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Original Paper

Transcription factor SOX-5 enhances nasopharyngeal carcinoma progression by down-regulating *SPARC* gene expression

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Abstract

Nasopharyngeal carcinoma (NPC) is prevalent in south-eastern Asia, and its tumourigenesis is rather complex. The purpose of this research was to identify the pivotal genes that may be altered during the early stage of NPC progression. Eleven genes were selected by comparative microarray analysis of NPC versus normal nasomucosal cells. The expression of SPARC (secreted protein, acidic, cysteine-rich) was statistically significantly down-regulated in NPC cells. In exploring the mechanism underlying the decreased transcription of SPARC in NPC cells, we found that the transcription factor SRY (sex-determining region Y)-box 5 (SOX-5) is up-regulated in NPC cells. RNA interference of SOX-5 by short hairpin RNA (shRNA) in NPC cells caused a dramatic increase in SPARC and chromosome immunoprecipitation assay showed that SOX-5 can bind directly to the SPARC promoter, suggesting that SOX-5 acts as a key transcriptional repressor of SPARC. We further demonstrated that shRNA knockdown of SOX-5 suppressed the proliferation of NPC cells, as well as their migratory ability, which was also observed when SPARC was over-expressed in NPC cells. Alternatively, blocking SPARC with an antagonistic antibody reversed the effects of SOX-5 knockdown. In 66 NPC patients, over-expression of SOX-5 in tumour cells correlated clinically with poor survival. Our study suggests that SOX-5 transcriptionally down-regulates SPARC expression and plays an important role in the regulation of NPC progression. SOX-5 is a potential tumour marker for poor NPC prognosis.

Keywords: nasopharyngeal carcinoma (NPC); NPC progression; NPC prognosis; SOX5; SPARC

Introduction

Nasopharyngeal carcinoma (NPC) is defined as a malignant tumour of the squamous metaplastic epithelium lining the surface and crypts of the nasopharynx [1]. According to the World Health Organization [2], Chinese people living in South China, Hong Kong, Taiwan and Singapore have a high incidence of NPC. Among the possible aetiological factors identified for NPC are genetic susceptibility [3–5] and environmental risk factors [6–8]. Although Epstein–Barr virus (EBV) has been proposed to be closely associated with NPC [9–11], recent observations suggest that EBV may play a role as an enhancer, not an initiator or promoter, of NPC tumourigenesis [12–14].

Much research into NPC gene expression profiles has focused on the relationship between the expression of various genes and EBV infection. EBV LMP-1-transfected NPC cells showed elevated expression of interleukin-1 [15], viral interleukin-10 [16], GAGE-1 and -2 proteins, interferon-γ (IFNγ) [17] and Twist [18]. EBV also inhibits the expression of major histocompatibility complex (MHC) class I in NPC [19]. However, because EBV seems only to enhance NPC tumourigenesis, there must be many unidentified genes, unrelated to EBV, that also regulate NPC tumourigenesis.

Recently, some NPC marker genes were reviewed [20]. Microarray analysis demonstrated that the up-regulation of NF-κB2 and survivin expression is crucial in NPC tumourigenesis [21]. The Wnt signalling pathway is also abnormally regulated in NPC cells [21,22]. Among all the genes studied, only RAS association domain family protein 1A (RASSF1A) pathway has been studied in detail [23]. Therefore, our

knowledge of the regulatory pathways of the genes involved in NPC tumourigenesis is still limited.

To identify other genes involved in NPC tumourigenesis, we took advantage of cDNA microarray technology and NPC cell lines that we had already established [24–26]. We identified genes showing significant differential expression patterns in primary cultures of normal nasomucosal (NNM) epithelia and NPC cell lines. This differential gene expression was confirmed by quantitative RT–PCR (Q-RT–PCR). Here, we determined that the transcription factor (TF) SRY (sex-determining region Y)-box 5 (SOX-5) down-regulates the expression of the potential NPC tumour suppressor gene *SPARC* (secreted protein, acidic, cysteine-rich).

In mice, SOX-5 has two isoforms, a long form (SOX-5L) and a short form (SOX-5S). In humans, SOX-5 has three isoforms, A, B and C. Isoforms A and B both correspond to mouse SOX-5L. Isoform C corresponds to mouse SOX-5S. Mouse SOX-5L protein associates with SOX-6 and SOX-9 to activate the type II collagen gene [27]. However, there is no detailed functional study of either mouse SOX-5S or human SOX-5. Only recently, SOX-5 was found to be associated with human glioma [28] and seminoma [29]. SPARC protein was first described as a major constituent of bovine and human bone and as a protein secreted by proliferating cells *in vitro* [30]. SPARC was later identified as a protein associated with various cancers. In some cancers [31–36] SPARC may act as a tumour suppressor, but in other situations SPARC acts as an enhancer or oncogene [37–44].

Although both SOX-5 [45] and SPARC [46] are genes known for their involvement in bone development and in certain cancers, there is no evidence of any interaction between the translational products of these two genes. This study provides the first evidence that SOX-5 suppresses the downstream expression of the tentative oncosuppressor gene *SPARC* in NPC. Furthermore, the elevated expression of SOX-5 and the suppression of SPARC protein expression in NPC cells may play a role in enhancing the progression of NPC pathogenesis.

Methods

Culture of NPC cell lines and primary culture of NNM cells

For this and other methods indicated, see Supplementary Methods, available online at: http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2299.html

RNA extraction and cDNA preparation

See Supplementary Methods online.

Q-RT–PCR analysis of genes selected with microarray analysis

See Supplementary Methods online.

Statistical analysis of Q-RT–PCR results

See Supplementary Methods online.

Bioinformatic study of the promoter region

To address the question of why the expression levels of those genes were correlated, we analysed their promoter regions to identify any common TFs. The putative promoter region of each gene was obtained from the DBTSS database [47]. We then used the TRANSFAC database system [48] to identify any TFs that could bind to each promoter. Supplementary Table 2 (available online), lists the gene names and the primer sequences used in the Q-RT–PCR analysis of these TF genes.

Cloning the TF gene *SOX-5* and its downstream gene *SPARC*

See Supplementary Methods online.

Demethylation analysis

See Supplementary Methods online.

Transfection of NPC cells

See Supplementary Methods online.

Western blotting

See Supplementary Methods online.

Immunohistochemical staining and immunofluorescent staining

See Supplementary Methods online.

Chromatin immunoprecipitation (ChIP) assay of *SPARC* gene promoter region

See Supplementary Methods online.

Statistical analysis of the relationship between SOX-5 expression and the survival of NPC patients

Initially, we randomly retrieved 73 patients diagnosed as having NPC in the National Taiwan University Hospital between 1997 and 1998 to follow up their clinical courses for 9–10 years; however, seven NPC patients who died with causes other than NPC were ruled out of this test. The remaining patients were divided into four groups according to the SOX-5 scores and NPC types. SOX-5 scores were defined as the numbers of SOX-5-positive (SOX-5^+) nuclei in two separate view fields under $\times 400$ magnification in the NPC biopsy specimens. For example, if the total number of SOX-5^+ nuclei in two high-power magnification fields is 50, the SOX-5 score is 50. Group 1 includes patients with undifferentiated NPC type (WHO type IIb) and SOX-5 score ≤ 50. Group

2 includes patients with undifferentiated NPC but SOX-5 score >50. Group 3 includes patients with keratinizing squamous cell (WHO type I) or non-keratinizing squamous cell (WHO type IIa) NPC types (these two types are considered together as differentiated NPC) and SOX-5 score ≤50. Group 4 includes patients with differentiated NPC types but SOX-5 score >50. Kaplan–Meier log-rank test was applied to analyse the disease-free survival of these four groups. The significance level was set to 95% confidence intervals (CI).

Cell migration assay of transfected cells

See Supplementary Methods online.

MTT [3-(4,5-dimethylthiazo-2yl)-2,5 diphenol tetrazolium bromide] assay

Stably transfected NPC cells were seeded into 96-well plates at 2000 cells/well. To induce SPARC expression, the cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4 μg/ml doxycycline. After incubation for 0, 2, 3 or 4 days, the cells were subjected to an MTT assay as described previously [49]. The population doubling time was calculated from OD_{540} data in the MTT assay by linear regression. To test the effects of anti-FGFR-1 (QED Bioscience, San Diego, CA, USA) and anti-SPARC antibodies on the cell proliferation rate of *SOX5*-shRNA-transfected NPC-TW04 cells, the antibodies were added to the culture medium at a concentration of 4 or 16 μg/ml. After 2 days, the relative cell numbers were measured by MTT assay.

Invasion assay

See Supplementary Methods online.

Semi-quantitative RT–PCR

See Supplementary Methods online.

In vivo assay of xenograft growth

See Supplementary Methods online.

Results

Genes selected by microarrays and their Q-RT–PCR verification

From the results of microarray analysis, 11 differentially expressed genes were selected for further verification by Q-RT–PCR. For the first round of Q-RT–PCR, two NNM cell cultures and six NPC cell lines were used. The mRNA copy numbers of each gene relative to that of *GAPDH*, determined by Q-RT–PCR (Table 1), were subjected to Student's *t*-test to ascertain whether that gene was differentially expressed in NPC and NNM cells. Six genes, *FGFR1*, *IGFBP6*, *CMTM7*, *RPL37A*, *SPARC* and *UCHL1*, were all significantly down-regulated in the NPC lines. For the second round of Q-RT–PCR, the sample size was increased to five NNM cell cultures and seven NPC cell lines. After Student's *t*-test, only FGFR1 and SPARC showed significant differences in expression levels (Table 2).

FGFR1 and SPARC promoter regions contain the same potential binding sites for three transcription factors

FGFR-1 is a membrane receptor for fibroblast growth factor and SPARC is an extracellular matrix protein. They are not obviously related by their functions. Therefore, we speculated that they might share one or more TFs, which regulated their co-expression. Two possibilities were hypothesized. Either these common TFs normally up-regulate FGFR1 and SPARC expression but the TFs are themselves down-regulated in NPC cells, or these TFs act as suppressors for both genes and are themselves up-regulated.

To identify these hypothetical TF genes, the putative 10 kb promoter sequences of *FGFR1* and *SPARC* were determined by searching the DBTSS database. Then, the TRANSFAC database was searched with the promoter sequences of these two genes to identify any common TFs that could bind to both promoter regions. Three TFs, Nkx-2.5, SOX-5, and NRF-1, were thus found. After Q-RT–PCR and statistical analysis, only SOX-5 showed a significant differential expression

Table 1. Quantitative RT–PCR and statistical analysis of 11 genes selected from microarray assays

	2 NNM primary cultures		6 NPC cell lines		
Gene	**Average**	**STDV**	**Average**	**STDV**	***p* Value of *t*-test**
CITED2	2.2721504930	1.6877010550	0.7498703720	0.5827377440	0.076
FGFR1	4.2370415310	0.8984154960	0.0327979750	0.0295249130	<0.0001
IGFBP6	15.1234905500	4.7485417330	3.6085917160	3.7337841170	0.0114
CMTM7	0.4374653480	0.0575614880	0.1448557040	0.1099614670	0.0132
MEF2C	17.6669691300	24.8681921000	0.0575575700	0.1192640450	0.0778
TNFSF11	416.6550143000	588.5843652000	0.0000481661	0.0001153290	0.0779
PTPN21	2.0935376550	2.5892427030	0.3301621140	0.3254181550	0.0968
RPL37A	4981.7455950000	1931.4052070000	615.5103187000	858.7542617000	0.003
SPARC	328.9748257000	311.1045242000	0.0074010530	0.0119851850	0.0193
ADAMTSL4	234.5018176000	329.1176923000	1.0997575010	1.1089576820	0.0775
UCHL1	23.4488206500	1.9285766820	0.0000015054	0.0000032014	<0.0001

Table 2. Quantitative RT–PCR and statistical analysis of six significant genes after increasing sample sizes

	5 NNM primary cultures		7 NPC cell lines		
Gene	**Average mRNA copy numbers**	**STDV**	**Average mRNA copy numbers**	**STDV**	***p* Value of *t*-test**
FGFR1	3.7430601786	4.2016460306	0.0308268587	0.0274523380	0.0382
IGFBP6	12.0880771712	12.8159949804	3.2134864773	3.5651615550	0.1072
CMTM7	1.3475750188	2.5239999859	0.1510488690	0.1017091800	0.2299
RPL37A	2037.9850053302	2856.9958887137	555.1044587743	800.0569628000	0.2144
SPARC	222.3496794676	260.449397811	0.0500125081	0.1132689570	0.0439
UCHL1	36.1480105236	55.8372479047	0.0047651548	0.0126034320	0.1111

Table 3. Quantitative RT–PCR and statistical analysis of three common transcription factors shared by FGFR1 and SPARC putative promoters

	5 NNM primary cultures		7 NPC cell lines		
Gene	**Average mRNA copy numbers**	**STDV**	**Average mRNA copy numbers**	**STDV**	***p* Value of *t*-test**
NKX2-5	0.000239289	0.000272595	0.000519493	0.000322313	0.1458
NRF1	0.003878201	0.001733216	0.009878979	0.006249378	0.0659
SOX5	0.000185655	0.000161995	0.000540935	0.00020177	0.0088

pattern and was highly expressed in NPC cell lines (Table 3).

SOX-5 protein isoform C is over-expressed in NPC cell lines

SOX-5 has three isoforms, A, B and C, whose putative molecular weights are 84, 82 and 42 kDa, respectively. The primers designed for Q-RT–PCR identified all the isoforms, because it was difficult to design isoform-specific primers. Figure 1A shows the representative FLAG-labelled recombinant SOX-5C expressed in the NPC-TW01 cell line (Figure 1A, left lane) and the endogenous SOX-5C expressed in the NPC-TW04 cell line (Figure 1A, right lane; data for Western blotting of recombinant isoforms A and B are not shown). These results demonstrate that the antibody we used is specific for SOX-5 and identifies all SOX-5 isoforms. The single band in Figure 1A (right lane) has a molecular weight of about 51 kDa, whereas the left lane contains two bands of about 52 and 53 kDa, according to the size markers. These bands had molecular weights about 10 kDa higher than the putative molecular weight of SOX-5 isoform C (42 kDa), which may be the results of different degrees of posttranslational modification. Figure 1B further shows that the SOX-5 isoform expressed in all NPC cell lines was isoform C. No detectable isoform A or B was observed. In NNM cell lysates, only trace amounts of SOX-5 isoform C were detected. The expression of SOX-5 in NPC cells was not affected by EBV infection (data not shown). The EBV-infected NPC cell lysate was prepared as described in our recent publication [26].

SOX-5 protein expression in NPC biopsy specimens is related to the prognosis of NPC patients

Immunostaining for SOX-5 in NPC cell lines revealed nuclear staining in the majority of the cells (Figure 1C-b). To establish clinical studies, the clinical histories of 66 NPC patients were reviewed and SOX-5 immunostaining was applied to the primary tumour paraffin section from each patient. Figure 1C-c represents the result of SOX-5 immunostaining of NPC biopsy specimen from a patient who had survived >5 years with no recurrence had almost no SOX-5^+ tumour cells. The biopsy specimen from a patient with metastasis to the liver had many SOX-5^+ tumour cells (Figure 1C-d). Those SOX-5^+ cells were all tumour cells. No SOX-5^+ cells were seen in the stroma.

These 66 NPC patients were divided into four groups, as described in Methods. Kaplan–Meier log-rank test was applied to these four groups to obtain a disease-free survival plot (Figure 1D). From this plot, groups 2 and 4 both have SOX-5 scores >50 and seemed to have poorer prognosis than groups 1 and 3; the latter two groups had SOX-5 scores ≤50. This phenomenon was supported by the log-rank *p* values. Group 2 shows significant difference with groups 1 or 3, and insignificant difference between either groups 1 and 3 or groups 2 and 4. The odd feature here is that group 4 did not show a significant difference with either group 1 or group 3, probably due to too few cases in group 4 (only three cases). These data may further suggest a hypothesis that the critical factor affects the prognosis of NPC patients is the number of SOX-5^+ cell numbers, but not NPC pathological subtypes.

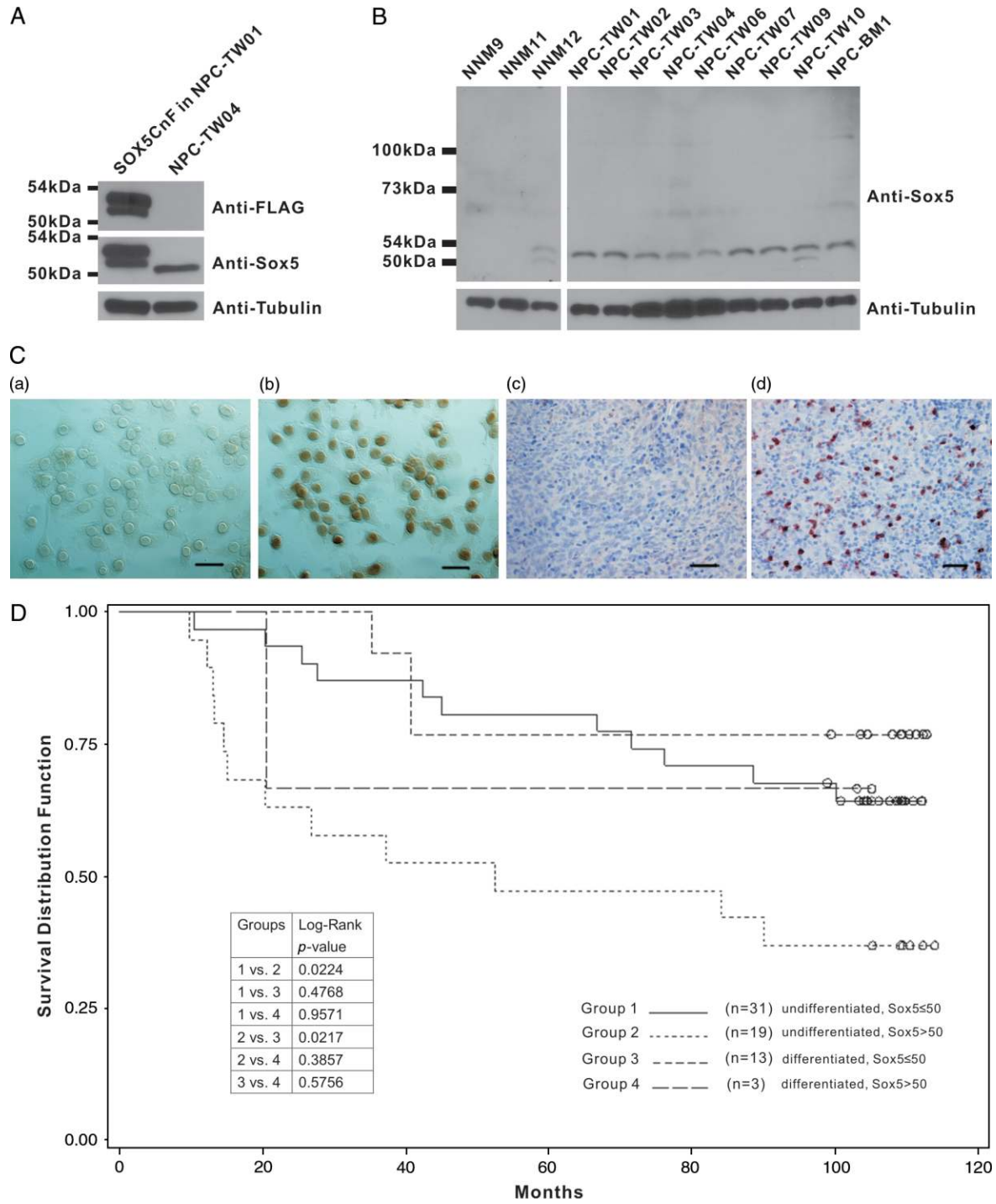

Figure 1. SOX-5 protein in normal nasomuscosal (NNM) cells, nasopharyngeal carcinoma (NPC) cell lines and NPC biopsy specimens. (A) Comparison of the western blotting of recombinant FLAG-labelled SOX-5C (labelled SOX5CnF) in NPC-TW01 cells and endogenous SOX-5 in NPC-TW04 cells. The endogenous SOX-5 in NPC cells is SOX-5C. (B) Western blot analysis of SOX-5 protein expression in NNM cells and NPC cell lines. NNM cells show only trace amounts of SOX-5C. However, SOX-5C is the dominant isoform in nine NPC cell lines. Neither primary cultured cells nor NPC cell lines show detectable isoform A or B. (C) Immunohistochemical staining of SOX-5 in the NPC-TW04 cell line and NPC biopsy specimens (bar = 160 μm); (a) negative control for NPC cell staining; (b) almost all the NPC cells show SOX-5$^+$ staining in their nuclei (brown); (c) a biopsy specimen from an NPC patient, who survived for longer than 5 years, shows almost no SOX-5$^+$ NPC cells; (d) another biopsy specimen, from another NPC patient with liver metastasis, shows many nuclear-stained SOX-5$^+$ NPC cells (brown colour). (D) A Kaplan–Meier plot of disease-free survival of total 66 NPC patients followed up in this study. Group 1 includes patients with undifferentiated NPC type (WHO type IIb) and SOX-5 score ≤50. Group 2 includes patients with undifferentiated NPC but SOX-5 score >50. Group 3 includes patients with differentiated NPC types (WHO type I and IIa considered together) and SOX-5 score ≤50. Group 4 includes patients with undifferentiated NPC types (WHO type I and IIa) but SOX-5 score >50. Only group 2 shows significant difference in survival compared with either group 1 or group 3. Due to the low case number in group 4 (only 3), the *p* value between groups 4 and 1, 2 or 3 all show no significant difference. Groups 1 and 3 with lower SOX-5 scores (≤50) also shows no significant difference

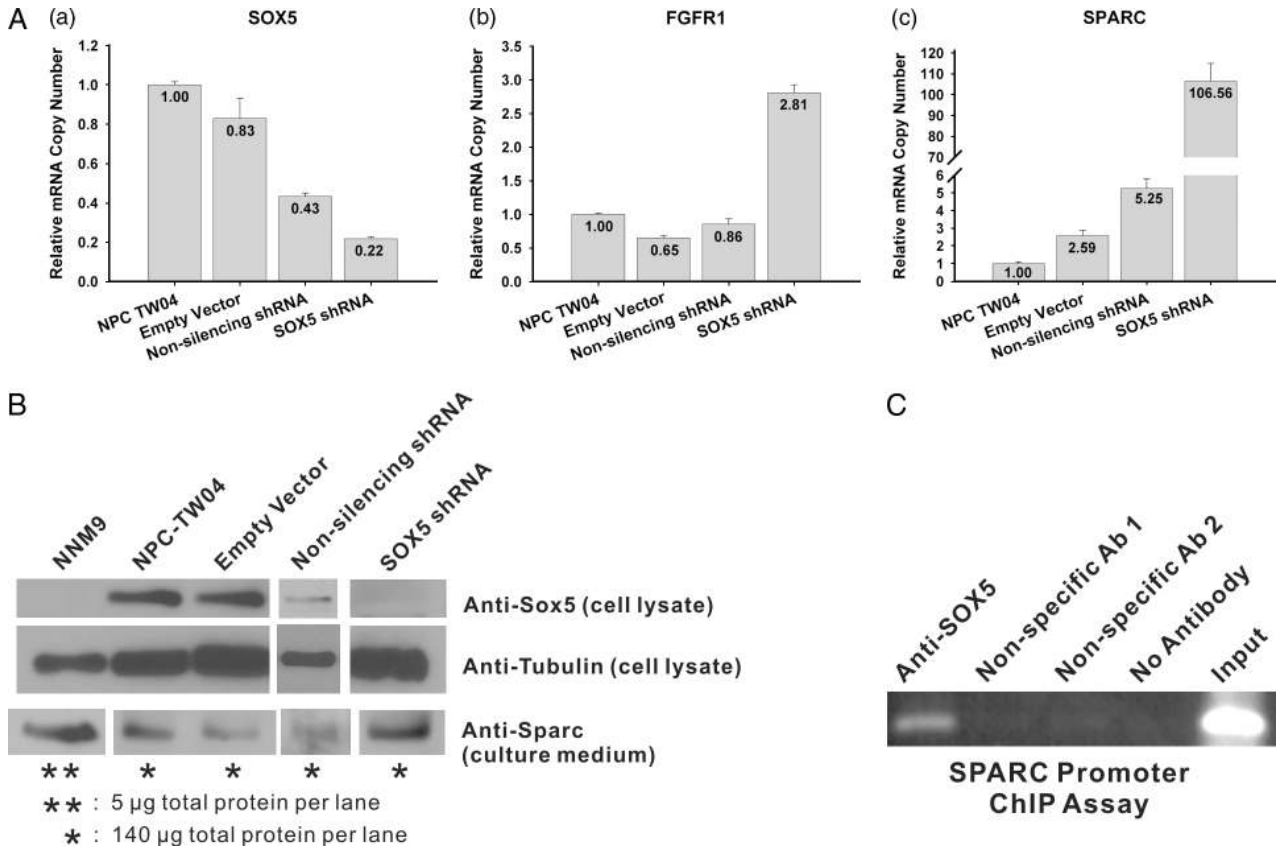

Figure 2. Effects of SOX-5 shRNA on *FGFR1* and *SPARC* gene expression. (A) Knockdown of (a) SOX-5, (b) increased FGFR1 and (c) SPARC mRNA levels. (B) SOX-5-specific shRNA down-regulated the expression of SOX-5 protein completely. SPARC protein also simultaneously increased significantly in the culture medium. (C) SPARC promoter chromatin immunoprecipitation (ChIP) assay. Only SOX-5-specific antibody can precipitate SPARC promoter region containing SOX-5 binding site

SOX-5 down-regulates *FGFR1* and *SPARC* gene expression

Because FGFR1 and SPARC were down-regulated and their common TF, SOX-5, was up-regulated in NPC cell lines, we hypothesized that SOX-5 down-regulates the expression of FGFR1 and SPARC. Our results show that when SOX-5 mRNA was knocked down by SOX-5-specific shRNA in NPC cells (Figure 2A-a), the mRNA expression levels of both FGFR1 (Figure 2A-b) and SPARC (Figure 2A-c) increased. The mRNA expression of SPARC, in particular, increased over 100-fold and SPARC protein was detected in the culture medium by western blotting (Figure 2B).

Since hypermethylation in the promoter region can down-regulate SPARC expression [33,36], NPC cells were treated with the demethylation reagent 5-aza-2′-deoxy-cytidine. However, this treatment did not induce the expression of SPARC protein (see Supplementary Figure 1 online). On the other hand, chromatin immunoprecipitation (ChIP) assay of the SPARC promoter region containing the SOX-5 binding site (Figure 2C) showed that SOX-5 could bind to SPARC promoter region directly. These finding suggests that the down-regulation of SPARC protein in NPC cells is not due to the hypermethylation of the SPARC promoter region, but by the elevated expression of SOX-5.

Functional analysis of SOX-5 in the NPC-TW04 line

When SOX-5 shRNA was transfected into NPC cells, an increase in the cell population doubling time was observed (Figure 3A). In a scratch migration ability test, knocking down SOX-5 impaired the migratory ability of the NPC-TW04 cell line (Figure 3B).

By adding anti-SPARC antibody, but not by adding anti-FGFR-1 antibody, into the culture medium, the cell proliferation rate was elevated by up to 48% (Figure 3C). This result supports the hypothesis that the effects of SOX-5 down-regulation are exerted via SPARC protein.

Functional analysis of SPARC in the NPC-TW01 line and in SCID mice bearing NPC xenografts

SPARC protein was abundant in both NNM culture medium and cell lysates (Figures 2B, 4A). In NPC cells, none was detectable in cell lysates (Figure 4A), although a small amount of SPARC was detected in the culture medium (Figure 2B). In NPC biopsy specimens, no SPARC immunoreactivity was observed in the tumour cells, but was only seen in some of the stromal cells (see Supplementary Figure 2 online). When SPARC over-expression was induced by doxycycline, both the culture medium and the cell lysate contained a large quantity of SPARC protein

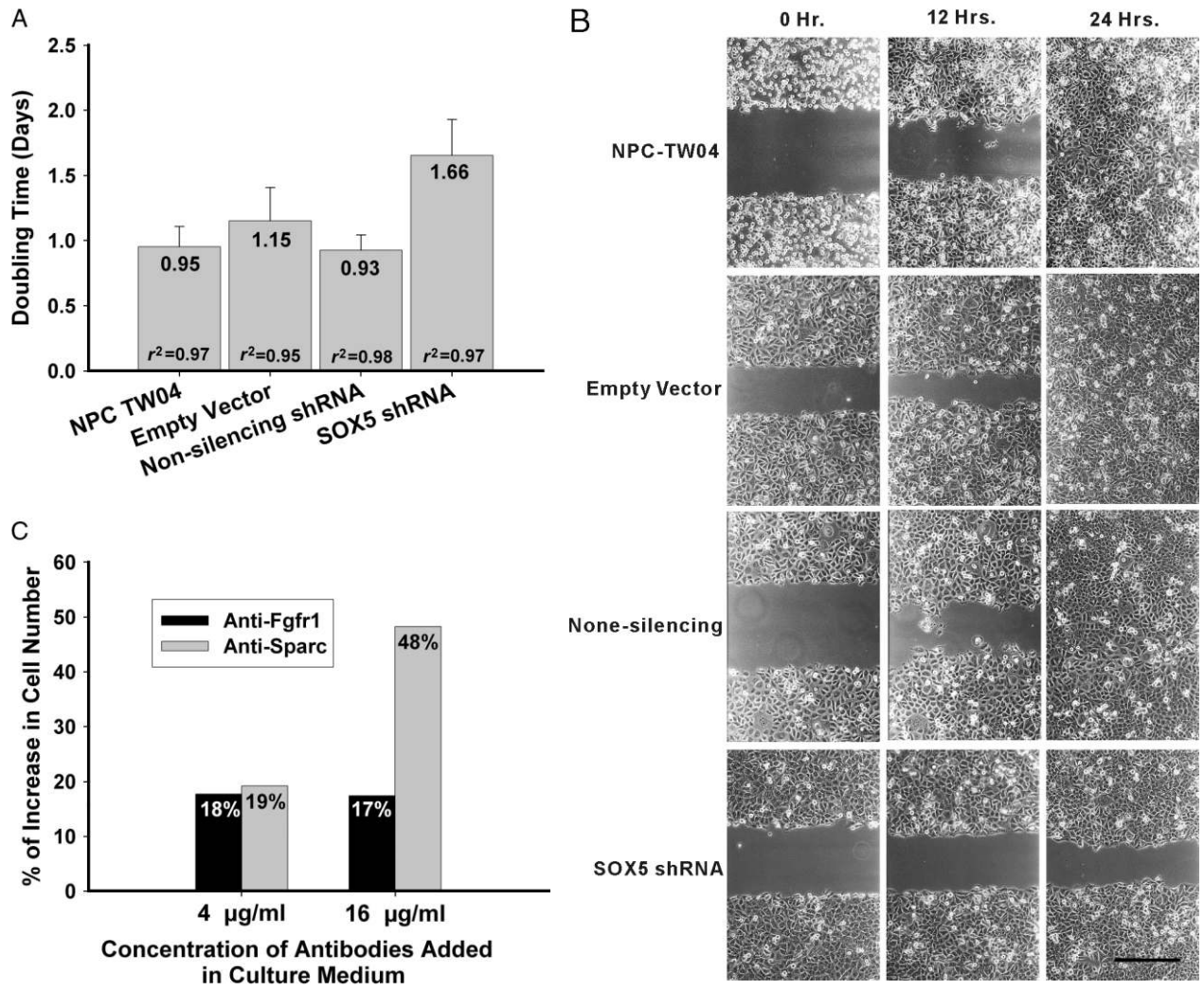

Figure 3. The effect of knocking down SOX-5 on NPC cell growth and migration. (A) SOX5-shRNA-transfected NPC cells displayed a prolonged cell population doubling time of over 12 h. (B) Scratch wound tests revealed significantly reduced cell migratory capacity in SOX-5 knockdown NPC-TW04 cells (bar = 2 mm). (C) Incubation of SOX5-shRNA-transfected NPC cells with anti-SPARC antibody at a concentration of 16 μg/ml for 2 days significantly increased the cell proliferation rate. No such effect was observed with anti-FGFR-1 antibody

(Figure 4B). Inside the cells, the SPARC protein was mainly distributed in the cytoplasm (Figure 4C).

Similar to SOX-5 knockdown, the over-expression of SPARC in NPC cells also extended the cell population doubling time (Figure 5A) and impaired the cells' migratory capacity (Figure 5B). SPARC over-expression in NPC-TW01 cells also dramatically retarded the cells' invasive capacity to about 10% of that of the control cells (Figure 5C).

In animal experiments, the sizes and weights of the xenografts were much smaller when SPARC over-expression was induced (Figure 6A). Immunostaining of the xenografts showed that SPARC protein was found only in regions of SPARC-over-expressing tumour cells (Figure 6B-b2), whereas the stroma revealed marked desmoplasia (Figure 6B-b1, b2). Of the six SCID mice bearing NPC xenografts without doxycycline induction, all showed large multiple metastatic nodules distributed on the serosa of the visceral organs contrary to the other six mice with SPARC expression induced (data not shown).

Discussion

Previously, we used cDNA microarray analysis to compare the gene expression patterns of NPC and NNM epithelia, and identified some differentially expressed gene groups [26]. In the present study, we proposed a novel way of analysing microarray data to identify potential pivotal transcription factors (TFs) that may down-regulate the expression of NPC oncosuppressor genes in NPC pathogenesis.

The TF gene identified here was *SOX-5*. In testicular seminomas, SOX-5 has been correlated indirectly with the invasive growth of the tumour [29]. Recently, SOX-5 protein was identified as a tumour antigen of glioma [28]. These findings, together with our observation of an increased number of SOX-5-expressing tumour cells in advanced NPC (Figure 1C-d), all suggest that SOX-5 expression may be up-regulated in various advanced tumour types and that SOX-5 may be a potential tumour marker for advanced grades of malignancy. This conclusion is strongly supported

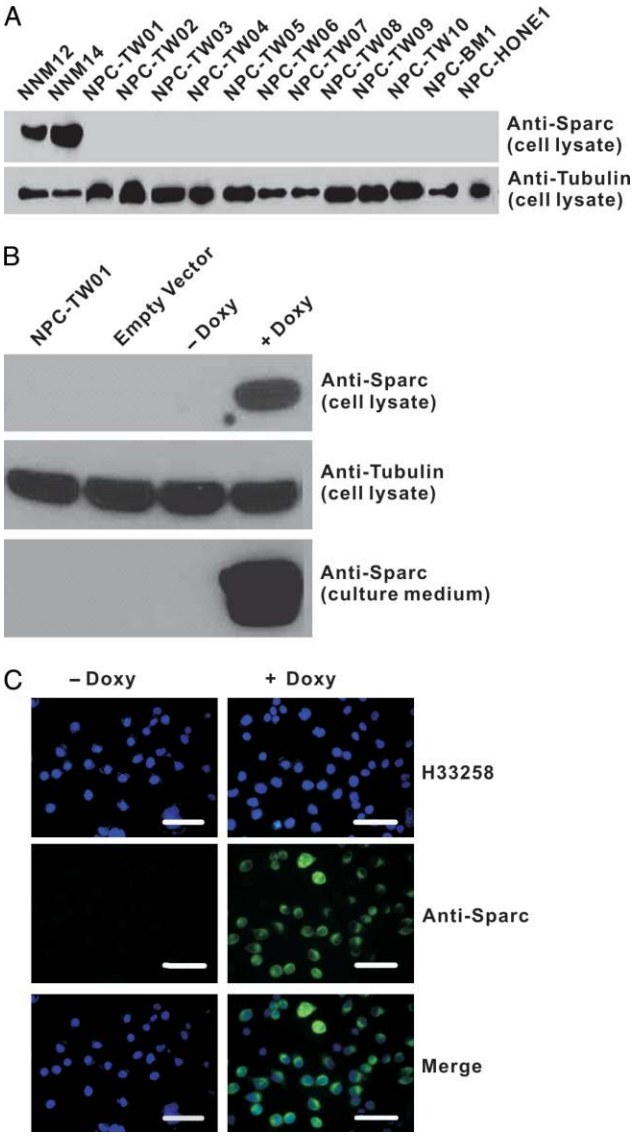

Figure 4. Expression of SPARC protein in NNM cells, the NPC-TW01 cell line, and NPC-TW01-pBIG2i–SPARC transfectants. (A) NNM cells showed strong SPARC protein expression. None of the NPC cells expressed detectable SPARC protein. (B) NPC-TW01 pBIG2i-empty-vector transfectants and NPC-TW01 pBIG2i–SPARC transfectants without doxycycline induction showed no detectable SPARC protein. Only NPC-TW01 pBIG2i–SPARC transfectants induced with 4 μg doxycycline showed strong SPARC protein expression in both the cell lysate and culture medium. (C) Immunofluorescent staining of NPC-TW01 pBIG2i–SPARC-transfected cells, with or without doxycycline induction; SPARC protein expression was detectable in the cytoplasm of most doxycycline-induced NPC cells (nucleus, blue; $SPARC^{+}$, green; bar = 333 μm)

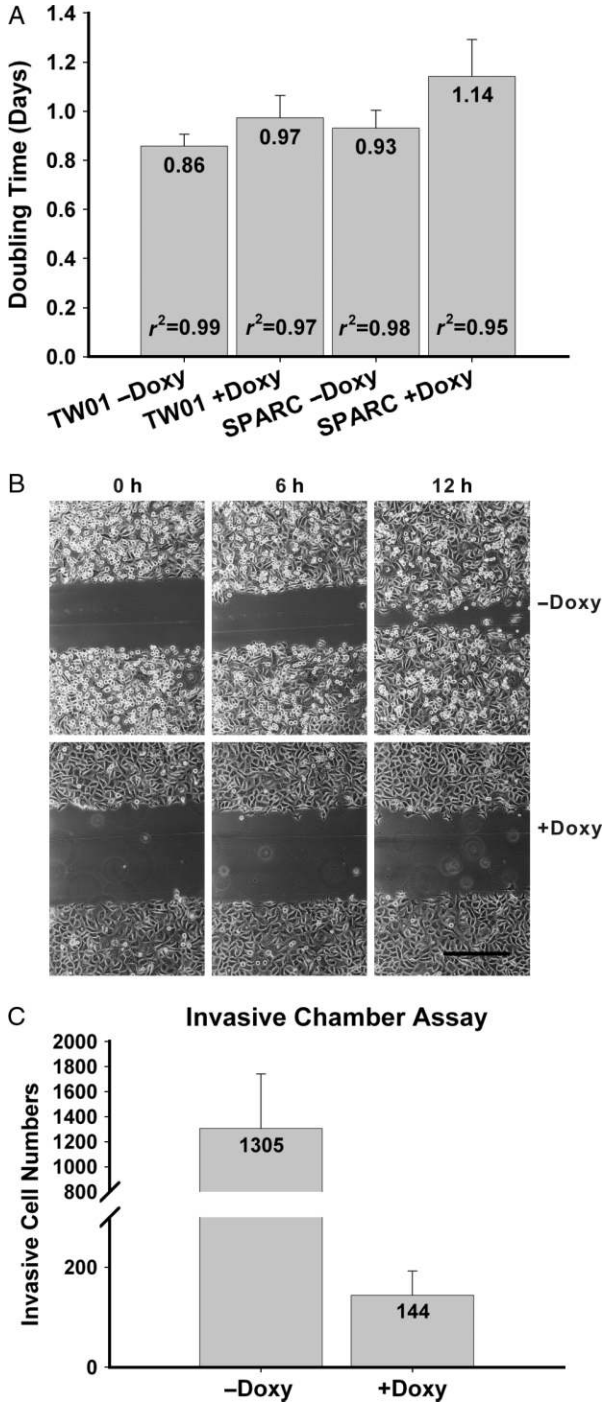

Figure 5. Effects of SPARC over-expression on the NPC-TW01 cell line. (A) SPARC over-expression, induced by 4 μg/ml doxycycline, slowed cell proliferation and increased the cell population doubling time. However, doxycycline alone did not have a significant effect on cell proliferation. (B) SPARC over-expression reduced the cell migratory capacity (bar = 2 mm). (C) Effects of SPARC on NPC-TW01 cell invasiveness. In a Matrigel invasion chamber assay, SPARC over-expression significantly reduced the NPC cell invasion capacity up to 10-fold. The mean number of invasive uninduced cells was 1305. When SPARC over-expression was induced with doxycycline, the mean number of invasive cells was reduced to 144

by the clinical data analysis that increase of SOX-5 expressing tumour cell numbers has a poor prognosis for patients' survival (Figure 1D).

Although SOX-5 is an essential TF for chondrogenesis [50] and SPARC expression is induced by the transcription factor Runt-related transcription factor 2 in osteoclasts [51], the relationship between SOX-5 and SPARC is not clear. The facts that (a) SOX-5 is markedly expressed in NPC cells (Figures 1B, 2B, Table 3), whereas SPARC protein was only weakly detectable in the NPC cell culture medium and cell lysate (Figure 4A, B); (b) SOX-5 knockdown can up-regulate SPARC expression (Figure 2A-c, 2B); and

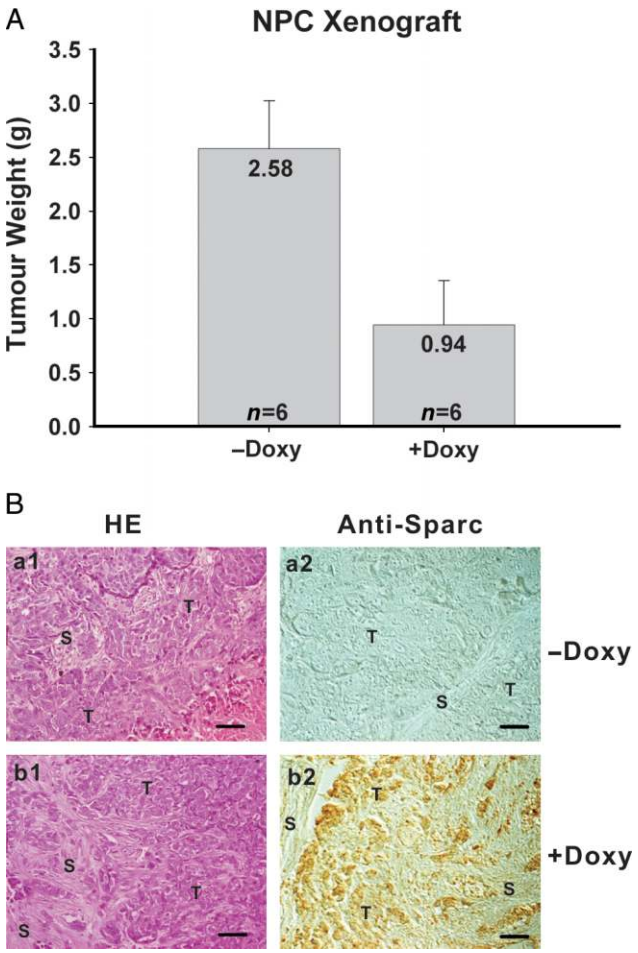

Figure 6. *In vivo* study of SPARC-over-expressing NPC-TW01 cells. (A) Over-expression of SPARC, induced with 4 μg/ml doxycycline in the drinking water, significantly reduced xenograft tumour sizes 34 days after NPC-TW01 pBIG2i–SPARC-transfected cells were injected subcutaneously into NOD SCID mice. (B) Haematoxylin–eosin and immunohistochemical staining of a xenograft tumour paraffin section (bar = 160 μm). Tumour mass without doxycycline induction showed no sign of desmoplasia (a1) and no SPARC protein expression (a2); tumour mass with doxycycline induction showed regions of desmoplasia (b1, b2) and strong SPARC protein expression in tumour cells (T in b2, brown). No SPARC protein immunostaining was visible in the stroma (S)

(c) SOX-5 protein can bind directly onto the SPARC promoter region (Figure 2C), all indicate that SOX-5 is an upstream TF that down-regulates SPARC expression.

SPARC, a matricellular protein, is a known tumour suppressor with antiproliferative effects, and modulates cell migration in certain cancer types [31–36]. These observations are consistent with our results that the over-expression of SPARC protein in NPC cells extends the cell population doubling time (Figure 5A), impairs cell migratory ability (Figure 5B) and reduces tumour cell invasiveness (Figure 5C). Contrary to these observations, in glioblastoma cell lines, SPARC can delay tumour growth, but it can also induce cell migration and invasion via the uPA–uPAR signalling pathway [34]. These phenomena suggest that SPARC plays different roles in cancer progression in different tumour cell types and acts via different signal transduction pathways. Our findings that SPARC over-expression in NPC cells can slow cell proliferation, retard cell migration, reduce cell invasiveness (Figure 5A–C) and also retard NPC xenograft tumour growth (Figure 6A) suggest that SPARC acts as a tumour suppressor in NPC pathogenesis.

In conclusion, in the present experiments, we have demonstrated that the TF *SOX-5* acts as an oncogene, playing an important role in regulating the progression of NPC pathogenesis. SOX-5 is expressed in all NPC cell lines so far examined, and in many tumour cells of advanced cases of NPC. These results imply that SOX-5 protein may be a potential tumour marker for NPC prognosis and a molecular target for chemotherapy. SOX-5 exerts its effects on NPC progression by suppressing other oncosuppressor genes, especially *SPARC*. The function of *SPARC* in NPC is as an oncosuppressor that suppresses NPC tumour growth.

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Supplementary material

Supplementary material may be found at the web address: http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2299.html

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