**FEZF2, a novel 3p14 tumor suppressor gene, represses oncogene EZH2 and MDM2 expression and is frequently methylated in nasopharyngeal carcinoma**

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**Nasopharyngeal carcinoma (NPC)** is an Epstein-Barr virus-associated tumor prevalent in southern China and southeast Asia, with the 3p14–p12 locus reported as a critical tumor suppressor gene (TSG) region during its pathogenesis. We identified a novel 3p14.2 TSG, **FEZF2** (FEZ family zinc finger 2), for NPC. **FEZF2** is readily expressed in normal tissues including upper respiratory epithelium, testis, brain and ovary tissues, as well as in immortalized nasopharyngeal epithelial cell line NP69, but it is completely silenced in NPC cell lines due to CpG methylation of its promoter, although no homozygous deletion of **FEZF2** was detected. 5-Aza-2′-deoxycytidine treatment restored **FEZF2** expression in NPC cell lines along with its promoter demethylation. **FEZF2** was frequently downregulated in NPC tumors, with promoter methylation detected in 75.5% of tumors, but only in 7.1% of normal nasopharyngeal tissues. Restored **FEZF2** expression suppressed NPC cell clonogenicity through inducing G1/M cell cycle arrest and apoptosis and also inhibited NPC cell migration and stemness. **FEZF2** acted as a histone deacetylase-associated repressor downregulating multiple oncogenes including **EZH2** and **MDM2**, through direct binding to their promoters. Concomitantly, overexpression of **EZH2** was frequently detected in NPC tumors. Thus, we have identified **FEZF2** as a novel 3p14.2 TSG frequently inactivated by promoter methylation in NPC, which functions as a repressor downregulating multiple oncogene expression.

**Introduction**

Cancer is a disease caused by accumulated genetic and epigenetic changes (1). Abnormal activation of oncogenes and/or inactivation of tumor suppressor genes (TSGs) due to these alterations confer precancerous cell growth advantages, further leading to tumor initiation and progression (2,3). Nasopharyngeal carcinoma (NPC) is a malignancy virtually 100% associated with Epstein-Barr virus infection and shows unique geographical and ethnic distribution with high incidence in southern China and southeast Asia (4). Genetic alterations in tumors, such as homozygous deletion or loss of heterozygosity, pinpoint potential loci for critical TSGs (1). A recent new model of cancer gene island phenomenon was proposed, revealing that hemizygous deletions preferentially occur in small gene islands harboring high density of TSGs to maximize the proliferative advantage of tumor cells (5). Loss of 3p (3p21 and 3p14–p12) is common in NPC (4) and other tumors including lung (6), esophageal (7) and breast (8) cancers. Several critical TSGs within this region, inactivated by genetic and/or epigenetic mechanisms, have been identified for NPC and other tumors, such as **FHIT** (3p14.2) (9), **PTPRG** (3p21–p14) (10), **ADAMTS9** (3p14.1) (11), **RASSF1A** (3p21.3) (12) and **MLH1** (3p21.3) (13). We have also refined several minimal deleted regions in 3p including 3p14–p12 in NPC, using 1 Mb array-based comparative genomic hybridization (aCGH), and identified some functional TSGs, like **BLU** (14), **PLCD1** (15) and **DLEC1** (16), as well as a novel 3p14 candidate tumor suppressor **FEZF2** (FEZ family zinc finger 2, also known as **ZNFS132** or **ZFP312**).

First isolated as a neural-specific gene, **Fezf2** is specifically expressed in the forebrain of Xenopus, zebra fish and mouse, and regulates forebrain development through controlling neuronal differentiation (17–24). **FEZF2** belongs to the **FEZF** (forebrain embryonic zinc finger) protein family, which includes another member **FEZF1** (also known as **ZNFS132B**). Like **FEZF2**, **FEZF1** also plays a critical role during neural development (21,25). Recent data demonstrated that human **FEZF1** acts as an oncogene mediating the transcription activation of **K-RAS**, thus contributing to gastric cancer progression (26). However, the role of **FEZF2** in human tumorigenesis is still unknown.

In this study, we examined the epigenetic alteration of **FEZF2** in NPC and further characterized its tumor suppressive functions and the underlying molecular mechanisms during NPC pathogenesis.

**Materials and methods**

**Cell lines and tumor samples**

A series of NPC and glioma cell lines were used (27,28). Cell lines were cultured in RPMI 1640 (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum. Immortalized, non-transformed normal epithelial cell line NP69 was used as a control (29). Cell lines were obtained either from the American Type Culture Collection or from our collaborators. RNA samples of three normal nasopharyngeal biopsy tissues were described previously (30). DNA samples of normal nasopharyngeal and primary NPC tissues were described previously. All the NPC tissues were Epstein-Barr virus positive (28). Nasal swab DNA samples from NPC patients were also used. C15, C17 and C18, three nude mice-passaged undifferentiated NPC tumors from North Africans, were used (31). Paired RNA samples of tumors and adjacent normal tissues were obtained from BioChain (Newark, CA) or Stratagene (Santa Clara, CA). Human normal tissues RNA were purchased from Stratagene, BioChain or Chemicon (Billerica, MA).

**Array-based comparative genomic hybridization**

The aCGH was carried out as reported previously (32), using 1 Mb resolution whole-genome arrays from Sanger Institute (Cambridge, UK) with 3040 bacterial artifical chromosome/P1-derived artificial chromosome clones. Log2 ratio ranging from −0.2 to −0.7 was considered as hemizygous deletion.

**Semiquantitative and quantitative reverse transcription–PCR**

Total RNA was extracted using TRI reagent. Reverse transcription (RT) using random hexamer and RT–PCR using Go-Taq (Promega, Madison, WI) were performed as described previously (33). Primers used for RT–PCR were listed in Supplementary Table S1, available at Carcinogenesis Online.

**Quantitative real-time PCR** was performed as described previously (31). SYBR Green master mix (Applied Biosystems, Grand Island, NY) was used. The expression of target genes in **FEZF2**-transfected cells was normalized to those transfected with vector control. Glyceraldehyde 3-phosphate dehydrogenase (**GAPDH**) was used as an internal control. The sequences of primers used in quantitative RT–PCR will be provided upon request.

**Bisulfite treatment and promoter methylation analysis**

Bisulfite modification of DNA, methylation-specific PCR (MSP) and bisulfite genomic sequencing (BGS) were conducted according to our...
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The specificity of MSP primers was tested first using DNA samples without bisulfite treatment. Thirty-five cycles of PCR reaction were performed in MSP using primers amplifying methylated gene allele, with 40 cycles for reaction using primers amplifying unmethylated gene allele. PCR products amplified using BGS primers were cloned into pCR-TOPO vector (Invitrogen), with 6–10 colonies randomly chosen and sequenced. Primers used for MSP and BGS were listed in Supplementary Table S1, available at Carcinogenesis Online.

Demethylation treatment using 5-aza-2′-deoxycytidine and trichostatin A

A Treatment of NPC cell line using 5-aza-2′-deoxycytidine (Aza) and trichostatin A (TSA) was carried out as described previously (32). Briefly, cells were treated with 10 μM Aza (Sigma, Ronkonkoma, NY) for 72 h and harvested for DNA and RNA extraction. Alternatively, after 72 h of Aza treatment, cells were incubated with 100 ng/ml of TSA for additional 24 h.

Construction of FEZF2 expression vector

The full-length open reading frame of FEZF2 was amplified from human testis total RNA using Pfu polymerase (Stratagene) and cloned to the EcoRI and BamHI sites of pcDNA3.1 (+) vector, with a Flag tag added to its C-terminus. The sequence and orientation of FEZF2 open reading frame were confirmed. FEZF2 open reading frame was also cloned into pCMV-BD vector, with the DNA binding domain of GAL4 fused to the N-terminus of FEZF2 protein.

Monolayer and soft agar colony formation assays

For colony formation assay using monolayer culture, HNE1 and HONE1 cells were seeded in a 12-well plate at 1–2 × 10⁴ cells/well. The cells were then transfected with FEZF2 expression vector or empty vector using Fugene 6 (Roche, Basel, Switzerland). After 48 h of post-transfection, the transfectants were subcultured into six-well plates for selection with 400 μg/ml of G418 (Calbiochem, Darmstadt, Germany). After 2 weeks of selection, surviving colonies (>50 cells per colony) were stained with gentian violet and counted.

For soft agar assay, the transfected cells were suspended in growth medium containing 0.35% agar and 400 μg/ml of G418 in 24-well plates. Surviving colonies were photographed and counted after about 2 weeks of selection.

Immunoprecipitation

HEK293T cells were transfected with control vector or FLAG-FEZF2. After 48 h of transfection, cells were washed with ice-cold phosphate-buffered saline once and incubated with lysis buffer (50 mmol/lTris–HCl, pH 8.0, 150 mmol/l NaCl and 0.5% NP40) on ice for 30 min. After centrifugation, the supernatant was collected and 200 μg of total protein was applied for immunoprecipitation using 40 μl anti-FLAG M2 affinity gel (Sigma) at 4°C overnight. Precipitated protein was eluted with 150 μg/ml of 3xFLAG peptide at 4°C for 1 h, and then used for immunoblotting analysis with 5% cell lysate as input control.

For immunoprecipitation using histone deacetylase 1 (HDAC1) antibody, 200 μg of total protein lysate was incubated with 1 μg HDAC1 antibody at 4°C overnight. The lysate was then incubated with 20 μl Protein G Sepharose beads at 4°C for 4 h. Sepharose beads were washed three times using RIPA lysis buffer and resuspended in 4x sodium dodecyl sulfate protein loading buffer. After boiling for 5 min, the supernatant was collected and applied to immunoblotting analysis with 5% cell lysate as input control.

Western blot

Western blot was carried out as described previously (34). Membranes were incubated with primary antibody at 4°C overnight, followed by incubation with secondary antibody at room temperature for 1 h. Immunoreactive bands were detected using western blot luminol reagent (GE Healthcare, Waukesha, WI). The antibodies used were anti-FEZF2 (ab69436; Abcam, Cambridge, UK), anti-FLAG (F7425; Sigma), anti-HDAC1 (5356; Cell Signaling, Boston, MA), anti-MDM2 (sc-813; Santa Cruz, Dallas, TX), anti-p53 (M7001; Dako, Glostrup, Denmark), anti-α-tubulin (MS-581; Thermo Lab Vision, Kalamazoo, MI) and anti-GAPDH (MAB374; Millipore, Billerica, MA).

Flow cytometry analysis

Cells stably transfected with FEZF2 or empty vector were collected, washed twice with phosphate-buffered saline and stained for 1.5 h at 37°C using 50 μg/ml propidium iodide (Sigma). Cells were then washed with phosphate-buffered saline and applied to flow cytometry analysis using Beckman Cell Lab SC Quanta. Results were analyzed using ModFit LT.

Wound healing assay

Wound healing assay to evaluate cell migration ability was performed as described previously (35). Briefly, cells were transfected with empty vector or FEZF2 construct and allowed to grow until confluent (>95%). Cell scratches were then created using 20 μl sterile tips and washed twice with culturing medium. After indicated time points of incubation, cells were imaged under a phase contrast microscope. The experiments were performed in duplicate.

Immunofluorescence

Immunostaining of HONE1 and HNE1 cells was performed as described previously (28). Briefly, the cells were seeded on coverslips and transfected with FLAG-FEZF2 plasmid. After 24 h of transfection, cells were fixed and incubated with primary antibody anti-FLAG M2 (F3165; Sigma) at 4°C overnight, and then stained with secondary fluorescein isothiocyanate-conjugated antibody (F0313; Dako, Glostrup, Denmark) at 37°C for 1 h. Cell nuclei were then stained with 4′,6-diamidino-2-phenylindole and imaged using a confocal microscope (Leica TCS SP5; Leica Microsystems, Mannheim, Germany).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was carried out using ChIP-IT Express Kit from Active Motif (#53008; Carlsbad, CA). For each ChIP reaction, 25 μg of total chromatin was incubated with 20 μl of Protein G magnetic beads and 1 μg of FLAG-M2 antibody (F3165; Sigma) at 4°C overnight. Both input and precipitated DNA were purified with QIAamp DNA Mini Kit (Qiagen, Valencia, CA) for subsequent real-time PCR. The relative enrichment of precipitated DNA was normalized to its input. Following primers were used for ChIP assay: MM2CHIIPF, 5′-CATTTGTTGACAATTCCAGC and MM2CHIIPR, 5′-TGGAAACCTGCAGAATGGC; EZH2CHIIPF, 5′-AAAGGGTTGGGATGGG and EZH2CHIIPR, 5′-AAACGGAGTCTCACGTC and GAP1CHIIPF, 5′-TATCGAGTCAGGCTACAG and GAP1CHIIPR, 5′-GGCTTCCGGTTAGTACAC.

Dual-luciferase reporter assay

Cells were co-transfected with indicated reporter constructs together with either FEZF2 expression vector or empty vector in 24-well plate. After 48 h of transfection, cells were collected and analyzed by the Dual-Luciferase Assay Kit (Promega, Madison, WI). Each experiment was repeated in triplicate wells for three times independently.

Immunohistochemistry

Immunohistochemistry was performed using the ChemMate EnVision Detection Kit (#K500705; Dako, Carpinteria, CA) according to manufacturer’s instruction. Primary EZH2 antibody used for staining was from Zymed (#18–7395; Invitrogen, Grand Island, NY). A total of 67 primary NPC and 17 normal nasopharynx or chronic nasopharyngitis cases were used. Cells with strong nuclear staining were taken as EZH2 positive. Percentage of EZH2 positive cells was scored by a pathologist blindly in five random fields (100 cells per field) for each sample.

Statistical analysis

Results were presented as mean ± SD. Statistical analysis was carried out with Student’s t-test and P < 0.05 was considered as statistically significant.

Results

Identification of FEZF2 as a novel 3p14.2 TSG candidate for NPC

To search for novel TSGs for NPC, we refined 3p14–p12, a critical TSG hotspot at 3p, as a major deleted region in NPC cell lines using 1 Mb aCGH (Figure 1A). Hemizygous deletions represented by three bacterial artificial chromosome clones (bA170K19, bA204J18 and bA108A8), all located at 3p14.2, were detected in all NPC cell lines studied (Supplementary Figure S1, available at Carcinogenesis Online), indicating the existence of critical TSGs within this locus. We thus examined the expression profile of candidate genes residing in this locus by semi-quantitative RT–PCR. It was found that FEZF2 was silenced in all five NPC cell lines (Figure 1D). However, FEZF2 was readily expressed in normal larynx and trachea tissues (Figure 1B), normal nasopharyngeal tissues (Figure 1C) and immortalized nasopharyngeal epithelial cell line NP69 (Figure 1D). Downregulation of FEZF2 in NPC cell lines, compared with normal cells, was further confirmed by quantitative real-time PCR (Figure 1H) and western blot at the protein level (Figure 1I).

We also evaluated FEZF2 expression in other human normal tissues. FEZF2 was highly expressed in both adult and fetal brain
tissues, consistent with its important role in the development of neural system. In addition, \textit{FEZF2} expression was also observed in adult testis and ovary tissues, whereas barely detectable in other human tissues (Figure 1B). The expression of \textit{FEZF2} in upper respiratory tract tissues and its silencing in NPC cell lines suggested that \textit{FEZF2} is likely a candidate tumor suppressor for NPC.

Silencing of \textit{FEZF2} due to its promoter CpG methylation in NPC

As TSGs can be inactivated by genetic deletions, we first determined the homozygous deletion of \textit{FEZF2} in NPC. Deletion of \textit{FEZF2} exon 2 was assessed by multiplex genomic DNA-PCR, using \textit{GAPDH} as an internal control. Results showed no homozygous deletion of \textit{FEZF2} in NPC cell lines and normal peripheral blood mononuclear cell controls (Figure 1E), suggesting that other mechanism is responsible for \textit{FEZF2} silencing in NPC.

We thus studied whether promoter CpG methylation was involved in silencing \textit{FEZF2} in NPC. Bioinformatic analysis revealed a typical CpG island spanning \textit{FEZF2} promoter and exon 1 is shown at the bottom. Each vertical bar represents a CpG site. MSP primer sites and BGS region are labeled. (B) \textit{FEZF2} expression in human normal adult and fetal tissue panel by RT–PCR, with \textit{GAPDH} as a control. Sk. Muscle, skeletal muscle. (C) \textit{FEZF2} is expressed in normal nasopharyngeal tissues as evaluated by RT–PCR. (D) \textit{FEZF2} was silenced by promoter methylation in NPC cell lines as determined by RT–PCR and MSP but expressed and unmethylated in immortalized normal cell line NP69. M, methylated; U, unmethylated. (E) Multiplex genomic DNA-PCR showed no homozygous deletion of \textit{FEZF2} in NPC cell lines, \textit{GAPDH} was used as a control. Normal peripheral blood mononuclear cells were used as positive controls. (F) High-resolution BGS methylation analysis of \textit{FEZF2} promoter. Each row of circles represented an individual promoter allele. Filled circle, methylated CpG site; open circle, unmethylated CpG site. (G) Pharmacologic demethylation restored \textit{FEZF2} expression in methylated and silenced NPC cell lines. A + T, treatment with Aza and TSA. (H) Quantitative RT–PCR further confirmed \textit{FEZF2} downregulation in NPC cell lines compared with normal tissues and its restoration after demethylation treatment. The expression level of each sample was normalized to internal control \textit{GAPDH}. Fold change of \textit{FEZF2} expression was calculated relative to that of larynx tissue. Experiments were performed three times independently. (I) Western blot showed downregulation of \textit{FEZF2} protein in NPC cell lines compared with normal trachea tissue. \textit{α}-Tubulin was used as a loading control.
methyltransferase inhibitor Aza, alone or in combination with HDAC inhibitor TSA. After Aza treatment, FEZF2 expression was restored (Figure 1G, upper panel and Figure 1H), accompanied by significant increase of unmethylated promoter alleles (Figure 1G, bottom panel). Demethylation of FEZF2 promoter in C666-1 and HONE1 cells was confirmed by BGS analysis (Supplementary Figure S2B, available at Carcinogenesis Online). Thus, promoter CpG methylation directly mediates FEZF2 silencing in NPC.

FEZF2 is frequently downregulated and methylated in NPC tumors

We also examined FEZF2 expression in paired tumor and adjacent normal tissues. FEZF2 was downregulated in larynx tumor compared with the adjacent normal larynx tissue (Figure 2A), but not expressed in other common tumors, including lung, colon, rectum, liver, kidney, breast and gastric tumors, as well as their adjacent normal tissues (Supplementary Figure S2C, available at Carcinogenesis Online).

Consistently, we observed frequent FEZF2 downregulation in primary NPC tissues (Figure 2A). We further detected FEZF2 methylation in 75.5% of primary NPC tumor tissues (37 of 49) and three NPC tumors passaged from nude mice, but only in 7.1% of normal nasopharyngeal tissues (1 of 14) (Figure 2B). FEZF2 methylation in NPC tumors was further confirmed by BGS (Figure 2C). Furthermore, FEZF2 methylation was detected in 75% (12 of 16) of nasal swab samples from NPC patients (Figure 2D), whereas only one of the seven nasopharyngitis tissues from healthy individuals was detected having weak methylation (Supplementary Figure S2D, available at Carcinogenesis Online). These results suggest that FEZF2 is frequently downregulated and methylated in NPC in a tumor-specific manner.

**FEZF2 as a transcriptional repressor**

FEZF2 contains an EH1 repressor domain and six tandem C2H2-type zinc fingers, which are highly conserved among its homologs (Supplementary Figure S3, available at Carcinogenesis Online). Immunofluorescence staining showed that FEZF2 is localized in the nucleus (Figure 3A and Supplementary Figure S4, available at Carcinogenesis Online). To determine whether FEZF2 acts as a transcriptional activator or repressor, a GAL4 luciferase reporter system was used. Fused to the GAL4 DNA binding domain, FEZF2 was recruited to the luciferase gene promoter containing five GAL4 binding sites. Results showed that BD-FEZF2 dramatically repressed the reporter activities in HNE1 and HONE1 cells. Moreover, FEZF2 repressed the reporter activity in a dose-dependent manner in HEK293T cells (Figure 3B). Furthermore, we found that FEZF2 physically associates with HDAC1 (Figure 3C and 3D), a common core component of repressor complexes like LSD-CoREST and...
FEZF2 is a transcriptional repressor. (A) Upper panel: Functional domains of FEZF2 protein. ZnF, zinc finger. Bottom panel: Confocal microscopy of FEZF2 subcellular localization in HONE1 cells. 4′,6-Diamidino-2-phenylindole was used for nucleus staining. Scale bar: 5 μm. (B) Lower left: GAL4 luciferase reporter assay showed that FEZF2 suppressed the transcription of reporter gene in HNE1 and HONE1 cells. *P < 0.05; **P < 0.01. Lower right: Transcription repression mediated by FEZF2 was dose dependent in HEK293T cells. (C and D) Co-immunoprecipitation assays showed that HDAC1 binds to the transfected (C) and endogenous (D) FEZF2 protein. IP, immunoprecipitation; IB, immunoblotting.

Fig. 3. FEZF2 is a transcriptional repressor. (A) Upper panel: Functional domains of FEZF2 protein. ZnF, zinc finger. Bottom panel: Confocal microscopy of FEZF2 subcellular localization in HONE1 cells. 4′,6-Diamidino-2-phenylindole was used for nucleus staining. Scale bar: 5 μm. (B) Lower left: GAL4 luciferase reporter assay showed that FEZF2 suppressed the transcription of reporter gene in HNE1 and HONE1 cells. *P < 0.05; **P < 0.01. Lower right: Transcription repression mediated by FEZF2 was dose dependent in HEK293T cells. (C and D) Co-immunoprecipitation assays showed that HDAC1 binds to the transfected (C) and endogenous (D) FEZF2 protein. IP, immunoprecipitation; IB, immunoblotting.

NuRD. These data suggest that FEZF2 is a transcriptional repressor and a component of some repressor complexes.

**FEZF2 suppresses NPC cell growth through inducing cell cycle arrest and apoptosis**

To investigate the potential tumor suppressive properties of FEZF2, we performed cell colony formation assays of methylated/silenced NPC cell lines, transfected either with an empty vector or a plasmid encoding FEZF2. Results showed that, compared with control groups, FEZF2 significantly inhibited the anchorage-dependent colony formation of monolayer-cultured NPC cells and also suppressed the anchorage-independent growth of NPC cells in soft agar (Figure 4A and 4B). Restored expression of FEZF2 in these cells was confirmed by RT–PCR (Figure 4B), which showed FEZF2 expression levels in transfected cells are similar to normal tissues, such as larynx and trachea.

We then studied the underlying mechanisms of FEZF2-mediated growth inhibition. Flow cytometry analysis showed an increase of...
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G2/M phase cell percentage in tumor cells stably transfected with FEZF2 compared with control cells (Figure 4C). In addition, we observed the downregulation of two critical cell cycle regulators (CDC25A and E2F3), which promote G2/M transition, by FEZF2 (Figure 4E). A sub-G1 peak was observed in FEZF2-transfected cells but not in control cells (Figure 4D), suggesting that FEZF2 expression induced tumor cell apoptosis. Immunofluorescence staining showed that some FEZF2-expressing cells had abnormal nucleus condensation and segmentation, a typical mark of cells undergoing apoptosis (Figure 4D). Moreover, the mRNA expression of two antiapoptotic genes, BCL2 and cIAP2, was downregulated in FEZF2-expressing cells (Figure 4E). Consistently, we also observed downregulation of BCL2 protein and upregulation of the key apoptosis regulator p53 by FEZF2 (Figure 4E).

We further found that p53 expression was detectable in most NPC cell lines, whereas only a single point mutation of p53 was reported in two NPC cell lines (Supplementary Figure S5, available at Carcinogenesis Online), indicating that epigenetic silencing of FEZF2 likely contributes critically to the perturbation of p53 signaling pathway in NPC cells. These results suggest that FEZF2 suppresses NPC cell growth through inducing cell cycle arrest and apoptosis.

**FEZF2 inhibits the migration and stemness of NPC cells**

We further found that FEZF2 inhibited the migration of NPC cells by wound healing assay (Figure 5A). Considering the pivotal role of FEZF2 in neural cell differentiation and the reported stem-like properties of NPC cells (32,35), we also assessed whether it regulates the...
stemness of NPC cells. Indeed, we observed the change of mesenchymal-like phenotype to epithelial-like phenotype in FEZF2-expressing NPC cells (Figure 5B) and the downregulation of several stem cell markers including **KLF4**, **CD44** and **BMI1**, by FEZF2 (Figure 5C). These data indicated that FEZF2 inhibits the migration and stemness of NPC cells.

**FEZF2 represses multiple oncogene expression including EZH2 and MDM2**

To identify cancer genes regulated by FEZF2, we examined the expression of a panel of oncogenes in NPC cells transfected with FEZF2 construct. Quantitative RT–PCR showed that the expression of multiple oncogenes, including **EZH2** and **MDM2**, was significantly downregulated in FEZF2-transfected HONE1 and HNE1 cells (Figure 6A; Supplementary Table S2, available at Carcinogenesis Online).

Recently, the genome-wide binding sites of zebrafish FEZF2 were characterized, with a core CnnCAnCn sequence as the putative consensus FEZF2 binding motif (36). As FEZF2 is evolutionarily well conserved, the human FEZF2 is likely to target similar sites in the genome. We thus analyzed the promoter regions of **EZH2** and **MDM2** and found multiple potential FEZF2 binding sites (the CnnCAnCn motif) in the promoter. We then performed ChIP assay and detected the recruitment of FEZF2 to **MDM2** and **EZH2** promoters (Figure 6B). Furthermore, the promoter activity of **MDM2** and its protein level was significantly decreased in FEZF2-expressing cells (Figure 6C). **EZH2** was also found to be upregulated in most NPC cell lines compared with normal immortalized nasopharyngeal cell line NP69, which is negatively correlated with **FEZF2** expression status in these cell lines (Figure 6D). Immunohistochemistry further confirmed the overexpression of **EZH2** in NPC tumors compared with normal nasopharyngeal
Fig. 6. FEZF2 represses multiple oncogene expression. (A) Real-time RT–PCR showed FEZF2 repressed multiple oncogenes expression in HONE1 and HNE1 cells. The gene expression level in vector-expressing cells was set to 1.00. Fold change of expression in FEZF2-expressing cells was calculated by normalizing to vector-transfected cells. GAPDH was used as an internal control. Data are shown as mean ± SD of three independent experiments. *P < 0.05; **P < 0.01. (B) Top panel: Putative FEZF2 binding sites at MDM2 and EZH2 promoters. Fragments amplified in ChIP–PCR were indicated by bottom black lines. Lower panel: ChIP–quantitative PCR showed enrichment of FEZF2 binding at MDM2 and EZH2 promoter regions. GAPDH was used as a negative control. The signal of precipitated DNA was normalized to their input DNA. *P < 0.05; **P < 0.01. (C) FEZF2 repressed MDM2 promoter activity and downregulated its protein expression, as determined by luciferase reporter assay and western blot, respectively. **P < 0.01. (D) RT–PCR showed upregulation of EZH2 in NPC cell lines compared with normal cell line NP69. Expression and methylation status of FEZF2 in these cell lines are also indicated at the bottom. (E) Representative immunohistochemistry results for EZH2 in normal nasopharynx and NPC tissues. Two magnifications (×20 and ×40) of the same sample are shown.
Discussion

Our aCGH study and others showed that 3p14 is one of the frequently deleted regions in NPC cell lines (32,37). Previously, several 3p14 genes located adjacent to FEZF2, like FHIT (9,38–40), PTPRG (10,41,42) and ADAMTS9 (11), have been identified as functional TSGs for NPC and other cancers. Our study demonstrated that FEZF2 is another functional novel 3p14 TSG. Thus, FHIT-PTPRG-FEZF2 appears to be a small TSG island at 3p14. Moreover, a study using cytogenetic detection method FISH even proposed 3p12.3–p14.2 deletion as a prognostic marker for advanced NPC patients (43).

Epigenetic alterations, especially promoter CpG methylation, have been shown to be as important as genetic abnormalities during tumor initiation and progression (44–46). We found that promoter methylation is responsible for FEZF2 silencing in NPC. Importantly, we observed frequent FEZF2 methylation in primary NPC tumor tissues and nasal swab samples from NPC patients, but not in normal nasopharyngeal tissues, indicating its methylation is tumor specific.

As FEZF2 is a critical gene for vertebrate forebrain development, we also examined whether it is a candidate TSG for NPC tumor. Through analyzing the microarray data from online database (Oncomine; Compendia Bioscience, Ann Arbor, MI) (47), we found that FEZF2 expression was significantly reduced in brain tumors compared with normal brain tissues (Supplementary Figure S6A, available at Carcinogenesis Online), with high-grade brain tumors showing lower FEZF2 expression than low-grade brain tumors (Supplementary Figure S6B, available at Carcinogenesis Online). We also found that FEZF2 expression levels in normal brain and glioblastoma tissues were negatively correlated with EZH2 levels (Supplementary Figure S6C, available at Carcinogenesis Online), indicating that FEZF2 silencing also contributes to EZH2 overexpression in brain tumors. In addition, we detected FEZF2 downregulation in glioma cell lines (Supplementary Figure S6D, available at Carcinogenesis Online), although no promoter methylation was observed, suggesting that other mechanism like genetic deletions might be responsible for its inactivation in these cell lines.

So far, two FEZF family genes, FEZF2 and FEZF1 (Supplementary Figure S7, available at Carcinogenesis Online), have both been linked to cancer development, although displaying distinct properties. FEZF1 acts as a onco gene promoting gastric cancer progression (26) and is activated by abnormal epigenetic modifications in gastric cancer (48). In this study, FEZF2 was found to possess tumor suppressor properties and is silenced by promoter methylation in NPC. The unique proline-rich domain of FEZF1 mediates its translocation to nucleus and represses the transcription of multiple oncogenes.

In conclusion, we identified and functionally characterized a novel 3p14.2 TSG, FEZF2, in NPC pathogenesis. FEZF2 is frequently silenced by promoter methylation in NPC, and functions as a TSG suppressing the NPC growth through inducing cell cycle arrest and apoptosis and inhibiting NPC cell migration and stemness. Finally, FEZF2 was shown to be an HDAC-associated transcription repressor, downregulating multiple oncogenic genes, which control cell cycle progression, apoptosis and cell stemness (Supplementary Figure S8, available at Carcinogenesis Online).

Supplementary material

Supplementary Figures S1–S8 and Tables S1 and S2 can be found at http://carcin.oxfordjournals.org/

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