

SHORT REPORT**Identification and characterisation of the porcine 7SK RNA polymerase III promoter for short hairpin RNA expression**David Cummins^{1,2}, Timothy J Doran^{1*}, Scott Tyack¹, Damian Purcell² and Jef Hammond¹¹CSIRO Livestock Industries, Australian Animal Health Laboratory, Geelong 3220, Australia, ²The University of Melbourne, Department of Microbiology and Immunology, Parkville 3010, Australia.*Correspondence to: Timothy Doran, Email: timothy.doran@csiro.au

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© Copyright The Authors: This is an open access article, published under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/2.0/uk/>). This license permits non-commercial use, distribution and reproduction of the article, provided the original work is appropriately acknowledged with correct citation details.**ABSTRACT**

The RNA polymerase III (pol III) type III promoters U6 and 7SK are routinely used to express short hairpin RNA (shRNA) molecules from a DNA construct. In this study, we identified, characterised and compared the porcine 7SK promoter in porcine (homologous) and non-porcine (heterologous) derived cell lines. The porcine 7SK small nuclear RNA (snRNA) was identified by alignment with known sequences and further characterisation of the upstream regions determined the presence of typical RNA pol III sequence motifs. The porcine 7SK (po7SK) promoter was cloned and a one-step PCR strategy used to construct shRNA expression cassettes. The 7SK promoter activity was quantified by knockdown of the exogenous reporter gene encoding the enhanced green fluorescent protein (EGFP). Results indicated the po7SK promoter was functional in both homologous and heterologous cells lines. The identification and characterisation of the porcine RNA pol III promoter will contribute to the area of RNAi delivery and further develop our understanding of RNA promoter structure and function.

KEYWORDS: RNAi, 7SK, U6, pol III, shRNA**INTRODUCTION**

RNA polymerase (pol) III type III promoters are the most commonly used promoters for the expression of short hairpin RNA (shRNA; Amarzguioui et al, 2005). Type III promoters such as U6 and 7SK are often preferred because they naturally express small, highly-abundant non-coding RNA transcripts referred to as small-nuclear RNAs (snRNAs). Type III promoters do not contain intragenic control regions, the promoter region is located entirely upstream of the transcription start site (+1) and transcription termination occurs at a defined tract of 4-5 thymidines (Geiduschek and Kassavetis, 2001). The pol III type III promoters contain sequence motifs located within regions referred to as the enhancer and core region (Schramm and Hernandez, 2002). The enhancer region consists of an octamer motif (OCT) and an *SphI* Post-

octamer Homology domain (SPH), while a proximal sequence element (PSE) and a TATA-like element form the core region (Schramm and Hernandez, 2002). The mouse U6 and human U6/H1 promoters are the most commonly used promoters for shRNA expression. Promoter activity may vary considerably for the family of U6 promoters, such as the five human U6 promoters which all have varied transcriptional efficiencies (Domitrovich and Kunkel, 2003).

More recently, researchers have identified the human 7SK promoter as the most efficient pol III promoter for the expression of short hairpin RNAs (shRNAs) *in vivo*, surpassing the activity of the human U6-1 promoter (Koper-Emde et al, 2004). Numerous 7SK pseudo-genes are known to exist, but only a single 7SK promoter is known to be present in the human (Murphy et al, 1987),

bovine (Lambeth et al, 2006), mouse (Moon and Krause, 1991) and chicken (Bannister et al, 2007) genomes.

With a large portion of the porcine genome sequence currently available and the conserved nature of the 7SK snRNAs (Humphries et al, 1987), we sought to identify, characterise and compare the porcine 7SK (po7SK) promoter for promoter efficiency relative to various known RNA pol III type III promoters.

MATERIALS AND METHODS

Cell culture and transfection

Swine testis (ST) cells (ATCC, CRL-1746) were maintained in Eagles Minimal Essential Media (EMEM) with 1% (w/v) sodium pyruvate and 0.25% (w/v) lactalbumin hydrolysate (LAH). Baby hamster kidney (BHK; ATCC, CCL-10) were grown and maintained in Basal Medium Eagle (BME) and 10% (w/v) tryptose phosphate broth (TBP). African green monkey kidney cells (VERO; ATCC, CCL-81) were grown and maintained in EMEM. All growth media used contained additional supplements including; 2 mM glutamine, 10 mM HEPES, 10% (v/v) fetal calf serum (FCS) with the addition of penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells were maintained in a 5% (v/v) CO₂ atmosphere at 37°C. Cells were harvested by washing twice in phosphate buffered saline-A (PBSA), followed by the addition of 0.25% (v/v) PBSA diluted trypsin. All cell lines were transfected at approximately 80-90% confluency in 8-well chamber slides (Nunc) for fluorescence microscopy, 24-well plates for flow cytometry analysis or 25 cm² culture flask for RNA isolation. Cells for microscopy and flow cytometry analysis were transfected with 0.5 µg of each plasmid using Lipofectamine 2000 (Invitrogen) according to manufactures instructions. When the cells were to be used for RNA isolation, 10 µg of plasmid DNA and 25 µl of Lipofectamine 2000 reagent was used.

Isolation of the porcine 7SK promoter

The po7SK promoter was PCR amplified from pig genomic DNA (Wizard Genomic DNA purification kit, Promega) using PCR platinum supermix (Invitrogen) and primers; po7SK-F (5'-GGCTCAGAGCCAGAGAAAA AC-3') and po7SK-R (5'-GAGGGCCTGAGGAAGGC CGC-3'). The 436 bp amplified PCR product was cloned into pGEM[®]-T Easy vector system (Promega), sequenced confirmed and named pGEM-po7SK.

The sequence was compared using mega-Basic Local Alignment Search Tool (mega-BLAST; Altschul et al, 1990). The putative po7SK promoter was deposited into GenBank under the accession number EU39883.

Construction of the porcine 7SK shRNA expression vectors

Expression vectors containing an EGFP shRNA (po7SK-EGFPsh) and an irrelevant shRNA control targeting the influenza virus nucleocapsid protein (NP; po7SK-NPsh) under the control of the po7SK promoter were constructed. The po7SK-EGFPsh and po7SK-NPsh expression vectors were constructed using the one-step PCR approach

described by Lambeth et al, 2005 (Supplementary Figure 1). Briefly, the pGEM-po7SK construct was used as template to amplify the po7SK promoter using a short forward oligonucleotide and a long reverse oligonucleotide containing complementary sequence to the po7SK promoter along with the sense, loop, antisense and stop sequence of the shRNA. The amplified PCR product containing the promoter and hairpin sequence was cloned into pGEM-T Easy. The EGFP shRNA expression vectors under the control of the bovine 7SK (pEZ-b7SKshEGFP), chicken 7SK (pch7SK-shEGFP) and mouse U6 (pmU6-shEGFP) promoter have previously been described (Lambeth et al, 2006; Bannister et al, 2007). The bovine, chicken and mouse EGFP shRNA expression constructs were renamed b7SK-EGFPsh, c7SK-EGFPsh and mU6-EGFPsh for this investigation.

Detection of expressed shRNA molecules

Small enriched RNAs were isolated from transfected ST cells at 72 hr post-transfection using the *mirVana* miRNA isolation kit (Ambion). Detection of expressed EGFP shRNA molecules was achieved using an RNase protection assay (RPA) using the RNA probe LL91 (named shEGFP probe in this investigation) described previously (Lambeth et al, 2006). RNA samples were prepared and hybridised with the radiolabelled (γ -³²P) shEGFP probe in solution overnight at 42°C. The shEGFP hybridised samples were RNase A/T1 treated as recommended. Samples were separated on a 15% (w/v) polyacrylamide (8M urea) gel and then exposed to Hyperfilm[™] ECL (Amersham Biosciences) within an EC-AWU cassette (Fuji) and placed at -80°C overnight. The film was developed using an X-ray processor FPM-100A (Fuji).

RNAi assay

EGFP expression was analysed at 48 hr post-transfection. Fluorescence microscopy images were captured using a Leica DM LB Fluorescence Microscope coupled to a Leica DC200F digital camera (Leica Microsystems) 100× magnification. Flow cytometry was used to measure the EGFP Mean Fluorescence Intensity (MFI) of each sample. Transfected cells were harvested using 0.25% (w/v) trypsin-EDTA, pelleted at 2000 rpm for 5 min, washed in phosphate buffered saline-A (PBSA) (Oxoid) and resuspended in FACS-solution (1% fetal calf serum (v/v) in PBSA). Analysis was performed using a FACScalibur (Becton Dickinson) fluorescent activated cell sorter and CELLQuest software (Becton Dickinson). The MFI was calculated as a percentage of the non-silencing control (po7SK-NPsh) for each cell line.

RESULTS

Identification and isolation of the porcine 7SK promoter

To identify the po7SK promoter, a bioinformatics search of the porcine genome using the 331 nucleotide human 7SK snRNA (GenBank Accession Number NR001445) was conducted. Several matches were identified with significant similarity observed. The porcine sequence (CU62466.2) showing the greatest similarity to the human 7SK snRNA was selected for further analysis and an alignment between the human, chicken and bovine 7SK

snRNA sequences carried out to identify the presence of the porcine full-length 7SK snRNA and delineate sequence similarities (Figure 1).

PCR was used to amplify 436 bp upstream of the porcine 7SK snRNA. Analysis of the cloned po7SK sequence by alignment predicted the following; TATA box at -25 to -32 bp, PSE at -47 to -67 bp, SPH domain at -194 to -212 and an OCT motif at -226 to -234. An alignment of the po7SK promoter sequence motifs are shown in Figure 2.

Detection of expressed EGFP shRNA

The activity of the putative po7SK promoter was verified by the detection of expressed EGFP shRNAs. An RNase protection assay was performed on ST cells transfected with the plasmids po7SK-EGFPsh, b7SK-EGFPsh, c7SK-EGFPsh, mU6-EGFPsh and negative controls pEGFP-N1 and po7SK-NSsh (Figure 3). A 19 nucleotide band was detected in the lanes representing the po7SK-EGFPsh, b7SK-EGFPsh, c7SK-EGFP and mU6-EGFPsh transfected ST cells. No bands were identified in the pEGFP-N1 or po7SK-NPsh control samples. These results demonstrated that the cloned po7SK promoter was functional as indicated by the detection of the expressed shRNAs.

Activity of the porcine 7SK promoter measured by EGFP knockdown

To determine the activity of the po7SK promoter, EGFP silencing experiments in ST, BHK and VERO cells were

conducted. The pEGFP-N1 construct was co-transfected with either po7SK-EGFPsh or the non-silencing control po7SK-NPsh. Given co-transfection of the shRNA expression plasmid and the reporter construct are considered to be 100% efficient for RNAi validation (Cullen, 2006), a reduction in EGFP fluorescence was considered a result of RNAi-mediated EGFP knockdown. In addition, the constructs b7SK-EGFPsh, c7SK-EGFPsh and mU6-EGFPsh were also co-transfected along with pEGFP-N1 and fluorescence microscopy used to observe EGFP knockdown (Supplementary Figure 2). The level of gene silencing was compared relative to the level of fluorescence observed in the pEGFP-N1/po7SK-NPsh control cells. Results indicated that the po7SK promoter induced specific knockdown of EGFP in all three cell lines. To quantify the level of EGFP knockdown mediated by po7SK compared with that of the other RNA pol III promoters, those constructs were co-transfected into the three cell lines as described above. At 48 hr post-transfection, cells were harvested and analysed for their MFI by flow cytometry (Figure 4). The MFI was calculated as a percentage of the pEGFP-N1/po7SK-NPsh co-transfected cells. The po7SK promoter was capable of silencing EGFP by 30% in VERO cells and by 80% in both BHK and ST cells. These results demonstrated that the po7SK promoter was functional in a range of cell types. However, it appeared that in each of the cell types the bovine, chicken and mouse RNA pol III promoters induced greater EGFP knockdown than the po7SK promoter.

po7SK snRNA	1	ggatgtgagggcgatctggctgcgacatctgtcaccgccattgatcgccagggttgattcggctgatctgg
h7SK snRNA	1
c7SK snRNA	1
b7SK snRNA	1t.....
po7SK snRNA	71	ctggctagggcgggtgtccccttccctccctcaccgctccatgtgctgctccctccogaagctgcgcgctcgg
h7SK snRNA	70
c7SK snRNA	71
b7SK snRNA	71
po7SK snRNA	141	cgaagaggacgaccatccccgatagaggagg-accgcttcttcggtaagggtatacagtagctgcgctc
h7SK snRNA	140g.....
c7SK snRNA	141t.....ac..t.....a.....
b7SK snRNA	141c.....-.....g.....
po7SK snRNA	210	ccctgctagaacctccaacaagctctcaaggctccatttgtaggagaaacgtagggtagtcaggcttccaa
h7SK snRNA	209
c7SK snRNA	210
b7SK snRNA	210a.g.....
po7SK snRNA	280	gactccagacacatccaaatgaggcgctgcatgtggcagctctgcctttctttt
h7SK snRNA	279g.....
c7SK snRNA	278g.....g.....a.....g.....a.g.....
b7SK snRNA	

Figure 1: Alignment of the 7SK snRNA. An alignment of the porcine (po7SK snRNA; CU62466.2), human (h7SK snRNA; NR001445), bovine (b7SK snRNA; CR810253) and chicken (c7SK snRNA; AJ890101) 7SK snRNA sequences using ClustalW (Chenna et al, 2003). Dot (.) represent identical base. Dash (-) represents gap/no base. Base changes are indicated with the appropriate base.

DISCUSSION

The identification and characterisation of promoters for the expression of shRNA molecules has been limited to species with known genome sequence. The recent release of the porcine genome sequence will enable the identification of porcine promoters for shRNA expression. Porcine related RNAi research has focused on silencing porcine specific viruses such as porcine reproductive and respiratory syndrome virus, porcine circovirus and porcine transmissible gastroenteritis virus using commercially available constructs and promoters (He et al, 2007; Sun et al, 2007; Zhou et al, 2007). In this investigation, we are the first to identify and characterise a porcine RNA polymerase III promoter and demonstrate its functionality for shRNA expression.

The conserved nature of the 7SK snRNA enabled the porcine 7SK snRNA to be identified by comparison with the human 7SK snRNA sequence (Humphries et al, 1987). Although many 7SK pseudogenes are known to exist, we were able to identify a full-length porcine snRNA sequence with high similarity to the human, chicken and bovine 7SK snRNA (Figure 1). Given that only a single functional 7SK promoter has been identified upstream of the 7SK snRNA in the human, bovine, chicken and mouse genomes (Murphy et al, 1987; Lambeth et al, 2006; Moon and Krause, 1991), it was likely that a functional promoter would be located upstream of the porcine 7SK snRNA. Typical pol III motifs TATA, PSE, OCT and SPH were identified in the po7SK and showed significant sequence identity to motifs in the h7SK, b7SK and c7SK promoters.

Activity of the po7SK promoter was determined by its ability to express EGFP shRNAs and silence plasmid expressed EGFP. A shRNA expression vector was constructed and tested in homologous and heterologous cell lines to test functionality, efficiency and specificity. Our results clearly demonstrated the promoter was functional through the detection of shRNAs (Figure 3) and silencing of the reporter gene EGFP (Figure 4). Although the po7SK was capable of inducing gene silencing at varying levels in different cell types, the po7SK promoter was not as efficient as the bovine 7SK, chicken 7SK or mouse U6 promoters in either porcine, hamster or monkey derived cells. This difference in efficiency is similar to a study by Bannister et al, (2007), who demonstrated that the chicken 7SK promoter was not as efficient as the b7SK or mU6 promoters in chicken DF1 cells. Previous studies (Lambeth et al, 2007; Koper-Emde et al, 2004) have shown 7SK promoters perform more efficiently in cell lines that are of the same species as the origin of the promoter. Greater silencing efficiencies for the po7SK promoter were not observed in this study or the study by Bannister et al, (2007), suggesting that promoter sequence is the most important factor for promoter activity rather than the species of promoter origin.

Comparison of the bovine and porcine 7SK promoter elements at the nucleotide level revealed significant similarities, although the activity of the bovine promoter was as much as 20% more effective at gene silencing than the porcine promoter. The TATA and PSE elements of the

bovine and porcine promoters are similarly spaced and match the consensus sequence at the same locations. The porcine promoter has greater sequence similarity to the SPH consensus than the bovine, although the bovine SPH is located upstream of the OCT domain. Perfect similarity and spacing is observed between the OCT and CACCC box for both the porcine and bovine promoters. Although the bovine elements are located an additional 10 bp upstream of the start site, relative to the porcine promoter. Co-dependence has been demonstrated for the OCT and SPH elements in *Xenopus laevis* tRNA^{sec} promoter and human U6 promoters for efficient enhancer activity (Myslinski et al, 1992; Kunkel et al, 1996). Whereas for the human 7SK, optimal efficiency is not dependent on the presence of an SPH region (Boyd et al, 2000). If this feature applies to both bovine and porcine 7SK promoter elements and given the SPH motif of the bovine promoter has been suggested to be located 5' to OCT motif, then the downstream spacing and sequence could be responsible for the increased promoter efficiency observed in the bovine promoter. Additionally, differences between the chicken and porcine sequence motifs were also observed. The largest difference is the absence of a CACCC box and a C/A substitution at position 1 (bp -222) in the chicken 7SK OCT motif. The CACCC box is a notable feature of 7SK promoters and has been reported to function in enhancing the transcriptional activity of the human 7SK promoter (Kleinert et al, 1990). Mutational studies within the OCT sequence of the human 7SK promoter have been shown to have the greatest effect on transcription (Boyd et al, 2000). Given there is greater sequence variation between the po7SK and c7SK, it would be hypothesised that promoter efficiency would be greatest for the porcine 7SK because of the more conserved nature of the sequence motifs. However, in this study we observed that the chicken 7SK performed marginally better in both BHK and VERO cell lines and was substantially more efficient in ST cells. This data further suggests that promoter sequence variation and not the species of promoter origin plays the major role in directing promoter activity.

CONCLUSIONS

In this report, we identified and characterised the po7SK promoter for the delivery of shRNAs. Analysis revealed that the po7SK promoter efficiently expressed shRNAs and induced gene silencing. The identification of additional porcine pol III promoters including the U6 family of promoters should provide greater insight into the mechanisms directing transcriptional efficiency between different promoter types within the same species. Furthermore, the identification and characterisation of new promoters will enable the development of tools for shRNA expression not only for porcine specific applications, but also for the field of RNAi.

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COMPETING INTERESTS

None declared.

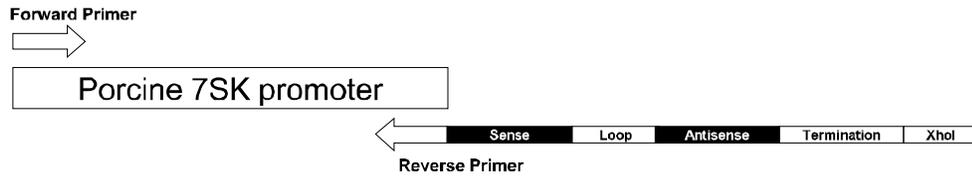
LIST OF ABBREVIATIONS

BHK: Baby hamster kidney
 BME: Basal Medium Eagle
 b7SK: bovine 7SK
 c7SK: chicken 7SK
 EMEM: Eagles Minimal Essential Media
 FCS: fetal calf serum
 HEPES: N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
 LAH: lactalbumin hydrolysate
 mU6: mouse U6
 OCT: octamer motif
 pol III: polymerase III
 po7SK: porcine 7SK
 PSE: proximal sequence element
 snRNA: small nuclear RNA
 SPH: post-octamer Homology domain
 ST: Swine testis
 VERO: African green monkey kidney cells

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One Step PCR Approach

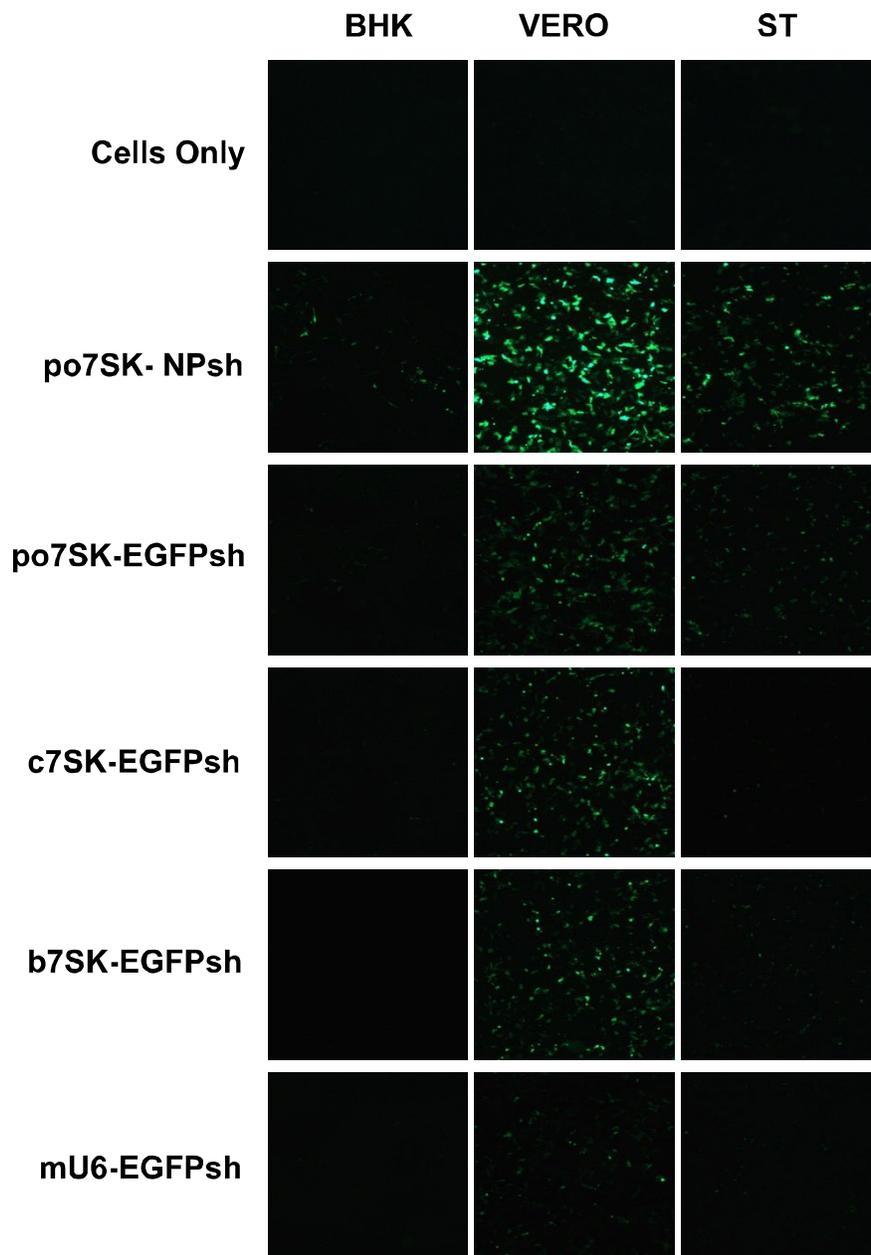


PCR Products



Primer	Sequence (5'-3')
po7SK-Fwd	GGCTCAGAGCCAGGAGAAAA
po7SK-EGFPsh-Rev	CTCGAGTTCCAAAAAGCTGACCCCTGAAGTTCATCTCTCTTGAAGATGAACTTCAGGGTCAGCGAGGGCCTGAGGAAGGCCGC
po7SK-NPsh-Rev	CTCGAGTTCCAAAAAGGATCTTATTTCTTCGGAGTCTCTTGAAGTCCGAAGAAATAAGATCCGAGGGCCTGAGGAAGGCCGC

Supplementary Figure 1: Construction of the porcine 7SK shRNA expression vectors: A one step PCR approach. Construction of the shRNA expression vector was achieved using a one step PCR approach. A regular forward primer and a long reverse primer, containing the complementary sequence of the 3' promoter (20 bp) and the hairpin sequence, the sense (19 bp), loop (9 bp), antisense (19 bp), termination (6 bp) and a restriction site.



Supplementary Figure 2: Knockdown of EGFP - fluorescence microscopy. Swine Testis (ST), Baby Hamster Kidney (BHK) and VERO cells were co-transfected with pEGFP-N1 and either a porcine (po7SK-EGFPsh), chicken (c7SK-EGFPsh), bovine (b7SK-EGFPsh) or mouse (mU6-EGFPsh) RNA pol III promoter construct expressing EGFP shRNAs. A porcine 7SK non-silencing control (po7SK-NPsh) was also used. EGFP suppression was analysed at 48 h post-transfection by fluorescence microscopy (Magnification $\times 100$).