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Profiling and Comparing Transcription Factors Activated in Non-Metastatic and Metastatic Nasopharyngeal Carcinoma Cells

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ABSTRACT

Transcription factors (TFs) are modulators of gene expression that are critically important in the establishment and progression of human cancers. In the current study, the activity profiles of TFs in a normal nasopharyngeal epithelial cell line and in nasopharyngeal carcinoma (NPC) cell lines were studied using oligonucleotide array-based TF assays. Compared to the normal epithelial cell line NP69, nine TFs in the non-metastatic NPC cell line (6-10B) and eight TFs in a metastatic NPC cell line (5-8F) were upregulated. Among upregulated TFs, Sp1, AP2, and ATF/CREB families exhibited relatively high activities in NPC cell lines. Transcription levels of Sp1, ATF-1, ATF-2, AP2α, AP2γ, and CREB1 were higher in 5-8F cells than in 6-10B cells. In addition, higher expression of the Sp1 target genes MMP-9 and VEGF was observed in 5-8F cells. Sp1 silencing reduced VEGF and MMP-9 expression. Inhibition of Sp1 expression and activity in 5-8F cells by mithramycin resulted in downregulated expression and secretion of MMP-9 and VEGF, concomitant with inhibition of cell migration and invasion. These results suggest that dynamic changes in TF activities occur in NPC cells and that these changes may play important roles in regulating the expression of genes associated with the development and progression of NPC. J. Cell. Biochem. 109: 173–183, 2010. © 2009 Wiley-Liss, Inc.

KEY WORDS: COMPARING; TRANSCRIPTION FACTOR; METASTASIS; NASOPHARYNGEAL CARCINOMA

The occurrence of nasopharyngeal carcinoma (NPC), a tumor derived from epithelium, is highly associated with genetic and environmental factors such as Epstein–Barr virus (EBV) infection. Activation of oncogenes and inactivation of tumor suppressor genes, accompanied by other genetic and epigenetic changes such as loss of heterozygosity and gene methylation, are involved in the carcinogenesis of this neoplasm. All of these changes can cause variation in expression and function of genes that directly or indirectly participate in signal transduction, and, as the endpoint of signaling pathways, induce activity state changes of transcription factors (TFs). Moreover, some of these genes are themselves TFs. Abnormal activity changes in TFs lead to aberrant gene expression, which is highly associated with development and progression of NPC. Thus, an understanding of mechanisms of aberrant gene expression controlled by TFs that are activated in NPC may help address the phenotypic and biological characterization of this tumor.

Expression and activity changes in TFs in NPC tissues and cell lines have been reported previously [Chen et al., 2001; Hui et al., 2002; Hsiao et al., 2003; Huang et al., 2006]. However, differential activities of TFs in metastatic and non-metastatic NPC cells have not been systematically documented. Acquisition of differential activity profiles of TFs in non-metastatic and metastatic NPC cell lines is important in elucidating the mechanism by which abnormal activity of TFs dysregulates expression of genes involved in NPC invasion and metastasis.

In this study, we employed a high-throughput oligonucleotide array-based TF assay method [Qiao et al., 2008], which allowed the simultaneous assay of multiple TFs, in order to obtain the activity profiles of TFs and compare TFs present in NP69, 6-10B, and 5-8F

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cells. Using this method, we identified 18 TFs whose activities changed in NPC cells. Although AP2, ATF/CREB, and Sp1 family TFs displayed high activities in both NPC cell lines, subsequent examination showed that differential expression of components of these three families occurred in the two NPC cell lines. We also found that two target genes of Sp1, MMP-9, and VEGF, were more highly expressed in 5-8F cells than in 6-10B cells. Inhibition of Sp1 expression and activity effectively downregulated MMP-9 and VEGF expression and blocked 5-8F cell migration and invasion. These results show that changes in the activities of specific TFs occur in NPC cells and help to identify the mechanism of regulation of target genes implicated in metastasis of this tumor as well as new molecular targets for treatment of this malignancy.

MATERIALS AND METHODS

CELL CULTURE AND TRANSIENT TRANSFECTION

NP69 cells are normal human nasopharyngeal epithelial cells and have been described previously [Tsao et al., 2002]; these were kindly provided by Prof. S. W. Tsao (Department of Anatomy, The University of Hong Kong). NP69 cells were cultured using keratinocyte serum-free medium (SFM) (Invitrogen, Carlsbad, CA) supplemented with L-glutamine, epidermal growth factor, and bovine pituitary extract. NPC cell lines 6-10B (low tumorigenic and non-metastatic ability) and 5-8F (the highest tumorigenic and metastatic ability) were cultured in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum and penicillin/streptomycin. Cells were maintained at 37°C in an atmosphere containing 5% CO_2 siRNA (GeneChem, China) transfections were performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. The siRNA oligonucleotide for Sp1 was 5′-AUC ACU CCA UGG AUG AAA UGA dTdT-3′ [Higgins et al., 2006]. The targeted DNA sequence of the negative control was 5′-TTC TCC GAA CGT GTC ACG T-3′ [Takemura et al., 2006].

NUCLEAR PROTEIN EXTRACTION

Nuclear extracts (NEs) were prepared using NE-PER® nuclear and cytoplasmic Extraction Reagents (Pierce) according to the manufacturer's instructions. Protease inhibitor cocktail (Roche) was added to extraction reagents. Extracts from NP69, 6-10B or 5-8F cells were used for TF activity array analysis, electrophoretic mobility shift assays (EMSAs) and examination of protein expression in Western blots. Cells were washed twice and harvested by scraping in cold PBS. Cell pellets were resuspended in 200 μl of cytoplasmic Extraction Reagent I with vigorous vortexing and incubated on ice for 10 min. Eleven microliters of cytoplasmic Extraction Reagent II was then added to the tubes. The tubes were vortexed vigorously for 5 s and incubated on ice for 1 min, then centrifuged at 16,000*g* for 5 min. The resulting pellets were resuspended in 100 μl nuclear extraction reagent and mixed by vortexing, followed by incubation on ice for 40 min. The mixture was centrifuged at 16,000*g* for 10 min and the supernatant fraction (NE) was collected and frozen in aliquots at −80°C. The protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce).

TF ACTIVITY ARRAY ANALYSIS

We performed TF assays based on oligonucleotide arrays (CapitalBio Corporation, Beijing, China) containing a set of oligonucleotides, the sequences of which corresponded to the consensus binding sequences of 270 TFs used as probes. For the array assay, NEs (10–15 μg) from NP69, 6-10B, and 5-8F cells were incubated for 10 min on ice with the binding buffers prior to the addition of the double-stranded TF-probe mixture. Binding was carried out in a final 20 μl reaction volume containing 10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 0.5 mM EDTA, 1 mM $MgCl_2$, 4% glycerol, 0.5 mM dithiothreithol, and 0.05 μg/μl poly(dI–dC) (Amersham Biosciences). After 1 h incubation at 30°C, samples were resolved on 2% agarose gels (Sigma, USA) in chilled 0.5 × TBE for 20–25 min at 120 V. The gel area containing the protein/DNA complex was excised and transferred to a 1.5-ml tube. DNA was collected from the gel slices using a QIAEX II® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. Single primer amplification (SPA) methods using Cy3- and Cy5-labeled T7 promoter sequence were used to amplify the target DNA. The amplification reaction (20 μl) contained a mixture of double-stranded probes as template, 1× PCR buffer (Mg2+ plus, TaKaRa, Dalian, China), 500 nM Cy3- or Cy5-labeled primers, 20 nM dATP, 20 nM dTTP, 20 nM dCTP, 20 nM dGTP, and 1 U Taq™ (TaKaRa). The amplification protocol was as follows: 95°C for 5 min; 30–35 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 53°C, and elongation for 20 s at 72°C; and a final extension at 72°C for 10 min. Two SPA products with Cy3- or Cy5-label were mixed and hybridized to microarrays in 3× SSC (450 mM sodium chloride/45 mM sodium citrate, pH 7.0), 0.2% SDS, 5× Denhart's, and 25% formamide in a volume of 12 μl. Hybridization was carried out at 42°C for 16–20 h followed by sequential washing at 42°C in 2× SSC plus 0.5% SDS for 10 min, and 0.2× SSC plus 0.1% SDS for 10 min. Immediately after washing, arrays were spun dry by centrifugation for 2 min at 1,500 rpm. Array images were acquired using a CapitalBio LuxScan™-10K (A) Dual Color Confocal Scanner (CapitalBio Corporation). The signal intensity of each of the spots in the scanned images was quantified using GenePix Pro 4.0 (Axon Instruments, Molecular Devices, CA). The absolute signal intensities of the spots were calculated by subtracting the background fluorescence intensity from the detected signal intensity of the spots. The analysis of arrays was replicated twice, exchanging the Cy3- and Cy5-label. The average ratio of each spot representing the difference in binding activity for each TF was calculated and is listed in Table I.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

EMSAs were performed according to the instructions provided with the Chemiluminescent Nucleic Acid Detection Module (Pierce). Briefly, 5–15 μg NE from NP69, 6-10B, or 5-8F cells was mixed with 1× binding buffer (10 mM Tris–HCl pH 7.5, 50 mM KCl, 1 mM DDT, 1 μg poly(dI–dC), 2.5% glycerol, 5 mM $MgCl_2$, 1 mM EDTA), with or without 50× or 200× unlabeled cold probe. Biotin-labeled probe (20–100 fmol/μl) was then added and the sample was incubated at room temperature for 30 min. Cold probe was incubated with NE for 15 min at room temperature before adding biotin-labeled probe. Free probe (without NE) was used as a negative control. The consensus probe sequences for Sp1 were ATT CGA TCG GGG CGG

TABLE I. TFs Showing Differential Activity

TF	Description/alternative name	6-10B/NP69	5-8F/NP69	5-8F/6-10B
AP-2	Activator protein 2	↑3.02	↑2.14	0.71
ATF	Activating transcription factor	↑2.03	↑1.45	0.71
CREB	cAMP responsive element binding protein			
Sp1	Specificity protein 1	↑1.85	↑1.95	1.05
GATA-1	GATA binding protein 1	↑1.82	↑1.42	0.78
FOX	Forkhead box	↑1.68	↑1.35	0.80
SRY	Sex-determining region Y, TDF, testis-determining factor	↑1.69	↑1.27	0.75
C/EBP	CCAAT/enhancer binding protein	↑1.54	1.09	0.70
PAX3	Paired box 3	↑1.53	↑1.30	0.85
RFX1	Regulatory factor X 1	↑1.40	1.04	0.74
ZIC2	Zinc finger protein 2	0.91	↑1.46	↑1.54
SRF	Serum response factor	↓0.31	↓0.33	1.06
NF-E2	Nuclear factor (erythroid-derived 2)	↓0.66	↓0.57	0.80
AP-1	Activator protein 1	↓0.66	↓0.57	0.80
STAT1	Signal transducer and activator of transcription 1	↓0.67	↓0.58	0.86
GABP	GA binding protein, NRF2	↓0.67	↓0.58	0.86
Elk-1	p62 ternary complex factor	↓0.73	↓0.61	0.83
AML	Acute myeloid leukemia protein	↓0.73	↓0.66	0.90
NF-κB	Nuclear factor-kappa-B	↓0.82	↓0.65	0.80

GGC GAG C; for ATF/CREB, AGA GAT TGC CTG ACG TCA GAG AGC TAG; and for AP2, GAT CGA ACT GAC CGC CCG CGG CCC GT. The mixture was loaded on a 6% native polyacrylamide gel and electrophoresed at 120 V in 0.5% Tris–borate–EDTA (TBE). The samples were transferred to a nylon membrane at 380 mA in 0.5% TBE for 45 min and subsequently fixed on the membrane by ultraviolet crosslinking. The biotin-labeled probe was detected using streptavidin-horseradish peroxidase (HRP), and the image was visualized on X-ray film (Kodak) using lumino/enhancer and substrate. The results were normalized to the amount of protein loaded.

REAL-TIME REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA was isolated from NP69, 6-10B, and 5-8F cells using TRIzol reagent (Invitrogen). Reverse transcription was carried out using the RT-PCR system (Promega). Real-time PCR analysis was performed in a 20-μl final reaction volume using SYBR Green I Supermix (Takara) according to the manufacturer's protocol. All reactions were run in triplicate on an iCycler IQ multicolor Detection System (BioRad) with the following cycling parameters: 95°C for 10 s followed by 40 cycles of 95°C for 5 s and annealing at 60°C for 15 s and final extension at 72°C for 20 s. All quantitations were normalized to the level of GAPDH RNA in the reaction. The comparative threshold cycle (CT)(ΔΔCT) method, which compares differences in CT values between common reference RNA and target gene RNA, was used to obtain the relative fold changes in gene expression. Primers for PCR were designed using Primer 3.0 online. Primer sequence for MMP-9 forward: ttgacagcgacaagaagtgg, reverse: gccattcacgtcgtccttat; VEGF forward: aaggaggagggcagaatcat, reverse: atctgcatggtgatgttgga; GAPDH forward: gagtcaacggatttggtcgt, reverse: ttgattttggagggatctcg. Primer sequences for members of AP2, ATF/CREB, and Sp1 families are listed in Table II. The results are expressed as mean ± SE.

TABLE II. Primer Sequences Used for RT-PCR Analysis of Expression of AP2, ATF/CREB, and Sp1 Family Members

Gene	Accession no.	Primer location and sequence	Product size (bp)
Sp1-F	NM_138473	299–318, tgcagcagaattgagtcacc	246
Sp1-R		525–544, cacaacatactgcccaccag	
Sp2-F	NM_003110	118–137, ccagggaggaagatgtcgta	191
Sp2-R		289–308, aggaggtgtcacagcagctt	
Sp3-F	NM_003111	691–710, tccagtcagcagatggtcag	227
Sp3-R		898–917, ttgggtttgaccaggaaaag	
Sp4-F	NM_003112	1127–1146, gaatctccctcctcctccac	250
Sp4-R		1357–1376, caatttgcacctgctgaaga	
ATF-1-F	NM_005171	321–340, caacctggttcagcagttca	197
ATF-1-R		498–517, tttctgccccgtgtatcttc	
ATF-2-F	NM_001880	1245–1264, ctccagctcacacaactcca	247
ATF-2-R		1472–1491, tgtttcagctgtgccacttc	
ATF-3-F	NM_001030287	874–893, caagtgcatctttgcctcaa	167
ATF-3-R		1021–1040, ccacccgaggtacagacact	
ATF-4-F	NM_182810	411–430, tcaaacctcatgggttctcc	226
ATF-4-R		617–636, gtgtcatccaacgtggtcag	
CREB1-F	NM_004379	93–112, gtgttacgtgggggagagaa	201
CREB1-R		274–293, gggctaatgtggcaatctgt	
AP2-α-F	NM_001032280	3325–3344, actggccatattggaagcag	165
AP2-α-R		3470–3489, tacgcctgggtaaagacagc	
AP2-β-F	NM_003221	5122–5141, ggtcgaatggaaaactcgaa	172
AP2-β-R		5274–5293, tcttgaggcaacatcagacg	
AP2-γ-F	NM_003222	1724–1743, agccttcactggttctgcat	207
AP2-γ-R		1911–1930, tggcccagggaatgtattta	

CELL WHOLE PROTEIN EXTRACTION

For total protein extraction, cells were lysed on ice for 30 min in lysis buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM NaF, 1 mM Na_3VO_4, and complete protease inhibitor cocktail). The cell lysates were then centrifuged at 12,000 rpm for 10 min, and protein contents were determined by BCA protein assay kit (Pierce).

WESTERN BLOT ANALYSIS

Protein extracts (50 μg) were resolved on 10% SDS–polyacrylamide gels. The proteins were transferred onto PVDF membranes, incubated with 5% skim milk at room temperature in TTBS (20 mM Tris–HCl, pH 7.5, 500 mM NaCl, 0.1% Tween-20), and then incubated at 4°C for 12 h with rabbit polyclonal primary antibody against Sp1 (PEP2), Sp2 (K-20), Sp3 (D20), and Sp4 (V-20) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Sp1 and Sp3 were each diluted 1:200; Sp2 and Sp4 were diluted 1:50. The mouse monoclonal anti-human β-actin antibody sc-47778 (Santa Cruz) was used 1: 200. Rabbit polyclonal primary antibody against VEGF (Boster Biological Technology, China) was diluted 1:200. After washed with TTBS, the membranes were incubated at 37°C for 1 h with goat anti-rabbit IgG and goat anti-mouse IgG secondary antibodies diluted 1:1,000 (Boster Biological Technology). The membranes were developed using the chemiluminescent substrate ECL detection system (Amersham Biosciences) and bands were visualized on X-ray film (Kodak). To measure secreted MMP-9 protein, the conditioned medium from each sample was collected and concentrated using a Centricon 10 microconcentrator (Amicon, Beverly, MA). The concentrates were subjected to protein analysis. Rabbit polyclonal primary antibody against MMP-9 (Boster Biological Technology) was diluted 1:200.

CELL CYTOTOXICITY ASSAYS

To determine cytotoxic effects of mithramycin (MIT, Amresco, Solon Industrial Parkway, OH) on 5-8F cells, cell cytotoxicity assays were performed using a CellTiter 96 AQ One Solution Cell Proliferation/Cytotoxicity Assay kit (MTS, Promega) according to the manufacturer's instructions. 5-8F cells were seeded in 96-well plates at 1×10^4 per well and cultured overnight. After washing three times with SFM, cells were treated with various concentrations of MIT (0.1–1.6 μmol/L) in 100 μl SFM for 24 or 48 h. Then, 20 μl/well of combined 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium/phenazine methosulfate solution was added. After incubation for 1 h at 37°C in a humidified 5% CO_2 atmosphere, the absorbance at 490 nm was recorded using an ELISA plate reader. The ratio of the number of dead cells to the number of viable cells in the control was calculated and plotted. The results are expressed as mean ± SD.

DETECTION OF VEGF AND MMP-9 BY ELISA

5-8F cells in subconfluent culture condition (~80–90% confluent) were washed and incubated with or without indicated concentrations of mithramycin for 24 h. The conditioned SFM was collected, centrifuged, and frozen at −70°C until assayed. The protein concentrations of total MMP-9 (pro- and active MMP-9) and VEGF in the culture supernatants were measured using ELISA kits (Boster Biological Technology) according to the manufacturer's instructions. The results are expressed as mean ± SE.

MIGRATION ASSAYS

Cell migration was evaluated using the scratch wound assay as previously described [Li et al., 2007]. 5-8F cells were cultured to 90% confluence and washed with SFM. The cell monolayers were then disrupted by scratching with a 10-μl plastic pipette tip. After the cells were treated with mithramycin at indicated concentrations for 24 h, the wound areas were photographed using an inverted microscope. The extent of migration (the furthest distance that cells migrated from the wound edge) was measured and was expressed as an average value measured from five independent microscope fields. The results are expressed as mean ± SD.

INVASION ASSAYS

Invasion assays were performed using Transwell® (Corning, Corning, NY) as previously described [Kwak et al., 2006]. Matrigel (Becton Dickinson, Bedford, MA) was diluted in cold SFM and 25 μg of the preparation was added to the upside of porous filters (pore size, 8 μm) and allowed to gel at 37°C overnight. After the coated filters were rehydrated with 100 μl medium, 1×10^5 cells in 200 μl SFM supplemented with 0.2% bovine serum albumin were seeded into the upper part of each chamber and were treated with the indicated concentrations of mithramycin, whereas the lower compartments were filled with 500 μl of culture medium. Following incubation for 24 h in a humidified incubator at 37°C with 5% CO_2, non-invaded cells on the upper surface of the filter were wiped off using a cotton swab, and the invaded cells on the lower surface of the filter were fixed and stained with hematoxylin. Invasiveness was determined by counting cells in four microscopic fields per well, where the extent of invasion was expressed as an average number of cells per microscopic field. The results were expressed as mean ± SD.

STATISTICAL ANALYSIS

All results are presented as mean ± SD or SE of three independent experiments. Statistical significance ($P < 0.05$) was determined using Student's t test.

RESULTS

TRANSCRIPTION FACTORS ACTIVATED IN NPC CELLS

As shown in Figure 1, some TFs displayed different activities in NP69, 6-10B, and 5-8F cells. By comparing the ratio of each spot in the arrays, 10 upregulated TFs and 8 downregulated TFs were identified in the two NPC cell lines (Table I). Among the upregulated TFs, AP2, ATF/CREB, and Sp1 families displayed differential activities in NPC cells compared with NP69 cells (Fig. 1). To confirm these results, EMSA was performed to detect AP2, ATF/CREB, and Sp1 family binding activity in vitro using the corresponding consensus DNA binding elements. As shown in Figure 2, the binding activities of AP2, ATF/CREB, and Sp1 were enhanced in NPC cells as compared to NP69 cells. Specific binding of TFs was confirmed by using a cold probe.

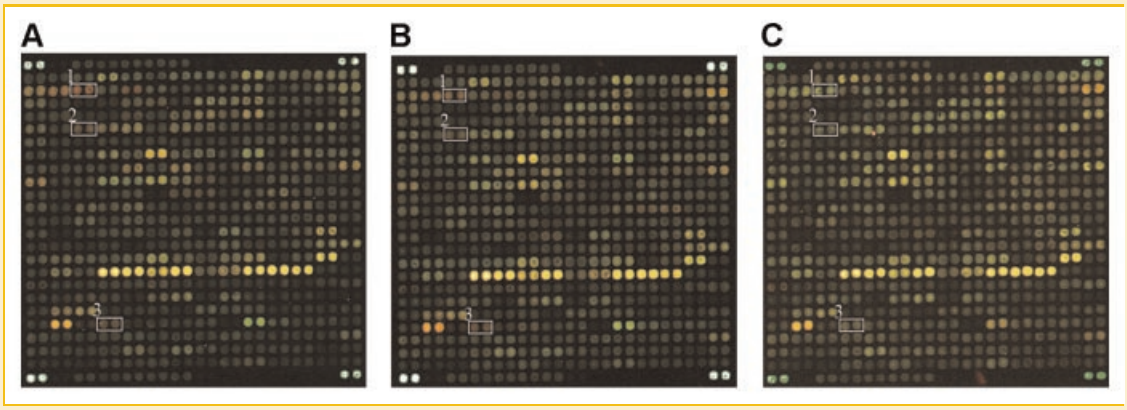

Fig. 1. Fluorescence images of arrays used for activity detection of TFs extracted from NP69 cells, 6-10B cells, and 5-8F cells. A: 6-10B versus NP69; B: 5-8F versus NP69; C: 6-10B versus 5-8F. The representative TFs showing upregulated activity are identified by boxes. 1: AP2, 2: ATF/CREB, 3: Sp1.

DIFFERENTIAL EXPRESSION OF MEMBERS OF AP2, ATF/CREB, AND Sp1 FAMILIES

Many studies have demonstrated that members of different TF families show different expression and function in various tumors; this led us to examine the expression status of different members among three families of TFs in NPC cell lines. The results of array and EMSA assays showed increased activities of TFs of the AP2, ATF/CREB, and Sp1 families in NPC cells; therefore, we also measured the mRNA levels of major genes of these three families of TFs by real-time PCR. To ensure specific amplification of homologous genes belonging to each TF family, sequences of PCR products were pre-blasted by complying with BLAST procedure of NCBI online (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). As shown in Figure 3A, the expression of AP2α and AP2β was markedly upregulated in 6-10B and 5-8F cells; AP2α expression in 5-8F was significantly higher than in 6-10B cells. Notably, AP2γ was apparently overexpressed only in 5-8F cells. These results suggest that AP2α and AP2γ may play important roles in regulating genes that function to initiate and/or maintain malignant and metastatic characteristics of 5-8F cells. With respect to the ATF/CREB family (Fig. 3B), we found that ATF1 increased gradually in NPC cells. Interestingly, ATF2 and CREB1 were downregulated in 6-10B cells but upregulated in 5-8F cells. In contrast, ATF-3 and ATF-4 expression were decreased in both the NPC cell lines. These data show that members of the ATF/CREB family present aberrant expression status in 6-10B and 5-8F cells. Further, the mRNA level of Sp1 was increased significantly in both NPC cell lines, Sp2 and Sp3 showed no evident difference between NP69 cells and NPC cell lines, and Sp4 was decreased in NPC cells (Fig. 3C).

Sp1 FAMILY PROTEIN AND VEGF AND MMP-9 EXPRESSION IN NPC CELLS

We previously reported that Sp1 is expressed with high abundance in NPC cells [Liu et al., 2008]. To further validate the results observed using RT-PCR, the protein levels of four members of the Sp1 family in the nucleus were examined. As shown in Figure 4A, nuclear Sp1 protein was increased in NPC cells, especially in 5-8F cells. Sp2 protein in the nucleus was very low, and no difference was observed in NPC cell lines. Surprisingly, Sp3 mRNA expression was not significantly different in 6-10B and 5-8F cells (Fig. 3C); however,

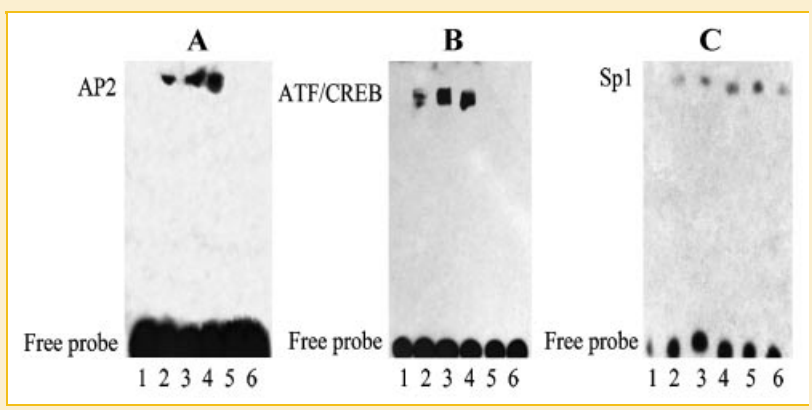

Fig. 2. Confirmation of DNA binding activity of AP2, ATF/CREB, and Sp1 families from nuclear extracts using EMSA. A,B: Lane 1, free probe; lane 2, NP69; lane 3, 6-10B; lane 4, 5-8F; lane 5, 6-10B + 200× cold probe; lane 6, 5-8F + 200× cold probe. C: Lane 1, free probe; lane 2, NP69 + 50× cold probe; lane 3, NP69; lane 4, 6-10B, lane 5, 5-8F; 6: 5-8F + 50× cold probe.

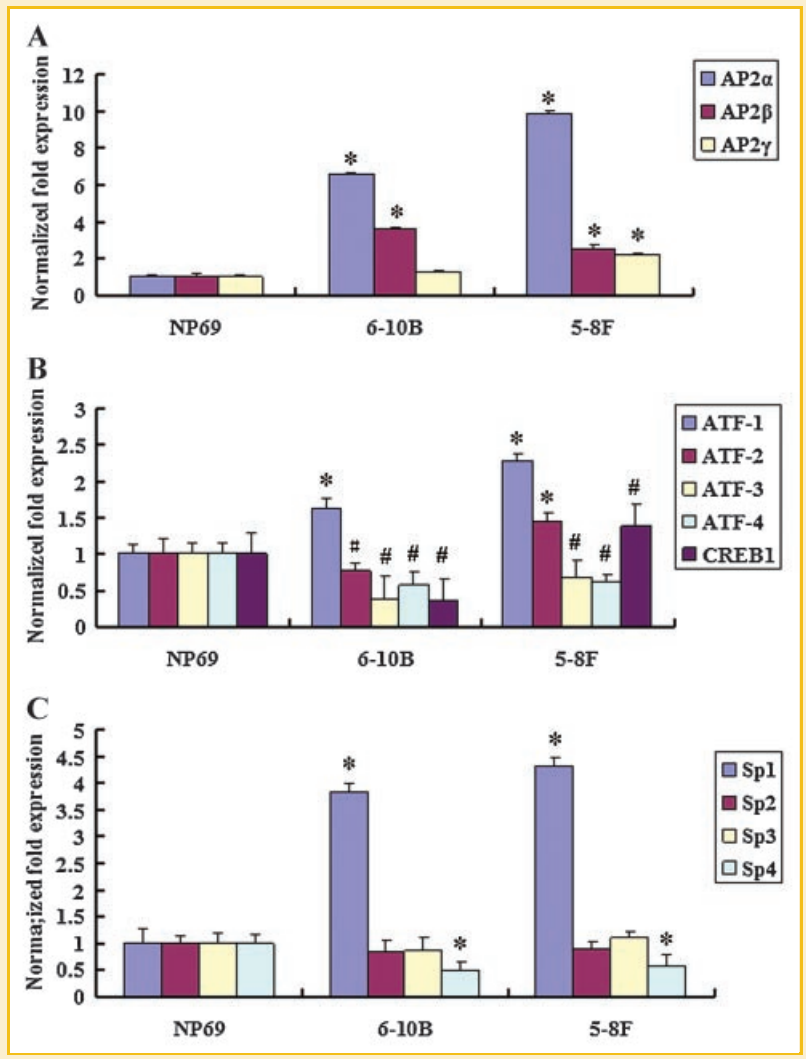

Fig. 3. The expression of members of the AP2, ATF/CREB, and Sp1 families of transcription factors in NPC cells as detected by RT-PCR. A: AP2; B: ATF/CREB1; C: Sp1. Quantitative reverse transcription-PCR was performed on amplified RNA to determine the expression of members of AP2, ATF/CREB, and Sp1 families of transcription factors. GAPDH was used as internal control for normalization. Means of fold changes and SE for each mRNA were calculated as described in Materials and Methods Section. The results are shown as the ratios of means of NPC cells compared with NP69 cells. $^{*}P < 0.01$; $^{\#}P < 0.05$.

the nuclear levels of Sp3 protein were elevated in both the NPC cell lines, similar to the tendency with Sp1. Sp1 levels were relatively more increased than those of Sp3. These data indicate that elevated expression of Sp1 may predominantly account for the increased activity of Sp1 family in NPC cells. In addition, Sp4 levels in NPC cell nuclei were decreased.

Overexpression of Sp1 or increased Sp1 binding activity has been implicated in upregulation of tumor invasion and metastasis-associated genes such as MMP-9 and VEGF [Sato and Seiki, 1993; Wu et al., 2005]. To explore the significance of overexpression and overactivation of Sp1 in 5-8F cells, which have high-metastatic capability compared to 6-10B cells, we measured the expression of MMP-9 and VEGF in these cells. As shown in Figure 4B, the expression of MMP-9 and VEGF in 5-8F cells was much higher than in 6-10B cells. These data suggest that the increased intranuclear expression and activity of Sp1 may be associated with higher expression of MMP-9 and VEGF in 5-8F cells.

Sp1 ACTIVATION IS INVOLVED IN VEGF AND MMP-9 EXPRESSION AND SECRETION IN 5-8F CELLS

To further determine whether inhibition of Sp1 directly affects VEGF and MMP-9 expression in 5-8F cells, 50 nM Sp1 siRNA was transfected into 5-8F cells. Sp1 siRNA significantly decreased Sp1 protein level, VEGF expression and MMP-9 secretion (Fig. 5A,B). We also applied mithramycin, an inhibitor of Sp1 TF [Jungert et al., 2006; Kwak et al., 2006]. At first, we incubated 5-8F cells in medium alone or in medium containing mithramycin at various concentrations ranging from 0.1 to 1.6 μmol/L for 24 and 48 h, respectively. The cytotoxic effect of mithramycin on 5-8F cells was assessed using MTS assay. Treatment with mithramycin produced dose-dependent cytotoxic effects on 5-8F cells. Based on calculated IC_{50} values for mithramycin (Fig. 6A), significant cytotoxicity occurred with treatment for 48 h. Thus, incubation with mithramycin at lower concentrations (0.1, 0.2, 0.4 μmol/L) for 24 h was used in subsequent experiments. As shown in Figure 6B, mithramycin effectively

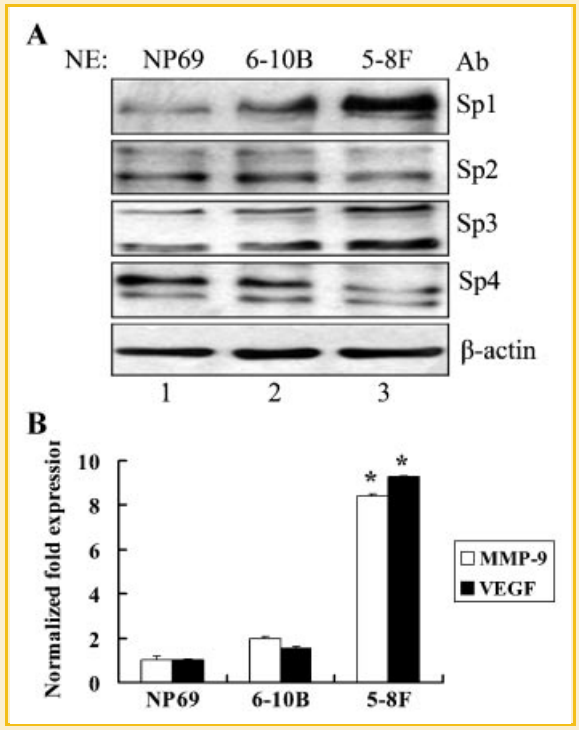

Fig. 4. Analysis of Sp1 family protein and VEGF and MMP-9 expression in NPC cells. A: Sp1 family protein levels of nuclear extract (NE) from NPC cells were determined by Western blot. Line 1: NP69 cells; lane 2: 6-10B cells; line 3: 5-8F cells. Antibodies are shown to the right: Sp1, Sp2, Sp3, Sp4, and β-actin. B: The expression of VEGF and MMP-9 regulated by Sp1 in NPC cells were detected by RT-PCR, and GAPDH was used as internal control for normalization. Means of fold changes and SE for each mRNA were calculated as described in Materials and Methods Section, and the relative fold expression is shown. $^{*}P < 0.01$ indicated as 5-8F cells versus 6-10B cells.

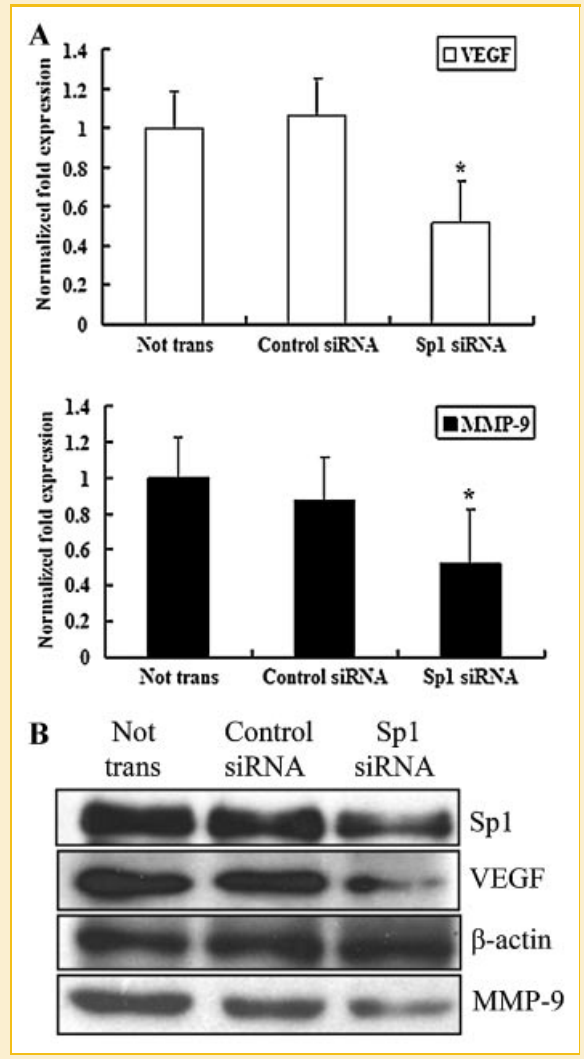

Fig. 5. Sp1 silencing inhibits VEGF and MMP-9 expression and secretion in 5-8F cells. Cells were transfected with Sp1 siRNA (50 nmol/L) or control siRNA for 24 h. A: VEGF and MMP-9 expression were detected by RT-PCR, and GAPDH was used as internal control for normalization. Means of fold changes and SE for each mRNA were calculated as described in Materials and Methods Section. $^{*}P < 0.05$ versus Not trans. B: Total protein lysates and conditioned medium were harvested, and the level of Sp1, VEGF, and MMP-9 proteins were determined using Western blot analysis. Antibodies are shown to the right: Sp1, VEGF, MMP-9, and β-actin were used as internal control.

inhibited Sp1 expression in a dose-dependent manner, concomitant with decreased VEGF expression in 5-8F cells. This result is consistent with previous reports showing an inhibitory effect of mithramycin on Sp1 and VEGF expression in human pancreatic cancer cells [Jia et al., 2007]. Mithramycin had no significant effect on Sp3 expression, while Sp1 decreased expression in nucleus was observed (Fig. 6C). Inhibition of Sp1 DNA binding activity by mithramycin was also seen (Fig. 6D). VEGF and MMP-9 expression and secretion in 5-8F cells were also reduced in a dose-dependent manner by mithramycin treatment (Fig. 6E,F). These results suggest that the inhibitory effect of mithramycin on the expression of VEGF and MMP-9 may have been mediated through downregulation of Sp1 expression and DNA binding activity.

INHIBITION OF Sp1 ACTIVATION IS INVOLVED IN INHIBITING 5-8F CELL MIGRATION AND INVASION

We next examined whether suppression of MMP-9 and VEGF gene expression affects migration and invasion activities of 5-8F cells. As expected, treatment with mithramycin significantly blocked migration (Fig. 7A) and invasion of 5-8F cells (Fig. 7B). These data therefore indicate that high expression and activity of Sp1 in 5-8F cells may, at least in part, be important in promoting the potentiality of invasion and metastasis through upregulating expression of MMP-9 and VEGF.

DISCUSSION

In this study, we screened activity changes in some TFs in NPC cell lines compared with normal epithelial cell lines by oligonucleotide array-based TF assay. Among abnormally activated TFs, AP2, ATF/CREB, and Sp1 families displayed obviously upregulated activities in NPC cells. It should be noted that though the expression state of some genes of these three families have been investigated in other

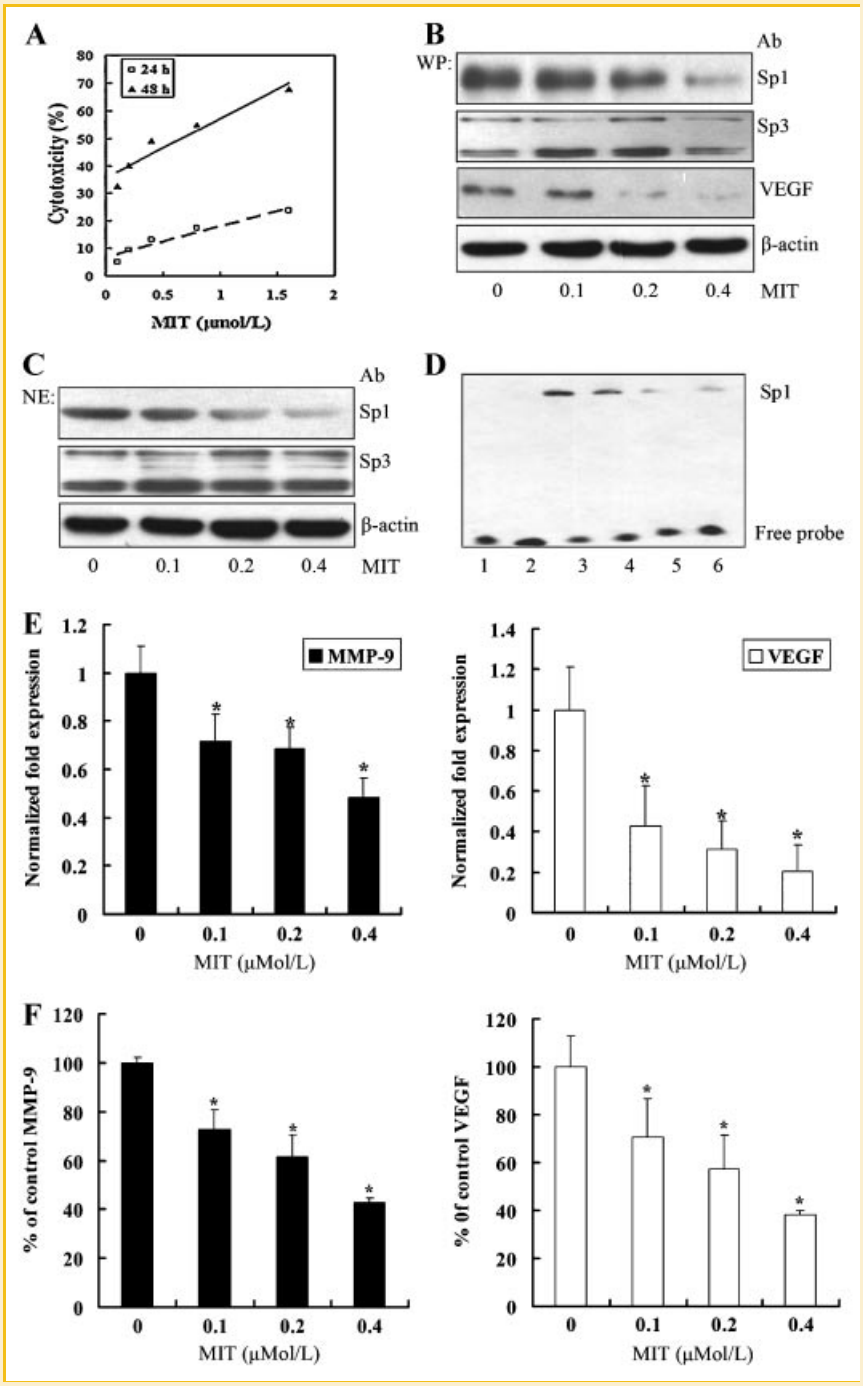

Fig. 6. Effect of mithramycin on Sp1 expression and VEGF and MMP-9 expression and secretion of 5-8F cells. 5-8F cells were incubated for 24 h in medium alone or a medium containing mithramycin at the indicated concentrations. A: Cytotoxic effect of mithramycin on 5-8F cells was assessed using MTS assay. B: Total protein lysates were harvested, and the level of Sp1, Sp3, and VEGF protein expression were determined using Western blot analysis. Antibodies are shown to the right: Sp1, Sp3, VEGF, and β-actin was used as internal control. C: Sp1 and Sp3 levels of nuclear extract (NE) from 5-8F cells were detected by Western blot. β-Actin was used as internal control. D: Sp1 activity in vitro was determined by EMSA. Lane 1, free probe; lane 2, control + 200× cold probe; lane 3, control; lane 4, 0.1 μmol/L MIT, lane 5, 0.2 μmol/L MIT; lane 6, 0.4 μmol/L MIT. E: VEGF and MMP-9 expression were detected by RT-PCR, and GAPDH was used as internal control for normalization. Means of fold changes and SE for each mRNA were calculated as described in Materials and Methods Section, and the relative fold expression is shown. F: Supernatants were collected after 5-8F cells were treated with mithramycin for 24 h. VEGF and MMP-9 secretion levels of 5-8F cells were analyzed using an ELISA assay kit, and the relative percentage compared with control is shown. MIT, mithramycin; $^{*}P < 0.05$ versus control.

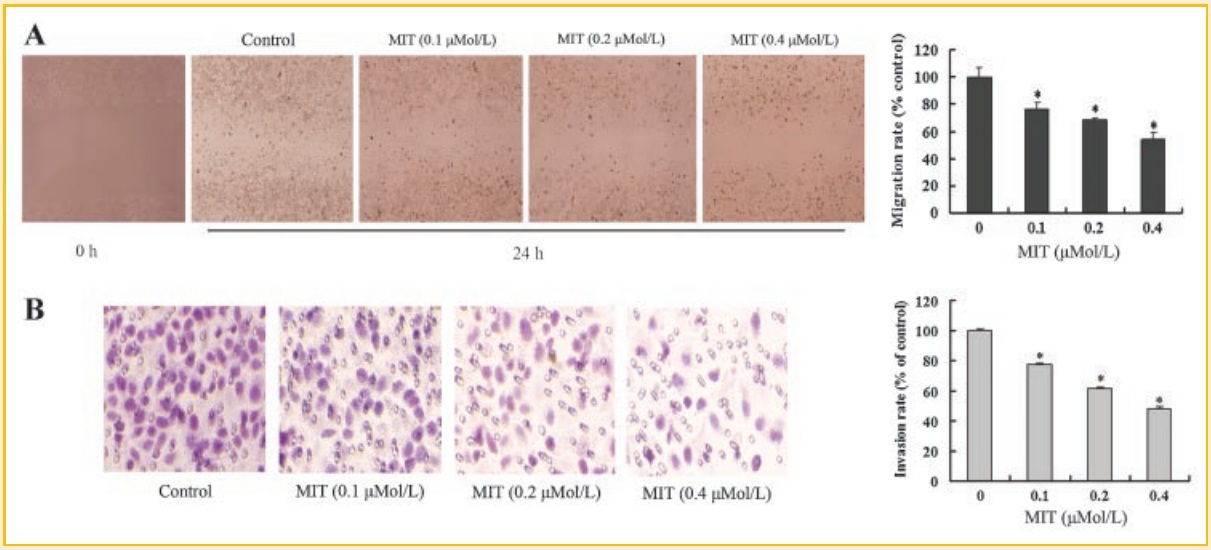

Fig. 7. Effect of mithramycin on migration and invasion of 5-8F cells. 5-8F cells were treated with mithramycin for 24 h. A: Cell migration was analyzed using the scratch wound assay. The migration distance was calculated as described in Materials and Methods Section. Migration rate was expressed as the ratio of migration distance between treated and untreated cells at indicated mithramycin concentrations. B: Invasion rate was determined by the ratio of means of cell numbers between treated and untreated cells at indicated mithramycin concentrations. MIT, mithramycin; $^{*}P < 0.05$ versus control.

tumors, they have not yet been completely characterized in NPC cells.

Studies have shown that members of the AP2 family act as tumor suppressor genes [Heimberger et al., 2005; Schwartz et al., 2007] or oncogenes [Paonessa et al., 2006; Orso et al., 2008] in tumors. When EGFRs were activated in AP-2 null keratinocytes of AP2 knockout mice, lack of AP2α elevated expression of epidermal growth factor receptor (EGFR) in differentiating layers of epidermis led to cell hyperproliferation [Wang et al., 2006]. However, it has also been reported that AP2α is significantly increased in proliferating keratinocytes of human squamous cell carcinoma [Oyama et al., 2002]. AP-2 transcriptional activity decreases in differentiated human epidermal keratinocytes, but remains unchanged in differentiation-insensitive squamous cell carcinoma cell lines [Popa et al., 2004]. In the present study, we found that AP2 activity was increased in NPC cells. Overexpression of AP2α was detected in both NPC cell lines studied, and a higher level of AP2α expression was seen in metastatic 5-8F cells. A previous report showed AP2β-mediated specific activation of human telomerase reverse transcriptase in non-small cell lung cancer cells [Deng et al., 2007]. In our study, AP2β was elevated in both NPC tumor cell lines, although there was no notable difference between the two cell lines. We found that AP2γ expression is apparently only increased in 5-8F cells. Jäger et al. [2003] demonstrated AP2γ-stimulated proliferation and impaired differentiation in an AP2γ transgenic mouse model, though no carcinoma development was observed. These results suggest a role of AP-2γ in the maintenance of a proliferative and undifferentiated state of cells. We speculate that AP2α and AP2β may be involved in gene regulation during the carcinogenic process of NPC, and that AP2γ may perform functions during the late phase of NPC.

Overexpression of ATF-1 in melanoma cells is thought to contribute to the acquisition of the metastatic phenotype [Jean et al., 2000]. MMP-2 expression induced by platelet-activating factor is related to ATF-1 and CREB1 activation via the p38 MAPK signal pathway in metastatic melanoma cells [Melnikova et al., 2006]. ATF-2 upregulation of MMP-2 expression via p38 activation induced an invasive phenotype in preneoplastic MCF10A human breast epithelial cells [Kim et al., 2007]. Our results showed that ATF-1 was overexpressed gradually in 6-10B and 5-8F cells. Interestingly, ATF-2 and CREB1 expression were decreased in 6-10B but increased in 5-8F. Elevation of phosphorylated ATF-2 was observed in NPC biopsies [O'Neil et al., 2008]. Based on our results, we presume that enhanced expression of ATF-1, ATF-2, and CREB1 in 5-8F cells is associated with high tumorigenicity and metastasis in 5-8F cells. In addition, a strong association between EBV infection and NPC has been widely accepted since specific viral oncogenes, latent membrane protein 1 and 2 (LMP1, LMP2) were critical to development of dysplasia and carcinoma in infected cells [reviewed by Young and Murray, 2003]. ATF-1, ATF-2, and CREB1 have been implicated in LMP1 and LMP2 expression in a B lymphocyte cell line [Sjöblom et al., 1998]. Because both NPC cell lines used in the present study were originally identified as EBV-positive cells [Song et al., 2002], we inferred that members of the ATF/CERB family not only give rise to gene expression variation underlying this tumor progression, but are also involved in the EBV carcinogenic process. Researches have indicated that ATF-3 and ATF-4 participate in tumor progression under conditions of hypoxia or anoxia [reviewed by Ameri and Harris, 2008]. We observed that transcriptional levels of ATF-3 and ATF-4 were decreased in both NPC tumor cell lines. We presume that these factors were not induced since, in our study, NPC cells were cultured in normoxic conditions lacking other stress factors.

Sp1 and Sp3 are ubiquitously expressed in a variety of tissues and cells. Usually, Sp1 plays a predominant role in gene regulation,

while Sp3 functions as a coactivator or suppressor, itself displaying very weak activity in transcriptional regulation [reviewed by Suske, 1999]. Sp1 is implicated in the development and progression of many tumors, and regulates a variety of genes associated with angiogenesis [Wu et al., 2005; Kanai et al., 2006], invasion and metastasis [Sato and Seiki, 1993; Sroka et al., 2007; Takami et al., 2007]. HER-2/neu induced interaction of Sp proteins with HDAC1 inhibits the expression of the metastasis suppressor RECK and increases MMP-9 secretion in NIH/3T3 cells and human HaCaT keratinocytes [Hsu et al., 2006]. In the current study, we found that activity of Sp1 family TFs was gradually increased in 6-10B and 5-8F cell lines. The progressive increase of Sp1 and Sp3 proteins in the nuclei of these cells further demonstrated the sustained high activity of Sp1 and Sp3 in NPC cells. In 5-8F cells, Sp1 exhibited higher apparent expression in the nucleus than Sp3. Moreover, higher expression of MMP-9 and VEGF, well-known target genes of Sp1 [Sato and Seiki, 1993; Wu et al., 2005], was observed in 5-8F cells than in 6-10B cells. As expected, inhibition of Sp1 expression and activity downregulated MMP-9 and VEGF expression, concomitant with suppression of migration and invasion of 5-8F cells. Therefore, we speculate that invasion and metastasis of 5-8F cells may be promoted, at least in part, through Sp1 over-activation of target gene transcription, though it is possible that other TFs also regulate these genes. Taken in this context, Sp1 may be an effective targeting molecule by means of which anti-tumor therapy could be used to block overexpression of metastasis-associated genes in this tumor. In addition, RECK expression in TW04 NPC cells was inhibited by LMP1 via the ERK/Sp1 signaling pathway [Liu et al., 2003], while its constitutive expression was stimulated by Sp1 in an EBNA2-independent pattern in a B lymphocyte line [Sjöblom et al., 1998]. We previously reported that Sp1 is required for activation of BRD7, a bromodomain gene, which is considered a candidate tumor suppressor gene in NPC; methylation of its promoter blocked Sp1 activation of this gene [Liu et al., 2008]. This implies that tumor suppressor gene silencing in NPC cells may disrupt the balance of normal gene expression regulated by Sp1 and facilitate expression of oncogenes which promote tumorigenesis and progression of NPC.

In summary, through the use of TF arrays, we identified differentially activated TFs in NPC cell lines. Differential expression of members of the AP2, ATF/CREB, and Sp1 families was observed in metastatic and non-metastatic NPC cell lines. Further studies revealed that overactivated Sp1 is crucial in promoting over-expression of MMP-9 and VEGF, which may be associated with the more aggressive phenotype of metastatic NPC cell lines. These results provide important clues to the regulatory mechanisms by which differential activities of TFs mediate the abnormal expression of target genes related to invasion and metastasis in NPC tumors.

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