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Identification of GPR65, a novel regulator of matrix metalloproteinases using high through-put screening

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ABSTRACT

Matrix metalloproteinases (MMPs) are over-expressed in nearly all cancers. To study novel regulatory factors of MMP expression in head and neck cancer (HNC), we screened a total of 636 candidate genes encoding putative human transmembrane proteins using MMP promoter reporter in a dual luciferase assay system. Three genes GPR65, AXL and TNFRSF10B dramatically activated the induction of MMP3 expression. The induction of MMP expression by GPR65 was further confirmed in A549 and/or FaDu cells. GPR65 mediated MMP induction under acidic conditions. The AP-1 binding site in MMP3 promoter was crucial for MMP3 induction. Moreover, the A549 cells infected by recombinant adenovirus of GPR65 showed accelerated cell invasion. In conclusion, we validate that GPR65 is vital regulatory genes upstream of MMP3, and define a novel mechanism of MMP3 regulation by proton-sensing G-protein-coupled receptors.

1. Introduction

The activation of invasion and metastasis is one hallmark of cancer [1]. Matrix metalloproteinases (MMPs) are the major contributing factors during metastasis [2,3]. To date, more than 20 MMPs have been discovered [2,4]. MMPs are a family of zinc-dependent endopeptidases that degrade structural components of the extracellular matrix (ECM) to assist tumor cell metastasis. Aberrantly high level of MMPs expression has been found in multiple human tumors [2]. In line with these findings, MMPs are considered potential targets for cancer therapy. In addition to degrading extracellular matrix to promote tumor metastasis, MMPs also play important roles in controlling cell growth, inflammation and angiogenesis [3]. These functions of MMP provide new insight for cancer treatment.

Abundant evidence supports the idea that MMP expression is mainly regulated at the transcriptional level [5]. In fact, the MMP promoters contain several cis-elements, allowing for the regulation of MMP gene expression by its binding to a diverse set of transactivators, including AP-1, PEA3, Sp-1, β-catenin/Tcf-4, and NF-κB. Up to date, many regulators involved in these pathways have been reported to modulate the expressions of MMPs and contribute to tumor development [5,6].

Head and neck cancer primarily arises from the oropharynx, oral cavity, hypopharynx and larynx [7]. It is the sixth most common cancer worldwide. Stokes et al. [8] have shown that the expressions of MMP1, MMP3, MMP10 and MMP13 elevate in HNC, Patients with regional lymph node and/or distant metastases show significantly higher levels of MMP9 expression than patients without any tumor metastases [9]. The expressions of MMP9 and MMP13 are significantly different between tonsil squamous cell carcinoma and normal control tissues. Furthermore, MMP13 expression is correlated with tumor invasion, and the expression of MMP9 is correlated with nodal metastasis [10].

In this study, we aimed to identify novel regulators of MMPs that are aberrantly expressed in HNC. Using MMP luciferase reporter gene, we screened a cDNA panels encoding putative human transmembrane proteins. GPR65 was identified to activate the transcription of MMP3. Furthermore, GPR65 and other family members were further investigated on the gene expression regulation and functional analyses.

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Table 1
PCR primers used in this study.

Gene symbol	Primer (5′–3′)	Vector	Inserted site
MMP1	pF1: ggcctcgagtatgtctggactgcagtggcacaga pR1: ggcaagctttggcctttgtcttctttctcagtgc	pGL3-Basic	XhoI/HindIII
MMP12	pF12: ggcctcgagtctccaacaacaaggtaaatatctgg pR12: ggcaagcttacggatcaattcagtttactgtgttc	pGL3-Basic	XhoI/HindIII
MMP2	pF2: cggggtacctctccaacttggctctctggctatc pR2: ccgctcgagaggtcctggcaatccctttgtatgt	pGL4.17	KpnI/XhoI
MMP3	pF3: cggggtaccctgggacttgggaaacatctttca pR3: ccgctcgaggtctctatgccttgctgtcttgcct	pGL4.17	KpnI/XhoI
MMP9	pF9: cggggtaccagtgacttgcccaaggtcacata pR9: ccgctcgagtgagggcagaggtgtctgactg	pGL4.17	KpnI/XhoI
MMP10	pF10: cggggtacctgtgctagactttcgtatggcag pR10: ccgctcgagactgcccttaccttctttgtctact	pGL4.17	KpnI/XhoI
MMP13	pF13: cggggtacctttagccttcatttcatttccatagac pR13: ccgctcgagatggtgatgcctggggactgtt	pGL4.17	KpnI/XhoI
F1	pF4: cggggtacctctatcaggctttcctctaaacttt pR4: ccgctcgaggtctctatgccttgctgtcttgcct	pGL4.17	KpnI/XhoI
F2	pF5: cggggtacctttccctgtatttcaatcaggac pR5: ccgctcgaggtctctatgccttgctgtcttgcct	pGL4.17	KpnI/XhoI
F3	pF6: cggggtaccggagaatcacttgagcccaga pR6: ccgctcgaggtctctatgccttgctgtcttgcct	pGL4.17	KpnI/XhoI
F4	pF7: cggggtaccggatggattctgttcttcaacttc pR7: ccgctcgaggtctctatgccttgctgtcttgcct	pGL4.17	KpnI/XhoI
MMP3ΔAP1	pF8: aaagcaaggaagctgcgggtgat pR8: ccgcagcttccttgctttcatcc	pGL4.17	KpnI/XhoI
MMP3ΔPEA3	pF9: ctaccaagagagattaatcactgtgttg pR9: tgattaatctctcttggtagaggagaaaac	pGL4.17	KpnI/XhoI
GPR65	pF16: gtggagttcctgatgttggt pR16: gcctggagaatgtgagtgg	–	–
Beta-actin	pF17: tgacgtggacatccgcaaag pR17: ctggaaggtggacagcgagg	–	–

2. Materials and methods

2.1. MMP reporter gene plasmid construction

The promoter regions of MMP1, MMP2, MMP3, MMP9, MMP10, MMP12 and MMP13 were PCR amplified. All PCR primers are shown in Table 1. PCR fragments were inserted into pGL3-basic or pGL4.17 (Promega) using *Xho*I/*Hind*III and *Kpn*I/*Xho*I sites, respectively. The luciferase reporter gene plasmid of MMP3 promoter is named for MMP3-Luc.

In order to find crucial regulatory elements in the MMP3 promoter, a series of 5′-deletion mutants and TRE-deletion mutant of the MMP3 promoter were constructed. MMP3-Luc plasmid was used as the template, and the primers used were listed in Table 1. The MMP3 promoter region is 2037 bp (–2002 bp to +35 bp). The mutated constructs F1, F2, F3 and F4 contained −1867 bp to +35 bp, −1644 bp to +35 bp, −1500 bp to +35 bp, and −849 bp to +35 bp of the MMP3 promoter, respectively. MMP3ΔAP1 and MMP3ΔPEA3 contained MMP3 promoter deleted of the AP1 site (−64 bp to −70 bp) and the PEA3 site (−201 bp to −216 bp), respectively [5,11]. To generate MMP3ΔAP1, primers pF3 and pR8 were used to amplify the amino terminal fragment, and primers pF8 and pR3 were used to amplify the carboxyl terminal fragment. Finally, pF3 and pR3 were used to amplify the truncated mutant alleles and the amino and carboxy terminal fragments generated in the previous PCR reactions were used as the template. The truncated and deleted mutants were subcloned into pGL4.17 using the *Kpn*I/*Xho*I sites. The procedure of MMP3ΔPEA3 construction was similar to MMP3ΔAP1, except that primers pF9 and pR9 were used.

2.2. Mammalian expression vectors of human proton-sensing GPRs

GPR65 was obtained from the transmemberane protein plasmid library (see below), and the other three known human proton-sensing GPCRs (GPR132, GPR4, GPR68), were obtained from Sino-GenoMax (China).

2.3. cDNA library screening

A human cDNA library used in this study has been described previously [12]. In this study, a cDNA sub-library comprised of 636 genes encoding transmembrane proteins was used for screening. The TMHMM servers (http://www.cbs.dtu.dk/services/TMHMM-2.0/) and GO annotation (http://www.geneontology.org) tools were used for analyzing the transmembrane proteins. SignalP (http://www.cbs.dtu.dk/services/SignalP/) was used to analyze the transmembrane helixes of the N-terminal signal peptides.

2.4. Cell culture

293T, FaDu and A549 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Neuronbc, China) supplemented with 10% fetal bovine serum. All cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

2.5. MMP promoter activity assay and high throughput screening

The relative luciferase activity (RLA) of MMP promoter-Luc was measured by dual luciferase activity assay as described previously [12,13]. The most sensitive MMP-Luc was selected for further screening. For screening, the dual luciferase assay and signal analysis were performed as mentioned above [12,13].

2.6. Acid stimulation and inhibitory assay

The pH of serum-free medium DMEM/F12 was adjusted to 5.9, 6.5 or 7.4 with hydrochloric acid (4 M) or sodium hydroxide (0.4 M) [14]. 293T cells were plated and transfected with four GPRs

as mentioned above. Eighteen hours after transfection, the culture media were replaced by DMEM/F12 (pH 6.5 or 7.4). The cells were harvested and used to detect RLA after stimulation for 6 h. For inhibitory experiments, DMEM/F12 (pH 5.9) was used for acidic stimulation for 6 h, and 1%, 0.75%, 0.5%, 0.25% and 0.13% of 1-butanol was added to the culture media at the same time of media replenishment.

2.7. Generation of recombinant adenovirus of carrying GPR65 gene (Ad-GPR65)

The Ad-GPR65 was generated according to the methods as described previously [15]. Briefly, GPR65 were sub-cloned into the adenoviral vector pAdxsi (Sinogenomax, China). The recombinant adenoviral construct was transfected into the packaging HEK293 cells. The packaging HEK293 cells were freeze–thawed for several times to release intracellular viral particles. The viral titer were tested in HEK293 cells and the plaque formation unit (pfu) was obtained using a plaque formation assay.

2.8. Western blot analysis

A total of 100 μg proteins were separated on 10% SDS–PAGE gels, and transferred to nitrocellulose membranes (Invitrogen, USA). The membrane was incubated with anti-MMP3 antibody (R&D, USA). After rinsed in TBST, the membrane was incubated with corresponding secondary antibody for 2 h. After washes, the blots were scanned using an Odyssey Imaging System (Li-COR Bioscience, Lincoln, USA). An anti-GAPDH antibody (Santa Cruz, CA) was used to confirm equal loading subsequently.

2.9. Quantitative real time PCR

The cDNA of transfected A549 and FaDu cells was used as the template. SYBR was used to label the double strand DNA. The PCR reaction was performed as recommended by the manufacturer. The primers for GPR65 and β-actin were listed in Table 1. The relative RNA level in each sample was determined by the 2(−Delta Delta C (T)) Method.

2.10. Transwell invasion assay

The A549 and FaDu cells seeded in 6-well plate were infected by Ad-GPR65 or Ad-null (a blank recombinant adenovirus as control) with a MOI (multiplicity of infection) of 100. After 24 h, the infected cells were harvested, diluted to 1×10^5 cells/ml, seeded into Matrigel Invision Chamber (24-well plate, 8.0 micron pore, BD, CA), and cultured for 24 h. After 48 h, the matrigel membrane were fixed in 4% paraformaldehyde for 20 min, and stained in 1% crystal violet for 20 min. Cells were examined under an inverted microscope (Olympus IX70).

2.11. Zymography

The serum-free medium of infected cells by Ad-null or Ad-GPR65 were harvested, and the protein in the medium was then concentrated using centrifugal filter devices (Millipore, Amicon ultra 10K device). The protein concentration was measured using a BCA protein assay kit. Then, 50 μg total proteins were loaded and separated in 10% polyacrylamide gels mixed with 1 mg/ml gelatin (SBH-Bio, Japan). Gelatinolytic activity was detected using coomassie brilliant blue R250 staining as described [16].

2.12. Immunohistochemistry analysis

Paraffin-embedded tissue blocks of laryngeal or hypopharyngeal cancer samples were obtained from the head and neck tumor specimen bank of Beijing Tongren Hospital. The slides were incubated with human GPR65 antibody (Abcam, Hongkong). The bound antibody was detected using the DAB reaction. The level of immunoreactivity was graded as described previously [15]. The grade for each sample was used for statistical analysis with rank sum test. SPSS 13.0 was used for statistical analyses. Images were taken with an Olympus IX70 microscope.

3. Results

3.1. High-throughput cell-based screening reveals a role for GPR65 in activating MMP3 promoter

To identify novel regulators of MMP expression in HNC, we constructed seven MMP promoter reporters including MMP1, MMP2, MMP3, MMP9, MMP10, MMP12 and MMP13, which were aberrantly expressed in HNC patients. These promoters had basal activities when compared to the vector controls (Supplementary Fig. 1). PMA, a protein kinase C activator, stimulated the transcriptional activities of these promoters. Among these promoters, MMP3 had the most sensitive activity, and was selected for high throughput screening.

Transmembrane proteins have great therapeutic potential. For example, GPCRs are the targets of more than 90% of small molecules [17]. Moreover, it is relatively easy to develop monoclonal drugs or diagnostic markers targeting membrane proteins [18].

Here we tested a library containing 636 candidate genes encoding membrane proteins for MMP3 induction. In the preliminary screening, three candidate genes AXL (AXL receptor tyrosine kinase), TNFRSF10B (tumor necrosis factor receptor superfamily, member 10b) and GPR65 (G-protein coupled receptor 65) greatly induced MMP3 expression (Fig. 1A). They were validated as MMP3 activators in another triplicate assays. In our assay, GPR65 was the most sensitive activator for MMP3 induction. But the relation between GPR65 and matrix metalloproteinases is still unknown. Therefore, GPR65 was selected for further analyses.

3.2. Induction of MMP expression by proton-sensing GPCRs

Quantitative PCR and western blot analysis were performed to further identify whether GPR65 up-regulate the expression of MMP3. Compared with the pCMV-SPORT6 vector control, GPR65 over-expression promoted the transcription of MMP3 by over eight folds in both A549 and FaDu cells (Fig. 1B). The enhanced expression of MMP3 was also validated at the protein level using western blot analysis (Fig. 1C).

The proton-sensing GPCR family comprises four members, including GPR65, GPR4, GPR132 and GPR68, which share similar structures and sense protons to induce intracellular inositol phosphate (IP) or cAMP formation under acidic conditions [19]. Therefore, we next explored whether these GPCRs activate MMP expression. GPR65 dramatically increased the transcription of MMP1 and MMP12, and moderately promoted the transcription of MMP10 (Supplementary Fig. 2). GPR4 could activate the transcription of MMPs in a similar way to GPR65. GPR68 activated the expression of MMP3, MMP10 and MMP12, and the activation was the strongest on MMP3. Amongst all four GPCRs, GPR132 had no effect on MMP expressions. The promoters of MMP2, MMP9 and MMP13 did not response well to the over-expressions all four GPCRs. MMP2 and MMP9 are gelatinases that degrade gelatin and collagen IV [2]. Zymography is widely used for detecting MMP9 and MMP2, but not as common in detecting other MMPs.

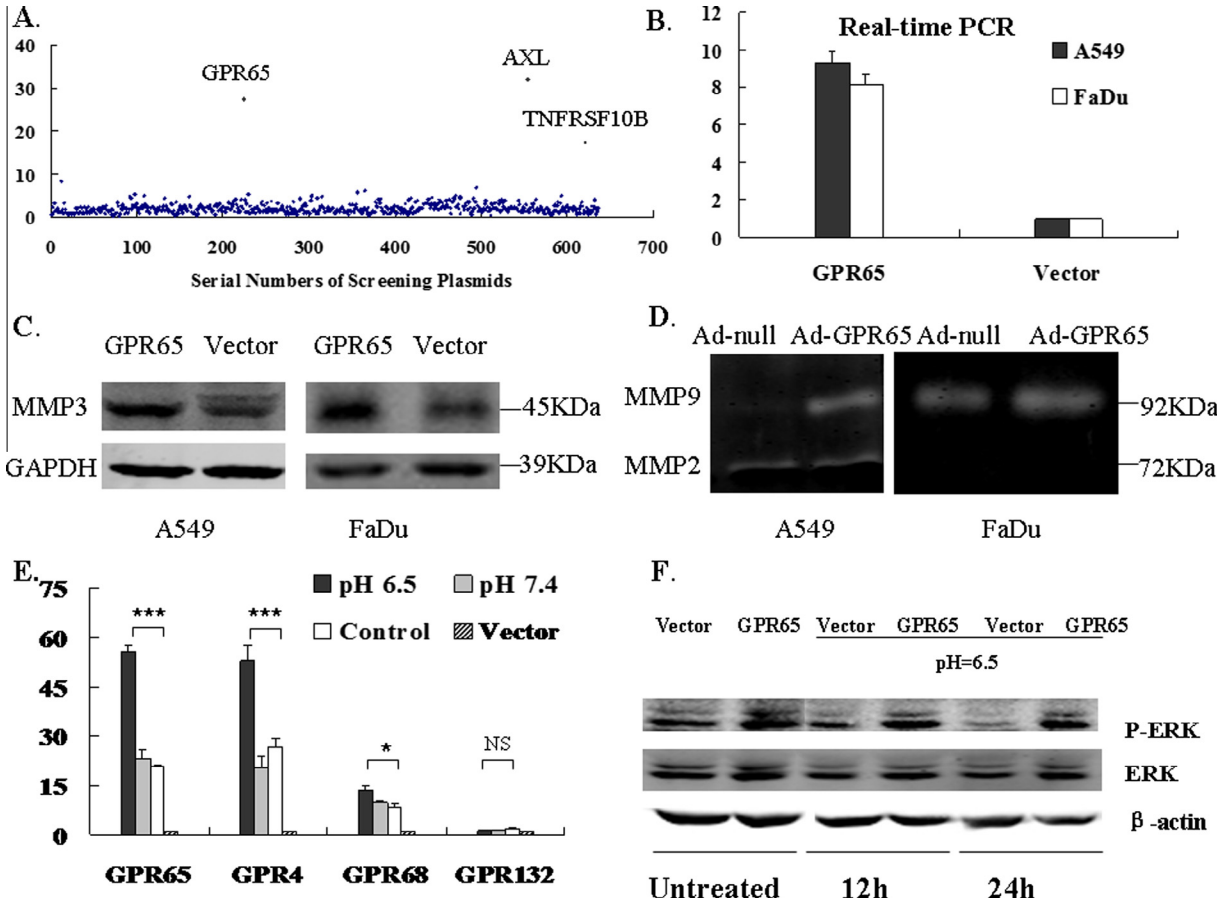

Fig. 1. MMP3-Luc based high throughput screening and induction of MMP expression by proton-sensing GPCRs or acidic stimulation. (A) 636 candidate genes encoding transmembrane proteins were tested for their ability to activate MMP3. Three clones (indicated with names) exhibited over 10-fold increase of luciferase activities compared with the blank vector control; GPR65 enhanced the RNA (B) and protein (C) expressions of MMP3 in A549 and FaDu cells. (D) Gelatin zymography revealed that GPR65 up-regulated the expression of MMP9 in A549 and FaDu cells. (E) Under acidic condition, GPR65, GPR4 or GPR68, but not GPR132, enhanced the transcriptional activity of MMP3 promoter in a dual-reporter assay system. (F) ERKs signaling pathway participated in the acidic stimulation of MMP3 promoter in A549 cells over-expressing GPR65.

Therefore, we performed a gelatinolytic zymography analysis uisng A549 and FaDu cells infected with Ad-GPR65. Upon infection, the A549 and FaDu cells showed increased expressions of MMP9 (Fig. 1D).

3.3. Acidic extracellular pH enhances MMP expression via ERK-signaling pathway in GPR65 or GPR4 over-expressed cells

Acidic extracellular pH induces the expression of MMP9 in mouse metastatic melanoma cells [14]. We showed that GPR65 and GPR4 greatly induced MMP3 expression at the transcriptional level in 293T cells. To examine whether extracellular pH stimulates MMP3 expression via regulation by GPCRs, we measured the transcriptional activity of MMP3 at different pH values. Compared with extracellular pH 7.4, the activity of MMP3 was enhanced by 2.86, 1.82 and 1.6-fold at pH 6.5 in cells over-expressing GPR65, GPR4, and GPR68, respectively. This result suggested that acidic extracellular pH enhanced MMP expression via proton-sensing GPCRs (Fig. 1E). However, no difference was observed between cells over-expressing GPR132 at pH 7.4 and pH 6.5. Further experiments showed that the enhancement in activity was significantly suppressed by 1-butanol, the phospholipase D inhibitor, in 293T cells over-expressing GPR65 [14], especially when the concentration was over 0.5% (Supplementary Fig. 3).

Previous reports have shown that ERK-signaling pathway mediates extracellular acidic pH signaling [14,20]. To evaluate the role of ERKs in the signal transduction of acidic pH stimulation of MMP3 by GPR65, we measured the phosphorylation levels (the active form) of ERKs. Under acidic condition, the phosphorylation of ERKs increased in A549 cells over-expressing GPR65 (Fig. 1F).

3.4. AP-1 is crucial for MMP3 induction by proton-sensing GPCRs

The transcriptional regulation of MMP expression involves many cis-elements and trans-factors, such as AP-1, PEA3, Sp-1, b-catenin/Tcf-4, and NF-κB [5]. Because only GPR4 and GPR65 strongly activated MMP3, we examined the effects of these two proteins on the activity of the mutant promoters (Fig. 2A). Deletion from −2002 to −1501 bp of the MMP3 promoter had no effect on MMP3 activation by GPR4 or GPR65, but further deletion from −1500 to −850 bp resulted in a slight decrease in MMP3 activation by GPR65 (Fig. 2B). When the region (from −1867 to −1644 bp) of MMP3 promoter was further deleted, the MMP3 activation by GPR4 decreased by about 50%, suggesting that this region might contain potential GPR4-dependent regulatory elements. Furthermore, when the AP-1 site was deleted, the transcriptional activation of MMP3 by both GPR4 and GPR65 dramatically decreased, whereas deletion of the two PEA3 sites had no effect on MMP3 activity. Further examination revealed that both GPR4 and GPR65 activated AP-1 (Fig. 2C). Therefore, the proximal AP-1 was an important regulatory element responsible for the activation of MMP3 by proton-sensing GPCRs.

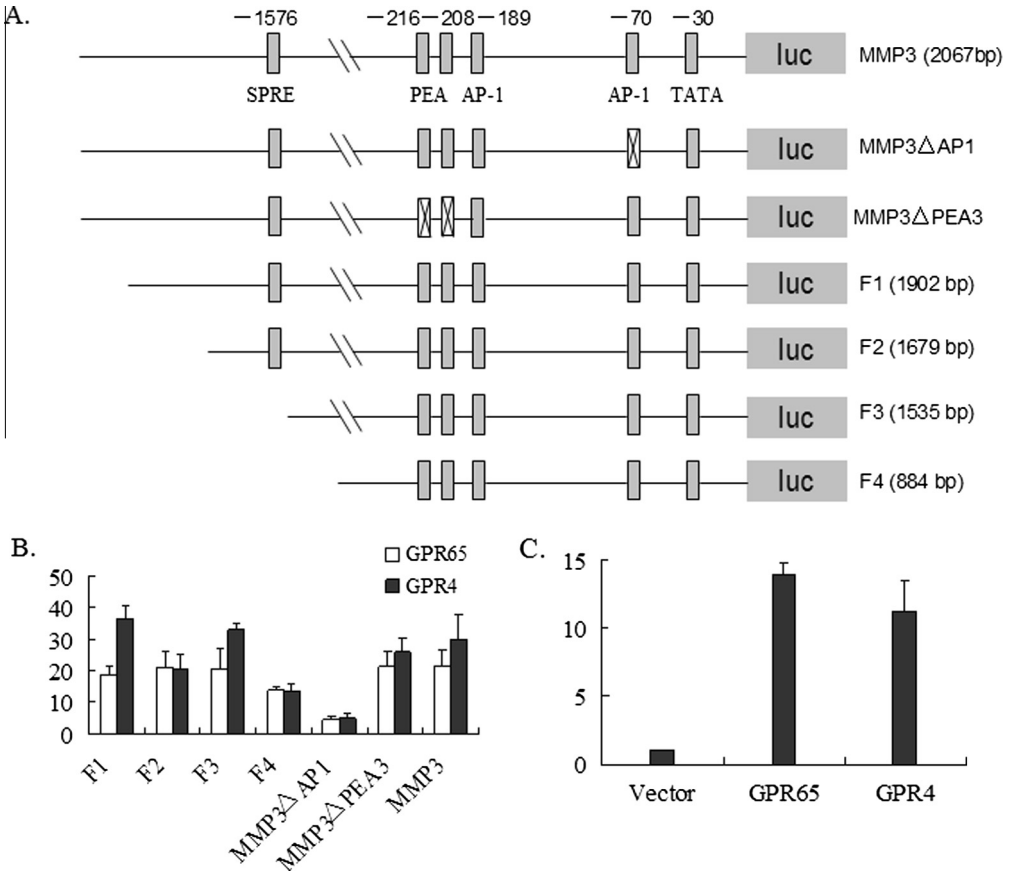

Fig. 2. AP-1 is crucial for MMP3 promoter activation by GPR65 and GPR4. (A) A schematic diagram of the transcription factor binding sites and MMP3 promoter deletion constructs. (B) By comparing the activities of these MMP3 promoter alleles, we revealed that AP-1 was the crucial element on MMP3 promoter regulated by GPR65 and GPR4. (C) Results of the double luciferase report assay showed that GPR4 and GPR65 activated AP1-Luc (Strategene), which is a luciferase report gene plasmid of AP-1.

3.5. Over-expression of GPR65 accelerates cancer cell invasion

Because MMPs are required for ECM remodeling during cancer cell metastasis, we next examine whether the over-expression of GPR65 reinforces the invasive capability of cancer cell using the transwell invasion assay. GPR65 dramatically reinforced the invasive ability of A549 cells, but not FaDu cells (Fig. 3). This was likely due to the assay system. The matrigel in the chamber we used was a mixture of structural proteins including collagen IV, laminin, and entactin. These components are subjective to degradation by MMP2 and MMP9. Our results suggested that GPR65 could up-regulate the expression of MMP9 in A549 but not FaDu cells.

3.6. GPR65 are over-expressed in laryngeal and hypopharyngeal cancer

We selected 12 cases of HNC. All cases have been re-confirmed as squamous cell cancer by two experienced pathologists (Table 2). Immunostaining results revealed that GPR65 was expressed in epithelial cells in all cases. No significant difference in GPR65 expression was found between the cancerous and peri-cancerous cells ($p = 0.096$). In cancer cells, GPR65 tended to be highly expressed. In interstitial tissues, GPR65 was expressed only at low levels in lymphocytes, plasma cells, and vascular endothelial cells. It was not detected in fibroblast cells (Fig. 4).

4. Discussion

In this study, we show that proton-sensing GPCRs regulate the expressions of MMP1, MMP2, MMP3, MMP9, MMP10 and MMP12. To date, four GPCRs that sense extracellular acidic stimuli have been identified. Among these extracellular proton sensors, GPR65 and GPR4 have the most significant regulatory effects on MMP expression. GPR65 is originally identified as an orphan GPCR that promotes apoptosis in thymocytes. Ishii et al. have subsequently revealed that GPR65 is an acid sensing GPCR [21]. GPR65 enhances tumor development by promoting the adaptation of cancer cells to the acidic environment and enhancing cell survival and proliferation [20]. In thymocytes and splenocytes explanted from receptor-deficient mice, GPR65 is critical for pH-dependent cAMP production. In contrast, GPR132 is dispensable for this process. GPCRs exhibit differential sensitivity to extracellular protons, and the expression of TDAG8 in immune cells may regulate the responses to acidic microenvironments [19].

Sin et al. have shown that GPR4 and GPR65 are over-expressed in a range of human cancer, such as breast, ovarian, colon, and liver and kidney tumors. In animal models, GPR4 and GPR65 trigger malignant transformation of normal mouse cell line, and play a role in driving or maintaining tumor formation [22]. Wyder et al. have found that GPR4-deficient mice have a significantly reduced angiogenic response to VEGF and reduction in tumor growth [23].

The extracellular acidosis of solid tumors is due to glycolysis metabolites accumulation. The acidosis is associated with tumor metastasis. Kato et al. have shown that extracellular acidic pH (5.4–6.5) can induce matrix metalloproteinase-9 (MMP-9) expression in mouse metastatic melanoma cells [14]. However, a link between proton-sensing GPCR and the expression regulation of MMP have not been established.

We found that the over-expressions of GPR65 and GPR4 *in vitro* enhanced the transcriptional activity of several MMPs in the

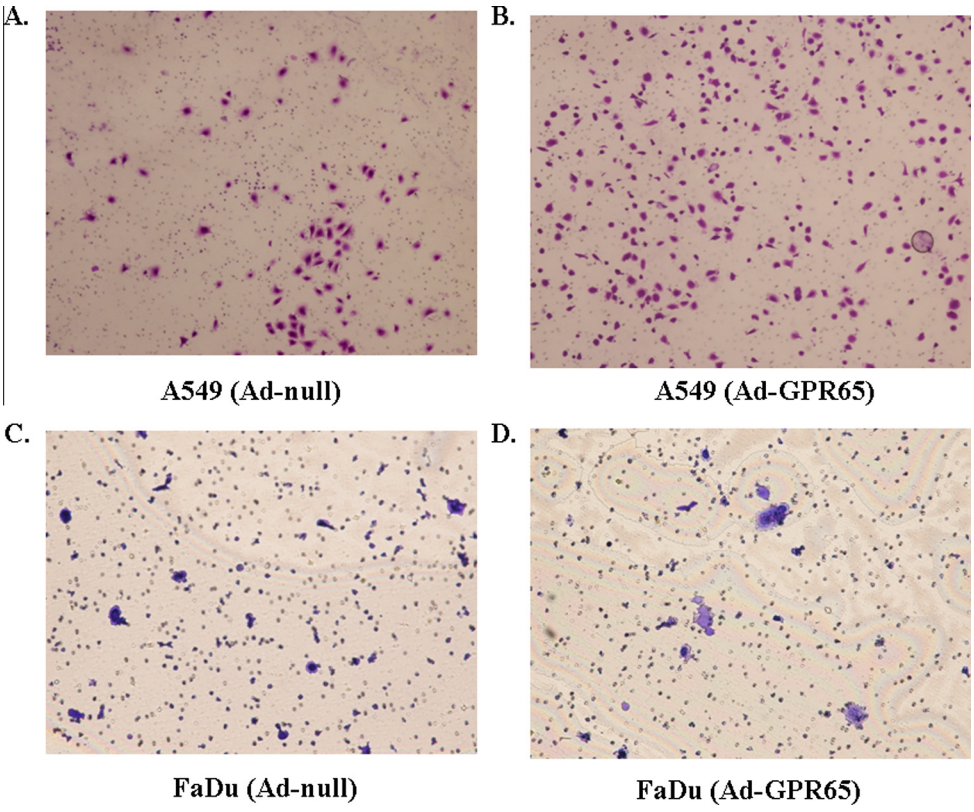

Fig. 3. GPR65 accelerated the invasion of A549 but not FaDu cells. The cells passing through the matrigel were stained with 1% crystal violet. The images were taken at 40× magnification. Three independent experiments were performed and similar results were obtained. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Clinical features of the HNC tumor samples and corresponding GPR65 expression in IHC.

Case	Gender	Age	Clinical and pathological diagnosis	TNM staging	Differentiation	IHC GPR65 expression score	
						CT	PCT
1	M	57	SGC	T2N1M0	Moderate	2	1
2	M	48	SGC	T2N0M0	Moderate	2	2
3	M	62	SGC	T1N0M0	High	3	2
4	M	64	SGC	T2N0M0	High	2	1
5	M	48	GC	T2N0M0	High	2	3
6	M	71	GC	T3N0M0	High	2	2
7	M	76	GC	T3N0M0	Moderate	2	3
8	M	54	HPC	T4aN2M0	Low	2	2
9	M	50	HPC	T3N2M0	Low	1	1
10	M	52	HPC	T2N2M0	High	2	3
11	M	64	HPC	T2N0M0	Low	1	3

CT: cancerous tissue; PCT: peri-cancerous tissue; SGC: superglottic cancer; GC: clottic cancer; HPC: hypopharyngeal cancer; All cases were squamous cell carcinoma. TNM staging is according to the AJCC cancer staging 7th edition. Wilcoxon signed ranks test showed $p = 0.527$ between CT and PCT.

dual-luciferase reporter system, western blot analysis, and zymography analysis. In addition, the AP-1 binding site at −70 bp played a key role in the induction of MMP3 by GPR4 and GPR65, and the region from −208 bp to −189 played a minor role in the regulation of MMP3 expression induced by GPR4. Which transcription factor mediates the activation needs to be further investigated. We also found that in acidic extracellular pH conditions, the expression of MMP3 was elevated, suggesting that MMP responded to extracellular pH, which was consistent with the results of previous studies [14]. This response was likely mediated by the increases in phosphorylation level and kinase activity of the components of the ERK-AP1 signaling pathway. Moreover, exogenous expression of GPR65 promoted the invasion of A549 cells, indicating the potential role of GPR65 in tumor metastasis. Therefore, we hypothesized that during tumor development, glycolysis metabolites resulted in the accumulation of acid, which bound to GPCRs and accelerated tumor metastasis via the up-regulation of MMPs. Therefore, the aberrantly high expression of GPCR in cancer makes a positive feedback loop to accelerate cancer cell metastasis.

Although several MMPs are regulated by GPCRs, which MMPs play a major role during the process of tumor cell invasion is still not clear. We validated at protein levels in A549 cells that MMP3 and MMP9 were up-regulated by the over-expression of GPR65. However, the up-regulation of only MMP3 was confirmed in FaDu cells. This suggests that the downstream regulation by

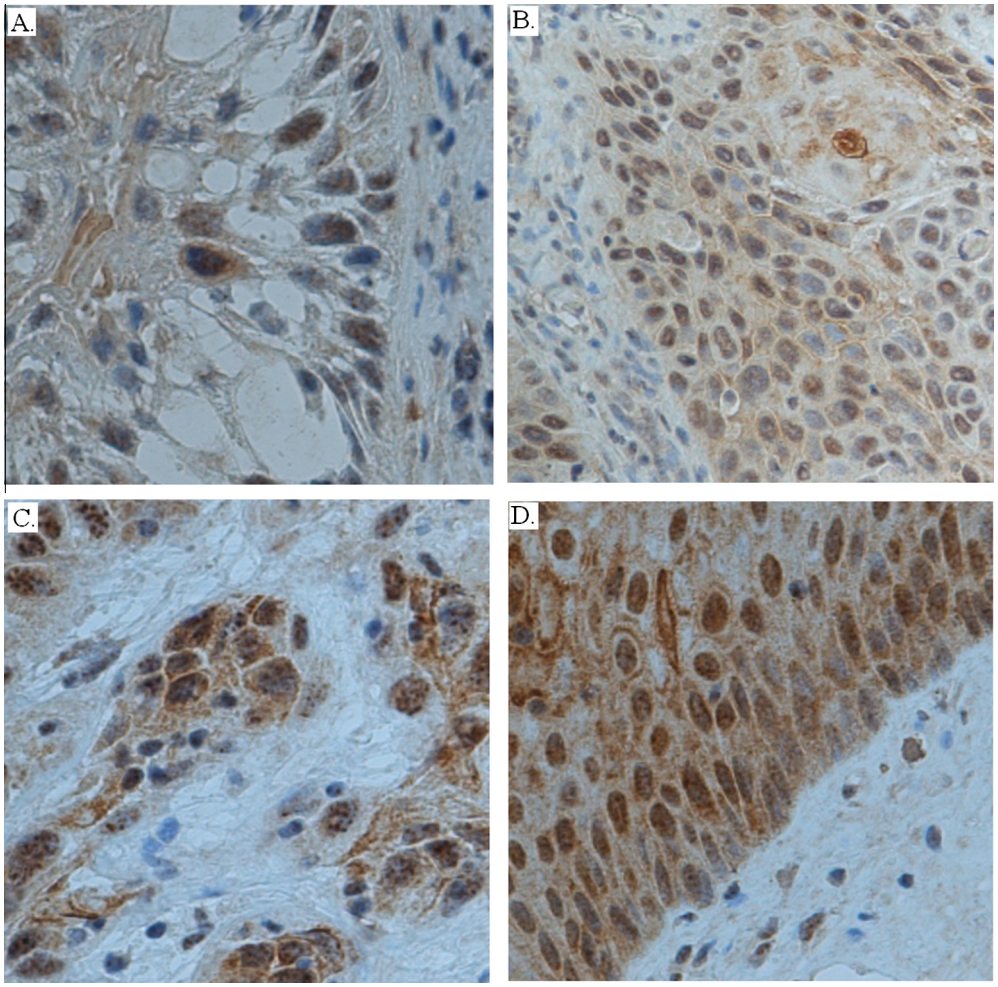

Fig. 4. Immunostaining of GPR65 in laryngeal and hypopharyngeal cancer. GPR65 was expressed at relatively high levels in epithelial cells of cancer and peri-cancerous cells, and at relatively low levels in the interstitial tissues. (A) Highly differentiated laryngeal cancerous tissue. (B) Moderately differentiated laryngeal cancerous tissue. (C) Low differentiated hypopharyngeal cancerous tissue. (D) Peri-cancerous tissue of laryngeal cancer.

GPR65 over-expression was cell type dependent. MMP3, also known stromelysin-1, is over-expressed in a wide variety of tumor types. MMP3 has wide substrate specificity for various extracellular matrix components, targeting most proteins and proteoglycans except for fibrillar collagens. Sternlicht et al. have shown that the expression of MMP3 or MMP14 in the mammary gland results in spontaneous breast cancers [24]. However, it remains unclear whether MMP3 plays a role in accelerating HNC development.

In conclusion, we validated that GPR65 and GPR4 are the vital regulatory genes of MMP3 and defined a previously uncharacterized mechanism for MMP3 expression regulated by proton-sensing GPCRs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.065.

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