and at the same time belong to the cluster of the genes which in LMET cells respond to hypoxia differently than in HMET cells (Cont'd)

Gene	Migration	Survival	HMET/LMET (Hpx)	HMET/LMET (Nmx)
IGFBP6	+	+	14.22	30.48
HIF1AN	+		2.57	3.74
ETV5	+		5.43	8.57

NOTE: Relevance to cell migration and survival based on published data are indicated (+, increase; −, decrease) as well as differential expression shown as fold change between average value in HMET/LMET under normoxia or hypoxia, respectively.

adhesion pathway, retinoic acid, and WNT pathways (Supplementary Fig. S6). Sox9 is a positive regulator of expression of *CXCR4* from CXCR4 pathway, *FN1, GRB7, PAK3, PIK3R1* from the focal adhesion pathway, *RHD10, RETSAT*, and *RXRA* from the retinoic pathway, and *SMAD3* and *FZD7* from the canonical WNT pathway. SOX9 was also shown to be a negative regulator of *FYN* and *RHOB* expression in CXCR4 pathway, and *WNT11* from the WNT pathway. FYN interacts with FAK (27), a kinase that plays a central role in directed cell movement. The Akt pathway focuses survival signals from other pathways regulated by SOX9: focal adhesion pathway via at least *GRB7*, *PAK3*, and *PIK3R1*gene. All-*trans*-retinoic acid is an important regulator of epithelial differentiation, embryogenesis, and cancer (28). *RXRA* as well as at least 2 genes (*RDH10* and *RETSAT*) linked to the biosynthesis of retinoic acid were identified as targets of SOX9 regulation in this setting (Fig. 5A). Potential points of cross-talk between the regulatory pathways are depicted in Supplementary Fig. S6.

Oxygen presence affected methylation of the *SOX9* promoter in an inverse manner in relation to gene expression: hypoxia-reduced methylation induced downregulation of *SOX9* expression in LMET cells. Interestingly, methylation of the *SOX9* promoter in HMET cells was only weakly sensitive to the oxygenation status of the cells analogous to the marginal alterations in gene expression of *SOX9* itself and other genes of the gene cluster (e.g., *VEGF*) (Supplementary Fig. S2B compare with Supplementary Fig. S2B). These data show that differential methylation of the *SOX9* promoter is at least in part responsible for the differential expression of *SOX9* in LMET versus HMET cells. Finally, increased expression of *SOX9* and *VEGF* mRNA was found in human pancreatic adenocarcinoma specimens as compared with normal pancreatic tissue (Fig. 6), suggesting that SOX9 may help drive expression of *VEGF in vivo*. Differential methylation of the *SOX9* promoter may be responsible in part for the regulation of *SOX9* seen. A reduction in *SOX9*-

Downloaded from mcr.aacrjournals.org on October 24, 2021. © 2014 American Association for Cancer Research.

Published OnlineFirst December 3, 2013; DOI: 10.1158/1541-7786.MCR-13-0351

promoter methylation correlated with increased expression of *SOX9* and *VEGF* expression in patient tumor samples.

We are aware that our experimental model is dealing with one couple of isogenic cell lines supported with the results of animal experiments. Because we assume that the mechanism of overcoming of HIF-1α regulation via SOX9 has more general significance, we tried to verify our data obtained on cell lines LMET and HMET in paired samples of primary tumor versus metastasis. We searched for gene array data of analyzed patient's specimen comparing primary pancreatic tumors and metastasis for SOX9-based regulation patterns. In our Supplementary Material, we added the respective results of the GEO database from the manuscript of Mozon and colleagues (29). Unfortunately, the majority of such data were generated on gene array platforms incompatible to the one used in this manuscript. However, a small amount of gene array data generated on a HG-U133A platform revealed an upregulation of *CXCR4* and *LAMC* genes as well as a downregulation of *FYN*, *PIK3R1*, *ARHGAP*, *COL3A1*, and Sox2 in metastases in comparison to primary tumors. We obtained similar data using LMET and HMET cells. Yu and colleagues (30) demonstrated by RNA sequencing of pancreatic circulating tumor cells that the WNT and CXCR4 signaling are important pathways for the establishment of metastases comparable to own results about the genes *WNT11*, *CXCR4*, and *CTNNB1*. A recently published report demonstrated the upregulation of *SOX9* in colorectal carcinoma samples in comparison to normal tissue specimens. This regulation correlated with upregulation of β-catenin and PPAR-γ expression in cell cultures (31). Both those genes are members of the *SOX9* target gene cluster identified in this study. In case of β-catenin, a direct binding of *SOX9* to the promoter was demonstrated via ChIP (Supplementary Fig. S1B). The data presented here support the general relevance of *SOX9* regulation for overcoming the lack of HIF-1α–based hypoxic induction of gene expression in micrometastasis followed persistent regulation of SOX9 target genes in metastases. SOX9 may represent an important new therapeutic target for the treatment of pancreatic carcinoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: P. Camaj, P.J. Nelson, C.J. Bruns
Development of methodology: P. Camaj, E.N. DeToni, C.J. Bruns
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P. Camaj, S. Krebs, H. Blum, K.-W. Jauch, C.J. Bruns
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P. Camaj, C. Jäckel, S. Krebs, H. Blum, K.-W. Jauch, P.J. Nelson, C.J. Bruns
Writing, review, and/or revision of the manuscript: P. Camaj, E.N. DeToni, P.J. Nelson, C.J. Bruns
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.J. Bruns
Study supervision: P.J. Nelson, C.J. Bruns

Acknowledgments

The authors thank M. Hofstetter (Clinical Biochemistry Group, Medizinische Klinik und Poliklinik IV, University of Munich, Germany) and A. Tischmacher (Department of Surgery, Department of Gastroenterology, Munich University Medical Center, Munich, Germany) for the excellent technical assistance.

Grant Support

This work was supported by grants of Deutsche Forschungsgemeinschaft NE 648/2-4 to P.J. Nelson and by grants from the Deutsche Forschungsgemeinschaft (DFG BR 1614/7-1 to C.J. Bruns; DFG SPP1190 "Tumor Vessel Interface" to C.J. Bruns and P.J. Nelson).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 3, 2013; revised October 29, 2013; accepted November 11, 2013; published OnlineFirst December 3, 2013.

References

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. CA Cancer J Clin 2009;59:225–49.
2. Foster JW. Mutations in SOX9 cause both autosomal sex reversal and campomelic dysplasia. Acta Paediatr Jpn 1996;38:405–11.
3. Sudbeck P, Scherer G. Two independent nuclear localization signals are present in the DNA-binding high-mobility group domains of SRY and SOX9. J Biol Chem 1997;272:27848–52.
4. Sudbeck P, Schmitz ML, Baeuerle PA, Scherer G. Sex reversal by loss of the C-terminal transactivation domain of human SOX9. Nat Genet 1996;13:230–2.
5. Malki S, Nef S, Notarnicola C, Thevenet L, Gasca S, Mejean C, et al. Prostaglandin D2 induces nuclear import of the sex-determining factor SOX9 via its cAMP-PKA phosphorylation. EMBO J 2005;24:1798–809.
6. Robins JC, Akeno N, Mukherjee A, Dalal RR, Aronow BJ, Koopman P, et al. Hypoxia induces chondrocyte-specific gene expression in mesenchymal cells in association with transcriptional activation of Sox9. Bone 2005;37:313–22.
7. Seymour PA, Freude KK, Tran MN, Mayes EE, Jensen J, Kist R, et al. SOX9 is required for maintenance of the pancreatic progenitor cell pool. Proc Natl Acad Sci USA 2007;104:1865–70.
8. Bruns CJ, Harbison MT, Kuniyasu H, Eue I, Fidler IJ. *In vivo* selection and characterization of metastatic variants from human pancreatic adenocarcinoma by using orthotopic implantation in nude mice. Neoplasia 1999;1:50–62.
9. Sempere LF, Korc M. Shining the spotlight on shed KRAS in pancreatic cancer. Cancer Biol Ther 2008;7:361–3.
10. Gysin S, Rickert P, Kastury K, McMahon M. Analysis of genomic DNA alterations and mRNA expression patterns in a panel of human pancreatic cancer cell lines. Genes Chromosomes Cancer 2005;44:37–51.
11. Immervoll H, Hoem D, Kugarajh K, Steine SJ, Molven A. Molecular analysis of the EGFR-RAS-RAF pathway in pancreatic ductal adenocarcinomas: lack of mutations in the BRAF and EGFR genes. Virchows Arch 2006;448:788–96.
12. Rejiba S, Wack S, Aprahamian M, Hajri A. K-ras oncogene silencing strategy reduces tumor growth and enhances gemcitabine chemotherapy efficacy for pancreatic cancer treatment. Cancer Sci 2007;98:1128–36.
13. Smyth GK. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 2004;3:Article3.
14. Ho Sui SJ, Fulton DL, Arenillas DJ, Kwon AT, Wasserman WW. oPOSSUM: integrated tools for analysis of regulatory motif overrepresentation. Nucleic Acids Res 2007;35:W245–W52.
15. Seeliger H, Camaj P, Ischenko I, Kleespies A, De Toni EN, Thieme SE, et al. EFEMP1 expression promotes *in vivo* tumor growth in human pancreatic adenocarcinoma. Mol Cancer Res 2009;7:189–98.

Downloaded from mcr.aacrjournals.org on October 24, 2021. © 2014 American Association for Cancer Research.

Published OnlineFirst December 3, 2013; DOI: 10.1158/1541-7786.MCR-13-0351

16. Imtiyaz HZ, Williams EP, Hickey MM, Patel SA, Durham AC, Yuan LJ, et al. Hypoxia-inducible factor 2α regulates macrophage function in mouse models of acute and tumor inflammation. J Clin Invest 2010;120:2699–714.
17. Mazzatti D, Lim FL, O'Hara A, Wood IS, Trayhurn P. A microarray analysis of the hypoxia-induced modulation of gene expression in human adipocytes. Arch Physiol Biochem 2012;118:112–20.
18. Kelder T, van Iersel MP, Hanspers K, Kutmon M, Conklin BR, Evelo CT, et al. WikiPathways: building research communities on biological pathways. Nucleic Acids Res 2012;40:D1301–7.
19. Ito T, Nakayama T, Ito M, Naito S, Kanematsu T, Sekine I. Expression of the ets-1 proto-oncogene in human pancreatic carcinoma. Mod Pathol 1998;11:209–15.
20. Shah SA, Potter MW, Hedeshian MH, Kim RD, Chari RS, Callery MP. PI-3′ kinase and NF-κB cross-signaling in human pancreatic cancer cells. J Gastrointest Surg. 2001;5:603–12.
21. Yao Z, Okabayashi Y, Yutsudo Y, Kitamura T, Ogawa W, Kasuga M. Role of Akt in growth and survival of PANC-1 pancreatic cancer cells. Pancreas 2002;24:42–6.
22. Michieli P. Hypoxia, angiogenesis and cancer therapy: to breathe or not to breathe? Cell Cycle 2009;8:3291–6.
23. Tang RF, Wang SX, Peng L, Zhang M, Li ZF, Zhang ZM, et al. Expression of vascular endothelial growth factors A and C in human pancreatic cancer. World J Gastroenterol 2006;12:280–6.
24. Brahimi-Horn C, Pouyssegur J. The role of the hypoxia-inducible factor in tumor metabolism growth and invasion. Bull Cancer 2006;93:E73–80.
25. Buchler P, Reber HA, Buchler M, Shrinkante S, Buchler MW, Friess H, et al. Hypoxia-inducible factor 1 regulates vascular endothelial growth factor expression in human pancreatic cancer. Pancreas 2003;26:56–64.
26. Galmiche L, Sarnacki S, Verkarre V, Boizet B, Duvillie B, Fabre M, et al. Transcription factors involved in pancreas development are expressed in paediatric solid pseudopapillary tumours. Histopathology 2008;53:318–24.
27. Cary LA, Chang JF, Guan JL. Stimulation of cell migration by overexpression of focal adhesion kinase and its association with Src and Fyn. J Cell Sci 1996;109:1787–94.
28. Napoli JL. Physiological insights into all-trans-retinoic acid biosynthesis. Biochim Biophys Acta 2012;1821:152–67.
29. Monzon FA, Lyons-Weiler M, Buturovic LJ, Rigl CT, Henner WD, Sciulli C, et al. Multicenter validation of a 1,550-gene expression profile for identification of tumor tissue of origin. J. Clin. Oncol. 2009;27:2503–8.
30. Yu M, Ting DT, Stott SL, Wittner BS, Ozsolak F, Paul S, et al. RNA sequencing of pancreatic circulating tumour cells implicates WNT signalling in metastasis. Nature 2012;487:510–3.
31. Panza A, Pazienza V, Ripoli M, Benegiamo G, Gentile A, Valvano MR, et al. Interplay between SOX9, β-catenin and PPARγ activation in colorectal cancer. Biochim. Biophys. Acta 2013;1833:1853–65.

Downloaded from mcr.aacrjournals.org on October 24, 2021. © 2014 American Association for Cancer Research.

Published OnlineFirst December 3, 2013; DOI: 10.1158/1541-7786.MCR-13-0351

AACR American Association for Cancer Research

Molecular Cancer Research

Hypoxia-Independent Gene Expression Mediated by SOX9 Promotes Aggressive Pancreatic Tumor Biology

Peter Camaj, Carsten Jäckel, Stefan Krebs, et al.

Mol Cancer Res 2014;12:421-432. Published OnlineFirst December 3, 2013.

Updated version	Access the most recent version of this article at: doi:10.1158/1541-7786.MCR-13-0351
Supplementary Material	Access the most recent supplemental material at: http://mcr.aacrjournals.org/content/suppl/2013/12/03/1541-7786.MCR-13-0351.DC1

Cited articles	This article cites 30 articles, 6 of which you can access for free at: http://mcr.aacrjournals.org/content/12/3/421.full#ref-list-1
Citing articles	This article has been cited by 3 HighWire-hosted articles. Access the articles at: http://mcr.aacrjournals.org/content/12/3/421.full#related-urls

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, use this link http://mcr.aacrjournals.org/content/12/3/421. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.

Downloaded from mcr.aacrjournals.org on October 24, 2021. © 2014 American Association for Cancer Research.