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Repression of porcine endogenous retrovirus infection by human APOBEC3 proteins

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ABSTRACT

It has been shown that porcine endogenous retrovirus (PERV) can infect human cells, indicating that PERV transmission poses a serious concern in pig-to-human xenotransplantation. A number of recent studies have reported on retrovirus interference by antiviral proteins. The most potent antiviral proteins are members of the APOBEC family of cytidine deaminases, which are involved in defense against retroviral attack. These proteins are present in the cytoplasm of mammalian cells and inhibit retroviral replication. To evaluate the inhibition of PERV transmission by human APOBEC3 proteins, we co-transfected 293T cells with a PERV molecular clone and human APOBEC3F or APOBEC3G expression vectors, and monitored PERV replication competency using a quantitative analysis of PERV *pol* genes. The replication of PERVs in cells co-expressing human APOBEC3s was reduced by 60–90% compared with PERV-only control. These results suggest that human APOBEC3G and APOBEC3F might serve a potential barrier function against PERV transmission in xenotransplantation.

1. Introduction

Xenotransplantation from nonhuman animals offers a potential resolution of shortages of human donor organs [1]. Due to ethical considerations, breeding characteristics, compatibility of organ sizes, and physiology, pigs are the preferred donors for xenotransplantation [2]. However, some obstacles to their use in xenotransplantation remain. Immunological rejection of pig organs by humans is an obvious problem in xenotransplantation. Infection associated with porcine endogenous viruses that exist naturally in the porcine genome is also a major concern [1,3]. PERVs have drawn significant attention because all pig strains have been found to carry many copies of these viruses in their germ line DNA [4–6].

Various strategies have been developed and employed to minimize the possibility of PERV transmission, including identification of low-virus producing pigs [7] and the use of RNA interference techniques [8–10]. Recently, a number of studies have reported retrovirus interference by antiviral proteins. Within the APOBEC family, APOBEC3 (A3) proteins, which are a type of cytoplasmic cytidine deaminase, especially inhibit retroviral infection [11,12]. Sheehy et al. reported on the antiviral function of APOBEC3G (A3G) against infection and replication of human immunodeficiency virus [13,14]. A3 proteins interact with the nucleocapsid (NC) of HIV and become incorporated into new virions during maturation [15]. Cytosine-to-uracil mutations then occur on newly synthesized minus-strand cDNAs during viral reverse transcription. Such mutations are registered as G-to-A hypermutation in the plus strand DNA, so A3-containing HIVs cannot replicate properly in the newly infected host [16,17].

To test the ability of human A3 proteins to inhibit gamma retrovirus PERVs in human cells, we cloned APOBEC3F (A3F) and A3G genes from human peripheral blood mononuclear cells (PBMCs), and analyzed their effect on PERV infectivity in human cell lines.

2. Materials and methods

2.1. Isolation of human A3 cDNAs

Human A3G and A3F genes were isolated from normal human PBMCs by reverse transcription-polymerase chain reaction (RT-PCR). RT reactions were performed with mRNA from PBMCs using 200 units of SuperScript II reverse transcriptase (Invitrogen, NY,

Abbreviations: PERV, porcine endogenous retrovirus; HIV, human immunodeficiency virus; NC, nucleocapsid; A3, APOBEC3; A3F, APOBEC3F; A3G, APOBEC3G; PBMCs, peripheral blood mononuclear cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescence protein; DMEM, Dulbecco's modified Eagles medium; CDA, catalytic deaminase domains; CD, catalytic domain.

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USA), and then amplified with A3G or A3F specific primer sets using KAPATaq DNA polymerase (Kapa Biosystems, MA, USA). The primer sets used for amplification of the full-length A3F and A3G cDNAs were A3F-F/A3F-R (5′-gttaaccaccatgaagcctcacttcaga-3′ and 5′-ctcgagctacttgtcatcatcgtccttgtagtcaatctcctgcagcttg-3′), and A3G-F/A3G-R (5′-gttaaccaccatgaagcctcacttcaga-3′ and 5′-ctcgagctacttgtcatcatcgtccttgtagtcgttttcctgattctgga-3′), respectively. Underlined characters indicate the restriction enzyme sites, *Hpa*I and *Xho*I, used for subcloning into the expression vector. The FLAG tag (DYDDDDK, shaded boxes) was fused to allow detection of expressed proteins. Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 5′-accacagtccatgccatcac-3′ and 5′-tccaccaccctgttgctgta-3′) were used for normalization of PCR amplifications. Cloned human A3F and A3G genes were deposited at GenBank ID JF262037 and JF262036.

2.2. Construction of human A3 expression vectors

Amplified cDNAs of human APOBEC3s were directly cloned into the pGEM-T Easy Vector (Promega, WI, USA). TA-cloned A3 genes were digested with *Hpa*I and *Xho*I, and the fragments were subcloned into the pLL3.7 lentiviral vector, which expresses green fluorescence protein (GFP) gene [18]. The commonly used expression vector, pcDNA3.1, was also used for A3 gene expression. Expression of A3s was confirmed by Western blot analysis using an anti-FLAG M2 monoclonal antibody (Sigma, MO, USA).

2.3. Production of A3-harboring PERVs

The human 293T cell line (CRL-11268; American Type Culture Collection, VA, USA) was maintained in Dulbecco's modified Eagle's medium (DMEM; Hyclone Laboratories, UT, USA) supplemented with 10% fetal bovine serum (Hyclone Laboratories, New Zealand) at 37 °C in a humidified 5% CO_2 atmosphere. A3G- and A3F-containing PERVs were created by co-transfecting 293T cells with the PERV-B molecular clone (465D1, EU523109) [19] and A3G or A3F expression plasmids. The pLL3.7 lentiviral vector and pcDNA3.1 were used as expression vectors for A3 proteins. For transfections, 10 μg of the PERV-B molecular clone was co-transfected with 5 μg of pLL3.7 (pcDNA3.1), pLL-A3F (pc-A3F), or pLL-A3G (pc-A3G). Four hours after transfection, supernatants were removed and replaced with growth medium, and cells were incubated for an additional 48 h. Virus-containing supernatants from transfected cells were collected, filtered to remove cell debris, and subjected to ultracentrifugation (35,000 rpm) on 20% and 30% sucrose cushions (SW41, L-90 K, Beckman).

PERV production was detected by performing RT-PCR using RNAs from PERV-transfected cells or supernatants. The specific primer pair described in a previous study was used to detect the PERV *pol* gene [19,20]. 18s rRNA primers were used as an internal control [21].

2.4. Quantitative analysis of gDNA

For infection with APOBEC-containing PERVs, 5×10^4 normal 293T cells were plated on 6-well tissue culture plates. Two hours before infection, cells in test wells were washed with serum-free DMEM. 293T cells were inoculated with the same amounts of PERVs by incubating for 24 h in the presence of polybrene (8 μg/ml). Infected cells were washed with phosphate-buffered saline, followed by the addition of 3 ml of fresh culture media. At 72 h post-infection, gDNA isolated from infected 293T cells was used to detect PERV *pol* genes integrated into gDNA. Quantitative PCR reactions consisting of 500 ng of gDNA, 10 pmole/l primers, and 2 × SYBR Premix *Ex Taq* in a total volume of 20 μl were run on a Rotor Gene 3000 (Corbett Life Science, Australia). PCR conditions were as follows: 95 °C for 15 min, followed by 40 cycles of 94 °C for 10 s, 60 °C for 10 s, and 72 °C for 20 s. The amount of amplified PERV *pol* PCR product was calculated using Rotor-Gene 6 software. Reactions were performed in duplicate in two independent repetitions, and the degree of infection was expressed as the level of integration into 293T cell gDNA.

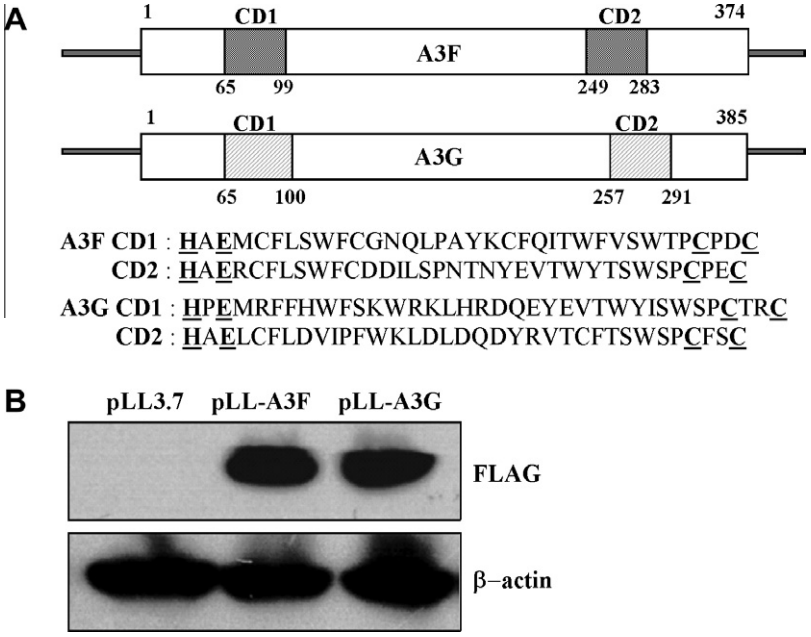

Fig. 1. Isolation of human A3F and A3G cDNAs from human PBMCs. (A) Diagrams of the A3 catalytic domains and amino acid residues, based on a sequence analysis of deduced amino acids. Underlined characters indicate H-X (any amino acid)-E-X_{23-28}-PC-X_{2-4}-C conserved motifs, which are involved in deamination of cytosine. CD; catalytic domain, consisting of CD1 (N-terminal CDA domain) and CD2 (C-terminal CDA domain). (B) Expression of pLL-A3F and pLL-A3G lentiviral vectors. Western blot analysis was carried out using an anti-FLAG M2 monoclonal antibody. A β-actin antibody was used as a loading control.

3. Results

3.1. Isolation of human A3s from PBMCs

Full-length A3F and A3G genes were isolated from human PBMC mRNA by RT-PCR. Following cloning into the T vector, the nucleotide sequences of the cDNAs were analyzed. The A3F and A3G genes used in this study were 99.5% and 99.6% homologous with reference sequences NM_145298 (A3F, 1122 bp) and NM_021822 (A3G, 1155 bp), respectively. The A3F and A3G proteins have two conserved catalytic deaminase domains (CDA) [12]. An analysis of the deduced amino acid sequence of cDNAs revealed the presence of conserved forms of catalytic domain 1 (CD1), which is responsible for encapsulation [22,23], and CD2, which is the catalytically active domain [16,24,25]. The CD1 and CD2 domains of A3F correspond to the sequences HAEMCFLSWFCGNQLPAYKCFQITWFVSWTPCPDC (amino acid residues 65–99) and HAERCFLSWFCDDILSPNTNYEVTWYTSWSPCPEC (amino acid residues 249–283), respectively. The CD1 and CD2 domains of A3G correspond to the sequences HPEMRFFHWFSKWRKLHRDQEYEVTWYISWSPCTRC (amino acid residues 65–100) and HAELCFLDVIPFWKLDLDQDYRVTCFTSWSPCFSC (amino acid residues 257–291), respectively. CD1 and CD2 domains of both proteins are 35 amino acids in length and display typical characteristics of CDAs (Fig. 1A). Underlined characters denote the CDA domain consensus sequence (or conserved motifs).

3.2. Expression of human A3s with PERV virions

Isolated human A3 cDNA clones were subcloned into the lentiviral expression vector (pLL3.7) for production of pLL-huA3F and pLL-huA3G. The plasmids pc-huA3F and pc-huA3G were also generated by cloning into the commonly used expression vector pcDNA3.1. Expression of A3 genes in 293T cells was confirmed by Western blotting (Fig. 1B).

Three types of viruses were produced via co-transfection of A3 expression vectors with the PERV-B molecular clone: PERV-A3F (PERV-pLL-A3F or PERV-pc-A3F), PERV-A3G (PERV-pLL-A3G or PERV-pc-A3G), and PERV-Vec (PERV-pLL3.7 or PERV-pcDNA3.1). RT-PCR was used to titrate PERVs in cell lysates (Fig. 2A) and supernatants (Fig. 2B). PERVs were detected in cell lysates and supernatants under all conditions except normal 293T supernatants (negative control), and similar amounts of PERVs were detected regardless of the A3 types used in co-transfection. Efficient transduction of lentiviral PERV-Vec (pLL3.7 only), PERV-pLL-A3F and PERV-pLL-A3G vectors in 293T cells was confirmed by GFP fluores-

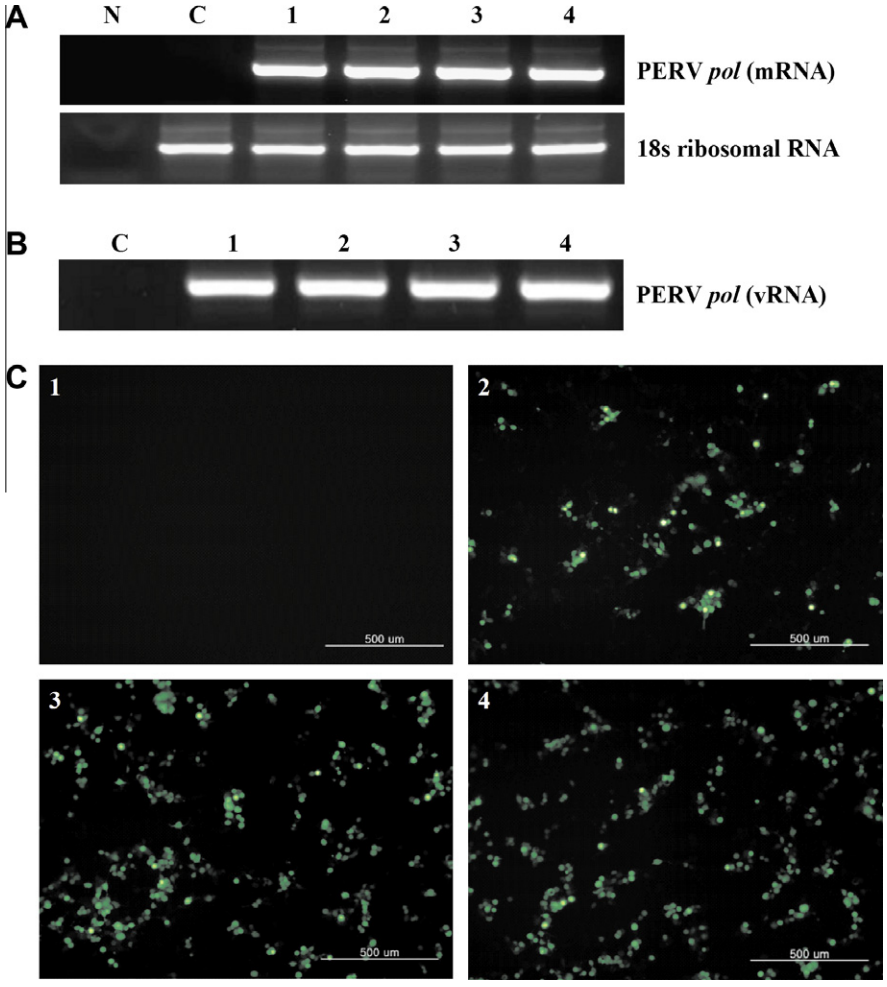

Fig. 2. Co-expression of PERV molecular clone with A3s. PERV-B was detected in co-transfected cells (A) and supernatants (B). PERV particles were produced by co-transfection of a PERV-B molecular clone with A3 expression vectors. Quantities of virus particle were confirmed by detection of the PERV *pol* gene in mRNA (A) and viral RNA (B). N; no template control, C: normal 293T cell lysate (mRNA) and supernatant (viral RNA); lane 1: PERV-B only; lane 2: PERV-pLL3.7, Co-transfected PERV molecular clone with pLL3.7, lane 3: PERV-pLL-A3F; Co-transfected PERV molecular clone with pLL3-A3F, lane 4: PERV-pLL-A3G; Co-transfected PERV molecular clone with pLL-A3G. Infection of PERVs harboring APOBEC3 proteins expressed from a lentiviral vector (C). 293T cells were infected with (1) PERV-B, (2) PERV-Vec (pLL3.7 vector only), PERV-pLL-A3F (3), or (4) PERV-pLL-A3G viruses, as described in Section 2. GFP was expressed from a pseudo packaged mRNA of lentiviral vector.

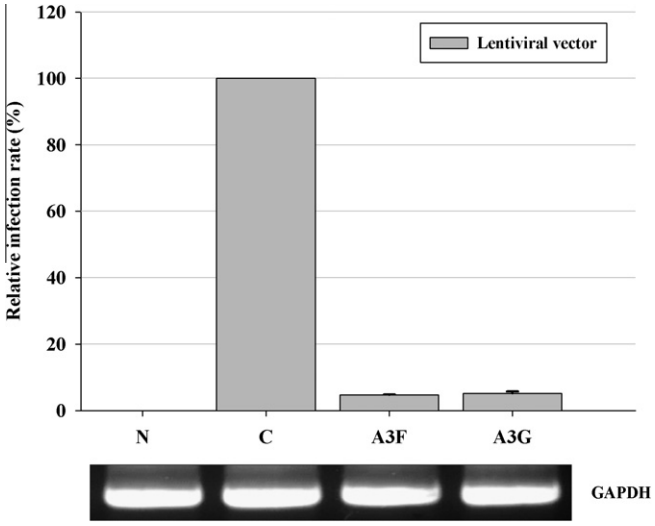

Fig. 3. Reduction of PERV infectivity by APOBEC3 expressed from a lentiviral vector. gDNA samples were purified from the four groups of 293T cells (Normal, PERV-Vec, PERV-A3F, and PERV-A3G) and analyzed by PCR for integrated PERV *pol* gene. The *y*-axis represents the relative integration rates in all analyzed samples. N: normal 293T cell gDNA; C: PERV-Vec (pLL3.7); A3F: PERV-pLL-A3F, A3G: PERV-pLL-A3G. Error bars represent mean ± standard deviation. The lower panel showed the amplification of GAPDH.

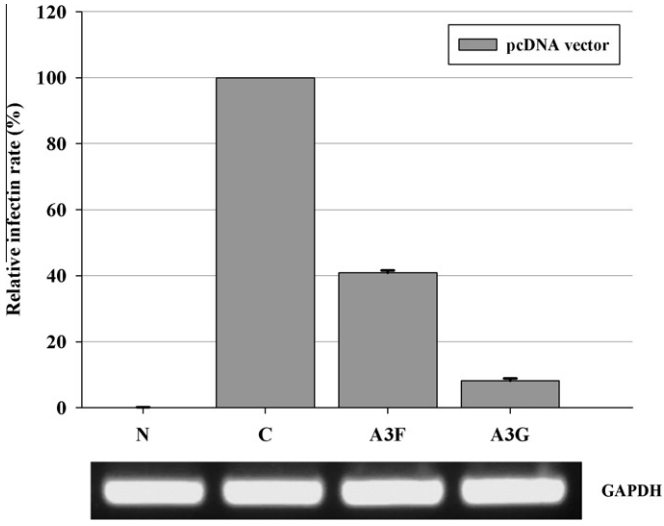

Fig. 4. Reduction of PERV infectivity by APOBEC3 expressed from a pCDNA3.1 vector. gDNA samples were purified from the four groups of 293T cells (Normal, PERV-Vec, PERV-A3F, and PERV-A3G) and analyzed by PCR for integrated PERV *pol* gene. The y-axis represents the relative integration rates in all analyzed samples. N: normal 293T cell gDNA; C: PERV-Vec (pcDNA3.1); A3F: PERV-pc-A3F, A3G; PERV-pc-A3G. Error bars represent mean ± standard deviation. The lower panel showed the amplification of GAPDH.

cence (Fig. 2C). These results showed that the LTR, present at both ends of the pLL3.7 vector, includes packaging signals and confirmed that simultaneous expression with PERV results in packaging within the virion. These results further support the infectivity of the PERV virus detected from the supernatant.

3.3. Reduction of PERV infectivity by APOBEC3s

A3 protein-containing PERV-B viruses were generated through co-transfection of the PERV-B molecular clone (465D1) and A3 expression plasmids. Viral titers within the culture supernatant were calculated using real-time PCR. Repetitive tests revealed that the titer of each harvested virus was approximately 1×10^4 particles/ml (data not shown). To test the effect of A3s in PERV replication, we infected 293T cells with equal amounts (1×10^4) of each PERV. The gDNAs of 293T cells infected with PERV-Vec, PERV-A3F, and PERV-A3G were extracted 48 h post-infection. Figs. 3 and 4 show a quantitative analysis of PERV *pol* integration into 293T gDNAs. The number of copies of *pol* genes integrated into gDNA purified from cells infected with PERV-Vec only was quantified and compared to the integration rate of PERV-B containing A3F or A3G proteins; PERV-Vec values were set as the 100% control (i.e., no reduction in PERV-B infectivity). Compared to infection with PERV-Vec, the infectivity of co-transfected PERVs was decreased by more than 90%. Specifically, for PERVs co-transfected with A3F and A3G using the lentiviral vector system, the infection rates dropped to 4% and 5%, respectively ($P < 0.0001$) (Fig. 3) Similar results were obtained using the pcDNA vector system. In this case, the infection rate for PERV-pc-A3G was ~8.1%, a similar reduction comparable to that of A3G in the lentiviral vector. Although the infectivity rate for PERV-pc-A3F was somewhat less (~40%), it also showed PERV reduction statistically ($P < 0.0001$) (Fig. 4).

4. Discussion

The purpose of these studies was to evaluate the suppression of PERV by A3 proteins as a type of innate immunity. We demonstrated the antiviral activity of A3s against PERV infection. PERV is a zoonotic factor in xenotransplantation. A3F and A3G are known to have antiviral effects by virtue of their cytidine deamination activity, which edits viral cDNA. By co-transfecting cells with human A3F or A3G and the PERV-B molecular clone, it was possible to produce infective PERV virions containing antiviral proteins. As a result of incorporation of A3s, PERV infectivity was dramatically reduced in new host cells. Collectively, the results of this study confirm that the antiviral proteins A3F and A3G inhibit PERV infection of human 293T cells.

Bishop et al. (2006) reported that A3G proteins are able to exert antiviral effects in the absence of deamination activity (i.e., without cDNA editing) [26]. They showed that although mutation of conserved Cys, His, or Glu residues in the C-terminal CD of A3F and A3G resulted in a loss of CDA activity, such mutations only modestly affected antiviral potency against HIV-1. The CD domains of human A3F and A3G used in this study also included conserved His, Cys, and Glu residues. Therefore, it is possible that the reduction in PERV-B infectivity may be attributable not only to C-to-U changes due to deamination, but also may reflect the influence of additional antiviral mechanisms. In addition, Glu67, Phe70, and Tyr91 residues of the N-terminal CD domain, and Glu259, Phe262, and Tyr282 residues of the C-terminal CD domain of A3G have been reported to play important roles in proton transfer, which is required for zinc ion coordination as well as CDA activity. The YYFW motif (amino acids 124–127), which is present in the A3G isolated here, influences A3G virion encapsidation [27].

We observed that the degree to which A3s reduced PERV infectivity was similar regardless of the gene-delivery vector systems used (pLL3.7 lentiviral vector and nonviral pcDNA3.1 expression vector). The consistent reduction of PERV infectivity by plasmid DNA-based A3 indicates that the suppression of PERV infectivity could be mainly attributed to the increased expression of exogenously transfected A3 genes, rather than lentiviral vector-related artifacts. Moreover, in the perspective of vector safety issues, A3 gene-encoding plasmid DNA can be preferentially developed as a gene therapy modality against PERV infections.

Although the apparent antiviral mechanism of APOBEC3s against retroviral infection requires further study, the results suggest the human A3 proteins in human cell can be a natural barrier against PERV in the xenotransplantation.

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References

[1] S. Magre, Y. Takeuchi, B. Bartosch, Xenotransplantation and pig endogenous retroviruses, Rev. Med. Virol. 13 (2003) 311–329.
[2] M.K. Schneider, J.D. Seebach, Xenotransplantation literature update: February–March, Xenotransplantation. 17 (2010) 256–260.
[3] J.A. Fishman, Infection in solid-organ transplant recipients, N. Engl. J. Med. 357 (2007) 2601–2614.
[4] C. Patience, Y. Takeuchi, R.A. Weiss, Infection of human cells by an endogenous retrovirus of pigs, Nat. Med. 3 (1997) 282–286.
[5] T. Argaw, A. Ritzhaupt, C.A. Wilson, Development of a real time quantitative PCR assay for detection of porcine endogenous retrovirus, J. Virol. Methods 106 (2002) 97–106.
[6] R. Mang, J. Maas, X. Chen, et al., Identification of a novel type C porcine endogenous retrovirus: evidence that copy number of endogenous retroviruses increases during host inbreeding, J. Gen. Virol. 82 (2001) 1829–1834.
[7] B.A. Oldmixon, J.C. Wood, T.A. Ericsson, et al., Porcine endogenous retrovirus transmission characteristics of an inbred herd of miniature swine, J. Virol. 76 (2002) 3045–3048.
[8] J. Ramsoondar, T. Vaught, S. Ball, et al., Production of transgenic pigs that express porcine endogenous retrovirus small interfering RNAs, Xenotransplantation. 16 (2009) 164–180.
[9] B. Dieckhoff, B. Petersen, W.A. Kues, et al., Knockdown of porcine endogenous retrovirus (PERV) expression by PERV-specific shRNA in transgenic pigs, Xenotransplantation. 15 (2008) 36–45.
[10] S. Miyagawa, S. Nakatsu, T. Nakagawa, et al., Prevention of PERV infections in pig to human xenotransplantation by the RNA interference silences gene, J. Biochem. 137 (2005) 503–508.
[11] R.S. Aguiar, B.M. Peterlin, APOBEC3 proteins and reverse transcription, Virus Res. 134 (2008) 74–85.
[12] R. Goila-Gaur, K. Strebel, HIV-1 Vif, APOBEC, and intrinsic immunity, Retrovirology 5 (2008) 51.
[13] J. Wang, J.M. Shackelford, N. Selliah, et al., The HIV-1 Vif protein mediates degradation of Vpr and reduces Vpr-induced cell cycle arrest, DNA Cell Biol. 27 (2008) 267–277.
[14] A.M. Sheehy, N.C. Gaddis, J.D. Choi, et al., Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein, Nature 418 (2002) 646–650.
[15] B.D. Gooch, B.R. Cullen, Functional domain organization of human APOBEC3G, Virology 379 (2008) 118–124.
[16] A. Jarmuz, A. Chester, J. Bayliss, et al., An anthropoid-specific locus of orphan C to U RNA-editing enzymes on chromosome 22, Genomics 79 (2002) 285–296.
[17] R.S. Harris, M.T. Liddament, Retroviral restriction by APOBEC proteins, Nat. Rev. Immunol. 4 (2004) 868–877.
[18] D.A. Rubinson, C.P. Dillon, A.V. Kwiatkowski, et al., A lentivirus-based system to functionally silence genes in primary mammalian cells. Stem cells and transgenic mice by RNA interference, Nat. Genet. 33 (2003) 401–406.
[19] N.Y. Kim, D. Lee, J. Lee, et al., Characterization of the replication-competent porcine endogenous retrovirus class B molecular clone originated from Korean domestic pig, Virus Genes 39 (2009) 210–216.
[20] D. Lee, N.Y. Kim, G.E. Bae, et al., Transmissible infection of human 293T cells with porcine endogenous retroviruses subgroup a from NIH-miniature pig, Transplant Proc. 40 (2008) 3742–3745.
[21] J.G. Hashimoto, A.S. Beadles-Bohling, K.M. Wiren, Comparison of RiboGreen and 18S rRNA quantitation for normalizing real-time RT-PCR expression analysis, Biotechniques 36 (2004) 54–56. 58–60.
[22] S. Opi, H. Takeuchi, S. Kao, et al., Monomeric APOBEC3G is catalytically active and has antiviral activity, J. Virol. 80 (2006) 4673–4682.
[23] F. Navarro, N.R. Landau, Recent insights into HIV-1 Vif, Curr. Opin. Immunol. 16 (2004) 477–482.
[24] K. Xie, M.P. Sowden, G.S. Dance, et al., The structure of a yeast RNA-editing deaminase provides insight into the fold and function of activation-induced deaminase and APOBEC-1, Proc. Natl. Acad. Sci. USA 101 (2004) 8114–8119.
[25] J.E. Wedekind, G.S. Dance, M.P. Sowden, et al., Messenger RNA editing in mammals: new members of the APOBEC family seeking roles in the family business, Trends Genet. 19 (2003) 207–216.
[26] K.N. Bishop, R.K. Holmes, M.H. Malim, Antiviral potency of APOBEC proteins does not correlate with cytidine deamination, J. Virol. 80 (2006) 8450–8458.
[27] H. Huthoff, M.H. Malim, Identification of amino acid residues in APOBEC3G required for regulation by human immunodeficiency virus type 1 Vif and Virion encapsidation, J. Virol. 81 (2007) 3807–3815.