The ORF4 protein of porcine circovirus type 2 antagonizes apoptosis by stabilizing the concentration of ferritin heavy chain through physical interaction

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INTRODUCTION

Porcine circovirus type 2 (PCV2), a pathogenic strain of porcine circovirus (PCV), has been identified as the primary aetiological agent of porcine circovirus-associated disease in swine. The mechanism of PCV2 pathogenesis remains largely unknown. A newly identified viral protein of PCV2, ORF4, has been suggested to be involved in virus-induced apoptosis. However, there is still no information regarding the molecular mechanism by which ORF4 regulates apoptosis. In this study, we reveal that a physical interaction between the PCV2 ORF4 protein and ferritin heavy chain (FHC) in the cytoplasm of host cells reduced the cellular concentration of FHC. The ORF4-mediated reduction of FHC inhibited reactive oxygen species accumulation in PCV2-infected cells. Consequently, the ORF4 protein inhibited apoptosis in host cells. This may be the first report to describe the mechanism of ORF4 cytoprotection against apoptosis during the early stages of PCV2 infection.
yeast- or bacteria-based two-hybrid assay (Timmusk et al., 2006; Liu et al., 2007; Finsterbusch et al., 2009; Lv et al., 2015b), although the details of these interactions remain unknown at this time. To date, however, the molecular basis of PCV2 pathogenesis remains elusive, and there are still no data on the molecular mechanism of the ORF4 protein regulating apoptosis.

With regard to apoptosis, PCV2 or its ORF3 protein reportedly induced apoptosis in cultured PK15 cells, mouse splenic macrophages and porcine peripheral blood mononuclear cells (PBMCs), and this process appeared to be dependent on caspase-3 and caspase-8 activity but not on caspase-9 activity (Kiupel et al., 2001; Liu et al., 2005; Lin et al., 2011). Indeed, PCV2-induced B-lymphocyte depletion has also been ascribed to apoptosis (Shibahara et al., 2000), although contradictory evidence suggests decreased cell proliferation rather than apoptosis as the reason for this hallmark of PCV2 infection (Mandrioli et al., 2004). Interestingly, the newly characterized ORF4 protein was found to play a role in suppressing caspase activity and modulating the host immune system via regulation of CD4+ and CD8+ T lymphocytes during PCV2 infection (He et al., 2013). Hence, PCV2 may prevent apoptosis by expressing its own anti-apoptotic gene to complete its propagation in the early stage of PCV2 infection, but in the late stage, it promotes apoptosis either to kill uninfected immune cells or to induce the breakdown of infected cells in the host and, thereby, facilitate viral dissemination. If so, it is necessary to further clarify whether and how the ORF4 protein is involved in the anti-apoptotic mechanism, as all the functions of ORF4 described above are based on indirect results.

In this study, we demonstrated that the PCV2 ORF4 protein has an anti-apoptotic function by stabilizing the concentration of the ferritin heavy chain (FHC) through physical interaction. These results will contribute to elucidating the molecular mechanisms of early infection by PCV2.

RESULTS

ORF4 Per se is not anti-apoptotic but antagonizes PCV2-induced apoptosis

Previously, the anti-apoptotic activity of PCV2 ORF4 was assessed using an ORF4-deficient PCV2 mutant clone (He et al., 2013). To directly confirm this result, PK15 stable cell lines expressing either GFP–ORF4 fusion proteins or GFP alone were constructed and subjected to flow cytometry analysis. As shown in Fig. 1(a), expression of GFP and the GFP–ORF4 fusion proteins was visible under an inverted fluorescence microscope (Nikon), but no green fluorescence was detected in the mock-transfected PK15 cells. Immunoblotting using an anti-GFP antibody indicated that PK15 cells transfected with pEGFP-ORF4 and the pEGFP-C1 plasmid expressed proteins of approximately 33 kDa and 27 kDa, respectively (Fig. 1b). However, no immunoreactive band was visualized in the negative control cells. These results confirmed the expression of the PCV2 ORF4 protein in PK15 cells. The results of flow cytometry indicated that the ORF4 protein alone did not have an apoptosis-promoting effect, but its anti-apoptotic effect was also low, suggesting that the anti-apoptotic role of ORF4 is achieved through the process of PCV2 infection. To clarify this issue, various PK15 cell lines were infected with the PCV2 virus. As shown in Fig. 1(c, f), apoptotic activity induced by PCV2 in PK15 cells was significantly higher than that produced by PCV2 in ORF4-expressing cells. However, the level of apoptosis detected in PCV2-infected ORF4-expressing cells was still higher than that produced in the mock-infected cells. These results indicate that the ORF4 protein is able to partially antagonize PCV2-induced apoptosis.

ORF4 has no impact on the pro-apoptotic function of ORF3

It has been reported that the ORF3 protein is critical for PCV2-triggered apoptosis (Liu et al., 2005). To further clarify whether the ORF4-triggered anti-apoptotic effect relies on the inhibition of ORF3-induced pro-apoptotic functions, PK15 cells were infected with recombinant lentiviruses expressing intact ORF3 and subjected to flow cytometry analysis at 72 h after infection. As shown in Fig. 2(c, d), apoptosis induced by overexpression of ORF3 in ORF4-expressing cells was similar to that in control cells. Subsequent real-time quantitative RT-PCR analysis demonstrated that there was no significant difference in the levels of ORF3 mRNA among lentivirus-infected PK15 cells (Fig. 2e). These data suggest that the ORF4 protein is probably not involved in ORF3-induced apoptosis, although a recently published study contradicts these results as two ORF4-deficient PCV2 viruses showed significantly higher ORF3 transcription levels than WT PCV2 virus (Gao et al., 2014).

FHC interacts with ORF4 in the cytoplasm of PK15 cells

Evidence for the interaction between ORF4 and FHC has recently been demonstrated as described above (Lv et al., 2015b). However, it is still unclear whether the endogenous FHC binds the ORF4 protein within host cells, especially in PK15 cells. To clarify this issue, we performed immunofluorescence assays with the relevant antibodies using confocal microscopy and co-immunoprecipitation (co-IP) assays with anti-FHC antibodies and Western blotting with anti-DsRed. Co-localization of ORF4 and FHC in the cytoplasm of PK15 cells was clearly visible as shown in Fig. 3(a), and endogenous FHC precipitated DsRed–ORF4 but not DsRed alone, which is shown in Fig. 3b, indicating that endogenous FHC interacts with the PCV2 ORF4 protein in the cytoplasm of host cells.
Fig. 1. The PCV2 ORF4 protein antagonizes PCV2-induced apoptosis. (a) Fluorescence detection of the GFP–ORF4 fusion protein and GFP alone expressed in PK15 cells (×200). (i) Mock-infected PK15 cells. (ii) Stable GFP-protein-expressing PK15 cells. (iii) Stable GFP–ORF4-protein-expressing PK15 cells. (b) Immunoblotting analysis of the GFP–ORF4 fusion protein in PK15 cells. Proteins extracted from the GFP–ORF4-expressing and control cells were subjected to Western blotting using an anti-GFP polyclonal antibody. (c) Representative results of the detection of apoptosis by flow cytometry in the GFP–ORF4-expressing and control cells. (d) The mean percentage of apoptosis in three independent experiments displayed in (c).
FHC is involved in PCV2-induced apoptosis

FHC, the primary iron storage factor, acts as an effective anti-apoptotic molecule that protects cells from apoptosis induced by a variety of pro-apoptotic stimuli (Brouard et al., 2000; Berberat et al., 2003). However, the effect of FHC on PCV2-triggered apoptosis is unknown. To clarify this issue, we investigated the impact of FHC expression on apoptosis in PCV2-infected cells using flow cytometry. As shown in Fig. 4, lentivirus-mediated overexpression of FHC significantly reduced the number of apoptotic cells in PCV2-infected cells.

Fig. 2. ORF4 is not involved in ORF3-induced apoptosis. (a) Confirmation of lentivirus infection by fluorescence detection of the GFP reporter expressed in PK15 cells (×100). (i) Mock-infected PK15 cells. (ii) PK15 cells infected with recombinant lentiviruses expressing intact ORF3. (iii) Stable GFP–ORF4-expressing PK15 cells infected with recombinant lentiviruses expressing intact ORF3. (b) Confirmation of ORF3 protein expression in PK15 cells by Western blot analyses using an anti-Flag monoclonal antibody. (c) Flow cytometry analysis of PK15 cells infected with recombinant lentiviruses expressing intact ORF3. (d) The mean percentage of apoptotic cells from flow cytometry analysis. (e) Expression of ORF3 mRNAs. PK15 cells were infected with the lentiviruses indicated at an m.o.i. of 1. Total cellular mRNA was harvested at different timepoints post-infection and was measured by real-time quantitative RT-PCR. Results are presented as the mean±SD (n=3). PI, Propidium iodide.
infected cells, compared with untreated cells. Conversely, knockdown of FHC by short hairpin RNA (shRNA) (Fig. 4c, d) markedly increased the number of apoptotic cells (Fig. 4e, f). Taken together, these results support the hypothesis that FHC is involved in PCV2-induced apoptosis.

ORF4 stabilizes the concentration of FHC in PCV2-infected cells

Recently, the anti-apoptotic effect of FHC was reported to be dose-dependent as shown by the induction of toxicity at doses higher than the protective range and the limited effect at lower doses (Berberat et al., 2003). Therefore, we hypothesized that the interaction between ORF4 and host FHC may be important in the regulation of FHC expression. To confirm this hypothesis, FHC mRNA in PCV2-infected PK15 cells and mock-infected cells was first measured by real-time quantitative RT-PCR at the time points indicated, and mRNA expression curves were plotted. As shown in Fig. 5(a), the FHC transcription levels in PCV2-infected PK15 cells were significantly higher than those in mock-infected cells (P<0.05), which indicated that PCV2 infection of PK15 cells activates the transcription of FHC. These data are consistent with previous results showing that FHC is induced downstream of NF-κB (Pham et al., 2004), because PCV2 infection strongly induces the activation of NF-κB (Wei et al., 2008). Additionally, the FHC mRNA levels in PCV2-infected PK15 cells, ORF4-deficient PCV2-infected PK15 cells and PCV2-infected ORF4-expressing PK15 cells at 72 h post-infection were also measured by real-time quantitative RT-PCR. As shown in Fig. 5(b), similar levels of FHC mRNA were identified in all three groups, which suggested that the interaction between ORF4 and FHC may not influence FHC transcription. Finally, cell lysates from the above-mentioned experimental groups were subjected to Western blot analyses. As shown in Fig. 6, the protein level of FHC in PCV2-infected ORF4-expressing PK15 cells was significantly lower than that in mock-infected cells (P<0.001), the protein level of FHC in ORF4-deficient PCV2-infected PK15 cells was significantly higher than that in mock-infected cells (P<0.001), and the protein level of FHC in PCV2-infected PK15 cells was also significantly lower than that in ORF4-deficient PCV2-infected PK15 cells (P<0.01), indicating that the ORF4 protein can reduce the FHC protein levels in host cells. Notably, the facilitated reduction of the FHC protein band in PCV2-infected and PCV2-infected ORF4-expressing PK15 cells was attributed to the gradual increase in another immunoreactive band, which was located over the FHC protein band (Fig. 6a). These observations suggest that facilitated reduction of the FHC by ORF4 is likely achieved through protein modification. The combined results indicate that a direct interaction between ORF4 and host FHC may control the FHC protein levels in virus-infected cells. Considering the important role of FHC in apoptosis, these results suggest that the PCV2 ORF4 protein antagonizes apoptosis by stabilizing the concentration of FHC in PCV2-infected cells.

ORF4 suppresses reactive oxygen species (ROS) accumulation in PCV2-infected cells

ROS can mediate cell death triggered by a variety of stimuli, including TNFα, ceramide, radiation and chemotherapeutic agents (Pham et al., 2004). Recently, a time-dependent increase in ROS during PCV2 infection has been reported (Chen et al., 2012), and this progression was shown to alter NF-κB activity (Chen et al., 2012). Activation of the NF-κB pathway promotes the expression of pro-inflammatory...
molecules, such as TNFα, E-selectin, P-selectin, ICAM-1 and VCAM-1 (Berberat et al., 2003), and is thus a crucial component of the cellular defence against PCV2 infection (Zhang et al., 2013). Interestingly, FHC is induced downstream of NF-κB and is identified as an essential mediator of TNFα-triggered apoptosis via control of ROS induction (Pham et al., 2004).

Given the above results, we speculated that the interaction between ORF4 and host FHC may influence ROS accumulation during PCV2 infection. To investigate this hypothesis, the production of ROS was measured 72 h after the infection of
PCV2 infection has been shown to induce apoptosis in cells (Kiupel et al., 2001; Liu et al., 2005), and the major mechanism underlying PCV2-mediated apoptosis has also been clarified (Liu et al., 2007). However, PCV2 must antagonize apoptosis to replicate prior to cell death due to its extensive dependence on the host cell machinery. The ORF4 protein of PCV2 has been proposed as an anti-apoptotic protein (He et al., 2013; Gao et al., 2014) and even a molecule down-regulating host cell signalling (Choi et al., 2015); therefore, defining how ORF4 antagonizes apoptosis during the early stages of PCV2 infection may explain how PCV2 manipulates apoptosis to facilitate its replication.

Currently, several porcine proteins have been identified as binding partners of the ORF4 protein using a yeast mating approach (Lv et al., 2015b). Among these, FHC has been shown to bind exogenous ORF4 in transfected cells. FHC can act as an anti-apoptotic protein that protects cultured cells from apoptosis induced by a variety of stimuli (Berberat et al., 2003) and is therefore the most likely molecule to be involved in inhibition of apoptosis triggered by PCV2 ORF4. In particular, a previously published study reported that FHC is induced downstream of NF-κB and inhibits TNFα-induced apoptosis by suppressing ROS (Pham et al., 2004). As activation of the NF-κB signalling pathway and production of ROS were reportedly detected in PCV2-infected PK15 cells (Wei et al., 2008; Chen et al., 2012), the interaction between ORF4 and host FHC may be responsible for inhibition of apoptosis by PCV2 during its early infection.

To confirm this possibility, we first demonstrated that the ORF4 protein itself is not anti-apoptotic but antagonizes PCV2-induced apoptosis (Fig. 1), and that ORF4 has no impact on the pro-apoptotic function of ORF3 (Fig. 2). Interestingly, a recent report suggested that the PCV2 ORF4 protein may prevent virus-induced apoptosis by restricting ORF3 transcription (Gao et al., 2014). Therefore, the data reported here are inconsistent with previous results. However, our hypothesis is a possibility, as the previous results are derived from an indirect presumed method. We then focused on the interaction between ORF4 and host FHC and demonstrated that ORF4 physically interacted with endogenous FHC in the cytoplasm (Fig. 3). This observation provides further evidence supporting the idea that ORF4-mediated inhibition of apoptosis is achieved through its physical interaction with host FHC. To confirm this hypothesis, we explored the role of FHC in PCV2 infection. The results indicated that FHC is involved in PCV2-induced apoptosis, as shown by the reduced apoptosis in FHC-overexpressing cells and the increased apoptosis in FHC knockdown cells (Fig. 4). Given previous reports that the anti-apoptotic effect of FHC was dose-dependent (Berberat et al., 2003), we determined whether the PCV2 ORF4 protein affects the cellular concentration of FHC during PCV2

DISCUSSION

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infection. The results clearly indicated that expression of ORF4 leads to a significant reduction in FHC protein but no change in FHC mRNA (Figs 5 and 6). Notably, there was a larger immunoreactive band in addition to the FHC band (Fig. 6a), suggesting that stabilization of FHC at low concentrations of ORF4 may be associated with modification of the FHC protein through their physical interaction. These results were further confirmed by analyses of ROS production in various PK15 cells infected with PCV2 or PCV2Δ (Fig. 7).

In summary, the mechanism by which the PCV2 ORF4 protein antagonizes apoptosis in PCV2-infected cells was defined in this study. The ORF4 protein physically binds host FHC, resulting in the reduction of FHC protein levels in host cells. Reduction of FHC concentration further inhibits the accumulation of ROS in host cells, leading to reduced apoptosis. This report may be the first explanation of the mechanism by which ORF4 protein confers cytoprotection against apoptosis during the early stages of PCV2 infection.

**METHODS**

**Vectors, viruses and cells.** The pEGFP-C1 eukaryotic expression vector was purchased from Clontech (USA). The pcDNA3.1(+) eukaryotic expression vector was purchased from Invitrogen. The eukaryotic expression plasmid pDsRed–ORF4 was maintained in our laboratory (Lv et al., 2015b). The PCV2 virus used in this study was originally isolated from a pig with naturally occurring postweaning multisystemic wasting syndrome in the Guanzhong region of China (Tang et al., 2011). PCV-free PK15 cells, which were kindly provided by Qing-hai Tang, Nanyang Normal University, PR China, were cultured in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) supplemented with 10% FBS (Gibco) at 37°C in a 5% CO₂ incubator (Tang et al., 2013). HEK293T cells were cultured as described previously (Zhang et al., 2015).

**Antibodies and reagents.** A mouse anti-GFP polyclonal antibody was purchased from Sungen Biotech. A mouse anti-GAPDH monoclonal antibody and a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody were purchased from Sigma-Aldrich. Rabbit anti-FHC antisera and HRP-conjugated goat anti-rabbit antiserum were purchased from Abcam. A rabbit anti-DsRed polyclonal antibody was purchased from Clontech. A goat anti-rabbit IgG H&L (Alexa Fluor-488) was purchased from Abcam. Hoechst 33342 was purchased from Beyotime.

**Construction of the recombinant plasmid and establishment of stable cells expressing GFP–ORF4 and GFP.** Amplification of the coding sequence of the PCV2 ORF4 gene was accomplished by using a pair of primers (F-EcoRI, 5’-GGGAATTCTATGACGTGTACT-3’ and R-BamHI, 5’-ATGGATCC-3’).
TCAGGGGACACCCGAGTTG-3'). After purification, the amplified product was cloned into the pEGFP-C1 eukaryotic expression vector. By confirmation by enzymic digestion and DNA sequencing, the recombinant plasmid was named pEGFP-ORF4. PK15 cells grown in 12-well plates were transfected with pEGFP-ORF4 and a pEGFP-C1 control vector using TurboFect (Thermo). After incubation at 37°C for 48 h, cultures were propagated in selection media containing 1000 μg G418 ml⁻¹ for 2 weeks. When all control cells showed evidence of death in the presence of the selection agents, cultures transfected with pEGFP-ORF4 were propagated for another two weeks in selection media containing 500 μg G418 ml⁻¹. After confirmation by immunoblotting, the resultant PK15 cell lines stably expressing either GFP-ORF4 or GFP alone were used for the follow-up studies.

**Cell apoptosis detection by flow cytometry.** To determine the impact of ORF4 protein expression on apoptosis in the established cell lines, annexin V-PE and propidium iodide (PI) staining and flow cytometry were performed. Briefly, cells grown in six-well culture dishes were treated with trypsin, washed three times with PBS and resuspended to a concentration of 0.5–1.0 × 10⁶ cells ml⁻¹. After the addition of 0.2 μg of PE-tagged annexin-V and 100 μl binding buffer, cells were incubated at 25°C in the dark for 30 min. After washing with PBS, 0.25 μg PI and 400 μl binding buffer were added sequentially. Finally, apoptotic cells were examined by a Coulter Epics XL FACS (Beckman).

**Lentivector construction and lentivirus production.** Recombinant lentiviruses expressing intact ORF3 and FHC were constructed as previously described with slight modifications (Zhang et al., 2015). Briefly, the cDNA for the PCV2 ORF3 and porcine FHC proteins were cloned into the over-expression lentivector pCDH-CMV-MCS-EF1-GreenPuro (CD513B-1) (SBI) to generate the LV-ORF3 and LV-FHC plasminids using the primer pairs ORF3-F (5'-CGGAAATTCATGGTAACCATCC-3') and ORF3-R (5'-CGGAAATTCATGGTAACCATCC-3'). After the addition of 0.2 μg of PE-tagged annexin-V and 100 μl binding buffer, cells were incubated at 25°C in the dark for 30 min. After washing with PBS, 0.25 μg PI and 400 μl binding buffer were added sequentially. Finally, apoptotic cells were examined by a Coulter Epics XL FACS (Beckman).

**Immunoblotting analysis.** Western blot analysis was performed as described elsewhere with some modifications (He et al., 2013). Briefly, protein samples were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore). After blocking with 5% skimmed milk, the membranes were probed with the primary antibodies indicated at room temperature for 2 h, followed by incubation with HRP-conjugated secondary antibodies. Finally, immunoreactive bands were visualized by chemiluminescence using an enhanced ECL Western blotting analysis system (Thermo). The cellular protein GAPDH served as an internal control.

**Real-time quantitative RT-PCR.** Real-time quantitative RT-PCR was performed to measure the relative quantification of gene expression as described previously (Lv et al., 2015a). Briefly, total RNA was isolated from PK15 cell samples using TRIzol (Invitrogen) and was used as a template for reverse transcription with a PrimeScript RT reagent kit (TaKaRa) according to the manufacturer’s recommendations. The real-time quantitative RT-PCR assays were performed with the primer pairs F-ORF3 (5'-CTTCTACATCGGCCATCTG-3')/R-ORF3 (5'-GACAAAGGAGGTAGCCTTG-3') and F-HFC (5'-CCGCGGATGATGTGGCTTTG-3')/R-HFC (5'-GGTTTCATGATCCTGGAAGG-3'). The real-time relative expression level of genes was calculated using the 2⁻ΔΔCt method with porcine β-actin as an internal control (Livak & Schmittgen, 2001).

**Confocal microscopy and co-IP assays.** To determine whether the ORF4 protein interacts with endogenous FHC, confocal microscopy was performed as previously described with slight modifications (Lv et al., 2015b). To further confirm this result, PK15 cells grown in six-well plates were transfected with pDsRed-ORF4. As a negative control, the empty pDsRed-Monomer-C1-transfected cells were used. At 48 h post-transfection, the cells were washed twice with PBS and treated with lysis buffer (containing PMSF) for 30 min on ice. After centrifugation, the supernatants were subjected to immunoprecipitation using rabbit anti-FHC antiserum for 2 h at 4°C. After the addition of 30 μl of protein G Sepharose (GE healthcare) at 4°C for 2 h, the immune complex was

### Table 1. shRNA inserts

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<th>shRNA</th>
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washed three times with ice-cold lysis buffer and subjected to SDS-PAGE followed by Western blot analysis.

**Construction of the ORF4-deficient PCV2 mutant clone.** Recombinant plasmid pSK-W containing the complete genome of PCV2 was constructed by our lab (Lv et al., 2015a). To generate the infectious PCV2 mutant clones, a specific mutation altering the start codon of ORF4 from ATG to ACG was introduced into the cloned PCV2 genome with a set of mutagenic primers, F(5’-AAGACTAATGTACAGTQGT-TAGTCCTCCACATG-3’) and R(5’-GATTGGAAGACTAAGCGTG-TACATTGAGCTT-3’) (mutant site is underlined), according to the protocol of a site-directed gene mutagenesis kit (Boytom). After excision from the pSK-W plasmid and re-circularization by ligation, the resultant DNA mixture was transfected into PCV-free PK15 cells using TurboFect (Thermo). After incubation at 37°C for 24 h, fresh medium containing 300 mM D-glucosamine was added. After 48 h, three successive freeze-thaw cycles were performed on the genome-transfected PK15 cells to harvest viral stocks.

**Assay of intracellular ROS.** ROS levels were measured with a cell superoxide anion in situ assay kit (Genmed). Nitro blue tetrazolium (NBT) from this kit is reduced by ROS to blue formazan, which was then measured at 650 nm with a microplate reader (Multiskan FC, Thermo). PK15 cells were seeded in 96-well plates at a density of 5x10^4 per well, infected with PCV2 or PCV2Δ at an m.o.i. of 1 at 4 h post-seeding and incubated at 37°C for 72 h. PK15 cells pre-incubated with 50 µM arachidonic acid (Sigma) for 30 min and mock-infected PK15 cells were used as controls. After addition of 20 µl NBT and incubation at 37°C for 4 h, 100 µl of fixative solution was added to cultures, followed by 100 µl of lysis buffer. After incubating at room temperature for 5 min, the light absorbance was measured at 650 nm.

**Statistical analysis.** All data are expressed as the mean±s.d. The results were analysed with Student’s t-test, and P<0.05 was regarded as statistically significant.

**ACKNOWLEDGEMENTS**

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