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Oncogenicity of the developmental transcription factor Sox9

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Abstract

SOX9, a high mobility group (HMG) box transcription factor, plays critical roles during embryogenesis and its activity is required for development, differentiation and lineage commitment in various tissues including the intestinal epithelium. Here, we present functional and clinical data of a broadly important role for SOX9 in tumorigenesis. SOX9 was overexpressed in a wide range of human cancers, where its expression correlated with malignant character and progression. Gain of *SOX9* copy number is detected in some primary colorectal cancers. SOX9 exhibited several pro-oncogenic properties, including the ability to promote proliferation, inhibit senescence and collaborate with other oncogenes in neoplastic transformation. In primary MEFs and colorectal cancer cells, SOX9 expression facilitated tumor growth and progression whilst its inactivation reduced tumorigenicity. Mechanistically, we have found that Sox9 directly binds and activates the promoter of the polycomb protein Bmi1, whose upregulation represses the tumor suppressor Ink4a/Arf locus. In agreement with this, human colorectal cancers showed a positive

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correlation between expression levels of SOX9 and BMI1 and a negative correlation between SOX9 and ARF in clinical samples. Taken together, our findings provide direct mechanistic evidence of the involvement of SOX9 in neoplastic pathobiology, particularly in colorectal cancer.

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INTRODUCTION

SOX9 (sex-determining region Y (SRY)-box 9 protein) is a member of the SOX family of transcription factors. These are developmental regulators that possess HMG box DNA-binding domains as well as other conserved features, such as transactivation domains (1). However, their action on target genes is context dependent, relying on other transcription factors with which they may directly interact for specificity (1). SOX proteins coordinate disparate functions, such as maintaining stem cell properties, lineage restriction and terminal differentiation, through precise temporal and spatial expression patterns that differ between particular cell types and tissues (2). In particular, SOX9 plays a pivotal role in a number of developmental processes and its levels need to be strictly controlled for normal embryogenesis. In humans, *SOX9* heterozygous mutations result in Campomelic Dysplasia (CD), a syndrome characterized by severe skeletal malformations, defects in the CNS and several other organs, frequent XY female sex reversal, and perinatal lethality (3). In mice, *Sox9* homozygous null mutant embryos die around embryonic day 12 (E12) due to heart defects, while heterozygotes die around birth with phenotypes similar to human CD patients, although without sex reversal (4). In addition, duplications of the *Sox9* gene, or its deliberate misexpression have been linked with XX male sex reversal and fibrosis-related disorders (5, 6), and demonstrate that dysregulation of the gene can cause disease. Further analyses have shown that SOX9 is crucial for Sertoli cell differentiation, chondrogenesis, neural crest development and differentiation of some of its derivatives, as well as, for the development of specific cell types and lineages within the CNS, pancreas, prostate intestine, skin, pituitary, heart, kidney and sensory systems (7).

Colorectal cancer is the third leading cause of cancer-related deaths world-wide and its incidence is steadily increasing (8). Colorectal carcinomas derive from the intestinal epithelium, which is constantly self-renewing and requires a high rate of cell division to maintain epithelial homeostasis (9). It is becoming increasingly clear that mutations in developmentally regulated genes can cause the initiation and progression of these cancers. Particularly, genes constituting the Wnt signaling pathway are crucial for the maintenance of undifferentiated progenitors in the crypts and for the maintenance of the postmitotic Paneth cells. Wnt activating mutations are often found in colorectal cancers, frequently targeting the tumor suppressors APC or Axin2 or the oncogene β-catenin (10). Members of other important developmental programs such as Notch and BMP are also deregulated in colorectal cancers (11).

Despite the importance of SOX9 in multiple developmental programs, its role in cancer remains unclear. On the one hand, SOX9 is often overexpressed in cancers of the skin, prostate, lung and brain (12-15). On the other hand, SOX9 may behave as a tumor suppressor, at least in some melanomas (16). This ambiguity also concerns colorectal cancers where SOX9 has been found overexpressed (17) but at the same time it has been reported to reduce the tumorigenicity of HT-29Cl.16E colorectal cancer cells (18). A number of observations suggest that Sox9 could be a critical player in the homeostasis of colorectal epithelium. In particular, Sox9 is localized in proliferating crypt cells and promotes stem/progenitor cell proliferation and it is required for Paneth cell differentiation in the intestinal epithelium during development and in adult stages (19-21). Also, Sox9 is a downstream effector and a regulator of the Wnt pathway in the regulation of intestinal epithelium homeostasis (22). Beyond the intestinal epithelium, Sox9 is also a target of BMP,

Notch or Hedgehog in different processes such as chondrogenesis or regulation of stem cells from several tissues (7). SOX9 is activated in bladder injury repair and constitutively upregulated in bladder cancer (23). Given that activated stem cells are required for injury repair, and that chronic injury increases the risk of forming cancer, SOX9 might contribute to carcinogenesis through effects on stem cells. Furthermore, Sox9 promotes survival and provides competence for epithelial-to-mesenchymal transition (EMT) in the neural crest (24), and contributes to cancer cell invasion and migration in urothelial carcinomas (23). In this work, we directly test the role of SOX9 in cancer and reveal new cellular and molecular mechanisms that reinforce and explain its involvement in tumor biology, and particularly in colorectal cancer.

Materials and Methods

Mice and primary mouse cells and cell lines

Mice were housed in the pathogen-free barrier area of the MRC National Institute for Medical Research in London and of the Spanish National Cancer Research Center (CNIO), Madrid. The pCAGGS-ERT2iCre line was generated as follows; the chicken beta-actin enhancer/CMV promoter fragment of the plasmid pCAGGS was released with SalI/XhoI digestion and inserted into the SalI site upstream of the PBS.ERT2iCrepA plasmid, generating the expression plasmid CAGGSET. The plasmid was sequenced using an on-site sequencing service. The transgene fragment was released with SalI/NotI digestion, and was purified for pronuclear injection into fertilized eggs of B10/CBAF1 mice. *Sox9*$^{Flox/flox}$ and *Z/Sox9* mice were maintained on a mixed C57BL6/129 genetic background. Isolation, culture, and assays with mouse embryo fibroblasts (MEFs) were carried out as previously described (25). For detailed methods see Supplementary Data.

Human cancer cell lines were obtained from the American Type Culture Collection and cultured under recommended conditions in DMEM medium supplemented with 10% fetal bovine serum at 37°C and 5% CO_2. The cell lines were tested for mycoplasma contamination and the levels of expression of oncogenes and tumor suppressors for the various lines correlated with the ATCC profiles of these cells. For *SOX9* or *Bmi1* knockdown by shRNA, cells were transfected using Lipofectamine (invitrogen), according to instructions of the reagent supplier. Cells were cultivated in the presence of puromycin for 3 weeks. Cells were transfected using two different *Sox9* shRNAs (Origene, *sh1* or *sh75* and *sh2* or *sh73*) and shRNA especific for BMI1. A non-specific shRNA (*pRS*) was used as a control.

RNA and protein analysis

Total RNA was extracted with Trizol (Life Technologies). Reverse transcription was performed using random priming and Superscript Reverse Transcriptase (Life Technologies), according to the manufacturer's guidelines. Quantitative real-time PCR was performed using Absolute SYBR Green mix (Thermo Scientific) in an ABI PRISM 7500 thermocycler (Applied Biosystems). Variations in input RNA were corrected by subtracting the number of PCR cycles obtained for ß-actin. For information on primers see Supplementary Data.

Immunoblots were performed following standard procedures. Typically, equal amounts of protein (30 μg) were separated on 15% SDS polyacrylamide gels and blotted onto nitrocellulose membranes (BioRad). For detection of human and mouse SOX9 we used AB5535 (Chemicom) and sc-20095 (Santa Cruz); for detection of Ink4a (p16^{Ink4a}), we used M156 antibody (Santa Cruz); for detection of Arf (p19^{Arf}), we used R562 antibody (Abcam); for p21, we used sc-397 (Santa Cruz); for Bmi1, we used Clone F6 antibody

(Millipore) and for β-actin, we used mouse monoclonal antibody AC-15 (Sigma). For secondary antibodies, we used HRP-linked anti-rabbit or HRP-linked anti-mouse (DAKO), both at a 1:2000 dilution. Detection was performed by chemiluminescence using ECL (Amersham).

For immunofluorescence, cells were fixed with 4% paraformaldehyde for 15 min, and washed with PBS supplemented with 0.2% Triton X-100 and 1% FCS, for 5 min at 4°C. Subsequent to blocking for 1h with PBS and 1% FCS, cells were incubated with P-H3 (Chemicom) antibody for 2h. Secondary antibodies were from Jackson. Nuclear DNA was stained with a 4% PBS buffered paraformaldehyde solution containing 10 μg/ml 4′6-diamidino-2-phenylindole (DAPI, Sigma).

Chromatin immunoprecipitation (ChIP) assays—ChIP assays were performed according to standard procedures (see supplementary information). DNA fragments were immunoprecipitated with antibodies against Sox9 (Chemicon), H3K4me3, H3K9me3 and H3K27me3 (Millipore). The immunoprecipitated DNA was extracted and subjected to PCR amplification with primers directed against the proximal promoter of *Bmi1* or *Sox9* respectively.

Histopathology and immunohistochemistry

Murine tissues were dissected, fixed in 10%-buffered formalin (Sigma) and embedded in paraffin. Three-micrometer-thick sections were stained with hematoxylin and eosin (H&E). Antibodies used for immunohistochemistry analysis included rabbit polyclonal anti-Sox9 (1:200 dilution, Millipore); A Dako automated system was used for the immunostaining after antigen retrieval in a PTLink (Dako).

Tumor samples and array CGH datasets

Human Tissue Microarrays (TMAs) were obtained from the CNIO Tumor Bank Unit. Human colorectal carcinoma samples and data from patients were provided by the Basque Biobank for Research-OEHUN (http://www.biobancovasco.org) and two independent transcriptome profiling datasets previously described (26, 27). The raw data are accessible through the NCBI's Gene Expression Omnibus public repository for microarray data (accession numbers GSE25071 and GSE24550). Samples were processed following standard operation procedures with appropriate ethical approval. The array-comparative genomic hybridization (CGH) data were recently generated (28). Here, the NimbleGen Human Whole-Genome Array CGH Analysis v1 (Roche Diagnostics, Mannheim, Germany), was applied, which provides measurements from 385 000 unique genomic loci.

In vivo carcinogenesis assays

For 3-methylcholanthrene (3MC)-induced carcinogenesis, 3-6 month old mice received a single intramuscular injection, in one rear leg, of 40 μl of 3MC (Sigma) dissolved in sesame oil (Sigma) at a concentration of 25 μg/μl. Mice were observed on a daily basis until tumors reached 12 mm. For nude mice, neoplastically transformed MEFs or human cells were harvested with trypsin/EDTA and resuspended in PBS. Cells (1×10^6) were injected subcutaneously into both flanks of nude mice (8 weeks old). Mice were observed on a daily basis for up to 40 days and external calipers used to measure tumor diameters at the indicated time points from which tumor volume was estimated.

Data evaluation

Data are presented as mean values ± S.E.M. with the number of experiments (n) in parenthesis. Unless otherwise indicated, statistical significance (p-values) was calculated

using the Student́s t -test. Asterisks (*, **, and ***) indicate statistical significance ($p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively).

RESULTS

SOX9 is overexpressed in a wide range of human cancers

We first analyzed SOX9 levels by immunohistochemistry on a multitumor tissue microarray (TMA) containing human cancers derived from a diverse range of tissues. Compared to normal control tissue, SOX9 staining showed more intense and widespread staining in all cancer types, with the exception of lymphomas. According to frequencies of SOX9 positive tumors, cancers can be broadly classified as highly positive (brain, pancreatic, colorectal, lung and prostate cancers), medium (sarcomas, thyroid and breast cancers), and low (kidney cancers) (Table Suppl. 1). The staining was generally observed in the nucleus with varying intensities among cells and similar results were obtained using two different primary antibodies against SOX9. To obtain more compelling evidence, we decided to analyze tumor TMA of specific tissues. More than 70% of lung, ovary, colon, prostate, pancreatic, neurofibroma, MPNST and medulloblastomas displayed moderate to robust expression of SOX9 (Table Suppl. 2, Fig. Suppl. 1). This may reflect the concept that cancer cells exhibit some features corresponding to the stem/progenitor cells of their tissues of origin and it is notable that SOX9 has been associated with the stem cell compartments of most of the corresponding normal tissues (see Introduction). Quantification of the number of cancers containing more than 25% of SOX9 positive cells using an automated scanning microscope and computerized image analysis system (Ariol SL-50; Genetix) generated essentially identical results (Table Suppl. 2, Fig. Suppl. 2). Together, these data demonstrate that SOX9 is frequently expressed in many types of human cancers, and particularly in those derived from tissues where SOX9 plays a role in their development and homeostasis.

SOX9 overexpression in colorectal cancer

To further characterize SOX9 expression in human cancer, we decided to focus our attention in colorectal cancers. Colorectal cancer arises through an ordered sequence of genetic and epigenetic events in the adenoma-carcinoma development. It typically begins with the formation of non-invasive benign adenomatous polyps, which ultimately leads to the development of invasive carcinomas with metastatic capacity (29). Our studies showed significantly higher levels of SOX9 in both adenomas and carcinomas when compared to normal colon tissue ($P = 0.0001$) (Fig. 1A,B, Suppl. Table 3). In particular, we observed SOX9 overexpression in 18 of 24 (75%) adenomas and 91 of 110 (83%) carcinomas. The frequency of SOX9 overexpression was equivalent at protein and mRNA levels reflecting increased transcription. Analysis of *SOX9* mRNA levels in colorectal adenoma and carcinoma samples revealed that *SOX9* was upregulated by an average 2 and 3.5 fold in colon adenomas and carcinomas respectively compared to non-tumor colonic mucosa (Fig 1C). Furthermore, when we analyzed *SOX9* levels in relation to tumor stage, we found that higher levels of *SOX9* mRNA were associated with advanced tumor stage (Fig. 1D). High levels of *SOX9* were found both in carcinomas of the colon and rectum (Fig. Suppl 3). From high resolution array-CGH data we found that some carcinoma samples (7%) had low copy number gain at the distal part of chromosome 17, including the *SOX9* gene (Fig. 1E, Fig. Suppl 4). The gain is observed at low level and in few samples implying that copy number change is a plausible but not exclusive mechanism, for the increase of SOX9 expression in colorectal carcinoma. Consistent with the tissue data, SOX9 was generally highly expressed in human colorectal cancer cell lines, with levels ranging from 1.5 to 30 times higher than in normal colonic mucosa (Fig. 1F,G, Fig. Suppl. 3). In particular, SOX9 expression was remarkably higher in SW620 than in SW480 (Fig. 1F,G), two cell lines established from lymph node metastasis and its primary colon carcinoma respectively from the same patient,

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further supporting the role of SOX9 in tumor progression and malignancy in colorectal cancers.

Finally, we examined the expression of Sox9 in colorectal cancers in mice. Immunohistochemistry revealed strong expression of Sox9 in colitis-associated colon tumors obtained from mice treated with azoxymethane/dextran (AOM/DSS) (Fig. 1H). In summary, these findings confirm that SOX9 is expressed at a high level in human and murine colorectal cancers and cancer-derived cell lines and that SOX9 expression correlates with tumor progression in this type of cancer. Our findings support the use of SOX9 as a potential tumor and prognostic marker in colorectal cancer.

SOX9 promotes proliferation

Enhanced proliferation, immortalization, and neoplastic transformation are important stages in the conversion of a cell with normal growth into one that is malignant. Thus, regulators of these cellular phenomena play a major role in tumorigenesis. To test whether SOX9 may be involved in these processes, we first analyzed the biological consequences of altering Sox9 expression levels in primary mouse embryonic fibroblasts (MEFs) derived from two lines of transgenic mice that provide gain and loss of Sox9 function respectively. In particular, we used mice carrying a conditional *Sox9-IRES-EGFP* transgene (*Z/Sox9*) in which Cre recombinase-mediated excision of a stop cassette results in the stable expression of Sox9 and EGFP (30). MEFs were established from embryos hemizygous for a β-*actin Cre* transgene alone (referred to here as *wt*) or hemizygous for both *Z/Sox9* and β-*actin Cre* (referred to as *Z/Sox9tg*) and we confirmed elevated levels of Sox9 protein and mRNA (Fig. 2A,B). Of note, the level of overexpression of Sox9 in the transgenic MEFs (30-fold higher) is within the range of overexpression observed in human colorectal tumor cell lines (Fig 1F), suggesting that the levels of overexpression achieved with the transgene are of biological relevance. To study the effect of *Sox9* gene inactivation, we established MEFs from embryos homozygous for a conditional null allele of *Sox9* (*Sox9*$^{flox/flox}$) that also carried a broadly expressed *Cre-ERT2* transgene (*pCAGGS-ERT2iCre*) (31). Cre-mediated excision of *Sox9* after treatment with 4-hydroxy tamoxifen (4-OHT) significantly diminished the basal levels of Sox9 present in normal MEFs (Fig. 2C,D). However, Sox9 expression was not completely lost reflecting inefficiency in the Cre-mediated deletion. Expression of other *Sox* gene family members (*Sox2, Sox8* and *Sox10*) was not affected by gain or loss of Sox9 indicating no cross talk or compensatory changes (Fig. Suppl. 5). Having established these cellular models of gain and loss of *Sox9*, we analyzed their proliferation. *Z/Sox9tg* cells exhibited a higher rate of proliferation compared to *wt* cells (Fig. 2E), whereas *Sox9*$^{\Delta/\Delta}$ MEFs grew slower than their corresponding controls (Fig. 2F). Next, we examined whether Sox9 regulates cell cycle progression by FACS analysis. The numbers of *Z/Sox9tg* cells in S-phase were higher and those in G1 lower when compared to *wt* cells (Fig. 2G). Conversely, the number of *Sox9*$^{\Delta/\Delta}$ MEFs in S-phase was reduced compared to controls (Fig. 2G). Together, our results support the idea that Sox9 regulates proliferation in primary cells controlling the progression of the cell cycle from G1 to S phase.

Having established that SOX9 promotes proliferation in MEFs, we moved to a more relevant cellular setting by analyzing human colorectal cancer cells. We focused on HCT116 and SW620 because they express very high levels of SOX9 (Fig. 1 G). Sublines were derived after stable introduction of two independent *SOX9* shRNAs. We obtained similar results but we present the protein and mRNA data obtained with the most efficient shRNA (*sh1)* (Fig. 2H,I). Downregulation of SOX9 levels resulted in a reduction in proliferation as measured by cell growth curves and phospho-histone H3 (P-H3) immunostaining (Fig. 2J,K). No significant differences were observed in apoptosis in HCT116 cells (data not shown). These observations suggest that SOX9 contributes to the proliferation of human colorectal cancer cells.

Sox9 bypasses senescence and immortalizes primary cells

Next, we analyzed the effect of Sox9 in cell senescence. This is a fundamental mechanism triggered in primary cells after a finite number of cell divisions or in response to oncogenic stress that limits cellular lifespan and constitutes a barrier against cellular immortalization *in vitro* and *in vivo* (32). In contrast, cancer cells are essentially immortal if cultured under optimal conditions. *Z/Sox9tg* and *wt* MEFs were cultured using a 3T3 serial passage protocol designed to study senescence. Strikingly, *Z/Sox9tg* MEFs bypassed senescence and continued proliferating, whereas control MEFs entered senescence at around passage 3-4 (Fig. 3A). Consistent with this result, *wt* cells seemed enlarged and with flat morphology, typical of senescent cells, meanwhile *Z/Sox9tg* exhibited normal fibroblast morphology (Fig. 3B). Reduced proliferation and subsequent senescence of primary cells is partly due to the progressive accumulation of Ink4A and Arf tumor suppressors ($p16^{Ink4a}$ and $p14^{ARF}$/$p19^{Arf}$; p14 when referred to the human protein and p19 when referred to the murine protein) encoded in the *Cdkn2a* locus (33). This prompted us to ask whether Sox9 may contribute to proliferation and senescence modulating Ink4a and Arf expression. Importantly, early-passage *Z/Sox9tg* MEFs displayed decreased basal levels of Ink4a and Arf, while the opposite was true for early-passage $Sox9^{\Delta/\Delta}$ MEFs (Fig. 3C, Fig. Suppl. 6). It is well established that Arf stabilizes p53, another major regulator of senescence (33). In accordance with Arf expression, *Z/Sox9tg* MEFs contained lower p53 basal expression (Fig. 3C). In addition, the expression of Ink4b and DcR2, established markers of senescence (34), was inversely correlated with Sox9 levels (Fig. Suppl 6).

Next, we evaluated the consequences of altering *Sox9* in response to oncogene-induced senescence. High levels of oncogenic *H-RasV12* result in the activation of a protective response that limits proliferation and enhances resistance to neoplastic transformation in primary MEFs (35). In contrast to *wt* MEFs, *Sox9* overexpressing cells were able to proliferate and generate colonies even in the presence of *H-RasV12* (Fig. 3D). The *Ink4a/Arf* locus is one of the most important defensive mechanisms against oncogenic stress. In particular, *H-RasV12* upregulates both Ink4a and Arf (35). We hypothesized that Sox9 could modulate their expression in response to this oncogene. The levels of Ink4a and Arf were lower in *Z/Sox9tg* cells in the presence of *H-RasV12* compared to *wt* cells, although *H-RasV12* was still capable of upregulating Ink4a and Arf in *Z/Sox9tg* cells (Fig. 3E, Fig. Suppl 6). Conversely, $Sox9^{\Delta/\Delta}$ MEFs showed higher Ink4a and Arf levels in response to *H-RasV12* (Fig. 3E, Fig. Suppl 4). Moreover, the expression of other established markers of senescence such as p53, Ink4b or DcR2 inversely correlated with Sox9 in cells transduced with *H-RasV12* (Fig. 3E, Fig. Suppl. 6). In order to study the contribution of the members of the *Ink4a/Arf* locus underlying the proliferation and senescence phenotypes seen in cells with altered levels of Sox9 expression, we compared *Ink4a/Arf* null MEFs that had been retrovirally transduced with a plasmid expressing *Sox9* or the empty vector. We found no difference in their rates of proliferation (Fig. Suppl. 7), revealing that the role of Sox9 in proliferation and senescence is dependent at least in part, on Ink4a and Arf. In summary, our results suggest that Sox9 is a novel regulator of senescence *in vitro,* and its activation is sufficient to escape both serial passage and oncogene-induced senescence, partly through modulating the expression of Ink4a and Arf tumor suppressors.

It is well known that senescence occurs *in vivo* as a barrier against tumor progression. Indeed, tumor senescent cells are prevalent in a variety of pre-malignant tumors while progression to malignancy requires evading senescence. This paradigm has been well characterized in the *K-RasG12V* conditional mouse model, where low-grade pre-malignant lung adenomas are strongly positive for senescence markers and high-grade invasive malignant adenocarcinomas are essentially negative (34). In this context, immunohistochemical analysis of Sox9 expression revealed remarkable high levels of expression exclusively in lung adenocarcinomas, while normal tissue and pre-malignant

adenomas were for the most part SOX9 negative (Fig. 3F). Moreover, the most intensely Sox9 positive cells were observed in morphologically more malignant and aggressive areas of the adenocarcinomas, particularly at their growth edge and in endobronchial invasions. This observation suggests the existence of an inverse correlation between the expression of Sox9 and senescence markers *in vivo* and further indicates that Sox9 expression is associated with tumor progression and malignancy.

Sox9 cooperates with Ras oncogene in neoplastic transformation

Extensive cultivation of primary *wt* MEFs usually favors the amplification of rare pre-existing mutant cells that overgrow senescent cells. . Loss of the Cdkn2a locus or loss of p53 are the most characteristic events responsible for the immortalizaiton of MEFs. In agreement with the role of Sox9 as a negative regulator of Cdkn2a, 3 analyzed immortal Sox9tg MEFs retained functional p53 (data not shown). Established cultures of such immortal (passage 15) cells were found to express higher levels of Sox9 compared to those at early passage (passage 1) (Fig. 4A,C). This prompted us to analyze Sox9 expression in neoplastically transformed cells. For this, we retrovirally transduced primary *wt* and *Z/Sox9tg* MEFs with a combination of *E1a* and *H-Ras V12* oncogenes (*E1a/Ras).* Transformed cells contained increased Sox9 expression compared to non-transformed, with the highest levels in *Z/Sox9tg* cells (Fig. 4B,C). This is likely to reflect increased transcription and not protein stability, as we found that, compared to normal control MEFs, *Sox9* mRNA levels were augmented by around 10 and 15 fold in immortal and transformed *wt* cells and between 40 and 70 times respectively in *Z/Sox9tg* cells (Fig. 4D,E). In contrast, SOX9 expression was significantly decreased in $Sox9^{\Delta/\Delta}$ transformed cells (Fig. 4F). These data demonstrate that endogenous levels of Sox9 increase with progressive stages of tumorigenesis, such as spontaneous immortalization and neoplastic transformation, which emphasizes the likely physiological significance of Sox9 in these processes. Moreover, the levels of Sox9 in *Z/Sox9tg* cells at early passage were comparable to endogenous Sox9 levels at immortal and neoplastic stages in *wt* cells reinforcing the concept that the degree of overexpression achieved with the transgene is of biological relevance.

Histone modifications play an important role in the regulation of gene expression. Methylation of histone 3 lysine 4 (H3-K4me) is associated with gene activation while trimethylation of H3-K9 and H3-K27 (H3K9me3 and H3K27me3) are linked with gene silencing (36). To examine whether Sox9 overexpression is associated with histone modifications on the promoter region, we performed ChIP analysis against those histone marks in *E1a/Ras* transformed cells. We observed an enrichment of the gene activation mark histone H3 lysine 4 trimethylation (H3K4me3), whereas the repressive marks H3K9me3 and H3K27me3 completely disappeared from the *Sox9* promoter in transformed MEFs (Fig. 4G). These results show that Sox9 upregulation in neoplastic cells correlates with chromatin changes, thus indicating another mechanism for its upregulation.

To further substantiate the oncogenic properties of Sox9, we explored the behavior of cells by altering *Sox9* in neoplastic transformation. To test whether Sox9 collaborated with oncogenes to trigger cell transformation, we used *wt* and *Z/Sox9tg* MEFs transduced with *E1a/Ras.* Importantly, *Z/Sox9tg* MEFs gave rise to 5 times more foci than *wt* cells (Fig. 4H). In contrast, $Sox9^{\Delta/\Delta}$ MEFs formed 3 times fewer foci in response to *E1a/Ras* (Fig. 4I). Since the ability of cells to grow independently of adhesion is a feature of cancer cells, we analyzed whether the growth of *E1a/Ras* transformed cells was anchorage-independent by plating cells in soft agar. Similar results were obtained with *Z/Sox9tg* generating more colonies (Fig. 4J). Together, our findings indicate that *Sox9* deregulation promotes proliferation, inhibits senescence and collaborates in malignant transformation in primary MEFs. Finally, to investigate the oncogenic activity of SOX9 in human colorectal cancer cells, we addressed its ability to promote anchorage-independent cellular growth using the

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same soft agar assay. We first increased SOX9 expression by transiently transfecting a plasmid carrying *Sox9-IRES-EGFP* in SW480 cells. Cells overexpressing SOX9 formed higher number of colonies than cells carrying the empty vector (Fig. 4K). In contrast, the growth of SW620 and HCT116 in soft agar was inhibited by 50% with SOX9 knockdown (Fig. 4L). In summary, our observations demonstrate that SOX9 modulates phenotypes associated with cancer in human colorectal cancer cells.

SOX9 promotes tumorigenesis *in vivo*

Next, we explored the hypothesis that Sox9 is relevant to tumor formation and growth *in vivo*. First, we studied whether the increased sensitivity to neoplastic transformation observed in *Z/Sox9tg* cells *in vitro* translates into an increased tumorigenicity *in vivo*. Consistent with this idea, pools of *E1a/Ras*-transformed *Z/Sox9tg* cells gave rise to tumors at notably higher rates than *E1a/Ras* transformed *wt* cells when transplanted in nude mice (Fig. 5A, Fig. Suppl. 8). To further substantiate whether Sox9 contributes to tumor growth, we repeated the same experiment with pools of $Sox9^{flox/flox}$ and $Sox9^{\Delta/\Delta}$ MEFs. Conversely, fewer than 20% of mice injected with *E1a/Ras* transformed $Sox9^{\Delta/\Delta}$ cells generated tumors at day 10 compared to 45% with control cells, and the average tumor volume was also markedly reduced (Fig. 5B, Fig. Suppl. 8). These results further support the idea that Sox9 possesses oncogenic properties and facilitates tumorigenesis *in vivo*. To provide further evidence for the contribution of Sox9 to tumorigenesis *in vivo*, we took advantage of mice carrying both *Z/Sox9* and *pCAGGS-ERT2iCre* transgenes (*Z/Sox9c*). Groups of $Z/Sox9c^{+}$ and $Z/Sox9c^{-}$ mice (that is, receiving 4OHT to activate the transgene or ethanol as a control, respectively) were injected with the carcinogen 3-methylcholanthrene (3MC) to evaluate their susceptibility to fibrosarcoma, a cancer type which reveals strong staining of Sox9 (Fig. 5C). In this context, $Z/Sox9c^{+}$ mice were notably more susceptible to cancer formation than their $Z/Sox9c^{-}$ littermates (Fig. 5D). In particular, 6 months after 3MC treatment 100% of $Z/Sox9c^{+}$ mice developed fibrosarcoma compared to only 55% of $Z/Sox9c^{-}$ control mice. Moreover, the median survival of $Z/Sox9c^{+}$ (4.5 months) was significantly decreased compared to control mice (5.9 months) (logrank test p= 0.03) (Fig. 5D). Finally, to evaluate whether the pro-tumorigenic role of SOX9 could be extended to human cells, we performed similar analysis using human colorectal cancer cell lines. We used HCT116 and SW620, in which we have previously shown (see above) that knockdown of SOX9 impairs cell proliferation. These results were paralleled by an attenuation of tumorigenicity (Fig. 5E,F, Fig. Suppl. 8). In summary, our findings support the notion that Sox9 facilitates tumor formation and progression *in vivo* and indicate a functional involvement of SOX9 in the tumor establishment and/or development of colorectal human cancer.

Sox9 directly regulates *Bmi1* expression

With respect to the mechanisms underlying the phenotypes seen in cells with altered levels of Sox9 expression, we have seen that *Sox9* activity modulates the expression of Ink4a and Arf tumor suppressors (see Fig. 3). The Polycomb-group protein Bmi1, a well-established oncogene, plays an important role in proliferation, senescence and carcinogenesis through its ability to repress the transcription of the *Ink4a/Arf* locus (37). We hypothesized that *Sox9* might regulate *Ink4a/Arf* expression via Bmi1. We therefore analyzed Bmi1 expression in Sox9 gain and loss of function MEFs finding that Sox9 overexpression in *Z/Sox9tg* MEFs resulted in increased Bmi1 compared to controls while $Sox9^{\Delta/\Delta}$ MEFs had lower levels (Fig. 6A). Sox9 is known to function as a transcriptional activator in several contexts, and consistent with this, the analysis of *Bmi1* mRNA in early-passage MEFs indicated that Sox9 modifies its levels in a manner that parallels the changes in protein (Fig. 6B). These results suggest that Sox9 exerts its effect on proliferation and senescence, at least in part, through regulation of Bmi1-Ink4a/Arf expression.

With respect to transformed cells, Bmi1 paralleled Sox9 upregulation in response to *E1a/Ras* induced transformation, with *Z/Sox9tg* MEFs presenting the highest levels of Bmi1 (Fig. 6C,D) and $Sox9^{\Delta/\Delta}$ MEFs the lowest (Fig. 6E,F). Next, we tested the possibility that Sox9 modulates Bmi1 expression by direct transcriptional control. To test this, we performed chromatin immunoprecipitation (ChIP) assays that revealed readily detectable Sox9 bound to the *Bmi1* promoter in *Z/Sox9tg* but not in *wild-type* control MEFs (Fig. 6G). In addition, ChIP assays also demonstrated that Sox9 interacts with the *Bmi1* promoter in transformed cells (Fig. 6G). Our data show that *Bmi1* is a novel direct downstream target of Sox9 in primary and transformed MEFs and may therefore represent a key mechanism mediating the role of Sox9 in tumorigenesis.

It has been previously reported that high levels of BMI1 are linked with progression and poor prognosis in colorectal carcinoma (38, 39). The results in MEFs prompted us to address whether SOX9 may contribute to colorectal carcinogenesis through *BMI.* First, we found that BMI1 expression mirrors SOX9 expression in human colorectal carcinomas. Thus, the same tumor regions that strongly expressed SOX9 also contain high levels of BMI1 (Fig 7A). Analysis of mRNA levels revealed that *SOX9* upregulation correlated with increased *BMI1* levels in colon carcinomas compared to non-tumor colonic mucosa (Fig 7B). Furthermore, the correlation analysis showed a significant association between *SOX9* and *BMI1* expression in colorectal carcinomas (Fig 7C, Fig. Suppl. 9). In addition, *SOX9* mRNA levels correlated negatively with *ARF* (Fig. Suppl. 9). Next, we extended this association to human colorectal cancer cell lines. BMI1 was strongly expressed in SW620, with high levels of SOX9, and it was weak in SW480, with low levels of SOX9 (Fig. 7D). Conversely, INK4a expression is very high in SW480 and low in SW620. These results show that regulatory interactions between SOX9, BMI1 and INK4a/ARF are likely to be critical during the initiation and progression of colorectal cancers.

Next, we checked whether SOX9 regulates BMI1 and INK4a expression in colorectal cancer cells. Indeed, *Sox9* ectopic overexpression increased BMI1 levels accompanied by decrease in INK4a while *SOX9* knockdown diminished BMI1 expression and increased INK4a (Fig. 7E). Paralleling the results observed in MEFs, *SOX9* regulated *BMI1* expression at transcriptional level in colorectal cancer cells (Fig. 7F,G). In order to test the role of BMI1 and INK4a in SOX9 regulation of cancer cells, we stably inactivated *BMI1* and overexpressed *INK4a* in SW620 cells, which possess high levels of SOX9. The shRNA-mediated inhibition of BMI1 and the restoration of INK4a significantly impaired proliferation and the number of colonies in soft agar (Fig. 7H,I), while SOX9 levels were unchanged (Fig. 7J,K). Together, these data provide direct experimental support for the role of SOX9 in cancer by regulating the expression of BMI1 and INK4a/ARF.

Discussion

SOX9 is a critical gene for embryonic development and tissue homeostasis in several organs, with a role in cancer not totally understood yet. Our results show that its role in human cancer is context dependent. We found SOX9 overexpressed in a wide range of human cancers, particularly in tissues where it plays critical roles in their development and in stem/progenitor cells such as colon, ovary, pancreas, prostate, lung, peripheral nerves and brain (Table Suppl. 1,2). Nevertheless, our results extend these findings to fibrosarcomas, breast, and thyroid cancers (Table Suppl. 1), where SOX9 is not known to be involved during normal physiology. On the other hand, SOX9 seems to be unaffected in lymphoma and kidney cancers and it has been reported to behave as a tumor suppressor in some melanoma cases (16), although its expression is upregulated in others (41-43). In addition to ourselves, others have also observed SOX9 overexpression in cancers from tissues such as prostate, lung, skin carcinomas and brain (12-15) indicating that de novo or increased

expression of SOX9 in cancer is likely to be positively selected rather than a bystander event.

In line with this idea, our results show that SOX9 is overexpressed in colorectal cancers and exhibits important functions in the regulation of colorectal cancer cells. The high levels might reflect the origin of the tumors from the expansion of SOX9-positive stem/progenitor cells. The increased SOX9 expression detected in early pre-malignant adenomas together with the role for Sox9 in stem/progenitor cells in the intestinal epithelium (21) and the evidence that transformation of such cells is the initiating step in colorectal tumorigenesis (44) support this hypothesis. Alternatively, the high levels of SOX9 could reflect genetic or epigenetic alterations and/or other types of deregulation. We identified altered SOX9 chromosomal copy number in colorectal cancers. These might not be exceptional cases. Amplification of 17q24.3, the chromosomal region containing *SOX9*, has been found in cancers such as prostate, MNPSTs, neuroblastoma, medulloblastoma, breast and ovarian cancer (45-49), all of them cancer types in which we have also identified SOX9 as highly expressed (Table Suppl. 1,2). These results support the idea that SOX9 copy number change might be relevant for a subgroup of samples among several malignancies. At the same time, a few cases of *SOX9* duplication are reported in the literature that are the cause of sex reversal and brachydactyly-anonychia (50, 51), but to our knowledge these individuals have no developed cancer. However, this is consistent with ectopic expression of Sox9 in mice, for which tumor incidence is not increased in comparison with controls (52), but together with a transforming agent, activated oncogene or event (such as chronic injury) higher levels of Sox9 significantly increase the rate of developing tumors. SOX9 expression in cancer might also be due to loss of repression. The changes in chromatin modifications that we found are consistent with altered upstream regulation. In line with this idea, SOX9 expression in colon cancer cell lines is controlled by Wnt signaling (19), a pathway in which constitutive activation generally initiates colorectal cancers (44).

Mechanistically, we observed that Sox9 activity promotes proliferation and its upregulation is sufficient to bypass senescence in primary MEFs. Furthermore, SOX9 regulates proliferation and facilitates tumor growth in colorectal cancer cells. It has been shown that Sox9 promotes proliferation and prevents differentiation in intestinal stem cell/progenitors, acting downstream of Wnt signaling (20, 22). These results support the idea that the mechanisms governing self-renewal and differentiation are overlapping with those regulating proliferation and senescence, and are consistent with a role for SOX9 in early stages of colorectal tumorigenesis. Furthermore, we found that SOX9 levels increase with advanced tumor stages in colorectal cancers and it cooperates with activated *K-Ras*, an oncogene frequently found mutated during colorectal cancer development (53), to facilitate transformation and tumor progression. It is noteworthy that high levels of SOX9 are associated with lower survival and adverse prognosis (17), and amplification of the 17q24.3 region correlates with the transition from primary colorectal cancer to metastasis (54). Collectively, these data indicate that SOX9 is important in colorectal cancer progression and support its use as a potential diagnostic and prognostic marker in colorectal cancers.

BMI1 is a key oncogene implicated in enhancing proliferation, stem cell self renewal, evading senescence, and favoring multiple cancer types partly through its ability to repress the transcription of INK4a and ARF (55). Nevertheless, BMI1 plays also a role in tumorigenesis through an INK4a/ARF-independent mechanism (56, 57). We found that Sox9 directly binds the *Bmi1* promoter, it regulates BMI1 expression in MEFs and colorectal cancer cells and its expression is associated with *BMI1* levels in primary colorectal carcinomas. Moreover, *BMI1* inactivation partly rescues the phenotypes seen in cancer cells with high levels of SOX9. These data indicates that SOX9 oncogenic activity is achieved at least in part by its ability to regulate the expression of BMI1 and shows that

SOX9-BMI1 interactions are likely to be critical events in colorectal tumor development and progression. In agreement with this, it is well known that Bmi1 is a stem/progenitor cell marker involved in the transformation of stem/progenitor cells in several tissues including intestinal epithelium (55, 58). High levels of BMI1 have been observed in benign adenomas and malignant carcinomas, and its expression has been linked with progression, lower survival and poor prognosis in colorectal cancers (38, 39, 59). Moreover, both genes, *SOX9* and *BMI1,* have been linked to tumorigenic epithelial-to-mesenchymal transition, a mechanism causing cancer metastasis, and that occurs in the most advanced colorectal cancers (40, 60). Finally, we provide evidence that some of the Sox9-Bmi1 functions are dependent on Ink4a and Arf, whose reduced expression has been associated with BMI-1 overexpression in human colorectal cancers (61). In summary, our observations provide direct experimental support for the oncogenic activity of SOX9, its relevance at different stages of tumorigenesis, and the mechanisms involved, particularly in colorectal cancers. The relevance of this pathway may be extended to other cell types and tissues where these proteins are involved in their normal physiology and deregulated in cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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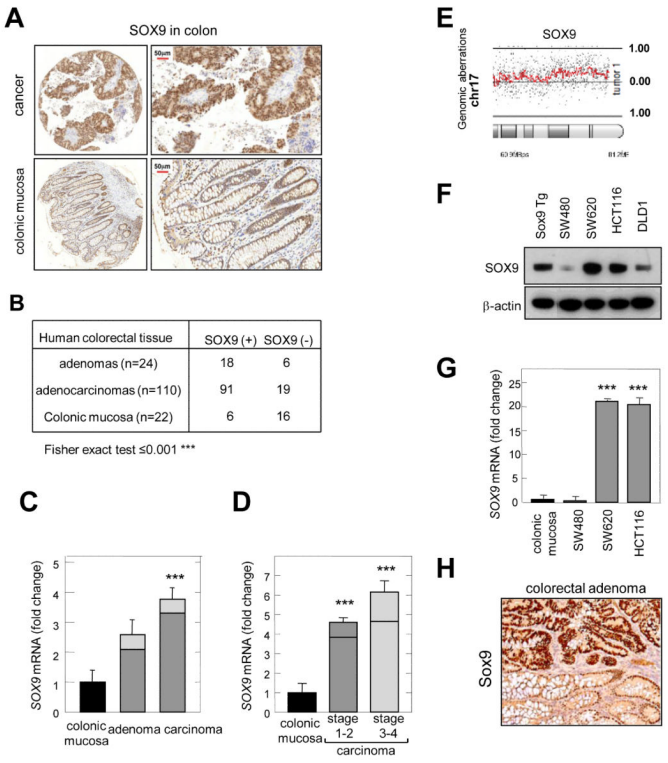

Figure 1. High levels of SOX9 in colorectal cancers

A: Representative images of SOX9 expression at different magnification in sections from human colorectal carcinoma and normal colonic mucosa from TMA (n=34). **B:** Quantification and statistical analysis of immunohistochemical staining of SOX9 in human colorectal adenomas and carcinomas. **C:** *SOX9 m*RNA levels were assayed in a set of primary human colorectal adenomas (n=6) and carcinomas (n=79), with 50 and 84% respectively displaying *SOX9* upregulation. Samples from normal colonic mucosa from the same patients (n=25) were used as controls. The figure shows *SOX9* average expression in all the cases (n=6 and n=79 lower section, intense grey), and in tumors that are *SOX9* positive (n=3 and n=66 upper section, weak grey). **D:** *SOX9* mRNA expression correlates with advanced tumor stage based on the American Joint Committee on Cancer (AJCC) TNM stage criteria. *SOX9* expression was measured in colorectal carcinomas in stage 1-2 (total n=24 lower section, and with *SOX9* upregulation n=20 upper section); and stage 3-4 total n=9 lower section, and with *SOX9* upregulation n=7 upper section) and control normal colonic mucosa (n=25). **E:** Representative example of CGH showing gain of chromosomal section where *SOX9* is located in a colorectal adenocarcinoma.

F-G: High levels of SOX9 were detected in several human colorectal cancer cell lines by western blot and qRT-PCR. **H:** Representative example showing high levels of Sox9 in azoxymethane/dextran (AOM/DSS) induced colorectal adenocarcinoma in mice (n=10).

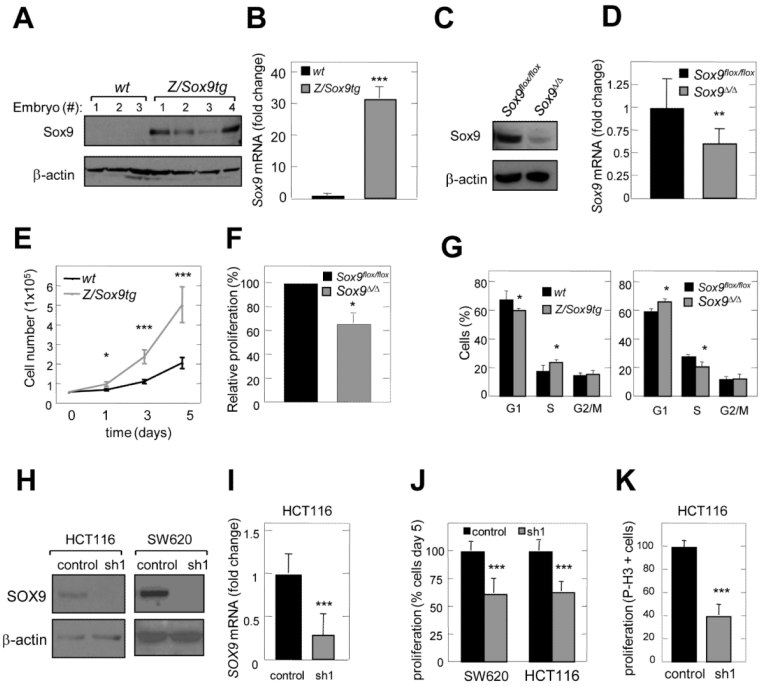

Figure 2. Analysis of MEFs with *Sox9* gain and loss of function

A-D: Sox9 protein and RNA levels in *Z/Sox9tg* MEFs and *Sox9*$^{flox/flox}$ MEFs at early passage. Increased Sox9 was observed in independent *Z/Sox9tg* cultures **A:** by western blot and **B:** qRT-PCR (n=9) compared to *wt* embryos (n=7). In contrast, **C:** immunoblots and **D:** PCR determinations showed decreased Sox9 in *Sox9*$^{\Delta/\Delta}$ MEFs treated with 4OHT for five days, at protein (representative of four independent MEF assays) and RNA levels (n=6). PCR data were normalized to β-actin expression and are expressed relative to gene expression levels in *wild-type* and ethanol treated (*Sox9*$^{flox/flox}$) cells respectively.

E-F: *Sox9* regulates proliferation in primary MEFs. 2.5×10^4 cells early-passage *wt* (n=6), *Z/sox9tg* (n=7) *Sox9*$^{\Delta/\Delta}$ (n=7) were plated and cell numbers counted at the indicated time points. Proliferation of *Sox9*$^{\Delta/\Delta}$ is indicated relative to the number of *Sox9*$^{flox/flox}$ cells at day five. **G:** Cell cycle profile of early passage *wt, Z/sox9tg* and *Sox9*$^{\Delta/\Delta}$ (n=3) was examined by flow cytometry and percentages of cells in G0/G1, S and G2/M phase are indicated in the table.

H-K: Characterization of colorectal cancer cells with *SOX9* stable knockdown. HCT116 and SW620 were transiently transfected with *pRS* empty vector (control) or *SOX9* shRNA (*sh1*), cultured for 2-3 weeks in the presence of 2 μg/μl puromycin and the pool of selected cells examined for SOX9 expression; **H:** Immunoblots and **I:** PCR determinations are representative of 3 different experiments. *SOX9* stable knockdown impairs proliferation. **J:** 2.5×10^4 *sh1* and control transfected cells were plated and proliferation was quantified counting cells at day five. *sh1* transfected cell proliferation is presented relative to control transfected cells (n=6). **K:** Number of P-H3 s*h1* cancer positive cells (n=3) was quantified and are indicated relative to the number of control transfected positive cells.

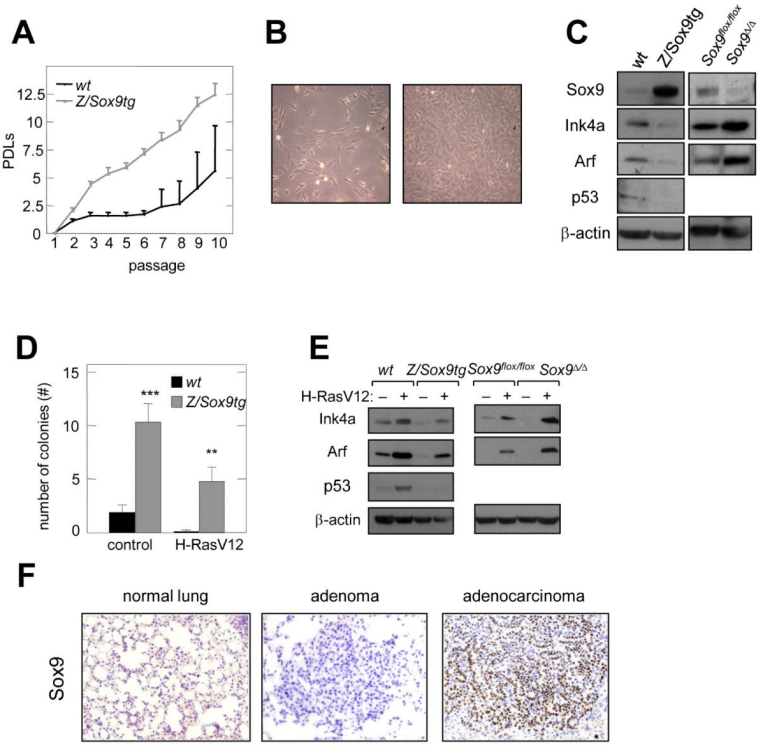

Figure 3. Sox9 regulates senescence

A: Sox9 overexpression is sufficient to bypass serial passage induced senescence. Cultures derived from wild-type (n=13) and *Z/Sox9tg* (n=14) embryos were maintained according to the Serial 3T3 protocol. The graph represents the accumulated number of doublings that each genotype has undergone at every passage **B:** Representative image of the morphology of *wt* and *Z/Sox9tg* MEFs at passage 4.

C: *Sox9* inversely regulates the expression of senescence markers. Early passage *Z/Sox9tg* contains lower Ink4a, Arf and p53 protein expression than *wild-type* cells while $Sox9^{\Delta/\Delta}$ have higher levels of Ink4a and Arf. Immunoblots are representative of at least three assays with independent MEF cultures. **D:** *Sox9* cooperates with Oncogenic Ras. *Z/Sox9tg* MEFs are partially resistant to *H-RasV12* induced cell cycle arrest *in vitro. H-RasV12* transduced *wt* and *Z/Sox9tg* MEFs (2×10^3 cells) (n=4) were plated, and the number of foci scored after three weeks. **E:** Detection of senescence marker protein expression in H-*RasV12* transduced MEFs with different levels of Sox9 were obtained 3 days after selection and are representative of 3 independent cultures. **F:** Representative examples of Sox9 expression from normal lung, adenomas and adenocarcinomas shows high levels of Sox9 exclusively in malignant lung adenocarcinomas obtained from *K-RasG12V;Cre* mice (n=9).

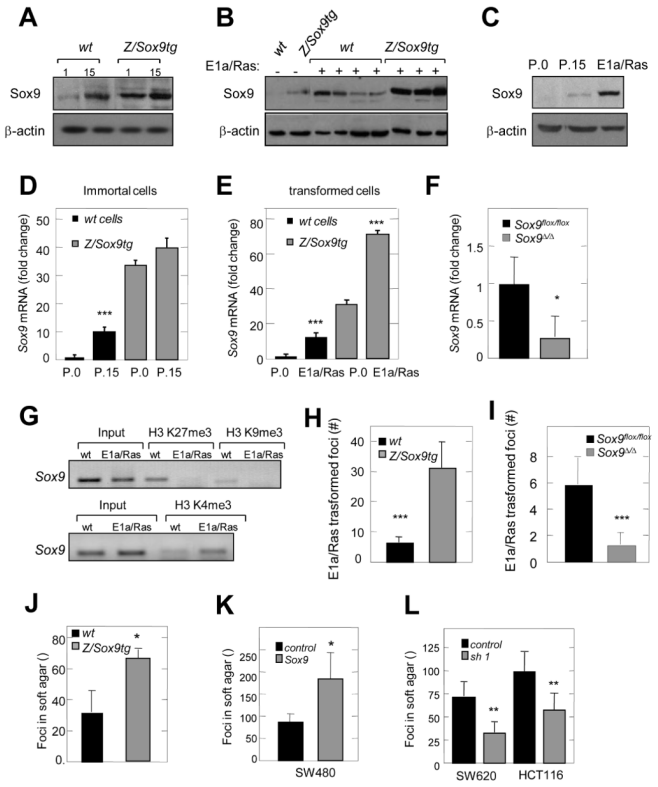

Figure 4. Sox9 is involved in spontaneous immortalization and oncogenic transformation *in vitro*

A-F: Sox9 accumulation with different stages of tumorigenesis. **A,C,D** Spontaneously immortalized cultures and **B,C,E)** *E1a/Ras* transformed *wt* and *Z/Sox9tg* cells express higher levels of Sox9 compared to primary cells. Immunoblots are representative of three and five assays respectively with independent MEF cultures. **F:** *E1a/Ras* induced expression of Sox9 is lower in $Sox9^{\Delta/\Delta}$ MEFs (n=2).

G: ChIP analysis of *Sox9* promoter using H3K4me3, H3K9me3 and H3K27me3 antibodies. Representative images from 4 different assays

H-L: Sox9 cooperates with oncogenes in neoplastic transformation. **H,J:** *Z/Sox9tg* MEFs are more sensitive to neoplastic transformation. *E1a/Ras* transformed *wt* and *Z/Sox9tg* MEFs were plated in plastic (2×10^3 cells), or in soft agar (2×10^5 cells), and the number of foci scored after three and two weeks respectively. Data correspond to the average and standard error of 14 and 4 independent MEF cultures. **I:** *Sox9* inactivation provides resistance to neoplastic transformation in $Sox9^{flox/flox}$;*CreERT2* MEFs and K,**L:** SW480 transiently transfected with *Sox9* and SW620 and HCT116 cancer cells were plated in soft agar (2×10^5 cells), and the number of foci scored after two weeks. Data are mean values ± S.E of, at least, 3 independent experiments.

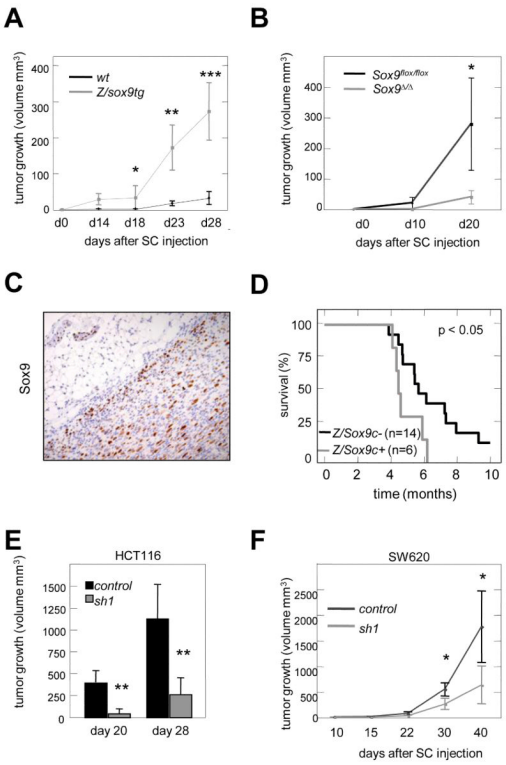

Figure 5. Sox9 regulates tumorigenesis *in vivo*

A-B: Sox9 levels modulate tumor growth in *E1a/Ras* neoplastically transformed MEFs. **A:** Pool of *E1a/Ras* transformed *wild type* (n=12) and *Z/Sox9tg* (n=17) cells were injected subcutaneously into nude mice, and the growth of the tumors scored at the indicated time points. **B:** Pool of $Sox9^{flox/flox}$ transformed MEFs were treated for five days *in vitro* with ethanol or 4OHT, and $Sox9^{flox/flox}$ and $Sox9^{\Delta/\Delta}$ (n=16) were subcutaneously injected into nude mice and tumor growth scored at the indicated time points. **C:** Representative image of high levels of Sox9 in a fibrosarcoma derived from *wt* mice (n=10). **D:** Kaplan-Meier representation of the survival curves of $Z/Sox9c^{-}$ (n=14) and $Z/Sox9c^{+}$ (n=6) mice. Statistical significance of the difference in $Z/Sox9c^{+}$ lifespan relative to $Z/Sox9c^{-}$ mice was assessed using the logrank test (p=0.03 *).

E-F: *SOX9* inactivation decreases tumor growth in human colorectal cancer cells. Sublines of *control* or *sh1SOX9* HCT116 and SW620 colorectal cells were injected into the flanks of nude mice (n=6) and tumor growth was measured at the indicated time points.

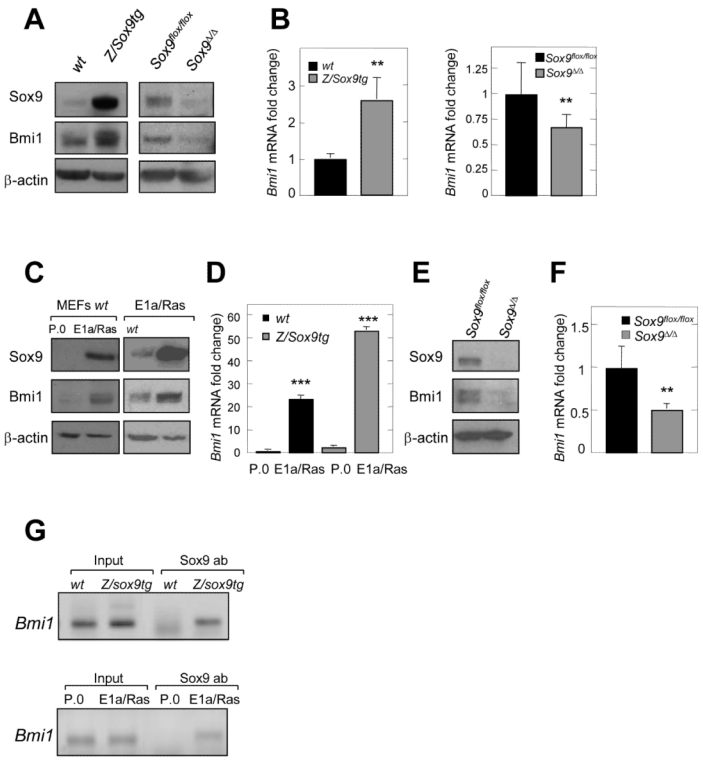

Figure 6. Sox9 directly regulates Bmi1 activity

A,B: Sox9 regulates Bmi1 expression in MEFs. **A:** Bmi1 expression correlates with Sox9 in *Z/Sox9tg* and $Sox9^{\Delta/\Delta}$ cells at protein and **B:** mRNA levels. Immunoblots are representative of four assays with independent MEF cultures and RNA levels are mean values ± S.E. of (n=7) embryos. **C-F:** Detection of Bmi1 expression in *E1a/Ras* transformed MEFs varying Sox9 levels. **C,E:** Representative inmunoblots of five assays with independent MEF cultures. **D,F:** *Bmi1* mRNA levels in transformed *Z/Sox9tg* (n=5) and $Sox9^{flox/flox}$;*CreERT2* MEFs (n=2).

H-I: Sox9 binding to the endogenous *Bmi1* promoter. **H:** Representative example of ChIP analysis in early passage *wild-type* Vs *Z/Sox9tg* MEFs **I:** and in transformed *wild-type* MEFs. Images are representative of three assays with independent MEF cultures.

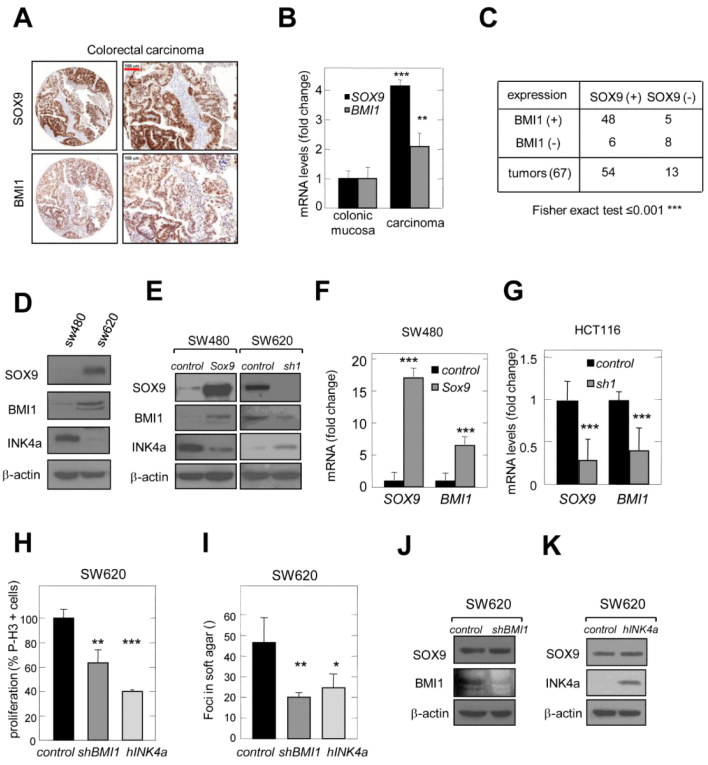

Figure 7. SOX9-BMI1-INK4a/ARF pathway in human colorectal cancer

A: Representative image of SOX9 and BMI1 correlative expression at different magnification in sections from a human colorectal carcinoma TMA (n=34). SOX9 strong staining is observed in 27 carcinomas with 25 of them showing high levels of BMI1. **B,C:** *SOX9* and *BMI1* mRNA expression were assayed in an independent set of human primary colorectal carcinomas (n=33) and control colonic mucosa (n=25). We found SOX9 overexpression in 27 samples with 23 of them showing high levels of BMI1 by qRT-PCR. **C:** Statistical analysis of the expression of SOX9 and *BMI1* in these two sets of human colorectal cancers (n=67). SOX9 is positively associated with BMI1 (Fisher exact Test ≤0.001 ***).

D: Representative images of SOX9, BMI1 and INK4a expression in colorectal SW480 and SW620 cancer cell lines indicate that high levels of SOX9 concomitant with BMI1 together with low INK4a are associated with tumor malignancy. **E:** *SOX9* regulates the expression of BMI1and INK4a. Left, *Sox9* ectopic expression by transient transfection in SW480 cells increased BMI1 and diminished INK4a levels three days after transfection. Right, *sh1SOX9* SW620 cells contained lower BMI1 and higher INK4a levels than control cells. Immunoblots are representative of at least three different assays. **F,G:** SOX9 regulates *BMI1* transcriptionally in SW480 and HCT116 cells (n=2).

H-K: SW620 stably transfected with *ShBMI1 or hINK4a.* **H:** Impaired proliferation of cells with sh*BMI1* or *hINK4a* quantified by numbers of P-H3 positive cells (n=3). **I:** *ShBMI1, hINK4a* or *control* SW620 cells were plated in soft agar (2×10^5 cells), and the number of foci scored after two weeks. Data are mean values ± S.E of, at least, 3 independent experiments. **J**: SOX9 and BMI1 were assessed in *ShBMI1* SW620 cells. Immunoblots are representative of two different assays. **K**: SOX9 and INK4a were assessed in *hINK4a* SW620 cells. Immunoblots are representative of two different assays.