Identification and comparison of the porcine H1, U6, and 7SK RNA polymerase III promoters for short hairpin RNA expression

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Abstract

Background RNA polymerase III is an essential enzyme in eukaryotes for synthesis of tRNA, 5S rRNA, and other small nuclear and cytoplasmic RNAs. Thus, RNA polymerase III promoters are often used in small hairpin RNA (shRNA) expression. In this study, the porcine H1, U6, and 7SK RNA polymerase III type promoters were cloned into a pcDNA3.1(+) expression vector containing a shRNA sequence targeting enhanced green fluorescent protein (EGFP). PK and DF-1 cells were cotransfected with the construction of recombinant interference expression vector and the EGFP expression vector, pEGFP-N1. The average fluorescence intensity of EGFP in transfected cells was measured by fluorescence microscopy and flow cytometry. Real-time PCR was used to detect expressed shRNAs and the relative expression of EGFP, to confirm the activity of the promoters.Results The results showed that the activity of porcine 7SK promoter is stronger than the U6 promoter, which is in turn stronger than porcine H1.Conclusions This study confirms that the porcine H1 promoter is effective for expression of shRNA, and may be an excellent tool to knock down gene expression in pigs for functional genomics studies. The results also lay a foundation for the development of porcine RNAi technology and genetically modified porcine research.

Background

RNA polymerase III is an essential enzyme in eukaryotes that synthesizes tRNA, 5S rRNA, and some small nuclear and cytoplasmic RNAs.Thus, RNA polymerase III promoters are often used in small hairpin RNA (shRNA) expression(1). Many RNA polymerase III promoters have been discovered, including human U6, 7SK, and H1(2–4); mouse U6, 7SK, and H1 (5, 6); bovine7SK, U6–1, U6–2, and U6–3(7, 8); chicken U6, 7SK, and H1(9–11); and pig U6, 7SK(12, 13), and successfully cloned and transfected into mammalian or avian cells for *in vitro* verification.

Pigs are very similar to humans in terms of anatomy, physiology, nutrition, and metabolism and are also important livestock animals. With the development of transgenic technology, genetically engineered pig has become an irreplaceable research model(14). RNA interference (RNAi) is a sequence-specific post-transcriptional gene silencing technology that has a wide range of applications in functional genomic research and development of new medicines for cancer and virus treatment(15). RNAi techniques in genetically modified animals for disease resistance have shown some progress, however most studies have focused on mouse models and are at a cellular level. Thus, breeding genetically modified RNAi-producing pigs for disease resistance will have broad prospects for agricultural application and further developmental studies (16).

Promoter selection is an important issue for optimal shRNA expression. Most studies of promoters for shRNA expression have focused on human, mouse, or chicken RNA polymerase III type U6 or 7SK promoters, with very little research on porcine promoters, especially H1. The high levels of expression of the U6 and 7SK promoters saturate the microRNA level in the host cell, which can cause cytotoxicity and tissue damage (17, 18). Thus, the weaker H1 promoter may cause less damage and represent a good alternative (11). In this study, we sought to first clone and identify the porcine H1 promoter and compare

its promoter efficiency to porcine U6 and 7SK promoters, which are known RNA polymerase III promoters in PK and DF–1 cells, respectively. This study provides an experimental tool for the development of porcine RNAi technology and transgenic pig research.

Results

Characterization of the porcine H1 promoter

The 341-bp human H1 RNA sequence (GenBank NR002312) was used to search the porcine genome (http://www.ncbi.nlm.nih.gov/genome/84). The BLASTN search showed the human H1 RNA sequence, from nucleotides 2–338, had 91% identity to a region (nucleotides 246274–246611) of a *Sus scrofa* chromosome 7 contig (GenBank NW_003535236.2). After searching for sequence motifs characteristic of type III RNA pol III promoters [19], a 255-bp sequence was amplified and cloned. Sequence analysis identified four elements typical of a Pol III promoter: an octamer motif (OCT; bp −92 to −100), an *Sph*I post-octamer homology domain (SPH; bp −71 to −90), a proximal sequence element (PSE; bp −70 to −53), and a TATA box (bp −32 to −29) (Fig. 1A). All four elements were highly similar to the consensus sequences common to human, mouse, and chicken H1 promoters (Fig. 1B). The full sequence of the 255-bp PCR product containing the porcine H1 promoter was deposited in GenBank under accession number KC176454.

Analysis of porcine H1 promoter activity

To analyze the ability of the porcine H1 promoter to express a shRNA for silencing of EGFP expression, we conducted an assay to determine the level of EGFP in transfected PK–15 and DF–1 cells.The extent of EGFP expression silencing was directly observed through fluorescence microscopy. Images showed that the fluorescence intensity of PK–15 and DF–1 cells 48 h after cotransfection with pEGFP-N1/ppH1-shEGFP was weaker than with pEGFP-N1/ppH1-shNS or pEGFP-N1 only. However, this reduction in fluorescence was not as great as was observed with pEGFP-N1/pp7SK-shEGFP or pEGFP-N1/ppU6-shEGFP cotransfection(Fig.2A). The percent of mean fluorescence intensity (MFI%), relative to the cotransfected control pcH1-NSsh/pEGFP-N1, of expressed EGFP was determined by flow cytometry.The MFI of pEGFP-N1/ppH1-shEGFP cotransfected cells was lower than pEGFP-N1/ppH1-shNS cotransfected PK–15 and DF–1 cells 48 h post-transfection (Fig. 2B).This decrease was not as evident as for pEGFP-N1/pp7SK-shEGFP, or pEGFP-N1/ppU6-shEGFP, which had the lowest MFI% of any of the combinations tested in this study. QPCR to determine the relative expression of EGFP, the result was consistent with the two experiments above(Fig.2C).

To prove the silencing of EGFP was specifically due to the RNAi, the expression of the EGFP shRNA was detected in transfected cells by qRT-PCR. The EGFP shRNA was detected only in those samples that were transfected with the EGFP shRNA vectors, and not in ppH1-shNS or untransfected cell controls. Compared with expression of EGFP shRNA in the pp7SK-shEGFP and ppU6-shEGFP cells, expression of the EGFP shRNA in ppH1-shEGFP transfected cells was weaker ($P < 0.05$ and $P < 0.01$, respectively) (Fig. 2D).

In order to study whether the H1 could be used to knock-down endogenous gene expression in porcine cells, GAPDH and β-actin expression in transfcted PK–15 and DF–1 cells was dected by qPCR. The results showed that H1 could not knock-down of the porcine genes(Fig.3A and B)

CCK–8 colorimetric assay measured the actual levels of cellular toxicity that U6 and 7SK, as well as H1, generated in porcine cell linesThe results show thatthe high levels of expression of the U6 and 7SK promoters saturate the microRNA level, which can cause much more cytotoxicity than H1 promoters both in the PK–15 and DF–1 cells(Fig.4)

Discussion

Expression of shRNAs from the human H1 promoter has been investigated both *in vivo* and *in vitro(*4), and the chicken H1 promoter has also been cloned and tested *in vitro* in PK–15 and DF–1 cells(11). However, the pig H1 promoter is yet to be cloned and discussed. Production of a genetically modified pig expressing shRNA under the control of the pig-specific H1 promoter is an important biotechnological step. Like other RNA polymerase III promoters, human, mouse and chicken H1 have typical elements that include the DSE, containing an OCT motif and a SPH site, the PSE, and a TATA motif.

Lambeth *et al.* found that bovine 7SK has cross-species transcriptional activity in multiple cell lines that is higher than bovine and murine U6(7). Human 7SK *in vitro* interference was shown to be better than U6–1(20), while subsequent studies of the chicken Pol III promoter did not appear similar to the results of Chen *et al(*21). Our study also indicated the transcriptional activity of pig 7SK is higher than U6, but the general rule that 7SK silencing is stronger than U6 only among large mammals requires further investigation. Mäkinen *et al.* showed that human H1 activity is weaker than the human U6 promoter in endothelial cells and mouse brain tissue(22).Our study also indicated that porcine H1 transcriptional activity is weaker than U6 and 7SK, which is consistent with other reports.

The human RNase P gene encodes the human ribonuclease P enzyme, which transcribes other genes with the H1 promoter. We determined that the human and porcine RNAse P RNAs shared 91% sequence homology. Cloning of the upstream region of the porcine H1 promoter revealed that the *cis*-acting DSE, containing OCT and SPH motifs, PSE, and TATA motif all displayed a high level of similarity to the consensus of the human, mouse, and chicken H1 promoter sequences.

There are a variety of methods for detection of siRNA, microRNA, and other small RNAs.Northern blotting is the most commonly used method, but its specificity and sensitivity are not high,while the stem-loop qRT-PCR method can be used to accurately quantify unmodified single-stranded siRNA, and the 3' end of two prominent double-stranded siRNAs (Silencer siRNA and LNA-modified Silencer Select siRNA), at the molecular, cellular, and whole animal levels, which solved the difficult problem of assessing the efficiency of siRNA delivery, distribution, and stability(21). This method proved successful in the current study in confirming that the silencing of EGFP was specifically due to the RNAi. The EGFP shRNA was detected only in those samples that were transfected with the EGFP shRNA vectors, and not in ppH1-shNS or untransfected cell controls.

We first cloned the porcine H1 promoter and constructed an shRNA expression vector to verify its function by expressing an shRNA that targets EGFP through transfection in mammalian and avian cell lines. After sequencing and software analysis, we showed that the cloned porcine H1 promoter core sequence contains control elements typical of RNA polymerase III type promoters. We also proved that expression of shRNA under the control of the porcine H1 promoter caused gene-specific knockdown of an exogenous EGFP reporter gene in both PK–15 and DF–1 cells, although the effect was not as pronounced as with the porcine U6 and 7SK promoters. This study shows that the porcine RNA polymerase III type H1 promoter may provide an excellent tool to knock down gene expression in pigs for functional genomic studies, and lays a foundation for the development of porcine RNAi technology and transgenic porcine research.

Conclusions

In this study, we sought to first clone and identify the porcine H1 promoter and compare its promoter efficiency to porcine U6 and 7SK promoters, which are known RNA polymerase III promoters in PK and DF–1 cells, respectively. This study provides an experimental tool for the development of porcine RNAi technology and transgenic pig research.

Methods

Cloning of the porcine H1 promoter

Porcine genomic DNA was isolated from pig kidney (PK–15) cells using a genomic DNA purification kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. PCR reactions were carried out in a 25 μl reaction volume containing 5 ng of genomic DNA, 12.5 μl of Q5® High-Fidelity DNA Polymeras (Takara, Dalian, China), and 200 nM each of primers pH1-FP and pH1-RP (Invitrogen, Carlsbad, CA, USA), under the following PCR conditions: 95°C for 5 min, 35 cycles of 95°C for 30 s, 55.4°C for 30 s, 72°C for 30 s, and a final extension of 5 min at 72°C. The 255 bp PCR product was cloned into the pMD–18T vector system (Takara), sequenced (Invitrogen, Carlsbad, CA, USA), and named TpH1.

Design and synthesis of small interfering RNA

Small interference RNAs, targeting the enhanced green fluorescent protein (EGFP) gene, were designed using online software (http://rnaidesigner.invitrogen.com/rnaiexpress), and *Hin*dIII and *Bam*HI restriction sites were introduced at both ends of the sequence. Oligonucleotides were synthesized by Shanghai Health Bioengineering (Shanghai, China). The two pairs of small interfering RNA fragments were annealed to produce shRNA.

Construction of a porcine H1 expression vector.

pcDNA3.1(+) vector was digested with *Nde*I and *Hin*dIII to remove the eukaryotic cytomegalovirus and T7 promoters. The pH1 promoter fragment was removed from the TpH1 plasmid by digestion with *Nde*I and *Hin*dIII and purified. This purified pH1 promoter fragment was ligated into the *Nde*I and *Hin*dIII sites of pcDNA3.1(+) and verified by DNA sequencing (Invitrogen). The recombinant plasmid was named pcDNA3.1-pH1. Next, the designed shRNA targeting EGFP was connected with pcDNA3.1-pH1, the sequence was verified (Invitrogen), and the construct plasmid was named ppH1-shEGFP.

EGFP shRNA expression vectors under the control of the porcine promoters 7SK (pp7SK-shEGFP) and U6 (ppU6-shEGFP), and a negative control non-silencing expression vector (ppH1-shNS), were constructed as for ppH1-shEGFP above. Sequences of all primers used in this study are listed in Table 1.

Cell culture and transfection

PK–15 cells and chicken embryo fibroblasts (DF–1) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), containing 10% fetal bovine serum (PAA Laboratories, Linz, Austria), 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37°C with 5% atmospheric CO_2. PK–15 cells and DF–1 cells were transfected at approximately 80–90% confluence in 24-well plates. Cells were transfected with 2 μl of Lipofectamine 3000 reagent (Invitrogen), according to the manufacturer's instructions. Cells in each well plates were co-transfected with 400 ng of shRNA expression plasmid containing one kind of promoter (H1, or U6 or 7SK, respectively) and together with 400 ng of EGFP expression vector (pEGFP-N1), diluted in 500 μl Opti-MEM (Invitrogen). The combination of ppH1-NSsh/pEGFP-N1 was used as a negative control. Six hours post-transfection, medium was replaced. Each sample was repeated in triplicate.

Detection of EGFP expression

EGFP fluorescence was detected 48 hours post-transfection by fluorescence microscopy at 100× magnification. After cells were digested with pancreatin (PAA Laboratories), each well was suspended in PBS and the average fluorescence intensity of each well was measured using flow cytometry. Total RNA was extracted from each well using an RNA total kit (Invitrogen), then two micrograms of RNA were reverse transcribed using a GoldScript cDNA synthesis kit (Invitrogen) with random primers. Quantitative real-time PCR (qRT-PCR) was performed using a SYBR GREEN kit (Roche,NY, USA) according to manufacturer's instructions, with primers EGFP-FP and EGFP-RP to amplify EGFP from PK and DF–1 cells, on a Roche LightCycler 480 system (Roche). Pig GAPDH and Chicken beta-actin were used as controls for PK–15 and DF–1cells, respectively. Standard curves using the two control genes were constructed to assist with actual efficiency calibration, though relative gene expression was calculated for the EGFP gene transcription. Each reaction was performed in triplicate.

Detection of shRNA expression

RNA was extracted from transfected PK and DF–1 cells with a MirVana miRNA Isolation Kit (Applied Biosystems, Foster City, CA, USA), then 2 μg of RNA was reverse transcribed using a TaqMan Micro RNA

Reverse Transcription Kit (Applied Biosystems). According to the methods of Liu(19), qRT-PCR primers (RT-qPCRFP and RT-qPCRRP), universal stem-loop structure sequence (SLP-C) (Takara), minor groove binder probe (MGB-B), and siRNA (Si-C) were designed and synthesized (Shanghai Health Bioengineering). Ten-fold serial dilutions of a known copy number of Si-C were used to construct a standard curve (copy number from 10 to 1.0×10^8) using a Roche LightCycler 480 system. The standard curve was used to determine the shRNA expression in transfected cells. Reactions contained 1 μl cDNA, 2.5 μl 10× ExTaq buffer (Takara), 2 μl dNTPs (2.5 mmol/l) (Takara), 3 μl $MgCl_2$ (25 mmol/l) (Takara), 0.5 μl Probe (10 pmol/l), 1 U Ex Taq HS (Takara), and 200 nM of primers RT-qPCRFP and RT-qPCRRP, in a total volume of 25 μl. Each reaction was performed in triplicate.

Detection of the porcine genes expression in transfcted PK–15 and DF–1 cells by qPCR

Porcine genomic DNA was isolated from transfcted PK–15 cells using a genomic DNA purification kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. QPCR was performed using a SYBR GREEN kit (Roche) according to manufacturer's instructions, with primers GAPDH and beta-actin to respectively amplify GAPDH and beta-actin from transfcted PK–15 and DF–1 cells, on a Roche LightCycler 480 system (Rochester, NY, USA)

Detection of the cytotoxicity of shRNA expression in PK–15 and DF–1 cells by CCK–8 kit.

Three column were selected randomly from each transfected plasmid and the control PK–15 cells respectively, added 80μl of CCK–8 solution (5mg/ml), incubation at 37°C with 5% atmospheric CO_2 for 4 hours. The supernatant was discarded, and 600μl of DMSO was added in each column and shocked for 10 mins. Then columns were measured by Microplate Reader with 450nm absorbance wavelength and recorded the results.

Sequence analysis

Sequence alignments were performed using NCBI (http://www.ncbi.nlm.nih.gov/). Genomic sequences containing the human H1 (NT_026437.12), mouse H1 (NW_001030552.1), chicken H1 (JF912377), or porcine H1 promoter were identified by BLAST analysis, using the human H1 ribonuclease P RNase sequence (NR_002312) as a query(18). The core areas of transcription factor binding in the porcine H1 promoter were identified with TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html).

Statistical analyses

All experimental results are expressed as the mean ± standard deviation (S. D.). All experiments were performed in triplicate. Kruskal-Wallis test was used for statistical analysis.

Abbreviations

ppH1-shNS: negative control non-silencing expression vector; ppH1-shEGFP: pCDNA3.1-porcine H1-shRNA targeting EGFP;ppU6-shEGFP: pCDNA3.1-porcine U6-shRNA targeting EGFP;pp7SK-shEGFP:

pCDNA3.1-porcine 7SK-shRNA targeting EGFP

Declarations

Ethics approval and consent to participate

This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Harbin Veterinary Research Institute.

Consent for publication

Not applicable

Availability of data and material

All data generated or analysed during this study are included in this published article

Competing interests

The authors declare that there is no conflict of interest regarding the publication of this article.

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Authors' contributions

Hai-Chang Yin and Qing-Wen Meng designed the experiments. Hai-Chang Yin and Wei Wang performed the experiments. Hai-Chang Yin wrote the paper. Xin-Yu Chen analyzed the data and drew the graphs.

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Tables

Table 1 Oligonucleotides used in this study

Primer name	primer sequence(5′–3′)
pH1-FP	ACGCATATGAACATCGACGTCATCAACCAC
pH1-RP	TTTAAGCTTATGAGCTTCTCTCCGCCCTA
pU6-FP	ACGCATATGCGGGGTGGGTCAATAGGCAA
pU6-RP	TTTAAGCTTCACGACGGCTGGGTCTTCTCA
p7SK-FP	ACGCATATGGGCTCAGAGCCAGGAGAAAAAC
p7SK-RP	TTTAAGCTT GAGGGCCTGAGGAAGGCCGC
EGFP-FP	AGCCGCTACCCCGACCACAT
EGFP-RP	CGGTTCACCAGGGTGTCGCC
β-actin–FP:chicken	CAGAGCAAGAGGGGCATC
β-actin–RP:chicken	AGGTAGTCGGTCAGGTCC
GAPDH –FP:porcine	ACATGGCCTCCAAGGAGTAAGA
GAPDH –RP:porcine	GATCGAGTTGGGGCTGTGACT
Si-C	GCAGCACGACUUCUUCAAG
SLP-C	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCTTGAA
RT-qPCR-FP	ACACTCCAGCTGGGGCAGCACGACTTCTT
RT-qPCR-RP	CTCAAGTGTCGTGGGTCGGCAA
MGB-B	TTCAGTTGAGCTTGAA

Figures

Pig H1 promoter region

GGTCCCTCTCCCGCGCGCAGCCATGGATCCCTAACATCGACGTCATCAACCACTTCCTAGGAATCTGCCGGCGCAGTCTCAA
GGCGGGAACATGTATTGCGCGTGCGTCCTGGAGCGAAGCTGCCTGCACCGGACGGGGACGAACGCGCCACAATATTTGCAT
GTCGCAATGTCTTATGGGAAATCATAACTATGAAATCTCTCTACTTATGGGAGTTTTATAAGTGCTCACAGAAGCAGTTTTCCT
ACGT

	OCT	SPH	PSE	TATA
CONSENSUS	ATGCAAAT	YYWCCCRNMATSCMYYRCRN	TCACCNTAANYRAAAAT	TATA
Human H1	-90 ATGCAAAT -97	-69TTTCCCAGAACACATAGCGA-88	-68TCACCATAAACGTGAAAT-51	-30 TATA-27
Mouse H1	-96 ATGCAAAT-89	-66TTTCCCACAAAGCACAGCGC-85	-65TCACCCTAAACGTAAAAT-48	-28TATA-25
ChickenH1	-87ATGCTAAT-94	-64TTACCCGGCATGCTCTGCGG-83	-62TCGCCCTACTCTAAAAG-46	-26TATA-23
Porcine H1	-92ATGCAAAT-100	-71TCCCATAAGACATTGCGA -90	-70TCACCATAACTATGAAAT-53	-32 TATA-29

Figure 1

(A) Porcine H1 promoter region containing four cis-acting elements typical of a Pol III promoter: the distal sequence element (DSE) comprising SPH and OCT, the proximal sequence element (PSE), and TATA motif. (B) Conservation of H1 promoter elements. SPH, OCT, PSE, and TATA sequences of human, mouse, chicken, and pig are shown.

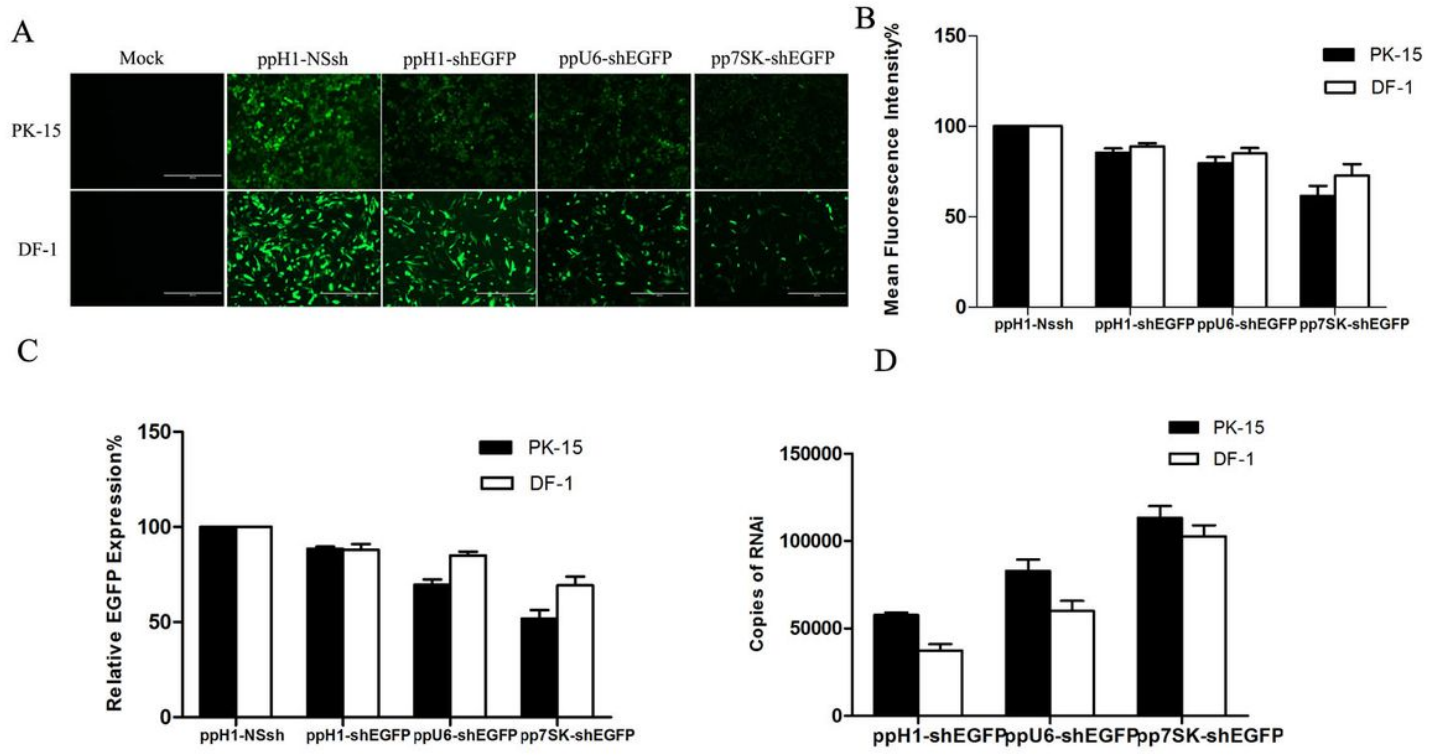

Figure 2

(A) Fluorescence microscopy images of EGFP fluorescence in PK-15 and DF-1 cells transfected with either EGFP alone or in combination with a vector expressing EGFP shRNA under the control a various promoters at 48 h post-transfection. The scale represented 400μm. (B) The relative percent of mean fluorescence intensity (MFI%) of expressed EGFP, as determined by flow cytometry in PK-15 and DF-1 cells at 48 h post-transfection. (C) QPCR quantification of the relative expression of EGFP in PK and DF-1 cells at 48 h post-transfection. (D) Expression of the EGFP shRNA was detected in transfected PK and DF-1 cells by qRT-PCR at 48 h post-transfection. Cells that were transfected with pcH1-NSsh represent a non-silencing negative control.

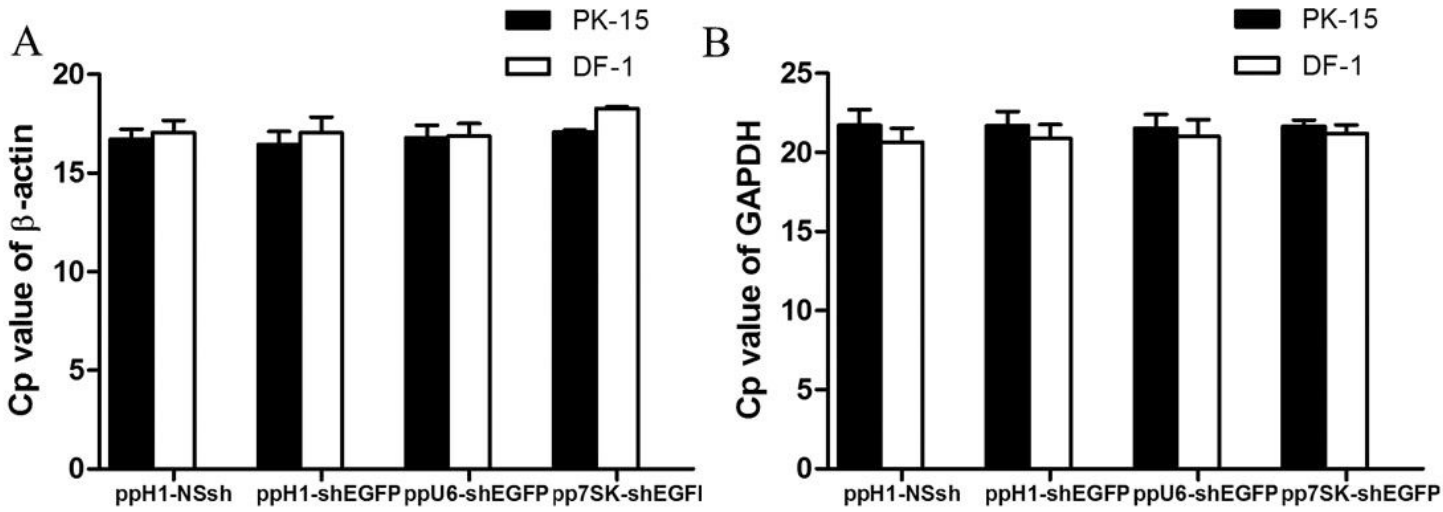

Figure 3

(A) The expression of GAPDH in PK-15 and DF-1 cells at 48 h post-transfection.were determined by qPCR.
(B) The expression of β-actin in PK-15 and DF-1 cells at 48 h post-transfection were determined by qPCR.

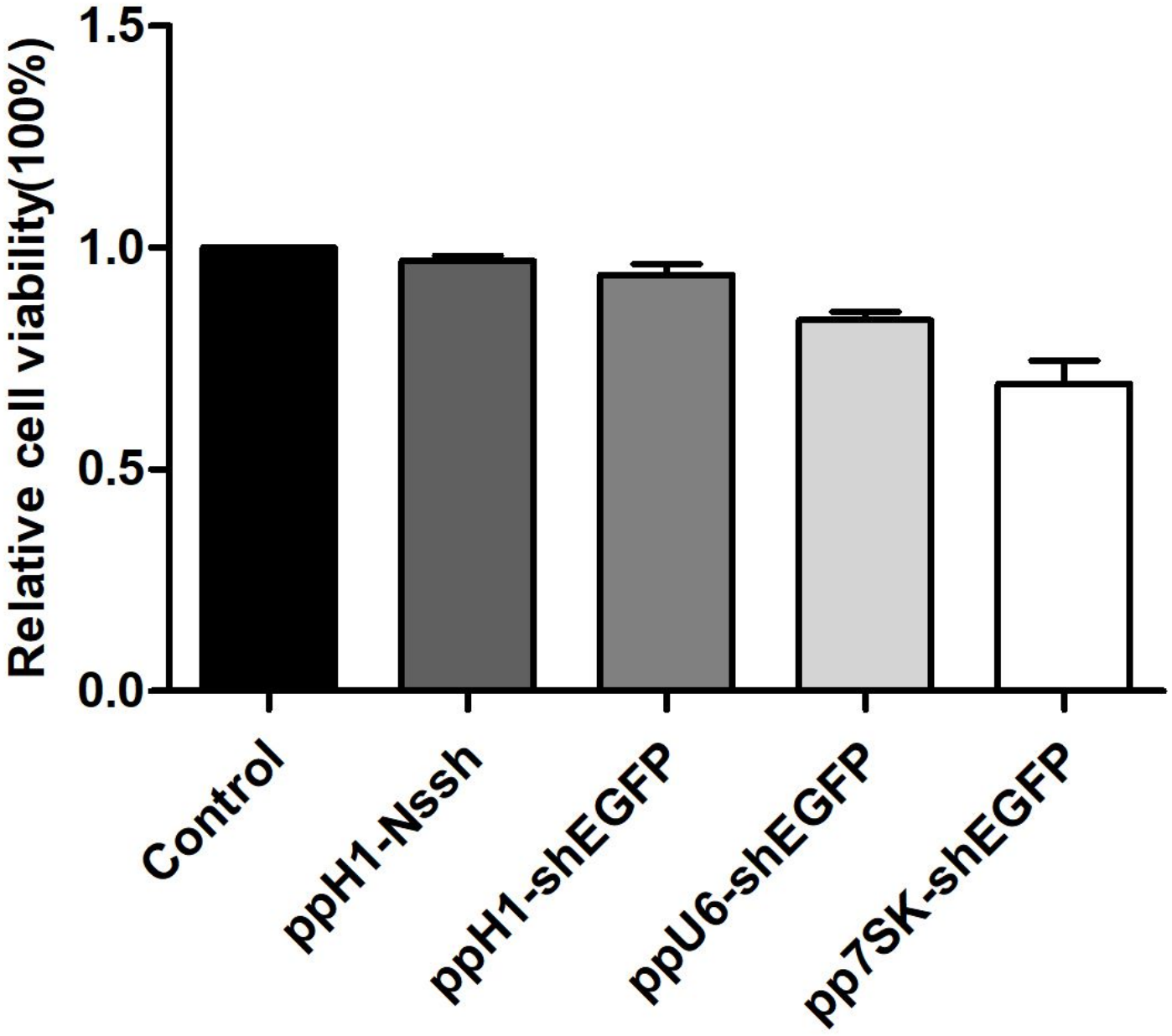

Figure 4

The actual levels of cellular toxicity that various promoters were measured by CCK-8 colorimetric assay.