Target gene prediction and pathway analysis of miRNA targeted AMPK involved in lipid accumulation of bovine granulosa cell luteinization

S Prastowo\textsuperscript{1,2}, N Widyas\textsuperscript{1}, A Ratriyanto\textsuperscript{1,2}

\textsuperscript{1}Animal Science Department, Universitas Sebelas Maret, Surakarta, Indonesia
\textsuperscript{2}Center for Biotechnology and Biodiversity Research and Development, Universitas Sebelas Maret, Surakarta, Indonesia

Corresponding author: prastowo@staff.uns.ac.id

Abstract. Luteinization is process of turning granulosa into luteal cells to produce progesterone. It’s indicated by lipid accumulation which controlled by AMPK gene, the expression is controlled by miRNAs at pre and/or post transcriptional level. Previously, 4 miRNAs namely miR-19b, miR-130, miR-101, and miR-19a were predicted targeted to AMPK. For that, this study aimed to identify board target of these miRNAs to genes in specific metabolic pathway. An \textit{in silico study} was performed using online molecular databases that are miRDB (www.mirdb.org) and DAVID Bioinformatic Resource (https://david.ncifcrf.gov/) and Genomes (KEGG) pathway (https://www.genome.jp/kegg/pathway.html). Result shows that miR-19b, miR-130, miR-101, and miR-19a targeted to 1121, 738, 859, and 1117 respectively. A total 3835 genes were analyzed, resulted five annotated functional group namely coiled coil, serine/threonine-protein kinase, ATP-binding, nucleotide-binding, and kinase. According to gene ontology analysis, genes were grouped at nucleoplasm, cytoplasm, nucleus, golgi apparatus, and transcription factor complex process. Moreover, pathway analysis found five top KEGG pathways that are FoXO, MAPK, cAMP, Sphingolipid, and prolactin signaling. Two genes namely RAF1 and AKT3 were involved in all pathways. It is concluded the expression of miRNA group targeted to AMPK affect to complex cellular metabolism pathway direct and/or indirectly.

1. Background
Ovarian follicle luteinization is post ovulation process which turning granulosa into luteal cell. As generally known, luteal cells produce progesterone to initiate and support further pregnancy [1]. This process is indicated by the accumulation of lipid in theca and granulosa cells [2]. In here, lipid act as source of cholesterol and crucial for hormone production in steroidogenesis [3]. Progesterone is steroid hormone which plays a key role in reproductive events. Its functions are to induce embryo implantation [4], affected to embryo development, embryo survival, increase maternal recognition of pregnancy, increase pregnancy rates, initiate, establish and maintenance pregnancy [5]. Therefore, any disturbance of luteinization, would lead to pregnancy failure [6] related to embryonic loss which is become main problem in dairy and beef cattle fertility.

Similar with other biological process, lipid accumulation is controlled by the gene expression of AMPK as master regulator of energy balance. Its work by sensing the [AMP] : [ADP] concentration in
the cell [7], and officially known as protein kinase AMP-activated (https://www.ncbi.nlm.nih.gov/). Low expression of AMPK lead to the accumulation of lipid, whilst high AMPK expression would improve lipid oxidation to meet the energy demand [8–10]. This phenomenon has been demonstrated in bovine embryo, when low AMPKA1 expression was associated with low mitochondrial activity lead to lipid accumulation [11]. Moreover, low mitochondrial activity could be a hallmark of cell oxidative stress which affect to the Reactive Oxidative Stress (ROS) and antioxidant activity [12,13]. In normal cell, AMPK could mediating hormone responsiveness [14,15]. For example, the inhibition of AMPK activity affect to the granulosa cell proliferation through follicle-stimulating hormone (FSH) stimulation [16]. Active form of AMPK have been demonstrated to cause FSH downregulation and decrease steroidogenesis [17]. Other reports also shows that active AMPK in mammals inhibit progesterone and/or estrogen secretion by granulosa cell [18,19].

miRNAs are non-coding RNAs with post-transcriptional regulator gene expression functions [20,21]. 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 [22]. Gene can be targeted by single or many miRNA or vice and versa. Previously, we have predicted 4 miRNAs namely miR-19b, miR-130, miR-101, and miR-19a which targeted to AMPK in bovine granulosa cell luteinization [23]. For that, this study aimed to identify the board target of those miRNAs to genes in specific metabolic pathway or biological process by performing insilico study using online algorithm molecular databases. And also, it could be a way to do a preliminary validation study of miRNA targeted gene before validated in the wet lab study.

2. Methods

2.1. miRNAs-gene target prediction

An online database for miRNA target prediction namely miRDB (http://www.mirdb.org/) was employed to predict the target genes of miRNAs. The potential genes targeted were mined using human genes database due to these selected miRNAs [23] are completely identical with bovine miRNA sequence. In this study, genes targeted with less than 60 target prediction score were excluded from further analysis, and only top five group of genes functional annotation, ontology and biological pathway were selected.

2.2. Genes functional annotation and clustering

Following genes target mining, gene functional annotation and clustering was analyzed using DAVID Bioinformatic Resource (https://david.ncifcrf.gov/).

2.3. Pathway mapping

For pathway mapping, KEGG Database (https://www.genome.jp/kegg/pathway.html) was used to identify the representative biological pathway. Moreover, to find specific genes which involve in the common pathways, Venn diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/) was used.

3. Result and discussion

To identify the metabolic pathway of selected miRNAs in the previous study [23], we employing miRDB algorithms to predict the potential target genes. A total of 3835 genes were found to be targeted by miR-19b, miR-130, miR-101 and miR-19a. The detail number of targeted genes predicted by each individual miRNAs and top 10 official gene symbol were listed in Table 1. As we can see, each individual miRNA having different number of targeted genes. It is know that miRNA bind to the 3’UTR of specific gene, namely seeding region, and suppress the function of gene [24]. Indeed, one miRNA could have only one or multiple seeding region in the target gene. The greater number of
miRNA seeding region, it will have more change to suppress the function of particular gene by
decrease the stability or inhibit translation of messenger RNAs (mRNAs) [25].

Table 1. List of total number of targeted genes and the name of top 10 genes predicted.

| Name of miRNA | Number of genes | Official symbol of top 10 genes |
|---------------|-----------------|--------------------------------|
| miR-19b       | 1121            | ESR1, ATXN1, ZMYND11, ATG14, SLC24A3, SHANK1, LRP2, HCFC2, SECISBP2L, ACBD5 |
| miR-130       | 738             | GJA1, CPEB1, SKIDA1, SLAIN1, CLIP1, MYBL1, ESR1, RAP2C, KLF7, PIK3CB |
| miR-101       | 859             | BICRA, MYCN, ATXN1, ABHD17C, TET2, ARID1A, STC1, MFSD6, FBN2, CDK8 |
| miR-19a       | 1117            | ESR1, ATXN1, ZMYND11, ATG14, SLC24A3, SHANK1, LRP2, HCFC2, SECISBP2L, ACBD5 |
| **Total**     | **3835**        |                                |

Next, the genes list was uploaded to DAVID Bioinformatic database for functional annotation. At
this step, we identify that the listed genes were annotated (five top groups) in coiled coil, serine/threonine-protein kinase, ATP-binding, Nucleotide-binding, and kinase group (Table 2). Interestingly, we found set of genes in serine/threonine-protein kinase. It is known that serine/threonine-protein kinase is the agent which responsible for phosphorylation process in mammals [26] lead to activated AMPK [27].

Table 2. Genes functional annotation.

| Group                             | P-Value   | Number of genes involved | Official symbol of top 10 genes |
|-----------------------------------|-----------|--------------------------|--------------------------------|
| Coiled coil                       | 2.00x10^{-17} | 373                     | ARFIP1, ARID4B, ABCC9, ATP12A, ATRX, BDP1, BICD2, BBS7, CLIP1, CEBPA |
| Serine/threonine-protein kinase   | 5.80x10^{-07} | 49                      | AKT3, AAK1, GRK6, NEK7, PDIK1L, RAF1, WEE1, ACVR1, ACVR2B, BMP1R1B |
| ATP-binding                       | 6.20x10^{-07} | 138                     | PFKB3, AKT3, AAK1, ABCA1, ABCA5, ABCB7, ABCC3, ABCC5, ABCC9, ABCE1 |
| Nucleotide-binding                | 1.40x10^{-06} | 172                     | PFKFB3, ARL8B, AKT3, AAK1, ABCA1, ABCA5, ABCB7, ABCC3, ABCC5, ABCC9 |
| Kinase                            | 1.40x10^{-06} | 83                      | PFKFB3, AKT3, AAK1, EPHA5, EPHB3, FYN, FASTK, GRK6, IDNK, JAK2 |

According to the ontology classification, we identify set of genes which were located in nucleoplasm, cytoplasm, nucleus, golgi apparatus, and transcription factor complex. The number and name of genes involved were listed in Table 3. Following that, KEGG pathway analysis showed genes which were involved in five top pathways namely FoXO signaling, MAPK signaling, cAMP signaling, Sphingolipid signaling and prolactin signaling (Table 4). Among these pathways, we found two genes namely RAF1 and AKT3 were involved in all pathways (Figure 1, Table 5), and these genes were belonging to threonine protein kinase which known as the activator of AMPK.

One of the fundamental requirements of cells is to balance the energy between ATP consumption and ATP generation. In here AMPK act as cellular energy sensor that is activated when modest decreases in ATP production result in relative increases in AMP or ADP. In response of energy demand, AMPK promotes catabolic pathways to generate ATP, and inhibits anabolic pathways.
AMPK also act as signalling pathway which is important for cell growth and metabolism [28] via controlling the transcription factors [29]. Many transcription factors play a key role in cellular differentiation, regulated by phosphorylation, ubiquitination, acetylation/deacetylation and interactions between two or more proteins controlling multiple signaling pathways [30].

Table 3. Gene ontology.

| Classification                | P-Value     | Number of genes involved | Official symbol of top 10 genes                                                                 |
|-------------------------------|-------------|--------------------------|--------------------------------------------------------------------------------------------------|
| Nucleoplasm                   | 5.90x10⁻¹⁰  | 214                      | MTRR, PFKFB3, ADAM12, ARFGEF1, AFF3, AKT3, ARID1A, ARID2, BDP1, BRAF1                            |
| Cytoplasm                     | 5.00x10⁻⁰⁷  | 419                      | MTRR, ARFIP1, ARL8B, AFF3, ALS2CL, ASAP1, BDP1, BCL3, BBX, BAMB1                               |
| Nucleus                       | 1.10x10⁻⁰⁵  | 381                      | ALG2, ARID4B, ARID5B, ATAD2B, AGFG1, BCL3, BCL9, BAG5, BRIP1, BACH2                            |
| Golgi apparatus               | 5.60x10⁻⁰⁵  | 88                       | PXYLPl, AKT3, ABCA1, ATP8A1, ATP2Cl, BCL9, BICD2, FAM20B, GABARAPL1, LRP2                      |
| Transcription factor complex  | 1.00x10⁻⁰⁴  | 35                       | E2F2, E2F7, E2F8, FOS, NAA15, PBX3, PKN0X1, RBL2, RCOR1, RCOR3                                 |

Table 4. Top five KEGG pathway involved.

| Pathway                      | P-Value     | Number of genes involved | Official symbol of top 10 genes                                                                 |
|------------------------------|-------------|--------------------------|--------------------------------------------------------------------------------------------------|
| FoXO signaling pathway       | 8.20x10⁻¹⁰  | 38                       | AKT3, BCL2L11, FBXO32, GABARAPL1, KRAS, KLF2, RBL2, RAF1, SETD7, SMAD4                         |
| MAPK signaling pathway       | 2.60x10⁻⁰⁸  | 54                       | AKT3, FOS, GNA12, KRAS, MECOM, RAP1A, RAP1B, RASGRP3, RASA1, RAF1                              |
| cAMP signaling pathway       | 1.50x10⁻⁰⁷  | 44                       | AKT3, ATP1B1, ATP2B2, FOS, RAP1A, RAP1B, RAF1, RAPGEF4, SOX9, ACOX3                            |
| Sphingolipid signaling       | 1.50x10⁻⁰⁷  | 32                       | AKT3, FYN, GNA12, GNAQ, KRAS, RAF1, BDKRB2, CERS2, CERS6, DEGS1                               |
| Prolactin signaling pathway  | 1.70x10⁻⁰⁷  | 24                       | AKT3, FOS, JAK2, KRAS, RAF1, SOS2, TNFRSF11A, CCND1, CCND2, ESR1                               |

In our result, we identify five top signaling pathway which interact with AMPK that in agreement with several previous reports. For example, AMPK reported directly regulating FoXO transcription [31] and AMPK has important role in promoting p38 MAPK activation [32]. The activity of AMPK was reported to be regulated by cAMP [33] and in Sphingolipids, AMPK has direct interaction in controlling energy homeostasis due to sphingolipid regulate activities related to including mitochondrial function and cell death [34]. Lastly, previous study showed that inhibition of AMPK activity lead to inhibit cell growth and prolactin secretion [35].
Table 5. Genes involved in the same pathway.

| Pathway               | Total | Gene name                                      |
|-----------------------|-------|-----------------------------------------------|
| FoXO signaling        | 2     | RAF1, AKT3                                    |
| MAPK signaling        |       |                                               |
| Prolactin signaling   |       |                                               |
| Sphingolipid signaling|       |                                               |
| cAMP signaling        |       |                                               |
| FoXO signaling        | 1     | KRAS                                          |
| MAPK signaling        |       |                                               |
| Prolactin signaling   |       |                                               |
| Sphingolipid signaling|       |                                               |
| MAPK signaling        | 1     | FOS                                           |
| Prolactin signaling   |       |                                               |
| cAMP signaling        | 2     | RAP1B, RAP1A                                  |
| MAPK signaling        |       |                                               |
| Sphingolipid signaling|       |                                               |
| FoXO signaling        | 7     | BCL2L11, RBL2, KLF2, FBXO32, GABARAPL1, SETD7, SMAD4 |
| MAPK signaling        | 3     | RASGRP3, RASA1, MECOM                         |
| cAMP signaling        | 5     | ACOX3, ATP2B2, RAPGEF4, SOX9, ATP1B1           |
| Sphingolipid signaling| 6     | FYN, CERS6, GNAQ, CERS2, BDKRB2, DEGS1         |
| Prolactin signaling   | 6     | SOS2, CCND2, JAK2, CCND1, TNFRSF11A, ESR1     |

4. Conclusions
In this study, we are able to demonstrated that miRNA(s) have board targeted genes which control different metabolism pathway. The expression of specific group of miRNA targeted to AMPK would affect to other complex organizations of cellular metabolism pathway by direct or indirect interaction.
Moreover, the finding in this study support previous result that selected miRNAs were target AMPK metabolism pathway. However, further wet lab study needs to be employed to validating this result.

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