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Research article

The luciferase reporter system of the MMP12 endogenous promoter for investigating transcriptional regulation of the human MMP12 gene

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ABSTRACT

Background: Matrix metalloproteinase 12 (MMP12), a member of MMPs, can take lots of roles including extracellular matrix component degradation, viral infection, inflammation, tissue remodeling and tumorigenesis. To explore the transcriptional regulation of MMP12 gene, a sensitive luciferase reporter HEK293 cell line for endogenous MMP12 promoter was generated by CRISPR/Cas9 technology.
Results: The HEK293-MMP12-T2A-luciferase-KI cell line was successfully established by CRISPR/Cas9 technology. The sequencing results indicated that one allele of the genome was proven to have a site-directed insertion of luciferase gene and another allele of the genome was confirmed to have additional 48 bp insertion in this cell line. The cell line was further demonstrated to be a sensitive reporter of the endogenous MMP12 promoter by applying transcription factors STAT3, AP-1 and SP-1 to the cell line. The reporter cell line was then screened with bioactive small molecule library, and a small molecule Tanshinone I was found to significantly inhibit the transcriptional activity of MMP12 gene in HEK293-MMP12-T2A-luciferase-KI cell line by luciferase activity assay, which was further confirmed to inhibit the expression of MMP12 mRNA in wild-type HEK293 cells.
Conclusions: This novel luciferase knock-in reporter system will be helpful for investigating the transcriptional regulation of MMP12 gene and screening the drugs targeting MMP12 gene.
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1. Introduction

Matrix metalloproteinases (MMPs), consisting of a family of zinc-dependent endopeptidase, play an essential role in the regulation of both cell-extracellular matrix and cell–cell interactions. MMPs have a great impact on many physiological processes, such as proliferation and apoptosis of cells, tissue remodeling, hematopoiesis regulating, wound repair and angiogenesis [1]. While MMPs were also found to be closely related to the process of epithelial-mesenchymal transition (EMT) and pathological conditions in tumor invasion and metastasis, drugs targeting MMPs individually could improve the treatment of many diseases [2]. Among MMPs, matrix metalloproteinase 12 (MMP12) is a 22-kDa secretory proteinase that is predominantly secreted by macrophage, but has less or no expression in normal tissue [3,4]. MMP12 is able to degrade the components of extracellular matrix to facilitate tissue remodeling [5,6]. It has been found that MMP12 is highly expressed in most tumors, such as squamous cell cancer [7], none small cell lung cancer [8], gastric cancer [9], ovarian cancer [10] and hepatocellular carcinoma [11]. The up-regulated expression of MMP12 can increase cancer cell migration, invasion, metastasis and angiogenesis, and high levels of MMP12 expression have been reported to be related to poor clinical outcome [12]. However, the detailed mechanisms involving MMP12 biological activity remain unclear.

Genome editing technology has been wildly utilized for gene functional analysis and therapeutic application in animals. Compared with transcription activator-like effector nucleases (TALENs) and zinc finger nucleases (ZFNs), clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system provides an easier and efficient way for gene targeting [13,14,15,16]. CRISPR/Cas9 is artificially engineered from the type II acquired immune system in bacterium, which can achieve highly flexible and specific genome editing in eukaryotic cell [17]. Single guide RNA (sgRNA) can guide the CRISPR/Cas9 to modify target genome by matching targeted DNA sequence [17]. CRISPR/Cas9 recognizes DNA sequences and cuts DNA at specific sites to generate double-strand breaks (DSBs) by

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recognizing 20- or 24-nt sgRNA. Then, the insertion or deletion of gene at fixed site can be achieved via non-homologous end joining (NHEJ) or homologous recombination (HR) repair mechanism [18].

The aim of this study was to establish a luciferase reporter knock-in system of HEK293 cell line using CRISPR/Cas9 technology for studying transcriptional regulation of the human MMP12 gene. In this system, the luciferase reporter gene was regulated by endogenous MMP12 gene promoter. The established stable cell line with an efficient reporter under the control of endogenous MMP12 would greatly facilitate the study of the function of MMP12 gene, and provide a powerful tool for screening drugs targeting MMP12 gene.

2. Materials and methods

2.1. Cell line and cell culture

HEK293 cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Gaithersburg, MD) supplemented with 10% (vol/vol) FBS, 100 g/ml streptomycin and 100 U/ml penicillin G (Gibco BRL, Gaithersburg, MD).

2.2. Vector construction

To construct Cas9/sgRNA vector, four gene-specific sgRNAs targeting the region after MMP12 gene stop codon were designed based on the websites (http://crispr-era.stanford.edu/index.jsp). The sequences of four sgRNAs are shown in Table 1. Each sgRNA was then cloned into the pUC19/U6 vector (kept in Xia's lab). The resultant vectors were called pU6-MMP12-sgRNAx (x stands for 1–4). Next, the sgRNA with highest cutting efficiency was ligated into pUC19/CMV-Cas9-SV40pA according to the method described previously [19]. The obtained vector was called pCMV-Cas9-SV40pA-U6-sgRNA-SV40pA.

To generate donor vector, human genomic DNA was used for a template to generate the DNA fragment of up or down homologous arm by PCR. According to the methods reported previously [19], the resultant donor vector called pUC19/MMP12 was constructed successfully, which was composed of an up homologous arm (835 bp) followed by a T2A-luciferase reporter cassette and a positive selection cassette CMV-eGFP-T2A-Neomycin-SV40pA, a down homologous arm (1112 bp) followed by a negative selection cassette PGK-TK-T2A-mCherry-SV40pA. The primers used for PCR amplification of the homology arms are listed in Table 2.

2.3. T7E1 assay

In order to test the targeting efficiency of the sgRNAs, HEK293 cells were transfected with 3 μg pUC19/CMV-Cas9 and 6 μg pU6-MMP12-sgRNAx (x stands for 1–4) in 60 mm plates (2×10^6 per dish), pU6-control sgRNA was used as a negative control. Then, the nested-PCR method was used to amplify the target sequence fragment (TSF) based on the genomic DNA extracted from the HEK293 cells transfected with the plasmids above for 72 h. Next, the PCR products were purified after denaturation and annealing, and the purified products were subjected to T7E1 assay. The primers used for the PCR amplification of the TSF are shown in Table 3.

Table 1
The primers for sgRNA targeting MMP12.

Primer	Sequence
P1:MMP12 Knock-in sgRNA1 for	5′-ACCGctctaagtagtggtacactg-3′
P2:MMP12 Knock-in sgRNA1 reverse	5′-AAACcagtgtaccactacttagag-3′
P3:MMP12 Knock-in sgRNA2 for	5′-ACCGggtaacaccacttgtgtcct-3′
P4:MMP12 Knock-in sgRNA2 reverse	5′-AAACaggacacaagtggtgttacc-3′
P5:MMP12 Knock-in sgRNA3 for	5′-ACCGctaggctacacacaacccca-3′
P6:MMP12 Knock-in sgRNA3 reverse	5′-AAACtggggttgtgtgtagcctag-3′
P7:MMP12 Knock-in sgRNA4 for	5′-ACCGgcatggtaagcacatcattc-3′
P8:MMP12 Knock-in sgRNA4 reverse	5′-AAACgaatgatgtgcttaccatgc-3′

Table 2
The primers used for the amplification of donor in the study.

Primer	Sequence
P9:MMP12 Knock-in Up Arm Nest for	5′-ggcccaggatttttccctga-3′
P10:MMP12 Knock-in Up Arm Nest reverse	5′-gtggtacactgaggacatagca-3′
P11:MMP12 Knock-in Up Arm for *Cla* I	5′-cATCG ATggttgtctagcaggcagagg-3′
P12:MMP12 Knock-in Up Arm reverse *Spe* I	5′-gACTA GTacaaccaaaccagctattgc-3′
P13:MMP12 Knock-in Down Arm Nest for	5′-ggccaagtccatttcaagctg-3′
P14:MMP12 Knock-in Down Arm Nest reverse	5′-aactacagttctggcaggct-3′
P15:MMP12 Knock-in Down Arm for *Sal* I	5′-cGTCG ACtaactcaggagggaggcgtt-3′
P16:MMP12 Knock-in Down Arm reverse *Bgl* II	5′-aAGAT CTagtccacaaggtagacagtcc-3′

2.4. Generation HEK293-MMP12-T2A-luciferase-KI cell line

To further construct a reliable HEK293 cell line with site-specific integration of luciferase gene which under the regulate of an endogenous promoter of MMP12 gene, 8 μg of pUC19/MMP12 donor and 4 μg of pCMV-Cas9-U6-MMP12-sgRNA were co-transfected into HEK293 cells in Opti-MEM I reduced serum medium using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The cells were selected in selection medium containing 200 g/ml Geneticin (G418) (Invitrogen, Waltham, MA, USA) and 0.5–1.0 mg/ml Ganciclovir (GCV) (Sigma, St. Louis, MO, USA) for 48–72 h post transfection, the stable cell lines were then obtained through limited dilution after a round of positive and negative selection, the luciferase activity of each clone was detected by luciferase assay kit. The constructed cell line was named HEK293-MMP12-T2A-luciferase-KI.

2.5. Validation of luciferase expression regulated by endogenous MMP12 promoter in HEK293-MMP12-T2A-luciferase-KI cell line

To verify if the expression of luciferase reporter gene in the HEK293-MMP12-T2A-luciferase-KI cell line is regulated by the endogenous MMP12 gene, two transcription factors including STAT3 and AP-1, which have been previously reported to significantly improve MMP12 transcription [20,21], were cloned by PCR. Meanwhile, transcription factor SP-1, targeting MMP12 promoter, was predicted by GENE CARD software and cloned by PCR. Then, STAT3, AP-1 and SP-1 were separately ligated into the eukaryotic expression vector pUC19/CMV (stored in Xia's laboratory). These plasmids were named pUC19/CMV-STAT3, pUC19/CMV-AP-1 and pUC19/CMV-SP-1, respectively. 0.5 μg of each vector carrying a transcription factor above was transfected into HEK293-MMP12-T2A-luciferase-KI cell line or wild-type HEK293 cells for 48–72 h, and luciferase activity of HEK293-MMP12-T2A-luciferase-KI cells was detected by using luciferase assay kit (Promega, Madison, WI). Whereas, the expression of endogenous MMP12 gene in HEK293 cells was analyzed by real-time PCR. Glyceraldehyde phosphate 3-dehydrogenase (GAPDH) was used as an endogenous control. The primers specific for real-time PCR of MMP12 and GAPDH are listed in Table 4.

Table 3
The primers for the amplification of TSF.

Primer	Sequence
P17:MMP12 Knock-in TSF Nest for	5′-tgactggctgttggtagacg-3′
P18:MMP12 Knock-in TSF Nest reverse	5′-ccagcttgaaatggacttggc-3′
P19:MMP12 Knock-in TSF for	5′-ttcctactccaacgtatcacca-3′
P20:MMP12 Knock-in TSF reverse	5′-acttggccatttctgcaaac-3′

Table 4
The primers used for RT-PCR in the study.

Primer	Sequence
P21:hMMP12 Real-time PCR for	5′-ggaatcctagcccatgctttt-3′
P22:hMMP12 Real-time PCR reverse	5′-cattacggcctttggatcact-3′
P25:hGAPDH for	5′-gcaccgtcaaggctgagaac-3′
P26:hGAPDH reverse	5′-tggtgaagacgccagtgga-3′

2.6. PCR-based detection of the genotype of HEK293-MMP12-T2A-luciferase-KI cell line

To prove whether the reporter gene was integrated into the specific site of genome, genomic DNA extracted from the reporter HEK293 cell line was used for PCR template for the detection of the integration of exogenous gene in the genome. The predicted 5.6 kb large fragment was amplified from the targeted allele with integrated reporter gene in the MMP12 gene, and a predicted 3.2 kb small band was amplified from the wild-type MMP12 gene using 5′ primer P17 (shown in Table 3) and 3′ primer P14 (shown in Table 2). The predicated 2.0 kb up homologous arm was amplified from the genomic DNA extracted from clone 3 using 5′ primer P9 located in genomic region before up homologous arm and 3′ primer P23 that was specific to luciferase gene, and a predicted 1.3 kb down homologous arm was also amplified from clone 3 using 5′ primer P24 that was specific to SV40 gene and 3′ primer P14 located in genomic DNA after down homologous arm. All PCR fragments above were separately ligated into pGEMT-easy vector and sequenced. All primers used for amplifying up or down homologous arm were shown in Table 5.

2.7. Screening of small molecule drugs in the HEK293-MMP12-T2A-luciferase-KI cell line

To identify drugs targeting MMP12 gene, 30 bioactive small molecule drugs which were unknown to affect MMP12 gene was purchased from Selleck Chemicals, Houston, USA. The standard small molecule drug screening was performed by using a single concentration (10 μM) of drugs in HEK293-MMP12-T2A-luciferase-KI cell in 96-well plates (5×10^4 cells per well) for 48 h. Then the drugs affect the luciferase activity of the reporter cells with significant changes will be further validated in HEK293 cells by analyzing the expression of endogenous MMP12 using RT-PCR.

2.8. Statistical analysis

The statistical analysis and graphs plot were performed using GraphPad Prism software version 5. Results are expressed as means values ± Standard Deviation (SD), all data were analyzed by Student's t-test. Value of $P < 0.05$ was considered as significantly different from the control group.

Table 5
The primers used for detecting the integration of foreign gene in the genome.

Primer	Sequence
P9:MMP12 Knock-in up integration detection for	5′-ggcccaggatttttccctga-3′
P23:MMP12 Knock-in up integration detection reverse	5′-cgtcggtaaaggcgatggt-3′
P24:MMP12 Knock-in down integration detection for	5′-cctctacaaatgtggtatggctgat-3′
P14:MMP12 Knock-in down integration detection reverse	5′-aactacagttctggcaggct-3′

3. Results

3.1. Generation of HEK293-MMP12-T2A-luciferase-KI cell line

To target a luciferase reporter gene into the genome controlled by the MMP12 gene promoter in HEK293 cell line using CRISPR/Cas9 technology, four target sgRNAs were designed in the genome, which target the downstream region behind the stop codon of MMP12 gene (Fig. 1A). T7E1 assay showed that sgRNA3 had the greatest target activity (Fig. 1B). Then, pCMV-Cas9-SV40pA-U6-sgRNA3-SV40pA vector was generated by cloning sgRNA3 expression cassette into pUC19/CMV-Cas9 vector (Fig. 1C). According to the method reported previously (10), pUC19/MMP12 donor vector was generated, which included a T2A-luciferase reporter gene controlled by MMP12 endogenous promoter, a positive selection cassette CMV-eGFP-T2A-Neomycin-SV40pA and a negative selection cassette PGK-TK-T2A-mCherry-SV40pA (Fig. 1D). Then, pUC19/MMP12 donor and pCMV-Cas9-U6-MMP12-sgRNA3 were co-transfected into HEK293 cells followed by the selection of G418 and GCV for 28 d, we observed eGFP expression but undetectable expression of mCherry (Data are not shown). Through limited dilution, 30 clones were selected for luciferase activity assay (Fig. 2A). Subsequently, we selected 5 clones with higher luciferase activity for analysis by PCR through 5′ primer P17 (shown in Table 3) and 3′ primer P14 (shown in Table 2). A 5.6 kb large fragment amplified from the clones indicated that the exogenous gene was specifically inserted into an allele in the genome, and a 3.2 kb small band amplified from the clones which was identical to that amplified from wild-type HEK293 cells, suggesting another allele was not integrated by exogenous gene in the genome (Fig. 2B). To further confirm the site-specific integration of the exogenous gene, the two bands amplified with the primers located outside the homologous arms from clone 3 with highest luciferase activity were separately ligated into pGEMT easy vector for sequencing (Fig. 2C). The sequencing results indicated that the targeted integration of the foreign gene included the sequence of T2A-luciferase-CMV-eGFP-T2A-Neomycin cassette (Fig. 2D–G). And the sequenced result of 3.2 kb small band was proven to contain the insertion of 48 bp DNA sequence in the targeted region of the genome (Fig. 2H). The results indicated that the luciferase reporter gene controlled by MMP12 promoter had been specifically inserted into the target region of the genome. The obtained cell line from clone 3 was called HEK293-MMP12-T2A-luciferase-KI cell line.

3.2. The effect of transcription factors and drugs on the luciferase expression of HEK293-MMP12-T2A-luciferase-KI cell line

To test whether the luciferase activity was regulated by the MMP12 promoter in HEK293-MMP12-T2A-luciferase-KI cell line, the eukaryotic expression vector carrying transcription factors STAT3, AP-1 or SP-1 were constructed and transfected into the reporter cell line, respectively. The results demonstrated that luciferase activities were increased in cells treated with STAT3, AP-1 or SP-1 expressing vector compared with the control group, indicating that MMP12 transcriptional activity was up-regulated by STAT3, AP-1 or SP-1 transcription factors in HEK293-MMP12-T2A-luciferase-KI cells (Fig. 3A). Similar results were also observed for the expression of endogenous MMP12 that was up-regulated by three transcription factors in wild-type HEK293 cells (Fig. 3B).

Subsequently, a traditional Chinese medicine library was purchased to screen drugs which regulate the activity of endogenous MMP12 promoter in HEK293-MMP12-T2A-luciferase-KI cell line by luciferase activity assay. Interestingly, we found that the luciferase activity has dramatic decline in the reporter cells treated with Tanshinone I for 48 h compared with the control group (Fig. 3C), and the mRNA

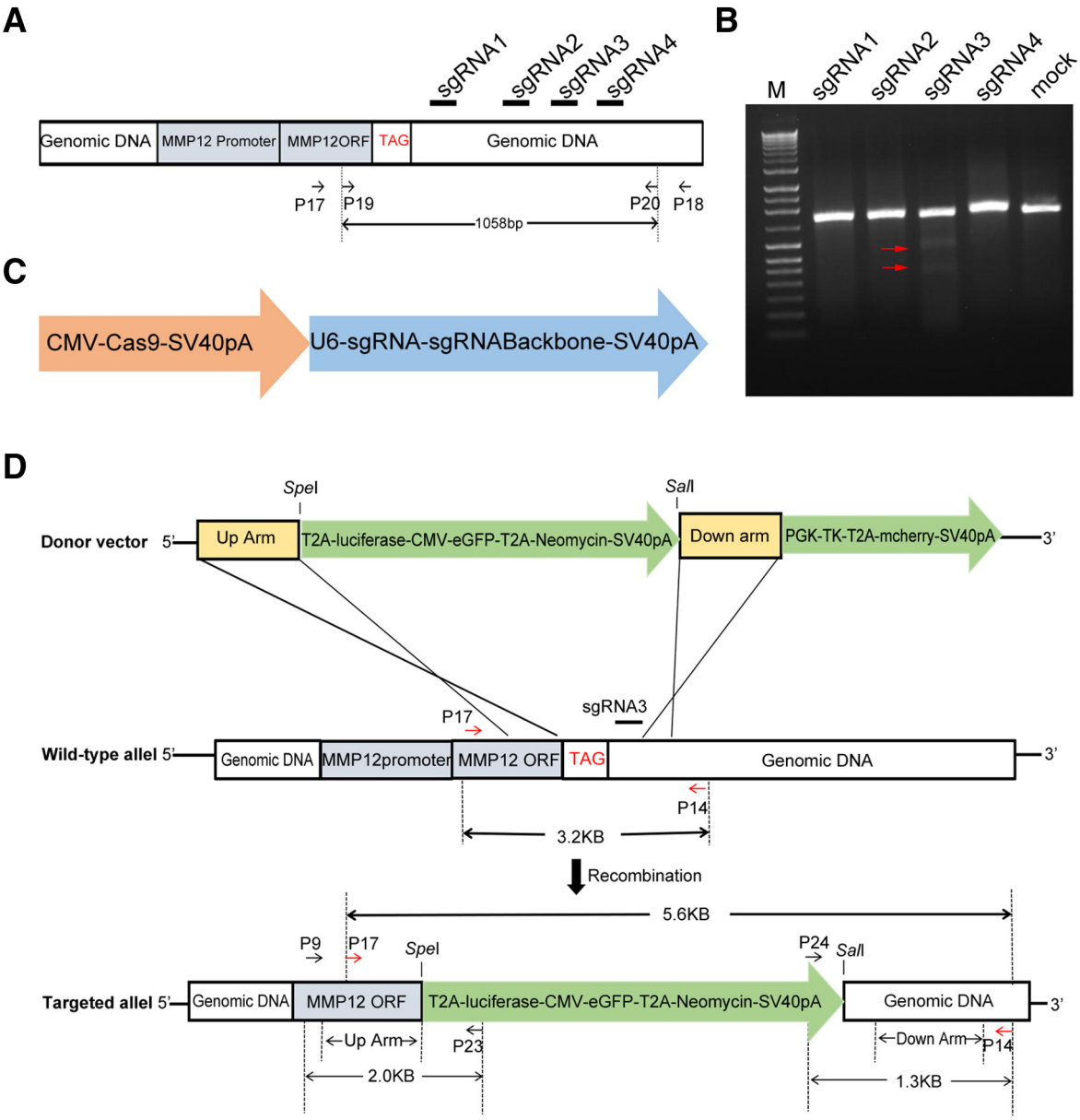

Fig. 1. Schematic of a luciferase report gene knocked in the human MMP12 gene locus. (A) Designed four sgRNAs for gene targeting at the MMP12 locus and the location of PCR primers (arrows) used to amplify the TSF for T7E1 assay are depicted. (B) Screening of sgRNA biological activities by T7E1 assay. Expected DNA bands cleaved by T7E1were shown here (arrows). (C) Cartoon of targeting vector. Targeting vector contains a U6 promoter for sgRNA expression and a CMV promoter for Cas9 expression. (D) Schematic diagram of the site-specific integration of exogenous gene in the genome, targeted allele indicates the site-specific integration of donor vector in the wild-type allele.

expression of MMP12 gene down-regulated by Tanshinone I was also validated in wild-type HEK293 cells (Fig. 3D). These results demonstrated that Tanshinone I could inhibit the transcriptional activity of MMP12 gene.

4. Discussion

MMP12 plays an important role in extracellular matrix degradation and involving in tumor cell migration and metastasis. It is an important role in tumorigenesis, has been considered as a potential target for cancer therapy [22]. In this study, CRISPR/Cas9 successfully mediated site-specific knock-in of the luciferase reporter gene into gene locus in HEK293 cells, and the resultant reporter cell line would have a great potential for investigating the transcriptional regulation of MMP12 gene and exploring the therapeutic drugs for affecting MMP12 gene expression.

A luciferase reporter of knock-in cell line will provide a sensitive, easy and rapid way to explore the expression of the endogenous target gene compared with conventional technology [23]. So far, to rapidly obtain a knock-in cell line with the targeted integration of exogenous gene in the genome is still a challenge. In this study, we developed a reliable site-specific integration strategy to construct a stable knock-in cell line with high efficiency using a donor vector with positive and negative selection combined with CRISPR/Cas9 technology.

A luciferase reporter gene was integrated into the locus at the terminal codon of human MMP12 through homologous recombination mediated by CRISPR/Cas9 technology. The reporter gene was fused with MMP12 gene via T2A peptide, which was controlled by endogenous MMP12 promoter, and two proteins were equally produced by self-cleavage peptide T2A. We confirmed the targeted integration of exogenous gene into the genome by PCR

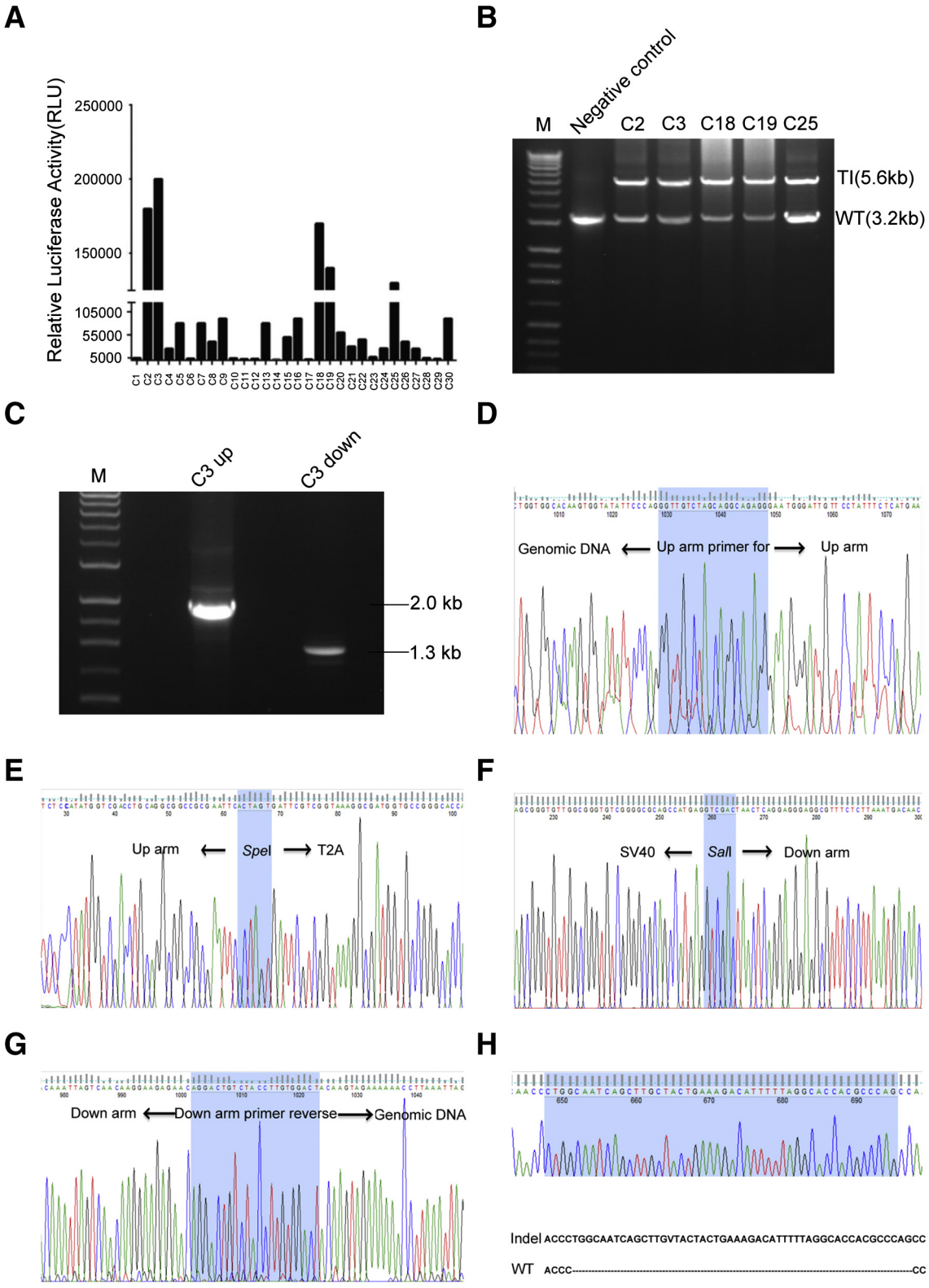

Fig. 2. Analysis of HEK293-MMP12-T2A-luciferase-KI cell line. (A) Targeted clones of HEK293 cells were selected by luciferase activity assay. (B) Genotyping of different stable cell clones with high luciferase activity were detected by PCR assay using primer P17 and P14. Both 3.2 kb band from wild type (WT) and 5.6 kb band from targeted integration (TI) are shown in each clone. Lane 1: marker; lane 2: wild-type HEK293 cell; lanes 3–7: the clones with high luciferase activity. (C) PCR analysis of clone 3 (HEK293-MMP12-T2A-luciferase-KI cells). Lane 1: marker; lane 2: bands amplified with 5′ primer P9 (shown in Table 5) located in genomic DNA before up homologous arm and 3′ primer P23 (shown in Table 5) that was specific to luciferase gene to detect the mutant MMP12 allele of up homologous arm; lane 3: bands amplified with 5′ primer P24 (Table 5) that was specific to SV40 gene and 3′ primer P14 (shown in Table 5) located in genomic DNA after down homologous arm to detect the mutant MMP12 allele of down homologous arm. (D–G) Analysis of site-specific integration of the exogenous gene in targeted locus by sequencing of the fragments amplified from clone 3. Primers P9 and P23 (Table 5) were used for up integration detection of 5′ terminal sequences (D) and 3′ terminal sequences (E), primers P24 and P14 (Table 5) were used for down integration detection of 5′ terminal sequences (F) and 3′ terminal sequences (G). (H) Sequencing results of the lower band of clone 3 using the primers P17-P20 (Table 3). The lower band contained a 48 bp non-homologous end joining (NHEJ) product (underlined) in the MMP12 locus. The location of PCR primer binding sites are depicted in Fig. 1D.

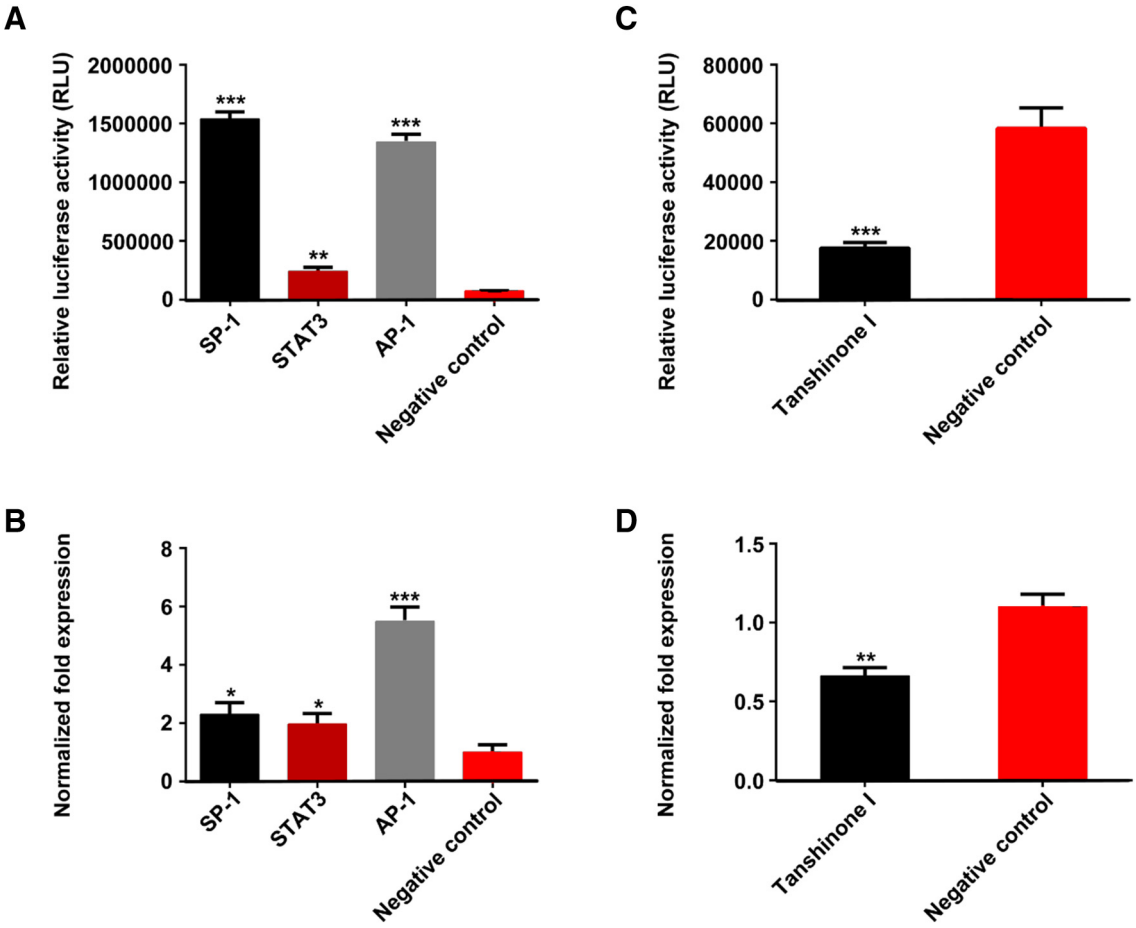

Fig. 3. The effect of transcription factors and Tanshinone I on MMP12 gene expression. (A) Luciferase activity analysis of HEK293-MMP12-T2A-luciferase-KI cell line treated by different transcription factors; (B) The endogenous MMP12 mRNA expression in HEK293 cells transfected with transcription factors by RT-PCR. (C) Luciferase expression analysis of HEK293-MMP12-T2A-luciferase-KI cell line treated with Tanshinone I; (D) The endogenous MMP12 mRNA expression in HEK293 cells treated with Tanshinone I by RT-PCR. All primers used for RT-PCR were listed in Table 4. Data, mean ± SEM. * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$ by t-test after repeated measures.

and sequencing. To verify the reliability of HEK293-MMP12-T2A-luciferase-KI cell line, STAT3 and AP-1 that have been reported to up-regulate the MMP12 promoter activity [20,21], were tested in the knock-in cell line, the results indicated that luciferase activities were increased by two transcription factors. Interestingly, SP-1 targeting MMP12 promoter predicted by GENE CARD software was demonstrated to up-regulate the expression of luciferase in the knock-in cells. Similar results for the expression of endogenous MMP12 with three transcription factors were achieved in wild-type HEK 293 cells. These results suggested that we have successfully generated HEK293-MMP12-T2A-luciferase-KI cell line, the luciferase activity of the reporter cell line was well correlated with its transcriptional activity of endogenous MMP12 promoter. Besides, a drug Tanshinone I was screened from a library of small molecule drugs, which was demonstrated to down-regulate the luciferase activity in this knock-in cell line and the expression level of MMP12 mRNA in HEK293 cells. In this screening, we found that Tanshinone I could inhibit the transcriptional activity of MMP12 gene, which might be considered as a potential candidate for cancer therapy in future.

In summary, we established a luciferase knock-in reporter system which can actually reflect the expression level of endogenous MMP12 gene in this study. This novel system will be helpful in investigating the functional roles of MMP12 in promoting inflammation and initiating tumorigenesis, screening small molecule chemicals for cancer therapy, and provides a new experimental method for cancer research.

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Declaration of competing interest

The authors declare no conflicts of interest.

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