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Function and regulation of AMP-activated protein kinase in in vitro development and lipid metabolism of preimplantation bovine embryos

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Dedicated to

My beloved family

Function and regulation of AMP-activated protein kinase in in vitro development and lipid metabolism of preimplantation bovine embryos

One of the major problems associated with the in vitro production of bovine embryos is lipid accumulation due to the presence of serum in culture media. AMP-activated protein kinase (AMPK) is well documented as a master regulator of energy metabolism, particularly lipid metabolism, in mammalian cells. The present study aimed to explore the potential role of the AMPK pathway in bovine embryos related to lipid accumulation. For this, the whole study was divided into 3 experiments. In experiment 1, the AMPK activity of in vitro produced blastocysts in the presence or absence of serum was analyzed and associated with lipid metabolism phenotypes. In experiment 2, 5-aminoimidazole-4-carboxamide riboside (AICAR) was applied to enhance AMPK activity during in vitro culture, to investigate its effect on embryo development rate, blastocyst cell number, AMPK activity, mitochondrial activity and the expression of lipid metabolism genes. Furthermore in experiment 3, the post transcriptional regulatory mechanism of AMPKA1 gene was investigated in bovine granulosa cells. The results showed that the presence of serum suppressed the expression of genes in the AMPK pathway (AMPKA1, STK11) and mitochondrial activity (CPT1, PGC1A), while the expression of lipid synthesis gene (ACC) was significantly increased. In addition to the reduced activity of AMPK, the presence of serum increased the level of ROS accumulation followed by low mitochondrial activity in resulting blastocysts. The application of AICAR during embryo culture showed no impact on embryo development rate but significantly reduced blastocyst cell number. The presence of AICAR increased the expression AMPKA1, STK11, CPT2, PGC1A, NQO1 genes, while ACC was suppressed. In experiment 3, *in silico* study predicted 11 miRNAs as potential regulators of AMPKA1. The miRNA profiling in granulosa cells showed that miR-33b is the most abundant miRNA accompanied by low expression of AMPKA1 and high lipid droplet accumulation. Luciferase assay validated the binding of miR-33b and the 3'UTR of AMPKA1. Inhibition of miR-33b resulted in up regulation of AMPKA1 and significantly increases PGC1A gene expression. Moreover, miR-33b was highly expressed in blastocysts derived from culture with serum, while AMPKA1 showed a reverse pattern compared to without serum. Altogether, the present study demonstrates that expression of AMPK and lipid metabolism pathway in blastocysts were affected by the presence of serum in culture media leading to accumulation of lipid.

Funktion und Regulation der AMP aktivierte Proteinkinase in der in vitro Produktion und dem Lipidmetabolismus von präimplantierten Embryonen

Ein Hauptproblem bei der in vitro Produktion von bovinen Embryonen ist die Lipidanreicherung, die durch das im Kulturmedium enthaltene Serum entsteht. AMP aktivierte Proteinkinase (AMPK) ist ein Hauptregulator für den Energiemetabolismus vor allem des Lipidmetabolismus in Säugetierzellen. Das Ziel der vorliegenden Studie war die Rolle des AMPK Signalwegs in bovinen Embryonen in Verbindung zur Lipid Anreicherung zu untersuchen. Dafür unterteilte sich die vorliegende Studie in 3 experimentelle Ansätze. Im ersten Experiment wurde die AMPK Aktivität in in vitro produzierten Blastozysten in der An- und Abwesenheit von Serum analysiert und in Verbindung zum Lipidmetabolismus gesetzt. Im zweiten Experiment kam AICAR für die Verbesserung der AMPK Aktivität während der in vitro Kultur zum Einsatz. Dabei sollte der Effekt von AICAR auf die embryonale Entwicklungsrate, Blastozystenzellzahl, AMPK Aktivität, Mitochondrienaktivität und die Expression von Genen des Lipidmetabolismus untersucht werden. Zusätzlich wurden im dritten Experiment post transkriptionelle regulatorische Mechanismen des AMPKA1 Genes in bovinen Granulosazellen untersucht. Die Ergebnisse zeigten, dass das Serum die Expression der Gene des AMPK Signalwegs (AMPKA1, STK11) und der Mitochondrienaktivität (CPT1, PGC1A) unterdrückt, wohingegen die Genexpression der Lipidsynthese (ACC) signifikant erhöht war. Zusätzlich zu der reduzierten AMPK Aktivität erhöht das Serum das ROS Level gefolgt von einer geringen Mitochondrienaktivität in den Blastozysten. Die AICAR Zugabe während der Embryokultur zeigte keinen Einfluss auf die embryonale Entwicklungsrate, aber eine signifikant reduzierte Blastozystenzellzahl. AICAR erhöhte die Genexpression von AMPKA1, STK11, CPT2, PGC1A sowie NQO1 und unterdrückte die Genexpression von ACC. Die in Experiment drei durchgeführte *in silico* Untersuchung erbrachte 11 miRNAs als potentielle AMPKA1 Regulatoren. Die miRNA Profilanalyse in Granulosazellen zeigte, dass die miR-33b am höchsten detektiert war woraus eine geringe AMPKA1 Expression sowie eine hohe Lipidtröpfchenanreicherung resultierte. Durch den Luciferase Assay konnte die Bindung zwischen der miR-33b und der 3'UTR des AMPKA1 überprüft werden. Eine miR-33b Hemmung resultierte in einer Hochregulierung des AMPKA1 und einen signifikanten Anstieg der PGC1A Genexpression. Außerdem war die miR-33b von in Serum kultivierten Blastozysten hoch exprimiert, wohingegen AMPKA1 ein gegenläufiges Muster im Vergleich zu serumfreien Blastozysten zeigte. Zusammenfassend veranschaulicht die vorliegende Studie, dass die AMPK Expression und der Signalweg des Lipidmetabolismus in Blastozysten durch das Serum im Kulturmedium beeinflusst werden sowie zur Lipidanreicherung führt.

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List of abbreviations

A	Adenine
ACC	Acetyl-CoA carboxylase alpha
Ago	Argonaute
AICAR	5-aminoimidazole-4-carboxamide riboside
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
AMPKA1	Protein kinase, AMP-activated, alpha 1 catalytic subunit
AMPKA2	Protein kinase, AMP-activated, alpha 2 catalytic subunit
ANOVA	Analysis of variance
ATP	Adenosin triphosphate
bp	Base pair
BSA	Bovine serum albumin
BSAFAF	Bovine serum albumin fatty acid free
C	Cytosine
CT	Cycle threshold
CaMKK	Ca^{2+}/calmodulin-dependent protein kinases
cDNA	Complementray deoxyribonucleic acid
CoA	Coenzyme A
COC	Cumulus oocyte complex
CPT1	Carnitine palmitoyltransferase 1B
CPT2	Carnitine palmitoyltransferase 2
DAPI	4',6-diamidino-2-phenylindole
ddH_2O	Double distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
eCG	Equine chorionic gonadotropin
ECS	Estrus cow serum
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
ETC	Electron transport chain
FA	Fatty acid

FCS	Fetal calf serum
FSH	Follicle stimulating hormone
G	Guanine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
hCG	Human chorionic gonadotropin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IU	International Unit
IVC	In vitro culture
IVF	In vitro fertilization
IVM	In vitro maturation
IVP	In vitro production
LB	Luria bertani
LD	Lipid droplet
LH	Luteinizing hormone
LOS	Large offspring syndrome
MAPK	Mitogen activated protein kinase
miR-33b	Micro RNA 33b
miRNA	Micro RNA
mRNA	Messanger RNA
mtDNA	Mitochondrial DNA
NQO1	NAD(P)H ,nicotinamide adenine dinucleotide (phosphate) dehydrogenase, quinone 1
OD	Optical density
Oxphos	Oxidative phosphorylation
PBS	Phosphate buffer saline
PGC1A	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha;
PVA	Polyvinyl alcohol
qRT-PCR	Quantitative real time PCR
RFU	Relative fluorescent unit
RISC	RNA-induced silencing complex
ROS	Reactive oxygen species
rpm	Revolutions per minute
SOF	Synthetic oviduct fluid
SREBP1	Sterol regulatory element binding transcription factor 1
SREBP2	Sterol regulatory element binding transcription factor 2

STK11	Serine/threonine kinase 11
TCA	Tricarboxylic acid
TCM	Tissue culture media
TG	Triglycerides
U	Uracil
UV	Ultra violet
v/v	Volume by volume
x *g*	Gravity

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1 Introduction

Despite the success of embryo transfer from IVP technology in the presence of serum, many problems attributed to low quality of embryos still remain unsolved (Crosier et al. 2001, Lazzari et al. 2002, Smith et al. 2009, Viuff et al. 2001). One of the major problems of IVP embryos is low freezing tolerance which is associated with enhanced cytoplasmic lipid accumulation compared to those derived from in vivo or those cultured without serum (Abe et al. 2002, Gomez et al. 2008). Excess lipid formation is suggested to come from culture environment which modified internal embryo properties and lead to lipid accumulation (Abe et al. 2002, Abe and Hoshi 2003, Ferguson and Leese 1999, Gomez et al. 2008). Addition of serum to embryo culture media provides beneficial components for embryo development such as nutrients, growth factors and antioxidants which subsequently increased blastocyst formation (Oliveira et al. 2006, Rizos et al. 2003, Sudano et al. 2011, Wrenzycki et al. 2001). On the other hand, serum has been shown to cause harmful effects on embryo metabolism by increasing the production of oxidative stress leading to reactive oxygen species (ROS) accumulation which alter mitochondrial structure and function (Abe et al. 2002, Cagnone and Sirard 2014, Crosier et al. 2001, Thompson et al. 2007). Reports have noted that oxidative stress has a negative effect on early embryonic development by altering gene expression as well as embryo metabolism (Balasubramanian et al. 2007, Harvey et al. 2002, Rinaudo et al. 2006), lowering embryo quality, increasing apoptotic rate and fragmentation (Khurana and Niemann 2000b, Liu and Keefe 2000) and also increasing intracellular lipid accumulation (Abe et al. 2002, Barcelo-Fimbres and Seidel 2007a, Sudano et al. 2011).

Intracellular lipid accumulation is a hallmark of cellular stress. It is mediated by oxidative stress which hampers mitochondrial integrity and activity in excessive ROS production (Alfaradhi et al. 2014, Lee et al. 2013, Tanaka et al. 2008). Oxidative stress has been reported as the causative factor for mitochondrial dysfunction (Bonnard et al. 2008, Lockman et al. 2012) or *vice versa* (Kumari et al. 2014, Murphy 2013). Excessive generation of ROS has been reported to modulate mitochondrial function through β-oxidation process (Van Hoeck et al. 2013b). Moreover, the homeostasis of lipid metabolism is controlled by mitochondrial activity where low activity is demonstrated to be associated with increased lipid accumulation (Somfai et al. 2011). To the best of our knowledge, the mechanism of intracellular lipid accumulation in embryos derived from in vitro culture is still unclear. However, several lines of evidences documented that embryos are able to take up fatty acid as the basic of lipid

molecules, which are available in culture environment (Barcelo-Fimbres and Seidel 2007a, Ferguson and Leese 1999, Sata et al. 1999). Fatty acids are metabolized by mitochondrial β-oxidation and when their supply is plentiful there is an overload of mitochondria activity (Koves et al. 2008) that results in elevated ROS production (Burton et al. 2003). In bovine embryos, high ROS level increases lipid accumulation and lipid stress pathway (Cagnone and Sirard 2014, Gomez et al. 2008) by reducing mitochondrial activity (Amin et al. 2014). Moreover, it is known that mtDNA is sensitive to ROS and any disruption resulted in mitochondrial dysfunction (Yakes and Van Houten 1997).

Recent study in our group showed that blastocysts derived from alternate culture of preimplantation embryo in vitro and in vivo, resulted in differential expression of protein kinase, AMP-activated, alpha 1 catalytic subunit (PRKAA1) or namely AMPKA1 (Gad et al. 2012), which is part of the AMPK heterotrimetric complex (Carling 2004). AMPK is reported as the master regulator of metabolic energy balance in several mammalian cells and acts as a master metabolic regulator of lipid, glucose and protein metabolism. It is a direct regulator of ACC gene activity which is responsible for *de novo* lipid synthesis (Hopkins et al. 2003). AMPK is regulated by the ratio of AMP:ATP rather than the intracellular depletion of ATP or accumulation of AMP (Corton et al. 1994). AMPK is activated by energetic stress and it promotes catabolic metabolism and inhibits anabolic reactions (Agathocleous and Harris 2013). The active form of AMPK has been prove to suppress lipogenic genes and to control lipid production in mice and human skeletal muscle (Li et al. 2011, Thomson and Winder 2009), regulate glucose metabolism in diabetes (Viollet et al. 2009) and decrease obesity (Xu et al. 2012) in humans. However, the inactive form increases lipid synthesis (Hopkins et al. 2003) and lipid accumulation by suppressing mitochondrial activity (Picard et al. 2012). In addition, AMPK is required for mitochondrial biogenesis and activities (Zong et al. 2002) and also reported to be antioxidant activator in response to oxidative stress (Austin and St-Pierre 2012, Finkel 2006).

Lipid homeostasis in embryos can be modulated by the presence of chemicals in culture media and these chemicals will act on lipid metabolism pathway (Barcelo-Fimbres and Seidel 2007b, Barcelo-Fimbres and Seidel 2007a). AICAR is one of permeable chemicals which are capable of stimulating AMPK activity and have diverse effect on glucose metabolism, lipid metabolism and protein synthesis (Hardie et al. 2003, Smith et al. 2005). It has been extensively used to enhance AMPK activity in many types of cells with various physiological

processes to control lipid and glucose metabolism (Hawley et al. 2003, Imai et al. 2006, Li et al. 2011, Merrill et al. 1997, Thomson et al. 2007).

Previous studies have demonstrated that in vitro culture media affect the pattern of bovine embryo gene expression (Niemann and Wrenzycki 2000, Saadeldin et al. 2011, Sagirkaya et al. 2006) and subsequent differences in metabolic status (Khurana and Niemann 2000a). For instance, addition of serum to bovine culture media significantly increases the expression level of genes related to apoptosis (Bax) and oxidative stress (MnSOD, SOX), meanwhile the expression of genes related to gap junctions (Cx43) and maternal recognition of pregnancy (IFN-tau) was decreased (Rizos et al. 2003). As reviewed by Cortessis et al. (2012) gene expression is influenced by environment through epigenetic process. At least 3 processes are involved in epigenetic regulatory mechanisms that are DNA methylation, histone modification and miRNA (Fabbri et al. 2007, Feil and Fraga 2011, Hu et al. 2014, Lennartsson and Ekwall 2009). In the field of miRNA studies, miRNA control gene expression through sequences complementary to mRNA (Krol et al. 2010). MicroRNAs control many cellular processes by modulating the precise amount of proteins either by promoting mRNA degradation or repressing mRNA target translation (Siomi and Siomi 2010). In addition to that, Kropp et al. (2014) revealed that miRNAs are secreted from pre-implantation embryos into culture media and the miRNA expression may correlate with the developmental competence of the embryo which is reflected in gene expression. In this regard miRNA display a fundamental role in early bovine embryonic development which is important to regulate folliculogenesis, fertilization, early embryonic survival and blastocyst development (Tripurani et al. 2011, Tripurani et al. 2013).

Considering the fact that AMPK is the master regulator of lipid metabolism, mitochondrial biogenesis and mitochondrial activity, in this study it is hypothesized that the activity of AMPK during embryo development mediates lipid accumulation in embryos cultured in vitro. Consequently, the present study aimed to explore the role of the AMPK during in vitro bovine embryo production. In order to test that hypothesis, 3 experiments were performed. In the first experiment, the expression of AMPK and its lipid metabolism related genes were investigated in different types of culture media. In the second experiment, the activity of AMPK was modulated using AICAR as exogenous activator to explore its effect on embryo metabolism in terms of lipid accumulation. Moreover, in order to investigate the regulatory mechanism of the AMPKA1 gene, in the third experiment potential candidate of miRNAs were identified and validated for their interaction with the target gene.

2 Literature review

2.1 In vitro production of bovine embryos

The increasing demand of milk and meat products all over the world makes dairy and meat industries have a huge economic value. The continuous supply of these products relies on animal reproduction system which functions to increase population and accelerate genetic gain between generations. One of technologies that can be used to improve both aims is IVP of bovine embryos. Since the first calf was born from an IVP embryo (Brackett et al. 1982), many progress and improvements have been made so far. One of those improvements is the use of in vitro matured oocytes fertilized by frozen sperm, that allows mass production of embryos by in vitro protocol (Ball et al. 1983, Parrish et al. 1986). Combined with bull genetic selection, semen sexing, estrus synchronization and embryo cryopreservation, IVP embryos becomes a valuable genetic material to enhance cattle population and genetic merit simultaneously compared to other assisted reproduction technologies.

The IVP of bovine embryo is well known to be established from IVM, IVF and IVC steps. In the IVM step, immature oocytes are aspirated from small follicles (3-5 mm) and matured in maturation medium which is supplemented with 10% fetal FCS and gonadotropin hormones (FSH, LH) under 5% CO_2 at 37-38°C. This process will take place 20-24 h of incubation and will complete its maturation process signaled by extrusion of a first polar body. Thus matured oocytes are ready to be fertilized. As widely practiced today, the semen that is used for fertilization is frozen semen. The best quality of motile sperm is separated by a Percoll gradient, as common system. In general, two types of media are used for IVF i.e. tyrode's albumin lactate pyruvate (TALP) based medium or a SOF based medium with or without glucose. Moreover, heparin is also added to the IVF medium aiming to increase sperm motility and induce capacitation which increase the successful of fertilization (Fukui et al. 1990). Fertilization is performed by co-incubating oocytes with sperm for 18-20 h. Afterwards, presumptive zygotes were transferred to IVC media for embryo development until they are ready for embryo transfer or storage (Galli et al. 2003). During culture in vitro all necessary nutrients, waste metabolism removal and toxic protection effect are provided by culture media. However, culture media and protein source in post fertilization influence embryonic gene expression subsequent to development potential of in vitro produced embryos (Gad et al. 2012, Rizos et al. 2003, Sagirkaya et al. 2006, Van Hoeck et al. 2013a, Warzych et al. 2007, Wrenzycki et al. 2001).

2.1.1 In vitro culture of embryos

Instead of in vivo, production of embryo by in vitro systems has been capable to produce embryos for transfer or research purposes. It is offering a flexibility of protocol and capable to do mass production of bovine embryos. All of the in vitro system relies on culture media which provide all important nutrition for embryo development. Embryo culture media are containing nutrients and buffer systems to support cell growth and in the same time prevents the cell metabolite toxicity during culture. Even though embryo production has been developed 40 years ago, further studies are required to develop culture media which meet the precise requirements of embryo metabolism. In this regard, with respect to resulting blastocyst quality, in vitro culture media are reported to be major factors while oocytes quality is important for blastocyst yield (Rizos et al. 2002). Over the last decade, there are two types of embryo culture media, basal media, that have been widely used are tissue TCM-199 and SOF. TCM-199 is a complex and undefined medium, while SOF is a simple and relatively well defined medium. TCM-199 was designed particularly for somatic cells cultured in vitro rather than for mammalian embryo culture. Therefore, embryo generated from SOF medium shows greater similarity to in vivo on the basis of gene expression patterns than those generated by the TCM system (Wrenzycki et al. 2001). In addition to the basal medium, IVC systems with and without serum are the two major types widely applied in IVP protocols (Gstraunthaler 2003).

Despite the ability of culture media to support embryo development during IVP, there is ample evidence showing differences between embryos derived from in vitro and in vivo with respect to morphology and molecular aspects. During the last few decades, many types of IVC media have been developed by varying embryo nutrient combination (Leese 2012). Several lines of evidence reviewed by Gardner et al. (2000b) show the plasticity of embryo metabolism to its surrounding environment, which enables mammalian embryos to develop in a wide variety of culture conditions. However, further adaptation of this process leave a signature in embryonic gene expression and subsequently influence on embryo metabolism, developmental competence and even affect the resulting offspring. Previous studies have documented that culture environment influence embryonic gene expression (Block et al. 2009, Gad et al. 2012, Rizos et al. 2003, Sagirkaya et al. 2006, Wrenzycki et al. 2001), cryotolerance (Abe et al. 2002, Gad et al. 2012, Gomez et al. 2008) and pregnancy rates following transfer (Block et al. 2009, Lim et al. 2007). In addition to that, differences in metabolism (Khurana and Niemann 2000b), especially high lipid content (Abe et al. 2002,

Gomez et al. 2008), organelle structure (Crosier et al. 2001) and chromosomal abnormalities were observed in resulting embryos (Lonergan et al. 2004). The IVC systems were also reported to influence fetal and placental development, indicate large of spring syndrome (LOS), particularly by increase birth weight and placenta abnormalities (Farin et al. 2001, Lazzari et al. 2002).

2.1.2 Serum addition in embryo culture media

Although it is undefined and its composition varies from batch to batch, the use of serum is widely applied as a supplement in various culture media. Serum was obtained from sterile liquid fraction of clotted blood and widely used for in vitro culture considered being rich of components which are support cells survival and living of mammalian cells in vitro. In the market, there are 5 types, based on sources, of serum available namely fetal bovine serum, newborn calf serum, calf serum, donor bovine serum and adult bovine serum (categorized by EMEA 1793 & Ph.Eur. 2262 & 9CFR Australia and United States origin).

Serum in embryo culture media has been shown to be beneficial as it is a premium source of nutrients for embryo development such as amino acids, pH buffer, molecule carries and maintenance the surface tension of the culture media (Gardner 2008). Addition of serum to embryo culture media is beneficiary for embryo development such as nutrients, growth factors and antioxidants. Moreover, it is well documented that the addition of serum during embryo culture resulted in increased blastocyst formation rate and development kinetics when compared to culture without serum system (Gutierrez-Adan et al. 2001, Oliveira et al. 2006, Rizos et al. 2003, Sudano et al. 2011, Wrenzycki et al. 2001). However, different development kinetics have been reported to affect the abundance of specific transcripts in later periods which is suggested due to different energy substrate during embryo culture and might affect the implantation rate of the resulting embryo (Khurana and Niemann 2000a). The difference in developmental speed is also associated with the alteration of gene expression, which is important for physiological embryonic process (Dode et al. 2006, Oliveira et al. 2006, Ripamonte et al. 2012).

On the other hand, serum addition also has been shown to cause harmful effects on embryo metabolism (Abe et al. 2002). Serum addition is reported to increase ROS (Rizos et al. 2003). The accumulate of ROS then alter mitochondrial structure and function (Abe et al. 2002, Cagnone and Sirard 2014, Crosier et al. 2001, Rizos et al. 2003, Thompson et al. 2007), alter

gene expression as well as embryo metabolism (Balasubramanian et al. 2007, Harvey et al. 2002, Rinaudo et al. 2006), reduce embryo quality by elevating blastocyst apoptotic rate and fragmentation (Khurana and Niemann 2000b, Liu and Keefe 2000) and increase intracellular lipid accumulation (Abe et al. 2002, Barcelo-Fimbres and Seidel 2007a, Sudano et al. 2011). This has been evidenced by dark colour of in vitro produced blastocysts compared to their in vitro counterpart (Abe et al. 2002, Gad et al. 2012, Gomez et al. 2008). In vitro produced embryos have a modified lipid metabolism, which may account for different in membrane structure thus increasing their sensitivity to oxidative stress and their high sensitivity to cryopreservation (Abe et al. 2002, Gomez et al. 2008).

Previous studies suggested that lipid accumulation in in vitro embryo is due to the incapability of mitochondria to metabolize the abundant lipid in form of fatty acids in culture media (Abe et al. 2002, Barcelo-Fimbres and Seidel 2007b, Ferguson and Leese 1999, McEvoy et al. 2000). From that point of view, one factor which influenced lipid accumulation is the presence of abundant energy source in culture media that came from the addition of serum. As reviewed by Gstraunthaler (2003) serum contains protein, glucose and cholesterol that can be used as energy. In addition to that, Sata et al. (1999) showed that fatty acids, particularly linoleic acid (LA; C18:2), is the most abundance component in serum. Moreover, McKeegan and Sturmey (2011) stated that different types of fatty acids play different role in developing mammalian oocytes and early embryos by affecting metabolism, oxidative stress, membrane composition, cell signaling and gene expression. The effect of individual fatty acids has been reported in previous studies. For example, 100 μM LA in the maturation medium affected bovine oocytes maturation rates, increased prostaglandin E2 (PGE2) secretion from COC, had significantly lower cleavage and blastocyst rate compared to control medium without serum (Marei et al. 2010). The presence of LA is also reported to reduce mRNA expression of glutathione peroxidase 1 (GPX1) and superoxide dismutase 1 (SOD) (Khalil et al. 2013) and to increase triacylglycerol accumulation (Carro et al. 2013) in bovine oocytes. Moreover, supplementation of LA can induce molecular change of oocytes associated with oocyte maturation leading to decreased developmental potential. Addition of palmitic acid (C16:0) or stearic acid (C18:0) during oocyte maturation had negative effects on maturation, fertilization and cleavage rate, blastocyst yield and embryo cryotolerance (Leroy et al. 2005, Shehab-El-Deen et al. 2009). More apoptotic cumulus cells were also observed in cumulus-oocyte complexes matured in the presence of these two fatty acids (Leroy et al. 2005).

Serum supplementation, the common method for in vitro embryos production, is effective but having high variability composition from batch to batch production. In addition to that, using serum incites specific issues concerning on scientific and animal welfare, particularly the fetal bovine serum (FBS) regarding its harvest and production methods, recommends to avoid using serum in culture media (Brunner et al. 2010). Effort to omit serum from culture media has been developed, for example, by Keskintepe and Brackett (1996) and Wydooghe et al. (2014). Many component of serum may be eliminated by using bovine serum albumin (BSA) as protein source and the macromolecular requirement for embryo culture is often satisfied by BSA. Culturing embryo with BSA is possible without affecting blastocyst yield and quality. Furthermore it could improve embryo survival after freezing (Gomez et al. 2008, Mucci et al. 2006). However, several lines of evidences show a lower development rate (Holm et al. 1999, Rizos et al. 2003) of blastocyst derived in vitro without serum compared to with serum. In addition, early develop of blastocysts reported to induce higher pregnancy rates compared to the later one (Barrenetxea et al. 2005, Hasler 2000, Jacob et al. 2012, Muthukumar et al. 2013).

2.2 Embryo energy metabolism

During preimplantation development, embryos undergo significant changes in their physiology. Along with that, embryo needs to uptake nutrients and then convert them into energy to support the physiological change (Gardner 1998). In general, nutrients for embryo development come from internal storage or external source in culture media. Several studies have been reported that glucose, pyruvate, lipid and amino acids are used as energy substrate in embryos at preimplantation stages (Ferguson and Leese 2006, Gardner et al. 2001, Rieger et al. 1992). A previous study (Khurana and Niemann 2000a) reported that the presence of glucose during IVC is beneficial for embryo development. In addition to that, consumption of glucose, pyruvate and oxygen were relatively low at zygote until 16-cells stages, while its uptake will be significantly increased during compaction and cavity formation started at morula until blastocyst (Khurana and Niemann 2000b). Lipid and its derivate, triglycerides and fatty acids, are reported to be the key energy source during bovine oocyte maturation and preimplantation embryo development (Dunning et al. 2010, Ferguson and Leese 2006). Addition of amino acids in the culture media positively affects bovine blastocyst development (Lee and Fukui 1996, Pinyopummintr and Bavister 1996). Recent study (Van Hoeck et al. 2011) also reported that amino acids are important nutrient which has influence on the regulation of embryo metabolism. The amino acid turnover in the culture medium could be

used as non-invasive marker indicating the embryo viability (Hemmings et al. 2012, Sturmey et al. 2010).

The nutrient breakdown and metabolism status of embryos can be used as indicator of embryo quality. According to the metabolism status, embryos cultured in vitro having higher oxidative phosphorylation (oxphos) rates and more lactate compared to the in vivo condition (Khurana and Niemann 2000b). The embryo cultured in vitro also reported having high glycolysis rate, when fresh embryo in vivo was transferred into in vitro culture media (Lane and Gardner 1998). Low embryo glycolytic rate reported close to in vivo develop blastocyst and had higher viability that those with abnormal elevated levels of glycolysis (Lane and Gardner 1996). These studies indicated the influence of IVC media in control embryo metabolism status. It's also widely accepted that culture media is the stress factor for embryo during IVC. For instance, in vitro embryo produced with elevated or different types of fatty acids shows higher amino acid turn over, which means more noise in metabolism, compared to the normal ones (Van Hoeck et al. 2011). In here, noisier embryo indicated the more deviation of metabolism from normal, thus embryo demanding more energy for its metabolism. It also can be stated that high energy demand of in vitro embryo is indicator of stress response from culture media. At this point of view, based on metabolic indicator, shows that preimplantation embryo survival is best served by a relatively low level of metabolism, which support quite embryo metabolism hypothesis (Baumann et al. 2007, Leese 2002).

2.2.1 ATP as energy source for embryo development

ATP is the major energy currency in preimplantation stages of embryos; it's produced in mitochondria and relies on oxphos process (Leese 2012, Sturmey and Leese 2003, Thompson et al. 1996). Similar to other mammalian cells, the consumption of available glucose, pyruvate, lipid and amino acids were used to generate ATP. Previous study showed that ATP production is essential for bovine embryo development (Thompson et al. 2000). Disturbance of ATP production through mitochondrial inhibition reported impaired blastocyst development (Dunning et al. 2010, Wakefield et al. 2011), meanwhile improved mitochondrial activity positively affect embryo developmental competence (Dunning et al. 2010, Dunning et al. 2011).

In mammalian cells, ATP generation occurs via oxphos or glycolysis pathway. Oxidative phosphorylation occurs in mitochondria and requires oxygen, meanwhile glycolysis occurs

without oxygen in cytoplasm. Oxidative phosphorylation generates more ATP than glycolysis in each round of reaction (30 ATP vs 4 ATP; respectively). The process of ATP production depends on the availability of Acetyl Co-A as the starting material. In general, pyruvate and fatty acids are converted into Acetyl Co-A in mitochondria. Thereafter, Acetyl Co-A goes to TCA cycle in oxidative phosphorylation process, as machinery which relies on ETC (e^-) and proton pumps (H^+), to generate ATP (Alberts et al. 2002). Electron transport chain made by 5 groups of protein complexes that coded by mtDNA. First of 4 proteins complex (I - IV) are responsible for carrying out the protein function, while the 5th complex (V) is responsible for ATP production at final step. Brief flow of ATP generation in mitochondria is illustrated in figure 1.

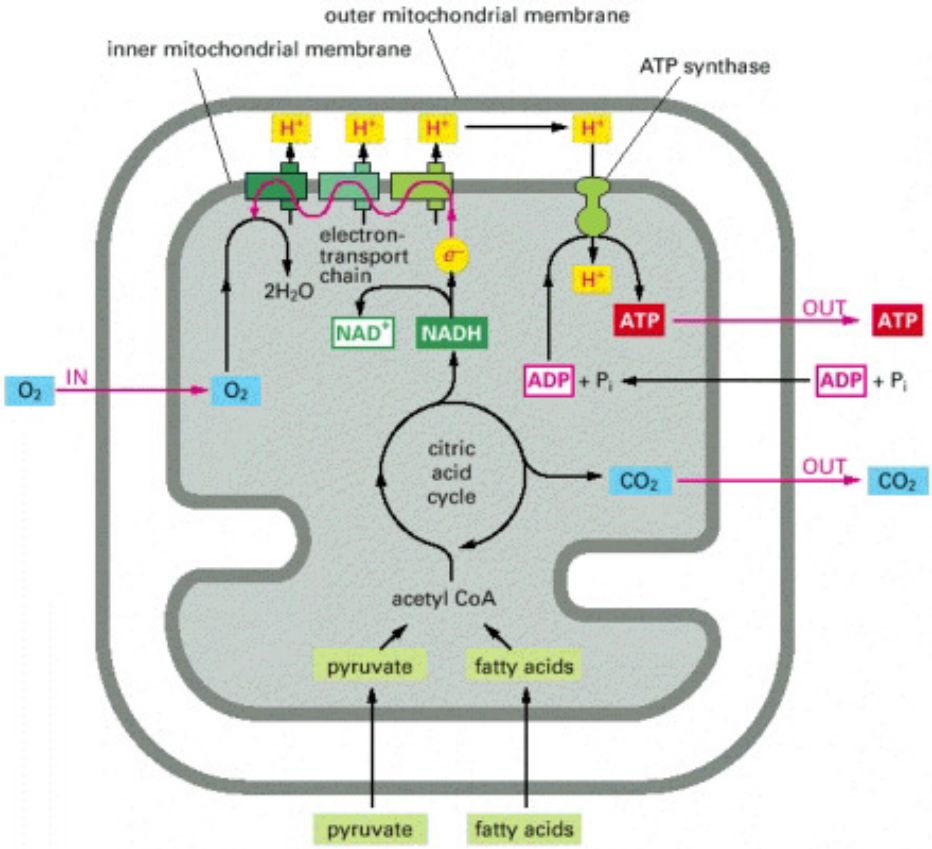

Figure 1: A summary of energy generating metabolism in mitochondria as illustrated by Alberts et al. (2002).

The TCA cycle initiated by oxidation of Acetyl Co-A and resulting electron transferred to nicotinamide adenine dinucleotide (NAD^+) and flavin adenine dinucleotide (FAD) to produce NADH and $FADH_2$. These electrons are donated to the ETC at complexes I and II respectively. Electrons are shuttled from complex I and II to complex III via ubiquinone and

from complex III to complex IV via cytochrome c. The two electrons are finally donated to the molecular oxygen (1/2 O_2) to generate H_2O. Incomplete, one-electron reduction of oxygen (O_2) can occurs at complexes I, II, and III subsequently produce superoxide (O_2^-). At this point, superoxide (source of ROS) production is increased with the concentration of oxygen and electron donor. By electron transport, different current leads to proton pumping across inner mitochondrial membrane create a gradient, which is used to produce ATP. In addition to that, mitochondria also produce ROS as by product during ATP production. The rise level of $NADH/NAD^+$ is a major factor leading to increased mitochondrial ROS production. When the breakdown of metabolites in the TCA cycles exceeds the capacity of ETC to fully acquire the resulting electron, thereafter ROS production is increased. In this regards, it can be stated that electron leaking occur due to over capacity of ETC in energy conversion process (Murphy 2009, Turrens 2003). In addition, the presence of excessive ROS might cause developmental arrest and apoptosis in the blastocyst due to at preimplantation stage mammalian embryos is very sensitive to oxidative stress (Dumollard et al. 2009).

2.2.2 ATP and cells energy balance

In almost all mammalian cell, the cellular functions are depends on the presence of ATP. ATP content is known to reflect the balance between energy production and consumption. ATP as energy currency, generated from diverse source of nutrient in the cells. It is need to be kept in mind that ATP is an energy coupling, not a fuel or energy storage for further use. The use of ATP in cell is initiated by hydrolysis of ATP into ADP or AMP. The following reaction shows how the ATP breakdown.

$$ATP \rightarrow ADP + P_i$$

$$2ADP \leftrightarrow ATP + AMP$$

In normal energy demand, ATP converted into ADP and P_i (phosphate). At the times when more energy is required, ATP releases 2 phosphates producing AMP and a chain of two phosphates namely pyrophosphate. In the condition when ATP hydrolyzed into ADP, ATP spending one phosphate. Immediately ADP is recycled and charged into ATP, by hooking one phosphate in mitochondria. This cycle of spending and charging phosphate for ATP is continuously cycled, to ensure that cell has enough energy to maintain the homeostasis condition. Meanwhile in the condition of ATP hydrolyzed into AMP, AMP is produced in two steps. First, hydrolysis of ATP produces ADP, and then the reaction catalyzed by

adenylate kinase to produce AMP. In addition to that, ATP transfers one phosphate molecules to AMP producing 2ADP, then ADP goes into TCA cycle then converted into ATP (Alberts et al. 2002). In this regards, the presence of [AMP] is a much more sensitive indicator of a cell's energetic state than [ATP]. Normally cells have a far higher [ATP] (5 to 10 mM) than [AMP] (<0.1 mM) (Nelson and Cox 2005). As the [ATP] is typically 30 to 100 times higher than that of [AMP], a small decrease in [ATP] will be manifested as a much larger percentage increase in [AMP] (Gardner et al. 2000b). For instance Nelson and Cox (2005) calculate the chemical equation balance if [ATP] drops by 10% and producing [ADP] and [AMP] in the same amount, the relative change in [AMP] is much greater (Table 1).

Table 1: Relative changes in [ATP] and [AMP] when ATP is consumed.

Adenine nucleotide	Concentration before ATP depletion (mM)	Concentration after ATP depletion (mM)	Relative change
ATP	5.0	4.5	10%
ADP	1.0	1.0	0
AMP	0.1	0.6	600%

One important mediator of the process regulate by AMP is AMPK, which responds to an increase in AMP concentration by phosphorylating key proteins, thereby regulating their activities. The rise in AMP concentration may be caused by a reduced nutrient supply on an increased exercise. The action of AMPK increases glucose transport and activates glycolysis and fatty acid oxidation, while suppressing energy requiring process such as the synthesis of fatty acids, cholesterol and protein (Nelson and Cox 2005). AMP which is accumulated as the result of ATP breakdown binds to and activates phosphorylase in purpose of activating AMPK. In the contrary at times when ATP levels are adequate, ATP blocks the allosteric site that AMP binds, then inactivates phosphorylase to deactivate the AMPK (Hardie and Hawley 2001, Hawley et al. 1996). In this regards, ATP is important as an on-off switch signal that control chemical reaction in the cell.

2.3 Mitochondria of preimplantation bovine embryos

In every mammalian cell, mitochondria are the powerhouse, which serves ATP as energy. Mitochondria are highly dynamic organelles whose length, shape, size and number are

controlled by fusion and fission process to ensure mitochondrial functional state (Detmer and Chan 2007). It is essential for the cellular adaptation in response to cellular metabolic status, stress and other intracellular or environmental signals (Palikaras and Tavernarakis 2014). In this regard, mitochondria proper function is crucial to the maintenance of cellular homeostasis. Mitochondria are structured by outer membrane, inner membrane and matrix. The outer membrane functions as a cover, freely permeable to facilitate molecules and ion transport in and out the mitochondria. The inner membrane or called cristae, structurally layered and folds where many chemical reactions occur to generate ATP. Fatty acid β-oxidation, amino acid oxidation and TCA cycles are the chemical reaction, which occurs in this surface. Therefore, more surfaces in this part mean more energy that can be generated. The matrix contains fluid where many enzymes, soluble metabolic intermediates, ions, ATP, ADP, P_i, ribosome and mtDNA lies (Alberts et al. 2002, Nelson and Cox 2005).

2.3.1 Mitochondrial development in oocytes and embryos

Mitochondria have an important role as central determinants of developmental competence of oocytes and preimplantation embryo (Van Blerkom 2009). It is unique due to it has its own genetic material (mtDNA), which maternally transmitted and amplified during oogenesis (Cummins 1998). Since embryonic mitochondrial replication does not occur until after the hatched blastocyst stage, mature oocytes, fertilized oocytes and early cleavage stage embryos are dependent on the function of the mitochondrial pool, which is present at ovulation time (Cummins 2000, Spikings et al. 2006). Consequently, any adverse influence on mitochondria will negatively impact embryo development, particularly by excessive ROS level.

During embryo preimplantation stages there are no replication of mtDNA (Cummins 1998, Cummins 2001). For that, the presence of mitochondrial biogenesis protein, PGC1A, is important to ensure mitochondria to be normally function (Austin and St-Pierre 2012). PGC1A influences in organelle remodeling by maintenance mitochondrial internal properties, thus maintaining ATP production. In addition, PGC1A also increase mitochondrial capabilities in scavenging ROS, which is harmful in excessive level (Austin and St-Pierre 2012, Finkel 2006). Different mitochondrial structure during embryo preimplantation was demonstrated in embryo cultured under serum system (Abe et al. 2002, Crosier et al. 2001) as the result of excessive of ROS production (Gomez et al. 2008). Moreover, increased level of ROS in bovine embryos is followed by low mitochondrial activity and low PGC1A expression level (Prastowo et al. 2014), means reduced ATP production.

2.3.2 Mitochondrial ROS production during embryo metabolism

Several line of evidence reviewed by Murphy (2009) and Samoylenko et al. (2013) show that mitochondria are the important source of ROS. ROS is byproduct of mitochondrial metabolism and normally produced during ATP generation. In excessive level, ROS causing oxidative stress due to imbalance between the ROS generation and scavenging, antioxidant, mechanism. The abundant generation of ROS during early embryo development is detrimental (Harvey et al. 2002, Rizos et al. 2003). The ROS hamper mitochondrial DNA structure and subsequently cause their dysfunction (Abe et al. 2002, Crosier et al. 2001). That in turn leads to decrease mitochondrial oxidation capacity and TCA cycle and subsequently induces excessive lipid droplet formation (Lee et al. 2013, Schrauwen and Hesselink 2004, Wang et al. 2009). This is accompanied by inactivation of enzymes that are essential for energy production and detoxification of ROS resulting in modulated mitochondrial functions (Kumari et al. 2014). The over generation of ROS is known to affect the capability of embryos to metabolize lipids (Abe et al. 2002, Abe and Hoshi 2003, Barcelo-Fimbres and Seidel 2007a, Barcelo-Fimbres and Seidel 2007b, Cagnone and Sirard 2014).

ROS produced during IVC post fertilization was reported to alter the mtDNA. It is demonstrated that mtDNA copy number during embryo development is sensitive to environmental factors (McConnell and Petrie 2004). The mtDNA encodes essential enzymes for oxphos reaction (Chandrasekaran et al. 1997, Marusich et al. 1997, Shoubridge 2001). For that, any defect on mtDNA can induce metabolic dysfunction and disturbs embryo development (Wilding et al. 2009). Oxidative stress induced mitochondrial dysfunction/damage by attacking mtDNA due to it is susceptible to mutation and lack of histones (Cui et al. 2012, Lenaz et al. 2002, Stuart and Brown 2006). Mitochondrial morphology changes after oocyte maturation might be caused by the of lack of mtDNA replication (Bavister and Squirrell 2000).

An increase of ROS has been measured in mouse embryo in vitro compared to in vivo (Goto et al. 1993), in vitro embryo derived from 5% O_2 compared to 20% in mouse (Kwon et al. 1999), porcine (Karja et al. 2006, Kitagawa et al. 2004) and bovine (Amin et al. 2014). The presence of high glucose concentration also increases ROS production in mouse (Trocino et al. 1995) and porcine (Karja et al. 2006) embryo. Another study also found an increasing oxidative stress and down regulation of antioxidant response gene during embryo development in vitro in the presence of serum compared to without serum, high glucose

concentration and in vitro compared to in vivo (Cagnone et al. 2012, Gad et al. 2012, Rizos et al. 2003). Those results underlined the factors that are affecting ROS production in the embryo produced in vitro. In addition, the role of mitochondria in lipid homeostasis also has been emphasized strongly in the recent study. It is reported that mitochondrial dysfunction can lead to disorder of lipid metabolism and triglyceride accumulation (Vankoningsloo et al. 2005). Mitochondrial dysfunctions are mostly caused by defects in the enzymes which are involved in mitochondrial respiration and oxidative phosphorylation (Wallace 1999). It is reported that mitochondrial dysfunction is indicated by more truncated mtDNA, and the consequences are the decrease in mitochondrial membrane potential, rate of ATP synthesis, and cellular ATP/ADP (Porteous et al. 1998). Defective mitochondrial function is also found to be the signal of mammalian aging. This aging was reported to increase fat accumulation by reducing ±40% of mitochondrial oxphos, which results in less ATP concentration in cell (Canto and Auwerx 2011).

2.4 AMPK as energy metabolism regulator

AMPK is known as a master regulator of metabolic energy balance in a wide range of tissue and cells (Carling 2004, Carling et al. 2011). Moreover it is a central component of a cellular signaling system that regulates multiple metabolic enzymes and pathways in response to reduced intracellular energy level (Hong et al. 2003). AMPK is controlling the ratio of AMP:ATP (Corton et al. 1994) and its activity is regulated by high ratio of AMP:ATP. AMPK also documented by Hopkins et al. (2003) as the master regulator of lipid, glucose and protein metabolism. In addition, it has been reported that AMPK is important for mitochondrial biogenesis and activities (Zong et al. 2002) in response to oxidative stress (Austin and St-Pierre 2012). The role of AMPK as central regulator of glucose, protein and lipid metabolism is illustrated in figure 2.

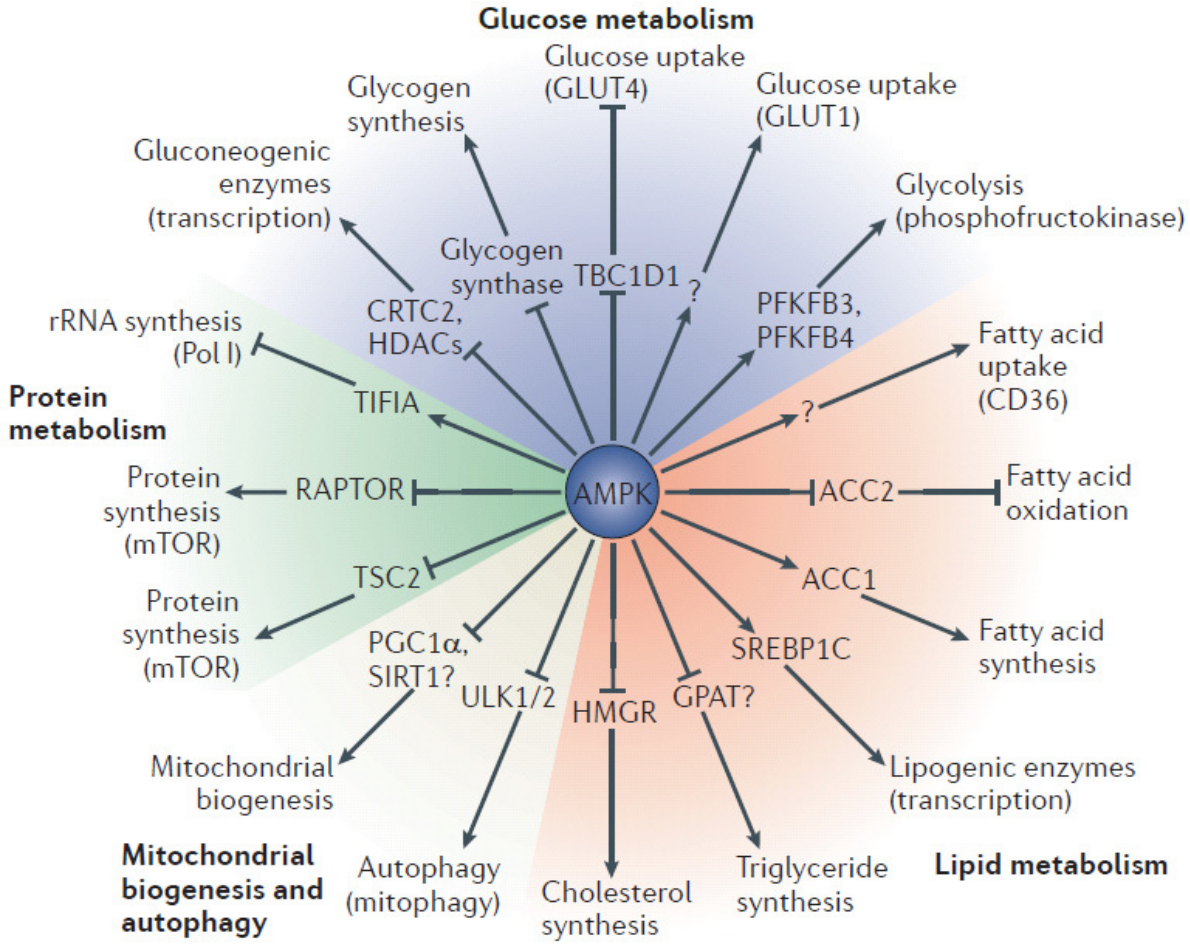

Figure 2: The effect of AMPK on cellular metabolism (Hardie et al. 2012).

The regulation of AMPK to mitochondrial activity has been reported in human (O'Neill et al. 2013, Wu et al. 2014) and also in mice (Dugan et al. 2013). Regarding to this, down regulation of AMPK is followed by low mitochondrial activity, low mitochondrial biogenesis and low antioxidant system (Austin and St-Pierre 2012, Hopkins et al. 2003, Li et al. 2011, Zong et al. 2002). Growing evidences showed that AMPK become one of promising target in combating diseases related to mitochondrial dysfunction such as diabetes, obesity and cancer in human research area (Bokko et al. 2007, Faubert et al. 2013, Hardie 2013, Hawley et al. 2003, Kim et al. 2014, Zhang et al. 2009).

2.4.1 AMPK structure and its activity

Based on its structure, AMPK is heterotrimetric complex, which consists of 3 subunits namely α, β, and γ. The α subunit (two isoforms AMPKA1, AMPKA2) is catalytic, whether β (two isoforms AMPKB1, AMPKB2) and γ (three isoforms AMPKG1, AMPKAG2,

AMPKAG3) are the regulatory subunits (Carling 2004). The α subunit contains the kinase domain at the N terminus and C-terminal regulatory domain containing an auto inhibitory region that inhibits protein kinase in the absence of AMP. The β subunit act as scaffold which function as α and γ subunit binding site and γ subunit acts as the binding site of AMP. In activated AMPK, AMP bind to the γ subunit and stabilized the connection between α-γ and then α subunit will be phosphorylated the target protein (Hardie and Hawley 2001).

AMPK is regulated by the ratio of AMP:ATP rather than the intracellular depletion of ATP or accumulation of AMP (Corton et al. 1994). AMPK is activated upon an increase in the AMP to ATP ratio, which reflects the energy status of the cell (Canto and Auwerx 2011). AMPK is regulated allosterically by AMP and reversible phosphorylation. Threonine-172 (Thr172) within the catalytic subunit α was identified as the major site for phosphorylation in AMP-activated protein kinase (Hawley et al. 1996). AMPK became active if Thr172 in the α subunit is phosphorylated and leads to increase its activity for several hundred folds. Mutation of Thr172 site is reported to decrease more than 90% of AMPK activity following treatment with protein phosphatases (Stein et al. 2000), this study shown that Thr172 involved in the sensitivity of AMPK complex to AMP. Control of Thr172 phosphorylation is the major mechanism for AMPK regulation because the binding of AMP to AMPK and activation of its Ser/Thr protein-kinase domain have been well characterized. The agent responsible for the phosphorylation process, in mammals, is the kinase LKB1 or CaMKKβ and it is promoted by AMP that binding in AMPK γ subunit. Binding of AMP to the regulatory γ subunit triggers phosphorylation of the catalytic α subunit by the upstream kinase LKB1 and the activated kinase switches on ATP generating catabolic pathways while in the same time switching off ATP requiring processes (Hardie 2005). In inactive AMPK, the dephosphorylation is promoted by a high level of ATP and the releasing phosphate is to be mediated by protein phosphatases such as PP2a and PP2c (Oakhill et al. 2011, Xiao et al. 2011).

Liver kinase B1 (LKB1) or also known as serine/threonine kinase 11 (STK11) is a master of kinase in most mammalian tissues (Hawley et al. 2003, Shackelford and Shaw 2009). This gene, which encodes a member of the serine/threonine kinase family, regulates cell polarity and functions as a tumor suppressor. LKB1 encodes a serine-threonine kinase that directly phosphorylates and activates AMPK (Thomson et al. 2007). Over nutrition and hyperglycemia suppress LKB1-AMPK signaling pathway that leads to obesity or diabetic condition (Shackelford and Shaw 2009). In addition to that, it's also reported that LKB1

activity is 50% down regulated subsequent to decrease AMPK activity by the present of palmitate and linoleate. In addition to that, LKB1 is also reported to be the regulator of fatty acid in mice muscle (Thomson et al., 2007) and adipocytes (Gormand et al. 2011) by inhibiting acetyl-CoA carboxylase (ACC) and regulating, malonyl-CoA level. LKB1 is also reported to regulate glucose and lipid metabolism (Imai et al. 2006). These studies demonstrated that AMPK activity decreased by the high concentration of FA (Watt et al. 2006). Similarly, Zhou et al. (2007) also reported in human ovarian that inhibition of fatty acid synthesis causes AMPK activation. These report indicated that the presence of FA would decrease the AMPK activity. Active AMPK suppresses the expression of lipogenesis associated genes such as fatty acid synthase, pyruvate kinase and ACC. In contrast, active AMPK increase of FA oxidation and suppress lipogenic enzyme (Dzamko et al. 2008, Lee et al. 2006, Zhou et al. 2001). Once AMPK got activated, AMPK regulates a number of transcriptional factors and signals the transduction protein, such as acetyl CoA carboxylase, hepatic nuclear factor and MAPK. Activated AMPK leads to control enzyme expression of gluconeogenesis, glucose 6-phosphatase and phosphoenolpyruvate carboxylase through LKB1 activities (Imai et al. 2006). Moreover, AMPK can be activated through other kinases, such as CaMKK and transforming growth factor-β-activated kinase. However, activation through this kinase is only restricted in specific cells and different tissue (Carling et al. 2008, Witczak et al. 2008).

2.4.2 AMPK and its role in lipid metabolism

Active forms of AMPK have been demonstrated to suppress lipogenic genes and control lipid production in mice (Li et al. 2011), fat metabolism in human skeletal muscle (Thomson and Winder 2009), regulate of glucose metabolism in diabetes (Viollet et al. 2009) and to decrease obesity (Xu et al. 2012). However, inactive form of AMPK increases lipid synthesis (Hopkins et al. 2003) and lipid accumulation due to down regulation of mitochondrial activity (Picard et al. 2012). AMPK is activated by energetic stress then promotes catabolic and simultaneously inhibits anabolic reactions (Agathocleous and Harris 2013). Active AMPK promotes fatty acid oxidation and inhibits fatty acid synthesis (Dzamko et al. 2008, Lee et al. 2006, Osler and Zierath 2008, Viollet et al. 2009, Zhou et al. 2001). These previous reports, displayed that AMPK is important signal for switch on or off cell metabolism, catabolism and anabolism, to generate or to store energy in cell. It's governed how the cells behave in response to the energy demand.

The rate-controlling enzymes for cholesterol and fatty acid biosynthesis are phosphorylated and inactivated by the AMP-activated protein kinase. When this protein kinase is activated, either through phosphorylation or in response to the binding of its allosteric activator 5′-AMP, the two major pathways responsible for the synthesis of lipids from acetyl-CoA both are inhibited. AMPK activation is resulting in the inhibition of energy-consuming biosynthetic pathways (such as fatty acid synthesis in liver and adipocytes, cholesterol synthesis in liver, protein synthesis in liver and muscle and insulin secretion from β-cell) and the activation of ATP-producing catabolic pathways (such as fatty acid uptake and oxidation in multiple tissue, glycolysis in heart and mitochondrial biogenesis in muscle). AMPK can also modulate transcription of specific genes involved in energy metabolism, thereby exerting long-term metabolic control (Viollet et al. 2009).

The rates of cholesterol and triglyceride biogenesis are predominantly controlled by SREBP (Horton et al. 2002). Sterol Regulatory Binding Protein (SREBP) mediated the expression of lipogenic genes. SREBP is transcription factor that regulate the gene expression of FA and cholesterol metabolism. SREBP family is divided into three types they are SREBP-1a, SREBP-1c and SREBP-2. The SREBP 1-a function is to activate all SREBP responsive genes. To the date it is known that SREBP is the direct transcription of more than 30 genes for uptake and synthesis cholesterol, FA, TG, and Phospholipids (Horton et al. 2002). Two forms of SREBP are SREBP-2 and SREBP-1c, which preferentially regulate the lipogenic genes involved in sterol biosynthesis and fatty acid synthesis. SREBP-1c is responsible in the gene related to FA, phospholipids and TG synthesis, meanwhile SREBP-2 acts in cholesterol biosynthesis and LDL receptor. Whereas SREBP-1c stimulates transcription of genes required for fatty acid synthesis, such as acetyl-CoA carboxylase and fatty acid synthase.

Activation of SREBP-1c affected lipid accumulation in heart cells (Marfella et al. 2009), in mouse liver (Shimomura et al. 1999) and in mouse kidney cell (Wang et al. 2005). High activity of SREBP-1c is accompanied by an insulin resistance (Wang et al. 2005). In the other side, increasing activity of SREBP-2 resulted in an increase of cholesterol efflux *induces by* the expression of low-density lipoprotein receptor (LDLR) and HMG-CoA reductase (HMGCoR), which increases low-density lipoprotein (LDL) cholesterol uptake and nascent cholesterol biosynthesis (Van Rooyen and Farrell 2011). Mechanisms of SREBP work depend on the SCAP and Insulin Signal (Insig). SCAP deficient in mice showed an 80% reduction in basal rates of cholesterol and FA synthesis, decreasing mRNAs encoding biosynthetic

enzymes (Matsuda et al. 2001). Insig-1 effectively blocks the transcription of SREBP-1c, preventing the activation of the genes for lipid biosynthesis (Chen et al. 2011).

The connection between AMPK-SREBP1 pathway in lipid accumulation has been demonstrated in previous research (Jung et al. 2011), where SREBP1 and ACC activity were suppressed by the abundance of AMPK. Recent study in cell culture and animal model demonstrated that AMPK suppress the SREBP activity in hepatic cells, resulted in lowering lipid synthesis and decreased insulin resistance (Li et al. 2011). AMPK interact directly with SREBP-1c and 2. AMPK inhibits the cleavage and transcriptional activity of SREBP by direct phosphorylation and resulted effect is lowering FA/TG and cholesterol biosynthesis. Previous study (Yap et al. 2011) reported that activation of AMPK suppress hepatic SREBP-1c transcription by inhibiting endogenous liver X receptor (LXR) ligand production and processing. Moreover, stimulation effect of AMPK reported lowering lipid and inhibiting LDLR activity (Lian et al. 2011, Zang et al. 2006).

2.4.3 Artificial modulation of AMPK activity

AMPK can be activated artificially by using diabetic treatment drug such as AICAR ,5-aminoimidazole-4-carboxamide riboside (Hawley et al. 2003, Imai et al. 2006, Li et al. 2011, Merrill et al. 1997, Thomson et al. 2007) and metformin (Stephenne et al. 2011, Zang et al. 2004). AICAR is pharmacological cell permeable molecule which has been extensively used to increase AMPK activity in a wide variety of cells under the various physiological processes. In principle, AICAR is imidaZole monophosphate (ZMP) which its structure similar to AMP inside the cell. By increasing the AMP concentration, artificially, AMPK can be activated.

In the field of animal reproduction, the use of AICAR to increase AMPK activity has been well documented in hen preovulatory follicle (Tosca et al. 2006), mouse embryo (Eng et al. 2007), mouse oocytes (Chen et al. 2006, Downs et al. 2010) and bovine oocytes (Bilodeau-Goeseels et al. 2007). Activation of AMPK in mouse oocytes to regulate its maturation (Chen and Downs 2008, Downs et al. 2002, Downs et al. 2010, LaRosa and Downs 2006), while in bovine oocytes, AICAR inhibits nuclear maturation (Bilodeau-Goeseels et al. 2007). AICAR also has been reported to have various effect on the cellular function, such as either induce (Li et al. 2008, Zang et al. 2008) or inhibit (Giri et al. 2006) cell differentiation, up regulate embryonic stem cells pluripotency (Adamo et al. 2009, Shi et al. 2013), reduce lipid

accumulation and in parallel inhibits cell proliferation (Giri et al. 2006, Jose et al. 2011). Another effect of AICAR is the enhancement of mitochondrial activity for ATP production through AMPK activity. Therefore, AICAR is more effective in cells with low steady-state ATP content and a high proliferation rate (Jose et al. 2011) such as cancer or tumor cells. In addition, enhanced mitochondrial activity through β-oxidation reduced cytoplasmic lipid droplet (Somfai et al. 2011). Lipid droplet in term of lipid metabolism in oocytes is closely associated with mitochondria in regards with ATP production in the process called β-oxidation (Dunning et al. 2010, Dunning and Robker 2012, Sturmey et al. 2006).

2.5 miRNAs in animal reproduction and energy metabolism

miRNAs are non-coding RNAs with post-transcriptional regulator gene expression functions (Krol et al. 2010). It is a class of small (19–24 nt) regulatory RNA and controls many developments and cellular process by modulating the precise amount of proteins in a cell either by promoting degradation or repressing of mRNA target translation (Siomi and Siomi 2010). There are more than 28.000 miRNAs have been listed in miRBase (http://www.mirbase.org/ released at February 2015). Its regulates a wide range of biological processes through the recognition of complementary sequences between miRNAs and their target genes, and have essential role during animal development (Liu et al. 2010).

As explained by Carletti and Christenson (2009), the miRNA biogenesis begins in the nucleus and initiated with the transcription of miRNA gene in the help of RNA polymerase II providing long precursor transcripts know as pri-miRNA. Mediated by Drosha and DGCR8A, pri-miRNA proceeds to processing into hairpin structure called pre-miRNA which has a shorter sequence. The pre-miRNA then transported to cytoplasm that facilitated by a transport enzyme called exporting 5, and then followed by editing process by DICER resulted in a matured miRNA. The mature miRNA will be approximately 22 nt and ready to target specific mRNA. In its action, mature miRNA and Ago protein form RISC, a ribonucleoprotein complex, mediating post-transcriptional gene activity by bind to the 3-UTR of mRNA. miRNA control down regulation of gene expression thorough mRNA translation inhibition and degradation (Beilharz et al. 2009, Huang et al. 2007, Li et al. 2013). Several reports also indicated that miRNA can be functioning as post transcriptionally stimulates gene expression (Ma et al. 2010, Place et al. 2008, Vasudevan et al. 2007). In this regards, it can be underlined that presence of miRNA in cell regulation is to fine tune or orchestrated the protein level.

2.5.1 miRNA regulate animal reproduction process

Regulation of gene expression plays a central role in defining cell fates and controlling organ formation which is transcriptionally controlled by coordinating the expression of selected genes at specific times and places (Suh and Blelloch 2011). Moreover, the identification and characterization of differentially regulated genes especially in bovine oocytes and preimplantation embryos are required to explore which is important in maturation, fertilization, early cleavage and even long-term development process (Cui and Kim 2007). So far, the bioinformatics prediction indicated that mammalian miRNAs able to regulates 30% of all protein-coding genes (Filipowicz et al. 2008). In bovine research area, miRNA expression have been observed, for example, in sperm (Amanai et al. 2006), maturated oocytes (Tesfaye et al. 2009), ovaries (Hossain et al. 2009), zygotes (Svoboda and Flemr 2010) and preimplantation embryos (Kues et al. 2008). The correct regulation of mRNA expression by miRNAs is believed to be important for early development. Detailed analyses of gene expression profiles during embryogenesis will become valuable tools to a better understanding of basic cellular and molecular mechanisms that control early development, thus allowing the better strategies for improving the efficiency or use in other technologies such as transgenic and cloning.

Table 2: The role of miRNA in animal reproduction process.

miRNA name	Gene	Animal	Related reproduction process	Status	Reference
miR-106a, Let-7	MYC WEE1A	Bovine	Oocytes	Predicted	(Miles et al. 2012)
miR-205		Bovine	Preimplantation embryo	Predicted	(Abd El Naby et al. 2013)
miR-212	FIGLA	Bovine	Preimplantation embryo	Validated	(Tripurani et al. 2013)
miR-196a	NOBOX	Bovine	Preimplantation embryo	Validated	(Tripurani et al. 2011)
miR-27b, miR-372, miR-382	ACVR1, ID2	Equine	Pre-ovulatory granulosa cells	Predicted	(da Silveira et al. 2014)
miR-376a	PCNA	Mouse	Oocyte apoptosis	Validated	(Zhang et al. 2014)
miR-34c	BCL-2	Mouse	First cell division in zygote	Validated	(Liu et al. 2012)
miR-29a	NASP	Mouse	Epididymal cell proliferation	Validated	(Ma et al. 2012)
miR-145	ACVR1B	Mouse	Granulosa cell proliferation	Validated	(Yan et al. 2012)

Table 2: Cont.

miRNA name	Gene	Animal	Related reproduction process	Status	Reference
miR-320	E2F1, SF-1	Mouse	Granulosa cell proliferation and steroidonegesis	Validated	(Yin et al. 2014)
miR-224	SMAD4	Mouse	Granulosa cell proliferation and steroidonegesis	Validated	(Yao et al. 2010)
miR-224	Ptx3	Mouse	Granulosa cell expansion	Validated	(Yao et al. 2010)
miR-200a	PTEN	Mouse	Uterus	Validated	(Shen et al. 2013)
miR-34a	INHBB	Porcine	Granulosa cell apoptosis	Validated	(Tu et al. 2014)
miR-136-3p	LHR	Rat	Granulosa cells	Validated	(Kitahara et al. 2013)
Let-7, miR-22	ESR1, CYP19A1, SOX9	Sheep	Estrogen-signalling pathway during fetal testicular development	Predicted	(Torley et al. 2011)

In animals, most of miRNA control gene expression by binding on the 3-UTR sequence of mRNA target at nucleotides number 2-8 known as seeding region (Ambros 2004, Bartel 2009, Zhou et al. 2013). Several studies, (listed in Table 2), shows the function of miRNA in animal reproduction, however, information about its function is limited compared to miRNA that studied in human area especially for cancer model. However it shows a promising area for future research in evaluating miRNA functional effect on gene translation subsequent to biological pathways in livestock. Another importance of miRNAs is as their role as molecular biomarker or potential target for diseases therapy needs to be developed in further. In animal production area, growing evidence shows that miRNA involvement in regulating gene expression subsequent to animal production traits, need to be underlined and given more attention in the further. For instance, Govindaraju et al. (2012) found different miRNAs expression between bulls with high and low fertility. Other studies also reported that miRNA is involved in milk production (Jabed et al. 2012) and control postpartum period (Fatima et al. 2014) of the cow. Therefore, more research needs to be completed for understanding the miRNA role and its application in animal agriculture (McDaneld 2009).

2.5.2 miRNA regulate energy metabolism

Growing evidences have been demonstrated that miRNA is involved in control of metabolism by sensing variation of nutrients, hormones and cytokines. These variations of stimuli effect on the miRNA biogenesis and its expression, leads to the cause of changes in gene expression which lies in metabolism pathway (Dumortier et al. 2013). Regulations of different miRNAs in different type of cell and organism have been reported in the previous studies. For example, miR-278 (Teleman et al. 2006) in Drosophila, miR-26a in rectal cancer cells (Chen et al. 2014), miR-223 in human cardiac cells (Lu et al. 2010) and miR-33a/b in human hepatic cells (Ramirez et al. 2013) were involved in glucose metabolism. Another studies, documented that miR-33a/b (Davalos et al. 2011, Goedeke et al. 2013), miR-27b (Vickers et al. 2013), and miR-224 (Peng et al. 2013) were involved in lipid and fatty acid metabolism. These studies, display to us the role of miRNA in controlling cell metabolism. However, to the best of our knowledge little studies have been made in the regulation of miRNAs in animal reproduction especially the one that dealing with energy metabolism in embryo research area.

3 Material and methods

In order to explore the function and role of AMPK in in vitro bovine embryos and its association with embryo development and lipid accumulation, three experiments were performed as follows:

Experiment 1: Expression of AMPK and lipid metabolism genes in bovine embryos under different culture environments

To determine the AMPK gene expression and its association with lipid accumulation in embryos, the first experiment was divided into 3 parts as indicated in figure 3. As indicated, the expression of genes in AMPK pathway (AMPKA1, AMPKA2), lipid metabolism (ACC) and mitochondrial activities (CPT1, PGC1A) were quantified using qRT-PCR method. Moreover, lipid droplets and its quantity from blastocysts derived in vitro vs. in vivo were evaluated in the first part of experiment 1. Due to the difference of in vitro vs. in vivo is the presence of serum, in the second part of experiment bovine embryos were produced with and without serum. Embryos from 2-, 8- and 16-cell were collected at 31, 72, and 168-192 h post insemination aiming to know the temporary expression of AMPK and its related lipid metabolism genes. In this part, expression of genes in AMPK pathway (AMPKA1, AMPKA2, STK11), lipid metabolism (SREBP2, SREBP1, ACC), antioxidant (NQO1) and mitochondrial activity (CPT1, PGC1A) were quantified in each stage of embryos development. In addition, mitochondrial activities of embryo cultured with and without serum were detected at blastocyst stage. In order to know whether different culture media affecting AMPKA1 activity, embryos were cultured under different macromolecule supplementation in the third part of experiment. The expression level of genes in AMPK pathway (AMPKA1, AMPKA2), lipid metabolism (ACC) and mitochondrial activities (CPT1, CPT2, PGC1A) were quantified. Moreover, the embryo development competent, ROS production level, mitochondrial activity, protein expression of AMPK and AMPK phosphorylation at Thr-172 and lipid content were evaluated at blastocyst stage.

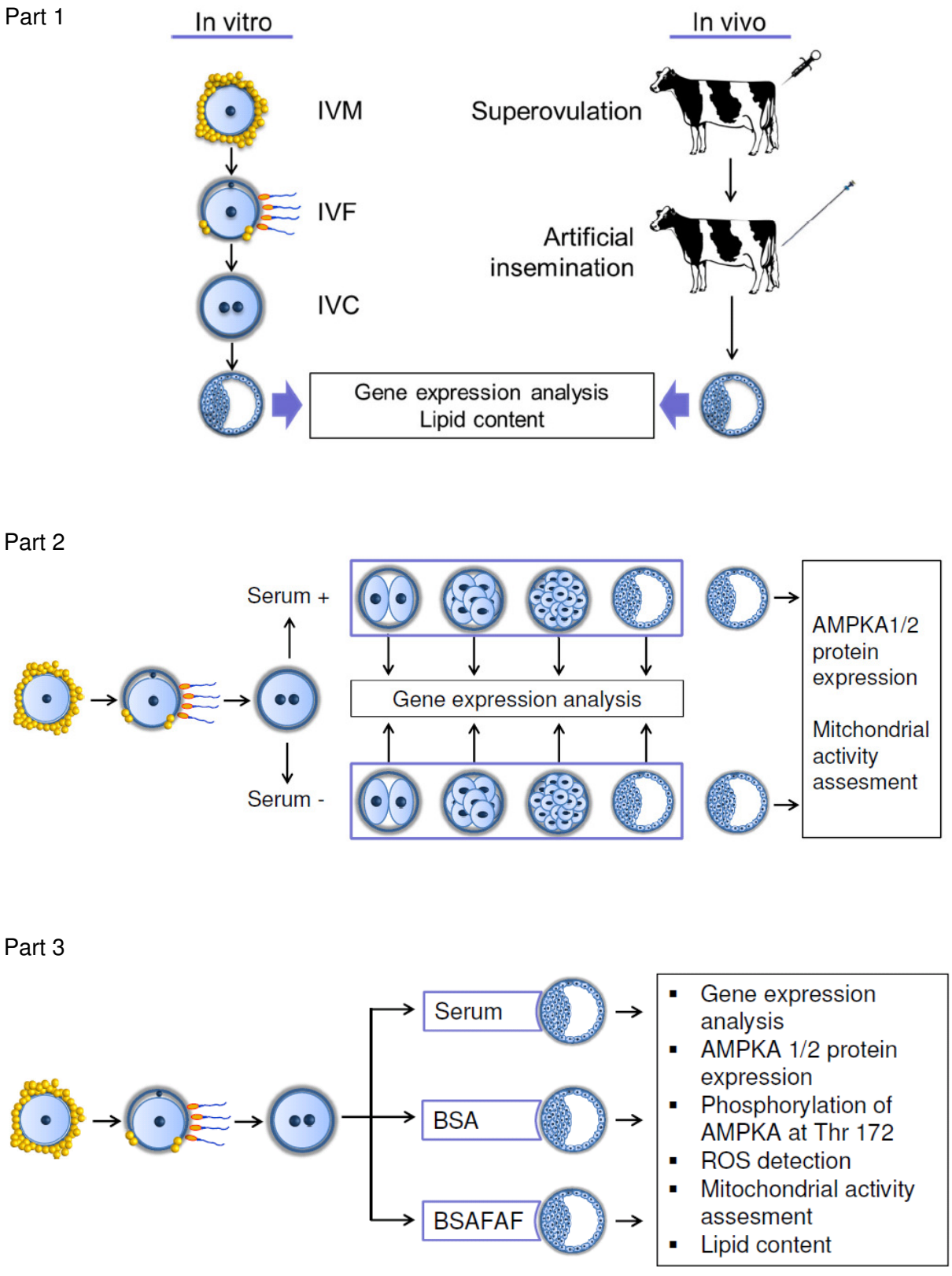

Figure 3: Brief workflow of experiment 1.

Experiment 2: Modulation of AMPK activity by using AICAR as exogenous activator

In the effort to modulate AMPK activity, AICAR was applied in the embryos culture media. Initially, 4 dosages of AICAR (0, 1, 10, 100 mM) were applied and followed by AMPKA1 gene expression analysis at blastocyst stage. The initial study then allowed to select one AICAR dosage that gives higher AMPKA1 expression. As indicated in figure 4, expression level of genes in AMPK pathway (AMPKA1, AMPKA2), lipid metabolism (ACC), antioxidant (NQO1) and mitochondrial activities (CPT1, CPT2, PGC1A) were quantified. Moreover, the embryo development competent, blastocyst cell number, protein expression of AMPKA, mitochondrial activity and lipid content were evaluated at blastocyst stage.

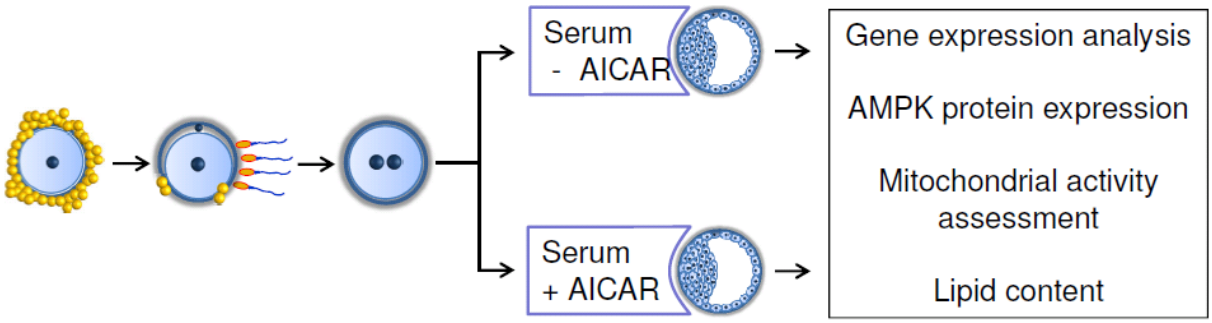

Figure 4: Brief workflow of experiment 2.

Experiment 3: The potential post transcriptional regulation of AMPKA1 by miRNA in bovine granulosa cells as the model

This experiment aimed to know the regulatory mechanism of AMPKA1 using miRNA. Due to miRNA target validation can't perform directly in the embryo, bovine granulosa cells were selected as the model. As indicated in figure 5, this study was initiated with *in silico* study using bioinformatics approach to select potential candidate of miRNA targeted to AMPKA1. For further target validation, potential candidate of miRNAs were profiled from granulosa cells sample which cultured with and without serum system. miRNA target validation was performed through plasmid construction, miRNA co-transfection and luciferase assay.

Once the miRNA candidate is validated, the miRNA mimic and inhibitor were transfected into granulosa cells to know its effect on gene and protein expression level of AMPKA1, followed by gene expression analysis of lipid metabolism (ACC) and mitochondrial activities (CPT1, PGC1A). Moreover, cultured granulosa cells in the presence of AICAR followed by

AMPKA1 quantification were performed for comparison purposes. Moreover, validated miRNA targeted to AMPKA1 also profiled in blastocysts sample derived from culture in the presence or absence of serum, aiming to know its expression for further functional study at embryo stage.

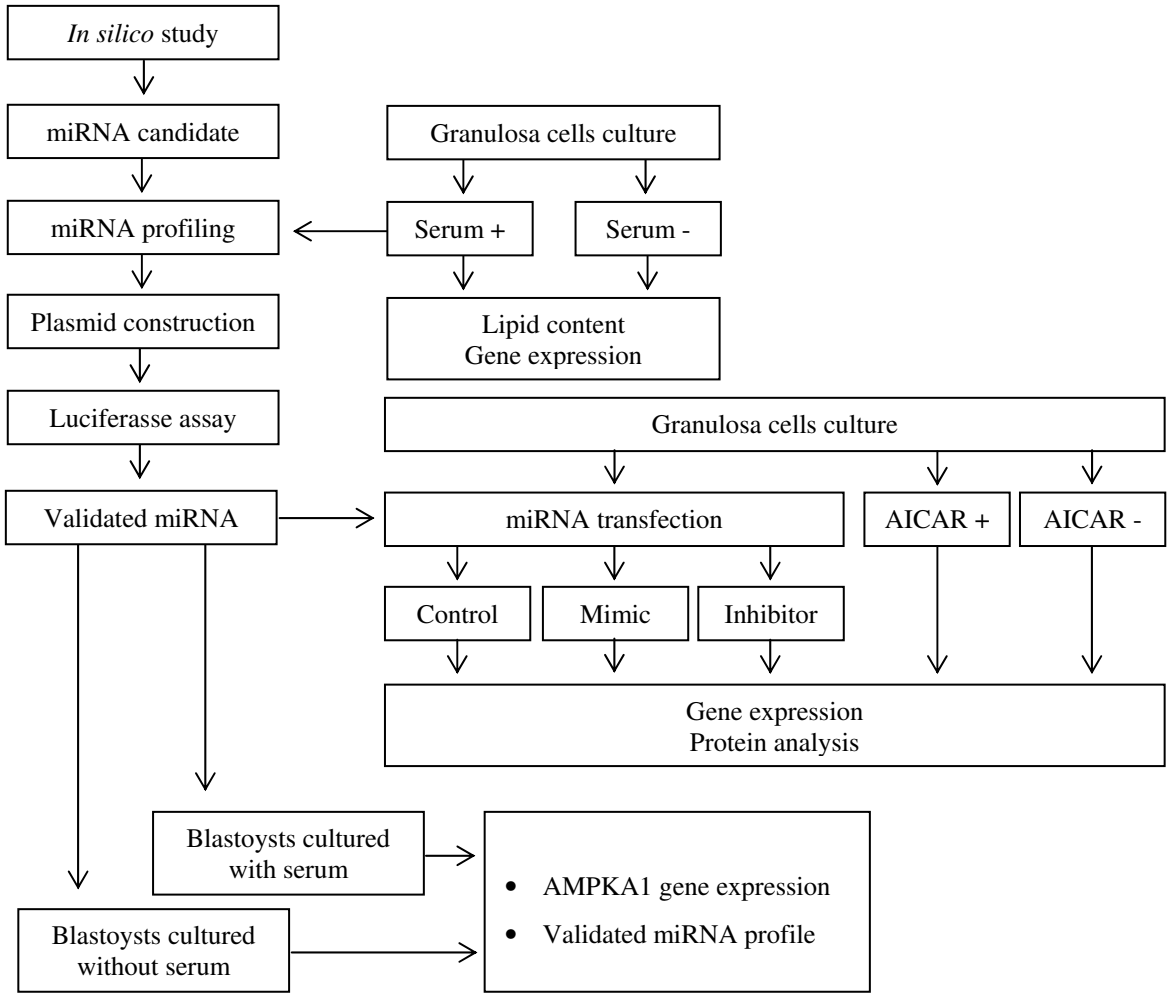

Figure 5: Brief workflow of experiment 3.

3.1 Materials

3.1.1 List of chemicals and kits

During the whole experiment, various types of chemical and kit were used and supplied from different companies. The use of those chemicals and kits were based on the protocol supplied or with minor modification if necessary until it's stated.

Chemical and kit	Manufacture / supplier
10x PCR Buffer S complete	Genaxxon bioscience GmbH, Germany
Acturus® PicoPure® RNA isolation	Life Technologies, USA
Agarose	Carl Roth GmbH, Karlsruhe, Germany
AICAR	Sigma-Aldrich Chemie GmbH
Alexa Flour® 568 secondary antibody	Life Technologies, USA
Ampicillin	Sigma-Aldrich Chemie GmbH, Munich, Germany
AMPKA1/2 antibody (H-300)	Santa Cruz Biotechnology
Bromophenol blue	Sigma-Aldrich Chemie GmbH, Munich, Germany
BSA	Sigma-Aldrich Chemie GmbH, Munich, Germany
BSAFAF	Sigma-Aldrich Chemie GmbH, Munich, Germany
Calcium L-lactate hydrate	Sigma-Aldrich Chemie GmbH, Munich, Germany
Chloroform	Sigma-Aldrich Chemie GmbH, Munich, Germany
Cloprostenol	Essex, Munich, Germany
Competent cell, *E. coli* bacterial strain	Promega, Germany
Deoxynucleotide Set, 100 mM	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
DL-Dithiothreitol	Sigma-Aldrich Chemie GmbH, Munich, Germany
DMSO	Sigma-Aldrich Chemie GmbH, Steinheim, Germany

DNA digesting enzyme, SacI	New England Biolabs, USA
DNA digesting enzyme, Xbal	New England Biolabs, USA
DNA Ladder Fermentas Gene Ruler™ 1 kb Plus	Thermo Scientific, USA
Dual-Luciferase® Reporter Assay System	Promega, USA
Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham	Sigma-Aldrich Chemie GmbH, Munich, Germany
EDTA	Sigma-Aldrich Chemie GmbH, Munich, Germany
Estrumate	Essex Pharma GmbH, Munich, Germany
Ethanol	Roth, Karlsruhe, Germany
Ethidium bromide	Sigma-Aldrich Chemie GmbH, Munich, Germany
Ethylene glycol	Sigma-Aldrich Chemie GmbH, Munich, Germany
ExiLENT SYBR® Green Master mix	Exiqon, USA
Fetal Calf Serum	Gibco, Karlsruhe, Germany
First Strand cDNA Synthesis Kit	Thermo Scientific, USA
Formaldehyde solution	Sigma-Aldrich Chemie GmbH, Munich, Germany
FSH, Folltropin	Stimufol, Liege, Belgium
Fungizone® Antimycotic	Life Technologies, USA
GenElute™ Plasmid Miniprep Kit	Sigma-Aldrich Chemie GmbH, Munich, Germany
Gentamycin sulphate	Sigma-Aldrich Chemie GmbH, Munich, Germany
Glacial Acetic Acid	Sigma-Aldrich Chemie GmbH, Munich, Germany
Glucose	Sigma-Aldrich Chemie GmbH, Munich, Germany
Glycerol	Sigma-Aldrich Chemie GmbH, Munich, Germany
Glycin	Carl Roth GmbH, Karlsruhe, Germany
Green Fluorescence Plasmid (GFP), pmaxGFP® 0.5 µg/µL	Amaxa biosystems, USA

Hefeextrakt	Carl Roth GmbH, Karlsruhe, Germany
Heparin sodium salt	Sigma-Aldrich Chemie GmbH, Munich, Germany
Hepes	Sigma-Aldrich Chemie GmbH, Munich, Germany
Hypotaurine	Sigma-Aldrich Chemie GmbH, Munich, Germany
Intergonan 6000® (eCG)	Intervet, Boxmeer, The Netherlands
IPTG	Sigma-Aldrich Chemie GmbH, Munich, Germany
Isopropyl alcohol	Sigma-Aldrich Chemie GmbH, Munich, Germany
iTaq™ Universal SYBR® Green Supermix	Bio-Rad Laboratories, USA
iTaq™ Universal SYBR® Green Supermix	Bio-Rad, Germany
KCl	Carl Roth GmbH, Karlsruhe, Germany
KH_2PO_4	Carl Roth GmbH, Karlsruhe, Germany
L-Glutamine	Sigma-Aldrich Chemie GmbH, Munich, Germany
LigaFast™ Rapid DNA Ligation System	Promega, USA
Lipofectamine® 2000 Transfection Reagent	Life Technologies, USA
Mg^{2+}	Sigma-Aldrich Chemie GmbH, Munich, Germany
Mineral oil	Sigma-Aldrich Chemie GmbH, Munich, Germany
miRCURY LNA™ microRNA PCR, Polyadenylation and cDNA synthesis kit II	Exiqon, USA
miRCURY LNA™ Universal RT microRNA PCR, SYBR Green master mix	Exiqon, USA
miRNeasy® Mini Kit	Qiagen, Germany
Mitotracker® Red CMXRos	Life Technologies, USA

Mounting Medium with DAPI VECTASHIELD®	VECTOR Laboratories, United Kingdom
Na_2HPO_4	Carl Roth GmbH, Karlsruhe, Germany
NaCl	Fisher Scientific GmbH, Germany
$NaHCO_3$	Sigma-Aldrich Chemie GmbH, Munich, Germany
NaOH	Carl Roth GmbH, Karlsruhe, Germany
Oil Red O	Sigma-Aldrich Chemie GmbH, Munich, Germany
Opti-MEM® Reduced Serum Medium, GlutaMAX™ Supplement	Life Technologies, USA
Ovogest 5000® (hCG)	Intervet, Boxmeer, The Netherlands
PCR Primer set	Eurofins
Penicillamine	Sigma-Aldrich Chemie GmbH, Munich, Germany
Penicillin-Streptomycin	Life Technologies, USA
Percoll	Sigma-Aldrich Chemie GmbH, Munich, Germany
pGEM®-T Easy Vector Systems	Promega, Germany
pmirGLO Dual-Luciferase miRNA Target Expression Vector	Promega, Germany
PVA, Poly(vinylalcohol)	Sigma-Aldrich Chemie GmbH, Munich, Germany
QIAquick PCR Purification Kit	Qiagen, Germany
Rnase-Free Dnase Set	Qiagen, Germany
SacI DNA restriction enzyme	New England Biolab, USA
Secondary Antibody, Alexa Flour® 568 Goat Anti-Rabbit IgG (H+L)	Life Technologies, USA
Sodium pyruvate	Sigma-Aldrich Chemie GmbH, Munich, Germany
Sucrose	Sigma-Aldrich Chemie GmbH, Munich, Germany
Taq Polymerase S	Genaxxon bioscience GmbH, Germany
TCM-199 medium	Sigma-Aldrich Chemie GmbH, Munich, Germany

Tris(hydroxymethyl)-aminomethane	Carl Roth GmbH, Karlsruhe, Germany
Triton X-100	Carl Roth GmbH, Karlsruhe, Germany
Trypton/Pepton	Carl Roth GmbH, Karlsruhe, Germany
Universal cDNA Synthesis Kit II	Exiqon, USA
Urea	Sigma-Aldrich Chemie GmbH, Munich, Germany
Vectashield, mounting medium	Vector Laboratories, UK
WST-1 Cell Proliferation Assay Kit	Cayman Chemical, USA
XbaI DNA restriction enzyme	New England Biolab, USA
Xgal, Blue-White Select™ Screening Reagent	Sigma-Aldrich Chemie GmbH, Munich, Germany
Xylene Cyanol	Sigma-Aldrich Chemie GmbH, Munich, Germany

3.1.2 Reagents and media preparation

The following reagent and media were prepared with ddH_2O and when it is necessary pH was adjusted with NaOH or HCl. In addition, solutions or buffers that dealing with cell culture systems needs to be autoclaved at 120°C for 20 minutes or filtered through 0.2 µm filter.

Media or solution	Chemical ingredients	Amount / volume
10x TBE buffer	$(HOCH_2)_3CNH_2$ (Tris base)	108 g
	H_3BO_3 (Boric acid)	55 g
	EDTA (0.5 M)	40 ml
	ddH_2O	up to 1000 ml
10x PBS	NaCl	8.77 g
	Na_2HPO_4	1.50 g
	NaH_2PO_4	2.04 g
	ddH_2O	up to 1000 ml
10x TAE buffer	Tris	48.5 g

	acetic acid	11.4 ml
	EDTA 0.5 M (pH 8.0)	20 ml
	ddH_2O	up to 1000 ml
Agarose loading buffer	Bromophenol blue	0.0625 g
	Xylencyanol	0.0625 g
	Glycerol	7.5 ml
	ddH_2O	25 ml
Cell culture medium	Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham	44 ml
	FCS	5 ml
	Penicillin-Streptomycin	0.5 ml
CR1-aa culture medium	Calcium lactate	273.0 mg
	Streptomycin sulphate	3.9 mg
	Penicillin G	1.9 mg
	NaCl	315.6 mg
	Potassium chloride	11.2 mg
	Sodium hydrogen carbonate	105 mg
	Sodium pyruvate	2.2 mg
	L-Glutamine	7.3 mg
	Phenol red solution	100 µl
	Sodium hydrogen carbonate	80 mg
dNTP solution	dATP (100 mM)	10 µl
	dGTP (100 mM)	10 µl
	dTTP (100 mM)	10 µl
	ddH_2O	400 µl
	Fungizole	0.5 ml

EDTA 0.5 M (pH 8.0)	EDTA	186.1 g
	ddH_2O	1000 ml
	NaOH (pH adjusting)	-
Glucose 2 M	Glucose	36 g
	ddH_2O	100 ml
Glycine 0.3 mmol/l (stock solution)	Gylcine	0.02252 g
	PBS (CMF)	10 ml
Glycine working solution	Gylcine stock	100 µl
	PBS-PVA	9.9 ml
Guanidin-ethanol solution	Guanidin	1,43 g
(50 ml)	95% Ethanol	up to 50 ml
In vitro culture medium	CR1aa	50 ml
	Hemi-calcium lactate	273 mg
	Streptomycin sulphate	3.9 mg
	Penicillin G	1.9 mg
	Sodium chloride	315.6 mg
	Potassium chloride	11.2 mg
	Sodium hydrogen carbonate	105 mg
	Sodium pyruvate	2.2 mg
	L-Glutamine	7.3 mg
	Phenol red solution	100 µl
	Sodium hydrogen carbonate	80 mg
In vitro fertilization medium (Fert-TALP medium)	$CaCl_2.2H_2O$	29.4 mg
	KCl	23.9 mg
	$MgCl_2.6H_2O$	10.1 mg
	$NaH_2PO4.H_2O$	5.5 mg
	Lactic Acid (Sodium salt; 60% w/w	186 µl

	syrup)	
	NaCl	666 mg
	$NaHCO_3$	210 mg
	Na Pyruvate	1.0 ml
	Penicillamine	2 mM
	Hypotaurine	1 mM
	Epinephrine	250 µM
	BSA	600 mg
	Gentamycin 50 µg/ml	100 µl
	ddH_2O	up to 100 ml
In vitro maturation medium	TCM-199	99 ml
(modified parker medium)	Hepes	149 mg
	$NaCHO_3$	25 mg
	OCS (heat inactivated)	12%
	L-glutamine	0.5 mM
	Pyruvate	0.2 mM
	Gentamycin sulphate	50 µl/ml
	FSH	10µl/ml
KCl 1 M	KCl	74.55 g
	ddH_2O	100 ml
LB Agar	NaCl	4.0 g
	Peptone	4.0 g
	Yeast extract (Hefeextrakt)	2.0 g
	Agar	8.0 g
	ddH_2O	400.0 ml
	NaOH 1 M (pH: 7)	280 µl

Lysis buffer	Tris pH 7.6	50 mmol/L
	NaCl	150 mmol/L
	SDS	0.1%
	NP-40	1%
	Protease and phosphatase inhibitor cocktails	1 mg
Mg^{2+} 2 M	Mg^{2+}	4.861 g
	ddH_2O	100 ml
Modified tissue culture medium (oocyte collection medium)	TCM-199 medium	ready solution
	Hepes	4.4 mM
	$NaCHO_3$	33.9 mM
	Pyruvate	2 mM
	Calcium lactate	2.9 mM
	Gentamycin sulphate	55 mg/ml
NaCl 0.9% (saline)	NaCl	9.0 g
	ddH_2O	1000 ml
NaCl 1 M	NaCl	58.44 g
	ddH_2O	100 ml
PBS Ca and Mg free (CMF) 10x	NaCl	8.0 g
	KCl	0.2 g
	Na_2HPO_4	1.15 g
	KH_2PO_4	0.2 g
	ddH_2O	1000.0 ml
	95% Etanol	up to 50 ml
PBS-PVA	PBS	50 ml
	PVA	150 mg

SOC Media	Trypton	8.0 g
	Yeast extract (Hefeextrakt)	2.0 g
	NaCl 1 M	4.0 ml
	KCl 1 M	3.4 ml
	ddH_2O	400.0 ml
	Mg^{2+} 2 M	4.0 ml
	Glucose 2 M	4.0 ml
Urea/DTT (10M urea, 50 mM DTT)	Urea	3 g
	DTT	250 µl
	ddH_2O	3 ml

3.1.3 List of equipment

The equipment used in the present study is listed below.

Equipment name	Manufacture / supplier
Analytical Balance Mettler Toledo XS603S	Mettler Toledo GmbH, Switzerland
Autoclave Systec 3870 ELV	Tuttnauer Europe b.v. The Netherlands
Chemidoc XRS (Bio-Rad) instrument	BioRad, Munich, Germany
Clean bench Heraeus Herasafe HSP 12	Heraeus Instruments, Germany
Clean bench KOJAIR	Kojair Tech Oy, Vippulla, Finland
CO_2 incubator (MCO-17AI)	Sanyo, Japan
Confocal Microscope CLSM-710	Carl Zeiss, Germany
Cover glass 24x24 mm	Marienfeld, Germany
Cover glass 24x60 mm	Marienfeld, Germany
CryotubeNunc	Roskilde, Germany

CytoOne 96-Well Plate	Starlab, Switzerland
Diagnostic Microscope Slides	Thermo Scientific, Germany
Digital pH-Meter Type 643	Knick, Germany
Electrophoresis unit	BioRad, Munich, Germany
Falcon tube 15ml Cellstar®	Greiner Bio-One GmbH, Frickenhausen, Germany
Falcon tube 50ml Cellstar®	Greiner Bio-One GmbH, Frickenhausen, Germany
Four well dishes for embryo culture	Nunc, Roskilde, Denmark
GenomeLab GeXP Genetic Analysis System	Beckman Coulter, Germany
Grant Bio PMR-30 Platform Rocker (rotational schaker)	Keison International Ltd, UK
Haemocytometer Set, Neubauer	Marienfeld, Germany
Ice Flaker Scotsman AF80	Scotsman® Ice Systems, England
Incubated and Refrigerated ShakerMaxQ 6000 Stackable	Thermo Scientific, USA
Incubator Memmert BE 600	Memmert GmbH, Germany
Injection Needle Sterican® 18G x 1½”	Braun Melsungen AG, Germany
Injekt luer Solo	B. Braun Medical Inc., Pennsylvania, USA
Inverted microscope, ECLIPSE TS100	Nikon, Japan
Labnet Hermle Z200 M/H benchtop micro centrifuge	Labnet International, Inc., USA
Labnet Hermle Z233 MK Refrigerated Centrifuge	Labnet International, Inc., USA
Lab-Tek II Chamber Slide w/Cover 154941 CC2	Thermo Fisher Scientific, USA

Glass Slide Sterile	
Liquid nitrogen container	Taylor-Wharton, USA
Luminometer plate MICROTEST™ Flat Bottom Tissue Culture, Treated by Vacuum Gas Plasma	Becton Dickinson Labware Europa, Le Pont De Claix, France
Magnetic Stirer IKA Big Squid White 115V IKA 3050009	IKA Werke GmbH & CO. KG, Staufen, Germany
Micropipette PIPETMAN Classic™ 0.2-2 µL	Gilson Inc, France
Micropipette PIPETMAN Classic™ 100-1000 µL	Gilson Inc, France
Micropipette PIPETMAN Classic™ 10-100 µL	Gilson Inc, France
Micropipette PIPETMAN Classic™ 20-200 µL	Gilson Inc, France
Micropipette PIPETMAN Classic™ 2-20 µL	Gilson Inc, France
Microscope slide 25x75x1.0 mm	Thermo Scientific, Germany
Millipore Milli-Q A10) Ultra-Pure Water Purifier (FMQS6V0TY)	EMD Milipore Corporation, USA
Mini-Centrifuge Sprout®	Heathrow Scientific, USA
MyCyler Thermal cycler	Bio-Rad Laboratories, USA
Mµlti®-SafeSeal®Tubes, natural	Carl Roth GmbH+Co. KG, Karlshure, Germany
NanoDrop 8000	Thermo Scientific, USA
Orbital shaker KL2	Edmund Bühler, Tubingen, Germany
PCR Strip Tubes, PCR-0208-C	Axygen, USA
Precision Hot Plates PRÄZITERM	I.M.P Vienna, Austria
RT-PCR ABI PRISM® 7000 DS	Applied Biosystems, USA
RT-PCR plate MicroAmp® Fast Optical 96 well	Applied Biosystems, USA

Spectrophotometer Ultraspec2100 pro	GE Healthcare Europe GmbH, Freiburg, Germany
Sub-Cell GT Cell and PowerPac Basic 300V Power Supply Gel electrophoresis	Bio-Rad Laboratories, Inc., USA
The Universal Microplate Luminometer Centro LB 960	Berthold Technologies, Germany
Thermal Cycler MyCycler™	Bio-Rad Laboratories, Inc., USA
Thermo Max Elisa plate reader	MWG-Biotech Germany
Ultra low freezer (-80°C)	Labotech GmbH, Göttingen, Germany
Ultratip Blue (100-1000 µl)	Greiner Bio-One GmbH, Germany
Ultratip White (1-10 µl)	Greiner Bio-One GmbH, Germany
Ultratip Yellow (10-200 µl)	Greiner Bio-One GmbH, Germany
Universal Hood II	Bio-Rad Laboratories, Inc., USA
UV Transluminator (Uvi-tech)	Uni Equip, Martinsried, Germany
Vortex Certomat MV	B. Braun Biotech International GmbH, Melsungen, Germany
X-Clear Advanced Polyolefin StarSeal	Starlab International GmbH, Hamburg, Germany

3.1.4 List of software

During the experiments, the following software was used:

Software name	Link / source
ENSEMBL genome browser	http://www.ensembl.org/
GraphPad Prism 5	GraphPad Software, San Diego California USA
NCBI Blast	http://www.ncbi.nlm.nih.gov/blast

Pimer3	http://bioinfo.ut.ee/primer3-0.4.0/
Quantity One® 1-D analysis software	Bio-Rad
QuickCalcs molarity calculator	http://www.graphpad.com/quickcalcs/Molarityform.cfm
Weight to molar quantity (for nucleic acids)	http://molbiol.ru/eng/scripts/01_07.html
Zen 2011	http://microscopy.zeiss.com/
miRanda	http://www.microrna.org/microrna/home.do
miRBase	http://www.mirbase.org/
miRecords	http://mirecords.biolead.org/
MikroWin 2000	http://www.mikrotek.de/content/view/19/56/
TargetScan	http://www.targetscan.org/
PICTAR	http://www.pictar.org/
DNA reverse and complementary sequence	http://www.bugaco.com/calculators/dna_reverse_complement.php
FINDTAR3	http://bio.sz.tsinghua.edu.cn/
ImageJ v.1.48	http://bio.sz.tsinghua.edu.cn/
Softmax Pro v4.0	http://www.moleculardevices.com/Products/Software/SoftMax-Pro.html
Serial Cloner v.2.6.1	http://serialbasics.free.fr/Serial_Cloner.html
GenomeLab System v.10.0.30	https://www.beckmancoulter.com/wsrportal/wsr/research-and-discovery/products-and-services/genetic-analysis-systems/index.htm

3.2 Methods

3.2.1 Sample production

There are two types of sample that is produced during this study. First is bovine embryos derived from in vivo and in vitro, and the second one is bovine granulosa cells cultured in vitro.

3.2.1.1 In vivo production of bovine embryos

The part of experiment that deals with animals was performed and handled according to the animal protection laws of Germany. In vivo blastocysts were produced as described previously (Tesfaye et al. 2004). Simmental heifers were super ovulated with 2000 IU of eCG by intra muscular injection at Day 9 of estrus cycle. The superovulation followed by double injection of PGF2α in 8-10 h interval at Day 13 in a purpose to induce a luteolysis. On Day 15, 3000 IU of hCG were administrated intravenously to the cows, followed by two times of artificial insemination using frozen-thawed semen. In this study, semen for artificial insemination and IVF come from a commercial bull. At Day 7 after insemination, in vivo blastocysts were flushed out by a non-surgical uterine flushing method. Consequently blastocysts were pooled in groups of 10, snap frozen with lysis buffer in liquid nitrogen and stored at -80°C until further use for gene expression analysis.

3.2.1.2 In vitro production of bovine embryos

The processes of in vitro production consist of 4 main parts namely oocytes collection, in vitro maturation, in vitro fertilization and in vitro culture, respectively. The entire steps were performed in Animal Biotechnology Laboratory, Frankenforst Research Station, University of Bonn. Bovine ovaries were collected from local abattoir (Bernhard Frenken GmbH Vieh und Fleischhandel Schlachthof Düren) and transported to the laboratory while keeping it warm (37°C) in saline solution (0.9% NaCl) within 1-2 h after the collection. Immediately after arrived, ovaries were then washed 3 times with 37°C saline and 1 time with 70% ethanol for 30 seconds respectively. The COCs were aspirated by using 18G needle attached on 10 ml syringe containing 2 ml aspiration medium, then pooled in 50 ml tubes and kept warm at 37°C. The COCs that are surrounded by more than three compact layers of cumulus cells were selected and separated under stereomicroscope using a glass pipette. Briefly, selected COCs were transferred to TCM-199 medium that was supplemented with 12% ECS (heat inactivated).

The maturation process of selected COCs was initiated by 3 times washing in pre warmed maturation medium then COCs were grouped in each 50. Briefly, COCs were transferred into four well dishes containing 400 µl pre-incubated maturation medium and covered by mineral oil. Oocytes were then incubated for 22-24 h at 37°C 5% CO_2. Prior fertilization, two times washing in fertilization medium was performed. Afterward, oocytes were fertilized by co-incubated with $2x10^6$ spermatozoa/ml in 400 µl of fertilization medium. The best qualities of motile sperm were selected from top layer of percoll gradient. Sperm were collected by

centrifugation method at 10.000 rpm for 10 minutes, followed by 2 times washing and finally re-suspended in a fertilization medium. Fertilization process will take place for 18 h at 37°C 5% CO_2.

Presumptive zygotes were collected in 15 ml falcon tube containing 1 ml culture medium then denuded from cumulus cells. After a series of washing, in order to remove dead sperm and cumulus cells, 50 zygotes were then grouped and transferred into 4 well dishes each containing 400 µl IVC medium (SOF) covered by mineral oil. The first cleavage of embryos was expected within 48 h post fertilization, following with incubation for consecutive days to produce different stages of preimplantation embryos according to the experimental design and analysis purposes.

In the second part of experiment 1, presumptive zygotes were randomly transferred into 2 types of culture media. First group is zygotes that cultured in the presence of 10% OCS (with serum) and the second one was cultured in media with the addition of PVA 0.1% (without serum). Resulting embryos at 2-, 8-, 16-cell and blastocyst stage were harvested. All embryos sample were snap frozen with lysis buffer in liquid nitrogen and stored at -80°C for RNA isolation subsequently to gene expression analysis. Moreover, in the experiment 1 part 3, zygotes were subsequently divided into 3 groups then transferred to culture medium supplemented with different macromolecules source namely 10% serum, 0.3% BSA and 0.3% BSAFAF. For all embryo culture media, essential amino acids and non-essential amino acids were supplemented for each 10 µl/ml. Zygotes were cultured until Day 9 of blastocyst stages for development rate calculation. Blastocysts which develop at Day 7 were snap and frozen at -80°C for RNA isolation. Part of the resulted blastocyst also used for phenotype analysis such as lipid content, mitochondrial activity, ROS detection and protein detection by immunohistology method.

3.2.1.3 AICAR application in bovine embryo culture media

In this study, AICAR was used in attempt to increase AMPK activity in embryos artificially. Briefly, 25 mg AICAR was diluted in 2.5 ml sterile ddH_2O resulted 10 mg/ml AICAR as stock solution. Considering of AICAR molecular weight (MR: 258.23), a calculation with the help of molarity calculator was performed to prepare 1 mM AICAR as working solution by diluted AICAR stock in the culture medium followed by filtration. Desired AICAR working solution concentration (0, 1, 10 and 100 µM) then can be prepared by the same dilution method and applied to bovine in vitro culture media in the presence of serum. We treated

embryos by AICAR from zygote until blastocyst stage. Result from this preliminary treatment, allowed us to select one AICAR concentration which shows the highest AMPKA1 gene expression compared to the other groups. The selected concentration is then applied in IVP of embryo and resulted blastocyst were pooled and snap frozen with lysis buffer and stored at -80°C for RNA extraction and for specific phenotype analysis.

3.2.1.4 Bovine granulosa cells culture

Granulosa cells were collected from 2-8 mm growing follicle by aspirate the follicular fluid then pooled in 15 ml falcon tube containing 5 ml PBS without Ca^{2+} and Mg^{2+}. Thereafter, cell debris was let settled down for 15 minutes. To collect granulosa cells, 500 x *g* of centrifugation followed by discard flow through were performed for 5 minutes. Pooled granulosa cells were then centrifuged and the resulted pellet was washed two times and re-suspended in cell culture media. Number of granulosa cells were counted using haemocytometer and its viability were assessed by trypan blue staining. Final granulosa cells suspension was delivered into 24 wells tissue culture plate and viable cells were seeded in concentration $2x10^5$ cells/ml. Granulosa cells were cultured in F12 culture medium supplemented with 10% FCS or 0.3% BSAFAF, 200U/mL penicillin streptomycin and 100mg /mL fungizone. Culture media were changed in every 24 h. In this granulosa cells culture work, Day 0 set as 24 h, Day 1 as 48 h and Day 2 as 72 h post seeding. Cells confluency was visually observed by counting the proportion of attached cells to the bottom of culture dish. In addition, in each observed day, granulosa cells were harvested using 400 µl of EDTA after the removal of culture media and two times washing with PBS using pipetting method. Cells were then incubated at 37°C for 10 minutes followed by gently rocking backward and forward. Detached cells were then transferred into 15 ml falcon tube, pooled from 3 to 6 wells and centrifuged at 1000 x *g* for 5 minutes followed by discarding the supernatant. Afterward, cells pellet were washed two times using PBS and centrifuged with the same previous speed. Thereafter, granulosa cells were stored in -80°C for further RNA isolation work.

3.2.2 RNA isolation

During the experiment, two types of RNA isolation kit were used. Total RNA from embryos sample were isolated using Acturus® PicoPure® RNA isolation kit, while total RNA from granulosa cells were isolated using miRNeasy® Mini Kit.

3.2.2.1 Total RNA isolation from preimplantation stage of embryos

Ten embryos from each preimplantation stage were pooled in one tube as one replication and 3 replicates were needed for each group. Total RNA from embryos was isolated using PicoPure RNA isolation kit and performed according to the manufacture protocol. Briefly, 75 µl of extraction buffer was added to the tube, which is containing embryo sample followed by well mixing by repeated pipetting. Thereafter, samples were incubated in 42°C for 30 minutes and before the end of incubation time purification columns were prepared by pipetting 250 µl of conditioning buffer. The purification columns then incubated for 5 minutes at room temperature followed by columns centrifugation at 13250 rpm for 1 minute. At the end of incubation time, 75 µl of ethanol 70% was added to the samples then mixed well by repeat pipetting. Afterward, the cells extract, which is a mix between samples and ethanol was transferred to preconditioned purification column then centrifuge at 1000 rpm for 2 minutes to bind RNA. And then the column is immediately centrifuged at 13250 rpm for 30 seconds. Columns were then continued to the washing step by pipetting 100 µl wash buffer 1 and centrifuge at 9500 rpm for 1 minuet. An additional of DNA digestion step was performed afterward, by mixing 5 µl of DNase I with 35 µl of RDD buffer then adding the mixture to the purification column followed by 15 minutes incubation at room temperature. By the end of incubation time, 40 µl of wash buffer 1 was pipetted into column and centrifuge at 9500 rpm for 15 seconds. This process then followed by two times washing step with 100 µl washing buffer 2. The first washing step was followed by centrifugation at 9500 rpm for 1 minute, and then the second step followed by centrifugation at 13250 rpm for 2 minutes. After that, any residual buffer was observed in the column. At this point if there was any fluid remaining, 1 minute re-centrifugation at 13250 rpm will be performed. Columns were then transferred to the new 0.5 ml micro collection tubes and 12 µl of elution buffer was pipetted directly onto the middle of column membrane followed by 1 minute incubation at room temperature. To collect the total RNA, columns were centrifuged at 3250 rpm followed by 13250 rpm and each centrifugation is for 1 minute. Total RNA concentration was determined by NanoDrop and stored in -80°C for further use.

3.2.2.2 Total RNA isolation from granulosa cells

Total RNA was isolated from three pools of granulosa cells as a replicate by using miRNeasy mini kit. Briefly, 200 µl of QIAZOL was added into each granulosa cells samples followed by gently vortex until all the cells were diluted. Thereafter, 5 minutes incubation at room temperature was performed. By the end of incubation time, 200 µl was added followed by short vortex, 2 minutes incubation and 15 minutes centrifugation at 4°C. Keeping in mind, all

centrifugation steps in this protocol were performed at 12.000 x *g*. Five hundred µl of upper aqueous phase was transferred to a new collection tube and immediately 750 µl of ethanol 100% was added followed by gently mixing by repeat pipetting. Resulted mixtures then transferred to RNeasy Mini Spin Column, followed by 30 seconds centrifugation at room temperature. The step was repeated until the rest of remaining sample. Afterward, 1 time washing by 700 µl RWT buffer and 2 times with RPE were performed and in each step followed by centrifugation at room temperature for 1 minutes and discard flow through. Columns were then transferred into fresh collection tube and centrifuged at maximal speed to dry the columns for 2 minutes. Later on columns were leaved open for 1 minute and transferred to a non-stick RNase free microfuge tube. Carefully, 30 µl of RNAse free water was added into center of column membrane, followed by incubation for 1 minute at room temperature and 1 minute centrifugation to collect RNA. RNA concentration then determined by Nanodrop and stored at -80°C for further work.

3.2.2.3 Small RNA isolation from bovine embryos

For miRNA profiling in bovine blastocysts sample, small RNA was isolated using miRNAeasy kit. The protocol was as identical as total RNA isolation in granulosa cells. Briefly 20 embryos were pooled into one replicates per groups and each group contain 3 replicates. Before RNA extraction protocol started, the tube containing blastocysts were thawed and centrifuged at room temperature for 1 minute. Afterward, normal steps RNA isolation as mentioned in granulosa cells was performed.

3.2.3 First strand cDNA synthesis

Adjusted total RNA concentration was converted into first strand cDNA synthesis in 20 µl volume reaction. The entire chemicals used in this procedure were ordered from Thermofischer Company namely First Strand cDNA Synthesis Kit. Briefly, chemicals were thawed, mixed and stored on ice before use. Total RNA concentration from its original stock was adjusted at 1 ng per reaction for RNA from cells and 200 ng for RNA from embryos, by adding nuclease free water (supplied by company). After that, 0.5 µl of Oligo-dT and 0.5 µl Random Primer were added, mixed well by pipetting, followed by short centrifugation and incubation at 65°C for 5 minutes. In the meantime, a master mix containing 4 µl of 5x reaction buffer, 1 µl of RiboLock RNase inhibitor, 2 µl of 10 mM dNTP mix and 2 µl of M-MulV reverse transcripts were mixed by gently pipetting. At the end of incubation time, 9 µl of master mix was added to each reaction followed by incubation at 37°C for 60 minutes, then

70°C for 5 minutes to terminate the reaction. At this point, cDNA product can be directly preceded to gene quantification or stored at -20°C for further use.

3.2.4 Transcription level quantification

The mRNA expression levels were quantified by qRT-PCR by using relative standard curve method. For that, serial dilution for each gene was prepared accordingly by a series of gene cloning and DNA sequencing work.

3.2.4.1 Gene specific cloning for serial dilution

Primer 3.0 software was used to design specific gene primers, and then primers were ordered from Exiqon Company. The primers sequence was listed in table 3. Primers were diluted with ddH_2O treated with UV to 1 mmol/µl as stock solution, and working solution was prepared at 0.1 mmol/µl. Specific gene sequences was amplified in PCR thermocycler and bovine gDNA was used as the template. The PCR product then proceeds to DNA sequencing process, once it is confirmed a series of ligation work were performed.

Table 3: Details of primers used for gene expression analysis.

Gene name	Accession number (NCBI)		Primer sequence 5'-3'	Annealing temperature (°C)	Product size (bp)
AMPKA1	NM_001109802.2	F	AGCCCTTCCTTCTCTTGCTC	55	246
		R	AGGATGCCTGAAAAGCTTGA		
AMPKA2	NM_001205605.1	F	AGCAGAGGTCTGGTTCCTCA	55	155
		R	TGAGACAGAGGACGACATGC		
STK11	XM_003586293.2	F	TTTACAGAGGGCGAGCTGAT	53	207
		R	GATTTTGACGGCTCTTCTGC		
ACC	NM_174224.2	F	CTCTTCCGACAGGTTCAAGC	55	248
		R	ACCATCCTGGCAAGTTTCAC		
CPT1	NM_001034349.2	F	CTGGGACTCAACACAGAGCA	53	285
		R	CTTCTTGATGAGGCCTTTGC		
CPT2	NM_001045889.1	F	CACAACATCCTGTCCACCAG	54	209
		R	CCTTCCAAGGCATCAAACAT		

Table 3: Cont.

Gene name	Accession number (NCBI)		Primer sequence 5'-3'	Annealing temperature (°C)	Product size (bp)
NQO1	NM_001034535.1	F	AACCAACAGACCAGCCAATC	54	154
		R	CACAGTGACCTCCCATCCTT		
PGC1A	NM_177945.3	F	GGCAATTGTCAGGTTGGAGT	55	296
		R	AAAAGTCACGTCGGCCATAC		
GAPDH	NM_001034034.2	F	CCAGGGCTGCTTTTAATTCT	55	166
		R	ATGGCCTTTCCATTGATGAC		
M13	-	F	TTGTAAACGCGGCCAGT	59	
		R	CAGGAAACAGCTATGACC		

Briefly, ligation was set up by mixing 4 µl PCR product, 3 µl 2x Rapid Ligation buffer, 0.5 µl pGEM®T Easy Vector plasmid and 0.5 µl T4 DNA ligase. The ligation components were then gently mixed by pipetting followed with overnight incubation at 4°C to get the maximum number of ligation product. On the following days, ligated PCR products were transformed to Escherichia coli (*E.coli*) as competent cell. Slowly, frozen competent cell was thawed on ice, while 8 µl of ligation product was prepared in 15 ml falcon tube. Sixty µl of competent cell was carefully transferred into the falcon tube by very gently pipetting then placed on ice for 20 minutes. Afterward, competent cell was shock heated in water bath at 42°C for 90 seconds and returned immediately to an ice bucket for 2 minutes. Moreover, 750 µl of SOC media, without ampicillin, was added to the tubes containing cell, and followed by shaking incubation (100 rpm) at 37°C for 90 minutes. In the meantime, sterile bended glasses were prepared to spread previous incubated cells over LB agar plates. Afterward, LB agar plates were prepared by spreading IPTG and X-gal, each 20 µl, onto its surface by using of bended glass, then allowed absorbing for 10-20 minutes under laminar flow.

At the end of incubation time, 400 µl of transformed cell was spread over prepared LB agar plate. The agar plate then stored under the hood for 15 minutes followed by incubation at 37°C for 13-15 h. Expected insert colonies were screened by observe white and blue colonies. Visible white colonies, minimum 5, were picked up and diluted in SOC media containing 4 µl/ml of ampicillin for further multiplication in shaking incubator (100 rpm) at 37°C. In

parallel, part of diluted colonies and also blue colonies for non-inserted control, were amplified using M13 primers in normal PCR reaction. Part of resulted PCR product then analyzed in agarose gel.

Selected colonies, based on product size estimation in agarose gel, were briefly transferred to 15 ml falcon tube containing 8 ml SOC media with ampicillin and incubated in the shaking incubator (100 rpm) at 37°C for overnight. On the following day, plasmids were harvested using GenElute Plasmid Miniprep Kit according to the supplied protocol. In the plasmid isolation work all centrifugation process was performed at 13.000 x *g*. Briefly, falcon tube containing plasmid and SOC media were centrifuged for 5 minutes and the supernatant was discarded. Afterward, 200 µl re-suspension solutions was added and gently mixed by pipetting followed by transfer to 2 ml new collection tube. The steps were followed by adding 200 µl lysis solutions, and mixed by 5 times tube inversion then allowed it to clear for 5 minutes. The next step is adding 350 µl of neutralization solution and mixed it also by inversion, followed by centrifugation for 10 minutes. Meanwhile, binding columns were prepared by adding 500 µl preparation solution and centrifuged for 1 minuet then discard flow through. The clear part was briefly transferred into the binding column then centrifuged for 1 minute. Subsequent steps by adding 500 µl optional wash solution and 750 µl washing solution were respectively performed and each step was followed by centrifugation and discards the flow through. Columns were then dried by additional centrifugation at maximum speed for 1 minute and then transferred to fresh collection tube. Thereafter, plasmids were collected by adding 50 µl of elution buffer into columns, followed by 1 minute centrifugation. Plasmid concentration was determined using NanoDrop and serial dilution was prepared in 10^9-10^1 concentration. Prepared serial dilution for each gene then stored at -20°C for further gene expression quantification.

3.2.4.2 Quantitative real time PCR

The mRNA expression level was quantified using qRT-PCR analysis. Specific primers (Table 3) and its combination was performed in 20 µl reaction which is composed by 2µl sample, 8µl master mix (primer + water) and 10µl SYBR green fluorescent detection dye under an ABI PRISM®7000 instrument (Applied Biosystems, USA). The thermal cycler program was set at 95°C for 3 minutes for initial denaturation step, followed by 40 cycles of 15 seconds at 95°C and 45 seconds at 60°C then followed by melting curve. The amount of transcripts in each gene was normalized to GAPDH as internal control.

3.2.5 DNA sequencing

To confirm the amplified sequence of PCR product, samples were sequenced using CEQ™ 8000 Series Genetic Analysis System (Beckman Coulter). Briefly, 5 µl of DNA sample was cleaned by adding 1 µl of Exosap-IT and incubated at 37°C for 30 minutes. ExoSap-IT was then deactivated at 80°C for 15 minutes. Samples were then amplified in PCR machine using forward or reverse primer in 16 µl reaction consist of 5 µl ddH_2O, 5 µl DNA template, 2 µl forward or reverse primer (specific for each DNA sample) and 4 µl DTCS (Dye Terminator Cycle Sequencing) mix. For this purpose, PCR programs were run at 96°C for 2 minutes, 50°C for 2 minutes and 60°C for 4 minutes respectively for 30 cycles. Afterward, PCR product then transferred to a sterile 1.5 ml micro centrifuge and mixed gently by pipetting up and down. In the meantime, fresh stop solution consist of 2 µl 3M NaOAc (pH : 5.2), 2 µl 100 mM EDTA (pH : 8.0) and 1 µl glycogen were freshly prepared.

In brief, 5 µl of stop solution was added to PCR product and mixed with 60 µl of ethanol 98% in a sterile tube by vortex. Afterward, 15 minutes centrifugation at 4°C was performed and supernatant was removed using micropipette. This step then followed by two times washing with 200 µl ethanol 70%, and each washing step was accompanied by 5 minutes centrifugation at 4°C. The PCR products were then dried in vacuum dry machine for 10 minutes and continued for 5 minutes at 35°C. The resulted pellet then re-suspended in 40 µl SLS (Sample Loading Solution). After 2 minutes incubation, samples were transferred to a CEQ sample plate and covered with mineral oil. In the other side, fresh CEQ separation buffers also need to be prepared and filled into the well. The DNA reading program was initiated according to the manufacturer instruction and resulted sequence was analyzed using NCBI Blast.

3.2.6 Immunofluorescence staining of AMPKA protein

Immunohistochemistry was performed to detect AMPK protein in blastocysts sample. Briefly blastocysts were fixed in 4% paraformaldehyde for overnight at 4°C and on the following day two times washing with glycine in PBS (GPBS) was performed. Afterward, samples were treated by 0.5% (v/v) Triton-X100 in PBS for 3 h at room temperature. Blastocysts were then incubated in 3% donkey serum in PBS at room temperature for 1 h, followed by incubation at 4°C for overnight in primary antibodies against AMPKA1/2 (H-300 Santa Cruz, dilution 1:100) to detect AMPK protein expression. In the next day, samples were washed two times

in PBS and secondary antibody Alexa Flour® 568 (Invitrogen, dilution 1:300) goat anti-rabbit IgG (H+L) was applied by incubating the sample for 1 h incubation at 37°C. After two additional wash in PBS, samples were fixed and mounted on a slide glass using Vectashield (H-1200) containing DAPI. Images were captured under laser confocal scanning microscope LSM 710 (Carl Zeiss, Germany) and Zen 2010 software was used to process the images.

3.2.7 Immunofluorescence staining of AMPKA phosphorylation site

In order to detect AMPK protein activity which known active by phosphorylation process, AMPK phosphorylation site at Thr172 was stained by immunohistochemistry method in blastocyst sample. Briefly blastocysts were fixed in 4% paraformaldehyde for overnight at 4°C followed by two times washing in GPBS. The following process is as same as in AMPK protein staining protocol, however the difference is the use of primary antibody against phospho-AMPKA (p-AMPKA Thr172, 40H9, rabbit monoclonal antibody, Cell Signaling Technology, dilution 1:100).

3.2.8 Assessment of embryo mitochondrial activity

Activity of mitochondria was assessed using dyes that are accumulated in mitochondrial membrane, represent electron gradient at ATP production in oxidative phosphorylation process (Chazotte 2009). Mitochondrial activity was visualized by MitoTracker® Red CMXRos (M7512 Invitrogen) as described by the previous study (Amin et al. 2014). Briefly, blastocysts were incubated with 200nM MitoTracker Red (Mt-Red) for 45 minutes, followed by two times washing with PBS-PVA 0.1%. Blastocysts were then fixed in 4% formaldehyde at 4°C for overnight. Thereafter, samples were washed two times with PBS-PVA 0,1% and mounted on slide glass with Vectashield (H-1200) containing DAPI. Samples were visualized under laser scanning confocal microscope, LSM 710 (Carl Zeiss, Germany), and appropriate wavelength for the Mt-Red (579-599 nm) was used to acquire and photograph the images at x40 lens magnification. Images were then processed using Zen 2011 software.

3.2.9 Blastocyst ROS detection

ROS level in blastocysts sample were determined using cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Life Technologies, USA). Briefly, 10 blastocysts were incubated in 400 µl of 5 µM H_2DCFDA for 20 minutes at 37°C in dark

conditions followed by two times washing with PBS-PVA 0.01%. Thereafter, the blastocyst ROS level was evaluated under inverted fluorescence microscope (Leica DM IRB, Wetzlar, Germany), and images were captured using green fluorescence filter which emitting 517-527 nm wave length.

3.2.10 Embryo lipid droplet staining and quantification

Oil red was applied to blastocyst sample in purpose to stain lipid droplet in the cytoplasm. Briefly, embryos were removed from culture media followed by 2 times in PBS-PVA 0.1%. Afterward, blastocysts were fixed for overnight with formaldehyde 10% at 4°C. In the following day, embryos were washed 2 times in PBS-PVA 0.1% and then incubated for 10 minutes in isopropanol 60%. Oil red was applied to embryos for 1 h followed by 4 times washing with PBS-PVA 0.1%. For quantitative measurement of lipid contents, oil red in blastocyst was eluted with isopropanol 100%. Briefly, stained blastocysts were incubated in rotational shaking machine at 100 rpm for 1 h leading to diffusion of lipid bound oil red out of the embryos into the surrounding isopropanol, and eluted oil red was quantified under spectrophotometer (Ultraspec 2100pro, GE Healthcare Europe, Germany) at 490 nm wavelength. Finally, absolute values for optical densities were calculated to relative values.

3.2.11 Assessment of blastocyst cell number

Cell number was scored by counting an individual blastocyst on each group of treatment. Briefly 20 blastocysts were removed from culture medium and washed 2 times in drop of PBS-PVA by transferring embryo from drop to drop. Then, blastocysts were fixed in paraformaldehyde 4% for 10 minutes, followed by three times washing in drop of PBS. Blastocysts were then mounted on clean microscope slide glass, stained with DAPI (0.01µg/µL in PBS) for 10 minutes and gently pressed with 16x16 mm cover glass. The blastocyst nucleus which emitting blue signal was counted under fluorescent microscope.

3.2.12 miRNA target prediction (*in silico* study)

miRNA target prediction was performed in bioinformatics database that are miRBase, PICTAR, miRanda and TargetScan. Common miRNAs, which were found in at least 3 databases, were selected as candidate then profiled in the granulose cell samples. Then the selected miRNA, which is the most abundance, was uploaded into FINDTAR3 web based

software along with 3'UTR of AMPKA1 in purpose to predict its binding site (Ye et al. 2008). Thereafter, selected binding site then proceeded to 3'UTR primer design for plasmid construction.

3.2.13 miRNA expression profiling

The expression level of miRNAs targeted to AMPKA1 was profiled in qRT-PCR using Universal RT microRNA PCR SYBR Green master mix. To do that, small RNA was converted into small cDNA using miRCURY LNA™ Universal RT MicroRNA PCR system. All these processes were performed according to the protocol supplied.

3.2.13.1 Small cDNA synthesis

Total RNA concentration that carries small RNA was adjusted to 100 ng. In brief, 5x reaction buffer was gently thawed and placed on ice. The RNA spike-in was re-suspended by adding 40 µl nuclease free water and mixed by vortex prior usage then were left on ice for 15-20 minutes. Immediately before use, enzyme mix was thawed by gently flicking the tube and placed it on ice. Master mix was set up for each reaction in 20 µl total volume containing 4 µl 5x reaction buffer, 9 µl nuclease free water, 2 µl enzyme mix, 1 µl synthetic spike-in control and 4 µl template total RNA. The master mix then gently dispersed into nuclease free tubes, followed by RNA template in each tube. This step is followed by mixing the reaction in a very gentle way by pipetting and continued with a short spin down. Samples were incubated at 42°C for 60 minutes followed with heat inactivation at 95°C for 5 minutes and immediately cooled down to 4°C. By the end of the incubation time, cDNA were placed on ice and diluted to 20 folds by adding nuclease free water and stored in -20°C.

3.2.13.2 miRNA expression analysis

In brief, diluted small cDNA and SYBR green were thawed for 15-20 minutes and placed on ice. At this point, SYBR green master mix vials must be protected from light by covering it with aluminum foil. The PCR reactions were set up in 10 µl volume by pipetting 5 µl SYBR green master mix, 1 µl PCR primer mix and 4 µl cDNA templates into each well of PCR plate. After that, 2 minutes centrifugation at 1000 rpm was performed to ensure that all ingredients are mixed well. The RT-PCR was performed by setting up the program in a real time PCR machine with an initial denaturation process at 95°C for 10 minutes, 40 cycles amplification process at 95°C for 10 seconds then 60°C for 1 minute, and a default program for the melting curve analysis. Three reference miRNAs (U6, Snord48 and 5S) were used for

normalization and plotted as reference by geometric mean. Relative quantification of each miRNA expression was calculated with the $2^{-\Delta\Delta Ct}$ method and data were presented in a graph.

3.2.14 miRNA target validation

To validate miRNA which targeted to AMPKA1, luciferase assay method was performed. The luciferase assay work was initiated by plasmid construction followed by luciferase activity measurement.

3.2.14.1 Plasmid construction

In this study, pmirGLO was selected as carrying vector considered it has luciferase promoter sequence and its activity will be measured in luciferase assay. The DNA insert will be cloned in multiple cloning region of pmirGLO as illustrated in figure 6. Initially, plasmid was constructed virtually in Serial Cloner software. Briefly, AMPKA1 3'UTR contain miRNA binding sites were uploaded to the software then primers were designed by adding restriction enzyme sequence. In the other side, a mismatch of oligonucleotide was designed in the same way. The mismatch oligonucleotides were designed according to the method in the previous study (Eades et al. 2011). Virtual PCR, DNA digestion and ligation were performed respectively aimed to ensure the primers and mismatch oligonucleotide perfectly cloned in pmirGLO multiple cloning regions. In the present study, selected region of AMPKA1 3'UTR was amplified using bovine gDNA in normal PCR reaction. Moreover, mismatch oligonucleotide was annealed using annealing buffer which supplied by Promega. Thereafter, amplified DNA and annealed sequence were digested using restriction enzyme and purified using QiaQuick PCR Purification Kit. Then, the concentration of purified plasmid was measured in NanoDrop. At this step, purified DNA can be proceed to ligation step immediately or stored at -20°C.

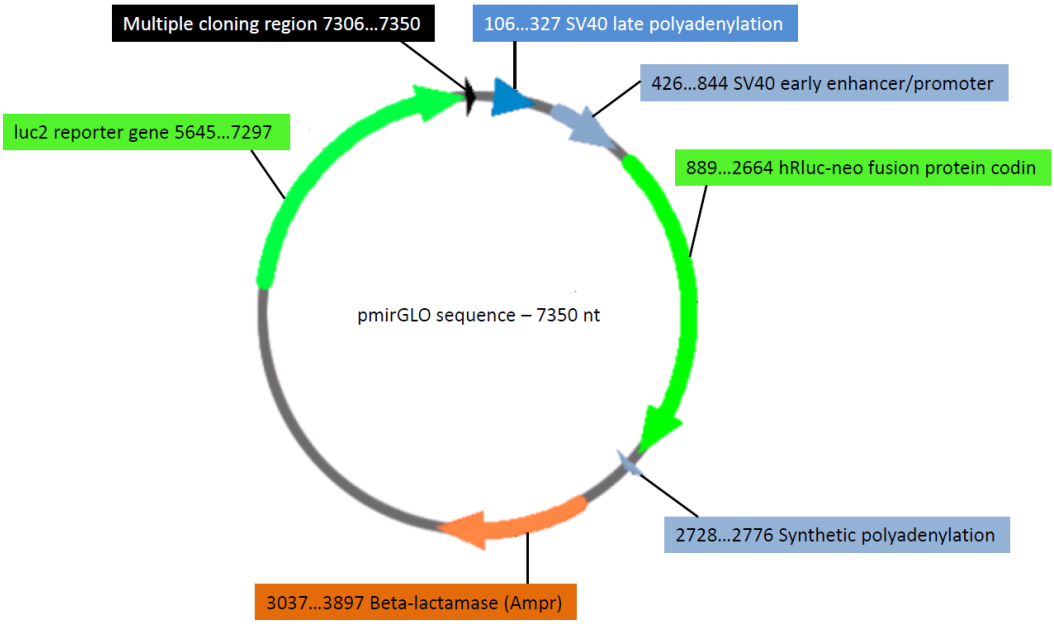

Figure 6: pmirGLO plasmid and its features.

In the present work, ligation was performed in 10 µl reaction using T4 DNA ligase according to the supplied protocol. By the end of the ligation step, 2 types of plasmid namely pmirGLO-AMPKA1-3'UTR-Wild Type (WT) and pmirGLO-AMPK1-Mismatch (MT) will be constructed. The wild type plasmid is carrying miRNA binding site, while the mismatch carries the mutated one. Purified DNA in the previous step was ligated using linearized pmirGLO with concentration 3:1. In order to achieve maximum ligation product, 4°C incubation for overnight was performed. On the next day, ligated DNA was transformed into competent cell and platted in agar medium for 12-14 h. The transformation protocol, bacterial spreading, colony picking followed by bacterial growing were identical as mentioned in gene cloning for serial dilution methods. In regards to screen inserted plasmid contain intended DNA sequence, 10 bacterial colonies were picked up on the following days, and insertion result were confirmed by PCR using pmirGLO primer which amplify multiple cloning site. Product size comparison displayed between colonies in agarose gel was the information for colonies selection. Inserted plasmids will have a greater product size compared to the non-inserted one. Thereafter, inserted colonies were inoculated in SOC media followed by incubation in a shaking chamber, 100 rpm at 37°C, for further plasmid isolation. Moreover, 5 µl of PCR product also prepared for DNA sequencing. Once clone sequences were verified, plasmids were harvested by using GenElute™ Plasmid Miniprep Kit and concentration was measured in NanoDrop. At this point, plasmids can be proceeds directly into co-transfection work or stored at -20°C for further use.

3.2.14.2 Luciferase assay

Luciferase activity was measured using Dual-Luciferase® Reporter Assay System according to the manufacture protocol. Initially, effective dosage of transfection reagent was selected by transfect granulosa cells with different dosage of Lipofectamine that combined with fluorescence green plasmid (pmaxGFP® 0.5 µg/µl). Thereafter the most effective dosage was used for further granulosa cells transfection. In the transfection part, briefly, granulosa cells were seeded at 2 x 10^5/µl in 24 wells plate. After cells confluency reach 80-90%, cells were co-transfected between constructed plasmid either wild type or mutant with miRNA mimic using Lipofectamine followed by incubation for 24 h at 37°C. On the following days, cell culture media was removed and cells were rinsed using PBS. Afterward, 100 µl of 1x passive lysis buffer was added into each well of cell culture plate then gently shake for 15 minutes at room temperature to harvest cell lysate. Lysate were then transferred to a clean tube, and at this point cell lysate can be stored at -80°C of immediately proceed to luciferase activity assay.

Luciferase assay was initiated with reagent II (LAR II) and Stop & Glo reagent preparation. Luciferase assay reagent was prepared by adding 10 ml of luciferase assay buffer II to lyophilized luciferase assay substrate. Afterward, LAR II can be stored at -80°C or immediately used. Meanwhile, Stop & Glo reagent were prepared by adding 2.1 ml of 50x Stop & Glo substrate to 105 ml of Stop & Glo buffer followed by vortex for 10 seconds. Once it is prepared, Stop & Glo reagent can be stored at -20°C for 15 days. In this study luminometer Berthold Centro LB 960 was used to measure luciferase reporter activity. Injector 1 and 2 in luminometer machine were set to disperse 100 µl of LAR II and Stop & Glo respectively. Briefly, 20 µl of cell lysate were dispersed in each well of luminometer plate then loaded in luminometer machine. For luciferase signal measurement, luminometer was set at 2 seconds delay and 10 seconds read time. The resulted luciferase activity data were then analyzed using MikroWin 2000 software, where renilla luciferase activity was normalized to the firefly luciferase activity. Normalized luciferase activity was compared to the un-transfected cells to ensure the transfection protocol is in proper way. In this assay we were measuring luciferase activity of un-transfected cell, transfected with WT plasmid and transfected with MT plasmid.

3.2.15 miRNA mimic or inhibitor transfection

In the aim to study the regulatory mechanism of AMPKA1 post transcriptionally, granulosa cells were transfected with validated miRNA inhibitor. In this study, 50 nM (in total) of miRNA inhibitor in 550 µL culture medium was used in each well of 24 wells plate. To achieve that concentration, cells were cultured in 500 µL culture media, and 50 µl as the complex, 25 µl containing Lipofectamine and 25 µl containing miRNA, were gently added into each well. In addition cells were seeded in 24 wells plates for 2 days prior transfection at confluency around 80-90%. Fifty nM of miRNA was transfected in each 24 well plate using MEM reduced serum system and granulosa cells were continued for incubation in F12 medium plus 10% FCS for the next 24 h. On the following day, cells were harvested and store at -80°C until RNA isolation and protein extraction.

3.2.16 Protein isolation from granulosa cells and Western blot analysis

Granulosa cell proteins were isolated from organic phenol portion during RNA isolation using miRNeasy kit as protocol supplied by the company. Briefly, 300 µl of 100% ethanol was added, mixed and incubated for 2-3 minutes at room temperature. Following centrifugation at 2000 x *g* at 4°C, supernatant which containing protein fraction was transferred into a new cap lock tube then 1.5 ml isopropanol was added, and followed by 10 minutes incubation at room temperature. Samples were then centrifuged at 12.000 x *g* for 10 minutes at 4°C and supernatant was removed. At the following step, all centrifugation and incubation was performed at room temperature until it stated. Immediately, 2 ml of guanidine ethanol was added followed by 20 minutes incubation. By the end of the incubation time, samples were centrifuged at 7500 x *g* and discarded the flow through. Afterward, 2 ml of 100% ethanol was added, vortexes, incubated for 20 minutes and centrifuged at 7500 x *g* for 5 minutes followed by discarding the supernatant. Final centrifugation was performed at 13.000 x *g* for 5 minutes, followed pellets air dried for 10 minutes. Fifty µl of urea-DTT solution was added and pellet was broken up using long needle. Another 450 µl of urea-DTT was also added to the previous solution, followed by incubation for 1 h. After incubation time, protein solutions were transferred into a new collection tube and centrifuged at 10.000 x *g* for 10 minutes. Then supernatant that containing protein was transferred to a new collection tube. Afterward, 500 µl of urea-DTT solution was added and incubated at 95°C for 3 minutes followed by a transfer into a new collection tube and centrifuged at 10.000 x *g* for 10 minutes. Thereafter,

supernatant was transferred into a new collection tube and NanoDrop was used to determine its protein quantity.

For Western blot analysis, protein from each sample were loaded and resolved in 12% SDS-PAGE polyacrylamide gels (Bio-Rad, Corp., Hercules, CA, USA) then transferred to nitrocellulose membranes (Biotrace NT, Pall life Sciences, Pensacola, FL, USA). Membranes were blocked using 5% non-fat dried milk in TBST for 1 h at room temperature and incubated with polyclonal antibodies raised in rabbit against AMPKA1/2 dilution 1:500 at 4°C for overnight. On the following days, after subsequent washing in PBST (5 times each 5 minutes), membranes were incubated with secondary antibodies anti-rabbit for 1 h at room temperature with continuous agitation. In here, primary and secondary antibodies were diluted in 0.1 X Roti-Block buffer. Afterward, membranes were washed in PBST for 5 times each for 5 minutes, then incubated for 5 minutes in chemiluminescent substrate (Thermo Scientific, Waltham, USA) and immunoreactive proteins were visualized under Chemidoc XRS (Bio-Rad) instrument.

3.3 Statistical analysis

In all experiments, samples were produced minimum 3 times and each represents biological replicate. Data were statistically analyzed using Statistical Analysis System (SAS) version 9.1 (SAS Institute Inc., Cary, NC, USA), while graphs were generated using GraphPad Prism 5.0 for Windows, GraphPad Software, San Diego California USA. Mean differences between groups were statistically analyzed using *t-test*. In experiment which has more than 2 groups, their mean differences were compared using one-way ANOVA followed by post hoc multiple pairwise comparisons using *Student Newman Keuls's* (SNK) method. Finally, data were presented as means ± standard deviation (SD) in all experiments and $p<0.05$ was considered to be significant.

4 Results

4.1 AMPK activity and lipid metabolism in bovine embryos under different culture environments

4.1.1 Expression pattern of AMPK gene and lipid accumulation in blastocysts derived in vitro vs. in vivo

Based on transcriptome analysis of blastocysts derived from in vitro vs. in vivo culture, the AMPKA1 was found to be down regulated in blastocysts which is spend longer time in in vitro culture environment compared to in vivo (Gad et al. 2012). On the pathway analysis (by IPA software) it was shown that lipid metabolism was the top affected pathway (FC≥2; $p<0.05$). Therefore, genes in AMPK pathway (AMPKA1, AMPKA2), lipid metabolism (ACC) and mitochondrial activity genes (CPT1, PGC1A) were selected to investigate their expression in in vitro vs. in vivo blastocysts. As the result, AMPK catalytic subunit α, AMPKA1 and AMPKA2, were significantly lower in blastocysts derived from in vitro compared to those derived from in vivo. In contrast, expression of ACC showed significantly higher when embryos were cultured in vitro. Meanwhile the expression of mitochondrial activity genes, CPT1 and PGC1A, were significantly higher in blastocysts derived in vivo (Figure 7).

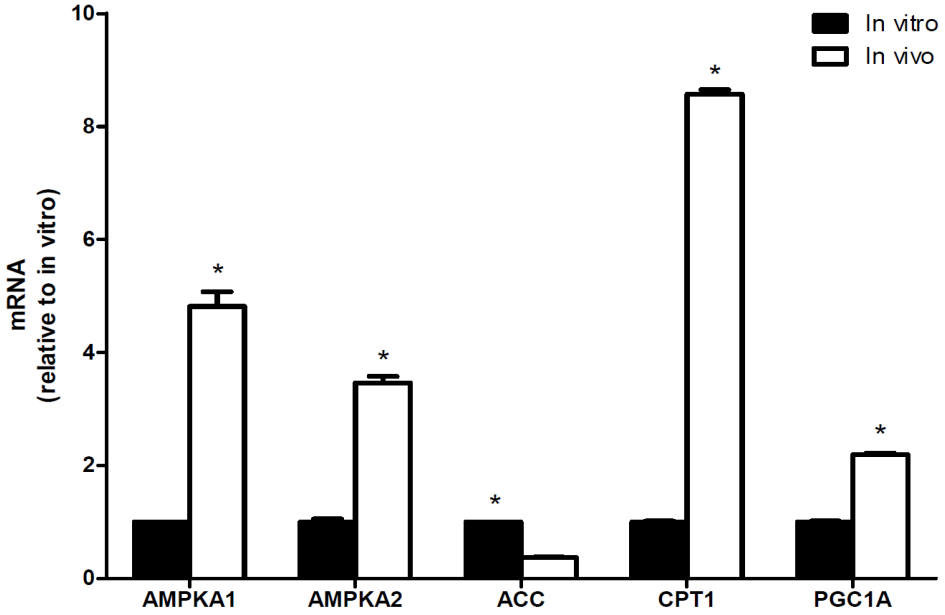

Figure 7: AMPK and its related lipid metabolism genes in blastocysts derived in vitro vs. in vivo. Asterisk (*) represent statistically significant differences ($p<0.05$).

Lipid staining analysis showed a higher lipid droplets stained in blastocysts derived from in vitro than those derived from in vivo (Figure 8A and B). Quantification of these accumulated lipid showed a twofold higher lipid level in IVP embryos than in vivo ones (Figure 8C).

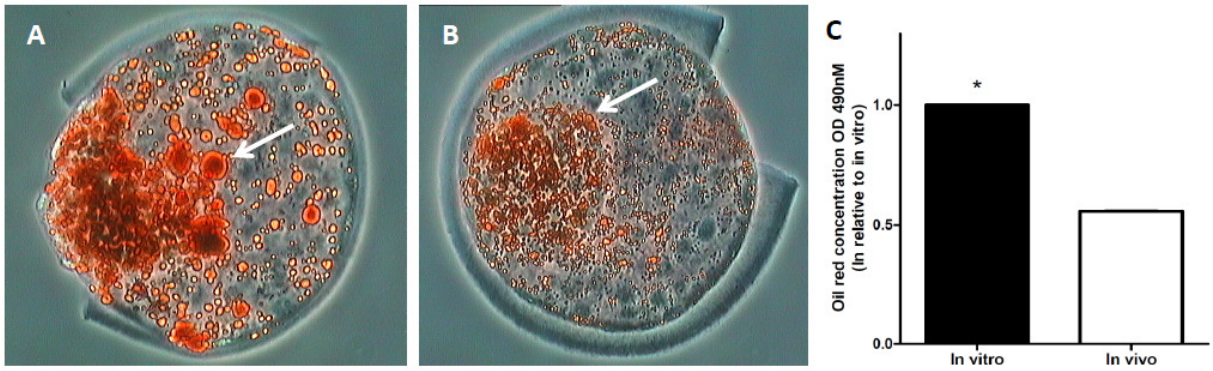

Figure 8: Lipid droplet accumulation as stained by oil red in in vitro (A), in vivo (B) blastocysts and quantified lipid concentration (C). Lipid droplet showed by arrow and asterisk (*) represent statistically significant differences ($p<0.05$).

4.1.2 Expression pattern of AMPK and its lipid metabolism related genes in embryos cultured in vitro in the presence or absence of serum

Taking into account that the widely used protocol for embryo production is IVC and in order to reduce lipid effect which is abundant in serum, we used PVA fatty acid free as the source of macromolecule and as a model of in vitro culture without serum. For that, the gene expression of embryos cultured in the presence or absence of serum at 2-, 8-, 16-cell and blastocyst stages were compared. In this part, genes in AMPK pathway (AMPKA1, AMPKA2, STK11), lipid metabolism (SREBP2, SREBP1, ACC), antioxidant (NQO1) and mitochondrial activity (CPT1, PGC1A) were selected.

The result revealed that expression of AMPKA1 transcript at 2- and 8-cell stage (Figure 9) in embryos cultured in the presence of serum was significantly lower compared to those cultured without serum. Meanwhile, expression of AMPKA1 at 16-cell and blastocysts were significantly higher in embryos cultured without serum, while there was no significant difference in AMPKA2 expression between groups at 2- to 16-cells stage. In addition to that, higher values of AMPKA2 were obtained at blastocysts stage when embryos cultured without serum. The expression of STK11, responsible for upstream kinase protein, showed

significantly higher expression from 2-cell to blastocyst stage in embryo cultured without serum.

In SREBP1 and ACC were express significantly higher when embryos were cultured in presence of serum. SREBP2 showed a reciprocal pattern in 2-cell stages, with no differences in 8- and 16-cell stage on those cultured in the presence or absence of serum. The NQO1, CPT1 and PGC1A showed higher expression in the absence of serum compared to those with serum (Figure 9).

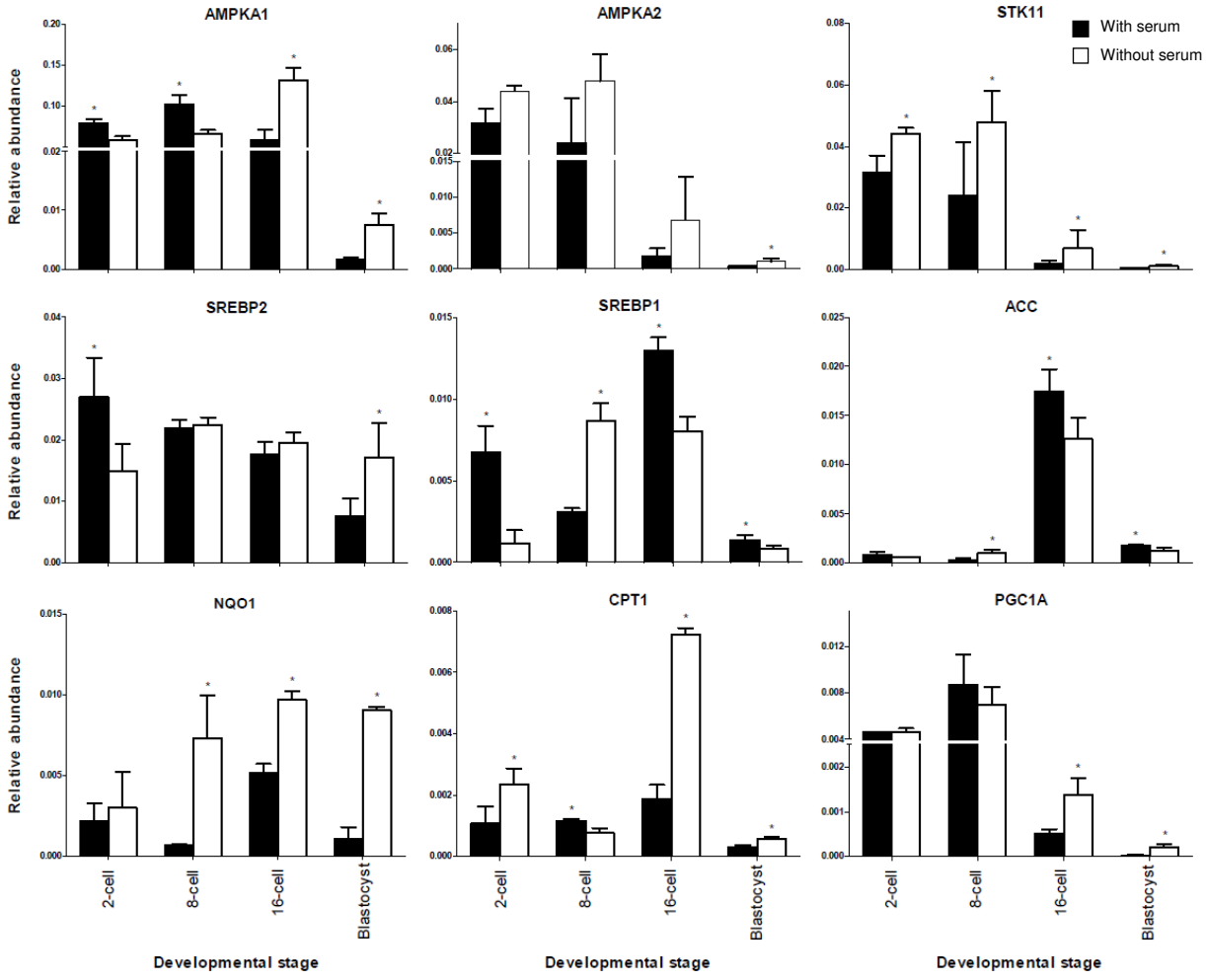

Figure 9: Temporal expression of AMPK and lipid metabolism in various development stages of preimplantation bovine embryos produced in vitro in the presence (black bar) and absence of serum (white bar). Asterisk (*) represent statistically significant differences ($p<0.05$).

Considering solely the blastocyst stages, the gene expression of AMPK was confirmed with protein analysis expression. As the result, blastocyst cultured in the presence of serum

expresses lower AMPK protein compared to without serum. As evidence, lower red fluorescence signals were observed in blastocysts derived from with serum compared to its counterpart (Figure 10).

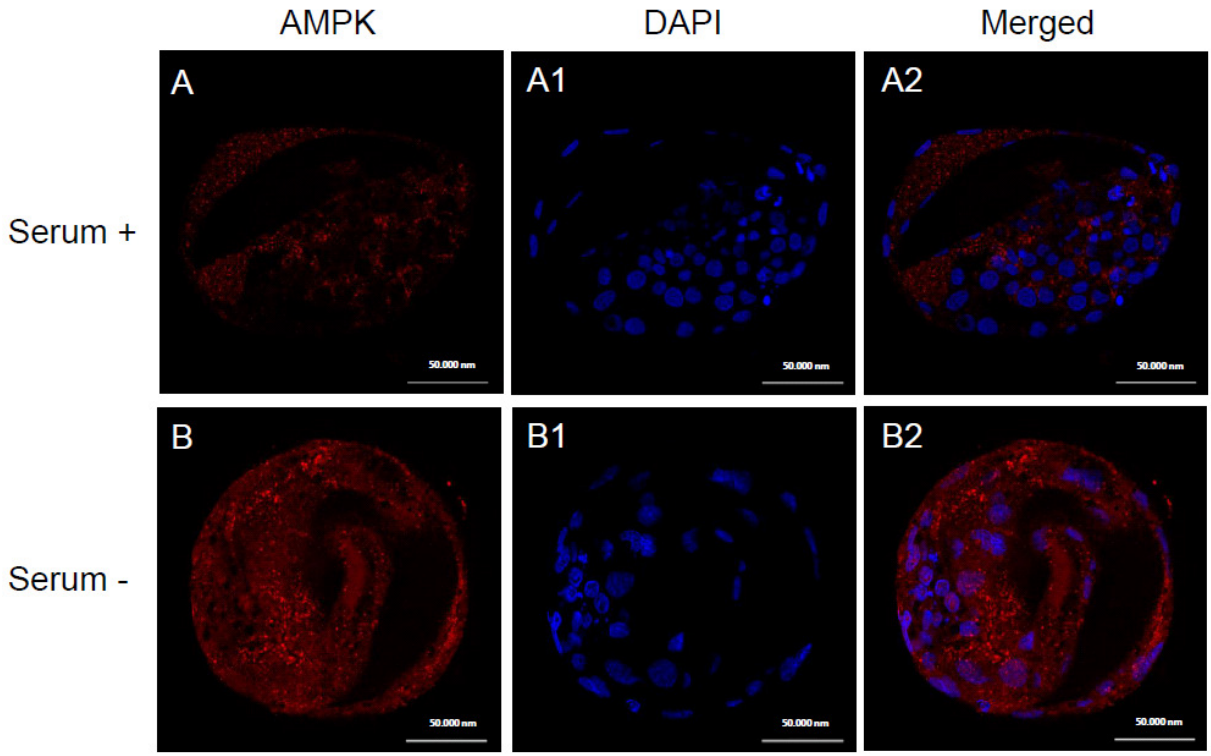

Figure 10: Protein expression of AMPK in bovine blastocysts produced in the presence or absence of serum. Red signal shows AMPK protein (Label A, B) while blue signal is nuclear cells stained by DAPI. Scale bars, 50 µm.

4.1.3 Mitochondrial activity of embryo cultured in vitro in the presence or absence of serum

Here the mitochondrial activity of bovine blastocysts cultured in the presence or absence of serum were compared. Blastocysts cultured in the presence of serum exhibit lower mitochondrial activity as compared to the absence of serum. This was evidenced by, higher red fluorescence signal observed in blastocysts derived from culture without serum compared to those derived from the culture on the presence of serum (Figure 11).

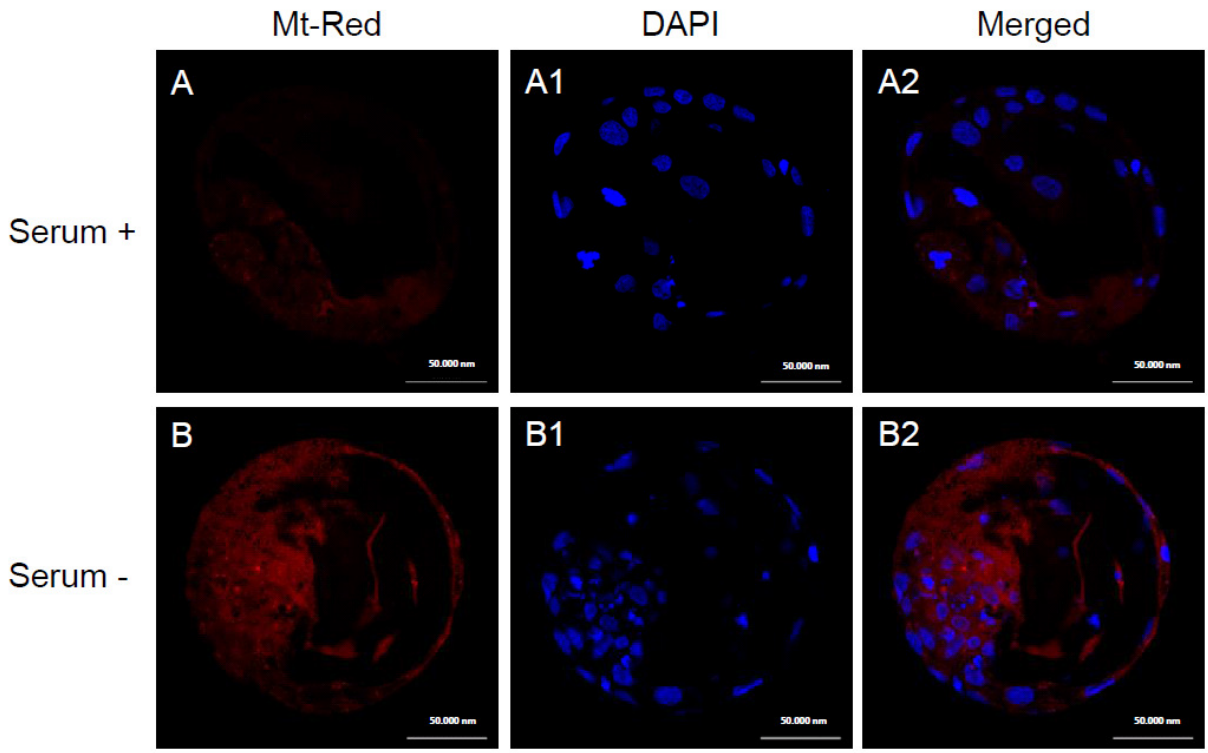

Figure 11: Mitochondrial activity detection of blastocysts cultured in the presence or absence of serum. Red signal in label A and B represent MitoTracker, blue signal A1 and B1 represent DAPI. Scale bars, 50 µm.

4.1.4 Developmental competence of embryos cultured in vitro with different macromolecules supplementation

Taking into account that serum contains abundant fatty acids (Brunner et al. 2010, Gstraunthaler 2003, Sata et al. 1999), different macromolecules which contain different amount of fatty acids were used, to investigate their effect on the expression of AMPK and lipid metabolism genes. For this, embryos were cultured in media with abundant fatty acid (serum), intermediate fatty acid (BSA) and free fatty acid (BSAFAF).

Embryos cultured in the different of macromolecules supplementation were compared in terms of cleavage, blastocyst rate and development speed (Tabel 4). There were no significant differences in cleavage rate between these three groups. On the other hand, embryos cultured in the presence of serum reached to blastocyst stage at Day 7 significantly faster compared to BSA and BSAFAF media. This difference then disappears in Day 8 and Day 9. Moreover, embryos cultured in the presence of serum developed faster compared to the other two groups.

Table 4: Developmental rates of bovine blastocysts produced with different macromolecules supplementation.

Developmental rate	Serum		BSA		BSAFFA	
	n	%	n	%	n	%
Matured oocytes	872	-	809	-	827	-
Cleavage	683	78.33	654	80.84	665	80,41
Blastocyst Day 7	246	28.2[a]	137	16.93[b]	164	19.83[b]
Blastocyst Day 8	306	35.09	228	28.18	245	29.63
Blastocyst Day 9	340	38.99	271	33.50	282	34.10
Kinetic development (Day 7/Day 9)	-	72.35[a]	-	50.55[b]	-	58.16[b]

[a,b] Value with different superscripts indicate within rows differ significantly.

4.1.5 AMPK and lipid metabolism related gene expression in embryos cultured in vitro with different macromolecules supplementation

The AMPKA1 expression of blastocysts cultured with different macromolecules supplementation was significantly lower in embryos derived from IVC in presence of serum compared those cultured in BSA or BSAFAF, while AMPKA2 didn't show any differences between groups (Figure 12). Adversely, ACC showed a significant higher expression in embryos cultured in the presence of serum compared to those cultured in the other two groups. The expression of CPT1, CPT2 and PGC1A were significantly lower in embryos cultured in the presence of serum compared to those cultured in BSA or BSAFAF groups.

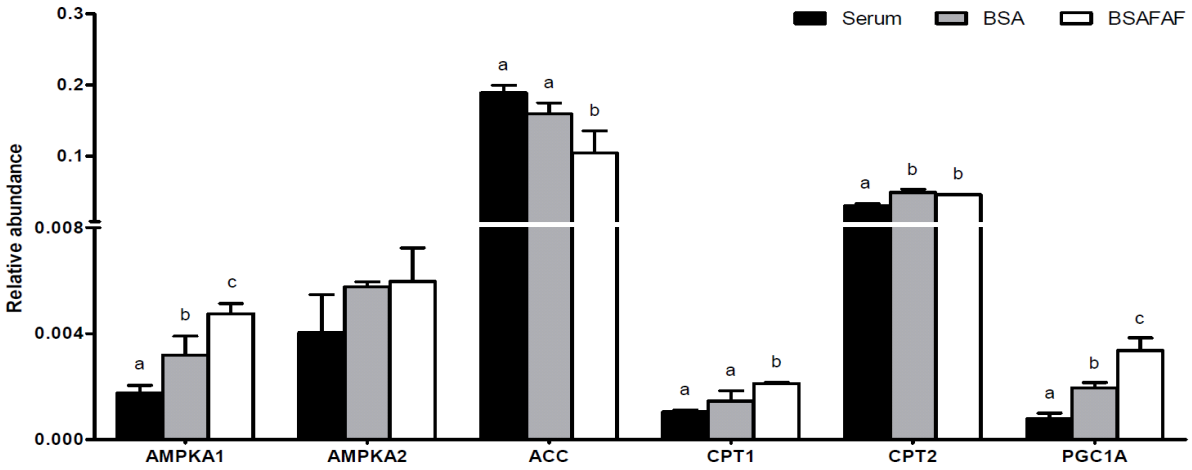

Figure 12: Expression of AMPK pathway in blastocysts produced in vitro with serum (black bar), BSA (grey bar) and BSAFAF (white bar). [a,b]Different superscripts indicate significant difference among groups ($p<0.05$).

Immunofluorescence staining of AMPK protein supported the gene expression data. The lowest signal intensity of AMPK was detected in blastocyst cultured in the presence of serum compared to the other two groups (Figure 13). Due to AMPK protein activity depend on phosphorylation process, therefore we detect the phosphorylation site of AMPKA at Threonin-172 (p-AMPKA Thr172) at blastocyst stage. As result, the lowest fluorescent signal intensity of p-AMPKA Thr172, indicate low AMPK activity or low phosphorylation, was detected in blastocysts cultured in the presence of serum. Meanwhile the highest AMPK activity was detected in blastocysts cultured with BSAFAF (Figure 14).

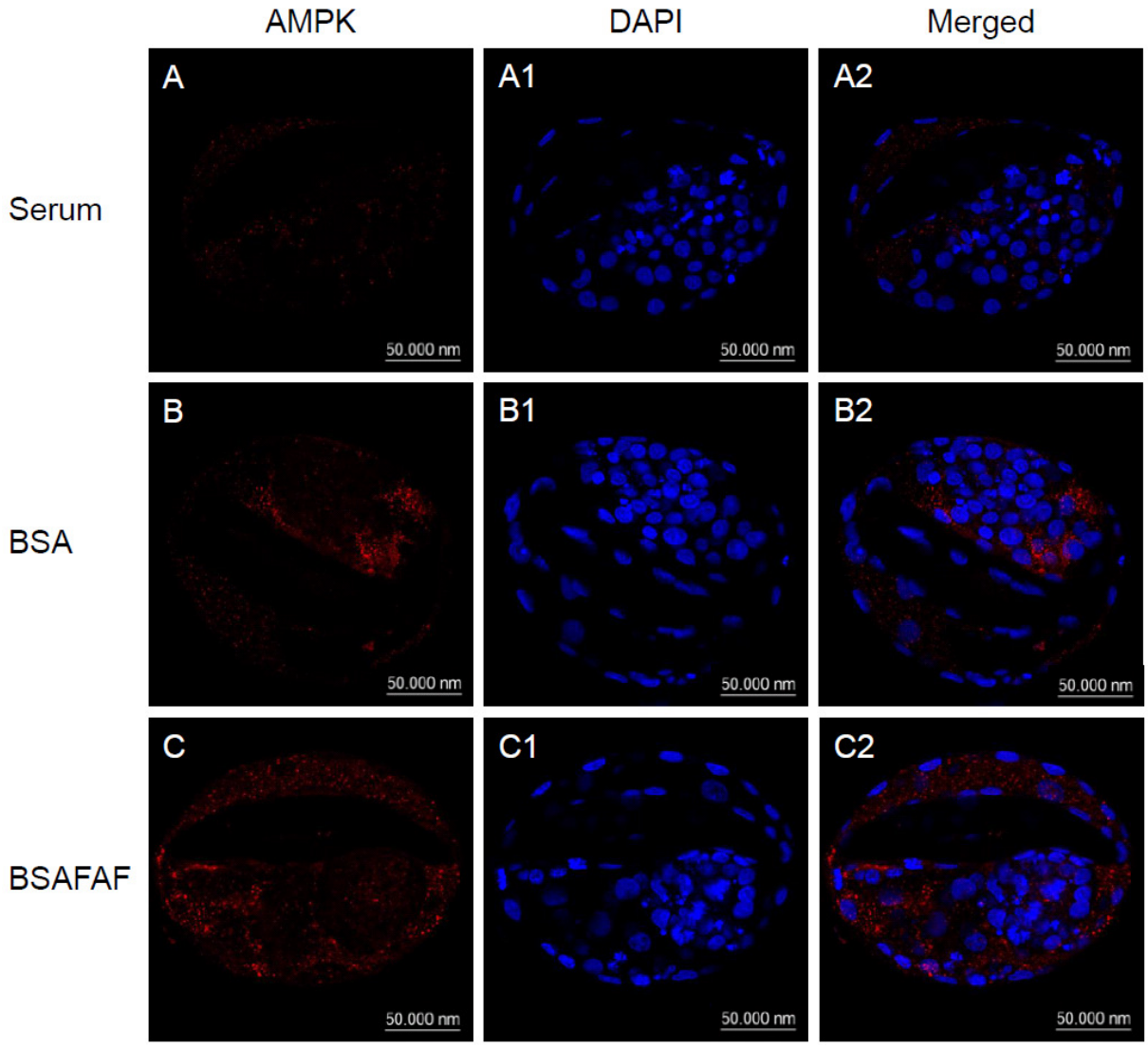

Figure 13: Protein expression of AMPK in bovine blastocysts produced with serum, BSA and BSAFAF. Red signal showed AMPK protein (Label A, B, C) while blue signal is nuclear cells stained by DAPI. Scale bars, 50 µm.

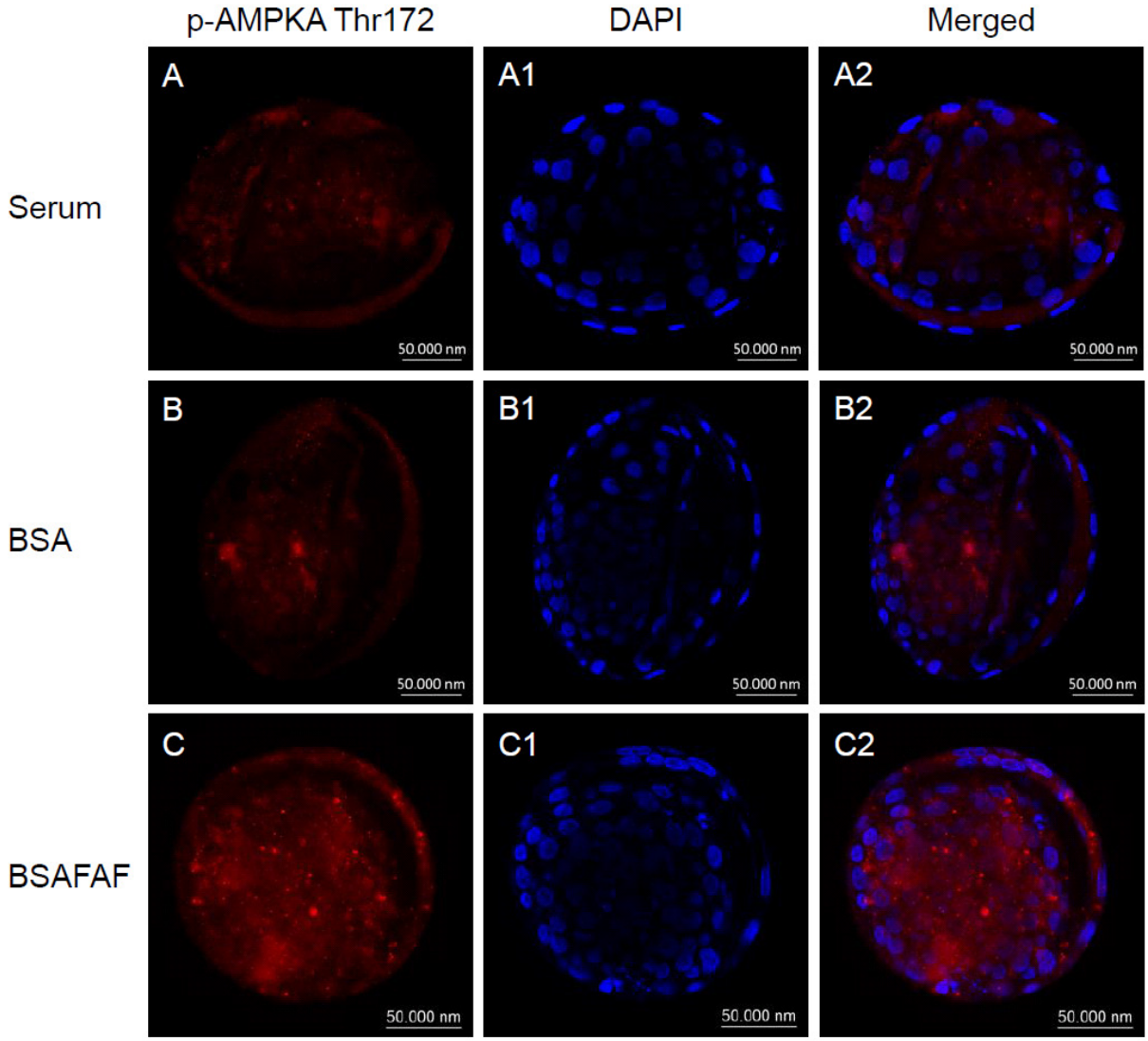

Figure 14: Protein expression of phosphorylated AMPKA at Thr172 (p-AMPK Thr172) in bovine blastocysts produced with serum, BSA and BSAFAF. Red signal showed p-AMPK protein (Label A, B, C) while blue signal is nuclear cells stained by DAPI. Scale bars, 50 µm.

4.1.6 ROS level, mitochondrial activity and lipid accumulation in blastocysts derived from in vitro culture with different macromolecules supplementation

The ROS level between groups was compared by visual evaluation of fluorescence signal exhibited by resulting blastocyst. The result demonstrated a brighter green fluorescence signal intensity which represents more ROS in blastocysts derived from culture in the presence of serum compared to the other two groups (Figure 15).

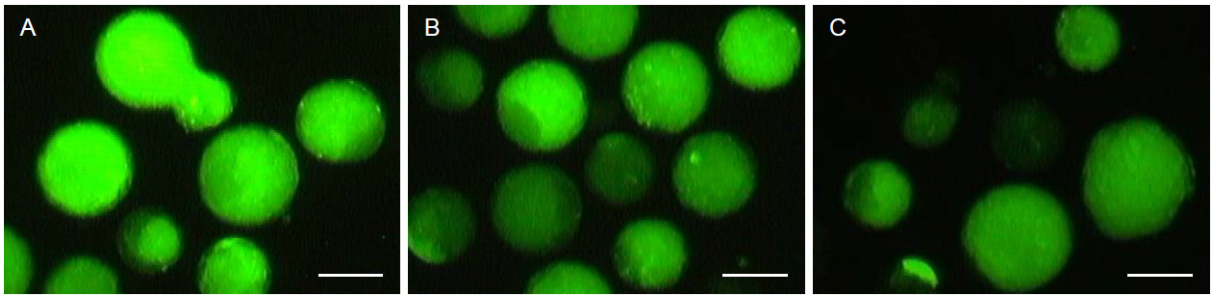

Figure 15: ROS level of blastocysts produced with serum (A), BSA (B) and BSAFAF (C). Brighter green signal indicated more ROS production. Scale bars, 70 µm.

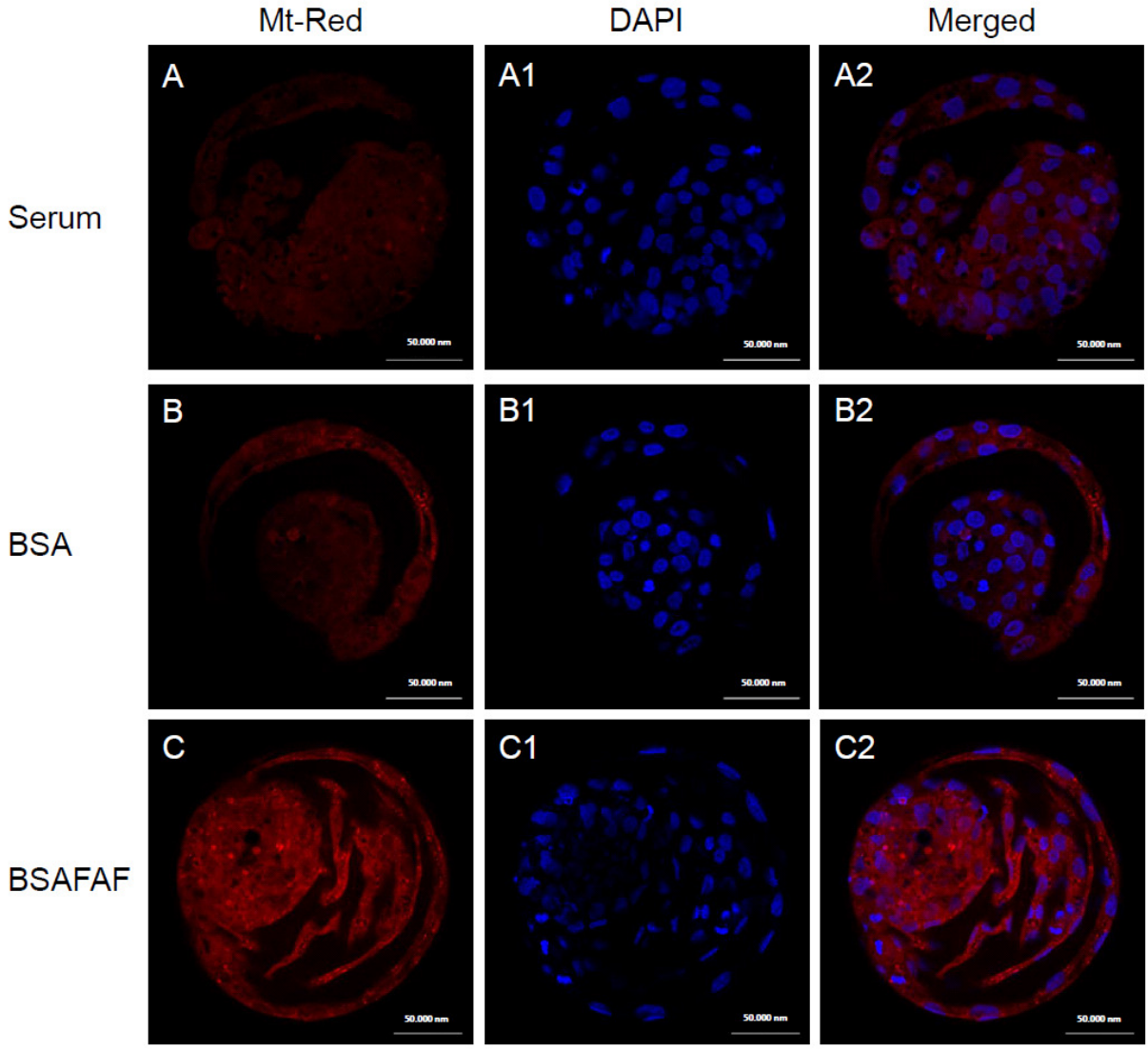

Figure 16: Mitochondrial activity of bovine blastocysts produced with serum, BSA and BSAFAF. Mitochondria were stained with MitoTracker Red (Label A, B, C) and nucleus was stained with DAPI. Scale bars, 50 µm.

Moreover, the increased level of ROS was accompanied by the reduction of mitochondrial activity. In this regards, blastocysts cultured in the presence of serum exhibit lowest mitochondrial activity, while blastocysts cultured with BSAFAF showed the highest one. Low intensity of red florescence detected in blastocysts derived from culture in the presence of serum and higher intensity was observed in BSAFAF group (Figure 16). Investigation of lipid accumulation showed that blastocysts cultured in vitro in the presence of serum to show the highest lipid level compared to those cultured in BSA or BSAFAF (Figure 17).

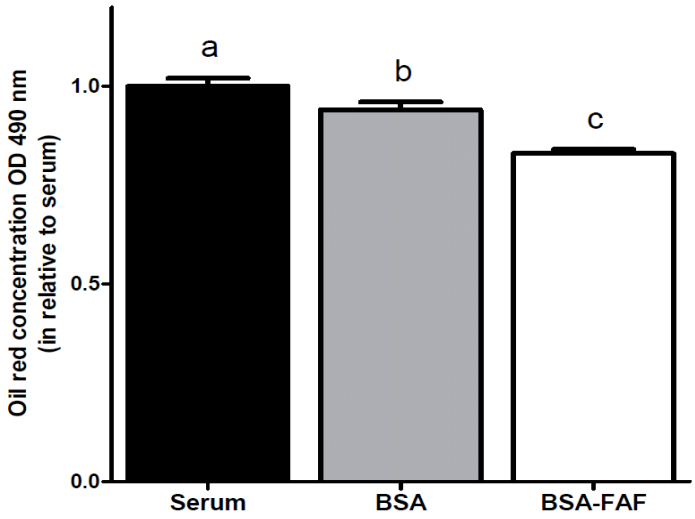

Figure 17: Lipid content of blastocysts produced in vitro with serum, BSA and BSAFAF. [a,b] Different superscripts indicate significant difference among groups ($p<0.05$).

4.2 Modulation of AMPK using AICAR as exogenous activator

In an effort to reduce lipid accumulation through AMPK activity modulation, AICAR as AMPK exogenous activator was applied in bovine embryos culture media. Compared to the lower concentration levels, 100 µM AICAR has been found to increase significantly AMPKA1 gene expression as presented in figure 18.

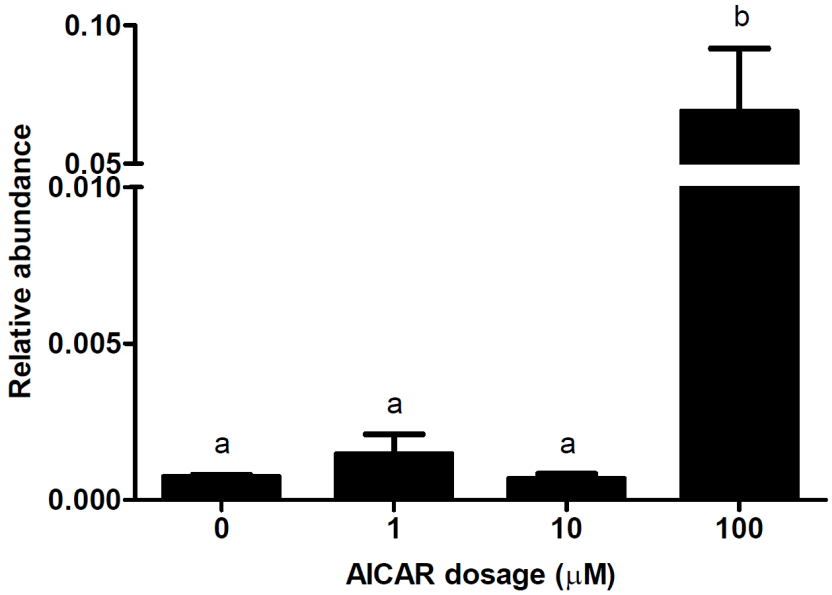

Figure 18: AMPKA1 gene expression of blastocysts cultured with different AICAR dosage. [a,b]Different superscripts indicate significant difference among groups ($p<0.05$).

4.2.1 Effect of AICAR on embryos development competence and blastocyst cell number

As shown in table 5, there is no significant difference of blastocysts development rate. However, the development speed of blastocyst cultured with AICAR was 3.83% lower compared to the control. On the other hand, blastocyst derived from culture with AICAR had significantly lower cell number compared to those without AICAR (116 ± 11.98 vs. 132 ± 14.83; respectively).

Table 5: Bovine embryo development rate of in vitro culture with and without AICAR.

Culture medium	Matured oocytes	Cleavage		Blastocyst rate						Development speed
				Day 7		Day 8		Day 9		
	n	n	%	n	%	n	%	n	%	%
Serum	464	374	80.60	141	30.39	183	39.44	200	43.10	70.50
Serum + AICAR	478	383	80.13	140	29.29	186	38.91	210	43.93	66.67

4.2.2 Effect of AICAR on AMPK expression and its related lipid metabolism genes

As shown in figure 19, the expression of AMPKA1, STK11, PPP2CA were significantly higher in blastocysts derived from culture with AICAR compared to without AICAR. Meanwhile the ACC expression is significantly lower in blastocysts with AICAR. On the expression of NQO1, CPT2, PGC1A are express significantly higher in AICAR treatment. Moreover, the presence of AICAR in culture media didn't show any effect on the CPT1 expression level.

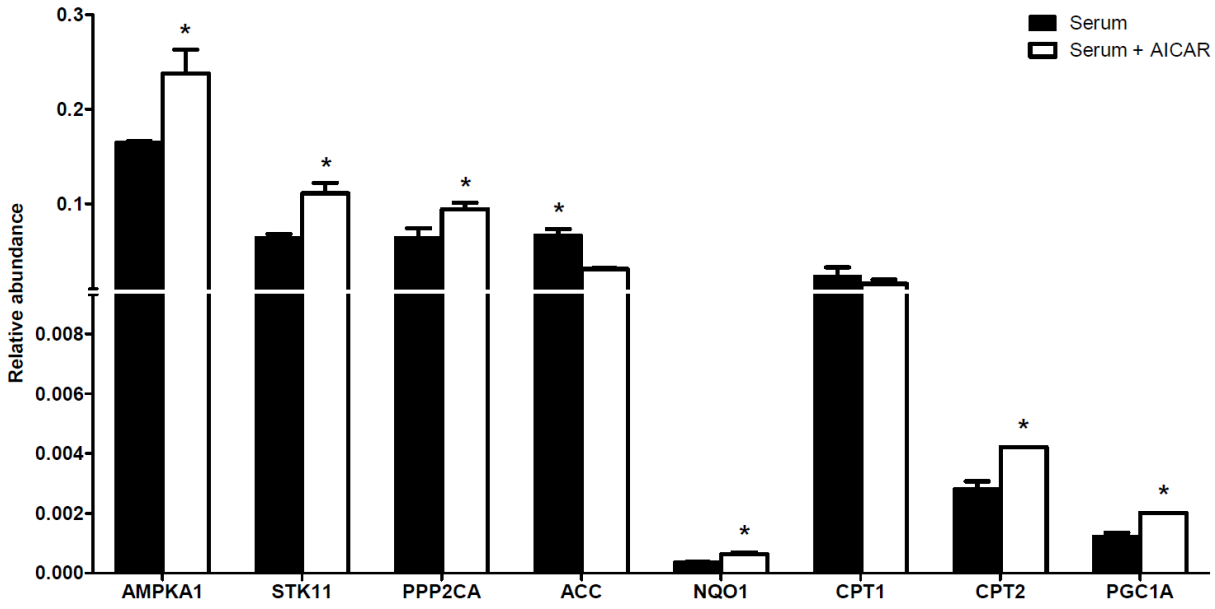

Figure 19: AMPK and its related lipid metabolism genes expression in blastocysts produced with and without AICAR. Asterisk (*) represent statistically significant differences ($p<0.05$).

4.2.3 Effect of AICAR on AMPK protein expression and mitochondrial activity

Alteration on AMPKA1 gene expression due to AICAR treatment at protein level was validated using immunofluorescence. AICAR increases AMPK protein abundant as evidence by higher fluorescent signal intensity was detected in blastocysts cultured with AICAR than that without AICAR (Figure 20). Moreover, AICAR also show to increase mitochondrial activity of blastocyst stage embryos. Visually, blastocysts derived from culture with AICAR exhibit stronger fluorescent signal compared to the absence of AICAR (Figure 20). In addition to that, fluorescent signal quantification of AMPK protein and MitoTracker (mitochondrial activity) confirmed the difference significantly (Figure 21).

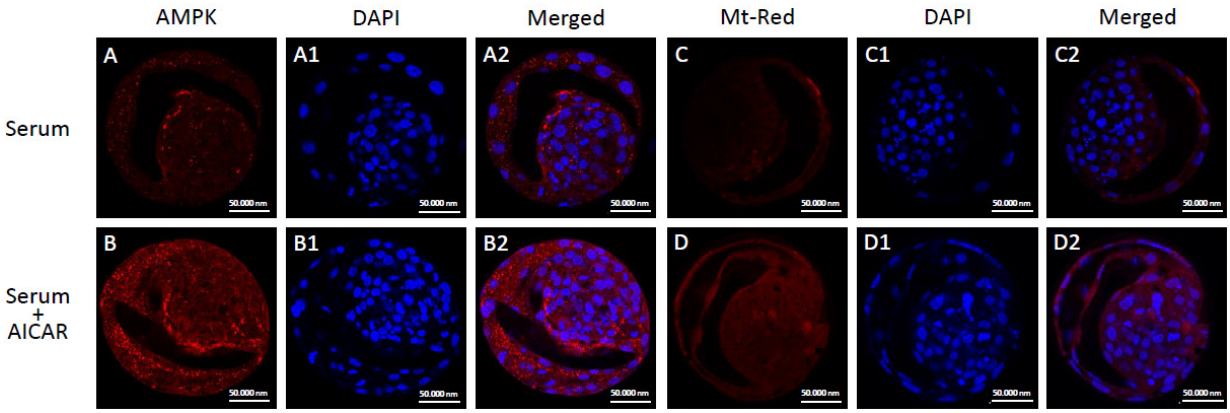

Figure 20: AMPK protein expression and mitochondrial activity assessment in blastocysts cultured with and without AICAR. Red signal showed AMPK protein (Label A, B); MitoTracker (Label C, D) while blue signal is nuclear cells stained by DAPI. Scale bars, 50 µm.

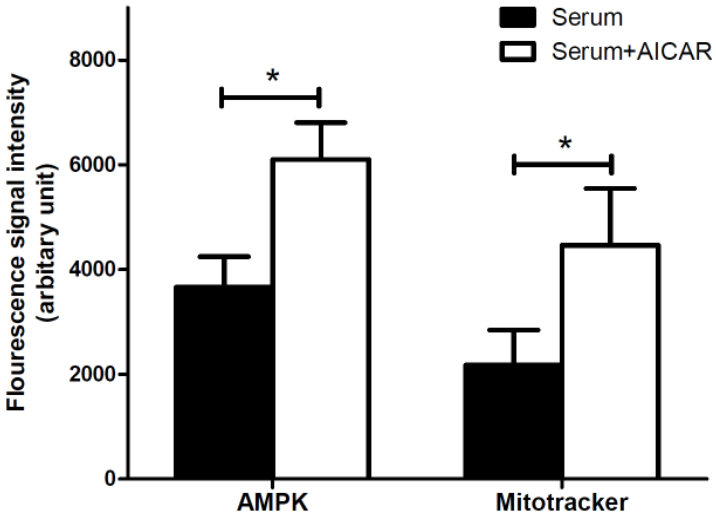

Figure 21: Fluorescent signal intensity quantification of AMPK and MitoTracker from blastocysts cultured with and without AICAR. Asterisk (*) represent statistically significant differences ($p<0.05$).

4.2.4 Effect of AICAR on lipid accumulation of embryo cultured in vitro

Here, the effect of AICAR treatment on blastocysts lipid content was investigated. The result showed that blastocysts derived from in vitro with AICAR treatment has 8.9% lower lipid content compared to without AICAR (Figure 22). However, this result difference was not statistically significant ($p>0.05$).

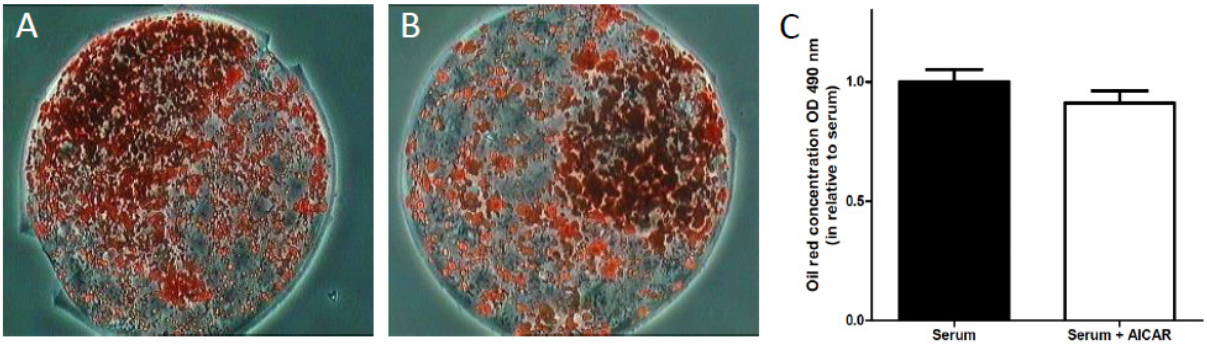

Figure 22: Lipid droplet accumulation as stained by oil red of bovine blastocysts cultured without (A), with (B) AICAR and quantified lipid content (C).

4.3 Potential post transcriptional regulation of AMPKA1 by miRNA

4.3.1 AMPKA1 expression and lipid accumulation of granulosa cell cultured in the presence and absence of serum

In order to investigate the post-transcriptional regulatory mechanism of AMPKA1 in embryo by miRNA, granulosa cell culture model was used to validate the miRNA – mRNA interaction. There are two reasons why granulosa cells were used as cell model. First, it is the closest cells to oocytes and second its luteinization process which marked by lipid accumulation post ovulation. Moreover, the luteinization process can be easily mimicked by IVC under the presence of serum (Kayani et al. 2009). In addition, miRNA target validation to specific genes cannot perform directly in embryos, therefore cell model is needed to bridge the gap.

This study was initiated by cultured granulosa cells in the medium with serum or without serum (BSAFAF). Granulosa cells cultured with serum shows rapid development (more confluence) from Day 0 to Day 2 when compared to without serum (Figure 23). This means the serum gives better support for cells growth which is similar to our result in the development rates of embryos cultured under serum system.

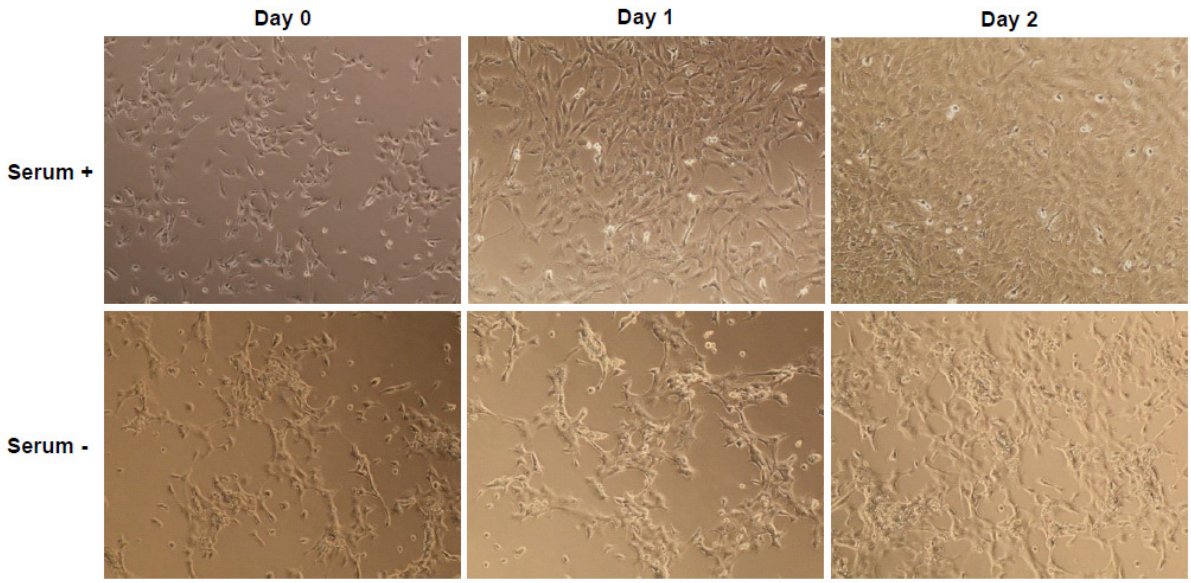

Figure 23: The confluency of bovine granulosa cells cultured in the presence and absence of serum. Original magnification 20x.

The AMPKA1 was found significantly down regulated in Day 2 compared to Day 0 and Day 1 of culture (Figure 24A). More lipid droplets were accumulated in granulosa cell cultured with serum compared to without serum. This result was confirmed by oil red quantification, where culture in the presence of serum significantly increases lipid content at Day 2 (Figure 24B and 24C).

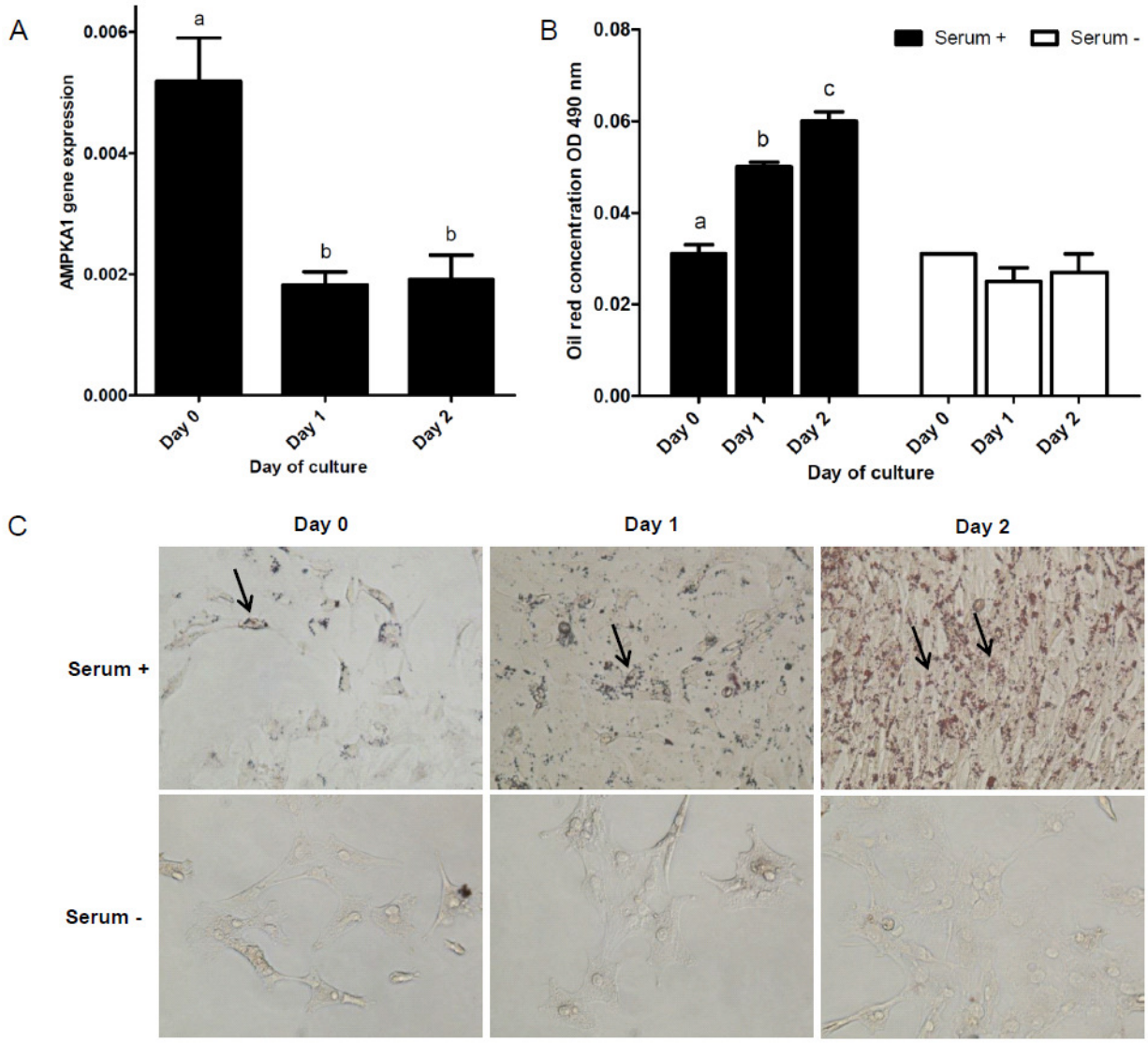

Figure 24: AMPKA1 gene expression (A), lipid content (B) and stained lipid droplet by oil red (C) in bovine granulosa cells cultured in the presence or absence of serum. Lipid droplet shown by arrow (original magnification 40x). [a,b]Different superscripts indicate significant difference among groups ($p<0.05$).

The initial result indicated that the presence of serum in culture media, down regulate AMPKA1 gene expression and increases lipid accumulation across culture time of bovine granulosa cell. Next, we performed *in silico* analysis aiming to predict miRNA which is potentially targeted to AMPKA1.

4.3.2 *In silico* analysis and miRNA profiling

A series of *in silico* analysis using bioinformatics tools including mirBase, PICTAR, mirWalk, miRanda and Targetscan were performed to identify and select miRNAs which

potentially targeted to AMPKA1. As a result in table 6, 11 miRNAs were chosen based on similarity sequence comparison between human (hsa) and bovine (bta). The reason behind this is due to the most available miRNA primer in the market is designed for human miRNA.

Table 6: Bovine miRNA primer and its similarity to human miRNA sequence.

miRNA	Sequence	Accession number	Similiarity (%)
bta-mir-101	UACAGUACUGUGAUAACUGAA	MIMAT0003520	
hsa-miR-101-3p	UACAGUACUGUGAUAACUGAA	MIMAT0000099	100
bta-mir-130	CAGUGCAAUGUUAAAAGGGCAU	MIMAT0009223	
hsa-miR-130a-3p	CAGUGCAAUGUUAAAAGGGCAU	MIMAT0000425	100
bta-mir-148b	UCAGUGCAUCACAGAACUUUGU	MIMAT0003814	
hsa-miR-148b-3p	UCAGUGCACUACAGAACUUUGU	MIMAT0000759	100
bta-mir-152	UCAGUGCAUGACAGAACUUGGG	MIMAT0009238	
hsa-miR-152	UCAGUGCAUGACAGAACUUGG	MIMAT0000438	95.45
bta-mir-301a	CAGUGCAAUAGUAUUGUCAAAGCAU	MIMAT0009276	
hsa-miR-301a-3p	CAGUGCAAUAGUAUUGUCAAAGC	MIMAT0000688	92
bta-mir-301b	CAGUGCAAUGAUAUUGUCAAAGCAU	MIMAT0009277	
hsa-miR-301b	CAGUGCAAUGAUAUUGUCAAAGC	MIMAT0004958	92
bta-mir-33a	GUGCAUUGUAGUUGCAUUGCA	MIMAT0009294	
hsa-miR-33a-5p	GUGCAUUGUAGUUGCAUUGCA	MIMAT0000091	100
bta-mir-33b	GUGCAUUGCUGUUGCAUUGC	MIMAT0009295	
hsa-miR-33b-5p	GUGCAUUGCUGUUGCAUUGC	MIMAT0003301	100
bta-mir-144	UACAGUAUAGAUGAUGUACUAG	MIMAT0009234	
hsa-miR-144-3p	UACAGUAUAGAUGAUGUACU	MIMAT0000436	90.90
bta-miR-19a	UGUGCAAAUCUAUGCAAAACUGA	MIMAT0004336	
hsa-miR-19a-3p	UGUGCAAAUCUAUGCAAAACUGA	MIMAT0000073	100
bta-miR-19b	UGUGCAAAUCCAUGCAAAACUGA	MIMAT0004337	
hsa-miR-19b-3p	UGUGCAAAUCCAUGCAAAACUGA	MIMAT0000074	100

To select the most abundance of miRNA among candidates, expression profiling analysis in granulosa cells samples that cultured in the presence of serum from Day 0 to Day 2 was performed. To do that, 3 miRNA reference genes (U6, Snord48 and 5S) and geometrical mean from those genes was used for miRNA expression normalization (Figure 25A). As indicated in figure 25B, all miRNAs showed a certain expression pattern over the day of culture. However, in Day 2 of culture we found miR-33b to be is the most abundant one compared to the other miRNA. In addition, when compared to AMPKA1 gene expression, miR-33b express in the opposite direction (Figure 24A) but similar pattern with lipid accumulation (Figure 24C). Based on this result, therefore, miR-33b was selected and followed with target validation and functional study.

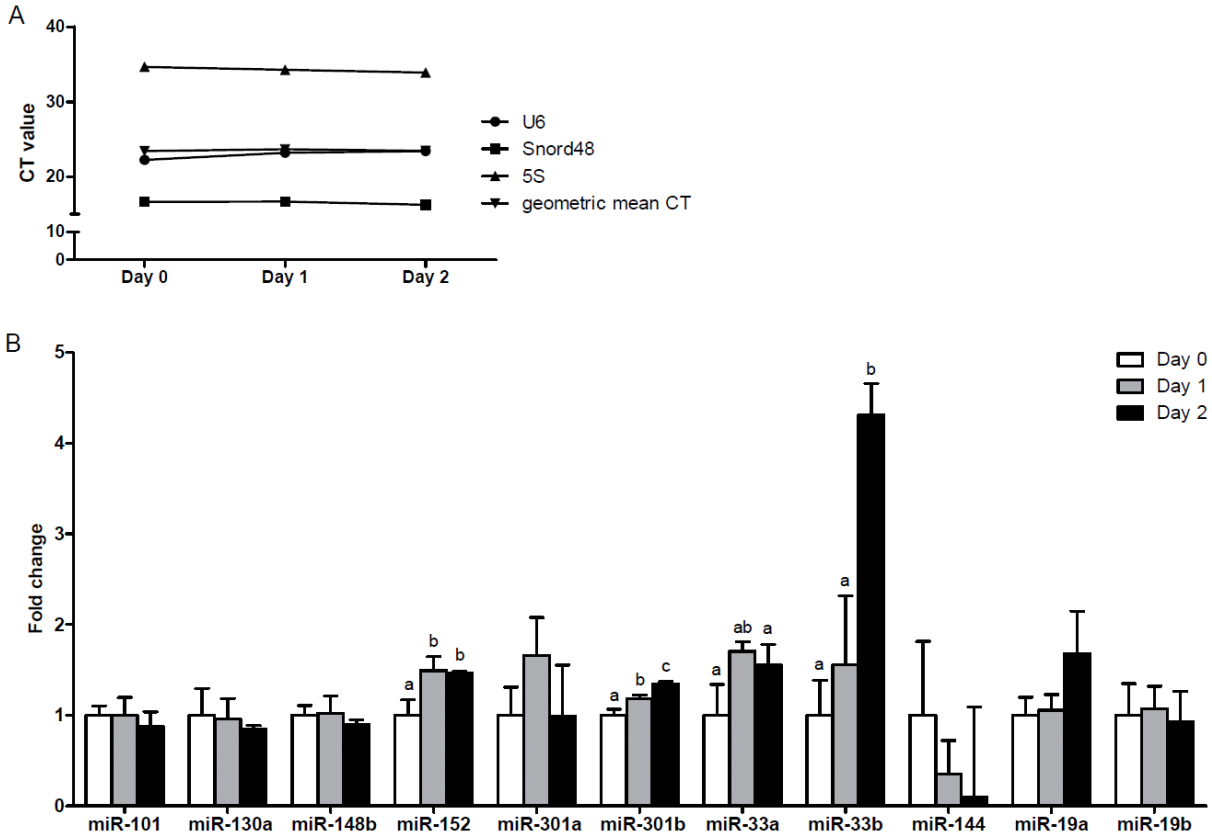

Figure 25: The stability of miRNA reference genes across culture time (A) and miRNA candidate expression profiling in granulosa cells cultured in the presence of serum (B). [a,b]Different superscripts indicate significant difference among groups ($p<0.05$).

The miR-33b target validation to AMPKA1 was initiated by analyzing the miR-33b binding site in 3'UTR of AMPKA1. The AMPKA1 3'UTR and miR-33b sequence were uploaded into

online prediction software FINDTAR 3.0 (http://bio.sz.tsinghua.edu.cn/) which give score, ΔG and alignment or binding site position (Ye et al. 2008). Thereafter, 3 binding sites of miR-33b in AMPKA1 3'UTR were found as illustrated in figure 26. This work then followed by binding site position mapping and finally two position 2426-2449 and 2553-2571 were selected as DNA template (150 bp). To amplify the DNA template and to construct plasmids, *in silico* analysis using Serial Cloner v.2.61 software was performed, aiming to design specific primer and choose the appropriate restriction enzyme for cloning purposes. To ensure the designed primers are working, virtual PCR and ligation were also performed. As the result, specific primer carrying restriction enzyme sequence are generated as presented in table 7.

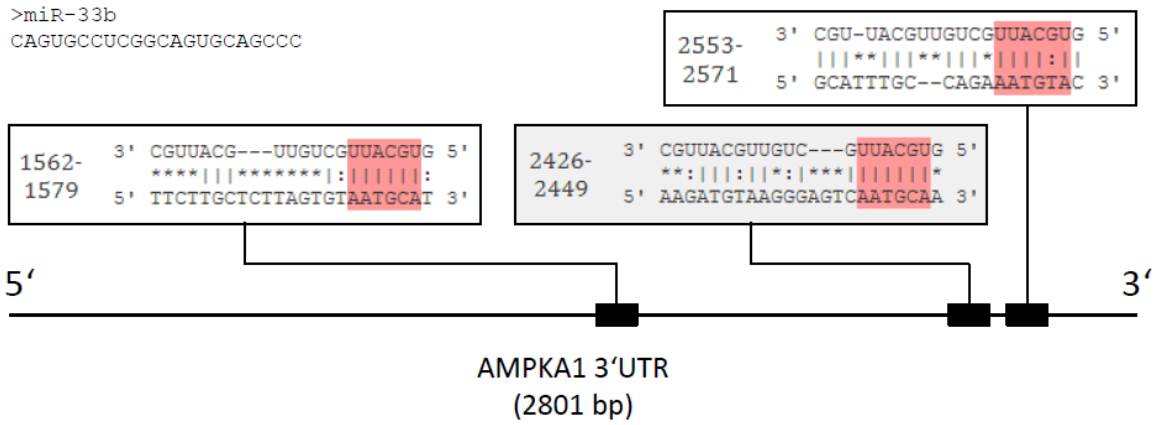

Figure 26: miR-33b binding site (red shadow) on AMPKA1 3'UTR predicted by FindTar 3.0 software.

Table 7: Details of primers used for plasmid construction.

Primer name	sequence (5'-3')
AMPKA1 3'UTR forward (SacI)	GC**GAGCTC**AAGATGTAAGGGAGTCAATGC
AMPKA1 3'UTR reverse (XbaI)	GC**TCTAGA**ACAGTACATTTCTGGCAAATG
Mismatch sense (SacI)	GC**GAGCTC**AAAGATGTATAAGGCATTTGCCAG AACGTT**A**CTGTTTCTAGA
Mismatch antisense (XbaI)	**TCTAGA**AACAGTAACGTTCTGGCAAATGCCTT ATACATCTTTGAGCTCGC

Bold and underlined shows the restriction enzyme sequence

4.3.3 AMPKA1 3'UTR - miR-33b plasmid construction

In order to test whether miR-33b could regulate the predicted binding sites of AMPKA1-3'UTR, luciferase assay was performed by construct plasmid which carries AMPKA1-3'UTR binding site. Insert DNA carrying miR-33b binding site were *in silico* cloned to construct AMPKA1-3'UTR WT (Wild Type). A DNA fragment which carry mismatch miR-33b binding site was also constructed by mutating 3 nucleotide bases and cloned to construct AMPKA1-3'UTR MT (Mismatch). Both constructed DNA were cloned in the multiple cloning region using two restriction enzymes namely Xbal and Sacl (Figure 27).

To construct plasmid in the "wet" lab, DNA fragment of mir-33b AMPKA1-3'UTR was amplified in normal PCR reaction using primer containing specific restriction enzyme (Table 7). All DNA fragment were sequenced to confirm the sequence and the confirmed one then proceed to ligation process in pmirGLO to construct intended plasmid.

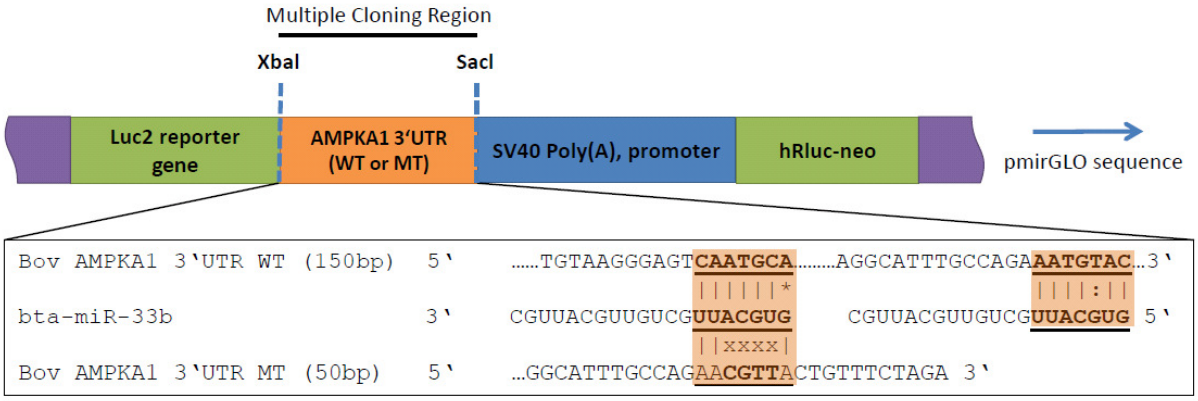

Figure 27: A DNA insert containing miR-33b binding site in multiple cloning region of pmirGLO plasmid.

4.3.4 miR-33b - AMPKA1 target validation by luciferase assay

To perform luciferase assay, the work was initiated by selecting the lipofectamine dosage, by transfecting granulosa cell using pmaxGFP and observed the result under fluorescent microscope. Figure 28 shows that 5 µl of lipofectamin in 550 µl culture media gives the highest transfection efficiency as confirmed in fluorescence signal quantification (Figure 28E). This finding allowed us to select 5 µl of lipofectamine dosage for transfection work.

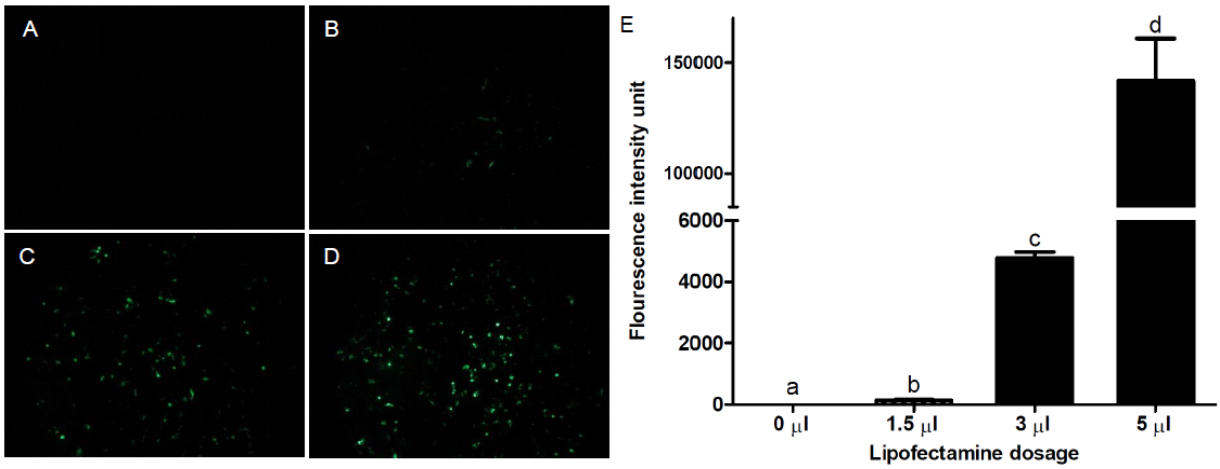

Figure 28: Transfection efficiency with different lipofectamine dosage, 0 µl (A), 1 µl (B), 3 µl (C), 5 µl (D) and green florescence signal quantification (E). Green signal means cells transfected with pmaxGFP. [a,b]Different superscripts indicate significant difference among groups ($p<0.05$).

Thereafter, AMPKA1-3'UTR plasmid co-transfected with miR-33b were performed at Day 2 of cultured granulosa cells. As the result, the presence of miR-33b was found to decrease luciferase activity in AMPKA1-3'UTR WT, meanwhile AMPKA1-3'UTR MT was not affected significantly (Figure 29). This result shows that miR-33b was valid targeted to 3'UTR of AMPKA1 mRNA in bovine granulosa cells.

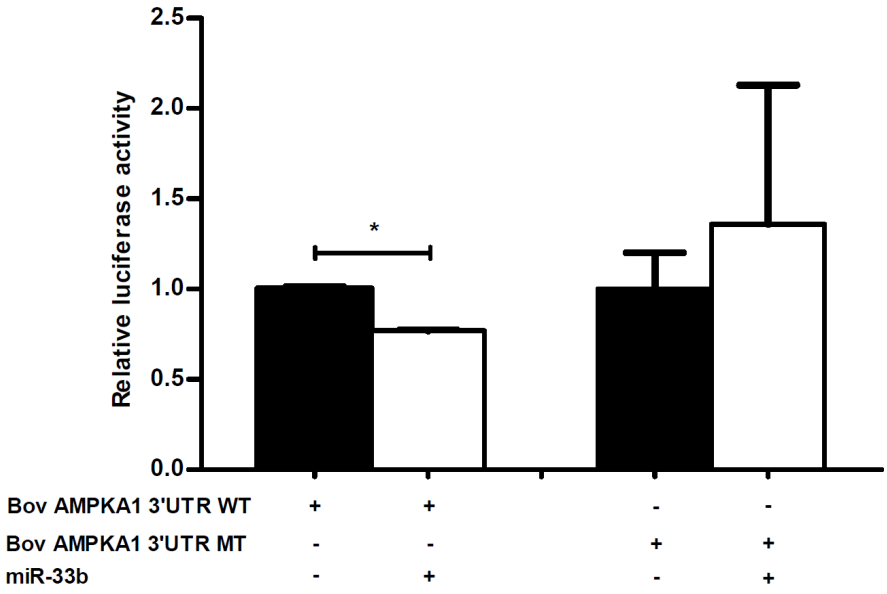

Figure 29: miR-33b targeted bovine AMPKA1 3'UTR as validated by luciferase activity assay. Asterisk (*) represent statistically significant differences ($p<0.05$).

4.3.5 The effect of miR-33b inhibitor on AMPKA1 gene expression in granulosa cells

In this study, abundance of miR-33 inhibits AMPKA1 gene expression in bovine granulosa cells. Therefore, it is suggested that inhibition of miR-33 will be beneficial to increase AMPKA1 activity. To answer that question, granulosa cells were transfected with 50 nM miR-33 inhibitor at Day 2 of culture followed with 24 h incubation and AMPKA1 gene expression analysis. Moreover, to confirm the effect of miR-33b inhibitor on AMPKA1 activity, granulosa cells culture with 0.5 mM AICAR treatment was also performed. In this study, presence of miR-33b inhibitor significantly increases AMPKA1 expression as pattern shows in AICAR (Figure 30A and 30B). This result was confirmed in Western blot analysis where miR-33b inhibitor has increased protein level of AMPKA1 compared to control, however when it is compared to AICAR treatment, its expression is lesser (Figure 30C).

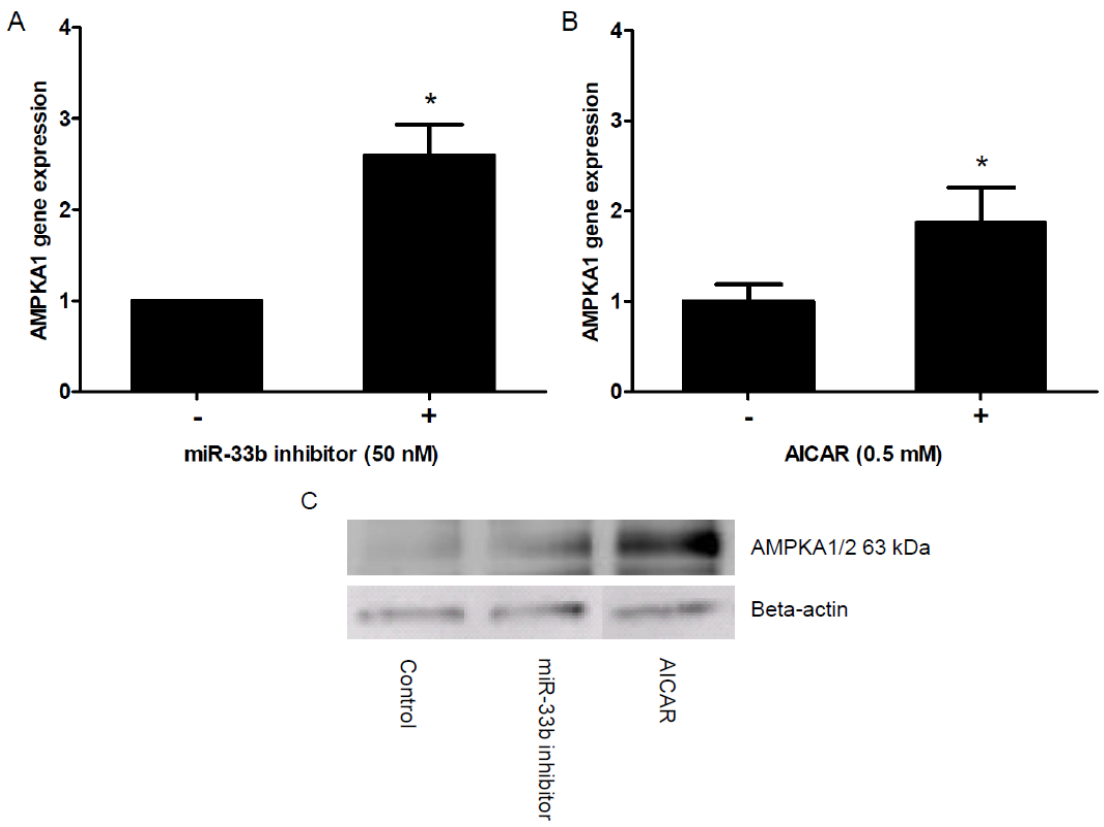

Figure 30: Effect of miR-33b inhibitor (A), AICAR treatment (B) on AMPKA1 gene expression and AMPKA1/2 protein level by Western blot analysis (C). Asterisk (*) represent statistically significant difference $p<0.05$.

4.3.6 The effect of miR-33b inhibitor on lipid metabolism and mitochondrial activity gene expression in granulosa cells

As indicated in figure 31, inhibition of miR-33b significantly increases ACC expression compared to control (without transfection). In the mitochondrial activity genes expression, inhibition of miR-33b didn't affect to CPT1 but significantly increase PGC1A compared to control.

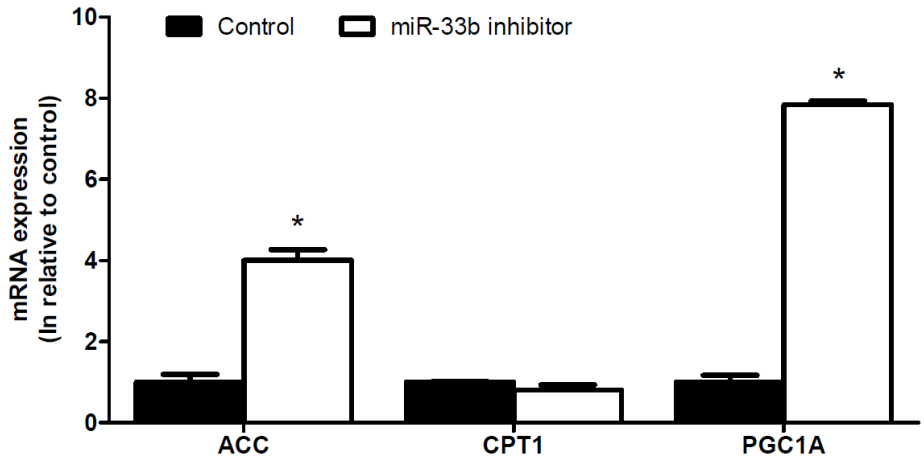

Figure 31: Effect of miR-33b inhibitor on genes related to AMPK pathway in bovine granulosa cells. Asterisk (*) represent statistically significant differences ($p<0.05$).

4.3.7 miR-33b expression in blastocysts derived from in vitro cultured in the presence and absence of serum

Taking into account that miR-33b has been validated to target AMPKA1 in bovine granulosa cells and considering the aim to know its role during embryo culture in the further study, the expression of miR-33b in blastocysts sample were analyzed. For that, miR-33b expression was profiled in blastocysts derived from in vitro in the presence or absence of serum. Instead of serum, BSAFAF was used as macromolecules source. As the result, miR-33b significantly higher abundant in blastocysts cultured with serum compared to without serum (Figure 32A) and AMPKA1 gene was showed in the contrary expression (Figure 32B).

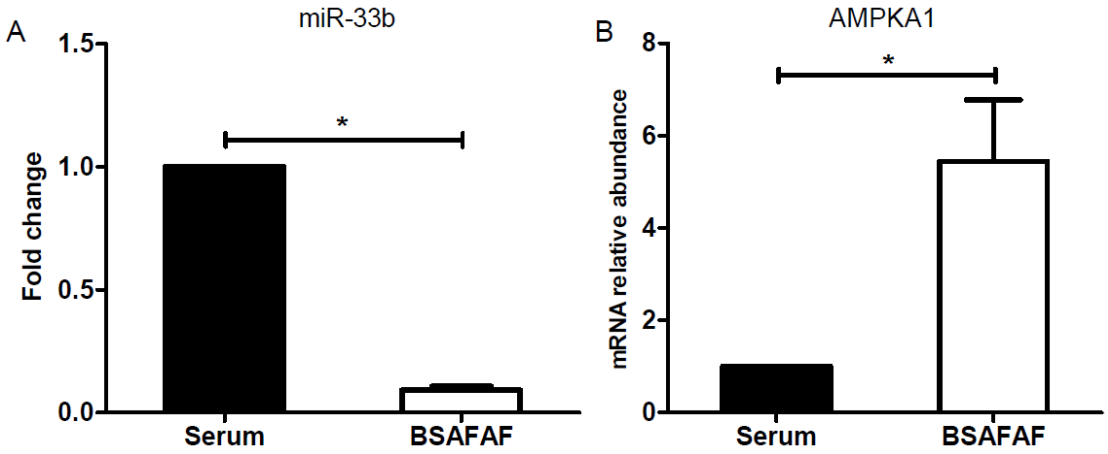

Figure 32: miR-33b (A) and AMPKA1 (B) expression of blastocysts cultured in the presence and absence of serum. Asterisk (*) represent statistically significant differences ($p<0.05$).

5 Discussion

5.1 AMPK activity and lipid metabolism related genes in in vitro bovine embryos under different of culture environment

It is well known that the supplementation of serum to culture media results in lipid accumulation, enhances ROS production and alters mitochondrial condition subsequently lead to lower cryotolerance of blastocyst. The molecular background behind this phenomenon is far from known. Recently, the AMPK pathway was identified as differentially expressed in bovine blastocyst derived from alternate in vitro vs. in vivo culture environment (Gad et al. 2012). In that study, blastocyst which spends longer time under in vitro environment showed more AMPKA1 down regulation compared to in vivo. Since AMPK pathway has been reported as master regulator of energy metabolism in a wide range of tissues and cells (Carling 2004, Carling et al. 2011), this study aimed to analyze its potential role in bovine embryos cultured in different culture environment in order to elucidate the molecular mechanism of AMPK pathway activity in regulation of embryo phenotype.

5.1.1 In vitro culture environment affect AMPK expression

In the first part of experiment, gene expression level of gene in AMPK pathway was compared. The result showed that AMPKA1 and AMPKA2 were found down regulated in blastocysts derived from in vitro. Meanwhile, the lipid metabolism gene, ACC (represent lipid synthesis), which is known as the direct target of AMPK shows an opposite expression. These results are in agreement with previous studies showing that down regulation of AMPK activates lipogenic genes (Li et al. 2011, You et al. 2004). It is reported that increased abundance of ACC enhances malonyl-CoA which in turn increases the conversion of free fatty acids into lipids (Dzamko et al. 2008, Hardie 1989, Ruderman and Prentki 2004).

The down regulation of AMPKA1 and AMPKA2 was accompanied by lower expression of mitochondrial activity genes, CPT1 (lipid oxidation) and PGC1A (mitochondrial biogenesis), in vitro derived blastocysts. Earlier studies have been reported that reduction in AMPK activity causes lower activity of PGC1A (Dugan et al. 2013). Noteworthy, PGC1A has been reported to balance mitochondrial density and to protect cells from ROS by induction of ROS-detoxifying enzymes (Finkel 2006, O'Donnell et al. 2013, St-Pierre et al. 2006). Moreover, PGC1A has been reported to control CPT1 which is already identified to be the rate-

controlling enzyme in fatty acid oxidation (Louet et al. 2002). Low abundance of PGC1A went along with low expression of CPT1 of blastocysts derived in vitro when compared to fully in vivo derived blastocysts in our experiments. Thus, AMPK mediated down regulation of PGC1A and CPT1 is being in line with previous hypothesis that enhanced lipid accumulation of in vitro derived embryos is caused by the incapability of mitochondria, reduced mitochondrial activities, to metabolize lipid in culture media (Abe et al. 2002, Barcelo-Fimbres and Seidel 2007b, Ferguson and Leese 1999, McEvoy et al. 2000).

The lipid content of in vitro vs. in vivo derived blastocysts confirmed the gene expression data. Results clearly showed a significantly higher lipid content in embryos derived from in vitro compared to in vivo, in agreement with several previous studies (Abe et al. 2002, Abe and Hoshi 2003, Barcelo-Fimbres and Seidel 2007b, Sudano et al. 2011). It has been well documented that culture environment affects embryo gene expression which is important for its physiological and metabolism (Badr et al. 2007, Lonergan et al. 2003a). For instance, Lonergan et al. (2003b) demonstrated that in vitro culture environment significantly increase the expression of genes that are responsible for apoptosis, oxidative stress and mitochondrial activity compared to in vivo condition.

5.1.2 The presence of serum during bovine embryo negatively affect AMPK activity

In addition to the other factors, the difference between in vivo vs. in vitro environment is especially the present of serum in culture media that suggested could have resulted to the observed differences with respect to AMPK gene expression and mitochondrial activity. Thus, we aimed to check whether addition of serum to culture medium could lead to similar effects with respect to the expression of AMPK and lipid related metabolism genes as well as lipid content.

Result showed that the expression of genes in AMPK pathway, lipid metabolism and mitochondrial activity to vary in expression of stages before 16-cell. On the contrary, stages beyond 16-cell showed a stable expression. These results support the previous observations where embryo development is depend on early embryonic genome activation (EGA) involving protein synthesis (Ma et al. 2001, Memili and First 2000). It is also reported by Khurana and Niemann (2000b) that the pattern of metabolic activity of in vitro embryo is relatively constant before 16-cell stage, as evidence by no difference with respect to the rate of oxidation before that stage. In addition to that, oxidative processes were associated to

mitochondrial development during embryogenesis. However, in early development of embryos, mitochondria are starting to elongate at 4-cell stage and fully functioning at the blastocyst stage (Cummins 2002). Consistent with the previous evidences, expression of genes related to AMPK pathway, lipid metabolism and mitochondrial activity in this study showed variation before 16-cell stage. It seems that, particularly before EGA, embryos development depends on its maternally derived transcript.

Considering solely the blastocyst stage, results revealed that expression of genes in AMPK pathway of in vitro embryos cultured in presence or absence of serum resembled the trend in expression as observed for in vitro vs. in vivo derived embryos. The expression of AMPKA1 and AMPKA2 in blastocysts cultured with serum are down regulated and confirmed with the expression of STK11 or namely LKB1 (upstream kinases) as the sign of AMPK activity as reported by Towler and Hardie (2007). This study also showed that lipid accumulation and mitochondrial activity went along with expression of ACC, CPT1, NQO1 and PGC1A are in perfect harmony. In addition to that, the expression of lipid metabolism transcription factor, (SREBP1, SREPB2) also support that result.

Low expression of AMPK in bovine blastocysts derived from in vitro with serum, accompanied by up regulation of lipid synthesis as well as down regulation of mitochondrial activity. This result was in accordance with the hypothesis that enhanced lipid accumulation in IVP derived embryos is caused by the incapability of mitochondria to metabolize abundant free fatty acids in culture media (Abe et al. 2002, Barcelo-Fimbres and Seidel 2007b, Ferguson and Leese 1999, McEvoy et al. 2000). Comparing mitochondrial activity, with vs without serum, supports those previous studies. It is showed that blastocysts in the present of serum exhibit lower mitochondrial activity compared to without serum. In line with that, Cagnone and Sirard (2014) revealed a high lipid stress pathway is significantly associated with mitochondrial dysfunction especially in its ability to oxidase fatty acids which is abundant in serum. Moreover, previous studies (Abe et al. 2002, Abe and Hoshi 2003, Crosier et al. 2001) have been reported the defect of mitochondrial structure lead to lipid accumulation when embryos cultured under serum system. Due to many growing evidences in the recent year showed that AMPK is influencing the mitochondrial activities, AMPK became one of the most promising target in combating diseases caused by mitochondria dysfunction such as diabetes, obesity and cancer in human research area (Bokko et al. 2007, Faubert et al. 2013, Hardie 2013, Hawley et al. 2003, Kim et al. 2014, Zhang et al. 2009).

5.1.3 Abundant fatty acid in culture environment induces lipid accumulation through reducing AMPK activity during bovine embryo culture

Taking into account the result of first and second part of experiment 1, the result proved that serum supplementation to culture media causes change in AMPK gene expression. Significant down regulation of AMPK gene and mitochondrial activity in blastocysts cultured in vitro in the presence of serum implicating its role for lipid accumulation. To that regards, we suggest that down regulation of AMPK and its effects on the AMPK pathway programs early embryo to a rather anabolic mode as reflected by higher ACC expression as well as reduced mitochondrial biogenesis and/or activity due to down regulation of CPT1 and PGC1A. Although the mode by which addition of serum to culture medium causes down regulation of AMPK is not resolved, activity of AMPK was reported by Zhou et al. (2007) to be negatively correlated with fatty acid synthesis. That could implicate that excess presence of free fatty acids within serum could result in increased fatty acid synthesis within embryo and thus to be the cause leading to low AMPK activity.

In order to investigate whether free fatty acids or whether factors other than free fatty acids are leading to change the expression of genes in AMPK pathway subsequently to lipid accumulation and mitochondrial dysfunction, bovine embryos were cultured in media supplemented with different macromolecules with or without fatty acids (BSA vs. BSAFAF) as well as in serum supplementation. Based on the supplementation of different macromolecules in culture media, no difference with respect to embryo development rate was observed (Table 4). However, embryos cultured in the presence of serum showed faster development kinetics compared to the other groups. It is in agreement with results where serum increase the production of blastocyst rate compared to those in the absence of serum (Gutierrez-Adan et al. 2001). The early develop blastocysts reported by Barrenetxea et al. (2005) and Muthukumar et al. (2013) induces higher pregnancy rates compared to the later one. However, the differences in development speed reported to be associated with alteration of gene expression via altering the abundance of specific transcript which is important to control embryo development competence (Dode et al. 2006, Ripamonte et al. 2012).

The present study showed that embryos cultured in BSAFAF accumulate lower amounts of lipids than their counterparts cultured in BSA or serum being in agreement with previous studies (Abe et al. 2002, Gomez et al. 2008). Moreover, embryos cultured in presence of serum exhibited higher ROS level compared to culture in BSAFAF with culture using BSA as

supplement exhibiting intermediate values. Thus, ROS level were found to be much higher in embryos cultured in the presence of fatty acids (BSA) compared to those derived from fatty acid free culture (BSAFAF). Importantly, addition of fatty acids to culture medium decreased mitochondrial activity as observed for embryos cultured in presence of serum in our study. These results are in line with previous studies reporting an incapability of embryos to metabolize lipids due to low mitochondrial activity goes along with excessive levels of ROS (Abe et al. 2002, Abe and Hoshi 2003, Barcelo-Fimbres and Seidel 2007a, Barcelo-Fimbres and Seidel 2007b, Cagnone and Sirard 2014). Abundant generation of ROS during early development is generally suggested to be detrimental to the embryos due to negative effects on mtDNA structure (Harvey et al. 2002, Rizos et al. 2003) subsequently causing mitochondrial dysfunction (Abe et al. 2002, Crosier et al. 2001). That in turn leads to reduced ATP production levels and TCA cycle (Wang et al. 2009), subsequently induce lipid droplet formation and accumulation (Lee et al. 2013) by reducing the mitochondrial oxidation capacity (Schrauwen and Hesselink 2004). This is accompanied by inactivation of enzymes that are essential for energy production and detoxification of ROS that in turn caused disabling of the mitochondrial ROS scavenging machinery, result in modulated mitochondrial functions (Kumari et al. 2014).

NQO1, which belongs to one of antioxidant enzyme, is found to be down regulated in blastocysts cultured with serum compared to without serum in experiment 1 part 2. This finding is in line with the previous studies where in in vitro derived bovine embryos need more antioxidant to face oxidative stress conditions (Rizos et al. 2003). Moreover, excess ROS production has been reported to cause fragmentation of mitochondria and results in functional defects with respect to oxidation capacity, size, number and density of mitochondria (Jheng et al. 2012) which could explain the low capability of the corresponding embryos to metabolize lipids. Thus, it is suggested that a harmful of encasing high ROS levels, mitochondrial dysfunction and enhanced lipid accumulation is a consequence of enhanced free fatty acids during in vitro culture.

Moreover, the present result confirmed that expression of genes in the AMPK pathway resembled the trend of expression when culture medium was supplemented with fatty acids as observed for media supplemented with serum. Lipid accumulation, mitochondrial dysfunction and ROS level were in perfect agreement with expression of genes under study of the AMPK pathway. Again, AMPKA1 and AMPKA2, PGC1A and CPT1 were significantly down regulated meanwhile ACC showed up regulation in embryos cultured in presence of free fatty

acids (Figure 12). This result, however, was not only restricted on the RNA level but also accompanied with lower abundance of the AMPKA1/2 protein (Figure 13). In addition, lower activity of AMPK catalytic sub unit α indicated at phosphorylation site of Threonine-172 (p-AMPK Thr172), was demonstrated in embryos cultured with free fatty acids (Figure 14).

As mentioned before, activity of AMPKA has been reported to be dependent on the AMP:ATP ratio in cells with relative high ratio of AMP:ATP leading to higher activity of AMPK (Corton et al. 1994). When fatty acid supply is plentiful, there is an up regulation of mitochondrial activity and increase in oxidative capacity (Iossa et al. 2002, Mollica et al. 1999), which is suggested to result in elevated ROS level as well as elevated ATP levels (Burton et al. 2003). Taking into account that the AMP:ATP ratio senses activity of AMPK (Corton et al. 1994), oversupply of ATP would lower the AMP:ATP ratio, chemical equation balance (Nelson and Cox 2005), and thereby lowering AMPK activity resembling a self-controlling cycle. Conclusive to this study findings, it was reported by Tomas et al. (2002) in muscle cells that increased activity of AMPK causes inhibition of ACC which decreases malonyl-CoA concentration and increases fatty acid oxidation by β-oxidation suggested to result in lipolysis and therefore lower lipid accumulation. Lipolysis by β-oxidation, however, results in generation of ATP which is required to power the pre-implantation stages of development. Thus it is speculate that AMPK might be a key-enzyme precluding exorbitant lipolysis by depicting a negative feedback mechanism. Considering that embryo lipid droplets are an endogenous reserves for energy generation (Sturmey et al. 2009), such a mechanism would avoid overriding generation of ATP by utilization of these endogenous reserves guarantying that ATP production and ATP consumption are balanced. Conclusively, internal fatty acids have been considered as energy resource for the first days of development with embryos of species remaining unattached for a relative longer time in pig and cow embryos. Consequently, embryo will dependence on AMPK activity by sensing AMP:ATP ratio that warrants economic utilization of these internal energy stores which guaranty the embryo can develop largely based on its internal energy stores without the need of external fatty acids. That hypothesis is further supported by the finding that inhibition of triglyceride metabolism in bovine embryo prevented development to the blastocyst stage (Ferguson and Leese 2006) whereas culture in fatty acid free conditions did not prevent development to the blastocyst stage as shown by the present study as well as numerous others. Therefore, lipid droplets should be largely metabolized until later developmental stages as observed for in vivo derived blastocysts within this study.

On the other side, bovine embryos are capable of taking fatty acids from serum (Sata et al. 1999) and that has been reported to mask changes in endogenous reserves (Ferguson and Leese 1999). In the condition of embryos cultured under serum, high uptake of fatty acid resulted abundance ATP through exceeding capacity of TCA cycle and ETC resulted more ROS production (Tarazona et al. 2006). When exogenous fatty acid supply is plentiful, there is up regulation of mitochondrial activity increasing oxidative stress (Iossa et al. 2002, Mollica et al. 1999) which is suggested to result in elevated ROS level (Burton et al. 2003) as well as elevated ATP levels that decreasing AMP:ATP ratio and therefore AMPK activity as consequence of energy balance system (Nelson and Cox 2005). As a consequence, profound inactivation of AMPK would subsequently stop further utilization of endogenous lipid stores but would favor lipogenic processes instead. Low activity of AMPK however might not prevent uptake and metabolize the exogenous fatty acids. Thus, high abundance and uptake of exogenous fatty acids might bypass the endogenous feedback system keeping AMPK activity on low levels due to high ATP levels as a consequence of imbalance between excessive ATP generation and moderate ATP consumption. Moreover, low AMPK activity would result in lower activity of PGC1A which in turn decrease mitochondrial biogenesis and/or activity.

Moreover, a longer period culture in vitro under serum or free fatty acids during in vitro embryo culture over 7 days, higher oxidative stress due to higher ROS as the result of mitochondrial activity would cause mitochondrial dysfunction/damage by attacking mtDNA (Cui et al. 2012, Lenaz et al. 2002, Stuart and Brown 2006). Since mtDNA encode for enzymes essential for oxidative phosphorylation (Chandrasekaran et al. 1997, Marusich et al. 1997, Shoubridge 2001), any defect of it could cause metabolic dysfunctions at the blastocyst stage and would impact subsequent embryo viability (Wilding et al. 2009). In addition, mitochondrial dysfunction also reduces capabilities to metabolize fatty acids thorough β-oxidation. Therefore, fatty acids taken up from the culture environment or serum are diverted into lipid synthesis resulting in increased accumulation of triglycerides as observed previously (Boren and Brindle 2012, Henique et al. 2010, Hurd et al. 2007) implicating that lipid accumulation also reflects mitochondrial dysfunction as speculated earlier (Korge and Weiss 2006). Based on that and considering this study result, a model as illustrated by figure 33 was summarized which demonstrating the relationship between AMPK activity, lipid accumulation, generation of ROS and mitochondrial activity during embryo culture in vitro in the presence of serum.

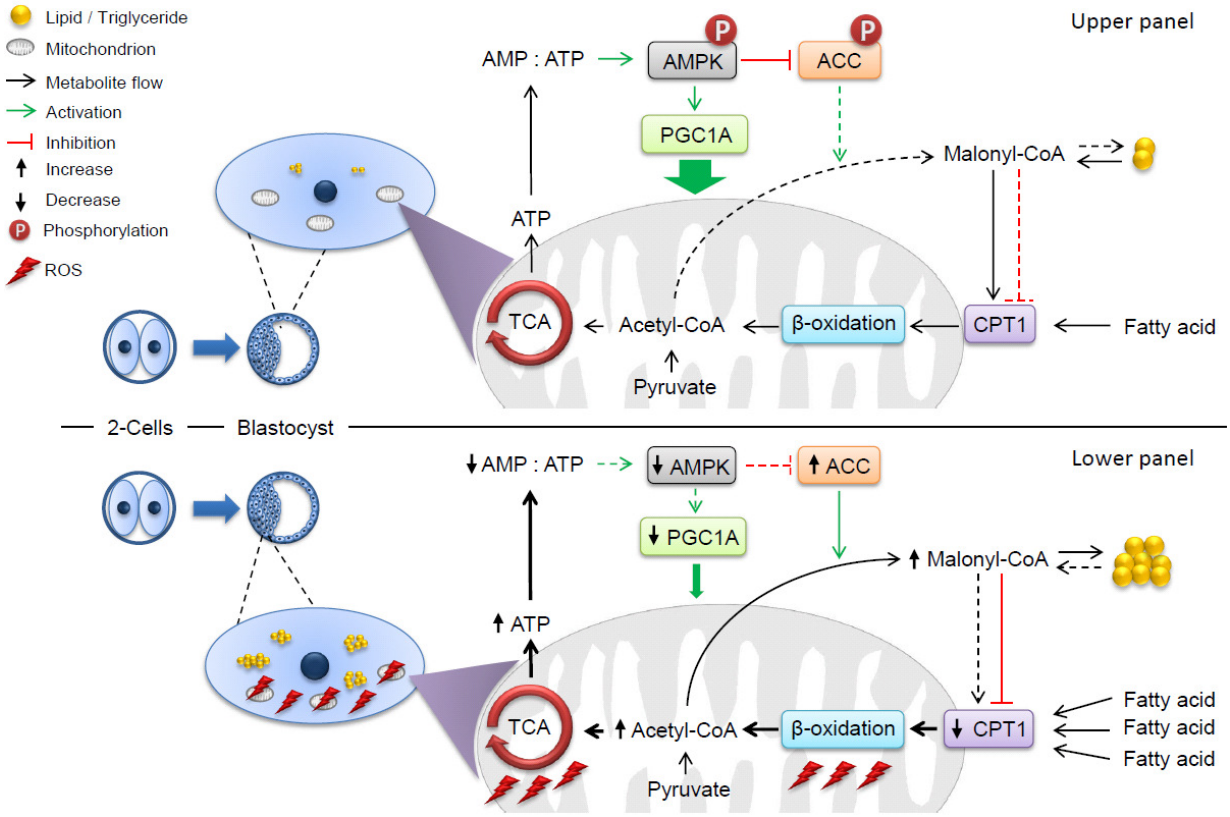

Figure 33: Hypothetical model of AMPK mediated lipid metabolism in bovine embryo cultured in vitro. Under low fatty acid condition (upper panel), AMPK is more active and reducing ACC expression level by phosphorylation process. Thus the conversion of FA into lipid through Malony-CoA generation is less, dominantly Acetyl-CoA goes to TCA cycle which maintains ATP production. Thereafter, AMPKA promote PGC1A expression which is responsible to maintain mitochondrial condition and enhance oxidative stress response mechanism. In the contrary, culture environment with abundance fatty acid (lower panel) reduce AMPK activity which promote ACC expression subsequent to enhance conversion of Acetyl Co-A into Malonyl-CoA leading to lipid accumulation. Moreover, continuous and plenty supply of fatty acids increase β-oxidation followed by exceed TCA which resulting more ROS. As the consequence, high ROS level lowering mitochondrial activity and AMPK activity. Thereafter, low AMPK inhibits PGC1A expression which is involved in CPT1 regulation and responsible for mitochondrial biogenesis.

Taken altogether, the present study has unraveled a special role of the AMPK pathway during early bovine embryo development. The environmental conditions known to cause lipid accumulation, mitochondrial dysfunction and high ROS level were reflected by down

regulation of AMPKA1, PGC1A and CPT1 resembling a reduced lipolytic trend and up regulation of ACC in parallel resembling an increased lipogenic trend in these embryos. Thus, expression of these genes reflected an anabolic direction of lipid metabolism. Of impact, present study results also indicate that active AMPK might be the key-enzyme precluding exorbitant lipolysis by depicting a negative feedback mechanism controlled by the AMP:ATP ratio. Moreover present study underlined the relevance of AMPK pathway in establishing an anabolic/lipogenic in bovine embryo phenotype and the key is the inability of mitochondria to metabolize available amount of fatty acids. However, further studies should be conducted to investigate distinct fatty acids relevant for this alteration of AMPK pathway or by restoring the mitochondrial activity or condition. Restoring mitochondrial activity or condition, therefore, using antioxidants or through AMPK activation has been beneficial for ATP production (Hawley et al. 2003, Imai et al. 2006, Merrill et al. 1997, Schafer et al. 2009). For that, keeping mitochondria normally function during pre-implantation embryo development is a prerequisite to high embryo quality and subsequent developmental outcomes.

5.2 AMPK activity modulation of in vitro bovine embryos using AICAR supplementation

According to the first experiment, the present study found that AMPK activity is associated with mitochondrial activities and lipid accumulation. Previous studies reported that modulation of AMPK activity becomes a strategy to improve mitochondrial function and to reduce lipid accumulation (Cui et al. 2012, Duchen and Szabadkai 2010, Taylor and Turnbull 2005). Therefore, enhancing AMPK activity subsequent to rescuing mitochondrial condition would be one strategy to reduce lipid accumulation especially in embryo in vitro production under serum system. Ample evidences, shows that down regulation of AMPK can be artificially activated by using chemical compound such as AICAR (Imai et al. 2006, Merrill et al. 1997, Thomson et al. 2007) or metformin (Stephenne et al. 2011, Zang et al. 2004). In this study, AICAR was used because of its has promising therapeutic potential to increase AMPK activity in regards to manipulate lipid metabolism and reducing lipid accumulation as well in the field of diabetic research (Boon et al. 2008, Koistinen et al. 2003) and obesity research area (Drake et al. 2010, Yang et al. 2012). Moreover, previous study also reported that AICAR able to increase AMPK activity in matured bovine oocytes (Bilodeau-Goeseels et al. 2007), mouse oocytes (Downs et al. 2010), mouse blastocyst (Eng et al. 2007), rat muscle (Kaushik et al. 2001) and hen granulosa cell (Tosca et al. 2006). However, the exact effect of

AICAR treatment on bovine embryo development in vitro especially in reducing lipid accumulation through modulation of AMPK activity is still need to be elucidated.

5.2.1 Effect of AICAR on blastocyst development rate and cell number

One of the aim in IVP technology is to produce blastocyst which having good quality to result in subsequent pregnancies. Top quality of in vitro embryo generally considered by high blastocyst cell number, good morphology and finally the implantation or pregnancy rate (Balaban et al. 2000, Gardner et al. 2000a). Despite many factors which influence implantation successful rate, speed of development to be one of indicator in which fast developing embryo induce more pregnancy compared to the slower one (Hasler 2000, Jacob et al. 2012, Muthukumar et al. 2013). For instance, Shapiro et al. (2000) demonstrated that pregnancy rates were higher when women transferred with expanded blastocysts compared to less developed one (43% vs. 17%; respectively). In addition to that, Archer et al. (2003) and Edgar et al. (2000) documented that reduction of implantation potential is influenced by the loss number of blastomere in human embryos. From these reports, blastocyst cell number is one of phenotype which can be a useful marker for pregnancy rate expectation. In this regard, serum application in embryo culture media is more effective than other macromolecules to improve development rate and cell number of resulted bovine blastocyst (Iwata et al. 2003, Lazzari et al. 2002).

Initially, 100 µM AICAR concentrations showed the highest AMPK activity in blastocyst. Several studies reported different AICAR dosage to activate AMPK, for instance 2 mM in rat muscle (Kaushik et al. 2001), 750 µM in HeLa cell line (Jose et al. 2011), 1 mM in hen granulosa cell (Tosca et al. 2006), 1 µmol/l in mouse oocytes (Eng et al. 2007), 200 µM in mouse oocytes (Downs et al. 2010) and 1 mM in bovine oocytes (Bilodeau-Goeseels et al. 2007). In agreement with that, this study showed that AICAR enhance AMPKA1 activity in blastocysts derived from in vitro culture. In this regard, AICAR is imidazole monophosphate (ZMP) which its structure similar to AMP, abundance of ZMP inside the cell change [AMP]:[ATP] subsequent promote AMPK activity. Moreover, sensitivity of cell in respond to AICAR looks like depend on the cell type, presumably the different ratio of [AMP]:[ATP] threshold in each type of cell which able to activate AMPK.

The cell growth inhibition as the effect of activated AMPK by AICAR was demonstrated in this study. This was indicated by lower kinetic development speed and significantly less

blastocyst cell number at Day 7 compared to control (Table 5). Similarly, inhibitory effect of AICAR in nuclear maturation of bovine oocytes has been demonstrated in the previous study (Bilodeau-Goeseels et al. 2007). AMPK is the suppressor of cell proliferation by controlling variety of cellular events in normal cells as well as in tumor and cancer cells (Bode and Dong 2004). AMPK activation overcomes the growth-stimulatory signaling via activation of p53-p21 axis as well as inhibition of mTOR signaling, resulting in cell growth arrest or suppression of cell proliferation by disturbing cell cycle (Motoshima et al. 2006). The p21 or CDKN1A which is known as cyclin-dependent kinase inhibitor of CDK2 and CDK4 complexes, regulates cell cycle progression and cell proliferation. The expression of CDKN1A is tightly controlled by p53 (TP53) and its induction leads to cell cycle arrest (Gartel and Radhakrishnan 2005, Harper et al. 1993). Cell with deficient of CDKN1A showed extensive cytochrome C release, mitochondrial membrane depolarization and caspase activation. On the contrary, increased level of CDKN1A causing cell cycle arrest and inhibiting DNA damage-induced apoptotic (Gartel and Radhakrishnan 2005, Harper et al. 1993). By blocking deregulated cell cycle progression, CDKN1A can influence the sensitivity of mitochondria to pro-apoptotic signals in DNA damage which is one factor induced cancer cells as demonstrated by Le et al. (2005). Based on the previous finding, the presence of AICAR in long period of bovine culture embryos might be inducing mitochondrial stress which increasing CDKN1A expression and associated with caspase 3 activity (Li et al. 2005). In addition, AICAR inhibit cell growth in term of cell proliferation primarily through inhibition of cholesterol and fatty acid synthesis mediated by increased level of AMPK activity.

5.2.2 Effect of AICAR treatment on AMPK activity and lipid accumulation of bovine blastocysts

As mentioned before, the attachment of AMP is allosteric modulator and the principal event to trigger AMPK activity. Under conditions of high [AMP], dephosphorylation of AMPK is inhibited by protein phosphatase. The upstream kinase (LKB1) then phosphorylates AMPK and makes it a better site for AMP attachment (Foretz et al. 2010). However, when protein phosphatase dephosphorylates AMPK, its make a worst site for AMP but good for ATP attachment, thus AMPK is deactivated. In this study, the expression pattern of PPP2CA, protein phosphatase, after AICAR treatment seems to be independent from AMPK activity. Its expression was in the same trend and not involved in the dephosphorylation of AMPK. For that we suspect other protein phosphatase, for example PP2A (Benziane et al. 2012, Wu et al.

2007) and SiT4 (Ruiz et al. 2011), might be involved in this process. These proteins phosphatase are need to be analyzed to see the potential of enhanced AMPK activity through its inhibition on bovine embryo.

In the expression of lipid metabolism and mitochondrial activity gene, the present study demonstrated that AICAR reduces expression of ACC and simultaneously increases PGC1A and NQO1 expression. This result shows that enhancement of AMPK is capable to induce mitochondrial activity (Figure 20) and it is displayed in the reduction of lipid accumulation (Figure 22). As previously described, ACC is a critical enzyme that controls lipid synthesis and oxidation (Beckers et al. 2007). It is well known that AMPK directly phosphorylates and inactivates ACC subsequently suppress malonyl-CoA production. The suppression of malonyl-CoA then accelerates the entry of long-chain acyl-CoA into mitochondria which mean an increase of lipid oxidation, thus reduce lipid accumulation (Long and Zierath 2006). Moreover, PGC1A was reported to increase mitochondrial density and ROS detoxifying enzyme (Finkel 2006, O'Donnell et al. 2013, St-Pierre et al. 2006). In addition, Marmolino et al. (2010) demonstrated that AMPK kinase stimulation resulted in a faster up-regulation of PGC1A and SOD (antioxidant gene). In this regard AICAR treatment is able to rescue the mitochondrial biogenesis and its activity (Dugan et al. 2013).

Blastocysts derived from in vitro culture with serum seem to have different structure including to dysfunction of mitochondria compared to either in vitro without serum or in vivo (Abe et al. 2002, Crosier et al. 2001). The AICAR treatment during embryo culture is one of intervention strategies by using small molecules to rescue the mitochondrial dysfunction. Small molecules therapies and metabolic manipulation generally aim to increase the capacity for ATP synthesis, bypass mitochondrial defect, stimulate mitochondrial biogenesis and reduce oxygen species level (Schiff et al. 2011). Another strategy that can be applied in effort to modulate mitochondrial function is to manipulate nutrient content in culture media (Schiff et al. 2011). For instance, Sudano et al. (2011) has combined low serum concentration and mitochondrial metabolic regulator to reduce lipid accumulation. Moreover, manipulation strategies in this regards can be expected as the magic bullet to improve mitochondrial condition during in vitro culture under serum. However, the way of applying that manipulation strategy with respect to embryonic development competence as well as gene expression and resulted offspring need to be elucidating accordingly in more experiment data. For instance, result in this study suggested that modulation of AMPK activity using AICAR treatment adversely impact on embryo development by decreasing blastocyst cell number.

Reduction of blastocyst cell number gives an information about of AICAR toxic effect that needs to be emphasized. However, the use of specific molecule to enhance or reduce embryo metabolism become a promising strategies in the effort to reduce lipid accumulation.

Taking into account that lipid metabolism, particularly lipid oxidation, depends on mitochondrial activity, the manipulation processes which keep mitochondria normally function will be more effective in lowering lipid accumulation. Serving the embryo with either endogenous or exogenous antioxidant could be an indirect way to overcome lipid accumulation problem. Enhancing endogenous antioxidant level in blastocyst cultured in vitro has been proved to increase mitochondrial activity and lowering lipid accumulation (Amin et al. 2014). Meanwhile application of exogenous antioxidant, for instance melatonin in bovine embryo (Wang et al. 2014), enhances blastocyst quality in term of development rate and cryotolerance. Other growing evidence also demonstrated that supplementation of antioxidant in culture medium give positive effect on embryo development as compared to control without antioxidant (Ali et al. 2003, Hosseini et al. 2009, Lim et al. 1997, Livingston et al. 2004, Luvoni et al. 1996, Orsi and Leese 2001, Ozawa et al. 2006). Understanding the factor and consequences of mitochondrial dysfunction and its complexity especially in bovine embryo develop in vitro, becomes a way to develop a rational treatment strategy. Indeed, the main consideration is the quality of resulted embryo and its ability to induce pregnancies. In this regards, appropriate regulation of energy is very critical for embryo development and viability. Thus the plasticity of embryo to adapt with the various culture environments still open many possibilities to develop better culture strategies which consider the accepted embryo physiology.

5.3 Post transcriptional regulation of AMPKA1 by using miRNA in bovine granulosa cells as a model

Growing evidences showed that miRNAs are involved in various mammalian physiological processes. Studies from our group reveal the present and dynamic expression of bovine miRNA in ovary (Hossain et al. 2009), COC's and preimplantation embryo (Abd El Naby et al. 2013, Tesfaye et al. 2009), granulosa cells (Salilew-Wondim et al. 2014), follicular fluid (Sohel et al. 2013) and placenta (Hossain et al. 2014). In addition, ample evidences demonstrated the fundamental role of miRNA which play in early embryonic development. For example, Tripurani et al. (2011) reported that miR-196a regulates bovine newborn ovary homeobox gene (NOBOX) which is important for folliculogenesis and blastocysts

development. miR-212 demonstrated to regulates transcription factor in the germline alpha (FIGLA) which is essential for fertilization and early embryonic survival in bovine embryo (Tripurani et al. 2013). Moreover, miR-17 families are evidenced control stem cells differentiation in mouse embryo (Foshay and Gallicano 2009). These works, give a display about the exploration area of miRNA function, especially in the effort to explain the mechanism of specific gene that having possibilities to solve problems in animal reproduction. As demonstrated in experiment 1, lipid accumulation in embryo culture in vitro in the presence of serum is associated with the down regulation of AMPKA1. Therefore, this study was aimed to know the AMPKA1 post regulatory mechanism in bovine granulosa by using specific miRNA.

5.3.1 Effect of serum on candidate miRNAs in granulosa cells

The presence of serum gives better support in granulosa cells development as observed in cells confluency. The same result was reported by Langhout et al. (1991) that presence of serum increases granulosa cell number compared to those cultured without serum. Abundance of growth factor, insulin and antioxidant in serum, by far, to be beneficially for cell growth. Moreover, lipid droplet accumulation in Day 2 of granulosa culture with serum was also observed. As reported in the previous studies, the presence of serum during cell culture reported turn granulosa and theca cells into luteal cells (Kayani et al. 2009, Roberts and Skinner 1990) which indicated by formation of lipid droplet (Nelson et al. 1992). This luteinization process was histologically evidenced by Hansel et al. (1987), that granulosa cells will turn into large luteal cell while theca cells become the small one. Moreover, based on morphological examination small luteal cell characterized by large oval nucleus, flattened and some lipid droplet, meanwhile large luteal cell having small spherical nuclei, not flatted and loaded with numerous lipid droplets (Nelson et al. 1992).

In the present study, low expression of AMPKA1 (Figure 24) followed by the abundance of miR-33b (Figure 25) in granulosa cells, indicated its post regulatory mechanism on AMPKA1 mRNA. Similarly, activity of luciferase construct containing AMPKA1-3'UTR in our study was suppressed by the presence of miR-33b and the regulation was abolished by mutation in miR-33b binding site indicating the specific binding of miR-33b to AMPKA1 mRNA. It is documented that miR-33a/b is expressed in many types of cells across different species (Goedeke et al. 2013) and it has been reported to play important role in lipid homeostasis (Rayner et al. 2011a, Rayner et al. 2011b, Shao et al. 2014, Taniguchi et al. 2014). In this

regards, miR-33a/b reduces fatty acid oxidation through negative regulation on AMPK protein (Davalos et al. 2011). Therefore, inhibition of miR-33b expression is potential to increase AMPKA1 activity and reduce lipid accumulation as well.

5.3.2 Regulation of AMPKA1 by miR-33b

Inhibition of miR-33b resulted in increasing of AMPKA1 expression in this study. This result is in agreement with the previous studies that antagomir-33 increases the expression of gene related to cholesterol transport (ABCA1), mitochondrial activity (CROT, CPT1A) and energy balance (AMPKA1) (Rayner et al. 2011b, Rayner et al. 2011a). Lines of evidence reviewed by Fernandez-Hernando and Moore (2011) shows that miR-33 family regulate lipid homeostasis through metabolism of cholesterol, fatty acid, glucose and also insulin signaling that related to energy production in cell (Hardie and Pan 2002). Therefore, inhibition of miR-33 expression in regards to regulate lipid metabolism became a promising way to treat metabolic disorder.

Several evidences showed that increased level of ACC enhance malonyl-CoA subsequent to lipid accumulation (Dzamko et al. 2008, Hardie 1989, Ruderman and Prentki 2004). In the contrary, low expression of ACC lowering malonyl-CoA and increasing fatty acid oxidation respectively (Dzamko et al. 2008, Hardie 1989, Ruderman et al. 2003, Ruderman and Prentki 2004). In this case, the presence of malonyl CoA is the limiting factor of fatty acid oxidation by inhibiting CPT1 activity which transporting fatty acid into mitochondria where β-oxidation takes place. Together with CPT2, CPT1 works in mitochondria internal membrane to facilitate fatty acid translocation (Bonnefont et al. 2004). However, this study showed that stimulation of AMPKA1 through miR-33b inhibition shows an enhanced ACC expression and has no effect on CPT1 expression. Indeed, high level of ACC keeps malonyl-CoA in high level explained no different on CPT1 expression. As mentioned before, ACC is AMPK direct target and both works by phosphorylation (Carling et al. 2008, Carling et al. 2011, Long and Zierath 2006). Due to in this experiment we use granulosa cells which undergo luteinization process, therefore lipid accumulation is a normal process. In this regards lipid synthesis is controlled by high expression of ACC due to lipid is the source of cholesterol for steroidogenesis (Drouineaud et al. 2007).

The result of present study demonstrated that inhibition of miR-33b resulted in stimulation of AMPKA1 activity and increase PGC1A expression simultaneously. Reported by Marmolino

et al. (2010) stimulation of AMPK activity resulted in fast up-regulation of PGC1A and positively enhance mitochondrial activity by increasing its density and its capabilities to scavenging ROS (Finkel 2006, O'Donnell et al. 2013). Despite the different expectation of ACC and CPT1 expression, this study clearly demonstrated the tight connection between AMPKA1 and PGC1A as well as in experiment 1 and 2.

According to the finding in this study, it is possible to speculate that down regulation of AMPKA1 which lead to lipid accumulation is due to the abundance of miR-33b in blastocyst cultured in the presence of serum (Figure 32). That result emphasized possibility to study the effect of miR-33b inhibition in effort to enhance AMPKA1 in bovine embryo. However, to elucidate miR-33b specific functions, a functional study in bovine embryo need to be performed. Due to the presence of zona pellucida, it is needed to consider carefully in regard to miRNA delivery strategies, technique, efficiency and toxic effect during embryo development (Joo et al. 2014). So far, the known technique to deliver miRNA into zygote is by microinjection (Tripurani et al. 2011, Tripurani et al. 2013) and free injection method also being reported (Joo et al. 2014). Recently, functional study of genes by miRNAs becomes a growing area in different research field. However, little research has been conducted to understand gene function post transcriptionally in embryo development to explain underlying molecular mechanism. Huge efforts are needed to explore this area, especially in mammalian embryos as general or in bovine as the focus model.

6 Summary

The major problem associated with in vitro production of bovine embryos in the presence of serum is lipid accumulation resulting in low cryotolerance compared to those derived from in vivo or without serum. It is suggested that lipid accumulation occurs from culture environment which modifies internal embryo properties, however its molecular mechanism is largely unknown. A recent study in our group, found AMPKA1 down regulation, part of AMPK heterotrimetrix complex, in a transcriptome profile of blastocyst derived in vitro compared to in vivo. Taking into account that AMPK is a lipid metabolism regulator, it is suggested that down regulation of AMPK is one factor which is responsible for elevated lipid accumulation.

In order to know the role of AMPK on lipid metabolism during IVC of bovine embryos, the first experiment was divided into 3 parts. In experiment 1 part 1, two groups of blastocysts were produced by in vivo and in vitro, followed by expression analysis of genes in AMPK pathway (AMPKA1, AMPKA2), lipid metabolism (ACC) and mitochondrial activities (CPT1, PGC1A). Three biological replicates, each containing 10 embryos per group, were used for total RNA isolation using Arcturus®PicoPure®RNA isolation kit. Furthermore, cDNA synthesis was performed in 20 µl reaction volume and mRNA transcripts were measured in qRT-PCR using relative standard curve method and normalized by GAPDH. Moreover, blastocyst lipid content was stained with oil red and spectrophotometer was used to quantify the lipid content. In the second part of experiment 1, embryos were produced with and without serum followed by gene expression analysis of genes in AMPK pathway (AMPKA1, AMPKA2, STK11), lipid metabolism (SREBP1, SREBP2, ACC), antioxidant (NQO1) and mitochondrial activities (CPT1, PGC1A). Moreover, AMPKA protein expression at blastocyst stage was analyzed using immunofluorescence method and mitochondrial activity was detected using MitoTracker under confocal laser scanning microscope. To investigate whether macromolecules supplementation affected AMPKA1 down regulation, in experiment 1 part 3 blastocysts were produced using different macromolecules namely serum, BSA and BSAFAF. Blastocyst development rate and expression of genes in AMPK pathway (AMPKA1, AMPKA2), lipid synthesis (ACC), mitochondrial activities (CPT1, PGC1A) were compared between groups. In addition, protein expression analysis, phosphorylation site of AMPKA at Thr172, ROS level and mitochondrial activity were also performed. Result of first experiment showed that compared to blastocysts derived from in vivo or in the absence of serum, the expression level of AMPKA1 was

significantly ($p<0.05$) lower in blastocysts produced with serum. The present study found that down regulation of AMPKA1 mRNA level increased the lipid content compared to in vivo, BSA and BSAFAF. A similar trend was confirmed in which higher ($p<0.05$) mRNA level of ACC and low expression of CPT1, NQO1 and PGC1A in culture with serum was found. Moreover, low phosphorylation site of AMPK, high level of ROS and low mitochondrial activities were followed by lipid accumulation in blastocysts cultured in the presence of serum (abundant fatty acid), in contrast to blastocysts derived from culture with BSAFAF (less fatty acid) supplementation. Results of the first experiment demonstrated the relationship of AMPK activity, generation of ROS, mitochondrial activity and lipid accumulation during embryo culture in vitro. Collectively, this study underlined the relevance of the AMPK pathway in establishing an anabolic/lipogenic bovine embryo phenotype and notably the inability of the mitochondria to metabolize the abundant lipid in culture environment. Therefore, altering AMPK activity during in vitro embryo culture might be beneficial to reduce lipid accumulation.

In the effort to reduce lipid accumulation of in vitro derived blastocyst through AMPK activity modulation, AICAR was applied in embryo culture media. In thi part of experiment, bovine blastocysts were produced in culture media supplemented with 100 µM AICAR, followed by investigation of embryo development rate, blastocyst cell number, gene expression, AMPK protein analysis, mitochondrial activities and lipid accumulation. As the result, AICAR treatments showed no difference in embryo development rate but significantly ($p<0.05$) reduced blastocyst cell number compared to those not supplemented with AICAR. AICAR was found to increase ($p<0.05$) the expression of genes in AMPK pathway (AMPKA1, STK11), antioxidant (NQO1) and mitochondrial activities (CPT2, PGC1A), the lipid synthesis gene (ACC) was downregulated ($p<0.05$). In addition, AMPKA protein and mitochondrial activities were enhanced by AICAR treatment and reflected in the trend of blastocysts lipid content reduction.

In order to elucidate the potential regulatory mechanism of AMPKA1 by miRNA, specific miRNA that potentially targeted to AMPKA1 were predicted, profiled and validated in experiment 3. For that, a series of *in silico* analysis was performed and 11 miRNAs were predicted to target AMPKA1. In the miRNA profiling analysis using three reference genes (U6, Snord48 and 5S) miR-33b was found to be the most abundant one compared to other miRNA candidates. Similar result was demonstrated in luciferase activity of plasmid containing AMPKA1-3'UTR where its activity was suppressed by the presence of miR-33b

and the regulation was abolished by mutation in miR-33b binding site which indicate the specific binding of miR-33b to AMPKA1 mRNA. In the effort to increase AMPK activity, 50 nM of miR-33b were transfected to granulosa cells followed by gene expression analysis of AMPKA1, ACC, CPT1 and PGC1A. As a result, inhibition of miR-33b significantly ($p<0.05$) increases AMPKA1, ACC and PGC1A, however expression of CPT1 was not affected. High level of ACC expression suggested a granulosa luteinization process.

Altogether, the present study showing culture dependent down regulation of AMPKA1 resulted in enhanced lipid accumulation due to reduced mitochondrial activities. The result of present study also highlights that AMPKA1 activities can be modulated by using artificial activator and specific miRNA inhibitor.

7 Zusammenfassung

Das Hauptproblem der in vitro Produktion von bovine Embryonen ist die durch Serum entstehende Lipidanreicherung sowie die daraus resultierende verringerte Kyrotoleranz im Vergleich zu den in vivo oder ohne Serum entstehenden Embryonen. Es wird angenommen, dass die Lipidanreicherung unter Kulturbediungungen die inneren embryonalen Eigenschaften modifiziert. Allerdings sind die dafür verantwortlichen molekularen Mechanismen immer noch unklar. Die neuesten Studien unsere Arbeitsgruppe ergaben, dass AMPKA1, ein Teil des AMPK heterotrimetrix Komplex, in in vitro Blastozyten im Vergleich zu in vivo entstandenen Blastozyten runter reguliert war. Vor dem Hintergrund, dass AMPK ein Regulator des Lipidmetabolismus ist, kann angenommen werden, dass die runter Regulierung des AMPK ein Faktor für die erhöhte Lipidanreicherung sein könnte.

Um die Rolle des AMPK im Lipidmetabolsimus während der IVK von bovine Embryonen zu verstehen, wurden in der vorliegenden Studie drei Experimente durchgeführt. Im ersten Experiment wurden zwei Gruppen aus in vivo und in vitro produzierten Blastozyten für die Expressionsanalyse von Genen des AMPK Signalwegs (AMPKA1, AMPKA2), des Lipidmetabolismus (ACC) und der Mitochondrienaktivität (CPT1, PGC1A) eingesetzt. Insgesamt wurden drei biologische Wiederholungen verwendet und jede bestand aus 10 Embryonen. Die RNA Isolierung erfolgte mittels dem Arcturus®PicoPure®RNA Kit und für die anschließende cDNA Synthese wurde ein Reaktionsvolumen von 20 µl eingesetzt. Mittels qRT-PCR und der relativen Standardkurvenmethode wurde die mRNA Expressionsanalyse durchgeführt. Die anschließende Normalisierung erfolgte mit dem Houskeeping Gen GAPDH. Zusätzlich wurde der Lipidgehalt der Blastozyten mit der Oil Red Anfärbung und mit Hilfe des Spektrophotometers untersucht. Im zweiten Teil des ersten Experiments wurden Embryonen mit und ohne Serum erzeugt und die Expression von Genen des AMPK Signalwegs (AMPKA1, AMPKA2, STK11), des Lipidmetabolismus (SREBP1, SREBP2, ACC), der Antioxidant- (NQO1) und Mitochondrienaktivitäten (CPT1, PGC1A) analysiert. Zusätzlich wurde die AMPKA Proteinexpression der Blastozysten mittels Immunfluoreszen bestimmt und die Mitochondrienaktivität durch MitoTracker unter dem Konfokalmikroskop untersucht. Um festzustellen, ob Makromolekülergänzungen die runter Regulierung des AMPKA1 beeinflussen, wurden im dritten Teil des ersten Experiments Blastozysten durch die Zugabe unterschiedlicher Makromoleküle, die Seren BSA und BSAFAF, produziert. Die Blastozysten Entwicklungsrate und die Expression der Gene des AMPK Signalwegs (AMPKA1, AMPKA2), der Lipidsynthese (ACC), der Mitochondrienaktivität (CPT1,

PGC1A) wurden zwischen den Gruppen verglichen. Zusätzlich wurden Analysen zur Proteinexpression, für die Bestimmung der Phosphorylierungsstelle im AMPKA am Thr172, des ROS Levels und der Mitochondrienaktivität durchgeführt. Die Ergebnisse des ersten Experiments zeigten, dass im Vergleich zu in vivo Blastozysten oder ohne Serum kultivierte Blastozysten das AMPKA1 Expressionsniveau in Serum kultivierter Blastozysten signifikant ($p<0,05$) geringer war. Die vorliegende Studie erbrachte, dass die AMPKA1 runter Regulierung den Lipidgehalt verstärkte im Vergleich zu in vivo, BSA und BSAFAF produzierten Blastozysten. Ein ähnlicher Trend bestätigte sich mit einem höheren ($p<0,05$) ACC mRNA Level und einer geringeren Genexpression von CPT1, NQO1 und PGC1A bei der Zugabe von Serum. Zusätzlich konnte eine geringe Phosphorylierung des AMPKA, ein hohes ROS Level und eine geringe Mitochondrienaktivität identifiziert werden, gefolgt von einer Lipidanreicherung in Blastozysten die mit Serum (reich an Fettsäuren) produziert wurden, im Vergleich zu Blastozysten die unter der BSAFAF (geringer Fettsäureanteil) Zugabe erzeugt wurden. Die Ergebnisse des ersten Experiments zeigten einen deutlichen Zusammenhang der AMPK Aktivität mit der ROS Entwicklung, der Mitochondrienaktivität und der Lipidanreicherung während der in vitro Embryokultur. Insgesamt hebt diese Studie die Relevanz des AMPK Signalwegs bei der Entstehung eines anabol/lipogenen bovinen Embryos hervor sowie die Unfähigkeit der Mitochondrien den Lipidüberfluss unter Kulturbedingungen zu metabolisieren. Deshalb könnte eine veränderte AMPK Aktivität während der in vitro Kultur nützlich für eine reduzierte Lipidanreicherung sein.

Mit dem Ziel die Lipidanreicherung in in vitro produzierten Blastozysten, durch die Veränderung der AMPK Aktivität, zu reduzieren, wurde AICAR dem Embryokulturmedium zugefügt. In diesem Teil des Experiments wurden bovine Blastozysten in Kulturmedium das mit 100 µM AICAR angereichert war, produziert. Im Anschluss daran wurde die Embryoentwicklungsrate, die Blastozystenzellzahl, die Genexpression, die AMPK Proteinexpression, die Mitochondrienaktivität und die Lipidanreicherung untersucht. Dabei zeigte sich, das die Zugabe von AICAR keine Veränderungen in der Embryoentwicklungsrate, aber eine signifikant ($p<0,05$) reduzierte Blastozystenzellzahl im Vergleich zu den AICAR unbehandelten Blastozysten bewirkte. Weiterhin, erhöhte AICAR ($p<0.05$) die Expression von Genen des AMPK Signalwegs (AMPKA1, STK11), der Antioxidant- (NQO1) und Mitochondrienaktivität (CPT2, PGC1A) und reduzierte sie bei Genen der Lipidsynthese (ACC) ($p<0.05$). Durch die AICAR Zugabe waren zusätzlich die AMPKA Protein- und Mitochondrienaktivitäten verbessert, das spiegelt den reduzierten Trend des Lipidgehalts in Blastozysten wieder.

Um den potentiellen regulatorischen Mechanismus des AMPKA1 durch miRNA zu verdeutlichen, wurde nach spezifischen miRNAs deren Zielgen möglicherweise AMPKA1 sein könnte, gesucht. Dafür wurden eine Reihe von *in silico* Untersuchungen durchgeführt und 11 miRNAs ermittelt. In der miRNA Profilanalyse, unter Verwendung von drei Referenzgenen (U6, Snord48 und 5S), war die miR-33b im Vergleich zu den anderen Kandidaten miRNAs am meisten angereichert. Ein ähnliches Ergebnis erbrachte die Bestimmung der Luciferaseaktivität des AMPKA1-3'UTR Plasmids, wobei dessen Aktivität durch die miR-33b Präsenz unterdrückt und die Regulierung durch die Mutation in der miR-33b Bindungsstelle abgeschwächt war. Dieses deutet auf die spezifische Bindung der miR-33b mit der AMPKA1 mRNA hin. Mit dem Ziel, die AMPK Aktivität zu erhöhen, wurden 50 nM miR-33b in Granulosazellen transfiziert und die Genexpression von AMPKA1, ACC, CPT1 und PGC1A überprüft. Das Ergebnis zeigte, dass die Unterdrückung von miR-33b ($p<0.05$) die Expression von AMPKA1, ACC und PGC1A signifikant erhöhte, während kein Einfluss auf die CPT1Genexpression festgestellt wurde. Die hohe ACC Genexpression deutet auf den natürlichen Granulosaluteinisierungsprozess hin.

Zusammenfassend zeigte die vorliegende Studie, dass die kulturbedingte runter Regulierung des AMPKA1 in einer erhöhten Lipidanreicherung durch die reduzierte Mitochondrienaktivität resultiert. Ebenfalls heben die Ergebnisse dieser Studie hervor, dass die AMPKA1 Aktivität durch den Einsatz von künstlichen Aktivatoren und spezifischen miRNA Inhibitoren moduliert werden kann.

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10 Curriculum Vitae

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Publication and contributions

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