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microRNA 21-Mediated Suppression of Sprouty1 by Pokemon Affects Liver Cancer Cell Growth and Proliferation

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ABSTRACT

Transcriptional repressor Pokemon is a critical factor in embryogenesis, development, cell proliferation, differentiation, and oncogenesis, thus behaving as an oncogene. Oncomine database suggests a potential correlation between the expressions of Pokemon and Sprouty1. This study investigated the regulatory role of Pokemon in Sprouty1 expression and the effect on liver cancer cell growth and proliferation, revealing a novel miR-21-mediated regulatory circuit. In normal (HL-7702) and cancer (QGY-7703) liver cell lines, Sprouty1 expression is inversely correlated with Pokemon levels. Targeted expression or siRNA-mediated silencing showed that Pokemon is a repressor of Sprouty1 expression at both mRNA and protein levels, but Pokemon cannot affect the promoter activity of Sprouty1. Sprouty1 is a target of miR-21 and interestingly, we found that miR-21 is up-regulated by Pokemon in liver cancer cells. Luciferase reporter assays showed that Pokemon up-regulated miR-21 transcription in a dose-dependent manner, and ChIP assay exhibited a direct binding of Pokemon to the miR-21 promoter at −747 to −399 bp. Site-directed mutagenesis of the GC boxes at −684 to −679 bp and −652 to −647 bp of miR-21 promoter abolished the regulatory activity by Pokemon. Furthermore, we found that the modulation of Pokemon and miR-21 expression affected the growth and proliferation of liver cancer cells QGY-7703. In summary, our findings demonstrate that Pokemon suppresses Sprouty1 expression through a miR-21-mediated mechanism, affecting the growth and proliferation of liver cancer cells. This study recognized miR-21 and Sprouty1 as novel targets of the Pokemon regulatory network. J. Cell. Biochem. 114: 1625–1633, 2013.

KEY WORDS: microRNA 21; POKEMON; SPROUTY1; LIVER CANCER; siRNA

Pokemon, also known as LRF, OCZF, and FBI-1, is encoded by the ZBTB7A gene. Pokemon is a member of the POK protein family, which plays a critical and pleiotropic function in cellular differentiation [Pessler et al., 1997; Davies et al., 1999; Kukita et al., 1999; Morrison et al., 1999; Maeda et al., 2005a]. Human Pokemon gene, localized on chromosomal regions 19p13.3, is widely expressed in adult tissues and cell lines. Pokemon is also overexpressed in some human cancers, such as lung, breast, colon,

Abbreviations used: TF, transcription factor; miR-21, microRNA 21; miRNA, microRNA; siRNA, short-interfering RNA; anti-miR-21(anti-21), microRNA 21 inhibitor; NC, negative control; ChIP, chromatin immunoprecipitation; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Conflict of interest: nothing to declare.

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and liver cancers and diffuse large B-cell lymphomas (DLBCLs), non-Hodgkin's lymphoma (NHL), follicular lymphomas, and diffuse malignant mesothelioma [Wei et al., 2006; Apostolopoulou et al., 2007; Aggarwal et al., 2010]. POK family of proteins contain an amino-terminal BTB/POZ structure domain and a carboxy-terminal Krüppel type zinc finger domain [Schubot et al., 2006]. BTB/POZ domain forms complexes with histone-deacetylases (HDAC), and carboxyl terminus mediates the binding to special DNA sequence. The special structure renders its capability of playing a key and pleiotropic function in the embryogenesis, development, differentiation, and oncogenesis [Chen et al., 2003]. Pokemon plays a crucial role in the carcinogenic process through suppressing the tumor suppressor pathway ARF-p53 [Maeda et al., 2005b]. Pokemon is located in the upstream of many tumor suppressor genes and oncogenes, thus being an effective target of oncotherapy.

Recent studies have shown that Pokemon is regulated by microRNAs (miRNAs) [Poliseno et al., 2008] and meanwhile, is also a regulator of many miRNAs [Verduci et al., 2010]. miRNAs are short ribonucleic acid (20–25 nucleotides noncoding RNAs) molecules found in eukaryotic cells. miRNAs are produced from RNA polymerase II-transcribed precursors by the RNase III family enzymes Drosha and Dicer [Kim, 2005]. miRNAs are post-transcriptional regulators that bind to complementary sequences on target messenger RNA transcripts (mRNAs), usually resulting in translational repression or target degradation and gene silencing [Bartel, 2004, 2009]. miRNAs have essential roles in development, cell differentiation, proliferation, apoptosis, and chromosome structure [Mraz et al., 2009]. Some miRNAs can modulate tumor formation as a potential oncogene, called oncomiR [He et al., 2005]. miR-21 is one of the first miRNAs identified as an oncomiR. As most of the targets of miR-21 are tumor suppressors, miR-21 is associated with human cancers, such as breast [Si et al., 2006], brain [Chan et al., 2005], lung [Volinia et al., 2006], prostate [Volinia et al., 2006], ovaral [Iorio et al., 2007], liver [Meng et al., 2007], and colon cancers [Asangani et al., 2007]. Informed research demonstrated that the transcription factors (TFs) and miRNAs extensively interact with each other and the biological functions of miRNAs may be wired in the regulatory network topology [Yu et al., 2008].

Sprouty1 was a member of the endogenous inhibitors of the Ras/mitogen-activated protein kinase pathway and was discovered in a Drosophila genetic screen designed to identify components in fibroblast growth factor (FGF)-induced tracheal branching. Lack of Sprouty leads to random "sprouting" of tracheal tubules [Hacohen et al., 1998; Casci et al., 1999; Kramer et al., 1999; Reich et al., 1999]. Sprouty1 may play an important role in the remodeling of branching tissues and preventing cell transformation. Down-regulation of this gene may reduce the threshold for cells to become malignant [Kwabi-Addo et al., 2004; Lo et al., 2004; Fong et al., 2006]. Mammalian Sprys have been shown to particularly function as negative regulators in FGF signaling during vertebrate embryonic development. The Sprouty1 expression is downregulated in cancer cells, such as breast [Lo et al., 2004], prostate [Kwabi-Addo et al., 2004], and liver cancers [Fong et al., 2006]. Recent study has shown that Sprouty1 expression is regulated by miR-21 [Thum et al., 2008]. In this study, we show that Pokemon down-regulates Sprouty1 expression through miR-21 mediated mechanism, stimulating cell growth and proliferation. This report identifies a novel regulatory mechanism through which Pokemon exerts its oncogenic role in liver cancer cells.

MATERIALS AND METHODS

REAGENTS

Human mature miR-21 inhibitor (anti-miR-21), and short-interfering RNA (siRNA) (si-Pokemon RNA [Jeon et al., 2008; Zu et al., 2011]) were synthesized by GenePharma (Shanghai, China). All the restriction and modifying enzymes, PrimeScript RT Master Mix Pertect Real Time, and SYBR® *Premix Ex Taq*™ (Tli RNaseH Plus) were from TaKaRa Biotechnology (Dalian) Co., Ltd. TRIzol Reagent, Dynabeads Protein G, Lipofectamine™ 2000 (Invitrogen). Dulbecco's modified Eagle's medium-high glucose (DMEM-HG), modified form RPMI-1640 medium, and fetal bovine serum (FBS) (HyClone). Opti-MEM I Reduced Serum Media (GIBCO). miRcute miRNA isolation kit, miRcute miRNA first-strand cDNA synthesis kit, miRcute miRNA qPCR detection kit (SYBR) were from TIANGEN BIOTECH (BEIJING) CO., LTD., China. Anti-Pokemon antibody (F9304, Sigma–Aldrich). Anti-Pokemon antibody (ab36606), Control IgG antibody (ab37373), Anti-Sprouty1 antibody (ab56670) (Abcam). β-actin antibody, HRP-labeled Goat Anti-Rabbit IgG(H + L), HRP-labeled Goat Anti-Mouse IgG (H + L) were from Beyotime (China). Dual-Luciferase Reporter Assay (Promega).

CELL CULTURE

All cells used in this study were obtained from the Cell Bank of Chinese Academy of Sciences. HL-7702 cells were grown in RPMI 1640 supplemented with 10% FBS, QGY-7703 cells were grown in Dulbecco's modified Eagle's medium-high glucose supplemented with 10% FBS. All cells were incubated at 37°C in a humidified chamber supplemented with 5% CO_2.

PLASMIDS

Pokemon-expressing plasmid pcDNA3.1(−)/Pokemon and negative control (NC) pcDNA3.1(−) vector were present in our laboratory. Sprouty 1 promoter pluc-Sprouty1 (2,000 bp upstream the transcription start site 1) was amplified with the primer pair forward 5′-CGAAGATCTAACGTGCGGAATCGGCTAAG-3′ where the *Bgl*II site is underlined, and reverse 5′-GGGAAGCTTTTCGGACAATCCCATCACCATA-3′ where the *Hin*dIII site is underlined (Temperature annealing 60°C). To construct a plasmid expressing Sprouty1 pcDNA3.1(−)/Sprouty1, we amplified a 960 base pair DNA fragment from 293T cell genomic DNA using PCR primers: forward 5′-GG GGTACCATGGATCCCCAAAATCAACA-3′ where the *Kpn*I site is underlined, and reverse 5′-CCC AAGCTTTCATGATGGTTTACCCTGA-3′ where the *Hin*dIII site is underlined (temperature annealing 52°C). miR-21 human promoter (747 bp upstream the transcription start site1) was amplified with the primer pair forward 5′-CCCTCGAGTAGAACCTCAGTAATCCG-3′ where the *Xho*I site is underlined and reverse 5′-GGAAGATCTGGCAAGTTAACGAAAAGAA-3′ where the *Bgl*II site is underlined (temperature annealing 50°C) and subcloned in the pGL4.10 vector, generating the pluc-M21 plasmid.

SITE-DIRECTED MUTAGENESIS OF THE miR-21 PROMOTER

To investigate the role of Pokemon-binding sites on transcription, mutations were introduced into the miR-21 promoter sequence using the site-directed mutagenesis PCR. The mutagenic primers used were: pluc-M21-m1, 5′-AGCCGGGCACTACAGT*T*T*T*T*GCT TGCTCACGGTGC-3′; pluc-M21-m2, 5′-TGCTGTGCCAGGGC GT*T* T*T*T*TGCTGGCGACTAGGG-3′ where the asterisks indicate the mutated bases.

TRANSFECTION

Transfection of QGY-7703 cells and HL-7702 cells were performed with Lipofectamine 2000 following the manufacturer's protocol. Briefly, the cells were seeded in six-well plates at 50% confluence on the day before transfection. 3.5 μg of Pokemon-expressing plasmid or control vector was used for each transfection in antibiotic free Opti-MEM medium. Transfection the si-Pokemon RNA or scrambled siRNA at 75 nM was used for each transfection. Transfection the anti-miR-21 or NC at 50 nM was used for each transfection. For quantification of Pokemon, Sprouty1, and miR-21 expression, cells were collected 48 h after transfection.

EXPRESSION ANALYSIS OF miR-21

miRNA was extracted from $0.5–1.0 \times 10^6$ cells with miRcute miRNA isolation kit following the manufacturer's recommendation. The RT reaction was carried out following the miRcute miRNA first-strand cDNA synthesis kit. Mature miR-21 was quantified using miRcute miRNA qPCR detection kit according to the manufacturer's instructions. Oligonucleotides (5′-GTAGCTTATCAGACTGATGTT GA-3′, 5′-CTTCGGCAGCACATATACTAA AAT-3′) were used as forward primers, respectively, for miR-21 and U6 in the real-time amplification mixtures. All reactions were performed in triplicate, and data were analyzed using U6 as internal control. The relative expression SE of three independent experiments in triplicate is shown.

REAL-TIME PCR ANALYSIS

Total RNA was extracted from 5×10^5 cells using the TRIzol Reagent. After DNase treatment, 1 μg of total RNA was retro-transcribed using PrimeScript RT Master Mix Pertect Real Time following the manufacturer's instructions. Real-time PCR was carried out with 7500 Real time PCR System (Applied Biosystems). Pokemon and Sprouty1 transcripts were quantified using SYBR® *Premix Ex Taq*™ and the following primers: Pokemon, forward (5′-GAAGCCCTACGAGTGCAACATC-3′), reverse (5′-TGGTTCTTCAGG TCGTAGTTGTG-3′); Sprouty1, forward (5′-GCAGTGGCAGTTCGT-TAGTT-3′), reverse (5′-TGT CTGTGCTCGTAGTTATTATTC -3′); all reactions were performed in triplicate. Transcript values were normalized with those obtained from the amplification of GAPDH (internal control) with the following primers: forward (5′-AGCCT-CAAGATCATCAG CAATG-3′), reverse (5′-TGTGGTCATGAGTCCT TCCACG-3′) for SYBR Green analysis.

WESTERN BLOT ANALYSIS

Samples of 5×10^5 cells were lysed (10 mM Hepes, pH 7.9, 1 mM EDTA, 10 mM KCl, 0.1% Nonidet P-40, 1 mM PMSF, 1 mM AEBSF, 800 nM Aprotinin, 50 μM Bestatin, 15 μM E64, 20 μM Leupeptin, 10 μM Pepstatin A). Proteins (30–50 μg/lane) were separated on 12% SDS–polyacrylamide gels and transferred to nitrocellulose membranes. Immunoblots were probed with the following primary antibodies: anti-Pokemon (1:2,000), anti-Sprouty1 (1:200), and anti-β-actin (1:2,000). Signals were revealed after incubation with recommended secondary antibody HRP-labeled Goat Anti-Mouse or Rabbit IgG (H + L) using ECL.

LUCIFERASE ACTIVITY ASSAY

Luciferase assay was carried out to assess the effect of Pokemon on Sprouty1 and miR-21 promoter activity as described previously [Zu et al., 2009]. QGY-7703 cells were seeded in 24-well plates and transfected with pcDNA3.1(−)/Pokemon and pluc-Sprouty1, or pluc-miR-21, or mutational plasmids, and pRL-TK plasmid as internal control. Forty-eight hours later luciferase activity was measured in cell lysates using a dual-luciferase report assay system according to the manufacturer's protocol and analyzed with a Microplate Luminometer (Beckman Coulter, Fullerton, CA).

CHROMATIN IMMUNOPRECIPITATION ASSAYS

The binding Pokemon to the miR-21 promoter in vivo was analyzed by ChIP assays. QGY-7703 cells were used in ChIP assay. Cells were treated with formaldehyde (final 1%) to cross-link Pokemon to the miR-21 promoter. The remainder of the ChIP assay procedure was performed as reported previously [Chung et al., 2006]. To amplify the proximal and distal promoter regions of the miR-21 gene, PCR reactions of the immunoprecipitated DNA were carried out using the following sets of oligonucleotide primers: promoter region −747 to −399 bp, forward primer, 5′-TAGAACCTCAGTAATCCG-3′ and reverse primer, 5′-GGGTG TTTCCCTATAATC-3′ and −391 to −1 bp, forward primer, 5′-TGCTGTTTGGTCTCAGTA-3′ and reverse primer, 5′-GGCAAGTTAACGAAAA GAA-3′ and −519 to −172 bp, forward primer, 5′-GGACATTGCAGGGTCCTC-3′, and reverse primer, 5′-CAGC CCAAATCACTAATAAGAA-3′.

CELL GROWTH ASSAY

Twenty-four hours after transfection with Pokemon-expressing plasmid pcDNA3.1(−)/Pokemon, vector control, anti-miR-21 and pcDNA3.1(−)/Pokemon + anti-miR-21, respectively, or with pcDNA3.1(−)/Pokemon, vector control, pcDNA3.1(−)/Sprouty1 and pcDNA3.1(−)/Pokemon + pcDNA3.1(−)/Sprouty1, respectively, the cells were seeded into 24-well plates at 2.5×10^4 cells/well or into 96-well plates at 5,000 cells/well. The cell number was calculated microscopically for five successive days to determine cell growth. The MTT assay was used to determine cell proliferation.

STATISTICAL ANALYSIS

Data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA). Statistical differences were determined by unpaired *t*-test, with values of $P < 0.05$ considered statistically significant. Each experimental point in the graph represents the mean SE of at least three independent experiments.

RESULTS

POKEMON SUPPRESSES THE EXPRESSION OF SPROUTY1, BUT DOES NOT AFFECT ITS PROMOTER ACTIVITY

We first examined the expression of Sprouty1 and Pokemon in liver cancer cells QGY-7703 and normal liver cells HL-7702. As shown in Figure 1A,B, the levels of Sprouty1 and Pokemon expression were found to be negatively correlated. The expression level of Pokemon was low in normal liver cells, but overexpressed in liver cancer cells. By the contrast, the expression level of Sprouty1 was high in normal liver cells, but was low in liver cancer cells.

To understand the underlying regulation, transfection experiments were performed to determine the role of Pokemon in the expression of Sprouty1. QGY-7703 cells with abundant Pokemon expression were transfected with si-Pokemon RNA to knock down the expression of Pokemon, while HL-7702 cells with a basal level of Pokemon were transfected with Pokemon expression vector to increase its level. Pokemon-specific siRNA successfully knocked down its expression

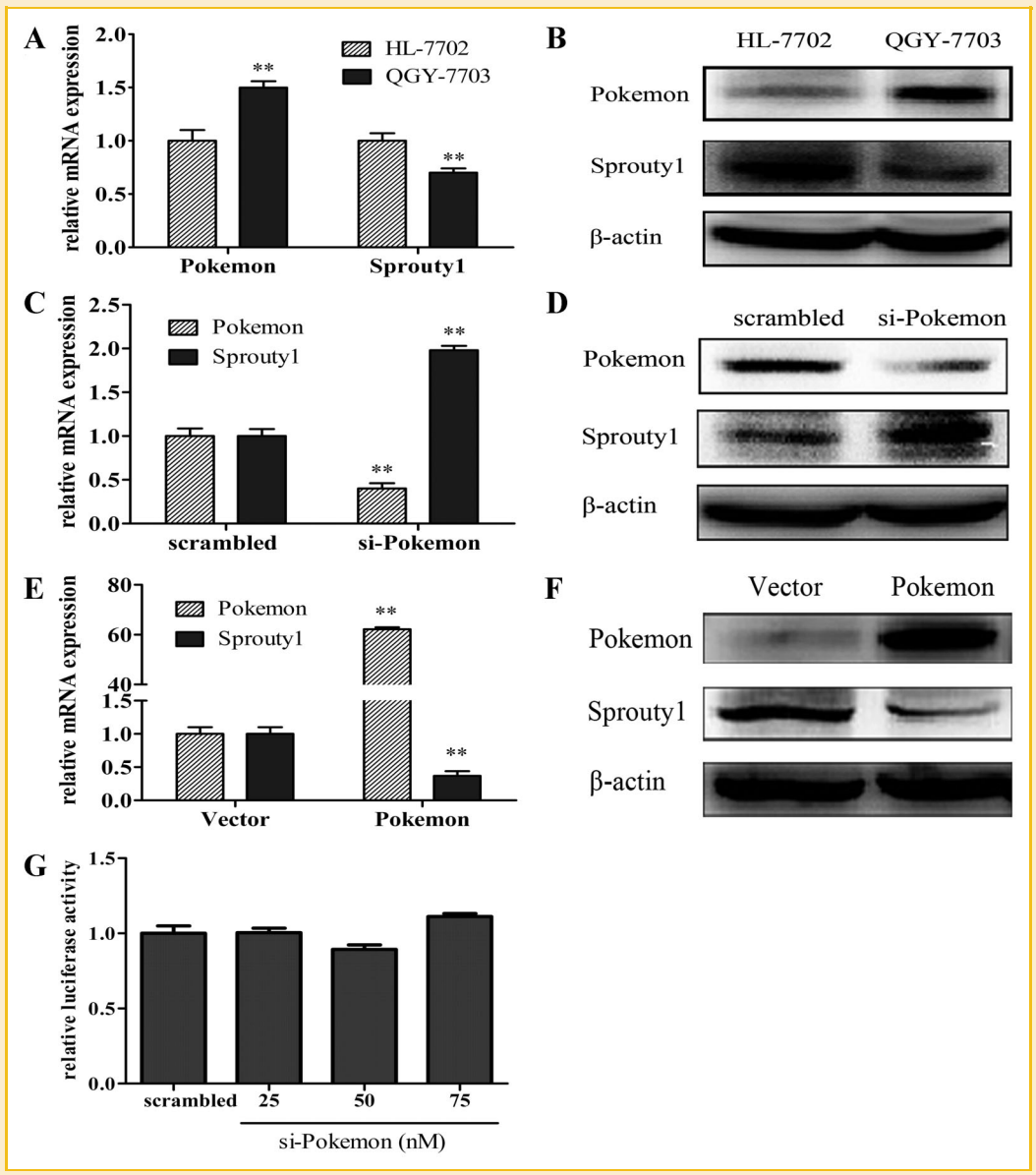

Fig. 1. Pokemon suppresses the expression of Sprouty1, but does not affect its promoter activity. A: Real-time PCR indicating Pokemon and Sprouty1 mRNA expression in HL-7702 cells and QGY-7703 cells. Experiments were performed in triplicate ($^{**}P < 0.01$ vs. HL-7702). B: Western blot assay indicating Pokemon and Sprouty1 protein expression in HL-7702 cells and QGY-7703 cells. QGY-7703 cells transfected with Pokemon siRNA and scrambled siRNA for 48 h were harvested for real-time RT-PCR (C, $^{**}P < 0.01$ vs. scrambled) and Western blot analysis (D). HL-7702 cells transiently transfected with Pokemon expression vector and control vector for 48 h were used for expression by real-time RT-PCR (E, $^{**}P < 0.01$ vs. Vector) and Western blot analysis (F). The values reported in the real-time PCR analysis were the mean of three independent transfection experiments. G: Pokemon did not affect Sprouty1 promoter activity in QGY-7703 cells. Sprouty1 promoter (pLuc-Sprouty1) was cotransfected with si-Pokemon RNA, and luciferase activity was measured with pRL-TK plasmid as an internal control. These data suggest that Pokemon did not affect promoter activity of Sprouty1.

and in turn led to an increase of Sprouty1 (Fig. 1C,D). Similarly, transient expression of Pokemon in HL-7702 cells down regulated Sprouty1 expression (Fig. 1E,F). Taken together, these results suggest that Pokemon suppresses Sprouty1 expression.

We further explored the mechanism how Pokemon regulates the expression of Sprouty1. The promoter region of Sprouty1, containing 2,000 bp upstream of the transcription start site, was cloned and used to drive the expression of luciferase reporter in QGY-7703 cells. As shown in Figure 1G, Pokemon did not affect the promoter activity of Sprouty1, which suggesting TF Pokemon has not direct effect on Sprouty1 promoter activity.

POKEMON UP-REGULATES miR-21 EXPRESSION IN QGY-7703 CELLS

Sprouty1 is a target of miR-21, so we investigated the role of Pokemon in miR-21 expression. Liver cancer cells QGY-7703 and normal liver cells HL-7702 were used for this analysis. As shown in Figure 2A, the levels of miR-21 and Pokemon expression were found to be highly correlated. In cancer cells QGY-7703, the expression levels of both Pokemon and miR-21 were high.

To test the regulation of miR-21 expression by Pokemon, we silenced Pokemon expression in QGY-7703 cells by si-Pokemon RNA with a scrambled siRNA as a NC. Meanwhile, we ectopically expressed Pokemon in HL-7702 cells with low endogenous levels. As shown in Figure 2B, Pokemon knockdown by siRNA led to a decrease of miR-21 expression in QGY-7703, whereas miR-21 was up-regulated by Pokemon delivery in HL-7702 cells (Fig. 2C). Taken together, these results suggest that Pokemon up-regulates miR-21 expression.

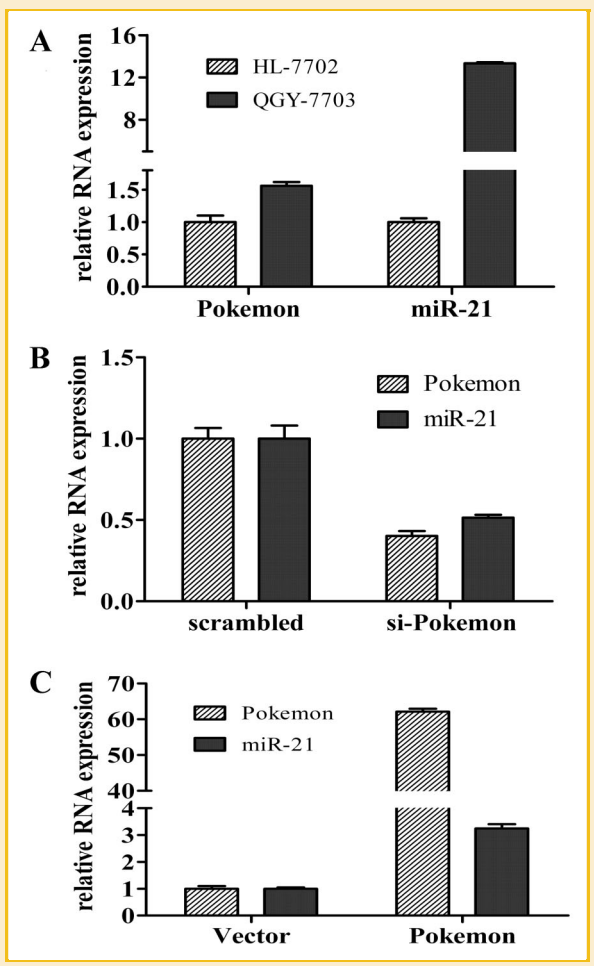

Fig. 2. Pokemon up-regulates miR-21 expression in QGY-7703 cells. A: Real-time PCR analysis of Pokemon and miR-21 expression in QGY-7703 cells and HL-7702 cells (**$P < 0.01$ vs. HL-7702). B: QGY-7703 cells were transfected with si-Pokemon RNA or scrambled siRNA. After 48 h, total RNA was extracted, and real-time PCR was performed to quantify Pokemon and miR-21 transcripts (**$P < 0.01$ vs. scrambled). C: HL-7702 cells were transfected with Pokemon expression plasmid and control vector. After 48 h, total RNA was extracted, and real-time PCR was performed to quantify Pokemon and miR-21 transcripts (**$P < 0.01$ vs. Vector).

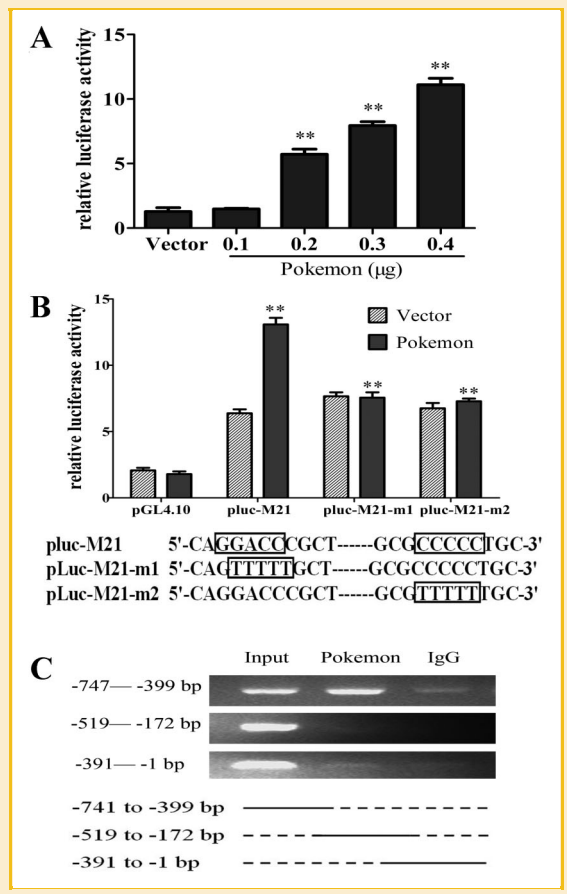

Fig. 3. Pokemon regulates miR-21 expression via DNA sequence-specific binding to its promoter. A: Regulation of miR-21 promoter activity by Pokemon in QGY-7703 cells. miR-21 promoter (pLuc-M21) was cotransfected with Pokemon expression plasmid, and luciferase activity was measured with pRL-TK plasmid as an internal control (**$P < 0.01$ vs. Vector). B: Upper, miR-21 promoter-luciferase reporter plasmids, including wild-type miR-21 promoter and site-directed mutants at putative binding sites, pLuc-M21-m1 (−684 to −679 bp) and pLuc-M21-m2 (−652 to −647 bp). Lower, schematic representation of the wild-type and mutational miR-21 promoters cloned in luciferase reporter vector (**$P < 0.01$ vs. Vector). C: Chromatin immunoprecipitation (ChIP) assay shows that Pokemon binds to miR-21 promoter in QGY-7703 cells. Rabbit immunoglobulin G was used as a negative control. Lower panel: scheme of promter fragments for ChIP assay.

POKEMON REGULATES miR-21 EXPRESSION VIA DNA SEQUENCE-SPECIFIC BINDING TO ITS PROMOTER

miR-21 is intergenic and has its own promoter. To confirm its expression regulation by Pokemon, we did a luciferase reporter assay. miR-21 promoter was cloned and used to drive luciferase reporter gene expression in QGY-7703 cells. Results showed that co-transfection of Pokemon in QGY-7703 cells efficiently stimulated miR-21 promoter activity (Fig. 3A). Promoter motif analyses recognized two GC boxes GACCC and CCCCC, located at −684 to −679 bp and −652 to −647 bp in miR-21 promoter, which are putative binding sites of Pokemon. Therefore, we mutated these two consensuses and investigated the regulatory activity of Pokemon. As shown in Figure 3B, replacing GC boxes with TTTTT abrogated the promoter's responsiveness to Pokemon in QGY-7703 cells. These data suggest that Pokemon controls miR-21 transcription by a sequence-specific manner.

A ChIP assay confirmed the direct binding of Pokemon to miR-21 promoter. As shown in Figure 3C, miR-21 promoter fragment was amplified by polymerase chain reaction from chromatin precipitated by an anti-Pokemon antibody, but not by nonspecific immunoglobulin G. These data suggest that Pokemon up regulates miR-21 expression through direct binding to its promoter region at −741 to −399 bp upstream of the transcription start site.

POKEMON MODULATES SPROUTY1 VIA miR-21 REGULATION

As demonstrated above, Pokemon suppresses Sprouty1 expression (Fig. 1C–F), but has not effect on its promoter activity (Fig. 1G). Loss-of-function experiments showed that the transfection of anti-miR-21 increases Sprouty1 level as compared with transfection with a NC (Fig. 4A,B). As a target of miR-21, therefore, the Sprouty1 expression suppression by Pokemon may be mediated by miR-21. Indeed, we found that the Sprouty1 expression reduction induced by Pokemon was partially counterbalanced by the cotransfection with anti-miR-21 (Fig. 4C). Therefore, it appears that Pokemon up-regulates the expression of miR-21 that in turn down-regulates Sprouty1 expression. Both miR-21 and Sprouty1 are a part of the Pokemon regulatory network.

SPROUTY1 SUPPRESSION BY POKEMON OR miR-21 STIMULATES LIVER CELL GROWTH AND PROLIFERATION

To gain more insight of Pokemon-miR21-Sprouty1 axis into the role in cell growth and survival, QGY-7703 cells were treated with vector control, pcDNA3.1(−)/Pokemon, anti-miR-21 and pcDNA3.1(−)/Pokemon + anti-miR-21. Cell growth and proliferation assay results showed that up-regulation of Pokemon stimulated cell clonogenic growth and proliferation and the down-regulation of miR-21 inhibited cell these behaviors. When miR-21 was inhibited, Pokemon cannot affect QGY-7703 cells growth and proliferation (Fig. 5A,B). QGY-7703 cells were treated with vector control, pcDNA3.1(−)/Pokemon, pcDNA3.1(−)/Sprouty1 and pcDNA3.1(−)/Pokemon + pcDNA3.1(−)/Sprouty1. Results showed that the up-regulation of Sprouty1 inhibited cells clonogenic growth and proliferation. Co-transfection of Pokemon and Sprouty1 in QGY-7703 cells cannot affect cell growth and proliferation (Fig. 5C,D). Therefore, it appears that Sprouty1 suppression by Pokemon or miR-21 stimulates QGY-7703 cells growth and proliferation.

DISCUSSION

Transcription of miRNAs, in common with all genes transcribed by RNA polymerase II, can be modulated by TFs and an extensive combinatorial interaction among miRs and between miRs and TFs

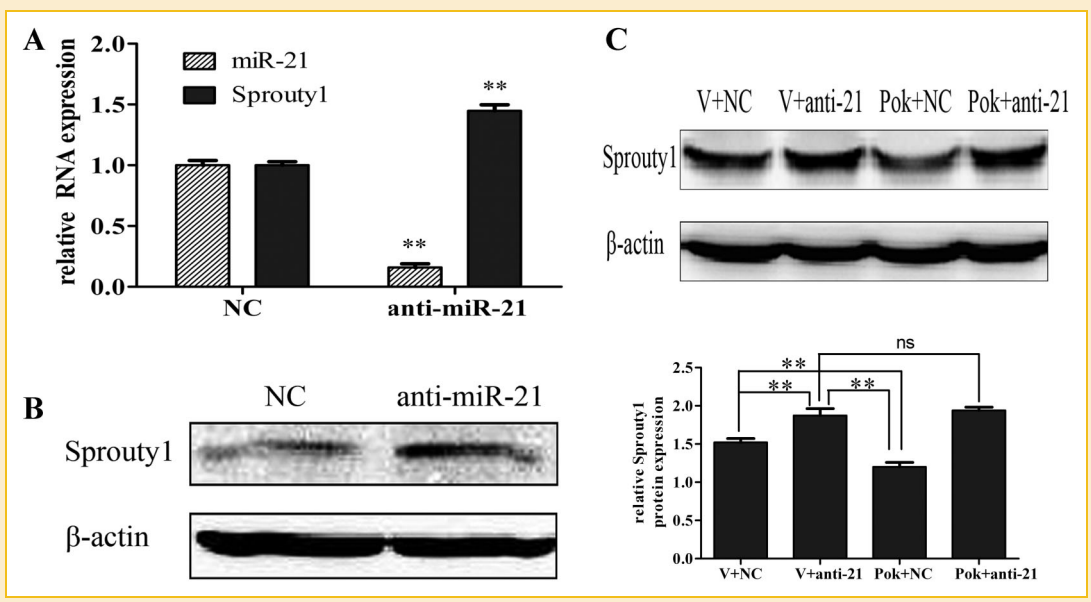

Fig. 4. Pokemon modulates Sprouty1 via miR-21 regulation. QGY-7703 cells transiently transfected with miR-21 inhibitor (anti-miR-21) and negative control (NC) for 48 h were tested for the expression of Sprouty1 by real-time PCR (A, $^{**}P < 0.01$ vs. NC) and Western blot analysis (B). C: QGY-7703 cells were co-transfected with vector control (Vector) + NC, Vector + anti-miR-21, Pokemon-expressing vector (Pokemon) + NC, Pokemon + anti-miR-21. After 48 h, cell lysates were used for Western blot assay. ns, not significant; $^{**}P < 0.01$ versus control (Pok + NC).

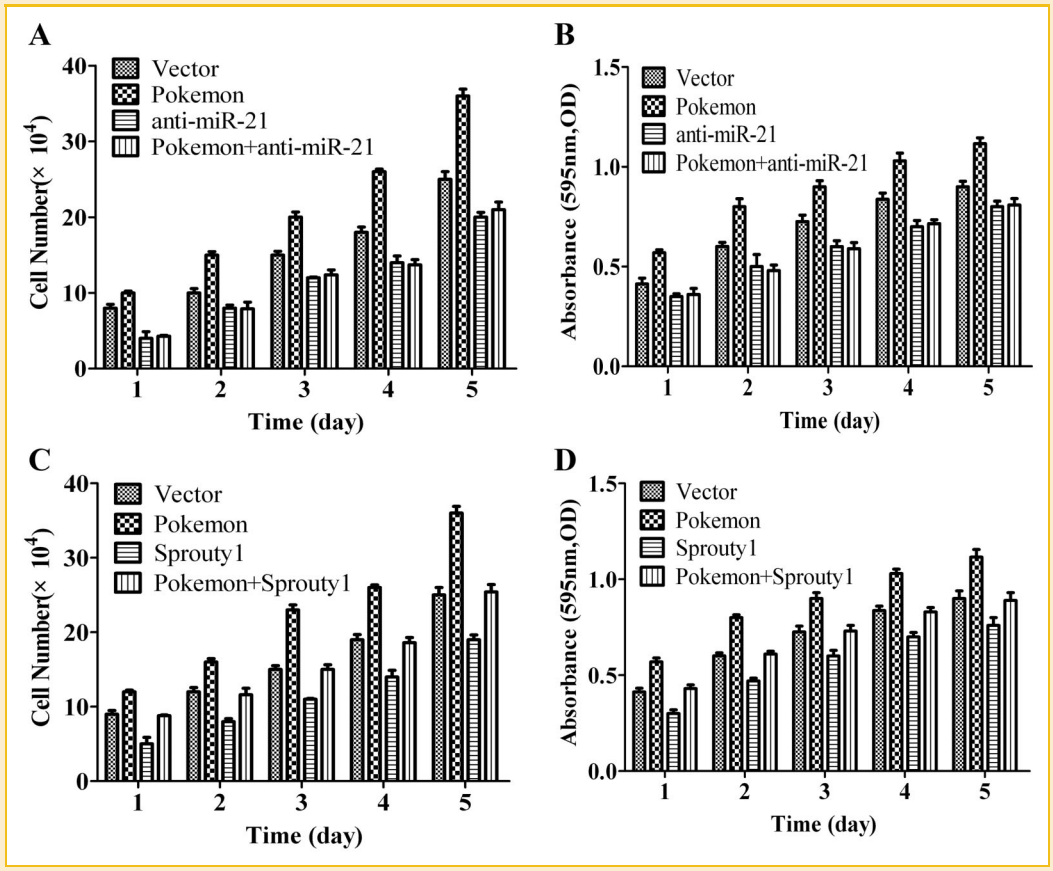

Fig. 5. Sprouty1 suppression by Pokemon or miR-21 affects cell growth and proliferation. QGY-7703 cells were transfected with vector control, pokemon overexpression plasmid pcDNA3.1(−)/Pokemon, anti-miR-21 and pcDNA3.1(−)/Pokemon + anti-miR-21 or with vector control, pcDNA3.1(−)/Pokemon, Sprouty1 overexpression plasmid pcDNA3.1(−)/Sprouty1 and pcDNA3.1(−)/Pokemon + pcDNA3.1(−)/Sprouty1. After 48 h, cells were trypsinized and seeded for cell growth curve (A,C) and MTT assay (B,D).

has been reported [Shalgi et al., 2007]. For instance, Pokemon is post-transcriptionally regulated by miR-20a, and on the other hand, Pokemon regulates miR-290 expression [Poliseno et al., 2008; Pitto et al., 2009]. Recent studies showed that Pokemon-dependent miRNAs miR-28 and miR-505 control MEF proliferation and survival by targeting ASF/SF2, suggesting the existence of Pokemon-miRNAs axis in cellular homeostasis, in addition to the Pokemon-p53 pathway [Verduci et al., 2010].

Several researchers reported that the expression of Sprouty family members is deregulated in cancer. Kwabi-Addo et al. [2004] have shown that Sprouty1 expression is decreased in human prostate cancer, afterwards, Lo et al. [2004] pointed out that Sprouty1 and Sprouty2 are deregulated in breast cancer, and Sprouty2 is also down-regulated in hepatocellular carcinoma be reported by Fong et al. [2006]. However, the mechanism of Sproutys down-regulation is still unknown. In this study, we found that Pokemon and Sprouty1 expression is negatively correlated in human liver cancer cells, and Pokemon can downregulate the expressions of Sprouty1 mRNA and protein, but has not effect on promoter activity of Sprouty1. Several studies have confirmed that Sprouty1 is a target of miR-21 [Thum et al., 2008; Hatley et al., 2010]. With gain-of-function and loss-of-function experiments, we demonstrated that Sprouty1 is under the control of miR-21. For these reasons, we hypothesized that Pokemon might modulate Sprouty1 via miR-21. In this study, we show that Pokemon is able to modulate the promoter activity of the miR-21 gene (Fig. 3), suggesting a direct control of miR-21 expression by Pokemon. Fortunately, we found a direct interaction of Pokemon with the promoter of the miR-21 by binding to the GC boxes.

A number of targets for miR-21 have been experimentally validated and most of them are tumor suppressors, such as PTEN [Meng et al., 2007], PDCD4 [Asangani et al., 2007], Tropomyosin [Zhu et al., 2007], Sprouty1 [Thum et al., 2008], Sprouty2 [Sayed et al., 2008], rendering miR-21 as an oncomir. Proto-oncogene Pokemon is overexpressed in some human cancers, playing a crucial role in the carcinogenic process. In this study, we demonstrated that Pokemon modulates miR-21 expression in liver cancer cells and enriches its oncogenic role. Further studies showed that miR-21 was causatively involved in growth and proliferation of liver cancer cell QGY-7703. Together these results may imply that Pokemon exerts its pro-proliferation activity not only by repressing ARF-MDM2-p53 [Maeda et al., 2005a], but also by miR-21-Sprouty1 axis (Fig. 6). This finding reveals a new signaling pathway of Pokemon-mediated oncogenesis and advances understanding of miR-21 regulatory mechanisms.

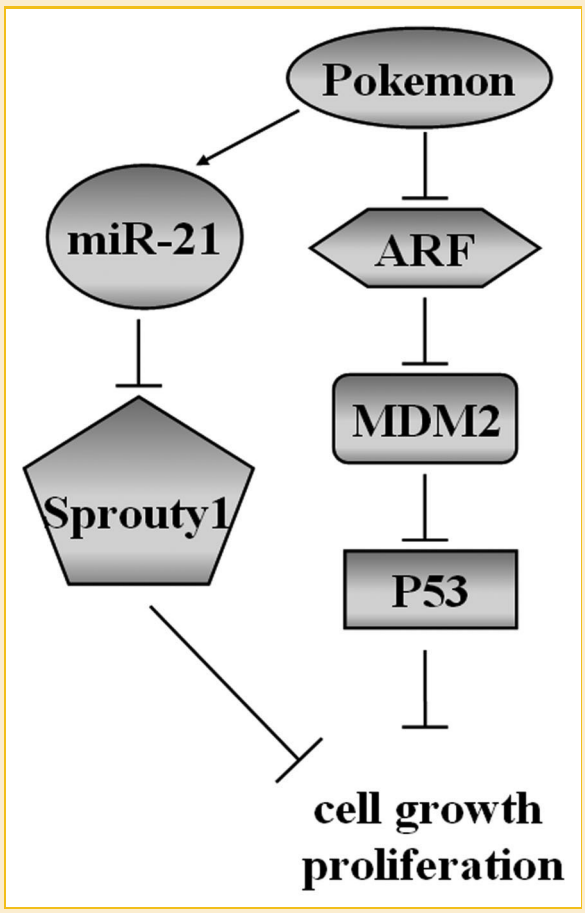

Fig. 6. Proposed roles of the Pokemon/miR-21/Sprouty1 axis (modified from Maeda et al., 2005b). The expression of miR-21 induced by Pokemon affects Sprouty1 levels and reinforces the role of Pokemon in cancer cell growth and proliferation.

Pokemon is overexpressed in liver cancer cell and inhibits the expression of Sprouty1 via mediating miR-21 expression, promoting cell growth and proliferation. This study found that miR-21 and Sprouty1 are new members of the Pokemon regulatory network, and specifically recognized a new oncogenic pathway of Pokemon, that is, Pokemon-miR21-Sprouty1. These findings may serve as a new therapeutic entry point for liver cancer and illustrate a broad therapeutic potential of miRNAs.

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