ผลและกลไกของเชื้อไวรัสพีอาร์อาร์เอสไทป์ 1 และไทป์ 2 ต่อการยอมให้ซึมผ่านระหว่างเซลล์และการอยู่รอดของเซลล์เยื่อบุมดลูกสุกร

นายดรัณ รักอาชีพ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Effects and mechanisms of porcine reproductive and respiratory syndrome virus (PRRSV) type 1 and type 2 on the permeability and viability of porcine endometrial epithelial cells

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Animal Physiology
Department of Veterinary Physiology
Faculty of Veterinary Science
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Thesis Title	Effects and mechanisms of porcine reproductive and respiratory syndrome virus (PRRSV) type 1 and type 2 on the permeability and viability of porcine endometrial epithelial cells
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ดรัณ รักอาชีพ : ผลและกลไกของเชื้อไวรัสพีอาร์อาร์เอสไทป์ 1 และไทป์ 2 ต่อการยอมให้ซึมผ่านระหว่างเซลล์และการอยู่รอดของเซลล์เยื่อบุมดลูกสุกร (Effects and mechanisms of porcine reproductive and respiratory syndrome virus (PRRSV) type 1 and type 2 on the permeability and viability of porcine endometrial epithelial cells) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. สุทธาสินี ปุญญโชติ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร. ฉัตรศรี เดชะปัญญา, ดร. สุพจน์ วัฒนะพันธ์ศักดิ์, หน้า.

ไวรัสพีอาร์อาร์เอสทั้งสองชนิด คือ ไทป์ 1 และไทป์ 2 ซึ่งทำให้เกิดระบบสืบพันธุ์ล้มเหลวพบว่ามีความสัมพันธ์กับรอยโรคบริเวณที่มีการฝังตัวของตัวอ่อน การสูญเสียความแข็งแรงและการทำหน้าที่ของเยื่อบุมดลูกส่วนต่อมของแม่สุกรอาจกระทบต่อการขนส่งอาหารไปให้ลูก งานวิจัยนี้มีเป้าหมายที่จะตรวจสอบผลโดยตรงของไวรัสพีอาร์อาร์เอสไทป์ 1 และไทป์ 2 ต่อความอยู่รอดและการทำหน้าที่เป็นปราการของเยื่อบุมดลูก โดยใช้เยื่อบุมดลูกสุกรส่วนต่อม การเปรียบเทียบเส้นทางในการติดเชื้อและสายพันธุ์ของไวรัสไปพร้อมกับประเมินความรุนแรงของไวรัสต่อเซลล์เยื่อบุทางเดินสืบพันธุ์จะถูกพิจารณาด้วย

เซลล์เยื่อบุมดลูกสุกรถูกแยกจากมดลูกสุกรอายุ 4-6 เดือนซึ่งได้รับจากฝูงสุกรที่ไม่พบการระบาดของโรคพีอาร์อาร์เอส (จำนวนตัวอย่างจาก 7 สุกร) นำมาเลี้ยงในอาหารเลี้ยงเชื้อมาตรฐานที่มีซีรัมลูกวัวร้อยละ 5 จนกระทั่งเซลล์โตร้อยละ 90 ของภาชนะ ไวรัสพีอาร์อาร์เอสไทป์ 1 และไทป์ 2 ซึ่งถูกแยกสดใหม่และคำนวณปริมาณไวรัสที่ทำให้เซลล์ติดเชื้อได้ร้อยละ 100 ในปริมาตร 2 มิลลิลิตร นำมาบ่มที่ด้านยอดหรือด้านฐานเป็นเวลา 1 ชั่วโมง สังเกตการเกิดซีพีอีทุกวัน ประเมินความสามารถในการซึมผ่านด้วยสารโมเลกุลใหญ่ FD-4 และวัดค่าความต้านทานทางไฟฟ้าของเยื่อบุผิว (TER) ณ วันที่ 0, 2, 4 และ 6 หลังจากทำให้ติดเชื้อ วัดการแสดงออกของไทต์จังก์ชันยีนซึ่งประกอบไปด้วย คลอดิน1, 2, 3, 4, 5, 7, 8 และโซนูลาออกคลูดิน1 ด้วยวิธี qPCR เปรียบเทียบกับ GAPDH ณ วันที่ 4 หลังทำให้ติดเชื้อ และวัดการอยู่รอดของเซลล์ด้วยวิธี MTT และย้อมสี annexin V/PI ที่วันที่ 0, 2, 4 และ 6 หลังทำให้ติดเชื้อ

ผลการศึกษาพบว่าเชื้อไวรัสพีอาร์อาร์เอสทำให้เซลล์เยื่อบุมดลูกเกิดซีพีอีชนิดต่าง ๆ ได้หลังจากเริ่มบ่มเชื้อไว้ การบ่มไวรัสไทป์ 2 ทางด้านยอดของเซลล์ทำให้ความต้านทานของเยื่อบุลดลง และเพิ่มการซึมผ่านของ FD-4 ในวันที่ 4 ($p<0.05$) บ่งชี้ถึงการติดเชื้อไวรัสไทป์ 2 ทำให้เกิดความเป็นพิษต่อเซลล์เยื่อบุมดลูก ในทางตรงข้ามการติดเชื้อไวรัสทั้งสองสายพันธุ์ที่ด้านฐานไม่ส่งผลให้เกิดความเสียหายใด แต่สามารถรักษาความแข็งแรงของเยื่อบุได้จนถึงวันที่ 6 และไม่พบการเปลี่ยนแปลงของการซึมผ่านของ FD-4 ($p<0.05$) ไวรัสทั้งสองสายพันธุ์ดูเหมือนจะเพิ่มการทำงานของไทต์จังก์ชันโดยเพิ่มการแสดงออกของโปรตีนที่สร้างความแข็งแรงคือ คลอดิน5 แต่ลดการแสดงออกของโปรตีนที่ทำให้เกิดช่องคือ คลอดิน7 นอกจากนี้ยังพบว่ามีไทต์จังก์ชันยีนอื่นที่เป็นเป้าหมายของไวรัสแตกต่างกันไปตามสายพันธุ์ ได้แก่ คลอดิน3 และ 8 เป็นเป้าหมายของไวรัสพีอาร์อาร์เอสไทป์ 1 ส่วน ZO-1 เป็นเป้าหมายของไวรัสพีอาร์อาร์เอสไทป์ 2 อย่างไรก็ตามการเปลี่ยนแปลงการแสดงออกของไทต์จังก์ชันยีนไม่สัมพันธ์กับการผลของไวรัสต่อการทำงานของไทต์จังก์ชัน ส่วนผลของการอยู่รอดของเซลล์ พบว่าไวรัสไทป์ 2 เพิ่มจำนวนเซลล์เยื่อบุมดลูก แม้ว่าการตายแบบ necrosis จะเกิดไปด้วยกันในวันที่ 2 และ 4 ($p<0.05$) โดยในวันที่ 2 กลุ่มที่ติดเชื้อไวรัสไทป์ 1 ตรวจพบการตายแบบ necrosis เล็กน้อย ผลของการติดเชื้อไวรัสต่อการตายและการเพิ่มจำนวนเซลล์จะกลับเข้าสู่ปกติในวันที่ 6 หลังจากติดเชื้อของทุกกลุ่มการทดลอง อย่างไรก็ตามเซลล์เยื่อบุมดลูกสุกรที่ฟื้นตัวจากติดเชื้อไวรัสนั้นจะมีการทำงานที่เป็นปกติยังเป็นที่น่าสงสัย การศึกษาครั้งนี้สามารถสรุปได้ว่าไวรัสพีอาร์อาร์เอสไทป์ 2 ก่อโรคในแง่ของการทำให้เกิดการรั่วของเยื่อบุและการตายของเซลล์ได้รุนแรงกว่าไวรัสไทป์ 1 และไวรัสยังสามารถทำให้เกิดความผิดปกติที่การแสดงออกของไทต์จังก์ชันและทำให้เกิดการตายของเซลล์แบบ necrosis ดังนั้นหากมีการปนเปื้อนเชื้อไวรัสพีอาร์อาร์เอสที่เยื่อบุมดลูกก็จะมีความเสี่ยงให้เกิดความเสียหายและนำไปสู่ความล้มเหลวในการสืบพันธุ์ได้

ภาควิชา สรีรวิทยา
สาขาวิชา สรีรวิทยาการสัตว์
ปีการศึกษา 2560

ลายมือชื่อนิสิต .....
ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
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5875308931 : MAJOR ANIMAL PHYSIOLOGY
KEYWORDS: CELL DEATH, PERMEABILITY, PORCINE ENDOMETRIAL EPITHELIAL CELLS, PRRSV, TIGHT JUNCTION

DRAN RUKARCHEEP: Effects and mechanisms of porcine reproductive and respiratory syndrome virus (PRRSV) type 1 and type 2 on the permeability and viability of porcine endometrial epithelial cells. ADVISOR: ASSOC. PROF. SUTTHASINEE POONYACHOTI, DVM, Ph.D., CO-ADVISOR: ASSOC. PROF. CHATSRI DEACHAPUNYA, Ph.D., SUPHOT WATTANAPHANSAK, DVM, Ph.D., pp.

Both PRRSV types 1 and 2 revealed the sign of reproductive disorders associated with a lesion at implantation sites. Impairment of maternal glandular endometrium cell integrity and function by PRRSV infection may impact a proper nourishing fetus. This research was aimed to examine the effects of PRRSV type 1 and type 2 directly on the viability and barrier function of the endometrium using porcine glandular endometrial epithelial cell culture (PEG). The comparison of the route and the strain of PRRSV infection coinciding with their virulent in reproductive epithelia were considered.

PEG cells isolated from the 4-6 months old PRRSV-free-herd gilts (n=7 pigs) were cultured in standard media DMEM with 5% fetal bovine serum until 90% confluent. The fresh isolated PRRSV type 1 and type 2 (at $TCID_{100}$/2 ml) inoculated to either apical or basolateral site of PEG cells for 1 h. The cytopathic effect (CPE) was observed daily. The permeability assessment of barrier function, the measurement of FITC dextran 4 kDa (FD-4) flux and transepithelial electrical resistance (TER), were performed at 0, 2, 4 and 6 days post infection (dpi). The expression of TJ protein genes; *Cldn 1, 2, 3, 4, 5, 7, 8* and *ZO-1* were detected by real-time qPCR and normalized with *GAPDH* at 4 dpi. The viability was determined by MTT assay and annexin V/propidium iodide (PI) assay at 0, 2, 4 and 6 dpi.

All PRRSV inoculations to PEG cells produced a various CPE formation upon the inoculation. Apical inoculation with PRRSV type 2 decreased TER and increased FD-4 flux at 4 dpi ($p<0.05$). This indicates the cytotoxicity of PRRSV type 2 infection. In contrast, basolateral inoculation of PRRSV type 1 and 2 had no effects but maintained the integrity of the TJ barrier reflected by the stable of FD-4 flux through 6 dpi ($p<0.05$). Both viruses seem to increase the barrier function by up-regulating the barrier builder TJ *Cldn5* but down-regulating the pore-forming TJ *Cldn7* ($p<0.05$). The additional TJs gene was differently a target of PRRSV type 1 and 2 infections; *Cldn3* and *Cldn8* for PRRSV type 1 vs. *ZO-1* for PRRSV type 2. However, the alteration of TJs expression induced by PRRSV was not relevant to their effects on TJs barrier functions. In the viability test, PRSSV type 2 increased cell proliferation, although the necrotic cell was concomitantly detected at 2 and 4 dpi ($p<0.05$). A few necrotic cells were also founded by PRRSV type 1 infection at 2 dpi. All of the cytotoxicity or proliferative effects were recovered at 6 dpi in all PRRSV infected PEG. However, whether the recovery PEG cell has a proper function is suspicious. The conclusion from our findings is PRRSV type 2 has more severity on the TJs barrier leaky and cell death than type 1 PRRSV in porcine endometrium. Since PRRSV can disturb TJs mRNA expression associated with necrosis, mucosal contamination or inoculation with PRRSV may be at risk or associated with PRRSV-induced reproductive disorders.

Department:	Veterinary Physiology	Student's Signature	.....
Field of Study:	Animal Physiology	Advisor's Signature	.....
Academic Year:	2017	Co-Advisor's Signature	.....
		Co-Advisor's Signature	.....

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%	Percent
ºC	Degree Celsius
±	Plus and minus
µg	microgram
µM	micromolar
µm	micrometer
A	Surface area
Å	Angstrom
$A_{570, 620}$	Absorbance 570, 620 nm
ANOVA	Analysis of variance
APHCA	Animal Production and Health Commission for Asia and the Pacific
AV	Annexin V-FITC
bp	Base pair
C_0	Initial concentration
Caspase	Cysteine-aspartic proteases
CD	Highly pathogenic PRRSV
cDNA	Complementary deoxyribonucleic acid

Cldn	Claudin
cm	centimeter
cm^2	Square centimeter
CPE	Cytopathic effect
CSFV	Classical swine fever virus
C_t	Threshold cycle
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dpi	Day post infection
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohemorrhagic *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ERK	Extracellular signal-regulated kinase
FasL	Fas ligand
FBS	Fetal bovine serum
FD	Fluorescein isothiocyanate dextran
FITC	Fluorescein isothiocyanate
g	Gram

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GE	Glandular epithelium
GP	Glycoprotein
h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
hNEC	Human nasal epithelial cell
hpi	Hour post inoculation
HP-PRRSV	Highly pathogenic PRRSV
i.e.	That is
IBD	Inflammatory bowel disease
IFN	Interferon
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL	Interleukin
JAM	Junction adhesion molecule
LE	Luminal epithelium
LPS	lipopolysaccharide

MA104	African green monkey kidney cell line MA104
MAPK	Mitogen-activated protein kinase
MARC-145	African green monkey kidney cell line MARC-145
MDCK	Madin-Darby canine kidney cell
min	Minute
ml	Milliliter
MLV	Modified live vaccine
mm	Millimeter
mM	Millimolar
mm^2	Square millimeter
mRNA	Messenger ribonucleic acid
mW	Milliwatt
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
mV	Millivolt
MW	Molecular weight
nm	Nanometer
Nsp	Non-structural protein
OD	Optical density

ORF	Open reading frame
PAMPs	Pathogen-associated molecular pattern
P_{app}	Apparent permeability coefficient
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PEG	porcine glandular endometrial epithelial cell culture
PI	Propidium iodide
PKC	Protein kinase C
PRRS	Porcine reproductive and respiratory syndrome
PRRs	Pattern recognition receptors
PRRSV	Porcine reproductive and respiratory syndrome virus
PS	Phosphatidylserine
Q	Quadrant
RNA	Ribonucleic acid
ROCK	Rho-associated protein kinase
RSV	Respiratory syncytial virus
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction

SC	Syncytial cell
sec	Second
SEM	Standard error of mean
Sn	Sialoadhesin
$TCID_{100}$	100% tissue culture infectious dose
TER	Transepithelial electrical resistance
TJ	Tight junction
TLR	Toll-like receptor
TNF	Tumor necrosis factor
U	Unit
VC	Vacuole
ZO	Zonula occludens
Ω	ohm

CHAPTER I
INTRODUCTION

1.1 Background and rationale

Porcine reproductive and respiratory syndrome virus (PRRSV) is an epidemic disease leading to the economic loss worldwide. PRRSV is an enveloped, single-stranded positive sense RNA virus in *Arteriviridae* family (Snijder and Meulenberg, 1998). The virus causes pathogenesis in many ages of pigs, including fetuses, piglets, and growing pigs. The significant clinical signs of PRRSV infection are the severe respiratory infection such as pneumonia, but not the reproductive organ infection (Lunney et al., 2010). However, the PRRSV infected sows produced the weak-born piglet, slow-growth, abortion, and the surviving sows will be reservoir for transmitting the virus to other pigs repeatedly (Lunney et al., 2010). PRRSV was first discovered in Europe, and so-called EU strain or type 1 while PRRSV type 2 strain was eventually discovered in United State of America and named as US strain (type 2). However, the pathogenesis and virulence of PRRSV on the reproductive failure (i.e. infertility and abortion) have not been clearly indicated.

The control of PRRSV in the field using the modified live vaccine (MLV) or autogenous vaccine prepared from the homologous PRRSV to the field strain has been successful for the decrease of the respiratory infection and viremia. However, the stillborn fetus and weak-borne piglets is persisted and recirculated PRRSV to their herds. Unfortunately, the vaccinated gilts revealed a higher number of PRRSV-positive cells in the myometrium/endometrium than the unvaccinated gilts (Karniychuk et al., 2012). Since the mechanism of PRRSV-induced reproductive failure is poorly understood, the safety or the success of MLV for the treatment of reproductive organ

disorders is intrigued. PRRSV strain that has the ability to recirculate or cause the abnormalities of reproductive organs should be investigated.

In the previous studies, when PRRSV was inoculated to fetuses or pregnant sows, the infected fetuses and pregnant sows showed the viremia and PRRSV replication in the endometrium/fetal implantation sites with the apoptosis of surrounding cells at the late gestation. The apoptosis of endometrium/fetal placental cells were found. The mechanism is mediated by PRRSV in fetal blood circulation crossing the barrier to the maternal endometrium induced the apoptosis of fetal/maternal cells. It has been suggested that either the inflammation induced by PRRSV-infected macrophages via viremia or transplacental route causes the reproductive failures induced by PRRSV (Karniychuk and Nauwynck, 2009; Karniychuk and Nauwynck, 2013). Fundamentally, PRRSV is very restricted, or so called tropism, for some subsets of macrophages, which is a little subpopulation inside the endometrium (Van Gorp et al., 2008; Karniychuk and Nauwynck, 2009). Thus, the evidence has not been satisfied to explain the crucial role of PRRSV in replication and translocation between maternal and fetal membrane. Recently, our preliminary data have revealed the expression of PRRSV receptors, sialoadhesin and CD163, which allows PRRSV to infect and replicate in the porcine endometrial cell culture. It raised the possibility that the endometrium/placental tissues, which provide the nutrient and the defense mechanism for the mother and fetuses, may be the target of PRRSV infection.

Endometrium, the inner layer along the reproductive tract, is an implantation site of the fetus and significance for the development of the placenta. The endometrial epithelium has the polarity property; the apical site of epithelium contacts the uterine lumen; and the basolateral site locates closely to the blood circulation. Tight junction

proteins (TJs) are the membrane protein that play role in the epithelial polarity, barrier mechanism and ion selective permeability function. Claudins and zonula occludens 1 (ZO-1) complex proteins are the major TJs performing both the permeability to select the macromolecules and prevention from the pathogen invasion (Gaetje et al., 2008; Gunzel and Yu, 2013). The impairment of epithelium integrity, causing the increase in permeability and allowing the invasion of pathogens, is predominantly caused by the alteration of claudins and ZO-1 expression and function in the porcine endometrium (Anderson and Van Itallie, 2009; Kiatprasert et al., 2015). The interruption of these structures and functions, i.e. the loss of integrity or death of endometrial cells by PRRSV may contribute to the pathogenesis of PRRSV on the reproductive failures and weak-born piglets in herds.

Upon the infection, PRRSV initiates the synthesis of many structural and non-structural proteins (Nsp), which are the important factors that render them to replicate (Van Gorp et al., 2008; Karniychuk and Nauwynck, 2009; Music and Gagnon, 2010; Karniychuk and Nauwynck, 2013; Zhang and Yoo, 2015). Since the genotypes of PRRSV strain are different, the different virulence of PRRSV on the clinical signs has been indicated in many studies. In addition, the time-courses and route of infections have been suggested (Cano et al., 2009; Han et al., 2013; Linhares et al., 2015). However, up to date, the virulence of PRRSV relevant to reproductive disorders has never been discriminated. Moreover, the problems of reproductive failures relevant to different routes of infection, fetal/maternal route vs viremia in which PRRSV infected at apical vs basolateral side of the endometrial epithelium have not yet identified (Karniychuk et al., 2011). The use of inappropriate PRRSV strain for MLV or autogenous vaccine may be at risk.

For the viral infection, cell death induced by viral infection at the proper time is important for either combating or spreading of the virus in the host cells. By theory, cell death is classified to non-programmed (necrosis) and programmed cell death (apoptosis). PRRSV is capable to induce apoptosis in macrophages, endothelial and placental membrane of PRRSV-infected cells (Karniychuk et al., 2011; Wang et al., 2015). Additionally, *in vivo* studies, PRRSV has an ability to increase the histopathological lesions with the apoptosis at the maternal-fetal interface (Novakovic et al., 2016). *In vitro* studies have been indicated that PRRSV induced apoptosis by Nsps activating apoptotic mediating enzymes, caspase-3, -8 and -9 pathways (Ma et al., 2013; Yuan et al., 2016). Although there are studies about PRRSV induced the apoptosis in endometrial lining at the implantation site in pregnant sows (Karniychuk and Nauwynck, 2013), the examining of PRRSV affecting the immune cells has never been performed. That is because the interplay between endometrium/fetal membrane and surrounding immune cells is very complex and difficult to understand.

In the present study, we are attempting to demonstrate that the epithelial barrier and the replication of PRRSV of maternal tissues are associated with the loss in the integrity or death of maternal endometrial cells. PRRSV directly affecting the tight junction barrier system and inducing the death (necrosis/apoptosis) of primary cultured porcine endometrial epithelial cells will be emphasized. The comparison of the effects of type 1 vs. type 2 PRRSV strains via mucosal vs. blood-borne route of infection will be investigated in order to clarify the principal passage of PRRSV. Our information might elucidate the safety of MLV in gilts and pregnant sows, and valuable for the eradicating plan for PRRSV-induced reproductive disorders in herds. Although, the highly pathogenic PRRSV (HP-PRRSV) was recently discovered, it is dismissed from this study. Since the identification of the evolution of type 1 to HP-PRRSV is still controversial, as

well as the virulence of HP-PRRSV are greater than type 1 strain (Han et al., 2017), using and handling HP-PRRSV may be at risk.

1.2 Research Objectives

1. To determine the epithelial barrier properties and functions directly affected by PRRSV type 1 and type 2 inoculation via apical and basolateral routes in primary cultured porcine endometrial epithelial cells.

2. To determine the viability of epithelial cells induced by PRRSV type 1 and type 2 in primary cultured porcine endometrial epithelial cells.

1.3 Research Hypothesis

PRRSV type 1 and type 2 infections via in apical and basolateral routes change the tight junction protein expression and function relating to the increased permeability and affect the viability (proliferation or cell death) of primary cultured porcine endometrial epithelial cells. These alterations are associated with PRRSV-induced endometrial dysfunction.

1.4 Expected Benefits and Application

1. This research will provide the benefit of physiological change, including permeability of the porcine endometrium that are infected by PRRSV type 1 and type 2 in different routes.

2. This research will be advantage of understanding the pathogenesis of PRRSV affected viability or apoptosis in the primary cell of porcine endometrial epithelium.

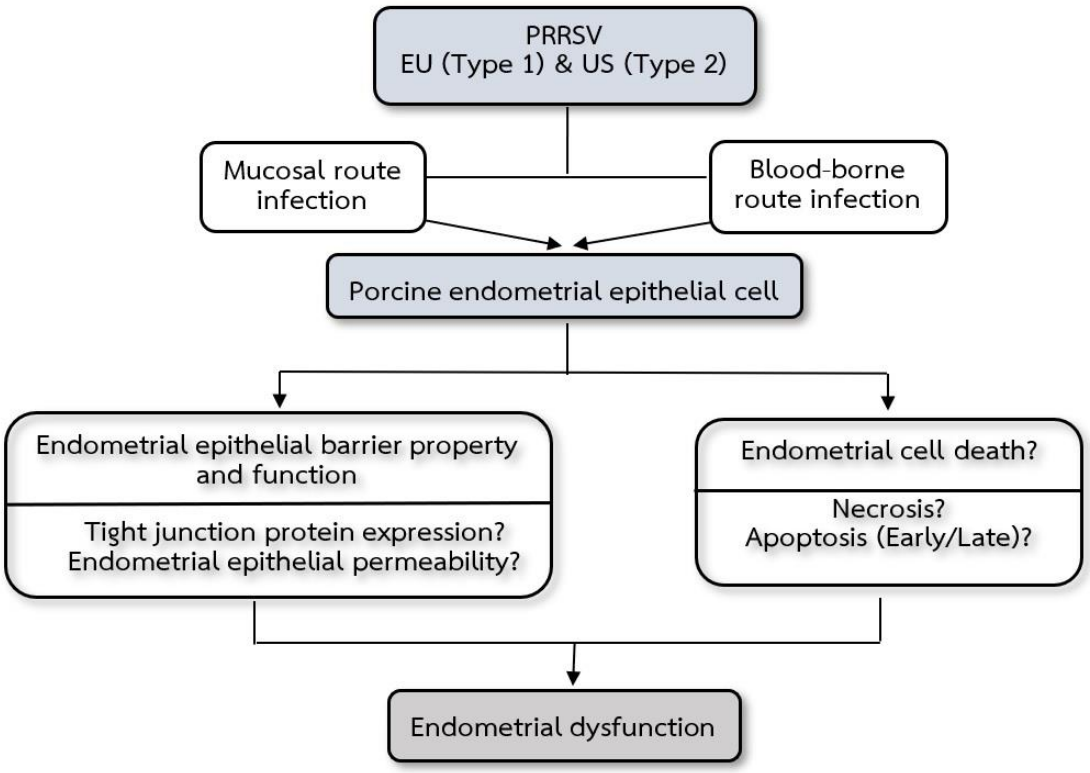
3. This research will be the basic information and a model to study PRRSV-infected PEG cell.

4. This research will be the useful data for awareness and controlling PRRSV in porcine reproductive system.

1.5 Places of study

Department of Veterinary Physiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand and Department of Physiology, Faculty of Medicine, Srinakharinwirot University, Bangkok, Thailand.

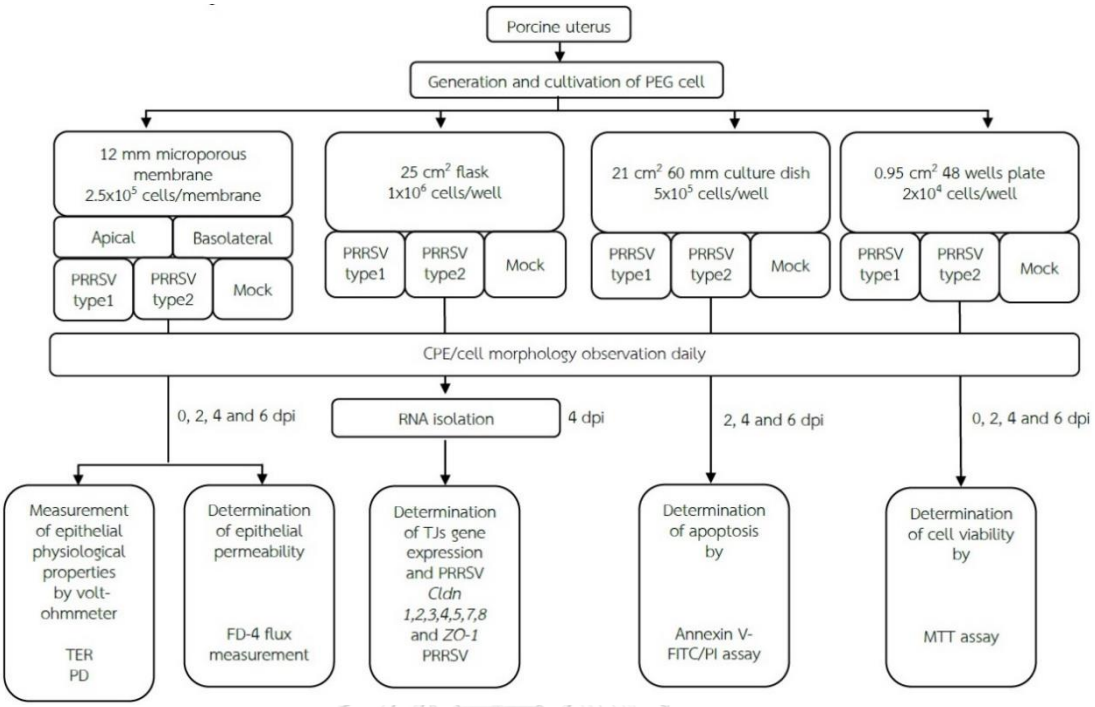
1.6 Conceptual Framework



1.7 Keywords

Cell death, Permeability, Porcine endometrial epithelial cells, PRRSV, Tight junction.

1.8 Research design



CHAPTER II

LITERATURE REVIEWS

2.1 Porcine uterus

The uterus is an important organ for fetal implantation, development and parturition. However, uterus lumen is opened and encountered to the invaded microorganisms, such as bacteria, fungi, protozoa and virus, that can cause infertility, abortion or systemic infectious diseases (Givens and Marley, 2008).

Porcine uterus is anatomically located at the bilateral uterine horns (bicornuate) starting from the cervix to fallopian tube. The uterine wall is histologically composed of 3 layers; the mucosal (endometrium), the muscle (myometrium) and the serosal (perimetrium) layers. The endometrium consists of 2 zones, (1) the superficial functional layer (*stratum funtionale*) lining with the simple columnar cell type, and (2) the deeper basal layer (*stratum basale*) that functions to support and restore the functional layer (Lorenzen et al., 2015). Basically, the epithelial cells lining endometrium are divided into luminal epithelium (LE) and glandular epithelium (GE), following the developmental processes. The development of uterine gland is called adenogenesis. Endometrial adenogenesis is initiated after birth, when LE develops proliferation and invaginates to the mesenchyme following with the LE differentiated to the uterine gland. GE will be developed into simple epithelial tubes which distribute among the stroma after birth. The endometrial glands and endometrial fold develop continuously under the regulation of sex steroids. However, the timing of uterine maturity which is capable to support the pregnancy differs among the species. For the porcine endometrium, it is fully developed at post-natal 120 days, suggesting that maturity of porcine uterine wall is entirely age of puberty (Cooke et al., 2013). The existence and function of LE and GE are essential for the accomplishment of the

pregnancy, including the preparation for implantation, maintenance of pregnancy and parturition (Filant and Spencer, 2014). It has been suggested that endometrial glands are required for establishment and maintenance of pregnancy, since the supplies of conceptus and fetal development are directly from uterine blood or biosynthetic activity of uterine endometrium (Cooke et al., 2013). Alteration or dysfunction of endometrial secretion may account for the failure of pregnancy or fetal development. Besides the physiological function in reproductive events, the endometrium plays an important role in the innate immunity, including a tight junction barrier, a mucus lining, the secretion of antimicrobial peptides and cytokines (Hickey et al., 2011). The impairment of the endometrium may cause the pathogen invasion leading to reproductive organs disorders including infertility.

To date, the primary cells have been used to replace the animal used protocol. The primary cells of the porcine glandular epithelium have been previously performed as a good glandular endometrial model for reproductive physiology and pathophysiology (Vetter et al., 1997; Deachapunya and O'Grady, 1998; Deachapunya et al., 1999; Deachapunya and O'Grady, 2001; Palmer-Densmore et al., 2002; Deachapunya et al., 2008). Porcine glandular endometrial epithelial cells (PEG cells) are isolated from porcine uterus. The important characteristics of PEG cell consist of cytokeratin positive cell and tight junction protein, which function as similar to those in the intact uterus (Deachapunya et al., 2008; Kiatprasert et al., 2015; Srisomboon et al., 2017). The confluent cells are monolayer classified as tight epithelium that had the maximal transepithelial electrical resistance up to 2870 ± 54 Ω cm^2. However, the paracellular permeability of PEG cells can respond and be regulated by many factors such as endotoxin, viral nucleic acid and sex steroids (Deachapunya et al., 2008; Kiatprasert et al., 2015; Srisomboon et al., 2017). In addition to the paracellular transport, PEG cell has the transcellular transport that is significance for pregnancy

receptivity and the malnutrition of the fetus, i.e. chloride, and sodium and nutrient absorption and secretion (Deachapunya and O'Grady, 1998; Deachapunya et al., 2008).

2.2 Innate immunity of porcine endometrium

The innate immune system is the non-specific immune system that initiates the response to microbes. The function of innate immunity consists of prevention, control and elimination of the pathogens. The innate immune effector cells and molecules are fully functional before infection; therefore, they can immediately respond to microbes. The cells that are responsibility in the innate immune system include neutrophils, macrophages, dendritic cells, natural killer cells, mast cells and eosinophils. The essential role of innate immunity is to combat the opportunistic invasion of virus, bacteria and parasite.

In addition, the physical barriers as the first line mechanism against the pathogens are important innate immune system. The physical barriers, especially an epithelium, provide the selective permeable and preventive functions. Each epithelial cell contacts together with many tight junction proteins (Van Itallie and Anderson, 2013). Moreover, the loss of epithelial integrity, leading to the increased paracellular transport has been indicated to affect to the physiological function of transepithelial secretion and absorption in many organs (Frizzell and Hanrahan, 2012). As aforementioned, endometrial transport dysfunction may be relevant to inadequate nutritional supply to the fetus and may be the cause of stillborn or weak-borne offspring.

2.3 Tight junction proteins

In general, tight junction (TJ) are the intercellular junctional complex of proteins which interact together or with other proteins. The porcine endometrium is formed by the single layer of columnar cell produced by GE. The space between cells is adhered by epithelial junctional complex consisting of TJ proteins, adherens junction and desmosome. These complexes perform to be the fence and barrier. The paracellular transports are worked by the structure near the apical surface, mainly TJ (Markov et al., 2017). An important function of TJ is the maintenance of barrier integrity. The alteration of TJ protein expression has been indicated to be a part of the pathogenesis of many diseases (Guttman and Finlay, 2009). In the tight junction complex, claudins are the critical sealing proteins, and act as the backbone of TJ (Fig. 1). The important proteins that interact with claudins are zonular occludens (ZO) which are built of the scaffolding structure. The carboxyl terminus of claudin anchors to bind the first PDZ-domain of ZO-1 and connect to ZO-2 and ZO-3 proteins (Fig. 1) (Ulluwishewa et al., 2011). ZO-1 is capable to regulate many transcription factors. However, the *ZO-1* expression is provided an inverse result with the cell proliferation in the cancerous study (Gonzalez-Mariscal et al., 2014) suggesting that the appropriate numbers of TJ protein of ZO-1 may be significant.

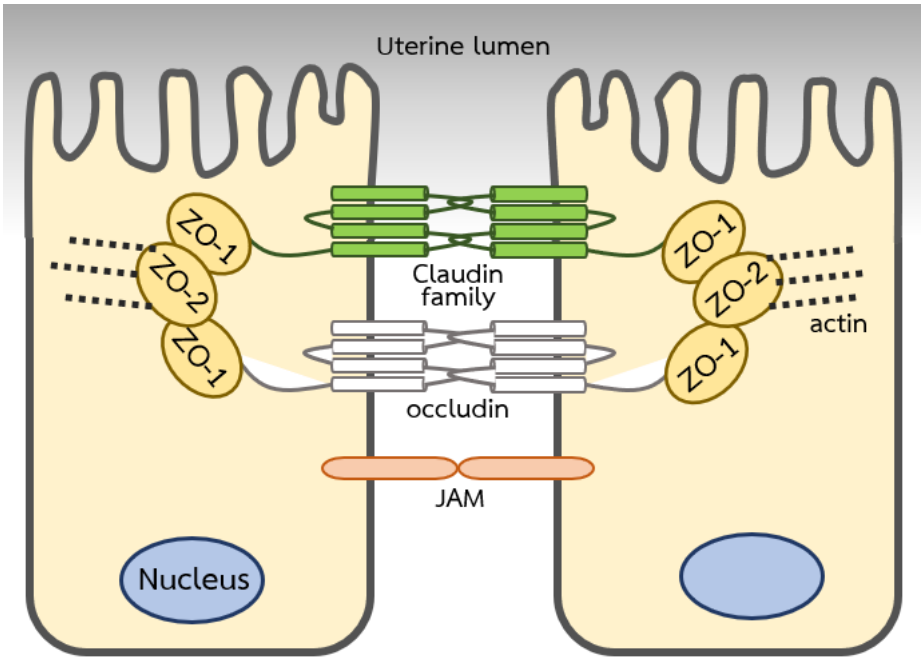


Figure 1 The model shows the protein components of tight junction proteins of endometrial epithelial cell. Tight junction consists of three classes of transmembrane proteins; claudin, occludin and JAM. Claudin and occludin attach to plaque proteins, ZO-1 and ZO-2 that linked to actin filaments (Reviewed by Ulluwishewa et al., 2011).

Up to date, the number of different claudin proteins have been classified. The different epithelium appears to express multiple and different types of claudins (Gunzel and Yu, 2013). Human reproductive tracts express *Cldn1, 3, 4, 5* and *7* mRNA (Gaetje et al., 2008). The expression of claudin-1, -2, -3 and -4 proteins in the porcine endometrium are dynamic by the estrus cycle under the regulation of the sex steroid hormones (Bailey et al., 2010). *In vitro* study of the immortalized porcine glandular endometrial epithelial cell culture (PEG) revealed the mRNA expression of claudin-1, -3, -4, -7 and -8 (Kiatprasert et al., 2015). Generally, claudin-1, -3, -4 and -8 are the barrier builder type of TJ. Claudin-2 and -7 are the pore-forming proteins which are responsible for cation and anion permeable selectivity, respectively. Claudin-5

performs the function as the blood brain barrier in an endothelial cell, but the function in other epithelial tissues has remained unclear (Gunzel and Yu, 2013). The changes of TJ protein expression, which particular is the decrease of barrier builder or the increase of pore-forming TJ protein, leading to the increase in epithelial permeability in many organs including the endometrium (Gunzel and Yu, 2013; Kiatprasert et al., 2015).

In general, the paracellular spaces are selectively permeable to ions, nutrient, substance and pathogen. The selectivity depends on the functions of the TJ protein complex. The TJ pore sizes are ~4-8 Å in radius. It functions to regulate the paracellular permeability of epithelia. TJ pore sizes vary in the types of tissue, and their molecular components are also different (Zihni et al., 2016). In order to determine the TJ function, the measurement of transepithelial electrical resistance (TER) and solute flux permeability can be performed in both *in vitro* study of the stripped mucosal tissues and monolayer epithelial cell culture, and *in vivo* model. However, unlike the stripped tissue or *in vivo* model, the solute small molecules and electrical currents can pass the isolated cell monolayer, which can be monitored well without the obstacle of the other supporting cells or muscle layers. The increase in paracellular permeability associated with low TER and high solute flux reflects the loss of tightness of epithelium or the leakage of TJ. The phenomena are so called “leaky epithelia” which may be characteristics of epithelium in either normal or abnormal physiological functions (Anderson and Van Itallie, 2009). However, the leaky epithelia may be associated with the invasion of pathogens leading to the consequence of the systemic infection.

Many pathogens can disrupt the TJs, leading to an increase in paracellular permeability and polarity defects. The mechanism of TJs disruption is not only mediated by the damage of TJs, but also altering of TJs types or composition ratio. Enterohemorrhagic *Escherichia coli* (EHEC), an enteric pathogen, during the infection

can increase claudin-2 expression in *in vivo* model of C57B1/6J mice. The augmentation of *Cldn2* gene expression was proposed as a mechanism to the decrease of TER in the colon (Roxas et al., 2010). *Clostridium perfringens*, the cause of food poisoning associated with leaky epithelia, release the *C. perfringens* enterotoxin (CPE) that can decrease the claudin-4 of TJs complex in infected Madin-Darby canine kidney I (MDCK) cells (Sonoda et al., 1999). For the viral infection, respiratory syncytial virus (RSV), the serious pediatric respiratory tract disease in infants, considerably affects at the TJs by up-regulating the barrier builder TJ protein gene expressions. The RSV induced *occludin*, *Cldn4 and ZO-1* expression in live human nasal epithelial cells (hNECs) producing the higher polarity of alveolar cells than natural cells (Tsutsumi et al., 2011). The increased TER in the RSV-infected cells facilitate cellular secretion of propagating RSV, which is leading to viral spreading. In general, tight junctions seem to efficiently restrict most the bacteria, however they could not limit the viral particles from penetrating into deeper tissues. Unfortunately, some pathogens have developed specific strategies, which is possibly the cellular or immunological responses of host cells, i.e. the induction of tight junction protein expression. The alteration of mismatch or imbalance of TJ protein types may disrupt these TJs structures. This phenomena may be part of the pathogenesis of viral infection causing organ disorders and allowing themselves viral particles and the other pathogen invading to the systemic circulation (Guttman and Finlay, 2009). Focusing on the reproductive organs infection, human immunodeficiency virus (HIV) directly decreases the TER of the female reproductive tract by disturbing the TJ proteins. The depletions of *Cldn2* and *ZO-1* mRNA expression are related to this phenomenon (Lu et al., 2014). Interestingly, the change of TER and FITC dextran-4 (FD-4) flux has been reported with PEG treated which lipopolysaccharide (LPS) in our previous study (Kiatprasert et al., 2015). Although LPS does not penetrate the cells, it can reduce the TER after incubation for 48 h. This result

is correlated with down-regulation of *Cldn8* and up-regulation of *Cldn7* and *ZO-1* mRNA expression (Kiatprasert et al., 2015). In addition, PRRSV inoculation affecting the porcine endometrial permeability and TJs has not been studied. Meanwhile, TER and FD-4 flux are the reliable model for the study of epithelial permeability. Therefore, *in vitro* model of primary porcine endometrial cell which is novel and may be challenged to clarify the PRRSV related reproductive disorders.

2.4 PRRSV and their pathogenesis

PRRS is an epidemic disease leading to economic loss worldwide. The virus can cause pathogenesis in many ages of pigs as well as the fetus, piglet, and growing. The clinical signs of PRRSV infection consist of pneumonia, weak-born piglet, slow-growth, and abortion. PRRSV was firstly discovered in Europe called EU strain (type 1, EU) and later in United States of America named US strain (type 2, NA). Type 1 and type 2 of PRRSV share 50-60% sequence identity (Dokland, 2010). Although type 1 and type 2 viruses present similar clinical signs, type 2 strain has more virulence in respiratory distress than type 1 strain. In addition, type 2 PRRSV is accomplished to shed the virus titer for longer periods than type 1 infected-pigs (Han et al., 2013). However, the surviving swine will be a reservoir and transmits virus to other pigs repeatedly.

The etiology of PRRS is porcine reproductive and respiratory syndrome virus (PRRSV), which is an enveloped, single-stranded positive sense RNA virus in *Arteriviridae* family, order *Nidovirales*. PRRSV RNA genome consists of at least 9 open reading frames (ORFs) which encode structural proteins and non-structural proteins (Nsps). Structural proteins contain glycoproteins (GPs) and unglycosylated membrane proteins (M, N, E) which play important role in the viral structure, host cell infection and immunogenicity (Fig. 2) (Music and Gagnon, 2010).

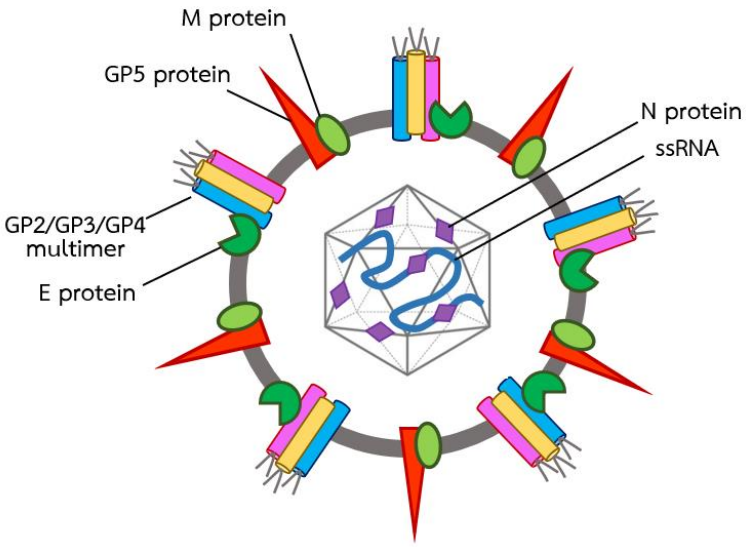


Figure 2 The model shows the structure of PRRSV particle. PRRSV structure proteins consist of M/GP5 heterodimer and GP2/GP3/GP4/E multimer at the viral envelop. PRRSV nucleic acid (ssRNA) is inside and surrounded by N protein (Reviewed by Music and Gagnon, 2010).

Since ORF1a and ORF1b on the PRRSV RNA encoding Nsps had been detected, other ORFs encoding both structural and nonstructural proteins was also indicated. Both structural and non-structural protein encoded by different ORFs had specific roles for surviving and replication of PRRSV in host cells (Music and Gagnon, 2010). Moreover, the homology and heterology of Nsps transcribed by different PRRSV strain are important for virulent and pathogenesis of PRRSV infection (Music and Gagnon, 2010). In general, varieties of Nsps are synthesized when the virus is replicated. Nsp1 has been reported to inhibit the phosphorylation of IκB and consequence suppressed the activation NF-κB leading to suppress interferon (IFN) production in PRRSV-infected macrophages (Song et al., 2010). At least three nonstructural proteins, Nsp1, Nsp2, and Nsp11, have been identified to play roles in the immunosuppression by suppressing

the IFN-γ production or inducing the interleukin (IL) -1 blockers, IL-1R (Sun et al., 2012). In the enterocytes, Nsp4 recognized as the viral enterotoxin of rotavirus induces the release of intracellular calcium and disrupts TJ resulting in paracellular leakage (Parashar et al., 2013).

PRRSV was shown to entry and replicate in the nasal epithelial cell explants at 72 hours post inoculation (hpi) via the PRRSV tropism cells founded at airway epithelia. However the epithelial cell barrier of the nasal mucosa seems to be resistant to type 1 strain, but not type 3 (highly-virulent strain) (Frydas et al., 2013). Recently, porcine endometrial cells revealing PRRSV receptors and serving as PRRSV cell tropism may be major target for the pathogenesis of reproductive disorders in PRRSV. The reproductive epithelial lining necrosis, damages and dysfunction in PRRSV-infected sows could not successfully explained, although the reproductive system is also a target of PRRSV infection. However, the effects of PRRSV had been indicated at the implantation site which consists of fetal/maternal membrane (Karniychuk and Nauwynck, 2013). The fetal death caused by PRRSV infection had been indicated to be relevant to virus-induced lesion, i.e. apoptosis, at maternal endometrium and fetal membranes (Karniychuk et al., 2011). However, the apoptotic or necrotic cells could not be successfully followed in pregnant sows.

2.5 Pathogenesis of PRRSV on cell death and apoptosis

Cell death is an essential mechanism that can control the homeostasis of cells. It can be categorized to non-programmed and programmed cell death. The well-known non-programmed cell death is necrosis, in which the cellular content is uncontrolled releasing and leading to death of cells. In contrast, programmed cell deaths consist of various stages (Fink and Cookson, 2005).

Generally, apoptosis is an active programmed process of the cells, which eliminate unwanted cells following the physiological function or pathogen-induced condition. The characteristics of apoptosis are nuclear condensation and the fragmentation of cell membranes. The fragmented apoptotic cells, called apoptotic bodies, will be further degraded by phagosomes. These processes occur without an inflammation. However, the materials from degradation and phagocytosis can be recycled (Fink and Cookson, 2005). The pathways of apoptosis have been categorized to 2 pathways; extrinsic pathway and intrinsic pathway. The extrinsic pathway is initiated by death receptor on cell surface bound to a soluble ligand, such as tumor necrosis factor (TNF) and Fas ligand (FasL) (Gupta et al., 2006). The downstream regulation of the extrinsic pathway is stimulated by caspase-8. The intrinsic pathway or mitochondrial-dependent pathway is activated by mitochondrial breakdown. These processes release cytochrome c which turns out to stimulate the activation of caspase-9. Finally, the activated caspase-8 and caspase-9 from both pathways will activate the caspase-3 enzyme which has the activity to degrade DNA fragmentation or organelles (Miller and Fox, 2004). Basically, apoptosis is divided into 2 phases consist of early and late, which can be determined by the changes of plasma membrane property. The early phase of apoptosis is changed by translocation of phosphatidylserine (PS) from the inner side of the membrane lipid bilayer to outer surface side, but the membrane integrity is still stable. At this stage, the cell is viable, even though DNA breakdown is initially presented. Apoptosis is further proceeded with increased in membrane permeability. The macromolecules can pass through the membrane of cells at the late phase. This stage is classified as late apoptosis or alternating necrosis, in which the cell character is difficulty to differentiate (Sgonc and Gruber, 1998). However, necrosis or non-programed cell death can be confirmed by many assays, including MTT. Meanwhile, the early stage of apoptosis should be discriminated and revealed, since

the cells in this stage are unhealthy and proceed to death. The common method for detecting the early stage of apoptosis is Annexin V-conjugated fluorochrome (Annexin V-FITC; AV) which can bind to PS present at the outer layer of membrane lipid bilayers. The combination used of Annexin V-FITC with propidium iodide (PI) could discriminate the early stage apoptosis from the late stage apoptosis when cells are stained with Annexin V-FITC without penetration of propidium iodide (PI) to the nucleus (Sgonc and Gruber, 1998). This assay has been used to detect apoptosis induced by many pathogens, including virus-induced disease (Clarke and Tyler, 2009). However, this assay could not distinguish cell undergone apoptosis from necrosis, since their cell membrane are loss of integrity, allowing Annexin V-FITC and PI to penetrate.

There are many pathogens, such as virus, bacteria, fungi and parasites that induce apoptosis in the host cells. Virus is the important pathogen that habitually induce apoptosis (Clarke and Tyler, 2009) in both *in vitro* and *in vivo* (Miller and Fox, 2004). Apoptosis induced by PRRSV is found in many organs such as lung, lymphoid tissue, and implantation site of the placenta (Sur et al., 1998; Karniychuk et al., 2011). Moreover, each strain of PRRSV is capable to induce apoptosis in different site of inoculation; for example, PRRSV type 1 can induce apoptosis in fetal implantation site of the placenta (Karniychuk et al., 2011). However, apoptosis of infected piglet thymus was predominantly induced by PRRSV type 2 (Wang et al., 2015). For the mechanism of apoptosis induced by PRRSV, it has not been yet clarified because PRRSV has many compositions that can cause the apoptosis of host cells. One of suspected factors is the major envelope protein of PRRSV, the GP5, in particular. GP5 has been reported that can persuade the apoptosis phenomenon (Fernandez et al., 2002; Music and Gagnon, 2010). However, the present study argued that GP5 only mediated the invasion and recognition of the host cell, but not associated with apoptosis (Ma et al., 2013). In contrast, the non-structural proteins (Nsps), including Nsp4 and Nsp10, are one of the

crucial constituents that induce apoptosis in infected cells (Ma et al., 2013; Yuan et al., 2016). Nevertheless, PRRSV-induced apoptosis is an interesting topic to investigate, especially in endometrium. There are a few reports that provide the information about PRRSV induced apoptosis in macrophages, endothelial and endometrium at the implantation site (Karniychuk et al., 2011). Even though, at the feto/placental sites, only 1-3 apoptotic cells were detected in the connective tissues which is a distance from the maternal endometrium (Karniychuk et al., 2011). The evidence could not be satisfying for the account of the pathogenesis of PRRSV on the reproductive disorders. It intrigues us to find out whether the apoptosis in various stages, including necrosis in endometrium can be induced by PRRSV. It may be the cause of reproductive problems induced by PRRSV, i.e. late term abortion, stillborn and weak-borne piglets, if the death of PEG is widespread. However, it is hardly detected in the *in vivo* because of the complexity of organs.

CHAPTER III
MATERIALS AND METHODS

3.1 Materials

All cell culture containers, including 48-, and 96-well plate of transwell microporous filters and 60 and 100 mm cell culture dishes, were obtained from Costar (Corning, MA, USA). All cell culture media and supplements, including Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), collagenase (type 1), 0.25% trypsin-0.53 mM ethylenediaminetetraacetic acid (EDTA), kanamycin, penicillin-streptomycin and fungizone were purchased from Gibco (Grand Island, NY, USA). All high purity grade salts, insulin, non-essential amino acid, L-glutamine, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and FD-4 (FITC-Dextran; MW=4 kDa) were purchased from Sigma Chemical Co., (St Louis, MO, USA). TRIzol® reagent and diethylpyrocarbonate (DEPC) were purchased from Invitrogen™, Thermo Fisher scientific, CA, USA. cDNA synthesis kit was purchased from Biorad (iScript, Biorad, CA, USA). The qPCR kit was purchased from Primer design (Primer design, Chandler's Ford, UK). The commercial multiplex PCR kit for contaminated samples were purchased from Qiagen (Qiagen, Leipzig, Germany). The specific primer sets were designed and purchased from Biosearch Technologies (Biosearch Technologies, CA, USA) for *Cldn1, 3, 4, 7, 8* and Lab Eurofins (Operon, Eurofins, Hvidovre, Denmark) for *Cldn2, 5* and Lab iScience for *ZO-1* (iScience Technology, Bangkok, Thailand). The apoptosis kits including of Annexin V FITC and PI were obtained from ImmunoTools (Gladiolenweg 2, Friesoythe, Germany).

3.2 Porcine glandular endometrial epithelial cell (PEG) isolation

The 4-6 months old gilts (n=7 pigs), were euthanized at the slaughterhouse in Klong Toey, Bangkok, Thailand, in member countries of the Animal Production and Health Commission for Asia and the Pacific (APHCA). Porcine uteruses were immediately removed and preserved in porcine ringer's solution (concentration in mM; 130 NaCl, 6 KCl, 3 $CaCl_2$, 0.7 $MgCl_2$, 20 $NaHCO_3$, 0.3 NaH_2PO_4, 1.3 Na_2HPO_4, pH 7.4 and 4ºC) during transportation. The PEG isolation protocol of Deachapunya and O'Grady was used in this experiment (Deachapunya and O'Grady, 1998). Briefly, the uterus was washed in Ca^{2+} and Mg^{2+} free phosphate buffer saline (PBS) containing 100 µg/ml kanamycin sulfate, 100 U/ml penicillin and 100 mg/ml streptomycin. After the muscle layer was stripped out, the mucosa was minced and digested by collagenase type 1 in Dulbecco's Modified Eagle Medium (DMEM) for 24 h in 5% CO_2 at 37ºC. The uterine gland was isolated from the digested tissue by a 250 µm pore size mesh filter. The purification of the uterine glandular endometrial cells from surface endometrial cells were isolated by size using the gravitational sedimentation technique. Endometrium contaminated *Mycoplasma spp.*, swine fever or PRRSV were observed and excluded by multiplex qRT-PCR detection kit (Microplasma 16s Ribosomal RNA Gene genesig® Standard kit, Primerdesign, Camberley, UK; Virotype® CSFV RT-PCR kit, QIAGEN, Leipzig, Germany; Virotype® PRRSV RT-PCR kit, QIAGEN, Leipzig, Germany). The uterine glands were plated in 100 mm culture dish and incubated in growing media with 10% fetal bovine serum (FBS) in DMEM with 100 µg/ml kanamycin sulfate, 100 U/ml penicillin and 100 mg/ml streptomycin at 5% CO_2, 37ºC until 90% confluent. The growing PEG cells were counted and seeded in appropriate containers for each objective of the study. For all studies, PEG cell was maintenance in 5% FBS DMEM with 100 µg/ml kanamycin sulfate, 100 U/ml penicillin and 100 mg/ml streptomycin throughout the experiments. The refreshed medium was changed in every 2 days until confluent

(approximately 4-6 days). Upon the confluents, PEG cells were dissociated from cell culture dish with 0.25% trypsin with 1 mM EDTA (0.25% trypsin/EDTA), and sub-cultured to the appropriate cell culture plate for each experiment. Briefly, PEG cells plated in 12 mm microporous filtered membranes were infected with PRRSV in either apical or basolateral side, and subsequently performed the measurement of electrical parameters and the permeability test. PEG cells growing in the 25 cm^2 culture flask were used for the evaluation of the tight junction mRNA expression. The 48 well (0.95 cm^2) plate was used to maintain cells for viability assay. Apoptosis assay was determined in PEG cells seeded in 60 mm (21 cm^2) culture dish. In each experiment, PEG cell plates were randomized into 3 groups; PRRSV type 1 inoculation, PRRSV type 2 inoculation, and mock inoculation (Mock).

3.3 PRRSV isolation and inoculation in PEG cell

After the lesion in the lungs of carcasses confirmed by the pathologist, type 1 and type 2 PRRSV were isolated from the lung of dead pigs. The confirmation and discrimination of type 1 vs. type 2 were finally courtesy performed and reported by Dr. Suphot Watanapansak's laboratory at Farm Animal Hospital, Faculty of Veterinary Science, Nakorn Pathom using qRT-PCR. In addition, the field contamination of PRRSV, swine fever and *Mycoplasma spp* was checked by commercial multiplex qRT-PCR kit as aforementioned test for PEG isolation. The lungs that positive to any contaminant were excluded from these experiments. For the PRRSV isolated in this study, the modified protocol was performed following Meng and colleagues (Meng et al., 1996). Lung with the lesion of PRRSV was weighed about 2.3 grams and incubated in cold DMEM 15 ml. PRRSV-infected lung was homogenized to liberate PRRSV to the medium. The homogenized tissue was centrifuged at 10,000 g at 4°C for 10 minutes 2 times. In

order to avoid bacterial contamination, supernatant was sieved through 0.2 μm filter. Then 15 ml of DMEM was added to filtered supernatant containing PRRSV. The volume of PRRSV infected media was primarily calculated by examining in the standard PRRSV target cells, the derived monkey kidney MARC-145 cell line. The volume at the concentration of PRRSV at $TCID_{100}$/2ml were infected to PEG cells. PEG cells were incubated with the viral infected media for 1 h at 5% CO_2, 37°C. Subsequently, PEG cell was washed and incubated with the fresh maintaining media. During the experiment, all PEG cells were observed for cell morphology/cytopathic effect (CPE) daily and refreshed media every 48 h.

3.4 Determination of PRRSV infection in PEG cells

To determine the PEG cells infected by PRRSV, PRRSV protein expression was revealed by immunohistochemistry (IHC) in PEG cells paralleled with the PRRSV target cell MARC-145. Polyclonal rabbit-anti-PRRSV GP5 antibody (Biorbyt Ltd., Cambridge, UK) was used as a primary antibody. Followed the manufacturer's protocol, the cell culture microporous membranes were removed from chamber at 2 dpi, washed with 0.1% tween in PBS and immersed in 4% paraformaldehyde in PBS pH 7.4 for 24 h. After removing and washing out the fixing solution with PBS, fixed membrane was incubated with primary antibody at dilution 1:100 in dissolving solution 0.1% tween in PBS at the 4°C for 4 h. The membranes were washed with 0.1% tween in PBS and proceeded for HRP-streptavidin secondary antibodies, using mouse-anti-rabbit IgG (Vectastain, Vector laboratories, Inc., CA, USA; dilution 1:2000) for 1 h at room temperature. Then conjugated HRP was added and incubated 30 mins at room temperature. Finally, they were stained with DAB substrate and counter stained with hematoxillin (Histostain-SP, Invitrogen, CA, USA). The processed sample was mounted on the slide and sealed

cover slip with modified 10% glycerol in PBS using as the mounting solution (Glycerol, Invitrogen, CA, USA). PRRSV GP5 positive cells, which were golden brownish color were observed under the light microscope digital camera (BX50F and UC50, Olympus, Tokyo, Japan). The positive result was examined in all inoculated PEG experiments compared to MARC-145. The negative result was also performed without primary antibody incubation in the same sample.

3.5 Measurement of epithelial physiological properties

After PEG cells at 2.5 x 10^5 cells were seeded and maintained on 12 mm transwell for 7 days. Before PRRSV inoculation, transepithelial electrical resistance (TER) and potential difference (PD) was measured by the volt-ohmmeter coupled to Ag-AgCl chopstick electrode (Millicell®ERS-2 Voltohmmeter, Merck KGaA, Darmstadt, Germany) daily. PEG cells expressing TER about 1,400-1,800 Ω.cm^2 were considered confluent and chosen for inoculation. The TER that measured before PRRSV inoculation would be considered as 0 days post inoculation (dpi) or initial TER. After PRRSV media was inoculated to PEG cells, TER and PD were measured at 2, 4 or 6 dpi.

As the initial TER from each pig were variety, the data were normalized with the percent changes of TER at observed day from the initial TER (at 0 dpi) using the equation (1):

$$\% \text{ changes of TER} = \frac{(\text{TER at observed dpi} - \text{TER at 0 dpi})}{\text{TER at 0 dpi}} \times 100$$

3.6 Measurement of epithelial permeability

At the 2, 4 and 6 dpi, PEG cells were proceeded for epithelial permeability after the measurement of TER and PD. The apical to basolateral transport of fluorescein isothiocyanate dextran MW= 4 kDa (FD-4) across the PEG cell monolayer was assessed. Before adding the FD-4, PEG cell in 12 mm transwell was incubated with 1 ml of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer saline solution (containing of 25 mM HEPES, 120 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 25 mM $NaHCO_3$, 15 mM glucose), pH 7.4 at the basolateral side. The apical side was then incubated with FD-4 at 1 mg/ml, 0.5 ml in pH 7.4 HEPES buffer saline solution in a dark, 37°C, 5% CO_2. After 24 h of incubation, media from both compartments were collected, transferred and measured for the amount of FD-4 using a fluorescence 96-wells plate reader (Synergy^{TM} HT, BioTek, VM, USA). The excitation and emission wavelengths respectively at 485 and 528 nm, which are specific for fluorescein isothiocyanate, were respectively applied. All experiments were done in triplicate at 37°C. The apparent permeability coefficient (P_{app}; $h^{-1}.mm^{-2}$) reflecting cumulative transport of the amount of FD-4 from apical side to basolateral side was calculated according to the following equation:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A \times C_0}$$

where dQ/dt (mg/h) is the rate of appearance of FD-4 on the basolateral side, A is the surface area of the monolayers (mm^2), and C_0 (mg/ml) is the initial concentration of fluorescent applied on the apical side.

3.7 Measurement of tight junction protein gene expression

3.7.1 Total RNA isolation

PRRSV-infected PEG cell at $1x10^6$ cells in 25 cm^2 flask was collected at 4 dpi. The manufacturer's instruction of TRIzol® reagent was performed for RNA isolation. Briefly, 500 µl TRIzol® reagent was added in each flask for cell lysis. Chloroform at the volume of 100 µl was added. The separation of nucleic acid from protein contaminant centrifuged at 12,000 g, 4°C for 15 min (Micro Centaur Plus, MSE, London, UK). The upper layer containing nucleic acid was collected and precipitated with 250 µl of 100% isopropanol. After centrifugation, the RNA pellet was washed with 75% ethanol in diethylpyrocarbonate (DEPC). Finally, the dry pellet was diluted to 10:1 (TRIzol®: RNase free water). The RNA concentration was measured by NanoDrop (NanoDrop™, Thermo Fisher scientific, MA, USA). The purity of RNA was concerned with the OD_{260}/OD_{280} ratio that should be between 1.8-2.0.

3.7.2 cDNA synthesis

cDNA synthesis kit (iScript, Biorad, CA, USA) was used to convert RNA following to manufacturer's protocol. Briefly, 3 µg of RNA was mixed with *cDNA* synthesis kit reagents and converted using Thermocycler (Biometra® T-Gradient Thermoblock Biometra, Jena, Germany). The reaction was transformed to cDNA using the following cycle 25°C 3 min, 46°C 20 min, and 95°C 1 min. cDNA concentration was determined for assurance by NanoDrop (NanoDrop™, Thermo Fisher scientific, MA, USA). The purity of cDNA products was performed with the OD_{260}/OD_{280} ratio that should be about >2.0.

3.7.3 qRT-PCR analysis

Quantitative real time polymerase chain reaction (qRT-PCR) was applied by Thermocycler (CFX96^{TM}, Biorad, CA, USA) to measure the mRNA expression of tight junction protein. The mRNA expression tight junction protein consisting of *Cldn1, 2, 3, 4, 5, 7, 8 and ZO-1* was investigated using SYBR green based qPCR kit, qRT-PCR kit reagent (Precision FAST 2x MasterMix, Primerdesign, Chandler's Ford, UK). The specific primer sets used in the present study were designed by Biosearch Technologies (Biosearch Technologies, CA, USA) for *Cldn1, 3, 4, 7, 8* and Lab Eurofins (Operon, Eurofins, Hvidovre, Denmark) for *Cldn2, 5* and Lab iScience for *ZO-1* (iScience Technology, Bangkok, Thailand) (Table 1). Followed the manufacturer's protocol, 3 µg of cDNA was mixed with the specific primer and the qRT-PCR kit reagent. The reaction was set up for denaturing at 95°C 20 sec, annealing at 60°C 30 sec and extension at 72°C 20 sec in order. The PCR reaction was amplified for 40 cycles totally. Melting curve analysis and 1.5% agarose gel electrophoresis were performed to confirm the specificity of the amplification products. The numbers of cycle that start detecting the real-time signal (SYBR green) of each PCR samples, the threshold cycle (C_t), were recorded. The calculation of *Cldn* gene expression products was normalized with the endogenous control porcine glyceraldehyde 3-phosphate dehydrogenase (porcine *GAPDH*) gene expression using the equation $2^{-\Delta Ct}$, where the ΔC_t is calculated by following equation;

$$\Delta C_t = C_t \text{ of } Cldn \text{ gene} - C_t \text{ of } GAPDH \text{ gene}$$

The alteration of *Cldn* gene expression induced by PRRSV was calculated as fold changes from the mock group using a modification of $2^{-\Delta\Delta Ct}$ equation (Livak and Schmittgen, 2001), where the different ΔC_t of gene is calculated by following equation;

$$\Delta\Delta C_t = \Delta C_t \text{ of PRRSV inoculation} - \Delta C_t \text{ of Mock}$$

Table 1 Sequence of specific primer sets of porcine tight junction protein and control genes.

Gene	Primer sequences (5' -> 3')	Accession number	Product size (bp)
Cldn1	F: CCCGGTCAATGCCAGATATG R: CACCTCCCAGAAGGCAGAGA	NM_001161635.1	80
Cldn2	F: CTCCCTGATAGCTGGGATCATC R: CCTGATAGGCATCGTAGTAGTTGGA	NM_001161638.1	80
Cldn3	F: CATTATCACAGCGCGGATCA R: CGTACACTTTGCACTGCATCT	NM_001160075.1	86
Cldn4	F: GTGTAAGGTGCTACCGCTGATTC R: AGGGCCATTCTGGAGTCACA	NM_001161637.1	80
Cldn5	F: ACCGGCGACTACGACAAGAA R: GCCCTCCAAAGCGGAGTT	NM_001161636.1	93
Cldn7	F: CCATGACTGGAGGCATCATTT R: GACAATCTGGTGGCCATACCA	NM_001160076.1	80
Cldn8	F: TGGGAAGGACTGTGGATGAAC R: AGAGCCAGCAGGGAATCGTA	NM_001161646.1	80
ZO-1	F: CTTGACCCTAGACAGCACCCTGA R: GTGCATCATAGGCAGGAGTGGA	XM_005659811.1	163
GAPDH	F: GGACCAGGTTGTGTCCTGTGA R: TCCACCACCCTGTTGCTGTAG	NM_001206359.1	143

3.8 Determination of cell viability

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed to evaluate the viability of PEG cell following the methods of Freimoser and co-workers (Freimoser et al., 1999). Briefly, the PEG cell at $2x10^4$ cells were plated and maintained on 48-well plate (0.95 cm^2). After inoculation with PRRSV at day 0, 2, 4 and 6 dpi, 62.5 µg of MTT diluted in DMEM was added to each well of 48-well plate and incubated for 3 h. The reagent was removed, and dimethyl sulfoxide (DMSO) was used to dissolve the crystal formazan for 1 h. The dissolved formazan solution was transferred to 96-well plate and measured by microplate reader (Epoch, BioTek, VM, USA) at the wavelength 570 and 690 nm. The OD_{570} unit specific for formazan absorbance subtracted by OD_{620} unit of non-specific background absorbance were calculated as OD unit (A_{570}-A_{620}). The value of OD unit (A_{570}-A_{620}) would be relevant to the viable PEG cells.

3.9 Determination of PRRSV-induced necrosis and apoptosis

Annexin V-FITC/propidium iodide (PI) assay was performed to evaluate the apoptosis of the PEG cell using the conventional protocol. Briefly, the PEG cell at $5x10^5$ cells plated and maintained in 60 mm (21 cm^2) culture dish was harvested by 0.25% trypsin with 1 mM EDTA (0.25% trypsin/EDTA) at day 2, 4 and 6 dpi. Trypsinization reaction was stopped by 5% FBS+DMEM, and then washed by binding buffers: PBS with 8.2 mM Ca^{2+}, pH 7.4. The cell suspension was centrifuged at 300 g for 5 min. Annexin V-FITC and PI (Annexin V FITC-conjugated, ImmunoTools, Friesoythe, Germany) diluted in binding buffers were incubated with the cell pellets in the dark for 15 minutes at room temperature. Flow cytometry (Guava EasyCyte™ Systems, Merk, MA, USA) was performed to detect the apoptotic cells. After the emitting 60 mW with an argon laser

at 488 nm, the specific band pass filters of 525 ± 30 nm or 583 ± 26 nm was used for determining the Annexin V-FITC or PI positive-stained cells, respectively. Data of 50,000 cells was recorded by Guava EasyCyte™ Systems. Negative staining of both Annexin V-FITC and PI represented a living cell. Early apoptosis of cell was counted for the Annexin V-FITC-positive staining cells, but not PI-positive staining cells. Late apoptosis was counted of double-staining with Annexin V-FITC and PI cells, whereas the necrosis were only PI-positive staining cells, according to the methods of Hickey and co-workers (Hickey et al., 2011). The percentages of cells in different stages were calculated from the sum of all cells using InCyte™ software version 3.1 (Merck, Darmstadt, Germany) (Fig.3). All experiments were done in triplicate for each pig.

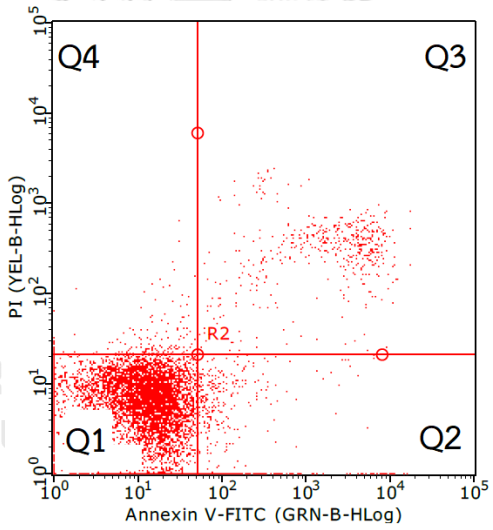


Figure 3 The quadrant plot of death analysis performed by InCyte™ software. The plot depicted the population of PEG cells staining with Annexin V-FITC and/or PI detected by the flow cytometry. Cells depicted in Q1, Q2, Q3 and Q4 were represented as a living cell, early apoptosis, late apoptosis and necrosis, respectively.

3.10 Statistical analysis

All of the data were shown in as mean ± SEM from at least 7 pigs. Graphpad Prism 5.0 (Graphpad software Inc., CA, USA) was used to perform statistical analysis. Two-way repeated measure *ANOVA* followed by Bonferroni post-hoc analysis was performed for comparison of the different PRRSV strain at each observed dpi. In some experiments with specific observed dpi, mRNA expression and Annexin V-FITC/PI assay, one-way ANOVA was used instead, to compare the different effect of PRRSV strain inoculation. The Newman-Kuel post-hoc test was following performed as randomized complete block design. Significant difference was considered at 95% degree of freedom ($p<0.05$).

CHAPTER IV
RESULTS

4.1 Determination of PRRSV infection in PEG cells

In the present study, we attempted to use the primary endometrial epithelial cell culture as a model for PRRS infection. The infectivity of PRRSV to PEG cells was firstly standardized and declared by the immunohistochemistry (IHC) of intracellular viral protein expression or the cytopathic effects (CPE) of PEG cells. Shortly after PRRSV type 1 or type 2 ($TCID_{100}$/2ml) inoculating to PEG cells, phase-contrast photograph showed that CPE, the syncytial formation (SC; Fig. 4B and 4F), had been generated at 2 dpi (Fig. 4). In addition, afterward some PRRSV-infected cells had generalized vacuoles in the cytoplasm (VC; Fig. 4C). For the detection of viral particles entry or replication, the dark-brown immunostaining of PRRSV glycoprotein 5 (GP5) co-localized in syncytial and surrounded cells (SC; Fig. 4F). Mock inoculation did not show the GP5 positive cell (Fig. 4A). Most of PEG cells (>50%) were indicated positive to GP5 in all PRRSV inoculation groups starting at 2 dpi. The PRRSV-immunoreactive cells and CPE (syncytial formations and vacuoles) indicated the infectivity of the virus to PEG cells. The infectivity of PRRSV and anti-GP5-antibody used in this study was standardized with the standard model of PRRSV-target cells MARC-145. All MARC-145 inoculated with PRRSV demonstrated the immunoreactivity similar to infected PEG (dark-brown staining; Fig. 4E), whereas mock-inoculated MARC-145 had negative immuno-staining (blue staining with no dark-brown staining; Fig. 4D).

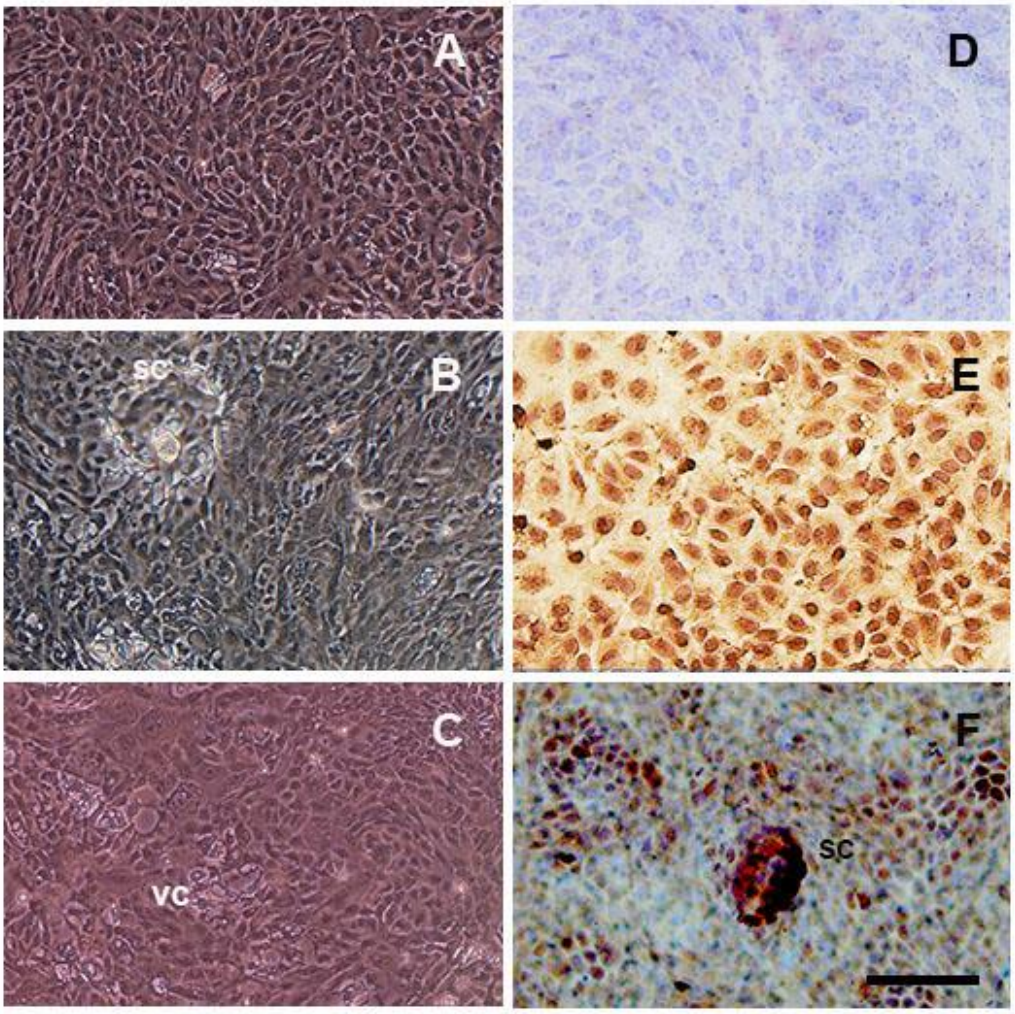


Figure 4 The cytopathic effects and intracellular viral protein of type 2 observed at 2 dpi in PEG cells. The phase-contrast photomicrographs of cytopathic effects (CPE) in PRRSV-inoculated PEG cells (B-C) compared with Mock (A). Syncytial formations (SC) and vacuoles (VC) were presented only in PRRSV-inoculated group (B and C). Immunohistochemistry (IHC) detection of PRRSV GP5 protein in PEG cells (F) was shown in dark-brown color. The PRRSV-immunoreactive cells in PEG were standardized with the negative (D) and positive control (E) in PRRSV permissive cells MARC-145 inoculated with mock or PRRSV, respectively. Scale bar = 200 μm.

4.2 Effects of PRRSV inoculation on the electrical parameter of PEG cells

The baseline numbers of TER across the monolayer PEG cells at 0 dpi were in the range of 1,400-1,800 $\Omega.cm^2$ (Table 2). The initial TER of PEG seemed to be individually different among pigs. Even though TER was daily performed, changes of TER were significantly detected at 2, 4 and 6 dpi. In order to eliminate the individual variation of initial TER numbers, the TER of PEG after PRRSV inoculation was calculated and reported as percent changes from the initial TER at 0 dpi. The apical PRRSV type 2 inoculation, but not type 1, decreased the resistance of PEG cells at 4 and 6 dpi by 30% compared with mock (Fig. 5A; $p<0.05$). Conversely, PRRSV inoculation in basolateral site did not significantly show the alteration the TER from mock (Fig. 5B; $p>0.05$). The TER of all groups was slightly decreased from initial to 6 dpi (Table 2 and Fig. 5). The TER was significantly different in time and interaction between the group of experiment and time ($p<0.05$).

To examine whether the PRRSV inoculation affected the net charges of ion at the apical site compared with the basolateral site, the potential difference (PD) was measured by volt-ohmmeter. The PD of all PEG including mock, type 1 and type 2 inoculated cells was approximately 40-60 mV up to the end of the experiment (Table 2 and Fig. 6). The statistical analysis of PD in PRRSV apical and basolateral inoculation showed no significant differences between strains, times or routes of inoculation compared to mock (Fig. 6; $p>0.05$).

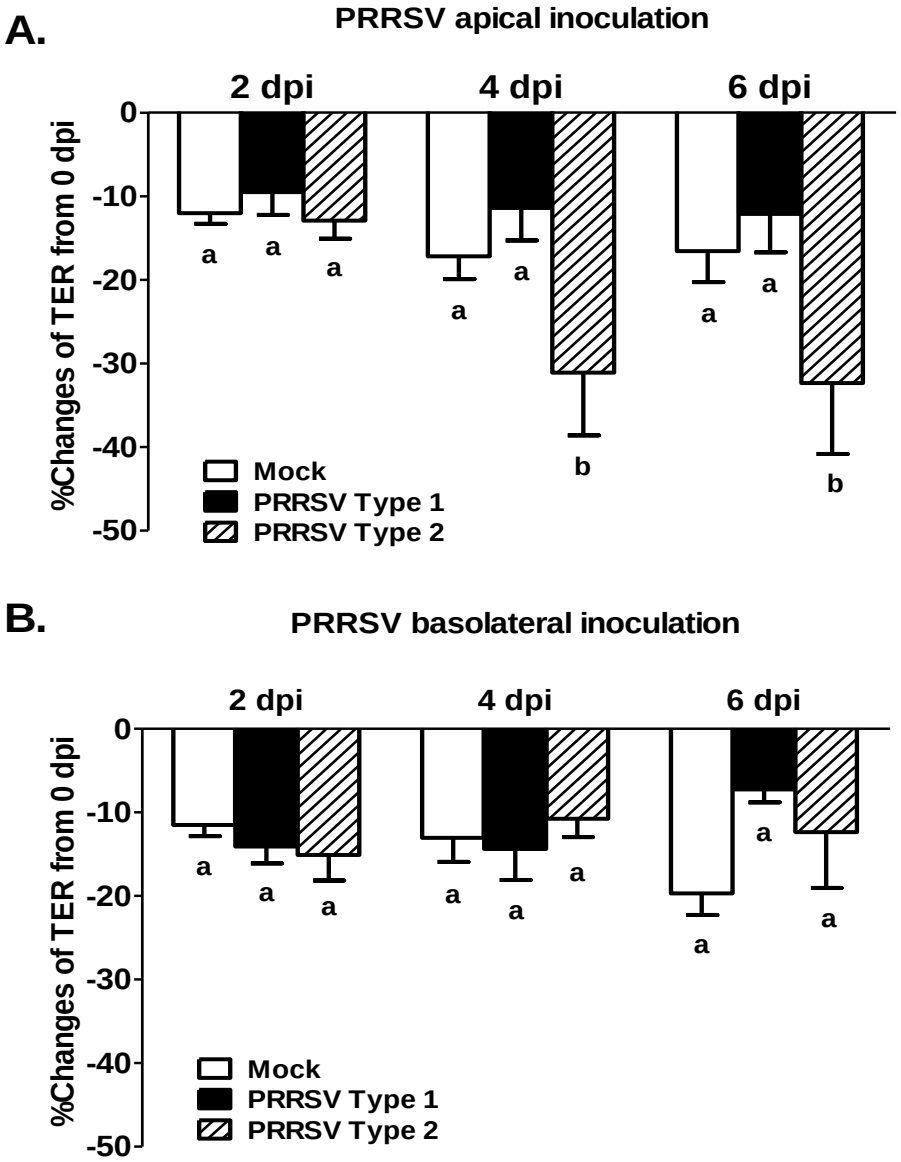


Figure 5 Effects of PRRSV inoculation on the transepithelial electrical resistance (TER) of PEG cells. After the PEG cells cultured in transwell microporous with the standard media, they were (A) apically or (B) basolaterally inoculated with vehicle (Mock), PRRSV type 1 or type 2. The TER was measured by volt-ohmmeter at 0, 2, 4 and 6 dpi. Bar graph represents mean ± SEM of percent changes of TER on the observed day from TER of 0 dpi each experiment was done triplicate from 7 pigs. Bar graph with different letters (a, b) indicates significantly different at p value < 0.05 by two-way ANOVA followed by Bonferroni post-hoc test.

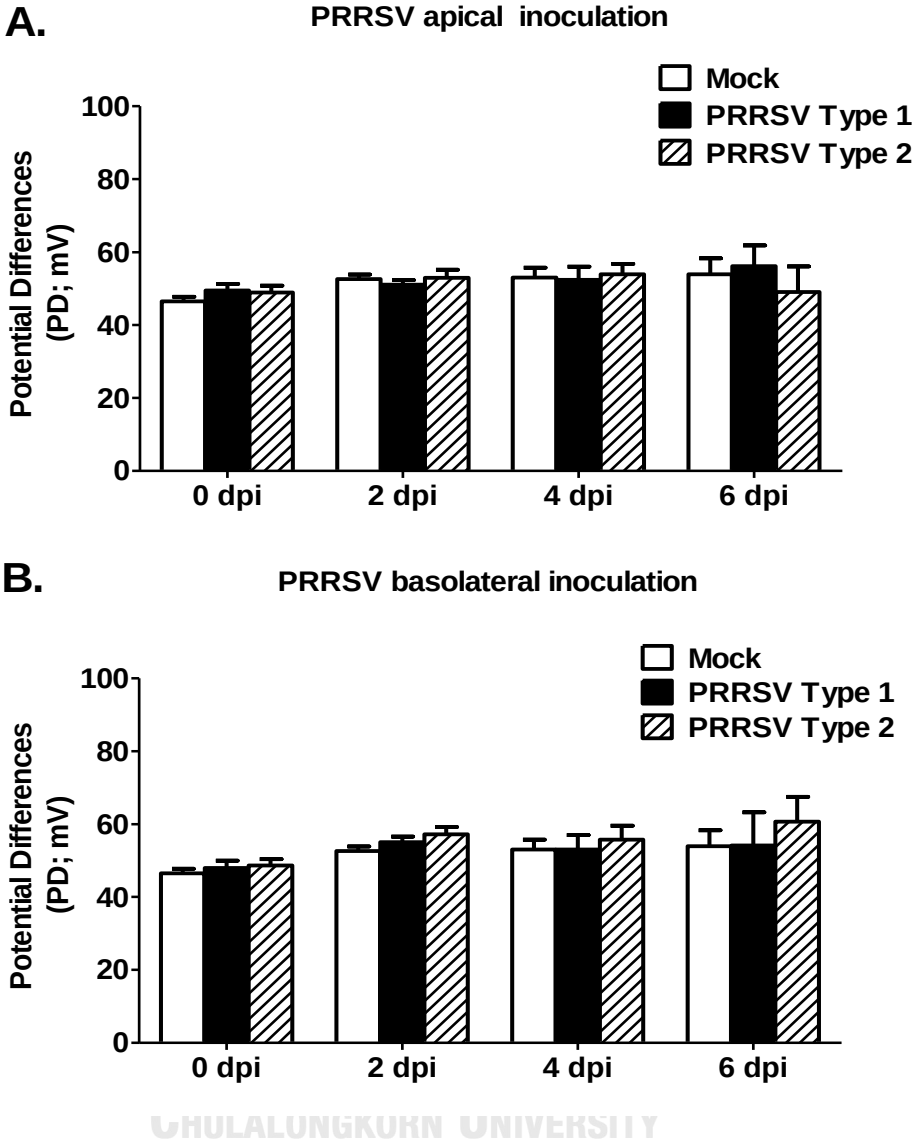


Figure 6 Effects of PRRSV inoculation on the potential differences of PEG cells. After the PEG cells cultured in transwell microporous with the standard media, they were (A) apically or (B) basolaterally inoculated with vehicle (Mock), PRRSV type 1 or type 2. The potential differences (PD) were measured by volt-ohmmeter at 0, 2, 4 and 6 dpi. Bar graph represents mean ± SEM of PD (mV) performed triplicate in each experiment from 7 pigs. Bar graph showed no significantly different by two-way ANOVA followed by Bonferroni post-hoc test.

Table 2 Effects of PRRSV inoculation on the electrical properties of PEG cells. Value represents mean ± SEM of TER or PD value measured at 0, 2, 4 and 6 dpi in triplicate cell culture of 7 pigs.

	TER ($\Omega.cm^2$)				PD (mV)			
Treatment	0 dpi	2 dpi	4 dpi	6 dpi	0 dpi	2 dpi	4 dpi	6 dpi
Mock	1647.22±51.17	1456.14±37.05	1393.76±41.88	1354.28±91.38	46.45±1.26	52.61±1.29	52.99±2.77	53.94±4.34
Apical inoculation								
PRRSV type 1	1531.03±50.54	1413.17±18.24	1386.40±55.71	1358.80±114.23	49.48±1.79	51.03±1.23	52.41±3.59	56.09±5.79
PRRSV type 2	1662.03±75.87	1444.43±37.80	1274.25±73.58	1144.50±71.52	48.96±1.85	52.94±2.20	53.91±2.86	49.06±6.97
Basolateral inoculation								
PRRSV type 1	1611.67±65.18	1393.90±24.65	1386.55±45.88	1380.50±158.64	47.94±2.01	55.06±1.55	52.99±3.99	54.18±9.06
PRRSV type 2	1701.83±73.41	1424.10±29.47	1403.60±52.94	1350.80±131.10	48.64±1.77	57.20±2.03	55.75±3.86	60.70±6.81

4.3 Effects of PRRSV inoculation on epithelial permeability of PEG cells

To further evaluate whether the PRRSV inoculation on PEG had the ability to increase epithelial permeability, paracellular transport of macromolecular fluorescein isothiocyanate dextran (FD-4; MW=4 kDa) across the PEG monolayers was measured at 2, 4 and 6 dpi. After 1 h inoculation with mock or PRRSV containing media, FD-4 (1 mg/ml) in HBSS buffers were incubated in the apical side of transwell and the accumulation of FD-4 in the basolateral side up to 24 h were measured. Figure 7 showed the apical-to-basolateral transport of FD-4 across PEG monolayers as the apparent coefficient permeability of FD-4 (P_{app} of FD-4; $h^{-1}.mm^{-2}$). Under normal condition, PEG cells defined as 0 dpi revealed the characteristic of tight epithelia, in which P_{app}=0 $h^{-1}.mm^{-2}$ (n=60 cell cultures from n=7 pigs). It indicates the suitable properties of PEG for the study model of PRRSV infection. However, P_{app} value of all PEG cells had increased depending upon time of observation (6 dpi) (Fig. 7). The apical inoculation of PRRSV type 1 showed the similar P_{app} value compared to mock. But, PRRSV type 2 inoculation at the apical side significantly enhanced the cumulative transport of FD-4, P_{app} value at 4 dpi was 10 folds greater than mock and PRRSV type 1 inoculated PEG (Fig. 7A; $p<0.05$). In the observation of PRRS inoculating at the basolateral side, all PRRV-inoculated PEG hardly detected the signal of P_{app} accumulation at the basolateral inoculation (Fig. 7B). In contrast, in mock, an amount of P_{app} was significantly higher than basolateral PRRSV-inoculated PEG cells at 6 dpi, in particular (Fig. 7B; $p<0.05$). This result was significantly different in time and interaction between the group of experiment and time ($p<0.05$).

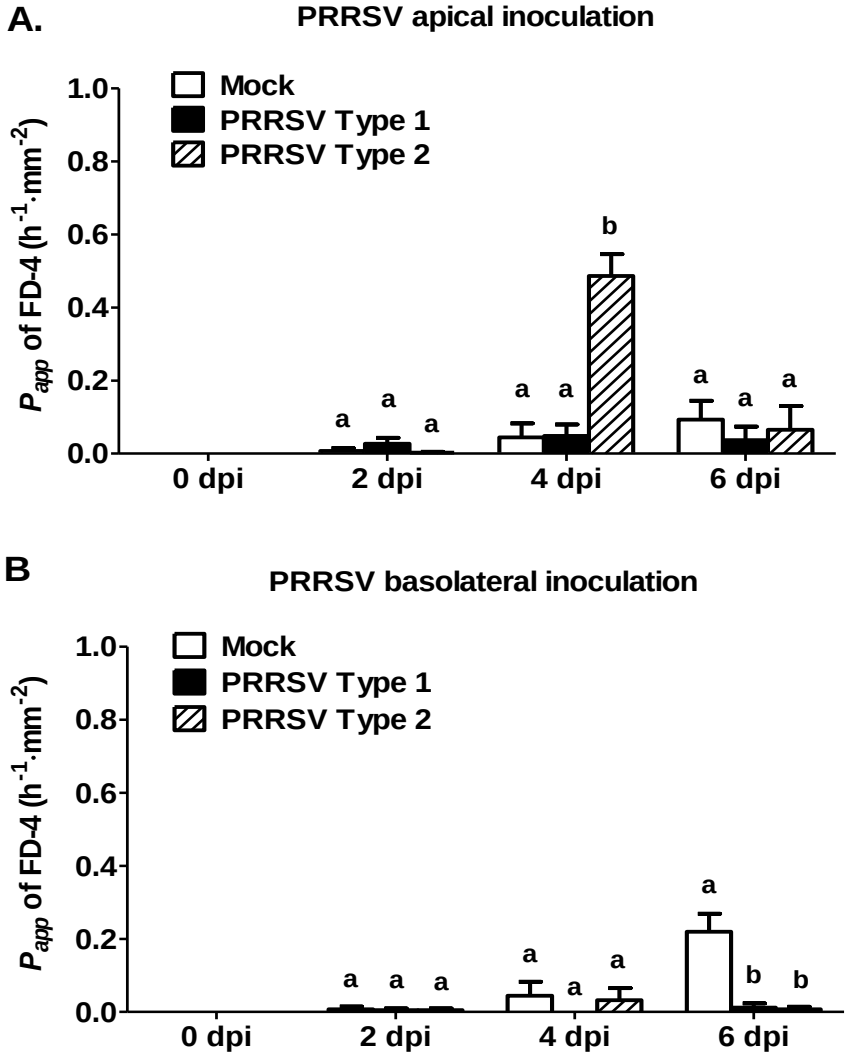


Figure 7 Effects of PRRSV inoculation on epithelial permeability of PEG cells. The apparent permeability coefficient (P_{app}) on apical (A) or basolateral (B) inoculation in PEG cells. After the PEG cells cultured in transwell microporous with the standard media and inoculated with vehicle (Mock), PRRSV type 1 or type 2, at 0, 2, 4 and 6 dpi, FD-4 was incubated on apical site for 24 h. Bar graph represents mean ± SEM of FD-4 appearance at the basolateral site compared to the apical site (P_{app}, $h^{-1}.mm^{-2}$) by fluorometry in triplicate cell culture of 7 pigs. Bar graph with different letters (a, b) indicates significantly different at p value < 0.05 by two-way ANOVA followed by Bonferroni post-hoc test.

4.4 Effects of PRRSV inoculation on tight junction gene expression of PEG cells

Next was to determine whether the tight junction protein that regulated paracellular permeability was a PRRSV target gene. Since the alteration of paracellular permeability induced by PRRSV had been revealed at 4 dpi, the gene expression of tight junctions (TJs); *Cldn1, 2, 3, 4, 5, 7, 8 and ZO-1* were assessed by quantitative reverse transcriptase PCR or RT-qPCR at 4 dpi. The specific PCR products of the expected based pair length at the 40 cycles using the 3 µg of cDNA synthesized from the RNA of all samples were firstly considered using the 1.5 % agarose gel electrophoresis. Eventually, the comparison among the TJs gene expression, the cycle threshold (C_t) values obtained from real-time PCR of each gene were examined and normalized to those of the housekeeping gene *GAPDH*. Under normal condition, PEG cells maintained in the standard media with 5% FBS differently expressed TJs mRNA, *Cldn7* > *Cldn4* > *Cldn5* > *Cldn*1 > *Cldn*3 > *Cldn*2 > *Cldn*8 (Table 3). However, as seen in the figure 9 the expression of TJs and its scaffold *ZO-1* at 4 dpi are much lesser than *GAPDH*. The effect of PRRSV altered TJs mRNA expression on PEG cell was calculated by the fold changes from mock ($2^{-\Delta\Delta Ct}$). PRRSV type 1, but not type 2, up-regulated *Cldn3* (2.69 ± 0.73) and *Cldn8* (2.38 ± 0.11) significantly (Fig. 8; $p<0.05$). On the other hand, *ZO-1* was stimulated by PRRSV type 2 (4.34 ± 0.80; $p<0.05$). Both strains of PRRSV activated the expression of *Cldn5* (Fig. 8; 8.32 ± 1.42 by type 1 vs. 6.39 ± 1.64 by type 2) and decreased *Cldn7* (Fig. 8; 0.26 ±0.07 by type 1 vs. 0.24 ± 0.10 by type 2). In this study, PRRSV inoculation had no effect on the expression *Cldn1* and *Cldn2* compared to mock at 4 dpi.

Table 3 Cldn1, 2, 3, 4, 5, 7, 8 and ZO-1 expression of non-PRRSV-inoculated PEG cells. Value represents mean ± SEM of 2-ΔCt performed by RT-qPCR at 4 dpi in triplicate cell culture inoculated with vehicle (Mock) of 7 pigs.

Porcine TJs gene	Tight junction mRNA expression normalized to GAPDH (2-ΔCt)
Cldn1	0.0088 ± 0.0028
Cldn2	0.0002 ± 0.0001
Cldn3	0.0047 ± 0.0020
Cldn4	0.0405 ± 0.0200
Cldn5	0.0338 ± 0.0335
Cldn7	0.0769 ± 0.0326
Cldn8	0.0001 ± 0.000
ZO-1	0.0015 ± 0.0005

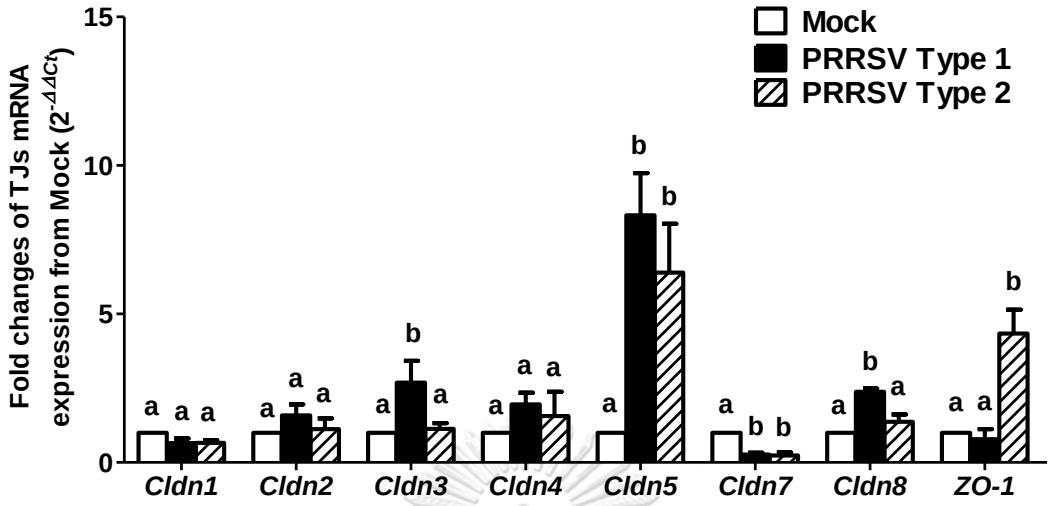


Figure 8 Effects of PRRSV inoculation on TJs gene expression of PEG cells. After cultured in the standard media, PEG cells were inoculated with vehicle (Mock), PRRSV type 1 or type 2. Total RNA was isolated at 4 dpi for determining of Cldn1, 2, 3, 4, 5, 7, 8, ZO-1 and GAPDH by qPCR. Bar graphs show mean ± SEM (n=7 pigs) of the fold changes of TJs mRNA expression from mock using the $2^{-\Delta\Delta Ct}$. Data of threshold cycle (C_t) of TJs gene were normalized to those of reference gene GAPDH and represented as ΔC_t. Experiments were conducted in triplicate cell culture. Bar graph with different letters (a, b) indicates significantly different at p value < 0.05 by one-way ANOVA followed by multiple comparison Newman-Kuel post-hoc test.

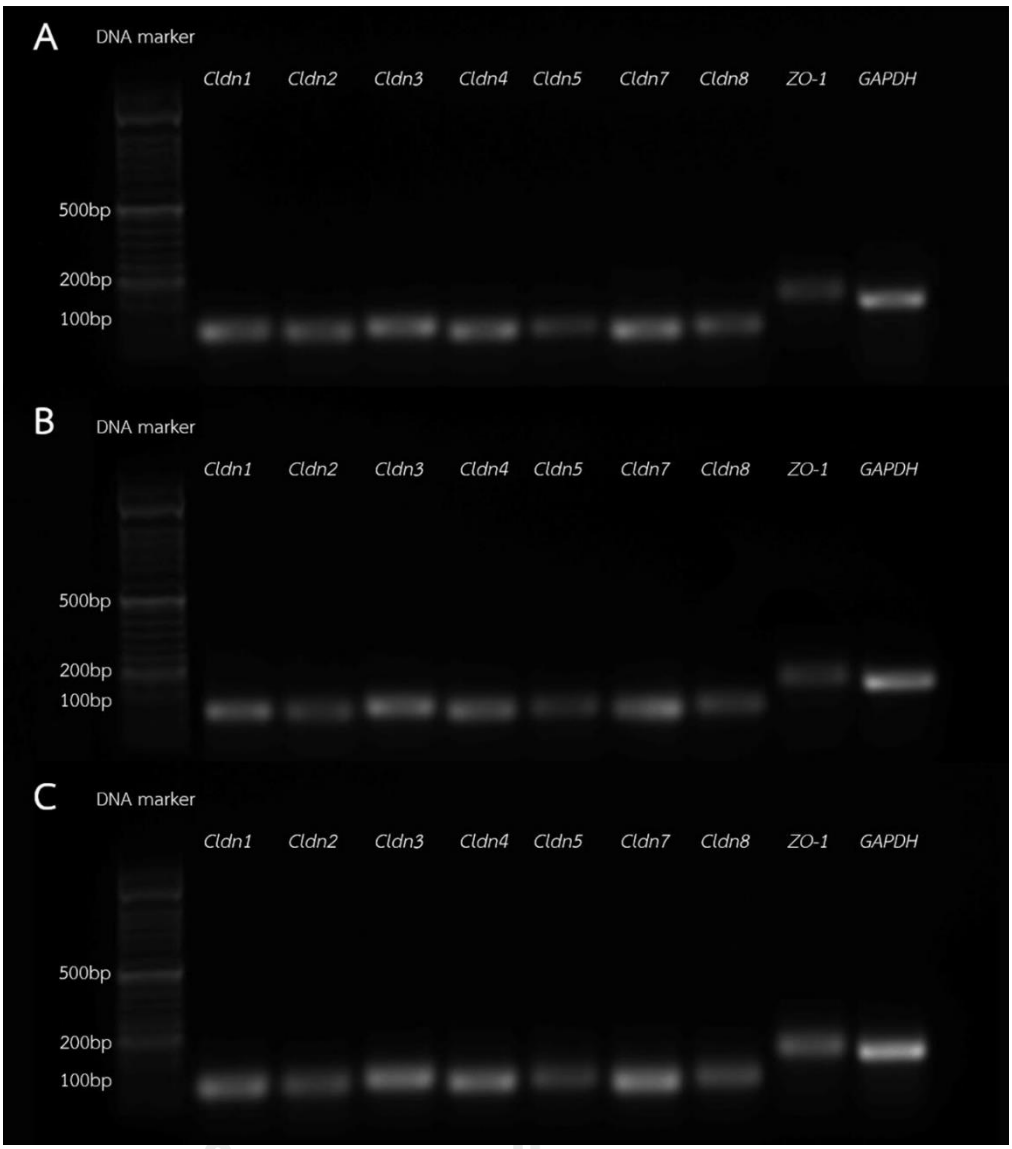


Figure 9 1.5% agarose gel electrophoresis show the amplification products of TJs gene expression of mock (A), type 1 (B) and type 2 (C) inoculation including *Cldn1* (80bp), *Cldn2* (80bp), *Cldn3* (86bp), *Cldn4* (80bp), *Cldn5* (93bp), *Cldn7* (80bp), *Cldn8* (80bp), *ZO-1* (163bp) and *GAPDH* (143bp). Represented bands were performed using 3 µg of cDNA from samples of 7 pigs. The PCR reaction was amplified for 40 cycles totally.

4.5 Effects of PRRSV inoculation on the viability of PEG cells

To address whether PRRSV can induce the death of cells as seen in the alveolar macrophages, the MTT cell viability assay was carried out after viral inoculating PEG at 2, 4 and 6 dpi. MTT assay at 0 dpi prior to PRRSV inoculation was performed when the cells reaching 90% confluent had OD $_{(A570-A620)}$ values of 1.5 units. Type 1 and type 2 PRRSV increased the OD value at 2 and 4 dpi by 20% (Fig 10. $p<0.05$). However, the cell viability numbers induced by PRRSV type 1 were less than PRRSV type 2 and was not significantly different from mock (Fig. 10; *p>0.05*). The increased in OD reflects the more activity of mitochondrial enzymes and the proliferation of PEG cells. However, the increased numbers during 4-6 dpi did not last as it returned to the baseline value at 6 dpi (Fig 10. $p>0.05$). The MTT result was significantly difference in time (*p<0.05*) but did not in the interaction between group and time.

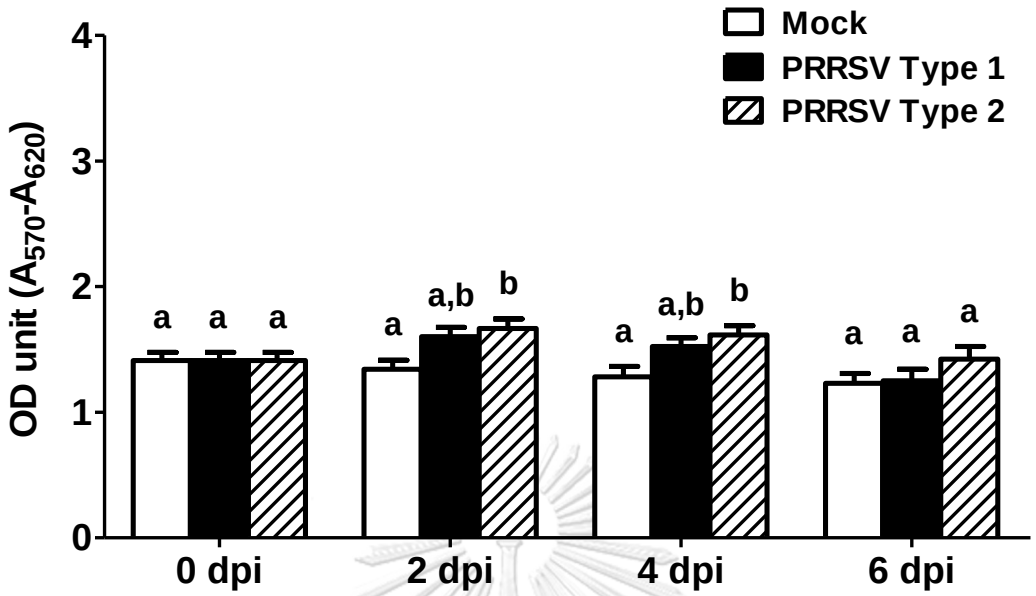


Figure 10 Effects of PRRSV inoculation on the viability of PEG cells. After the PEG cells cultured in 48-well plate with the standard media, they were inoculated with vehicle (Mock), PRRSV type 1 or type 2. The viability of PEG cells was assessed by MTT assay at 0, 2, 4 and 6 dpi. Bar graphs demonstrate mean ± SEM of the OD unit (A_{570}-A_{620}) of dissolved solution from MTT assay in triplicate cell culture of 7 pigs. Bar graph with different letters (a, b) indicates significantly different at *p* value < 0.05 by two-way ANOVA followed by Bonferroni post-hoc test.

To examine whether PRRSV-inoculated PEG cells underwent apoptosis, the apoptotic cell characteristics were evaluated by Annexin V FITC (AV) and propidium iodide (PI) staining. Flow cytometry revealed the similarity in viable PEG cell population with no staining with AV/PI in all groups (approximately 70-80%; Q1) (Fig. 11A). The viability of non-infected or PRRSV-infected cells were not reduced up to 6 dpi (Fig. 11A). In Q2 of plot analysis, cells positive with AV but negative with PI counted as early apoptotic cells were equivalent in all groups (Fig. 11A; $p>0.05$). It suggests that PRRSV inoculated cells did not undergo apoptosis differences from mock. Late apoptotic and necrotic cells were also considered in Q3 and Q4, as the percentages of PI-positive cells with or without AV positive (Fig. 11A). It showed that PRRSV type 1 and type 2 inoculated PEG cells, increased the number of necrotic cells at 2-4 dpi (Q4; Fig. 11B-C; $p<0.05$). No significant changes in the percentages of early and late apoptotic cells of mock compared to PRRSV-inoculated PEG cells (Q3; Fig. 11B-D; $p>0.05$).

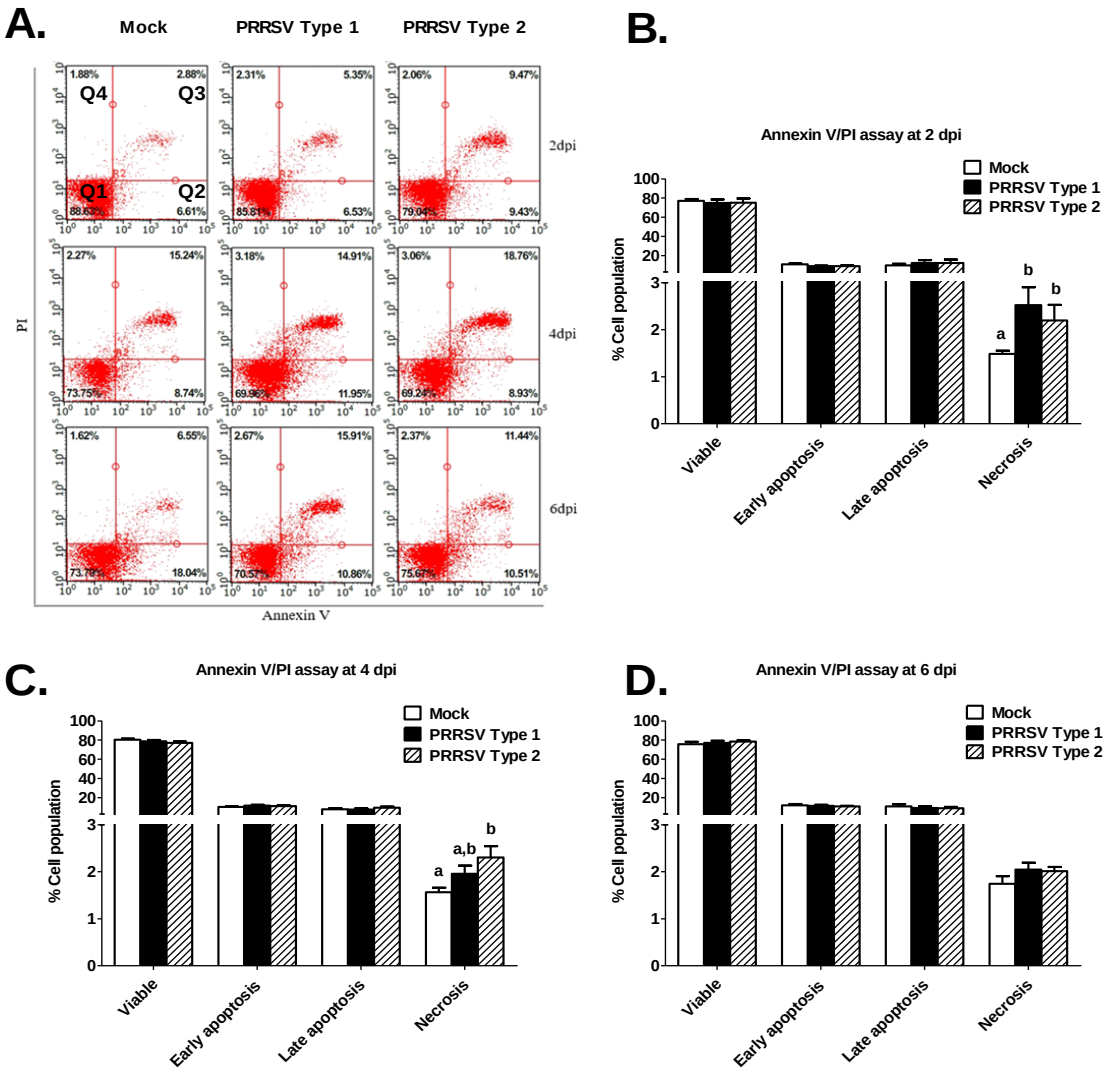


Figure 11 Effects of PRRSV inoculation on the apoptosis of PEG cells. After the PEG cells were cultured in 60 mm culture dish with the standard media, they were inoculated with vehicle (Mock), PRRSV type 1 or type 2. Representative plots of cell death analysis determined by flow cytometry at 2, 4 or 6 dpi were shown in (A). PEG (50,000 cells/samples) was stained with Annexin V-FITC and PI. The percentage of cells in each quadrant of Q1-Q4 were respectively quantified as mean ± SEM of viable, early apoptotic, late apoptotic and necrotic cells in bar graph (B-D) at each dpi. Experiments were conducted in triplicate cell culture from 7 pigs. Bar graph with different letters (a, b) indicates significantly different at p value < 0.05 by one-way ANOVA followed by multiple comparison Newman-Kuel post-hoc test.

CHAPTER V
DISCUSSION

The objectives of this research were to evaluate and to compare the alterations in permeability and cell death of PEG cell-infected with two variated factors of PRRSV, i.e., strains (type 1 vs type 2) and routes (mucosa vs blood-borne). These present results showed that mucosal infection with PRRSV type 2 induce more severe epithelial leaky than type 1. Instead, basolateral (blood-borne) PRRSV infection with type 1 or type 2 enhances the tightening of TJ barriers equally. The underlying mechanism seems to be mediated by the genomic effects of PRRSV infection on tight junction gene expression. However, the TJ gene targets of type 1 and type 2 PRRSV are different. In addition, the leaky epithelia induced by apical PRRSV infection relevant to cell death or necrosis. Nevertheless, the PRRSV effects on PEG cell death and TJs barrier leaky were not continuous but recovered within 6 days post inoculation. The evidences may explain host-viral interaction of PRRSV infection during the viral entry toward the viral replication, in which the consequent response may continuously disturb organ function throughout the reproductive cycles.

The primary cell culture of porcine glandular endometrial epithelial cell (PEG cell) used in this research followed the protocol of Deachapunya and O'Grady (Deachapunya and O'Grady, 1998). The recent isolated PEG culture was standardized by determining the morphological characteristics and electrical properties of PEG cells consistent with the previous study. Briefly, as seen in the first part of this study, PEG cells cultured under normal condition clearly revealed the purified nucleated rounded cells, which can be declared as a glandular epithelium (Rehfeld et al., 2017). Unlikely, the stroma of connective tissues or surface endometrium is more flattened and loosen than the glandular endometrium (Rehfeld et al., 2017). The PEG cells produced high

TER throughout the experiments, which additionally confirmed the purity of endometrial epithelial cell culture. The successful culture of tight PEG monolayer provides us a suitable *in vitro* model to study the direct effect of PRRSV on porcine endometrium.

In addition, in our preliminary trial, PEG cells were found to express the specific receptor to PRRSV, CD163 and sialoadhesin (CD169), which are required for permissive to PRRSV infection. Like the standard PRRSV permissive cell line study such as African green monkey kidney cell line (MA104, MARC-145 and CL2621), PRRSV could not be extending replicated and maintained in PEG as nearly as *in vivo* (Martinez-Lobo et al., 2011). Although these PRRSV permissive cell lines were susceptible to the virus, but they did not express CD169 (Duan et al., 1998). Therefore, they could not be as a good model for the study of mechanism and pathogenesis of PRRSV infection. Moreover, cytopathic effects observed in MA104 or MARC-145 were low and only a few focal (Kim et al., 1993). Previous studies revealed two phases, primary and secondary permissiveness, of PRRSV infection mechanism in a MARC-145 cell. The primary effect induced by PRRSV on the permissive cell was a little. In contrast, the secondary phase of PRRSV in MARC-145 is the outcome of infected cells transmitted to non-permissive PRRSV free cells, which appear as CPE (Cafruny et al., 2006). The mechanism was indicated as a cell-to-cell interaction with cytoskeleton is an unessential factor to massive infection leading to death of MARC-145 cells. The phenomena seem to be dissimilar to the pathogenesis of PRRSV infection in *in vivo*.

In the present study, cytopathic effects produced by PRRSV in PEG cells was a focal lesion, and subsequently propagating and forming the giant syncytial cells. The PRRSV-immunoreactive staining was propagated over the PEG confirming the infectivity of the virus to PEG. Interestingly, the PRRSV-positive particle was mostly located in the

syncytial cells. The surrounded cells showed many vacuoles (Fig. 3). The evidence indicates the potential of PRRSV to replicate and degenerate PEG cells. In disagreement with MTT assay, which can determine the degeneration or death of cells, it showed the increase in cell proliferation of PRRSV-inoculated cell instead.

Basically, the host cells infected with virus are proceeded to be dead or survive. The outcome of CPE produced by virus usually reflects the activity of virus in host cells. However, the virus that causes the cell degeneration or cell lysis may not be only the cytotoxic effect of the virus but may be the cellular mechanism of virus on the host for spreading their particles. In addition, the cellular survival after cytopathic virus infections may not be the recovered of cells, but it plays importance of the persistent infections. Perhaps, the host response to virus in this way is to protect themselves from viral elimination (Heaton, 2017). The mechanism is established through a variety of pathways, such as the autophagy and proliferation (Heaton, 2017).

Certainly, different viral families have the typical character interaction with their host cells. Most RNA viruses, including PRRSV can be replicated and persistent in host cell (Bierk et al., 2001; Griffin, 2010). Therefore, the unique factors produced by host in response to virus were suspected. During the viral entry, replication and evasion, the structural protein composed on the envelop or the nucleocapsid protein of PRRSV particles and non-structural proteins transcribed by PRRSV nucleic acids in host cells have been associated with host interaction with PRRSV. To date, mysterious evasion of host immunity and the persistence of PRRSV were studied, focusing on 4 components produced or expressed by PRRSV, which are N protein, Nsp1, Nsp2 and Nsp11 (Yoo et al., 2010; Rascón-Castelo et al., 2015). They are all immunogenic viral proteins, and tolerance are neutralized by host. The persistent infection in PEG cells at long-term infection and/or the infected PEG cells may possibly produce the

mechanism against PRRSV. In particular, Nsp1 and Nsp2 are very significant since both proteins can interact with host innate immune response to virus such as the production of IFN and the development of cell-mediated immunity. In addition, their sequence proteins are different among PRRSV strains and with other members of family *Ateriviridae* (Dokland, 2010). Therefore, their symptoms, sign and severity are very typical for PRRSV infection.

Many cytokines, either anti-viral cytokine type 1 interferon (IFN) (Sang et al., 2010) or the inhibitory cytokine IL-10 (Suradhat et al., 2003) released by immune cells has been indicated to be modulated by PRRSV. These cytokine play important role in the evasion and replication mechanisms of PRRSV (Yoo et al., 2010). The inhibition of IFN and the induction of IL-10 released by porcine peripheral blood mononuclear cells (PBMCs) have been largely contributed to immunosuppression allowing PRRSV persistent (Suradhat et al., 2003; Rascón-Castelo et al., 2015). However, in the uterine horn, the resident macrophages were a little, thus the indicated cytokines are released in low level. The circulating PRRSV-infected macrophages that redistributed to feto-placental epithelium via blood vessels was suggested as the pathogenesis of PRRSV replication in the reproductive organs (Karniychuk et al., 2011). In the present model of PEG, it is the purified glandular endometrial isolated cell, and it does not contain any immune cells including macrophages. However, the cellular responses, i.e. cell degeneration, proliferation remained to be seen in the PRRSV-infected target cells, suggesting the pathogenesis of PRRSV is not restricted to macrophages.

Focusing on the direct effect of PRRSV on the isolated PEG model, the present study was the first report of PRRSV infection in the endometrial epithelial cells other than macrophages. We employed the measurement of paracellular permeability by TER and FD-4 flux which are techniques widely used in the pharmaceuticals for drug

absorption testing in the intestinal epithelial cell monolayers are used in our study since 2015 (Natoli et al., 2012; Kiatprasert et al., 2015). In contrast to the study of PRRSV inducing the CPE, in which performed in the cell culture dish, PEG cells were cultured and able to form the monolayer on the filtered membrane. The filter-grown PEG monolayer can separate the epithelium into two compartments, apical and basolateral. This technique not only allows us to inoculate PRRSV at the different route, but also evaluates macromolecule transport across epithelium, which represents tight junction barrier function (Kiatprasert et al., 2015).

In this study, apical inoculation of PRRSV type 1 and type 2 had different severity on increased paracellular permeability as evidenced by decreased TER and increased FD-4 flux. In fact, different PRRSV strains were frequently demonstrated the different severity in an *in vivo* study. Particularly, PRRSV type 2 had been indicated to cause the disease distinctly in late term gestation gilts (Nielsen et al., 2002; Scortti et al., 2006). In consistent with others, the result of the present study indicates that apical infection with type 2 PRRSV is more pathogenic than type 1 in terms of the leakiness of paracellular permeability. As aforementioned, the differences between type 1 and type 2 pathogenicity depend on the heterologous protein and genes, specifically ORF5, Nsp1b and Nsp2 (Han et al., 2006).

The underlying mechanism of the epithelial leakiness induced by apical PRRSV type 2 infection was further studied. The paracellular permeability of epithelium, including glandular endometrium is controlled by tight junction protein complex. Factors and signals that modulate the paracellular permeability in long term has been suggested to influence TJs claudin expression patterns (Anderson and Van Itallie, 2009; Gunzel and Yu, 2013; Kiatprasert et al., 2015). In this study, the expressions of TJs in the basal condition of PEG cells seem to be predominantly characterized as an ion

permeable epithelium because the high level in *Cldn7* expression was expressed. Meanwhile, the barrier builder proteins, Cldn4 and Cldn5, are expressed only a half of *Cldn7* expression. Differently with our previous study, the immortalized PEG cells express *Cldn1, 4, 7, 8* and *ZO-1* equally Kiatprasert et al., 2015). The immortalized PEG cells can be represented as the normal cells, however the PEG was derived from the mutation of normal glandular endometrial cell which is possible to have some alteration of cell biology from the primary cell culture PEG.

In the present PEG model, the decreased pore-forming *Cldn7* expression and increased barrier builder *Cldn3, 5, 8* and *ZO-1* expression were found in response to PRRSV infection. In other words, PRRSV infected cells had a genomic effect toward the tightening epithelia. However, we could not detect the increase of TJ barrier function induced by PRRSV type 1 infection in this study. Based on the TER value, the nature of PEG cells is classified as a tight epithelium which does not allow the paracellular transport of FD-4. Detection of the further decrease in the permeability of FD-4 in PRRSV infection could not be possible. Although, the tightening of epithelia attributes the strength of tissue barrier, it may be the strategy of the viral spreading. Such as respiratory syncytial virus (RSV) infection, up-regulation of the barrier builder TJ protein gene expressions, *occludin*, *Cldn4 and ZO-1* expression in live human nasal epithelial cells (hNECs) produces the higher polarity of alveolar cells than the natural cells (Tsutsumi et al., 2011). The increased TER in RSV-infected cells facilitates cellular secretion of propagating RSV. The highly polarized PEG after recovery from PRRSV infected should be in attendance.

However, the leaky PEG caused by PRRSV type 2 was in our focus. It seems to be a characteristic of the cytotoxic effect of viral infection and may be the predominant pathogenesis of PRRSV infection. In this study, PRRSV type 2, but not type 1, infection

produced the leaky endometrium. Generally, the down-regulation of barrier builder proteins and/or the up-regulation of pore-forming proteins should be associated with the increase of epithelial leakiness. For instance, the evidence of our previous study in the immortalized PEG cell inoculated with LPS induce the leakiness mediated by the down-regulated builder barrier *Cldn8* but up-regulated pore-forming *Cldn7* (Kiatprasert et al., 2015). However, the leakiness induced by PRRSV type 2 was not correlated with the TJ gene expression. The barrier-builder *Cldn5* and its core protein *ZO-1* was up-regulated while the pore-forming *Cldn7* was down-regulated in the corresponding PRRSV type 2-infected cells. Certainly, the up- and down-regulation of TJs gene or protein expression would not be assured for the TJ barrier function, unless the TJs proteins were localized at the TJ structure. In addition, the over expression of mismatch claudin and ZO-1 may cause the disassociated formation of TJs complex (Anderson and Van Itallie, 2009).

The significance of the modulatory effects PRRSV may be either the strategy of virus to facilitate their process or the homeostasis process of host response to invading pathogens. It is possible that the target of virus-host interaction, PRRSV-glandular endometrium, are claudins which are the first line mechanism to attack PRRSV and can encourage downstream signaling pathways. Previously, claudin and other TJs protein have been indicated as the underlying mechanism of many pathogens. For instance, Clostridium perfringens could directly bind to claudin-4 and -5 proteins, and cause downregulation of *Cldn1*, *4* and *5* correlating with an important disease in human (Sonoda et al., 1999). Hepatitis C virus infection downregulated *Cldn1* and *occludin* promoting viral entry (Liu et al., 2009). The alteration of claudin in PEG cell may be associated with viral entry process. Besides direct stimulation, the previous studies show the pathogenesis of TJs indirectly via the immunomodulatory process. Tumor necrosis factor alpha (TNF-α) releasing in response to pathogens, particularly on the

bacterial sepsis, can separate junction adhesion molecules (JAM) in intestinal epithelia (Nusrat et al., 2000). In addition, Interferon gamma (IFN-γ) the anti-viral cytokine is important mediator to increase epithelial permeability and indirectly reduce Cl^- secretion in inflammatory bowel disease (IBD) (McKay and Baird, 1999; Nusrat et al., 2000). The *TJs* expression mechanisms in response to invading pathogens has been indicated previously to predominantly involve with ROCK, PKCs and MAPK pathway (Lu et al., 2014). However, the complete mechanism that can modulate the epithelial function have not been yet clarified and need to be further investigated.

Besides epithelial leakiness, PRRSV type 2 infection induced cytotoxic to PEG cells at 2 and 4 dpi. Other *in vitro* studies showed that the PRRSV permissive cell, MARC-145 cells infected with PRRSV, was vastly necrosis at 48 h (Miller and Fox, 2004). Lee and Kleiboeker found that PRRSV induced cell death including apoptosis and necrosis at 48 h and 60 h post infection (Lee and Kleiboeker, 2007). In agreement with their study, our finding showed necrosis induced by PRRSV at 2 and 4 dpi (Fig. 10), in which the time was later than those in the previous study. Remarkably, PRRSV induced apoptosis in PRRSV permissive cell line was observed at early infection within 48 h (Ma et al., 2013; Li et al., 2016). Moreover, Nsp4 and Nsp10 of PRRSV initially induced apoptosis at 6 hpi in MRC-145 cells (Yuan et al., 2016). The early apoptosis induced by PRRSV in PEG cells may be occurring; however, the annexin V/PI assay was initially determined at 2 dpi. Thus, the monitoring of apoptosis at the early period (0-48 h post inoculation) should be considered in the further investigation.

The apoptosis induced by PRRSV is an important argument because the initiation of apoptosis pathway can be stimulated by PRRSV directly and indirectly. And the removal process of apoptotic cells may be potential for host to remove unwanted cells. GP5, Nsp4 and Nsp10 are discovered to associate with both extrinsic and intrinsic

pathways of apoptosis directly (Fernandez et al., 2002; Music and Gagnon, 2010; Ma et al., 2013; Yuan et al., 2016). When the PRRSV is replicating in host cell, the transcribed ORF5 was induced to synthesis of many proteins including of p25. The protein p25 has been indicated to directly turn on the major pro-apoptotic proteins, p53 mediating the intrinsic pathway of apoptosis. However, this apoptotic pathway mediated by p25 protein could not be down-regulated by the induction of anti-apoptotic Bcl-2 expression (O'Brien, 1998; Roulston et al., 1999). Moreover, pro-inflammatory cytokines induced by PRRSV, such as tumor necrosis factor (TNF), can also induce apoptosis indirectly (Rath and Aggarwal, 1999). Interestingly, PRRSV stimulates *TNF* expression in various cells differently through the toll-like receptor signaling pathway (Lopez-Fuertes et al., 2000; Wang et al., 2007; Subramaniam et al., 2010). In PEG cell, the data about cytokines modulated by the PRRSV need to be explored in the future.

To date, PRRSV has been shown to process some pathway that prevent them being eliminated by host cells, i.e. the autophagy process (Li et al., 2016). Even though autophagy may benefit for the host cell to eliminate virus, it may advantage for virus to recycle the essential proteins in replication process. The process of autophagy during the PRRSV infection facilitates invading of host immune response or favoring its replication have to be determined in the further study (Shintani and Klionsky, 2004). MTT assay evaluates the viable cells which are remained the function of mitochondrial enzyme. This method is more accurate than manual cell count (Freimoser et al., 1999). Increasing of formazan dye means that PEG cells are proliferating, and/or the enzyme activities are raised. In the study of MTT assay, all PRRSV infection seems to maintain the longevity of PEG cell compared to the control, in which cell viability was gradually decreased from 0-6 dpi (Fig. 9). Moreover, PRRSV type 2 increased the OD unit of formazan indicating its stimulatory effects on proliferation or maintain the population of PEG (Fig. 9). The key signaling pathway mediated by various PRRSV structural and

non-structural proteins (i.e. Nsp2, 4 or 11) interacts with intracellular targets of host, including gene activation, post-translation gene activation. Moreover, PRRSV-induced autophagy may be the mechanism to serve the cell materials and cell survival (Shintani and Klionsky, 2004; Zhivotovsky and Orrenius, 2010).

Focusing on the cell proliferation, the major member of mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) pathway mediating PRRSV-induced CPE, viral replication and cell proliferation had been suggested (Zhou and Zhang, 2012). Nevertheless, ERK pathway-modulated PRRSV infection has not been clearly understood. The proliferation of epithelial cells may cause a complexity of the disease. For example, infection of intestinal mucosa causes crypt hyperplasia (Savidge et al., 1996). Human respiratory syncytial virus (RSV) stimulates cell proliferation at respiratory tract epithelium (Prince et al., 1978). Even though cell proliferation represents physiological defects, the new generating cells are the normal mechanism of tissue growth and repairing. The basolaterally PRRSV inoculated cells seem to remain the ability of permeability until late infection. This result may associate with cell proliferation without cell necrosis. The long-term effects of PRRSV in PEG cells have to be clarified and compared with *in vivo* study in the future.

In conclusion, this research is the first-time to investigate the effect of PRRSV in PEG cells. We found that PRRSV can modulate TJs gene expression which perhaps could not be explained as the underlying mechanism of the leakiness or tightening of TJs barrier of PEG. The virus infection stimulates both cell viability and apoptosis coinciding with the changes of paracellular permeability. In which, leaky epithelia and cell viability dysfunction may associate with the pathogenesis of late term abortion and stillbirth. Moreover, the development in PEG cell may lead to a good model to study about PRRSV infection. This research will provide the pathogenesis of PRRSV in

the reproductive system which may help us to improve the therapeutic strategies for management of re-circulated PRRSV in herds.

CHAPTER VI
CONCLUSION

This research demonstrated the effect of PRRSV type 1 and type 2 on the permeability and the viability of isolated porcine glandular endometrium cell culture model. The CPE and GP5 positive cell exhibited the infectivity of PRRSV on PEG cell. PPRSV infection, particularly type 2 had cytotoxic effect on permeability and viability. But it was subsequently recovered. Even though, PRRSV type 1 infection had a little effect on viability, it changed TJs profile of PEG to the character of tight epithelia. Nevertheless, both strains of PRRSV had genomic effect on the different group of TJs genes.

The conclusions could be indicated as follows:

1. PEG cell can be directly infected by PRRSV.
2. *Cldn3*, *5*, 7 and *8* are the target gene of PRRSV type 1. *Cldn5*, *7* and *ZO-1* are the target gene of PRRSV type 2. Both viruses up-regulated barrier builder TJ *Cldn5* and down-regulated pore-forming TJ *Cldn7*.
3. Both strains of PRRSV did not affect the number of viable PEG, even though the necrotic cells induced by PRRSV were detected by flow cytometry. PRRSV infection may induce necrosis earlier than 2 dpi; and the host-viral interaction process may replace the new cells in which we could not indicate in this study.
4. Apical PRRSV infection induced the leakiness of PEG cells, whereas basolateral infection maintained the tight of epithelia until the end of experiment.
5. PRRSV type 2 had more severity than type 1 to decrease permeability caused by necrosis and/or TJs disturbance.

The present study showed the different effect between PRRSV type 1 and type 2 on the permeability and the viability of PEG cells. The apical route of infection plays a pivotal role in the pathogenesis of PRRSV in reproductive organs. Effects and mechanisms of PRRSV may be associated with PRRSV-induced reproductive disorders.

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APPENDIX

VITA

Dran Rukarcheep was born on January 17th, 1989 in Nakhon Pathom province, Thailand. He graduated with Degree of Bachelor of veterinary science, Chulalongkorn University, Bangkok, Thailand in 2013. He had been worked as veterinarial in animal hospital since 2013 for 2 years. Dran is recently pursuing a master's degree in animal physiology program. His works is in the field of animal physiology and immunology in reproductive system at Department of Veterinary Physiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. He is interested in molecular science of reproductive system with aim of improving animal reproduction which interrupted by a pathogen. His thesis advisor is Associated Prof. Dr. Sutthasinee Poonyachoti who supported and gave the counsel for him all the time. Moreover, he also attended the poster presentation in the topic "Effect of porcine reproductive and respiratory syndrome virus (PRRSV) type 1 and type 2 on the viability of porcine endometrial epithelial cell culture" of Chulalongkorn university veterinary conference (CUVC) 2018 which was accepted to publish in Thai Journal Veterinary Medicine Volume 48.

During the study, he has got many experiences in training of animal use for scientific works from institute of animal for scientific proposes development (IAD). He was trained about chemical safety and chemical waste from Chulalongkorn University. He practiced the cell culture techniques and PCR techniques from Gibthai Co., Ltd. He studied the staining techniques including immunohistochemistry (IHC), immunocytochemistry (ICC), immunofluorescent assay (IFA) and other histopathological works from faculty of science, Mahidol University. He has the experience about primary cell isolation, cell line culture and management in laboratory more than 2 years.