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*Original Article*

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***Abstract***

Porcine circovirus type 2 (PCV2) is one of the most important swine viral diseases in Thailand and worldwide. While the interaction between PCV2 and pig immune system has been largely explored, the mechanisms by which PCV2 replicates in porcine immune cells and alters host cellular signaling pathways remain incomplete. In this study, extracellular regulated kinase (ERK) phosphorylation form was shown to slightly increase in PCV2-infected peripheral blood mononuclear cells (PBMCs) at 48 hr post infection. To demonstrate the role of ERK MAPK signaling, PBMCs were divided into three groups including control, virus, and virus with the mitogen-activated protein kinase (MEK) specific inhibitor, U0126, to prevent ERK phosphorylation. U0126 at 10 $\mu$M completely inhibited ERK activation in the porcine PBMCs at 48 and 72 hr. Interestingly, the inhibition of ERK MAPK significantly impaired PCV2 replication in the PBMCs. In summary, our results unraveled the role of ERK MAPK signaling during PCV2 replication in porcine immune cells. These findings provide important information on how PCV2 interacts with host cells and influences host immune response.

**Keywords:** ERK MAPK, PCV2, replication, peripheral blood mononuclear cells

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## Introduction

PCV2 is a DNA virus associated with post-weaning multisystemic wasting syndrome (PMWS), porcine respiratory disease complex (PRDC) and porcine dermatitis and nephropathy syndrome (PDNS). PCV2 is one of the important viral diseases which affect pig production worldwide (Allan et al., 1998; Allan and Ellis, 2000; Opriessnig et al., 2007; Gillespie et al., 2009). The characteristics of PCV2 infection are lymphoid depletion and altered pattern of cytokine responses in lymphoid cells leading to immunosuppression and multiple opportunistic infections (Darwich et al., 2003; Kekarainen et al., 2010; Segalés, 2012). Economic losses associated with PMWS include weight loss and high mortality if co-infections with other viruses or bacteria infection occur in the same herd (Ellis et al., 2003; Ellis et al., 2008; Sofia et al., 2008; Gillespie et al., 2009;)

Two types of PCV have been characterized including PCV type 1 (PCV1) and PCV type 2 (PCV2) (Meehan et al., 1998). PCV1 is a non-pathogenic virus that infects porcine kidney cell line (PK15 cells) without causing clinical diseases in swine, while PCV2 is associated with all porcine circovirus-associated diseases (Ellis, 2014). To establish infection, recent studies have reported that PCV2 activates multiple host cellular pathways including TLR/MYD88/NF-κB, PI3K/Akt, and MAPKs (Wei et al., 2012; Duan et al., 2014). Among these, all three MAPK signaling pathways including JNK1/2, p38 and ERK1/2 have been shown to regulate PCV2 replication in porcine PK15 cells (Wei and Liu, 2009; Wei et al., 2009). In addition to PCV2, ERK MAPK also regulates porcine reproductive and respiratory syndrome virus replication and modulates cytokine production (Wei and Liu, 2009; Lee and Lee, 2010; Hou et al., 2012; Zhou and Zhang, 2012; Ren et al., 2016). One of the main cellular targets for PCV2 replication is porcine peripheral blood mononuclear cells (PBMCs) (Gilpin et al., 2003; Pensaert et al., 2004). While the interaction between PCV2 and pig immune system has been largely explored, the mechanisms by which PCV2 replicates in the porcine PBMCs and alters cellular signaling have not yet been fully investigated.

In this study, whether ERK MAPK signaling implicates in viral-host interaction in porcine PBMCs and whether this interaction affects PCV2 viral progeny production were determined.

## Materials and Methods

***Reagents and experimental animals:*** Three, thirty-day-old specific pathogen free (SPF) pigs were maintained at the animal research facility, Faculty of Veterinary Medicine, Kasetsart University (Kamphaeng Saen Campus, Nakhon Pathom province). The protocol for laboratory animal use and care was approved by the Chulalongkorn University Animal Care and Use Committee, Chulalongkorn University (IACUC number 13310019, 1431086). The specific primary antibodies purchased were as follows: anti-phospho-p44/42 ERK and anti-p44/42 ERK from Santa Cruz Biotechnology (Dallas, Texas, USA). Horseradish peroxidase (HRP)-conjugated secondary antibody was purchased from Sigma (St Louis, Missouri, USA). The ERK MAPK inhibitor, U0126, was purchased from Calbiochem (San Diego, California, USA). All other chemicals and reagents were laboratory grade.

***Culture and maintenance of porcine circovirus type 2:*** Porcine circovirus type 2 strain ISU 31 was derived from an infectious cDNA clone (Lekcharoensuk et al., 2004). The virus was propagated in porcine kidney cell line (PK15) and maintained in minimal essential medium (MEM) supplemented with 5% heat inactivated fetal bovine serum (FBS) at 37°C with 5% $CO_2$. To determine viral replication, PBMCs were infected with PCV2 at a multiplicity of infection (MOI) of 0.1. Infectivity levels of PCV2 in the PBMCs were determined by the amount of viral DNA using quantitative PCR.

***Isolation and in vitro stimulation of porcine PBMCs:*** Whole bloods were collected from the experimental pigs in heparinized containers and were separated by centrifugation on a Ficoll-Hypaque gradient density 1.077 (GE healthcare, Pittsburgh, Pennsylvania, USA). Collected PBMCs were mixed in RPMI plus 10% fetal bovine serum (FBS) and plated at a density of $6x10^6$ cells per well in a 24-well plate (Nantakhruea et al., 2013).

***Effect of MAPK inhibitor on porcine PBMC viability and PCV2 replication:*** PBMCs were plated in a 24-well plate overnight as described previously (Nantakhruea et al., 2013). The cells were incubated with U0126, a ERK MAPK inhibitor, at various concentrations ranging from 1 to 40 $\mu$M or with an equivalent volume of dimethyl sulfoxide (DMSO) as a control. Cell cytotoxicity was determined at 24 and 48 hr after treatment using trypan blue exclusion assay. Live and dead cells were loaded and counted on a hematocytometer in triplicate and compared with each appropriate control treatment. PCV2-infected PBMCs were incubated with U0126 for 1 hr and were maintained at 37°C with 5% $CO_2$ for another 24 hr. Percentage of viable cells was calculated from 200 counted cells and compared to relevant control group.

***Immunoblotting for phospho-ERK protein detection:*** The porcine PBMCs were collected by centrifugation and washed twice with PBS. Cell pellets were incubated in lysis buffer following standard protein extraction protocol. Afterward, cell lysates were separated on SDS-PAGE acrylamide gels and transferred onto nitrocellulose membranes using semi-dry transfer protocol (Surachetpong et al., 2009). The membranes were blocked in non-fat dry milk and were incubated with specific phospho-p44/42 ERK and p44/42 ERK primary antibody with concentration as previously described (Wei and Liu, 2009). Subsequently, the membranes were incubated in HRP-conjugated secondary antibody overnight. To reveal antibody bound proteins, the membranes were incubated with chemiluminescent reagent (Pierce, Waltham, Massachusetts, USA) and measured with C-DiGit western blot scanner (LICOR, Lincoln, Nebraska, USA). Level of phospho-p44/42 ERK was normalized to the p44/42 ERK protein.

***Quantification of viral replication and pathogenesis:*** The PBMCs were incubated with U0126 for 1 hr prior to infection and then inoculated with PCV2 at 0.1 MOI. The cells were washed twice and maintained in fresh medium containing U0126 or DMSO for 24, 48 and 72 hr. To quantify the amount of viral genome replication, total DNA was extracted from lysates of the infected cells using a DNA extraction kit (QIAGEN, Valencia, California, USA) according to the manufacturer's protocol. Amplification of genomic DNA was performed by a quantitative PCR thermocycler CFX96 (BioRad, Hercules, California, USA) with the PCV2 specific forward primer PCV2-119 5'-ATGCCCAGCAAGAAGAATGGAAGAAG-3' and reverse primer PCV2-450 5'-AGGTCACTCCGTTGTC CTTGAGATC-3'. The qPCR condition included denaturation at 95˚C for 2.30 min, followed by 40 cycles at 95˚C for 5 sec, 56˚C for 30 sec, and 72˚C for 30 sec. The amount of viral genomic DNA was quantitatively compared between the U0126-treated cells relative to the DMSO-treated control group.

***Statistical analysis:*** Difference in viral DNA was determined using multiple group comparisons by the one-way analysis of variance (ANOVA) and Bonferroni multiple comparison test. All statistical analyses were performed using Prism software version 5.0.1 (GrapPad, San Diego, California, USA). A P value of <0.05 was considered to be statistically significant.

## *Results*

Prior to testing the effect of U0126 on virus replication, it is important to determine whether U0126 has no direct effect on PBMC viability. As a result, in this study, freshly isolated PBMC was cultured with U0126 at concentrations ranging from 1, 10 and 40 $\mu$M for 24 and 48 hr. The percentage of viable cells was measured by trypan blue exclusion assay. The control group was treated with DMSO at the concentration equal to 40 $\mu$M inhibitor. A total of 200 PBMCs were counted from three independent experiments. Although there was a slight increase in the number of cell death in the U0126-treated cells at 40 $\mu$M, the percent of viable cells in all U0126-treated groups were not statistically different from the untreated control group (Table 1).

**Table 1** Effect of ERK MAPK inhibitor (U0126) on PBMC viability

| Group | Percent survival (%) | |
|---|---|---|
| | 24 hr | 48 hr |
| Control | 100 | 100 |
| U0126 1 μM | 98.1 | 97.3 |
| U0126 10 μM | 97.1 | 93 |
| U0126 40 μM | 97 | 92.4 |

In this study, the immunoperoxidase monolayer assay (IPMA) was used to demonstrate PCV2 infection in porcine PBMCs. The antibody specific to ORF2 capsid protein of PCV2 (Lekcharoensuk et al., 2004) was used to stain and demonstrate PCV2 antigen in the positive PBMCs. The cells were inoculated with different amounts of PCV2, ranging from 0.01 to 1.0 MOI. At 72 hr post infection (hpi), virus-infected cells were abundantly found in 0.1 MOI inoculation (Fig. 1). In contrast, no positive cells were observed in the control PBMCs. This finding confirms that PCV2 was able to replicate in porcine PBMCs.

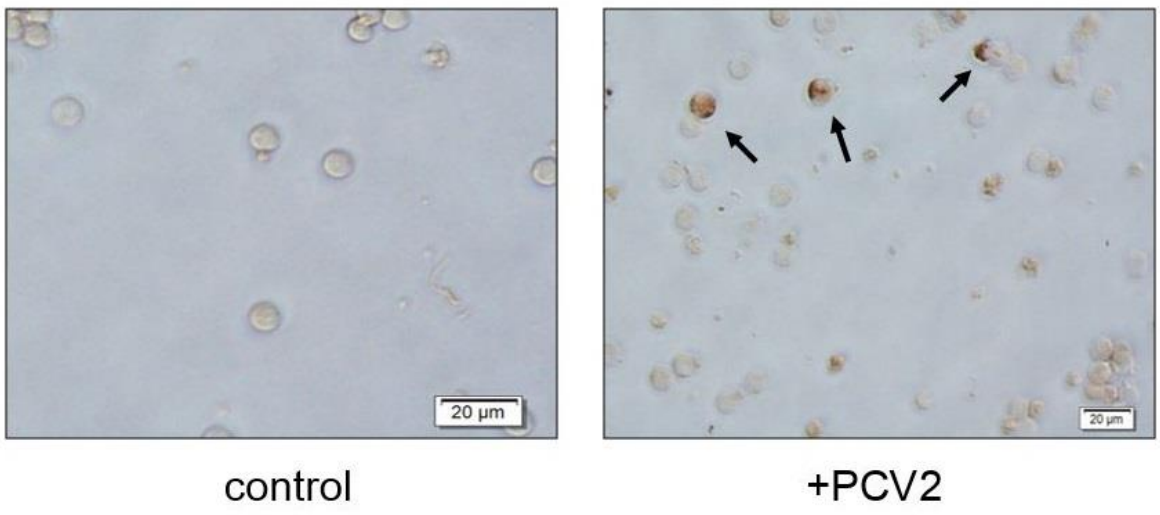


**Figure 1** **PCV2 infection in porcine PBMCs.** PBMCs were inoculated with PCV2 for 72 hr. The PCV2 positive cells (arrows) were demonstrated by immunoperoxidase monolayer assay (IPMA) using antibody specific to PCV2 capsid protein. PBMCs were inoculated with PCV2 at 0.1 MOI. The figures are representatives of three individual experiments.

To determine whether ERK MAPK participates in PCV2 replication, immunoblot analysis was used to determine the level of ERK MAPK activation in the presence or absence of PCV2 infection. The amounts of loading protein in each sample were demonstrated by coomassie blue staining (Fig. 2a). Moreover, the level of phospho-ERK in each sample was normalized to total ERK protein. At 48 hpi, ERK MAPK phosphorylation slightly increased in the infected PBMCs compared to the uninfected control cells (Figs. 2a, 2b). The slight reduction in ERK phosphorylation in the virus-infected cells was observed at 72 hpi (Fig. 2b, 2c). To further explore the role of ERK1/2MAPK in PCV2-infected cells, U0126 was used to inhibit ERK1/2 phosphorylation. U0126 has been shown as a highly selective inhibitor for MEK1 and MEK2, an upstream signaling molecule of ERK1 and ERK2 MAPK (Hotokezaka et al., 2002). Notably, U0126 was found to completely block ERK1/2 phosphorylation in the PCV2-infected cells at both 48 and 72 hpi (Figs. 2b, 2c).

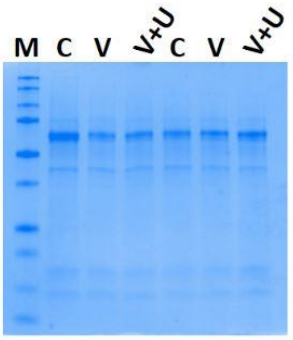


Fig 2a

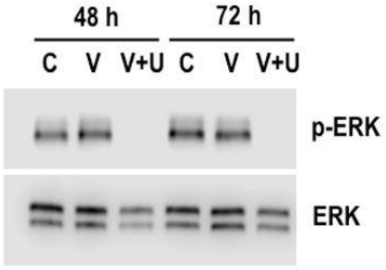


Fig 2b

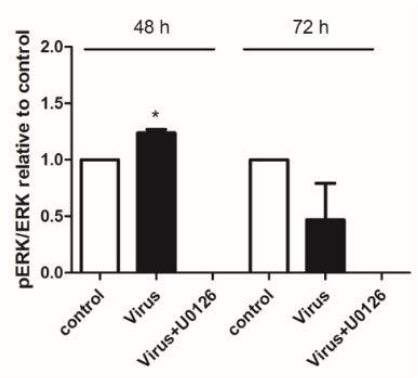


Fig 2c

**Figure 2** **Level of ERK phosphorylation in PBMCs inoculated with PCV2.** The amount of total proteins is demonstrated on coomassie blue staining gel (2a). ERK phosphorylation in PCV2-infected cells was determined by immunoblot analysis (2b). Total ERK (ERK) protein was used as a loading control. The relative change in ERK phosphorylation in virus-inoculated cells was normalized to uninfected cells (2c). C = control, V = PCV2-infected PBMC, V+U = PCV2-infected PBMC + U0126

The quantitative PCR was then used to assess the amount of PCV2 virus at 48 hpi (Fig. 3a) and 72 hpi (Fig. 3b) following virus inoculation in the U0126-treated cells. The primers used in this study were specific to ORF2 capsid protein of PCV2. At 48 and 72 hpi, the amount of PCV2 in the PBMCs was 443 to 2099 fold compared to the non-inoculated control cells. Interestingly, the cells treated with U0126 at concentrations ranging from 1 to 40 $\mu$M had lower PCV2 replication (P<0.05) at 72 hpi. However, only 40 $\mu$M U0126 treatment at 48 hpi had lower virus load relative to the control cells (P<0.05).

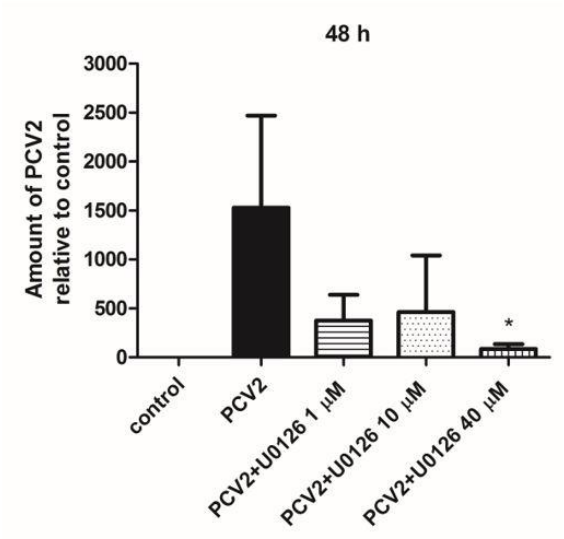


Fig 3a

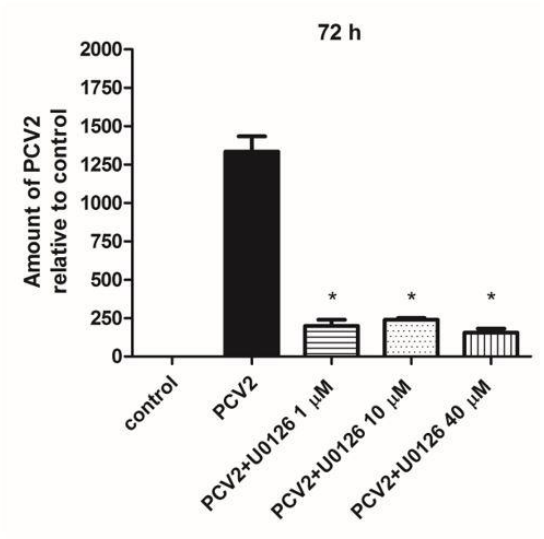


Fig 3b

**Figure 3** **Inhibition of ERK MAPK signaling reduced PCV2 replication in PBMCs.** Cells were treated with U0126 at concentrations ranging from 1 to 40 $\mu$M followed by PCV2 inoculation at MOI 0.1. At 48 and 72 hpi, DNA were extracted and processed for quantitative PCR analysis with specific primers against ORF2 capsid gene. (* = P<0.05)

## *Discussion*

Porcine circovirus type 2 infection is associated with high morbidity, clearly emphasizing the need for further knowledge of pathogen-host interactions. Despite extensive research on PCV2, little is known about the intracellular signaling pathways that participate in PCV2 pathogenesis in porcine white blood cells. This study reveals the role of ERK MAPK signaling on PCV2 replication in porcine PBMCs. The activation of ERK MAPK was notified at 48 hpi, but not at 72 hpi. In PK-15 cells, it was shown that ERK and Elk-1 phosphorylation promoted PCV2 replication (Wei and Liu, 2009). The mechanism behind this observation has not been investigated in porcine PBMCs, but it is probable that other host cellular proteins that regulate via MEK/ERK pathway may participate in the virus replication. For example, replication of classical swine fever virus (CFSV) depends on interaction between mitogen-activated protein kinase/extracellular regulated kinase (MEK1/2 and ERK1/2) and Janus kinase-signal transducer and activator of transcription (JAK/STAT) signaling pathway (Wang et al., 2016). It is possible that ERK pathway may indirectly impact other host proteins or cytokines that affect PCV2 viral progeny production. Upon infection, PCV2 induces interleukin-10 (IL-10) production through PI3K/Akt and p38 MAPK pathways in porcine alveolar macrophages (Dai et al., 2016). IL-10 is an immunosuppressive cytokine that inhibits pro-inflammatory cytokine production and cellular immune response (O'Garra et al., 2004). Modulation of this immunosuppressive cytokine could facilitate PCV2 replication in the host cells. In fact, elevated IL-10 in pigs was demonstrated in chronic and persistent PCV2 infection (Doster et al., 2010). Both PCV2a and PCV2b genotypes induced early IL-10 production in porcine PBMCs regardless of PCV2 infection status (Fort et al., 2010).

In pigs, the role of MAPK pathways including ERK, p38 and JNK1/2 pathways has been largely explored in multiple virus infection. All three MAPK pathways are required for active replication of PRRSV and PCV2 in porcine cells (Wei and Liu, 2009; Wei et al., 2009; Lee and Lee, 2010, 2012). To unravel the role of these pathways during virus replication, specific inhibitors are applied to inhibit MAPK activation. Using a small inhibitor (U0126) that specially target MEK1/2 signaling, an upstream activator of ERK1/2 is commonly applied for blocking MEK/ERK signaling pathway. Our study reveals that the specific MEK inhibitor, U0126, which prevents ERK phosphorylation dose, could dependently block PCV2 replication at 48 and 72 hpi. Likewise, these observations are consistent with PCV2 replication in PK15 cells (Wei and Liu, 2009). Also, the phosphorylation of ERK demonstrated to occur at 48 hr in the present study could clearly affect PCV2 replication at 72 hpi. However, it is worthwhile to note that the effect of U0126 on PCV2 replication may indirectly occur via other signaling proteins or PBMC proliferation. Such effects have not been explored in this study. Collectively, our data provide important evidence that ERK1/2 signaling not only plays an important role during PCV2 replication in PK15 cells, but also participates in PCV2 replication in porcine PBMCs. Understanding the molecular mechanisms during virus infection could lead us to the novel knowledge of the target of cellular signaling and pathogenesis of viruses in porcine cells. For instance, a transcriptomic study revealed that multiple signaling pathways regulated gene expression in porcine PBMC in response to PRRSV vaccine (Islam et al., 2017). In addition to PCV2 and CSFV, ERK activation is critical for porcine reproductive and respiratory syndrome virus (PRRSV) and porcine epidemic diarrhea virus replication (Lee and Lee, 2010; Kim and Lee, 2015). Such examples demonstrated the interplay between various swine viruses and host cellular networks which could determine the clinical outcome of virus infection.

In conclusion, PCV2 infection could induce ERK MAPK activation, which is important for PCV2 replication. These data extended our knowledge of ERK MAPK pathway in the PCV2 replication in porcine immune cells. These new findings may be utilized to design better vaccine for PCV2 prevention or improve the understanding of virus-host interaction.

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# บทคัดย่อ

## การตอบสนองทางภูมิคุ้มกันของเซลล์สุกรที่ติดเชื้อ porcine circovirus type 2 ผ่านระบบสื่อสาร ERK MAPK

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เชื้อเซอร์โคไวรัสในสุกรจัดเป็นโรคติดเชื้อไวรัสที่สำคัญในประเทศไทยและทั่วโลก ถึงแม้จะมีการศึกษาเกี่ยวกับปฏิสัมพันธ์ระหว่างไวรัสและระบบภูมิคุ้มกันสุกรจะมีรายงานจำนวนมาก การศึกษาเกี่ยวกับกลไกที่ไวรัสเพิ่มจำนวนในเซลล์ภูมิคุ้มกันสุกรและการเปลี่ยนแปลงของการสื่อสารภายในเซลล์เหล่านี้ยังมีจำกัด ในการศึกษาครั้งนี้ระดับฟอสโฟไลเลชั่นของ ERK MAPK ในเซลล์เม็ดเลือดขาวสุกร polymorphonuclear cells (PBMC) เพิ่มขึ้นเล็กน้อยที่ 48 ชั่วโมงภายหลังการติดเชื้อไวรัสเซอร์โคไวรัสชนิด 2 การศึกษาบทบาทของระบบสื่อสาร ERK MAPK ในเซลล์ PBMC ด้วยสารยับยั้ง MEK (U0126) ที่ระดับ 10 ไมโครโมลสามารถยับยั้ง ERK MAPK ได้อย่างสมบูรณ์ทั้งที่ 48 และ 72 ชั่วโมง ที่น่าสนใจคือการยับยั้ง ERK MAPK ช่วยลดปริมาณเชื้อเซอร์โคไวรัสชนิด 2 ในเซลล์ PBMC อย่างมีนัยสำคัญ โดยสรุปผลการศึกษาครั้งนี้แสดงถึงบทบาทของ ERK MAPK ต่อการเพิ่มจำนวนของเซอร์โคไวรัสชนิด 2 ในเซลล์ PBMC สุกรและแสดงให้เห็นถึงปฏิสัมพันธ์ระหว่างเชื้อเซอร์โคไวรัสชนิด 2 และการตอบสนองทางภูมิคุ้มกันของเซลล์สุกร

**คำสำคัญ:** ERK MAPK เซอร์โคไวรัสชนิด 2 การแบ่งตัว เซลล์เม็ดเลือดขาว

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