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A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Role of progesterone associated with PGRMC1 on preimplantation development of in vitro produced porcine embryos

돼지 체외생산 수정란의 착상 전 발달에서 PGRMC1을 통해 프로게스테론이 미치는 효과

February, 2020

By

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농생명공학부

조만호

Abstract

Role of progesterone associated with PGRMC1 on preimplantation development of in vitro produced porcine embryos

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Progesterone is a steroid hormone well known for its significant role in the reproduction process of mammals. Numerous studies have reported on the regulation of progesterone during implantation, pregnancy and parturition, but there are fewer studies on progesterone in relation to the early stages of embryo development. In the present study, we investigated the effects of progesterone during the development of *in vitro* produced porcine embryos. First, gene expression of various progesterone receptors in the *in vitro* produced porcine embryos were analyzed. PGRMC1 and PGRMC2 (progesterone receptor membrane component 1 and 2) showed distinct expression. Next, the embryos were treated with two concentrations of progesterone (10 nM and 100 nM) for two different durations (from day 0 and from day 4) to compare the developmental rates, cell numbers, and apoptosis rates of day 7 blastocysts. The

experimental groups in both durations showed similarly increased blastocyst cell numbers and decreased apoptosis rates when treated with 100 nM progesterone. Furthermore, the expression levels of PGRMC1, PGRMC2, PAIRBP1 (plasminogen activator inhibitor RNA-binding protein 1), and apoptosis-related genes were examined in blastocysts and, showed significant increases in the 100 nM treatment group compared to the control group. Subsequently, the embryos were treated with the PGRMC1 inhibitor, AG-205, and developmental rates, cell numbers, and apoptosis rates of day 7 blastocysts were compared. In addition, 100 nM progesterone was treated simultaneously with AG-205 to test if the inhibition effect is relieved by progesterone. Groups treated with 1 μM and 2 μM AG-205 showed decreased cell numbers and increased apoptosis rates in day 7 blastocysts compared to the control group. We also confirmed the recovery of inhibition by 100 nM progesterone. In conclusion, the present study indicated that progesterone positively affects the development of *in vitro* produced preimplantation porcine embryos by increasing cell proliferation and decreasing apoptosis via PGRMC1-involved actions. However, the detailed mechanisms of PGRMC1 need further elucidation.

Key words: *in vitro* produced porcine embryos, embryo development, progesterone, PGRMC1, blastocyst, apoptosis

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BAD	BCL2 associated agonist of cell death
BAK	BCL2 antagonist killer
BAX	BCL2-associated X protein
BCL2	B-cell lymphoma-2
Bcl-w	BCL2-like protein 2
Bcl-xL	B-cell lymphoma-extra-large
BH	BCL2 homology
BID	BH3-interacting domain death agonist
BIK	BCL2 interacting killer
BL	Blastocyst
BSA	Bovine serum albumin
CASP3	Caspase 3
cDNA	complementary DNA
COCs	Cumulus oocyte complexes

CRADD	CASP2 and RIPK1 domain containing adaptor with death domain
Ct	Threshold cycle
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
FSH	Follicle stimulating hormone
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
IVC	*In vitro* culture
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
IVP	*In vitro* production
LH	Luteinizing hormone
MAPRs	Membrane associated progesterone receptors
Mcl-1	Myeloid cell leukemia 1
mPRs	membrane progestin (progesterone) receptors
mRNA	messenger ribonucleic acid

mTBM	modified Tris-buffered medium
PAIRBP1	Plasminogen activator inhibitor RNA-binding protein 1
PAQRs	Progestin and adipoQ receptors
PCR	Polymerase chain reaction
PFF	Porcine follicular fluid
PGRMC1	Progesterone receptor membrane component 1
PGRMC2	Progesterone receptor membrane component 2
PR	(classical) progesterone receptor
PZM-3	Porcine zygote medium-3
qPCR	quantitative polymerase chain reaction
RNA	Ribonucleic acid
S.E.M.	Standard error of the mean
SERBP1	Serpine mRNA binding protein 1
TCM-199	Tissue culture medium 199
TdT	Terminal deoxynucleotidyl transferase
TL-HEPES-PVA	Tyrode's lactate - HEPES - polyvinyl alcohol
TUNEL	Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling

UTP	Uridine triphosphate
UV	Ultraviolet

LITERATURE REVIEW

1. *In vitro* production of embryos

Early embryo development in mammal has been considered as the basis for many fields of animal researches, and holds essential roles in animal reproduction, transgenesis, and stem cell studies (Laible, Wei, and Wagner 2015; Houdebine 2005). However, investigating the developmental process of early embryos in mammal is challenging due to a number of technical difficulties. For instance, obtaining the early stages of embryos from *in vivo* is strenuous because the embryos are often located in the oviduct until morula stage, and retrieving them non-surgically is not easy. This brought forth the necessity of producing embryos *in vitro* from the beginning, i.e. from unfertilized gametes (Lonergan and Fair 2014).

IVP (*in vitro* production) of mammalian embryos consists of three major processes (Coy and Romar 2002; Abeydeera 2002). They are IVM (*in vitro* maturation) of oocytes, IVF (*in vitro* fertilization) of matured oocytes and sperm, and IVC (*in vitro* culture) of fertilized embryos. Successful IVP of embryos were reported in numerous mammalian species, and although they share some common aspects within the overall process, each species differ in many detailed measures since they all have distinct features in embryo development.

In vitro maturation

IVM can be broadly explained by the maturation of two components of oocyte, nuclear and cytoplasm, with the support of cumulus oophorus. Nuclear maturation is typically characterized by the germinal vesical breakdown for meiotic resumption to the M2 stage and re-arrest in metaphase. Whereas cytoplasmic maturation refers to other changes that would prepare the oocyte for fertilization, which include relocation of organelles and exocytosis of cortical granules (Brackett 1985; Abeydeera 2002). During this process, cumulus oophorus (i.e. cumulus cells) that surround the maturing oocyte undergo a maturation of their own. They expand and mucify to reduce of gap junctions while participating in the promotion of glycolysis and stimulation of sterol biosynthesis, coordinating bi-directional amino acid transport with the oocyte to support its development (Evian Annual Reproduction Workshop 2011).

In vitro fertilization

The basic steps of IVF begin with sperm preparation. Freshly ejaculated sperm needs to go through a number of processes before fusing with the oocyte nucleus, i.e. capacitation, binding to zona pellucida, and acrosome reaction. The most important among these

to be considered in sperm preparation of IVF is acquiring the capacitated state of the sperm. Capacitation is a series of changes that sperm go through to obtain its motility and fertility involving restabilization of the membrane and reorganization of sperm space (Topfer-Petersen, Petrounkina, and Ekhlasi-Hundrieser 2000). This is mainly achieved by a substance called heparin *in vivo*, but *in vitro*, caffeine is most commonly used. After capacitation, the sperm interact with the matured oocyte via zona pellucida for acrosome reaction and penetration (Wortzman, Gardner, and Evans 2006).

In vitro culture

IVC is probably the most important step of all three processes, for it constitutes the longest period in IVP. The key aspect of IVC would be the culture media that house the embryos for as long as 7-8 days. Although many years ago it was deemed impossible to culture embryos past the 2 to 4-cell stage, there have been many researches within the last few decades reporting of multiple improvements in the embryo culture media that can overcome the 2, 4-cell block and further sustain the preimplantation development up to the BL (blastocyst) stage (Nielsen and Ali 2010). This was mainly accomplished by producing culture media that mimic the composition

of *in vivo* oviductal fluid, since early stage embryos generally reside in the oviduct up to the morula stage. However, it is still considered that the *in vitro* produced blastocysts are of less quality compared to their *in vivo* counterparts, and this is thought to be because of many suboptimal conditions of *in vitro* culture (Li et al. 2007).

Many attempts were made to resolve this long-standing issue by supplementing the culture media with various substances that are found to be in the oviductal fluid or are known to reduce harmful influences of *in vitro* culture conditions. The substances include hormones, and growth factors (Kwak et al. 2012; Takeuchi et al. 2017), and even extrinsic compounds like ginsenoside (Kim et al. 2016) or polyphenol (Salzano et al. 2014), although trials of supplementing progesterone during embryo culture are scarce. But it has been reported that progesterone is a steadily secreted hormone in the oviduct and uterus through the early periods of preimplantation development *in vivo* (Martyniak, Franczak, and Kotwica 2018; Peralta et al. 2005; Magness and Ford 1983). Thus, a further investigation was necessary regarding the effects of progesterone upon early embryo development.

2. Progesterone

In order to begin with discussing about progesterone, it is necessary to describe the basis of progesterone, which is steroid hormone. Steroid hormones are hydrophobic (lipophilic) compounds characterized by a common basic structure of cyclopentane-perhydro-phenantrene, which is a 'four-ring system' formed by 17 carbon atoms. Particularly, steroid hormones related to reproductive systems are referred to as sex steroids and are separated into three groups depending on their number of carbon atoms (Taraborrelli 2015). Each group are named as estranes (C18 steroids consisting of 18 carbons), androstanes (C19 steroids consisting of 19 carbons) and pregnanes (C21 steroids consisting of 21 carbons). Progesterone is included in the pregnanes group of C21 steroids, and was first described in detail in 1933 (Allen 1935).

It is known that the key biosynthetic pathways of producing steroid hormones are the same in an organism, independent of where it is manufactured, although the expression of organ-specific enzymes often decide the precise type and amount of each hormone. The initial step starts with the common precursor, cholesterol, which is produced by the conversion of two acetyl-CoA molecules and normally transported through the plasma membrane and to the

mitochondria (Vladic 2012). Cholesterol is then converted to pregnenolone by P450scc (cholesterol side-chain cleavage enzyme) and is released from the mitochondria to the cytosol (Stocco and Clark 1997) to follow either of the two metabolic pathways known as the $\Delta 5$ and $\Delta 4$ pathway. $\Delta 5$ pathway begins with the formation of 17-hydroxy-pregnenolone to the synthesis of dehydroepi-androsterone and to 5-androstene-3β,17β-diol (androstenediol). Whereas $\Delta 4$ pathway starts with pregnenolone converting to progesterone by 3β-hydroxysteroid dehydrogenase and $\Delta 5,4$-isomerase, later to be subsequently transformed to 17α-hydroxy-progesterone, androstenedione, and testosterone (Miller 2008). In summary, progesterone is a type of steroid sex hormone consisted of 21 carbons and is produced from cholesterol by the $\Delta 4$ pathway.

Progesterone in reproduction

Progesterone is widely studied as the key component practically at every stages of the female reproductive system of mammals. It is particularly well characterized in the uterus, where it mainly participates in the central physiological roles of implantation and most importantly, the maintenance of pregnancy by regulating the cyclical proliferation and differentiation of uterus endometrium. More

specifically, progesterone helps the nesting of blastocysts to the uterus wall at implantation period and allows the transition of proliferative phase to secretory phase during the onset of pregnancy in the uterus (Taraborrelli 2015). The regulation of uterus during pregnancy is achieved by the combined action of estrogen and progesterone, and the progesterone takes part first in promoting the proliferation of uterine cells and later in endometrial maturation, which are uterine vascularization and decidualization (Vladic 2012).

Novel progesterone actions

However, over the years many studies have reported the novel influences of progesterone in various other tissues, organs and biological processes such as ovary, mammary gland, embryo, central nervous system, cardiovascular system and cancer (Graham and Clarke 1997; Sitruk-Ware 2018). Some of the essential roles of progesterone in the above areas are described in the table below:

Table 1. Effects of progesterone in various tissues

Tissue	Regulations	References
Ovary	• Ovulation	(Robker, Akison, and Russell 2009)
Mammary Gland	• Cell proliferation • Lobular–alveolar development for milk secretion	(Macias and Hinck 2012)
Embryo	• Oocyte maturation • Preimplantation development	(Ferguson, Kesler, and Godke 2012) (Zhang and Murphy 2014)
Central Nervous System	• Neuroregeneration on traumatic and ischemic brain damage • Myelin repair and neuroprotective effects on Alzheimer's disease	(Sayeed and Stein 2009) (Irwin, Solinsky, and Brinton 2014)
Cardiovascular System	• Decreasing blood vessel vasoconstriction • Inhibiting coronary hyperactivity	(Thomas and Pang 2013)
Cancer	• Mediating proliferation and differentiation of breast cancer cells • Major suppressor of endometrial tumor	(Hurd et al. 1995) (Yang, Thiel, and Leslie 2011)

3. Progesterone receptors

Classical progesterone receptor

For decades, many influences induced by progesterone were only thought to be mediated by nuclear progesterone receptor. Naturally, they were all explained through the way of 'classical' steroid hormone actions, which involved progesterone diffusion into the nuclear to

bind to its receptor and act as a transcriptional factor that would modulate the gene expression of the cell to generate an effect. This PR (classical progesterone receptor), like other steroid hormone nuclear receptors, belong to a superfamily of ligand-activated transcription factors, consisting of a highly conserved DNA-binding region, with a carboxy-terminal ligand-binding region and a variable amino-terminal region (Beato et al. 1989). It has two major isoforms, PR-A and PR-B, that are encoded from the same gene by distinct promoters at two binding sites and differs in their amino-terminal region (Grimm, Hartig, and Edwards 2016). They share two transcription AF (activating functional) domains (AF-1, AF-2), but PR-A being 164 amino acids shorter, lacks the AF-3 of PR-B isoform (Kastner et al. 1990). This change makes these two isoforms differentiate in their localization and functional aspects (Hill et al. 2012). The actions of PRs are mediated by ligand binding and forming a ligand-receptor complex that subsequently recognize specific progesterone response elements that are typically localized in promoter regions of progesterone- responsive genes (Jacobsen and Horwitz 2012), with the involvement of a number of transcription factors that can activate/inhibit the PRs, including AP1 and STATs (Abdel-Hafiz and Horwitz 2014).

Non-classical progesterone receptor

Over the years, accumulating evidences of diverse rapid steroid effects were reported, and explanations through PRs and their classical actions were viewed insufficient in many ways (Losel and Wehling 2003), even after PR-B was found to be capable of activating cytoplasmic signaling cascades involving tyrosine kinase SRC/RAS/ERK/MAPK pathway (Boonyaratanakornkit et al. 2001). These rapid progesterone responses were often referred to as 'non-classical' which signified that the actions take place within a shorter time frame (seconds to minutes) compared to the classical actions, and that they did not require transcriptional modifications or genomic regulations. Therefore, a list of specific criteria were made to distinguish these non-classical actions with classical actions. The criteria are as follows: i) rapid actions that are incompatible with transcriptional activation and translation, ii) being insensitive to inhibitors of transcription or translation, iii) lacking response to nuclear receptor antagonists, iv) occurring in cells that do not have nuclei, v) induction even when conjugated with cell-impermeable molecules (Losel et al. 2003). Results from the past studies clearly showed that non-classical actions of progesterone participate in modulating a number of diverse physiological processes. These include oocyte development (Peluso 2006), acrosome reaction (Baldi

et al. 1995), cell proliferation in cancer cells (Albrecht et al. 2012), and apoptosis which are induced via alterations in intracellular cAMP levels or activation of SRC/MAPK pathways (Garg et al. 2017).

Membrane progesterone (progestin) receptors

mPRs (membrane progesterone receptors) consist one of the families of receptors known for mediating membrane associated non-classical actions of progesterone. Belonging to a larger family of proteins called PAQRs (progestin and adipoQ receptors), mPRs were first identified and cloned in fish ovaries (Patino and Thomas 1990), but were soon discovered in various mammals including mouse, sheep, pig, and human (Dressing et al. 2011). Their structure comprise of seven transmembrane domains that are similar with the G-protein coupled receptors (Zhu, Bond, and Thomas 2003). Three isoforms are best-known among mPRs, which are mPRα, mPRβ, and mPRγ. Each show distinct functions and expression levels in different tissues, but in general, all participate in the modulation of signal transduction cascades such as stimulation of ERK 1/2 and p38 MAPK pathways, or intracellular Ca^{2+} mobilization, upon progesterone binding (Ashley et al. 2006). They are also reports of mPRs contributing to proliferation and metastasis of breast cancer

cells (Dressing and Thomas 2007), hypermotility of human sperm (Thomas, Tubbs, and Garry 2009), expressed in multiple female reproductive organs such as uterine endometrium and fallopian tube (Fernandes et al. 2005). They were also found to be associated with oocyte maturation in porcine ovaries (Qiu et al. 2008).

Membrane-associated progesterone receptors

Another family of non-classical action mediating receptors are MAPR (membrane-associated progesterone receptors) in which PGRMC1 and PGRMC2 (progesterone receptor membrane component 1 and 2) belong. MAPRs share a similar heme-binding domain that resembles a cytochrome b5 domain fold, which functions with cytochrome P450 (Mifsud and Bateman 2002). Of the lesser known of the two is PGRMC2, despite its first cloning in human in 1998 (Gerdes et al. 1998), lack greatly for studies compared to PGRMC1. PGRMC2 is highly homologous to PGRMC1 in structure (Chen et al. 2010), containing both the cytochrome b5-like domain and SH2 target sequence at the C-terminal (Wendler and Wehling 2013), but is different at the N-terminal transmembrane domain (Ryu, Klein, and Zanger 2017). It is expressed in various tissues, also currently known to bind and interact with cytochrome P450 proteins, and is

related to cell migration of cancer cells (Albrecht et al. 2012).

On the other hand, PGRMC1 is a far better studied receptor consisted of a characteristic cytochrome b5 domain with a single N-terminal transmembrane domain and three binding spots for SRC homology domains (Cahill 2007). It was initially isolated and purified from porcine hepatocyte tissue (Meyer et al. 1996; Falkenstein et al. 1998) and is known to act via sumoylation or by constituting complexes with other proteins to take part in diverse pathways such as inhibition of TCF/LEF (T cell factor/lymphoid enhancer factor) activity (Peluso, Lodde, and Liu 2012). Furthermore, PGRMC1 is viewed as a very versatile protein possessing a wide spectrum of physiological functions that include progesterone signaling, cholesterol/steroid biosynthesis (steroidogenesis), homeostasis, cellular metabolism, promotion of autophagy, cell cycle regulation, cell survival/death responses (apoptosis) as well as cell proliferation (Peluso et al. 2006; Losel et al. 2008; Rohe et al. 2009; Peluso et al. 2014; Kim et al. 2018).

Out of all the roles above, PGRMC1 is most extensively studied in the female reproductive tract, especially in the ovary and granulosa cells (Peluso 2006). Although few studies have indicated the influence of PGRMC1 in cell mitosis (Lodde and Peluso 2011) and

follicle assembly (Nilsson, Stanfield, and Skinner 2006), most studies have examined its feature in apoptosis (Peluso et al. 2006; Peluso, Romak, and Liu 2008; Peluso et al. 2009; Peluso et al. 2010). Its anti-apoptotic effects are proposed to be in association with PAIRBP1 (plasminogen activator inhibitor RNA-binding protein 1, also known as SERBP1 or RDA299) (Peluso et al. 2004), which perform through regulation of intracellular Ca^{2+} and protein kinase G activation (Peluso, Liu, and Romak 2007), as PGRMC1 being the actual progesterone binding site.

4. Apoptosis

Apoptosis is a form of self-destruction mechanism for the cell, often called cell suicide, which is concerned with numerous biological events including embryogenesis, homeostasis and often generated for removing unwanted or damaged cells from the system (Kerr, Wyllie, and Currie 1972). Unlike necrosis where severe inflammation and damage to the surrounding cells are accompanied, apoptosis is a well-programmed type of death pathway that results in a complete removal of the target cell with minimal degrading cellular contents or cytokine release, preventing any vigorous immune response (Kanduc

et al. 2002). Range of characteristic morphological changes that occur during apoptosis include reduction of cell size, characteristic condensation of chromatin and cytoplasm, invagination of plasma membrane to a grape-like feature, DNA fragmentation and lastly the fragmentation of the cell itself to many small membrane-bound apoptotic bodies (Kroemer et al. 2009). Eventually, the fragmented apoptotic cells are subsequently phagocytosed by the neighboring cells.

Apoptosis detection methods

Numerous experimental methods are present for the detection of apoptosis of each stages, for example, detection of the exposed phosphatidylserine by a specific ligand called annexin V (linked with fluorescent conjugates) which recognizes the externalized phosphatidylserine after modification of membrane asymmetry of apoptotic bodies (Banfalvi 2017). Another widely used method would be TUNEL (TdT dUTP nick-end labeling) assay which detects the DNA degradation occurring at the late stages of apoptosis, based on the ability of TdT (terminal deoxynucleotidyl transferase) labelling blunt ends of DNA breaks without the need of a template. TUNEL assay exploits enzymatic histochemical labelling of dUTPs to free

3'OH terminals of fragmented DNA to be visualized under immunohistochemical techniques such as UV light (Kyrylkova et al. 2012).

Pathways to apoptosis

Apoptosis can proceed in two pathways: extrinsic or intrinsic. Extrinsic pathway is triggered by external stimuli such as death receptor ligand signaling, whereas intrinsic pathway results from internal stimuli such as severe DNA damage or cell cycle regulation (Fulda and Debatin 2006). In the extrinsic pathway, a death receptor–ligand association leads to caspase 8 activation which initiates the intrinsic pathway of mitochondrial outer membrane disruption or directly activates caspase 3, one of the most well–studied executioner protein of the final stages of apoptosis, to initiate cell degradation. On the other hand, the intrinsic pathway begins with the onset of apoptosis–related proteins of BCL2 family. Activation of pro–apoptotic BAX and BAK proteins is a critical step that will permeabilize the mitochondrial outer membrane to release apoptosis–inducing proteins including cytochrome c from the inter–membrane space of mitochondria. Released cytochrome c facilitates the formation of apoptosome protein complex and activate caspases,

which will then cleave pro-caspase 3 for action (Li et al. 1997; Birkinshaw and Czabotar 2017).

BCL2 family

As stated above, a group of proteins referred to as BCL2 family proteins holds great importance during the initiation stages of apoptosis, especially the intrinsic pathway. Upon discovery of the presence and its anti-apoptotic function in acute lymphoblastic leukemia (Tsujimoto et al. 1984; Pegoraro et al. 1984), BCL2 (B-cell lymphoma-2) protein was found to be analogous to many other proteins with similar roles regarding apoptosis. Furthermore, this so-called BCL2 family was soon classified into three subfamilies depending on the structural homology of the conserved motifs, known as BH (BCL2 homology) 1-4 motifs (Aouacheria et al. 2013), and the anti/pro apoptotic function of each protein (Tsujimoto 1998). The first subfamily including BCL2 (Vaux, Cory, and Adams 1988), Bcl-xL (B-cell lymphoma-extra-large) (Boise et al. 1993), Bcl-w (BCL2-like protein 2), Mcl-1 (myeloid cell leukemia 1) etc. exert anti-apoptotic (pro-survival) activity and share homology within all four BH motifs (BH1 through BH4). The second subfamily represented by BAX (BCL2-associated X protein) (Oltvai, Milliman,

and Korsmeyer 1993), BAK (BCL2 antagonist killer) (Chittenden et al. 1995) are the major players for the permeabilization of mitochondrial outer membrane, exerting pro-apoptotic activity and sharing homology at BH1 to BH3. The third subfamily includes BIK (BCL2 interacting killer), BID (BH3-interacting domain death agonist), BAD (BCL2 associated agonist of cell death) (Yang et al. 1995) etc. and is known to exert pro-apoptotic activity in both directly and indirectly as in activating BAX and BAK or suppressing the activities of anti-apoptotic BCL2 proteins. This third group of proteins only share homology within the BH3 motif (Tsujimoto 1998). Thus, from the above classification, researchers found the necessity of BH4 for anti-apoptotic function and BH3 for pro-apoptotic function (Huang, Adams, and Cory 1998; Dai et al. 2011). Furthermore, studies reported that the hetero-dimerization between anti-apoptotic and pro-apoptotic members of BCL2 proteins was the key to inhibit the biological activity of their opposite partners (Birkinshaw and Czabotar 2017).

Apoptosis in embryos

Apoptosis commonly occur in mammalian embryonic cells as in other types of cells, and although apoptosis is responsible for some

very important functions during preimplantation development (Jacobson, Weil, and Raff 1997), apoptosis of *in vitro* cultured embryos are generally recognized to represent the cellular response to suboptimal conditions and stress during development (Betts and King 2001). Thus, various aspects and levels of apoptosis in mammalian preimplantation embryos, such as genetic regulation, signaling pathways, proliferation, and morphology have been investigated in various past studies (Levy et al. 2001). Several possible causes include chromosomal abnormalities, imbalance or inappropriate ratio of growth factors or hormones, and incorrect composition of culture media etc. (Warner and Brenner 2001). These apoptosis processes in mammalian embryos are most frequently observed during blastocyst stages. Genetic and morphological changes of apoptosis were reported in various species such as mouse, human, pig and cattle, and even though apoptotic cells are phagocytosed by neighboring blastomeres, some can lead to secondary necrosis which then severely affects the entire embryo (Hao et al. 2003; Fabian, Koppel, and Maddox-Hyttel 2005). The most common morphological abnormality is known as blastomere fragmentation, and an excessive amount of fragmentation in preimplanation embryos are often used as indicators of poor embryo quality (Mateusen et al. 2005).

Apoptosis related with autophagy

Autophagy is a form of cell death that involves the formation of autophagosomes, which are double-membrane vesicles with organelles that eventually fuse with lysosomes for catabolism. Autophagy is regarded as a cellular survival mechanism as it is found to occur during stressful conditions, for instance, starvation, to re-use the cell's organelles (Levine and Yuan 2005; Mizushima et al. 2008). Different proteins that were originally known to mediate either autophagy or apoptosis are now reported as 'dual-functioning', and the key protein that mediates both of these pathways are Beclin 1. Beclin 1 is one of the most essential autophagic protein that is well known for its role with Vps34 complex (Funderburk, Wang, and Yue 2010). This is due to the structural feature that Beclin 1 possess, which is its BH3 domain. As explained above, BH3 domain is recognized as the major region that induce the pro-apoptotic regulation of many proteins in BCL2 family (Feng et al. 2007). Thus, anti-apoptotic proteins of BLC2 family, such as BCL2, Bcl-xL, Mcl-1 etc., can inhibit autophagy and induce apoptosis by binding to the BH3 domain of Beclin 1. Similarly, they can also dissociate with Beclin 1 in stressful conditions to activate autophagy (Pattingre et al. 2005; Decuypere, Parys, and Bultynck 2012). In this sense, autophagy is perceived as an anti-apoptotic mechanism (Marquez

and Xu 2012). In addition, caspase families of apoptosis pathways are also found to cleave Beclin 1. As a result, autophagosome formation is inhibited, leading to inhibition of autophagy (Wirawan et al. 2010).

INTRODUCTION

Progesterone is a steroid hormone known for its critical roles in many stages of mammalian reproduction, including implantation (Graham and Clarke 1997; Yoshinaga 2014), pregnancy (Vallet and Christenson 2004; Szekeres-Bartho, Halasz, and Palkovics 2009; Moon et al. 2014), and parturition (Vladic 2012; Vannuccini et al. 2016). Although it is less known, progesterone also participates in the process from gametogenesis to the development of embryo, including follicle development (Nilsson, Stanfield, and Skinner 2006), oocyte maturation (Shimada and Terada 2002; Yamashita et al. 2003), sperm activation (Baldi et al. 1995), and embryo development (Gallego et al. 2009; Zhang and Murphy 2014). The progesterone effect during preimplantation embryo development is still an area of study that needs more attention.

There have been several studies reporting that progesterone is constantly secreted in the oviduct and uterus of pigs (Robertson and King 1974; Stefanczyk-Krzymowska et al. 1998; Magness and Ford 1983) during the time when embryos develop from zygotes to late blastocysts. These observations imply that progesterone is likely to have significant influence within this period of embryo development, which has been investigated in several mammalian species, including mouse (Roblero and Izquierdo 1976; Warner and Tollefson 1978) and cattle (Larson, Krisher, and Lamb 2011; Ferguson, Kesler, and

Godke 2012) by focusing on the progesterone effects on early embryo development during *in vitro* culture. However, similar studies regarding porcine embryos have yet to be reported.

In addition to the studies on progesterone, there have also been many continuous investigations on specific progesterone receptors. For decades, many influences caused by progesterone have been suggested to be mediated only by the nuclear-located progesterone receptor. This well-known PR of the nuclear steroid hormone receptor family is activated upon progesterone binding, and it acts as a transcription factor to directly affect the target genes with progesterone response elements (Jacobsen and Horwitz 2012; Hill et al. 2012; Grimm, Hartig, and Edwards 2016). However, accumulating evidence has suggested that the explanations with only the nuclear progesterone receptor are insufficient for many rapid progesterone-induced effects (Gellersen, Fernandes, and Brosens 2009; Garg et al. 2017).

This issue was resolved by the discovery of multiple novel receptors of progesterone, which are now generally referred to as 'nonclassical' progesterone receptors (Bramley 2003; Dressing et al. 2011). These receptors have a distinct mode of action. Unlike PR, nonclassical progesterone receptors mainly locate on the plasma

membrane or in the cytosol of the cell, and upon progesterone binding, they induce signaling cascades that mediate protein kinase activities. These cascades eventually affect the cell in ways that are not restricted to the transcriptional regulation of genes (Garg et al. 2017). The most comprehensively studied among these receptors are mPRs and MAPRs. The mPR family belongs to a larger family of proteins called PAQRs, and they were first identified and cloned in fish ovaries (Patino and Thomas 1990), followed by identification in various mammals including mouse, pig, and human (Zhu, Bond, and Thomas 2003; Dressing et al. 2011). Three isoforms are best-known among mPRs, namely mPR α, mPR β, and mPR γ. The MAPR family includes PGRMC1 and PGRMC2 (Meyer et al. 1996; Chen et al. 2010), which share a similar heme-binding domain that resembles a cytochrome b5 domain fold (Mifsud and Bateman 2002) and function with cytochrome P450 (Ryu, Klein, and Zanger 2017).

Studies of novel progesterone effects in response to nonclassical progesterone receptor actions have reported various anti-mitotic and anti-apoptotic effects resulting from progesterone and its receptors in granulosa/luteal cells and oocytes (Guo et al. 2016; Peluso et al. 2014; Qiu et al. 2008; Thomas, Pang, and Dong 2014). These results also suggest the possibilities of analogous effects in porcine embryos. However, similar progesterone-related studies

regarding porcine embryos are scarce.

Therefore, the objective of this study was to examine the influences of progesterone during early development (from zygote to blastocyst) of *in vitro* produced porcine embryos in relation to progesterone receptors. We examined the gene expression levels of different progesterone receptors in *in vitro* produced porcine embryos and treated them with two concentrations of progesterone for different periods to compare the developmental rates and qualities of late blastocysts. The expression levels of PGRMC1, PGRMC2, PAIRBP1, and apoptosis-related genes were investigated in blastocysts. Finally, an inhibition and rescue assay using the PGRMC1 inhibitor AG-205 and progesterone was performed to verify PGRMC1 involvement and progesterone effects.

MATERIALS AND METHODS

1. Chemicals and reagents

Unless stated otherwise, all chemicals used in the present study were obtained from Sigma-Aldrich (St Louis, MO, USA). Progesterone (#P0130, purity ≥ 99%) and AG-205 (#A1478, purity ≥ 99% by high performance liquid chromatography) were diluted in DMSO (dimethyl sulfoxide) and stored at -20℃ until use. The supplements were mixed with PZM-3 (porcine zygote medium 3) to obtain the final concentration.

2. Recovery of oocytes and *in vitro* maturation

Ovaries were collected from prepubertal gilts at Dodram abattoir (Anseong, Kyunggi Province, South Korea) and transported to the laboratory in physiological saline within 2 h of extraction. The follicular fluid and COCs (cumulus-oocyte complexes) within 3 to 6 mm diameter follicles were aspirated using an 18-gauge needle attached to a 10 mL syringe and then pooled into 50 mL conical tubes to obtain sediments. After sedimentation, the supernatant was discarded, and the sediment was washed once with TL-HEPES-PVA medium. Then, oocytes with intact, unexpanded cumulus cell layers and granulated cytoplasm were selected under an inverted microscope for IVM. The selected COCs were cultured for 44 h at 39.8℃, 5% CO_2 and 100% humidity in TCM-199 (Tissue Culture

Medium 199; Life Technologies, Carlsbad, CA, USA) containing 10 ng/mL epidermal growth factor, 1 mg/mL insulin and 10% PFF (porcine follicular fluid). During the first half of the 44 h, COCs were treated with Q6 equine chorionic gonadotropin and human chorionic gonadotropin hormones (4 IU/mL; Intervet, Cambridge, UK), and COCs were cultured in hormone-free conditions for the latter half.

PFF was aspirated from 3 to 6 mm diameter follicles from prepubertal gilt ovaries and centrifuged at 4000 rpm for 30 min. The supernatant was then collected and filtered using 0.2 μm syringe filters. The prepared PFF was stored at -20℃ until use.

3. *In vitro* fertilization and culture

For sperm preparation, commercial boar semen samples from boars with proven fertility (Duroc) were supplied by the DARBI AI Center (Jochiwon, Sejong-si, South Korea). Delivered semen samples were diluted with DPBS (Dulbecco's Phosphate Buffered Saline) supplemented with 0.4% BSA (bovine serum albumin) and centrifuged at 1400 rpm for 3 min. This washing step was repeated twice for each sample. The sperm pellet was then re-suspended in mTBM (modified Tris-buffered medium) containing 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2 \cdot 2H_2O$, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate, and 0.8% (w/v) BSA to give a concentration of 2 x

10^6 cells/mL.

Upon completion of IVM, oocytes were removed from cumulus cells by repeated pipetting in TCM-199 with 1 mg/mL hyaluronidase. The denuded oocytes were then randomly placed in 50 μL mTBM droplets covered with prewarmed mineral oil in numbers of 20 to 30. Sperm suspension was then added to each drop to give a final sperm concentration of 2 x 10^5 cells/mL. After coincubation of gametes at 39.8℃, 5% CO_2 and 100% humidity for 4 h, oocytes were washed twice with DPBS supplemented with 0.4% (w/v) BSA, and any sperm attached to the oocytes were removed by repeated pipetting.

The washed oocytes were transferred to 500 μL of PZM-3 and cultured up to 7 days at 38.5℃ with an atmosphere of 5% CO_2, 5% O_2, and 90% N_2. Supplementation with 0.2% DMSO containing progesterone (10 nM and 100 nM), AG-205 (1 μM, 2 μM, 5 μM, 15 μM and 50 μM) or no other substances was provided to each experimental group at the beginning of or during culture. The cleavage rate, morula formation rate and blastocyst formation rate were evaluated under an inverted microscope at 2, 4 and 7 days after fertilization, respectively. Embryos that developed to or past the 2-cell stage at 2 days post-fertilization were defined as normally cleaved embryos.

4. Quantitative real time-PCR

Pools of *in vitro* produced embryos from each stage (2-cell, n=35; 4-cell, n=20; 8-cell, n=15; morula, n=10; and blastocyst, n=6) were processed with the Dynabeads mRNA DIRECT Kit (Invitrogen) according to the manufacturer's instructions. Shortly after mRNA extraction, cDNA (complementary DNA) was synthesized using a High-Capacity RNA-to-cDNA Kit (Applied Biosystems) following the manufacturer's protocols, and synthesized cDNA was stored at -20℃ until use.

For qPCR (quantitative real-time PCR), extracted cDNA samples were mixed with the DyNAmo HS SYBR Green qPCR Kit (Thermo Fisher Scientific) with 1 μM of each primer set listed in Table 1 added to complete a reaction volume of 10 μL. Amplification and detection were conducted using the ABI 7300 Real-Time PCR system (Applied Biosystems) under the following conditions: one cycle of 50℃ for 2 min and 95℃ for 10 min, followed by 40 cycles of denaturation at 95℃ for 15 sec and annealing/extension at 60℃ for 1 min. The dissociation curves were analyzed to confirm the specificity of the product. Relative expression levels of mRNA were analyzed by normalizing the Ct (threshold cycle) values of each gene to that of GAPDH (glyceraldehyde 3-phosphate dehydrogenase) via

the delta-delta Ct method. The list of primers is shown in Table 2.

TABLE 2. Primer sets used in qPCR

Genes	Primers	Sequence (5' -> 3')	Gene bank accession number	Product size (bp)
PR	Forward	CCCTAGCTCACAGCGTTTCT	NM_001166488.1	184
	Reverse	CACCATCCCTGCCAATATCT		
PGRMC1	Forward	ACCAACGTAGTCATCCACGG	NM_213911.1	187
	Reverse	GCCCCTCTCTCTGTCCAATC		
PGRMC2	Forward	AAACCAGGGGAAGAACCGTC	NM_001097521.2	150
	Reverse	ACCCAAAGACGCTGGACATT		
mPR α (PAQR7)	Forward	ATCCTCTCGAAGCTCCGTTC	NM_213739.1	131
	Reverse	CAGGGAGCCTAGATCACACAG		
mPR β (PAQR8)	Forward	ACGTCCTGTCCTCCATCACT	NM_213740.1	262
	Reverse	CGGTAACGGTACTTGGCGTA		
Bcl-xL	Forward	ACTGTGCGTGGAGAGCGTAG	AF216205.1	87
	Reverse	AGGTGGTCATTCAGGTAAGTGG		
BCL2	Forward	GAAACCCCTAGTGCCATCAA	NM_214285.1	196
	Reverse	GGGACGTCAGGTCACTGAAT		
CRADD	Forward	TTGCAGACGGGCACATGG	XM_021092768.1	125
	Reverse	CGTCAAGATCCCTTCCTGGT		
BAD	Forward	TTGCCAGCCGAGATTAACCC	XM_021082883.1	101
	Reverse	ACACGCGGGCTTTATTAGCA		
BAK1	Forward	CAGCCGACAGCGGAAAAC	AJ001204.1	109
	Reverse	GGTAGCCAAAGCCCAGAAGA		
CASP3	Forward	GAACTCTAACTGGCAAACCCAA	AB029345.1	84
	Reverse	CACTGTCCGTCTCAATCCCA		
PAIRBP1 (SERBP1)	Forward	GACAAGAAGGAGGAGACGCA	XM_003127935.4	212
	Reverse	CGGTCAATAATCGGTCTATCAACT		
GAPDH	Forward	TGCTCCTCCCCGTTCGAC	NM_001206359.1	100
	Reverse	ATGCGGCCAAATCCGTTC		

5. Cell counts and measurement of apoptosis

For fixation, blastocysts from day 7 of culture were fixed in 4% (w/v) paraformaldehyde at room temperature for 15 min and washed with DPBS supplemented with 0.4% BSA. The In Situ Cell Death Detection

Kit (Roche, Mannheim, Germany) was then used for the TUNEL assay according to the manufacturer's instructions. Following the last set of washes, blastocysts were mounted and stained overnight with ProLong™ Gold anti-fade reagent with DAPI (Invitrogen) on slides with cover glasses. Subsequently, blastocysts were visualized under a UV light using a micromanipulator equipped with differential interference contrast optics (eclipse TE2000-U; Nikon, Tokyo, Japan). Total cell numbers were determined by counting the number of nuclei stained blue with DAPI (4',6-diamidino-2-phenylindole), and nuclei displaying distinct red labeling that overlapped DAPI-staining were considered to be TUNEL-positive. Apoptosis rates were calculated by dividing the number of TUNEL-positive cells with the number of total cells.

6. Statistical analysis

Data obtained in this study were analyzed using the GraphPad Prism 6 statistical program (GraphPad Software). Developmental rate data were analyzed using two-way ANOVA and Dunnett's multiple comparisons test. The cell numbers, apoptosis rates and relative transcription levels of embryos were analyzed using one-way ANOVA and Tukey's multiple comparison's test or Dunnett's multiple comparisons test depending on the purpose of each analysis. All data

were expressed as the mean ± S.E.M. (standard error of the mean), and p-values less than 0.05 were considered statistically significant.

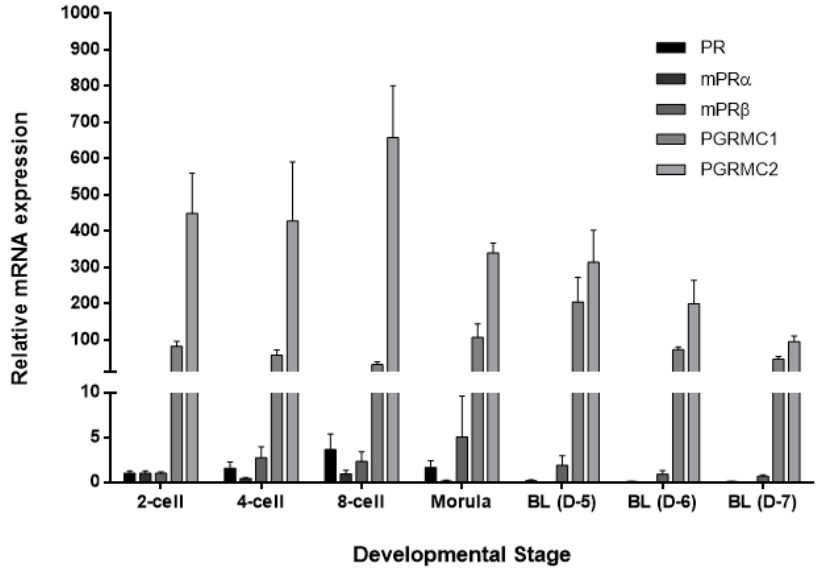
RESULTS

Determining the expression patterns of progesterone receptors in porcine embryos

Prior to progesterone treatment, we tested if the *in vitro* produced porcine embryos retain the required progesterone receptors. Figure 1a shows that gene expression levels of PGRMC1 and PGRMC2 were excessively higher (25− to 200−fold) than those of other progesterone receptors, with PGRMC2 maintaining the highest level in all developmental stages. In addition, the embryos expressed all of the investigated progesterone receptors before the morula stage of development, but the expression levels decreased through the blastocyst stage.

However, the expression pattern of PGRMC1 was distinct from the other receptors as shown in Figure 1b. PGRMC1 maintained a relatively constant level from zygote to 8−cell stage, and it increased significantly until the day 5 blastocyst stage and decreased back to its initial level by the day 7 blastocyst stage. In summary, different levels of gene expression were observed among the progesterone receptors, and the expression pattern of PGRMC1 differed from that of others.

(a)


Relative mRNA expression
1000
900
800
700
600
500
400
300
200
100
10
5
0
PR
mPRα
mPRβ
PGRMC1
PGRMC2
2-cell
4-cell
8-cell
Morula
BL (D-5)
BL (D-6)
BL (D-7)
Developmental Stage

(b)

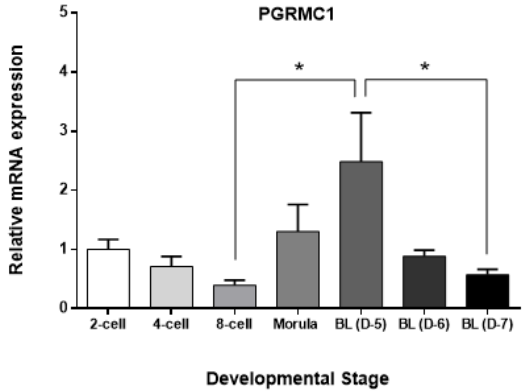

PGRMC1
Relative mRNA expression
5
4
3
2
1
0
*
*
2-cell
4-cell
8-cell
Morula
BL (D-5)
BL (D-6)
BL (D-7)
Developmental Stage

Figure 1. Gene expression levels of progesterone receptors in developmental stages of porcine embryos.

(a) Gene expression levels of PR, mPRα, mPRβ, PGRMC1, and PGRMC2 in subsequent developmental stages of embryos from 2-cell to BL (D-7). (b) Specific gene expression pattern of PGRMC1. Asterisks indicate significant difference ($p<0.05$) between groups. Values are means$\pm$S.E.M. of at least 3 replicates. BL, Blastocysts.

Identification of progesterone effects on early development of porcine embryos

Progesterone (10 nM and 100 nM) was dissolved in DMSO and used to treat the *in vitro* produced porcine embryos from day 0 (day of fertilization). Before the actual treatment, a preliminary test of DMSO toxicity was performed to verify that 0.2% DMSO had no influences on embryo development. Figure 2a clearly shows that the developmental properties of the 0.2% DMSO treatment group had no significant differences with the control group. Thus, 0.2% DMSO was used as the control for all following experiments.

As shown in Figure 2b, 10 nM and 100 nM progesterone showed no significant changes in morula formation rate at day 4 or blastocyst formation rate at day 7. However, there was an increase in the cleavage rate in the 10 nM treatment group. Furthermore, there was a significant increase in cell numbers (Figure 2c) and decrease in the apoptosis rate (Figure 2d) in day 7 blastocysts in the 100 nM progesterone treatment group. The group treated with 10 nM progesterone also showed increased cell numbers and decreased apoptosis rates in day 7 blastocysts, but the changes were insignificant compared to the nontreated control group.

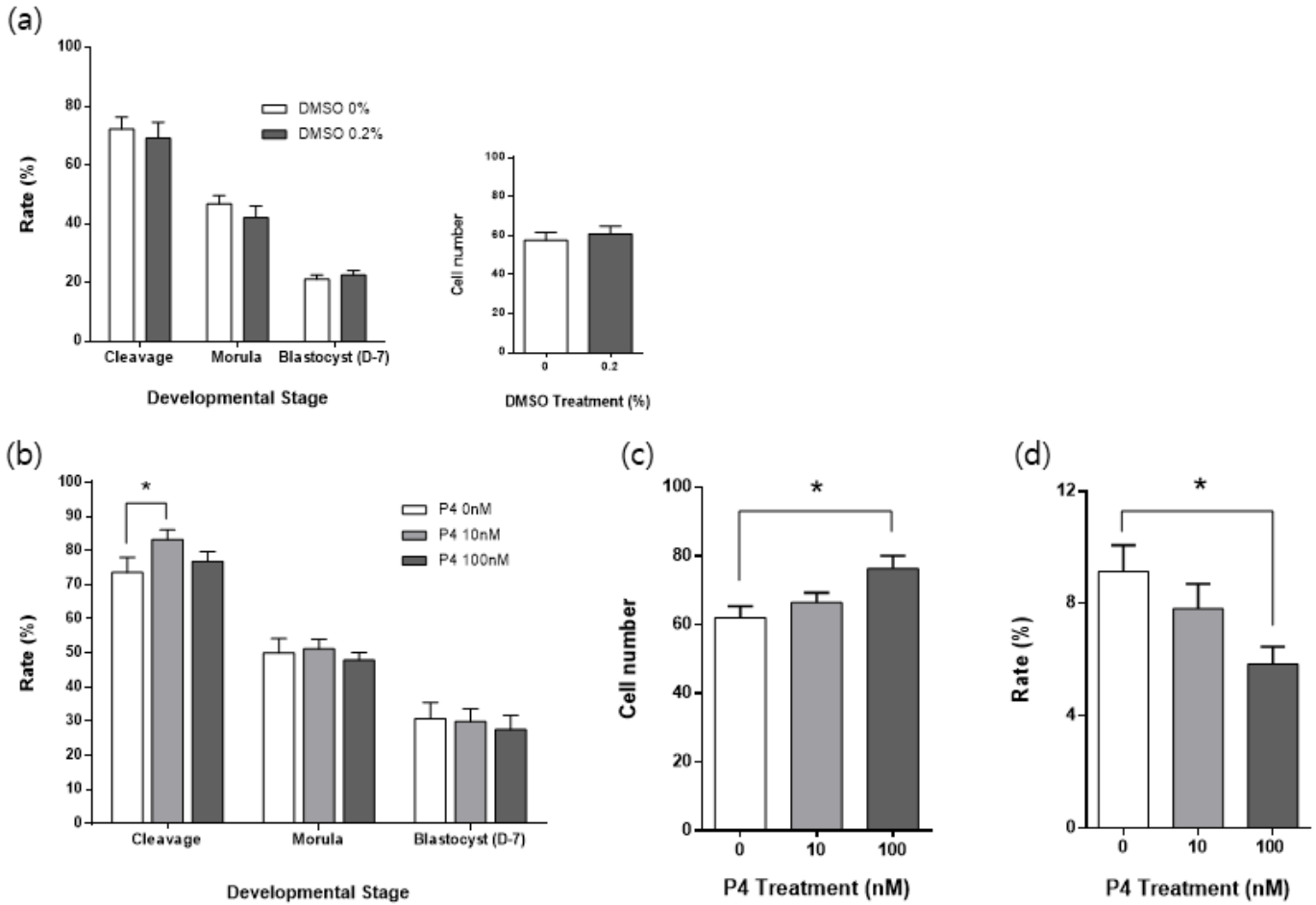

(a)
Rate (%)
DMSO 0%
DMSO 0.2%
Cleavage
Morula
Blastocyst (D-7)
Developmental Stage
Cell number
DMSO Treatment (%)
(b)
P4 0nM
P4 10nM
P4 100nM
Rate (%)
Cleavage
Morula
Blastocyst (D-7)
Developmental Stage
(c)
Cell number
P4 Treatment (nM)
(d)
Rate (%)
P4 Treatment (nM)

Figure 2. Effect of progesterone treatment from day 0 on porcine embryo development.

(a) Preliminary test of 0.2% DMSO toxicity on embryo developmental capacity. (b) Effect of progesterone treatment from day 0 on embryo developmental rates. (c) Cell numbers and (d) apoptosis rates of day 7 blastocysts derived from embryos treated with or without progesterone from day 0. Asterisks indicate significant difference ($p<0.05$) between groups. Values are means±S.E.M. of at least 3 replicates. P4, Progesterone.

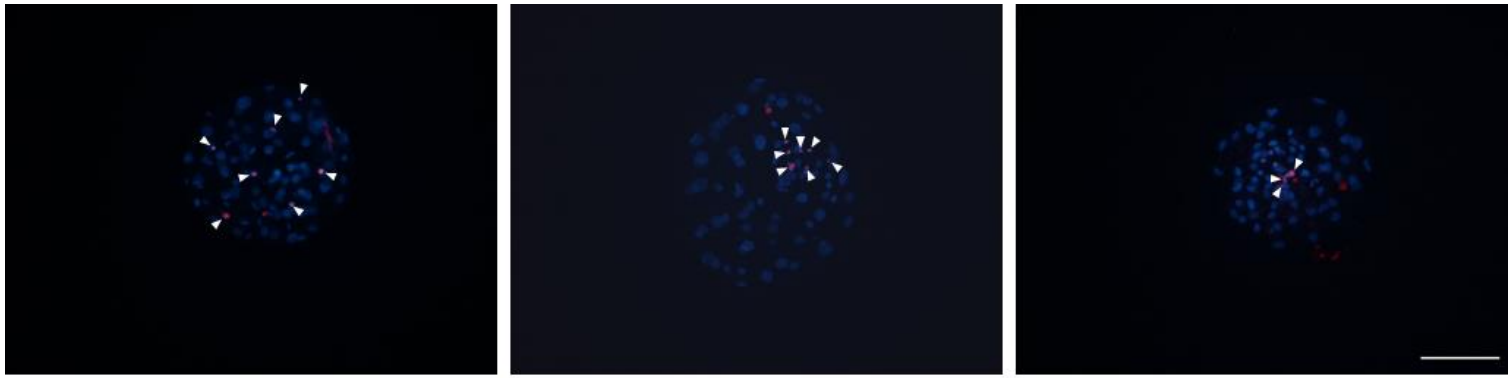


Figure 3. Apoptosis in blastocysts of porcine embryos treated with or without progesterone.

Day 7 blastocysts derived from embryos cultured in PZM-3 supplemented (a) without progesterone or (b) with 10 nM progesterone or (c) 100 nM progesterone. Nuclei (blue) are stained with DAPI and apoptotic nuclei (pink) are marked by arrows. Scale bar: 100 μm.

Verification of the action of PGRMC1

Progesterone (10 nM and 100 nM) was dissolved in DMSO and used to treat the *in vitro* produced porcine embryos from day 4. The outcomes appeared similar with those of day 0 treatment because, the developmental rates were indifferent from that of the control group (Figure 4a). There was a significant increase in cell numbers (Figure 4b) and decrease in the apoptosis rate (Figure 4c) in day 7 blastocysts in the 100 nM progesterone treatment group. However, there was no change in the cleavage rate in the 10 nM progesterone treatment group.

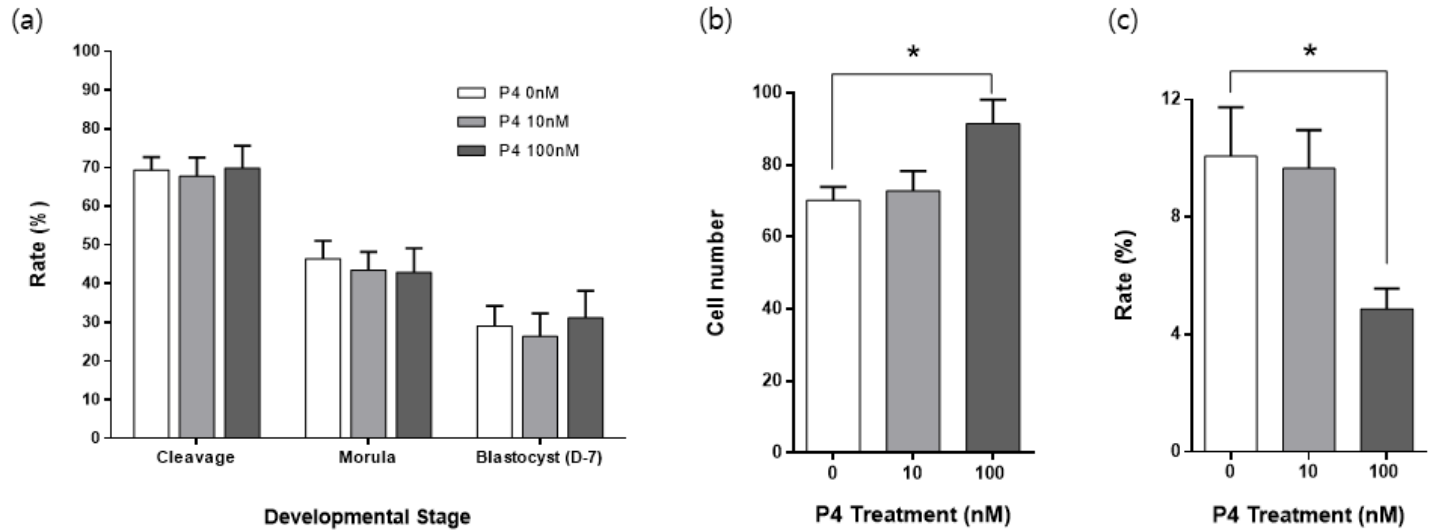

(a)
Rate (%)
100
90
80
70
60
50
40
30
20
10
0
P4 0nM
P4 10nM
P4 100nM
Cleavage
Morula
Blastocyst (D-7)
Developmental Stage
(b)
Cell number
100
80
60
40
20
0
*
0
10
100
P4 Treatment (nM)
(c)
Rate (%)
12
8
4
0
*
0
10
100
P4 Treatment (nM)

Figure 4. Effect of progesterone treatment from day 4 on porcine embryo development.

(a) Effect of progesterone treatment from day 4 on embryo developmental rates. (b) Cell numbers and (c) apoptosis rates of day 7 blastocysts derived from embryos treated with or without progesterone from day 4. Asterisks indicate significant difference ($p<0.05$) between groups. Values are means±S.E.M. of at least 3 replicates.

qPCR analysis of genes related to PGRMC1 action and apoptosis

Gene expression levels of PGRMC1, PGRMC2, and PAIRBP1 were observed in the control group and progesterone treatment groups via qPCR (Figure 5a). The transcript level of PGRMC1 in day 7 blastocysts in the 100 nM progesterone treatment group significantly increased compared to other groups, whereas PGRMC2 did not show any significant difference among groups. Furthermore, a well-known partner protein of PGRMC1, PAIRBP1, also showed a significant increase in gene expression level in day 7 blastocysts in the 100 nM progesterone treatment group.

In addition, the gene expression levels of six apoptosis-related genes were examined in the control group and 100 nM progesterone treatment group (Figure 5b). Among the six genes were two anti-apoptotic genes, Bcl-xL and BCL2, two pro-apoptotic regulator BH3-only genes, CRADD and BAD, and two pro-apoptotic executioner genes, BAK1 and CASP3. The expression levels of all genes, except for CRADD and BAK1, showed significant increases in day 6 blastocysts in the 100 nM progesterone-treated embryos, and these levels rapidly decreased to a similar level with the control group by the next day (day 7 blastocysts). When the Bcl-xL/BAK1 ratio was compared, the 100 nM treatment group showed a

significantly higher ratio in day 6 blastocysts (Figure 5c).

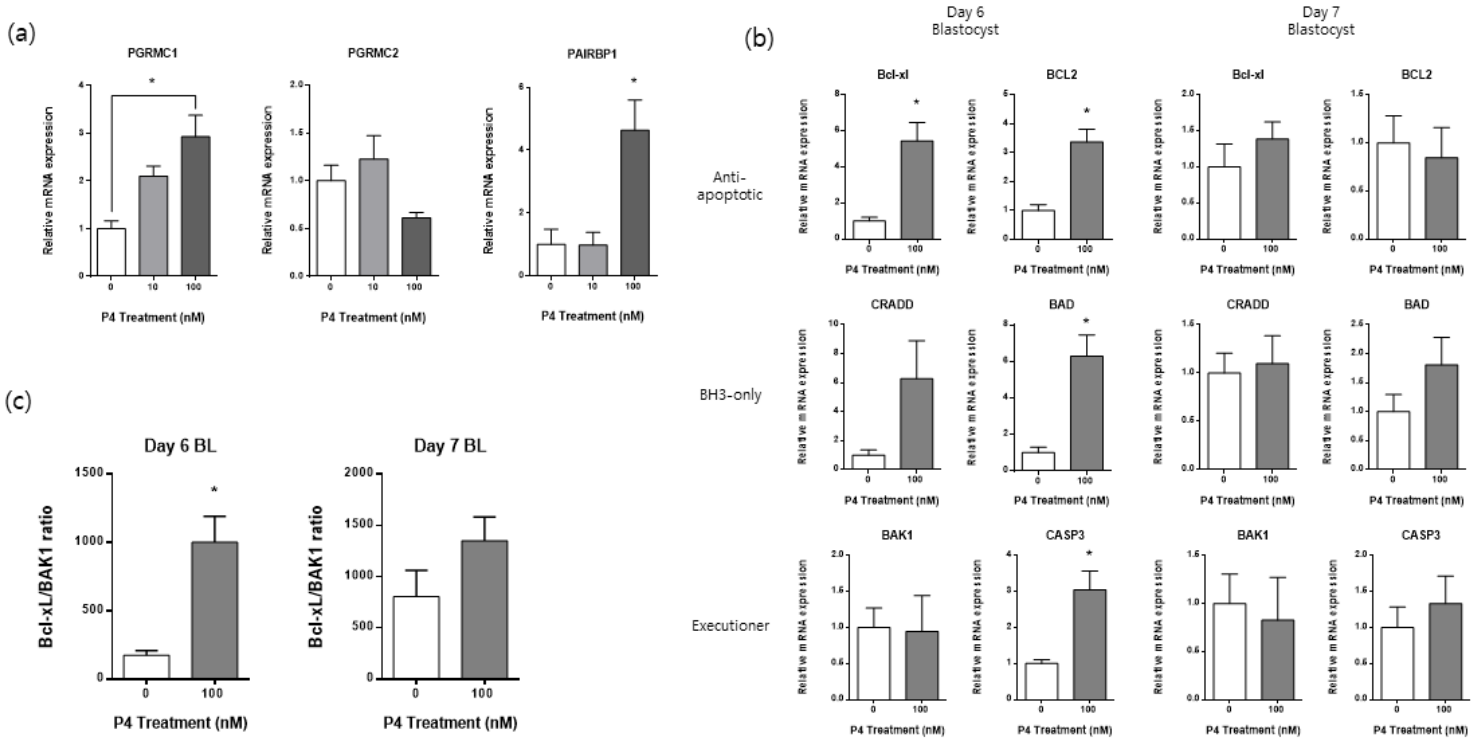

(a)
PGRMC1
PGRMC2
PAIRBP1
Relative mRNA expression
P4 Treatment (nM)
(b)
Day 6 Blastocyst
Day 7 Blastocyst
Anti-apoptotic
BH3-only
Executioner
Bcl-xl
BCL2
CRADD
BAD
BAK1
CASP3
(c)
Day 6 BL
Day 7 BL
Bcl-xL/BAK1 ratio

Figure 5. Gene expression levels of PGRMC1, PGRMC2, PAIRBP1, and apoptosis-related genes in blastocysts of porcine embryos treated with progesterone.

(a) Gene expression levels of PGRMC1, PGRMC2, and PAIRBP1 in day 7 blastocysts derived from embryos treated with or without progesterone from day 0. (b) Comparison of gene expression levels of apoptosis-related genes in day 6 and day 7 blastocysts derived from embryos treated with or without 100 nM progesterone from day 0. (c) Gene expression ratio of Bcl-xL/BAK1 in day 6 and day 7 blastocysts derived from embryos treated with or without 100 nM progesterone from day 0. Asterisks indicate significant difference ($p<0.05$) between groups. Values are means±S.E.M. of at least 3 replicates.

Inhibition assay to confirm the PGRMC1-associated progesterone effect

An inhibition assay was performed using AG-205, a widely used inhibitor of PGRMC1. Similar to DMSO, a preliminary test was performed to investigate the toxicity and determine the best treatment concentration of AG-205. AG-205 doses of 50 μM, 15 μM, 5 μM, 2 μM, and 1 μM were dissolved in DMSO and used to treat the *in vitro* produced porcine embryos, and the results showed that 50 μM and 15 μM AG-205 were detrimental to the embryos (data not shown). As shown in Figure 6a, 5 μM AG-205 had negative effects on the blastocyst formation rate in embryos, whereas 1 μM and 2 μM did not show this negative effect. In addition, 1 μM and 2 μM AG-205 did not show any significant difference with each other. Simultaneous treatment of AG-205 (1 μM and 2 μM) and 100 nM progesterone also showed no significant effect on embryo development (Figure 6b).

When 1 μM and 2 μM AG-205 were used to treat embryos without any additional progesterone, both treatment groups showed a decrease in cell numbers (Figure 6c) and an increase in the apoptosis rate (Figure 6d) in day 7 blastocysts compared to the control group. When 1 μM and 2 μM AG-205 were used along with

100 nM progesterone, the cell numbers and apoptosis rates recovered to a similar state as the control group in the 1 μM AG-205 group, whereas only the apoptosis rate was recovered in the 2 μM AG-205 group.

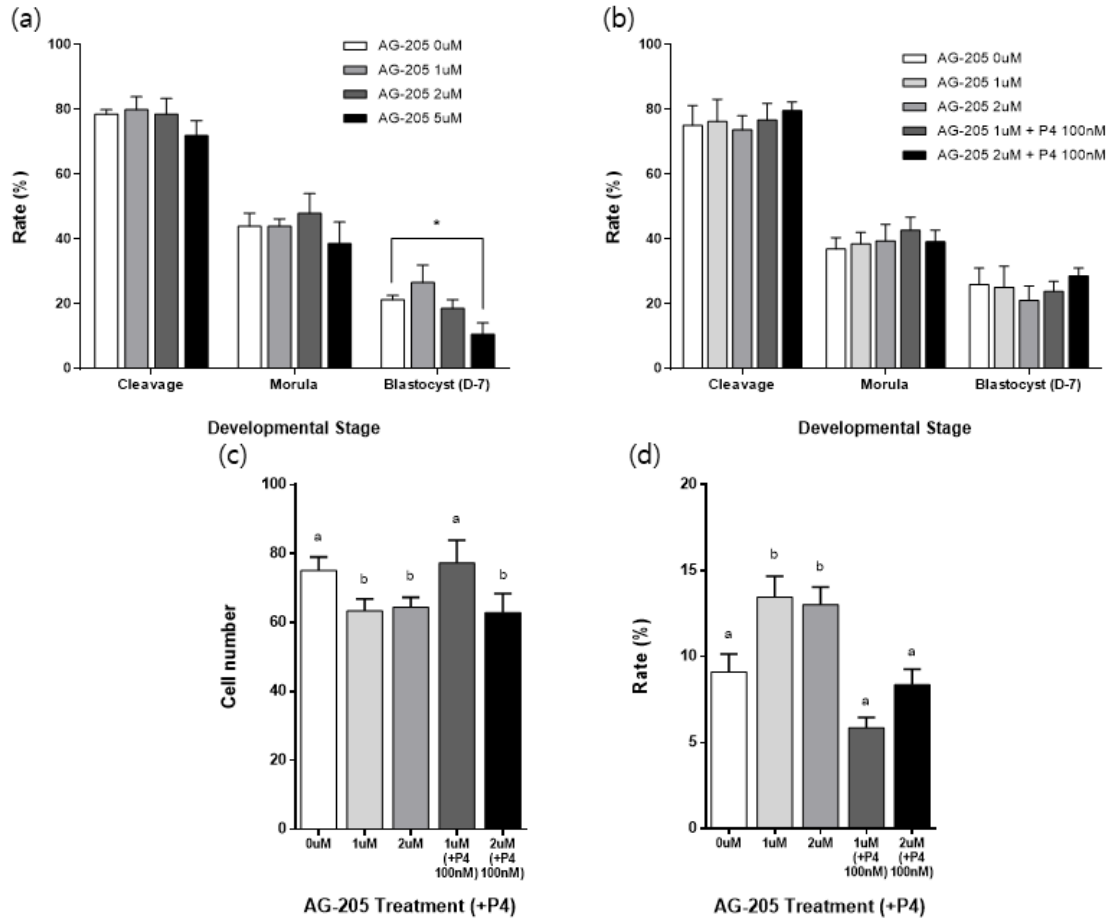

(a)
AG-205 0uM
AG-205 1uM
AG-205 2uM
AG-205 5uM
Rate (%)
Cleavage
Morula
Blastocyst (D-7)
Developmental Stage
(b)
AG-205 0uM
AG-205 1uM
AG-205 2uM
AG-205 1uM + P4 100nM
AG-205 2uM + P4 100nM
Rate (%)
Cleavage
Morula
Blastocyst (D-7)
Developmental Stage
(c)
Cell number
0uM
1uM
2uM
1uM (+P4 100nM)
2uM (+P4 100nM)
AG-205 Treatment (+P4)
(d)
Rate (%)
0uM
1uM
2uM
1uM (+P4 100nM)
2uM (+P4 100nM)
AG-205 Treatment (+P4)

Figure 6. Effect of AG-205 and progesterone treatment on porcine embryo development.

(a) Preliminary test of 1 μM, 2 μM, and 5 μM AG-205 toxicity on embryo developmental capacity. (b) Effect of 1 μM and 2 μM AG-205 treatment with or without 100 nM progesterone from day 0 on embryo developmental rates. (c) Cell numbers and (d) apoptosis rates of day 7 blastocysts derived from embryos treated with AG-205 from day 0. Some embryos were cultured simultaneously with 100 nM progesterone. Asterisks and different superscripts indicate significance difference ($p<0.05$) between groups. Values are means±S.E.M. of at least 3 replicates.

DISCUSSION

It is well known that progesterone is one of the major actors in animal reproduction. Progesterone is mainly produced and secreted from corpus luteum formed after ovulation, and it maintains uterus endometrium in an implantation-suitable state and interacts with gonadotrophins, such as FSH and LH, for cyclicity (Piette 2018). Moreover, the significance of progesterone during and after pregnancy for placenta development has been reported in various mammals (Spencer et al. 2004).

Apart from some of its primary roles during implantation and pregnancy, reports have revealed that progesterone also participates in the maturation of germ cells and fertilization as follows: oocyte maturation (Fair and Lonergan 2012), anti-apoptotic/anti-mitotic effect on granulosa/luteal cells (Grupen and Armstrong 2010; Rueda et al. 2000), ovulation (Robker, Akison, and Russell 2009), and regulation of sperm motility and acrosome reaction (Thomas, Tubbs, and Garry 2009). These findings indicate that progesterone is important in all stages of reproduction beginning from germ cell maturation to later parturition.

Studies regarding the effects of progesterone during preimplantation stages of embryo development are still insufficient compared to studies of other reproductive stages. According to

previous studies, progesterone exists in the blood and lymph of mammals after fertilization, and it is constantly secreted in oviduct and uterus during the early stages of embryo development (Martyniak, Franczak, and Kotwica 2018) and through blastocyst expansion/elongation (Geisert et al. 2017). These findings lead to a reasonable assumption that progesterone may have some significant influences in early embryo development.

Although only few studies regarding progesterone receptors in porcine embryos have been reported, most of them primarily focused only on PR. Therefore, we measured the gene expression levels of several well-established progesterone membrane receptors along with PR and compared them (Figure 1a). PR expression in *in vitro* produced porcine embryos from 2-cell to day 7 blastocyst stages was relatively consistent with the findings reported by Chingwen Ying *et al.* (Ying et al. 2000), who reported that PR transcripts were only detected at 2-cell to 4-cell stages and disappeared afterwards. In the present study, however, PR gene expression was also found in the 8-cell and morula stages. This difference may be due to technical limitations of the previous study because qPCR is more capable of measuring smaller amounts of genes than normal PCR.

The results of the different aspects of receptor expression revealed

some interesting factors. Qiu HB *et al.* (Qiu et al. 2008) and Masayuki Shimada *et al.* (Shimada et al. 2004) reported the actions of mPRβ and PR in porcine granulosa/cumulus cells, respectively. In the present study, the gene expression levels of PR, mPRα, and mPRβ were almost undetectable compared to those of PGRMC1 and PGRMC2, implying that these two receptors may have some important activities in preimplantation embryos. Thus, we hypothesized that if progesterone has influences on porcine embryo development, it would most likely be through PGRMC1 or PGRMC2 actions. The similar expression patterns observed for all the receptors, which showed significant changes before and after the 8-cell to morula stages, suggested that the expression levels of progesterone receptors are in accordance with the maternal zygotic transition of embryos (van Montfoort et al. 2014; Hou and Gorski 1993). Nevertheless, more studies need to be performed.

When two levels of progesterone (10 nM and 100 nM) were used to treat the embryos, the results differed between the two experiments with different treatment durations. The reason for treating progesterone from day 4 was to investigate the likelihood of PGRMC1 as the main regulator of the progesterone effect shown in day 7 blastocysts, which was in accordance with the distinct expression pattern of PGRMC1 shown in Figure 1. The intention was to reduce

the influences of PR, mPRα, mPRβ, and PGRMC2, which all showed decreased gene expression after day 4 of development, and to focus on the influence of PGRMC1.

The experiment with progesterone treatment from day 0 showed a significant increase in cleavage rate in the 10 nM treatment group, but the same result did not occur when progesterone treatment started from day 4. However, this change did not make any difference to the subsequent developmental capacity (morula and blastocyst formation rates) nor to the quality of blastocysts (cell numbers and apoptosis rates) (Figure 2b, 2c, and 2d). Thus, these experiments were not sufficient to conclude the influence of progesterone on the embryos. Because the effect only appeared in the day 0 treatment, it can be assumed that the progesterone effect on the cleavage rate is regulated by other progesterone receptors prevailing before day 4, and only at relatively low hormone concentrations (10 nM).

The quality of day 7 blastocysts, as by cell numbers and apoptosis rates, showed similar results in both experiments. There was a significant increase in cell numbers and a decrease in apoptosis rates in the 100 nM treatment group (Figure 2c, 2d, 3b, and 3c), which was in agreement with previous studies reporting the anti-apoptotic effects of progesterone on rat's spontaneously immortalized

granulosa cells and human granulosa cells (Peluso, Romak, and Liu 2008; Peluso et al. 2009). The results showing positive effects of progesterone on embryos at the 100 nM concentration are quite plausible because 100 nM is close to the physiological amount of progesterone secreted in the oviduct and uterus during the early porcine embryo development *in vivo* (Stefanczyk-Krzymowska et al. 1998).

Because progesterone promoted pro-proliferative and anti-apoptotic effects on early porcine embryos, PGRMC1, rather than PGRMC2, seemed like a more appropriate candidate. In addition, PGRMC1 is best known for its anti-apoptotic actions in various tissues. Correspondingly, the gene expression of PGRMC1 and PAIRBP1, a well-known partner protein associated with the anti-apoptotic actions of PGRMC1 (Peluso et al. 2004), showed an increased expression level in the 100 nM progesterone treatment group (Figure 5a). This finding suggested that progesterone stimulates the expression of PGRMC1 along with its activity by enhancing PAIRBP1 participation. In contrast, the gene expression of PGRMC2 showed no significant differences between treatment groups and the control group (Figure 5a). These data demonstrated that PGRMC1 is more likely to be related to the progesterone effects on embryos than PGRMC2.

The next step was to investigate several of the apoptosis-related genes to elucidate the mechanisms of the anti-apoptotic effects of progesterone on embryos. Unexpectedly, the expression levels of all the investigated genes had no significant difference in day 7 blastocysts between the control and 100 nM treatment groups, but some had significant increases in day 6 blastocysts (Figure 5b). These results were inconsistent with those reported in previous studies by Peluso *et al.* (Peluso et al. 2010), who demonstrated that the CRADD and BAD genes were downregulated by PGRMC1 in rat granulosa cells. A more likely mechanism of progesterone inducing an anti-apoptotic effect involves the upregulation of anti-apoptotic genes and downregulation of pro-apoptotic genes, but this was not the case.

The higher Bcl-xL/BAK1 ratio (Figure 5c) indicated that the anti-apoptotic effect of progesterone shown in Figure 2d and 4c was relevant, which agreed with several studies suggesting the 'rheostat model' of apoptosis initiation (Oltvai, Milliman, and Korsmeyer 1993; Perlman et al. 1999). Because the above data only consists of the expression levels of gene transcripts and not proteins, these data must be interpreted with caution as increased mRNA levels do not always lead to increased biological effects. Hence, the specific mechanism of the anti-apoptotic effect of progesterone on porcine

embryos needs to be further elucidated.

The last step was to confirm that PGRMC1 is involved with the pro-proliferative and anti-apoptotic actions of progesterone on embryos. Inhibition and rescue assays were performed by treating embryos with AG-205 in the presence or absence of 100 nM progesterone. AG-205 is a widely known inhibitor of PGRMC1 applied in numerous mammalian cell types, including ovary, granulosa cells, and cancer cells in cattle (Terzaghi et al. 2016), mice (Guo et al. 2016), and humans (Will, Liu, and Peluso 2017). AG-205 acts as an antagonist to progesterone, and when it binds to PGRMC1, AG-205 alters its spectroscopic structure to inhibit its actions (Ahmed et al. 2010). The concentrations used for the preliminary test of AG-205 toxicity were determined in reference to other studies, but optimum doses for embryos were much lower than for other tissues (Figure 6a). This finding may be due to undefined side effects of AG-205 or may suggest that PGRMC1 has roles that are more significant in the embryo compared to other cells, such as steroidogenesis.

The results of the inhibition assay indicated that the treatment of embryos with 1 μM and 2 μM AG-205 did not result in any impaired developmental capacity (Figure 6b), but the embryos had lower cell numbers and higher apoptosis rates compared to the control group

embryos (Figure 6c and 6d). AG−205 clearly reversed the effects of 100 nM progesterone treatment, which verified the action of PGRMC1. In addition, a rescue assay further confirmed the involvement of PGRMC1 by treating embryos with 100 nM progesterone in the presence of 1 μM and 2 μM AG−205. Although progesterone successfully relieved the anti−proliferative and pro−apoptotic effects of AG−205 in the 1 μM treatment group, the anti−proliferative effect failed to recover in the 2 μM treatment group (Figure 6c and 6d). Interpretation of these outcomes requires caution since the exact mechanisms of PGRMC1 in regulating proliferation and apoptosis are yet to be defined. Thus, we could only assume that there may be some unknown interactions between the downstream actions of PGRMC1 and AG−205 where 100 nM progesterone is sufficient to compete with 2 μM AG−205 for anti−apoptosis but is insufficient for pro−proliferative effects. Nevertheless, the inhibition and rescue assays provided clear evidence of PGRMC1 involvement in the action of progesterone.

In summary, by investigating the expression patterns of multiple progesterone receptor genes and analyzing the influences of progesterone in *in vitro* produced porcine embryos, the present study suggested that progesterone has positive effects on the embryo by enhancing proliferation and suppressing apoptosis in blastocysts.

Furthermore, these progesterone-induced actions involve the nonclassical progesterone receptor, PGRMC1, partly by altering the gene expression levels of apoptosis-related genes through undefined mechanisms. Therefore, this study will help improve the knowledge regarding the *in vitro* production and development of porcine embryos.

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SUMMARY IN KOREAN

프로게스테론은 포유동물의 번식과정에 걸쳐 중요한 역할을 수행한다고 알려진 대표적인 스테로이드계 호르몬이다. 이미 다수의 선행연구들을 통해 프로게스테론이 착상과 임신 및 분만 과정을 조절함이 밝혀져 있으나, 그에 비해 초기 배아 발달에 관련된 연구들은 부족한 상태이다. 따라서, 본 연구에서는 체외생산된 돼지 수정란에서 프로게스테론이 미치는 영향을 알아보고자 하였다. 먼저, 체외 수정된 돼지 배아에서 각 프로게스테론 수용체들의 유전자 발현률을 분석하였는데, PGRMC1과 PGRMC2가 다른 수용체들과 구분되는 독특한 발현 양상을 보임을 확인하였다. 이후, 배아 배양 과정에서 두 가지 농도(10 nM, 100 nM)의 프로게스테론을 두 기간(0일째부터 처리, 4일째부터 처리)으로 나누어 처리하며 배아 발달률, 7일차 배반포의 세포 수와 세포 자살 비율을 살펴보았다. 그 결과, 0일째와 4일째 처리군 모두 유사하게, 100 nM 프로게스테론 처리군에서 7일차 배반포의 세포 수가 증가하고 세포사멸 비율이 감소하였다. 추가적으로, 배반포의 PGRMC1, PGRMC2, PAIRBP1과 세포사멸 관련 유전자들의 발현률을 분석하였을 때, 대조군에 비해 100 nM 프로게스테론 처리군에서 유의미하게 발현률이 증가하는 것을 확인하였다. 이어 배아 배양 과정에서, PGRMC1 저해제인 AG-205를 처리한 뒤 배아 발달률, 7일차 배반포의 세포 수와 세포사멸 비율을 대조군과 비교하였고, 일부 실험군에 100 nM 프로게스테론을 함께 처리하여 AG-205의 저해

효과가 회복되는지도 살펴보았다. 그 결과, 1 μM과 2 μM의 AG-205를 처리한 실험군들에서 7일차 배반포의 세포 수가 감소하고 세포사멸 비율이 증가하는 것을 볼 수 있었고, 이러한 AG-205의 저해 효과가 100 nM의 프로게스테론을 함께 처리하였을 때 회복되는 것 또한 확인하였다. 결론적으로 본 연구를 통해, 프로게스테론이 체외생산된 돼지 수정란의 초기 발달 과정에서 배반포의 세포 수를 증가시키고 세포사멸을 감소시키는 긍정적 효과를 가지며, 이러한 프로게스테론 효과는 PGRMC1에 의해 매개됨을 검증하였다. 그러나, PGRMC1의 구체적인 작용 기전에 대해서는 추가적인 연구가 필요할 것으로 사료된다.

주요어 : 돼지의 체외생산 수정란, 배아 발달, 프로게스테론, PGRMC1, 배반포, 세포사멸

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